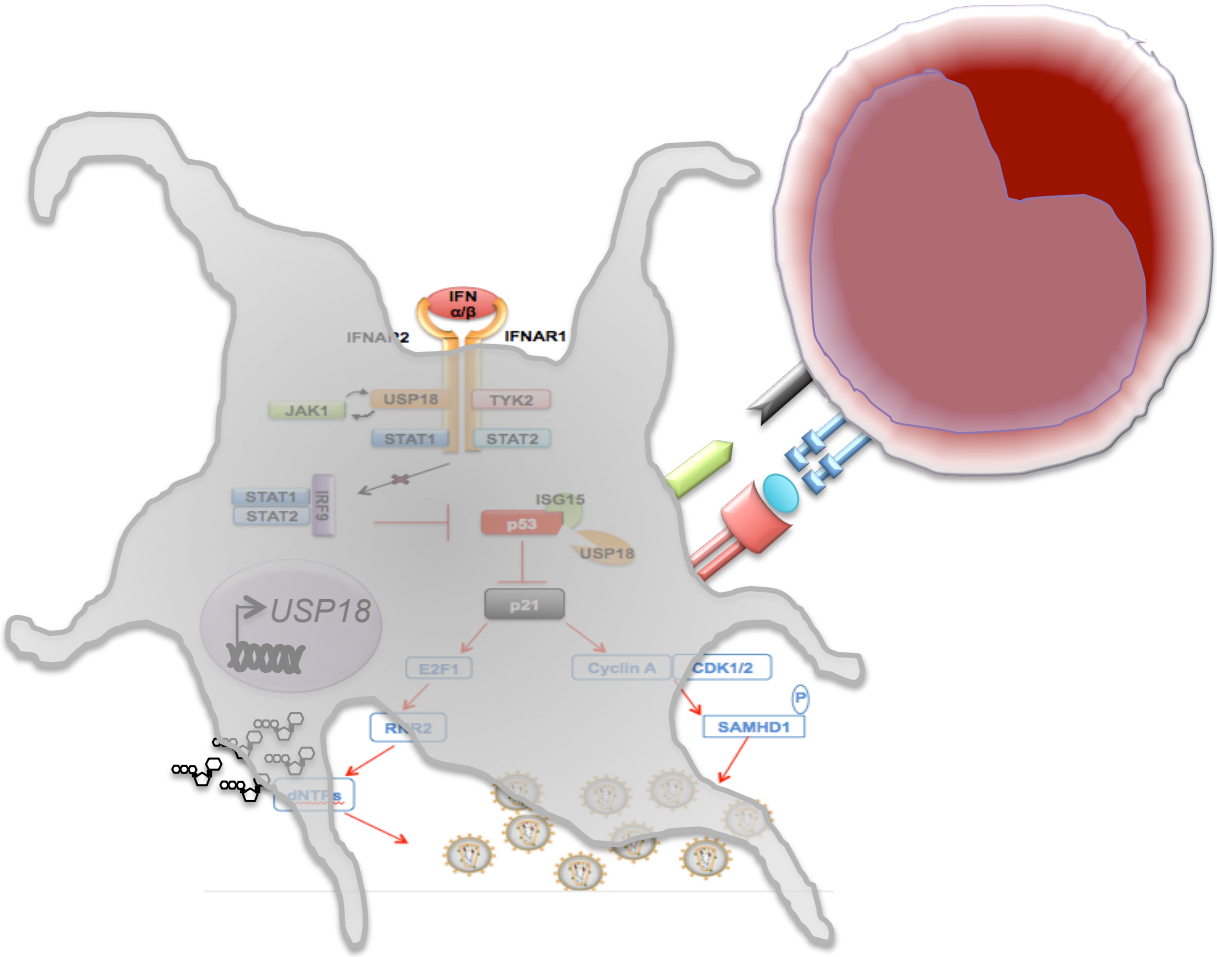

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The Unending War between HIV and the Intrinsic Innate and Adaptive Immune System: Dissecting the Role of USP18 (UBP43) and *H. pylori* Coinfection

Edmund Osei Kuffour

The Unending War between HIV and the Intrinsic Innate and Adaptive Immune System: Dissecting the Role of USP18 (UBP43) and *H. pylori* Coinfection

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Edmund Osei Kuffour
from Accra, Ghana

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from the Clinic for Gastroenterology, Hepatology and Infectious Diseases,
Medical Faculty at the Heinrich Heine University Düsseldorf

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Contents

Summary

The innate immune response against viruses is important for the antiviral host resistance, and it is formed in part by intracellular proteins. These factors are also called restriction or resistance factors, including ISG15 (Interferon-stimulated gene 15), the cyclin-dependent kinase (CDK) inhibitor p21 and the deoxynucleotide triphosphate (dNTP) triphosphohydrolase SAMHD1. Interferon can either induce or activate these factors as interferon (IFN)-stimulated genes (ISGs). Of its many functions, p21 regulates the antiviral activity of SAMHD1 and represses key enzymes involved in *de novo* dNTP biosynthesis. Lentiviruses like HIV-1 (human immunodeficiency virus type 1) circumvent the innate immune surveillance and establish disseminated infection in their host. The ability of HIV-1 to bypass the innate immune response may be mediated by cellular factors such as USP18, an ISG15-specific ubiquitin-like protease 43 that negatively regulates IFN signalling pathways.

In the event of an established infection, after bypassing innate immune recognition and sensing, HIV-1 preferentially targets and depletes memory CD4+ T cells of the adaptive immune system, resulting in AIDS and death, if the infection is not interrupted by antiretroviral therapy. In sub-Saharan Africa, where the prevalence and geography of HIV-1 and other pathogenic infections overlap, coinfection is highly probable with either beneficial or deleterious outcomes.

In this work, I present findings from three different projects I worked on, all focusing on the pathogenicity of HIV-1 infection. In the first project, I dissected the role of USP18 in HIV-1 infection and replication in innate immune cells, using the myeloid THP-1 cell line as a model *in vitro*. In my second project, I analysed and characterized for the different subsets of memory CD4+ T cells that are preferred target for HIV-1 infection and replication *in vivo*. Finally, I investigated the impact of *H. pylori* coinfection on immune modulation and clinical parameters in HIV-1-infected patients from Ghana.

Here, I demonstrate that USP18 enhances HIV-1 replication by abrogating the antiviral function of p21 in differentiated myeloid cells. USP18 downregulated p21 expression by accumulating misfolded dominant negative p53. By inactivating the p53 transactivation function, USP18 relieved p21 repression of key enzymes involved in *de novo* dNTP biosynthesis pathway, and inactivated SAMHD1 antiviral activity. Depletion of USP18 stabilized p21, activated SAMHD1 restriction function and blocked HIV-1 replication.

This work also shows that $CD25^{+}FOXP3^{+}$ memory $CD4^{+}$ T cells from HIV-1 infected patients contain more HIV DNA than their $CD25^{-}FOXP3^{-}$memory $CD4^{+}$ T cell subsets, likely because of relatively high HIV co-receptor CCR5 expression and a more proliferative stage with high $Ki67^{+}$ marker.

Finally, I demonstrate that *H. pylori* infection confers beneficial immunomodulatory effects that might predispose to slower disease progression in ART-naïve HIV-1-infected patients from Ghana.

Overall, I propose that the human USP18 is a novel factor that potentially contributes to HIV-1 infection and replication in innate immune target cells. A further study to elucidate how USP18 might modulate innate immune recognition and sensing of HIV-1 in primary macrophages is warranted. I also suggest that specific characteristics of $CD25^{+}FOXP3^{+}$ memory $CD4^{+}$ T cells might facilitate efficient HIV infection *in vivo* and passage of HIV DNA to cell progeny in the absence of active viral replication. Future work to quantify and characterize the relative contribution of this cell subset to plasma viremia *in vivo* is justified. Finally, I propose that *H. pylori* coinfection with HIV-1 confers beneficial immune modulatory outcome for patients. It would be interesting to understand the molecular and cellular mechanistic details to inform decisions on better therapeutic approaches to treatment of both HIV-1 and *H. pylori* infections.

Summary in German (Zusammenfassung)

Die Abwehr gegen Viren durch das angeborene Immunsystem wird unter anderem über die Expression von intrazellulären Proteinen vermittelt. Diese große Gruppe von Proteinen umfasst auch die Resistenz- und Restriktionsfaktoren, z. B. ISG15 (Interferon stimuliertes Gen 15), den Inhibitor von Cyclin-abhängigen Kinasen p21 und die Deoxynukleosidtriphosphat (dNTP) Triphosphohydrolase SAMHD1. Das Zytokin Interferon (IFN) kann diese Proteine induzieren oder aktivieren (z. B. IFN stimulierte Gene, ISGs). Neben anderen Funktioinen kann p21 die antivirale Aktivität von SAMHD1 regulieren und wichtige Enzyme der dNTP Synthese reprimieren.

Das humane Immundefizienz Virus Typ 1 (HIV-1) ist in der Lage die Abwehrmechanismen des angeborenen Immunsystems zu umgehen und im Menschen eine chronisch-progredierente Infektion zu etablieren. Die Fähigkeit von HIV-1 der angeborenen Abwehr zu entgehen könnte auch durch zelluläre Proteine wie z.B. USP18 bewirkt werden. USP18 ist eine De-ISGylase und ein negativer Regulator des IFN-Signalweges. Wenn HIV-1 eine chronische Infektion im Menschen etabliert hat und nicht durch antivirale Medikamente inhibiert wird, kommt es zu einer Zerstörung des adaptiven Immunsystems durch Depletion der Memory CD4+ T Zellen und in der Folge zu AIDS und Tod des Patienten. Im subsaharischen Afrika sind die Menschen nicht nur mit HIV-1, sondern auch mit anderen pathogenen Infektionen konfrontiert, die als Ko-Infektionen den Verlauf der HIV-Infektion beeinflussen können. In meiner Doktorarbeit habe ich mich mit drei Aspekten der HIV-1 Pathogenese beschäftigt. Im ersten Projekt habe ich die Rolle von USP18 bei der HIV-1 Infektion und Replikation in einer monozytären Zelllinie (THP-1 Zellen) untersucht. Im zweiten Projekt habe ich unterschiedliche Populationen von Memory CD4+ T Zellen in Patienten im Hinblick auf eine Suszeptibilität für HIV-1 analysiert und charakterisiert. Im letzten Teil der Arbeit wurde untersucht, ob eine Ko-Infektion mit *H. pylori* einen immunmodulatorischen Effekt bei Patienten mit HIV-1 Infektion in Patienten in Ghana vermittelt.

Die Ergebnisse weisen darauf hin, dass USP18 durch eine Repression der p21 Expression in Zellkultur eine “provirale” Wirkung auf HIV-1 Infektion bewirkt. Dabei blockt USP18 die p21 Expression durch die Induktion von fehlgefalteten dominant negativen p53 Protein. Reduzierte Mengen von p21 induzieren eine vermehrte dNTP Synthese und eine Inaktivierung der antiviralen Aktivität von SAMHD1. Umgekehrt führte eine Depletion der USP18 Expression zu erhöhten Konzentrationen von p21, verstärkter antiviraler Aktivität von SAMHD1 und einer reduzierten HIV-1 Replikation. In den Patientenanalysen konnte ich zeigen, dass CD25+FOXP3+ Memory CD4+ T Zellen mehr HIV-1 DNA enthalten als CD25-FOXP3+ Memory CD4+ T Zellen. Ursächlich dafür könnten eine höhere Expression des CCR5 HIV Ko-Rezeptors und eine höhere Zellproliferation, gemessen mit dem Ki67+ Marker, in den CD25+ Zellen sein. Schließlich deuten meine Ergebnisse darauf hin, dass eine Ko-Infektion mit *H. pylori* in Therapie-naiven Patienten zu einem langsameren Krankheitsverlauf führt.

Meine Daten unterstützen die Hypothese, dass USP18 ein wichtiger regulatorischer Ko-Faktor für die HIV-1 Replikation in Makrophagen ist. Zukünftige Studien sollten die Bedeutung von USP18 für die HIV-1 Infektion weiter untersuchen und den hier noch nicht beleuchteten Effekt auf das Sensing von HIV betrachten. Ich postuliere auch, dass CD25+FOXP3+ Memory CD4+ T Zellen eine noch unterschätzte Rolle für die HIV Pathogenese spielen. Ergebnisse der *H. pylori* Studie weisen darauf hin, dass es noch unbekannte Möglichkeiten gibt, um die HIV Pathogenese positiv zu beeinflussen.

Publications

This work is based on the publications below:

1. Osei Kuffour E, Schott K, Jaguva Vasudevan AA, Holler J, Schulz WA, Lang PA, Lang KS, Kim B, Häussinger D, König R, Münk C. 2018. USP18 (UBP43) abrogates p21-mediated inhibition of HIV-1. J Virol 92:e00592-18. https://doi.org/10.1128/JVI.00592-18.

2. Edmund Osei Kuffour, Renate König, Dieter Häussinger, Wolfgang A. Schulz, Carstn Münk. USP18 (UBP43) downregulates p21 antiviral function by accumulating misfolded dominant negative p53 that supports HIV-1 replication. Manuscript under preparation.

3. Chachage M, Pollakis G, Kuffour EO, Haase K, Bauer A, Nadai Y, Podola L, Clowes P, Schiemann M, Henkel L, Hoffmann D, Joseph S, Bhuju S, Maboko L, Sarfo FS, Eberhardt K, Hoelscher M, Feldt T, Saathoff E, Geldmacher C. 2016. CD25_ FoxP3_ memory CD4 T cells are frequent targets of HIV infection in vivo. J Virol 90:8954 –8967. doi:10.1128/JVI.00612-16.

4. Sarfo FS, Eberhardt KA, Dompreh A, Kuffour EO, Soltau M, Schachscheider M, et al. (2015) Helicobacter pylori Infection Is Associated with Higher CD4 T Cell Counts and Lower HIV-1 Viral Loads in ART-Naïve HIV-Positive Patients in Ghana. PLoS ONE 10(11): e0143388. doi:10.1371/journal. pone.0143388.

5. Kirsten Alexandra Eberhardt, Fred Stephen Sarfo, Albert Dompreh, Edmund Osei Kuffour, Christof Geldmacher, Mareike Soltau, Marei Schachscheider, Jan Felix Drexler, Anna Maria Eis-Hübinger, Dieter Häussinger, George Bedu-Addo, Richard Odame Phillips, Betty Norman, Gerd Dieter Burchard, Torsten Feldt; Helicobacter pylori Coinfection Is Associated With Decreased Markers of Immune Activation in ART-Naive HIV-Positive and in HIV-Negative Individuals in Ghana, Clinical Infectious Diseases, Volume 61, Issue 10, 15 November 2015, Pages 1615–1623, https://doi.org/10.1093/cid/civ577.

1. Introduction

We owe our current understanding of immunology in many ways to two main theories - clonal selection and pattern recognition. These concepts, interestingly, were formulated originally on theoretical grounds, and then later proven experimentally. The clonal selection theory, the first to be formulated, suggested an ingenious explanation of the function of the immune system, now considered as the adaptive immune system. In this paradigm, each lymphocyte was thought to display a structurally unique receptor, made at random during development in the central lymphoid organ to recognize its specific antigen. Upon recognition of its cognate antigen, this lymphocyte is selected for activation, clonal expansion, and differentiation into effector function. This theory further explained the concept of immune memory, which suggests that some of the antigen-stimulated cells further differentiate into memory cells, which do not necessarily carry out effector functions. However, upon latter encounter with the same specific antigen, they are more easily and readily activated into effector cells and can differentiate further into more memory cells (Ada, 2008, Lederberg, 1959, Billingham et al., 1953, Medzhitov, 2009, Medzhitov and Janeway, 2000, Medzhitov, 2010, Medzhitov, 2013).

The mechanism by which the immune system maintained tolerance of self-antigens was explained by the concept of clonal deletion or central tolerance of developing lymphocytes, a flip side of clonal selection. Here, it was thought that lymphocytes whose receptors recognize self-antigens are eliminated by apoptosis or by receptor editing in the central lymphoid organs (Burnet, F.M, 1959, Lederberg, J. 1959, (Alberts et al., 2008)). A basic inference from the concepts of clonal selection and deletion implied that any antigen could elicit an immune response as long as it is not a self-antigen (Billingham et al., 1953, Medzhitov, 2013). However, it became apparent that in the peripheral lymphoid organ, another form of immune tolerance occurred by a mechanism that could not be explained by clonal deletion (Medzhitov, 2013). Here, auto-reactive lymphocytes which escaped clonal deletion or receptor editing and

recognized their cognate self-antigen could still be tolerated by means of clonal inactivation or clonal suppression (Medzhitov, 2013). The mechanisms that helped to prevent allergies and autoimmunity in the peripheral lymphoid organ would be explained later on by Charles A. Janeway, Jr. in 1989 (Medzhitov, 2013, Medzhitov, 2009, Janeway, 1989). In his article, Charles A Janeway developed the theory of pattern recognition, which suggested that the distinct forms of the immune recognition, comprising the innate and adaptive system are fundamentally different in the roles they play in the immune system, but work synergistically (Medzhitov, 2013, Medzhitov, 2009, Janeway, 1989, Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002). He suggested that the innate immune recognition was based on non-clonal, germline-encoded receptors, which he termed pattern recognition receptors (PRR) (Medzhitov, 2013, Medzhitov, 2009, Janeway, 1989, Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002). These receptors were suggested to detect conserved components of microorganisms, or pathogen associated molecular patterns (PAMPs). The detection of PAMPs by PRR demonstrated the presence of microbial non-self antigens, originating from pathogens such as bacteria, virus, fungi and protozoa, which had the ability to trigger the activation of an innate immune system. The activated innate immune system subsequently activates the adaptive immune system (Medzhitov, 2013, Medzhitov, 2009, Bendelac and Medzhitov, 2002, Janeway and Medzhitov, 2002, Medzhitov and Janeway, 2000, Kabelitz and Medzhitov, 2007, Medzhitov and Janeway, 1997, Medzhitov, 2010). The activation of the adaptive immune system required a presentation of non-self antigen from an activated professional antigen presenting cell, a co-stimulatory signal, which interacts with its cognate receptor on the lymphocyte, a membrane bound adhesion molecule with which the innate and adaptive cells bind together to be activated and secretion of several types of cytokines that may determine which destination the activated lymphocyte is to go (Pasare and Medzhitov, 2005, Medzhitov, 2013, Medzhitov and Janeway, 2000, Soderberg et al., 2005, Medzhitov, 2009, Janeway and Medzhitov, 1998, Medzhitov and Janeway, 1997) (Fig. 1). Since the PRR of the innate immune cells are selected to specifically

recognize PAMPs of microbial origin and not self, they efficiently discriminate self from non-self antigen in the peripheral lymphoid organs. By so doing, they are able to maintain immune tolerance in the periphery by controlling the expression of co-stimulators and other signals that are required for lymphocytes activation (Medzhitov and Janeway, 2000). In the event where these co-stimulatory signals are absent from a professional antigen-presenting cell, the engaged lymphocyte cells are rendered clonally anergic, inactivated or suppressed (Alberts et al., 2008). Thus, the pattern recognition theory served to complement the clonal selection theory with the latter conferring antigen specificity and the former determining the origin of the antigen (Medzhitov, 2013). It is worth noting further that seminally important studies on viruses, have also helped to deepen our knowledge and understanding of immunology (Morrison et al., 1986, Braciale and Hahn, 2013, Zinkernagel and Doherty, 1974, Townsend et al., 1986).

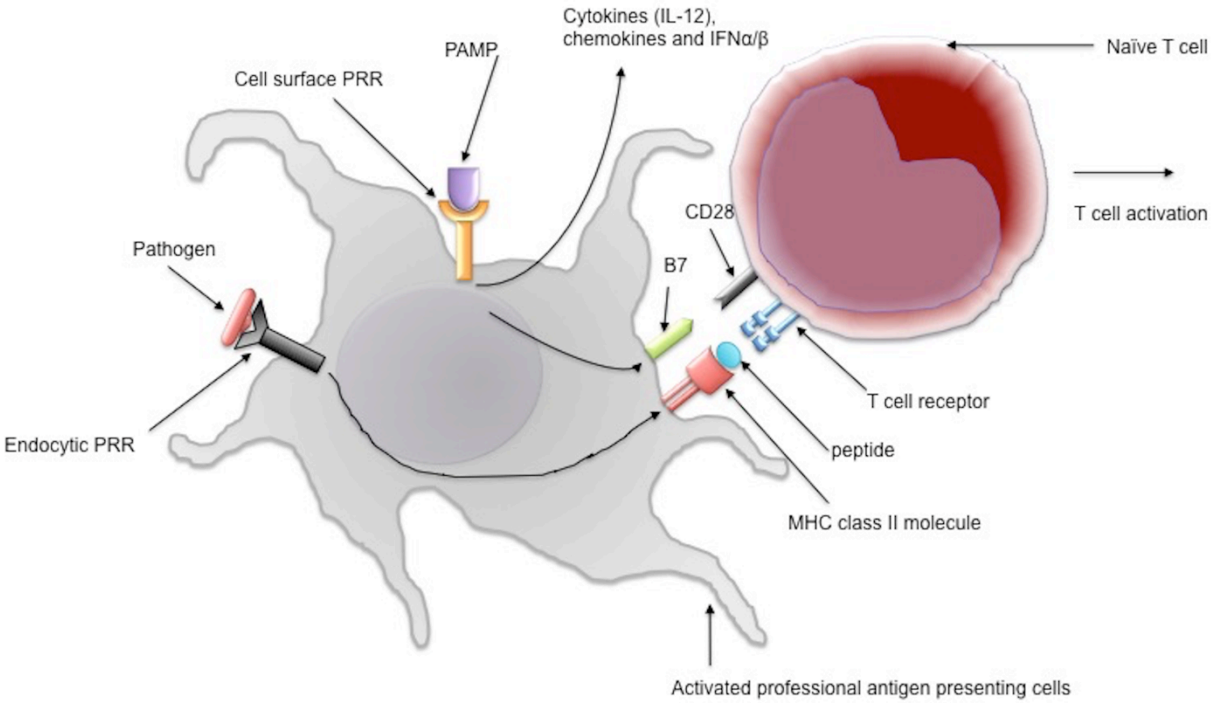

Figure 1. Pathogen recognition receptors (PRR) and the innate control of the adaptive Immune systems: Germ-line encoded PRRs, such as toll-like receptors, expressed on macrophages and dendritic cells are able to recognize and interact with the pathogen-associated molecular patterns (PAMPs) they encounter. The PRR-PAMP interaction generates signals such as cytokines, chemokines and costimulatory molecules that help to activate the adaptive immune system. Other cellular endocytic PRRs, such as

the macrophage mannose receptor, interacts with components of microbial cell walls and mediate the uptake and phagocytosis of the pathogens by these antigen-presenting cells. These cells process proteins derived from the pathogens they phagocytize in the lysosomes and generate antigenic peptides. These peptides form a complex with major-histocompatibility-complex (MHC) class II molecules on the surface of the antigen presenting cells. Cell surface receptors of naïve T cells, recognize and bind to the peptides and are activated for effector functions in the presence of cytokines, chemokines and costimulatory molecules, provided by the antigen presenting cells. Figure was conceived and reconstructed with permission from (Medzhitov and Janeway, 2000), copyright Massachusetts Medical Society.

1.1 The innate immune system

1.1.1 A brief description of the innate immune system

The innate immune system is an ancient form of immune defence against infections, probably present in all multicellular organisms, including plants (Janeway and Medzhitov, 2002). The vertebrate's innate immune system provides a general, non-specific first line of defence against invading pathogens. This consists of physical and chemical barriers, cell-intrinsic responses and mounts of innate immune responses against the infections, mediated by innate immune cells, such as professional phagocytic cells, including macrophages and neutrophils, dendritic cells, natural killer cells and the complement system (Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002). The host's immune response to invading pathogens relies on its innate immune cells' ability to recognize the conserved features of PAMPs that are neither of the host nor of innocuous environmental origin. These PAMPs include many types of molecules on microbial surfaces of bacterial, protozoan, fungi or viral origin (Table 1). An important feature of the vertebrate innate immune cells is their expression of the germ-line encoded PRR described above, whose specificities are predetermined genetically to detect the conserved products of microbial biosynthetic pathways (Janeway and Medzhitov, 2002, Medzhitov, 2009, Kopp and Medzhitov, 1999, Medzhitov, 2013). Once the PRRs identify their PAMP ligand, the innate

immune cells are triggered to perform their effector functions immediately without any delay for activation and proliferation, a feature of the adaptive immune cells (Fig. 1).

1.1.2 Types and functions of PRRs

A significant number of PRRs on innate immune cells have been identified over the years and this number keeps soaring with time as new tools become available to discover them (Table 1). These PRRs can be found on the cell surfaces, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Kopp and Medzhitov, 1999, Pasare and Medzhitov, 2005, Pasare and Medzhitov, 2004, Medzhitov, 2001, Medzhitov, 2013, Kabelitz and Medzhitov, 2007, Janeway and Medzhitov, 2002, Medzhitov, 2009, Iwasaki and Medzhitov, 2010, Iwasaki and Medzhitov, 2015, Akira et al., 2006, Takeuchi and Akira, 2010).

Table 1. PRRs and their PAMPs			
PRRs	Localization	PAMPs	Source of PAMPs
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria (*H. pylori*), viruses (HIV), parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria (*H. pylori*), viruses (HIV), self
TLR5	Plasma membrane	Flagellin	Bacteria (*H. pylori*)
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7	Endolysosome	ssRNA	Virus (*H. pylori*), bacteria, self
TLR8	Endolysosome	ssRNA	Virus (HIV), bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus (HIV), bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa (Toxoplasma gondii)
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD-1	Cytoplasm	iE-DAP	Bacteria (*H. pylori*)
NOD-2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, Fungi
Other PRRs			
AIM2	Cytoplasm	dsDNA	Viruses
DAI	Cytoplasm	dsDNA	Viruses
IFI16	Cytoplasm	dsDNA	Viruses (HIV)
cGAS	Cytoplasm	dsDNA	Viruses (HIV)
Tetherin	Plasma membrane	Viral envelope	Viruses (HIV)

Abbreviations: *PRR, pathogen-recognition receptor; TLR, toll-like receptor; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptors; MDA5, melanoma differentiation-associated gene 5; LGP2, laboratory of genetics and physiology 2; NLR, nucleotide binding, oligomerization domain (NOD)-like receptor, CLR, c-type lectin; AIM2, absent in melanoma-2; DAI, DNA-dependent*

activator of IRFs; IFI16, interferon-γ-inducible protein 16; cGAS, cyclic GMP-AMP synthase; dsRNA, double stranded RNA, LPS, lipopolysaccharide; ssRNA, single stranded RNA; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid; MDP, muramyl dipeptide; SAP130, spliceosome-associated protein 130; HIV, human immunodeficiency virus. Table was adapted from (Takeuchi and Akira, 2010) and modified with permission from the Elsevier publishing group.

Depending on their structure, the PRRs can be categorized into several families of proteins including Leucine-rich repeat domains (LRR), calcium-dependent lectin domains (C-type lectin domains) and the scavenger-receptor protein domains (Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002). Based on their function, they can be further sub-grouped into secreted, endocytic and signalling PRRs. Secreted PRRs serve as opsonins by binding to the microbial cell walls and flagging them for recognition by the complement system and phagocytes. The mannan-binding lectin (MBL), a member of the calcium-dependent lectin family belongs to this group of secreted PRR. (Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002). Endocytic PRRs occur on the surface of phagocytes. Following recognition of PAMP on a microbial cell, these receptors mediate the uptake and delivery of the pathogen into the lysosome to be destroyed. Pathogen-derived proteins can then be processed, and the generated peptides can be presented by macrophages or dendritic cells using their major-histocompatibility-complex (MHC) molecules on their surface (Fig. 1). Macrophages scavenger receptor (MSR) and Macrophage mannose receptor (MMR), also a member of the calcium-dependent lectin family is an example of the endocytic PRR (Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002).

Signalling receptors recognize PAMPs and activate signal-transduction pathways that induce the expression of a variety of immune response genes including pro-inflammatory cytokines. Among these are the well-characterized cell surface and endosome-associated Toll-like receptors (TLRs), which are found in both plants and animals (Kopp and Medzhitov, 1999, Pasare and Medzhitov, 2005, Pasare and Medzhitov, 2004, Kawai and Akira, 2011, Medzhitov, 2001, Takeuchi and Akira, 2010, Akira et al., 2006, Janeway and Medzhitov, 2002). TLRs are

type I transmembrane proteins, comprising a large extracellular domain, which contains a leucine-rich repeats that mediates the recognition of PAMPs, a transmembrane region, and a cytosolic Toll/IL-1 receptor (TIR) domain that activates downstream signalling pathways (Kopp and Medzhitov, 1999, Pasare and Medzhitov, 2005, Pasare and Medzhitov, 2004, Kawai and Akira, 2011, Medzhitov, 2001). Ten functional TLRs have been identified in humans with each detecting distinct PAMPs derived from viruses, bacteria, fungi and parasites (Kawai and Akira, 2011, Takeuchi and Akira, 2010, Akira et al., 2006) (Table 1). Following recognition of PAMPs, TLRs recruit a specific set of adaptor molecules that contain TIR domain such as MyD88 (myeloid differentiation primary-response gene 88) and TRIF (TIR-domain-containing adapter protein inducing IFNβ), and initiate a downstream signalling events, leading to the secretion of proinflammatory cytokines, including type I IFN, chemokines and antimicrobial peptides (Kawai and Akira, 2011). These innate immune responses recruit neutrophils, macrophages and dendritic cells into effector functions, inducing IFN-stimulated genes to either clear the infected pathogen directly or inducing the adaptive immune response, mediated by the dendritic cells (Kawai and Akira, 2011). Some endocytic PRRs may also induce innate immune signalling. An example is the C-type lectins receptors (CLRs). CLRs largely elicit inflammatory responses by recognizing fungal and bacterial PAMPs (Osorio and Reis e Sousa, 2011). The cytosolic retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, including RIG-1, MDA5 (melanoma differentiation-associated gene 5) and LGP2 (laboratory of genetics and physiology 2), are RNA helicases that recognize RNA species released into the cytoplasm in a variety of cell types and coordinate antiviral programmes via type I IFN induction (Loo and Gale, 2011). The nucleotide binding, oligomerization domain (NOD)-like receptor (NLR) family comprises more than 20 members. They are expressed intracellularly and respond to various PAMPs to trigger inflammatory responses. Several NLRs, including NOD1 and 2 are implicated in innate immune signalling following recognition of their ligands. NALP1 (NLRP1) and NALP3 (NLRP3) of the NLR family form the inflammasome along with the ASC (Apoptosis-associated

speck-like protein containing a caspase recruitment domain) and Caspase-I and mediate processing of pro-IL-1B to mature IL-1B for release (Elinav et al., 2011). DNA-dependent activator of IRFs (DAI) and interferon inducible gene 16 (IFI16) are cytosolic dsDNA sensors and induce type I IFN production whereas AIM2 (absent in melanoma-2) of the AIM-like receptors (ALR) recognizes dsDNA and induces the secretion of IL-1B (Kawai and Akira, 2011, Barber, 2011). The recent discovery of the cytosolic dsDNA sensor, cyclic GMP-AMP synthase cGAS, coupled with the ongoing determination of the signalling adapters, kinases, transcriptional factors, and regulators that control anti-retroviral innate immunity has provided exciting insights and developments into the viral immunology, but also major questions for future research (Dempsey and Bowie, 2015, van Montfoort et al., 2014, Hiscott, 2014b, Hiscott, 2014a). It is becoming apparent that a cross talk exists between these innate PRRs to elicit effective immune response against infections (Kawai and Akira, 2011, Takeuchi and Akira, 2010, Akira et al., 2006, Altfeld and Gale, 2015) (Table 1).

Following PRR-PAMPs interaction on innate immune cells, many effector functions occur, including activation of a group of blood proteins called complement. These complement systems are activated sequentially to mark microbes for macrophages and neutrophils to phagocytize. They have the ability to disrupt the membrane of target microbes and generate inflammatory responses. Macrophages, neutrophils and dendritic cells have the ability to neutralize infections by applying a combination of degradative enzymes, antimicrobial peptides and reactive oxygen species. Virus infected cells of the innate immune system produce several cytokines including type I IFNs, which trigger a series of cell responses to inhibit the viral replication and activate the killing activities of natural killer cells. Dendritic cells have the ability to ingest microbes at sites of infection and by transporting them to the peripheral lymph organs, they activate T cells of the adaptive immune system to make specific responses against the microbe (Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002, Medzhitov, 2009, Pasare and Medzhitov, 2005,

Pasare and Medzhitov, 2004, Iwasaki and Medzhitov, 2010, Medzhitov, 2013, Janeway and Medzhitov, 1998, Medzhitov, 2010, Medzhitov and Janeway, 1997, Kabelitz and Medzhitov, 2007, Soderberg et al., 2005, Iwasaki and Medzhitov, 2015, Bendelac and Medzhitov, 2002).

1.2 Adaptive Immune Response

The adaptive immune system is considered the second line of defence against invading pathogen. Cells of the adaptive immune system are called into effector function only when they are activated by cells of the innate immune system (Medzhitov, 2009, Pasare and Medzhitov, 2005, Pasare and Medzhitov, 2004, Iwasaki and Medzhitov, 2010, Medzhitov, 2013, Palm and Medzhitov, 2007, Janeway and Medzhitov, 1998, Medzhitov and Janeway, 1997, Kabelitz and Medzhitov, 2007, Soderberg et al., 2005, Iwasaki and Medzhitov, 2015, Bendelac and Medzhitov, 2002). These adaptive immune cells exhibit immunological memory, which allows them to provide specific and long-lasting protection against particular pathogens that called them into play. Many millions of lymphocytes clones make up the adaptive immune system, with cells in each clone having a unique cell-surface receptor that recognizes its cognate antigen (Ada, 2008). A variety of secreted signals such as cytokines, membrane-bound co-stimulatory signals and adhesion molecules from specialized cells, such as an antigen-mediated activated dendritic cell, are required to stimulate a lymphocyte to proliferate and differentiate into an effector function in the peripheral lymphoid organ. B-lymphocytes require such signals from Helper T cells, while dendritic cells provide these signals for the activation of T cells. An activated B lymphocyte secretes antibodies, which act over long distances, eliminating the pathogens and the toxins that induced them, while activated T cells act locally to eliminate the infected cells or help other effector cells to eliminate the pathogen. B and T lymphocytes are in continuous circulation in the blood stream and lymph, moving from one peripheral lymphoid organ to the other. Upon encountering an antigen, they are halted, activated, proliferated and

differentiated into memory cells, which exhibit stronger and efficient immune responses should they encounter the same pathogenic source again. Lymphocytes that encounter a self-antigen have their receptors altered by editing or eliminated by killing, inactivated or suppressed by regulatory T cells preventing autoimmunity (Alberts et al., 2008).

1.3 Brief History of HIV and its public health importance

The human immunodeficiency virus (HIV) is a pathogen with PAMPs that have the potential to engage innate and adaptive immune cells and stimulate their activation. HIV is the cause of Acquired Immunodeficiency Syndrome (AIDS). This disease was first recorded in 1981 in the United States of America (Gottlieb et al., 1981). Two groups later on independently isolated the virus in 1983 and identified it as the etiologic agent for AIDS (Barre-Sinoussi et al., 1983, Gallo et al., 1983). The term HIV was adopted in 1986 by a subcommittee of the International Committee on the Taxonomy of Viruses and has been retained till date (Coffin et al., 1986b, Coffin et al., 1986a).

HIV is thought to have been transmitted from nonhuman primates to humans in the 1900s due to use of these nonhuman primates as food (Peeters et al., 2014). This hypothesis is supported by nucleotide sequence similarities between several isolates of the distinct HIV-2 (human immunodeficiency virus type 2) and SIVs (simian immunodeficiency virus) from sooty mangabeys, and nucleotide sequence similarities between HIV-1 (human immunodeficiency virus type 1) and SIVs from chimpanzees (SIVcpz) and gorillas (SIVgor) (Sharp and Hahn, 2011). The known HIV-1 groups are thought to have arisen via at least two independent transmissions from the apes. For example, nucleotide sequence similarities exist between HIV-1 group M and N, and the SIVs from chimpanzee *Pan troglodytes troglodytes* (SIVptt), HIV-1 group P and O, and the SIVgor from the western lowland gorillas (*Gorilla gorilla gorilla*),

implicating these apes as the origin of the HIV-1 groups (Sharp and Hahn, 2011, Keele et al., 2006, Van Heuverswyn et al., 2006, Takehisa et al., 2009, Gao et al., 1999, D'Arc et al., 2015, Peeters et al., 2014).

HIV is primarily transmitted by sexual contact, but can also be transmitted by contaminated blood transfusion or use of contaminated syringes. According to the 2017 global HIV statistics, 36.9 million people were estimated to be living with the virus with up to 2.4 million new infection recorded in 2017. An estimated 35.4 million AIDS-related deaths have been recorded since the start of the HIV epidemic (UNAIDS 2017 Fact Sheet).

1.4 Description of HIV: Family and Genera

1.4.1 Retroviruses

HIV is a member of a large group of enveloped viruses called retroviruses (Table 2). This family of viruses obtained their name from their typical reverse transcription, characterized by the reverse flow of their genetic information from RNA to DNA and subsequent integration into the host genome (Telesnitsky, 2010, Kurth and Bannert, 2010). The reverse transcriptase enzyme, discovered in 1970, orchestrates the reverse transcription process (Temin and Mizutani, 1970, Baltimore, 1970). The family, *retroviridae* comprise seven genera, all of which have the basic proviral genomic structure made of the 5' *LTR* (Long terminal Repeat) –*gag* (group specific antigen) -*pol* (polymerase) -*env* (envelope) -3' *LTR* (Fig. 2A). These seven genera include: the spumaviruses, epsilonretroviruses, gammaretroviruses, deltaretroviruses, alpharetrovirues, betaretroviruses and lentiviruses (Table 2). These genera arise due to differences in their genomic organization, protein composition and architecture (Telesnitsky, 2010, Kurth and Bannert, 2010). The *gag-pol-env* genes of the retrovirus family encode for structural proteins and enzymes. In addition to these major genes, most retroviruses encode additional proteins

that exert influence at various stages of the life cycle and in pathogenesis (Flint et al., 2004) (Fig. 2B). Many aspects of retroviral replication are regulated at the DNA or RNA level by the specific cis or trans-active elements. Promoter activity of the 5' *LTR* of retroviruses generally initiates transcription and is terminated by a polyadenylation poly-adenylation (A) signal in the 3' *LTR*. Structural and regulatory proteins are translated from the full-length and spliced RNAs. The virions of retroviruses packages two identical full-length RNA genomes. These two RNA molecules are linked physically by hydrogen bonds and have a 5' cap and 3' poly-A, equivalent to the cellular mRNAs. The terminal region of the proviral 5' *LTR*, called U3, which drives transcription, and the terminal region of the 3' *LTR*, called U5, located downstream of the poly-A signal are absent in the genomic RNA. The "direct repeat" nature of the *LTR*s enables these regions to be duplicated during reverse transcription generating U3-R-U5, allowing for re-establishment of the complete proviral sequence. Another common feature of all exogenous retroviruses is their spherical structure with a diameter of about 100-150 nm. Their viral membranes are studded with glycoprotein spikes needed for attachment and entry into the target cells (Telesnitsky, 2010, Kurth and Bannert, 2010).

Table 2. The retroviral genera

Genus	**Type species**
Subfamily *Orthoretrovirinae*	
Alpharetrovirus	ALV, RSV
Betaretrovirus	MMTV
Deltaretrovirus	BLV, HTLV
Epsilonretrovirus	WDSV
Gammaretrovirus	MLV, FLV
Lentivirus	EIAV, FIV, SIV, HIV
Subfamily *Spumavirinae*	
Spumavirus	SFV, FFV

Abbreviations: *ALV, Avian leucosis virus; RSV, Rous sarcoma virus; MMTV, Mouse mammary tumour virus; BLV, Bovine leukaemia virus; HTLV, Human T-lymphotropic virus; WDSV, Walleye dermal sarcoma virus; MLV, Murine Leukaemia virus; FLV, Feline Leukaemia virus; EIAV, Equine infectious anaemia virus; FIV, Feline immunodeficiency virus; SIV, Simian immunodeficiency virus; HIV, Human immunodeficiency virus; SFV, Simian foamy virus; FFV, Feline foamy virus.*

1.4.2 Structure of the HIV

The HIV virus belongs to the *lentivirus* genus of the retroviral family. In addition to the three major structural genes, which characterize all retroviruses, the HIV genome encodes for additional auxiliary genes that exert their effects at different stages of the viral life cycle and the viral pathogenesis (Fig. 2B). HIV genes such as *rev*, encoded in the early stages of the viral life cycle, exert regulatory functions that are important for the replication of the virus. Other accessory genes such as *nef* (negative regulatory factor)*, vif* (viral infectivity factor)*, vpr* (viral protein r) and *vpu* (viral protein u) exhibit essential functions that are required for efficient production of the virus *in vivo*. The matured HIV particle is spherical in shape with a diameter of about 100 nm. The *gag* gene of the virus encodes for the matrix (MA), capsid (CA) and nucleocapsid (NC). The *pol* gene provides genetic information for the synthesis of the viral enzymes-protease (PR), reverse transcriptase (RT), ribonuclease H (RNase H) and integrase (IN). The *env* gene encodes for the two enveloped proteins, surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41). Two copies of ssRNA that are directly connected by hydrogen bond are complexly packaged in the core of the viral particle and are associated with a plethora of the viral NC, IN, RT and a cellular transfer RNA (tRNA) which is required for reverse transcription. A number of Vpr and Nef are co-packaged in the viral core, which is shielded by a layer of aggregated CA proteins. Exterior to the CA are matrix proteins, which are tethered to the inner layer of the viral membrane. The membrane of the virus is composed of lipid bilayer that is studded with the viral glycoproteins spikes gp120/41 (Telesnitsky, 2010, Flint et al., 2004, Kurth and Bannert, 2010) (Fig 2C).

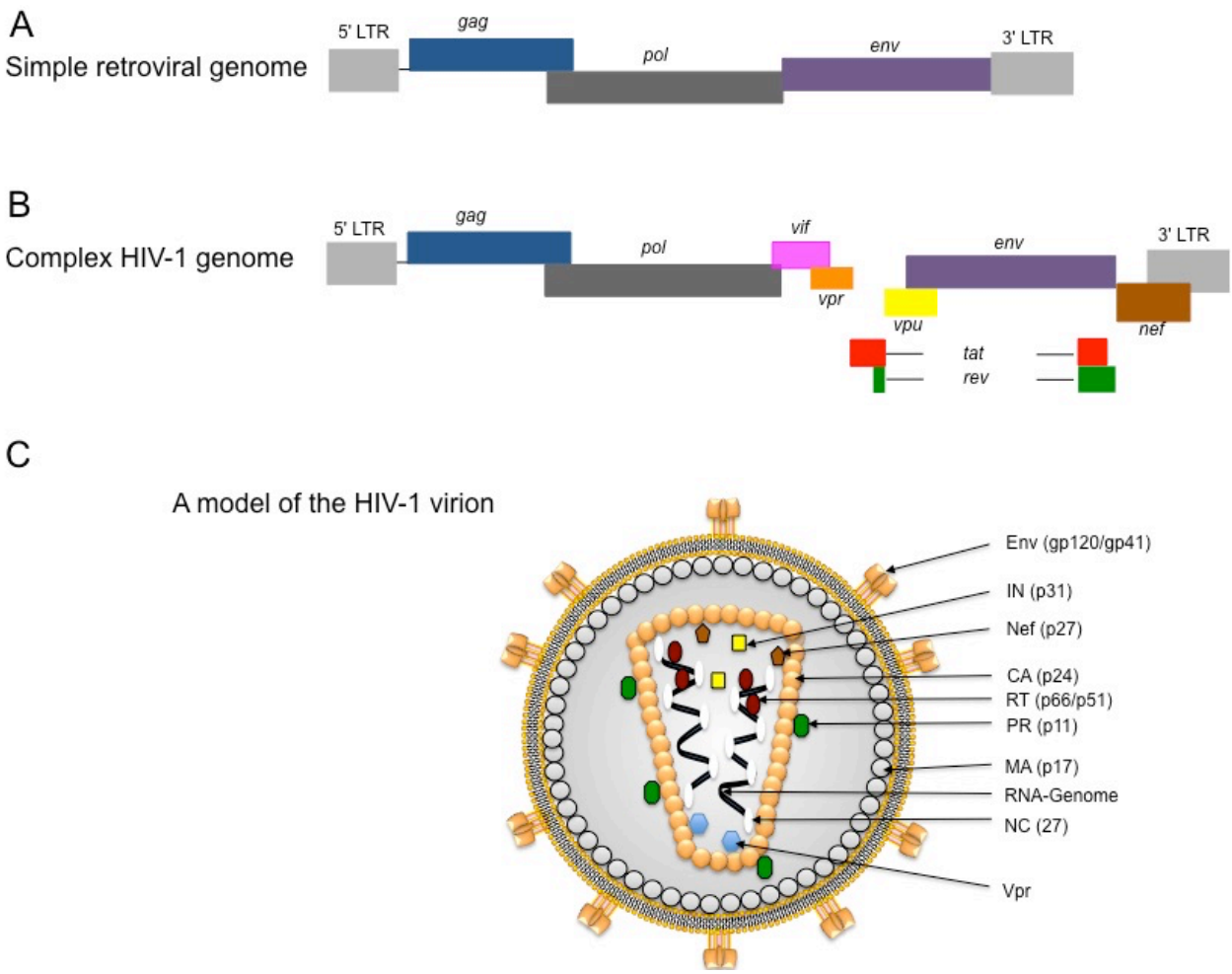

Figure 2. The retroviral genome and structure of the HIV-1 virion: (A) Schematic representation of a simple retroviral genome flanked by the 5' and 3' *LTR* regions (grey). The main structural genes characteristic of all retroviruses is depicted in blue (*gag*) charcoal (*pol*) and violet (*env*). The complex HIV-1 genome is illustrated in (B), flanked by 5' and 3' *LTR*s (grey). Apart from the structural genes common to all retroviruses (A), HIV-1 genome contains additional auxiliary genes, which are depicted in pink (*vif*), orange (*vpr*), yellow (*vpu*), brown (*nef*), red (*tat*) and green (*rev*). (C) A schematic representation of a matured structure of the HIV-1 virion. Figure was conceived and reconstructed with permission from (Kurth and Bannert, 2010).

1.4.3 Replication cycle of HIV

The host immune cells, such as CD4+ T cells, macrophages and dendritic cells expressing the cellular receptor CD4 (CD4+ cells) are the main targets of HIV infection (Telesnitsky, 2010, Melikyan, 2014, Swanstrom and Coffin, 2012, Kurth and Bannert, 2010). Replication of the virus occurs in several distinct stages, which are illustrated below (Fig. 3A).

1.4.3.1 HIV entry

HIV infection starts with the engagement of the viral envelope glycoprotein gp120 and the host membrane receptor CD4, which results in the formation of a bridging sheet, exposing a conserved co-receptor binding site on the glycoprotein. The interaction of the membrane-spanning co-receptor CC-chemokine receptor 5 (CCR5) or CXCR4 results in conformational changes that lead to the exposure of the fusion peptide in the TM (gp41). The fusion peptide is inserted in the target cell membrane, resulting in the formation of a helical hairpin structure where gp41 folds back on itself, drawing both membranes together for membrane fusion. The fusion of the viral and cellular membranes leads to the release of the viral capsid into the cytoplasm (Telesnitsky, 2010, Campbell and Hope, 2015, Kurth and Bannert, 2010).

1.4.3.2 HIV reverse transcription

The synthesis of the viral DNA from the viral genomic RNA is thought to begin in the capsid in the cytoplasm of the host cells following priming with intracellular dNTPs (Fig. 3B). This process may persist even when the replicative nucleoprotein complex enters the nucleus (Sumner et al., 2017, Telesnitsky, 2010, Kurth and Bannert, 2010). The reverse transcription process begins with synthesis of the minus-strand DNA by the viral reverse transcriptase. This process is primed by the incorporated partially unfolded host tRNA, which anneals to the complementary sequences in the primer-binding site (PBS) of the genomic RNA of a plus-strand polarity. This results in the synthesis of the minus-strand strong-stop DNA (-sssDNA) containing Repeat (R) and 5' un-translated (U5) sequences of about 100-150 bases, and subsequent RNase-H-mediated degradation of the viral RNA strand (R and U5 sequence). The viral –sssDNA-tRNA hybrid is transferred to the 3' end of the viral genome and anneals to the R sequence as RNA primer by a process called first strand transfer. Following minus-strand extension and viral RNA

degradation, a 3' polypurine tract (3'PPT), just upstream of the 3' LTR is used as an RNA primer to prime a plus-strand synthesis in all retroviruses. The resultant plus-strand strong-stop DNA becomes annealed to the 3' region of the extended minus-strand synthesis following RNA degradation and the second strand transfer. For HIV and foamy viruses, a second central polypurine tract (cPPT) is present in the integrase component of the *pol* ORF in the centre of the genome, which also primes a plus strand DNA synthesis (Fig. 3B). When the DNA strand initiated by 3'PPT meets the DNA strand, primed by the cPPT, a strand displacement occurs until the central termination sequence (CTS) is reached. Consequently, a single-stranded gap and a single-stranded DNA flap (central flap) of about 100 base pairs are formed with a copy of the long terminal repeat (*LTR*) at each end of the resultant viral cDNA (De Rijck and Debyser, 2006, Charneau et al., 1994, Shityakov et al., 2014) (Fig. 3B). Although it is not totally clear the biological relevance of the central flap in vivo, its impact on viral replication has been underscored in vitro (Shityakov et al., 2014, De Rijck and Debyser, 2006, Le Grice, 2012, Hungnes et al., 1992). The entire reverse transcription process is facilitated by two distinct enzymatic activities of the reverse transcriptase (RT), which include a DNA polymerase activity, which ensures appropriate dNTPs are incorporated into the RNA or DNA template and an RNase H activity that specifically degrades the RNA strand of the RNA: DNA hybrid (Telesnitsky, 2010, Telesnitsky and Goff, 1997, Hu and Hughes, 2012, Kurth and Bannert, 2010).

1.4.3.3 HIV integration

The capsid containing the synthesised reversed transcription complex in the cytoplasm migrates towards the nuclear pore in association with the microtubules and matures into the preintegration complex (PIC), where the capsid is believed to uncoat, releasing the PIC comprising of the viral DNA, integrase, capsid proteins and some cellular factors into the

nucleus of the target cell (Sumner et al., 2017, Telesnitsky, 2010, Telesnitsky and Goff, 1997, Lusic and Siliciano, 2017, Kurth and Bannert, 2010). Although the exact location of the capsid uncoating is not clear, there are models that suggest partial uncoating of the capsid in the cytoplasm before reaching the nucleus (Campbell and Hope, 2015). The PIC-associated integrase enters the nucleus and mediates the integration of the viral cDNA into the cellular genome. Indeed, several viral and cellular factors have been shown to influence the uncoating and entry of the PIC into the nucleus of the host cell (Lusic and Siliciano, 2017). The HIV capsid protein is considered as an important viral determinant for uncoating and PIC entry into the nucleus. Other viral and cellular proteins, including the MA, Vpr and cyclophilin A (CYPA) and RANBP2 respectively, play a role in the nuclear entry of the PIC on the cytosolic end of the nuclear pore complex (NPC) (Lusic and Siliciano, 2017). The host factor- transportin 3 (TNPO3), nucleoporin 153 (NUP153) and cleavage and poly-A specificity factor 6 (CPSF6) have been shown to mediate HIV import into the nucleus. The use of RANBP2, TNPO3 and NUP153 by HIV for entry into the nucleus is likely mediated by HIV interactions with CYPA and CPSF6 (Lusic and Siliciano, 2017). Upon entry into the nucleus through the NPC, the PIC reaches a complex and heterogenous environment that is said to provide anchoring sites for chromosomes (Lusic and Siliciano, 2017). From this periphery of the nucleus, the PIC-associated integrase is believed to identify a host chromosome target site, which may be of high gene density and transcriptional activity, likely mediated by the CPSF6, NUP153 and the lens epithelium-derived growth factor (LEDGF) (Lusic and Siliciano, 2017). The viral integrase then catalysis the integration of the provirus into the host genome by two independent reactions: first, the integrase multimerizes on both ends of the viral LTRs and excises two nucleotides from each of the 3' end of the linear viral DNA in a process called 3' processing. At the end of the 3' processing, a recessed and chemically reactive hydroxyl group is generated for insertion into the host chromosome.

Next, following entry of the PIC into the nucleus, the integrase binds to the host DNA and strand transfer occurs, resulting in a covalent linkage of the viral DNA into the target chromosome. After the disassembly of the strand transfer complex, the DNA recombination intermediate is repaired likely by host cellular enzymes, and the provirus, flanked by the host DNA, becomes an integrated part of the host cellular chromatin. (Telesnitsky, 2010, Lusic and Siliciano, 2017, Kurth and Bannert, 2010). It is thought that only a fraction of the viral cDNA is integrated into the host genome, leaving behind a large fraction of circular and full-length linear cDNA intermediates (van Montfoort et al., 2014).

1.4.3.4 HIV proviral transcription

In the absence of mitogenic stimuli, an integrated provirus is often transcriptional silent, under the control of epigenetic mechanisms, such as chromatin packaging via nucleosomes, which are formed by distinct histones. In activated target cells, basal transcription of the provirus is driven by the promoter and enhancer region of the U3 section of provirus' 5' LTR and mediated by the host cellular RNA polymerase II (RNAPII) (Telesnitsky, 2010, Kurth and Bannert, 2010). The proviral 5' LTR contains binding sites that recruit several host transcription factors such as NF-κB, NFAT and AP-1, which are induced in activated T cells or macrophages. NF-κB, for example, translocates to the nucleus, where it binds to the enhancer of the LTR and stimulates the initiation and elongation of HIV transcription (Telesnitsky, 2010, Kurth and Bannert, 2010). Although NF-κB is an important transcriptional factor and likely indispensable, it does not efficiently support expression of viral genes, hence the requirement for viral transcriptional transactivator protein Tat (Flint et al., 2004). HIV-Tat, Rev and Nef are among the early viral proteins synthesised from multiply spliced mRNA species of about 2-kb from the provirus transcription, mediated by the host cellular transcription factors. HIV-Tat is about 14kDa nuclear protein encoded by two exons. Once a sufficient amount of the Tat protein is synthesised, the

protein is imported to the nucleus, where it exhibits a positive feedback loop, mediating enhanced transcription of the proviral template upon binding to its RNA recognition site in nascent viral transcripts (Telesnitsky, 2010, Kurth and Bannert, 2010). Tat interacts with the proviral 5' LTR, and recruits several histone acetyltransferases (HATs), including the CREB-binding protein (CBP/p300) complex to the HIV-1 promoter, which acetylates the nucleosomes on the promoter, thereby relieving the 5' LTR transcriptional repression by the nucleosome (Romani et al, 2010). Further, HIV-Tat protein binds to an RNA stem-loop structure called transactivation response (TAR), located at the 5' end of nascent viral transcripts in a complex with the cyclin T subunit of pTEFb (positive-acting transcription factor b) and CDK9 (cyclin-dependent kinase 9). This complex leads to the phosphorylation of the C-terminal domain of RNAPII, promoting efficient transcriptional elongation and processing of full-length HIV-1 transcript by RNAPII (Telesnitsky, 2010, Kurth and Bannert, 2010).

The concerted effort of Tat, RNAPII and other viral and cellular factors results in the expression of many spliced pre-mRNAs, which are regulated by different splicing acceptors (SA) and donors (SD), situated in the viral genome (Asang et al. 2008; Stoltzfus and Madsen, 2006). The singly spliced mRNAs of about 4 kb in size encode for the structural and accessory proteins, including Env, Vif, Vpu and Vpu (Flint et al., 2004, Frankel and Young, 1998). Full-length mRNA encodes for Gag and Gag-Pol precursors and the genome of the virus. The Gag-Pol precursors are made by a mechanism of programmed ribosomal frameshifting during the Gag translation (Freed, 2015). Full-length and singly spliced HIV mRNAs are exported into the cytoplasm by a Rev-dependent cytoplasm-nucleus shuttling, mediated by an interaction between Rev and RRE (Rev-responsive element) (Flint et al., 2004, Fischer et al., 1995). The Rev protein possesses two signals, a nuclear localization signal (NLS) and a nuclear export signal (NES), which determines its localization (Pollard and Malim, 1998).

1.4.3.5 HIV assembly, release and maturation

The Gag precursor protein mediates the assembly and release of HIV (Fig. 3A). The uncleaved Gag protein contains the MA, CA, NC, p6 and two spacer peptides, denoted as SP1 and SP2, all with distinct functions for the forming virion. The MA domain is responsible for targeting Gag to the plasma membrane and promotes incorporation of the viral Env glycoproteins in the assembling virus, while the CA mediates Gag multimerization. The NC is responsible for recruiting the viral RNA genome into the assembling virion and mediates the assembly process, while the p6 domain recruits the endosomal sorting complex required for transport I (ESCRT-I) apparatus, which drives the membrane scission reaction required for release of the forming virion (Freed, 2015).

The HIV assembly begins with the recruitment of the full-length viral genomic mRNA by the NC domain of the Gag precursor protein. Gag begins to multimerize, a process, which is driven by CA domain, and then traffics through the cytosol to the plasma membrane by an unidentified pathway. The Gag precursor protein is attached to the plasma membrane in lipid raft microdomains through insertion of its amino-terminal myristate into the lipid bilayer and by direct interactions with phospholipid phosphatidylinositol-(4,5)-bisphosphate (Freed, 2015). HIV-Env glycoprotein is incorporated into the forming virion and then recruits ESCRT-I by a direct interaction between the PTAP motif in p6 and the tumour susceptibility gene 101 (TSG101) subunit of ESCRT-I. The YPXL motif in p6 of Gag also binds directly with the ESCRT-associated factor ALG2-interacting protein X (ALIX). As the process of budding progresses, other cellular factors, including the ESCRT-III and vacuolar protein sorting 4 (VPS4) complexes are recruited, which drive the membrane scission reaction needed for the release of the immature virion. Following release of the immature virion, the viral protease, encoded within the Gag-Pol polyprotein precursor, cleaves the Gag and the Gag-Pol polyprotein precursors,

triggering morphological transformations in the immature virion, leading to matured virion that is characterized with conical-shaped capsid (Fig. 3A) (Freed, 2015).

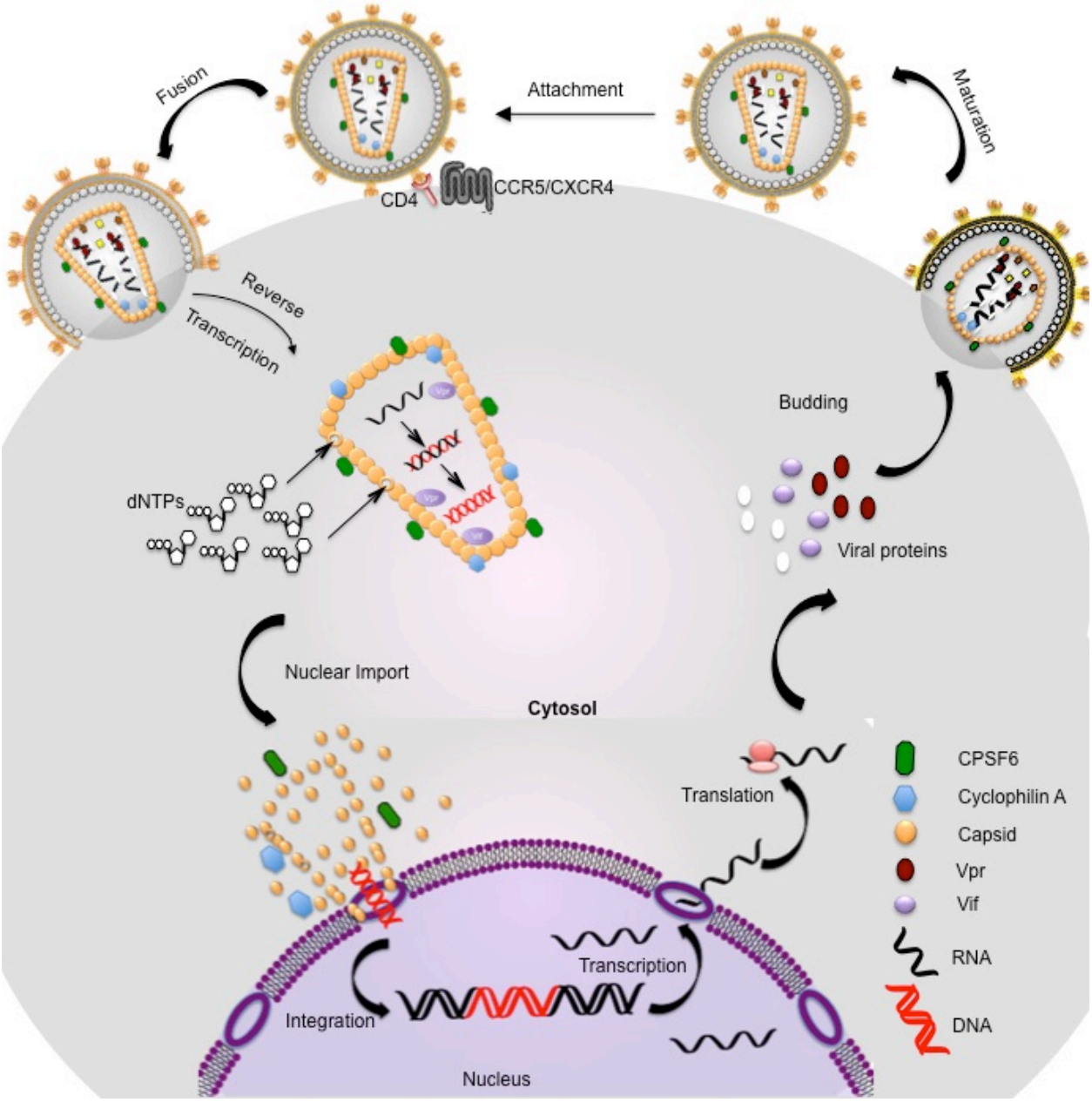

Figure 3A. A simplified replication cycle of HIV-1: (A) HIV-1 by its envelope glycoproteins gp120/gp41 trimers engages the cell surface receptor CD4 and co-receptor (CCR5 or CXCR4) for fusion and entry into its target cell. Upon fusion, the HIV-1 capsid is released into the cytosol of the cell and traverses through the cytoplasm to the nucleus via cellular microtubules, while the viral genome undergoes reverse transcription (Fig 3B), primed by nucleotides transport into the capsid cone. A highly organized capsid uncoating likely occur at the cytoplasm, nuclear pore complex, or in the nucleus, releasing the reversed transcribed DNA. The viral DNA integrates into the host genome, mediated by viral integrase and the provirus undergoes transcription and translation,

producing viral proteins. Newly synthesised viral proteins assemble at the plasma membrane, primed by the viral CA protein and immature virions bud off and are released, mediated by endosomal sorting complex required for transport (ESCRT) apparatus. The viral protease cleaves the structural polyproteins to form mature Gag proteins, generating new infectious virions. Figure was conceived and reconstructed with permission from (Sumner et al., 2017).

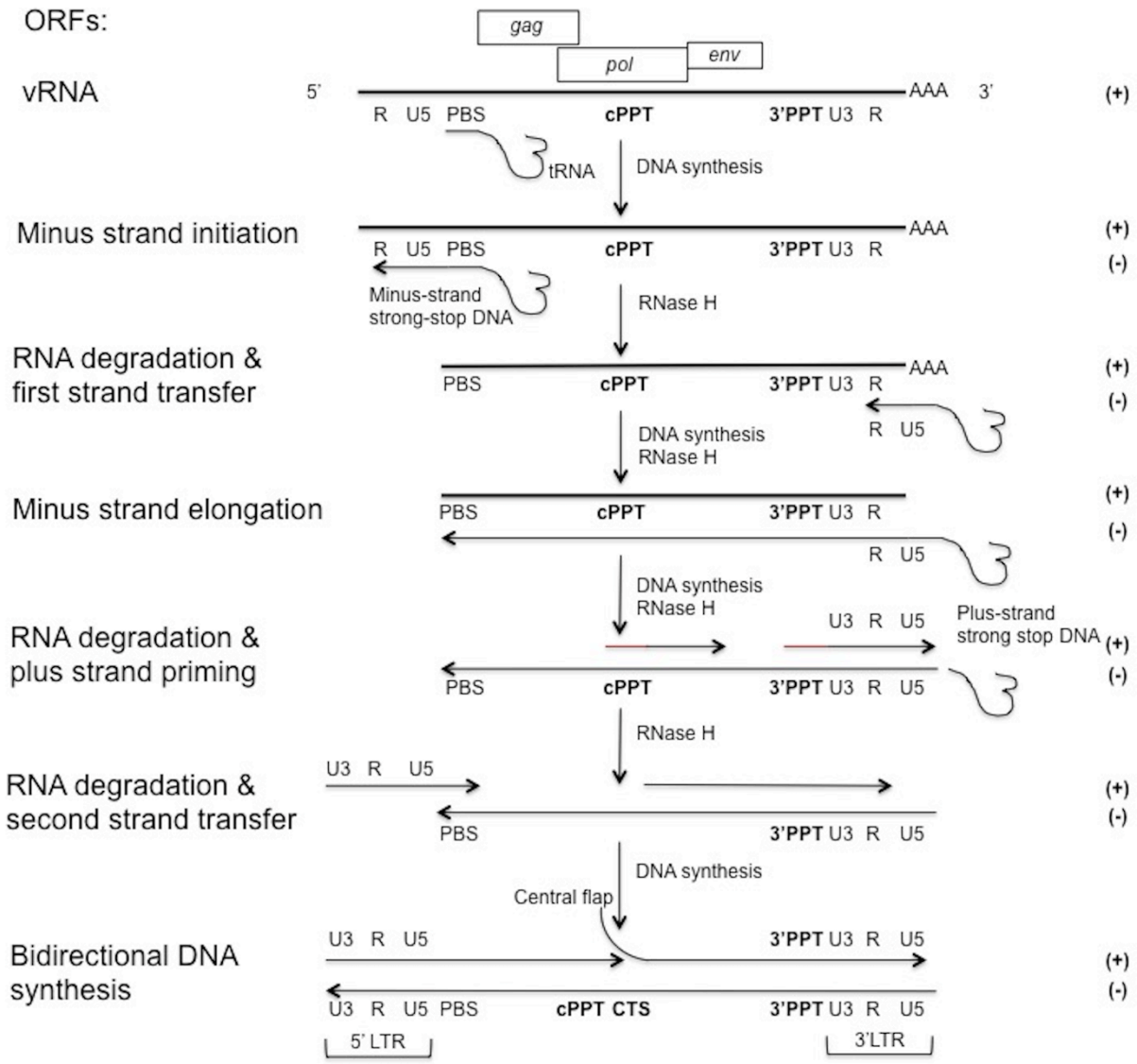

Figure 3B. HIV-1 reverse transcription: Illustrated above the viral RNA genome (vRNA) are open reading frames (ORFs) of the major structural genes common to all retroviruses including HIV-1. The reverse transcription process of HIV begins with synthesis of the minus-strand DNA by the viral reverse transcriptase. This process is primed by the incorporated partially unfolded host transfer RNA (tRNA), which anneals to the complementary sequences in the primer-binding site (PBS) of the genomic RNA of a plus-strand polarity. This results in the synthesis of the minus-strand strong-stop DNA (-sssDNA) containing Repeat (R) and 5' untranslated (U5) sequences of about 100-150 bases, and subsequent RNase-H-mediated degradation of the viral RNA strand (R and U5 sequence). The viral –sssDNA-tRNA hybrid is transferred to the 3' end of the viral genome and anneals to the R sequence as RNA primer by a process called first strand transfer. Following minus-strand extension and viral RNA degradation, cis-acting polypurine tracts- 3'PPT and cPPT, the former located upstream of the 3' LTR and the later in the integrase component of the *pol* gene, prime the synthesis of the plus-strand simultaneously. Following a plus-strand transfer of the 3'PPT-mediated plus strand strong stop DNA, DNA synthesis proceeds, displacing approximately 100 nucleotides of the cPPT-primed plus-strand DNA, but terminates abruptly at the central termination sequence (CTS), generating a single stranded gap and a single-stranded DNA flap denoted as a central flap,

with a copy of the long terminal repeat (LTR) at each end. Figure was conceived and reconstructed with permission from (Kurth and Bannert, 2010).

1.5 HIV Pathogenesis

An HIV virion, infecting a single CD4+ cells in or near the portal of entry is enough to establish a disseminated infection (Mogensen et al., 2010, Coffin and Swanstrom, 2013, Swanstrom and Coffin, 2012). The subsequent course of the infection is characterized by high viremia and drastic decline in CD4+ T cells and development of AIDS (Acquired Immunodeficiency syndrome), which is characterized by excessive immunodeficiency, susceptibility to deadly opportunistic infections and other symptoms, including dementia and wasting (Coffin and Swanstrom, 2013, Mogensen et al., 2010) (Fig. 4). A time course for HIV-1 infection can be categorized into four main phases; an eclipse phase, which lasts between one to two weeks and it is characterized by high replication of HIV and spread to proximal tissues and organs. An important feature of this phase is that no clinical symptoms are observed and neither viremia nor immune responses are detectable (Coffin and Swanstrom, 2013). CD4- and CCR5-expressing resident memory T cells in the mucosa are thought to be the first cells infected in the early infection and spread of the virus (Mogensen et al., 2010, Swanstrom and Coffin, 2012). Macrophages and dendritic cells are further potential targets of early HIV infection and spread, considering that these cells are also resident in the gut-associated lymphoid tissue (GALT) and express CD4 and CCR5 co-receptors. Although dendritic cells may not support productive infection, their ability to capture virus via their C-type lectin receptor (CLR) DC-SIGN and trans-infect CD4+ T cells in the lymphoid tissues implicates them in the spread of early HIV infection (Mogensen et al., 2010, Swanstrom and Coffin, 2012). A large population of the activated CD4+CCR5+ effector memory T cells of the GALT are thought to be preferentially infected and depleted during early HIV infection and spread, a phenomenon which is likely mediated by the direct killing by the virus or indirectly by Fas-mediated apoptosis or by pyroptosis (Swanstrom

and Coffin, 2012, Coffin and Swanstrom, 2013, Monroe et al., 2014, Doitsh et al., 2014). As the infection progresses with the onset of immunodeficiency, the virus evolves to use CXCR4 as a coreceptor, and to infect new cell types (Swanstrom and Coffin, 2012, Coffin and Swanstrom, 2013).

Two to four weeks following established infection, overt symptoms may begin to appear. These symptoms may show in a form of fever, flu-like symptoms and enlarged lymph nodes, characterizing an acute or primary infection. Further, relatively high levels of viremia with up to about 10^7 copies of viral RNA per millilitres (mL) of blood, and an expanding population of infected CD4+ T cells in the blood and in the lymph nodes can be measured. The high viremia, which characterizes this phase, is suggested to be due to lack of early immune response and expanding numbers of activated CD4+ T cells, which are further targets for HIV infection (Coffin and Swanstrom, 2013). At the point of peak viremia, the immune defences begin to appear in the form of antibodies against viral proteins and an influx of cytotoxic CD8+ T cells responses against the HIV-1 antigens, which may be expressed on infected cells. The influx of the cytotoxic CD8+ T cells and the appearance of humoral responses likely results in a drastic drop in viremia by a magnitude of about 100-fold or more, as well as a transient decline in CD4+ T cells in the blood, a characteristic feature ending the acute phase (Coffin and Swanstrom, 2013, Mogensen et al., 2010). Thus, acute HIV infection further leads to a selective and dramatic depletion of CD4+CCR5+ memory T cells from the GALT. This phenomenon could also be attributed to the CD8+ T cell mediated killing of infected CD4+ T cells or exhaustion of the activated CD4+ T cells and HIV-induced bystander CD4+ T cell death by pyroptosis (Altfeld and Gale, 2015, Doitsh et al., 2014, Monroe et al., 2014).

If the course of the infection is not interrupted by antiretroviral drugs, the acute phase proceeds to a typical chronic or clinical latency phase, which is characterized by slow but steady increasing levels of viremia in the order of about 1-100,000 RNA copies/mL referred to as “set

point" and a steady reconstitution of the CD4+ T cells count or gradual decline in the number of CD4+ T cells (Coffin and Swanstrom, 2013, Mogensen et al., 2010). Although the amount of the circulating CD4+ T cells in the blood appear to return to normal, CD4+ T cell numbers in the GALT remain severely reduced. This loss is thought to be largely irreversible with profound immunological consequences (Mogensen et al., 2010). Viral replication in the chronic phase still proceeds but at a slower level, infecting large numbers of CD4+ T cells. Patients in this phase may exhibit no symptoms (Mogensen et al., 2010, Coffin and Swanstrom, 2013). Another important feature of the chronic phase is a massive immune activation and an accelerated lymphocyte cell turnover, characterized by increased expression of CD38, HLA-DR and Ki67 with the CD38 serving as an important prognosis for disease progression (Hazenberg et al., 2003, Mogensen et al., 2010). Immune activation of the host cells is attributed to several factors including the ongoing viral replication and the influx of microbial translocation into the blood stream due to the breakdown of the mucosal barrier as a result of the profound immunological damage to the GALT (Klatt et al., 2013, Mogensen et al., 2010, Brenchley et al., 2006).

The end stage of the HIV infection is characterized by steady decline of the CD4+ T cells to a level (approximately 200 cells/μl) that can no longer maintain the control of opportunistic infections. Here, the immune control of viral replication is completely lost with increased viremia. A combination of factors including the effect of the viral replication, declining CD4+ T cell count, immune activation and perhaps other unidentified factors culminate in the development of AIDS, and subsequent death between two to ten years, if the patient is left untreated (Mogensen et al., 2010, Coffin and Swanstrom, 2013) (Fig. 4).

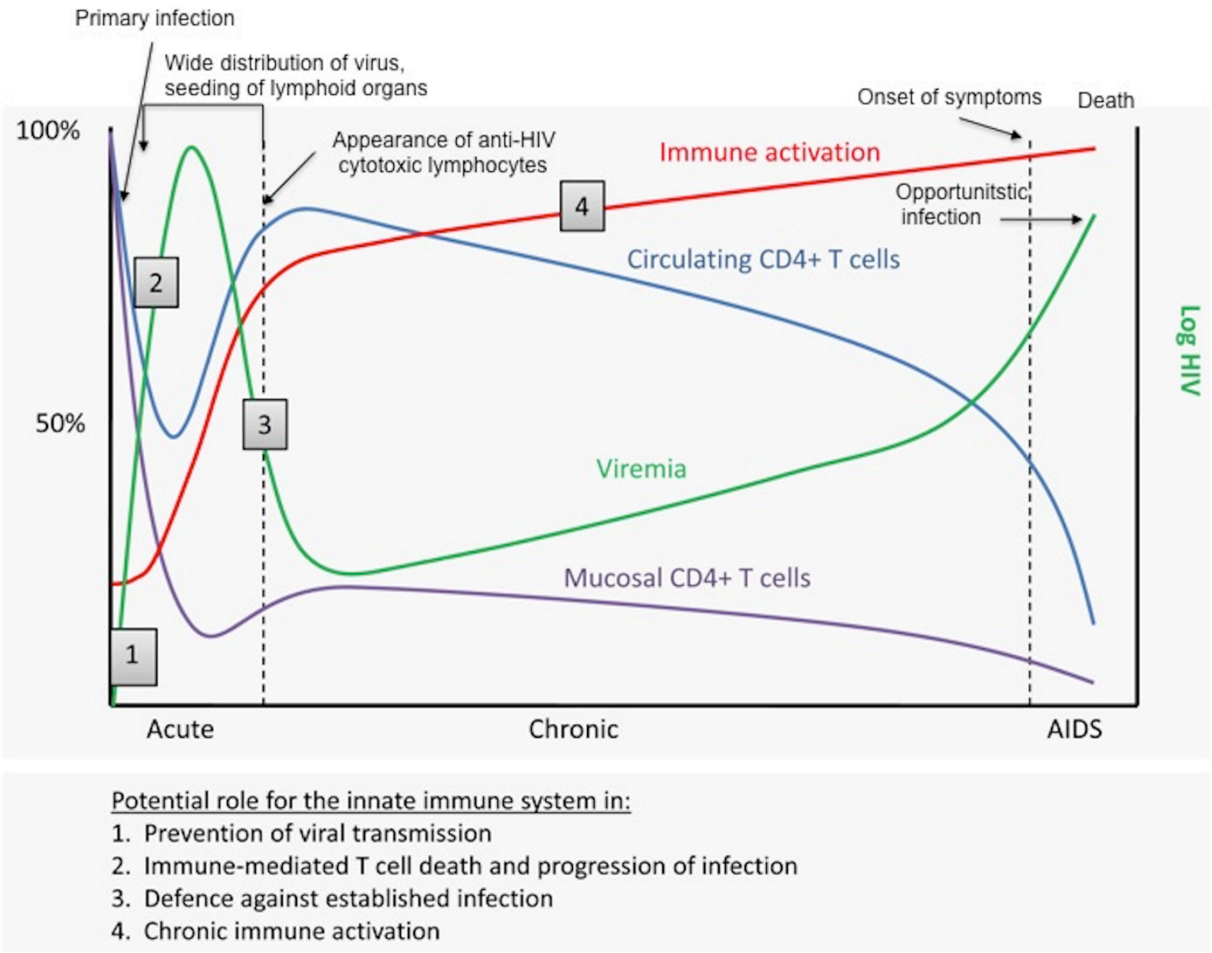

Figure 4. Potential roles of the innate immune system during HIV infection. (1) Following exposure at mucosal surfaces, HIV is transmitted with very low transmission efficiency, indicating that innate antiviral mechanisms are operative to prevent establishment of infection. (2) The early inflammatory response leads to recruitment and activation of various leukocytes, some of which serve as target cells for *de novo* HIV infection. (3) After acute infection, circulating viral load is generally decreased to a low level. This is mediated by the adaptive immune response, which is activated through processes driven by the innate immune response. Moreover, direct innate antiviral mechanisms contribute to control of virus replication during the chronic phase. (4) Persistent immune activation during chronic HIV infection involves activities stimulated by HIV-derived or opportunistic PAMPs through PRRs. Figure was adapted from (Mogensen et al., 2010) and modified with permission from the BioMed Central Ltd.

1.6 Innate immune response to HIV

The structure and distinct stages of the HIV replication cycle provide potential PAMPs that can be recognized and sensed by PRRs of infected cells. The PAMP-PRR interaction triggers a

signalling cascade in the infected cell that initiates innate intracellular antiviral effectors, which are aimed at restricting the replication and spread of the virus. These cell-intrinsic effectors propagate externally by the action of secreted factors such as chemokines and type I interferons (IFNs) (Altfeld and Gale, 2015, Mogensen et al., 2010, Jakobsen et al., 2015, van Montfoort et al., 2014, Sumner et al., 2017, Doyle et al., 2015) (Fig. 5). These secreted factors induce an antiviral state through upregulation of a plethora of interferon stimulated genes (ISGs), activate innate immune cells and attract them to the site of the infection and the peripheral lymphoid tissues (Altfeld and Gale, 2015, Schoggins and Rice, 2011, Schneider et al., 2014, Lenschow et al., 2007, Okumura et al., 2006). These antiviral innate immune cells can further contribute to the control of the viremia and modulate the quality of the adaptive immune response to the infection (Hertoghs et al., 2017, Gummuluru et al., 2014, Akiyama et al., 2015, Iwasaki and Medzhitov, 2015, Manel et al., 2010, Landau, 2014, Borrow and Bhardwaj, 2008). The concerted efforts of the PRR signalling, specific viral restriction factors, innate immune cells, innate-adaptive immune crosstalk and the viral evasion mechanisms, determine the outcome of the HIV-1 infection and immune responses and immunopathology (Altfeld and Gale, 2015).

Host immune response to HIV infection likely begins with the recognition and sensing of an extracellular virion (e.g., viral envelope) or viral molecules (e.g., reverse transcription product) inside uninfected or infected cells (e.g., gastric epithelial cells, T cells, macrophages, dendritic cells) at the portal of entry. The ensuing host-virus interaction mediates immune activation and determines the outcomes of immune responses, the control of the virus, chronic immune inflammation and immune pathology such as the death of the target cell. CD4+ T cells of the mucosal epithelium potentially represent the initial target of an infectious virion (Coffin and Swanstrom, 2013, Swanstrom and Coffin, 2012, Borrow and Bhardwaj, 2008, Mogensen et al., 2010, Miller et al., 2005, Zhang et al., 2004). These cells have the ability to recognize viral reverse transcription (RT) intermediates by their cytosolic PRR such as cGAS (cyclic GMP-AMP

synthase) and IFI16 (interferon inducible protein 16). In HIV-permissive activated CD4+ T cells, HIV-RT product sensing by cGAS induces the production of type I IFNs (Lahaye and Manel, 2015, Sumner et al., 2017, Vermeire et al., 2016, Xu et al., 2016), whereas in HIV-nonpermissive resting CD4+ T cells, abortive RT products likely lead to IFI16-mediated activation of the inflammasome pathway, leading to production of IL-1β and initiation of pyroptosis (Altfeld and Gale, 2015, Doitsh et al., 2014, Monroe et al., 2014). The proinflammatory cytokines and type I IFN released by the HIV-1-infected CD4+ T cells likely recruit other innate CD4+ target cells, including conventional (cDCs) and plasmacytoid dendritic cells (pDCs), monocytes and macrophages to site of infection (Altfeld and Gale, 2015, Hertoghs et al., 2017, Borrow and Bhardwaj, 2008, Borrow, 2011). Similarly, the HIV-1 envelope glycoprotein gp120 is a robust PAMP for the TLR-2 and TLR-4 on the surface of mucosal epithelial cells (Altfeld and Gale, 2015, van Montfoort et al., 2014). Even though mucosal epithelial cells may not be targets of HIV-1 infection, their recognition of HIV infection, induced by their PRR-gp120 interaction likely results in signalling in epithelial cells, triggering proinflammatory cytokines and chemokines production, which activates the innate immune cells and subsequently activates adaptive immune cells to the site of virus encounter (Altfeld and Gale, 2015, Guo et al., 2018). pDCs are able to take up HIV-1 by envelope protein interaction with CD4 and/or DC-SIGN without leading to productive infection (Swanstrom and Coffin, 2012, Borrow and Bhardwaj, 2008, Borrow, 2011) (Bhardwaj et al. 2012). Nevertheless, the engagement of pDCs CD4 with HIV-1 envelope, HIV-1 endocytosis and endosomal acidification is enough to trigger type I IFN production, likely mediated by its TLR-7 or -9 sensing of viral ssRNA in the endosomes of the pDCs, without the requirement for viral fusion or viral replication (Iwasaki and Medzhitov, 2015, Iwasaki and Medzhitov, 2010, Manel et al., 2010, Manel and Littman, 2011, Silvin and Manel, 2015, Lahaye and Manel, 2015, Sumner et al., 2017). Monocyte-derived dendritic cells (MDDCs), like pDCs, by their HIV-1 receptor expression, are also likely activated and recruited to the site of infection and are potentially infected but

inefficiently. They likely recognize and sense the ssRNA and dsDNA of the virus by endosomal TLR-7, 8 and cytosolic IFI16 and cGAS (likely aided by the nuclear protein NONO, when cGAS is in the nucleus) respectively (Sumner et al., 2017, Altfeld and Gale, 2015, Manel et al., 2010, Manel and Littman, 2011, Silvin and Manel, 2015, Lahaye and Manel, 2015, Landau, 2014, Guo et al., 2018, Lahaye et al., 2018, Gao et al., 2013). Thus, MDDCs likely augment the early response to HIV. However, their ability to migrate to the draining lymph nodes and to present antigens by their antigen bound MHC class II receptors enable them to potentially transmit the virus to activated CD4+ target cells (Borrow and Bhardwaj, 2008, Borrow, 2011). Long-lived macrophages support HIV-1 replication but at low levels. These cells are potentially recruited to the site of infection. These cells, by their TLR7/8 expression, recognize and sense ssRNA genome of HIV in the endosome, thereby triggering innate immune signalling and activation (Hofmann et al., 2016, Schlaepfer et al., 2006, Heil et al., 2004, Meier et al., 2008, Guo et al., 2018). Further, their expression of cytosolic cGAS, IFI16 and DDX3 (Dead-box helicase 3) enables them to recognize and sense the viral RT intermediates to trigger innate immune signalling, inducing a robust type I IFN induction (Sumner et al., 2017, Manel et al., 2010, Manel and Littman, 2011, Silvin and Manel, 2015, Lahaye and Manel, 2015). Other cellular PRR such as TLR-3, expressed on MDM (monocyte-derived macrophages) and MDDCs have been suggested to recognize dsRNA and engage the adapter TRIF (TIR-domain-containing adapter protein inducing IFNβ), allowing it to activate both NF-κB and interferon regulatory factor 3 (IRF3). Members of the RIG-1-like receptors (RLR), including RIG-1 and MDA-5 have also been suggested to sense the genomic RNA of HIV-1 (Loo and Gale, 2011, Kawai and Akira, 2011, Altfeld and Gale, 2015). RLRs signal through MAVS to activate the IKK and tank binding kinase 1 (TBK1) complexes to activate NF-κB and IRF3 and IRF7 (Loo and Gale, 2011). The NOD-like receptor family and the DNA-sensing associated protein AIM2 have all been implicated as PRR for innate immune sensing of PAMPs in MDMs and MDDCs (Sumner et al., 2017, Altfeld and Gale, 2015, Barber, 2011, Elinav et al., 2011).

In the cGAS / STING pathway, the hallmark of the HIV-PAMP and host-PRR interaction is an induction of a signalling cascade that is characterized by the production of a dinucleotide product, cyclic 2' 3' GMP-AMP (cGAMP) by cGAS, which functions as a second messenger to bind to STING to activate TBK1 and IKK-α/β and downstream IRF3, interferon regulatory factor 7 (IRF7) and NF-κB transcription factors. IFI16 mediates direct STING activation or signals to STING through an intermediate to drive pro-IL-1β, pro-inflammatory cytokines, chemokines and type I interferon induction. IFN signals back in infected cells and bystander cells through binding to the IFN-α/β receptor to drive further signalling through JAK-STAT pathway and activate ISGF3, the transcription factor complex STAT1-STAT2 and IRF9 to induce expression of a plethora of ISGs including anti-HIV-1 ISGs (Sumner et al., 2017, Altfeld and Gale, 2015, Doyle et al., 2015) (Fig. 5).

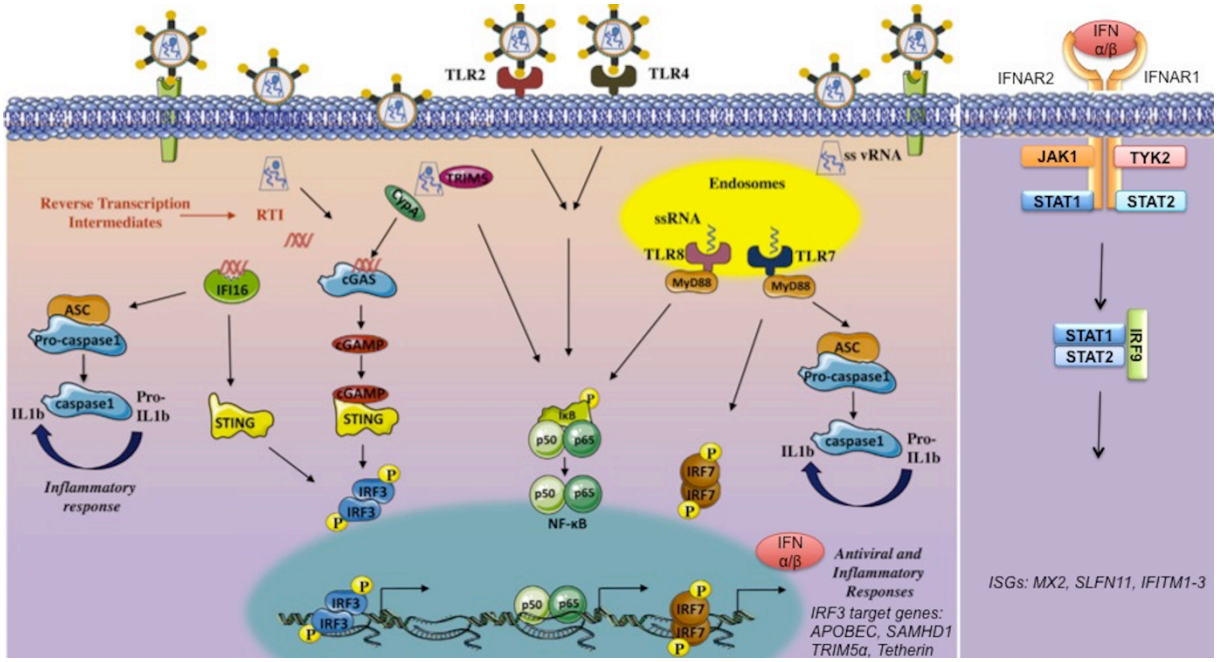

Figure 5. Sensing and innate antiviral response to HIV-1: During HIV-1 infection and replication, the host cell is exposed to viral envelope antigens, genomic ssRNA, reverse transcription intermediates (RTI), viral capsid and integrated provirus. Recognition of envelope antigen via TLR2 or TLR4 leads to NF-κB-mediated inflammatory responses. Recognition of genomic ssRNA via endosomal TLR7 or TLR8 induces NF-kB-dependent inflammatory responses, IRF7-dependent interferon responses, or activation of the ASC inflammasome, depending on the cell type. Recognition of ssRNA by RIG-I induces IRF3- dependent interferon responses. Recognition of HIV-1 capsid is mediated via Cyclophilin A and TRIM5α, resulting in IRF3-STING dependent interferon responses and NF-κB-dependent inflammatory responses. Recognition of dsDNA RTI by cGAS, IFI16 or another yet unknown sensor induces interferon response via the STING-IRF3 axis. Sensing by IFI16 can also result in activation of the ASC

inflammasome, resulting in release of IL-1b. (Figure was adapted from (van Montfoort et al., 2014) and modified with permission from the Elsevier publishing group).

1.7 ISGs as Restriction factors

Among the ISGs are restriction factors that mediate the innate immune control of the viral infection and spread. They function at almost every stage of the viral life cycle and the virus employs a combination of evasion and antagonistic strategies to achieve infection and replication.

1.7.1 TRIM5α

TRIM5α, a member of the large TRIpartite Motif (TRIM) family of proteins is upregulated by type I and II IFNs and exhibits a direct antiviral and antimicrobial activity (Sumner et al., 2017). It functions by targeting incoming retroviral capsid, soon after the virus enters the host cell and blocks infection before integration into the host genome. TRIM5α represents an important barrier to zoonotic retroviral transmission (Sumner et al., 2017, Doyle et al., 2015, Der et al., 1998, Schoggins and Rice, 2011, Schneider et al., 2014, Hertoghs et al., 2017, Jakobsen et al., 2015, van Montfoort et al., 2014, Harris et al., 2012). Until recently, it was thought that HIV-1 is poorly restricted by the human TRIM5α, likely due to the poor binding affinity of the human TRIM5α PRYSPRY domain to the HIV-1 capsid (Sumner et al., 2017). However, recent work by the Geijtenbeek lab demonstrates that HIV-1 is potently restricted by the human TRIM5α but in a cell type and viral entry pathway-specific manner (Sumner et al., 2017). TRIM5α from the rhesus monkey considerably inhibits HIV-1 likely due to its strong PRYSRY interaction with the HIV-1 capsid (Sumner et al., 2017).

TRIM5α forms complexes on the incoming viral core and activates its RING domain E3 ubiquitin ligase activity for the proteasomal degradation of the capsid, thereby enhancing uncoating and

blocking reverse transcription of the virus (Sumner et al., 2017). By interacting with viral capsid, TRIM5α may serve also as a capsid-PRR and mediate activation of the transcription factors NF-κB, AP-1, resulting in pro-inflammatory cytokine, inducing an antiviral state and modulation of adaptive immunity (Sumner et al., 2017, Harris et al., 2012, Iwasaki, 2012, Acchioni et al., 2015, Loo and Gale, 2007, Mogensen et al., 2010, Jakobsen et al., 2015, van Montfoort et al., 2014).

1.7.2 SAMHD1

SAMHD1 (sterile alpha motif and histidine-aspartate domain containing protein 1) is a well-characterized restriction factor that blocks retroviral replication in myeloid and resting CD4+ T cells. It is targeted by the SIV/HIV-2 accessory protein Vpx for polyubiquitination and proteasomal degradation by recruiting the host cell cullin-4 ligase substrate receptor DDB1- and CUL4-associated factor 1, DCAF1 (Laguette et al., 2011, Baldauf et al., 2012, Lahouassa et al., 2012, Hrecka et al., 2011, Descours et al., 2012, Simon et al., 2015). Unlike HIV-2, which has Vpx to counteract SAMHD1, HIV-1 possesses no accessory protein for SAMHD1 degradation. Why HIV-1 lacks an accessory protein to overcome SAMHD1 restriction function *in vivo* is currently not clear. SAMHD1 is a dNTPase that is thought to restrict HIV infection by lowering the dNTP concentrations below the level required to support viral DNA synthesis (Powell et al., 2011, Goldstone et al., 2011, St Gelais et al., 2012). SAMHD1 is expressed widely in diverse human tissues but it appears to only restrict HIV infection in non-dividing (cycling) cells. Cycling cells have high intracellular nucleotide levels that likely exceed the threshold that is depleted by SAMHD1 (Sumner et al., 2017). IFN induction and the expression of many restriction factors alone are sufficient to block the replication of HIV. However, the expression of SAMHD1 alone appears to be insufficient to confer resistance against HIV-1 in some cell types (Wittmann et al., 2015, Cribier et al., 2013, Sumner et al., 2017). In cycling cells, SAMHD1 is non-active against HIV replication likely because of its post-translational regulation by phosphorylation in a cell

cycle dependent manner. In cycling cells, SAMHD1 is inactivated by phosphorylation by the cyclin-dependent kinases (CDKs) at the C-terminal residue T592 (Cribier et al., 2013, White et al., 2013). This phenomenon has been attributed to its unstable tetramer structure and increased dissociation to catalytically inactive monomers and dimers (Arnold et al., 2015, Yan et al., 2013). In the primary MDM, phosphorylation of T592 of SAMHD1 and SAMHD1's antiviral activity appears to be very dynamic (Mlcochova et al., 2017). Macrophages have been proposed to exist in two states through which all of the cells periodically cycle, characterized by a typical G0 state with a lack of the cell cycle marker MCM2 (minichromosome maintenance complex 2), which have active and dephosphorylated SAMHD1 as well as resistance to HIV. The second state is described as G1-like, permissive to HIV-1 and characterized by expression of MCM2 and inactive phosphorylated SAMHD1 (Mlcochova et al., 2017, Sumner et al., 2017).

1.7.3 p21 and its transcription factor p53

SAMHD1 antiviral activity is further regulated by the cyclin dependent kinase inhibitor p21, which is involved in monocyte differentiation and maturation (Asada et al., 1999, Kramer et al., 2002, Xaus et al., 1999, Liu et al., 1996, Lloberas and Celada, 2009). p21 regulates SAMHD1 phosphorylation and antiviral function by overcoming the CDK activity in a cell cycle dependent manner (Cribier et al., 2013, Allouch et al., 2013, Pauls et al., 2014a, Pauls et al., 2014b, Abbas and Dutta, 2009, El-Deiry, 2016, Georgakilas et al., 2017). p21 is IFN inducible, and IFN-mediated induction results in cell cycle arrest (Hobeika et al., 1999, Xaus et al., 1999, Mandal et al., 1998, Katayama et al., 2007, Cribier et al., 2013). Host cellular factors that are important for the proper progression of the cell cycle are under the regulation of p21. E2F1, a transcription factor for RNR2 {(same as RRM2), a key enzyme involved in *de novo* biosynthesis pathway}, is tightly regulated by p21 (Allouch et al., 2013, Abbas and Dutta, 2009). By repressing E2F1 transactivation activity, p21 inactivates RNR2, thereby limiting the intracellular dNTP pool, which is required for viral and cellular DNA synthesis (Allouch et al., 2013, Valle-Casuso et al., 2017, Pauls et al., 2014b, Badia et al., 2016, Elahi et al., 2016).

The transcription, expression and activity of p21 are regulated in p53-dependent and independent pathways. Under non-stressed conditions, p21 transcripts are expressed at low levels and promote cell cycle progression. In the event of DNA damage or under other stressed conditions, p21 is highly upregulated mediated by p53 and likely other pathways (Jung et al., 2010, Abbas and Dutta, 2009, Georgakilas et al., 2017, El-Deiry, 2016). Upregulation of p21 mediates the arrest of the cell, DNA repair, senescence, aging and death by apoptosis depending upon the cellular context (Georgakilas et al., 2017, Abbas and Dutta, 2009, Jung et al., 2010, El-Deiry, 2016). Post-translationally, p21 expression is further regulated by ubiquitination and phosphorylation (El-Deiry, 2016, Abbas and Dutta, 2009). p21 is recognized by diverse E3 ubiquitin ligase complexes, such as SKP2 (S-phase associated protein 2), $CUL4^{CDT2}$ {CUL4A or CUL4B-DDB1-CDT2 (DDB1 is DNA damage-binding protein 1)} and APC/CCDC20 (anaphase-promoting complex (APC)-cell division cycle 20), which promote p21 proteolysis via the proteasome at distinct stages of the cell cycle under non-stressed conditions. These E3 ligase complex recognition factors promote ubiquitination and degradation of p21 only when p21 is complexed with cyclin E/CDK2 or cyclin A/CDK2, PCNA (proliferation cell nuclear antigen), or cyclin A/CDK1 or cyclin B/CDK1 (El-Deiry, 2016, Abbas and Dutta, 2009). In the absence of these complexes, p21 is degraded independent of ubiquitin in a cell-type dependent manner by interaction of its C-terminus with the C8α subunit of the 20S proteasome (Abbas and Dutta, 2009).

p53 is HIV-1 and IFN-inducible (Daniel et al., 2001, Cooper et al., 2013, Genini et al., 2001, Munoz-Fontela et al., 2016), and its expression and activity are also regulated by several post-translational modifications including ubiquitination, acetylation, phosphorylation and ISGylation, all of which likely impact p21 induction and function (Jung et al., 2010, Park et al., 2016, Huang et al., 2014, Menendez and Anderson, 2014). Inactivation of p53 by single point mutations, deletions and rearrangements in the p53 gene, a hallmark of many cancers, potentially impacts

p21 expression and affects the infection and replication of HIV (Kastenhuber and Lowe, 2017, Silva et al., 2014, Muller and Vousden, 2014, Ano Bom et al., 2012, Vikhanskaya et al., 2007, Rangel et al., 2014, Gannon et al., 1990, Bukholm et al., 1997, Kumari et al., 2014, Rangel et al., 2019, Joerger and Fersht, 2016, Vieler and Sanyal, 2018, Brady and Attardi, 2010, Ghosh et al., 2017, Unger et al., 1993, Sugimoto et al., 1992, Ishimaru et al., 2003, Lane, 1992, Candeias et al., 2016). Indeed, the absence of p53 decreases the expression of p21 and correlates significantly with the enhancement of HIV-1 infection and replication at the reverse transcription step (Shi et al., 2018, Kinnetz et al., 2017).

1.7.4 APOBEC3

Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 proteins (APOBEC3s or A3s) are members of the family of single-stranded DNA deaminases. The A3s are robustly induced by IFN, but some family members are constitutively expressed. The A3s restrict HIV primarily by repressing viral DNA synthesis via induction of mutations in the viral DNA resulting in replication incompetent proviruses (Sumner et al., 2017). Seven A3 enzymes have been identified in humans. These include, A3A, A3B, A3C, A3D, A3F, A3G and A3H, with A3G, being the most well characterized A3, and expressed in both CD4+ T cells and MDM. All of the A3s are active against HIV-1 except A3C, and A3A and A3B are not expressed in T cells (Refsland et al., 2010, Mariani et al., 2003, Koning et al., 2009). The A3s form important barriers against zoonotic retroviral transmissions. A3G and A3F antiviral activity is suppressed by the HIV-1 accessory protein Vif. Vif binds and mediate A3G and A3F degradation by the proteasome. This interaction is specific to human A3s, as Vif fails to bind to A3s of non-human primates and cannot mediate their degradation (Mariani et al., 2003, Schrofelbauer et al., 2004). To block HIV infection, A3G protein is incorporated into the viral particles through its interaction with cellular or viral RNAs bound to the nucleocapsid domain of the Gag polyprotein. In the absence of the

Vif, A3G suppresses DNA synthesis and catalyses the deamination of cytosine to form uracil in the minus-strand of the reverse-transcribed single-stranded DNA, resulting in G to A hypermutations in the plus strand of the viral DNA. The hypermutated proviral DNA that results is defective and unable to produce infectious progeny (Sumner et al., 2017, Harris et al., 2012, Jakobsen et al., 2015, van Montfoort et al., 2014, Simon et al., 2015, Sheehy et al., 2003, Sheehy et al., 2002, Acchioni et al., 2015).

1.7.5 Tetherin

Tetherin (BST2 or CD137) is an ISG product, expressed on the surface of HIV-1 target cells in response to IFN induction. Tetherin anchors newly produced HIV-1 virions to the host cell's plasma membrane to prevent the egress of the virion and the cell-cell spread of the infection. Virion-tetherin interaction initiates an intracellular signalling cascade downstream that activates NF-κB and drives pro-inflammatory cytokine production (Altfeld and Gale, 2015, Simon et al., 2015, Harris et al., 2012, Jakobsen et al., 2015, van Montfoort et al., 2014, Sumner et al., 2017, Swiecki et al., 2013, Neil et al., 2008).

1.7.6 Schlafen 11, IFITM

Schlafen 11 (SFLN11), IFITM and SUN2 are other restriction or resistance factors that have been found to mediate a block to HIV-1 replication. SFLN11 is a member of a protein family with structural similarity to the RNA-helicases. It serves to suppress viral protein synthesis at the late stage of the HIV infection. It exhibits its effect by binding to the tRNA to block the viral-mediated changes in the pool of the tRNAs available for HIV protein synthesis. High expression of SLFN11 in CD4+ T cells is associated with elite control of chronic HIV-1 infection (Sumner et al., 2017, van Montfoort et al., 2014, Acchioni et al., 2015, Jakobsen et al., 2013). IFITM1-3 are

transmembrane proteins that inhibit HIV infection at the entry step. IFITM proteins co-localizes with the HIV-1 Env and Gag and are packaged into the new virion, allowing them to block viral spread by limiting the entry of the virus in the next target cell (Sumner et al., 2017).

1.7.7 MX2

MXB (MX2), also an ISG, belongs to the dynamin-like GTPase superfamily that likely restrict primate lentiviruses before integration of the viral cDNA. MXB appears to exhibit antiviral activity against HIV-1 through direct interaction with the viral capsid, which may be dependent on HIV-1 cofactors such as cyclophilin a (CypA), which are recruited to the incoming capsids. It is thought that the recruitment of co-factors to the incoming virion targets it into a pathway where it may encounter MXB at the cytoplasmic face of the nuclear pore where MXB is localized, leading to a disruption of the viral uncoating process. MXB-capsid-CypA interaction may also serve to signal type I IFN induction and a block to viral replication (Sumner et al., 2017, Goujon et al., 2013, Kane et al., 2013, Liu et al., 2013b).

1.7.8 SERINC

SERINCs are multipass transmembrane proteins with five family members identified in humans. Recent work has demonstrated that SERINC5 may exhibit a strong pleiotropic antiretroviral activity against retroviruses including HIV, murine leukaemia virus (MLV) and the equine infectious anaemia virus (EIAV). SERINC5 is efficiently packaged into virions and blocks the entry of the viral core into target cells, thereby disrupting the fusion process of the incoming virus by an unidentified mechanism. It is able to promote neutralizing antibodies against HIV-1 virion (Firrito et al., 2018, Usami et al., 2015). (Fig. 6).

1.8 Immune evasion strategies of HIV

Although the presence and expression of host immune sensors and their subsequent signalling, induced by their engagement with HIV derived PAMPs, should, in theory, provide a hostile environment for the replication of virus, HIV has evolved to circumvent the immune responses generated against it by its host. Unlike other large DNA viruses such as the herpes or pox viruses, which harbour an armoury of proteins that are capable of disabling all branches of the innate immune response, HIV-1 carries only nine viral genes, with their proteins appearing to be multifunctional and dedicated to antagonizing its host defences (Sumner et al., 2017, Orzalli and Knipe, 2014, Manel and Littman, 2011, Strebel, 2013, Vermeire et al., 2016, Goff, 2013, Altfeld and Gale, 2015, Simon et al., 2015, Garcia-Sastre, 2017, Acchioni et al., 2015, Hertoghs et al., 2017). The HIV life cycle is composed of a complex series of immune evasion strategies that allow the virus to infect and spread between the target cells and between individuals. Immune evasion of the virus begins right at the point of entry into its target cells by rendering its Env protein insensitive to restriction abilities of the evolving IFITMs (Sumner et al., 2017).

The strong inhibitory activity of restriction factor SERINC5, against the fusion of virus to the host membrane is counteracted by the viral protein Nef, which is likely why this viral protein is synthesised early on in the viral transcription (Usami et al., 2015, Firrito et al., 2018). HIV is able to mask the conserved binding domains of its gp120 and avoids their exposure to neutralizing antibodies (Sumner et al., 2017). Upon fusion, the capsid is released into a hostile environment of the target cells, encountering the numerous innate restriction factors, following their activation by the viral PAMPs-host PRR. Nonetheless, the HIV employs several mechanisms to circumvent the cellular assault. While the capsid tranverses the hostile cytoplasm via recruitment of cellular factors including CypA, which may shield the virus from the restriction activities of TRIM5α, nucleotides are transported into the capsid cone through an electrostatic nucleotide transporter to enable reverse transcription (Sumner et al., 2017, Towers and

Noursadeghi, 2014). The synthesis of the viral cDNA in the core of the viral particle is likely shielded by CypA and CPSF6, preventing detection by the intracellular cytoplasmic DNA sensors including IF16, cGAS, NONO and PQPB1 and degradation by cellular exonucleases including TREX1 (Sumner et al., 2017, Altfeld and Gale, 2015, Towers and Noursadeghi, 2014, Lahaye et al., 2018, Yoh et al., 2015, Monroe et al., 2014, Gao et al., 2013, Doitsh et al., 2014). The presence of SAMHD1 limit intracellular dNTP required for reverse transcription. This restriction factor is however circumvented in the expression of the viral accessory protein X from HIV-2 and SIV lineage viruses. HIV-1 probably does not require an accessory protein to overcome SAMHD1 inhibition because there is likely often a window of opportunity for the virus to escape SAMHD1 restriction when it is phosphorylated, and SAMHD1 dNTP regulation likely provides the optimal dNTPs levels required for the viral replication in the capsid without inducing innate immune recognition and sensing in the event of excessive dNTP pool (Simon et al., 2015, Sze et al., 2013, Ballana and Este, 2015, Sumner et al., 2017, Plitnik et al., 2018). The ability of the APOBEC3s to suppress the viral cDNA synthesis by inducing hypermutation is overcome by their degradation by Vif (Sheehy et al., 2003, Sheehy et al., 2002, Desimmie et al., 2014, Mariani et al., 2003). Successful capsid uncoating may happen later at the nuclear pore complex, or in the nucleus in an organized manner, releasing the viral DNA, thus evading the inhibitory action of MXB (Sumner et al., 2017, Towers and Noursadeghi, 2014). Integration of the viral cDNA occurs close to the edge of the nucleus, a mechanism that may in a way prevent activating the host DNA's damage responses (Sumner et al., 2017, Lusic and Siliciano, 2017). Upon integration into the host genome, the provirus is undetectable to the host cell defences and may become transcriptionally silent or latent. Transcription of the provirus and its protein translation results in viral protein expression. The ability of SLFN11 to restrict viral protein synthesis is likely overcome by as at yet an unidentified mechanism (Sumner et al., 2017). Viral assembly occurs at the plasma membrane and immature virions bud off and are released. During maturation, the protease enzyme cleaves the structural Gag polyproteins to form mature

Gag proteins, resulting in the production of new infectious virions. The ability of tetherin to block the release of the virus is overcome by the Vpu-mediated degradation (Swiecki et al., 2013, Simon et al., 2015, Harris et al., 2012, Neil et al., 2008). In the event of virion sensing and signalling, the viral accessory proteins Vpu, and Vpr, as well as Vif, mediate the suppression of IRF3 signalling, thus escaping innate immune response (Sumner et al., 2017, Altfeld and Gale, 2015, Vermeire et al., 2016, Strebel, 2013) (Fig.6).

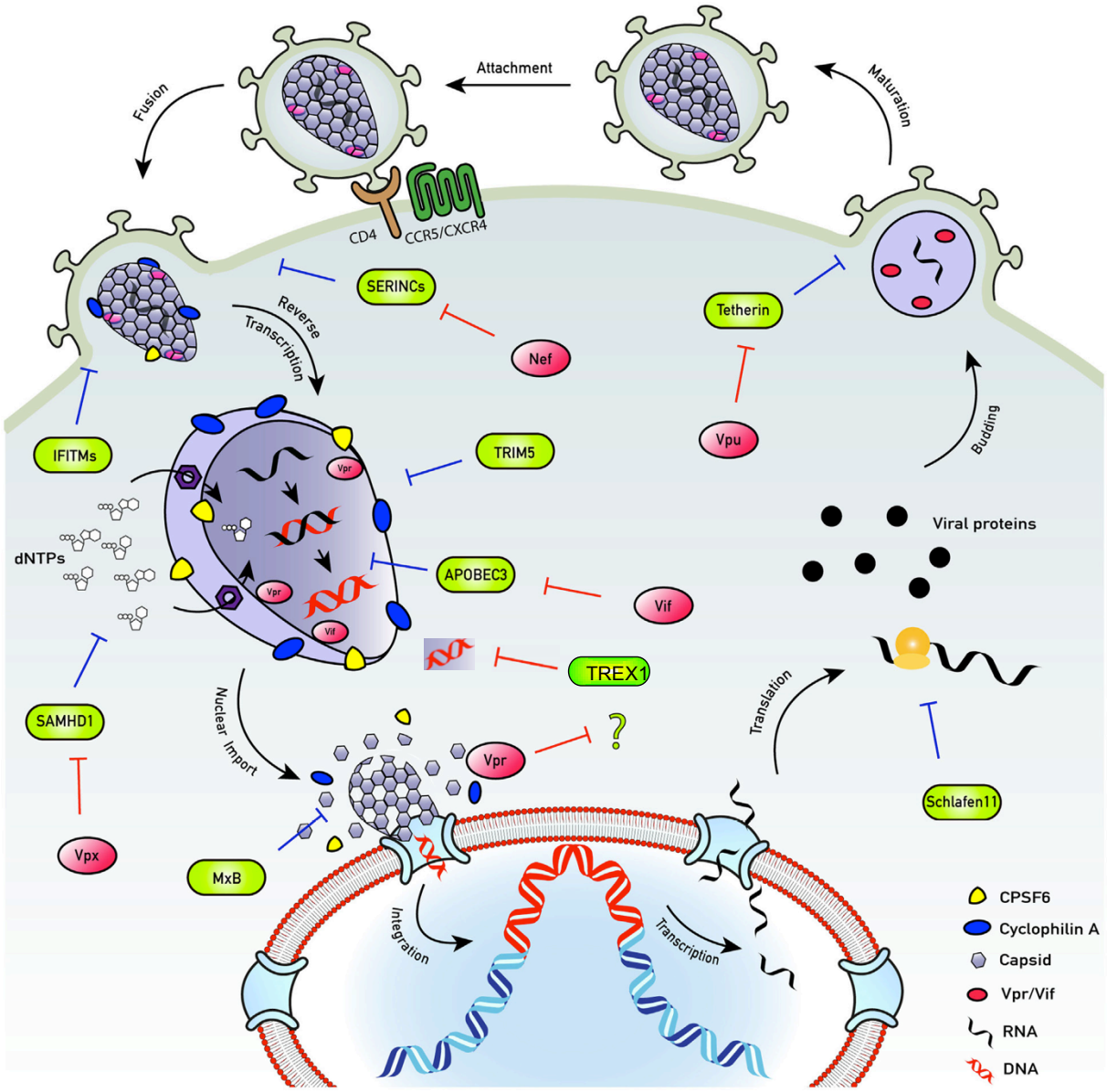

Figure 6. HIV-1 innate immune escape mechanisms at distinct stages of the viral life cycle: HIV life cycle comprises a complex series of immune evasion strategies that allow successful infection of host cells and transmission between them and between

individuals. To enter cells, HIV engages its envelope glycoprotein gp160 trimers with cell surface protein CD4 and co-receptor (CXCR4 or CCR5). Co-receptor usage allows conformational masking of conserved binding domains of gp120 and avoids their exposure to neutralizing antibodies. Upon fusion, capsid is released into the hostile environment of the cell where it encounters numerous innate restriction factors. However, HIV employs several mechanisms to overcome the cellular assault. While the capsid traverses the hostile cytoplasm, nucleotides are transported into the capsid cone through an electrostatic nucleotide transporter to fuel reverse transcription. Encapsidated DNA synthesis shields the viral genome from DNA sensors as well as exonucleases, e.g., TREX1. Capsid recruits cellular proteins cyclophilin A (blue) and CPSF6 (yellow), which have a role in preventing detection of the viral reverse-transcribed DNA by DNA sensors, e.g., cyclic GMP–AMP synthase (cGAS). Uncoating of successfully infectious cores may happen late, at the nuclear pore complex, or in the nucleus, in an organized manner and the viral DNA is released. The viral DNA integrates close to the edge of the nucleus to perhaps prevent activation of DNA damage responses. Once integrated, the provirus is invisible to the host cell defences and may become transcriptionally silent, or latent. Transcription and translation of the provirus result in viral protein expression. Viral assembly occurs at the cell surface. Immature virions bud off and are released. During maturation, the protease enzyme cleaves the structural polyprotein to form mature Gag proteins, resulting in the production of new infectious virions. SERINCs: prevent fusion of viral particles with target cells. Antagonized by Nef. IFITMs: impair virus entry into target cells. Antagonized by evolving IFITM3 insensitive Env proteins. TRIM5: forms a hexagonal lattice around the capsids. Targets them for proteasomal degradation and activates innate signalling. Antagonized by evolving TRIM5 insensitive viral capsid proteins. APOBEC3: suppresses viral DNA synthesis and induces mutations in the viral DNA. Antagonized by Vif-mediated degradation. SAMHD1: restricts infection by lowering nucleotide concentrations below those, which support viral DNA synthesis. Antagonized by Vpx-mediated degradation (SIVsm/HIV-2) or infection of inactive phospho-SAMHD1 positive cells (HIV-1). MxB: restricts HIV-1 nuclear entry and possibly integration. Schlafen 11: restricts HIV-1 protein translation. Tetherin: inhibits virus release from infected cells. Antagonized by Vpu-mediated degradation. Figure was adapted from (Sumner et al., 2017) and modified with permission from the author, Rebecca P. Sumner.

1.9 USP18

Other cellular factors that play important physiological roles in the innate immune homeostasis may also likely be hijacked by HIV to enhance its replication (Goff, 2013, Goff, 2007). Among them is the ubiquitin-like specific protease USP18 (UBP43). USP18 is a member of the ubiquitin-specific protease family that was first discovered as an ISG15-specific isopeptidase (Malakhov et al., 2002, Liu et al., 1999). The *USP18* gene encodes a 43-kDa protein with two functional domains. An ISG15-specific protease activity site at residue cysteine 64, and an interferon alpha receptor-2 (IFNAR2) c-terminal domain-binding site at residue L374. By interacting with the intracellular domain of IFNAR2, USP18 abrogates type I and III IFN signalling, replacing the JAK1 (Malakhova et al., 2006, Honke et al., 2016, Francois-Newton et al., 2011). Two isoforms of USP18 are found in humans, differing in their N-terminal region due to a non-canonical rare start codon CUG. Translation from this codon produces the full-length protein, while a canonical AUG start codon, downstream of the CUG results in an N-terminal truncated isoform, denoted as USP18-sf (Burkart et al., 2012). USP18 is predominantly a cytoplasmic protein. However, the USP18-sf appears evenly distributed in the cytoplasm and

the nucleus. Both proteins maintain their functional activity in terms of protease and interferon regulation function, but the USP18-sf is the main deISGylation enzyme for nuclear proteins and may serve other cell-specific functions *in vivo*. Additionally, because USP18-sf is controlled by two independent mechanisms, its regulation appears to be flexible (Burkart et al., 2012, Ketscher et al., 2015). USP18 is highly abundant in the liver, spleen, white matter microglia and the thymus, with low levels detected in the bone marrow, adipose tissue and the lung tissues

(Honke et al., 2016, Goldmann et al., 2015). High expression of this protein is observed in the myeloid lineage cells including CD169+ macrophages and bone marrow-derived dendritic cells, peritoneal macrophages and monocyte-derived macrophages. USP18 expression is also observed in splenic T and B-cells, with high abundance in naïve, effector/memory and natural regulatory T cells. A high expression is also detected in Th0, Th1 and Th17 T cells. USP18 is itself IFN-inducible and its expression levels are differentially regulated during T-cell activation, tolerance and effector differentiation. It is shown to mediate $CD11b^{+}$ dendritic cells (DCs) and Th17 cell development and survival. USP18 gene is robustly upregulated following viral infection or lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), or genotoxic stress (Honke et al., 2016, Ritchie et al., 2004). It is regulated by SKP2 dependent degradation by the proteasome in the absence of free ISG15 (Tokarz et al., 2004). The absence of USP18 heightens IFN-I and III signalling and it is associated with prolonged Janus-activated kinase/signal transducer and activator of transcription (JAK-STAT) signalling (Honke et al., 2016, Burkart et al., 2013, Francois-Newton et al., 2011). The absence of USP18 further prolongs ISG15-mediated ISGylation. USP18-deficient mice exhibit high sensitivity to treatment with IFN-1, poly I:C and LPS. Depletion of USP18 heightens NF-κB signalling induced by various TLR ligands (Honke et al., 2016). Apart from its ISG15-specific protease and IFN-I and III, USP18 likely exhibits an ubiquitinase activity (Yang et al., 2015). The expression of USP18 negatively regulates NF-κB signalling by targeting TAK1 and NEMO for deubiquitination through

distinct mechanisms as well as NFAT activation during Th17 differentiation by deubiquinating the TAK1-TAB1 complex (Yang et al., 2015, Liu et al., 2013a). By regulating type I and III IFN, USP18 enforces local replication of viruses to help activate the adaptive immunity. However, in the event of an infection with a virus being similar to an auto-antigen, the enforced viral replication may lead to an autoimmune disease (Honke et al., 2012, Honke et al., 2016, Honke et al., 2013) (Fig. 7). USP18 may exhibit important roles in tumorigenesis and other undefined cellular and physiological functions. Investigating and defining other cellular functions of USP18, and how it might influence the immune response to HIV infection and replication is one major focus of this thesis.

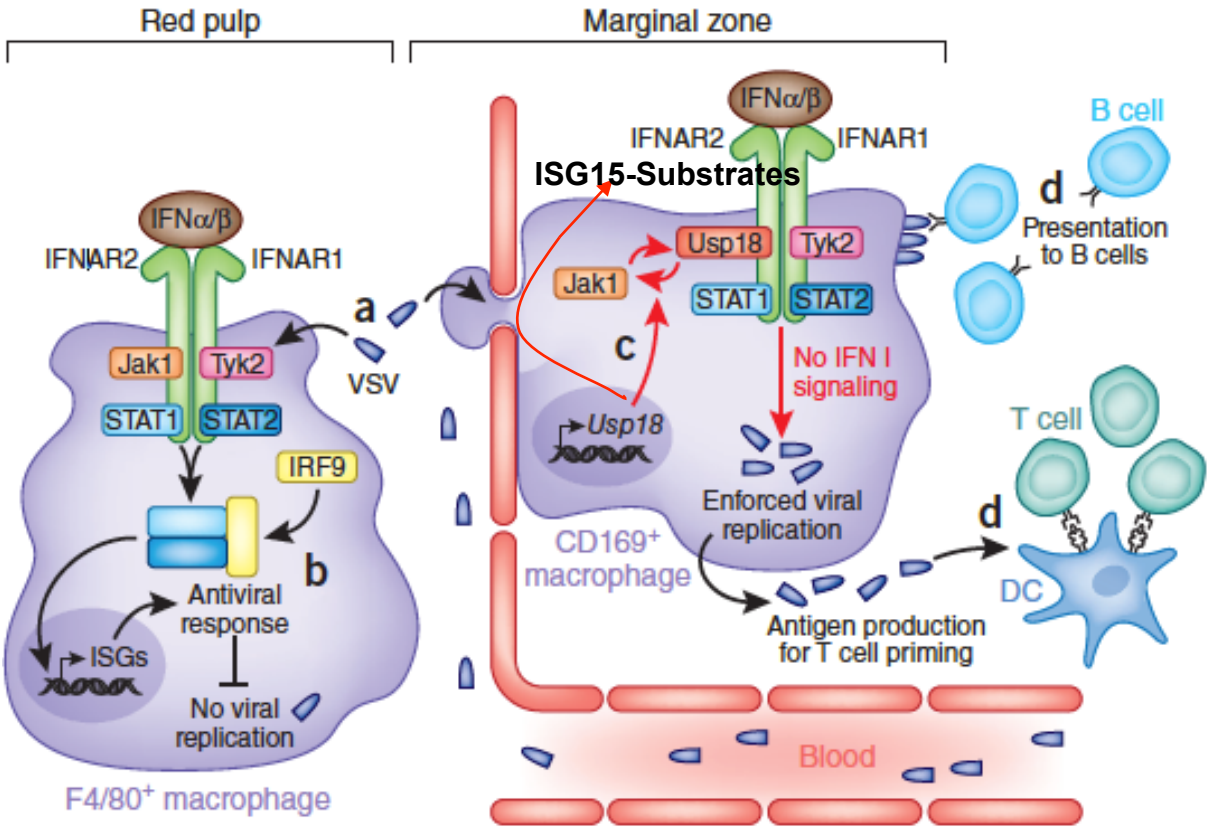

Figure 7. Inhibition of type I interferon signalling in CD169+ macrophages of the marginal zone through Usp18 enforces local virus production and improves activation of antiviral T cell and B cell responses. (A) After systemic infection with VSV, viral particles enter the spleen via the blood stream and 'preferentially' infect F4/80+ macrophages in the red pulp and CD169+ macrophages in the marginal zone. (B) Type I interferons trigger a potent antiviral intracellular response via IFNAR in F4/80+ macrophages of the red pulp to control the viral infection. (C) In contrast, CD169+ macrophages 'take the bullet', as Usp18-mediated inhibition of type I interferon signalling enforces viral replication in these cells. Usp18 functions as a negative regulator of type I interferon signalling by interfering with the binding of Jak1 to IFNAR2. (D) The production of live virus by CD169+ macrophages facilitates direct antigen

presentation of virus to B cells and generates sufficient antigen for processing and presentation via classical dendritic cells (DCs) for T cell priming. USP18 exhibits protease activity by de-conjugating ISG15 from its substrates. (Figure was adapted from (Ludewig and Cervantes-Barragan, 2011) and modified with permission from the authors and Springer Nature).

1. 10 HIV and *Helicobacter pylori* coinfection.

In the event of established, persistent HIV infection, coinfection with other pathogens is inevitable, especially in areas such as sub-Saharan Africa, where prevalence and geography of HIV and other pathogenic infections overlap. These coinfections have the potential to alter the course and pathogenesis of HIV infection (Karp and Auwaerter, 2007, Olmos et al., 2004, Radovanovic Spurnic et al., 2017, Sangare et al., 2011, Modjarrad and Vermund, 2010, Chang et al., 2013, Bell and Noursadeghi, 2018). *H. pylori* is a highly probable candidate for coinfection with HIV in sub-Saharan Africa. *H. pylori* exhibits a strong immune alteration in its host. Considering the robust immune modulatory effect of *H. pylori* infection, HIV/*H. pylori* coinfection may likely alter the disease course and pathogenesis of HIV (Radovanovic Spurnic et al., 2017, Nevin et al., 2014, Olmos et al., 2004).

1. 10.1 Epidemiology of *H. pylori*

H. pylori, formally called *Campylobacter pylori* is a spiral, gram-negative, microaerophilic bacterium, which was first identified in 2005 by Robin Warren and Barry Marshall as the aetiologic agent of chronic gastritis and peptic ulcer (Hellstrom, 2006, Kusters et al., 2006, Wroblewski et al., 2010). *H. pylori* persistently colonizes the human stomach, infecting about half of the world's population, and it is associated with gastric adenocarcinoma (Ishaq and Nunn, 2015, Wroblewski et al., 2010, Chmiela et al., 2017, Kusters et al., 2006). In sub-Saharan Africa, where HIV is endemic, more than 85% prevalence of *H. pylori* is recorded. Infection with *H. pylori* is associated with poor socioeconomic condition and designated as a disease of poverty. It is mainly acquired during childhood, and may persist for a lifetime, if left untreated.

The exact route of *H. pylori* infection is largely unknown, however, new infections have been speculated to occur as a consequence of direct human-to-human transmission, either via an oral-oral or faecal-oral route or both (Ishaq and Nunn, 2015, Wroblewski et al., 2010, Kusters et al., 2006, Khalifa et al., 2010). In many developing countries, where *H. pylori* prevalence can be more than 90% at a young age, development of ulcer or cancer is significantly lacking, although this be could be as result of under-reporting (Ishaq and Nunn, 2015, Wroblewski et al., 2010, Chmiela et al., 2017).

1. 10.2 Diagnosis of *H. pylori*

H. pylori infection can be detected either invasively from gastric specimens via histology or culturing, or it can be detected in a non-invasive way by antibody detection in the blood. It can also be detected from breath samples, urine and saliva. Stool antigen tests or urease activity are other possible means of detecting *H. pylori* (Kusters et al., 2006).

The ability of this bacterium to colonize the harsh conditions of the gastric mucosa is likely mediated by its ability to catalyse urease production and also its high motility, mediated by its flagella, which is thought to allow rapid movement toward the more neutral pH of the gastric mucosa (Montecucco and Rappuoli, 2001, Kusters et al., 2006, Wroblewski et al., 2010). Similar to other gram-negative bacteria, the *H. pylori* envelope comprises an inner cytoplasmic membrane, a periplasm with peptidoglycan and an outer membrane, which consists of phospholipids and LPS, which have the ability to either stimulate or evade immune response (Wroblewski et al., 2010).

1. 10.3 Symptoms of *H. pylori*

Chronic active gastritis is the primary disorder associated with *H. pylori* infection. The severity of this chronic inflammatory disorder and the intra-gastric distribution in the infected host is dependent upon a number of factors such as the *H. pylori* infected strain, host genetics and immune response, diet and the level of acid production (Polk and Peek, 2010, Wroblewski et al., 2010). The development of ulcer, gastric cancer and lymphoma are a consequence of the chronic inflammation induced by *H. pylori*. *H. pylori* virulence factors such as cytotoxin-associated gene A (*cag*), which encodes about a 140-kDa protein called CagA is highly immunogenic. Patients with *H. pylori* strains that encode this virulence factor usually have higher inflammatory response and are significantly more at risk for developing symptomatic outcomes, including peptic ulcers and gastric cancer (Chmiela et al., 2017, Ishaq and Nunn, 2015, Kusters et al., 2006, Polk and Peek, 2010, Wroblewski et al., 2010). About 50% of *H. pylori* strains secrete another virulence factor called vacoulating cytotoxin A (VacA). This factor, which encodes about 95-kDa protein, induces massive vacuolization in epithelial cells *in vitro*. VacA is also important in the pathogenesis of both peptic ulceration and gastric cancer. VacA has the ability to induce membrane channel formation, disrupt endosomal and lysosomal activity, and like other virulence factors, has been implicated in immune response to the bacteria (Chmiela et al., 2017, Kim and Blanke, 2012, Kusters et al., 2006, Montecucco and Rappuoli, 2001, Wessler and Backert, 2008, Wroblewski et al., 2010).

1. 10.4 Immune modulations by *H. pylori*

Immune modulation is a key feature in *H. pylori* infection. Although this bacterium induces a massive immune response in its host, this response seldom clears the infection, leading to a lifelong persistence of the bacteria (Kim and Blanke, 2012, Kusters et al., 2006, Montecucco and Rappuoli, 2001). It is believed that much of the pathology associated with *H. pylori* infection is a result of the host immune's response to the pathogen rather than a direct effect of the

bacteria (Khamri et al., 2010, Kusters et al., 2006, Montecucco and Rappuoli, 2001, Müller et al., 2011, Robinson et al., 2017, Wroblewski et al., 2010). Not only is *H. pylori* able to induce a strong immune response, but also, it is able to successfully down-regulate inflammation and to control the host's immune response, using its wide range of virulence factors (Gebert et al., 2003, Khamri et al., 2010, Kusters et al., 2006, Montecucco and Rappuoli, 2001, Robinson et al., 2017, Wroblewski et al., 2010, Chmiela et al., 2017).

Gastric epithelial cells (GECs) are primary targets for *H. pylori* infection, and likely contribute to immune recognition and sensing, and may be actively involved in the development of an acute and chronic immune inflammation (Kusters et al., 2006, Montecucco and Rappuoli, 2001, Müller et al., 2011, Robinson et al., 2017, Wessler and Backert, 2008). Gastric epithelial cells express innate immune PRRs that enable them to recognize and sense an infection (Fig. 8). However, available data suggest that *H. pylori* possesses an impressive ability to circumvent innate immune detection by the host's PRR, thereby preventing innate and adaptive immunity and ensuring persistence, a phenomenon which is shared also by HIV, albeit by a completely different mechanism of immune invasion (Müller et al., 2011, Kim and Blanke, 2012). *H. pylori,* by a mutation in its flagellin, circumvents recognition by TLR5, a specific GEC receptor for pathogenic bacteria flagellin (Müller et al., 2011). *H. pylori* LPS, predominantly comprising tetra-acylated lipid A variety, has such a poor bioactivity by over 1000-fold reduction compared to *E. coli* LPS. *H. pylori* LPS is a poor ligand for TLR4 and preferentially activates TLR2 signalling on GECs *in vivo*, activating NF-κB. However, rather than inducing pro-inflammatory response, *H. pylori*-LPS-TLR2 interaction results in anti-inflammatory response (Müller et al., 2011). The 5'-triphosphorylated RNA of *H. pylori* can be recognized and sensed by the intracellular receptor RIG-I and trigger a type I IFN in DCs, however, the outcome of this response in the control of *H. pylori* infection and associated immunopathology is yet to be explored (Müller et al., 2011). Unlike the fucosylated ligands of *Mycobacterium tuberculosis*, which activates a signalling

downstream of DC-SIGN and trigger pro-inflammatory cytokine, the DC-SIGN ligands of *H. pylori* actively disengages the signalling complex downstream of the C-type lectin, suppressing pro-inflammatory cytokine production (Müller et al., 2011). The cytosolic PRR, NOD1 of the NLR family recognizes the peptidoglycan of *H. pylori*. The introduction of peptidoglycan into the cytosol by *H. pylori* via its type IV secretion system or via outer membrane vesicles, stimulates NF-κB activation and type I IFN induction mediated by NOD1 recognition and sensing of iE-DAP (γ-D-glutamyl-meso-diaminopimelic acid) of peptidoglycan origin (Fig. 8). Interestingly, NOD1-mediated recognition and sensing of *H. pylori* is negatively regulated by the immunomodulatory glycoprotein olfactomedin 4 (OLFM4), a mechanism that is likely harnessed by *H. pylori* for immune escape (Müller et al., 2011) .

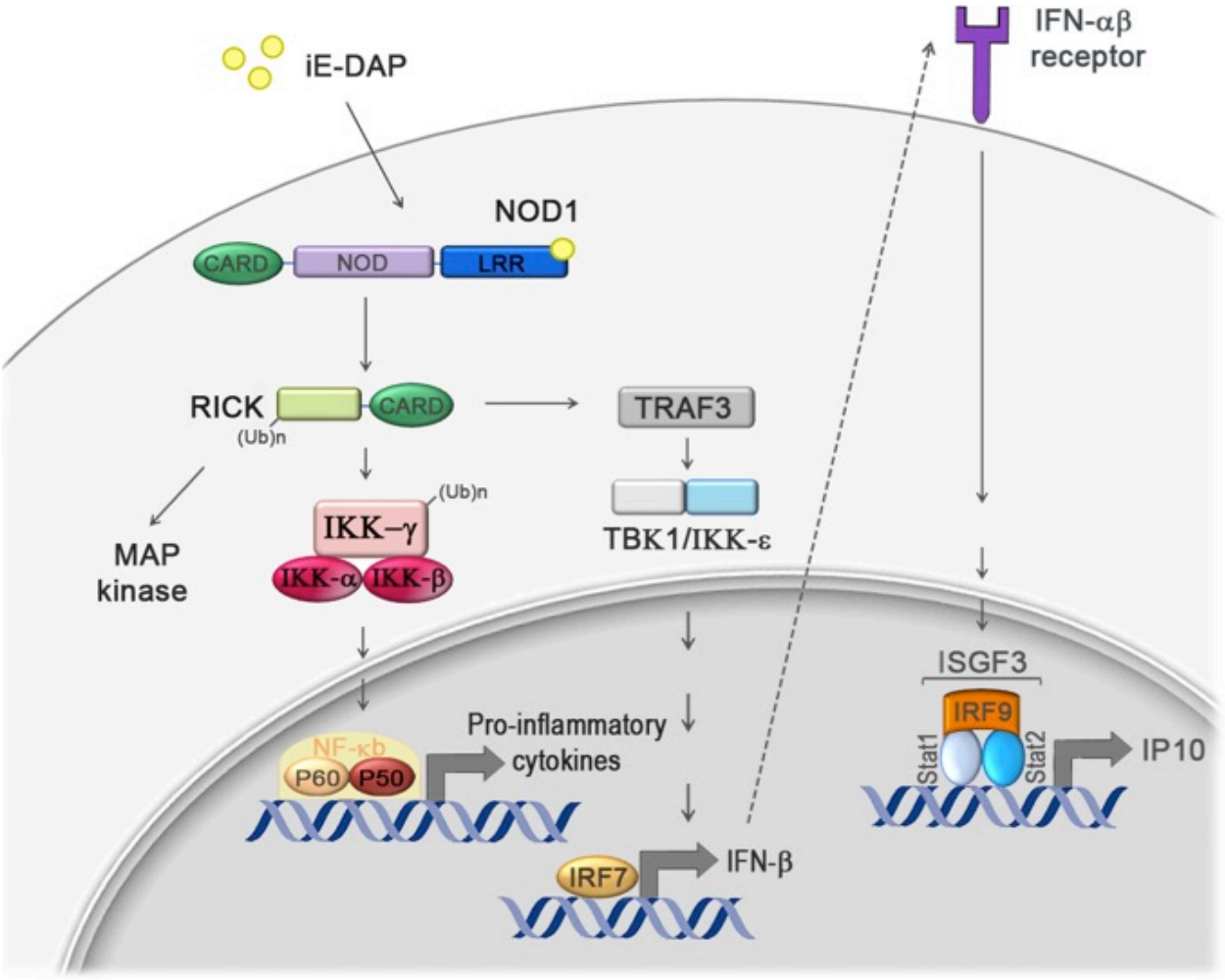

Figure 8. Schematic of NOD1 signaling pathways. NOD1 activation by its specific ligand iE-DAP activates at least two distinct signaling pathways. On the one hand, activated NOD1 triggers IFN-b production through the consecutive activation of RICK, TRAF3, TBK1/IKKε and IRF7. IRF7-dependent IFN-b production leads to the expression of IP-10 and other Th1 cytokines via transactivation of ISGF3 (Stat1-Stat2-IRF9 complex). On the other hand, NOD1 activation triggers the RICK and IKK-,α,β,γ complex-dependent activation of NF-κB and subsequent production of proinflammatory cytokines and chemokines such as IL-8. Whether NF-κB is indeed activated upon Nod1 ligation in all cell types, including epithelial cells remains not clear. Figure is adapted from (Müller et al., 2011) with permission from the author, Anne Müller the BioMed Central Ltd.

CD4+ T helper cells are critical for the control of experimental *H. pylori* infection. Pro-inflammatory response mediated by the Th1 and Th17 subtypes of CD4+ T cells contributes to protection against *H. pylori*. The effector functions of these cells, through their cytokine secretion and activation of other effector cells, likely contributes to chronic inflammation, injury and subsequent development of cancer (Blosse et al., 2018, Robinson et al., 2017). Th1 and Th17 lymphokines such as IFNγ and IL-17A respectively are enhanced in *H. pylori* infected

patients, and are associated with severe gastritis and peptic ulcer (Blosse et al., 2018, Khamri et al., 2010, Robinson et al., 2017). Despite the effector functions of these cells, the resolution of the infection in the host is usually not achieved. *H. pylori,* by its virulence factors, has been shown to impressively suppress the T cell mediated immunity. *H. pylori* virulence factor VacA, apart from inducing vacuolization of GECs is highly effective at inhibiting T cell activation, and proliferation by interfering with the T cell receptor/interleukin-2 (IL-2) signalling pathway at the level of the Ca2+-calmodulin-dependent phosphatase calcineurin (Blosse et al., 2018, Chmiela et al., 2017, Gebert et al., 2003, Khamri et al., 2010, Kim and Blanke, 2012, Kusters et al., 2006, Müller et al., 2011, Robinson et al., 2017, Wroblewski et al., 2010, Wessler and Backert, 2008, Montecucco and Rappuoli, 2001). Thus, VacA prevents the nuclear translocation of the T cell transcription factor NFAT, a global regulator of T cell responses, resulting in the downregulation of IL-2 gene transcription. Y-glutamyl transpeptidase (GGT), a low-molecular weight protein secreted by *H. pylori* also exhibits an inhibitory effect against T cell proliferation. GGT arrests antigen-activated T cells at the G1 phase of the cell cycle by interfering with G1 cyclin-dependent kinase activity, and disrupting the Ras signalling pathway (Müller et al., 2011). Apart from its ability to block T cell activation and proliferation, *H pylori* is thought to skew effector functions of T cells towards regulatory function. *H. pylori* infection induces dendritic and regulatory T cells (Tregs) with suppressive activity. It is thought that the paucity of TLR and NLR ligands that characterize *H. pylori* are probable means employed by this bacterium to avoid DC maturation, and by avoiding DC maturation, the bacteria can promote Treg effector function for its colonization and persistence. Indeed, it has been demonstrated that *H. pylori*-exposed DCs preferentially prime Tregs over Th1 and Th17 responses, thus failing to clear the bacteria. *H. pylori*-specific effector Th1 and Th17 cells are thought to be under the strict control of regulatory T cells. The extent of *H. pylori*-specific Tregs generation and effector functions appear to be dependent largely on the age at the time of infection (Blosse et al., 2018, Gebert et al., 2003, Khamri et al., 2010, Kusters et al., 2006, Müller et al., 2011, Robinson et al., 2017). Mice

infected with virulent *H. pylori* at old age developed gastritis and gastiric cancer precursor lesions as compared to mice that are exposed to *H. pylori* during neonatal period (Müller et al., 2011). Neonatal exposure to *H. pylori* leads to immunological tolerance, and mediated by regulatory T cells, which prevents the generation of *H. pylori*-specific T helper cell responses and protects from developing T cell driven immunopathologies (Müller et al., 2011). Apart from the inhibitory effect of *H. pylori* infection on effector Th1 and Th17 response, B cell effector functions are also impaired in the presence of *H. pylori*. *H. pylori* stimulates B cells into producing IL-10 with a suppressive function, rather than effector functions. B cell recognition and sensing of *H. pylori* is TLR2 and MyD88 dependent, inducing regulatory IL-10 secretion with immunosuppressive function. Thus the ability of *H. pylori* to survive in a hostile gastric mucosa of its host in the face of the robust and vigorous local and systemic immune response is largely due to its ability to circumvent detection by the host innate and adaptive immune machinery and its ability to downregulate their effector functions (Müller et al., 2011). The immunomodulatory properties of the pathogen and the reprogram of the immune system towards immunological tolerance assist the bacteria in establishing persistent infection (Blosse et al., 2018, Gebert et al., 2003, Müller et al., 2011, Robinson et al., 2017, Wessler and Backert, 2008) (Fig. 9).

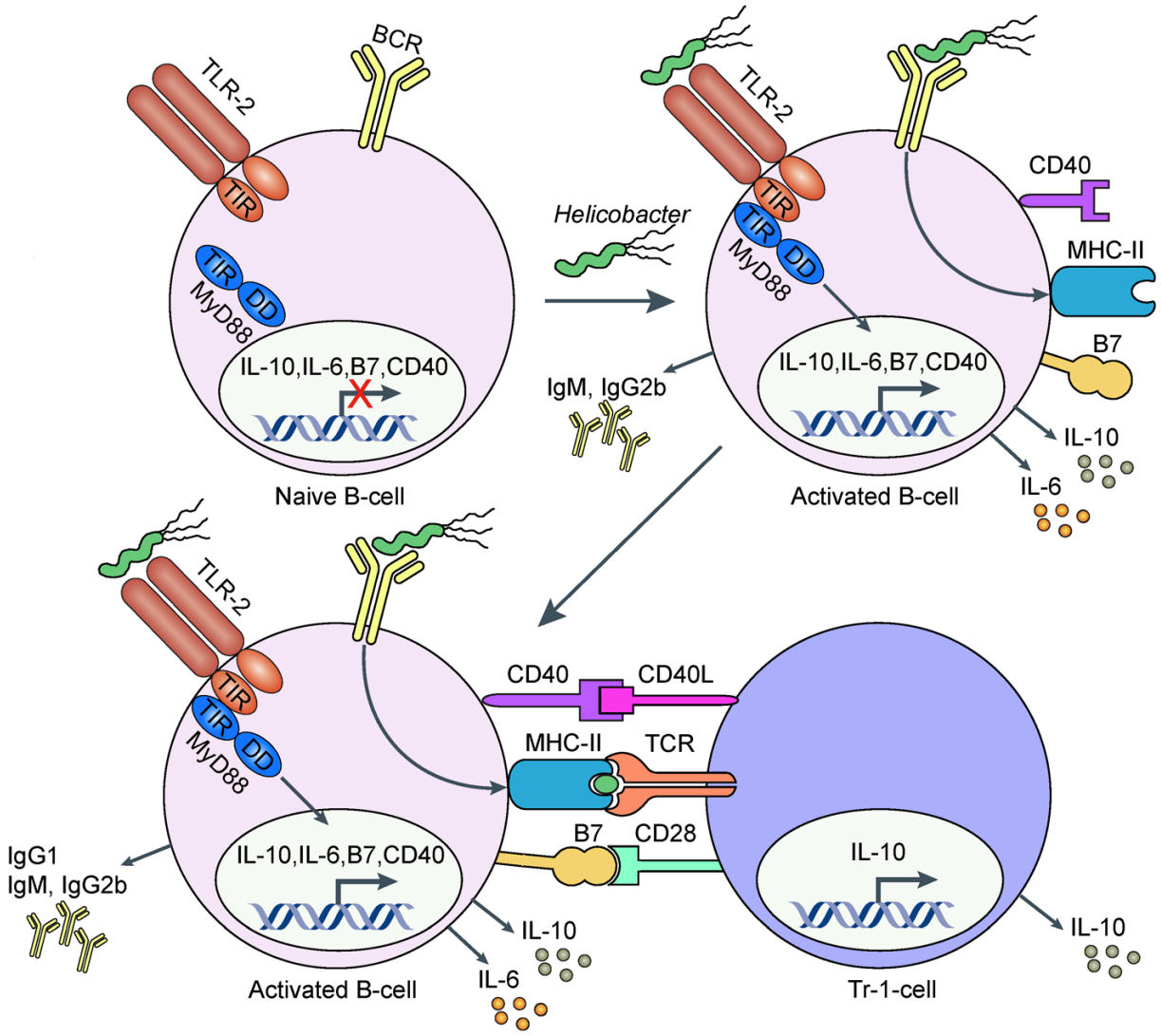

Figure 9. TLR-2-activated B-cells suppress Helicobacter-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. Schematic representation of the events occurring in the course of Helicobacter-specific activation of B-cells at the site of infection (i.e. the gastric mucosa) and/or in the draining mesenteric lymph nodes. Helicobacter TLR-2 ligands activate B-cells in a MyD88-dependent manner, which leads to the expression and surface exposure of CD80, CD86 (together referred to as B7 molecules), the increased expression of CD40, and the secretion of IL-10, IL-6 and moderate amounts of TNF-α (the latter is not shown here) as well as antibodies of the IgM and IgG2b subclasses. The interaction of activated B-cells and naive T-cells induces T-cellular IL-10 expression and suppressive activity in a manner dependent on a direct interaction between both cell types via CD40/CD40L, B7/CD28 and MHCII/TCR. IL-10 secreting T-cells are essential players in the prevention of excessive Helicobacter-associated immunopathology. Figure is adapted from (Müller et al., 2011), with permission from the author, Anne Müller and the BioMed Central Ltd.

Considering that activated and proliferating memory CD4+ T cells of the GALT are important targets of productive HIV infection *in vivo*, their effector regulation by circulating Tregs induced by *H. pylori* in the context of HIV/*H. pylori* coinfection may provide some level of protection

against the ongoing viral replication. Indeed, in sub-Saharan Africa where prevalence of both infections coincides, the likelihood of coinfection is very high, nevertheless, the impact of *H. pylori* on immune modulation and clinical parameters in HIV-1 infection has not been adequately explored. Available data suggests low prevalence of *H. pylori* among HIV infected patients, with the prevalence inversely correlating with CD4 count, with some data recording less than half of *H. pylori* infection in HIV-1 infected patients compared to uninfected controls (Nevin et al., 2014). Several reasons have been postulated to explain this mechanism of lower prevalence of *H. pylori* infection in HIV infected patients. But the most biologically plausible explanation is that *H. pylori,* likely require an intact CD4 milieu to colonize its host. Also, the chronic inflammation that characterizes the end stage of HIV infection provides unsuitable environment for the bacteria to drive (Nevin et al., 2014, Radovanovic Spurnic et al., 2017). Considering that *H. pylori* is acquired early in life, the acquisition of HIV-1 in the coinfection context comes later and the presence of *H. pylori* likely may provide some level of protection against infection. Indeed, infection of *H. pylori* has been associated with protection against allergy, chronic inflammatory and autoimmune diseases (Engler et al., 2014, Nevin et al., 2014, Papamichael et al., 2014, Arnold et al., 2012, Perry et al., 2010).

Research Objectives

The host resistance and restriction factors, such as ISG15, p21 and SAMHD1 respectively, represent integral part of the innate immune responses against viruses. These factors are generally stimulated by IFNs as ISGs, following recognition and sensing of the viruses. The CDK inhibitor p21, which is involved in monocyte differentiation and maturation, regulates the key enyzmes involved in *de novo* dNTP biosynthesis pathway and SAMHD1 antiviral function in a cell cycle dependent manner. p21 is itself regulated by p53-dependent and -independent pathways. HIV-1 is able to skip under the radar of the innate immune surveillance and establish disseminated infection in their host. The ability of HIV-1 to circumvent the innate immune response could be mediated by the presence of cellular factors. The ISG15-specific protease USP18 negatively regulates IFN signalling pathways and may influence HIV-1 infection and replication.

In the event of an established infection, after bypassing the innate immune system, HIV-1 preferentially infects and depletes memory CD4+ T cells resulting in AIDS and death if the infection is not interrupted by antiretroviral therapy. In sub-Saharan Africa, where the prevalence and geography of HIV-1 and other pathogenic infections overlap, coinfection is highly probable. More than half of the people living with HIV reside in sub-Saharan Africa, where over 85% of the population are infected by *H. pylori*, a gram-negative bacterium, which is responsible for gastritis and stomach ulcerations.

Considering that USP18 is the main regulator of IFN signalling pathway, and a specific protease of ISG15, all of which are required for successful innate immune defence against viruses, I asked whether USP18 could be a factor that contributes to HIV-1 infection and innate immune sensing escape, using the myeloid THP-1 cell line as model *in vitro*.

Although memory CD4+ T cells are the main targets of HIV-1 infection after bypassing the innate immune response, it remains to be clarified, which subset of these cells, *in vivo* are preferentially infected and contributes to plasma viremia. I therefore sought to identify and characterize the subset of memory CD4+ T cells that are preferentially infected by HIV-1 and contribute to HIV-1 plasma viral load

Finally, *H. pylori* exhibits a strong immune modulatory effect in its host, some of which appear beneficial. Considering the overlapping geography and prevalence of *H. pylori* and HIV-1, I asked whether coinfection was plausible, and if it is, how might *H. pylori* infection impact HIV-1 disease course in HIV-1-infected patients in Ghana.

In the different chapters, I provide some answers to these questions in the different publications from three different projects.

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CHAPTER I

USP18 (UBP43) Abrogates p21-Mediated Inhibition of HIV-1

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Edmund Osei Kuffour's contribution to this work:

1. Executed all the experiments in this work except the experiments for Fig. 4B and 4C, Fig. 6G - 6I and Fig. 7
2. Wrote the original draft of the manuscript

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Author:	Edmund Osei Kuffour,Kerstin Schott,Ananda Ayyappan Jaguva Vasudevan,Jessica Holler,Wolfgang A. Schulz,Philipp A. Lang,Karl S. Lang,Baek Kim,Dieter Häussinger,Renate König,Carsten Münk
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VIRUS-CELL INTERACTIONS

USP18 (UBP43) Abrogates p21-Mediated Inhibition of HIV-1

Edmund Osei Kuffour,[a] Kerstin Schott,[b] Ananda Ayyappan Jaguva Vasudevan,[a] Jessica Holler,[c] Wolfgang A. Schulz,[d] Philipp A. Lang,[e] Karl S. Lang,[f] Baek Kim,[c,g] Dieter Häussinger,[a] Renate König,[b,h] Carsten Münk[a]

[a]Clinic of Gastroenterology, Hepatology and Infectious Diseases, Medical Faculty, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

[b]Host-Pathogen Interactions, Paul-Ehrlich-Institut, Langen, Germany

[c]Department of Pediatrics, Emory University, Atlanta, Georgia, USA

[d]Department of Urology, Medical Faculty, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

[e]Department for Molecular Medicine II, Medical Faculty, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

[f]Institute of Immunology, University Hospital, University of Duisburg-Essen, Essen, Germany

[g]Department of Pharmacy, Kyunghee University, Seoul, South Korea

[h]Immunity and Pathogenesis Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA

ABSTRACT The host intrinsic innate immune system drives antiviral defenses and viral restriction, which includes the production of soluble factors, such as type I and III interferon (IFN), and activation of restriction factors, including SAMHD1, a deoxynucleoside triphosphohydrolase. Interferon-stimulated gene 15 (ISG15)-specific ubiquitin-like protease 43 (USP18) abrogates IFN signaling pathways. The cyclin-dependent kinase inhibitor p21 (CIP1/WAF1), which is involved in the differentiation and maturation of monocytes, inhibits human immunodeficiency virus type 1 (HIV-1) in macrophages and dendritic cells. p21 inhibition of HIV-1 replication is thought to occur at the reverse transcription step, likely by suppressing cellular deoxynucleoside triphosphate (dNTP) biosynthesis and increasing the amount of antivirally active form of SAMHD1. SAMHD1 strongly inhibits HIV-1 replication in myeloid and resting $CD4^+$ T cells. Here, we studied how USP18 influences HIV-1 replication in human myeloid THP-1 cells. We found that USP18 has the novel ability to inhibit the antiviral function of p21 in differentiated THP-1 cells. USP18 enhanced reverse transcription of HIV-1 by downregulating p21 expression and upregulating intracellular dNTP levels. p21 downregulation by USP18 was associated with the active form of SAMHD1, phosphorylated at T592. USP18 formed a complex with the E3 ubiquitin ligase recognition factor SKP2 (S-phase kinase associated protein 2) and SAMHD1. CRISPR-Cas9 knockout of USP18 increased p21 protein expression and blocked HIV-1 replication. Overall, we propose USP18 as a regulator of p21 antiviral function in differentiated myeloid THP-1 cells.

IMPORTANCE Macrophages and dendritic cells are usually the first point of contact with pathogens, including lentiviruses. Host restriction factors, including SAMHD1, mediate the innate immune response against these viruses. However, HIV-1 has evolved to circumvent the innate immune response and establishes disseminated infection. The cyclin-dependent kinase inhibitor p21, which is involved in differentiation and maturation of monocytes, blocks HIV-1 replication at the reverse transcription step. p21 is thought to suppress key enzymes involved in dNTP biosynthesis and activates SAMHD1 antiviral function. We report here that the human USP18 protein is a novel factor potentially contributing to HIV replication by blocking the antiviral function of p21 in differentiated human myeloid cells. USP18 downregulates p21 protein expression, which correlates with upregulated intracellular dNTP levels and the antiviral inactive form of SAMHD1. Depletion of USP18 stabilizes p21 protein expression, which correlates with dephosphorylated SAMHD1 and a block to HIV-1 replication.

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Address correspondence to Carsten Münk, carsten.muenk@med.uni-duesseldorf.de.

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Cellular antiretroviral restriction factors are an important component of the innate immune response against HIV-1 (1–3). Many of these proteins are induced or activated by interferons (IFNs) (4–6). These proteins can inhibit retroviral replication at different stages of the viral life cycle. However, viruses have evolved mechanisms that antagonize the restriction abilities of these factors (1, 2). For example, viral proteins such as HIV-1 Vif target APOBEC3 proteins for proteasomal degradation (1, 2), and VPX of the human immunodeficiency virus type 2 (HIV-2)/simian immunodeficiency virus (SIV) can induce depletion of SAMHD1 (7–9). VPX loads SAMHD1 onto an E3 ubiquitin ligase complex, cullin ring-finger ligase 4 (CRL4)-DDB1, via the substrate adapter cullin-associated factor 1 (DCAF1) to initiate its proteasomal degradation (7, 10–13). This results in a drastic increase in the intracellular deoxynucleoside triphosphate (dNTP) pool to a level required for efficient reverse transcription (14, 15).

In vitro, quiescent $CD4^+$ T cells, myeloid cells (including macrophages and dendritic cells), and phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells, express relatively high levels of SAMHD1 and restrict HIV-1 replication, likely because the deoxynucleoside triphosphohydrolase (dNTPase) activity of SAMHD1 leads to ablation of viral reverse transcription (14, 16–20). However, proliferating cells are highly permissive for infection and replication of HIV-1 (21–23). Together with upregulation of ribonucleotide reductase (RNR), downregulation of SAMHD1 expression and dNTPase activity during the S phase was considered a characteristic feature of proliferating cells (24, 25) until recently; Schott et al. (in 2018) and Yan et al. (in 2015) reported that SAMHD1 levels remain relatively unchanged during the cell cycle (26, 27).

The restriction ability of SAMHD1 is thought to be modulated in proliferating cells by phosphorylation at residue T592 by the cyclin A2/CDK1/2 complex and by dephosphorylation by the phosphatase PP2A-B55α during mitotic exit (21, 27–30). Intriguingly, the T592E mutant of SAMHD1, which mimics the phosphorylated form, is unable to restrict HIV-1 despite maintaining its ability to deplete the intracellular dNTP pool (28, 31). This finding suggests that SAMHD1 dNTPase activity is not sufficient to confer inhibition to retroviral replication. Thus, beyond its dNTPase activity, additional unidentified cellular regulatory mechanisms of SAMHD1 may be involved in its restriction of retroviruses.

Indeed, efficient replication of HIV-1 requires a sufficiently large cellular dNTP pool (32, 33), which is likely sustained by various cellular pathways (34, 35). *De novo* pathways synthesize new dNTPs, while salvage pathways recover nucleotides and their components from extracellular media and intracellular DNA degradation (34, 35). As a result, cycling cells exhibit higher dNTP levels compared to noncycling cells (15, 27, 34, 35). The CDK inhibitor p21 is one of the cellular factors regulating dNTP biosynthesis and is itself regulated during cell cycle. In cycling cells, p21 can be targeted for proteasomal degradation by the S-phase-associated protein 2 (SKP2) in a complex with cyclin A/E and CDK2 (36, 37). p21 blocks dNTP biosynthesis in monocyte-derived macrophages and dendritic cells by downmodulating the expression of the RNR2 subunit of ribonucleotide reductase, which is essential for the reduction of ribonucleotides to deoxynucleotides (23, 25, 38). On the other hand, the ability of p21 to inhibit cyclin/CDK activities likely regulates SAMHD1 antiviral activity by prohibiting the cyclinA/CDK1/2 phosphorylation of SAMHD1 at T592 (21, 22, 30, 39), suggesting that p21 regulates both *de novo* dNTP synthesis and the antiviral function of SAMHD1 (23, 39, 40).

Ubiquitin-like specific protease 18 (USP18, UBP43) is a cysteine protease that cleaves ISG15 (interferon-stimulated gene 15, a 17-kDa protein) from its conjugated targets. USP18 exerts both protease-dependent and -independent functions to balance immune responses in disease and nondisease states (41–46). USP18 is induced by IFNs, lipopolysaccharide, and viral infections and can modulate type I IFN responses (42). It acts as a negative regulator of NF-κB (nuclear factor "kappa-light-chain-enhancer" of

activated B cells) activation by inhibiting ubiquitination of TAK1 (TGF-β-activated kinase 1) and NEMO (NF-κB essential modulator) (47, 48). USP18 binds to IFNAR2 (IFN receptor 2) and, in an isopeptidase-independent manner, blocks IFN signaling by disrupting IFNAR2-JAK (Janus-activated kinase) binding (42, 49). In the absence of free ISG15, SKP2 promotes USP18 ubiquitination and degradation by the proteasome (50, 51).

Experimental knockout of USP18 enhances JAK/STAT (signal transducer and activator of transcription) signaling and increases ISGs with elevated levels of protein ISGylation, thus providing resistance to viral infections (3, 42, 43, 52). Recent work by Honke et al. (49) showed in 2012 that high expression of USP18 in murine CD169$^+$ macrophages in the splenic marginal zone was required to enforce a local replication of vesicular stomatitis virus (VSV), a negative-sense, single-stranded enveloped RNA virus belonging to the family *Rhabdoviridae*. The enforced viral replication was essential to provide adequate antigens for stimulation of a robust adaptive immunity to control the cytopathic virus infection (49). CD169$^+$ macrophages and dendritic cells are known targets of HIV-1 (53); we therefore sought to determine whether human USP18 might be a factor that influences HIV-1 replication in macrophages by using the monocyte-derived macrophage cell line THP-1 as a model.

RESULTS

USP18 is HIV-1 inducible, and its expression enhances viral replication in differentiated THP-1 cells. To evaluate the role of USP18 in HIV-1 infection, we generated THP-1 cells expressing USP18 at levels similar to those induced by IFN-β (Fig. 1A). HIV-1 infection upregulated USP18 expression, and this upregulation was even more robust in the presence of copackaged VPX (Fig. 1B). Expression of USP18 in undifferentiated THP-1 cells increased HIV-1 infection by up to 11-fold in the absence of type I IFN compared to the THP-1 control cells (Fig. 1C and D). The dose-dependent repression of HIV-1 by IFN-α (Fig. 1C) and IFN-β (Fig. 1D) in USP18-expressing THP-1 cells (THP-1.USP18) was significantly reduced compared to that of control cells (THP-1.Control). After PMA-induced differentiation, THP-1.USP18 and THP-1.Controls were transduced with a VPX-containing HIV-1 luciferase reporter virus produced in HEK293T cells, which had the ability to degrade SAMHD1 (Fig. 1E). In the presence of VPX, differentiated THP-1.USP18 cells showed significantly increased HIV-1 infection compared to THP-1.Control cells (Fig. 1F). Surprisingly, in the absence of VPX, USP18 overcame the SAMHD1 restriction, resulting in a $>$40-fold increase in HIV-1 infection (Fig. 1F). Thus, USP18 expression in the differentiated THP-1 cells mimicked the viral VPX function and allowed for higher infection even in the presence of increasing concentrations of IFN-α (Fig. 1G) and IFN-β (Fig. 1H) in the absence of VPX compared to the THP-1.Control cells.

USP18-mediated increase in HIV-1 infection is independent of USP18 isopeptidase activity. To test whether the USP18-dependent enhanced HIV-1 replication was a result of USP18 isopeptidase activity, we mutated the catalytic site residue, cysteine 64 to either alanine (A) or serine (S) (Fig. 2A). We tested the reactivity of USP18 and its mutants toward the catalytic core of ISG15 in an ISG15-vinyl sulfone (VS) probe. The wild-type (WT) USP18 reacted strongly with the catalytic core of ISG15, as indicated by an upward shift in the USP18 39-kDa band toward a size of about 72 kDa (Fig. 2B). As expected, the catalytic site mutants lost this enzymatic activity (Fig. 2B). However, like the WT USP18, the two active site mutants retained the capacity to enhance permissiveness to HIV-1 in undifferentiated (Fig. 2C) and differentiated (Fig. 2D) THP-1 cells. To test whether this effect of USP18 was specific to HIV-1 or general to lentiviruses, we transduced undifferentiated and PMA-differentiated THP-1.Control, WT USP18, and mutant USP18 cells with HIV-2-WT and HIV-2Δ*vpx* luciferase reporter viruses. We observed significantly higher HIV-2 infection in the undifferentiated WT and mutant USP18 cells compared to the THP-1.Control cells (Fig. 2E). As with HIV-1, both USP18 WT and mutant proteins increased HIV-2 infection in PMA-differentiated THP-1 cells by $>$7-fold (Fig. 2F) and additionally increased cell permissiveness in the presence of VPX (Fig. 2G).

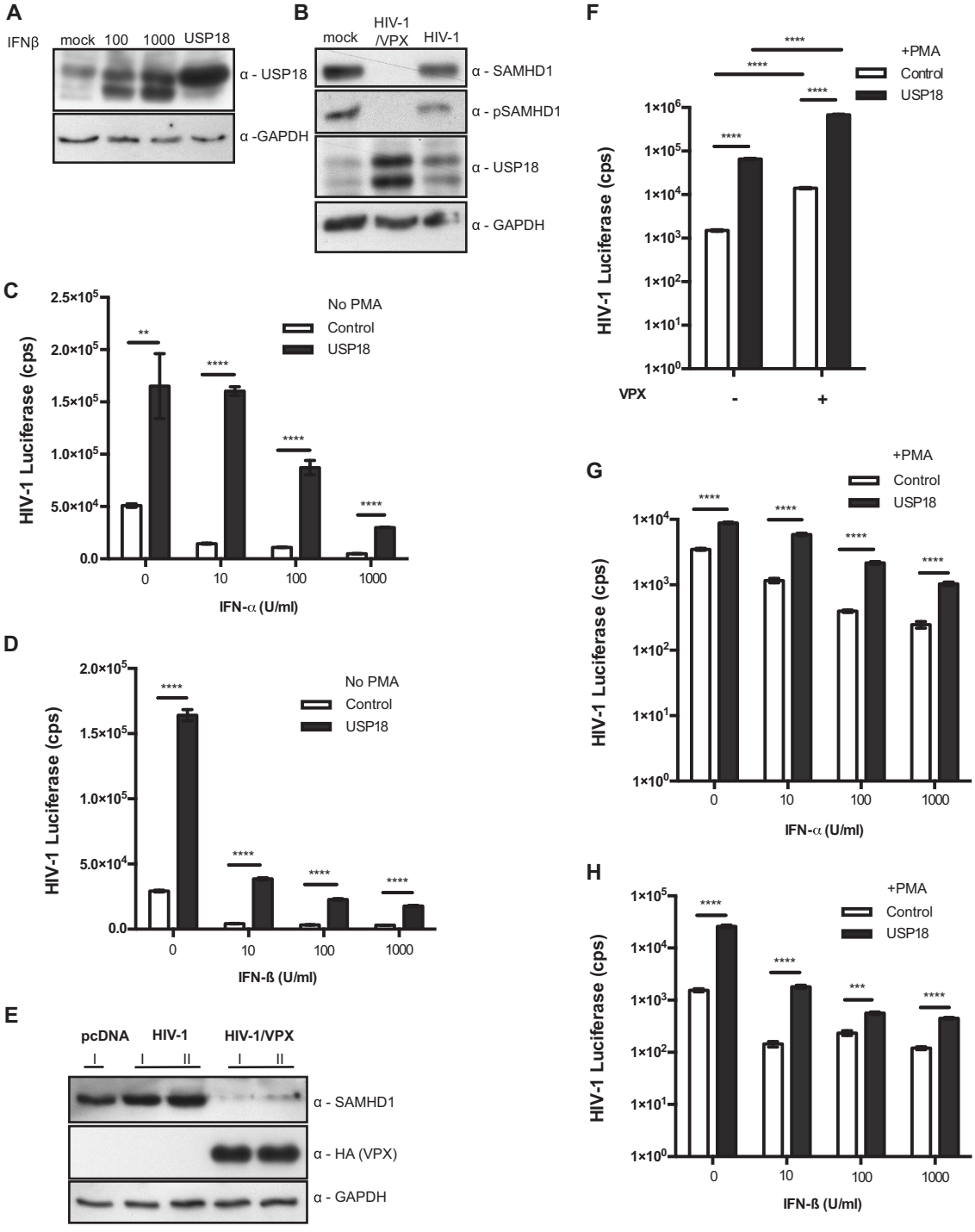

FIG 1 HIV-1 induces USP18 and USP18 expression enhances HIV-1 infection in PMA-differentiated THP-1 cells. (A) Immunoblot analysis of basal, IFN-β-induced, and stably expressing USP18 levels in THP-1 cells. (B) PMA-differentiated THP-1 cells were mock transduced (media) or HIV-1 transduced with or without copackaged VPX. At 72 h postransduction, cells were harvested and immunoblotted for endogenous total and phosphorylated SAMHD1, USP18, and GAPDH as a loading control. THP-1.USP18 and THP-1.Control cells were treated with different concentrations of IFN-α (C) and IFN-β (D) for 4 h and subsequently transduced with single-round HIV-1 luciferase reporter virus (E). At 72 h postransduction, the luciferase activity was measured. (F) In a related experiment, these cells were PMA differentiated for 48 h and subsequently transduced with HIV-1 luciferase reporter virus with or without the copackaged VPX produced in panel E, which had the ability to deplete SAMHD1. The same experiment was reproduced in the presence of different concentrations of IFN-α (G) and IFN-β (H). Mean differences of three replicates for each group in a single experiment was analyzed and compared between the groups by using a Student *t* test, with results expressed as means $\pm$ the SD. A *P* value of <0.05 was considered statistically significant (*). The higher the number of asterisks, the lower the *P* value. Each panel is representative of at least three independent experiments.

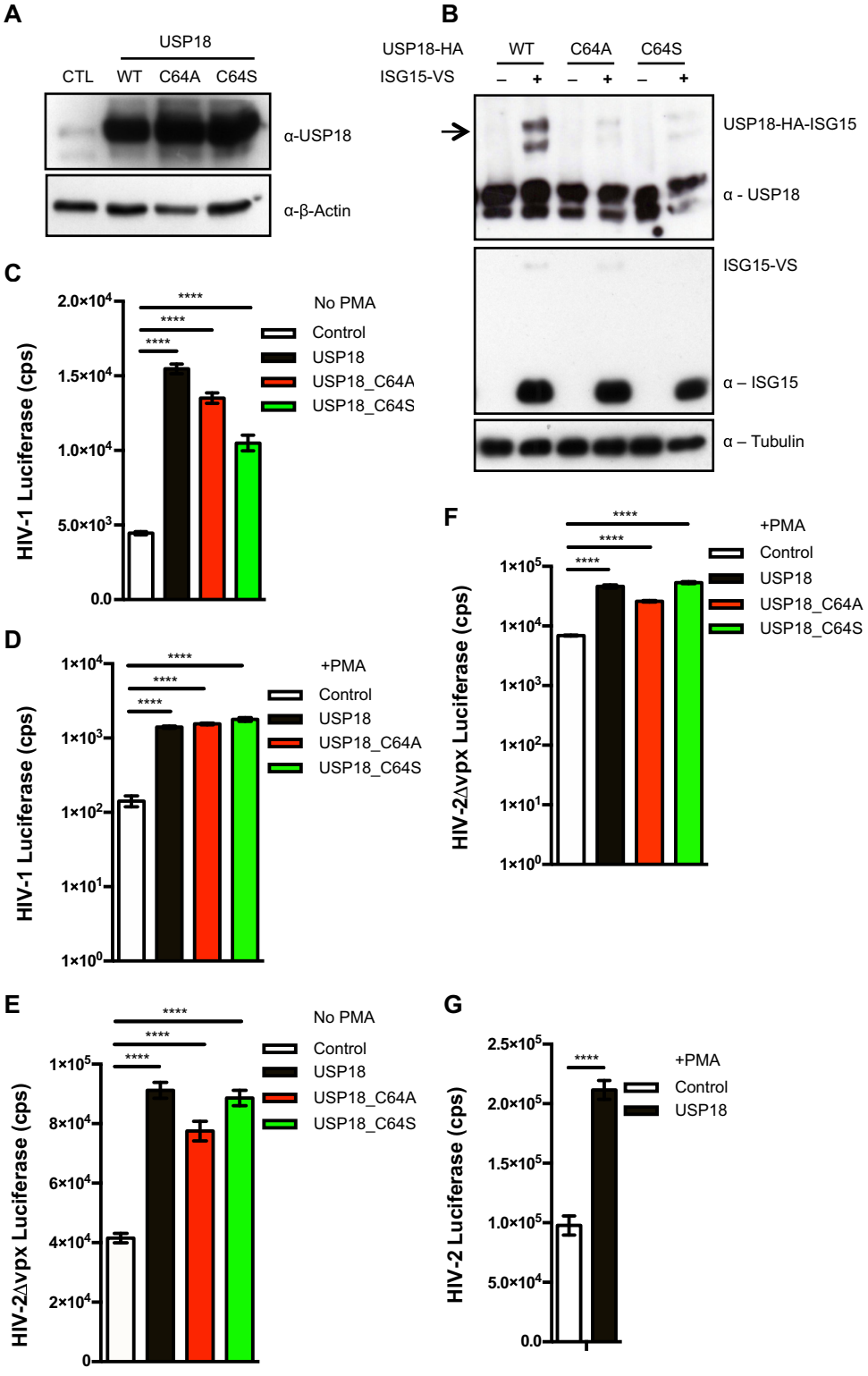

FIG 2 USP18 enhancement of HIV-1 infection is independent of USP18's isopeptidase activity. (A) Immunoblot of endogenous USP18 in THP-1 cells stably expressing pLOC empty vector control (CTL), WT USP18, and active site mutants (C64A, C64S), where the cysteine (C) at position 64 was mutated to alanine (A) or serine (S). (B) HA-tagged WT USP18 and its mutants were overexpressed in HEK293T cells, and protein lysates were incubated with ISG15-VS probe. Isopeptidase activity of USP18 toward ISG15 was revealed by the ISG15-VS adduct formation, which was detected by an upward shift in the USP18 band after immunoblotting with anti-USP18 antibody. THP-1.Control, THP-1.USP18 and its mutants (C64A, C64S) were transduced with a single round of HIV-1 luciferase reporter virus in an undifferentiated state (C) and in a PMA-differentiated state (D). The luciferase activity was measured at 72 h

(Continued on next page)

USP18 downregulates p21, which induces SAMHD1 phosphorylation in PMA-differentiated THP-1 cells. Recent evidence suggests that the antiviral activity of SAMHD1 is positively regulated by p21 via inhibition of the cyclin and CDK complex that inactivates SAMHD1 by phosphorylation (22, 23, 39, 40). To understand the molecular mechanism behind the USP18-mediated enhancement of HIV-1 replication, we tested the expression levels of p21 in undifferentiated and PMA-differentiated THP-1 cells in the presence or absence of type I IFN, which is a known inducer of p21 (21, 54, 55). Remarkably, USP18 induced a downregulation of the p21 protein in PMA-differentiated THP-1 cells (Fig. 3A), which could not be rescued by IFN (Fig. 3A and B). The expression levels of cyclin D1 (Fig. 3A) and cyclin D2 (Fig. 3B) appeared elevated in the THP-1.USP18 cells. IFN-β induced significantly higher levels of both cyclins in the PMA-differentiated THP-1.Control cells but no further increase in the THP-1.USP18 cells (Fig. 3A and B). We further tested the phosphorylation status of SAMHD1 in cycling and noncycling THP-1 cell lines. In the absence of PMA treatment, THP-1.Control, WT USP18, and mutant USP18 cells expressed similar levels of total and phosphorylated SAMHD1 (Fig. 3C and D). However, the phosphorylation signal of SAMHD1 almost disappeared in PMA-differentiated THP-1.Control cells (Fig. 3C and D) but was retained in the THP-1.USP18 cells (Fig. 3C and D), although the level was lower than in the nondifferentiated cells (Fig. 3C). The increase in phosphorylated SAMHD1 in the differentiated THP-1 cells was independent of the USP18 isopeptidase activity, as phosphorylated SAMHD1 was also increased in the THP-1.USP18.C64A and THP-1.USP18.C64S cells (Fig. 3C). A subsequent probe for interaction partners of p21 and SAMHD1, including cyclin A, SKP2, CDK2, and CDK4 (21, 29, 56), demonstrated similar levels of CDK2 (Fig. 3C) and CDK4 (Fig. 3D) but upregulated levels of cyclin A and SKP2 in the differentiated WT USP18 (Fig. 3C and D) and mutant USP18-expressing (Fig. 3C) THP-1 cells compared to the THP-1.Control cells. In contrast, the cycling cell lines expressed similar levels of these proteins (Fig. 3C and D). SAMHD1 activity is tightly controlled during the cell cycle (24, 27). Considering that p21 is regulated in a cell cycle-dependent manner and in turn regulates the SAMHD1 antiviral function, we analyzed the cell cycle status of our cell lines expressing USP18 by staining the DNA with propidium iodide. As expected, a significant population of the nondifferentiated cells was in the S phase (Fig. 3E) (24). However, treatment of the cells with PMA shifted the population toward the G_0/G_1 and G_2/M phases, with a conspicuously reduced S-phase population. No differences in the cell cycle populations were detected between the THP-1.USP18, mutant USP18, and control cells regardless of their differentiation status (Fig. 3E).

USP18 complexes with SKP2, cyclin A, CDK1, CDK2, and SAMHD1. To determine whether USP18 binds to SKP2 and other interacting partners of p21 (57), we tested the binding of SKP2 and SAMHD1 to USP18. HEK293T cells were either singly transfected or cotransfected with plasmids expressing hemagglutinin (HA)-tagged USP18, MYC-tagged SKP2, and FLAG-tagged SAMHD1. Cell lysates were immunoprecipitated with anti-HA beads and subsequently immunoblotted with anti-HA, anti-MYC, and anti-FLAG antibodies. The pulldown of USP18 precipitated SAMHD1 (Fig. 4A, lane 2), SKP2 (Fig. 4A, lane 3), and all three proteins complexed together (Fig. 4A, lane 4). Immunoprecipitation of SAMHD1 with anti-FLAG beads also pulled down SKP2 (Fig. 4A, lane 7), consistent with the observation of St Gelais et al. in 2014 (56). In a subsequent cotransfection of FLAG-SAMHD1 and pLOC-USP18, the cell lysates were immunoprecipitated with anti-FLAG beads and immunoblotted to detect the presence of endogenous cellular cyclin A2, CDK1, and CDK2 (Fig. 4B and C). The pulldown of SAMHD1 also precipitated USP18

FIG 2 Legend (Continued)
postransduction. In a related experiment, these cells were transduced with HIV-2Δ*vpx* in an undifferentiated state (E) and a differentiated state (F). (G) Similarly, the PMA-differentiated cells were transduced with HIV-2 luciferase reporter virus, which had an active VPX to degrade SAMHD1. Mean differences of three replicates for each group in a single experiment was analyzed and compared between the groups by Student *t* test, expressed as means ± the SD. A *P* value of <0.05 was considered statistically significant (*). The higher the number of asterisks, the lower the *P* value. Each panel is representative of at least three independent experiments.

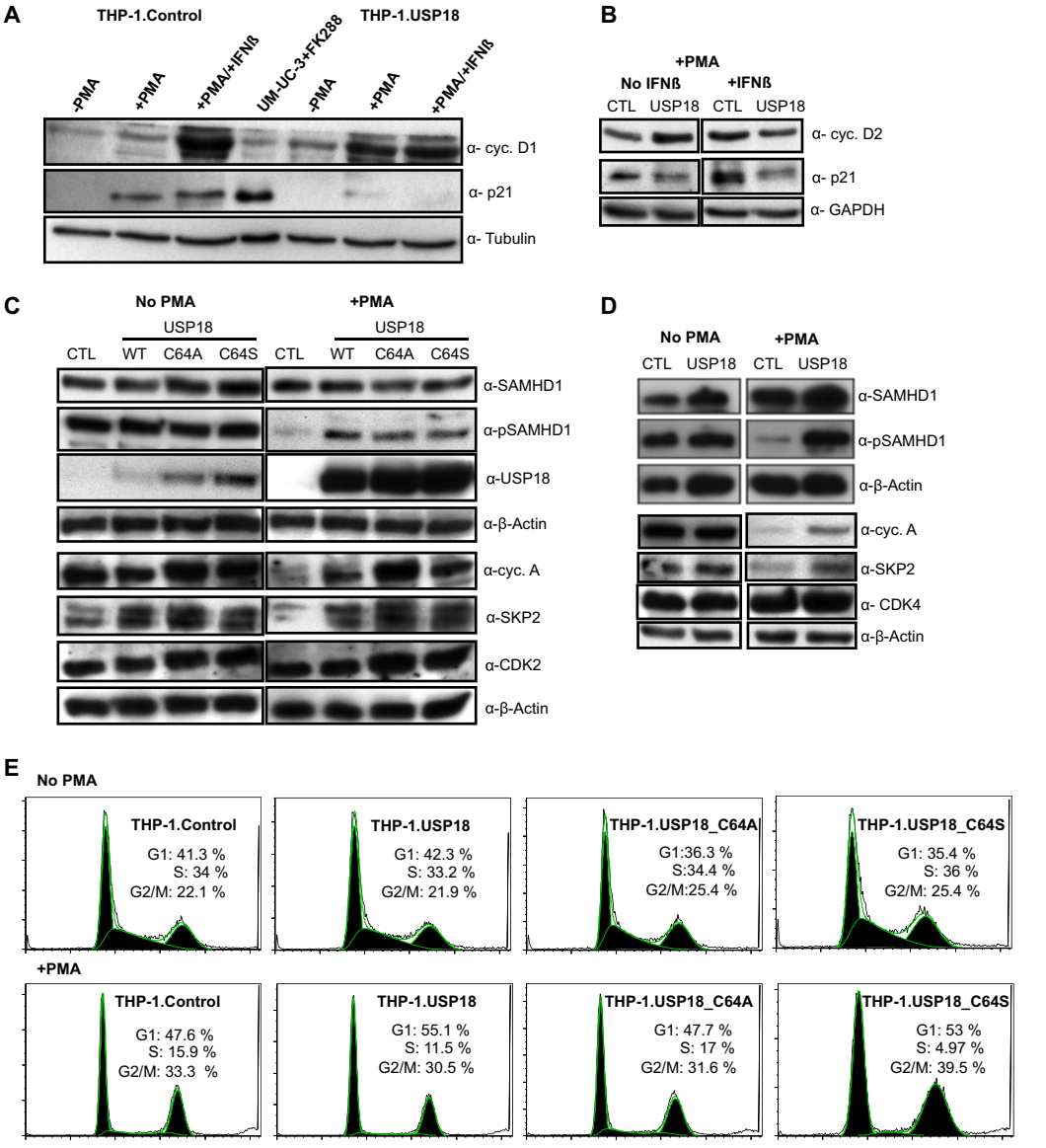

FIG 3 USP18 downregulates p21 expression, which induces phosphorylated SAMHD1 in PMA-differentiated THP-1 cells. PMA-differentiated and undifferentiated THP-1 control and THP-1.USP18 cells were stimulated with 1,000 U/ml of IFN-β. At 48 h posttreatment, the cells were lysed and immunoblotted for p21, cyclin D1 (A), and cyclin D2 (B) with tubulin or GAPDH as a loading control. Romidepsin (FK288)-induced p21 in a cancer cell line (UM-UC-3) was included in the experiment as a positive control. (C) Immunoblots of total and phosphorylated SAMHD1, USP18, cyclin A, SKP2, and CDK2 in PMA undifferentiated and differentiated THP-1.Control cells (CTL), WT cells, and mutant THP-1.USP18 cells with β-actin as a loading control. (D) An immunoblot of CDK4 was included in subsequent experiments. For clarity and conciseness of data presentation, blots of different proteins on the same membranes detected with different antibodies were cropped and juxtaposed. Each panel is representative of at least three independent experiments. (E) Cycling and noncycling THP-1.Control, THP-1.USP18, and active-site mutant (C64A and C64S) cells were fixed, treated with RNase A, and then stained thoroughly with propidium iodide and analyzed with flow cytometry. The percentages of cells in the G_1, S, and G_2/M phases were quantified by FlowJo. Each panel is representative of at least three independent experiments.

and endogenous cyclin A2, CDK1 (Fig. 4B), and CDK2 (Fig. 4C), suggesting a possible complex comprising all five proteins.

Presence or absence of USP18 regulates the level of p21. To further evaluate the mechanism of the USP18-mediated increase in HIV-1 replication, we knocked out USP18 in THP-1 cells by the CRISPR-Cas9 system (see Materials and Methods) and immunoblotted the PMA-differentiated cells for p21, total and phosphorylated SAMHD1, USP18, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the loading control (Fig. 5A). USP18KO cells contained significantly upregulated p21 pro-

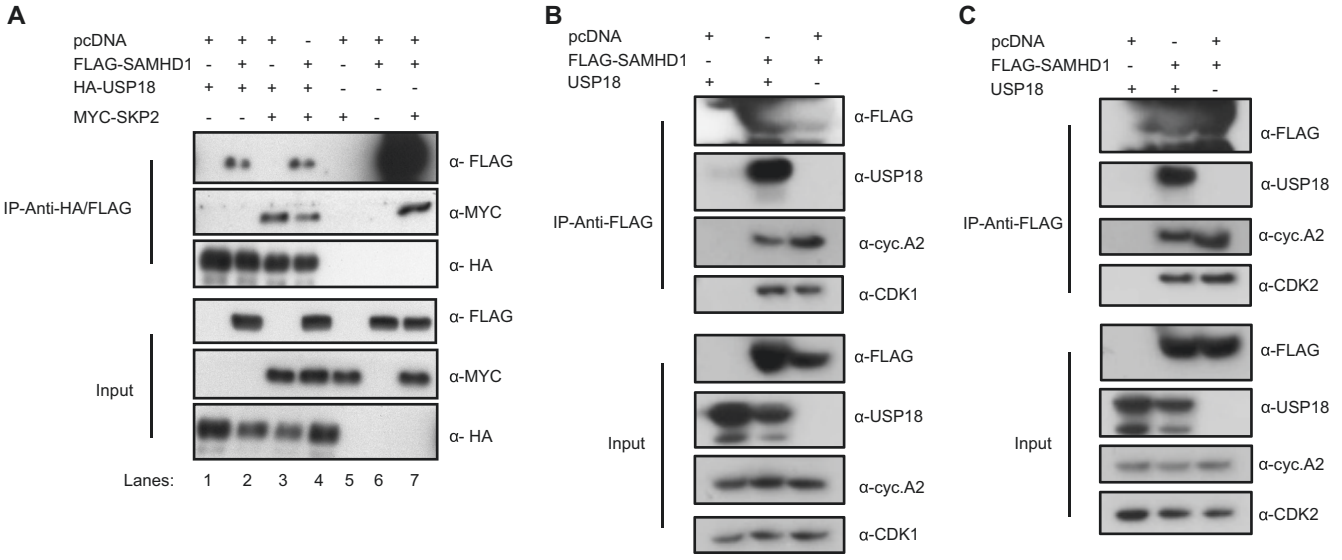

FIG 4 USP18 interacts with SKP2 and SAMHD1 and complexes with cyclin A2, CDK1, and CDK2. (A) Immunoprecipitation of HA-USP18, coexpressed with FLAG-SAMHD1 (lane 2), MYC-SKP2 (lane 3) or all three expression plasmids in a ratio of 1:1:1 (lane 4) using anti-HA affinity matrix beads. In parallel, FLAG-SAMHD1 was coexpressed with MYC-SKP2 and immunoprecipitated with anti-FLAG affinity matrix beads (lanes 7). Single and double transfections were supplemented with pcDNA3.1(+). Cell lysates were immunoblotted for USP18, SKP2, and SAMHD1, using their respective epitopped tags. (B) Immunoprecitation of FLAG-SAMHD1 coexpressed with pLOC-USP18 or without pLOC-USP18 and replaced by pcDNA3.1+. Proteins of HEK293T cells were immunoprecipitated with anti-FLAG affinity matrix beads and immunoblotted with FLAG and USP18 and endogenous expression of cyclin A2 and CDK1, cyclin A2 (B), and CDK2 (C). Each panel is representative of at least three independent experiments.

tein levels, which could be further enhanced by IFN-β treatment (Fig. 5A, lanes 3 and 4, and Fig. 5C). The highly induced p21 protein correlated significantly with reduced phosphorylated form of SAMHD1 to a level similar to exogenous IFN-β-induced dephosphorylated SAMHD1 (Fig. 5A, compare lanes 2 and 3) (21). The increased p21 protein levels also correlated significantly with diminished HIV-1 infection in the USP18KO THP-1 cells (Fig. 5B). In contrast, SKP2 protein levels were unchanged in the USP18KO THP-1 cells (Fig. 5C).

To understand how p21 downregulation by USP18 might affect HIV-1 replication in the absence of SAMHD1, we obtained SAMHD1KO THP-1 cells (58), and stably expressed USP18 or a vector control in these cells (Fig. 5D). The PMA-differentiated SAMHD1KO THP-1 cell lines were then immunoblotted for p21, total SAMHD1, USP18, and GAPDH as the loading control. USP18 overexpression significantly reduced p21 protein levels as seen before. The cells were further tested for HIV-1 infection following PMA differentiation. SAMHD1KO THP-1 cells demonstrated significantly enhanced levels of HIV-1 replication compared to wild-type THP-1 cells (Fig. 5E, compare white and black bars). The infection was further enhanced by USP18 in the absence of SAMHD1 by more than 100-fold (Fig. 5E, compare green and red bars). The significantly reduced p21 protein levels in the USP18 overexpressed SAMHD1KO THP-1 cells correlated strongly with upregulated SKP2 protein levels (Fig. 5F).

USP18 enhances HIV-1 replication at the reverse transcription step. To estimate the frequencies of HIV-1-infected THP-1.USP18 and control cells in a PMA-differentiated state, we generated USP18-expressing cells that lacked GFP expression by retroviral transduction compared to the lentiviral pLOC vector (Fig. 6A). This allowed us to infect the cells with an HIV-1.IRES-GFP reporter virus and to quantify the percentage of green fluorescent protein (GFP)-expressing cells (Fig. 6B). Flow cytometric analysis showed significantly increased frequencies of GFP^+ cells (6-fold) in the THP-1.USP18 cells compared to the vector control cells (Fig. 6B and C). To rule out a block at the membrane fusion step, we tested the HIV-1 VSV-G pseudotype fusion using a virion-based fusion assay as described previously (59, 60). Flow cytometric analysis allowed us to quantify the proportion of cells whose membranes were successfully fused by the

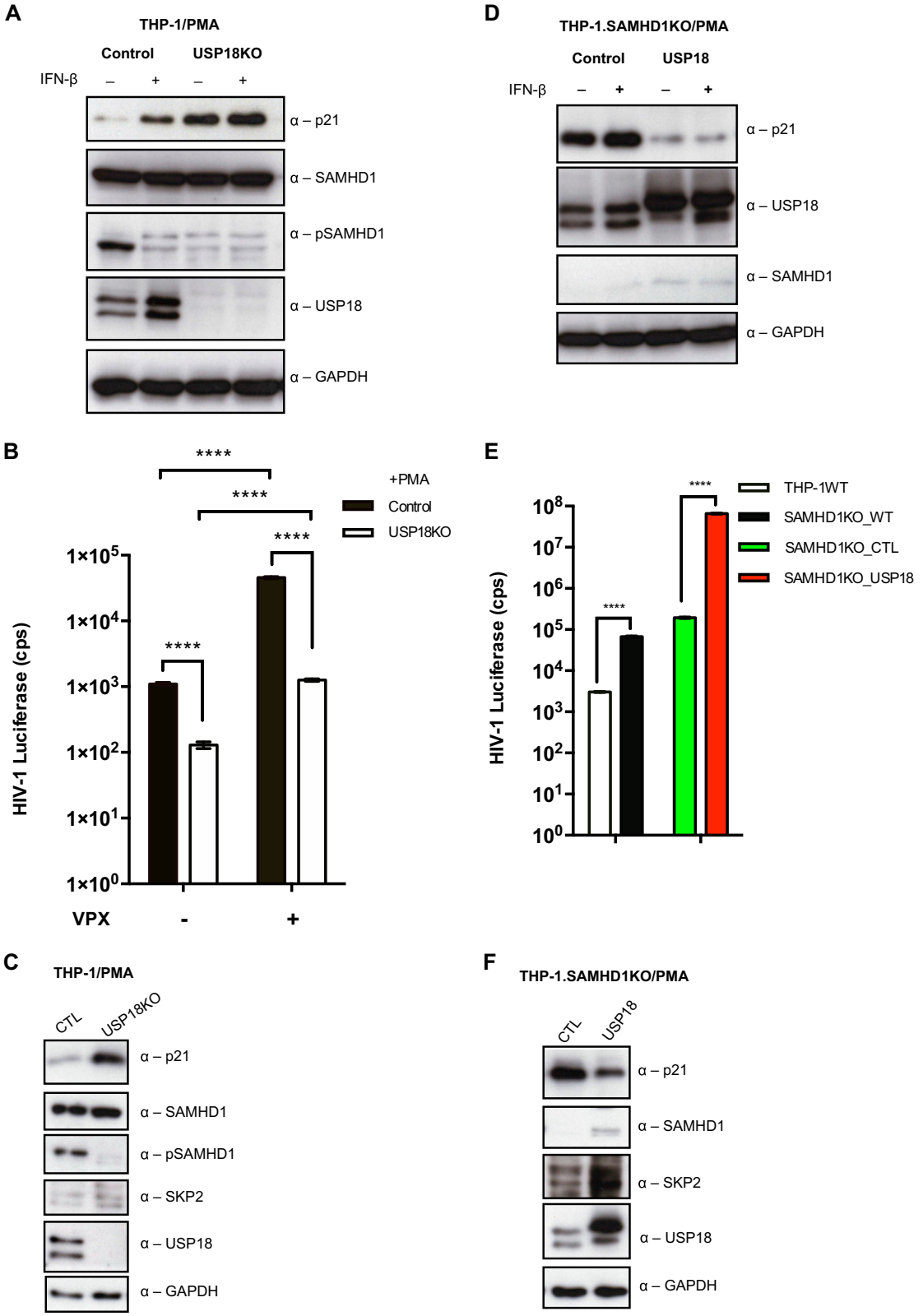

FIG 5 The presence or absence of USP18 regulates the level of p21. (A) PMA-differentiated USP18KO THP-1 cells and vector control cells were stimulated with 1,000 U/ml of IFN-β. At 24 h after stimulation, the cells were lysed and immunoblotted for the endogenous expression of p21, total and phosphorylated SAMHD1, USP18, and GAPDH as a loading control. (B) After 48 h of PMA treatment, the cells were also tested for HIV-1 replication and transduced with HIV-1 reporter virus in the presence or absence of copackaged VPX. (C) In a related experiment, PMA-differentiated cells USP18KO and control cells were treated with PMA and subsequently immunoblotted for p21, total, and phosphorylated SAMHD1, SKP2, USP18, and GAPDH as a loading control. (D) PMA-differentiated SAMHD1KO

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virus (Fig. 6D). No significant difference in virion fusion was observed between the THP-1.USP18 and control cells in both PMA-undifferentiated (Fig. 6E) and differentiated (Fig. 6F) states. In parallel, we tested these cells for luciferase activity 48 h postinfection with HIV-1 NL-LucR$^-$ E$^-$ reporter viruses. As expected, THP-1.USP18 cells demonstrated higher infection than the control cells (Fig. 6G). To test whether USP18 could relieve the block at the reverse transcription step in PMA-differentiated cells, we quantified the early and late reverse transcription products 12 h postinfection in the presence and absence of the antiretroviral drug nevirapine, a nonnucleoside reverse transcriptase inhibitor. Interestingly, the THP-1.USP18 cells contained significantly increased levels of early (Fig. 6H) and late (Fig. 6I) reverse transcription products compared to the vector controls.

USP18 upregulates intracellular dNTPs. p21 induction is known to reduce the intracellular dNTP pool by repressing key enzymes involved in *de novo* dNTP biosynthesis (23, 25, 34, 35, 39, 40). Because USP18 significantly downregulated p21 expression (Fig. 3A and 5D), we evaluated the impact of USP18 expression on the intracellular dNTP pool. Interestingly, dATP and dGTP levels were significantly upregulated in USP18-expressing THP-1 cells compared to controls (Fig. 7A) and, even more interestingly, USP18 significantly upregulated all four intracellular dNTPs in USP18-overexpressing SAMHD1KO THP-1 cells (Fig. 7B) compared to SAMHD1KO THP-1 cells without overexpression (Fig. 7B). Indeed, we could rescue the SAMHD1 block to HIV-1 replication by supplementing the differentiated THP-1 cells with deoxynucleosides (Fig. 7C).

DISCUSSION

USP18 is expressed at different levels in many mammalian tissues, including the liver, spleen, and thymus (41, 61). In addition, high expression is found in several innate immune cells, such as murine CD169$^+$ macrophages, bone marrow-derived dendritic cells, peritoneal macrophages, monocyte-derived macrophages, and white matter microglia, and it is differentially regulated during T-cell activation (41, 45, 47, 49, 61, 62). USP18 has been implicated in the innate immunity against bacteria and viruses (43, 44, 49, 52, 62). Our findings show that USP18 can be induced by HIV-1, an observation that has recently been independently confirmed in HIV-1-infected monocyte-derived macrophages (3). The expression of USP18 allowed for enhanced replication of HIV-1, HIV-2, and SIVmac (data not shown), enabling infection in noncycling THP-1 cells even in the presence of the potent restriction factor SAMHD1. The observed positive effect of USP18 is associated with the downregulation of the CDK inhibitor p21. USP18 overcame the inhibitory effect of p21 at the HIV-1 reverse transcription step likely by relieving the p21 block of SAMHD1 phosphorylation and by rescuing the p21-dependent repression of key enzymes of *de novo* dNTP biosynthesis (23, 25, 40). Thus, USP18 increased the supply of dNTPs for reverse transcription to take place. Moreover, the enhancement of HIV-1 infection by USP18 appeared to be independent of its isopeptidase activity, suggesting that de-ISGylation is not involved in overcoming the inhibitory effect of p21.

p21 is known as an important factor regulating cell growth, monocyte differentiation, survival, and maturation (63–65). Notably, its dysregulation is common in many cancers (57, 66). Many recent reports have highlighted HIV-1 inhibition by p21, which is likely achieved by its ability to regulate *de novo* dNTP biosynthesis and the cyclin-dependent kinases (CDKs) required for cell cycle progression (22, 23, 25, 34, 38–40, 54, 55, 63–65, 67–69). Despite the importance and the physiological relevance of the p21 protein, the mechanisms and the cellular factors required for the regulation of p21 *in vivo* are only partly understood. It is thought that p21 is regulated transcriptionally both

FIG 5 Legend (Continued)
THP-1 cells expressing the vector control and USP18 were immunoblotted for p21, USP18, SAMHD1, and GAPDH as a loading control. These cells were treated with 25 ng/ml PMA. (E) At 48 h posttreatment, the cells were transduced with a single round of HIV-1 reporter virus, and the luciferase activity was measured after 72 h. (F) In a related experiment, the cells were tested for SKP2 protein expression levels by immunoblotting the PMA-differentiated vector control and USP18 expressing SAMHD1KO THP-1 cells for p21, SAMHD1, SKP2, USP18, and GAPDH as a loading control. Each panel is representative of at least two independent experiments.

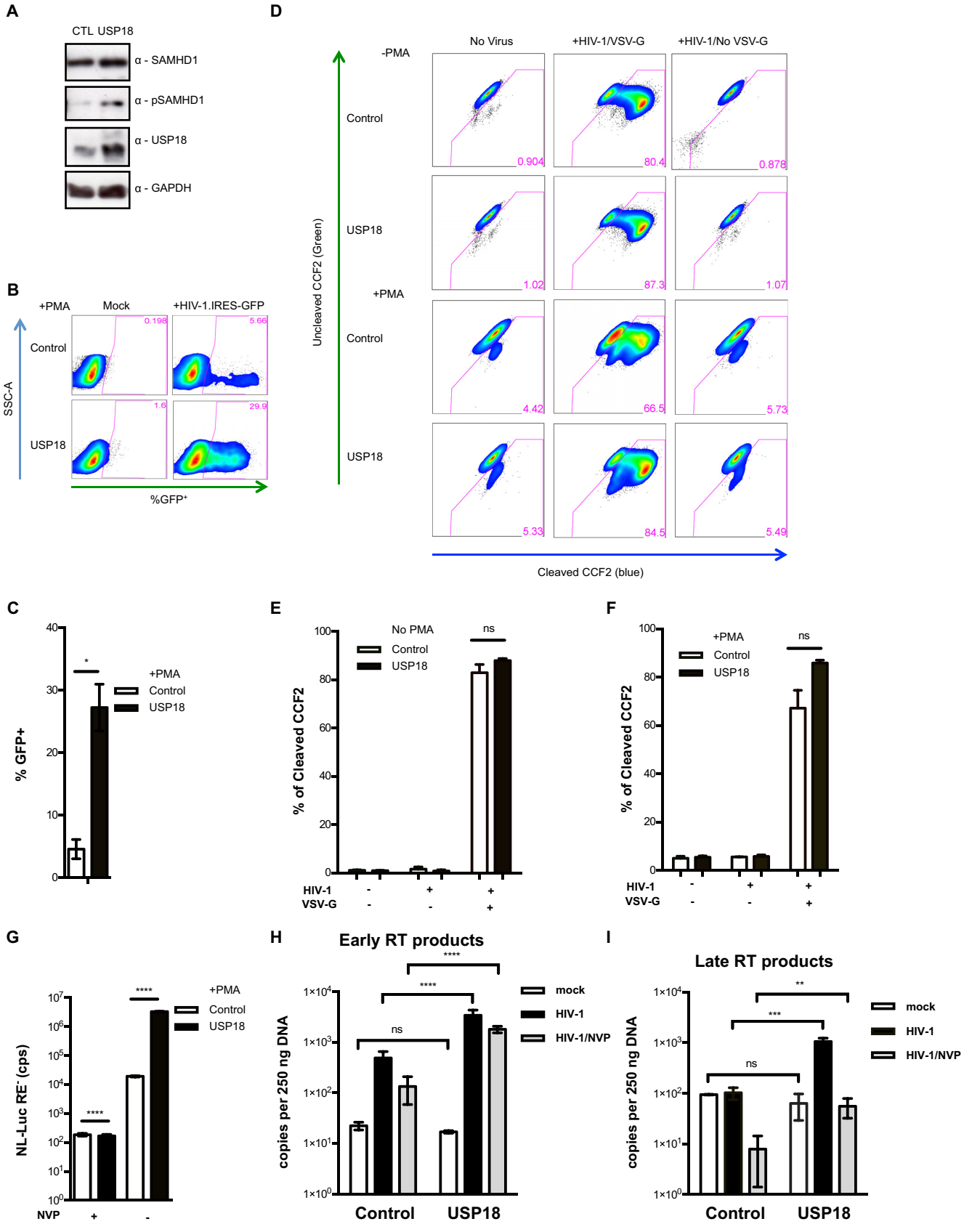

FIG 6 USP18 enhancement of HIV-1 infection occurs at the reverse transcription step. (A) Immunoblot of total and phosphorylated SAMHD1, USP18, and GAPDH as a loading control in retrovirus-based USP18 expression in THP-1 cells and controls. (B) Representative gating strategy for the frequencies of

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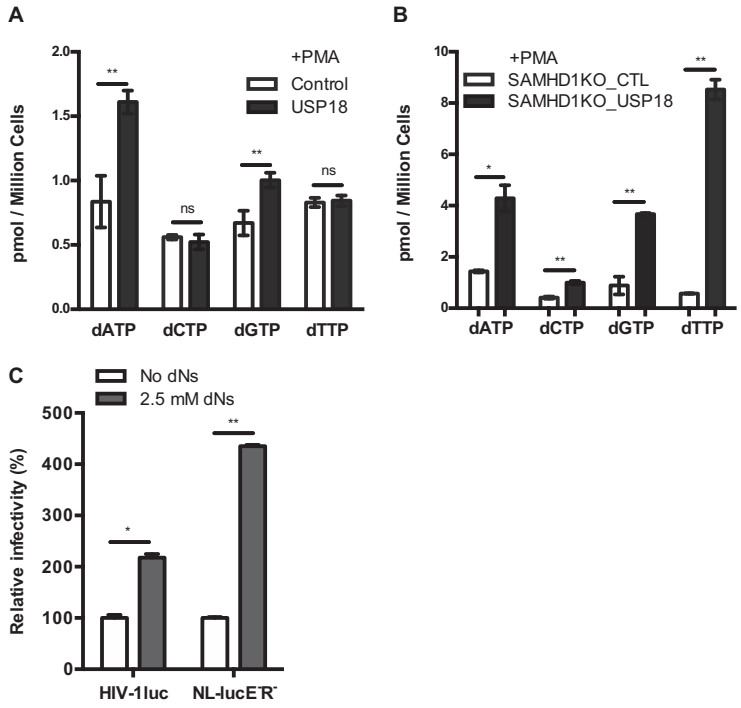

FIG 7 USP18 enhances HIV-1 replication by upregulating intracellular dNTP concentrations. (A) The intracellular levels of dATP, dCTP, dGTP, and dTTP were quantified by a single nucleotide primer extension assay in differentiated THP-1.Control, THP-1.USP18, SAMHD1KO_CTL, and SAMHD1KO_USP18 cells. Mean differences of three replicates for each dNTP level in a single experiment were analyzed and compared between THP-1.Control and THP-1.USP18 (A) cells, as well as between SAMHD1KO_CTL and SAMHD1KO_USP18 (B) cells, by a Student *t* test, and are expressed as means ± the SD. A *P* value of <0.05 was considered statistically significant (*). The higher the number of asterisks, the lower the *P* value. Each panel is representative of at least three independent experiments. (C) PMA-differentiated THP-1 cells were treated with 2.5 mM dNs and transduced with HIV-1 luciferase reporter virus (HIV-1luc) from the three-plasmid system (see Materials and Methods) and from the NL-LucE$^-$R$^-$ construct. At 48 h postinfection, the intracellular luciferase activity was measured. The values (i.e., for the luciferase activity) obtained in cells untreated with dNs were set as 100%, and the viral gene expression from cells treated with dNs was calculated relative to the untreated cells. The data are representative of two independent experiments.

in a p53-dependent and -independent manner; the p53-dependent manner is mediated by phosphorylation and also likely by ISG15 modification (36, 38, 57, 66, 70). p21 also undergoes extensive posttranslational regulation (36, 37, 57). In actively dividing cells, p21 is a highly unstable protein with a half-life of about 20 to 60 min (37, 57). G_1/S- and S-phase transition of the cell cycle requires ubiquitin-dependent degradation of p21, mediated by the E3 ubiquitin ligase complex substrate recognition factor SKP2, which promotes polyubiquitylation of p21 in a complex with CDK2 and cyclin E or A to initiate its proteasomal degradation (36, 57). However, another ubiquitin-independent proteolysis of p21 has been postulated, which may occur in a cell type-dependent manner (57, 71–73).

Here, we demonstrate that USP18 might be involved in the regulation of p21 protein

FIG 6 Legend (Continued)
GFP-expressing, PMA-differentiated THP-1.USP18 and control cells, transduced with HIV-1.IRES.GFP reporter virus at an MOI of 2 for 72 h. (C) The mean (n = 2) difference in the percentage of GFP$^+$ cells between the groups was analyzed by a Student *t* test. (D) Representative gating strategy for the frequencies of cleaved CCF2$^+$ (blue) in PMA-undifferentiated and differentiated THP-1.Control and USP18-expressing cells were transduced with HIV-1 pseudotyped virus with copackaged β-lactamase-Vpr with or without VSV-G. The mean (n = 3) difference in percentage cleaved CCF2$^+$ cells between the groups in an undifferentiated state (E) and a differentiated state (F) was analyzed by using a Student *t* test. (G) PMA-differentiated THP-1.USP18 and vector controls were transduced with HIV-1 NL-LucR$^-$E$^-$. At 48 h postransduction, the cells were measured for luciferase activity. Values are means ± the SD for three independent infections. Asterisks represent statistically significant differences (***, $P < 0.0001$). Similarly, cells were infected or uninfected (mock = media) in the presence or absence of nevirapine (NVP) for the quantification of early (H) and late (I) RT products by qPCR. At 12 h postransduction, the DNA of infected cells was isolated, and HIV-1 reverse transcripts were quantified by real-time qPCR. Each panel is representative of at least two independent experiments.

expression. The expression of USP18 dramatically diminishes p21 protein levels, which cannot be rescued by either PMA or type I IFN. On the other hand, the absence of USP18 stabilizes p21 protein and enhances its anti-HIV activity. How USP18 mediates p21 downregulation is currently not clear. Our data appear to exclude the involvement of ISG15 and rather support a mechanism involving SKP2-dependent regulation of p21 protein. USP18 likely recruits or retains SKP2 in an environment in which USP18 cannot be degraded by the proteasome due to the presence of free ISG15 (50, 51). By retaining SKP2, USP18 likely primes p21 ubiquitylation by SKP2, facilitating its degradation by the proteasome. The degradation of p21 thus retains cyclin A/CDK2 to phosphorylate SAMHD1 at residue T592 and potentially activates a *de novo* dNTP biosynthesis pathway in the differentiated THP-1 cells.

Overall, the identification of a novel function of USP18 in abrogating the antiviral activites of p21 underscores the importance of USP18 in the innate immune cells. *In vivo*, constitutive and HIV-1-induced expression of USP18 in innate target cells could potentially facilitate the replication of the virus and help it to escape the innate immune restriction mediated by p21. Further investigation to understand the mechanism of USP18-mediated downregulation of p21 and how this likely affects *de novo* dNTP biosynthesis is warranted and should be helpful for the design of better therapeutics for the control of HIV-1 replication in innate immune cells.

MATERIALS AND METHODS

Plasmids. The human USP18 open reading frame (ORF) was cloned into the pLOC lentiviral vector containing turbo GFP and blasticidin S resistance (Thermo Fisher Scientific, Inc., Darmstadt, Germany), using the NheI and SpeI restriction sites. An empty vector control was obtained by excising the ORF using the same sites and religating. The USP18 cDNA was alternatively cloned into the retroviral vector pMSCVneo (74) using the HpaI and XhoI restriction sites. All vector constructs were verified by sequencing and tested for USP18 protein expression. HIV-1 vector, pSIN.PPT.CMV.Luc.IRES.GFP (75, 76), HIV-2 virus containing HIV-2 construct (pHIV-2D4), and pHIV-2 Luc SV40 (previously called pHIV-2 SEW Luc SV40) have been described before (76). The HIV-1 construct psPAX2 was obtained from the NIH, AIDS Reagent Program repository. pRSV-Rev (77) and pMDLg/pRRE and pMD.G (77) have been described previously. HIV-1 NL-LucR$^-$ E$^-$, pMDLx g/pRRE, HIV-2$_{ROD}$, and SIVmac$_{239}$ VPX, cloned into pcDNA6/myc-His (Invitrogen/Life Technologies, Germany), were obtained from Nathaniel R. Landau (78). pHIT60 was kindly provided by Jonathan Stoye (79). The active site of human USP18 was mutated by site-directed mutagenesis from cysteine (C) 64 to alanine (A) or serine (S) to obtain pLOC-USP18-C64A or pLOC-USP18-C64S plasmids. HA- and V5-epitope-tagged-human USP18 were cloned into pcDNA3.1(+) at the HindIII and NotI restriction sites to obtain pcDNA3.1-HA-USP18 and pcDNA3.1-V5-USP18. C-terminal HA-tagged USP18 or V5-tagged USP18 was cloned into pLOC-empty vector at the NheI and SpeI restriction sites to obtain pLOC-HA-USP18 and pLOC-V5-USP18 plasmids.

Cell culture. THP-1 cells (ATCC TIB-202) (80) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (PAN-Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. HEK293T cells (ATCC CRL-3216) (81, 82), HOS cells (ATCC CRL-1543) (83), and TZM-bl cells (NIH, AIDS Reagent Program) (84–88) were maintained in Dulbecco modified Eagle complete medium (Biochrom, Berlin, Germany) supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cell lines were generated by transduction of THP-1 cells with lentiviral vectors made by cotransfection of pLOC-USP18, pLOC-USP18-C64A, pLOC-USP18-C64S, or pEV, together with psPAX2, pRSV-Rev, and pMD.G in HEK293T cells. Viral particles were layered on 2 ml 20% (wt/vol) sucrose, concentrated by ultracentrifugation at 284,061 $\times$ *g* (SW-41 rotor; Beckman Coulter, Krefeld Germany) for 2 h at 4°C and resuspended in RPMI. The cells were spinoculated at 1200 $\times$ *g* for 2 h at 30°C and selected using blasticidin S hydrochloride (Sigma-Aldrich, Taufkirchen, Germany). Blasticidin S-resistant cell pools were tested for GFP protein expression by fluorescence microscopy and for USP18 expression by using immunoblots. Alternatively, the cell lines were generated by retroviral transduction of THP-1 cells with particles generated from cotransfection of HEK293T with plasmid pMSCV-USP18 or pMSCV empty vector, together with pHIT60 for packaging and VSV-G (pMD.G) for entry. The retrovirus-based USP18 expressing THP-1 cells were selected using neomycin (G418-BC liquid, A 2912; Biochrom GmbH, Berlin, Germany). Neomycin-resistant cell pools were tested for USP18 expression by immunoblotting.

USP18 knockout by LentiCRISPRv2. Plasmids of LentiCRISPRv2 targeting *USP18* were constructed according to previously described protocols (89, 90). Briefly, complementary oligonucleotides containing the specific USP18 sgRNA sequences, including sequence TAATGAATGTGGACTTCACC, and overhangs complementary to the overhangs generated by BsmBI digestion of LentiCRISPRv2 were ligated into the BsmBI-digested LentiCRISPRv2 plasmid to generate the functional transfer vector. LentiCRISPRv2 plasmid lacking sgRNA sequence was used as an empty vector control. HIV-1 pseudotype virus containing the pLentiCRISPRv2 transfer vector, packaging plasmid psPAX2, and VSV-G were cotransfected into HEK293T cells. At 48 h posttransfection, viral supernatants were harvested, concentrated, and purified over 20%

(wt/vol) sucrose and resuspended in fresh RPMI media. THP-1 cells were transduced with the HIV-1 pseudovirus and, at 72 h postransduction, the cells were subcultivated in fresh media containing 2 μg/ml puromycin for selection over a period of 14 days. The selected cells were single cell cloned by serial dilution in a 96-well plate. Single cells were clonally expanded and tested for USP18 expression by immunoblot analysis. Cells that tested negative for USP18 expression were further analyzed by PCR amplification of genomic DNA flanking the CRISPR-targeted region. The forward primer 5′-CTGGTTGGT TTACACAACATTGGACAG-3′ and the reverse primer 5′-GATATTGAAGAGGTAAGACTGTTCTTCAGG-3′ were used to amplify exon 3. The gDNA amplicon was subsequently cloned into pJET1.2/blunt cloning vector and sequenced (CloneJET PCR cloning kit, K1232; Thermo Fisher). Multiple sequence alignments were performed using Vector NTI (91). SAMHD1 knockout THP-1 cells were obtained as a gift from Veit Hornung (58).

Virus production and transduction. HIV-1 luciferase reporter viruses were generated by transfecting HEK293T cells with 600 ng of either pNL-LucR$^-$ E$^-$ and 150 ng of pMD.G or alternatively, using 600 ng of pMDLg/pRRE or pMDLx g/pRRE, together with 250 ng of pRSV-Rev, 600 ng of pSIN.PPT.CMV. Luc.IRES.GFP, and 150 ng of pMD.G with or without pcDNA6/myc-His-VPX or HIV-2$_{rod}$ VPX using Lipofectamine LTX (Thermo Fisher Scientific, Schwerte, Germany), according to manufacturer's recommendations in a 6-well plates. For HIV-2, transfection consisted of 850 ng of pHIV-2D4, 150 ng of pMD.G, and 600 ng of HIV-2.Luc.SV40 with or without pcDNA6/myc-His-VPX or HIV-2$_{rod}$ VPX. Viral supernatants were collected at 48 h after transfection, concentrated (see above), treated with DNase I (ENO521; Thermo Fisher Scientific), and then titrated using TZM-bl or HEK293T cells. Cell lysates of transfected HEK293T cells were immunoblotted for endogenous SAMHD1 to confirm the degradation of SAMHD1 by VPX. PMA-differentiated (25 ng PMA/ml; Calbiochem, Darmstadt, Germany) and undifferentiated USP18-expressing and control THP-1 cells were treated with or without human type I IFNs: IFN-αA/D (Sigma-Aldrich) and IFN-β-1a (PBL Assay Science, Piscataway Township, NJ) at concentrations of 10, 100, and 1,000 U/ml for 4 h and subsequently transduced with HIV-1 or HIV-2 luciferase reporter viruses. The luciferase activity was measured 3 days later. All experiments were independently repeated at least three times in triplicates.

β-Lactamase-based virion fusion assay. A β-lactamase-containing HIV-1 fusion assay was performed as previously described (92). Briefly, β-lactamase containing HIV-1 pseudotype virus was generated by cotransfection of HEK293T cells with pMDLg/pRRE, pRSV-Rev, pSIN.PPT.CMV.Luc.iresGFP, and pMD.G for pseudotyping and pMM310 (59) for β-lactamase-Vpr chimeric protein expression. At 48 h posttransfection, viral supernatant was collected and concentrated by centrifugation over 20% (wt/vol) sucrose. For a negative-control viral particle, pMD.G (VSV-G) was replaced with pcDNA3.1+ to exclude background effect of the fluorescence substrate. Titers of the concentrated viral particles were determined on HEK293T cells. The viral particles were used to infect 10^6 PMA-differentiated and undifferentiated THP-1.USP18 and vector control at a multiplicity of infection (MOI) of 0.5 via spinoculation at 1,200 $\times$ *g* for 2 h at 30°C. The cells were incubated for additional 3 h at 37°C and then washed with serum-free media. To allow for fluorescence substrate uptake, the cells were resuspended in 1 ml of freshly prepared loading solution consisting of serum-free media, 10 mM HEPES, 1% probenecid, 0.015% solution A (CCF2-AM), and 0.08% solution B (100 mg/ml Pluronic-F127R, 0.1% acetic acid; GeneBLAzer detection kit; Invitrogen, Germany). The cells were subsequently incubated at 25°C in a 5% CO_2 incubator. At 16 h after incubation, the cells were washed thoroughly with phosphate-buffered saline (PBS), and the fluorescence was measured using BD FACSCanto II (BD Biosciences). Analysis was subsequently done using the FlowJo software version 9.9.6 (FlowJo LLC, Ashland, OR).

qPCR quantification of HIV-1 reverse transcripts. HIV-1 NL-LucR$^-$ E$^-$-pseudotyped viral particles were produced and titered in HEK293T cells. The particles were DNase I treated at 37°C for 1 h. PMA-differentiated THP1.USP18 or control cells were transduced with NL-LucR$^-$E$^-$ virus at an MOI of 0.5 in the presence or absence of 10 μM nevirapine, a gift from Henning Hofmann. Cells were harvested at 12 h postransduction (93) and stored at −80°C until processed for DNA isolation. 250 ng of total DNA was used for qPCR amplification of early and late HIV-1 reverse transcripts (early RT: forward, 5′-GTGC CCGTCTGTTGTGTGAC, and reverse, 5′-GGCGCCACTGCTAGAGATTT; late RT: forward, 5′-TGTGTGCCCGTC TGTTGTGT, and reverse, 5′-GAGTCCTGCGTCGAGAGAGC) (93). Assays were performed on a Roche LightCycler 96 (Hoffmann-La Roche, Ltd., Basel, Switzerland) using SYBR green (Applied Biosciences/Thermo Fisher, Inc.). All PCR data were adjusted to genomic GAPDH levels (GAPDH: forward, 5′-CATCATCCCTG CCTCTACTGG, and reverse, 5′-GGTCCACCACTGACACGTT). The data were normalized to a standard curve generated with proviral plasmid DNA serially diluted in HEK293T cell genomic DNA (94).

Transfection. For USP18, SKP2, and SAMHD1 interaction experiments, 10^6 HEK293T cells were cotransfected with 0.8 μg of HA-USP18, 0.8 μg of MYC-SKP2, and 0.8 μg of FLAG-SAMHD1-expressing plasmids in 6-well plates for 48 h. In a related experiment, 1.25 μg of FLAG-SAMHD1 was cotransfected with 1.25 μg of pLOC-USP18. Single and double transfections were supplemented with pcDNA 3.1+.

ISG15-VS probe reaction. HA-tagged wild-type human USP18 and its mutants (C64A and C64S) were transfected in HEK293T cells. At 48 h posttransfection, cells were lysed in 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 250 mM sucrose, 1 mM dithiothreitol (DTT) using glass beads (44, 95). Lysates (20 μg) were incubated with 1 μg of HA-ISG15-VS probe (Boston Biochem) for 1 h at 37°C. ISG15 and USP18 and its mutant proteins were separated on a sodium dodecyl sulfate (SDS) gel and immunoblotted for ISG15, USP18, and tubulin as a control using rabbit anti-ISG15 (ab 36765; Abcam, Germany) and rabbit anti-USP18 (D4E7; Cell Signaling, Frankfurt am Main, Germany) respective antibodies and mouse anti-tubulin (1:8,000, dilution, clone B5-1-2; Sigma-Aldrich).

Immunoblot analysis. PMA-differentiated WT, SAMHD1KO, and USP18KO THP-1 cells, as well as PMA-differentiated and undifferentiated THP-1.USP18, mutant and control cells were lysed in radioim-

munoprecipitation assay buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail set III [Calbiochem], and phosphatase inhibitor cocktail [Roche, Mannheim, Germany]). USP18 expression cells were detected by rabbit anti-human USP18 at a 1:1,000 dilution (D4E7; Cell Signaling). SAMHD1 expression was detected by rabbit anti-human SAMHD1 at a 1:1000 dilution (12586-1-AP; Proteintech, Manchester, United Kingdom). Phosphorylated SAMHD1 was detected by rabbit anti-human phosphorylated SAMHD1 (T592, 8005; ProSci, Biocat, Heidelberg, Germany) at a 1:1,000 dilution. Cell cycle regulators were detected by rabbit anti-human cyclin A (sc-751, H-432; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), cyclin D1 (sc-753, H-295; Santa Cruz Biotechnology), goat anti-human cyclin D2 (sc-181-G, C-17; Santa Cruz Biotechnology), CDK2 (sc-163-G, M2; Santa Cruz Biotechnology), or CDK4 (sc-260-G, C-22; Santa Cruz Biotechnology) using a 1:500 dilution. Endogenous and overexpressed SKP2 was detected using rabbit anti-human SKP2 (4358; Cell Signaling) at a 1:500 dilution and human endogenous $p21^{CIP1}$ was detected using mouse anti-human $p21^{CIP1}$ (556430, SX118; BD Pharmingen, Heidelberg, Germany) at a 1:500 dilution. Overexpressed FLAG-tagged SAMHD1 and HA-tagged USP18 in transfected HEK293T were detected by using mouse anti-FLAG at a 1:1,000 dilution (M2; Sigma-Aldrich) and mouse anti-HA at a 1:7,500 dilution (MMS-101P; Covance, Münster, Germany), respectively. Overexpressed c-MYC-tagged SKP2 in HEK293T was detected by mouse anti-c-MYC at a 1:500 dilution (9E10, MCA2200; Bio-Rad Abd Serotec, Puchheim, Germany). Tubulin and β-actin were detected using mouse anti-tubulin antibody (1:8,000, dilution, clone B5-1-2; Sigma-Aldrich) and rabbit anti-β-actin-linked HRP (5125, 13E5; Cell Signaling), respectively. GAPDH was detected with goat anti-GAPDH at a 1:10,000 dilution (EB06377; Everest Biotech, VWR, Darmstadt, Germany).

In other experiments, endogenous cyclin A2, CDK1, and CDK2 were detected using rabbit anti-human cyclin A2 (18202-1-AP; Proteintech; 1:500 dilution), rabbit anti-human Cdc2 p34/CDK1 (H-297, sc-747; Santa Cruz; 1:500), and rabbit anti-human CDK2 (78B2, 2546; Cell Signaling; 1:500), respectively, after immunoprecipitation of overexpressed SAMHD1-FLAG cotransfected with USP18. Secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse antibody (α-mouse-IgG-HRP; GE Healthcare, Munich, Germany), donkey anti-rabbit (α-rabbit-IgG-HRP; GE Healthcare), and rabbit anti-goat antibody (α-rabbit-IgG-HRP; Santa Cruz), and blots were developed with ECL reagents (GE Healthcare).

Immunoprecipitation. To determine SKP2 and SAMHD1 binding to USP18, HEK293T cells were singly or cotransfected with expression plasmids of 0.8 μg of HA-USP18, SKP2-MYC, and FLAG-SAMHD1 in a ratio of 1:1:1. Single and double transfections were supplemented with pcDNA3.1(+). After 48 h, the cells were lysed in immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.8% NP-40, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride solution [Sigma-Aldrich], and protease inhibitor cocktail set III [Calbiochem]). The lysates were cleared by centrifugation. The supernatant were incubated with 20 μl of anti-HA affinity matrix beads and anti-HA affinity resin beads (Roche) at 4°C for 2 h. The samples were washed four times with IP buffer on ice. Bound proteins were eluted by boiling the beads for 5 min at 95°C in reducing sample buffer. Interaction between MYC-SKP2 and FLAG-SAMHD1 was determined by single or cotransfection of their respective plasmids in HEK293T cells at a concentration of 0.8 μg each. Cell lysates were immunoprecipitated with 10 μl anti-FLAG affinity resin beads (Biotool; Absource, Munich, Germany), followed by incubation for 2 h at 4°C and then washed six times. Immunoblot analysis and detection were performed as described previously. To analyze the interaction of USP18, cyclin A, CKD1/2, and SAMHD1, FLAG-SAMHD1 was cotransfected with pLOC-USP18. HEK293T cells were harvested at 48 h posttransfection and lysed in 200 μl of NET lysis buffer (50 mM Tris/HCl [pH 7.4], 150 mM NaCl, 15 mM EDTA [pH 7.4], 1% NP-40 containing protease and phosphatase inhibitors)/dish for 30 min on ice. Lysates were centrifuged at 17,000 $\times$ *g* for 15 min at 4°C. For preclearing, lysates were incubated with 25 μl Protein G Sepharose 4 Fast Flow (GE Healthcare) in 500 μl of Tris-buffered saline (TBS) plus 0.1% NP-40 (containing protease inhibitor) for 1.5 h at 4°C. After centrifugation, 200 μl of NENT100 (20 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA [pH 7.4], 0.1% NP-40, 25% glycerol) plus 1 mg/ml bovine serum albumin was added to precleared lysates, which were subsequently incubated with 25 μl of anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 h at 4°C. Beads were washed twice with 300 μl NENT300 (20 mM Tris [pH 7.4], 300 mM NaCl, 1 mM EDTA [pH 7.4], 0.1% NP-40, 25% glycerol) and twice with 300 μl of TBS plus 0.1% NP-40, each time for 2 min at 4°C under constant rotation. Bound immune complexes were released in 25 μl of 2$\times$ sample buffer through boiling (95°C, 5 min).

Cell cycle analysis. THP-1.USP18, mutant cells, and controls were treated with 25 ng/ml PMA, and 3 days after differentiation, the cells were harvested and resuspended in fresh RPMI media, washed with PBS, and fixed with 70% ice-cold ethanol. At 24 h after fixation, the cells were washed thoroughly and treated with RNase A for 30 min at 37°C. The cells were then stained with propidium iodide for 30 min at 4°C in the dark and analyzed at a 488-nm excitation wavelength by flow cytometry (FACS Canto II; BD Biosciences, Heidelberg, Germany). The data were evaluated using FlowJo software version 9.8.2 (Tree Star, San Carlos, CA).

dNTP quantification. Cellular dNTPs were extracted according to a previously described protocol (32). Briefly, 2 $\times$ 10^6 PMA-differentiated THP-1.USP18, THP-1.Control, and SAMHD1 knockout THP-1.USP18 and THP-1.Control cells were harvested and washed with cold PBS and lysed in ice-cold 0.2 ml of 65% (vol/vol) aqueous methanol. Lysates were heated at 95°C for 3 min and clarified by centrifugation at 18,800 $\times$ *g* for 3 min. Supernatants were transferred into new sterile tubes and dried using a SpeedVac (Eppendorf GmbH, Hamburg, Germany) at 30°C. The dried dNTPs were then resuspended and quantified using primer extension assay as described earlier (32). The required linear range of the dNTP assay was between 2 and 32% of the primer extension. The intracellular dNTP concentrations (pmol) were based on 10^6 cells.

Exogenous dN treatment. Wild-type THP-1 cells were differentiated with 25 ng/ml PMA. At 48 h posttreatment, the cells were treated with or without deoxynucleosides containing 2.5 mM concentrations (each) of 2′-deoxyadenosine monohydrate (dA), dC (Sigma), dT, and dG (Abcam) 40 min prior to infection and subsequently transduced with HIV-1, generated from a three-plasmid system and NL-LucE$^-$R$^-$ (with or without deoxynucleosides [dNs]). After 24 h, fresh medium was exchanged and, at 48 h postinfection, the intracellular luciferase activity was measured.

Statistical analysis. Data were analyzed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA). The study groups were compared using a two-tailed, unpaired Student *t* test, and a *P* value of <0.05 was considered statistically significant. The data represent means $\pm$ the standard deviations (SD), as indicated in the figures.

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E.O.K., C.M., K.S.L., and P.A.L. conceptualized the study. E.O.K. and C.M. designed the methodology. E.O.K., K.S., A.A.J.V., and J.H. performed experiments. E.O.K., C.M., R.K., K.S., J.H., B.K., W.A.S., and D.H. interpreted the results. E.O.K. wrote the original draft of the manuscript, and E.O.K., C.M., R.K., K.S., K.S.L., A.A.J.V., B.K., and W.A.S. reviewed and edited the manuscript. C.M. and E.O.K. acquired funding. D.H., K.S.L., R.K., P.A.L., and W.A.S. obtained resources. D.H. and C.M. supervised the study.

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CHAPTER II

USP18 (UBP43) downregulates p21 antiviral function by accumulating misfolded dominant negative p53 that supports HIV-1 replication

Edmund Osei Kuffour, Renate König, Dieter Häussinger, Wolfgang A. Schulz, Carstn Münk. USP18 (UBP43) downregulates p21 antiviral function by accumulating misfolded dominant negative p53 that supports HIV-1 replication. Manuscript under preparation.

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1. Executed all the experiments in this work
2. Wrote the original draft of the manuscript

USP18 (UBP43) downregulates p21 antiviral function by accumulating misfolded dominant negative p53 to support HIV-1 replication

Edmund Osei Kuffour[1], Renate König[2,3], Dieter Häussinger[1], Wolfgang A. Schulz[4#], Carsten Münk[1#]
[1]Clinic for Gastroenterology, Hepatology and Infectious Diseases, Medical Faculty, Heinrich-Heine University Düsseldorf, Düsseldorf, 40225, Germany
[2]Host-Pathogen Interactions, Paul-Ehrlich-Institut, Langen, 63225, Germany
[3]Immunity and Pathogenesis Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA
[4]Department of Urology, Medical Faculty, Heinrich-Heine University Düsseldorf, Düsseldorf, 40225, Germany

Running Title: USP18 accumulates dominant negative p53 to downregulate p21

[#]To whom correspondence should be addressed:
Wolfgang A. Schulz, Department of Urology, Medical Faculty, Heinrich-Heine University Düsseldorf, Düsseldorf, 40225, Germany; Wolfgang.Schulz@uni-duesseldorf.de; Tel. +49 (0) 211-81-18966

Carsten Münk, Clinic for Gastroenterology, Hepatology and Infectious Diseases, Medical Faculty, Heinrich-Heine University Düsseldorf, Düsseldorf, 40225, Germany;
carsten.muenk@med.uni-duesseldorf.de; Tel. +49 (0) 211-81-10887; Fax. +49 (0) 211-81-15431.

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ABSTRACT
Innate immune cells, including macrophages and dendritic cells dominate early immune responses to lentiviruses, such as HIV-1. HIV-1 recognition and sensing by pathogen recognition receptors, *e.g.* the cytosolic sensor cGAS, induces intracellular signaling cascades that culminates in the production of type I interferon (IFN)-α/β in the innate immune cells. IFN-α/β signals back via the IFN-α receptor 1 and 2 (IFNAR1/2), inducing a plethora of IFN stimulated gene (ISGs), including ISG15 and p21.

p21 is a host intrinsic antiviral factor, involved in monocyte differentiation and maturation. p21 inhibits HIV-1 replication by inactivating the dNTP biosynthesis pathway and activating the restriction factor SAMHD1. p21 is under the transcriptional regulation of the genome guardian p53. p53 requires modification by ISG15 for degradation of its misfolded dominant negative version. Point mutations, deletions and rearrangements in the p53 gene, a hallmark of many cancers, inactivate its capacity to transactivate p21.

USP18 is an ISG15-specific isopeptidase that negatively regulates IFN signaling. We showed previously that USP18 contributes to HIV-1 replication by abrogating p21 antiviral function.

Here, we demonstrate a mechanism by which USP18 mediates p21 downregulation. USP18, by its protease activity accumulates misfolded dominant negative p53, which downregulates p21 mRNA and protein synthesis in differentiated THP-1 macrophage cells. Downregulation of p21 expression activates key enzymes involved in *de novo* dNTP biosynthesis, expanding the dNTP pool for enhanced HIV-1 replication.

This work thus underlines the importance of USP18 in HIV-1 infection in innate immune cells and can be considered for therapeutic approaches that block viral dissemination.

Introduction
Innate immune cells, including macrophages and dendritic cells, possess germ-line encoded pathogen recognition receptors (PRRs), that enable them to recognize and sense conserved features of human immunodeficiency virus type 1 (HIV-1)-derived pathogen associated molecular patterns (PAMPs) (1-10). The host-

PRR and HIV-1-PAMP interaction triggers innate immune signaling in the infected cells that culminates in the production of type I and III IFNs, including IFN-α/β. IFN-α/β production signals back via the transmembrane IFN α receptor 1 and -2, driving more IFN-α/β production and induction of interferon stimulated genes (ISGs), which help to block the replication and spread of the virus (4,10). Among the induced ISGs are restriction factors, including the APOBEC3s, MX proteins, TRIM5a, SAMHD1, SERINCs and tetherin (BST2), which block the viral replication at distinct stages of the viral life cycle (9-15). Apart from the plethora of restriction factors produced as ISGs, other host intrinsic antiviral factors, which may serve as resistance factors are also produced or activated. Among them are ISG15, p21/$^{Waf1/Cip1/Sdi1}$ (here after p21), and p53 (*TP53*), a well-described tumor suppressor important in maintaining the integrity of the host genome (16-31).

p21 is a downstream effector of p53. As a cyclin-dependent kinase (CDK) inhibitor, p21 mediates cell cycle arrest, DNA repair, senescence, aging and cell death by apoptosis (32). p21 is involved in monocyte differentiation, maturation and induced pluripotent stem cell reprogramming (32-37). p21 is induced by HIV-1 and its expression inhibits HIV-1 and related viruses in monocyte-derived macrophages (MDMs) and dendritic cells (MDDCs) (20,32,34,36-44). Upregulation of p21 in CD4+ T cells in HIV-1-infected elite controllers is associated with control of viremia (45,46). p21 affects early HIV-1 replication by regulating key enzymes involved in *de novo* dNTP (deoxyribonucleotide triphosphate) biosynthesis (41,42). It inhibits HIV-1 replication by blocking transcriptional activation of R2 subunit of ribonucleotide reductase (RNR2, also known as RRM2) by the host transcription factor E2F1 (41,42). As an inhibitor of several cyclin/CDK complexes, p21 further blocks HIV-1 replication by promoting dephosphorylation and activation of SAMHD1 restriction function (47-52) and by inhibiting CDK2-dependent phosphorylation of the HIV-1 reverse transcriptase (45). Experimental downregulation of p21 results in increased HIV-1 infection (46). The transcription, expression and activity of p21 are regulated via p53-dependent and independent pathways (43,53-56). Under stress conditions, such as DNA damage or viral infection, p21 is highly upregulated, likely mediated by the induction of p53 and other pathways (32,43,54,57,58). p21 expression is regulated post-translationally by ubiquitination and phosphorylation (32,43,54). Diverse E3 ubiquitin ligase complexes recognize p21 and mediate its degradation. These include SCF^{SKP2} {SKP1-CUL1-SKP2 (S-phase associated protein)}, CUL4^{CDT2} {CUL4A or CUL4B-DDB1-CDT2 (DDB1 is DNA damage-binding protein 1)} and APC/CCDC20 {anaphase-promoting complex (APC)-cell division cycle 20}, which promote p21 proteolysis via the proteasome at distinct stages of the cell cycle under non-stressed conditions. These E3 ligase complexes promote ubiquitination and degradation of p21 only when p21 is complexed with cyclin E/CDK2, cyclin A/CDK2, PCNA (proliferation cell nuclear antigen), cyclin A/CDK1 or cyclin B/CDK1 (43,54). In the absence of these complexes, p21 is degraded independently of ubiquitin in a cell-type dependent manner by interaction of its C-terminus with the C8α subunit of the 20S proteasome (54). p53 expression and activity are also regulated by several post-translational modifications including ubiquitination, acetylation, phosphorylation and ISGylation, all of which likely impact p21 induction and function. Single point mutations, deletion and rearrangement of the p53 gene affect p21 transcription and thus potentially impact HIV-1 infection and replication. Indeed, the absence of p53 decreases p21 expression and correlates significantly with the enhancement of HIV-1 infection and replication at the reverse transcription step (18,19). Moreover, p53 is itself activated by HIV-1 infection and its expression likely inhibits HIV-1 long terminal repeat (LTR) promoter activity (21,59-62).

IFN-inducible ubiquitin-like specific protease USP18 (UBP43) negatively regulates type I and III IFN signaling pathways (63-65). USP18 targets ISG15 (a 17-kDa protein) and cleaves it from its conjugated proteins (64-67). USP18 functions in a protease-dependent or –independent manner to balance immune responses in disease and non-disease states (64,65,67-71). It negatively regulates NF-κB (nuclear factor "kappa-light-chain-enhancer" of activated B cells) activation by inhibiting ubiquitination of TAK1 (TGF-β-activated kinase 1) and NEMO (NF-κB essential modulator), implicating USP18 as a

deubiquitinase (72,73). By interacting with IFNAR2, USP18 blocks IFN signaling by disrupting IFNAR2-JAK 1 (janus-activated kinase 1) binding in an isopeptidase-independent manner (65,74,75). In the absence of free ISG15, USP18 is targeted for ubiquitination and proteasomal degradation by SKP2 (76). USP18 depletion by experimental knockout enhances JAK/STAT (signal transducer and activator of transcription) signaling and increases ISGs with upregulated levels of protein ISGylation (65,69,70,77,78).

We recently demonstrated that USP18 is HIV-1 inducible and that the expression of the protein enhanced HIV-1 replication. The enhanced HIV-1 replication was mediated by USP18-induced downregulation of p21, which correlated with increased dNTP levels and phosphorylation of the inactive form of SAMHD1 (79). Here, we investigated the molecular mechanisms behind the USP18-mediated downregulation of p21 and its resultant elevation of dNTP levels and increased phosphorylated SAMHD1, in the myeloid THP-1 cells.

Results

USP18 relieves p21 repression of E2F1 and *de novo* dNTP biosynthesis pathway. We recently showed that USP18 enhances HIV-1 replication by inactivating p21 antiviral function (79). To understand the molecular mechanisms by which USP18 mediates p21 downregulation and how this enhances HIV-1 replication, we explored mRNA and protein expression levels of p21 (Fig. 1A and 1B), and downstream effector proteins of p21 in PMA-differentiated wild-type and SAMHD1 knock-out (KO) THP-1 cells expressing USP18 (Fig 1C). Interestingly, p21 expression was not only downregulated at the protein level, but also strongly at the transcriptional level in wild-type THP-1 cells expressing USP18 compared to vector control cells (Fig. 1A and B). p21 mRNA levels were reduced by about threefold (Fig. 1A), and this effect was even more prominent (more than 30-fold downregulation) in the absence of SAMHD1 in the THP-1.USP18 cells (Fig. 1B). We tested how USP18-mediated downregulation of p21 might affect downstream proteins that are regulated by p21. Interestingly, key enzymes of *de novo* dNTP biosynthesis were all significantly upregulated in the SAMHD1KO THP-1.USP18 compared to the control cells (Fig. 1C). USP18-mediated low p21 expression correlated strongly with upregulated total and phosphorylated CDK2, RNR2, E2F1 and TYMS in SAMHD1KO THP-1 cells expressing USP18 compared to their controls (Fig. 1C). The presence of IFN-β (1000 U/ml) could not alleviate this effect, except to reduce slightly the level of E2F1 (Fig. 1C). We further tested whether p21 downregulation was rescued by the proteasome inhibitor MG132. Indeed, p21 protein levels increased significantly by MG132 even in the SAMHD1KO THP-1.USP18 cells (Fig. 1D) and in activated primary PBMCs (Fig 1E). Surprisingly, however, expression levels of USP18 were reduced in both SAMHD1KO THP-1 and primary cells by MG132 treatment (Fig. 1D and 1E)). Bafilomycin, a lysosomal inhibitor had no effect on p21 upregulation either in the presence or absence of MG132 (Fig. 1D and 1E).

USP18 induces misfolded dominant negative p53 in myeloid THP-1 cells. Considering that p21 mRNA was drastically reduced in the SAMHD1KO THP-1.USP18 cells, we reasoned that transcription factors inducing p21, including the tumor suppressor p53 might have been negatively affected by USP18. We therefore tested for the mRNA and protein expression of p53 in the PMA-differentiated SAMHD1KO THP-1.USP18 and control cells. The USP18 expressing SAMHD1KO THP-1 cells showed slight increase in p53 mRNA by about two-fold compared to the control cells (Fig. 2A). Surprisingly, however, SAMHD1KO THP-1.USP18 cells showed dramatic accumulation of p53 whereas control cells exhibited undetectable levels of p53 proteins in cell lysates and low levels after immunoprecipitations (Fig. 2B and C). To understand why p53 would accumulate in the USP18 expressing cells, but fail to induce p21 expression, we sought to characterize these accumulated p53 proteins using conformation-specific antibodies after immunoprecipitation (53,80,81) (Fig. 2A). Interestingly, the monoclonal antibody PAb240 that recognizes an epitope exposed by activating mutations or denaturation (81), detected misfolded p53 in the USP18 expressing cells, but not in the vector control cells in an immunoprecipitation assay (Fig. 2B), and in total cell lysates without immunoprecipitation (Fig. 2C).

It is thought that newly synthesized misfolded p53 can gain new functions, acting as a

dominant negative factor against the wild-type p53 or drive transcriptional activation of proliferation associated genes (53,82-87). It is therefore imperative that misfolded dominant negative p53 proteins are degraded by the 20S proteasome, a process that requires modification by ISG15 (53,55). To understand whether the accumulated misfolded p53 was a result of the ISG15-specific protease activity of USP18, we tested for p53 expression in the SAMHD1KO THP-1 cells stably expressing active site mutants of USP18 (79). Interestingly, unlike wild-type USP18 cells, SAMHD1KO THP-1 cells with USP18 active site mutants (C64A and C64S) failed to accumulate the misfolded dominant negative p53 proteins (Fig. 2D). Moreover, the lack of these p53 proteins correlated with high p21 expression, similar to the vector controls (Fig. 2D). Although the USP18 active site mutants did not induce misfolded dominant negative p53, they could still enhance HIV-1 replication, (Fig. 2E), suggesting that there are other unidentified HIV-1 enhancing pathways mediated by USP18, independent of its protease activity. Indeed the lack of the accumulated misfolded dominant negative p53 proteins, and the increased p21 expression in cells with the active site mutants of USP18 also correlated with a decline in the expression of total and phosphorylated CDK2 and the key enzymes involved in *de novo* dNTP biosynthesis, similar to that observed in the control cells (Fig. 2F).

"Gain of function" mutant p53 support HIV-1 replication. THP-1 cells have two different p53 alleles, one wild-type and another containing a 26 base pair deletion in exon 5 (Fig. 3A). This latter variant (CΔTp53) was cloned from THP-1 cells and expressed in 293T cells in comparison to wild-type p53, a single amino acid inactive mutant (R273H), which is found in many cancers (82,84,87-90) and a C-terminal DNA binding regulatory domain (RD) deletion mutant (RDΔTp53), which has an intact DNA-binding domain, similar to the wild-type and was included as an additional control. The 26 base pair deletion causes a frame shift resulting in an approximately 25-kDa truncated protein (Fig. 3B and 3C). Interestingly only the wild-type p53 protein exhibited increased p21 induction compared to all the mutants and the vector control (Fig. 3B). We next expressed the wild-type p53 and its mutants in the SAMHD1KO THP-1 cells and checked for p53 expression. The mutant p53 cells maintained stable expression of p53 and remained viable. On the contrary, the wild-type p53 expressing SAMHD1KO THP-1 cells died overtime in culture (Fig. 3C). To analyze whether mutant p53 proteins support HIV-1 replication, SAMHD1KO THP-1 cells expressing wild-type p53 and its mutants were transduced with HIV-1 reporter virus. The wild-type and the RD domain deletion mutant p53 reduced HIV-1 infection, however, cells expressing the single amino acid variant (R273H) or the 26 base pair deletion mutant of p53 were highly susceptible to HIV-1 infection (Fig. 3D).

HIV-1 induces p53. To confirm that HIV-1 can induce p53 expression, we transduced SAMHD1KO THP-1 cells with HIV-1 vector particles at different time points. We indeed observed p53 induction 72 h after transduction (Fig. 4A). However, the p53 band disappeared at later time points (Fig. 4A). To further confirm this observation in a different myeloid cell line, we transduced differentiated and undifferentiated BlaER1 cells with HIV-1 and checked for p53 and p21 induction after 48 h. Interestingly, HIV-1 induced p53 expression in both differentiated and undifferentiated BlaER1 cells, which correlated with increased p21 induction, with high expression in the presence of VPX (91) from human immunodeficiency virus type 2 (HIV-2), which degrades SAMHD1 (Fig. 4B) (92-94).

Discussion

Mounting evidence suggests p21 as an important host intrinsic innate immune resistance factor against lentiviruses (18,38-42,45,46). p21-mediated inhibition of lentiviruses is explained by its ability to induce an active form of SAMHD1 and to repress key enzymes involved in *de novo* dNTP biosynthesis pathway (39-42). We have previously shown that the ISG15-specific protease USP18 has the ability to potently abrogate p21-mediated inhibition of lentiviruses (79).

Here, we demonstrate that USP18 by its protease activity accumulates p53 proteins that are dysfunctional in inducing p21 mRNA and protein synthesis in differentiated myeloid THP-1 cells. The accumulated p53 exhibited a

phenotype that is characteristic of misfolded dominant negative p53. By accumulating dysfunctional p53, USP18 relieves p21 of its antiviral function and activates key enzymes involved in *de novo* dNTP biosynthesis pathways. Thus, USP18 promotes conducive environment for HIV-1 replication.

Synthesis of p21 mRNA and protein is under transcriptional regulation of p53 as well as p53-independent pathways (32,43,54). p53 is an important tumor suppressor, recognized for its role in inducing cell cycle arrest or apoptosis in response to a plethora of different cellular stress signals (18,19,32,56,57). In its quest to maintain the integrity of the genome of its host, p53 acts as a transactivator of many genes encoding cell cycle regulators, including p21, which is required for ensuring DNA repair and proper progression of the cell cycle (32,43,57).

Under stressed conditions, including genotoxic-induced DNA double-strand breaks, DNA-dependent protein kinase (DNA-PK) and ATM signal a DNA damage response (95-100). This process likely causes the stabilization and posttranslational modifications of p53 by phosphorylation and acetylation, processes that lead to p53 activation (53,56,58,95,96,101). The increased expression and activation of p53 proteins transactivates p21 mRNA and protein expression, inducing cellular arrest and repair of the damaged DNA (24,82,102,103). Following repair of DNA damage, p53 is likely targeted for the proteasome either by ubiquitin-dependent MDM2-mediated degradation (24,32,56,82,102,103) or by ISGylation dependent degradation (24,53,55). Dysregulation of p53 is the hallmark of many tumors likely caused by mutations, deletions and rearrangements in the p53 gene (32,84,86,90,102-107). Mutant p53 proteins lose their transactivation function. Apart from their inability to induce transcriptional activation of their target genes, "Gain of function" of mutant forms of p53 negatively regulates the wild-type p53 function, which impacts the expression of its target genes (53,82,84,86,87,90,105-108).

p53 and its downstream effector gene, p21 are both HIV-1- and type I IFN-inducible (16,20,22,24,44,58,79,109,110). Induction and activation of p53 by HIV-1 possibly occurs at the level of the viral cDNA integration into the host genome. The HIV-1-integrase-mediated double strand break likely signals a DNA damage response mediated by the activation of DNA-PK and ATM (58,109,111,112). DNA-PK activation is thought to phosphorylate and stabilize p53 for its transactivation function, thereby inducing p21, which likely induces cell cycle arrest or cell death by apoptosis if the DNA repair machinery is unsuccessful (58,95,109).

Depending upon the cellular context, modification of p53 by ISG15 may either mediate its transactivation or degradation by the proteasome (24,53,55,56). Under cellular stressed conditions, p53 is modified by ISG15 for enhanced transactivation of p21 transcription, a process that is reversible by the ISG15-specific protease USP18 (56). Further, newly synthesized misfolded dominant negative p53 proteins also require ISG15 modification for degradation by the proteasome, a mechanism that is also reversible by USP18 (24,53,55).

Human ISG15, and USP18 are also induced by HIV-1 infection, type I IFNs and genotoxic stress (23,24,29,53,63-65,69,70,79). The sequential or parallel induction and expression of ISG15, USP18 and p53 in response to these stimulants are likely not due to chance, but probably reflect a feedback regulatory mechanism between these proteins. Indeed, it is shown that *ISG15* is a downstream target gene of p53 (53,56,113), and the expression of ISG15 is likely required for the enhancement of the p53 transactivation function or for the degradation of nascent misfolded p53 (24,53,55,56). It is not yet known if USP18 gene contains promoter elements for p53-mediated expression or vice versa. However, the stable expression of USP18 by lentiviral transduction of myeloid THP-1 cells induces a strong accumulation of p53 that appears dysfunctional for driving p21 mRNA and protein synthesis in differentiated THP-1 cells. This accumulated p53 exhibits a phenotype that is characteristic of misfolded dominant negative p53 (53). Upon expression of active site mutants of USP18, p53 did not accumulate, suggesting that the misfolded proteins were targeted for proteasomal degradation by ISG15-mediated modification. In contrast, the presence of wild type USP18 abrogated the ISG15-mediated degradation of the misfolded p53, which apparently had the ability to inactivate

the wild-type p53 function, as evidenced by decreased p21 mRNA and protein levels. Indeed, it is known that, p53 mutants can form prion-like amyloid structures that accumulate in cells, which promote the wild-type p53 to adopt conformational changes that inactivate its function and are propagated in a prion-like manner (83,87,89,114-116).
THP-1 cells possess a 26-base pair deletion in exon 5 of one allele of *TP53*, which leads to a frameshift that introduces an early stop codon, and translates into about 25-kDa protein, with no suggested activity (117). The other allele appeared intact with no alterations and translates into a 53-kDa protein. However, in the presence of USP18, p53 in the differentiated SAMHD1KO THP-1.USP18 cells failed to transactivate p21 mRNA and protein synthesis, implicating it as a misfolded dominant negative prion-like aggregate.

It is not clear which factor initially signaled p53 transcription and translation in the myeloid THP-1 cells leading to its accumulation in response to USP18 in the differentiated THP-1 cells. However, it is tempting to speculate that, the transduction of the cells using lentiviral vectors must have activated the DNA damage response genes, including DNA-PK and ATM, following integration of the lentiviral vectors into the cell genome. Indeed, we did observe the induction of p53, when we transduced THP-1 and the monocyte derived trans-differentiated BLaER1 cells, which have an intact p53 gene with HIV-1 with and without VPX (Fig. 4A and 4B). The induction of p53 correlated with high expression of p21 and even more robustly in the presence of VPX in the trans-differentiated BLaER1 cells, suggesting that the absence of SAMHD1 via VPX-mediated degradation heightens the extent of p53 stimulation possibly by DNA-PK or ATM and most probably by IFN following recognition and sensing of viral reverse transcripts (24,58,91-95,97,109,118,119). Thus, the newly synthesized misfolded p53 proteins in empty vector controls and the active site mutant USP18 cells might have been cleared by the ISG15-mediated proteasomal degradation, however, USP18 by its protease activity, retained accumulated misfolded dominant negative p53

By regulating p21 transcription, p53 indirectly affects the replication of lentiviruses that require dividing cells with large pools of dNTPs and inactive SAMHD1, and highly phosphorylated reverse transcriptase enzymes (18,19,21,22,32,38,43,53-62). It was recently shown that p53 and its downstream gene p21 could block early and late stages of HIV-1 replication in MDMs (18,19,62). Thus, the inability of the accumulated p53 proteins in the SAMHD1KO THP-1.USP18 cells to transactivate p21 mRNA and protein synthesis relieved p21 of its antiviral function, thereby enlarging the intracellular dNTP pool through activation of *de novo* dNTP biosynthesis, which allowed increased replication of HIV-1. Although the USP18 active site mutants did not accumulate dysfunctional p53 and exhibited increased p21 levels; these cells could still support enhanced HIV-1 replication, suggesting an additional p53-independent mechanism of enhanced HIV-1 replication mediated by USP18. Furthermore, many mutants of p53 have been shown to promote HIV-1 replication by activating HIV-LTR driven transcription (120). Consistent with this, DNA-binding domain inactive p53 mutant (R273H), as well C-terminal truncated mutants of p53 supported high HIV-1 replication in differentiated THP-1 cells, while the wild type and the RD deletion mutant reduced HIV-1 replication.

Overall, we provide evidence that USP18 is a factor that significantly contributes to HIV-1 infection in innate myeloid THP-1 cells by relieving p21 of its inhibitory function. Further work to explore p53-independent enhancement of HIV-1 replication and how USP18 might modulate innate immune recognition is warranted.

Experimental procedures

Plasmids

Lentiviral pLOC and pcDNA3.1(+) vectors containing the open reading frames (ORF) for human wild-type *USP18* gene, USP18 active site mutants (C64A and S), with and without HA and V5 epitopes, and turbo Green Fluorescent Protein (GFP) were generated as described before (79). The human p53 ORF and its single amino acid mutant (R273H), cloned into pBC12 plasmids were amplified and cloned into pLOC using the SpeI and AscI restriction sites. HA and V5-tagged p53 and its R273H mutant (R273H) were cloned into pcDNA3.1(+)

using the HindIII and NotI restriction sites. C-terminal DNA binding regulatory domain (RD) deletion mutant (RDΔTp53) of p53, expressing an approximately 39-kDa protein and a 26 base pair deletion mutant (CΔTp53) in exon 5 of the *TP53* gene that expresses a C-terminal truncated protein in THP-1 cells were amplified from cDNA, which was synthesized from genomic RNA from THP-1 cells. Forward primer: 5'-GTGACACGCTTCCCTGGAT, reverse primer: 5'-GAGTTCCAAGGCCTCATTCA were used for the PCR amplification of RDΔTp53 and forward primer: 5'-GTGACACGCTTCCCTGGAT and the reverse primer: 5'- GAGTTCCAAGGCCTCATTCA were used for CΔTp53 amplification. The PCR amplicons were cloned into pJET1.2/blunt Cloning Vector (CloneJET PCR Cloning Kit, K1232, Thermofisher Scientific, Karlsruhe, Germany), tagged with HA- and V5-epitopes, and then sequenced. Multiple sequence alignments were performed. The HA- and V5-tagged p53 and its mutants were further cloned into pLOC and pcDNA3.1(+) and their expression was confirmed by immunoblotting after sequencing. pSIN.PPT.CMV.Luc.IRES.GFP, which expresses the firefly luciferase and IRES.GFP, HIV-1-gag-pol plasmids, pMDLg/pRRE, pMDLx/pRRE, $SIVmac_{239}$ VPX, pRSV-Rev and the vesicular stomatitis virus glycoprotein, pMD.G expressing plasmids have been described before (79). The HIV-1 construct psPAX2 was obtained from the NIH, AIDS Reagent Program repository.

Cell culture

Wild-type THP-1 cells (ATCC TIB-202) (122) and SAMHD1KO THP-1 cells (47) expressing the pLOC-empty vector, the human USP18 and its active site mutants (C64A and S) were generated as described before (79). These cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (PAN-Biotech, Aidenbach, Germany) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 5 µg/mL of blasticidin S hydrochloride (Sigma Aldrich, Taufkirchen, Germany) except for the SAMHD1KO THP-1 stable cell lines, which were supplemented with 2 µg/mL of blasticidin S hydrochloride. All cell lines were cultured at 37˚C in a humidified atmosphere with 5% CO_2. HEK293T (ATCC CRL-3216) (123,124) were maintained in Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany), supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. BlaER1, a human B-cell precursor leukemia cell line stably expressing the myeloid transcription factor C/EBPα-ER-IRES-GFP, was obtained as a kind gift from Thomas Graf (125). The cells were maintained in RPMI 1640 medium (PAN-Biotech), supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin. The BlaER1 cells were trans-differentiated as previously described (126). Briefly, the cells were treated with 10 ng/mL of IL-3, 10 ng/mL of M-CSF and 100 nM of β-estradiol in RPMI medium for a maximum of 7 days at 37^{o}C, in 5% CO_2 for trans-differentiation into monocytes. Peripheral blood mononuclear cells (PBMCs) were extracted by centrifugation of whole blood on a Ficoll/Hypaque density gradient (Biocoll Separating Solution, Biochrom AG). The isolated PBMCs were washed in Dulbecco's phosphate-buffered saline (DPBS) and resuspended in RPMI, supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and stored at 37˚C in a humidified atmosphere with 5% CO_2. 1 x 10^6 cells were seeded in 6 well plates and stimulated with 1000 µg/mL of PHA (Sigma Aldrich) and 30 U/mL of IL-2 (Sigma Aldrich) for three days and subsequently used for experiments. Each 1x10^6 activated PBMCs, SAMHD1KO THP-1.USP18 or control cells were treated with MG132 (5 µM) (474790, Calbiochem) or Bafilomycin A1 (BafA1) (10 nM) (J61835, Alfa Aesar, Thermofisher Scientific, Karlsruhe, Germany), or with DMSO (A3672,0250, Pan Reac, AppliChem, Darmstadt, Germany) as a control. 16 h post-treatment, the cells were harvested and immunoblotted for p21 and GAPDH or α-Tubulin as a loading controls.

Virus production and transduction. HIV-1 luciferase reporter viruses were generated as described before (79). Briefly, 1x10^6 HEK293T cells were transfected with 800 ng of pMDLg/pRRE or pMDLx/pRRE (with 300ng of VPX), together with 350 ng of pRSV-Rev, 800 ng of pSIN.PPT.CMV.Luc.IRES.GFP, 250 ng of pMD.G using Lipofectamine LTX (Thermo Fisher Scientific), according to manufacturer's recommendation in 6-well

plates. 48 h after transfection, viral supernatants were collected and concentrated as described before (79), treated with DNase I (ENO521, Thermo Fisher Scientific) and then titrated using HEK293T cells. Wild-type and SAMHD1KO THP-1 cells expressing USP18 and vector controls were treated with 25 ng/mL of PMA (phorbol 12-myristate 13 acetate) (Calbiochem). 48 h after differentiation, the cells were either harvested for immunoblotting or treated with and without 1000 U/mL of human IFN-β (pbl assay science, New Jersey, USA) for 24 h and subsequently transduced with HIV-1 luciferase reporter viruses. Luciferase activity was measured two days later. All experiments were independently repeated at least three times in triplicates, unless stated otherwise.

qRT-PCR

Total genomic RNA from $1x10^6$ PMA-differentiated wild-type and SAMHD1KO THP-1 cells, expressing USP18 or controls was isolated using Qiagen RNeasy mini kit (#74106, Qiagen, Hilden Germany) according to the manufacturer's recommendation. One µg RNA was transcribed into cDNA using RevertAid H-Minus First Strand cDNA Synthesis Kit (K1631, Themofisher Scientific Inc.), according to the manufacturer's recommendation. RT-PCR amplification of p21, p53 and, as a control, HPRT1 (Hypoxanthine Guanine Phosphoribosyltransferase) transcripts was done using 1x Sybr Green PCR Master Mix (4309155, Applied Biosciences, Thermofisher Inc.) using the ViiA7 Real-Time PCR System (Applied Biosciences, Thermofisher Inc.). The data was analyzed using comparative Ct mean and normalized to HPRT1. The following primers were used for the amplification of p21 transcripts, forward primer: 5' TGGAGACTCTCAGGGTCGAAA, reverse primer: 5' GGCGTTTGGAGTGGTAGAAATC. For p53 transcripts, the following primers were used, forward primer: 5' GCCCAACAACACCAGCTCCT, reverse primer: 5' CCTGGGCATCCTTGAGTTCC. For HPRT1 transcripts, the following primers were used: forward primer: 5'-TGCTGAGGATTTGGAAAGGGT, reverse primer: 5'- GGGCTACAATGTGATGGCCT

Transfection

$1x10^6$ HEK293T cells were transfected with expressing plasmids for V5-tagged wild-type p53, R273H, RDΔTp53 and CΔTp53 mutants of p53 in 6-well plates, using lipofectamine LTX (Thermofisher Scientific Inc.). 48 h post-transfection, the cells were harvested and immunoblotted for V5 or p53 expression.

Immunoblot analysis

PMA-differentiated wild-type and SAMHD1KO THP-1 cells, expressing USP18, its active site mutants (C64A and C64S) and their controls, SAMHD1KO THP-1 cells, expressing p53 and its mutants (R273H, RDΔTp53 and CΔTp53), were lysed in radioimmunoprecipitation assay (RIPA) buffer as described before (79). USP18 expression was detected by rabbit anti-human USP18 at a 1:1000 dilution (D4E7, Cell Signaling, Frankfurt am Main, Germany). SAMHD1 expression was detected by rabbit anti-human SAMHD1 at a 1:1000 dilution (12586-1-AP, Proteintech, Manchester, United Kingdom). p21 was detected using either mouse anti-human p21 (556430, SX118, BD Pharmingen, Heidelberg, Germany) at a 1:500 dilution or rabbit anti-human p21 antibody (#2947,12D1, Cell signaling) at a ratio of 1:1000. Total and phosphorylated CDK2 expressions was detected with goat anti-human CDK2 (sc-163-G, M2, Santa Cruz Biotechnology) using in a 1:500 dilution and rabbit anti-human phospho-CDK2 (#2561, Cell signaling) at a 1:1000 dilution respectively. All endogenous and overexpressed p53 proteins were detected with mouse anti-human p53 (Ab-6, DO-1, OP43, Oncogene), unless stated otherwise. Misfolded dominant negative p53 protein expression was detected with mouse anti-human p53 antibody (Ab-3, PAb240, OP29, Oncogene) at a dilution of 1:500 under non-denaturing conditions. RNR2 expression was detected using rabbit anti-human RRM2 antibody in a 1:1000 dilution (62-363, ProSci, Biocat, Heidelberg), TYMS expression was detected mouse anti-human TYMS (MAB4130, clone TS106, Merck, Darmstadt, Germany) in a 1:500 dilution. E2F1 expression was detected with mouse anti-human E2F1 (KH95, sc-251, Santa Cruz Biotechnology, Inc.) in a 1:500 dilution.

α-Tubulin and GAPDH were detected using mouse anti-tubulin antibody (1:8,000, dilution, clone B5-1-2; Sigma-Aldrich) and goat anti-GAPDH at a 1:10,000 dilution (EB06377,

Everest Biotech, VWR, Darmstadt, Germany) respectively. Horseradish peroxidase-conjugated sheep anti-mouse antibody (α-mouse-IgG-HRP; GE Healthcare, Munich, Germany), donkey anti-rabbit (α-rabbit-IgG-HRP; GE Healthcare) and rabbit anti-goat antibody (α-rabbit-IgG-HRP; Santa Cruz) were used as secondary antibodies and blots were developed with ECL chemiluminescence reagents (GE Healthcare).

Immunoprecipitation

$1x10^6$ SAMHD1KO THP-1 cells expressing the empty vector, USP18 and its active site mutants were lysed with buffer A (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM NEM, 1 mM Sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 0.2% Triton X-100 and one tablet of protease inhibitor cocktail (Calbiochem) in 10 ml of the lysis buffer. The lysates were precleared at 4^oC at full speed for 25 minutes and subsequently incubated with mouse anti-human p53 (Ab-6, DO-1, OP43, Oncogene) for 2 h at 4^oC and then with 20 µl of protein A/G plus agarose (Santa Cruz) for additional 2 h with end-over-end rotation. The samples were then washed 4 times and bound proteins were eluted and immunoblotted with mouse anti-human p53 antibody (PAb240, Ab-3, OP29, Oncogene) against an epitope on the misfolded dominant negative p53 proteins.

Statistical Analysis

Data was analyzed using Graphpad prism version 6 (Graphpad Software Inc. La Jolla, USA). The study groups were compared using a two-tailed, unpaired student's *t*-test and a *P*-value of <0.05 was considered statistically significant. Data are represented as mean ± SD as indicated.

Ethical Approval

The blood bank of the Heinrich-Heine-University Hospital, Düsseldorf provided buffy-coats from anonymous blood donors after the ethics committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf (Reference number: 4767R-2014072657) approved use of these samples for the study.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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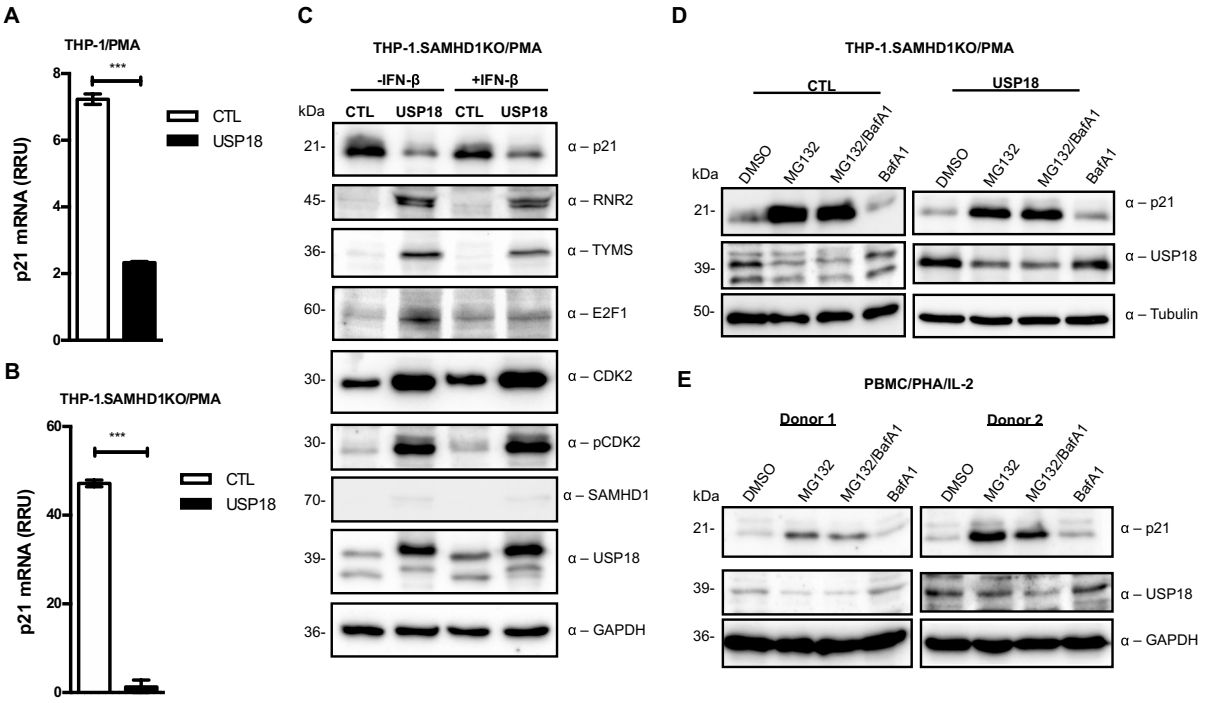
Figure 1


A
THP-1/PMA
***
p21 mRNA (RRU)
CTL
USP18
B
THP-1.SAMHD1KO/PMA
***
p21 mRNA (RRU)
CTL
USP18
C
THP-1.SAMHD1KO/PMA
-IFN-β
+IFN-β
kDa
CTL
USP18
CTL
USP18
21-
α – p21
45-
α – RNR2
36-
α – TYMS
60-
α – E2F1
30-
α – CDK2
30-
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70-
α – SAMHD1
39-
α – USP18
36-
α – GAPDH
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USP18
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MG132/BafA1
BafA1
21-
α – p21
39-
α – USP18
50-
α – Tubulin
E
PBMC/PHA/IL-2
Donor 1
Donor 2
kDa
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MG132
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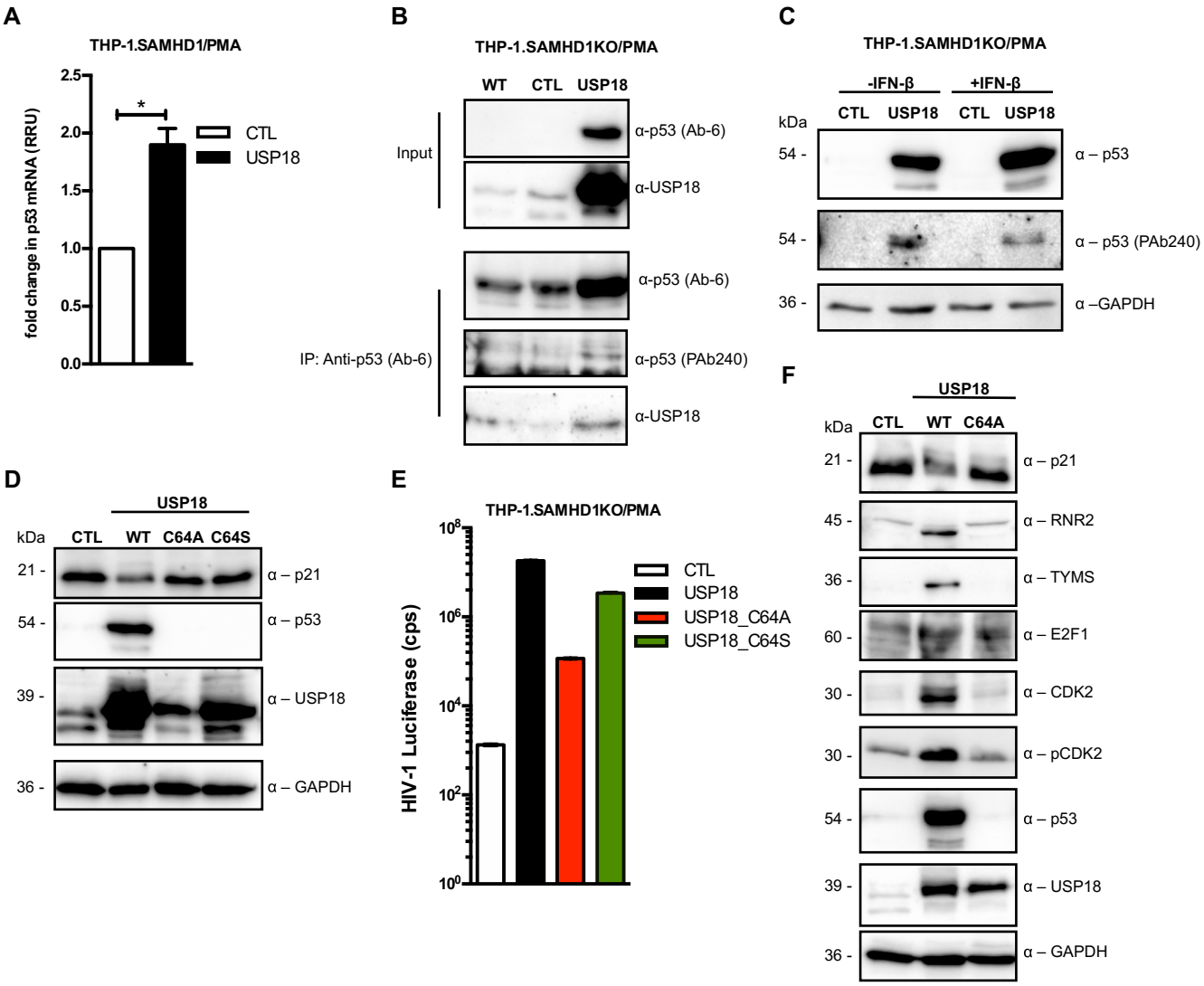
BafA1
DMSO
MG132
MG132/BafA1
BafA1
21-
α – p21
39-
α – USP18
36-
α – GAPDH

Fig. 1. USP18 relieves p21 repression of E2F1 and *de novo* dNTP biosynthesis. Total RNA from PMA-differentiated wild-type (**A**) and SAMHD1KO THP-1 cells (**B**) expressing USP18 and vector controls were isolated and quantified for p21 mRNA expression using qRT-PCR and normalized to HPRT1 (RRU= 7 and 2 for THP-1.USP18 and control, 47 and 2 for SAMHD1KO THP-1.USP18 and control cells respectively). (**C**) PMA-differentiated SAMHD1KO THP-1.USP18 and controls were treated with or without 1000 U/ml of IFN-β. 24 h post-treatment, the cells were lysed and immunoblotted for p21, RNR2, TYMS and E2F1 expression. Further, total and phosphorylated CDK2, SAMHD1, USP18, and GAPDH as loading control were detected, using the respective antibodies. (**D**) PMA-differentiated SAMHD1KO THP-1 cells over-expressing USP18 and its vector controls were treated with DMSO, MG132 (5 μM) or Bafilomycin (BafA1, 10 nM) or both. 16 h post-treatment, the cells were lysed and immunoblotted for p21, USP18 with GAPDH as a loading control. (**E**) In a related experiment, activated PBMCs from two different donors were treated with DMSO, MG132 or BafA1 or both and immunoblotted for p21, USP18 and GAPDH as a loading control. The average of the "Ct mean" for two independent experiments expressed as mean ± SD was compared between the groups in panel A and B by Student's *t* test. (*) indicates $P < 0.05$. Each panel is a representative of at least two independent experiments.

Figure 2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THP-1.SAMHD1/PMA
fold change in p53 mRNA (RRU)
2.5
2.0
1.5
1.0
0.5
0.0
*
CTL
USP18
B
THP-1.SAMHD1KO/PMA
WT
CTL
USP18
Input
α-p53 (Ab-6)
α-USP18
IP: Anti-p53 (Ab-6)
α-p53 (Ab-6)
α-p53 (PAb240)
α-USP18
C
THP-1.SAMHD1KO/PMA
-IFN-β
+IFN-β
CTL
USP18
CTL
USP18
kDa
54 -
α – p53
54 -
α – p53 (PAb240)
36 -
α –GAPDH
D
USP18
kDa
CTL
WT
C64A
C64S
21 -
α – p21
54 -
α – p53
39 -
α – USP18
36 -
α – GAPDH
E
THP-1.SAMHD1KO/PMA
HIV-1 Luciferase (cps)
10^8
10^6
10^4
10^2
10^0
CTL
USP18
USP18_C64A
USP18_C64S
F
USP18
kDa
CTL
WT
C64A
21 -
α – p21
45 -
α – RNR2
36 -
α – TYMS
60 -
α – E2F1
30 -
α – CDK2
30 -
α – pCDK2
54 -
α – p53
39 -
α – USP18
36 -
α – GAPDH

Fig. 2. USP18 induces HIV-1 enhancing p53 in myeloid THP-1 cells. (**A**) p53 mRNA expression in the SAMHD1KO THP-1.USP18 and control cells was quantified by qRT-PCR, normalized to HPRT1 (RRU = 0.15 and 0.34 respectively). **(B)** Protein lysates from PMA-differentiated SAMHD1KO THP-1.USP18 and control cells were immunoprecipitated with anti-p53 (Ab-6) antibody and immunoblotted for misfolded dominant negative p53 proteins using an antibody that recognizes an epitope exposed by activating mutations or denaturation of p53 (PAb240) or a general anti-p53 (Ab-6). (**C**) PMA-differentiated SAMHD1KO THP-1.USP18 and control cells were treated with or without 1000 U/ml of IFN-β. 24 h post-treatment, the cells were lysed and immunoblotted for p53 and dominant negative, misfolded p53 (PAb240) with GAPDH as a loading control. (**D**) SAMHD1KO THP-1 cells transduced with wild-type USP18 or active site mutants (C64A and C64S) or the control cells were immunoblotted for p21, p53, USP18 and GAPDH as a loading control. (**E**) In a follow-up experiment, the cells in panel (**C**) were transduced with HIV-1 luciferase reporter virus. 48 h post-infection, the cells were lysed and luciferase activity was measured. **(F)** Wild-type USP18, active site mutant C64A and the control cells in SAMHD1KO THP-1 cells were immunoblotted for p21, RNR2, TYMS and E2F1. Further probes for total and phosphorylated CDK2, p53, USP18 and GAPDH as a loading control were probed using the respective antibodies. Each panel is a representative of at least three independent experiments.

Figure 3

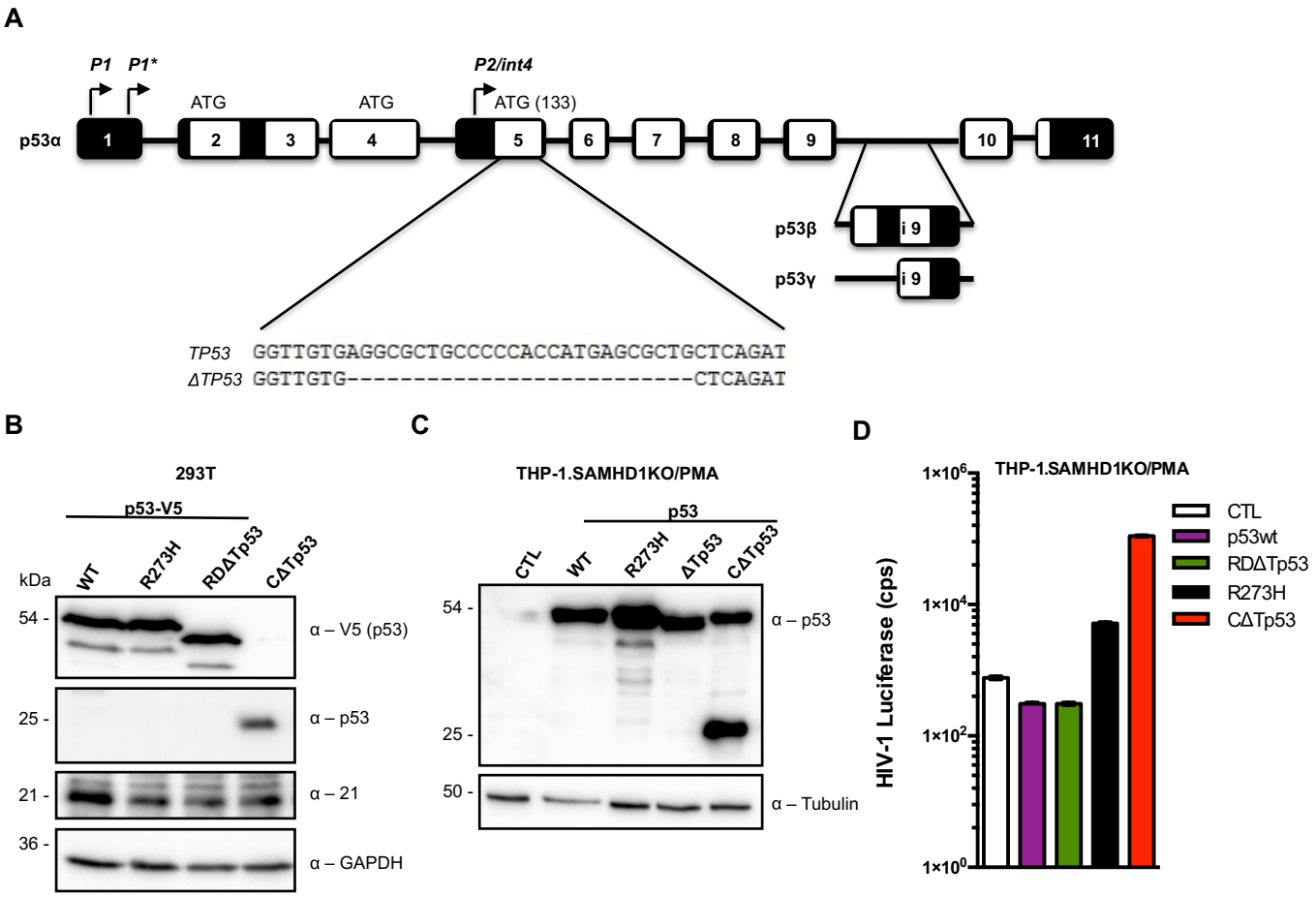

A
P1
P1*
P2/int4
ATG
ATG
ATG (133)
p53α
1
2
3
4
5
6
7
8
9
10
11
p53β
i 9
p53γ
i 9
TP53 GGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGAT
ΔTP53 GGTTGTG-----------CTCAGAT
B
293T
p53-V5
kDa
WT
R273H
RDΔTp53
CΔTp53
54 -
α – V5 (p53)
25 -
α – p53
21 -
α – 21
36 -
α – GAPDH
C
THP-1.SAMHD1KO/PMA
p53
CTL
WT
R273H
ΔTp53
CΔTp53
54 -
α – p53
25 -
50 -
α – Tubulin
D
THP-1.SAMHD1KO/PMA
HIV-1 Luciferase (cps)
1×10^6
1×10^4
1×10^2
1×10^0
CTL
p53wt
RDΔTp53
R273H
CΔTp53

Fig. 3. "Gain of function" p53 mutants promote HIV-1 replication. (A) Total RNA from wild-type THP-1 cells was isolated, reversed transcribed and p53 cDNA was amplified. The amplified p53 cDNA was cloned and sequenced for mutations and deletions in the gene. A 26 base pair deletion in one allele of the p53 gene in the THP-1 cells was confirmed using genomic DNA sequence of the p53 gene. (**B**) V5-tagged wild-type p53, single amino acid (R273H) mutant p53, RD domain mutant (RDΔTp53) and a C-terminal truncation mutant (CΔTp53) of p53 were expressed in 293T cells and lysates were immunoblotted for anti-V5, 25-kDa truncation mutant p53, p21 and GAPDH as a loading control. (**C**) pLOC plasmids expressing wild-type p53, R273H, RDΔTp53, or CΔTp53 were stably expressed in the THP-1 cells. These cell lines were subsequently differentiated by 25 ng/ml of PMA and immunoblotted for p53 and GAPDH as a loading control. (**D**) The wild-type p53 and its mutants (R273H, RDΔTp53 or CΔTp53) expressed in SAMHD1KO THP-1 cells were transduced with HIV-1 luciferase reporter virus. 48 h after transduction, luciferase activity was measured. Each panel is a representative of at least two independent experiments.

Figure 4

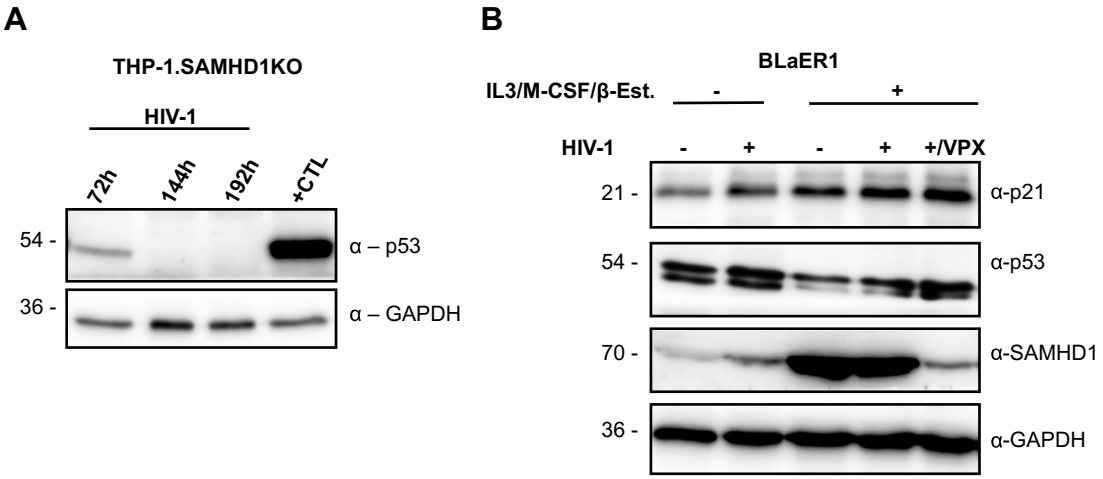
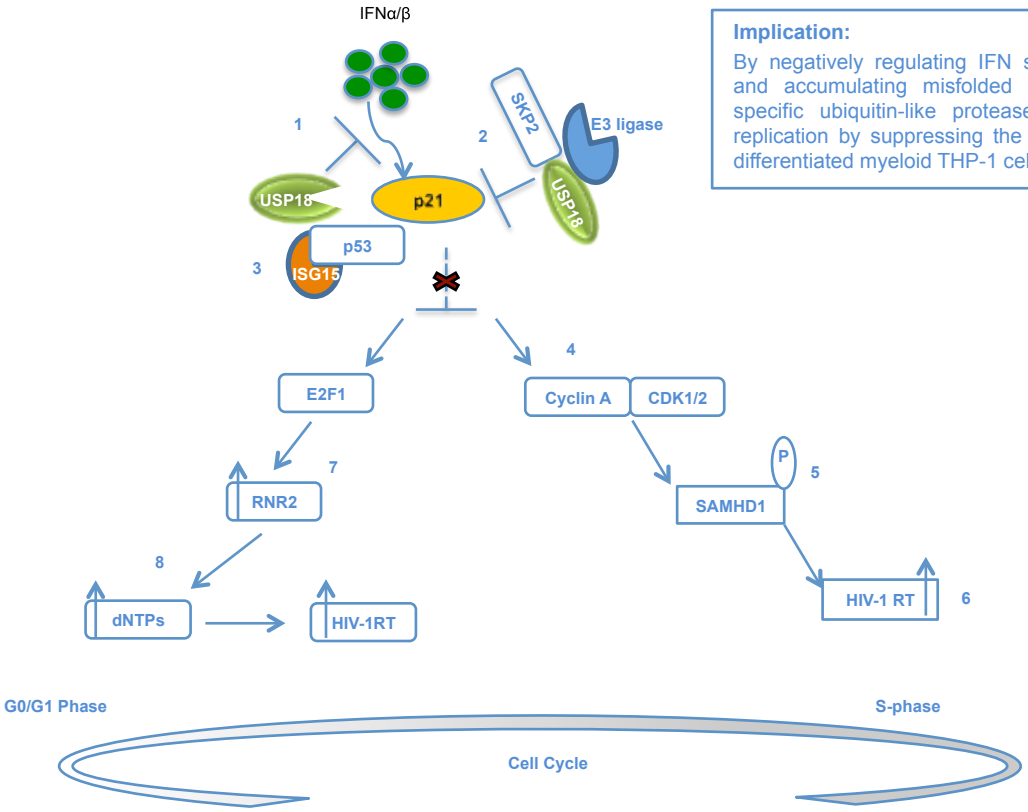

A
THP-1.SAMHD1KO
HIV-1
72h
144h
192h
+CTL
54 -
α – p53
36 -
α – GAPDH
B
BLaER1
IL3/M-CSF/β-Est.
-
+
HIV-1
-
+
-
+
+/VPX
21 -
α-p21
54 -
α-p53
70 -
α-SAMHD1
36 -
α-GAPDH

Fig. 4. HIV-1 induces p53 and p21 in trans-differentiated BlaER1 cells. (**A**) SAMHD1KO THP-1 cells were transduced with HIV-1 at an MOI of 2 at different time points. After each time point, the cells were harvested, lysed and immunoblotted for p53 with GAPDH as a loading control. Cell lysate from SAMHD1KO THP-1 cells overexpressing p53 was included in the samples as a positive control (+CTL). (**B**) BlaER1 cells were trans-differentiated into monocytes using IL3, M-CSF and β-estradiol. On day 7 after trans-differentiation, the BlaER1 trans-differentiated monocytes were transduced with HIV-1 luciferase reporter virus with or without VPX from $SIVmac_{239}$ at an MOI of 2. 48 h after transduction, the cells were lysed and immunoblotted for p21, p53, SAMHD1 and GAPDH as a loading control.

Figure 5



Implication:

By negatively regulating IFN signalling, complexing with SKP2 and accumulating misfolded dominant negative p53, ISG15-specific ubiquitin-like protease 43 (USP18) enhances HIV-1 replication by suppressing the antiviral function of p21 in PMA-differentiated myeloid THP-1 cells.

Fig. 5. Proposed Model for USP18-mediated downregulation of p21 expression and antiviral function. Type I IFN up-regulates p21 expression in THP-1 cells. The expression of USP18 (1) negatively regulates IFN induction of p21, likely retains SKP2, a recognition factor for the E3 ligase complex, which targets p21 for proteasomal degradation (2). By its protease activity, USP18 further accumulates misfolded dominant negative p53, which requires modification by ISG15 for the proteasome (3). Thus, the negative regulation of type I IFNs, the retention of SKP2 and the accumulation of misfolded dominant negative p53, mediated by USP18 downregulates p21 expression, which is thereby unable to block the effect of cyclin A-CDK1/2 complex (4) on SAMHD1 phosphorylation (5), resulting in increase in HIV-1 replication (6). Also USP18-mediated downregulation of p21 relieves p21-mediated repression of E2F1 transactivation of RNR2 (7), resulting in increased production of intracellular dNTP pool, which is required for enhanced HIV-1 replication (8).

CHAPTER III

CD25_ FoxP3_ Memory CD4 T Cells Are Frequent Targets of HIV Infection In Vivo

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Edmund Osei Kuffour's contribution

1. Performed the experiments in Fig 3.
2. Wrote part of the method section of the paper

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Author:	Mkunde Chachage,Georgios Pollakis,Edmund Osei Kuffour,Kerstin Haase,Asli Bauer,Yuka Nadai,Lilli Podola,Petra Clowes,Matthias Schiemann,Lynette Henkel,Dieter Hoffmann,Sarah Joseph,Sabin Bhuju,Leonard Maboko,Fred Stephen Sarfo,Kirsten Eberhardt,Michael Hoelscher,Torsten Feldt,Elmar Saathoff,Christof Geldmacher
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CD25$^+$ FoxP3$^+$ Memory CD4 T Cells Are Frequent Targets of HIV Infection *In Vivo*

Mkunde Chachage,[a] Georgios Pollakis,[b] Edmund Osei Kuffour,[c] Kerstin Haase,[d] Asli Bauer,[a,e] Yuka Nadai,[e] Lilli Podola,[a,e] Petra Clowes,[a,e] Matthias Schiemann,[f,g] Lynette Henkel,[f] Dieter Hoffmann,[h] Sarah Joseph,[i] Sabin Bhuju,[j] Leonard Maboko,[a] Fred Stephen Sarfo,[k] Kirsten Eberhardt,[l] Michael Hoelscher,[e,m] Torsten Feldt,[c] Elmar Saathoff,[e,m] Christof Geldmacher[e,m]

NIMR Mbeya Medical Research Centre, Mbeya, Tanzania[a]; Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom[b]; Department of Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Dusseldorf, Germany[c]; Department of Genome Oriented Bioinformatics, Technische Universität München, Freising, Germany[d]; Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Munich, Germany[e]; Institute for Medical Microbiology, Immunology and Hygiene,[f] Clinical Cooperation Groups Antigen-Specific Immunotherapy and Immune-Monitoring,[g] and Institute of Virology,[h] Helmholtz Center Munich, Technische Universität München, Munich, Germany; MRC Clinical Trials Unit at UCL, London, United Kingdom[i]; Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany[j]; Kwame Nkrumah University of Science & Technology, Kumasi, Ghana[k]; Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany[l]; German Center for Infection Research (DZIF), Partner Site Munich, Munich, Germany[m]

ABSTRACT

Interleukin 2 (IL-2) signaling through the IL-2 receptor alpha chain (CD25) facilitates HIV replication *in vitro* and facilitates homeostatic proliferation of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells. CD25$^+$ FoxP3$^+$ CD4$^+$ T cells may therefore constitute a suitable subset for HIV infection and plasma virion production. CD25$^+$ FoxP3$^+$ CD4$^+$ T cell frequencies, absolute numbers, and the expression of CCR5 and cell cycle marker Ki67 were studied in peripheral blood from HIV$^+$ and HIV$^-$ study volunteers. Different memory CD4$^+$ T cell subsets were then sorted for quantification of cell-associated HIV DNA and phylogenetic analyses of the highly variable EnvV1V3 region in comparison to plasma-derived virus sequences. In HIV$^+$ subjects, 51% (median) of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells expressed the HIV coreceptor CCR5. Very high frequencies of Ki67$^+$ cells were detected in CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells (median, 27.6%) in comparison to CD25$^-$ FoxP3$^-$ memory CD4$^+$ T cells (median, 4.1%; $P < 0.0001$). HIV DNA content was 15-fold higher in CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells than in CD25$^-$ FoxP3$^-$ T cells ($P = 0.003$). EnvV1V3 sequences derived from CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells did not preferentially cluster with plasma-derived sequences. Quasi-identical cell-plasma sequence pairs were rare, and their proportion decreased with the estimated HIV infection duration. These data suggest that specific cellular characteristics of CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells might facilitate efficient HIV infection *in vivo* and passage of HIV DNA to cell progeny in the absence of active viral replication. The contribution of this cell population to plasma virion production remains unclear.

IMPORTANCE

Despite recent advances in the understanding of AIDS virus pathogenesis, which cell subsets support HIV infection and replication *in vivo* is incompletely understood. *In vitro*, the IL-2 signaling pathway and IL-2-dependent cell cycle induction are essential for HIV infection of stimulated T cells. CD25$^+$ FoxP3$^+$ memory CD4 T cells, often referred to as regulatory CD4 T cells, depend on IL-2 signaling for homeostatic proliferation *in vivo*. Our results show that CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells often express the HIV coreceptor CCR5, are significantly more proliferative, and contain more HIV DNA than CD25$^-$ FoxP3$^-$ memory CD4 T cell subsets. The specific cellular characteristics of CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells probably facilitate efficient HIV infection *in vivo* and passage of HIV DNA to cell progeny in the absence of active viral replication. However, the contribution of this cell subset to plasma viremia remains unclear.

AIDS is caused by human immunodeficiency virus (HIV) infection and is characterized by the failure of the immune system to control diverse opportunistic infections facilitated by the progressive loss of CD4 T cells. The rate of CD4 T cell depletion correlates with set point levels of HIV-1 viral load in plasma (1) and is critically dependent on ongoing viral replication. Antiretroviral therapy (ART) blocks viral replication, reverses CD4 T cell depletion (2), and reconstitutes immunity to most opportunistic pathogens. Replication of HIV within CD4 T cells significantly contributes to plasma viral load and thus to HIV disease progression (3). It is well established that intracellular HIV DNA loads *in vivo* are influenced by CD4 T cell differentiation (4–6), functional properties of CD4 T cells (7), and pathogen specificity (8–10) and that T cell activation and proliferation contribute to productive HIV infection of memory CD4 T cells (11–15). Together these results imply that, depending on their biological properties, different CD4 T cell subsets might differ in their susceptibilities to HIV infection and their contributions to virion production *in vivo*. Perhaps the best characterized CD4 T cell subset in this re-

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Address correspondence to Mkunde Chachage, mchachage@nimr-mmrc.org, or Christof Geldmacher, geldmacher@lrz.uni-muenchen.de.

gard is follicular CD4 T helper cells (Tfh), which are essential for germinal center formation and which reside in the periphery of B cell follicles within secondary lymphoid organs (reviewed in reference 16). Recent data demonstrate that Tfh cells are a major reservoir for HIV replication *in vivo* (17, 18) and contribute to persistent simian immunodeficiency virus (SIV) virion production even in elite controller, aviremic macaques (19). In viremic macaques, virion production appears to be less restricted anatomically (19) and other cell subsets are likely to contribute.

One such cell subset may be memory CD4 T cells expressing the interleukin 2 (IL-2) receptor alpha chain (CD25). Interception of IL-2 signaling, which is required for antigen-specific proliferation and survival of CD4 T cells (reviewed in reference 20) almost completely abrogates productive HIV infection in cell cultures stimulated *in vitro* (13, 21–23). Moreover, expression of CD25 defines a CD4 T cell population that efficiently supports productive HIV infection in lymphoid tissue explants (10, 14). *In vivo*, CD25 expression is characteristic for CD4 T cells (24–26) coexpressing the transcription factor forkhead box P3 (FoxP3) often referred to as regulatory T cells (T_{regs}). CD25$^+$ FoxP3$^+$ CD4 T cells can suppress the activation, proliferation, and effector functions of a wide range of immune cells, including CD4 and CD8 T cells (reviewed in reference 27), activities shown essential for the maintenance of self-tolerance but which can also impede the clearance of chronic infections (28, 29). The vast majority (>80%) of circulating CD25$^+$ FoxP3$^+$ CD4 T cells express the memory marker CD45RO (30, 31), and high frequencies of these cells coexpress the cell cycle marker Ki67 in peripheral blood (10 to 20%) and even more so in secondary lymphoid tissue (40 to 80%) (30, 32), indicating high levels of *in vivo* proliferation. The doubling time of memory CD25$^+$ FoxP3$^+$ CD4 T cells in humans is only 8 days, which is 3-fold and 25-fold less than that of memory and naive CD4 T cells, respectively (33). These specific cell characteristics and the proposed mechanism of constant IL-2-dependent homeostatic replenishment of this cell subset (33, 34) support the hypothesis that CD25$^+$ FoxP3$^+$ CD4 T cells are particularly susceptible to HIV infection *in vivo* and may contribute to plasma virus production in viremic HIV progressors—potentially driven by IL-2 secreted by autoantigen-specific T cells (35).

To address this hypothesis, we analyzed peripheral blood samples of HIV-positive (HIV$^+$) and -negative (HIV$^-$) individuals for CD25$^+$ FoxP3$^+$ CD4 T cell numbers and frequencies, expression of HIV coreceptor CCR5, and the cell proliferation marker Ki67 in relation to HIV infection. We have also assessed the levels of cell-associated viral DNA and the phylogenetic relationship between cell- and plasma-derived HIV envelope sequences relative to those of other memory CD4 T cell subsets. Confirming previous reports (36), our data show that high proportions of circulating CD25$^+$ FoxP3$^+$ CD4 T cells express the HIV coreceptor CCR5. Furthermore, memory CD25$^+$ FoxP3$^+$ CD4 T cells from HIV$^+$ subjects contained high frequencies of Ki67$^+$ cells and higher levels of HIV DNA than memory CD4 T cells that were CD25$^-$ FoxP3$^-$. However, a phylogenetic comparison of the highly variable HIV EnvV1V3 region between plasma- and cell-derived virus sequences did not allow definite conclusions about the cellular origin of plasma virions, because sequences from the two compartments behaved similarly and intermingled with no evidence of compartmentalization. Instead, we observed that the phylogenetic distance between plasma- and memory cell-derived viral sequences increases with the duration of HIV infection, with a simultaneous decrease in the proportion of detectable quasi-identical cell-plasma sequence pairs.

MATERIALS AND METHODS

Cohorts, study volunteers, and blood processing. The WHIS cohort comprised 361 adult volunteers who were enrolled in a prospective cohort (WHIS) that studies the interaction between HIV-1 and helminth infection in the Mbeya region in southwestern Tanzania. The WHIS cohort study is described in detail elsewhere (37). HIV status was determined using the HIV 1/2 Stat-Pak assay (Chembio Diagnostics Systems), and positive results were confirmed using an enzyme-linked immunosorbent assay (ELISA; Bio-Rad). Discrepancies between HIV 1/2 Stat-Pak and ELISA results were resolved by Western blotting (MPD HIV blot 2.2; MP Biomedicals). Forty milliliters of venous blood was drawn from each participant by the use of anticoagulant tubes (citrate phosphate dextrose adenine [CPDA], EDTA; BD Vacutainer). Absolute CD4 T cell counts were determined in anticoagulated whole blood using the BD Multitest IMK kit (BD Biosciences) according to the manufacturer's instructions. Blood samples were processed in less than 6 h after the blood draw. Frequencies of CD25$^+$ FoxP3$^+$ CD4 T cells and surface CCR5 expression were determined in fresh, anticoagulated whole blood as described below. The absolute numbers of CD25$^+$ FoxP3$^+$ CD4 T cells in the peripheral blood was calculated from the total CD4 T cell counts and the percentage of CD25$^+$ FoxP3$^+$ CD4 T cells. Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll centrifugation method and LeucoSep tubes (Greiner Bio One) according to standard protocols. For the HHECO and HISIS cohorts, PBMCs were isolated from 28 HIV-positive blood donors who were recruited from a previously described cohort (HHECO) at the Komfo Anokye Teaching Hospital in Kumasi, Ghana (38, 39), and PBMCs from the previously described HISIS cohort (40) were also isolated by centrifugation of heparinized venous blood on a Ficoll-Hypaque (Biocoll separating solution; Biochrom AG, Berlin, Germany) density gradient, prior to cryopreservation.

Ethics statement. Ethical approvals for the WHIS and HISIS cohorts were obtained from the Mbeya Regional and National Ethics Committees of the Tanzanian National Institute for Medical Research (NIMR)/Ministry of Health in Dar es Salaam, Tanzania, and from the ethics committee of the University of Munich. The HHECO study was approved by the appropriate ethics committees of the Kwame Nkrumah University of Science and Technology (Ghana) and of the medical association in Hamburg (Germany) (38, 39). Signed informed consent was obtained from all participants.

Characterization of CD25$^+$ FoxP3$^+$ CD4 T cells in fresh whole blood. Fresh anticoagulated whole blood samples from the WHIS cohort were incubated for 30 min using the following fluorochrome-labeled monoclonal antibodies (MAbs) for cell surface staining; CD3-Pacific Blue (BD), CD4-peridinin chlorophyll (PerCP)-Cy5.5 (eBioscience), CD25-phycoerythrin (PE)-Cy7 (eBioscience), and CCR5-allophycocyanin (APC)-Cy7 (BD). Red blood cells in samples were then lysed by incubating and washing the samples twice for 10 min with 1× cell lysis solution (BD). Intracellular FoxP3 was detected with FoxP3-Alexa Fluor 647 (eBioscience) according to the manufacturer's instructions. Cells were finally fixed with 2% paraformaldehyde prior to acquisition. Acquisition was performed on a FACSCanto II system (BD). Compensation was performed with antibody capture beads (BD) stained separately with the individual antibodies used in the test samples. Flow cytometry data were analyzed using FlowJo (version 9.5.3; Tree Star, Inc.).

Characterization of CD25$^+$ FoxP3$^+$ memory CD4 T cells. Cell surface markers of immune regulation and cell proliferation/cell turnover were stained on cryopreserved PBMCs of individuals from the HHECO cohort using anti-CD3-PerCP, anti-CD4-Pacific Blue, anti-CD45RA-Alexa Fluor 700, and anti-CD25-PE-Cy7 (BD Biosciences, Germany). The stained cells were later fixed and permeabilized (FoxP3 staining buffer set; eBiosciences) for intracellular staining using anti-FoxP3-PE (Bioleg-

end, Germany) and anti-Ki67-Alexa Fluor 647 (BD Biosciences, Germany). Flow cytometric data were acquired with an LSRII flow cytometer (BD Biosciences, Germany). Compensation was performed with antibody capture beads (BD CompBeads set anti-mouse Igκ; BD Biosciences, Germany), stained separately with the individual fluorochrome-conjugated monoclonal antibodies used in all samples. Flow cytometry measurements were analyzed using FlowJo version 9.6.2 (Tree Star, San Carlos, USA).

Cell sorting. Cryopreserved PBMCs from HIV$^+$ WHIS ($n = 15$) and HISIS ($n = 6$) participants were thawed and washed twice in prewarmed (37°C) complete medium (RPMI medium plus 10% heat-inactivated fetal bovine serum [GIBCO]) that was supplemented with Benzonase (5 U/ml; Novagen). Surface staining was performed with CD3-Pacific Blue, CD4-PerCP-Cy5.5, CD25-Pe-Cy7, and CD45RO-PE (BD) for 30 min in the dark at room temperature; intracellular staining was performed with FoxP3-Alexa Fluor 647 (eBioscience) and Helios-fluorescein isothiocyanate (FITC) (BioLegend) according to the CD25$^+$ FoxP3$^+$ CD4 T cell staining protocol mentioned above. Cell sorting was performed on a FACSAria cell sorter (BD) after gating on CD3$^+$ CD4$^+$ CD45RO$^+$ cells into "regulatory T cell populations" (CD25$^+$ FoxP3$^+$ Helios$^+$ and CD25$^+$ FoxP3$^+$ Helios$^-$) and memory populations (CD25$^-$ FoxP3$^-$ Helios$^+$ and CD25$^-$ FoxP3$^-$ Helios$^-$) (see Fig. 4A). Between 293 and 750,000 fixed CD4 T cells from each of the four different populations were collected, depending on the number of PBMCs available from each individual. Cells were collected on fluorescence-activated cell sorter (FACS) buffer consisting of phosphate-buffered saline (PBS) mixed with 0.5% bovine serum albumin (BSA; Sigma), 2 mM EDTA, and 0.2% sodium azide at pH 7.45. The median counts of fixed cells collected for each population were as follows: CD25$^+$ FoxP3$^+$ Helios$^+$ cells, 9,017 (median) and 3,931 to 14,412 (interquartile range [IQR]); CD25$^+$ FoxP3$^+$ Helios$^-$ cells, 4,381 (median) and 1,579 to 9,799 (IQR); CD25$^-$ FoxP3$^-$ Helios$^+$ cells, 2,646 (median) and 1,336 to 5,644 (IQR); and CD25$^-$ FoxP3$^-$ Helios$^-$ cells, 185,000 (median) and 79,000 to 315,000 (IQR). Sorted cells were then centrifuged at 13,000 rpm for 3 min, and the supernatant was removed. The cell pellet was stored at −80°C until further analysis.

Quantification of cell-associated HIV *gag* DNA. Quantification of cell-associated HIV *gag* DNA was performed as previously described (8) with minor modifications. Sorted CD4 T cell subsets were lysed in 30 μl of 0.1 mg/ml proteinase K (Roche) containing 10 mM Tris-Cl (pH 8) (Sigma) for 1 h at 56°C, followed by a proteinase K inactivation step for 10 min at 95°C. Cell lysates were then used to quantify cell-associated HIV DNA by quantitative PCR (qPCR) as previously described, with some modifications (10). Briefly, the *gag* primers and probe used were as follows: 783gag, forward primer, 5′-GAG AGA GAT GGG TGC GAG AGC GTC-3′ ($T_m > 60$); 895gag, reverse primer, 5′-CTK TCC AGC TCC CTG CTT GCC CA-3′ ($T_m > 60$); 6-carboxyfluorescein (FAM)-labeled probe 844gagPr, 5′-ATT HGB TTA AGG CCA GGG GGA ARG AAA MAA T-3′. They had been designed to optimally cover subtypes A, C, and D, which prevalent in the Mbeya Region (10). To quantify the cell number in each reaction mix, the human prion gene copy number was also assessed by qPCR. Prion primers and probe sequences were as follows: prion forward primer, 5′TGC TGG GAA GTG CCA TGA G-3′; prion reverse primer, 5′CGG TGC ATG TTT TCA CGA TAG-3′; probe, 5′FAM-CAT CAT ACA TTT CGG CAG TGA CTA TGA GGA CC-6-carboxytetramethylrhodamine (TAMRA) (41). Five microliters of lysate was used in a total reaction volume of 25 μl containing 0.8 μM *gag* primers or 0.4 μM prion primers, 0.4 μM probe, a 0.2 mM concentration of each deoxynucleoside triphosphate, 3.5 mM $MgCl_2$, and 0.65 U Platinum *Taq* in the supplied buffer. Standard curves were generated using the HIV-1 *gag* gene (provided by Brenna Hill, Vaccine Research Center, NIH, Bethesda, MD) and prion gene-containing plasmids. Real-time PCR was performed in a Bio-Rad cycler CFX96 (Bio-Rad): 5 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. To ensure comparability of the results, cell-associated *gag* DNA from the four different memory CD4 T cell subsets, which were sorted from one patient, were always quantified simultaneously. Cell-associated *gag* DNA in memory CD25$^+$ FoxP3$^+$ CD4 T cells and CD25$^-$ FoxP3$^-$ memory CD4 T cells independent of Helios expression was calculated as follows: $\sum$*gag* DNA load (Helios$^+$ cells + Helios$^-$ cells)/$\sum$sorted cells in 5 μl lysate (Helios$^+$ cells + Helios$^-$ cells).

Amplification and phylogenetic comparison of HIV envelope sequences from plasma and sorted cell populations. A highly variable envelope region spanning the V1 to V3 region (EnvV1V3; Hxb 6559 to 7320) was amplified using a nested-PCR strategy from 10 μl of lysed sorted cells (described above) or from plasma virus cDNA. HIV RNA was extracted with sample preparation system RNA on the m24sp automatic extraction instrument (Abbott Molecular, USA) in accordance with the manufacturer's instructions. The HIV cDNA was synthesized from 3 μl of extracted RNA using the reverse primer ACD_Env7521R (5′ATGGGAGGGGCATAYAT TGC) and the Superscript III reverse transcriptase (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Newly designed PCR primer pairs optimized for the detection of subtypes A, C, and D were used to amplify the EnvV1V3 region. The first-round PCR was performed with 10 μl of template in a 50-μl reaction mixture (0.5 μl [5 U] Platinum *Taq* [Life Technologies, Darmstadt, Germany], 2.0 mM primers [ACD_Env6420F, 5′CATAATGTCTGGGCYACACATGC; ACD_Env7521R, 5′ATGGGAGGGGCATAYATTGC], 3.5 mM $MgCl_2$, 4 μl of deoxynucleoside triphosphates [dNTPs]) at 95°C for 10 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and finally 7 min at 72°C. The second-round PCR was performed with 2 μl of first-round PCR product in a 50-μl reaction mixture (0.25 μl [2.5 U] AmpliTaq Gold [Life Technologies, Darmstadt, Germany], 2.0 mM ACD_Env6559F [5′GGGAYSAAAGCCTAAARCCATGTG] and ACD_Env7320R [GTTGTAATTTCTRRR TCCCCTCC], 2.0 mM $MgCl_2$, 4 μl of dNTPs) at 95°C for 10 min, followed by 45 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s, and finally 7 min at 72°C. The second-round PCR products were extracted from agarose gel and then cloned using the TOPO-TA Cloning kit for sequencing (Life Technologies, Darmstadt, Germany), including the precut vector pCR4.1 and One Shot chemically competent *E. coli* according to the manufacturer's instructions. EnvV1V3 sequences from 11 to 23 clones per population per subject were then sequenced unidirectionally using Mnrev primers at Eurofins Genomics (Ebersberg, Germany). In total, 384 EnvV1V3 sequences from 6 subjects were analyzed.

To assess the error rate of the applied nested-PCR strategy, the positive-control template (Du422, clone 1 [SVPC5]) (42) was endpoint diluted using a 10-fold dilution series and amplified as described above. The EnvV1V3 product from the last detectable dilution step was then cloned as described above. Sequences from 21 clones were analyzed and compared to the original Du422 template sequence.

Phylogenetic analyses. Nucleotide sequences were aligned with respect to the predicted amino acid sequence of the reference alignment extracted from the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) as previously described (43) Evolutionary analyses were conducted using MEGA6 software (44). The evolutionary history is inferred by using the maximum-likelihood method based on the general time-reversible substitution model (GTR+G) (45) and is rooted in previous outbreaks. Upon each analysis, the tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is presented next to the branches. The initial tree(s) for the heuristic search was obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value.

Next-generation sequencing (NGS). Library preparation from EnvV1V3 PCR second-round products was done using a TruSeq DNA PCR-free sample preparation kit (Illumina, Inc., San Diego, CA, USA) with 550 bp as the insert size in accordance with the manufacturer's instruction. The libraries were controlled with an Agilent Bioanalyzer high-sensitivity chip (Agilent Technologies) and sequenced using a MiSeq

TABLE 1 Characteristics of study subjects from different cohorts

Parameter	Value for indicated cohort		
	WHIS	HHECO	HISIS
No. of subjects	361	28	6
No. of HIV-positive subjects	103	28	6
No. of females	217	25	6
Age (yr), mean (SD)	34.3 (11.05)	38.8 (7.5)	28 (3.2)
Median no. of CD4 cells/μl (IQR)[a]	396 (265–603)	629 (444–900)	496 (231–707)
Median log no. of pVL copies/ml (IQR)[a]	4.67 (3.74–5.23)	1.59 (1.59–3.82)	4.9 (4.4–5.5)
No. of subjects (%) on ART[a]	3 (0.8)	20 (71.4)	0 (0)

[a] Data given for HIV-positive subjects only.

desktop sequencer (Illumina, Inc.) and MiSeq reagent kits v3 (Illumina, Inc.). The sequencing was done to 250 cycles in both directions. The produced reads were processed through a quality control pipeline that removed all reads containing unresolved positions or had a mean quality below 20. Furthermore, poly(A) tails and low-quality read ends were trimmed away. All reads that had lengths below 30 nucleotides (nt) after trimming were also excluded from further analysis. An initial mapping was created for each sample by placing the reads onto the HIV HXB2 reference sequence (GenBank accession no. K03455.1 [46]) using segemehl (version 0.1.6) (47). The difference parameter was set to 2 in order to increase the sensitivity, given that the origin of the sequences was a highly variable viral genome. Using an adapted samtools (version 0.1.19) (48) pipeline, we created a consensus sequence for each sample from the initial mapping to use as an individual reference for a second round of alignments. This was necessary, as the official HIV reference sequence is very divergent from our set of reads, and thus the initial mapping was only able to place an unsatisfyingly low number of reads onto this sequence. The second individual mapping was able to use a higher number of reads and to create sufficient alignments, which were used as input for the quasispecies reconstruction tool QuasiRecomb (49). This tool uses an expectation maximization algorithm not only to reconstruct the single sequences present in the viral population but also to assign their relative proportions.

Statistical analysis. Data analyses were performed using Prism version 4.0 software (GraphPad, Inc.). Comparisons of two groups were performed using the Mann-Whitney test. Comparisons of paired groups were performed using the Wilcoxon matched-pairs test. For correlation analyses, the Spearman *r* test, the Pearson two-tailed statistical test, or linear regression was used. Differences were considered significant at *P* values of <0.05. Tests used for statistical analysis are described in the figure legends.

Accession number(s). Newly determined sequences were submitted to GenBank under accession numbers KX689364 to KX689748.

RESULTS

Study subjects. Table 1 provides an overview of the subjects included in this study. A total of 258 HIV-negative and 103 HIV-positive adults (mean age, 34.3 years) from the WHIS cohort (37) were included in this study, of which 217 (60%) were female. The vast majority of HIV$^+$ subjects from the WHIS cohort were treatment naive (97%), with a median CD4$^+$ T cell count of 396.3 cells/μl and median $\log_{10}$ plasma viral load of 4.7 copies/ml. Twenty-eight subjects from the previously described HHECO cohort were included for the in-depth characterization of memory CD25$^+$ FoxP3$^+$ CD4 T cells (38, 39) (Table 1). PBMCs from 6 viremic HIV$^+$ subjects from the HISIS cohort (40) were used for the characterization of HIV infection within different memory T cell subsets.

Correlation between CD4 T cells and CD25$^+$ FoxP3$^+$ CD4 T cell counts in HIV-infected subjects. We first determined and compared the frequencies and absolute numbers of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells in fresh anticoagulated peripheral blood of HIV$^+$ (treatment naive, $n = 100$) and HIV$^-$ ($n = 258$) subjects from the WHIS cohort. A representative dot plot and gating of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells are shown in Fig. 1A. In HIV$^+$ individuals, compared to HIV$^-$ individuals, CD25$^+$ FoxP3$^+$ CD4$^+$ T cell frequencies were moderately increased (Fig. 1B) (for HIV$^+$, median, 2.5%; IQR, 1.5% to 4.5%; versus for HIV$^-$, median, 2.1%; IQR, 1.5% to 2.9%; $P = 0.03$), but absolute numbers of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells were significantly decreased, with median counts of 10.16 cells/μl (IQR, 4.88 to18.57 cells/μl) in HIV$^+$ subjects and 17.75 cells/μl (IQR, 11.06 to 24.56 cells/μl) in HIV$^-$ subjects ($P < 0.0001$) (Fig. 1C). Within HIV$^+$ subjects, there was a positive correlation between CD25$^+$ FoxP3$^+$ CD4$^+$ T cell counts and CD4 T cell counts ($P < 0.0001$, $r = 0.6152$) (Fig. 1D). Confirming previous reports (50–54), our data show that the depletion of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells is closely linked to the loss of CD4 T cells.

High frequencies of CD25$^+$ FoxP3$^+$ CD4 T cells express HIV coreceptor CCR5 and the cell cycle marker Ki67. In order to determine whether CD25$^+$ FoxP3$^+$ CD4$^+$ T cells could potentially support entry of HIV, we assessed the expression of the HIV coreceptor CCR5. Fresh anticoagulated whole blood was used for improved CCR5 staining. A representative plot is shown in Fig. 2A. A considerable proportion of CD25$^+$ FoxP3$^+$ CD4$^+$ T cells expressed CCR5 (median, 53.7%), which was higher than that previously observed in total memory CD4 T cells (median, 40%; data not shown). HIV infection was associated with a moderate decrease in the frequency of CCR5-positive CD25$^+$ FoxP3$^+$ CD4$^+$ T cells (Fig. 2B) (median, 50.9%, compared to 54.5%; $P = 0.01$).

We next studied the cell cycle status of memory CD25$^+$ FoxP3$^+$ and CD25$^-$ FoxP3$^-$ CD4$^+$ T cells in HIV-infected subjects and analyzed cellular Ki67 expression using cryopreserved PBMC samples ($n = 28$ from HHECO cohort [Table 1]). The representative dot plots for Ki67 staining in memory (CD45RA$^-$) CD25$^+$ FoxP3$^+$ and CD25$^-$ FoxP3$^-$ CD4$^+$ T cells are shown in Fig. 3A. HIV-infected study participants had very high frequencies of Ki67$^+$ memory CD25$^+$ FoxP3$^+$ CD4 T cells (median, 27.6%) (Fig. 3B) despite the majority of subjects from the HHECO cohort being on ART. Importantly, the frequencies of Ki67$^+$ cells detected were 6.7-fold higher in CD25$^+$ FoxP3$^+$ memory CD4$^+$ T cells than in CD25$^-$ FoxP3$^-$ memory CD4$^+$ T cells (median, 4.1%; $P < 0.0001$), consistent with high *in vivo* proliferation of memory CD25$^+$ FoxP3$^+$ CD4 T cells. Correlation analysis demonstrated a close association between the proportions of Ki67$^+$ CD25$^+$ FoxP3$^+$ and Ki67$^+$ CD25$^-$ FoxP3$^-$ memory CD4 T cells ($P = 0.005$, $r = 0.51$) (Fig. 3C), which is linked to the level of CD4 T cell depletion in HIV$^+$ subjects ($P = 0.1$, $r = -0.3$ [Fig. 3D], and $P = 0.02$, $r = -0.4$ [Fig. 3E]). Memory CD25$^+$ FoxP3$^+$ CD4 T cells may therefore potentially support CCR5-mediated viral entry and subsequent steps of the viral life cycle due to their high *in vivo* proliferation. The correlation between the frequency of Ki67$^+$ memory T cells and CD25$^+$ FoxP3$^+$ CD4 memory T cells and the fact that the losses of these cell subsets are closely linked

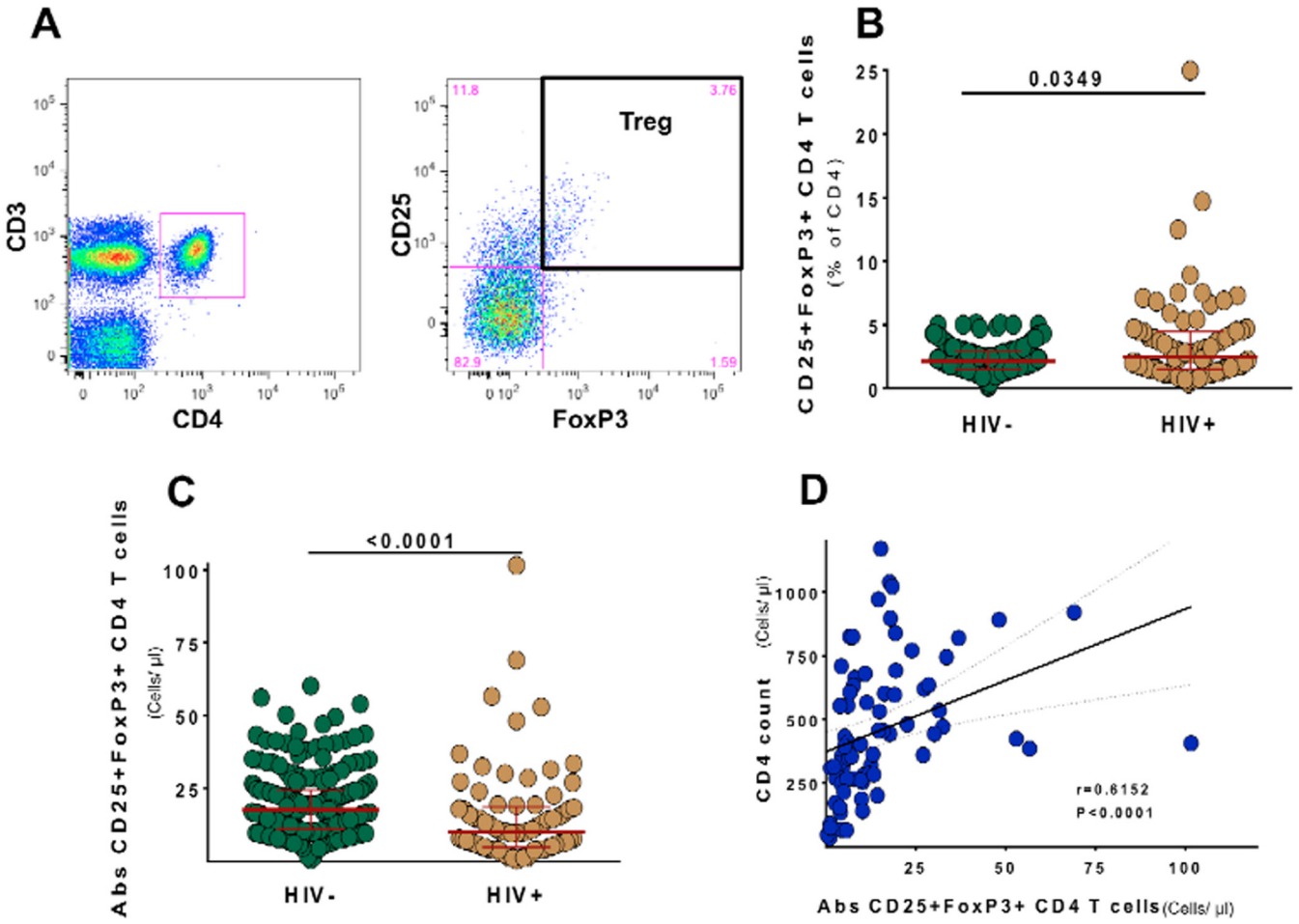

FIG 1 Frequencies and absolute numbers of $CD25^+$ $FoxP3^+$ CD4 T cells in the peripheral blood in relation to HIV infection. (A) Representative dot plots and gating strategy for the detection of regulatory T cells through CD25 and FoxP3 expression on $CD3^+$ $CD4^+$ T cells from fresh anticoagulated whole blood of WHIS subjects. (B and C) $CD25^+$ $Foxp3^+$ CD4 T cell frequencies (B) and absolute numbers (C) are compared between HIV^- and HIV^+ subjects. (D) Correlation analysis of absolute CD4 counts and $CD25^+$ $Foxp3^+$ CD4 T cell counts. Statistical analysis was performed using the Mann-Whitney test for comparison of groups and the Spearman *r* statistical test for correlation analyses.

support the proposed mechanism of constant replenishment of memory $CD25^+$ $FoxP3^+$ CD4 T cells from the memory CD4 T cell pool (30) also during HIV infection.

Memory $Helios^+$ and $Helios^-$ $CD25^+$ $FoxP3^+$ CD4 T cells are frequent targets for HIV infection *in vivo*. To determine *in vivo* HIV infection rates of memory $CD25^+$ $FoxP3^+$ CD4 T cells, we sorted four different subsets of $CD45RO^+$ memory CD4 T cells on the basis of their Helios, CD25, and FoxP3 expression (Fig. 4A) for 22 subjects (the WHIS cohort plus 6 subjects from the HISIS cohort [Table 1]) and quantified HIV *gag* DNA within the sorted subsets. Helios, an Ikaros transcriptional factor family member that is critical for the regulatory function of $CD25^+$ $FoxP3^+$ CD4 T cells (55–58), is a negative regulator of IL-2 signaling in $CD25^+$ $FoxP3^+$ CD4 T cells (59). A large fraction of $CD25^+$ $FoxP3^+$ CD4 T cells expressed the memory marker CD45RO in HIV^+ subjects (median, 87.3%; IQR, 71.85% to 93.55%), and most of these expressed Helios (median, 76.30%; IQR, 69.18% to 84.43%; data not shown), consistent with a regulatory cell function of this subset. In contrast, only a minor fraction of $CD25^-$ $FoxP3^-$ memory CD4 T cells expressed Helios (median, 1.65%; IQR, 1.15% to 2.75%). HIV *gag* DNA was detected in >80% of memory $CD25^+$ $FoxP3^+$ and $CD25^-$ $FoxP3^-$ CD4 T cells, with a 15-fold-higher median *gag* DNA load in $CD25^+$ $FoxP3^+$ memory CD4 T cells than in $CD25^-$ $FoxP3^-$ memory CD4 T cells [$\sum$($Helios^+$ cells + $Helios^-$ cells), 16,072 versus 1,074 copies/10^6 cells, respectively; $P = 0.003$] (Fig. 4B). From 16 subjects, we also determined the plasma viral load (pVL) and found a correlation between log cell-associated DNA *gag* in memory $CD25^-$ $FoxP3^-$ memory CD4 T cells and log pVL ($P = 0.025$, $r = 0.56$; data not shown). No such association was detected for memory $CD25^+$ $FoxP3^+$ memory CD4 T cells ($P = 0.1$, $r = 0.39$; data not shown).

Figure 4C shows the levels of HIV *gag* DNA within these memory CD4 T cell subsets further delineated by Helios expression. In comparison to the largest sorted memory CD4 T cell population in the blood ($FoxP3^-$ $CD25^-$ $Helios^-$), which contained a median of 154.4 HIV copies/10^6 cells (IQR, 0 to 10,241 copies/10^6 cells), the levels of HIV *gag* DNA were substantially increased in the other subsets: the $FoxP3^+$ $CD25^+$ $Helios^-$ CD4 T cells (119-fold increase; median, 18,407 copies/10^6 cells; IQR, 1,556 to 106,067 copies/10^6 cells; $P = 0.007$), the $FoxP3^-$ $CD25^-$ $Helios^+$ CD4 T cells (104-fold increase; median, 16,096 copies/10^6 cells; IQR, 837.9 to 47,903 copies/10^6 cells, $P = 0.029$), and the $FoxP3^+$

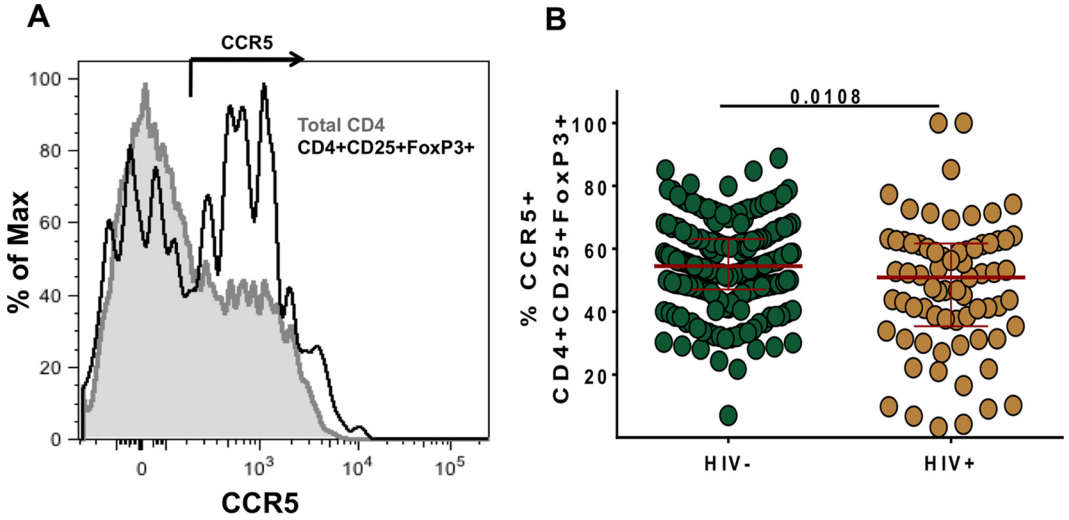

FIG 2 *Ex vivo* HIV coreceptor (CCR5) expression on CD25$^+$ Foxp3$^+$ CD4 T cells. (A) A histogram overlay of CCR5 expression on total CD4 T cells (gray) and CD25$^+$ Foxp3$^+$ CD4 T cells (black). (B) The frequencies of CCR5-expressing CD25$^+$ Foxp3$^+$ CD4 T cells are compared between HIV-negative and -positive subjects. For maximum staining sensitivity, fresh anticoagulated whole blood of individuals from the WHIS cohort was used to determine CCR5 expression on CD4 T cells. Statistical analysis was performed using the Mann-Whitney test.

CD25$^+$ Helios$^+$ CD4 T cells (26-fold increase; median, 4,106 copies/10^6 cells; IQR, 0 to 44,612 copies/10^6 cells; $P = 0.072$). Together these data demonstrate that CD25$^+$ FoxP3$^+$ memory CD4 T cells and in particular the small Helios$^-$ population contain high HIV DNA levels *in vivo*. Likewise, the small CD25$^-$ FoxP3$^-$ Helios$^+$ memory CD4 T cell population contained a substantially increased number of HIV DNA copies. In comparison, the main CD25$^-$ FoxP3$^-$ Helios$^-$ memory CD4 T cell subset (>90% of memory CD4 T cells in peripheral blood), of which high cell numbers were sorted for all 22 subjects, contained few and often a surprisingly undetectable number of *gag* DNA copies. Together these data suggest that CD25$^+$ FoxP3$^+$ and also CD25$^-$ FoxP3$^-$ Helios$^+$ memory CD4 T cells are frequent targets for HIV infection. However, the lack of correlation between plasma viral load and *gag* DNA loads in CD25$^+$ FoxP3$^+$ memory CD4 T cells is inconsistent with the hypothesis of significant plasma virus production by this cell subset.

Phylogenetic sequence analyses of the highly variable EnvV1V3 region in plasma virus and sorted memory CD4 T cell populations. To assess whether memory CD25$^+$ FoxP3$^+$ CD4 T cells could potentially contribute to plasma virion production, we compared the highly variable envelope V1V3 regions from cell DNA sequences (CD25$^+$ FoxP3$^+$ and CD25$^-$ FoxP3$^-$ memory CD4 T cell subsets) and plasma virus sequences in seven viremic subjects. The estimated HIV infection durations varied, from 9 months (subject H574), 27 to 30 months (subject H605), 1.3 to 3.3 years (subject 6233K12), above 3.2 years (subjects 3806A11, 8710U11, and 9440A11), and above 4.5 years (subject 8975T11). PCR-related sequence background variation was controlled for by using an endpoint diluted molecular clone of the subtype isolate Du422 clone 1. Ten of the 21 Du422 sequences did not contain any nucleotide substitutions compared to the template sequence, seven sequences had one substitution, and three sequences had two substitutions. Hence, the PCR protocol introduced only two or fewer nucleotide substitutions and no insertions or deletions in 95% of the amplicons. We therefore considered up to four substitutions between cell- and plasma-derived sequence variants as quasi-identical. EnvV1V3 amplicons containing clones from 6 of the 7 subjects were subjected to Sanger sequencing, and clonal sequences were analyzed using the maximum-likelihood method (Fig. 5A and B). In 4 of these 6 subjects (H574, H605, 6233K12, 9440A11) we found quasi-identical cell- and plasma-derived EnvV1V3 sequence pairs (Table 2). For subject H574 (HIV infected for 9 months), viral sequences were closely related to each other and sequences from all four sorted cell populations were closely related to those of plasma virus (Fig. 5B; Table 2). Of the cell-derived sequences, 11.4% (8 of 70) were quasi-identical to plasma-derived sequence variants. For subject H605 (infected 27 to 30 months), the closest sequence was derived from the "dominant" memory CD4 T cell subset (CD25$^-$ FoxP3$^-$ Helios$^-$; 3 substitutions) and 6.8% (3 of 44) of cell-derived sequences were quasi-identical to plasma-derived sequence variants. For subject 6233K12 (infected 16 to 38 months), only 1 of 53 EnvV1V3 sequences was quasi-identical to a plasma-derived sequence variant and was derived from the CD25$^-$ FoxP3$^-$ Helios$^+$ memory T cell subset. The three subjects, i.e., 8710U11, 8975T11, and 9440A11, were infected for at least 3.2 years, and the closest cell-derived sequences to a plasma-derived sequence variant had 32, 54, and 4 substitutions, respectively. Hence, we detected a single "quasi-identical pair" between cell- and plasma-derived EnvV1V3 sequences (derived from CD25$^+$ Helios$^+$ memory CD4 T cells) in only one of these three subjects. In summary, sequences derived from CD25$^+$ FoxP3$^+$ memory CD4 T cells (or those derived from the other sorted memory CD4 T cell subsets) were not preferentially clustering with plasma-derived sequence variants. Quasi-identical cell- and plasma-derived EnvV1V3 sequence pairs were generally infrequent, and their proportion decreased with HIV infection duration (Fig. 5C; $P = 0.03$, $r = -0.85$) as the nucleotide distances between cell- and plasma-derived sequences (Fig. 5D; $P = 0.02$, $r^2 = 0.84$) and between individual plasma-derived sequences (Fig. 5E; $P = 0.02$, $r^2 = 0.95$) increased. To ascertain the relatedness of the plasma sequences and the sequences isolated

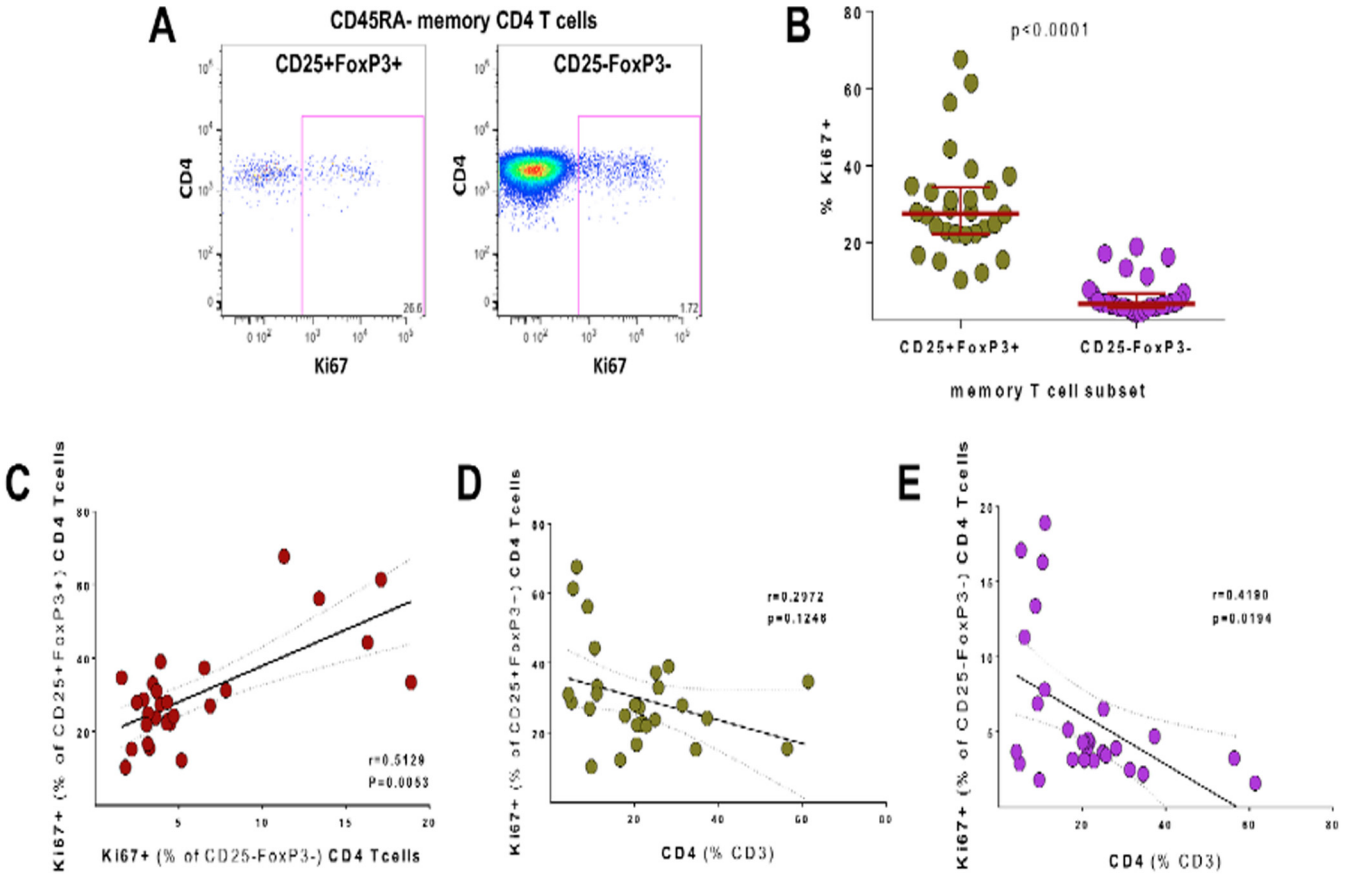

FIG 3 Ki67 expression in CD25$^+$ FoxP3$^+$ and CD25$^-$ FoxP3$^-$ memory CD4 T cells in HIV$^+$ subjects. (A) Representative dot plots for Ki67 staining. (B) Comparison of the frequencies of Ki67$^+$ cells in CD25$^+$ FoxP3$^+$ and CD25$^-$ FoxP3$^-$ memory CD4 T cells in HIV$^+$ subjects. (C) Correlation analysis of the frequency of Ki67$^+$ cells between CD25$^+$ FoxP3$^+$ (*y* axis) and CD25$^-$ FoxP3$^-$ (*x* axis) memory CD4 T cells. (D and E) Correlation analysis of the frequency of Ki67$^+$ cells among CD25$^+$ FoxP3$^+$ (D) and CD25$^-$ FoxP3$^-$ (E) CD4 T cells versus CD4 T cell frequencies (% of CD3). The analysis was done using cryopreserved PBMC samples from HIV$^+$ HHECO study participants. Memory status of CD4 T cells was determined by CD45RA staining. Statistical analysis was performed using the Mann-Whitney test for comparison of groups and the Spearman *r* statistical test for correlation analyses.

from the cell fractions, we estimated the nucleotide variation within each fraction. The estimation was performed using the neighbor-joining model with the Kimura 2-parameter method. The sequence diversity analyses showed that the sequence diversity in plasma was not different from the estimated diversity between the plasma- and cell-derived sequences (data not shown).

We also analyzed plasma- and cell-derived EnvV1V3 amplicons from two HIV$^+$ subjects (3806A11 and 9440A11) infected for more than 3.2 years by using next-generation sequencing to detect "rare" quasi-identical sequence pairs that we might have missed in the previous analyses. Between 780 and 10,000 EnvV1V3 sequences were first reconstructed using QuasiRecomb (49). The 50 most frequent sequences/population were aligned, and sequences were compared (Fig. 6). The closest cell-associated and plasma sequences were 6 and 14 nucleotide substitutions apart for subjects 3806A11 and 9440A11, respectively, inconsistent with a major contribution of the sorted peripheral memory CD4$^+$ T cell subsets to plasma virus production. BLAST searches of all plasma sequence variants against the 150 highest-frequency cell-derived variants (per sorted cell subset) identified the closest pairs as 4 (subject 3806A11, CD25$^-$ FoxP3$^-$ Helios$^+$) and 10 (subject 9440A11, CD25$^+$ Helios$^+$) nucleotides apart.

DISCUSSION

HIV plasma viremia predicts the rate of HIV disease progression (1, 60) and depends on active HIV replication in CD4$^+$ cells. Memory CD4 T cells are most probably the primary substrate for virus replication (11, 61–63). HIV infection rates differ substantially between different CD4 T cell subsets (4–6, 64). Recent data show that follicular T helper (Tfh) cells are a prime target for virus replication and contribute to virion production even in elite controller rhesus macaques (19) and most probably to plasma viremia (17). To what extent other CD4$^+$ cell subsets contribute to plasma virus production in viremic progressors is unclear. In various *in vitro* infection models, HIV replication is associated with IL-2 signaling and CD25 expression on stimulated CD4 T cells (10, 13, 14, 21–23). Because IL-2 is important for the homeostatic proliferation of the CD25$^+$ FoxP3$^+$ CD4 T cells (35, 65), and because of the high *in vivo* proliferation rates of this subset (32), we hypothesized that CD25$^+$ FoxP3$^+$ CD4 T cells constitute a prime target for HIV infection and may contribute to plasma virion production *in vivo*.

Consistent with a previous report, we showed that a large fraction of CD25$^+$ FoxP3$^+$ CD4 T cells express the HIV coreceptor

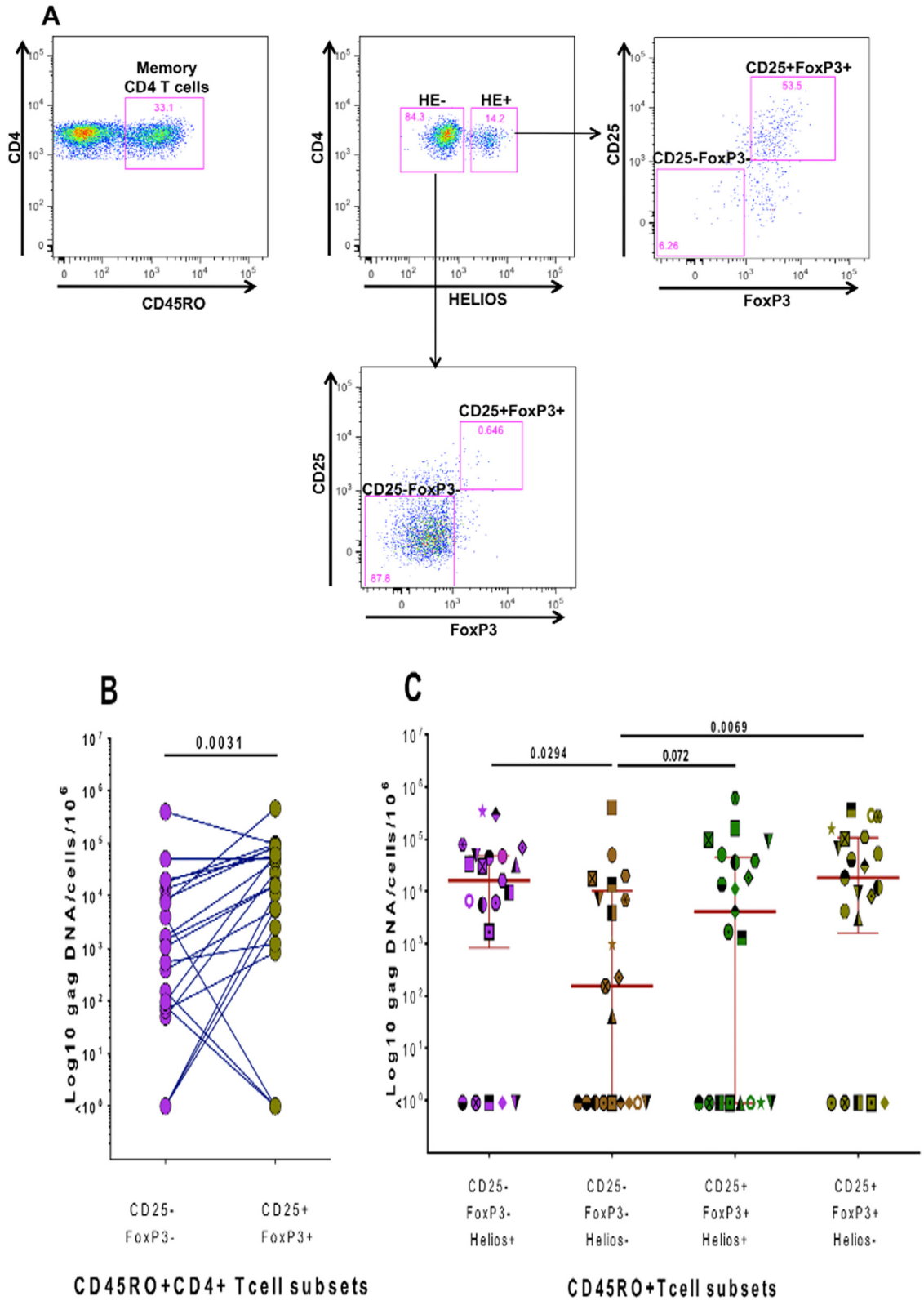

FIG 4 Quantification of cell-associated HIV *gag* DNA in sorted memory CD4 T cell subsets. (A) Gating/sorting strategy used to sort different memory CD4 T cell populations delineated by Helios, CD25, and FoxP3 expression. (B) Numbers of *gag* DNA copies/10^6cells detected in CD25$^-$ FoxP3$^-$ and CD25$^+$ FoxP3$^+$ memory CD4 T cells from 21 different subjects. (C) Numbers of *gag* DNA copies/10^6cells detected in these memory CD4 T cell subsets further delineated by Helios expression. *gag* DNA within different CD4 T cell populations of the same subject was quantified during the same real-time (RT)-PCR run. Cryopreserved PBMCs from the WHIS and HISIS cohorts were used for cell sorting. Statistical analysis was performed using the Wilcoxon rank matched-pairs test.

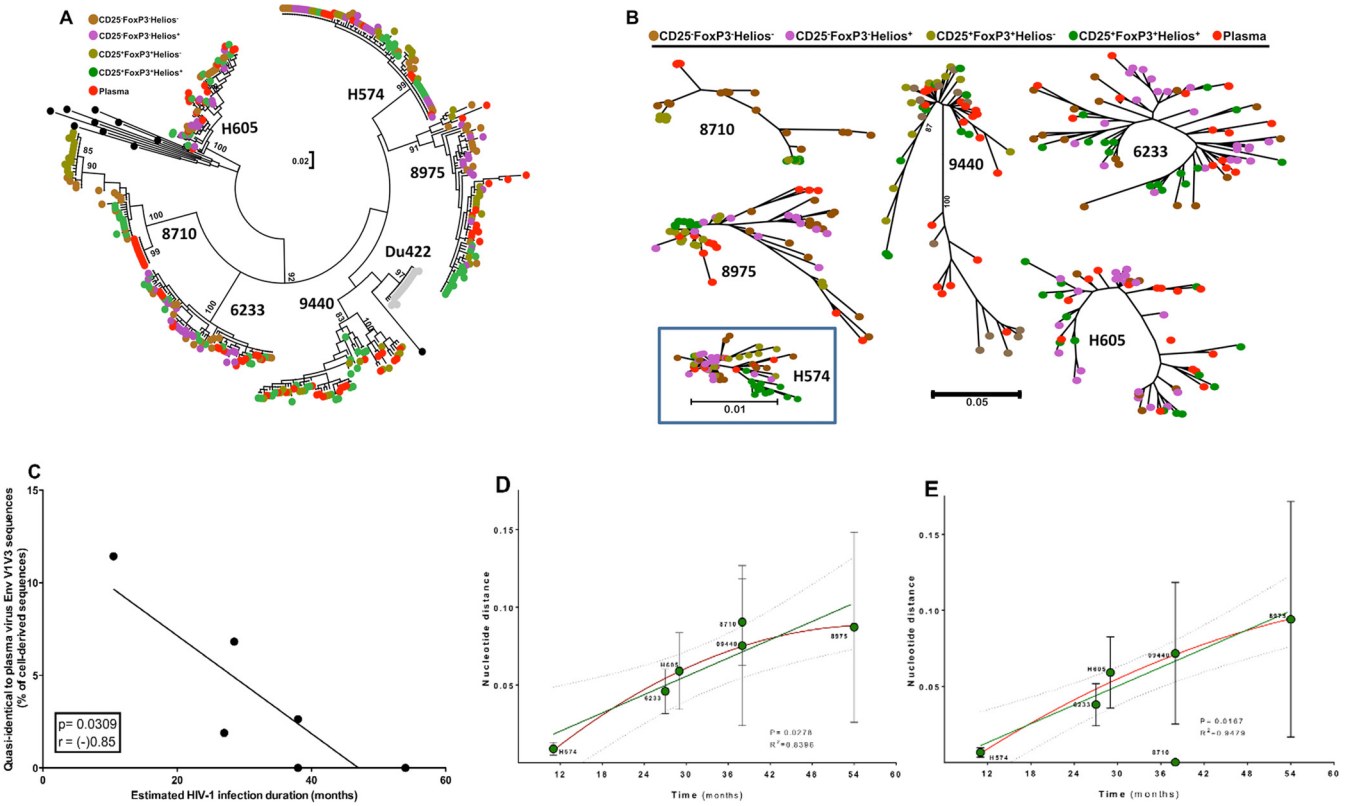

FIG 5 Phylogenetic relationship of HIV envelope sequences derived from plasma and sorted memory CD4 T cell populations. Plasma- and cell-derived sequences of the highly variable EnvV1V3 region (Hxb 6559 to 7320) were amplified, cloned, sequenced ($n = 384$, Sanger method), and analyzed for 6 viremic subjects from the WHIS and HISIS cohorts with differing HIV infection durations (H574, H605, 6233K12 [6233], 9440A11 [9440], 8710U11 [8710], 8975T11 [8975]). (A and B) The phylogenetic relationship was inferred by the maximum-likelihood method based on the general time-reversible substitution model (GTR+G). (C) Correlation between frequency of cell-derived sequences that were quasi-identical to plasma-derived sequences and the estimated infection duration. (D and E) Linear regression analysis (green line) between the distance of the EnvV1V3 sequences derived from plasma to the sequences extracted from the corresponding cellular fractions and the estimated duration of infection (D) and plasma sequence diversity plotted against the estimated duration of infection (E). The red line indicates a nonlinear analysis performed using a second-order polynomial equation taking into account the best-fit values. The evolutionary distances were computed using the Kimura 2-parameter method (77) and are given in units of the number of base substitutions per site, including both transitions and transversions. The rate variation among sites was modeled with a gamma distribution. The analysis was conducted in MEGA6 (44). No sequence diversity was observed in the subject 8710 plasma fraction, probably because the number of viruses sampled in each PCR was very low (Table 2). We therefore excluded the results for subject 8710 from the linear regression analysis. *P* and *r* values were calculated with the Pearson two-tailed statistical test.

CCR5 (35), potentially supporting viral entry. Although frequencies of $CD25^+$ $FoxP3^+$ CD4 T cells were slightly elevated in viremic, HIV^+ subjects, absolute cell numbers of this subset were significantly depleted, which confirms previously published data (50, 52, 66). A greater proportion of $CD25^+$ $FoxP3^+$ memory CD4 T cells from HIV^+ subjects expressed $Ki67^+$, with almost one-third of these cells "cycling" at any given time. This pattern, i.e., depleted cell counts despite increased fractions of $Ki67^+$ "cy-

TABLE 2 Key data of the EnvV1V3 phylogenetic studies and HIV infection duration for 6 viremic subjects

Subject ID	HIV infection duration (mo)	% of cell-derived sequences quasi-identical to plasma-derived sequences (*n*)	Mean no. of nt substitutions between plasma and cell-derived sequences	Cellular origin of closest sequence	No. of nt substitutions	Cellular origin of most distant sequence	No. of nt substitutions
H574	9–12	11.4 (8 of 70)	6	$CD25^+$ $FoxP3^+$ $Helios^-$	1	$CD25^+$ $FoxP3^+$ $Helios^+$	16
				$CD25^+$ $FoxP3^+$ $Helios^+$	1		
				$CD25^-$ $FoxP3^-$ $Helios^+$	1		
				$CD25^-$ $FoxP3^-$ $Helios^-$	1		
H605	27–30	6.8 (3 of 44)	39	$CD25^-$ $Foxp3^-$ $Helios^-$	3	$CD25^+$ $FoxP3^+$ $Helios^+$	32
6233K12	16–38	1.9 (1 of 53)	30	$CD25^-$ $FoxP3^-$ $Helios^+$	2	$CD25^-$ $FoxP3^-$ $Helios^+$	30
9440A11[a]	>38	2.6 (1 of 38)	46	$CD25^+$ $Helios^+$	4	$CD25^+$ $Helios^+$	76
8710U11	>38	0 (0 of 39)	57	$CD25^-$ $FoxP3^-$ $Helios^-$	32	$CD25^-$ $FoxP3^-$ $Helios^-$	67
8975T11	>54	0 (0 of 55)	53	$CD25^-$ $FoxP3^-$ $Helios^+$	54	$CD25^-$ $FoxP3^-$ $Helios^-$	86

[a] Cells (PBMCs) from this subject were sorted into four populations only on the basis of CD25 and Helios expression on memory (CD45RO) CD4 T cells.

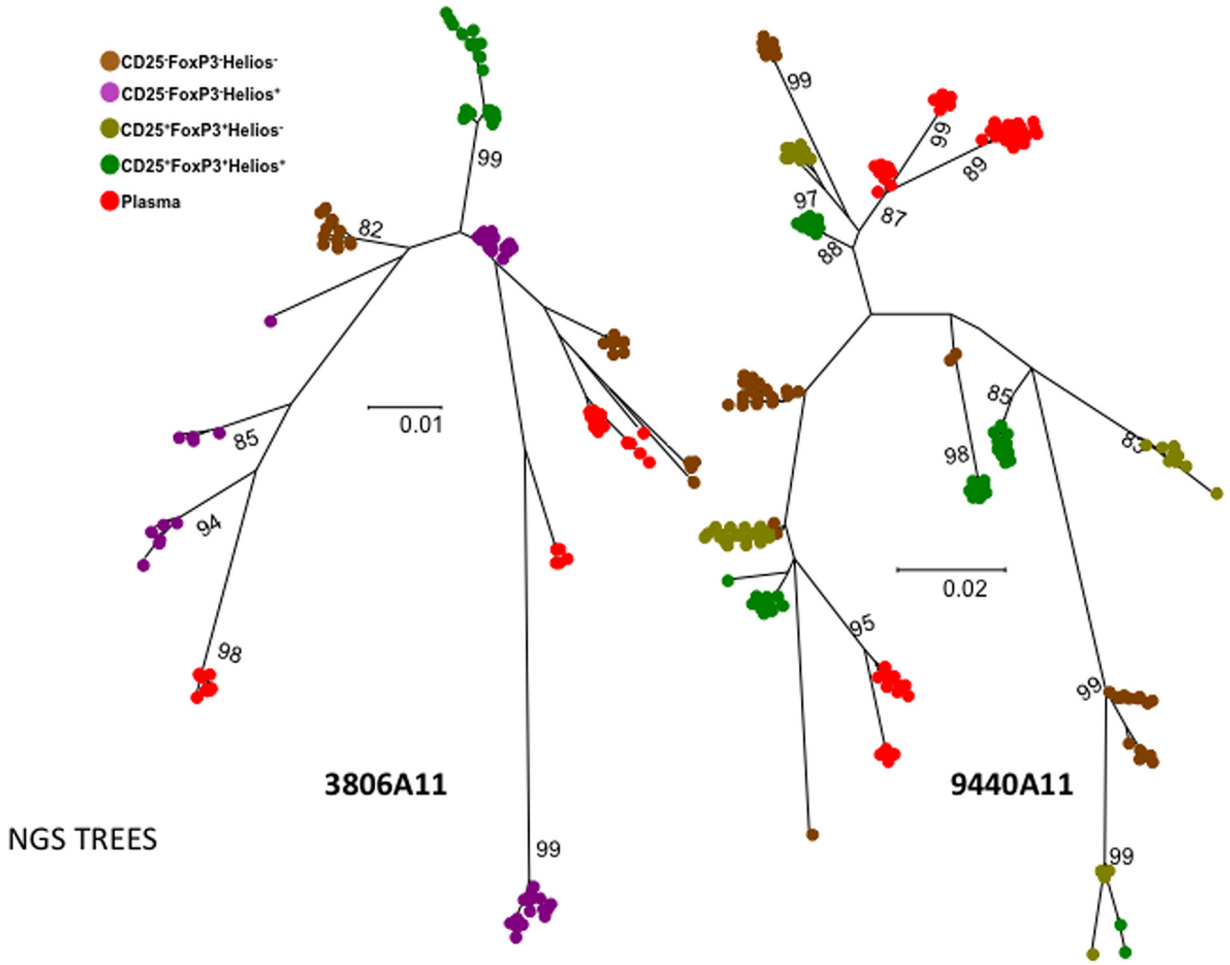

FIG 6 Phylogenetic analyses of HIV envelope sequences derived from plasma and sorted memory CD4 T cell populations using next-generation sequencing. Shown are the phylogenetic analyses of EnvV1V3 sequences from the 50 most frequently detected sequences derived from either plasma or the different sorted memory CD4 T cell subsets for two viremic subjects of the WHIS cohort. The phylogenetic relationship was inferred by the maximum-likelihood method based on the general time-reversible substitution model (GTR+G). EnvV1V3 amplicons were directly subjected to next-generation sequencing. Quasispecies reconstruction was performed using the software QuasiRecomb. The methods used are described in detail in Materials and Methods.

cling" cells, demonstrates that homeostasis of CD25$^+$ FoxP3$^+$ CD4 T cells is heavily perturbed by HIV infection. Furthermore, expression of CCR5 and high proportions of cycling cells within CD25$^+$ FoxP3$^+$ CD4 T cells should support both cell entry and reverse transcription of HIV, which is supported by the increased HIV DNA loads observed in memory CD25$^+$ FoxP3$^+$ CD4 T cells observed in this study (12, 36). Other reports show discrepant results regarding *in vivo* levels of HIV DNA in "regulatory" CD4 T cells that are typically defined by a CD25^{high} phenotype, instead of the definition using coexpression of CD25 and FoxP3 that we used (67–69). Tran et al. observed a higher infection rate in CD25^{high} than CD25$^-$ CD4 T cells (70) but did not exclude naive CD4 T cells, which are not susceptible to CCR5-tropic strains which predominate throughout most of the infection course. Of note, high *in vivo* proliferation of memory CD25$^+$ FoxP3$^+$ CD4 T cells might also potentially pass on proviral HIV DNA to the cell progeny in the absence of productive HIV infection during ART. Previous studies reported that CD25^{high} T cells (which were >99% FoxP3$^+$) release virus upon *in vitro* restimulation and have ~3-fold-higher HIV infection rates than other CD4 T cells upon *in vitro* activation (36, 70). Together these data suggest that CD25$^+$ FoxP3$^+$ CD4 T cells are a prime cellular target for HIV infection that might serve as an important HIV reservoir during ART.

We next wanted to address whether memory CD25$^+$ FoxP3$^+$ CD4 T cells could potentially contribute to plasma virion production. Because cell fixation complicates analyses of HIV transcription in sorted cell populations defined by intranuclear transcription factors (such as FoxP3), we decided to study the phylogenetic relationship between plasma- and cell-derived sequences within the highly variable EnvV1V3 region; if CD25$^+$ FoxP3$^+$ memory CD4 T cells significantly contribute to plasma virion production, EnvV1V3 DNA sequences derived from this cell population should often be quasi-identical or preferentially cluster with plasma-derived sequences. A previous study had reported rapid replacement of cell- and plasma-derived HIV sequences by an incoming superinfecting HIV strain (71), implying a highly dynamic exchange between these two compartments. In our study, the detection of quasi-identical sequence pairs derived from cells and plasma was rare and their fraction further decreased with infection duration, which is consistent with the broadening

of the viral reservoir with time. There was no clear pattern of phylogenetic clustering of the plasma virus with any of the cell subset-derived sequences we had sorted. In fact, cell-derived sequences did not "behave differently" from plasma-derived sequences, and sequences from both compartments intermingled. Our phylogenetic data therefore do not allow definite conclusions about the cellular origin of plasma virions. The high variability between individual plasma-derived sequences during chronic infection emphasizes that a huge number of infected cells must contribute to plasma virion production at any given time during chronic infection. It might therefore be difficult to determine the exact cellular origins of plasma virus through phylogenetic sequence analyses. Nonetheless, in our analyses of individual sequences, we did find several quasi-identical sequence pairs between plasma and $CD25^+$ $FoxP3^+$ CD4 T cells, indicating that they may contribute to the plasma viremia. One limitation of our study was that we used comparatively small amounts of PBMCs and plasma (compared to the total body amount) for phylogenetic analyses, and we therefore probably included insufficient numbers for detection of clusters of cell- and plasma-derived sequences. Virus sequences from very large amounts of specimen will need to be analyzed and will optimally include material from secondary lymphoid tissues for more conclusive answers. Secondary lymphoid tissues are thought to constitute the primary site for virion production (reviewed in reference 61). After ART interruption, the onset of viral RNA transcription in lymph nodes coincides with a rise in plasma viral load (72). $CD25^+$ $FoxP3^+$ CD4 T cells in secondary lymphoid organs contain high frequencies of $Ki67^+$ "cycling" cells with a significant capacity for IL-2 production and often express a $CD69^+$ "recently activated" phenotype (32), thereby differing from those in peripheral blood. A recent study detected colocalization of SIV p27 and FoxP3 expression in intestinal tissues using confocal microscopy (73). We therefore consider it likely that $CD25^+$ $FoxP3^+$ CD4 T cells in lymphoid tissues are targeted by HIV, but additional studies will be needed to define the role of $CD25^+$ $FoxP3^+$ CD4 T cells for plasma virion production *in vivo*.

We also sorted memory CD4 T cell populations depending on their Helios expression. Helios, an Ikaros transcriptional factor family member, is critical for the regulatory function of $CD25^+$ $FoxP3^+$ CD4 T cells (55–57) and for the prevention of autoimmunity (58). Helios modulates cell cycle progression and sustained cell survival through the regulation of genes involved in IL-2 signaling (58, 59). Helios expression is also linked to expression of a range of suppressive T cell markers and can be induced in CD4 T cells upon *in vitro* activation (74, 75). *In vitro*, dividing $CD25^+$ $FoxP3^+$ CD4 T cells coexpress Helios, while nondividing regulatory T cells lose expression of FoxP3 and Helios, suggesting that Helios is a marker of recently divided cells. In the same set of *in vitro* experiments, $CD25^-$ $Helios^+$ CD4 T cells were composed of highly activated "effector" memory cells (74). We detected higher median *gag* DNA loads in both Helios-positive (26-fold increased) and -negative (119-fold increased) $CD25^+$ $FoxP3^+$ memory CD4 T cells as well as in $CD25^-$ $FoxP3^-$ $Helios^+$ memory CD4 T cells (104-fold increased) than in $FoxP3^-$ $CD25^-$ $Helios^-$ memory CD4 T cells. It is remarkable that we often did not detect HIV DNA in this "dominant" memory CD4 T cell subset. A history of more frequent or recent cell divisions within $CD25^-$ $FoxP3^-$ $Helios^+$ memory CD4 T cells might have contributed to high HIV susceptibility in this memory cell subset, whereas removal of such cells in the sorted $CD25^-$ $FoxP3^-$ $Helios^-$ memory CD4 T cells may potentially explain the low HIV infection rates observed in this memory cell subset. "Nonactivated" circulating memory CD4 T cells are probably less susceptible and accumulate less HIV DNA over time than other memory CD4 T cell subsets with a history of *in vivo* proliferation. Helios-deficient regulatory CD4 T cells exhibit an activated phenotype, i.e., an increased capacity to secrete gamma interferon (IFN-γ) and to develop into nonanergic cells under inflammatory conditions (58, 76). The increased responsiveness to cellular activation in comparison to their $Helios^+$ counterparts' signaling may potentially explain the higher HIV DNA levels in $CD25^+$ $FoxP3^+$ $Helios^-$ memory CD4 T cells than in their $Helios^+$ counterparts. These data show that Helios and CD25/FoxP3 expression patterns are linked to different cellular HIV infection rates, consistent with a role of the IL-2 signaling pathway for HIV infection *in vivo*.

In conclusion, we find that homeostasis of $CD25^+$ $FoxP3^+$ CD4 T cells is heavily perturbed during HIV infection. High expression of HIV coreceptor CCR5 and *in vivo* proliferation potentially facilitate efficient HIV infection of memory $CD25^+$ $FoxP3^+$ CD4 T cells. Furthermore, high proliferative activity of this cell subset is likely to passage HIV DNA to cell progeny in the absence of active viral replication. This subset could therefore serve as an important viral reservoir during ART. Neither circulating memory $CD25^+$ $FoxP3^+$ CD4 T cells nor any of the other memory CD4 T cell subset-derived EnvV1V3 sequences preferentially clustered with plasma-derived sequences. Instead, sequences from the two compartments intermingled, and the genetic distance between and within the two compartments increased with infection duration, precluding a definite conclusion about the cellular origin of the plasma virus in this study.

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CHAPTER IV

Helicobacter pylori Infection Is Associated with Higher CD4 T Cell Counts and Lower HIV-1 Viral Loads in ART-Naïve HIV-Positive Patients in Ghana

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(Open access)

Edmund Osei Kuffour's contribution to this work:

1. Obtained patients' samples from study site
2. Analysed all patients' stool samples by ELISA, testing for *H. pylori* antigen
3. Data screening and preliminary statistical data analysis
4. Described the *H. pylori* diagnostic procedure in the method section

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RESEARCH ARTICLE

Helicobacter pylori Infection Is Associated with Higher CD4 T Cell Counts and Lower HIV-1 Viral Loads in ART-Naïve HIV-Positive Patients in Ghana

Fred Stephen Sarfo[1,2], Kirsten Alexandra Eberhardt[3], Albert Dompreh[2], Edmund Osei Kuffour[4], Mareike Soltau[3], Marei Schachscheider[3], Jan Felix Drexler[5], Anna Maria Eis-Hübinger[5], Dieter Häussinger[4], Emelia Efua Oteng-Seifah[6], George Bedu-Addo[1,2], Richard Odame Phillips[1,2], Betty Norman[1,2], Gerd Burchard[3], Torsten Feldt[3,4]*

1 Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, **2** Komfo Anokye Teaching Hospital, Kumasi, Ghana, **3** Clinical Research Unit, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany, **4** Clinic of Gastroenterology, Hepatology and Infectious Diseases, University Hospital Düsseldorf, Düsseldorf, Germany, **5** Institute of Virology, University of Bonn Medical Centre, Bonn, Germany, **6** Kumasi Centre for Collaborative Research, Kumasi, Ghana

* Torsten.Feldt@med.uni-duesseldorf.de

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Abstract

Background

Worldwide, there is a high co-endemicity of HIV and *H*. *pylori* infection and there is growing evidence that *H*. *pylori* co-infection is associated with parameters of HIV disease progression. The objective of this study was to investigate the prevalence of *H*. *pylori* infection, and the association with clinical, immunological and virological parameters in a large cohort of HIV-infected individuals and uninfected controls in a West African country.

Methods

HIV-patients (n = 1,095) and HIV-negative individuals (n = 107) were recruited at a university hospital in Ghana. *H*. *pylori* status was determined using stool antigen testing. HIV-related, clinical and socio-demographic parameters were recorded and analyzed according to *H*. *pylori* status.

Results

The prevalence of *H*. *pylori* infection was significantly lower in HIV-positive compared to HIV-negative individuals (51.5 vs. 88%, p<0.0001). In HIV patients, *H*. *pylori* prevalence decreased in parallel with CD4+ T cell counts. In ART-naïve HIV-infected individuals, but not in those taking ART, *H*. *pylori* infection was associated with higher CD4 cell counts (312 vs. 189 cells/μL, p<0.0001) and lower HIV-1 viral loads (4.92 vs. 5.21 log10 copies/mL, p = 0.006). The findings could not be explained by socio-demographic confounders or reported use of antibiotics.

Having no access to tap water and higher CD4+ T cell counts were identified as risk factors for *H. pylori* infection.

Conclusions

H. pylori prevalence was inversely correlated with the degree of immunosuppression. In ART-naïve individuals, *H. pylori* infection is associated with favorable immunological and virological parameters. The underlying mechanisms for this association are unclear and warrant investigation.

Introduction

Recently, the interplay between the Human immunodeficiency virus (HIV) and *Helicobacter pylori* (*H. pylori*) infection has attracted attention. A number of studies have reported lower *H. pylori* prevalence rates in HIV-infected compared to HIV-negative individuals [1,2]. This association is unexpected, since usually chronic infections are more commonly found in patients with advanced HIV disease. Furthermore, *H. pylori* infection is considered a disease of poverty, and poor socioeconomic status has been associated with rather disadvantageous outcomes of HIV infection [3]. The underlying mechanisms for this observed association are unclear. Most existing studies have important limitations such as small sample sizes thus preventing subgroup analyses and robust adjustment for confounders. In particular, information on socioeconomic variables, as putative confounders for *H.pylori* status is sorely lacking. As a consequence, interpretation and comparison of results are difficult and data published to date is partly inconsistent [2].

Considering the significant epidemiological and pathophysiological overlap of HIV and *H. pylori* infection, the investigation of possible interplay is of interest. Over the past few years it has become clear, that the gastrointestinal tract (GIT) plays an important role in the pathophysiology of HIV/AIDS. Chronic immune activation, associated with intestinal barrier dysfunction, has been identified as central pathomechanism in HIV disease [4]. *H. pylori* colonize the gastric and duodenal mucosae and induce a specific local and also systemic immune response, involving, among others, CD4+ T cells, dendritic cells, regulatory T cells (Treg) and Th17 cells, with all of these also playing a role in HIV pathogenesis [5–8].

The association of HIV and *H. pylori* co-infection has not been systematically studied in sub-Saharan Africa, where more than two thirds of HIV-infected individuals live, and where, at the same time, the vast majority of the population gets infected with *H. pylori* during childhood [9–11]. The objective of this study was to investigate the prevalence of *H. pylori* infection, and its association with clinical, immunological and virological parameters in a large cohort of HIV-infected individuals and uninfected controls in a West African country.

Materials & Methods

Study setting and recruitment

This cross-sectional study was conducted at the Komfo Anokye Teaching Hospital, a tertiary referral hospital in the Ashanti Region of Ghana. Between November 2011 and November 2012, consecutive adult HIV-infected patients presenting to the HIV outpatient clinic, and HIV-negative blood donors presenting to the blood bank of the hospital, were offered participation in the study. All participants gave a written informed consent prior to enrolment. The

study was conducted in conformity with the Helsinki declaration, and was approved by the appropriate ethics committees of the Kwame Nkrumah University of Science and Technology (Ghana) and of the medical association in Hamburg (Germany).

Data collection and measures

Demographic, socioeconomic, and clinical data, as well as a detailed medical history were recorded using standardized questionnaires, which were completed by trained study personnel. In particular, time since diagnosis of HIV infection, duration and kind of antiretroviral therapy (ART), co-medications, and clinical parameters were documented. Routine laboratory parameters were extracted from patient's folders. EDTA blood samples were obtained for the analysis of CD4/CD8 T cell counts, using a FACSCalibur® flow cytometer (Becton Dickinson, USA). HIV-1 and 2 antibody testing was done using the First Response® HIV-1/2 test (Premier Medical Corporation Limited, India) and the Genscreen® ULTRA HIV Ag-Ab Assay (Bio-Rad, France). EDTA plasma and native stool samples were freshly frozen at -80°C and transported to Germany on dry ice. Stool was tested for *H. pylori* using the RidaScreen® FemtoLab *H. pylori* stool antigen test (R-Biopharm AG, Germany). The sensitivity and specificity of this test has been described to be 98% and 96.7% in pediatric patients and 93% and 90% in adult patients [12,13]. HIV-1 viral load was measured using the RealTime HIV-1 PCR system (Abbott Diagnostics, Wiesbaden, Germany) according to the manufacturer's instructions. The same tests, except HIV-1 viral load analysis, were conducted for cases and controls.

Statistical analysis

Parametric variables were compared using the Student's t-test, non-parametric variables were compared using the Mann-Whitney U-test. Categorical data were analyzed using Chi-squared or Fisher's exact test. A multivariable logistic regression model was used to analyze the association between *H. pylori* infection and other demographic, clinical and laboratory parameters, using only parameters with a significance level of ≤ 0.05 in bivariate analysis and a correlation coefficient of ≤ 0.10 in the multivariate regression model. Missing data were excluded from analysis. Statistical analyses were conducted with SPSS version 19 software (IBM, Germany).

Results

Cohort characteristics

We recruited 1,095 HIV-positive individuals and 107 HIV-negative blood donors. Stool samples for *H. pylori* testing were available for 952 HIV-positive (86.9%) and 100 HIV-negative individuals (93.5%). HIV-positive, compared to HIV-negative individuals, were more often female, significantly older, had a lower BMI, lower socioeconomic status, lower CD4 and higher CD8 T cell counts (Table 1). The majority of HIV-infected participants were female (75.6%), and the mean age was 40 years. Approximately half of HIV-positive individuals (n = 500, 52.5%) were ART-naïve at the time of recruitment, 452 (47.5%) patients were receiving ART for a median duration of 45 months (IQR 19–69). Participants receiving ART, compared to ART-naïve participants, were more likely to be female, had a higher BMI, higher total absolute lymphocyte and CD4 T cell counts compared to ART-naïve HIV-positive participants (S1 Table).

H. pylori infection

The prevalence of *H. pylori* infection among HIV-negative individuals was significantly higher compared to HIV-positive individuals (88.0% vs. 51.5%, $p<0.0001$). In HIV-positive

Table 1. Comparison of demographic and laboratory characteristics of HIV-positive and HIV-negative participants.

Variable	HIV-positive	HIV-negative	p-value
	N = 952	N = 100	
Female gender, n (%)	720 (75.6)	66 (66.0)	0.04
Age (years), mean ± SD	40 ± 9.5	33 ± 12.3	<0.0001
Religion, n (%)[#]			0.12
Christian	814 (85.5)	86 (92.5)	
Moslem	120 (12.6)	6 (6.5)	
Traditional African religion	2 (0.2)	0 (0.0)	
Other	16 (1.7)	1(1.0)	
Educational level, n (%)[#]			<0.0001
Primary education	156 (16.4)	9 (9.7)	
Junior Secondary School	426 (44.7)	7 (7.5)	
Senior Secondary School	133 (14.0)	56 (60.2)	
Tertiary education	51 (5.4)	14 (15.1)	
No formal education	186 (19.5)	7 (7.5)	
Occupation, n (%)[#]			<0.0001
House wife	13 (1.4)	1(1.1)	
Farmer	78 (8.2)	2 (2.2)	
Trader	505 (53.0)	33 (35.5)	
Salary worker	60 (6.3)	27 (29.0)	
Others	114 (12.0)	4 (4.3)	
Currently unemployed	182 (19.1)	24 (25.8)	
Access to tap water, n (%)*	501 (52.6)	61 (63.5)	0.04
H. pylori test result, n (%)			
Positive	490 (51.5)	88 (88.0)	<0.0001
Negative	452 (47.5)	12 (12.0)	
Indeterminate	10 (1.0)	0 (0.0)	
BMI (kg/m^2), mean ± SD	23.1 ± 4.6	24.7 ± 5.0	0.002
T-cell populations, median (IQR)			
Total T-cell count/μL	1,381 (984–1,968)	1,460 (1,171–1,895)	0.13
CD4 T-cell count/μL	380 (173–596)	958 (786–1,161)	<0.0001
CD8 T-cell count/μL	914 (620–1,341)	439 (312–673)	<0.0001

BMI, Body mass index

[#] missing data for 7 participants of the HIV negative group.

* Missing data for 4 participants of the HIV-negative group.

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individuals, *H. pylori* prevalence declined in parallel with CD4+ T cell counts, from 64.8% in patients with more than 800 CD4 T cells/μL, to 41.4% in patients with less than 200 CD4 T cells/μL. The same trend was observed in HIV-negative individuals, without reaching statistical significance (Fig 1).

The characteristics of HIV-infected individuals according to *H. pylori* status are shown in Table 2. *H. pylori* co-infected HIV-positive patients were significantly less likely to have access to tap water (48.8 vs. 58.0%, p = 0.005) and less likely to have attained a tertiary level education (3.7 vs. 7.3%, p = 0.01). There were no significant differences in other demographic variables assessed, or in WHO clinical HIV disease stages (Table 3).

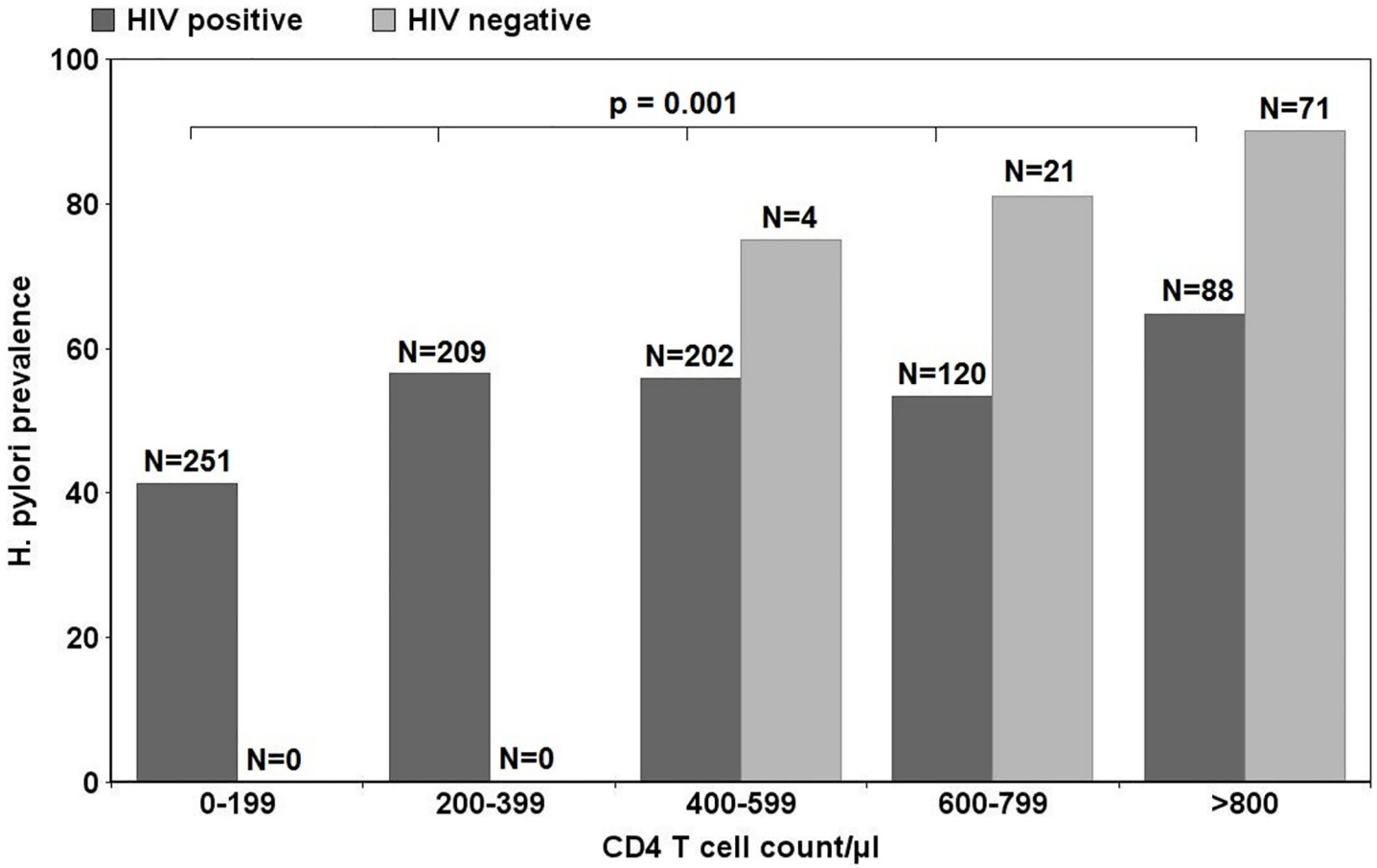

Fig 1. Comparison of *H. pylori* prevalence according to CD4 T cell count/µL for HIV-positive participants (p = 0.001, Chi-square test) and for HIV-negative individuals (p = 0.397, Chi-square test); N = Group sizes for CD4 T cell categories including *H. pylori* positive and negative participants.

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Associations between *H. pylori* infection and HIV clinical, immunological and virological parameters

Among ART-naïve HIV patients, those with *H. pylori* co-infection had higher CD4 T cell counts (312 vs. 189 cells/µl, p<0.0001), higher CD4/CD8 ratios (0.31 vs. 0.19, p<0.0001) and lower HIV-1 viral loads (4.92 vs. 5.21 log10 copies/ml, p = 0.006) compared to those without *H. pylori* co-infection. *H. pylori* positive patients in this group also had higher mean hemoglobin levels (11.1 vs. 10.4 g/dl, p = 0.01), and lower platelet counts (262.4 vs. 314.9 x1000/µl, p = 0.003), as shown in Table 3. There was no significant difference in the reported use of antibiotics in the 6 months before recruitment between *H. pylori* positive and negative individuals.

H. pylori infection was also not associated to increased frequencies of gastrointestinal symptoms in *H. pylori* positive, compared to negative patients, with weight loss (32.6% vs. 40%, p = 0.09), epigastric discomfort (10.0% vs. 9.4%, p = 0.81), and diarrhea (9.6% vs. 9.0%, p = 0.82) being the most common symptoms. In the HIV-infected, ART-exposed group, no significant associations between *H. pylori* status and CD4+ T cell count, HIV-1 viral load, or the proportion of patients with undetectable viral load were observed. However, significantly lower CD8+ T cell counts (858/µL vs. 990/µL, p = 0.02), and a trend towards higher CD4/CD8 ratios (0.55 vs. 0.49, p = 0.07), as possible indicator of decreased immune activation, were noted among those patients with *H. pylori* co-infection [14–17].

Table 2. Comparison of socio-demographic parameters of HIV-infected participants according to *H. pylori* status.

Variable	*H. pylori* positive	*H. pylori* negative	p-value
	N = 490	N = 452	
Female gender, n (%)	372 (75.9)	339 (75.0)	0.74
Age (years), mean ± SD	40 ± 9.4	40 ± 9.6	0.97
Religion, n (%)[#]			
Christian	410 (83.7)	396 (87.6)	0.12
Moslem	71 (14.5)	47 (10.4)	
Traditional African religion	2 (0.4)	0 (0.0)	
Other	7 (1.4)	9 (2.0)	
Educational level, n (%)[#]			0.08
No formal education	91 (18.6)	91 (20.1)	
Primary education	84 (17.1)	72 (15.9)	
Secondary education	297 (60.6)	256 (56.6)	
Tertiary education	18 (3.7)	33 (7.3)	
Occupation, n (%)[##]			0.75
House wife	6 (1.2)	7 (1.5)	
Farmer	42 (8.6)	36 (8.0)	
Trader	257 (52.4)	242 (53.5)	
Salary worker	27 (5.5)	33 (7.3)	
Others	64 (13.1)	48 (10.6)	
Currently unemployed	94 (19.2)	86 (19.0)	
Access to tap water, n (%)	239 (48.8)	262 (58.0)	0.005
Electricity in the household, n (%)	452 (92.2)	420 (92.9)	0.39
Television in household, n (%)	398 (81.2)	366 (81.0)	0.92
Owning a fridge, n (%)	336 (68.6)	322 (71.2)	0.89
Owning a car, n (%)	35 (7.1)	51 (11.3)	0.03

Analysis excludes 10 patients with indeterminate H. pylori result.

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Among HIV-negative controls, no differences in baseline characteristics, symptoms, or socio-demographic parameters were observed between individuals with and without *H. pylori* infection. A weak trend towards higher CD4/CD8 ratios was also observed in those HIV-negative individuals with *H. pylori* infection, compared to those without *H. pylori* infection (2.12 vs. 1.80, p = 0.13).

Logistic regression analysis of risk factors associated with H. pylori infection in HIV-positive individuals. Using a logistic multivariable regression model including parameters with $p \leq 0.05$ in the univariate analysis and a correlation coefficient of ≤ 0.1 in the regression model, only CD4+ T cell count (aOR 1.06, 95% CI 1.01–1.12, p = 0.012 for every 100 cells/μl higher) and having access to tap water (aOR 0.63, 95% CI 0.47–0.84, p = 0.002) were associated with *H. pylori* infection (Table 4). Significant predictors of *H. pylori* co-infection noted in univariate but not in multivariate analysis included use of anti-tuberculous therapy, current use of ART and use of co-trimoxazole. The risk ratio (RR) for *H. pylori* infection was 0.82 for those patients having access to tap water and 1.37 for those with >200 CD4 T cells/μl within the group of HIV-positive patients. No risk factors were identified to be associated with *H. pylori* infection in the HIV-negative group (data not shown).

Table 3. Comparison of clinical and laboratory parameters in HIV-positive and HIV-negative individuals according to H. pylori status.

Variable	ART-naïve group, n = 494			ART group, n = 448			HIV negative group, n = 100		
	H. pylori pos.	*H. pylori* neg.	p-value	*H. pylori* pos.	*H. pylori* neg.	p-value	*H. pylori* pos.	*H. pylori* neg.	p-value
	N = 239 (48.4%)	N = 255 (51.6%)		N = 251 (56.0%)	N = 197 (44.0%)		N = 88 (88.0%)	N = 12 (12.0%)	
Time since HIV diagnosis (months), median (IQR)	0.5 (0.5–3.5)	0.5 (0.5–1.0)	0.006	53 (24–82)	53 (25–74)	0.42	NA	NA	NA
Time on ART (months) median (IQR)	NA	NA		45 (18–70)	47 (22–68)	0.98	NA	NA	NA
WHO stage[#]			0.22			0.15	NA	NA	NA
1	109 (45.6)	118 (46.3)		139 (55.4)	117 (59.4)		NA	NA	
2	23 (9.6)	31 (12.2)		34 (13.5)	13 (6.6)		NA	NA	
3	28 (11.7)	38 (14.9)		35 (13.9)	28 (14.2)		NA	NA	
4	0 (0.0)	2 (0.8)		2 (0.8)	4 (2.0)		NA	NA	
No data	79 (33.1)	66 (25.9)		41 (16.3)	35 (17.8)		NA	NA	
Exposure to TB treatment, n (%)	21 (8.8)	41 (16.1)	0.01	24 (9.6)	17 (8.6)	0.87	0 (0.0)	0 (0.0)	NA
Currently on TB treatment, n (%)	8 (3.3)	25 (9.8)	0.004	1 (0.4)	2 (1.0)	0.58	0 (0.0)	0 (0.0)	NA
Previous TB treatment, n (%)	13 (5.4)	16 (6.3)	0.69	23 (9.2)	15 (7.6)	0.56	0 (0.0)	0 (0.0)	NA
Antibiotic use									NA
Antibiotic use past six months, n (%)	3 (1.3)	2 (0.8)	0.68	0 (0.0)	1 (O.5)	0.58	0 (0.0)	0 (0.0)	
Current use of co-trimoxazole, n (%)	62 (25.9)	80 (31.4)	0.18	49 (19.5)	48 (24.4)	0.25	0 (0.0)	0 (0.0)	
Self-reported symptoms*									
Epigastric discomfort	24 (10.0)	24 (9.4)	0.81	5 (2.0)	8 (4.1)	0.20	18 (21.4)	0 (0.0)	0.08
Anorexia	6 (2.5)	12 (4.7)	0.19	2 (0.8)	5 (2.5)	0.14	0 (0.0)	0 (0.0)	NA
Nausea and vomiting	9 (3.8)	16 (6.3)	0.11	2 (0.8)	6 (3.0)	0.07	2 (2.4)	0 (0.0)	0.68
Diarrhea	23 (9.6)	23 (9.0)	0.82	5 (2.0)	1 (0.5)	0.17	12 (14.3)	2 (16.7)	0.83
Weight loss	78 (32.6)	102 (40.0)	0.09	15 (6.0)	16 (8.1)	0.37	4 (4.5)	0 (0.0)	0.83
Body Mass Index (kg/m^2), mean ±SD	22.4 ± 4.1	21.9 ± 4.2	0.19	24.3 ± 4.5	23.8 ± 4.6	0.21	24.7 ± 5.0	24.6 ± 5.6	0.96
HIV-1 VL (log 10 c/mL), median (IQR)[§]	4.92 (4.09–5.51)	5.21 (4.59–5.63)	0.006	3.67 (3.10–4.57)	3.09 (2.31–4.71)	0.54	NA	NA	NA
T-cell populations, median (IQR)									
Total T-cell count/μL	1227 (867–1929)	1253 (794–1921)	0.94	1452 (1108–1934)	1584 (1151–2083)	0.05	1439 (1166–1910)	1520 (1318–1717)	0.79
CD4 T-cell count/μL	312 (128–508)	189 (75–403)	<0.0001	450 (270–643)	476 (272–654)	0.78	977 (792–1205)	861 (741–1008)	0.23
CD8 T-cell count/μL	832 (564–1336)	980 (595–1569)	0.29	858 (610–1230)	990 (697–1356)	0.02	436 (309–637)	585 (402–758)	0.18
CD4/CD8 ratio	0.31 (0.17–0.60)	0.19 (0.09–0.41)	<0.0001	0.55 (0.37–0.84)	0.49 (0.32–0.81)	0.07	2.12 (1.70–2.91)	1.80 (1.01–2.42)	0.13
WBC (x1000/μL), mean ± SD	4.99 ± 1.98	5.28 ± 2.09	0.35	5.0 (4.0–6.0)	5.0 (4.0–6.0)	0.90	NA	NA	NA
Hemoglobin (g/dL), mean ± SD	11.1 ± 1.79	10.4 ± 2.02	0.01	12.0 (11.0–13.0)	12.0 (11.0–13.0)	0.83	NA	NA	NA
Platelets (x1000/μL), mean ± SD	262.4 ± 97.2	314.9 ± 124.2	0.003	283 (224–333)	283 (228–330)	0.84	NA	NA	NA

Analysis excludes 10 patients with indeterminate *H. pylori* result. BMI, Body mass index; WBC, White blood cells; Hgb, Hemoglobin

[#] WHO clinical stage at recruitment, missing data for 147 patients of the ART-naïve group and 79 of the ART group.

[§] Missing viral load data for 14 H. pylori positive and 17 H. pylori negative participants

*Self-reported symptoms in the past 4 weeks, weight loss defined as significant for the patient, or loss of >10% of body weight. Diarrhea was defined as the passage of three or more loose or liquid stools per day

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Table 4. Univariate and multivariate logistic regression analysis of factors associated with *H. pylori* co-infection among HIV-infected individuals.

Predictor	Unadjusted OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Female gender	0.95 (0.71–1.28)	0.743		
			-	-
Age	0.10 (0.99–1.01)	0.968	-	-
Educational level	0.94 (0.85–1.03)	0.167	-	-
Access to Tap water	0.69 (0.53–0.89)	0.005	0.63 (0.47–0.84)	0.002
Intake of tuberculosis therapy	0.66 (0.43–0.10)	0.049	0.72 (0.46–1.12)	0.142
Use of co-trimoxazole	0.74 (0.55–0.10)	0.046	0.75 (0.53–1.04)	0.084
Use of ART	1.36 (1.05–1.76)	0.019	1.17 (0.86–1.59)	0.331
Duration on ART	1.00 (1.00–1.01)	0.535	-	-
Each 12-month increase				
T-cell CD4 count	1.07 (1.03–1.11)	0.001	1.06 (1.01–1.12)	0.012
Each increase of 100 cells/μL				
Viral load	0.91 (0.86–0.98)	0.007	-	-
Each increase of 1 log c/mL				

Parameters with a p-value $\leq$0.05 and a correlation coefficient of $\leq$0.1 between the parameters were included into the multivariate regression model.

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Discussion

This is the first and largest study to systematically investigate the interplay between *H. pylori* and HIV infection in sub-Saharan Africa, where both infections are highly co-endemic. We assessed the prevalence of *H. pylori* co-infection in a large cohort of unselected adult HIV-infected individuals and HIV-negative controls, and its association with clinical, immunological and virological parameters. We found a graded decrease in *H. pylori* prevalence in relation to the level of immune competence, being 88% in HIV-negative and 51.5% in HIV-positive individuals. Among HIV positive individuals, *H. pylori* prevalence declined in tandem with CD4+ T cell counts. A similar trend was observed in HIV-negative individuals, although statistical significance was not attained.

Our results are in accordance with previous epidemiologic studies, indicating a lower *H. pylori* prevalence in HIV-positive compared to HIV-negative individuals, and also among patients with AIDS compared to matched HIV-infected patients without AIDS [1,2]. However, the interpretation of existing studies is hampered by important limitations, such as small sample sizes which precluded subgroup analyses, and heterogeneous study populations, often including only patients with gastrointestinal symptoms [2]. Information on socio-demographic variables, as putative confounders for *H. pylori* status, often lacking in previous studies have been explored in the present study. Furthermore, studies including asymptomatic patients used serological tests to determine *H. pylori* status, which have been shown to be problematic especially in HIV-infected individuals [18]. *H. pylori* stool antigen tests, as employed in the present study, are non-invasive and have a proven high sensitivity and specificity, making them suitable tools for epidemiologic studies including HIV-infected individuals [12,13].

Although *H. pylori* is generally considered a disease of poverty and known to be associated with poor hygienic conditions, HIV-negative participants in our study, having a clearly higher *H. pylori* prevalence, ironically had indicators of a higher socioeconomic status. This suggests that the significant differences in *H. pylori* prevalence observed between the HIV positive and HIV negative participants may not be explained wholly by socioeconomic disparities. Indeed, the HIV negative participants had more frequent access to tap water compared to HIV-positive

individuals, and having no access to tap water was independently associated with the *H. pylori* infection in our study. Besides indicating poor sanitary conditions, the lack of access to tap water might also directly promote *H. pylori* acquisition by consumption of contaminated drinking water, e.g. from wells. An association between *H. pylori* and the consumption of water from wells has previously been reported from India, [19] and *H. pylori* has also been identified in drinking water samples from Pakistan by PCR [20].

We also found a significant graded decrease in *H. pylori* prevalence with the progression of immunodeficiency in HIV-positive individuals, with the same trend being observed in HIV-negative individuals, but without reaching statistical significance. The underlying mechanisms responsible for this association between immune competence and *H. pylori* prevalence are still unclear, although several hypotheses have been offered [2]. The most popular is that more frequent bacterial infections in HIV patients, especially those with advanced disease stages, lead to antibiotic treatment courses, probably resulting in unintended *H. pylori* eradication [2]. We found no association between *H. pylori* status and reported intake of antibiotics in the past six months before recruitment. Furthermore, only few patients reported taking antibiotics in this period of time, making it unlikely that the observed differences in *H. pylori* prevalence are explained by unintended eradication in our study population.

Antibiotic monotherapy has been reported to have only minor efficacy in *H. pylori* eradication [21]. Using a meta-analysis methodology, a pooled *H. pylori* eradication rate of 19% for monotherapy regimens has been reported [22]. In our study, Co-trimoxazole prophylaxis and tuberculosis therapy were associated with lower risk of *H. pylori* status in univariate, but not in multivariate logistic regression analysis. Co-trimoxazole has not been reported to have activity against *H. pylori*, and a culture medium containing trimethoprim and sulfamethoxazole has been developed to selectively isolate *H. pylori* from animal samples [23]. In contrast, it is known that rifampicin has activity against *H. pylori* [24]. A temporary suppression of *H. pylori* replication by concurrent tuberculosis treatment, or even clearance of the infection, is thus conceivable. However, it is to be noted that HIV patients with advanced disease are often prescribed Co-trimoxazole prophylaxis against opportunistic infections and are also more likely to receive anti-tubercular therapy for tuberculosis hence the observed lack of significant association in multivariate analyses between use of these antibiotics and risk of *H. pylori* co-infection. These findings suggest that progressive HIV disease rather than antibiotic usage may account for the diminution in frequency of *H. pylori* co-infection.

Another proposed hypothesis is that the maintenance of *H. pylori* infection requires an intact mucosal cellular immunity, and that the loss of the CD4+ T cell population in the gastric mucosa may prevent *H. pylori* persistence [2,25,26]. Hence the parallel decline of *H. pylori* prevalence with CD4+ T cell count would be consistent with this theory, although there is no evidence that impaired T cell immunity itself might cause a loss of *H. pylori* infection. CD4+ T cells have been shown to be increased in *H. pylori* gastritis, but gastric inflammation has been shown to correlate with lower *H. pylori* bacterial load, and pro-inflammatory genetic profiles are associated to lower *H. pylori* seroprevalence [27–29]. While Th1 and Th17-polarized effector T cell subsets are critical for the control of *H. pylori* infection, regulatory T cells have the ability to override this T cell driven immunity [30]. Although the alterations of gastric mucosal T cell immunity in the context of HIV infection are incompletely understood, HIV infection apparently rather impairs regulatory T cell suppressive capacity and is thus unlikely to directly promote *H. pylori* persistence [31]. Further studies are needed to dissect the interplay between systemic and local mucosal T-cell immunity and *H. pylori* persistence in the context of HIV infection.

H. pylori infection is linked to a number of adverse clinical effects, such as iron deficiency anemia, childhood growth faltering, other gastrointestinal infections and chronic diarrhea[32–

35]. In our study however, *H. pylori* infection was not associated to the presence of diarrhea, anemia, malnutrition or parasitic diseases (data not shown). Indeed a paradoxical protective effect of *H. pylori* infection against tuberculosis has been reported [36]. Furthermore, *H. pylori* infection is associated with enhanced Th1-type immune responses to TB antigens [37]. We have recently shown that *H. pylori* infection is associated with decreased markers of immune activation in ART-naïve HIV infected patients [38]. Considering that immune activation has been demonstrated to be one of the key mechanisms in HIV pathogenesis [14–17], it is tempting to speculate that *H. pylori* infection may influence susceptibility to HIV infection or the natural course of HIV disease. A large proportion of HIV-infected individuals worldwide are co-infected with *H. pylori*, hence such interaction could be relevant for the understanding of HIV immunopathology, and could also have public health implications, especially considering the ongoing efforts to develop an *H. pylori* vaccine [39].

There are some limitations of our study to be mentioned. The sample size of our HIV-negative group was smaller than that of HIV-positive individuals, and differed in terms of age and gender distribution. However, the main focus of this study was to analyze the effect of *H. pylori* within the group of HIV patients. Since we included unselected HIV patients, the group was heterogenous, among others, in terms of ART status and clinical stage of HIV disease. The group of patients taking ART in particular was heterogeneous, and we did not record details on the history and efficacy of ART in terms of CD4+ T cell recovery and virological suppression, limiting the informative value of the analysis in this subgroup. Importantly, the cross sectional study design did not allow for the investigation of causal relationships concerning the described associations.

In conclusion, we have shown that *H. pylori* infection is associated with higher CD4+ T cell counts and lower HIV-1 viral loads in ART-naïve patients. Our findings could not be explained by typical confounders as socioeconomic factors, time since diagnosis of HIV infection or unintended *H. pylori* eradication by antibiotic use for other infectious conditions. Considering the pathophysiological overlap of both chronic infections, the effects of *H. pylori* infection on the systemic immune response, and subsequently on the natural course of HIV disease, warrants further investigation employing prospective studies.

Supporting Information

S1 Table. Comparison of demographic, clinical and laboratory characteristics of HIV positive participants according to ART status.
(DOCX)

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Author Contributions

Conceived and designed the experiments: FSS KAE AD EOK MS MS JFD AME DH EEOS GBA ROP BN GB TF. Performed the experiments: FSS KAE AD EOK MS MS JFD AME DH EEOS TF. Analyzed the data: FSS KAE AD EOK TF. Contributed reagents/materials/analysis tools: AD JFD AME DH EEOS GBA ROP BN GB TF. Wrote the paper: FSS KAE AD EOK MS MS JFD AME DH EEOS GBA ROP BN GB TF.

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CHAPTER V

Helicobacter pylori Coinfection Is Associated With Decreased Markers of Immune Activation in ART-Naive HIV-Positive and in HIV-Negative Individuals in Ghana

This work was published in the journal of Clinical Infectious Diseases.

Kirsten Alexandra Eberhardt, Fred Stephen Sarfo, Albert Dompreh, Edmund Osei Kuffour, Christof Geldmacher, Mareike Soltau, Marei Schachscheider, Jan Felix Drexler, Anna Maria Eis-Hübinger, Dieter Häussinger, George Bedu-Addo, Richard Odame Phillips, Betty Norman, Gerd Dieter Burchard, Torsten Feldt; Helicobacter pylori Coinfection Is Associated With Decreased Markers of Immune Activation in ART-Naive HIV-Positive and in HIV-Negative Individuals in Ghana, Clinical Infectious Diseases, Volume 61, Issue 10, 15 November 2015, Pages 1615–1623, https://doi.org/10.1093/cid/civ577.

Edmund Osei Kuffour's contribution to this work:

1. Obtained patients samples from study site
2. Analysed all patients' stool samples by ELISA, testing for *H. pylori* antigen
3. Analysed all patients' PBMCs by multicolour flow cytometry, testing for markers for immune activation, exhaustion, senescence and regulation
4. Data screening and preliminary statistical data analysis
5. Wrote the method section of this work

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Helicobacter pylori Coinfection Is Associated With Decreased Markers of Immune Activation in ART-Naive HIV-Positive and in HIV-Negative Individuals in Ghana

Kirsten Alexandra Eberhardt,[1] Fred Stephen Sarfo,[2,3] Albert Dompreh,[3] Edmund Osei Kuffour,[4] Christof Geldmacher,[5,6] Mareike Soltau,[1] Marei Schachscheider,[1] Jan Felix Drexler,[7] Anna Maria Eis-Hübinger,[7] Dieter Häussinger,[4] George Bedu-Addo,[2,3] Richard Odame Phillips,[2,3] Betty Norman,[2,3] Gerd Dieter Burchard,[1,8] and Torsten Feldt[1,4]

[1]Clinical Research Unit, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; [2]Kwame Nkrumah University of Science and Technology, and [3]Komfo Anokye Teaching Hospital, Kumasi, Ghana; [4]Clinic of Gastroenterology, Hepatology and Infectious Diseases, Heinrich Heine University Düsseldorf, [5]Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (Ludwig Maximilian University [LMU]), [6]German Centre for Infection Research (partner site), Munich, [7]Institute of Virology, University of Bonn Medical Centre, and [8]Institute for Interdisciplinary Medicine, Asklepios Klinik St Georg, Hamburg, Germany

Background. *Helicobacter pylori* coinfection in human immunodeficiency virus (HIV) patients has been associated with higher CD4+ cell counts and lower HIV-1 viral loads, with the underlying mechanisms being unknown. The objective of this study was to investigate the impact of *H. pylori* infection on markers of T-cell activation in HIV-positive and HIV-negative individuals.

Methods. In a cross-sectional, observational study, HIV patients (n = 457) and HIV-negative blood donors (n = 79) presenting to an HIV clinic in Ghana were enrolled. Data on clinical and sociodemographic parameters, CD4+/CD8+ T-cell counts, and HIV-1 viral load were recorded. *Helicobacter pylori* status was tested using a stool antigen test. Cell surface and intracellular markers related to T-cell immune activation and turnover were quantified by flow cytometry and compared according to HIV and *H. pylori* status.

Results. *Helicobacter pylori* infection was associated with decreased markers of CD4+ T-cell activation (HLA-DR+CD38+CD4+; 22.55% vs 32.70%; $P = .002$), cell proliferation (Ki67; 15.10% vs 26.80%; $P = .016$), and immune exhaustion (PD-1; 32.45% vs 40.00%; $P = .005$) in 243 antiretroviral therapy (ART)–naive patients, but not in 214 patients on ART. In HIV-negative individuals, *H. pylori* infection was associated with decreased frequencies of activated CD4+ and CD8+ T cells (6.31% vs 10.40%; $P = .014$ and 18.70% vs 34.85%, $P = .006$, respectively).

Conclusions. Our findings suggest that *H. pylori* coinfection effectuates a systemic immune modulatory effect with decreased T-cell activation in HIV-positive, ART-naive patients but also in HIV-negative individuals. This finding might, in part, explain the observed association of *H. pylori* infection with favorable parameters of HIV disease progression.

Clinical Trials Registration. Clinicaltrials.gov NCT01897909.

Keywords. HIV/AIDS; *Helicobacter pylori*; immune activation; sub-Saharan Africa.

Human immunodeficiency virus (HIV) infection causes depletion of CD4+ T cells. Ongoing HIV viral replication results in progressive depletion of CD4+ T cells, expansion of CD8+ T cells, and, correspondingly, a low CD4+/CD8+ cell ratio [1, 2]. Antiretroviral therapy (ART) has led to a clear decline in morbidity and mortality among HIV-infected patients, mainly through its sustained suppression of HIV replication. However, treatment-mediated immune reconstitution is often incomplete, even after years of viral suppression [3]. Inflammation and T-cell activation remain elevated and CD4+ T-cell counts often fail to achieve normal levels [4]. Persistent immune activation is a hallmark

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Correspondence: Torsten Feldt, MD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, University Hospital, Heinrich Heine University Düsseldorf, Moorenstr. 5, D-40225 Düsseldorf (torsten.feldt@med.uni-duesseldorf.de).

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of HIV infection, resulting in exhaustion of the regenerative capacities of the immune system and consecutively in immunodeficiency and AIDS [5]. Furthermore, numerous non-HIV–related complications such as cardiovascular diseases, osteoporosis, neurocognitive decline, and non-AIDS associated cancer can be considered direct or indirect consequences of a chronic inflammatory status [6]. Several factors contribute to the chronic, generalized immune activation observed in HIV-infected individuals. In addition to HIV itself, HIV-mediated breakdown of the gut mucosal barrier and subsequent chronic exposure to intestinal microbial products such as lipopolysaccharide, exposure to other pathogens such as cytomegalovirus, or pyroptosis-induced cell death with subsequent release of proinflammatory cytokines might contribute to immune activation and HIV pathology [7, 8].

Helicobacter pylori is a gram-negative bacterium with a high prevalence of up to 85% in sub-Saharan Africa, the region that is also most affected by the HIV epidemic [9]. *Helicobacter pylori* is usually acquired during childhood and persistently colonizes the human stomach or duodenum [9]. The infection may contribute to the development of chronic gastritis, which can lead to peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma [10].

There is evidence that chronic *H. pylori* infection modulates the systemic immune response. Protective effects of *H. pylori* against the development of allergic asthma, inflammatory bowel diseases, and tuberculosis infection have been demonstrated [11–14]. It was shown that *H. pylori* infection is associated with enhanced interferon-gamma (IFN- γ) responses to tuberculosis [15]. On the other hand, *H. pylori* has been linked to a number of extraintestinal pathologies, including cardiovascular diseases, chronic urticaria, rosacea, Sjögren syndrome, and idiopathic thrombocytic purpura, in addition to pathologic changes of the gastric mucosa [16]. Furthermore, *H. pylori*–induced hypochlorydria leads to changes in the gastric microbiota composition, potentially resulting in an altered intestinal colonization and possible associations with pathogens such as *Shigella* or *Vibrio cholerae* [17, 18].

Potential regulatory properties on systemic immune response, especially on the activation of peripheral T lymphocytes, could be of particular interest for HIV pathology, since antiinflammatory drugs have been shown to be associated with a more favorable course of HIV disease [19, 20]. However, only a few studies with smaller sample sizes have investigated markers of activated peripheral regulatory T cells (Tregs) in *H. pylori*–positive persons, and those studies found inconsistent conclusions [21–23]. Furthermore, no data on associations between *H. pylori* and the systemic immune response and chronic inflammation in people living with HIV have been published to date. Hence, in the present study, we investigated the association between *H. pylori* infection and markers of immune activation (HLA-DR+CD38+), cell proliferation (Ki67), immune senescence (CD57), and immune exhaustion (PD-1) on T-cell subsets in a large HIV cohort and in HIV-negative controls in Ghana, West Africa.

METHODS

Study Design and Study Population

In this cross-sectional observational study, consecutive HIV-infected patients presenting to the HIV outpatient clinic and HIV-negative blood donors serving as controls were recruited between November 2011 and November 2012 at the Komfo Anokye Teaching Hospital, a tertiary referral hospital in Kumasi, Ghana. The appropriate ethics committees in Ghana and Germany approved the study. Written informed consent was obtained from all participants before enrollment in the study.

Data Collection and Laboratory Methods

Trained study personnel collected demographic and clinical data using a standardized questionnaire. Blood samples were collected in EDTA tubes for analysis of CD4+/CD8+ T-cell counts, using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, California). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation of heparinized venous blood on a Ficoll/Hypaque (Biocoll Seperating Solution, Biochrom AG, Berlin, Germany) density gradient. Cells were washed in phosphate-buffered saline and resuspended in Roswell Park Memorial Institute 1640 medium (both Gibco Invitrogen, Carlsbad, California) supplemented with heat-inactivated fetal calf serum (Biochrom AG, Berlin, Germany). PBMCs were cryopreserved and shipped to Germany on liquid nitrogen. EDTA plasma and native stool samples were freshly frozen and stored at −80°C until being transported to Germany on dry ice.

Stool was tested for *H. pylori* using the RidaScreen FemtoLab *H. pylori* stool antigen test (R-Biopharm AG, Darmstadt, Germany). HIV-1 viral load was measured using the RealTime HIV-1 polymerase chain reaction system (Abbott Diagnostics, Wiesbaden, Germany). The same tests, except viral load analysis, were applied to cases and controls.

Cell surface markers for immune activation and immune exhaustion/function were stained using a fluorochrome-conjugated mouse anti-human monoclonal antibody combination in a single panel: anti-CD3-APC-H7, anti-CD4-V500, anti-CD8-PerCP, anti-HLA-DR-FITC, anti-CCR7-Alexa-Flour-647 (CD197) (BD Biosciences, Heidelberg, Germany) and anti-CD38-PE-Cy7, anti-PD-1-V421, anti-CD57-PE, anti-CD45RA-Alexa-Flour-700 (Biolegend, Fell, Germany). In a second panel, cell surface markers of immune regulation and cell proliferation/cell turnover were stained using anti-CD3-PerCP, anti-CD4-Pacific Blue, anti-CD8-Alexa-Flour-700, and anti-CD25-PE-Cy7 (BD Biosciences, Heidelberg, Germany). The stained cells were fixated and permeabilized (FoxP3 staining buffer set, eBioscience,

Frankfurt a. M., Germany) for intracellular staining using anti-FOX-P3-PE (Biolegend, Fell, Germany) and anti-Ki-67-Alexa-Flour-647 (BD Biosciences, Heidelberg, Germany). Flow cytometric data were acquired using the LSRII flow cytometer (BD Biosciences, Heidelberg, Germany), and acquisition was set to 500 000 cells/sample for panel 1 and 1 000 000 cells/sample for panel 2. Compensation was conducted with antibody capture beads (BD CompBeads Set Anti-Mouse Ig, κ, BD Biosciences, Heidelberg, Germany), stained separately with the individual fluorochrome-conjugated monoclonal antibodies used in all samples. Flow cytometry measurements were performed in runs of 20 samples, each including samples of HIV-positive individuals and HIV-negative controls. Cutoffs for CD38 and HLA-DR expression were defined in an HIV-negative sample on the naive (CCR7+CD45RA+) T-cell population, typically expressing CD38, but only negligible amounts of HLA-DR, and uniformly applied to all samples of 1 run (Figure 1). A fluorescent minus one control experiment was done to confirm the gating strategy. Flow cytometry measurements were analyzed using FlowJo version 9.6.2 (Tree Star, San Carlos, California). The operator was blinded to participants' clinical and laboratory data. All samples of HIV-positive individuals and HIV-negative controls were processed according to the same protocols.

Statistical Analyses

Continuous variables were expressed as mean ± standard deviation or median (interquartile range [IQR]) and compared using the unpaired Student *t* test or the Wilcoxon rank sum test. Proportions were compared using either the χ^2 test or Fisher exact test as appropriate. A multivariable linear regression model was used to assess the association between the continuous outcome variables HLA-DR+CD38+ as activation marker of CD4+/CD8+ T cells and other laboratory, clinical, and demographic parameters. After assessing the Pearson correlation between the frequency of HLA-DR+CD38+CD4+ and HLA-DR+CD38+CD8+ T cells and age, gender, time since HIV diagnosis, use of co-trimoxazole or rifampicin in the last 6 months, and *H. pylori* status, only parameters with a *P* value ≤.1 were included in a linear multivariate regression model. The parameters CD4+ and CD8+ T-cell count and HIV-1 viral load, being directly linked to T-cell activation, were excluded. However, the linear multivariate regression model was alternatively calculated including those parameters. All *P* values were 2-sided, and *P* values <.05 were considered statistically significant. Statistical analyses were conducted with R 2.15.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Cohort Characteristics

A total of 1095 HIV-positive patients and 107 HIV-negative blood donors were recruited for the original cohort study, which sought to access the sociodemographic and clinical determinants of *H. pylori* coinfection among HIV-infected and non-infected individuals. Flow cytometry data were available for 457 (41.7%) HIV-positive and 79 (73.8%) HIV-negative participants. Compared with the original cohort, HIV-positive and negative patients with available flow cytometry data were not different from those without flow cytometry data in terms of gender, age, CD4+ cell count, or viral load.

Among patients with available flow cytometry data, HIV-positive individuals were older (40.4 vs 33.4 years; *P* < .001), had a lower CD4+/CD8+ ratio (0.42 vs 2.18; *P* < .001), and a lower prevalence of *H. pylori* infection (56.2% vs 87.3%; *P* < .001) compared with HIV-negative individuals. Markers of immune activation, senescence, exhaustion, and cell turnover on T-cell subsets differed significantly between HIV-positive and HIV-negative individuals (Table 1). Within the group of HIV-positive participants, approximately half of the participants (46.8%) were receiving ART; 53.2% were ART naive. Patients on ART were more likely to be female (81.3% vs 70.0%; *P* = .005), had a higher mean body mass index (24.3 vs 22.3; *P* < .001), a higher median CD4+ T-cell count (483 [IQR, 301–671] vs 269 [IQR, 105–448] cells/μL; *P* < .001), and were more frequently coinfected with *H. pylori* (62.2% vs 51.0%; *P* = .017) compared with ART-naive patients.

Markers of Immune Activation According to *H. pylori* Status

No differences in demographics were observed within the subgroups when compared according to *H. pylori* status (Table 2). Within the group of HIV-positive, ART-naive participants, those with *H. pylori* coinfection had significantly higher median CD4+ T-cell counts (312 [IQR, 135–484] vs 224 [IQR, 79–426] cells/μL; *P* = .024) and lower median HIV-1 viral loads (4.82 vs 5.18 log10 copies/mL; *P* = .004). Frequencies of HLA-DR+CD38-+CD4+ (22.55% vs 32.70%; *P* = .002), Ki67+CD4+ (15.1% vs 26.8%; *P* = .016), PD-1+CD4+ (32.45% vs 40.0%; *P* = .005), as well as Ki67+CD8+ (10.3% vs 16.6%; *P* = .031) and PD-1+CD8+ (36.15% vs 41.50%; *P* = .012) T cells were lower in individuals with vs without *H. pylori* coinfection (Table 3; Figure 2).

In the subgroup of HIV-positive participants on ART, *H. pylori* infection was associated with higher CD4+/CD8+ ratios (0.59 vs 0.43; *P* = .010) and a trend toward lower frequencies of CD25+FoxP3+CD4+ T cells (1.83% vs 2.44%; *P* = .059). No differences regarding markers for immune activation, exhaustion, or proliferation could be detected between *H. pylori*–positive vs *H. pylori*–negative individuals (Table 3). Interestingly, frequencies of CD4+ and CD8+ T cells expressing the activation markers HLA-DR+CD38+ were lower in HIV-negative blood donors with *H. pylori* compared with those without *H. pylori* infection (median 6.31 vs 10.40; *P* = .014 and 18.70 vs 34.85; *P* = .006, respectively) (Figure 2, Supplementary Figure 1). They also had a trend toward higher CD4+/CD8+ ratios (2.22 [IQR, 1.79–3.05] vs 1.77 [IQR, 1.01–2.38]; *P* = .087; Table 3).

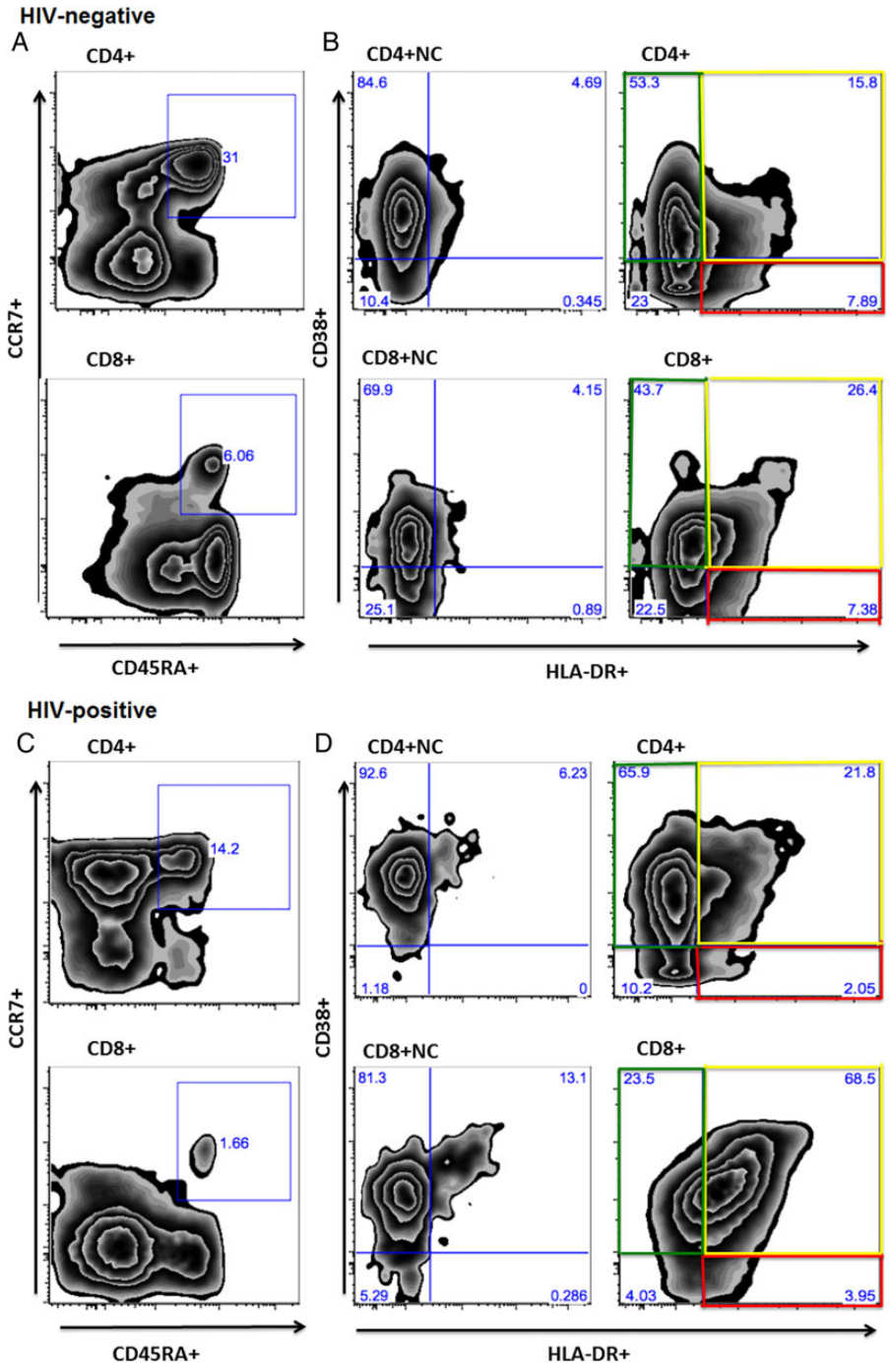

Figure 1. Gating strategy for HLA-DR and CD38 expression on peripheral CD4+ and CD8+ T cells of human immunodeficiency virus (HIV)–negative (panels *A* and *B*) and HIV-positive (panels *C* and *D*) individuals. Cutoffs for CD38 and HLA-DR expression were defined in an HIV-negative sample on the respective naive (CCR7+CD45RA+) T-cell population (panel *B*), typically expressing CD38 and only negligible amounts of HLA-DR, and uniformly applied to all samples of 1 run (panels *C* and *D*).

Independent Predictors of Immune Activation

In the multivariate linear regression analysis, a negative *H. pylori* status was identified as an independent risk factor for CD4+ T-cell activation in HIV-positive, ART-naive participants ($P = .008$). In HIV patients receiving ART, female gender and months since diagnosis of HIV infection, but not *H. pylori* infection, were associated with immune activation. Negative *H. pylori* status was identified as the only independent risk factor

Table 1. Immunological Parameters According to Human Immunodeficiency Virus Status

Variable, Median (Interquartile Range)	HIV Positive, n = 457	HIV Negative, n = 79
CD4+ T-cell count/μL	380 (184–567)	957 (769–1134)**
CD8+ T-cell count/μL	854 (610–1335)	420 (309–617)**
CD4+/CD8+ T-cell ratio	0.42 (0.21–0.69)	2.18 (1.69–2.96)**
HLA-DR+CD38+CD4+ (%)	17.90 (10.10–31.90)	6.69 (4.96–9.43)**
Ki67+CD4+ (%)	11.90 (6.98–26.25)	4.77 (3.80–5.75)**
PD-1+CD4+ (%)	34.10 (23.40–48.50)	31.00 (20.15–35.95)*
CD57+CD4+ (%)	16.30 (9.47–27.70)	12.80 (8.46–24.95)
CD25+Foxp3+CD4+ (%)	2.53 (1.50–4.43)	1.59 (1.03–2.26)**
HLA-DR+CD38+CD8+ (%)	40.60 (27.30–54.50)	19.20 (15.50–28.05)**
Ki67+CD8+ (%)	10.03 (6.04–17.48)	4.70 (3.55–5.88)**
PD-1+CD8+ (%)	30.40 (18.70–43.60)	15.30 (9.54–20.60)**
CD57+CD8+ (%)	50.20 (39.60–61.00)	61.10 (42.65–72.10)**

Abbreviation: HIV, human immunodeficiency virus.

*$P < .01$; **$P < .001$.

for increased CD4+ ($P = .001$) and CD8+ ($P < .001$) T-cell activation in HIV-negative individuals (Table 4). Also, after adjustment for CD4+ T-cell count and HIV-1 viral load, *H. pylori* infection was independently associated with decreased CD4+ T-cell activation in ART-naive HIV-infected individuals and with CD4+ and CD8+ T-cell activation in HIV-negative individuals (Supplementary Table 1).

DISCUSSION

Data from epidemiologic studies suggest that the prevalence of *H. pylori* infection is clearly lower in HIV-positive compared with HIV-negative individuals and that it further declines with the progression of immunodeficiency in HIV-infected patients. *Helicobacter pylori* coinfection is also associated with higher CD4+ T-cell counts and lower HIV-1 viral loads [24].

Several mechanisms have been proposed to explain this association. First, *H. pylori* infection itself could exert an effect on the progression of, or the susceptibility to, HIV infection. Recently, the importance of gastrointestinal microbiota as a determinant for the systemic immune response has been recognized, and a number of extraintestinal, immune-related implications of *H. pylori* infection have been reported [16, 17, 25]. Other explanations include the more frequent use of antibiotics in HIV patients, in particular those with more advanced HIV disease, leading to inadvertent eradication of *H. pylori*. Results from our original cohort study could, however, not explain differences in *H. pylori* prevalence by more frequent use of antibiotics or socioeconomic status in HIV-positive patients.

Table 2. Cohort Characteristics According to *Helicobacter pylori* Status

	HIV Positive, Antiretroviral Therapy Naive		HIV Positive on Antiretroviral Therapy		HIV Negative	
Characteristic	*H. pylori* Positive, n = 124	*H. pylori* Negative, n = 119	*H. pylori* Positive, n = 133	*H. pylori* Negative, n = 81	*H. pylori* Positive, n = 69	*H. pylori* Negative, n = 10
Female gender, n (%)	86 (69.36)	84 (70.59)	108 (81.20)	66 (81.48)	43 (64.18)	8 (80.0)
Age (y), mean ± SD	39.46 ± 9.58	41 ± 9.68	41 ± 8.50	40 ± 8.59	33 ± 13.32	33 ± 13.88
Body mass index (kg/m²), mean ± SD	22.38 ± 4.01	22.2 5 ± 4.88	24.59 ± 5.52	23.80 ± 4.61	24.55 ± 5.22	24.77 ± 6.13
Anti-tuberculosis treatment, n (%)	7 (5.65)	11 (9.24)	1 (0.75)	1 (1.24)	NA	NA
Co-trimoxazole, n (%)	27 (21.77)	39 (32.77)	33 (24.81)	19 (23.46)	NA	NA
Other antibiotics, n (%)	0	0	0	0	NA	NA
Months since diagnosis, median (interquartile range)	0.0 (0.0–2.8)	0.0 (0.0–3.0)	55.0 (26.5–83.5)	51.0 (27.25–75.5)	NA	NA

No significant differences were detected between *H. pylori*–positive vs *H. pylori*–negative individuals within each subgroup.

Abbreviations: HIV, human immunodeficiency virus; NA, not applicable; SD, standard deviation.

Table 3. Immunological Parameters According to *Helicobacter pylori* Status

Variable, median (interquartile range or %)	HIV Positive, Antiretroviral Therapy Naive		HIV Positive on Antiretroviral Therapy		HIV Negative	
	H. pylori positive, n = 124	*H. pylori* negative, n = 119	*H. pylori* positive, n = 133	*H. pylori* negative, n = 81	*H. pylori* positive, n = 69	*H. pylori* negative, n = 10
CD4+ T-cell count/μL	312 (135–484)	224 (79–426)*	505 (332–719)	448 (296–590)	958 (787–1169)	793 (737–980)
CD8+ T-cell count/μL	1186 (872–1739)	1326 (887–2034)	1462 (1092–2043)	1396 (1072–2072)	411 (304–548)	585 (412–811)
CD4+/CD8+ T-cell ratio	0.34 (1.75–5.49)	0.19 (0.09–0.42)**	0.59 (0.41–0.93)	0.43 (0.32–0.81)*	2.22 (1.79–3.05)	1.77 (1.01–2.38)
Viral load, log10 copies/mL	4.82 (4.09–5.43)	5.18 (4.57–5.67)**	1.59 (1.59–2.19)	1.59 (1.59–1.83)	NA	NA
HLA-DR+CD38+CD4+ (%)	22.55 (13.70–34.93)	32.70 (18.65–41.25)**	12.90 (7.30–20.60)	11.90 (7.76–18.90)	6.31 (4.94–8.15)	10.40 (8.67–15.15)*
Ki67+CD4+ (%)	15.10 (8.33–32.10)	26.80 (15.10–49.00)*	7.96 (5.48–12.00)	9.33 (6.98–13.50)	4.74 (3.75–5.73)	5.09 (4.26–6.51)
PD-1+CD4+ (%)	32.45 (23.33–48.73)	40.00 (28.35–55.30)**	31.80 (21.30–44.40)	31.70 (18.30–46.40)	30.90 (19.80–35.20)	35.95 (25.03–37.28)
CD57+CD4+ (%)	13.90 (9.39–25.73)	18.30 (9.70–29.50)	16.10 (9.32–26.90)	16.70 (10.00–30.30)	12.80 (8.16–24.50)	14.25 (11.25–39.78)
CD25+Foxp3+CD4+ (%)	2.54 (1.52–4.54)	3.28 (1.79–6.83)	1.83 (1.15–3.76)	2.44 (1.64–4.83)	1.59 (1.12–2.23)	1.46 (0.80–2.30)
HLA-DR+CD38+CD8+ (%)	49.25 (39.08–62.10)	52.90 (43.35–63.95)	27.90 (20.00–40.60)	26.00 (18.10–36.40)	18.70 (15.30–26.20)	34.85 (22.13–44.58)**
Ki67+CD8+ (%)	10.30 (7.88–15.90)	16.60 (11.20–23.10)*	6.04 (4.02–8.53)	9.27 (4.29–17.13)	4.58 (3.47–5.92)	5.19 (4.47–5.82)
PD-1+CD8+ (%)	36.15 (23.85–45.83)	41.50 (28.85–53.25)*	22.60 (14.50–34.80)	22.30 (16.10–32.80)	15.10 (9.57–20.40)	17.60 (6.48–23.175)
CD57+CD8+ (%)	44.85 (36.30–55.43)	45.80 (35.25–57.35)	54.40 (45.80–65.10)	52.80 (43.80–62.20)	60.00 (42.00–71.70)	66.65 (54.80–72.70)

Abbreviations: HIV, Human Immunodeficiency virus; NA, not applicable.

* $P < .05$; ** $P < .01$ compared between *H. pylori*–positive vs *H. pylori*–negative individuals within each subgroup.

There is no evidence that CD4+ T cells are needed for the maintenance of *H. pylori* infection or that the depletion of CD4+ T cells could lead to the loss of *H. pylori* infection. Rather, T-cell responses against *H. pylori* have been shown to be associated with gastric inflammation and protection against *H. pylori* infection [26]. The fact that the observed differences between individuals with vs without *H. pylori* infection were also observed in HIV-negative participants is another argument against this hypothesis.

It is known that the main target cells for HIV are activated CD4+ T lymphocytes, of which the majority is located in the lymphoid tissue of the gastrointestinal mucosa [7]. *Helicobacter pylori* might prevent the activation of CD4+ T cells for maintaining its own persistence in the gastric and duodenal mucosa via several mechanisms, thereby reducing the number of target cells susceptible for HIV infection and possibly slowing down the vicious circle of immune activation and HIV replication [27, 28].

The immune response to *H. pylori* infection is predominantly T-cell mediated, with Th1 and Th17 cells being major effectors [29]. *Helicobacter pylori* has evolved multiple mechanisms to evade adaptive immunity by interfering with antigen presentation and modulation of T-cell responses [30]. It has been shown that the *H. pylori* vacuolating toxin (VacA) directly inhibits T-cell activation by interfering with the maturation of dendritic cells and antigen presentation and by inhibiting activation-induced proliferation of T and B lymphocytes [30–33]. Apparently, *H. pylori* is able to induce Treg responses, while inhibiting Th17 responses [30, 34]. Treg cells are increased in the gastric mucosa of *H. pylori*–infected patients and attenuate the inflammatory response, among other mechanisms, by secreting the antiinflammatory cytokines transforming growth factor-β1 and interleukin-10 and thus facilitate the colonization of the stomach [21].

To date, it is not clear if those mechanisms are also relevant for the systemic immune response and thus could, in part, explain the decreased peripheral T-cell activation observed in this study. However, one study investigated the effect of *H. pylori* eradication on the cytokine profile of patients with chronic immune thrombocytopenia (cITP). Six months after eradication, those patients who achieved cITP remission showed a significant reduction in the concentrations of predominantly proinflammatory Th1- and Th17-associated cytokines and an increase in Treg- and Th2-associated cytokines [35]. Furthermore, it has recently been noted that *H. pylori* infection might trigger large intestinal microbiota changes, with possible implications for microbial translocation and immune activation [17, 25].

Helicobacter pylori infection, by modulating mucosal and systemic immunity, might influence susceptibility or the clinical course of other infections. Only 1 study has investigated the relationship between *H. pylori* seroprevalence and malaria incidence in Ugandan children; no evidence for a protective effect against malaria was found [36]. In contrast, a protective effect of

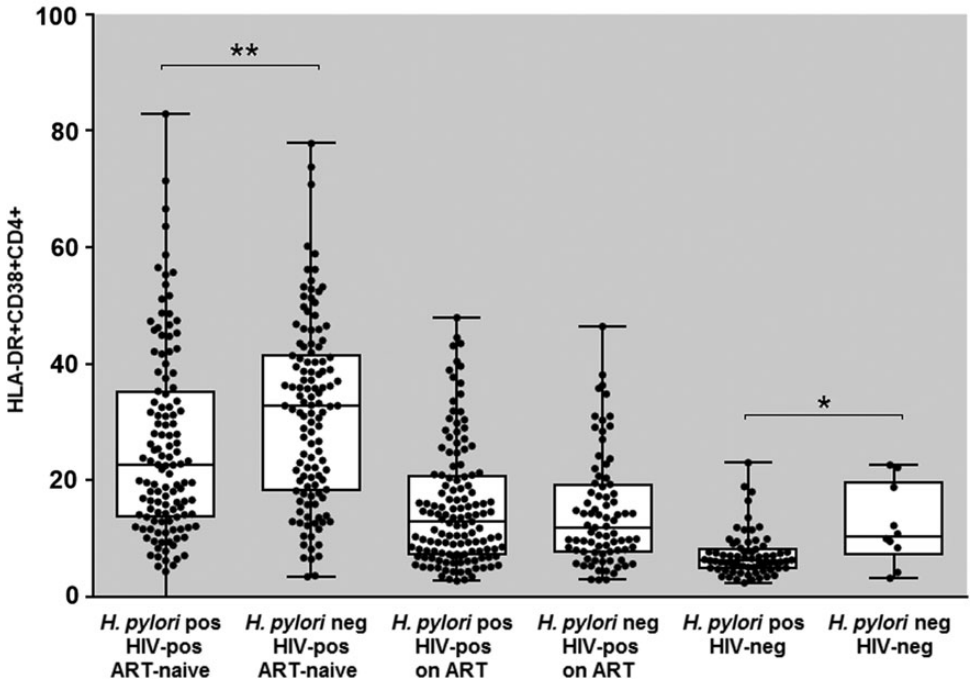

Figure 2. Boxplot scatter dot plot showing the proportion of activated CD4+ T cells (HLA-DR+CD38+CD4+) within the 3 subgroups of human immunodeficiency virus (HIV)–positive, antiretroviral therapy (ART)–naive patients, HIV-positive participants on ART and HIV-negative blood donors compared according to their *Helicobacter pylori* status. Intragroup comparisons were conducted using the Wilcoxon rank sum test, with *$P < .05$; **$P < .01$.

H. pylori against tuberculosis infection associated with enhanced IFN-γ responses has been reported in human tuberculosis case-contact cohorts and in monkeys that underwent a tuberculosis challenge [37].

Table 4. Univariate and Multivariate Analysis of Factors Associated With Increased Immune Activation

	HIV Positive, Antiretroviral Therapy Naive, n = 243				HIV Positive on ART, n = 214				HIV Negative, n = 79			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
Variable	*r*	*P* Value	β-Coef	*P* Value	*r*	*P* Value	β-Coef	*P* Value	*r*	*P* Value	β-Coef	*P* Value
Factors associated with increased HLA-DR+CD38+CD4+												
Female gender	−0.031	.636			0.072	.292			−0.050	.663		
Age, y	0.038	.552			−0.150	.029	0.104	.184	0.037	.747		
Anti-tuberculosis treatment	0.065	.315			0.070	.305			NA	NA		
Co-trimoxazole	0.009	.885			−0.074	.281			NA	NA		
Months since diagnosis	−0.137	.037	−0.134	.057	−0.290	<.001	-0.088	**<.001**	NA	NA		
Helicobacter pylori positive	−0.175	.006	−5.559	**.008**	0.044	.518			−0.359	.001	−4.923	**.001**
Factors associated with increased HLA-DR+CD38+CD8+												
Female gender	−0.074	.252			0.121	.078	4.997	**.049**	−0.138	.230		
Age, y	0.040	.534			−0.089	.193			−0.155	.179		
Anti-tuberculosis treatment	0.101	.115			0.094	.170			NA	NA		
Co-trimoxazole	0.096	.134			0.063	.361			NA	NA		
Months since diagnosis	−0.102	.120			−0.212	.002	−0.098	**.002**	NA	NA		
Helicobacter pylori positive	−0.075	.242			0.060	.382			−0.422	<.001	−14.098	**<.001**

The bold values represent $P < .05$.

Abbreviations: ART, antiretroviral therapy; β-Coef, multivariate linear regression coefficient (slope of regression line showing increase of outcome variable for every 1-unit increase in each predictor); HIV, human immunodeficiency virus; NA, not applicable; r, Pearson correlation coefficient.

This is the first study to systematically investigate the association between *H. pylori* infection and systemic immune activation in HIV-positive and HIV-negative individuals. Using multivariate regression analysis, *H. pylori* infection was associated with decreased markers of immune activation in CD4+ T cells and with decreased markers of immune exhaustion and cell turnover in CD4+ and CD8+ T cells in ART-naive HIV patients. Interestingly, *H. pylori* infection was also associated with decreased frequencies of activated CD4+ and CD8+ T cells in HIV-negative blood donors. This finding is remarkable considering the relatively small sample size of the HIV-negative control group and suggests that the observed correlation of *H. pylori* infection with decreased immune activation is not specific for HIV-infected individuals and that the observed association in HIV-infected participants is unlikely to be explained by confounders in the HIV-positive group. Furthermore, a higher level of T-cell activation in *H. pylori*–negative, HIV-uninfected individuals might potentially support the hypothesis that *H. pylori* infection decreases the susceptibility to HIV infection. Indeed, immune activation has been described as a risk factor for the acquisition of HIV infection in the CAPRISA 004 vaccination trial [38, 39]. In another trial, an association of CD8+ T-cell activation with increased risk of HIV infection was reported [40]. The authors noted that identifying causes for elevated innate immune activation could enable targeted prevention measures.

The failure to detect differences in the subgroup of HIV-infected individuals receiving ART might be explained by the markedly decreased baseline immune activation in those patients, together with the heterogeneity regarding duration and kind of ART. However, those patients with *H. pylori* infection had significantly higher CD4+/CD8+ ratios as an indicator for decreased immune activation compared with individuals without *H. pylori* coinfection.

There are limitations of our study to be mentioned. Most importantly, the causality of the observed associations cannot be established with the cross-sectional study design used. Longitudinal studies would be needed to explore the hypotheses that the risk of HIV acquisition is decreased in *H. pylori*–positive vs *H. pylori*–negative individuals and that *H. pylori* acquisition, respectively eradication, is associated with alterations in immune activation. The HIV-negative control group was smaller than the HIV-positive group, with a higher median age and most likely a lower risk for coinfections, making the intergroup comparison of immune activation problematic. However, the main focus of this study was the intragroup analysis of immune parameters according to *H. pylori* status. Overall, our findings support the hypothesis that *H. pylori* coinfection effectuates a systemic immune modulatory effect with decreased T-cell activation in HIV-positive, ART-naive patients and also in HIV-negative individuals. This might, in part, explain the observed association of *H. pylori* infection with favorable parameters of HIV disease progression and other extraintestinal effects.

The mechanisms of possibly beneficial immunomodulatory effects of *H. pylori* infection, which need to be characterized, might potentially represent a new therapeutic approach. At the same time, the high global burden of *H. pylori* infection warrants the development of vaccine or eradication strategies, emphasizing the definition of respective target groups.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Declaration

I hereby declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under the consideration of the 'Principles for the Safeguarding of Good Scientific Practice at Heinrich-Heine-University Düsseldorf. This dissertation does not contain any material published by any other person, except as acknowledged in the text. All sources of facts and figures have been duly acknowledged and with permission from the journal publishers and the corresponding authors. This dissertation has not been presented to any other faculty and no attempts to obtain a doctorate with this dissertation have been made.

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