12-2022

A Functional Study of Topological DNA Problem in Human T cells During Chronic Viral Infection

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A Functional Study of Topological DNA Problem in Human T cells During Chronic Viral Infection

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A dissertation

presented to

the faculty of the Department of Internal Medicine, Surgery and Biomedical Science

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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December 2022

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Keywords: topoisomerase, apoptosis, T cell dysregulation,
topological DNA damage, HBV, HCV, HIV

ABSTRACT

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T cells play an important role in adaptive immune system against viral infections, while premature aging and dysfunction of T cells induced by unrepaired DNA damages are always non-negligible snags during the long-term of fighting with chronic viral infections, such as Hepatitis B virus (HBV), Hepatitis C virus (HCV) or Human Immunodeficiency Virus (HIV) infection. In this dissertation, we investigated the role of topological DNA damage in reprogramming telomeric DNA damage responses (DDR), mitochondrial metabolisms, and T cell functions using $CD4^+$ T cells derived from individuals with chronic viral infections or healthy subjects treated with topoisomerase inhibitors. The healthy human T cells were treated with camptothecin (CPT) for mitochondrial topoisomerases I (Top1mt) or ICRF-193 or etoposide (ETP) for topoisomerases IIα (Top2α) as models. We found a significant suppression of Top2α and Top1mt protein levels and enzymatic activity in $CD4^+$ T cells in chronically HCV/HIV-infected patients compared to age and gender-matched healthy subjects, along with an accumulation of the topoisomerase cleavage complex (Topcc) in genomic DNA as well as mitochondrial DNA (mtDNA). Mechanistically, topoisomerase inhibition in healthy $CD4^+$ T cells caused topological DNA damage, telomere attrition, mitochondrial metabolic disorder and T cell apoptosis or dysfunction via inducing Topcc accumulation, PARP1 cleavage and failure in DNA repair, thus recapitulating T cell dysregulation in the setting of chronic viral infections. In addition, T cells from virally infected subjects with lower topoisomerase levels were vulnerable

to the inhibitor-induced cell apoptosis, indicating an important role for Top2α and Top1mt in preventing DNA topological disruption and cell death. These results demonstrate that accumulation of Topcc and topoisomerase deficiency lead to unrepaired DNA damage and render virally infected patients' T cells prone to senescence and apoptosis, thus contributing to mitochondrial metabolic disturbance or dysfunction in $CD4^+$ T cell during chronic HCV or HIV infection. This study reveals a novel mechanism by which topoisomerase deficiency promotes telomeric DNA or mtDNA damage and premature T cell aging, and provides a new therapeutic target for restoring the DNA topologic machinery protecting T cells from unwanted DNA damage and to maintain immune competence.

DEDICATION

This is dedicated to my beloved husband Dechao Cao for his endless love, supports and encouragements. It's also dedicated to my son Josiah Cao who has been my constant source of comfort and joy.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere appreciation to my research mentor who is also my committee chair, Prof. Zhi Q. Yao for providing invaluable guidance and continuous support throughout my Ph.D. study and research during the past five years. Besides, I would like to thank the rest of my committee members Prof. Jonathan P. Moorman, Prof. Krishna Singh, Prof. Shunbin Ning and Prof. Chuanfu Li for their insightful commons, experiential supervisions and thoughtful feedback.

I am extremely grateful to all our lab members: Dechao Cao, Lam Ngoc Thao Nguyen, Madison Schank, Sushant Khanal, Dr. Jinyu Zhang, Yi Zhang, James Adkins, Benjamin Pelton and our lab manager Xiao Y. Wu for their precious help during my graduate study. A special thanks to our lab senior scientist Dr. Ling Wang and Dr. Juan Zhao for their generous suggestions and has served as a constant resource of knowledge and encouragement during my research. My sincere thanks also go to Dr. Junping Ren, Dr. Yingjie Ji, Stella Ogbu, Dr. Zhengke Li, Lam Nhat Nguyen and Zheng D. Morrison who worked in our lab and helped me with my experiments.

Finally, my deepest gratitude to all the faculties and staff in the Department of Internal Medicine and the Biomedical Science graduate program. It was a previous opportunity and honor to work here in ETSU with all of you.

The research was supported by National Institutes of Health (NIH) grants: R01AI114748, R21AI138598, R21AI157909 and R15AG069544 (to Z.Q.Y.); VA Merit Review Award 1I01BX002670, 1I01BX004281 (to Z.Q.Y) and 5I01BX005428–02 (to J.P.M.); and Department of Defense Award PR170067 (to Z.Q.Y). This study is the result of work supported with resources and the use of facilities at the James H. Quillen Veterans Affairs Medical Center.

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CHAPTER 1. INTRODUCTION

Human T cells and Chronic Viral Infections

In adaptive immune responses, human T cells act as regulators and killers. When pathogens such as viruses or bacteria enter the bloodstream after passing through the protection of skin/mucosa, T cells are activated by pathogens and divide rapidly, while also releasing cellular cytokines to help destroy the intruders via inflammation. In general, acute infections can result in a brief surge of T cell protection, whereas persistent chronic infections typically produce immunological exhaustion and senescence due to T cell dysfunction, either premature disabled or depleted. T cells may enter a dysfunctional state known as T cell exhaustion if the immune system is unable to eliminate the foreign antigens. Furthermore, T cell exhaustion has been defined as weak antigen-specific T cell responses, and manifested as a decline in antiviral effector functions of antigen-specific T cells, such as a decrease in effector cytokine production, a decreased ability to eliminate infected cells, and impaired proliferation after antigen exposure (Penna et al. 2007; Dustin 2017; Osuch et al. 2020). HBV, HCV, and HIV are the major blood-borne pathogens that can establish chronic infection in humans by disrupting T cell homeostasis and surveilling functions. Compelling evidence suggests that increasing levels of chromosomal and mitochondrial DNA damages are observed in aging T cells from individuals with chronic HBV, HCV, and HIV infections (Farinati et al. 1999; Shawki et al. 2014; Awasthi et al. 2015; Seeger and Mason 2015; Mason et al. 2016; Nguyen et al. 2018; Yan et al. 2019). Hepatic cirrhosis is a common result of chronic hepatitis infection (HBV and HCV), which is the leading cause of hepatocellular carcinoma (HCC). Many studies show that regulatory T cells are involved in the modulation of HBV- and HCV-associated immune responses, thereby promoting the progression of HCC. Also, it becomes evidence that human chronic infectious diseases, such

as AIDS, hepatitis C, hepatitis B, and adult T-cell leukaemia/lymphoma (ATL) caused by human T-cell lymphotropic virus type 1 (HTLV-1), and cancers share the same mechanisms of T-cell exhaustion (Ahmed et al. 1984; Gallimore et al. 1998; Zinkernagel 2002; Barber et al. 2006; Urbani et al. 2006; Boni et al. 2007; Rehermann and Thimme 2019).

T cells and HBV Infection

The non-cytopathic HBV is a hepatotropic DNA virus. According to a WHO report from 2022, even though hepatitis B is a disease that may be prevented by vaccination, about 3,000 new HBV cases are reported in the United States annually, and more than 1 million individuals die each year worldwide from cirrhosis or HCC, while only 10% and 21% of people are aware that they have chronic hepatitis B or C, respectively (Miroux et al. 2010; Trépo et al. 2014).

Virus-specific CD4 and CD8 T cells are crucial for the control of HBV infection, however the antiviral immune response in individuals with chronic HBV infection is ineffective. Previous studies showed that patients with HBV infection had significantly lower CD4/CD8 ratios than healthy controls (Barnaba et al. 1983). A highly valuable HBV-tolerant transgenic mouse model was developed 20 years later, which may aid in understanding the human pathophysiology associated with HBV infection (Chisari et al. 1987; Roh and Kim 2003). It reveals, for the first time, a demonstration of the involvement of CD4 T cells with regulatory functions in the setting of HBV infection since it is sufficient to transfer this tolerance when CD4 T cells from tolerant mice are injected into immunocompetent mice (Chisari et al. 1987; Roh and Kim 2003; Thio et al. 2004). Since 2005, there has been an exponential increase in our understanding of the role that regulatory T cells (Tregs) play in human HBV infection. Functional study of Tregs has been made possible by isolating CD4/CD25 cells from peripheral blood of patients (Franzese et al. 2005; Xu et al. 2006; Miroux et al. 2010).

T Cells and HCV Infection

Around 1.5 million new cases of chronic hepatitis C virus are reported each year, infecting an estimated 58 million people worldwide. This disease results in about 400,000 death each year. Contrary to HBV, HCV is a RNA virus of the hepaciviral type. Besides, the HCV genome cannot incorporate into cellular DNA, necessitating its replication in order to survive (Pham and Michalak 2008).

Although both $CD4^+$ and $CD8^+$ HCV-specific T lymphocytes are frequently seen in peripheral blood and liver tissue, they are unable to eradicate the virus in the majority of patients and do not prevent re-infection with HCV, especially once chronic infection has been established (Rehermann 2009; Neumann-Haefelin and Thimme 2013; Heim and Thimme 2014; Nitschke et al. 2015). During the chronic phases of infection, a weak or non-persistent $CD4^+$ response notably inhibited by a significantly increasing of $CD4^+$ $CD25^+$ Tregs, while the HCV-specific $CD8^+$ T lymphocytes exhibit a functional defect, resulting in an alteration of their *in vitro* proliferation and Interferon gamma (IFN-γ) production (Wedemeyer et al. 2002; Bolacchi et al. 2006; Itose et al. 2009). Despite significant progress in treating chronic HCV infection in recent years, which is expected to reduce the extent of virus-related morbidity and mortality (Vermehren et al. 2018), the impact of anti-HCV treatment initiated during the chronic phase of infection on already-established T cell exhaustion is largely unknown and frequently inconclusive. Studies of T cell function in IFN-based therapy of chronic HCV infection have shown that it reduces the numbers and impairs the functional potential of antiviral T cells (Boni et al. 2007; Barnes et al. 2009; Abdel-Hakeem et al. 2010; Missale et al. 2012; Luxenburger et al. 2018), which means IFN-based treatment of chronic HCV infection does not result in the functional restoration of HCV-specific T cells (Missale et al. 2012; Larrubia et al. 2015). In

addition, the removal of viral proteins that block immunological responses, a quick decline in viral load, immune system activation with viral antigens, or any other possible technique for treating chronic HCV infection might theoretically reverse the functional T cell exhaustion (Li et al. 2005; Heim and Thimme 2014; Clausznitzer et al. 2016; Perpiñán et al. 2018). Additionally, the newly developed direct acting antiviral (DAA) agents significantly improved HCV-specific T cell responses after achieving sustained virologic responses (SVR).

T cells and HIV Infection

A pandemic of HIV infection affects more than 36 million people worldwide including 1.1 million US residents (Deeks et al. 2015; Galvani et al. 2018). The characteristic of HIV/AIDS is a progressive loss of $CD4^+$ T cells and host immunity, which together increase vulnerability to opportunistic infections, cancers, and finally mortality (Okoye and Picker 2013; Vijayan et al. 2017). Most patients with chronic HIV infection experience loss of immunological control over virus replication and chronically elevated viral replication. During the initial stages of infection, HIV-specific T cells prevent HIV viral replication. However, HIV-specific T lymphocytes exhaust themselves and lose their capacity to effectively attack infected cells while the viral antigen levels are still high.

While combined antiretroviral therapy (ART) effectively controls virus replication and increases life expectancy in HIV patients, it does not always result in complete $CD4^+$ T cell recovery (Gazzola et al. 2009; Lederman et al. 2011). ART-controlled individuals frequently exhibit both immunologic scarring and residual inflammation. The latter induces an inflammaging phenotype characterized by compromised mitochondrial functions, impaired T cell functions, and blunted vaccine responses (Malaspina et al. 2005; Patterson et al. 2014; Leung et al. 2017; Younes et al. 2018). HIV-induced inflammaging results in profound T senescence,

exhaustion, and dysfunction, which can cause increased viral infections, cancers, cardiovascular diseases, and neurodegenerative disorders, which are similar to what is seen in the elderly (Kirkwood 2005; Kaplan-Lewis et al. 2017). High viral antigen levels, potent pro-inflammatory immune activation, and a weakened T cell homeostasis during HIV infection, all make an important contribution to T cell exhaustion (Douek et al. 2002; Papagno et al. 2004). Therefore, HIV infection under ART management is regarded as a superb model of inflammaging in people, and it is crucial to understand the mechanisms behind T cell aging in patients with ART-controlled, virus-suppressed, latent HIV infection.

Human Topoisomerases in DNA Topological Damage and Repair

To ensure stably storing and transforming valuable genetic information, chromosomal DNA has a unique feature of double-helical structure. This structure is important for DNA replication, transcription, and recommendation. Given the nature of two intertwined DNA strands in chromosomes, almost all types of DNA activities can lead to topological entanglements that must be resolved to ensure genetic code normal transactions and cellular functions (Ye et al. 2010; Luo et al. 2015).

Topoisomerases are ubiquitously expressed enzymes that overcome topological problems in genomic DNA replication, transcription and repair (Watson and Crick 1953; Wang 2002). In order to prevent and correct these topological problems (knots and tangles), topoisomerases bind to and cut the DNA strands, allowing the DNA to be untangled, and after that, the DNA backbone is resealed (Maxwell and Gellert 1986; Deweese et al. 2009; Sissi and Palumbo 2010; Vos et al. 2011). A lack of topoisomerase activity can lead to incomplete replication, excessive supercoiling, persistent catenation, and even DNA knots. Failure to complete this catalytic process results in topoisomerase trapping on the broken DNA termini, generating topoisomerase

cleavage complexes (TOPcc) and PDB, a frequent event that occurs to induce cell apoptosis (Spitzner and Muller 1988; Björkegren and Baranello 2018).

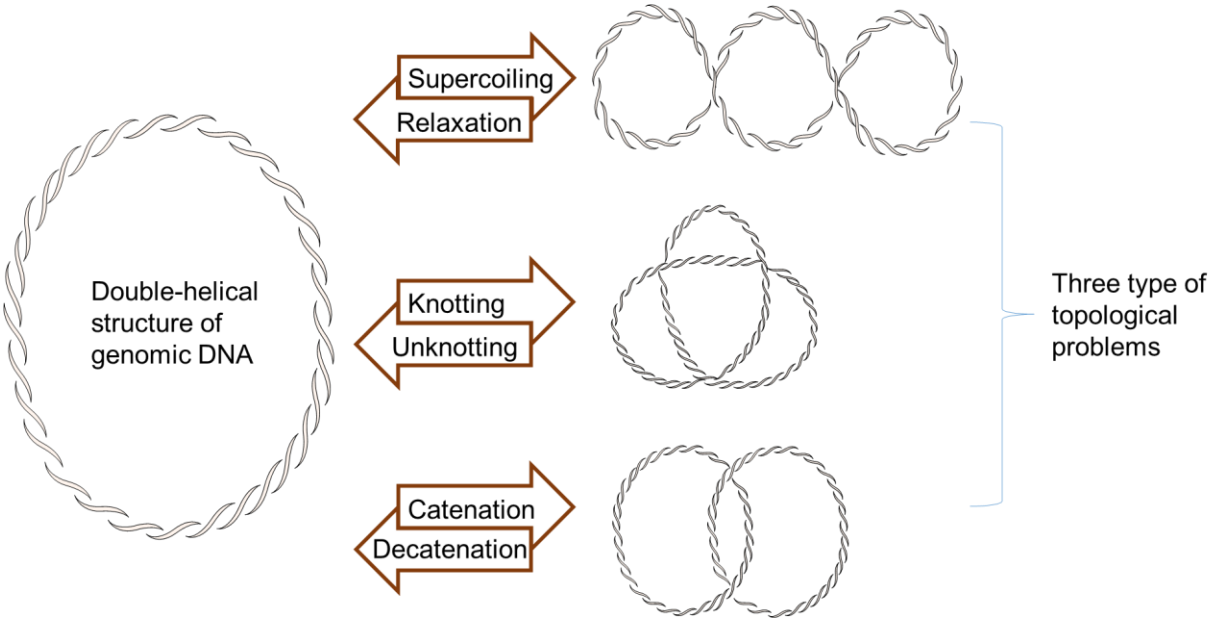

Figure 1.1 Cartoon representing biologically relevant topological structures of DNA. Schematics of the three topological DNA shapes that topoisomerases regulate and maintain are shown. The relative abundance of these species, which may be supercoiled/relaxed, knotted/unknotted, or catenated/decatenated.

Human Topoisomerase Function to Resolve and DNA Topologic Problems

There are three main types of topology: supercoiling, knotting, and catenation (Figure 1.1). Correspondingly, the human genome encodes three types of topoisomerases (type IA, type IB, and type IIA) to resolve such DNA entanglements. Also, there are different isoforms of topoisomerases that function at different locations, such as mitochondrial Top1 (Top1mt) and nuclear Top1 (type IB), Top2α and Top2β (type IIA), and Top3α and Top3β (type IA). These topoisomerases have unique functions and can synchronize each other to fulfill various cellular mechanisms and catalyze DNA activities (Watson and Crick 1953; Maxwell and Gellert 1986; Wang 2002; Deweese et al. 2009; Sissi and Palumbo 2010). Top I was the first topoisomerase

discovered in both prokaryotes and eukaryotes that can relax negative supercoils and catenate and decatenate nicked DNA (Wang 1971; Lima et al. 1994). It transiently cleaves and rejoins one strand of the DNA duplex to release the supercoiling and torsional tension introduced during DNA replication and transcription, and it introduces a single-strand break via transesterification at a target site in duplex DNA. The scissile phosphodiester is attacked by the catalytic tyrosine of the enzyme, resulting in the formation of a DNA-(3'-phosphotyrosyl)-enzyme intermediate and the expulsion of a 5'-OH DNA strand. The free DNA strand then rotates around the intact phosphodiester bond on the opposing strand, thus removing DNA supercoils. Finally, in the re-ligation step, the DNA 5'-OH attacks the covalent intermediate to expel the active-site tyrosine and restore the DNA phosphodiester backbone. Both TOP2α and TOP2β perform DNA decatenation and relax negatively supercoiled DNA, however TOP2α is essential for chromosome segregation while TOP2β is crucial for transcription in differentiated, non-dividing cells. (Wang 1971; Maxwell and Gellert 1986; Spitzner and Muller 1988; Lima et al. 1994; Sissi and Palumbo 2010; Pommier et al. 2016; Björkegren and Baranello 2018). Top3a is a type IA topoisomerase with catalytic activity of ATP-independent breakage of single-stranded DNA, followed by passage and rejoining. It is also required for mtDNA decatenation and segregation after completion of replication (Nicholls et al. 2018). Top3β is an enzyme catalyzes the transient breaking and rejoining of a single strand of DNA which allows the strands to pass through one another, thus relaxing the supercoils and altering the topology of DNA. The enzyme interacts with DNA helicase slow growth suppressor 1 (SGS1) and plays a role in DNA recombination, cellular aging, and maintenance of genome stability.

Notably, while topoisomerase function to resolve the topological entanglements in normal cell DNA replication and transcription, the insertion of viral or bacterial DNA into host

chromosomes also requires the action of topoisomerases. Many drugs, such as broad-spectrum fluoroquinolone antibiotics and chemotherapy drugs, operate through interference with the topoisomerases of bacteria or cancer cells and create PDB in chromosomal DNA that promote cell apoptosis or dysfunction (Roca et al. 1994; Pommier et al. 2010; Vos et al. 2011). Thus, although DNA topology is crucial for normal cell function, its disruption may lead to DNA damage response (DDR) and cell death.

Topological DNA Damage Repair Pathway in Human T cells

Top1 deficiency and Top1cc accumulation results in topological DNA damage. Generally, there are two pathways by which Top1cc can be removed and topological DNA damage be repaired. The first one is excision of Top1cc by Tyrosyl-DNA phosphodiesterase 1 (TDP1) (Murai et al. 2012) , and the another one is DNA cleavage by 3′-flap endonucleases, such as XPF-ERCC1 (Zhang et al. 2011). TDP1 was the first discovered eukaryotic enzyme that removes topoisomerase polypeptides (Yang et al. 1996). It acts as DNA end-cleansing enzyme, excising not only Top1cc but also a variety of 3'-end blocking lesions formed by endogenous or by exogenous DNA damaging agents (Zhou et al. 2009; Pommier et al. 2014; Kawale and Povirk 2018; Brettrager et al. 2019). Human TDP1 can also serve as a backup excision pathway for Top2cc (Murai et al. 2012), albeit much less efficiently than TDP2 (Nitiss et al. 2006; Murai et al. 2012). Meanwhile, XPF–ERCC1 functions as an alternative DNA repair pathway besides the PARP-TDP1 pathway in mammalian cells that repairs Top1cc by cleaving the DNA upstream from the Top1cc and generating "frank breaks" for the XPF–ERCC1-dependent activation of γH2AX (Zhang et al. 2011). Cells carrying SCAN1-TDP1 (TDP1H493R) are particularly trapped on the promoter and regulatory non-coding regions of mtDNA in mitochondria. When mitochondria were targeted specifically by the Top1 poison (mito-SN38) in nanoparticles,

trapped TDP1H493R-mtDNA complexes significantly increased.(El-Khamisy et al. 2007; Ghosh et al. 2019).

Tyrosyl DNA phosphodiesterase (TDP2) was originally discovered as the TRAF and TNF receptor associated protein (TTRAP) promoting MAPK/JNK/p38 signaling as well as inhibiting the activation of Nuclear factor- kappa beta (NF-kB) (Pype et al. 2000). It plays a critical role in cellular resistance to topoisomerase II-induced DNA damage. Human TDP2 is smaller than TDP1 with a molecular mass of 41 kDa (Schellenberg et al. 2012). The DNA repair pathways associated with TDP2 are also different from those of TDP1. To expose the DNA-5'-phosphotyrosyl bond, the cleavage complex must be denatured or proteolyzed prior to TDP2 activity. TDP2 separates the TOP2 polypeptide from the 5'-phosphate end. Direct ligation after annealing the two ends with the 4-base pair stagger, or double-strand break (DSB) repair mechanisms, can be utilized to repair the DNA break. (Shi et al. 2012).

Ordinarily, topological DNA entanglement is able to be excision by TDP, which require for inhibition of a DNA damage sensor called PARP1. However, increasing of PARP1 and decreasing of TDP protein were observed during topoisomerase inhibition, indicating that T cells fail to resolve such DNA repair and thus DDR is aroused, such as activation of the Ataxia telangiectasia mutated (ATM)/Checkpoint kinase 2 (CHK2) or Ataxia telangiectasia Rad3-related (ATR)/ Checkpoint kinase 1 (CHK1) pathways. In persistent DNA damage and repair process, ATM/CHK2 are depleted and deficient, which are observed in T cells repeatedly treated with Top1 or Top2α inhibitors or chronically infected with HBV, HCV, and HIV infection.

Consequences of Topological DNA Damage Induced By Topoisomerase Deficiency in Human T Cells

In studying the mechanisms of the DNA damage and repair signaling pathways involved in T cell dysregulation, our team found that both Top1 and Top2α are deficient/suppressed, at the post-transcriptional levels, and their enzymatic activities are also dampened, accompanied by the accumulation of TOPcc, DNA damage, and cell apoptosis during chronic HBV, HCV, and HIV infection. A number of consequences can be aroused by topoisomerase deficiency during chronic viral infection (Figure 1.2).

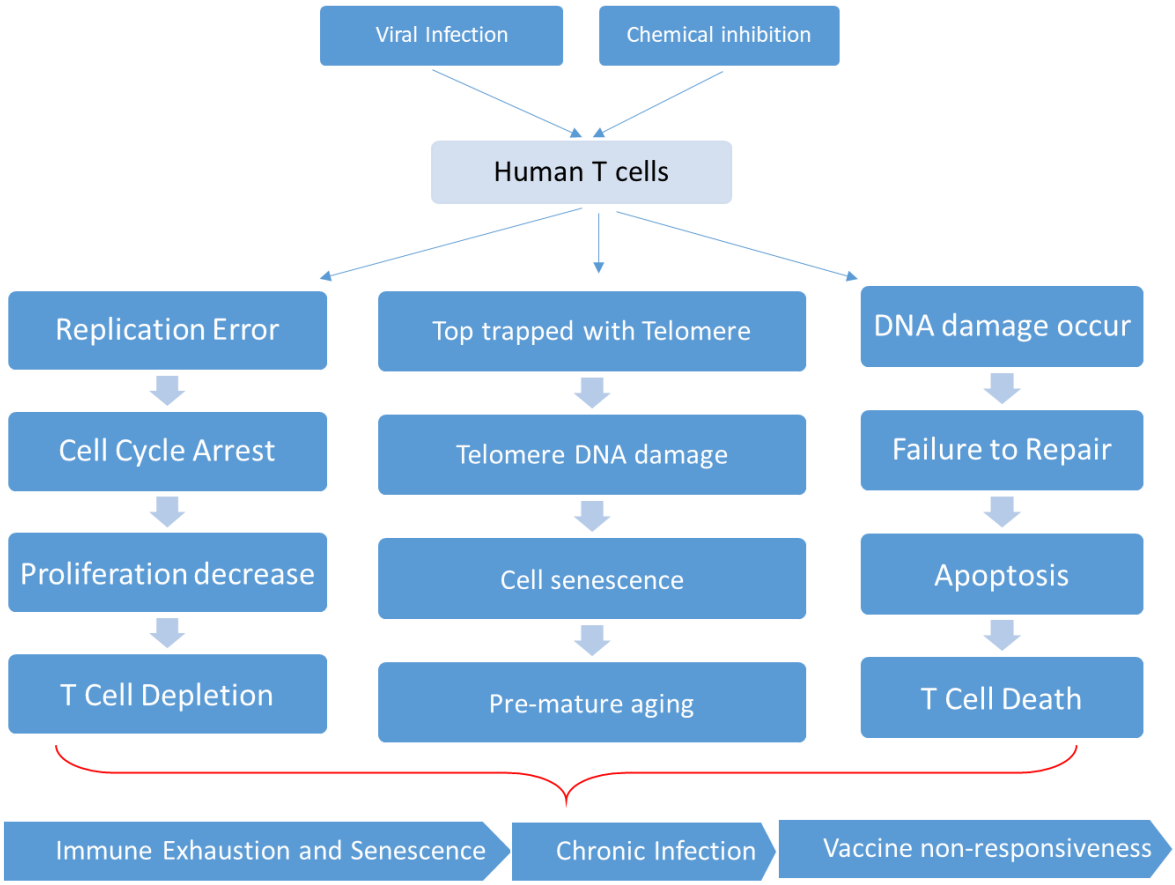

Figure 1.2 Consequences occurred in T cells induced by chemical inhibition or chronic viral infection. The image above illustrates three distinct effects that topological issues during viral infection might have on T cells. T cells cannot complete the entire cell cycle during mitosis when topological problems occur and cannot be fixed in a timely manner due to DNA replication errors. Then it may result in a general decrease in T cell proliferation, which would deplete the population of T cells. If a double-strand break (DSB) occurs near a telomere, it may cause

telomere DNA damage, which accelerates cell aging and senescence. Additionally, if the damage is not repaired, the cell may initiate the apoptosis program, which results in immediate cell death. Furthermore, it may result in immune exhaustion, immune senescence and vaccine non-responsiveness in this particular situation after a prolonged illness.

Cell Cycle Arrest

Cell proliferation is a vital indicator for understanding the mechanisms in action of certain genes, proteins and pathways involved in cell survival or death following pathogenic infections or exposing to toxic agents. Notably, Top2α depleted cells have a decreased cell proliferative capacity compared to healthy normal T cells, as it allows the cells bypassing the de-catenation checkpoint and proceed through mitosis without fully segregating their chromosomes (Adhikari et al. 2012; Schellenberg et al. 2012; Shi et al. 2012). Chemical inhibition of type II topoisomerase by ICRF-193 or the expression of a catalytically compromised type II topoisomerases leads to cell cycle arrest at the G2/M transition and the formation of chromosomal aberrations (Downes et al. 1994; Giménez-Abián et al. 2000). Even though there is no direct evidence for type I topoisomerases relate to cell cycle, a significantly inhibited T cell proliferation was observed in human T cells exposed to Camptothecin (CPT, a Top1 inhibitor) compared to control (Ji et al. 2019), suggesting that Top1 also plays an important role in DNA activities during cell proliferation.

Cell Senescence

As a telomere shelterin protein, TRF2 functions to protect telomeres from unwanted DNA damage, and its level was significantly inhibited during topoisomerase suppression (De Lange 2005; Cao et al. 2019; Ji et al. 2019). Our previous study shows that inhibition of TRF2 promotes telomere attrition and telomeric DNA damage that accelerates T-cell senescence and apoptosis (Nguyen et al. 2018; Nguyen et al. 2021). Consistently, in the topological DNA

damage studies, we demonstrate that topoisomerase inhibition can cause telomere loss via negatively regulating TRF2 and eventually induce T cell senescence. While our confocal data indicate Top2α localization on telomeres, whether TRF2 can directly or indirectly associated with Top2α and recruit it to telomeres remain an unknown question and warrant further investigation.

Apoptosis

We found that topoisomerase inhibition and TOPcc accumulation not only lead to telomere erosion via suppression of TRF2 and telomerase activity, but also involve in dynamic activation and depletion of ATM, an important DNA repair kinase (Ji et al. 2019; Dang et al. 2020). Apoptosis is a form of programmed cell death, which can be initiated by two pathways: the intrinsic and extrinsic pathways. The extrinsic pathway is activated by the engagement of transmembrane receptors, such as interactions between the Fas-Fas ligand, TNFα-TNF receptor, and TRAIL-TRAIL receptor. The intrinsic apoptosis induces mitochondrial outer membrane permeabilization (MOMP) and results in the mitochondrial DNA leaking into the cytosol through Bax/Bak channel. Since mitochondria DNA leaking is also a main cause triggering cell apoptosis, whether topological DNA damage is also occurring in mtDNA and induces activation of the Cyclic GMP-AMP synthase (cGAS)-Stimulator of interferon genes (STING) pathway still remain unclear. Caspase protein family is an essential component of both intrinsic and extrinsic apoptotic pathway. Our data show caspases 3 is significantly increased during topoisomerase inhibition (Ji et al. 2019; Dang et al. 2020), whether other caspases are directly regulated by topoisomerase inhibitions remains unknown.

Specific Aim

This study's overall aim is to clarify the processes through which chronic viral infection generates topological DNA damage that may result in T cell malfunction. We will use a sizable cohort of samples with chronic HBC, HCV, or HIV infection to research immune exhaustion and T cell dysregulation in humans, as these people have been recognized as excellent models.

Due to immune exhaustion and immune senescence, vaccine failure occurs more commonly in patients with chronic viral infections. Failure of the vaccine is significantly influenced by premature aging and dysfunction of T cells. Nearly all DNA activities can result in topological entanglements that need to be untangled in order to maintain regular genetic information exchange and cellular operations. Topoisomerases are a series of proteins that catalyze DNA processes like gene replication, transcription, and recombination by supercoiling, knotting, and catenating DNA. These functions complement one another to carry out numerous biological procedures.

We have recently concentrated on discovering biomarkers for T cell aging in HBV-, HCV- or HIV-infected persons to investigate the processes of T cell failure in virally infected individuals. When compared to age-matched HS, we discovered that people with HCV or HIV exhibit a severe impairment in CD4 T cell responses along with the overexpression of aging and exhaustion markers such as programmed death 1 (PD-1), T cell immunoglobulin domain 3 (Tim-3), killer cell lectin-like receptor subfamily G 1 (KLRG-1), and dual specific phosphatase 6 (DUSP-6). These findings imply that significant, virus-mediated, premature T cell aging may be the cause of T cell dysfunction mediated vaccine failure during chronic viral infection. Our long term goal is to elucidate the fundamental mechanisms underlying the activated/exhausted

immune phenotypes observed during chronic viral infection and to facilitate an immune response following vaccination in chronic virally infected patients by reversing T cell senescence and dysfunction.

In this study, we hypothesize that i) failure to repair telomeric DNA damage induced by suppression of Top2α and TDP2 deficiency triggers the accumulation of Top2cc, resulting in human T cell premature and senescence during chronic viral infection; and ii) Mitochondrial DNA damage induced by top1mt suppression plays a pivotal role in T cell dysfunction and apoptosis in HCV/HIV-infected subjects, and as such, restoring this DNA repair system may provide new opportunities for immunological competence maintenance and anti-aging protection of T cells. To test this hypothesis, we propose the following two independent but interrelated aims:

Aim 1. Determine the role of Top2α in telomeric DNA damage and T cell dysfunction in chronic HBV/HCV or HIV infection.

A) Evaluate the role of Top2α in regulating DNA damage repair and maintaining T cell functions during HBV/HCV or HIV infection.

B) Compare T cell homeostasis, senescence, and DNA damage in HS and Top2α inhibited samples.

The objectives of this aim are to determine the relationship of Top2α in preserving DNA integrity and T cell function during HBV, HCV, and HIV infection

Aim 2. Investigate the role of Top1mt in mitochondrial DNA damage and T cell dysfunction during chronic viral infection.

A) Explore the role of Top1mt in mtDNA damage and maintaining T cell functions in chronic viral infected subjects.

B) Characterize mitochondrial homeostasis and apoptosis in T cells and their relationships with mtDNA damage in Top1mt depletion subjects.

The objectives of these studies are to evaluate the role of Top1mt in regulating mtDNA damage repair and T cell exhaustion during HCV or HIV infection.

These translational studies are significant and timely because they will provide a working model for investigating mechanisms that may be fundamental to diminishing immune responses seen in a variety of chronic infectious diseases, including but not limited to HCV and HIV infection. For the purpose of creating strategies to enhance immune responses in the context of immunocompromised situations, understanding such processes is essential.

CHAPTER 2. INHIBITION OF TOPOISOMERASE IIA (TOP2A) INDUCES TELOMERIC DNA DAMAGE AND T CELL DYSFUNCTION DURING CHRONIC VIRAL INFECTION

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Keywords: DNA damage, HBV, HCV, HIV, T cell dysregulation, telomere erosion, topoisomerase IIA

Running title: Top2α and T cell dysfunction in viral infection

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Abstract

T cells play a critical role in controlling viral infection, however, the mechanisms regulating their responses remain incompletely understood. Here, we investigated the role of topoisomerase IIA (Top2α, an enzyme that is essential in resolving entangled DNA strands during replication) in telomeric DNA damage and T cell dysfunction during viral infection. We demonstrated that T cells derived from patients with chronic viral (HBV, HCV, and HIV) infection had lower Top2α protein levels and enzymatic activity, along with an accumulation of the Top2α cleavage complex (Top2cc) in genomic DNA. In addition, T cells from virally infected subjects with lower Top2α levels were vulnerable to Top2α inhibitor-induced cell apoptosis, indicating an important role for Top2α in preventing DNA topological disruption and cell death. Using Top2α inhibitor (ICRF193 or Etoposide)-treated primary T cells as a model, we demonstrated that disrupting the DNA topology promoted DNA damage and T cell apoptosis via Top2cc accumulation that is associated with protein-DNA breaks (PDB) at genomic DNA. Disruption of the DNA topology was likely due to diminished expression of tyrosyl-DNA phosphodiesterase 2 (TDP2), which was inhibited in T cells *in vitro* by Top2α inhibitor and *in vivo* by chronic viral infection. These results suggest that immune-evasive viruses (HBV, HCV, and HIV) can disrupt T cell DNA topology as a mechanism of dysregulating host immunity and establishing chronic infection. Thus, restoring the DNA topologic machinery may serve as a novel strategy to protect T cells from unwanted DNA damage and to maintain immune competence.

Introduction

T cells play a critical role in control of viral infection. In studying the role of T cell dysregulation in viral persistence in humans, we and others have previously shown that chronic

viral infections can cause premature T cell aging and immune senescence, as evidenced by the expression of aging markers and particularly, accumulation of DNA damage[1-18]. However, the underlying mechanisms remain unclear.

Given the nature of two intertwined DNA strands in chromosomes, almost all types of DNA activities, including gene replication, transcription, and recombination, can lead to topological entanglements that must be resolved to ensure genetic code normal transactions and cellular functions[19-21]. In order to prevent and correct these topological problems, topoisomerases bind to and cut the DNA strands, allowing the DNA to be untangled, and after that, the DNA backbone is resealed. Failure to complete this catalytic process results in topoisomerase trapping on the DNA termini, forming topoisomerase cleavage complex (TOPcc), and generating protein-linked DNA breaks (PDB), a frequent event that occurs to induce cell apoptosis[22, 23]. There are three main types of topology: supercoiling, knotting, and catenation. Correspondingly, the human genome encodes three types of topoisomerases (type IA, type IB, and type IIA) to resolve such DNA entanglements. Notably, the insertion of viral or bacterial DNA into host chromosomes also requires the action of topoisomerases. Many drugs, such as broad-spectrum fluoroquinolone antibiotics and chemotherapy drugs, operate through interference with the topoisomerases of bacteria or cancer cells and create PDB in chromosomal DNA that promote cell apoptosis or dysfunction[24-26]. Thus, although DNA topology is crucial for normal cell functions, its disruption may lead to DNA damage response (DDR) and cell death.

While inhibition of topoisomerases has been widely exploited to kill bacteria and cancer cells[24, 25], the role and mechanisms of topoisomerase in reprogramming DDR and altering the function of T lymphocytes, especially in the context of chronic viral infection, remain largely unknown. We have recently shown that topoisomerase I (Top1) is inhibited and causes topological

DNA damage and T cell senescence during chronic viral infections[9]. Here we further demonstrate that Topoisomerase IIA (Top2α) is significantly inhibited and play a critical role in reprogramming DDR and remodeling T cell function or apoptosis during chronic viral infections.

Results

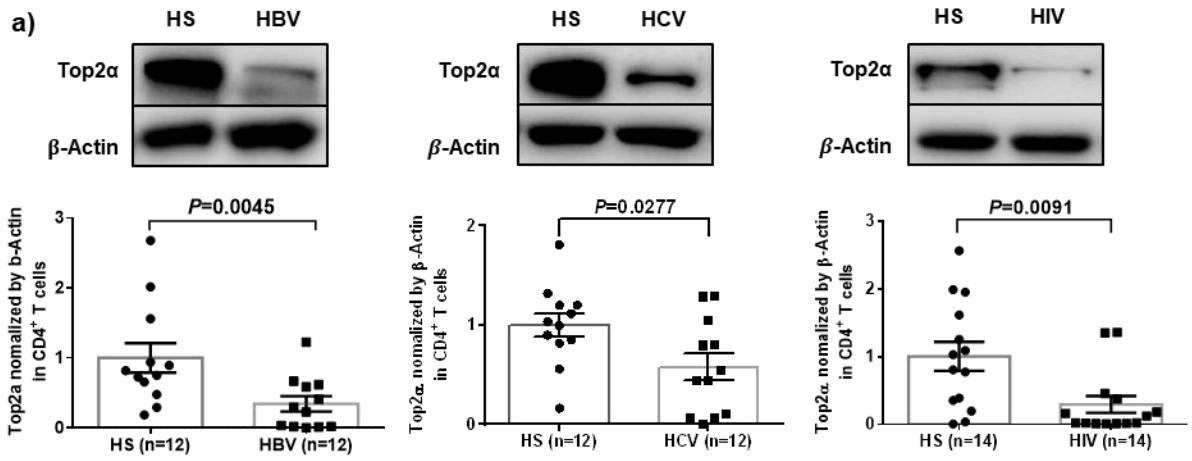
Top2α Expression and Activity Are Inhibited In CD4 T Cells during Chronic Viral Infections

Top2α is critical in unraveling the entangled DNA to prevent unwanted DNA damage and cell death[22]. As an initial approach to explore the role of Top2α in DNA damage and T cell apoptosis, we examined the levels of Top2α in CD4 T cells derived from individuals with chronic viral (HCV, HBV, HIV) infections. Since Top2a is only expressed in activated T cells, we examined Top2a expression in purified CD4 T cells stimulated with anti-CD3/CD28 for 3 days, followed by western blotting. As shown in **Fig.2.1a**, chronically HBV, HCV, or HIV-infected individuals exhibited a lower level of Top2α expression in CD4 T cells compared to age-matched healthy subjects (HS). To determine whether Top2α inhibition occurs at the transcriptional or post-transcriptional level, we measured Top2α mRNA by RT-PCR in CD4 T cells derived from the same subjects. As shown in **Fig.2.1b**, the mRNA levels of Top2α in CD4 T cells isolated from virus-infected patients remained unchanged compared to HS, indicating that Top2α inhibition occurs primarily at the post-transcriptional level during viral infection.

In addition to the Top2α expression, we employed a kDNA-based Top II Assay to measure Top2α activity. We found that the catenated kDNA was extremely large and could not migrate through a 1% agarose gel without being relaxed. In contrast, the monomeric DNA (2.5 kb) rapidly migrated through the gel as nicked open circular or fully closed circular rings. After optimizing concentrations of nuclear extracts, we compared Top2α activity using nuclear extracts from CD4

T cells isolated from virally infected subjects and HS. As shown in **Fig.2.1c** left panel, the linear kDNA (lane 1) and Top2α-relaxed kDNA (lane 2) served as a positive control and the kDNA treated with nuclear extracts of non-stimulated HS T cells served as a negative control (lane 3). The kDNA treated with the optimal concentration of nuclear extracts from T cell receptor (TCR)-stimulated T cells showed varying amounts of decatenated, closed circular DNA, and virally infected patients' samples (lane 5-7) had lower efficiency in relaxing kDNA compared to HS (lane 4). The relative Top2α activity in CD4 T cells derived from HBV, HCV, and HIV-infected individuals (normalized to HS) was summarized and is shown in **Fig.2.1c** right panel and shows that Top2α activity is inhibited in T cells during chronic viral infection.

To determine if Top2α inhibition results in Top2cc accumulation and entrapment in T cell chromosomes, we measured chromatin-associated Top2cc from CD4 T cells of virally infected patients by immunoblotting with a monoclonal antibody that specifically recognizes covalent Top2 α -DNA complexes. As shown in **Fig.2.1d**, an increased amount of Top2cc was detected in the genomic DNA of CD4 T cells isolated from HBV, HCV, and HIV-infected patients compared to HS. These results suggest that T cells from virus-infected patients have DNA topological problems, i.e., Top2α inhibition and Top2cc accumulation.



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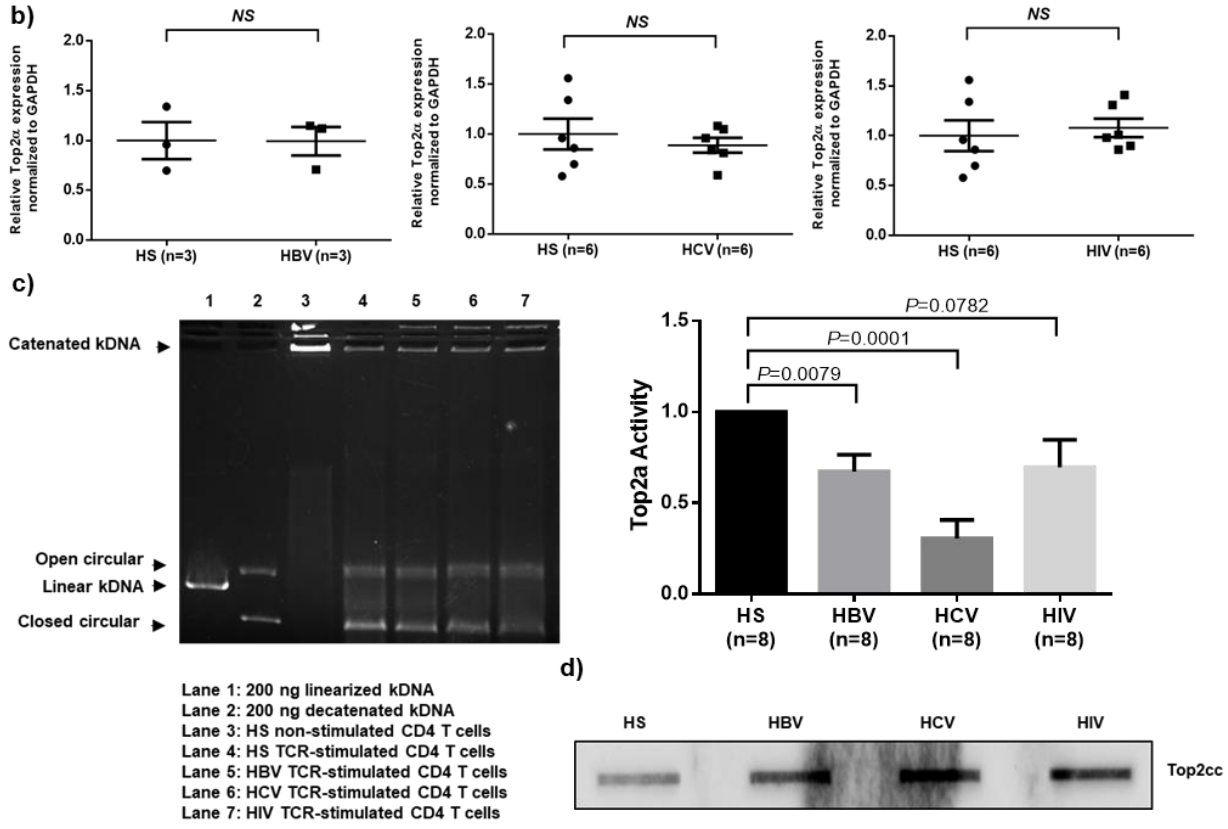


Fig.2.1. Inhibition of Top2α expression and activity in CD4 T cells during chronic viral infections. a) Top2α protein expression in CD4 T cells isolated from HBV, HCV, and HIV-infected individuals and healthy subjects (HS). Representative images and summary data of western blot are shown. The Top2α band intensity (lower panel) was normalized to β-actin and is presented relative to HS (n = number of subjects). b) Top2α mRNA levels, determined by RT-qPCR, in CD4 T cells isolated from virally infected individuals and HS. c) Top2α activity in CD4 T cells isolated from HBV, HCV, and HIV-infected individuals and HS. Representative images and summary data of Top2α-mediated digestion of kDNA substrate (normalized to TCRstimulated cells from HS) are shown (n = number of subjects tested). d) Top2cc was detected in genomic DNA isolated from CD4 T cells of virus-infected patients versus HS.

Top2α Inhibition Leads to T cell Apoptosis or Dysfunction

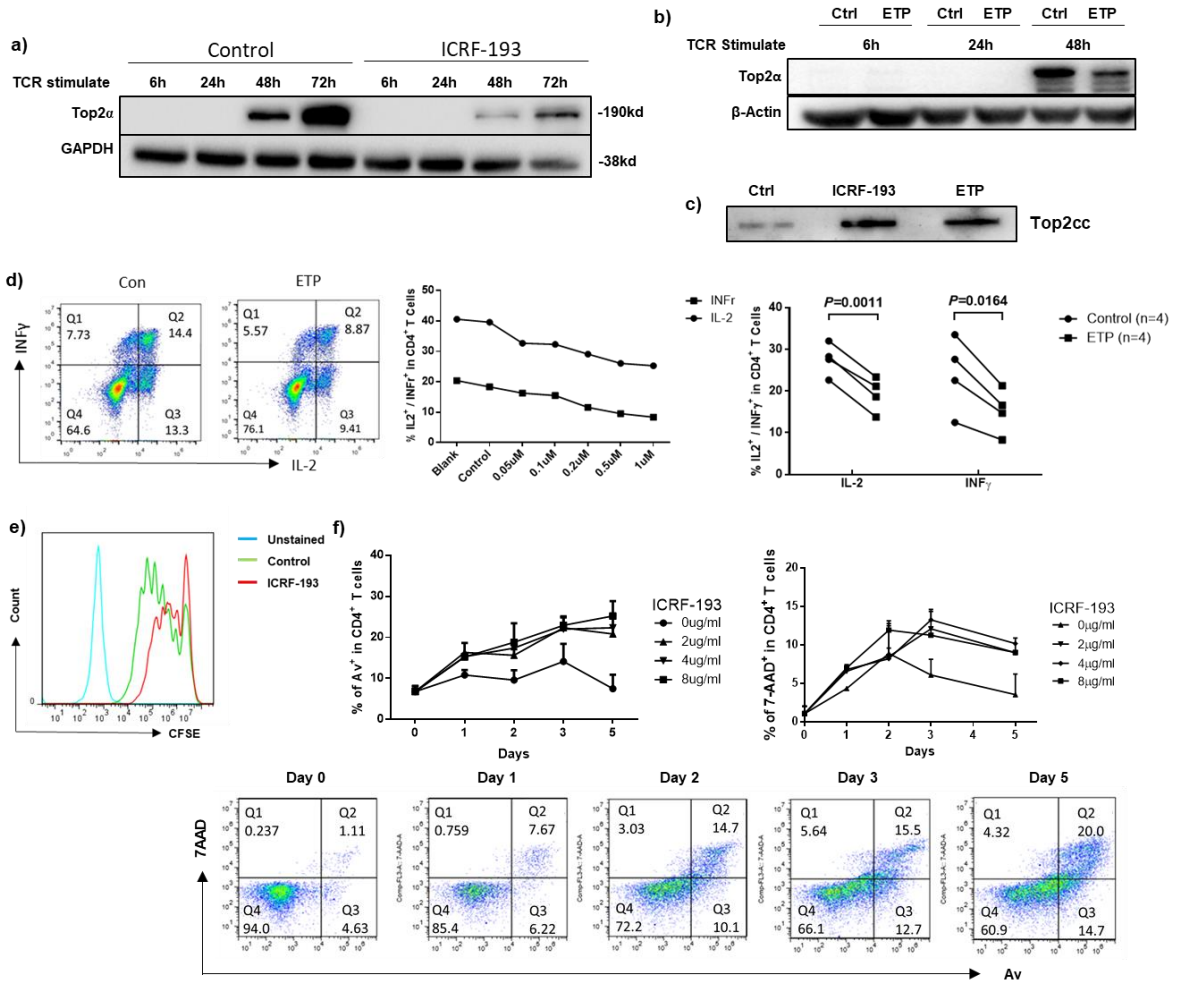
To determine the consequence of Top2α inhibition, we employed two distinct Top2α-targeting compounds in this study: Top2α poison Etoposide (ETP), which binds to and stabilizes Top2cc to prevent DNA re-ligation and generate PDB; and Top2α catalytic inhibitor ICRF193,

which inhibits ATP hydrolysis after strand passage and re-ligation and before the closed clamp conformation reopens[25-30]. Here, we employed Top2α inhibitor-treated primary CD4 T cells as a model to study the role and mechanisms of Top2α in T cell dysregulation. We first assessed the Top2α level in T cells treated with Top2α inhibitors. As shown in **Fig.2.2a-b**, Top2α protein was not detectable in resting primary CD4 T cells but was detected at 48 h after T cell stimulation. TCR-stimulated CD4 T cells showed Top2α inhibition by ICRF or ETP when compared to the DMSO-treated control. Also, Top2α inhibitor-treated CD4 T cells exhibited considerable Top2cc accumulation in their genomic DNA (**Fig.2.2c**). These results indicate that Top2α inhibition and Top2cc accretion in CD4 T cells treated with ICRF and ETP recapitulate our findings in CD4 T cells derived from virus-infected individuals.

We next measured the effect of Top2α inhibition on cellular functions. To this end, CD4 T cells were isolated from HS and exposed to various concentrations (0, 0.05, 0.1, 0.2, 0.5, 1 μM) of ETP in the presence of TCR stimulation for 3 days, followed by measuring intracellular IL-2 and IFN-γ production by flow cytometry. As shown in **Fig.2.2d**, IL-2 and IFN-γ productions were inhibited in a dose-dependent manner in CD4 T cells exposed to ETP. Also, a significant inhibition of T cell proliferation, determined by CFSE dilution in CD4 T cells, was observed in T cells exposed to ICRF compared to control with TCR stimulation (**Fig.2.2e**). In addition, we measured apoptosis of CD4 T cells exposed to various doses (0, 2, 4, or 8 μg/ml) of ICRF for different times (0, 1, 2, 3, or 5 days) in the presence of anti-CD3/CD28 (1 μg/ml) by flow cytometry analysis. As shown in **Fig.2.2f**, ICRF-treated T cells exhibited time-dependent increases in Annexin V (Av) and 7-Aminoactinomycin D (7AAD) staining compared to the DMSO-treated control.

Since T cells from virally infected patients exhibited lower levels of Top2α and enzymatic activity, we hypothesized that these cells are more vulnerable to Top2α inhibitor-mediated cell

apoptosis. We thus compared the apoptotic susceptibility of CD4 T cells derived from virus-infected patients and age-matched HS following ICRF treatment. Indeed, CD4 T cells isolated from HBV, HCV, and HIV-infected patients exhibited higher rates of cell apoptosis (Av^+) and death ($7\text{-}AAD^+$) upon exposure to a Top2α inhibitor compared to cells from HS (**Fig.2.2g**). Taken together, these results suggest that Top2α inhibition can cause T cell apoptosis or dysfunction, highlighting the role of Top2α in securing T cell survival and function, and providing a robust model to study topological DNA damage in human T cell dysregulation.



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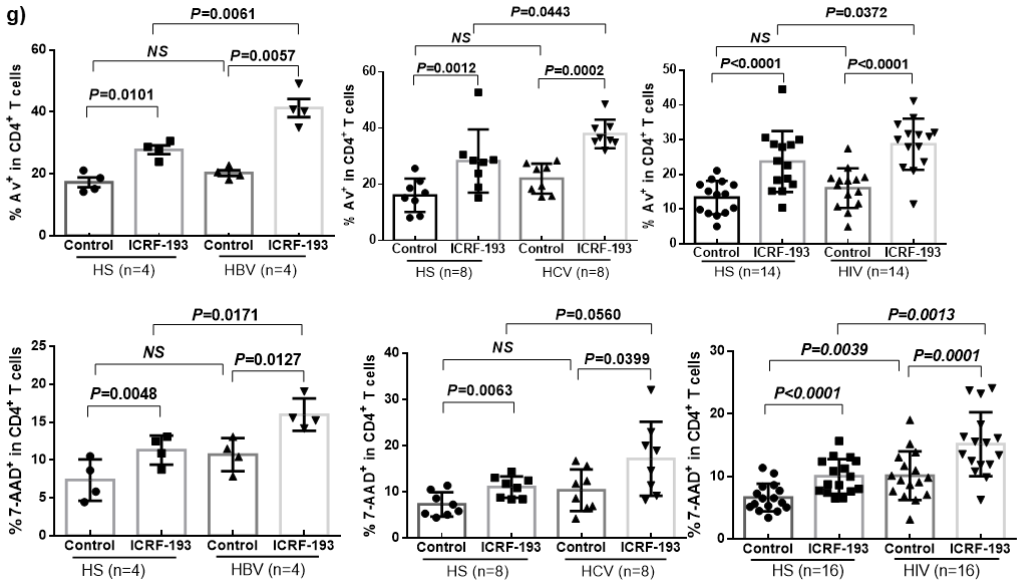


Fig.2.2. Top2α inhibition induces CD4 T cell apoptosis and dysfunction. a) Top2α levels in CD4 T cells treated with DMSO (con) or Top2α inhibitor (ICRF-193) in the presence of anti-CD3/CD28 stimulation for 6, 24, 48, and 72 h, were determined by immunoblotting. b) Top2α protein levels in CD4 T cells that were treated with DMSO (Cont) or Top2α inhibitor (ETP) with TCR (anti-CD3/CD28) stimulation for 6, 24, and 48h. c) Top2cc levels in genomic DNA from CD4 T cells that were exposed to DMSO control or Top2α inhibitor (ICRF orETP) for 72 h, were measured by immunoblotting. d) Representative dot plots and summary data of IL-2 and IFN-γ expression in TCR-stimulated CD4 T cells that were exposed to ETP or DMSO control. e) T cell proliferation, measured by CFSE dilution, in TCR-stimulated CD4 T cells in the presence of ICRF and DMSO control for 5 d. f) Av and 7-AAD staining of CD4 T cells that were treated with various concentrations of ICRF-193 for 1, 2, 3, and 5 days, determined by flow cytometry. g) The vulnerability of CD4 T cells that were derived from viral infected individuals and HS to the ICRF-193-induced apoptosis, were determined by flow cytometry.

Top2α Inhibition Induces Cell Apoptosis by Enhancing Topological DNA Damage That Extends to Telomeres

To determine if topological DNA damage is a major cause of T cell apoptosis, we treated primary CD4 T cells with ICRF-193 or DMSO in the presence of TCR stimulation for 3 days, followed by measuring the phosphorylated H2AX (γH2AX), a marker for DNA damage, as well as active caspase-3, a marker for cellular apoptosis, by flow cytometry and immunoblotting. As shown in **Fig.2.3a-b**, increased levels of γH2AX and caspase-3 were observed in ICRF-193-treated

CD4 T cells, indicating an increased apoptosis-associated topological DNA damage in T cells with Top2α inhibition.

Since telomeres play an important role in preserving chromosome integrity and cell longevity[31-34], we asked whether Top2α can also function at telomeres to maintain telomere integrity in proliferating T cells. To test this, we examined Top2α expression in primary CD4 T cells with or without TCR stimulation by microscopy. As shown in **Fig.2.3c**, the expression of Top2α was not detected in resting primary CD4 T cells without TCR stimulation. Similar to the immunoblot results (**Fig.2.2a**), after stimulation with anti-CD3/CD28 for 3 days, Top2α was detected in activated T cells, as shown by positive Top2α immunofluorescent staining in the nuclei of larger, activated T cells upon comparing the same bright-field imaging. Notably, confocal imaging from immunofluorescence-florescence in-situ hybridization (IF-FISH) showed that Top2α co-localized with a telomere probe (Tel-C-TAACCC) (**Fig.2.3d**), suggesting a role for Top2α in maintaining telomere integrity.

Human telomeres consist of triple guanine repeats (TTAGGG) that are sensitive to DNA damage[35, 36]. We hypothesized that Top2α inhibition-mediated genomic DNA damage may affect telomeres as we have previously shown for telomere erosion in T cells derived from virus-infected subjects[5-9]. To determine telomeric DNA damage in Top2 inhibitor-treated T cells, we measured the number of dysfunctional telomere-induced foci (TIF, a hallmark of telomeric DNA damage[37, 38]) by examining the co-localization of 53BP1/TRF1 (p53-binding protein 1/telomeric repeat-binding factor 1) using confocal microscopy. As shown in **Fig.2.3e**, the number of TIFs per nucleus was significantly higher in CD4 T cells exposed to ICRF or ETP compared to vehicle control. These results suggest that Top2α inhibition causes telomeric DNA damage and cell apoptosis.

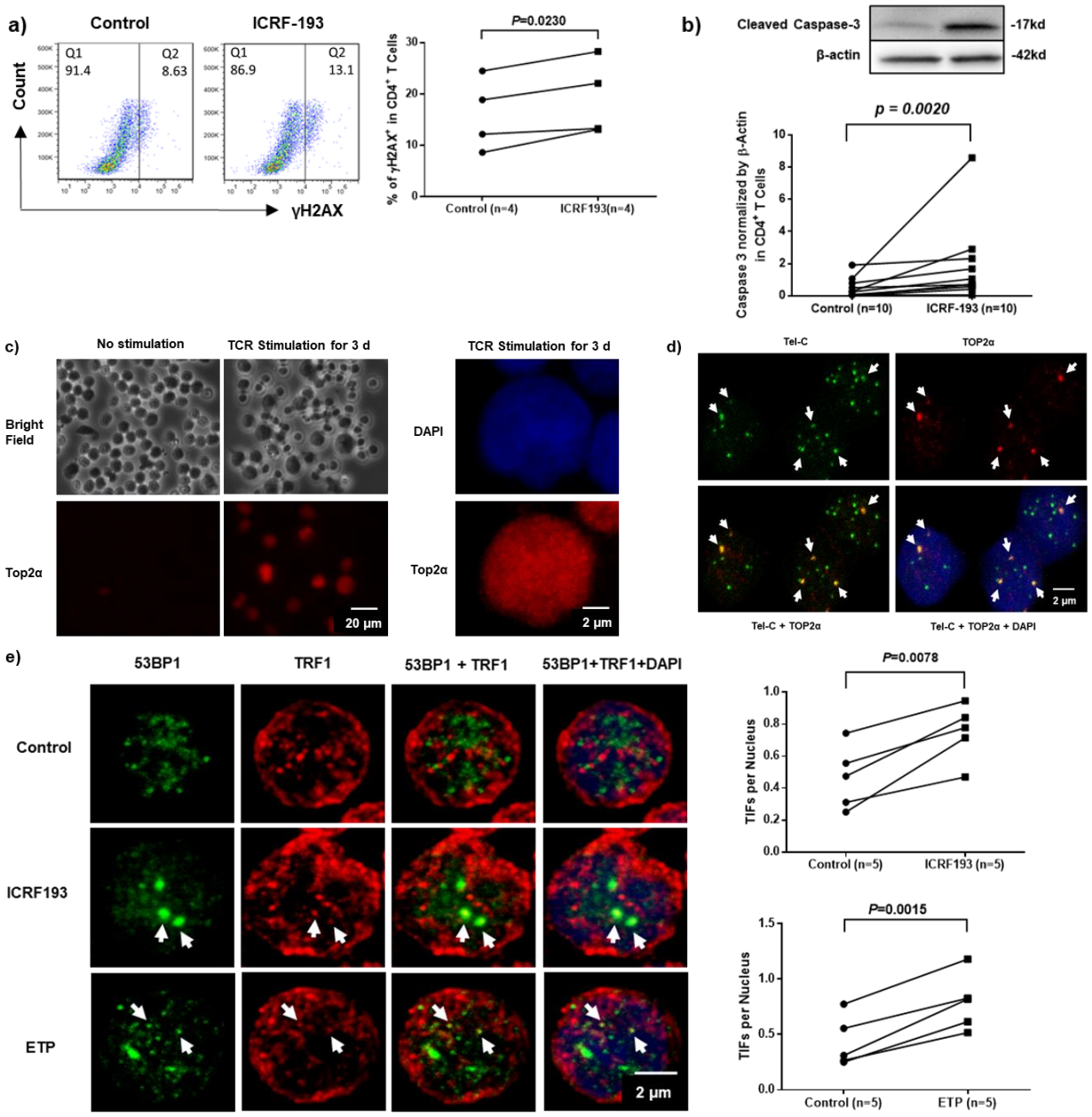


Fig.2.3. Induction of telomeric DNA damage in CD4 T cells that were treated with Top2α inhibitors. a) γH2AX levels in TCR-stimulated CD4 T cells treated with ICRF or DMSO control for 3 days, measured by flow cytometry. b) Active caspase-3 levels in TCR-stimulated CD4 T cells exposed to ICRF or DMSO control for 3 days, measured by immunoblotting. c) Top2α expression in TCR-activated CD4 T cells, determined by confocal microscopy. d) Co-localization of Top2α immunostaining with telomere probe signals in CD4 T cells, determined by confocal microscopy with white/dark and fluorescent fields. e) Representative images and summary data of the dysfunctional telomere-induced foci (TIFs) per nucleus in TCR-stimulated CD4 T cells exposed to ICRF, ETP, or DMSO control for 3 days, determined by confocal microscopy (n=number of subjects).

Top2α Inhibition Leads to Telomere Erosion via Suppression of Shelterin TRF2 and Telomerase Activity

To determine whether Top2α inhibitor-treated T cells mirror the telomere loss seen in patients with viral infections, we measured the telomere length in ICRF-treated T cells by Flow-FISH. T cells treated with ICRF-193 in the presence of TCR stimulation for 5 days showed significantly shorter telomeres compared to the control cells (**Fig.2.4a**).

Telomeres are protected by shelterin proteins[39, 40]. To explore the mechanisms of telomere erosion, we examined the integrity of the telomeric shelterin complex in T cells exposed to the Top2α inhibitor ICRF-193. As shown in **Fig.2.4b**, among the shelterin proteins examined, only telomeric repeat-binding factor 2 (TRF2) was significantly inhibited. TRF1 was also slightly down-regulated, but TRF1-interacting nuclear protein 2 (TIN2) was slightly up-regulated, while telomere protection protein 1 (TPP1), repressor/activator protein 1 (RAP1), and protection of telomere 1 (POT1) remained unchanged. These findings truly recapitulate the results we observed in CD4 T cells derived from HCV and/or HIV-infected patients[6, 7]. Since the primary functions of TRF2 are to protect telomeres from unwanted DNA damage and to recruit telomerase to telomeres, its inhibition may lead to telomere uncapping or deprotection as well as telomerase deprivation from telomeres.

Telomeres are replenished by telomerase[41-43]. To determine the role of telomerase in Top2α-mediated telomere inhibition, we measured the expression of human telomerase reverse transcriptase (hTERT, the catalytic subunit of telomerase) by RT-PCR and telomerase activities by a TRAP assay in ICRF-193-treated CD4 T cells. As shown in **Fig.2.4c**, ICRF treatment did not change hTERT expression, but remarkably inhibited telomerase activity, which again recapitulated our findings in T cells from patients with HCV or HIV infection.

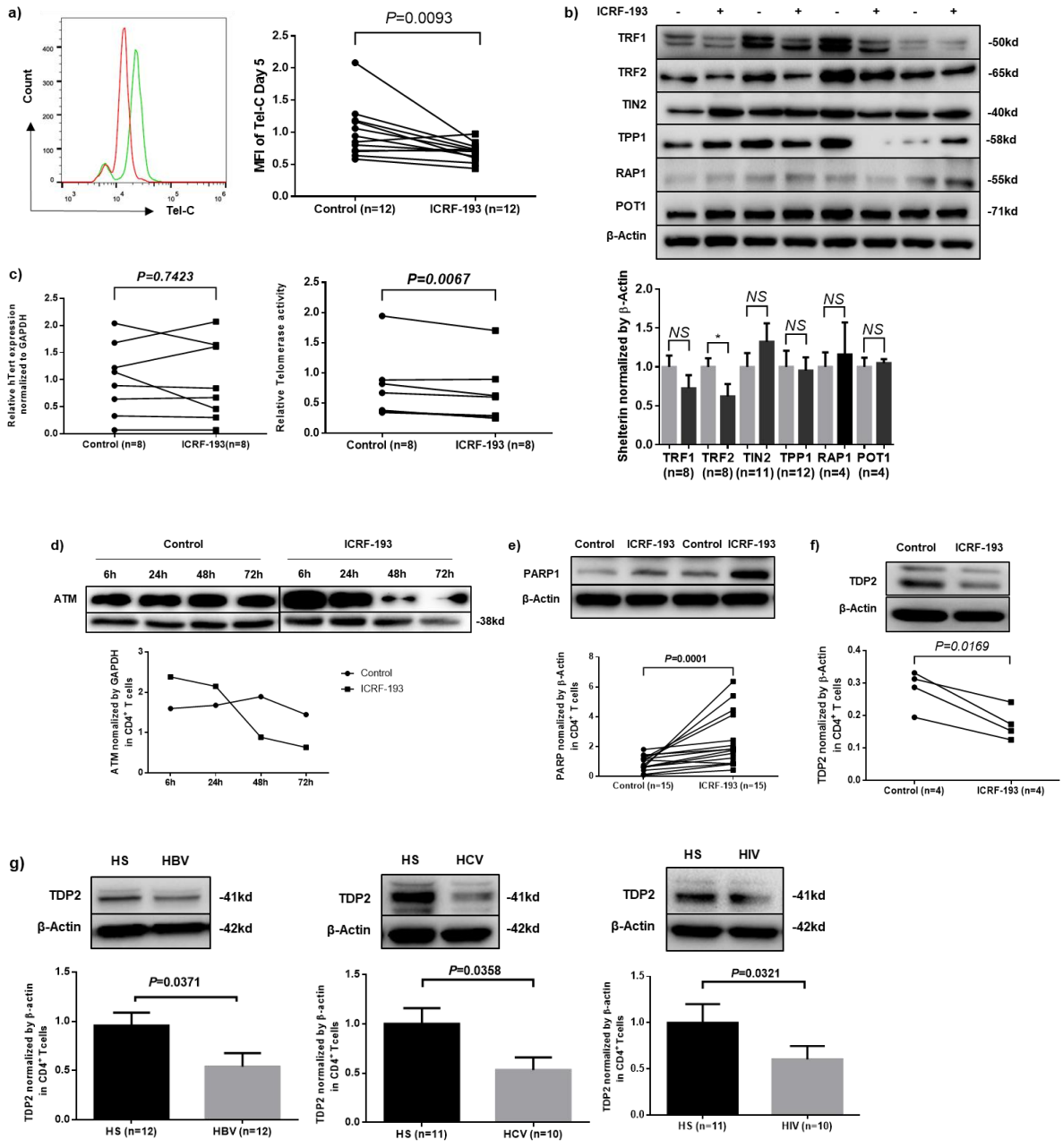


Fig.2.4. Top2α inhibition induces telomere erosion via suppressing shelterin proteins, telomerase activity, ATM, and TDP2. a) Telomere length (measured by Flow-FISH) in CD4 T cells treated with ICRF-193 or DMSO control for 5 days. b) Shelterin proteins (measured by immunoblotting) in CD4 T cells treated with or without ICRF-193. c) hTERT expression (measured by RT-qPCR) and telomerase activity (measured by TRAP assay) in CD4 T cells treated with or without ICRF. d) ATM expression (measured by immunoblotting) in TCR-stimulated CD4 T cells exposed to ICRF, or DMSO control for 6, 24, 48, and 72 h. e) TDP2 expression in TCR-activated CD4 T cells treated with ICRF or DMSO for 72 h. f) Cleaved PARP1 levels in TCR-activated CD4 T cells exposed to ICRF or DMSO for 72 h. g) TDP2

expressions in CD4 T cells isolated from HS and chronically HBV-, HCV, or HIV-infected individuals.

Top2α-Mediated Topological DDR Involves Dynamic Activation and Depletion of DNA Repair Kinases

DNA damage activates the protein kinase ataxia-telangiectasia mutated (ATM), an enzyme involved in repairing double-strand breaks (DSB) for cell survival[44-46]. To determine whether ATM is involved in Top2α-mediated topological DDR, we examined the expression kinetics of ATM in CD4 T cells following ICRF treatments. As shown in **Fig.2.4d**, ATM increased in response to DDR in the early phase (6-24 h) and then gradually diminished with longer ICRF-193 treatments (48-72 h). These results indicate that ICRF-193-induced, Top2α-mediated topological DDR involves dynamic activation and depletion of ATM. These are in line with our recent findings of ATM dynamics in healthy CD4 T cells treated with KML001 or Top1 inhibitor[8-9] and in CD4 T cells derived from patients with chronic HCV and/or HIV infection[5-7].

Top2α-Mediated Top2cc Accumulation and DDR Involves PARP1 Induction and TDP2 Inhibition

Top2α relaxes intertwined DNA by producing Top2cc, which can be trapped at enzyme-DNA crosslinks and causes PDB, whose removal depends on the tyrosyl-DNA phosphodiesterase-2 (TDP2) pathway[22, 23, 47]. Notably, Top1cc excision by TDP1 requires Poly ADP-Ribose Polymerase 1 (PARP1), an enzyme that catalyzes the transfer of ADP-ribose onto target proteins and plays an important role in cell apoptosis, DNA repair, and chromosomal stability[48, 49]. Specifically, PARP1 binds to and PARylates TDP1, leading to TDP1 stabilization and its recruitment at the sites of Top1cc-PDB to initiate the repair process[49, 50]. Whether PARP1 is involved in the TDP2-mediated Top2cc-PDB repair remains unknown. To determine whether Top2α inhibitor-induced DNA damage in T cells involves TDP2 suppression and PARP1

induction, we measured PARP1 and TDP2 in ICRF-treated CD4 T cells. As shown in **Fig.2.4e-2.4f**, the cleaved form of PARP1 was markedly induced while TDP2 was reduced by the treatment with Top2α inhibitor. Notably, TDP2 levels were also significantly inhibited in CD4 T cells derived from HBV, HCV, and HIV-infected subjects (**Fig.2.4g**), which are consistent with the data above showing Top2α inhibition, Top2cc accumulation, PARP1 induction, and apoptosis in these cells (**Fig.2.1-4**)[5-9].

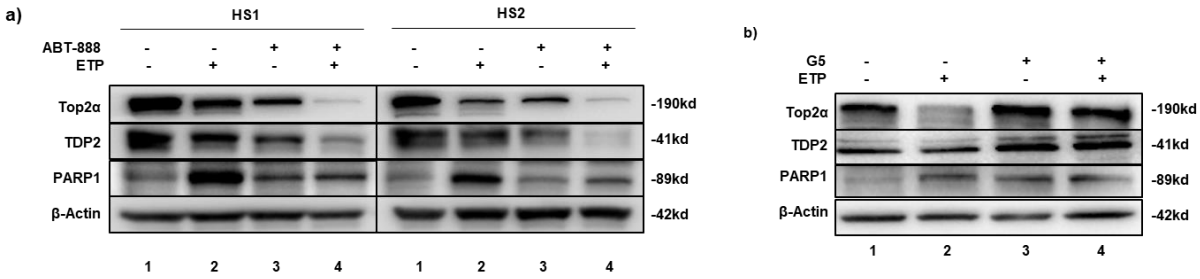
We next assessed whether inhibition of PARP1 could increase DNA damage in ETP-treated T cells. As shown in **Fig.2.5a**, compared to the DMSO-treated control (lane 1), ETP treatment (lane 2) inhibited Top2α and TDP2 expressions, but increased the cleaved form of PARP1. Notably, the PARP1 inhibitor (ABT-888)[51] diminished Top2α and TDP2 expressions, but increased PARP1 cleavage in DMSO-treated cells (lane 3) as well as in ETP-treated cells (lane 4), suggesting that PARP1 is involved in the ETP-mediated Top2α inhibition and TDP2-mediated DNA repair pathway.

We further hypothesized that Top2cc-mediated transcription blockade may trigger Top2α protein degradation by an ubiquitin-mediated mechanism. To test this hypothesis, we examined whether the ubiquitin degradation machinery could contribute to the observed Top2α protein inhibition and DNA damage-mediated apoptosis. As shown in **Fig.2.5b**, compared to the control (lane 1), ETP treatment decreased Top2α and TDP2 but increased the PARP1 levels (lane 2). Intriguingly, inhibition of ubiquitin by the isopeptidase inhibitor (G5), which causes depletion of free nuclear ubiquitin[52], prevented Top2α and TDP2 degradation in DMSO-treated cells (lane 3), especially in ETP-treated T cells (lane 4). We also investigated whether inhibition of the ubiquitin system could prevent DDR-mediated cell apoptosis. Treatment with G5 prevented further induction of PARP1 in ETP-exposed cells (lane 4) compared to DMSO-treated control

(line 3) and ETP-treated cells (lane 2), although G5 treatment alone (lane 3) increased level of cleaved PARP1 compared to cells exposed to DMSO (lane 1). Similarly, inhibition of proteolysis by the proteasome inhibitor MG132, which prevented ETP-induced Top2α and TDP2 degradation, also prevented ETP-induced accumulation of PARP1 (**Fig.2.5c**), suggesting that topological DNA damage depends on Top2α and TDP2 ubiquitination and proteasome degradation. Taken together, these results suggest that the ubiquitin-mediated proteolysis of Top2α and TDP2 is involved in the ETP-induced topological DNA damage.

TDP2 Inhibition Promotes CD4 T cell Apoptosis and Dysfunction

Since Top2α-mediated topological DNA damage can be repaired by TDP2, which is also inhibited in CD4 T cells treated with Top2α inhibitor *in vitro* and isolated from virally infected patients *in vivo* (**Fig.2.4**), we next determined the effect of the TDP2 inhibitor ZW-1226 on T cell survival and functions. ZW-1266 is a cell-permeable deazaflavin analog that selectively inhibits TDP2 activity and sensitizes cancer cells toward ETP-treatment[53]. While this drug did not affect the expression levels of TDP2 and Top2α in TCR-activated CD4 T cells (**Fig.2.5d**), TDP2 inhibition resulted in an increased level of γH2AX and Av staining, and decreased intracellular IL-2 and IFN-γ production (**Fig.2.5e-h**), suggesting that TDP2 is important for maintaining Top2α-mediated T cell DNA topology and cellular functions.



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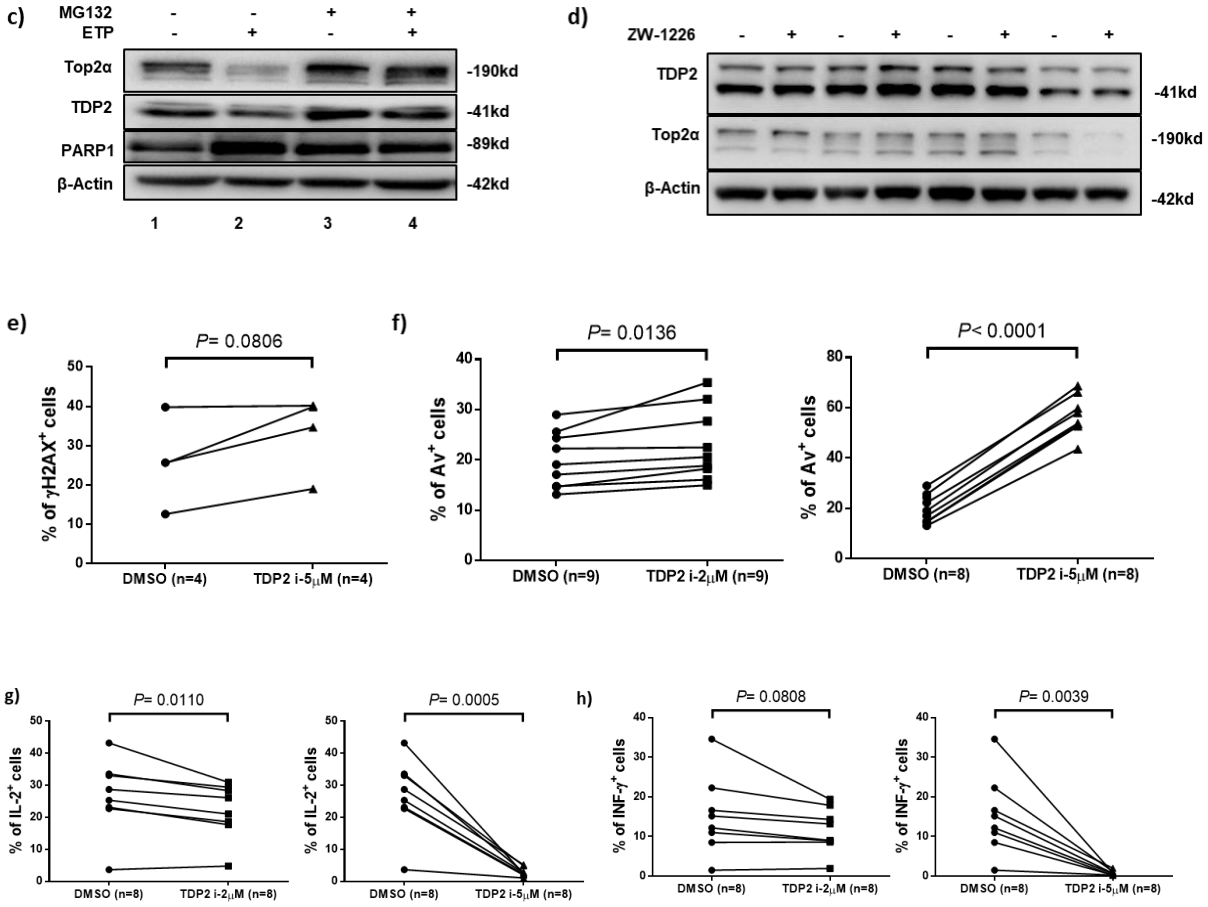

Fig.2.5. PARP1 and TDP2 are involved in the Top2α-mediated DNA damage and cell apoptosis. a) Immunoblotting of Top2α, TDP2, and PARP1 expressions in TCR-activated CD4 T cells in the presence or absence of Top2α inhibitor (ETP) or PARP inhibitor (ABT-888). **b)** Immunoblotting of Top2α, TDP2, and PARP1 expressions in TCR-stimulated CD4 T cells with or without ETP treatment in the presence or absence of ubiquitin isopeptidase inhibitor (G5). **c)** Immunoblotting of Top2α, TDP2, and PARP1 expressions in TCR-activated CD4 T cells with or without ETP treatment in the presence or absence of proteasomal inhibitor (MG-132). **d)** Immunoblotting of TDP2 and Top2α expressions in TCR-activated CD4 T cells exposed to TDP2 inhibitor (ZW-1226) for 3 days.**e**) Flow cytometric analysis of percentages (%) of $\gamma H2AX^+$ cells within TCR-activated CD4 T cells treated with TDP2 inhibitor (5 μM) or DMSO control for 3 days. **f-h)** Flow cytometric analysis of percentage (%) of Av^+, IL-2^+, and IFN-γ^+ cells within TCR-activated CD4 T cells treated with TDP2 inhibitor (2 μM, 5 μM) or DMSO control for 3 days.

Discussion

T cells play a pivotal role in controlling pathogenic infection and vaccine responses. During chronic viral infections, however, T cells are always dysregulated and often non-responsive to vaccines[1]. We and others have previously reported that T cells from chronically virus-infected individuals are prematurely aged due to accelerated telomere erosion[1-18], but the underlying mechanisms for T cell telomeric DNA damage remain unclear. Since Top2α is required to remove DNA supercoiling generated during cell proliferation, and Top2cc can become trapped during gene transcription to cause Top2cc-linked PDB due to TDP2 depletion[22, 23], we hypothesized that DNA topology in T cells may be affected during viral infections to trigger DDR as a mechanism of virus-induced immune evasion, and thus, persistent infection.

In the present study, we employed T cells isolated from virus-infected individuals and primary T cells treated with Top2α inhibitors as model to obtain molecular insights into the mechanisms underlying Top2α-mediated DNA damage and repair signaling. We demonstrated that: 1) T cells derived from chronically virus-infected individuals exhibit diminished Top2α enzyme expression and activity, leading to accumulation of Top2cc and DNA damage, including telomere erosion; 2) ETP or ICRF-induced Top2α inhibition, topological DNA damage, telomere uncapping and attrition, T cell apoptosis and dysfunctions recapitulate the phenotype seen in T cells during chronic viral (HBV, HCV, HIV) infections, highlighting the role of Top2α in maintaining telomeric DNA integrity and securing T cell survival or function; 3) Top2α inhibition occurs at the post-transcriptional level (likely via the ubiquitin-mediated proteolysis) and is related to TDP2 suppression, PARP1 induction, and Top2cc accumulation; 4) Top2α inhibition-mediated telomeric DDR involves telomere TRF2-uncapping, diminished telomerase activity, and a dynamic ATM activation followed by deprivation; and 5) T cells from virally infected subjects

with lower Top2α levels are more vulnerable to Top2α inhibitor-induced topological DNA damage and cell apoptosis, indicating an important role for Top2α in preventing unwanted DNA damage and securing cell survival.

Although the accumulation of DNA damage and the failure to repair it may affect cell survival and function, the molecular signaling pathways in T lymphocytes in the context of chronic viral infections are incompletely understood. Top2α cuts both strands of the DNA helix simultaneously in order to manage DNA entangles and supercoils[26]. Once cut, the ends of the DNA are separated, a second DNA duplex is passed through the break, and the cut DNA is then re-ligated. This process allows the Top2α to increase or decrease the linking numbers of a DNA loop by 2 units and promotes chromosome untying (**Fig.2.6a**). Etoposide is a semi-synthetic derivative of podophyllotoxin that forms a ternary complex with DNA and Top2α and prevents re-ligation of the DNA strands, and by doing so causes the DNA strands to break[27] (**Fig.2.6b**). ICRF-193 is a bisdioxopiperazine Top2α catalytic inhibitor that blocks Top2α turnover by trapping it in a closed clamp conformation and delays the cell cycle progression from metaphase in mammalian cells (**Fig.2.6c**), likely with the ability to damage DNA and trap the DNA-Top2α crosslinking complex28. ZW-1266 is a cell-permeable deazaflavin analog (**Fig.2.6d**) that selectively inhibits TDP2, but not TDP1 enzymatic activity in vitro, and strongly sensitizes cancer cells toward the treatment of ETP, a phenotype consistent with the TDP2 function loss and leads to the TDP2cc formation[53]. Cancer cells and highly proliferative T cells rely on Top2α more than other cells, because they divide more rapidly. Therefore, deficiency or inhibition of this enzyme causes errors in DNA topology and promotes cell apoptosis. Our results, in conjunction with our previous reports[5-9], support a model (depicted in **Fig.2.6e**), in which Top2α inhibition and Top2cc accumulation block transcription elongation, which triggers the ubiquitin-mediated Top2α

proteolysis and the generation of Top2cc-mediated PDBs. Defective repair of these PDBs by the DNA repair machineries such as TDP2 can lead to more DDR, which activates ATM kinase and phosphorylation of its downstream substrates such as CHK2 and p53. Activated ATM also activates 53BP1 and γH2AX assembly into nuclear DNA damage foci and promotes the ubiquitination of multiple signaling molecules in the process of DNA damage and repair. Notably, several E3 ligases have been reported for Top2α ubiquitination and proteasomal degradation[47]. Our findings that Top2α and TDP2 inhibitions, as well as γH2AX and PARP1 inductions in treated T cells, depend on Top2α ubiquitination suggest that they arise during the repair of Top2cc and this pathway feeds back to enhance Top2cc repair after Top2α-linked PDB induction. Moreover, it appears that PARP1 is involved not only in the process of TDP1-mediated excision of Top1cc[49, 50], but also in TDP2-mediated repair of Top2cc, since PARP1 inhibition alters Top2α-PDB induction following Top2cc stabilization. This Top2 model, similar to our Top1 model[9], is supported by our recent findings showing that ATM deficiency[5, 7], TRF2 uncapping6, and telomere targeting8 promote telomeric DNA damage and T cell senescence and apoptosis, as demonstrated in this study in T cells isolated from chronic viral infections or T cells treated with Top2α inhibitors.

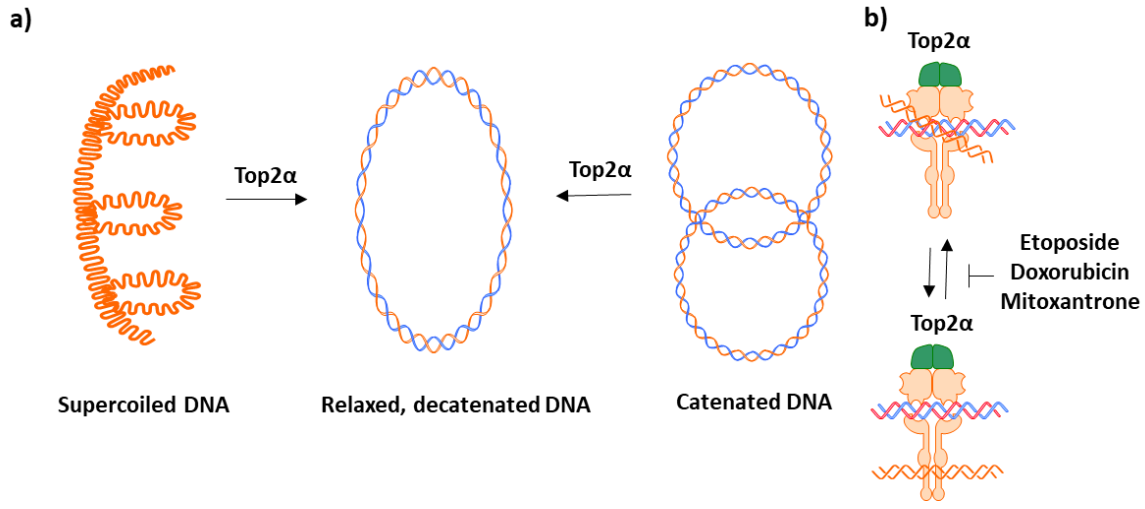

Fig.2.6 (continue)

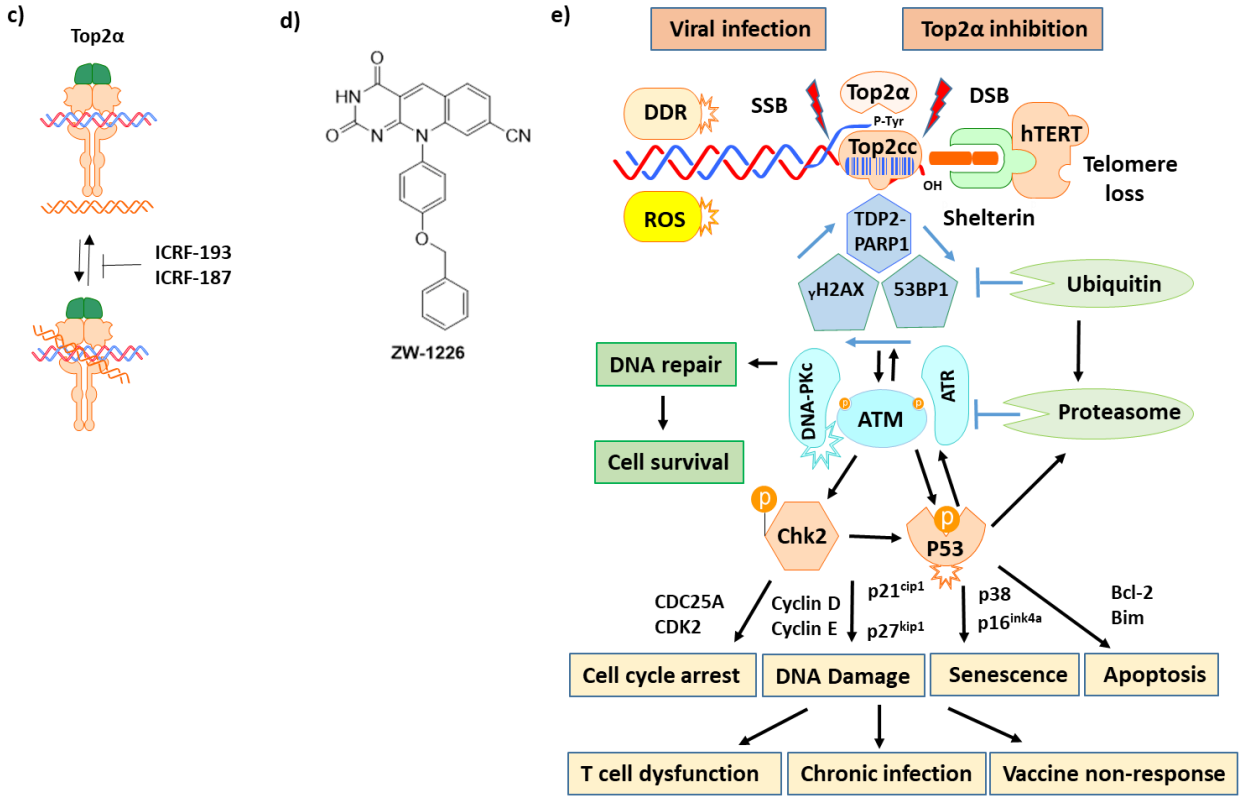

Fig.2.6. A working model for virus-induced disruption of DNA topology and in T cell dysregulation. a) A schematic representation of Top2α on the supercoiled, catenated chromosomal DNA. Top2α cuts both strands of the DNA helix in order to manage DNA tangles and supercoils. Once cut, the ends of the DNA are separated, a second DNA duplex is passed through the break, and the cut DNA is then re-ligated. This process allows Top2α to increase or decrease the linking number of a DNA loop by 2 units, and thus promotes chromosome disentanglement. b-c) A schematic representation showing ETP or ICRF in the Top2α-DNA crosslinks. Etoposide is a semisynthetic derivative of podophyllotoxin that forms a ternary complex with DNA and Top2α, and prevents re-ligation of the DNA strands, causing DNA strand breaks. ICRF-193 poisons DNA-Top2α cross-linking complexes and delays cell cycle progression to mitosis. d) Structure of ZW-1226, a TDP2 functional inhibitor. e) A schematic model of Top2α-mediated telomeric DDR and T cell dysregulation during chronic viral infection. The intertwined nature of two complementary DNA strands often leads to topological entanglements during DNA replication, transcription, and recombination that must be resolved to ensure normal DNA transactions and cell functions. In order to prevent and correct these types of topological problems, Top2α binds to DNA and cut two DNA strands simultaneously, allowing the DNA to be untangled or unwound. Based on our findings, the immunomodulatory virus (HBV, HCV, HIV) infection and/or ROS-generating inflammation can inhibit Top2α protein expression and enzyme activity, leading to Top2cc becoming trapped at the DNA break sites including telomere termini, and causing topological DNA damage, telomere loss, cell senescence, and apoptosis. This continuous regulatory cascade represents a novel molecular mechanism underlying CD4 T cell dysfunction, which contributes to the viral persistence and vaccine non-responsiveness in human viral infections.

While the mechanisms leading to Top2α inhibition during chronic viral infections remain unclear, multiple factors may play a role. Topological DNA damage might occur in proliferating T cells under physiological conditions, but Top2cc can be trapped under a broad range of pathological conditions, including Top2α inhibition by immuno-modulating viruses (HBV, HCV, and HIV), oxidative base damage by alkylation with carcinogenic compounds or antiviral agents, and ribonucleotide misincorporations during genetic activities in over-expanding T cells in response to low-grade chronic inflammation[19-24]. Therefore, these Top2α-linked PDBs may have a significant impact on replicative T cells, leading to reprogramming of the DDR and remodeling of T cell fate that arise from the defective removal of Top2cc due to deprivation of TDP2, deprotection of telomeres by shelterins[6], failure of telomere elongation by telomerase, and deficiency of DNA damage repair by ATM[5, 7]. T cells may particularly be prone and vulnerable to Top2α-mediated topological DNA damage and cell apoptosis as a result of high rates of cell turnover and oxygen consumption, which produce large amounts of reactive oxygen species (ROS) that we have recently shown significantly elevated in T cells from viral (HCV and HIV) infected individuals[5-7]. Indeed, ROS can stabilize Top2cc to cause topological DNA damage and activate the ATM signaling pathway[54]. Thus, our findings suggest a new concept in which topological DNA damage contributes to T cell senescence, apoptosis, and immune evasion during chronic viral infections.

It should be pointed out that while Top2α inhibition and Top2cc accumulation explain both telomeric DNA damage and cell apoptosis, they may also function as a double-edged sword that can result in both overwhelming cell death storm in acute infection and immune tolerance or immune suppression in chronic infection1. Nevertheless, these novel findings demonstrate, for the first time, the role of Top2α in DDR and shed light on the molecular aspects of immunomodulation

during human viral infections. Most importantly, this study provides a potential strategy for restoring impaired DNA topological machinery as a mean to improve T cell functions and vaccine responses against human viral diseases.

Materials and Methods

Subjects

The study protocol was approved by the institutional review board (IRB) of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN). Written informed consent was obtained from all subjects. The study subjects were composed of four populations: 27 chronically HBV-infected patients on antiviral treatment with undetectable viremia (HBV-DNA); 42 chronically HCV-infected patients prior to antiviral therapy; 56 latently HIV-infected patients on antiretroviral therapy (ART) with undetectable viremia (HIV-RNA); and 105 age-matched healthy subjects (HS). HS blood samples were provided by Physicians Plasma Alliance (PPA), Gray, TN, and were negative for HBV, HCV and HIV infections. The characteristics of the subjects recruited in this study are described in **Table 2.1**.

Table 2.1 Demographic Information of the Study Participants

Subjects	Numbers	Age (Mean)	Gender (M/F)	Viral load and other characteristics
HCV	42	29–68 (48)	36/6	135–6,696,048 IU/ml, 32 GT1, 6 GT2, 4 GT3
HBV	27	29–72 (42)	22/5	All on antivirals with undetectable HBV-DNA
HIV	56	22–70 (48)	46/10	All on ART with undetectable HIV-RNA
HS	105	21–65 (46)	75/30	All tested negative for HCV, HBV, and HIV

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density centrifugation (GE Healthcare, Piscataway, NJ). The $CD4^+$ T cells were isolated from PBMCs using the $CD4^+$ T Cell Negative Selection Kit and a MidiMACS™ Separator (Miltenyi

Biotec Inc., Auburn, CA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 100 IU/ml penicillin and 2 mM L-glutamine (Thermo Scientific, Logan, Utah) and maintained at 37°C and 5% CO_2 atmosphere.

Flow Cytometry

Intracellular IL-2, IFN-γ cytokine production, DNA damage marker γH2AX expression, CSFE-label CD4 T cell proliferation, telomere length measured by Flow-FISH, and cell apoptosis assay for Av/7AAD expression were analyzed by flow cytometry, as described previously[5-7]. The following reagents were used in the assays: PE-labelled Av, 7-AAD, IL-2, IFN-γ, γH2AX (BD), CD4-Alexa-647 (Bio legend), telomere probe Tel-C (TAACCC)-FITC (0.25 µg probe/mL, PNA Bio, Newbury Park, CA).

RNA Isolation And Real-Time RT-PCR

Total RNA was extracted from 1 x 10^6 CD4 T cells using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Bio-systems, Foster City, CA) per the manufacturer's instruction. Quantitative RT-PCR was performed in triplicate as described previously[5-7]. Gene expression levels were determined using the $2^{-\Delta\Delta ct}$ method. Values were normalized to the GAPDH level and are presented as fold changes. The PCR primer sequences are shown in **Table 2.2**.

Table 2.2 Primer Sequences Used in Real-time RT-PCR of This Study

Primers	Forward	Reverse
hTERT	5′-CCAAGTTCCTGCACTGGCTGA-3′	5′-TTCCCGATGCTGCCTGACC-3′
Top2α	5′-ACCATTGCAGCCTGTAAATGA-3′	5′-GGGCGGAGCAAAATATGTTCC-3′
GAPDH	5′-TGCACCACCAACTGCTTAGC-3′	5′-GGCATGGACTGTGGTCATGAG-3′

Immunoblotting

Since Top2α is only expressed in activated T cells, we examined Top2α level in purified CD4 T cells activated with anti-CD3/CD28 for 3 days by immunoblotting. Briefly, CD4 T cells (2 x 10^6) were purified from HCV, HBV, or HIV patients and HS as described previously[9]. Primary and secondary antibodies included Top2α, PARP1, TRF1, TRF2, TPP1, TIN2, RAP1, POT1, ATM, GAPDH, β-actin, and horseradish peroxide-conjugated antibody (Cell Signaling). Images were captured using ChemiDoc™ XRS+ System (Bio-Rad). Protein band intensity was quantitated by the Image Lab software (Bio-Rad).

Top2α Activity Assay

The activity of Top2α was measured using the Topoisomerase II Assay Kit (Cat #TG10001; Topogen Inc; Buena Vista, CO). Briefly, CD4 T cells were isolated, and cell extracts were prepared according to the manufacturer's instructions. Because nuclease activity may cause some degradation of the kDNA substrate and generate a smear of degradation products, we used a Top2α isolation kit to purify the nuclear extract. The purified extract was mixed with plasmid DNA substrate and reaction buffer for 30 min at 37°C, loaded to a 1% agarose gel with loading dye and then subjected to electrophoresis for 2 h at 5-10 V/cm before illuminating with a UV transilluminator. The intensity of the relaxed circular DNA was measured by densitometry.

Top2cc Detection

Top2cc was detected using the Human Topoisomerase ICE Assay Kit (Cat # TG1020-1; Topogen). The method of DNA purification was modified by combining the ICE Assay Kit and PureLink™ Genomic DNA Mini Kit (Cat #K182001; Thermo Fisher Scientific, Waltham, MA). Briefly, genomic DNA samples were extracted from cell pellets using buffers from the ICE assay

kit and then purified by the column of PureLink™ Genomic DNA Mini Kit. The DNA samples were loaded onto NC membrane by a vacuum pump and were incubated with primary anti-Top2cc antibody from the ICE assay kit, followed by western blotting as described above.

Confocal Microscopy

CD4 T cells were isolated and cultured as described above, followed by immunofluorescence staining using a method described previously[6]. The primary antibodies included Rabbit anti-53BP1, and mouse TRF1 (Thermo Fisher). The secondary antibodies included anti-rabbit IgG-Alexa Fluor 488 and anti-mouse IgG-Alexa Fluor 555 (Invitrogen). We also used Tel-C (TAACCC)-FITC probe for telomere staining. The cells were washed and mounted with DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL). Images were acquired with a confocal laser-scanning inverted microscope (Leica Confocal, Model TCS sp8, Germany).

Statistics

The data were analyzed using Prism 7 software and are presented as mean ± SEM. Differences between two groups were analyzed by independent Student's t-test or paired T-test. P-values of <0.05, or <0.01 were considered statistically significant or very significant, respectively.

Acknowledgments

This work was supported by National Institutes of Health grants R01AI114748 and R21AI138598; Veteran Administration Merit Review Awards 1I01BX002670 and 1I01BX004281; and Department of Defense Award PR170067 (to Z.Q.Y). We thank the NIAID AIDS Reagent Program for providing key reagents. This publication is the result of work supported with resources and use of facilities at the James H. Quillen Veterans Affairs Medical Center. The

contents in this publication do not represent the views of the Department of Veterans Affairs or the United States Government.

Competing interests

The authors declare no competing financial interests.

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CHAPTER 3. MITOCHONDRIAL TOPOISOMERASE 1 INHIBITION INDUCES TOPOLOGICAL DNA DAMAGE AND T CELL DYSFUNCTION IN PATIENTS WITH CHRONIC VIRAL INFECTION

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Keywords: HCV, HIV, mitochondrial dysfunction, topoisomerase I, T cell dysregulation

Running title: Mitochondrial Top1 inhibition and T cell dysregulation

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Abstract

T cells are crucial for controlling viral infections; however, the mechanisms that dampen their responses during viral infections remain incompletely understood. Here, we studied the role and mechanisms of mitochondrial topoisomerase 1 (Top1mt) inhibition in mitochondrial dysfunction and T cell dysregulation using CD4 T cells from patients infected with HCV or HIV and compared it with CD4 T cells from healthy individuals following treatment with Top1 inhibitor - camptothecin (CPT). We found that Top1mt protein levels and enzymatic activity are significantly decreased, along with Top1 cleavage complex (Top1cc) formation, in mitochondria of CD4 T cells from HCV- and HIV-infected patients. Notably, treatment of healthy CD4 T cells with CPT caused similar changes, including inhibition of Top1mt, accumulation of Top1cc in mitochondria, increase in PARP1 cleavage, and decrease in mtDNA copy numbers. These molecular changes resulted in mitochondrial dysfunction, T cell dysregulation, and programmed cell death through multiple signaling pathways, recapitulating the phenotype we detected in CD4 T cells from HCV- and HIV-infected patients. Moreover, treatment of CD4 T cells from HCV or HIV patients with CPT further increased cellular and mitochondrial reactive oxygen species (ROS) production and cell apoptosis, demonstrating a critical role for Top1 in preventing mtDNA damage and cell death. These results provide new insights into the molecular mechanisms underlying immune dysregulation during viral infection and indicate that Top1 inhibition during chronic HCV or HIV infection can induce mtDNA damage and T cell dysfunction. Thus, reconstituting Top1mt protein may restore the mtDNA topology and T cell functions in humans with chronic viral infection.

Introduction

T cells play a critical role in controlling viral infections; however, the molecular mechanisms that dampen their responses during viral infections remain incompletely understood. We have recently reported that chronic viral (HCV, HIV) infection can cause T cell aging, as evidenced by the increases in the aging markers, telomeric and mitochondrial DNA damage, and mitochondrial dysfunction[1–20]. Mitochondria are important cellular organelles responsible for energy production and oxidative metabolism and are a major source of reactive oxygen species (ROS). We have examined mitochondrial dysregulation in CD4 T cells during HCV or HIV infections and discovered that oxidative stress, caused by excess ROS production, induces mitochondrial injury and accelerates telomere erosion - a dual effect that can promote the T cell aging process and, eventually, cell death[8–12]. Thus, further investigation into how the mitochondrial machineries, especially mitochondrial DNA (mtDNA), are disrupted during chronic viral infections may provide new information regarding the mechanisms of CD4 T cell dysfunction and viral persistence[8,9].

Human mtDNA sequencing revealed 16,569 base pairs of 37 small circular genes that encode 13 mitochondrial proteins - all of which are involved in the processes of oxidative phosphorylation[21,22]. In essence, mtDNA damage disrupts the fine-tuned balance between ROS levels and ROS scavenging or antioxidative defenses by enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, resulting in mitochondrial dysfunction, cell senescence, and programmed cell death[22]. Notably, mtDNA replication, transcription, and recombination can lead to topological tangles that must be untangled to ensure normal gene transactions and cellular functions[23–25]. To prevent or correct these topological derangements, topoisomerases - the enzymes that modulate the topology of DNA to prevent their entanglements - bind to and cut the

double- or single-stranded DNA, allowing the DNA to be untangled. Failure to resolve the DNA knots can results in trapping of these enzymes at the target sites, thus generating topoisomerase cleavage complexes (Top1cc) and protein-linked DNA breaks (PDBs), and causing topological DNA damage and programmed cell death[25–27].

Topoisomerases also play an important role in viral or bacterial DNA insertion into the host chromosomes. Notably, some chemotherapeutic drugs and fluoroquinolone antibiotics interfere with the activity of topoisomerases in bacteria or cancer cells and create topological DNA damage, which promotes cell death[28–30]. Therefore, normal DNA topology is important for cellular functions, and its disruption can lead to topological DNA damage and cell death. We have previously reported that inhibition of topoisomerase 1 (Top1) and topoisomerase II alpha (Top2α) induces genomic DNA damage and T cell dysregulation during chronic viral (HCV, HBV, and HIV) infections[6,7]. Notably, there are different isoforms of Top1 enzymes, e.g., a nuclear Top1 (Top1nc, ~100 kDa) and a mitochondrial Top1 (Top1mt, ~75 kDa), which are thought to function at their respective locations. Whether inhibition of these Top1 isoforms occurs in mitochondria (the major powerhouse and ROS source in cells) and thus causes mitochondrial dysfunction and T cell dysregulation during chronic viral infections have yet to be investigated.

In the present study, we assessed the levels of Top1 expression in mitochondria of CD4 T cells from patients with chronic HCV or HIV infection. We also recapitulated the phenotype of Top1 inhibition using healthy CD4 T cells treated with camptothecin (CPT) to investigate the role of Top1 in remodeling mitochondria and functions of CD4 T cells. We found that Top1 levels and enzymatic activity are remarkably inhibited in mitochondria of CD4 T cells from patients with HCV or HIV infection and in healthy CD4 T cells treated with CPT, thus

implicating Top1 inhibition in promoting mitochondrial and T cell dysfunctions through regulating multiple cell death and metabolic pathways. These findings provide novel insights into the mechanism that disrupts mtDNA topology and its role in mitochondrial dysfunction and T cell dysregulation during chronic viral infection.

Results

Top1 Protein Levels and Enzymatic Activity Are Decreased In Mitochondria of CD4 T Cells in Patients with HCV or HIV Infection

We have previously reported that chronic viral infection induces T cell aging and immune senescence due to accelerated telomeric DNA damage and mitochondrial dysfunction[1-20]. Given the crucial role of DNA topology in maintaining mitochondrial integrity and cell function and viability[23–25], here we sought to determine the levels of Top1 protein in the mitochondria of CD4 T cells from chronically HCV- or HIV-infected patients. To this end, we first determined whether Top1 protein is present in mitochondria of CD4 T cells. We performed immunostaining in CD4 T cells using an antibody specifically targeting Top1mt, followed by confocal microscopy. As shown in **Fig. 3.1A**, Top1mt was primarily present in the mitochondria, as evidenced by the colocalization of Top1mt and mitotracker (mt, a mitochondrial marker) in the cytoplasm of these cells. We also used an antibody specifically targeting Top1nc for immunostaining and determined whether Top1nc protein can also localize in the mitochondria of CD4 T cells. Intriguingly, we found that Top1nc protein is also present in the mitochondria and co-localized with mt (**Fig. 3.S1A**). Notably, the fluorescent signals from Top1mt and Top1nc (green) as well as mt (red) and their co-localizations (yellow) were relatively lower in CD4 T cells from HCV or HIV patients compared with healthy subjects (HS) (**Fig. 3.1A, Fig. 3.S1A**).

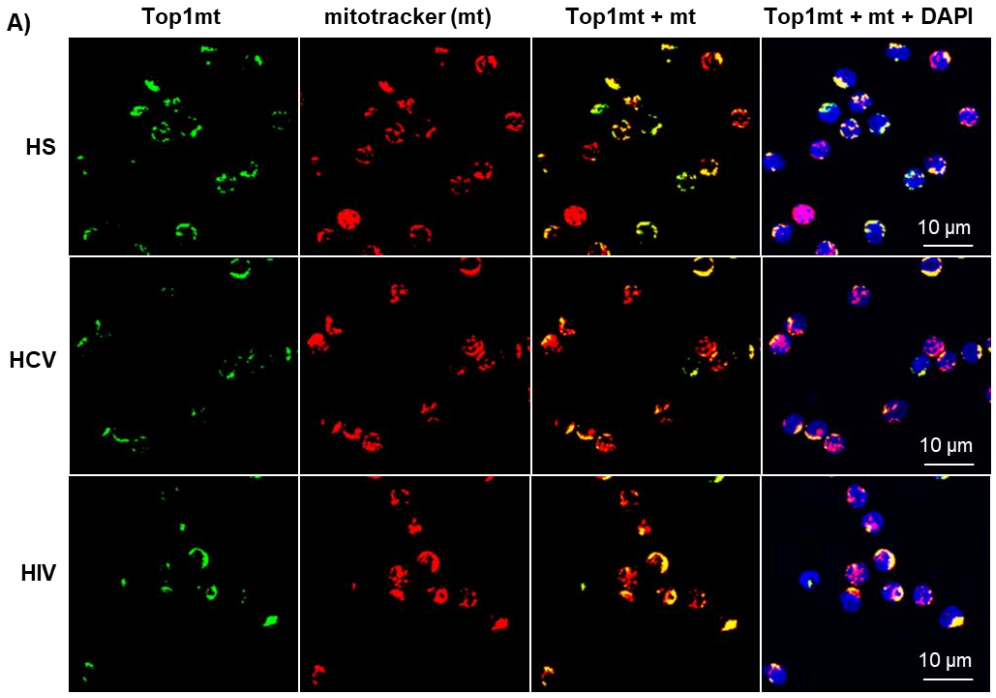
We then performed immunoblotting of Top1mt and Top1nc using whole cell lysates as well as fractionated mitochondrial and cytosolic extracts from CD4 T cells to determine the levels of Top1 proteins in these subcellular compartments. Mitochondrial heat shock protein 70 (mHsp70) is a mitochondrial resident protein which plays a critical role for protein translocation into the mitochondria. mHsp70 cooperates with Hsp10 and Hsp 60 and mediates essential functions for mitochondrial biogenesis, like protein folding and translocation into mitochondria[31,32]. mHsp70 and β-actin served as loading controls for mitochondria and cytosolic proteins, respectively. As shown in **Fig. 3.1B**, CD4 T cell lysates from HCV- or HIV-infected patients exhibited significantly lower levels of Top1mt protein compared with the age-matched, healthy CD4 T cells. Additionally, these cells exhibited significantly lower levels of Top1nc in both mitochondrial (M) and cytosolic (C) compartments compared with the healthy CD4 T cells (**Fig. 3.S1B**). To determine the relationship between Top1 levels and CD4 T cell numbers in patients with HIV infection, we measured Top1nc protein levels in mitochondrial and cytosolic extracts from antiretroviral therapy (ART)-treated HIV patients with CD4 T cell counts > 500 cells/μL (defined as HIV immune responders/HIV-IRs) and CD4 T cell counts < 500 cells/μL (defined as HIV immune non-responders/HIV-INRs)[8] and compared them with those from HS. As shown in **Fig. 3.S1C**, CD4 T cells from HIV-INRs exhibited the lowest Top1nc levels in both mitochondria and cytosol compared with CD4 T cells from HIV-IRs or HS, indicating that Top1nc protein levels correlate with CD4 T cell frequencies in HIV patients.

Human Top1 is a type 1B topoisomerase that can relax supercoiled DNA[25]. Thus, we used a plasmid (pHOT1)-based Top1 assay to measure Top1 enzymatic activity in CD4 T cells from HCV- and HIV-infected patients. We found that both mitochondrial (**Fig. 3.1C**) and cytosolic extracts (**Fig. 3.S1D**) from CD4 T cells derived from these patients failed to completely

relax the supercoiled plasmid DNA compared with CD4 T cells from HS, indicating a reduced level of Top1 enzymatic activity in the mitochondria and cytosol of CD4 T cells from patients with HCV or HIV infection.

To determine if the decrease in Top1 protein is due to its trapping at the mtDNA catalytic site, leading to Top1cc accumulation and then degradation, we used a monoclonal antibody that specifically targets covalent Top1-DNA complexes (but not free Top1 or DNA) and measured Top1cc by immunoblotting in mitochondria isolated from CD4 T cells[33]. We found a significantly higher amounts of Top1cc in mtDNA of CD4 T cells from HCV or HIV patients compared with HS (**Fig. 3.1D**).

Given our previous studies demonstrating premature CD4 T cell aging and dysfunction in chronic viral infections[1-20], the results described above suggest that topological DNA aberrancies (i.e., the decrease in Top1 protein and enzymatic activity and Top1cc accumulation) occur in the mitochondria of dysfunctional, senescent CD4 T cells during chronic HCV or HIV infections.



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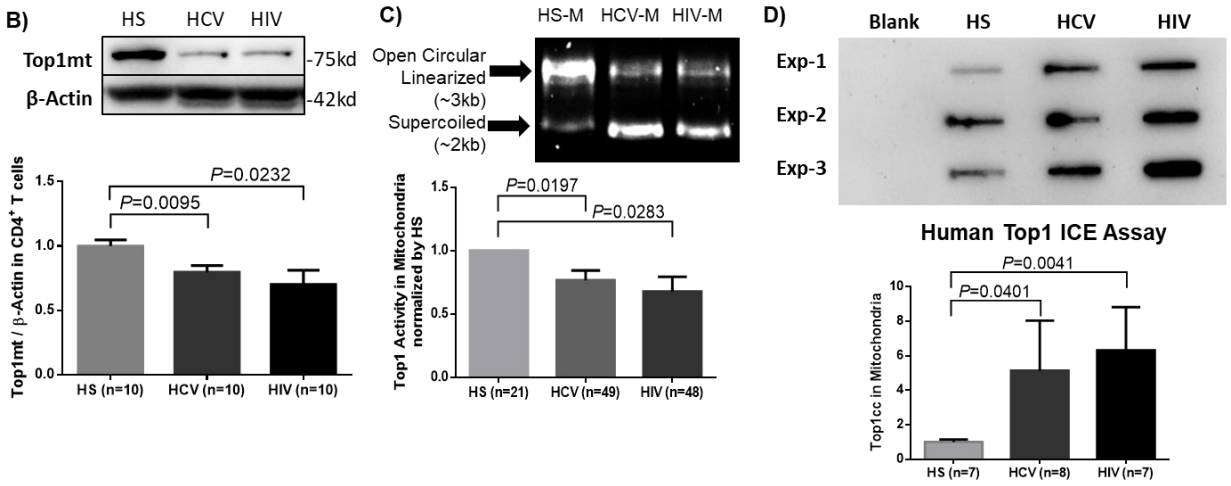


Fig.3.1 Top1mt expression and activity are inhibited in CD4 T cells during chronic viral infection. A) Top1mt protein (green), MitoTracker (mt, red), and their colocalization (yellow) in mitochondria of CD4 T cells from HCV- and HIV-infected patients and healthy subjects (HS). The nucleus was stained with DAPI, and representative confocal microscopy images were merged. B) Top1mt protein expression in CD4 T cells from HCV- and HIV-infected patients and HS. Representative images and summary data of Western blots are shown (n=10). The Top1mt band intensity was normalized by β-Actin. C) Top1 activity in mitochondria of CD4 T cells from HCV- and HIV-infected patients versus HS. Representative images and summary data of Top1-mediated digestion of supercoiled DNA substrate are shown. D) Top1cc was detected in mtDNA from CD4 T cells from HCV- and HIV-infected patients versus HS. Representative images and summary data of top1cc band intensity (normalized to HS) are shown.

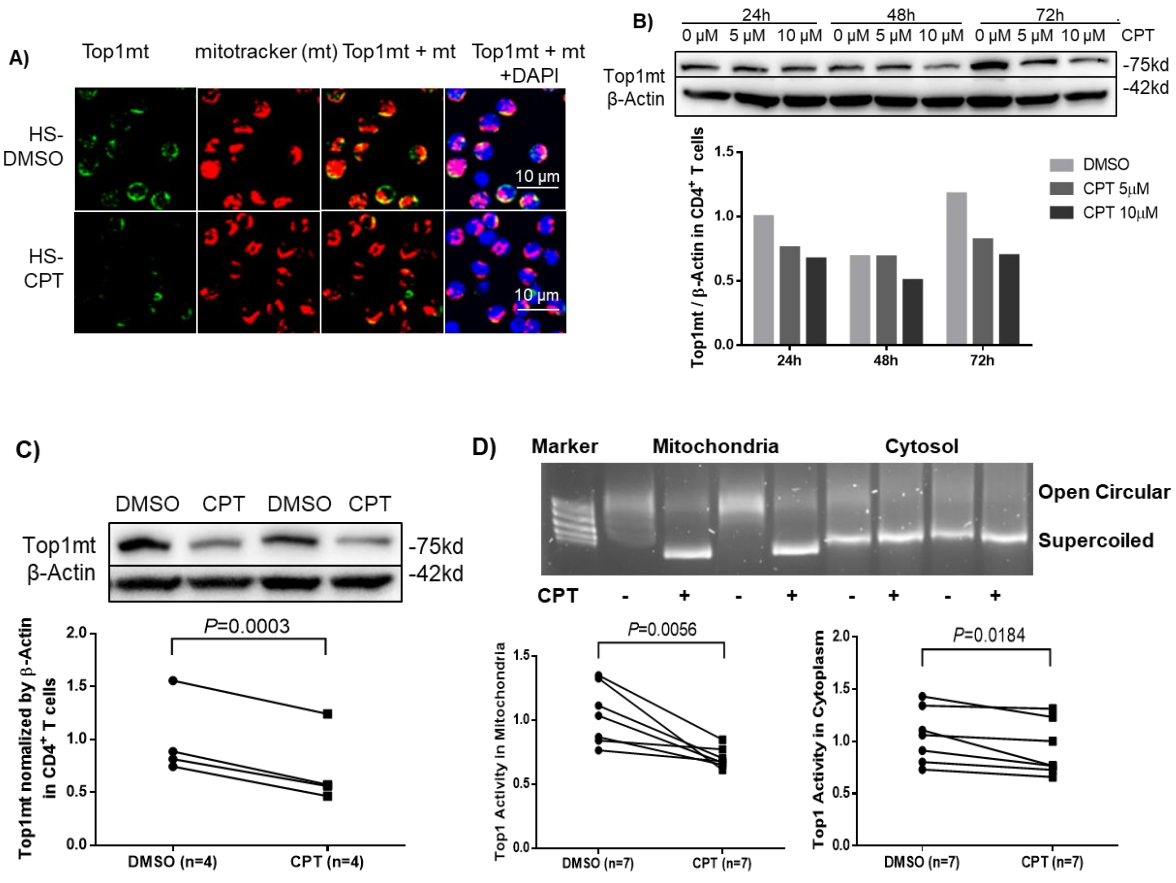
Top1 Inhibition by CPT Treatment Induces Topological mtDNA Damages in Healthy CD4 T Cells

We have previously shown that healthy CD4 T cells treated with CPT, which inhibits Top1 protein expression, exhibit topological DNA damage and cell dysfunction, recapitulating the phenotype of CD4 T cells from HCV or HIV-infected patients[6]. Mechanistically, CPT inhibits Top1 enzymatic activity by intercalating into the DNA at the catalytic site, leading to accumulation of the transcription-blocking Top1cc and PDB production, followed by degradation of the Top1cc and cell cytotoxicity[34]. To determine whether CPT treatment can lead to Top1 protein inhibition in mitochondria, we examined Top1 protein localization in CPT-

treated CD4 T cells from HS using confocal microscopy. As seen in CD4 T cells from HCV- and HIV-infected patients, Top1mt (**Fig. 3.2A**) and Top1nc (**Fig. 3.S2A**) proteins were primarily detected in the cytosol, co-localized with mitochondrial marker, and their levels decreased following CPT treatment compared with the dimethyl sulfoxide (DMSO) control treatment. We also employed immunoblotting to measure Top1mt and Top1nc protein levels in a mitochondrial extract from healthy CD4 T cells treated with CPT or DMSO. To do this, CD4 T cells from HS were treated with varying concentrations of CPT (0, 5, or 10 μM) for 24, 48, and 72 h. Significant inhibitions of Top1mt (**Fig. 3.2B-3.2C**) and Top1nc (**Fig. 3.S2B**) expression were observed following CPT treatment. Correspondingly, Top1 enzymatic activity was inhibited - more significantly in mitochondria than cytosol - in CD4 T cells treated with CPT compared with DMSO control, measured by the Top1 activity assay kit (**Fig. 3.2D**). In addition, Top1cc accumulation in mtDNA was higher in CPT-treated healthy CD4 T cells (**Fig. 3.2E**). These results indicate that inhibition of Top1 expression by CPT treatment in healthy CD4 T cells can recapitulate the mtDNA damage found in CD4 T cells from HCV- and HIV-infected patients (**Fig. 3.1**), and thus CPT represents a reliable model to study the mechanism of Top1-mediated mtDNA topological aberrancies and their role in T cell dysregulation during viral infection.

Trapped Top1cc can cause PDBs, which are removed by tyrosyl-DNA phosphodiesterase-1 (TDP1)[26,35–39]. Top1cc excision by TDP1 requires Poly ADP-Ribose Polymerase 1 (PARP1)[26,40,41]. Specifically, PARP1 interacts with TDP1 and is recruited to the sites of Top1cc-induced PDBs to initiate the DNA repair process. Failure to complete this process leads to unrepaired DNA damage, PARP1 cleavage, and cell dysfunction or apoptosis. To determine whether CPT-induced mtDNA damage in healthy CD4 T cells triggers PARP1 cleavage, we measured the PARP1 level in mitochondrial extracts from CD4 T cells treated with

CPT. As shown in **Fig. 3.2F**, the level of cleaved PARP1 was significantly increased in mitochondria, suggesting its involvement in mtDNA damage and cell apoptosis. Top1cc trapping at the Top1-DNA interface leads to Top1 inhibition and subsequent degradation of Top1-PDB complexes and, ultimately, topological DNA damage[31]. To determine the consequences of Top1 inhibition, we measured mtDNA content (mtDNA copy numbers normalized to nuclear DNA) by qPCR and found that its level was significantly lower in healthy CD4 T cells following CPT treatment (**Fig. 3.2G**). These CPT-induced changes are in line with our findings in CD4 T cells from HCV- and HIV-infected patients[8,9].



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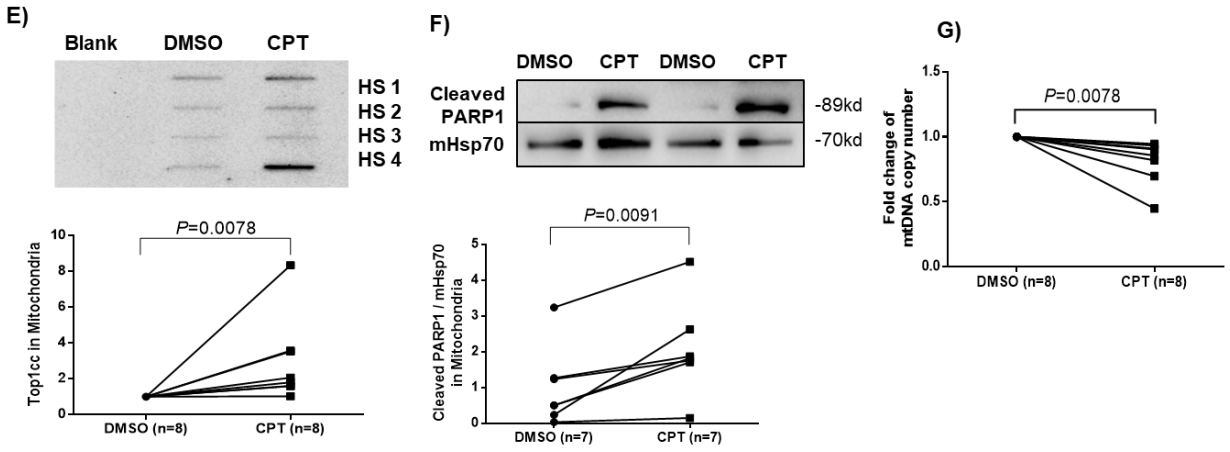


Fig.3.2. Top1 inhibition by CPT induces topological mtDNA damage in healthy CD4 T cells. A) Top1mt protein (green), MitoTracker (mt, red), and their colocalization (yellow) in mitochondria in HS-CD4 T cells treated with DMSO or CPT (10 μM) for 24 h and observed by confocal microscopy. The nuclei were stained with DAPI, and representative confocal microscopy images were merged. B) Immunoblotting of Top1mt in HS-CD4 T cells treated with CPT (5 μM or 10μM) or DMSO for 24 h, 48 h or 72 h. C) Immunoblotting and summary data of Top1mt in HS-CD4 T cells treated with 10μM CPT or DMSO for 24 h. Representative images and summary data from independent experiments (n=4) are shown. D) Top1 activity and summary data in mitochondria and cytosol in HS-CD4 T cells treated with CPT (5 μM) or DMSO for 24 h, determined by Top1 activity assay (n=7). E) Immunoblotting and summary data of Top1cc in mtDNA from HS-CD4 T cells exposed to CPT (5 μM) or DMSO for 48 h (n=8). F) Immunoblotting and summary data of PARP1 in mitochondria from HS-CD4 T cells treated to CPT (5 μM) or DMSO for 48 h (n=7). G) mtDNA copy numbers relative to nuclear DNA were determined by real-time RT-PCR in HS-CD4 treated with CPT (5 μM) or DMSO for 24 h (n=8).

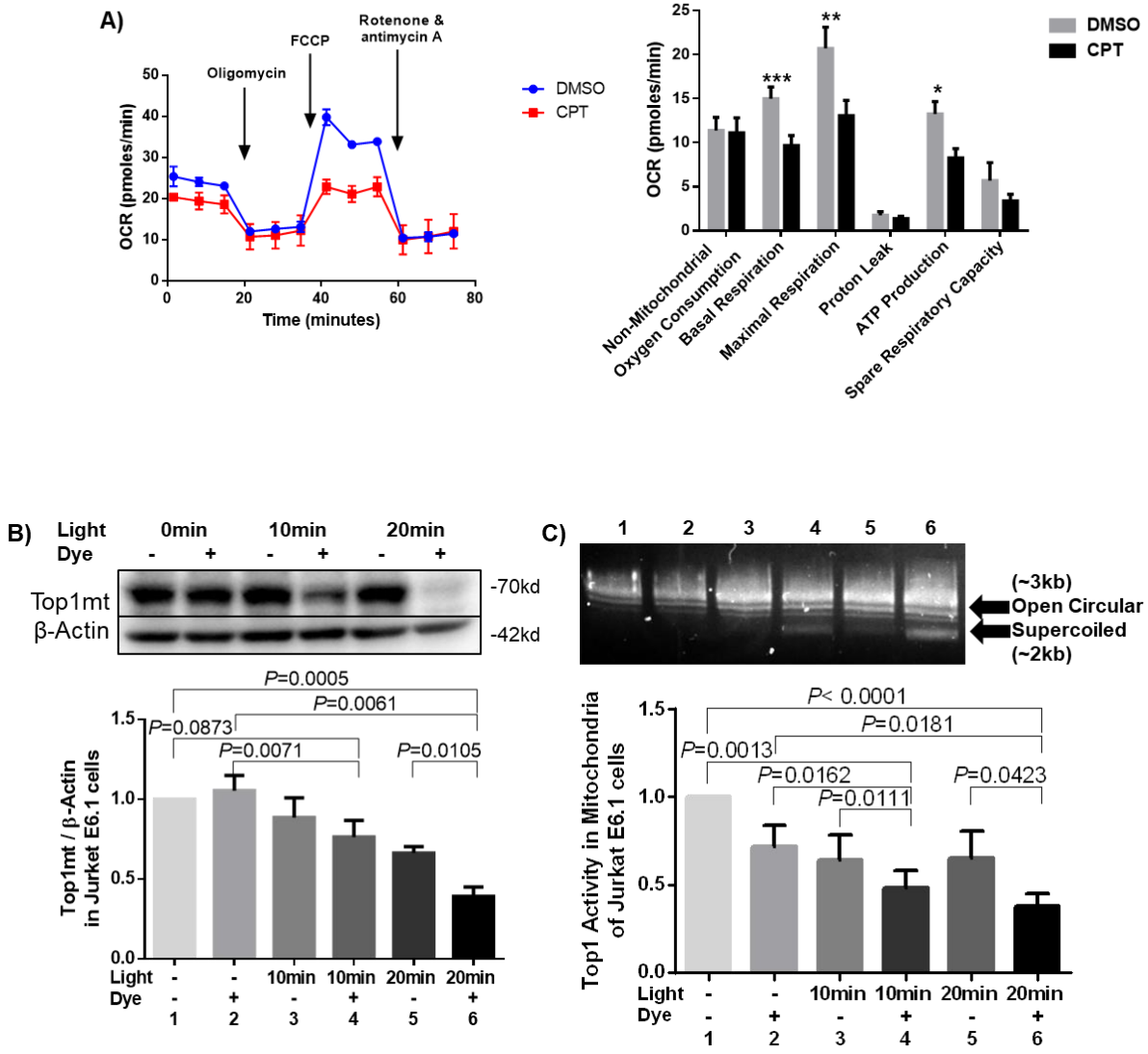
Top1 Inhibition in Healthy CD4 T Cells Induces Mitochondrial Oxidative Stress and Metabolic Dysfunctions

We next assessed whether CPT-induced Top1 inhibition affects T cell metabolic fitness. First, we evaluated mitochondrial function by measuring oxygen consumption rate (OCR) and ATP production in CPT-treated healthy CD4 T cells using Seahorse MitoStress Tests. In line with the presence of topological mtDNA damage, CPT-treated cells exhibited an impaired

mitochondrial function, as evidenced by the significant decrease in the levels of basal and maximal OCR and ATP production compared with cells treated with DMSO (**Fig. 3.3A**).

We have recently used a new chemoptogenetic method that can produce singlet oxygen ($^1O_2$) at specific organelles in CD4 T cells to show that oxidative stress promotes dual damage to mitochondria and telomeres in human T cells[12]. To exclude the possibility of a non-specific or cytotoxic effect by CPT drug treatment, we employed this innovative chemoptogenetic tool to specifically target and induce oxidative stress in mitochondria, followed by examining mitochondrial Top1 protein level and enzymatic activity. This method employs a specific cellular protein tagging with fluorescent mCerulean (mCer) and fluorogenic-activating peptide (FAP) - to detect the expression of the fusion protein in a specific cellular compartment[42,43]. FAPs have a high affinity to di-iodinated malachite green (MG2I, a photosensitizer dye), which produces singlet oxygen ($^1O_2$) only upon FAP binding and excitation with a light-emitting diode, thus triggering on-target oxidative damage without causing off-target injury[42–44]. As shown in **Fig. 3.3B** and **Fig. 3.S2C**, Top1mt and Top1nc levels were significantly reduced in a time-dependent manner in mitochondria of CD4 T cells following the exposure to light and MG2I dye compared with cells without these treatments. Also, Top1 enzymatic activity was significantly reduced in the mitochondria following the treatment with light plus dye compared with light or dye alone, or in cells without any treatment (**Fig. 3.3C**). Interferon regulatory factor 3 (IRF3), a transcriptional factor, plays a key role in innate responses against viral infection. Previous studies show that IRF3 is phosphorylated and activated by the Serine/threonine-protein kinase TBK1 mediated by innate immune adaptor proteins MAVS, STING and TRIF, thereby driving the production of type-I interferons[45]IRF3 was significantly inhibited in mitochondria (**Fig. 3.3D**), whereas cleaved PARP1, but not un-cleaved PARP1, was increased by this treatment

(**Fig. 3.3E**), indicating failed DNA damage repair and impaired cellular responses. These findings, in conjunction with our recent report showing mitochondrial dysfunction and cellular apoptosis following the same treatment[12], suggest that Top1 inhibition plays an important role in mitochondrial compromise and cell dysfunction.



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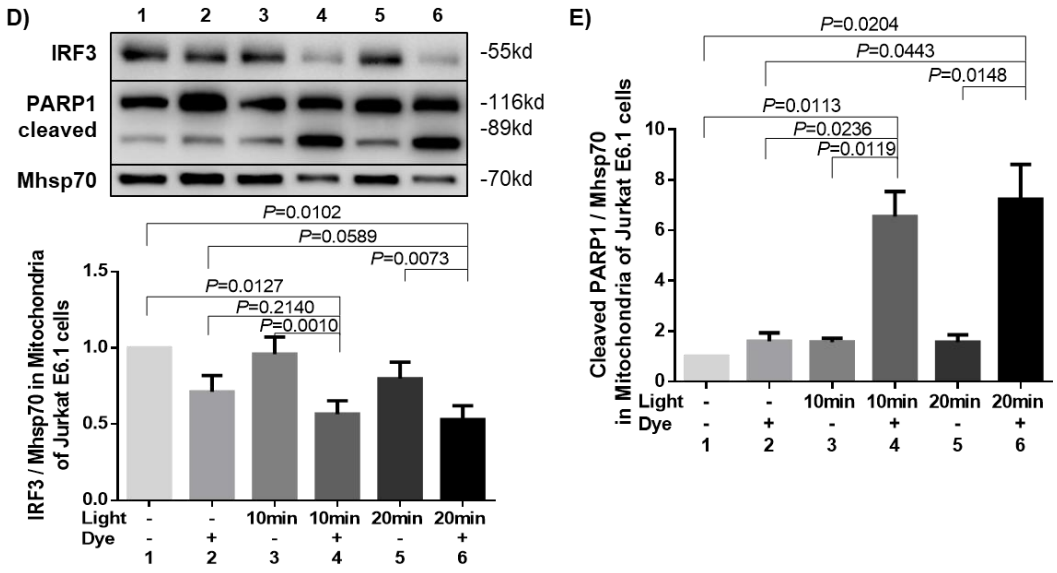

Fig.3.3. Top1 inhibition by CPT or cellular oxidative stress induces mitochondrial dysfunction in healthy CD4 T cells. A) Oxygen consumption rate (OCR) for non-mitochondrial oxygen consumption, basal respiration, maximal respiration, proton leak, ATP production, and spare respiratory capacity in HS-CD4 T cells treated with CPT (5 μM) or DMSO for 48 h. Representative images and summary data from independent experiments are shown. (n=7). B) Western blot analysis of Top1mt levels (normalized to β-Actin) in E6-1 cells transfected with /mito-FAP-mCer3 cells and treated with or without MG2I dye plus light for 10-20 min (n=5). C) Top1 enzymatic activity in mitochondria from E6-1/mito-FAP-mCer3 cells treated with or without MG2I dye plus light for 10 or 20 min (n=8). D) Representative Western blots and summary data of IRF3, and representative data of total PARP1/cleaved PARP1 levels in mitochondria from E6-1/mito-FAP-mCer3 transfected cells treated with or without MG2I dye plus light for 10 or 20 min (n=6). E) Summarized western blotting data of total PARP1, and cleaved PARP1 levels in mitochondria from E6-1/mito-FAP-mCer3 transfected cells treated with or without MG2I dye plus light for 10 or 20 min (n=4).

Top1 Inhibition in Healthy CD4 T Cells by CPT Induces Cell Dysfunctions via Disrupting The cGAS-STING Pathway

cGAS can sense DNA damage and trigger immune reactions via activating the adaptor protein STING on the endoplasmic reticulum (ER) surface[46–48]. STING then activates the transcription factors IRF3 and NF-κB, which translocate into the nucleus to turn on the

transcription of inflammatory cytokines[49,50]. Thus, DNA damage can trigger cGAS-STING activation, and the cGAS-STING pathway can link DNA damage to inflammation, cell senescence and dysfunction[46–50].

To investigate the mechanism involved in the CPT-induced topological DNA damage and cellular dysfunction, we examined the expression levels of cGAS/STING-related signaling molecules using quantitative RT-PCR. As shown in **Fig. 3.4A**, the levels of *cgas, sting1*, and *cxcl10* mRNA remained unchanged, whereas the levels of *icam1*, *csf2*, *il6*, and *il8* mRNA significantly decreased, and the levels of the pro-apoptotic *bax* mRNA significantly increased in healthy CD4 T cells treated with CPT compared with DMSO. We then measured the protein levels of these cytokines using supernatants of CD4 T cells exposed to CPT or DMSO for 2-3 days. As shown in **Fig. 3.4B**, IL-8 was significantly down-regulated, whereas TNFα was significantly upregulated in the CPT-treated CD4 T cells. We also measured intracellular IL-2 and IFN-γ expression by flow cytometry in CD4 T cells exposed to CPT or DMSO for 3 h, 6 h, or 48 h in following stimulation with PMA/ionomycin/brefeldin A for 4 h. Notably, both cytokines were significantly down-regulated in the CPT-treated CD4 T cells (**Fig. 3.4C**).

DNA damage dysregulates cell functions and triggers cell apoptosis by activating the cGAS/STING pathway[46–48]. Upon sensing nuclear or mitochondrial DNA damages, cGAS is recruited to the damaged DNA and activate STING via phosphorylation, leading to STING conformational changes to form transmembrane homodimers, which subsequently translocate from the ER to the Golgi apparatus[46–50]. This process is thought to free STING to activate TANK-binding kinase 1 (TBK1) and IRF3 via a phosphorylation-dependent mechanism[46–50]. Because cGAS and STING mRNA levels remained unchanged in CPT-treated healthy CD4 T cells (**Fig. 3.4A**), we assessed the protein levels of cGAS-STING-related signaling molecules in

CD4 T cells exposed to CPT (2-5 μM) or DMSO for 24 h using Western blotting. As shown in **Fig. 3.4D**, Top1 protein levels were consistently decreased in CPT-treated CD4 T cells. Interestingly, while cGAS protein was slightly changed, phosphorylation of STING (pSTING^{Ser366}) was significantly inhibited, and total STING levels remained unchanged in the CPT-treated cells. Correspondingly, the levels of pTBK1^{Ser172}, but not total TBK1, decreased in the CPT-treated healthy CD4 T cells. In addition, the levels of pIRF3^{Ser396} and total IRF3 expression were decreased in the CPT-treated cells. These results demonstrated that CPT inhibits the STING-related signaling molecules in healthy CD4 T cells.

Because CPT treatment in healthy CD4 T cells induced significant mtDNA damage, we asked whether cGAS is recruited to the mitochondria upon CPT-induced mtDNA damage. Confocal microscopy examination revealed that, while the cGAS signal (green) was slightly affected by the CPT treatment, its co-localization (yellow) with the mitochondrial marker mt (red) was increased compared with the DMSO control treatment (**Fig. 3.4E**). We then examined cGAS levels in the mitochondrial and cytosolic extracts. Interestingly, levels of cGAS protein were significantly higher in the mitochondrial fraction (**Fig. 3.4F**), but decreased in the cytosolic fraction (**Fig. 3.4G**) in CPT-treated healthy CD4 T cells, indicating cGAS translocation from the cytosol to mitochondria that are undergoing mtDNA damage. These novel findings - accumulation of cGAS in mitochondria with mtDNA damage and loss of cGAS in the cytosol - may explain why the STING-related signaling molecules were phosphorylated in the CPT-treated CD4 T cells.

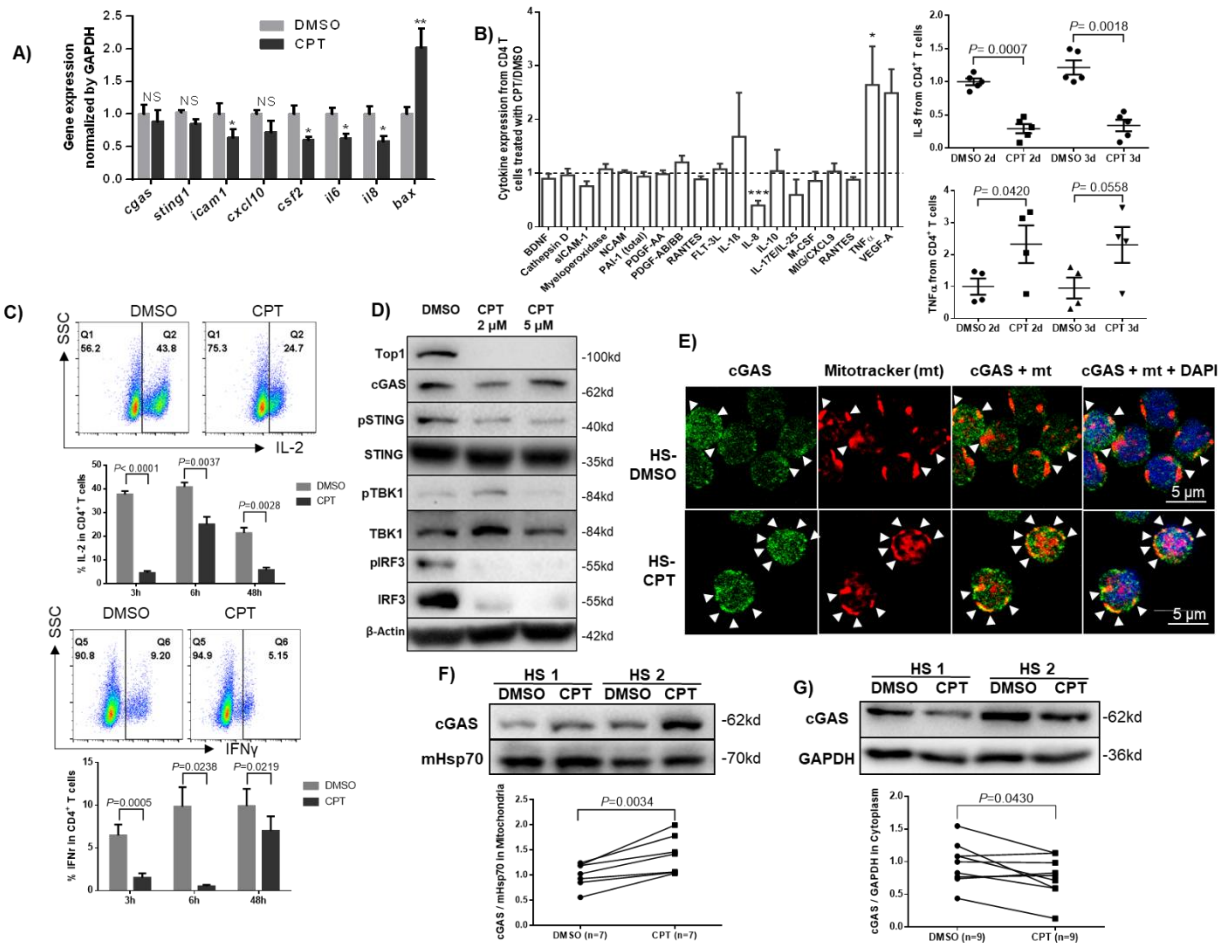

Fig.3.4. Top1 inhibition by CPT induces cell dysfunctions in healthy CD4 T cells via dysregulating the cGAS-STING pathway. A) mRNA levels of cGAS/STING-related signaling genes were determined by real-time RT-qPCR in HS-CD4 T cells treated with CPT (5 μM) or DMSO for 24 h (n=9). B) Cytokine array profile in cultured media of HS-CD4 T cells treated with CPT (5 μM) or DMSO for 48 h. Summary cytokine array data of IL-8 and TNFα in cultured media of HS-CD4 T cells treated with CPT (5 μM) or DMSO for 48 h and 72 h (n=5). C) Flow cytometry analysis of IL-2 and IFN-γ expression in HS-CD4 T cells treated with CPT (5 μM) or DMSO for 3 h, 6 h, and 48 h (n=4). D) Protein levels of cGAS-STING-related signaling genes in HS-CD4 T cells treated with CPT (2 μM or 5 μM) or DMSO for 24 h, determined by Western blot. E) Confocal microscopy images showing cGAS (Greengreen) and MitoTracker (Redmt, red) and their co-localization (yellow) in HS-CD4 T cells treated with CPT (5 μM) or DMSO for 24 h. F-G) Representative Western blots and summary data showing cGAS expression in the mitochondria (n=7) or cytosol (n=9) from HS-CD4 T cells treated with CPT (5 μM) or DMSO for 24 h.

Top1 Inhibition in HCV/HIV-CD4 T Cells By CPT Increases ROS Production and Enhances Apoptosis

To determine the consequences of topological mtDNA damage, we measured apoptosis in healthy CD4 T cells treated with CPT (5-10 μM) for 1, 2, and 4 days. Flow cytometry analysis showed increases in Annexin V (Av) and 7-Aminoactinomycin D (7-AAD) levels in a dose- and time-dependent manner in CPT-treated healthy CD4 T cells compared with DMSO control treatment (**Fig. 3.5A**). We have previously shown that dysfunctional CD4 T cells with mitochondrial compromise produce more ROS, which promotes cell apoptosis during chronic HCV or HIV infections[8,9,15,16]. Thus, we used flow cytometry to measure mitochondrial ROS production in CD4 T cells from HCV- and HIV-infected patients and HS following exposure to CPT. Mitochondrial ROS (MitoSOX) production significantly increased in CPT-treated HS-CD4 T cells (**Fig. 3.5B**), while total cellular ROS (CellROX) production significantly increased in CD4 T cells from HS as well as HCV or HIV patients following CPT treatment (**Fig. 3.5C**). Additionally, CPT-treated CD4 T cells from HCV or HIV patients produced higher levels of cellular ROS compared with those from HS.

Since CD4 T cells from HCV or HIV patients are deficient in mitochondrial Top1 protein and enzymatic activity and exhibit more mtDNA damage, cell dysfunction, and ROS production, we asked whether these cells would be more susceptible to CPT-induced apoptosis. We thus assessed apoptosis in CPT-treated CD4 T cells from HCV or HIV patients and compared it with HS. As shown in **Fig. 3.5D**, CPT-treated CD4 T cells from HCV or HIV patients exhibited higher rates of early (Av^+), late ($7\text{-}AAD^+$), and total (Av^+ $7\text{-}AAD^+$) apoptotic cell death compared with HS-CD4 T cells. These results suggest that the topological mtDNA damage caused by Top1 inhibition, if left unrepaired, may promote cellular ROS production and trigger

cell apoptosis and that CD4 T cells from HCV or HIV patients are more susceptible to cell death at least in part due to excess ROS production.

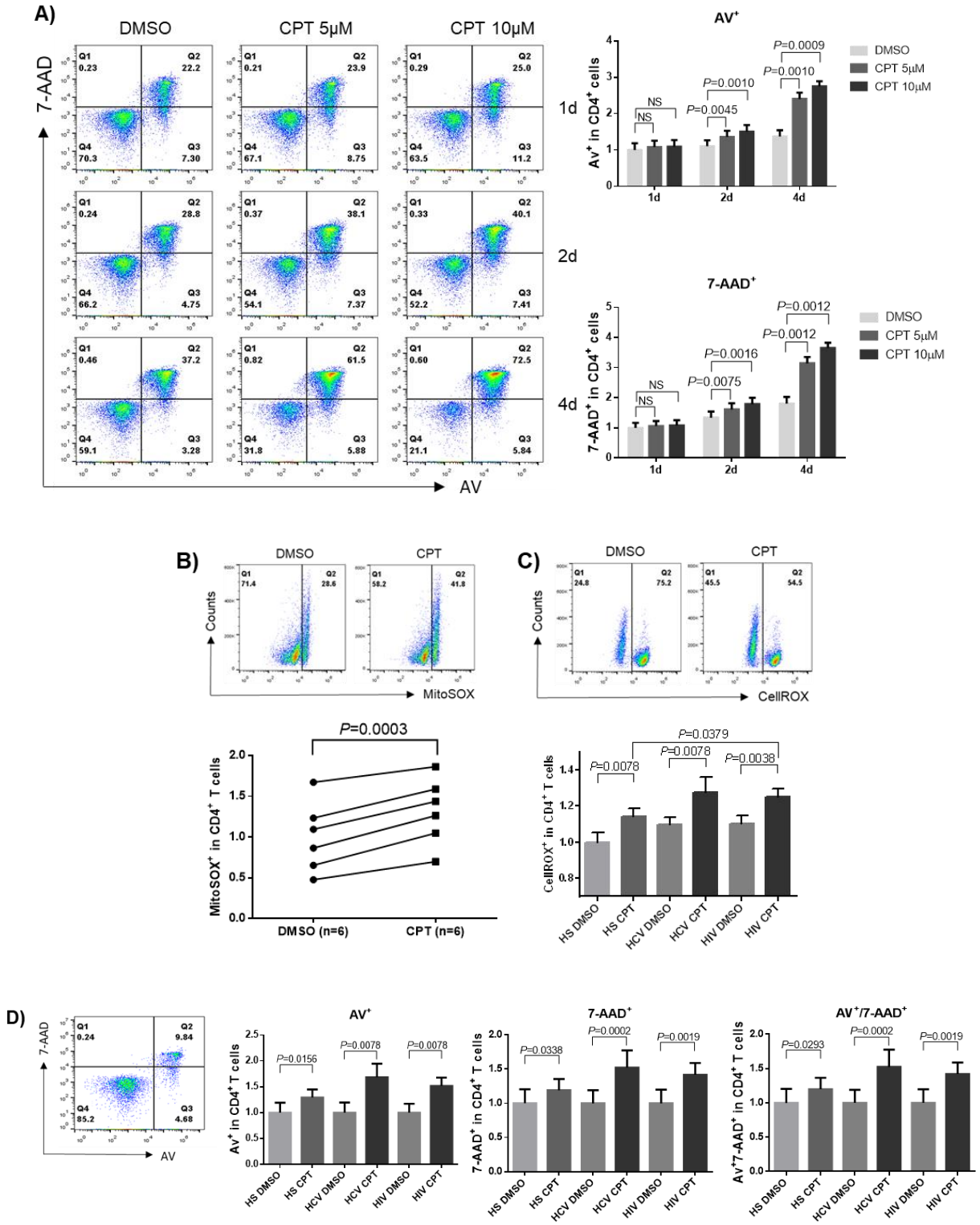

Fig.3.5. CD4 T cells from HCV- and HIV-infected patients are more susceptible to CPT-induced apoptosis than healthy CD4 T cells. A) Representative dot plots and summary data of Av and 7-AAD staining in HS-CD4 T cells treated with CPT (5 or 10 μM) for 1 d, 2 d, and 4 d, determined by flow cytometry (n=6). B) Normalized MitoSOX production in CD4 T cells from HS treated with CPT (5 μM) or DMSO for 48 h. MitoSOX was determined by flow cytometry (n=6). C) Cellular Normalized cellular ROS production in CD4 T cells from HCV and HIV patients, and HS that were treated with CPT (5 or 10 μM) or DMSO for 48 h. CellRox was determined by flow cytometry (n=8). D) Flow cytometry gating strategy and summary normalized data of Av and 7-AAD staining in CD4 T cells from HS, HCV patients and HIV patients that were treated with CPT (5 μM) or DMSO for 24 h (n=8).

Top1 Inhibition in Healthy CD4 T Cells by CPT Induces Cell Death via Multiple Signaling Pathways

CD4 T cell depletion is a hallmark of untreated HIV infection. We have previously reported that HIV infection induces CD4 T cell death through multiple death-related signaling pathways[4,5]. To elucidate the underlying mechanisms of CPT-induced CD4 T cell death, we examined alterations in cell death-related signaling molecules involved in cell apoptosis, pyroptosis, and ferroptosis using flow cytometry. Specifically, caspase-3 is activated by extrinsic and intrinsic pathways and plays a central role in cell apoptosis[51]. To determine whether apoptosis contributes to CD4 T cell depletion, we measured caspase-3 levels in healthy CD4 T cells exposed to CPT or DMSO for 24 h. Consistent with the increases in the levels of Av/7-AAD staining, caspase-3 levels were remarkably increased following CPT treatment compared with the DMSO control treatment (**Fig. 3.6A**), indicating that apoptosis plays a role in the CD4 T cell depletion that is triggered by Top1 inhibition.

Pyroptosis is a form of programmed cell death that occurs due to inflammation caused by infection with intracellular pathogens, such as viruses[52]. Pyroptosis requires the activation of caspase-1, which increases the secretion of IL-1β and IL-18 cytokines. When immune cells recognize viral infection, they release pro-inflammatory cytokines and then die by pyroptosis.

The cytokines released from pyroptotic cells promote injury of the surrounding bystander cells - a process that causes CD4 T cell depletion during HIV infection[53,54]. Similar to caspase-3, caspase-1 was significantly upregulated in CPT-treated healthy CD4 T cells compared with the DMSO control (**Fig. 3.6B**), suggesting that Top1 inhibition also promotes CD4 T cell depletion via pyroptosis.

Ferroptosis is an iron-dependent regulated cell death caused by the lethal accumulation of lipid-based reactive oxygen species (ROS)[55]. Glutathione peroxidase 4 (GPX4), as a lipid repair enzyme that converts lipid peroxides into non-toxic lipid alcohols, is the primary cellular mechanism of protection against ferroptosis (Ref 53). Another parallel protective pathway against ferroptosis is the oxidoreductase FSP1, which functions as an oxidoreductase to reduce non-mitochondrial coenzyme Q 10 (CoQ), thereby generates a potent lipophilic antioxidant to suppress the distribution of lipid peroxides[56,57].

To determine whether ferroptosis plays a role in CD4 T cell death due to Top1 inhibition, we measured GPX4 expression in healthy CD4 T cells exposed to CPT or DMSO for 24 h. GPX4 levels were decreased, indicating increased ferroptosis following CPT treatment (**Fig. 3.6C**). Taken together, these data demonstrate that Top1 deficiency in healthy CD4 T cells promotes programmed cell death via enhancing cell apoptosis, pyroptosis and ferroptosis.

To elucidate the mechanisms involved in the CD4 T cell programmed death following Top1 inhibition, we assessed the expression levels of pro- and anti-apoptotic proteins in CPT-treated healthy CD4 T cells using flow cytometry. Notably, BAX and BAD proteins are pro-apoptotic[58], whereas phosphorylated BAD ($pBAD^{Ser136}$) is anti-apoptotic - because canonic anti-apoptotic proteins such as BCL-2/BCL-XL form heterodimers with dephosphorylated BAD and triggers BAX-mediated apoptosis. When BAD is phosphorylated by AKT, BCL-2 is released and

inhibits the BAX-triggered apoptosis[59]. We observed a decrease in pBAD protein in CPT-treated cells (**Fig. 3.6D**). Moreover, we discovered that Survivin (survivin) and Mcl1 (two important anti-apoptotic proteins[60,61]) were remarkably downregulated in CPT-treated cells (**Fig. 3.6E-F**).

DNA damage responses (DDR) trigger specific signaling pathways that induce cell death, and the receptor-interacting protein kinase 1 and receptor-interacting serine/threonine-protein kinase 3 (RIPK1 and RIPK3) are crucial adaptor kinases at the crossroads of cell death[62]. Factors promoting survival and death compete with each other until one eventually dominates and dictates the cell fate[62]. We found that levels of both RIPK1 and RIPK3 were significantly decreased in healthy CD4 T cells following exposure to CPT compared with DMSO control treatment (**Fig. 3.6G-H**). Taken together, the above results suggest that multiple signaling pathways are involved in the CD4 T cell death caused by Top1 inhibition.

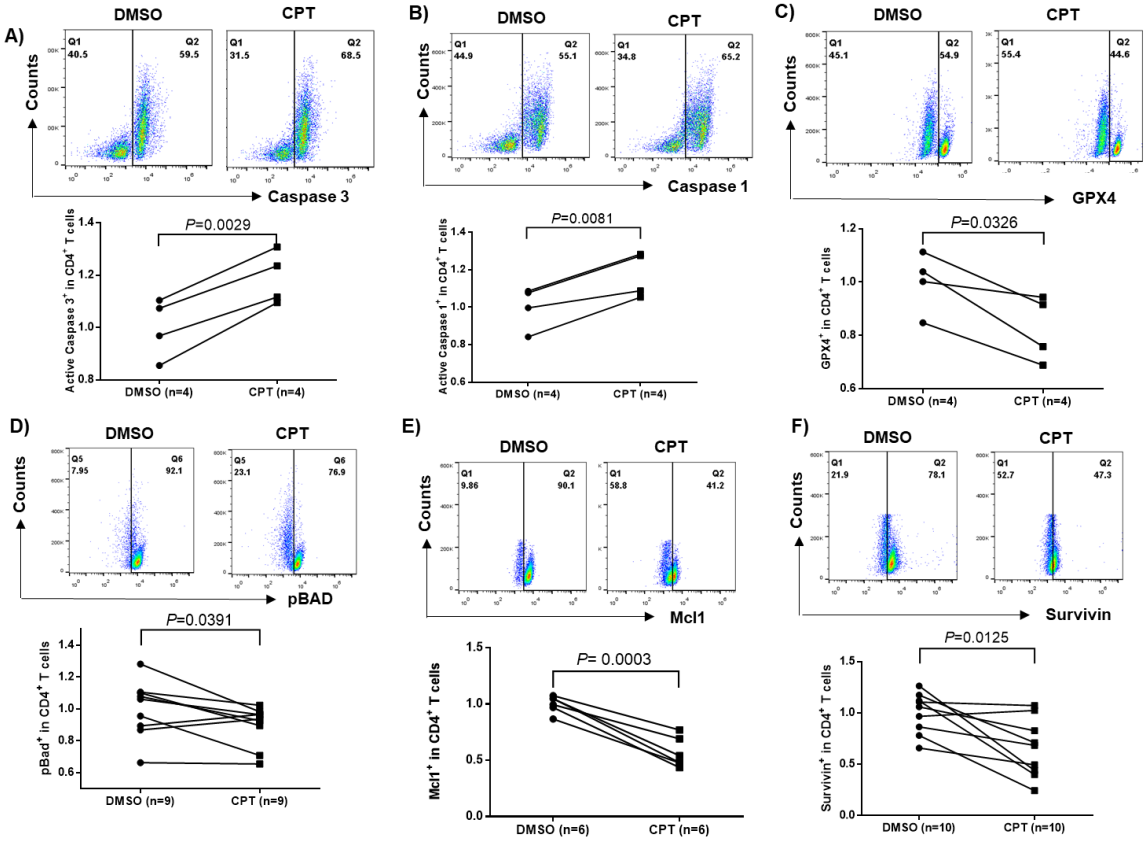

Fig.3.6 (continue)

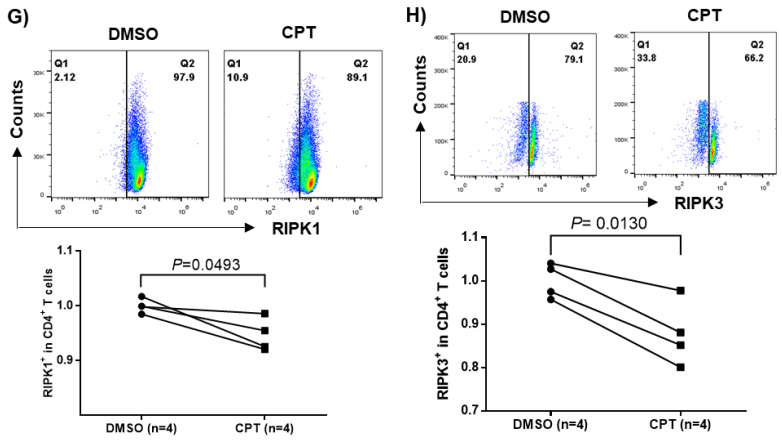

Fig.3.6. Top1 inhibition by CPT induces programmed cell death in healthy CD4 T cells via multiple cell death pathways. Healthy CD4 T cells were treated with CPT (5 μM) or DMSO for 24 h, followed by flow cytometry analysis of the expression levels of Caspase-3 (A), Caspase-1 (B), GPX-4 (C), p-BAD (D), Mcl-1 (E), BIRC-5 (F), RIP1 (G), and RIP-3 (H).

Discussion

CD4 T cells are crucial for controlling viral infections. However, chronic HCV or HIV infection blunts CD4 T cell functions and responses to vaccines (e.g., HBV vaccine)[1]. We have recently reported that CD4 T cells from patients with chronic HCV or HIV infection age prematurely or become senescent due to telomeric and mitochondrial DNA damage[1,2,11–20,3–10]. The mechanisms underlying this DNA damage and the failure to repair it remain unknown. Because Top1 enzymatic activity is required to remove the DNA tangles that are generated during cell proliferation, and because Top1cc accumulates and becomes trapped during DNA transcription and causes Top1-linked PDBs[25–28,30,33], we speculated that the topology mitochondrial DNA is altered due to Top1mt deficiency in mitochondria, leading to mitochondrial compromise and CD4 T cell dysfunction.

In the present study, we used CD4 T cells from patients with chronic HCV or HIV infection and healthy CD4 T cells, in which Top1 is inhibited by treatment with CPT, to examine Top1 inhibition and Top1cc formation in mitochondria and identify the molecular mechanisms underlying mtDNA damage and T cell dysfunction or cell death. We found that: 1) dysfunctional CD4 T cells from HCV- and HIV-infected patients exhibit lower levels of both Top1mt and Top1nc proteins and enzymatic activity, Top1cc accumulates in mitochondria, and the decrease in Top1 protein levels is associated with the degree of CD4 T cell depletion in HIV patients (HIV-INRs vs. HIV-IRs); 2) treatment of healthy CD4 T cells with CPT results in inhibition of Top1mt and Top1nc expression and enzymatic activity, Top1cc accumulation, and T cell dysfunction and apoptosis, recapitulating the phenotype we observed in HCV- and HIV-CD4 T cells and highlighting the role of Top1 in securing mtDNA integrity and maintaining T cell function and survival; 3) the mtDNA damage caused by Top1 inhibition in primary CD4 T cells leads to mitochondrial dysfunction - a process that could be recapitulated in CD4 T cells by oxidative injury induced by an innovative tool to specifically induce oxidative reactions at mitochondria; 4) these mtDNA damages lead to the translocation of cGAS from the cytosol into mitochondria, resulting in subsequent dephosphorylation or inactivation of STING signaling molecules and CD4 T cell dysfunction; 5) HCV- or HIV-CD4 T cells with Top1 inhibition are more susceptible to cell apoptosis, with excess mitochondrial and cellular ROS production; and 6) healthy CD4 T cells with Top1 inhibition undergo programmed death, triggered by multiple death signaling pathways. Taken together, these findings suggest that Top1 plays a pivotal role in preventing unwanted mtDNA damage and maintaining cell survival during chronic HCV or HIV infections.

We found that the accumulation of topological mtDNA damage affects cell survival and functions. Based on our results, we propose a model, as depicted in **Fig. 3.7**, where Top1 inhibition and Top1cc accumulation in mtDNA trigger Top1 proteolysis and Top1cc-linked PDB degradation, and thus topological mtDNA damage. Topological mtDNA damage leads to cGAS translocation from cytosol to mitochondria, resulting in dephosphorylation of STING, TBK1, IRF3 and thus inhibition of nuclear IRF3 and NF-κB-mediated gene transcription, cytokine expression, and cellular functions. This model is supported by our recent reports that deficiency of ATM (an important DNA damage and repair enzyme) during HCV or HIV infection promotes DNA damage, CD4 T cell dysfunction and apoptosis, likely due to the unrepaired DNA damage[4,5,15,16].

While Topological DNA damage can occur in replicating T cells under physiological conditions, Top1cc arises under multiple pathological conditions, such as viral (HBV, HCV, HIV, EBV, or CMV) infections, treatment with antiviral or chemotherapeutic agents, chronic inflammation, and oxidative reactions. As such, PDBs triggered by Top1cc degradation and Top1 inhibition may have a profound impact on CD4 T cell senescence, leading to the remodeling of the mtDNA damage response and reprogramming of CD4 T cell metabolism and apoptosis. These events may arise from defective removal of Top1cc and deficiency of DNA damage repair by ATM during HCV or HIV infection[15,16]. CD4 T cells are particularly prone and susceptible to Top1-mediated DNA damage and cell death due to the high rates of oxygen consumption, which enhances mitochondrial ROS production. In line with this, we have recently reported significant increases in ROS production in CD4 T cells from HCV or HIV patients[9,10].

Notably, ROS can promote Top1cc formation, causing DNA damage and ATM activation[63,64]. Although mitochondria are the cell powerhouses and a major source of ROS

production, the concept that mtDNA is particularly susceptible to the effects of ROS generated through the respiratory chain remains debated[24]. Indeed, mtDNA does not exhibit oxidative DNA damage more than nuclear DNA[23]. In contrast, we have recently found that mtDNA is more stable than nuclear and telomeric DNA in response to selective oxidative stresses[12]. Also, oxidative damage to DNA is repaired more efficiently in mitochondria than in the nucleus[25]. The mtDNA is packaged with protective proteins as the nuclear proteins that assemble the chromosomal structure[26]. Additionally, mitochondria possess an exclusive mechanism to maintain mtDNA integrity and stability via degrading the damaged mtDNA, followed by duplicating the repaired mtDNA. This mechanism does not exist in the nucleus and can only occur when multiple copies of mtDNA are present in mitochondria[27].

The cGAS-STING pathway is usually activated via sensing the damaged DNA in cytosol (which leaks from the nucleus) and plays an important role in regulating cell function and survival[46–50]. Intriguingly, our results indicate a unique ability of mtDNA (which are multiple, small circular DNAs and more stable relative to nuclear DNA) to recruit cGAS from the cytosol to mitochondria upon damage by Top1 inhibition, negatively affecting STING phosphorylation and its associated downstream signaling pathways in the ER and Golgi apparatus. In addition, we found that not only is Top1mt inhibited, but Top1nc is also located and inhibited in mitochondria in our cell model systems. This novel finding involving cCAS mitochondrial translocation and Top1nc mitochondrial localization and inhibition in primary CD4 T cells by CPT are significant. Given its novelty, we specifically used several experimental approaches to examine mitochondrial, cytosol, and nuclear fractions, and consistently detected Top1nc in the mitochondrial compartment. We believe that Top1nc is not exclusive to the cell nucleus and Top1mt is subject to CPT inhibition.

CPT induced a topological mtDNA damage event that is different from the nuclear DNA damage - which usually leaks into the cytosol to activate the cGAS-STING pathway and induce inflammatory responses[46–50]. Topological mtDNA damage may also activate the ATM pathway[4,5,15,16,64], and the outcome of mtDNA repair with mutation insertions may cause alterations in mtDNA encoding some mitochondrial proteins, which may affect the cell metabolism and/or fitness. If mtDNA damage remains unrepaired, it can activate multiple cell death signaling pathways, leading to programmed cell death. Hence, our study reveals a new model in which mtDNA topological damage contributes to the CD4 T cell aging, dysfunction, and apoptosis that occur during HCV or HIV infection.

Although study focused on HCV or HIV infections, our findings may be extended to other chronic viral infections, such as EBV and CMV. These viral infections lead to phenotypes similar to HCV or HIV infections; for example, T cell exhaustion and senescence. As observed in CD4 T cells, we expect to see similar changes in mtDNA topology in CD8 T cells, which often exhibit an even more exhausted and senescent phenotype. Importantly, topological DNA damage and repair may function as a double-edged sword, resulting in cell death during acute infection and immune tolerance during chronic infection. Nonetheless, this study reveals an important role for Top1 inhibition in promoting mitochondrial dysfunction and highlight the molecular aspects of immunomodulation in CD4 T cells that are caused by chronic HCV or HIV infections. This study also provides a potential strategy to restore impairment in mtDNA topology as a means to improve CD4 T cell functions during human viral diseases.

In summary, we showed that HCV or HIV infection inhibits Top1mt and Top1nc enzymes in CD4 T cells, leading to Top1cc entrapment in mtDNA, topological mtDNA damage, mitochondrial compromise, and T cell dysfunctions. Notably, we reproduced this mtDNA

disruption and T cell dysregulation phenotypes in healthy CD4 T cells *ex vivo* via CPT-induced inhibition of Top1 proteins. Notably, Top1nc is only found in the nucleus in mouse fibroblasts[65]. In addition, CPT targets Top1nc rather than Top1mt in yeast cells[66]. Our results showed that Top1nc is also localized in mitochondria in human CD4 T cells. Thus far, the function of Top1nc in the mitochondria of human CD4 T cells is unclear. It is also unclear whether the inhibition of Top1mt we observed in healthy CD4 T cells is due to a direct effect of CPT on Top1mt or due to CPT targeting Top1nc and causing DDR and cell death, which indirectly impacts Top1mt. In addition, since CPT treatment induce both Top1mt and Top1nc inhibition, the overexpression of Top1mt in HIV or HCV patient CD4 T cells and knockdown of Top1nc in healthy CD4 T cells need to be done to separate their effects on regulating topological DNA damage and T cell dysfunction. Thus, our study uncovers a novel mechanism for immunomodulation by viral infections, i.e., dysregulation of mtDNA topology and T cell functions. Thus, restoring mtDNA topology during chronic viral infection may rescue CD4 T cell functions.

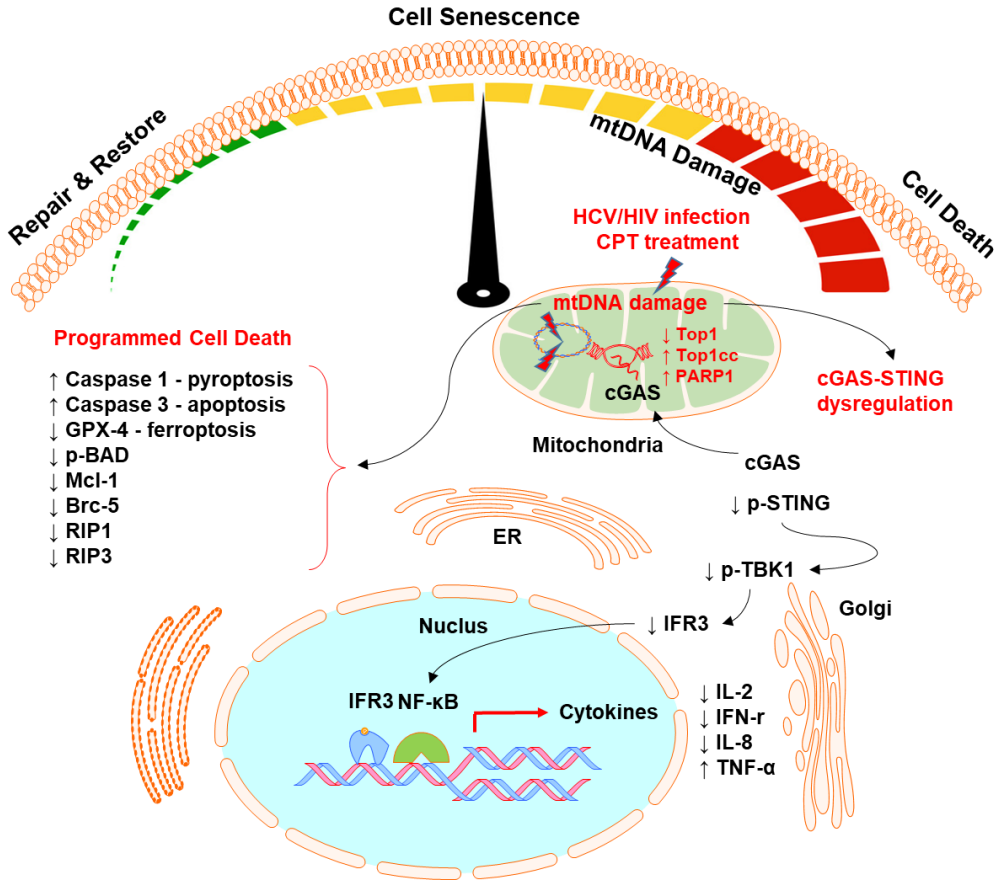

Fig.3.7. A working model for Top1-mediated topological DNA damage and T cell dysfunction. HCV/HIV infection or CPT treatment in healthy CD4 T cells can inhibit Top1 protein expression and enzymatic activity, leading to the accumulation of Top1cc and causing topological mtDNA damage, cell senescence, and programmed cell death through multiple signaling pathways. This topological mtDNA damage promotes cGAS translocation from cytosol to mitochondria, leading to dephosphorylation of STING, TBK1, IRF3, and thus inhibition of nuclear IRF3 and NF-κB-mediated gene transcriptions and cytokine expression. Topological mtDNA damage can also trigger multiple cell death or survival-related signaling pathways, leading to programmed cell death by apoptosis, pyroptosis, and ferroptosis. This regulatory cascade represents a novel molecular mechanism that underlies CD4 T cell senescence and dysfunction, which contribute to viral persistence and vaccine non-responsiveness during chronic HCV or HIV infection.

Materials and Methods

Study Subjects

The study protocol was approved by the joint institutional review board (IRB) of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson

City, TN). The study included three groups: 64 chronic HCV patients without antiretroviral therapy (ART); 83 HIV patients on ART) with undetectable HIV-RNA; and 76 age-matched healthy subjects (HS). HS blood samples were provided by BioIVT (Gray, TN) and were negative for HCV or HIV infections. The characteristics of the subjects enrolled in this study are described in **Table 3.1**.

Table 3.1. Demographic Characteristics of the Study Participants

Subjects	n	Age (Mean)	Gender (M/F)	Viral load and other characteristics
HCV	64	48	44/20	17,000-9,980,000 IU/ml, 44 GT1, 12 GT2, 8 GT3
HIV	83	43	72/11	All on ART with undetectable HIV-RNA
HS	76	41	56/20	All tested negative for HCV, HBV, and HIV

Cell Isolation and Culture

Mononuclear cells (PBMCs) were isolated from peripheral blood by Ficoll density centrifugation (Cat# 45-001-750, GE Healthcare, Piscataway, NJ). CD4 T cells were isolated from PBMCs using the CD4 T cell negative selection kit (Cat# 130-096-533, Miltenyi Biotec, Auburn, CA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Cat# S11050H, Atlanta Biologicals, Flowery Branch, GA), 100 IU/ml penicillin, and 2 mM L-glutamine (Cat# 25-030-081, Thermo Fisher Scientific, Waltham, MA) and maintained at 37°C in 5% CO_2 incubator.

Confocal Microscopy

$1x10^6$ CD4 T cells were stained with fluorochrome conjugated antibodies using a previously described method[12]. For Top1/MitoTracker staining, HS CD4 T cells were treated with 10μM CPT (Cat# SKU: TG4110, TopoGEN, Buena Vista, CO) or DMSO (Cat# D2650, Sigma-Aldrich, St. Louis, MO) for 2 days. For cGAS/MitoTracker staining, HS-CD4 T cells were treated

with 5μM CPT for 2 days. The primary antibodies included MitoTracker (Cat# M22425, Thermo Fisher Scientific, Waltham, MA), Rabbit anti-cGAS (Cat# 79978, Cell Signaling Technology, Danvers, MA), Rabbit anti-Top1mt (Cat# PA5-51660, Thermo Fisher Scientific, Waltham, MA) and mouse anti-Top1nc conjugated with Alexa Fluor 488 (Cat# ab223421, Abcam, Cambridge, MA). The secondary antibody included anti-rabbit IgG-Alexa Fluor 488 (Ca# 4412, Cell Signaling Technology). The cells were mounted with DAPI Fluoromount-G (Cat# D1306, SouthernBiotech, Birmingham, AL) and visualized with a confocal laser scanning inverted microscope (Leica Confocal, Model TCS sp8, Germany).

Isolation of Mitochondrial and Cytosolic Proteins

The Qproteome mitochondria isolation kit (Cat# 37612, Qiagen, Germantown, MD) was used to isolate and purify mitochondrial and cytosolic extracts according to the manufacturer's protocol. Briefly, approximately $5x10^6$ freshly isolated CD4 T cells were harvested and resuspended in 1 mL Lysis Buffer, followed by incubation on an end-over-end shaker for 10 min at 4°C. After centrifugation, the supernatant, which contained the cytosolic fraction was collected in a new tube and labeled as the 1st part of the cytosolic extract. Next, the pellets were resuspended in a 1.5 mL disruption buffer and centrifuged to separate nuclei and cytosol. The supernatants were centrifuged at 6000 x g, for 10 min to isolate the mitochondria in the pellet and the supernatant as the 2nd part of the cytosolic extract. To concentrate the combined cytosolic extract, an Amicon ultra-0.5 centrifugal filter unit (Cat# UFC503096, Millipore Sigma, St. Louis, MO) was used.

Western Blotting

CD4 T cells were treated with 2, 5, or 10 μM of CPT or DMSO control for different times and the mitochondrial and cytosolic extracts or whole cell lysates were lysed on ice in RIPA lysis

buffer (Cat# BP-407, Boston BioProducts, Ashland, MA) in the presence of protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail. Cat# 11836170001, Sigma). Protein concentration was measured by the Pierce BCA protein assay kit (Cat# 23225, Thermo Fisher Scientific). To obtain enough mitochondrial proteins for immunoblotting, cells were pooled from multiple subjects. Mitochondrial and cytosolic extracts and whole lysates of CD4 T cells were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk, 0.1% Tween-20 in Tris-buffered saline (TBS), and then incubated overnight with primary antibodies against Top1mt, Top1, pIRF3^{Ser396}, IRF3, PARP1, cGAS, pSTING^{Ser366}, STING, pTBK1Ser^{172}, TBK1, GAPDH, β-actin (Human-Reactive STING Pathway Antibody Sampler Kit #38866, Cell Signaling), and mHsp70 (Cat# MABS1955-100uL, Millipore Sigma). The membranes were incubated with appropriate horseradish peroxide-conjugated secondary antibodies (Anti-rabbit IgG, HRP-linked Antibody #7074, Anti-mouse IgG, HRP-linked Antibody #7076, Cell Signaling Technology, Danvers, MA), and the protein bands were developed and visualized with the Amersham ECL prime western blotting detection reagent (Cat# 45-002-401, GE Healthcare Bio-Sciences, Pittsburgh, PA). The protein bands were captured and quantified by the Chemi DocTM MP imaging system (Bio-Rad, Hercules, CA).

Top 1 Activity Assay

The enzymatic activity of Top1 was measured using the Top1 activity assay kit (Cat# TG1015–1, TopoGEN, Buena Vista, CO). Briefly, mitochondrial and cytosolic extracts were isolated from patients and HS CD4 T cells as described above. Mitochondrial or cytosolic proteins were mixed with plasmid DNA substrate and reaction buffer for 30 min at 37°C, diluted with Stopping Buffer containing protein loading dye, and electrophoresed on a 1% agarose gel for 2 h

at 5~10 V/cm. The supercoiled DNA bands were visualized with a UV transilluminator and quantified by densitometry.

Top1cc Detection

Top1cc was detected using the Human Topoisomerase ICE assay kit (Cat# TG1020–1, TopoGEN). The mtDNA purification protocol was modified by combining the ICE assay kit and the PureLink Genomic DNA Mini kit (Cat# K182001, Thermo Fisher Scientific). Briefly, mtDNA was isolated from mitochondrial extracts using extraction buffer from the ICE assay kit and then purified using purification columns from the PureLink Genomic DNA Mini kit. The DNA samples were loaded using a vacuum pump onto an NC membrane, which was incubated with primary anti-Top1cc antibody from the ICE assay kit, followed by Western blotting.

Flow Cytometry

Intracellular IL-2 and IFN-γ cytokine production, cell apoptosis assay for Av/7-AAD expression, cell death signaling molecules (Caspase-1, Caspase-3, GPX4, pBAD^{S136}, Mcl1, Survivin, RIPK1, and RIPK3), mitochondrial ROS production (MitoSOX™ Red Mitochondrial Superoxide Indicator. Cat# M36008) and cellular ROS production (CellROX™ Green Reagent, for oxidative stress detection. Cat# C10444) was determined by flow cytometry base on the guideline of the products, as described previously[5,12]. For IL-2 and IFN-γ cytokine production, antibodies anti-IL-2- FITC (Cat# 500304) and anti-IFN-γ-PE (Cat# 12-7029-42) antibodies (Biolegend, San Diego, CA) were used to stain purified HS CD4 T cells treated with DMSO or CPT for 3h, 6h, and 2 days. For apoptosis analysis, the cells were washed with DPBS and stained using PE Annexin V apoptosis detection kit I (Cat# 559763, BD Biosciences, San Jose, CA) in a 1X binding buffer according to the manufacturer's protocol. Controls for these assays included

unstained cells, isotype control antibodies, and single positive staining, which were used for gating and compensation. Samples were analyzed with a BD AccuriC6 Plus flow cytometer and FlowJo V10 software.

Cytokine Array

CD4 T cells isolated from 5 HS were cultured and treated with CPT (5 μM) or DMSO for 2 or 3 days. Approximately, 250 μl of the culture supernatants were collected for cytokine expression analysis. Briefly, Human Cytokine 48-Plex Discovery Assay (Cat# HD48) and Human Supplemental Biomarker 10-Plex Discovery Assay (Cat# HDHSB10) were performed by Eve Technologies (Calgary, AB Canada). Data were normalized to the expression levels in the DMSO-treated cells.

Seahorse XFp Cell Mito Stress Test

Seahorse XFp Cell Mito stress test (Cat# 103010-100, Agilent Technologies, Santa Clara, CA) was performed according to the manufacturer's protocol using an XFp instrument. CD4 T cells from healthy subjects were purified from PBMCs, cultured in complete RPMI-1640 medium with 10% FBS, and treated with 5 μM CPT or DMSO for 2 days. One day prior to the assay, Seahorse mini cartridges were hydrated overnight in a non-CO_2 incubator. On the day of the assay, the treated cells were seeded onto mini culture plates pre-coated for 1 h with poly-D-lysine (Thermo Fisher Scientific). Approximately 100,000 cells per well were cultured in Seahorse XF RPMI assay medium supplemented with 1.0 mM of glucose, 100 μM of pyruvate, and 1.0 mM of glutamine. The following inhibitors from the Cell Mito stress test kit were added to the culture media in this order: 2.0 μM of Oligomycin, 1.5 μM of FCCP, and 2.0 μM of Rotenone/Antimycin

A, and the related three sequential measurements were recorded. Data analysis was performed using the Seahorse Wave software and the Seahorse Mito stress test report generator.

Singlet Oxygen Induction in E6-1 Cell Line

The protocol for singlet oxygen induction in E6-1 cells and the treatment with light and dye was carried out as we previously described (Wang et al. 2021). Top1, IRF3, and total and cleaved PARP1 protein levels were measured by Western blot, and Top1 enzymatic activity was determined by the Top1a activity assay as described above.

Real-time qPCR

Mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) contents in genomic DNA were determined by real-time qPCR according to previously described methods[12]. Briefly, genomic DNA was extracted from CD4 T cells using the PureLink Genomic DNA isolation kit (Thermo Fisher Scientific). DNA concentration was measured by the Synergy H1 BioTek plate reader. The primers used for mitochondrial and nuclear DNA PCR are shown in **Table 2**. Approximately 25 ng of genomic DNA was used for the PCR reaction. The PCR cycling conditions were: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 62 °C for 60 s. The averages of mtDNA and nucDNA Cq values from triplicate reactions were calculated. The mitochondrial DNA content was determined using the following equations: $\Delta Cq = (\text{nucDNA Cq} - \text{mtDNA Cq})$; relative mitochondrial DNA content $= 2 \times 2\Delta Cq$ 30[67].

The expression of pro-apoptosis genes and the cGAS-STING-related genes was determined by real-time RT-qPCR. Total RNA was extracted from ~2×10^6 CD4 T cells treated with CPT for 48 h using the PureLink RNA Mini kit (Cat# 12183018A, Invitrogen), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Cat# 4368814, Applied

Biosystems, Foster City, CA) according to the manufacturer's instructions. The PCR reactions were performed in triplicate. The PCR primer sequences are shown in **Table 2**. The PCR conditions were the same as described above. Gene expression was calculated using the $2^{-\Delta\Delta ct}$ method, normalized to GAPDH level, and is presented as fold change.

Table 3.2. PCR Primers Used in This Study

mtDNA tRNALeu	5′-CACCCAAGAACAGGGTTTGT-3′	5′-TGGCCATGGGTATGTTGTTA-3′
nuDNA β2-microglobulin	5′-TGCTGTCTCCATGTTTGATGTATCT3′	5′- TCTCTGCTCCCCACCTCTAAGT3′
gapdh	5′-TGACGAAAGCTGATATGCAA -3′	5′-GAGCAGGAGAAACTCCATTT-3′
cgas	5′-AAGGATAGCCGCCATGTTTCT-3′	5′-TGGCTTTCAGCAAAAGTTAGG-3′
sting1	5′-AGCATTACAACAACCTGCTACG-3′	5′-GTTGGGGTCAGCCATACTCAG-3′
il6	5′-GGTACATCCTCGACGGCATCT-3′	5′-GTGCCTCTTTGCTGCTTTCAC-3′
il8	5′-AAGGAAAACTGGGTGCAGAG-3′	5′-ATTGCATCTGGCAACCCTAC-3′
icam1	5′-AGCTTCGTGTCCTGTATGGC-3′	5′-TTTTCTGGCCACGTCCAGTT-3′
cxcl10	5′-AGTGGCATTCAAGGAGTACC-3′	5′-TGATGGCCTTCGATTCTGGA-3′
csf2	5′-GCCAGCCACTACAAGCAGCAC-3′	5′CAAAGGGGATGACAAGCAGAAAG3′
ifnα	5′GTGAGGAAATACTTCCAAAGAATCAC3′	5′-TCTCATGATTTCTGCTCTGACAA-3′
ifi16	5′-GAAGTGCCAGCGTAACTCCTA-3′	5′-TACCTCAAACACCCCATTCAC-3′
bax	5′-TGGAGCTGCAGAGGATGATTG-3′	5′-CCCAGTTGAAGTTGCCGTCAG-3′

Statistics

The data were analyzed using Prism 7 software and are presented as mean ± SEM. Differences between two groups were analyzed by independent Student's t-test or paired t-test and by one-way ANOVA for multiple groups. P-values of <0.05 (or *), <0.01 (or **) and <0.001 (or ***) were considered statistically significant and very significant, respectively.

Acknowledgments and disclosures

This work was supported by National Institutes of Health grants R01AI114748, R21AI138598, R21AI157909, and R15AG069544 (to Z.Q.Y.); VA Merit Review Awards 1I01BX004281 (to Z.Q.Y) and 5I01BX005428–02 (to J.P.M.); DoD Award PR170067 (to Z.Q.Y). This publication is the result of work supported with resources and the use of facilities at the James

H. Quillen Veterans Affairs Medical Center. The contents in this publication do not represent the views of the Department of Veterans Affairs or the United States Government.

Author Contributions

X.D. and D.C. performed most of the experiments; J.Z., M.S., L.N.T.N., and S.K., performed some experiments. X.Y.W. and Y.Z. provided technical support. J.Y.Z., S.N., L.W., M.E., and J.P.M. offered intellectual input for troubleshooting and discussed the findings. Z.Q.Y. supervised the project and wrote the manuscript with the help of all other authors.

Competing interests

The authors declare no competing financial interests.

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Abbreviations

7-AAD	7-Aminoactinomycin D
AIDS	Acquired immune deficiency syndrome
AKT	Protein Kinase B

ATM	Ataxia telangiectasia mutated
ATP	Adenosine Triphosphate
Av	Annexin V
BAD	BCL-2-associated death promoter
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra-large
bp	Base pairs
cGAS	Cyclic GMP-AMP synthase
CMV	Cytomegalovirus
CPT	Camptothecin
CSF2	Granulocyte-macrophage colony-stimulating factor
CXCL10	C-X-C motif chemokine ligand 10
DAPI	4′,6-diamidino-2-phenylindole
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
FAP	Fluorogenic-activating peptide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX4	Glutathione peroxidase 4
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus

HIV-INRs	HIV immune non-responders
HIV-IRs	HIV immune responders
HS	Healthy subjects
ICAM-1	Intercellular Adhesion Molecule 1
IFI16	Gamma-interferon-inducible protein
IFN-γ	Interferon gamma
IL-18	Interleukin-18
IL-1β	Interleukin 1 beta
IL-2	Interleukin-2
IL6	Interleukin 6
IL8	Interleukin 8
IRB	Institutional review board
IRF3	Interferon regulatory factor 3
Mcl1	Induced myeloid leukemia cell differentiation protein
MG2I	Di-iodinated malachite green
mHsp70	Mitochondrial 70 kilodalton heat shock proteins
mtDNA	Mitochondrial DNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NS	No significance
OCR	Oxygen consumption rate
PARP1	Poly ADP-Ribose Polymerase 1
PBMC	Peripheral blood mononuclear cell
PDBs	Protein-DNA breaks

POT1	Protection of telomeres protein 1
RIPK1	Receptor-interacting protein
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TBS	Tris buffered saline
TDP1	Tyrosyl-DNA phosphodiesterase 1
TNFα	Tumor necrosis factor alpha
TOPcc	Topoisomerase-DNA covalent complexes
Top1 or Top1nc	DNA topoisomerase 1
Top1cc	Top1 enzyme–DNA covalent complexes
Top1mt	DNA topoisomerase I mitochondrial
TOP2α	DNA topoisomerase 2-alpha

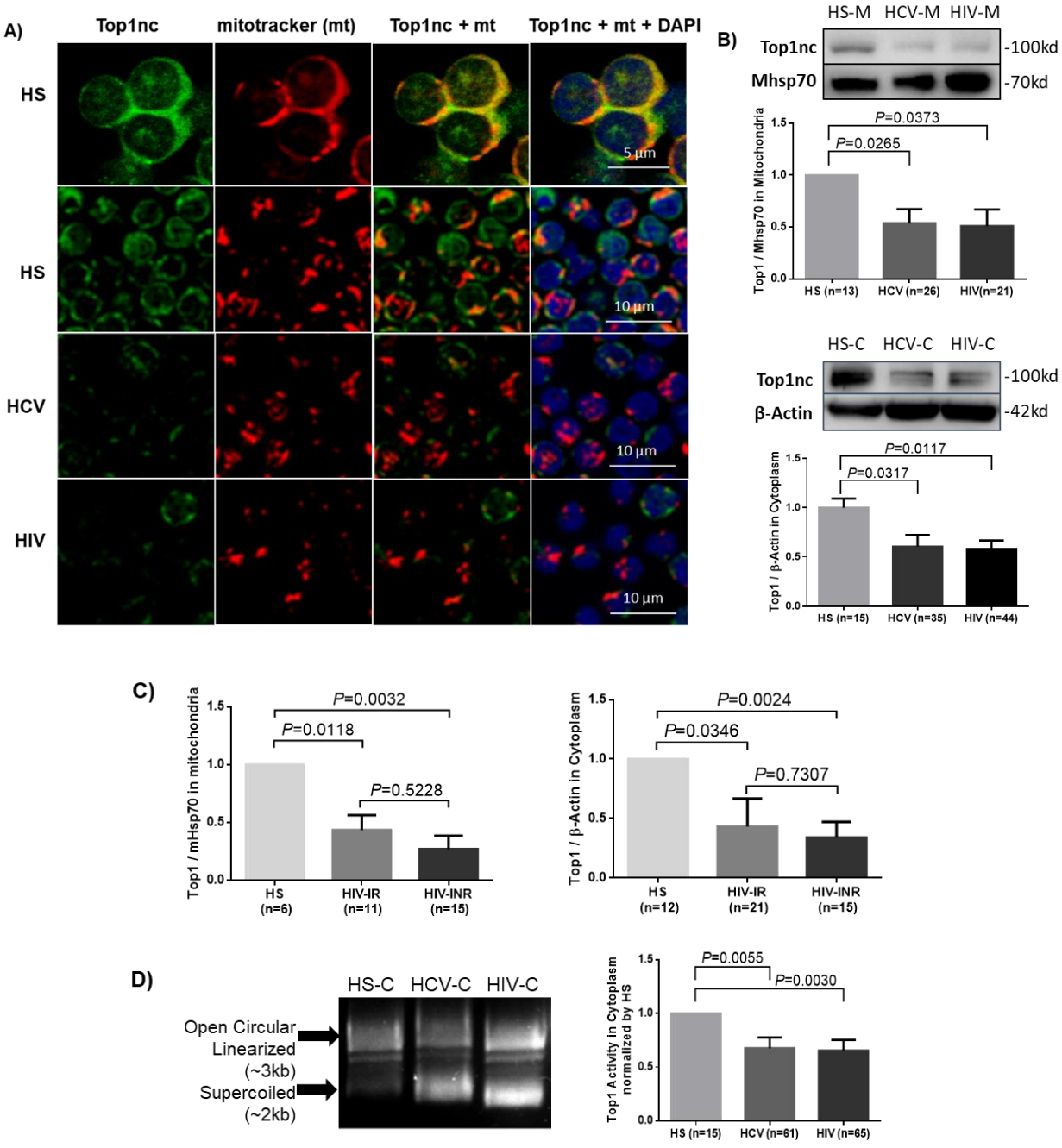

Fig.3.S1. Top1nc expression and enzymatic activity are inhibited in mitochondria and cytoplasm of CD4 T cells during chronic viral infection. A) Top1nc protein (green), MitoTracker (red), and their colocalization (yellow) in mitochondria of CD4 T cells from HCV- and HIV-infected patients and healthy subjects (HS). The nucleus was stained with DAPI, and representative confocal microscopy images were merged. The top panel shows representative

signals in HS-CD4 T cells with a smaller magnification. B) Immunoblotting of Top1nc protein in mitochondria (M) and cytoplasm (C) of CD4 T cells from HCV- and HIV-infected patients and HS. Representative blots and summary data are shown. Top1nc proteins bands were quantified and normalized to heat-shot protein 70 (Mhsp70) for mitochondria or β-actin for cytoplasm and is presented relative to HS (n = number of subjects). C) Immunoblotting of Top1nc protein in mitochondria and cytoplasm of CD4 T cells from HIV immune responders (HIV-IR; CD4 T cells > 500 cells/μL), HIV immune non-responders (HIV-INR, CD4 T cells<500 cells/μL), and HS. Summary data from 6-15 subjects are shown. D) Top 1 enzymatic activity in cytoplasm of CD4 T cells from HCV- and HIV-infected patients versus HS. Representative images and summary data of the digested supercoiled DNA substrate, resolved on 1% agarose gel, are shown.

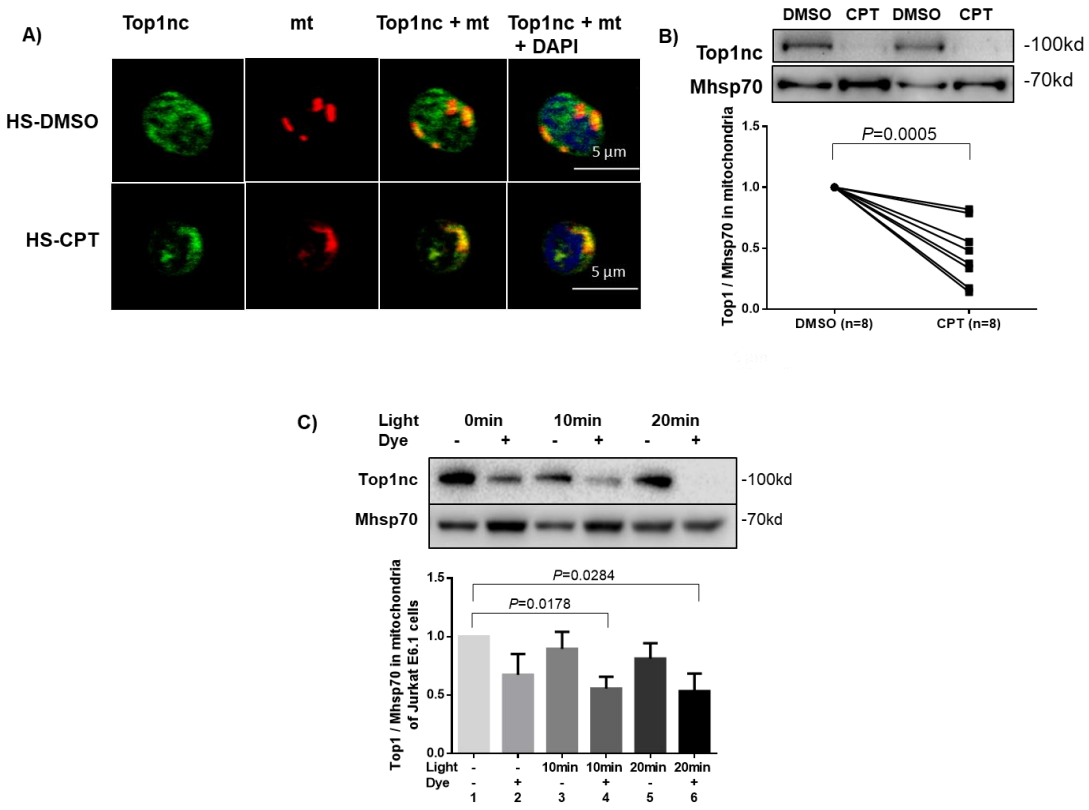

Fig.3.S2. Top1nc inhibition by CPT or cellular oxidative stress induces mitochondrial dysfunction in healthy CD4 T cells. A) Confocal microscopy images of Top1nc protein (green), MitoTracker (mt, red), and their colocalization (yellow) in mitochondria in HS-CD4 T cells treated with DMSO or CPT (5 μM) for 48 h. The nuclei were stained with DAPI, and representative images were merged. B) Immunoblotting and summary data of Top1nc in HS-CD4 T cells treated with 10 μM CPT or DMSO for 24 h. Representative blots and summary data are shown (n=8). C) Immunoblotting of Top1 protein (normalized to Mhsp70) in mitochondria from E6-1 cells transfected with /mito-FAP-mCer3 cells and treated with or without MG2I dye plus light for 10-20 min (n=10).

CHAPTER 4. SUMMARY AND FUTURE PERSPECTIVE

Summary

We and others have previously shown that chronic viral infection could cause premature T cell aging or immune senescence with accelerated telomere erosion, but the mechanisms underlying DNA damage and telomere loss remain unclear. For the first time to our knowledge, we discovered in this study that Top 2α was inhibited, and Top2cc was trapped in genomic DNA, in T cells from patients with chronic HBV, HCV, and HIV infection. We then used healthy T cells treated with ETP or ICRF-193 as a model to investigate the role and mechanisms of DNA topology in reprogramming telomeric DDR and T cell functions. We demonstrated that Top 2α inhibition caused topological DNA damage, telomere attrition, and T cell apoptosis or dysfunction by inducing Top2cc accumulation and failure in DNA repair, recapitulating T cell dysregulation in the setting of chronic viral infections. This study reveals a novel mechanism for immunomodulation by viral infections via disrupting DNA topology, telomere integrity, and T cell biology. As such, restoring the impaired DNA topologic machinery may offer a new strategy to maintain T cell function against human viral diseases.

Besides, understanding Top1mtcc triggered cellular senescence is essential to T cell immunocompetent reestablishment, as topoisomerase protein function clearly preserves mtDNA stability and cell proliferative potential. Therefore, we expect that modulation of Top1mt will reprogram mtDNA topology, remodel T cell functions, and restore vaccine responses.

The well-characterized patient cohort that we have developed over the past ten years to address this fundamental and clinically significant issue is the main resource accessible for this research. We have built a database and management system with patient information for hundreds of chronically infected people since we are prospectively collecting samples from

HBV, HCV, and HIV-infected subjects. As a result, this study is novel and strong in its concept, unique cohort and model system, and cutting-edge translational approaches to answer clinically relevant questions such as how chronic viral infection induces topoisomerases inhibition, mitochondrial defects, and T cell senescence, leading to an abnormal immune response; and, most importantly, whether reconstitution of the topoisomerases enzyme can prevent unwanted topological DNA damage and T cell senescence.

Future Perspective

First and foremost, studying the effects of topological DNA damage in T cell exhaustion and senescence and vaccine failure is a novel, unexplored area in human viral diseases. We speculate that reversing the topological damage during chronic viral infection including HBV, HCV and HIV may be a revolutionary answer of T cell exhaustion and senescence. By investigating topoisomerase protein expression and activity in HBV, HCV, and HIV subjects vs healthy subjects (HS) and by characterizing the roles and mechanisms of topoisomerases inhibition and Topcc accumulation, we can better understand how T cells are driven to undergo premature aging, leading to vaccine non-responsiveness in chronic viral infection. This study leads to an intersecting, novel field in topological DNA damage, mitochondria dysfunction, and T cell senescence concerning vaccine responses during chronic viral infection. Scientifically, characterization of the precise mechanisms regarding how topoisomerases is generated and its roles in the regulation of DNA damage as well as T cell senescence and mitochondria dysfunction are essential for improving the understanding of immune regulation during chronic viral infection. Moreover, a better understanding of the role of Topcc may provide novel therapeutic targets for immune dysfunction observed in, but not limited to, human chronic viral infection.

Second, topoisomerases are an increasingly recognized source of genome instability, but their full range of functions on DNA and RNA damages are barely understood. New directions include developing novel approaches such as RNA topology manipulation to understand how cells use and resolve knots, as well as the roles of topoisomerases in metabolism and mitochondria. Also, because topoisomerases can be potentially dangerous, it is likely that their activity is controlled and limited to specific sites of action. In this context, more research is needed to determine the molecular partners of topoisomerases within molecular machineries and how they are regulated by post-translational modifications in human T cells during chronic viral infection.

Finally, the precise location and roles of topoisomerases at centromeres and telomeres, as well as how topoisomerases interact with chromatin remodelling factors and the architecture of chromatin and chromosomes, are promising areas of research.

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APPENDIX: Abbreviations

53BP1	p53-binding protein 1
7AAD	7-Aminoactinomycin D
AMP	Adenosine Monophosphate
AIDS	Acquired immune deficiency syndrome
AKT	Protein Kinase B
ART	Antiretroviral therapy
ATM	Ataxia telangiectasia mutated
ATP	Adenosine Triphosphate
ATR	Ataxia telangiectasia Rad3-related
Av	Annexin V
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Birc-5	Baculoviral inhibitor of apoptosis repeat-containing 5
bp	Base pairs
CFSE	Carboxyfluorescein succinimidyl ester
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CMV	Cytomegalovirus

CPT	Camptothecin
CXCL10	C-X-C motif chemokine ligand 10
DAPI	4′,6-diamidino-2-phenylindole
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DSB	Double strain breaks
EBV	Epstein-Barr virus
ERCC1	DNA excision repair protein
ETP	Etoposide
FAP	Fluorogenic-activating peptide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Guanosine monophosphate
GPX-4	Glutathione peroxidase 4
H_2AX	Histone family member X
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HIV-INRs	HIV immune non-responders
HIV-IRs	HIV immune responders
HS	Healthy subjects

hTERT	Human Telomerase reverse transcriptase
HTLV-1	Human T-cell lymphotropic virus type 1
ICAM-1	Intercellular Adhesion Molecule 1
ICRF-193	4-[2-(3,5-Dioxo-1-piperazinyl)-1-methylpropyl]piperazine-2,6-dione
IFI16	Gamma-interferon-inducible protein
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IL-18	Interleukin-18
IL-1β	Interleukin 1 beta
IL-2	Interleukin-2
IRB	Institutional review board
IRF3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
mCer	Fluorescent mCerulean
Mcl-1	Induced myeloid leukemia cell differentiation protein
MFI	Mean florescence intensity
MG2I	Di-iodinated malachite green
Mhsp70	Mitochondrial 70 kilodalton heat shock proteins
miR	MicroRNA
MOMP	Mitochondrial outer membrane permeabilization
mtDNA	Mitochondrial DNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NS	No significance
p38	p38 mitogen-activated protein kinases
PARP1	Poly ADP-Ribose Polymerase 1
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PDB	Protein-DNA breaks
POT1	Protection of telomeres protein 1
RAP1	Ras-proximate-1 or Ras-related protein 1
RIP1	Receptor-interacting protein
RIP3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCAN1	Spinocerebellar ataxia with axonal neuropathy-1
SGS1	Slow growth suppressor 1
STING	Stimulator of interferon genes
SVR	Sustained virologic responses
TBK1	TANK-binding kinase 1
TBS	Tris buffered saline
TCR	T cell receptor
TDP1	Tyrosyl-DNA phosphodiesterase 1
TDP2	Tyrosyl-DNA phosphodiesterase 2
TIF	Telomere induced foci
TIN2	TERF1-interacting nuclear factor 2

TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
TOPcc	Topoisomerase-DNA covalent complexes
TOP1	DNA topoisomerase 1
Top1cc	Top1 enzyme–DNA covalent complexes
Top1mt	DNA topoisomerase I mitochondrial
Top1mtcc	Top1mt enzyme–DNA covalent complexes
TOP2α	DNA topoisomerase 2-alpha
TOP2β	DNA topoisomerase 2-beta
Top2cc	Top2 enzyme–DNA covalent complexes
TOP3α	DNA topoisomerase 3-alpha
TOP3β	DNA topoisomerase 3-beta
TPP1	Tripeptidyl-peptidase 1
TRAF	TNF receptor associated factors
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
TTRAP	TRAF and TNFreceptor-associated protein

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