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Role of ATM in T Cell Dysfunction During Chronic Viral Infections

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Role of ATM in T Cell Dysfunction During Chronic Viral Infections

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by

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ABSTRACT

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Hepatitis C virus (HCV) or human immunodeficiency virus (HIV) infection leads to a phenomenon of inflammaging, in which chronic infection or inflammation induces an immune aged phenotype with T cell dysfunction. Thus, HCV or HIV infection has been deemed as a model to study the mechanisms of T cell infammaging and viral persistence in humans. In this dissertation, T cell homeostasis, DNA damage and repair machineries were investigated in patients with chronic HCV or HIV infection at risk for inflammaging. We found a significant depletion in CD4 T cells, which was correlated with their apoptosis in chronically HCV/HIV-infected patients, compared to age-matched healthy subjects. In addition, virus-infected patients' CD4 T cells were prone to DNA damage that extended to chromosome ends (telomeres), leading to accelerated telomere erosion - a hallmark of senescence. Mechanistically, the DNA double-strand break (DSB) sensor MRE11, RAD50, and NBS1 (MRN) remained intact, but the DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) and its downstream checkpoint kinase 2 (CHK2) were significantly suppressed in T cells from HCV/HIV-infected individuals. Consistently, ATM/CHK2 activation, DNA repair, and cellular functions were also impaired in primary CD4 T cells following ATM knockdown, or exposure to the ATM inhibitor (KU60019), as well as in CD4 T cells co-cultured with HCV-infected hepatocytes, or a T cell line infected with HIV-1 in the presence of raltegravir *in vitro*, which recapitulates the biological effects observed in T cells in the setting of HCV/HIV infection *in vivo*. Importantly, ectopic expression

of ATM was essential and sufficient to reduce the DNA damage, survival deficit, and cellular dysfunction in T cells from both HCV and HIV-infected individuals. These results demonstrate that failure of DSB repair due to ATM deficiency leads to unrepaired DNA damage and renders virally infected patients' T cells prone to senescence and apoptosis, thus contributing to CD4 T cell loss or dysfunction during chronic HCV or HIV infection. This study reveals a novel mechanism by which ATM deficiency promotes telomeric DNA damage and premature T cell aging, and provides a new therapeutic target for inflammaging-induced immune dysfunction during chronic viral infection.

DEDICATION

Delicated to my parents and parents-in-law,

Shansen Zhao and Laifeng Wang, Anzhong Zhao and Bo Yu,

and most of all, to my beloved husband ZHILONG ZHAO

and my daughter EVELYN GRACE ZHAO

who have served as my inspiration.

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CHAPTER 1

INTRODUCTION

HCV Infection - An Excellent Model for Studying Viral Persistence in Humans

HCV is a parenterally-transmitted, hepatotropic virus that often causes inflammation of liver (hepatitis). Currently, there is approximately 170-200 millions of people worldwide and 4 millions of United States (U.S.) citizens are infected by HCV. Upon exposure, 15-25% of HCV-infected subjects can spontaneously resolve the acute infection, and 75-85% of infected subjects progress to chronic infection[1,2]. Chronic HCV infection is associated with an increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) (as showed in **Fig.1.1**), and is now the leading infectious cause of liver transplantation in the US[3]. According to CDC reporting[4], the number of HCV-related deaths has reached an all-time high, surpassing 60 other nationally reported infectious conditions combined. Thus, hepatitis C is the number one infectious disease leading to death in the U.S. Over the past 7-8 years, the successful development of highly effective direct-acting antiviral agents (DAAs) has led to a significant paradigm change in the treatment of HCV infection. While DAA has significantly improved the outcome of HCV treatment, these therapeutic cocktails are facing new issues such as viral mutation, relapse, and reinfection following therapy[5,6]. Additionally, the lack of a vaccine for this virus is a major hurdle to control this global infection.

Since HCV is highly efficient at establishing chronic infection via evasion of host immunity, it has become an excellent model to study the mechanisms of viral persistence in humans. Similar to the issues inherent to HCV infection, the failures to successfully manage many chronic infectious diseases and to effectively respond to vaccines stem from an incomplete

understanding of the host and pathogen interactions that may dampen the host immunity, thus this research is significant in that the mechanisms we will study may ultimately be applicable to multiple chronic infectious diseases.

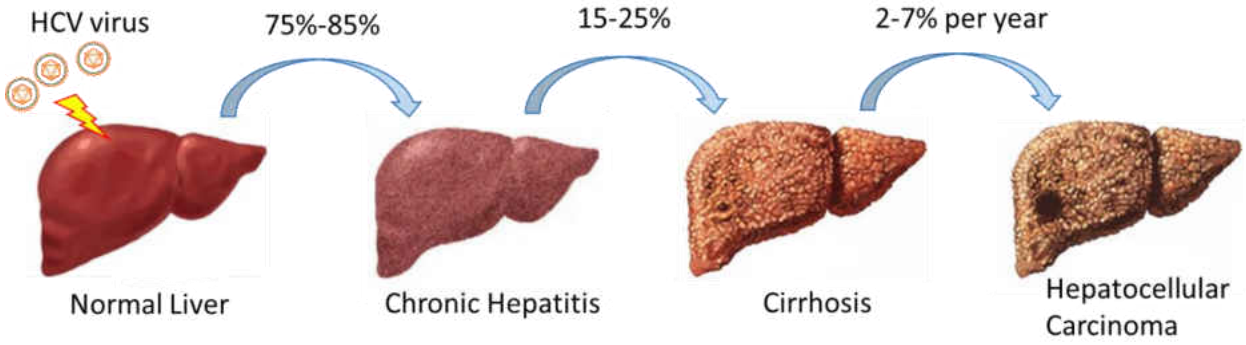

Fig.1.1 Natural history of HCV infection. (Modified from Osna NA 2017)
Chronic hepatitis develops among 75%-85% of HCV-infected subjects. HCV can cause repeated hepatocyte damage and result in cirrhosis with a rate of 15%-25% after 10-40 years. Among patients with liver cirrhosis, there is 2-7% annual risk of hepatocellular carcinoma.

HIV Infection - An Excellent Model for Studying Inflammaging in Humans

HIV-1 was first discovered in 1983, and is the cause of the acquired immunodeficiency syndrome (AIDS)[7,8]. There were approximately 36.9 million peoples worldwide living with HIV/AIDS in 2017. More than 1.1 million peoples in the U.S. are living with HIV today. Since 1997, highly active combined Antiretroviral Therapy (ART) has been used to treat HIV patients[9,10]. It is extremely effective at suppressing HIV replication, increasing CD4 T cell counts, and decreasing the HIV/AIDS-related morbidity and mortality[11-13]. However, current ART can only suppress, not eliminate, the HIV infection. Also, HIV patients on ART with undetectable viremia and reasonable CD4 T cell counts still harbor impaired immune functions, as evidenced by the shortened telomeres, low IL-2 production, poor cellular proliferative capacity, and blunted vaccine responses[14-24]. Importantly, ART-controlled HIV patients often

exhibit a state of chronic, low grade inflammation that can activate the immune system, and subsequently, drive T cell premature aging or senescence, a phenomenon called inflammaging[25-28]. Inflammaging is a major driver of increased susceptibility to infections, reduced responses to vaccines, increased incidences of cancers, cardiovascular disease, and neurodegeneration, similar to those often seen in the elderly[29-31]. The mechanisms regarding how inflammaging is developed and persists in virus suppressed, latent HIV infection, however, remain poorly understood.

Multiple factors may be involved in the mechanisms underlying inflammaging in ART-controlled, latent HIV infection. Specifically, HIV latency in the era of ART is characterized by the existence of viral reservoirs that prevent HIV-1 eradication and likely drive inflammaging[32,33]. Thus, HIV-mediated inflammaging could result from a myriad of insults (**Fig.1.2**) , including viral particles or viral proteins released from HIV-1 reservoirs, cell-secreted pro-inflammatory cytokines/chemokines, HIV-enhanced gut permeability or altered gut microbiota, frequent cytomegalovirus (CMV), Epstein–Barr virus (EBV), hepatitis B virus (HBV), and HCV coinfections, ART regimens, and other closely associated comorbidities including malignancies, or even social, personal stresses, and environmental factors[34-37]. Additionally, cumulative evidence has indicated a tight link between oxidative stress and inflammaging[38]. Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS) that cause damage to lipids, proteins and DNA, which may shape the homeostasis at various disease conditions[39-42]. In the setting of latent HIV infection, ROS within the cellular milieu during viral infection may also play an important role in driving inflammaging. The inflammaging imposed by HIV exposes the immune system, particularly, HIV-infected and bystander CD4 T cells, to unique challenges that lead to T cell exhaustion and senescence, similar to that observed in the elderly. Therefore, the virus-suppressed, latent HIV infection

can be deemed as an excellent model to study inflammaging in humans.

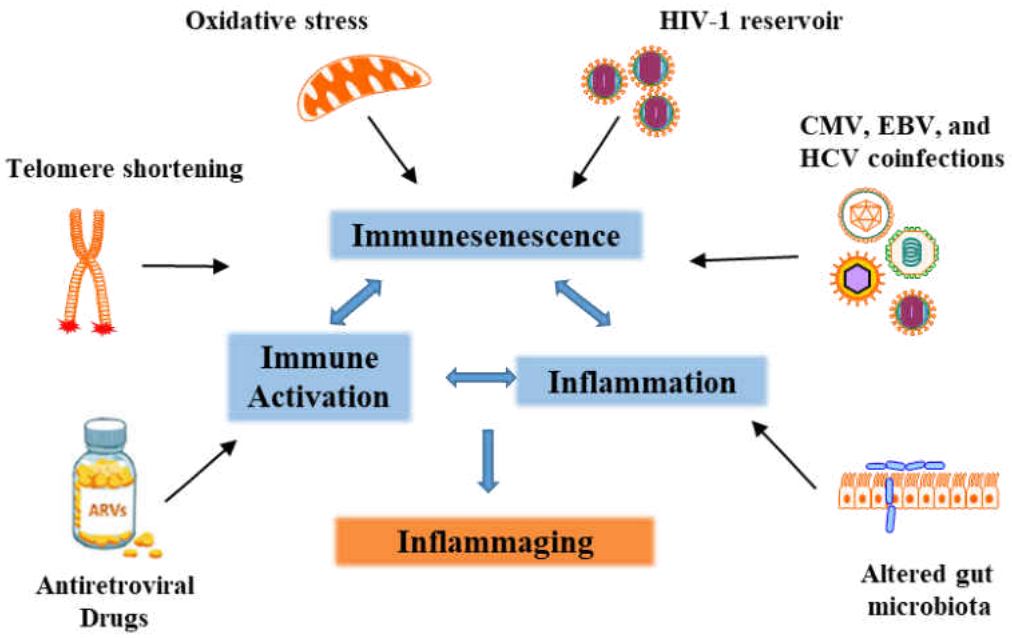

Fig.1.2. Multiple factors involve in the mechanisms underlying HIV-mediated inflammaging. (Modified from Hearps A 2016)

T Cell Aging or Senescence - A Phenomenon Often Seen in Chronic Viral Infection

Aging is associated with, and may be caused by, low-grade inflammation (inflammaging), which is a common denominator in the majority of age-related diseases[43-45]. Inflammaging is a major driver of increased susceptibility to infections, reduced responses to vaccines, increased incidences of cancers, cardiovascular diseases, and neurodegeneration, all of which contribute to comorbidity and mortality in the elderly[29-31]. The aged immune system, typically hypo-responsive to specific infection and vaccination, can be hyper-responsive or aberrant under non-specific inflammatory conditions. In human immune system, lymphocytes including CD4 helper T cells are long-lived, and thus are exposed to extensive genomic insults and replication pressure, making them vulnerable to aging-associated abnormalities. Recent

studies, including those reported by our lab, now link changes in immune aging directly to chronic DNA damage responses (DDR), which can lead to remodeling of immune responses[43-45].

In studying the role of CD4 T cell dysfunction in viral persistence, our lab, along with others have found that chronic viral (HCV, HIV) infection can accelerate premature T cell aging, as evidenced by overexpression of aging markers (such as KLRG1, CD57, $p16^{ink4a}$, and β-galactosidase), differential regulation of age-associated microRNA-21/-181a, and, notably, an accelerated loss of telomeres (**Fig.1.3.**), when compared to age-matched healthy subjects (HS)[17,46-58]. Accelerated telomere loss is indicative of excessive cell proliferative turnover or inadequate telomeric DNA maintenance. Telomeres are repeating hexameric sequences of DNA (GGGATT) that are found at the ends of linear chromosomes in association with a complex of proteins. Because of the enrichment of guanine (G) in telomeres, they are extremely sensitive to DNA damage, especially to the oxidative ROS-induced DDR. Telomere integrity is a key feature of the linear chromosomes that preserve genome stability and function; conversely, telomere erosion is a hallmark of cell aging or senescence(a quiescent, non-replicative state) that drives cell dysfunction or apoptosis[59,60]. While the telomere length is maintained in most cases by the telomerase that prolongs telomeric sequence[61-64], a telomere-bound protein complex (Shelterin) is required to protect telomeres from unwanted DDR[65-69]. Notably, several DNA repair pathways, including ATM (ataxia telangiectasia-mutated protein), ATR (ataxia telangiectasia and Rad3-related protein), and DNA-PKCs (DNA-dependent protein kinase catalytic subunit) are involved in the DDR[43,70-73]; however, how the telomeric DNA damage and repair signaling pathways are dysregulated in the context of viral infection remain largely unknown. It is thus important to elucidate the mechanisms underlying T cell senescence and inflammaging that is characterized by telomere erosion in individuals with chronic viral infection.

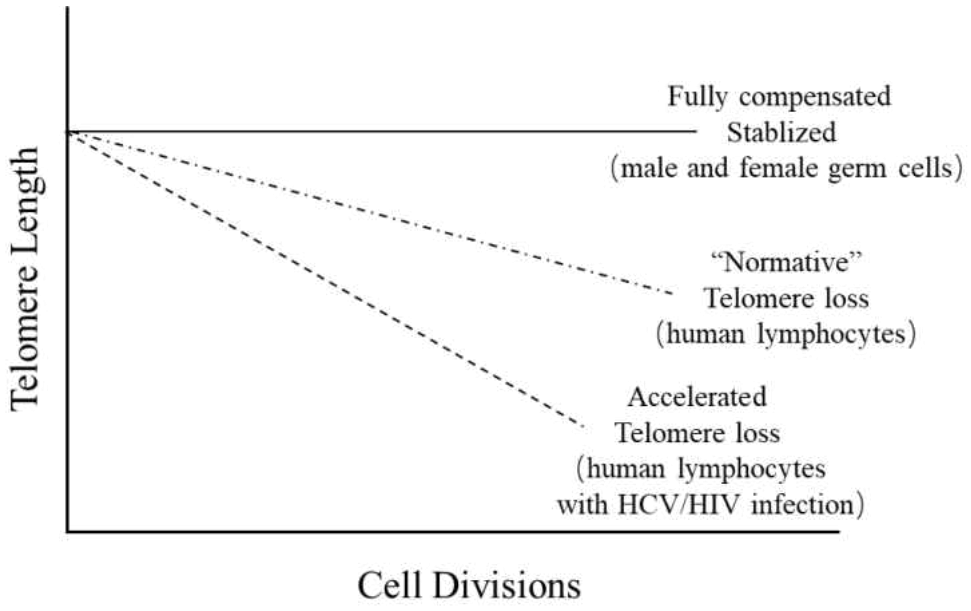

Fig.1.3. Relationship between cell division and changes in telomere length. (Modified from Richard J. Hodesa 1999)
The rate of telomere loss has been measured as 50–100 bp per cell division from *in vitro* analysis of cultured human lymphocytes. Under chronic HCV/HIV infection, the telomere loss can be as high as 250 bp per cell division and lead to T cell premature aging.

ATM - A Kinase Involves in Regulating DDR and Multiple Cellular Functions

Because DNA damage can cause telomere erosion, leading to premature cell senescence and/or apoptosis[59,74], we hypothesized that progressive CD4 T cell loss and failure of immune recovery during chronic viral infection could be, at least in part, due to a deficiency in the DNA repair that alters telomere integrity. Human CD4 T cells have a relatively long life span (150-160 days) and thus are exposed to a multitude of genotoxic stresses, leading to ~1% out of a pool of 300 billion T cells to be replaced daily[75]. To maintain genomic stability and cell survival, cells continuously recognize and respond to DNA damage by either activation of DNA damage checkpoints to arrest cell cycle progression and allow for repair, or, if the damaged DNA is beyond repair, to undergo apoptosis[76]. Notably, a major sensor of DSB is the MRN complex,

which subsequently recruits the protein kinase ATM, an enzyme critically involves in repairing DNA damage for cell survival (**Fig.1.4**)[77,78].

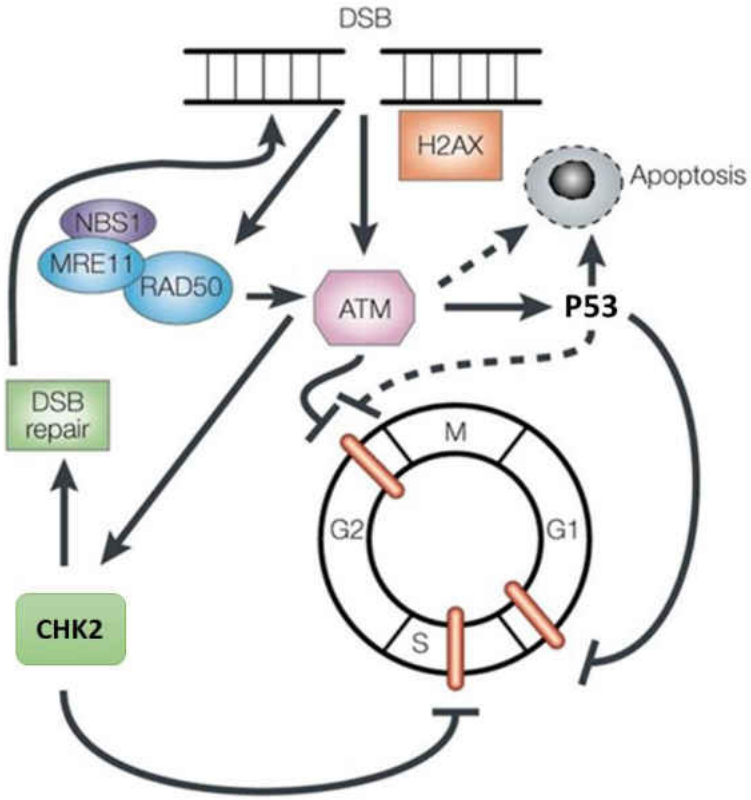

Fig.1.4. ATM protein kinase response to double stranded breaks. (Modified from Van Gent, D. C. 2001)
A major sensor of DSB is the MRN complex, which subsequently recruits the ATM protein kinase. Downstream targets of ATM phosphorylation include CHK2 and p53. By this process, ATM can influence DSB repair and cell-cycle progression, in addition to cell death through apoptosis. Because the ATM protein is one of most widely studied components in the cellular response to DNA damage, it has been placed in the center of this figure. However, numerous other factors, including ATR and DNA-PKc protein kinases, are essential for cell-cycle modulation after the presence of DNA damage, and some of its functions might be redundant with ATM. (The red bars indicate the three main cell-cycle checkpoints and dashed lines show that the effect is not a principal pathway).

ATM was originally identified in individuals with ataxia telangiectasis (AT), an autosomal recessive disorder that manifests with progressive ataxia, telangiectasia, immunodeficiency, genomic instability, and cancer predisposition[79]. It is a key kinase within the

DDR signaling cascade[80,81], and plays a unique role in lymphocyte biology, as programmed DDR participates in the gene remodeling process necessary for formation of a highly diverse TCR repertoire. ATM requires a signal for activation (usually a DNA damage signal), which promotes intermolecular auto-phosphorylation of ATM via the serine residue at position 1981, resulting in its inactive dimer to dissociate into active monomers[82]. Accumulation of damaged DNA activates the ATM cascades, along with ATR and DNA-PKc, all of which belong to the PI3K-related kinase (PIKK) family and are important for DNA reprogramming and T cell rearrangement [79-82]. However, how ATM is dysregulated in CD4 T cells in the setting of chronic viral infection remains unknown.

Specific Aims

The overall goal of this study is to elucidate the mechanisms by which chronic viral infection mediates DNA damage and telomere erosion that may lead to T cell dysfunction. To this end, we will employ a large cohort of individuals with chronic HCV or HIV infection because they have been deemed as great models to study inflammaging and T cell dysregulation in humans.

To explore the mechanisms of T cell dysfunction in virally infected individuals, we have recently focused on identifying biomarkers for T cell aging in HCV- or HIV-infected subjects. We found that HCV- or HIV-infected individuals exhibit a profound impairment in CD4 T cell responses, concomitant with overexpression of exhaustion and aging 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) in CD4 T cells

when compared to age-matched HS. These findings suggest that T cell dysfunction during HCV/HIV infection may be a result of profound, virus-mediated, premature T cell aging. Notably, this aging process, as in the elderly, not only involves antigen-specific effector and memory T cells, but also extends to naïve T cells that are the primary subsets responding to neo-antigens (vaccines). Importantly, these dysfunctional T cells also suffer telomere erosion - a hallmark of cell aging - indicating excessive proliferative pressure or telomeric DNA damage in chronic viral infection.

Telomere integrity is a key feature of linear chromosomes that preserves genome stability and function; whereas telomere erosion is a hallmark of cell aging or senescence that leads to cell dysfunction or apoptosis; thus telomeric DNA repair is essential to cell survial. While the telomere length is maintained in most cases by a telomerase that prolongs telomeric DNA, we found that telomerase activity is intact, whereas ATM, a DNA repair enzyme, is inhibited in CD4 T cells during HCV and HIV infection. How DNA damage repair signaling pathways are dysregulated in T lymphocytes in the context of HCV/HIV infection, and their roles in T cell aging and dysfunction, remain largely unknown.

In this study, we hypothesize that i) DNA damage and telomere loss play a pivotal role in T cell aging and dysfunction in HCV/HIV-infected subjects; and ii) lack of ATM-dependent DNA damage repair accelerates T cell aging and dysfunction in HCV/HIV infection, and as such, restoring this DNA repair machinery may open new avenues to protecting T cells from aging and maintaining immune competence. To test this hypothesis, we propose the following two independent but interrelated aims:

Aim 1. Determine the role of ATM in DNA damage and T cell dysfunction in chronic HCV infection.

A) Compare T cell homeostasis, apoptosis, and DNA damage in HS and HCV chronically-infected individuals.

B) Evaluate the role of ATM in regulating DNA damage repair and maintaining T cell functions during HCV chronic infection.

The objectives of these studies are to determine the relationship of DDR with T cell apoptotic death and the role of ATM in preserving DNA integrity and T cell function during HCV infection.

Aim 2. Investigate the role of ATM in telomeric DNA damage and T cell dysfunction during latent HIV infection.

A) Characterize T cell homeostasis, apoptosis and their relationships with telomeric DNA damage in $HIV^{+/-}$ subjects.

B) Explore the role of ATM in repairing telomeric DNA damage and maintaining T cell functions in $HIV^{+/-}$ subjects.

The objectives of these studies are to evaluate the role of ATM in regulating telomeric DNA damage repair and T cell function during latent HIV infection.

These translational studies are significant and timely in that they will provide a working model to explore mechanisms that may be fundamental to diminishing immune responses that

are observed in multiple chronic infectious diseases, including but not limited to HCV and HIV infection. Understanding such mechanisms is critical for developing approaches to improve immune responses in the setting of immunocompromised conditions.

CHAPTER 2

INSUFFICIENCY OF DNA REPAIR ENZYME ATM PROMOTES NAÏVE CD4 T CELL LOSS IN CHRONIC HEPATITIS C VIRUS INFECTION

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Running title**:** ATM deficiency leads to naïve T cell loss in HCV infection

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Abstract

T cells have a crucial role in viral clearance and vaccine response; however, the mechanisms regulating their responses to viral infections or vaccinations remain elusive. In this study, we investigated T-cell homeostasis, apoptosis, DNA damage, and repair machineries in a large cohort of subjects with hepatitis C virus (HCV) infection. We found that naive CD4 T cells in chronically HCV-infected individuals (HCV T cells) were significantly reduced compared with age-matched healthy subjects. In addition, HCV T cells were prone to apoptosis and DNA damage, as evidenced by increased 8-oxoguanine expression and γH2AX/53BP1-formed DNA damage foci - hallmarks of DNA damage responses. Mechanistically, the activation of DNA repair enzyme ataxia telangiectasia mutated (ATM) was dampened in HCV T cells. ATM activation was also diminished in healthy T cells exposed to ATM inhibitor or to HCV (core protein) that inhibits the phosphoinositide 3 kinase pathway, mimicking the biological effects in HCV T cells. Importantly, ectopic expression of ATM was sufficient to repair the DNA damage, survival deficit, and cell dysfunctions in HCV T cells. Our results demonstrate that insufficient DNA repair enzyme ATM leads to increased DNA damage and renders HCV T cells prone to apoptotic death, which contribute to the loss of naive T cells in HCV infection. Our study reveals a novel mechanism for T-cell dysregulation and viral persistence, providing a new strategy to improve immunotherapy and vaccine responses against human viral diseases.

Introduction

Hepatitis C virus (HCV) is a blood-born pathogen characterized by a high rate (> 80%) of chronic infection, which can progress to liver cirrhosis and hepatocellular carcinoma - a leading cause for liver transplantation [1]. Notably, HCV has evolved numerous strategies to evade host

immunity and harness virus persistence [1], providing an excellent model to study the mechanisms of virus-mediated host immune dysfunction in humans.We and others have previously reported that patients with chronic HCV infection, exhibit premature T cell aging, as demonstrated by overexpression of aging markers and telomere attrition- indicating excessive proliferative turnover or inadequate telomeric maintenance [2-6]. However, the molecular mechanisms that control T cell homeostasis and virus persistence in humans remain unclear.

T-cell homeostasis is tightly controlled, requiring a fine balance between influx of newly generated T cells from thethymus and efflux by consumption via T-cell apoptosis, and self-replication within the existing pools of T lymphocytes [7, 8]. With de ficient thymic influx in aging adults, the immune system responds to in vivo and in vitro challenges by expanding existing T cells, leading to increased proliferative turnover, telomere attrition, and cell apoptosis [7, 8]. We hypothesize that premature T cell aging not only involves virus-specific effector and memory T cells engaging in chronic viral infection, but may also extend to the compartment of naïve T cells that are unprimed by antigens. In support of this notion, broad regulatory anomalies, including the markers for T cell exhaustion and senescence, are found not only expressed on virus-specific T cells, but also on unprimed naïve T cells that have not yet engaged in immune responses[2-6, 9-14]. This notion is also supported by the observations that individuals with chronic viral (HCV or HIV) infection often have blunted vaccine responses, suggesting a broad and shared mechanism of immune dysregulation, particularly naïve CD4 T cell dysfunction, and vaccine non-responsiveness in virally infected individuals [2, 3, 15-19].

Human naïve T cells have a relatively long life span (150~160 days) and thus are exposed to a multitude of genotoxic stressors, leading to 1% of a pool of 300 billion T cells to be

replaced daily [7, 8]. Notably, naïve T cells are typically resistant to death receptor/ligand (Fas/Fas-L)-mediated apoptosis, pointing toward cell-internal signals as apoptosis initiators [20]. One of the internal stressors linked to apoptosis is damaged DNA, which is particularly important in senescent cells that have been chronically exposed to the endogenously generated reactive oxygen species (ROS) [21]. To maintain genomic stability and cell survival, cells continuously recognize and respond to this DNA damage which will either activate DNA damage checkpoints to arrest cell cycle progression and allow for repair, or, if the damaged DNA is beyond repair, undergo apoptosis [22].

A major sensor of DNA breaks is the MRN complex (MRE11, RAD50, and NBS1), which subsequently recruits the protein kinase ataxia telangiectasia mutated (ATM), an enzyme critically involved in repairing DNA double strand breaks (DSBs) for cell survival [23, 24]. ATM was originally identified in individuals with ataxia telangiectasis, an autosomal recessive disorder exhibiting progressive ataxia, telangiectasia, immunodeficiency, genome instability, and cancer predisposition [25]. ATM, accompanied by ataxia telangiectasia Rad3-related (ATR) and DNA-dependent protein kinase catalytic subunit c (DNA-PKc), is the pinnacle kinase of the DNA repair signaling cascade, and belongs to the phosphoinositide 3 kinase (PI3K)-related kinase family [26]. Accumulation of DNA DSBs activates ATM cascades, along with other DNA damage repair machineries, which are important for DNA reprogramming and cell remodeling.

To identify factors that perturb T cell homeostasis during HCV infection, we investigated the mechanism that controls T cell survival and DNA damage repair capabilities in primary CD4 T cells. We demonstrate that insufficiency of ATM leads to accumulation of DNA damage, rendering naive CD4 T cells sensitive to apoptosis and T cell loss, contributing to viral

persistence and vaccine non-responsiveness in chronic HCV infection.

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, USA). The study subjects were composed of two populations: 148 chronically HCV-infected individuals and 72 age-matched HS. Written informed consent was obtained from all participants. HCV patients were virologically positive for HCV RNA, prior to the antiviral treatment. Healthy subjects, derived from Physicians Plasma Alliance, Gray, TN, USA were negative for HBV, HCV, and HIV infection.

Cell Isolation and Culture

PBMCs were isolated from whole blood by Ficoll (GE Heathcare, Piscataway, NJ, USA) density centrifugation. Naïve and memory $CD4^+$ T cells were isolated from PBMCs using the Naïve or Memory $CD4^+$ T Cell Isolation Kit and a MidiMACS™ Separator (Miltenyi Biotec Inc., Auburn, CA). The isolated T cells were cultured in RPMI 1640 medium containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA), 100 IU/ml penicillin and 2 mM L-glutamine (Thermo Scientific, Logan, UT, USA) without mitogenic stimulation for 4 days at 37°C and 5% CO2 atmosphere. Cells were collected at day 0, day 2, or day 4 for detection of cell apoptosis and DNA damage. To test the role of pATM in repairing DNA damage and apoptosis, 10μM pATM inhibitor (KU60019, Abcam, Cambridge, MA) or dimethyl sulphoxide were added to the cultures for 48 h, followed by apoptosis and DNA damage analysis. To consolidate the

role of HCV in inhibiting ATM activation, purified naïve CD4 T cells were co-cultured with Huh7.5 cells with or without HCV infection, or 1μg/ml recombinant HCV core protein (Virogen, watertown, MA, USA) or control protein β-galactosidase (Virogen), in the presence or absence of 20 μM PI3K inhibitor (LY294002, Sigma) for 4 days, followed by flow cytometry or Western blot analysis for ATM/pATM expression, DNA damage, and cell apoptosis.

Flow Cytometry

For phenotypic analysis of naïve CD4 T cells, PBMCs were stained with CD4-APC, CD45RA-FITC (BioLegend, San Diego, CA, USA) antibodies or isotype controls. To quantify cell apoptosis, naïve or memory cells were purified, cultured, and collected at indicated days and stained with Av and 7-AAD using BD Pharmingen™ PE Av Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA). For intracellular staining, the cells were fixed and permeabilized with Foxp3 Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA), and stained with pATM (Ser1981)-PE antibody(BioLegend), ATM antibody (Abcam) and anti-Rabbit-IgG-Alexa Fluor 488 (Santa Cruz Biotechnology, Dallas, TX, USA), pCHK2 (Thr68)-PE antibody (eBioscience), 8-oxoguaine-FITC probe (OxyDNA Assay Kit, EMD Millipore, Billerica, MA).The stained cells were analyzed on AccuriTM C6 flow cytometer (BD, Franklin Lakes, NJ), and data were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR). Isotype control antibodies (eBioscience) and fluorescence minus one controls were used to determine the background levels of staining and adjust multicolor compensation as gating strategy.

RNA Isolation and Real-time RT-PCR

Total RNA was extracted from 1.0×10^6 cells with PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA), and cDNA was synthesized by using High Capacity cDNA Reverse Transcription Kit (Applied Bio systems, Foster city, CA) per manufacturer's instruction. Quantitative PCR were completed in triplicates following the conditions 95 °C, 10min and then 95°C, 15s; 60°C, 60s with 40 cycles. Gene expression was normalized to 18S ribosomal RNA and expressed as fold changes using the $2^{-\Delta\Delta ct}$ method. Primer sequences were shown in Table2.1.

Western Blotting

Naïve CD4 T cells purified from HCV-infected individuals and HS were lysed on ice in RIPA lysis buffer (Boston BioProducts Inc, Ashland, MA) in the presence of protease inhibitors (Thermo Scientific, Rockford, IL). The protein concentrations were measured by Pierce BCA protein assay kit (Thermo Scientific). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, which were blocked with 5% nonfat milk, 0.5% Tween-20 in Tris buffered saline, and incubated with the pATM (Ser1981) (D6H9), pBRCA1, pCHK1, and pCHK2 (Thr68) (C13C1) antibodies and βActin (8H10D10) antibodies (Cell Signaling, Danvers, MA). Appropriate horseradish peroxide-conjugated secondary antibodies (Cell Signaling) was then used and proteins were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare BioSciences, Pittsburgh, PA). Membranes were stripped and re-probed with MRE11, RAD50, NBS1, BRCA1, ATM (D2E2), γH2AX, PARP, CHK1, and CHK2 (D9C6) antibodies (Cell Signaling). Protein bands were captured and quantitatively analyzed by Chemi DocTM MP Imaging System (Bio-Rad System).

Table 2.1 Primer sequences to be used in the chapter 2

Name of the amplified genes	Primer sequences
ATM	F: 5'-TGGATCCAGCTATTTGGTTTGA-3'
	R: 5'-CCAAGTATGTAACCAACAATAGAAGAAGTAG-3'
p53	F: 5'-TCAACAAGATGTTTTGCCAACTG-3'
	R: 5'-ATGTGCTGTGACTGCTTGTAGATG-3'
MRE11	F: 5'-CTTGTACGACTGCGAGTGGA-3'
	R: 5'-TTCAC CCATCCCTCTTTCTG-3'
NBS1	F: 5'-TTGGTTGCATGCTCTTCTTG-3'
	R: 5'-GGCTGCTTCTTGGACTCAAC-3'
RAD50	F: 5'-CTTGGATATGCGAGGACGAT-3'
	R: 5'-CCAGAAGCTGGAAGTTACGC-3'
BRCA1	F: 5'-GGCTATCCTCTCAGAGTGACA-3'
	R: 5'-CTGATGTGCTTTGTTCTGGA-3'
CHEK1	F: 5'-GGTGAATATAGTGCTGCTATGTTGACA-3'
	R: 5'-TTGGATAAACAGGGAAGTGAACAC-3'
CHEK2	F: 5'-CCCAAGGCTCCTCCTCACA-3'
	R: 5'-AGTGAGAGGACTGGCTGGAGTT-3'
18S ribosomal RNA	F: 5'-CCTGGATACCGCAGCTAGGA-3'
	R: 5'- GCGGCGCAATACGAATGCCCC-3'

Confocal Microscopy

Naïve $CD4^+$ T cells were isolated and cultured as described above. Immunofluorescence staining was performed according to the reported method [39]. In brief, the cells were fixed in 2% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 in PBS for 10 min, blocked with 5% BSA in PBS for 1 h, and then incubated with rabbit anti-53BP1 antibody (Cell Signaling) and mouse anti-γ-H_2AX (Ser139) antibody (Biolegend) at 4 °C overnight. The cells were washed with PBS with 0.1% Tween-20 for three times, and then stained with anti-rabbit IgG-Alexa Fluor 488 and anti-mouse IgG- Alexa Fluor 555 (Invitrogen) at room temperature for 1 h, 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).

ATM Transfection

Purified naïve $CD4^+$ T cells from HCV patients were transfected with 2.5 μg pcDNA3.1 (a gift from Adam Antebi [48], Addgene plasmid # 52534), or pcDNA3.1(+)Flag-His-ATM wt (a gift from Michael Kastan [49], Addgene plasmid # 31985), or hATMS1981A mutant (a gift from Michael Kastan [31], Addgene plasmid # 32300), using the Human T Nucelofector Kit and Nucleofector I Device (Lonza, Allendale, NJ). 24 h post transfection, GFP fluorescence was observed under microscope and the GFP expression level was measured by Flow Cytometry. 48 h post transfection, transfection efficiencies were monitored by flow cytometry measuring the frequency of His^+ cells. Ectopic ATM expression was detected by western blot. Active caspase-3, γH2AX, and IL-2 expressions were assessed in the His-positive cells by Flow Cytometry.

Statistical Analysis

The data were summarized as mean ± SEM or median with interquartile range and analyzed using Prism 7 software. Comparisons between two groups were made using independent Student's t test or Mann Whitney test, paired T test or Wilcoxon matched-pairs signed rank test. Multiple groups were analyzed by one-way ANOVA test. P-values <0.05, <0.01, or <0.001 were considered statistically significant or very significant, respectively.

Results

Naïve CD4 T Cell Apoptosis and Loss in HCV-Infected Patients

As an initial approach to identify factors that perturb T cell homeostasis in HCV infection, we characterized the frequencies of primary T cells and their survival rate or susceptibility to apoptosis in individuals with chronic HCV infection (n=68) versus age-matched healthy subjects (HS) (n=38). We first analyzed total $CD4^+$, $CD45RA^+CD4^+$ (naïve), and $CD45RA^-CD4^+$ (memory) T cell frequencies in the peripheral blood mononuclear cells (PBMCs) using flow cytometry. As shown in **Fig.2.1A** (representative dot plots and summary data), while the total CD4 T cell numbers in PBMCs were slightly lower in HCV patients, the compartment of naïve CD4 T cells was significantly reduced (P<0.0001), whereas memory CD4 T cells were expanded in chronically HCV-infected subjects compared to HS. To exclude the possibility that the gated PBMCs may include some $CD4^-$expresing monocytes, we further gated on $CD3^+$ T cells, followed by analyzing $CD45RA^+CD3^+CD4^+$ (naïve) and $CD45RA^-CD3^+CD4^+$ (memory) T cell populations, which produced similar results; i.e., chronic HCV subjects exhibited a significant contraction of naïve T cell pools and expansion of memory T cells in their peripheral blood (data not shown). Notably, the loss of naïve CD4 T cells from chronically HCV-infected

subjects did not correlate with HCV genotype, viral load, or hepatic transaminase levels (data not shown). This observation is in line with the previous reports showing a reduced naïve CD4 T cell number - reflecting a state of immune activation and exhaustion in patients with chronic HCV infection[9, 10].

Apoptosis represents a major mechanism for controlling T cell homeostasis in adults [7]. The vast majority of CD4 T cells in vivo are in a resting state and, accordingly, do not undergo apoptosis. When removed from their natural resources and kept ex vivo, human T cells spontaneously and progressively go through the programmed cell death (spontaneous apoptosis). To explore whether apoptosis contributes to the naïve T cell loss in HCV-infected individuals, we purified $CD4^+CD45RO^-$ naïve and $CD4^+CD45RA^-$ memory T cells from $HCV^{+/-}$ subjects and cultured the cells without stimulation for 0, 2, 4 days, followed by measuring the Annexin V (Av) / 7-Aminoactinomycin D (7AAD) expressions. As shown in **Fig.2.1B** and **C**, although healthy T cells showed signs of apoptosis/death accrual, an increased T cell apoptosis and decreased cell survival rate were observed in HCV-infected patients, especially in the naïve CD4 T cell pools. By day 4 in culture without stimulation, 90% of healthy naïve T cells were alive, whereas only 75% of the HCV naïve T cells still remained survival. Notably, increased apoptotic propensity (Av expression) was inversely associated with cell survival rate, with HCV naïve T cells more prone to spontaneous apoptosis compared to the HS. By day 4 in culture without stimulation, HCV naïve T cells exhibited significant apoptosis compared with HS (~40% vs. ~20%, $P<0.05$), which negatively correlated with the cell numbers present in the peripheral blood from the same individuals (data not shown). These results suggest that apoptotic susceptibility of T cells from HCV-infected subjects may be one mechanism contributing to the disproportionate T cell loss, , whereas lack of thymic influx in aging adults and increase of naïve

T cell differentiation into antigen specific effector and memory T cells during persistent viral infection may be other mechanisms for the different outcomes of the two T-cell subsets.

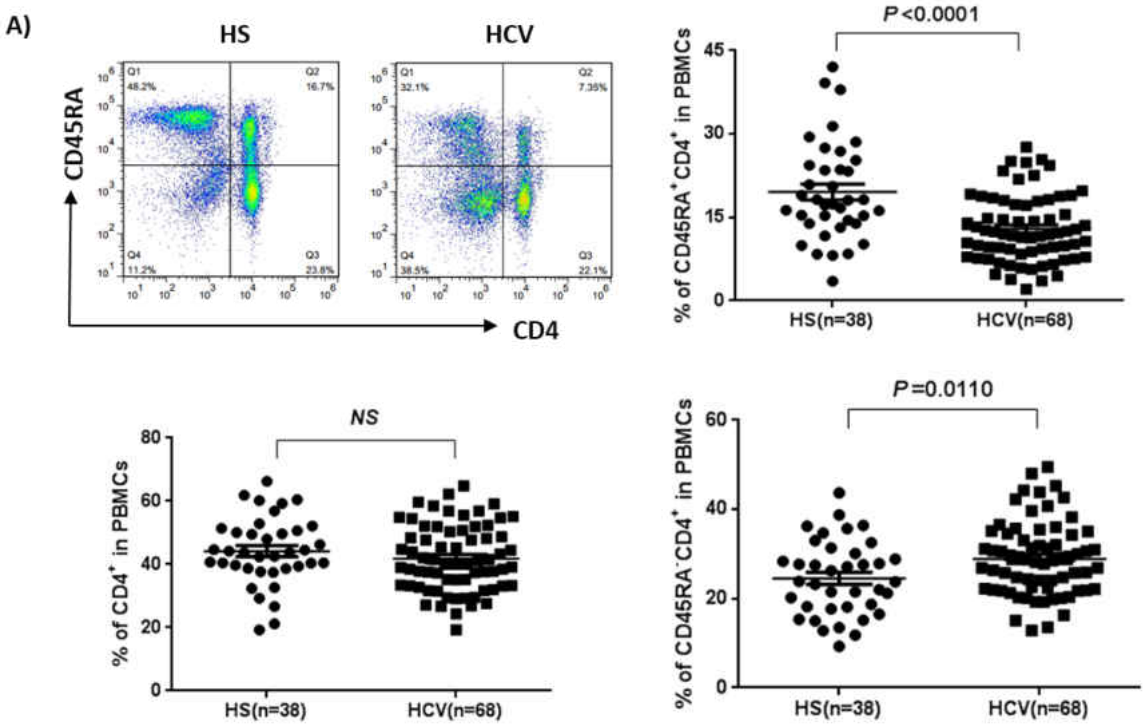
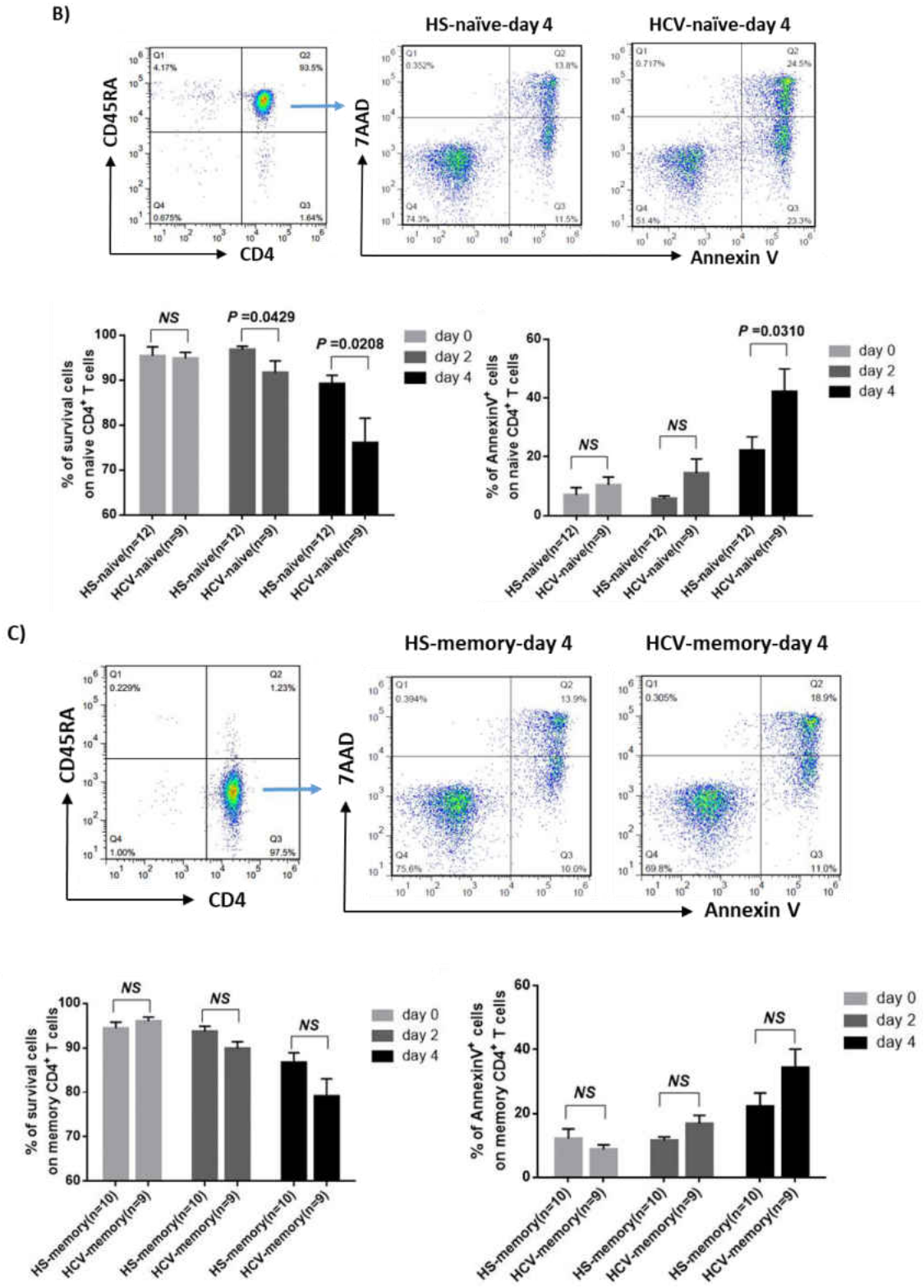


Fig.2.1. T cell homeostasis and apoptosis in HCV-infected patients versus age-matched HS. **A)** Naive CD4 T-cell loss in HCV patients vs. agematched HS. PBMCs isolated from HCV-infected patients and HS were analyzed using flow cytometry for T cell homeostasis. Representative dot-plots and summary data of the flow cytometry for the percentages of total $CD4^+$, $CD45RA^+CD4^+$ (naïve), and $CD45RA^-CD4^+$ (memory) T cell frequencies are shown. Each symbol represents one particular subject; the mean ± SE and p value of the statistical analysis are shown, NS = no significance. **(continued on the next page).** **B) & C)** Susceptibility of T cells to spontaneous apoptosis and death in HCV patients vs. HS. Naïve and memory CD4 T cells were purified from PBMCs of the subjects, cultured *in vitro* without stimulation for 0, 2, and 4 days, followed by flow cytometric analysis of Annexin V and 7AAD expression. Representative dot plots for the cell purity, gating strategy, and summary data for the percentages of survival cells as well as apoptotic cells are shown. n = the number of subjects studied in each group. NS = no significance. P value with significant changes are shown.

Fig.2.1 (continued)



DNA Damage in Naïve CD4 T Cells from HCV-Infected Patients

Why naïve CD4 T cells are susceptible to apoptosis and loss in virally-infected individuals is unclear. Unlike activated or memory T cells, resting naïve T cells typically do not express Fas surface receptor(**Fig.2.1SA**). In addition, blocking the extrinsic death pathways by disrupting Fas-Fas ligand, TNFα-TNF receptor, and TRAL-TRAIL receptor interactions in CD4 T cells did not affect cell apoptosis or death rates (data not shown), indicating they are resistant to the exogenous apoptotic pathway-mediated cell death, but sensitive to endogenous oxidative stress (particularly ROS-mediated genotoxicity) [20]. To assess endogenous DNA damage as a possible cause of impaired T cell survival, $CD4^+CD45RO^-$ naïve and $CD4^+CD45RA^-$ memory T cells were isolated from HCV-infected patients and age-matched HS, cultured *in vitro* without stimulation for 0-4 days, followed by DNA integrity anaysis by measuring the expression of 8-oxogunine (8-oxoG), a marker for DNA DSBs that are caused by excessive oxidative stress [21]. As shown in **Fig.2.2A** and **B** (representative overlaid histogram and summary data of flow cytometry), resting naive CD4 T cells derived from HCV-infected patients had significantly higher expression of 8-oxoG DNA bases compared to HS, indicating an accumulation of DNA lesions during chronic viral infection. After culturing cells without mitogenic or antigenic stimulation, both HCV and HS T cells showed increases in the expression of 8-oxoG. However, the extent of this increase in HCV naïve T cells was less than that in HS. This could be attributed to (i) the relatively high baseline 8-oxoG level in HCV naïve T cells at day 0, and (ii) the test saturation of 8-oxoG load in T cells under these culture conditions with oxidative DNA stress which could limit the 8-oxyG differences between HCV and HS T cells at day 4 in culture. In contrast, memory T cells from HCV and HS exhibited an overload of 8-oxoG at baseline. After culturing cells without stimulation, 8-oxoG levels only slightly increased, although HCV

memory T cells exhibited marginally higher DNA lesions than HS at baseline (day 0) and 4 days in culture. These data indicate that naïve T cells from HCV patients exhibit oxidative DNA DSBs that remains unrepaired during viral infection.

Following genotoxic insult, histone variant H2AX is recruited to the site of DNA-DSB and becomes phosphorylated at its C-terminal Ser-139 residue to form the γH2AX complex, which subsequently acts as a docking site for other mediators or adaptor proteins, such as 53BP1, to form microscopically visible nuclear focus (DNA damage foci) - a hallmark of DNA damage response (DDR) [27, 28]. To confirm that DNA damage occurs in T cells during HCV infection, we purified naïve and memory CD4 T cells from $HCV^{+/-}$ subjects, and compared DNA damage foci by examining the colocalization of γH2AX/53BP1 per nuclei using confocal microscopy. As shown in **Fig.2.2C** (representative imaging and summary data of confocal microscopy), the number of DNA damage foci was significantly higher in the naïve CD4 T cells freshly isolated from HCV patients compared with the HS. We also observed an increase in DNA damage foci in memory CD4 T cells derived from HCV patients versus HS (Supplementary **Fig 2S1B**). When the cells were cultured without stimulation for 4 days, the DNA damage foci increased, and were significantly higher in the naïve CD4 T cells from HCV patients compared with the HS. These results, in conjunction with the changes in T cell frequency and apoptotic death, suggest that unrepaired DNA damage is associated with T cell apoptosis and loss in individuals with chronic HCV infection, emphasizing the role of DNA damage repair to secure T cell survival.

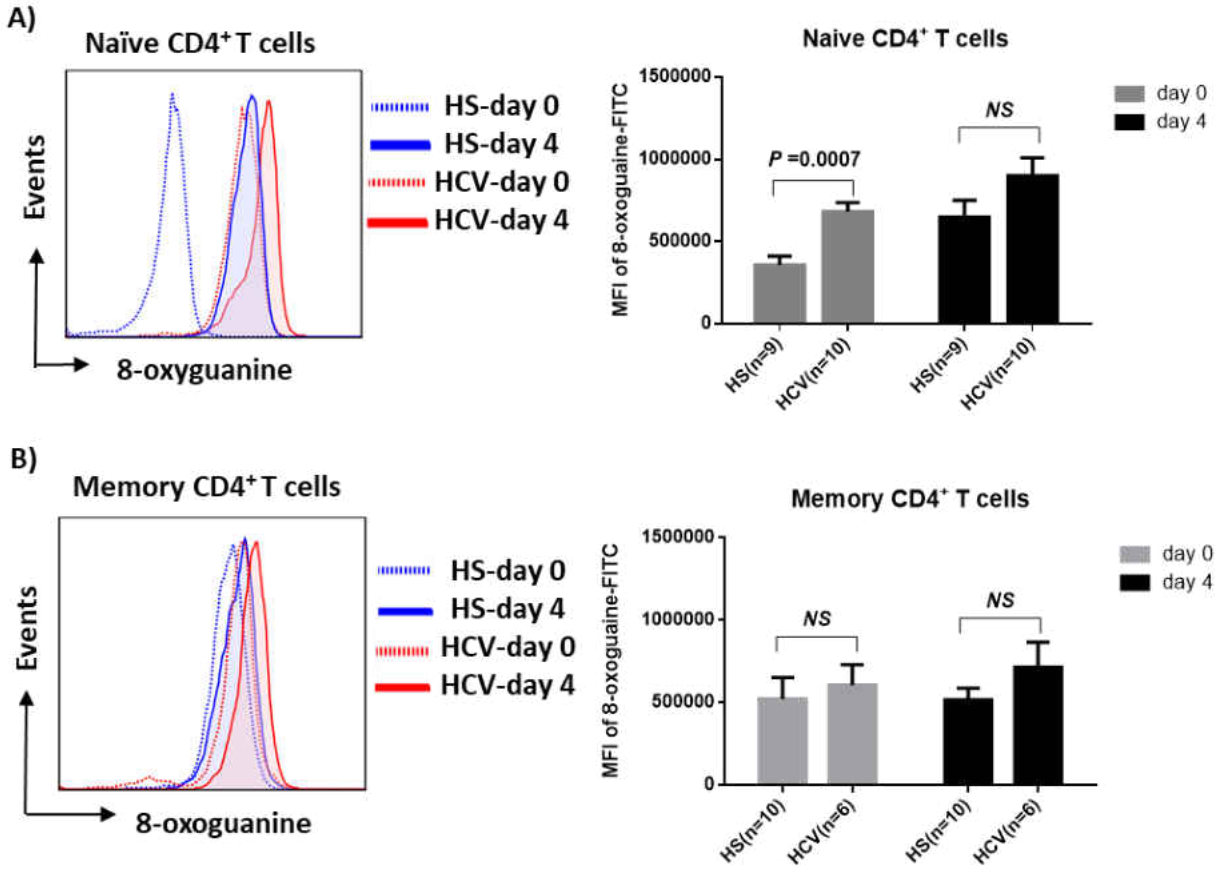
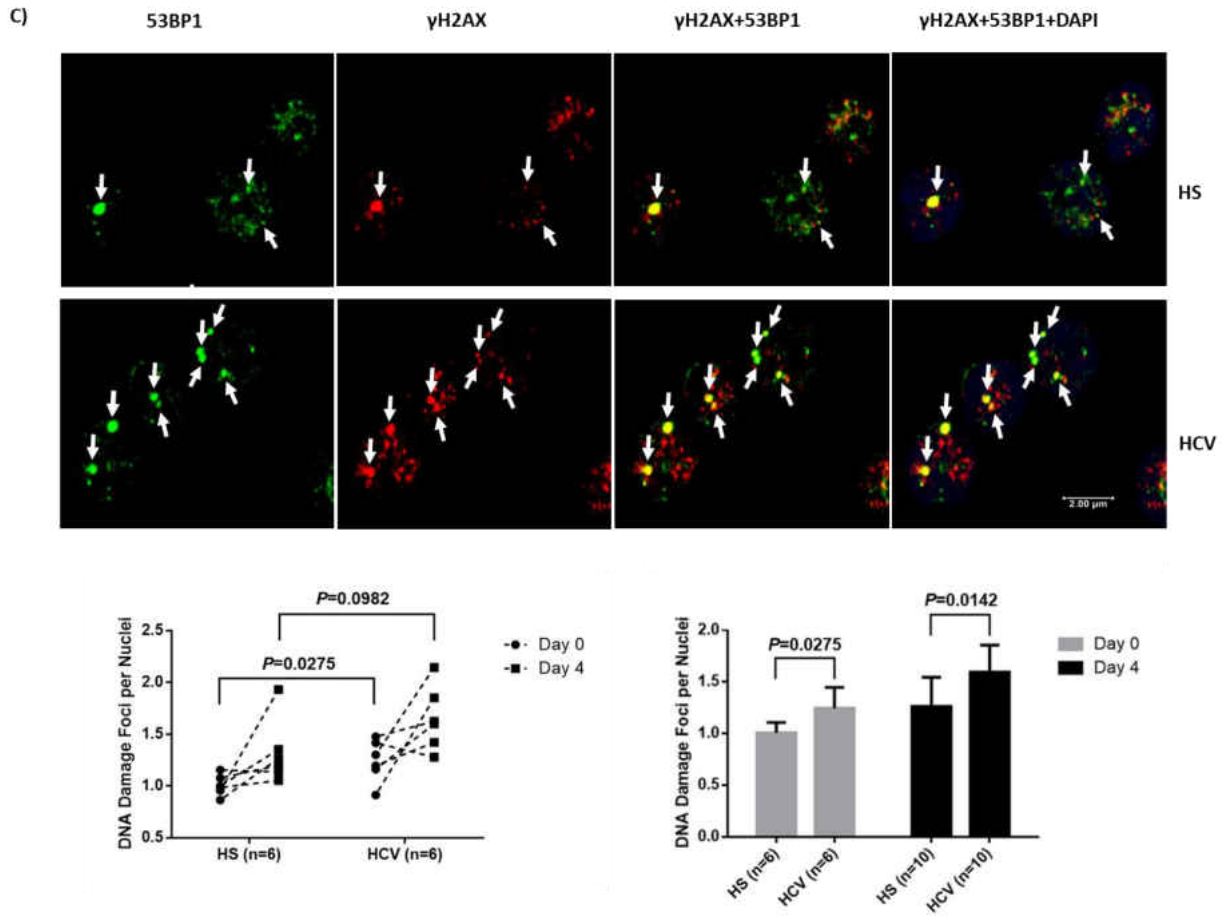


Fig.2.2 DNA damage in CD4 T cells from HCV-infected patients versus age-matched HS. A) & B) 8-oxyguanine expression in CD4 T cells. Naïve and memory CD4 T cells were purified from PBMCs of HCV-infected patients and HS, followed by flow cytometric analysis of 8-oxyguanine expression. Representative overlaid histogram and summary data for mean florescence intensity (MFI) of 8-oxyguanine expression are shown. **(continued on the next page). C)** Co-localization of 53BP1 and γH2AX in CD4 T cells. Naïve CD4 T cells were isolated from PBMCs of HCV patients and HS, cultured *in vitro* without stimulation for 0-4 days, followed by confocal microscopic analysis of 53BP1 and γH2AX co-localization in the nuclei. 100 cells were counted per subjects. N=number of subjects studied. P values of the DNA damage foci per nuclei in the two groups are shown in the summary data.

Fig.2.2 (continued)



DNA Damage Sensing and Repairing Machineries in Naïve CD4 T Cells from HCV-Infected Individuals

Accumulation of DNA-DSBs in CD4 T cells from HCV patients indicates that the DNA damage sensing and repairing machinery is disrupted. Essential components of this machinery include DNA damage sensors, such as MRN complexes (MRE11, RAD50, and NBS1), which recruit and mediate the DNA repair kinase ATM that can phosphorylate several downstream checkpoint proteins (such as p53, BRCA1, CHK1 and CHK2) [22-24]. To investigate the cellular machineries that contribute to the DNA damage repair, we examined mRNA transcripts and

protein expressions of these DDR molecules in CD4 T cells from HCV-infected patients and HS using realtime RT-PCR and western blotting or flow cytometry. As shown in **Fig.2.3A**, the mRNA levels of MRN complexes showed no difference or higher levels of MRE11, RAD50, and NBS1. In parallel, the protein levels of these DNA damage sensors in naïve CD4 T cells were also unchanged or slightly higher in HCV compared with HS (**Fig.2.3B**). Intriguingly, altrough the mRNA level of ATM was higher (**Fig.2.3C**), its protein level was lower; particularly, ATM phosphorylation (pATM) was significantly lower in HCV naïve CD4 T cells in the 4-day culture compared to the HS, as measured by flow cytometry (**Fig.2.3D**) and western blot (**Fig.2.3E**). Similarly, the mRNA expressions of ATM signaling molecules, P53, 53BP1, BRCA1, CHK1, and CHK2 remained unchanged or even higher in naïve CD4 T cells from HCV patients when compared to the HS (**Fig.2.3F**). However, pp53 protein was undetectable in resting naïve CD4 T cells without stimulation, whereas BRCA1 and CHK1 total and phosphorylated proteins remained unchanged (**Fig.2.3G**); CHK2 protein, especially pCHK2, was significantly suppressed in HCV T cells compared to HS as demonstrated by western blot and flow cytometry (**Fig.2.3H**). These results suggest that the DNA damage sensing machinery is intact, but the DNA repair (ATM/CHK2) pathway is inhibited, at the post-transcriptional levels, especially the phosphorylation process essential for its activation and function.

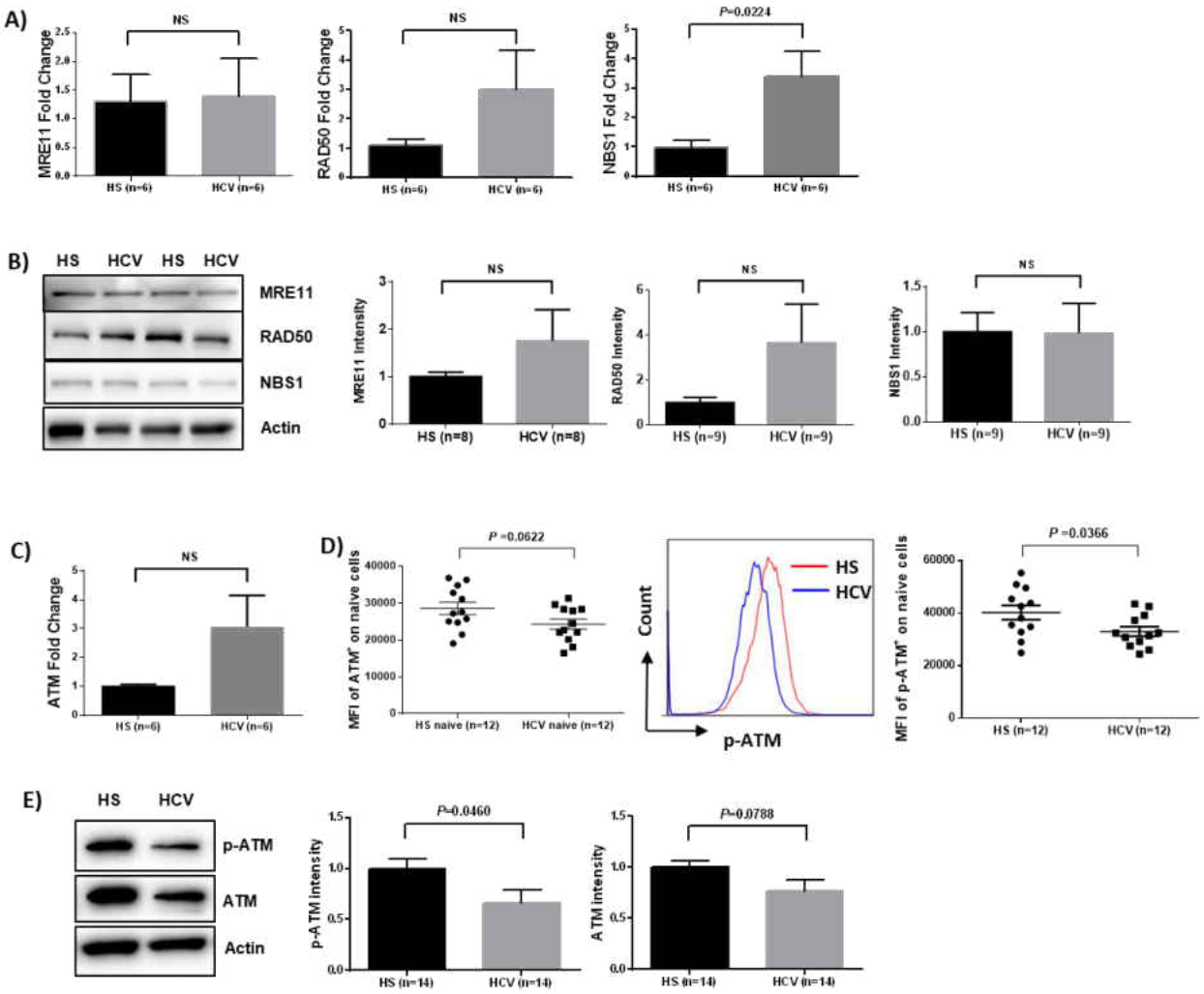
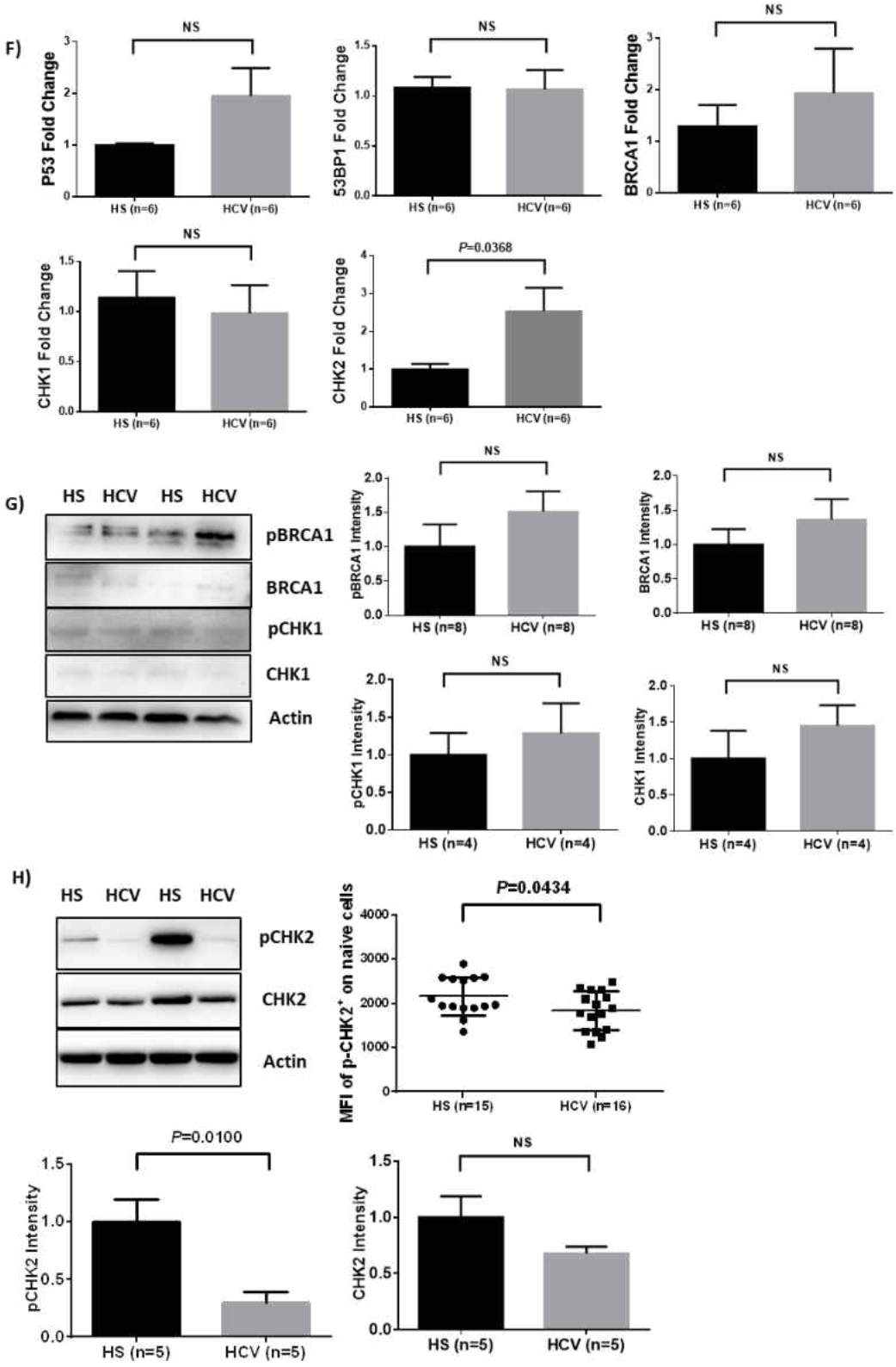


Fig.2.3. DNA damage repair ATM signaling pathway in T cells of HCV-infected patients vs age-matched HS.

A) DNA damage sensor MRN complex expressions in CD4 T cells. Naïve CD4 T cells were isolated from 6 HCV-infected patients and 6 age-matched HS, cultured in vitro without stimulation for 4 days, followed by real-time RT-PCR assay for MRE11, RAD50, and NBS1 mRNA expression. **B)** MRN protein expression detected by Western blot in naïve CD4 T cells derived from HCV patients vs. HS. Representative imaging and summary data from multiple subjects are shown. **C)** ATM mRNA expressions in CD4 T cells. Naïve CD4 T cells were isolated from the study subjects as indicated, cultured in vitro without stimulation for 4 days, followed by RT-PCR analysis for ATM mRNA level. **D&E)** Flow cytometry and western blot analysis for ATM total and phosphorylated protein expression in naïve CD4 T cells from HCV patients vs HS. **(continued on the next page). F-H)** ATM signaling molecule mRNA and protein expressions in CD4 T cells. Naïve CD4 T cells were isolated from the study subjects, cultured in vitro without stimulation for 4 days, followed by RT-PCR analysis for P53, BRCA1, CHK1 and CHK2 mRNA level, western blot assay for their total and/or phosphorylated protein levels. pCHK2 expression was confirmed by flow cytometry analysis.

Fig.2.3. (continued)



Role of HCV in Dampening ATM Activation and Its Effects on DNA Damage and Cell Apoptosis

As chronically HCV-infected patients often have other co-morbidities that may cause immune dysregulation, we examined the specific role of HCV in triggering DDR and promoting cellular senescence or apoptosis. We incubated naïve CD4 T cells with Huh7.5 cells with or without HCV infection, followed by measuring ATM activation. As shown in **Fig.2.4A** (immunofluorescence staining of Huh7.5 cells with or without infection by HCV JFH-1 strain), hepatocytes transfected with HCV RNA at 48 h showed positive expression of HCV core protein, whereas cells with mock transfection exhibited negative staining. Moreover, we detected HCV RNA in the supernatant of HCV-transfected cells (at 24 h as well as 48 h) but not mock-transfected cells (data not shown). Importantly, similar to the observations in HCV patients (**Fig.2.3B**), wherase the total ATM protein expression was not significantly decreased, the phosphorylation of ATM was markedly inhibited in the naïve CD4 T cells that were incubated with HCV^+ Huh7.5 cells compared with cells co-cultured with HCV^- Huh7.5 hepatocytes (**Fig.2.4B**). In addition, T cells exposed to HCV-expressing hepatocytes were more apoptotic, as demonstrated by a significant increase (P=0.0357) in Av/7AAD expression in CD4 T cells exposed to HCV compared with the negative control (data not shown).

We have previously shown that primary T cells treated with HCV core protein exhibited a senescent state, as evidenced by a higher level of the aging marker β-Galactosidase expression and shortened telomeres [5]. To further investigate whether HCV core-treated T cells have impairment in the DNA damage repair enzyme ATM, we exposed healthy naïve CD4 T cells to HCV core protein, which is secreted by virally infected hepatocytes and circulates in the

peripheral blood of HCV-infected patients and can dampen T cells through its interaction with the globulin head of C1q receptor expressed on the surface of T cells, thus delivering inhibitory signaling [29],. As shown in **Fig.2.4C**, compared to the β-gal control, treatment with HCV core protein for 5 days significantly inhibited the phosphorylation of ATM, but not total ATM protein in naïve CD4 T cells. In addition, HCV core-treated T cells exhibited an increased level of γH2AX (a marker of DNA damage), suggesting that insufficient pATM is associated with an increased DNA damage in HCV core-treated T cells.

ATM belongs to the PI3K family, and we have previously shown that HCV can induce T cell senescence by inhibiting the AKT/PI3K pathway [3]. To further explore the mechanisms that might be involved in inhibiting ATM activation, we treated naïve CD4 T cells with LY294002 (a potent PI3K-specific inhibitor) in the presence or absence of HCV core protein. As shown in **Fig.2.4D**, compared with the DMSO-treated control, T cells treated with 20μM LY294002 exhibited remarkable inhibition of pATM, along with decreases in pAKT. Moreover, the cleaved form of Poly (ADP-ribose) polymerase (PARP), an enzyme that catalyzes the transfer of ADP-ribose onto target proteins and plays an important role in DNA repair and cell survival, was significantly increased in T cells treated with the PI3K inhibitor. In addition, we assessed the effect of LY294002 on ATM inhibition in CD4 T cells co-cultured with $HCV^{+/-}$ Huh7.5 cells. As shown in **Fig.2.4E**, pATM expression in CD4 T cells in the presence of Huh7.5 hepatocytes without HCV infection was significantly inhibited by the PI3K inhibitor. pATM expression was further inhibited in naïve CD4 T cells incubated with HCV^{+} Huh7.5 cells, especially in the presence of PI3K inhibitor. These results suggest that HCV (core protein) can induce T cell DNA damage that is associated with impaired ATM via inhibition of the PI3K.

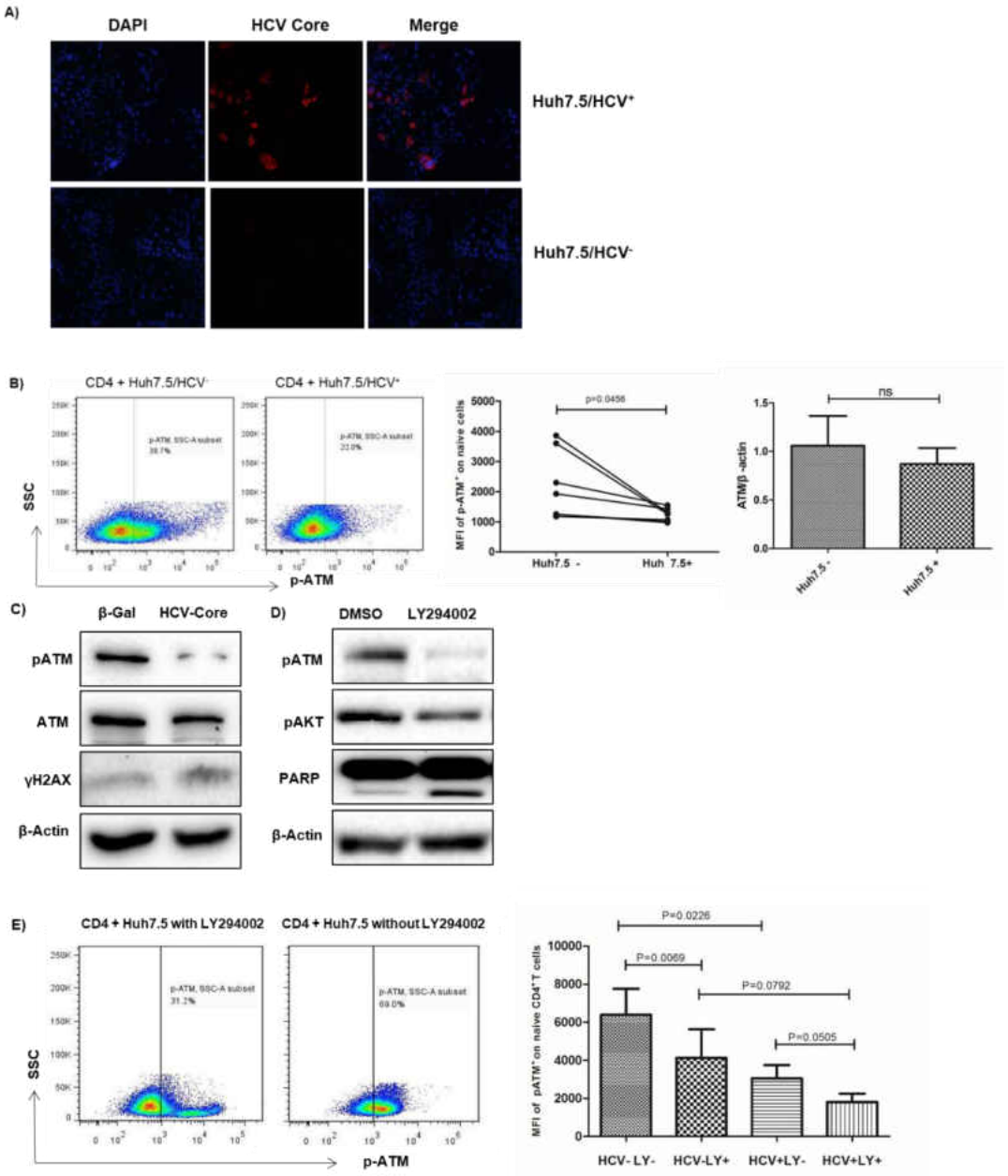


Fig.2.4. HCV inhibits ATM/pATM expressions via dampening PI3K pathway. A) HCV infection of Huh7.5 hepatocytes. Immunostaining of HCV core protein is positive in HCV-infected cells, but negative in HCV-uninfected cells. **B)** ATM/pATM is inhibited in naïve CD4 T cells co-cultured with Huh7.5 cells with HCV infection compared with those incubated with Huh7.5 cells without HCV infection. **C)** HCV core protein inhibits ATM/pATM and enhances γH2AX expressions in naïve CD4 T cells compared to the β-Gal control. **D)** PI3K inhibitor (LY294002) inhibits pATM/pAKT and enhances PARP expressions in naïve CD4 T cells compared to those treated with DMSO control. **E)** PI3K inhibitor (LY294002) inhibits pATM expressions in naïve CD4 T cells co-cultured with HCV+/- Huh7.5 cells compared to the cells without inhibitor treatment. Representative flow cytometry dot plots and summary data (n=8) with p value between the groups are shown.

Inhibition of ATM Phosphorylation in Naïve CD4 T cell Leads to DNA Damage and Apoptosis

The ATM signaling pathway is pivotal to the maintenance of genome integrity and cell survival [26]. To test the functionality of this DNA repair machinery in T cell survival, freshly isolated healthy naïve ($CD4^+CD45RO^-$) T cells were treated with a specific ATM inhibitor (KU60019, 10μM) for 48 h, followed by measuring DNA damage and cell apoptosis or death. As shown in **Fig.2.5A**, naïve T cells exposed to KU60019 showed an inhibition in ATM, in particular pATM, compared with the DMSO control. In addition, the phosphorylation of CHK2 protein, a downstream effector of ATM signaling pathway, was significantly inhibited by the treatment (**Fig.2.5B**). Importantly, T cells exposed to the ATM inhibitor exhibited an elevated γH2AX expression, suggesting an increase in DNA damage (**Fig.2.5C**). In addition, T cells treated with the ATM inhibitor exhibited an increase in DNA damage foci (γH2AX/53BP1 co-localization) compared with the control (**Fig.2.5D**). Moreover, ATM inhibition in naïve CD4 T cells resulted in considerately increased T cell apoptosis and death, as demonstrated by an increase in Av and 7ADD expression, as well as activated caspase-3 following treatment (**Fig.2.5E**). In essence, an insufficiency of ATM leads to greater DNA damage and cell apoptotic death, which may necessitate compensatory homeostatic proliferation and lead to telomere loss and premature senescence, particularly in the naive T-cell pool, a mechanism that can potentially contribute to the naïve T cell loss and poor immune (vaccine) responses in chronic HCV infection.

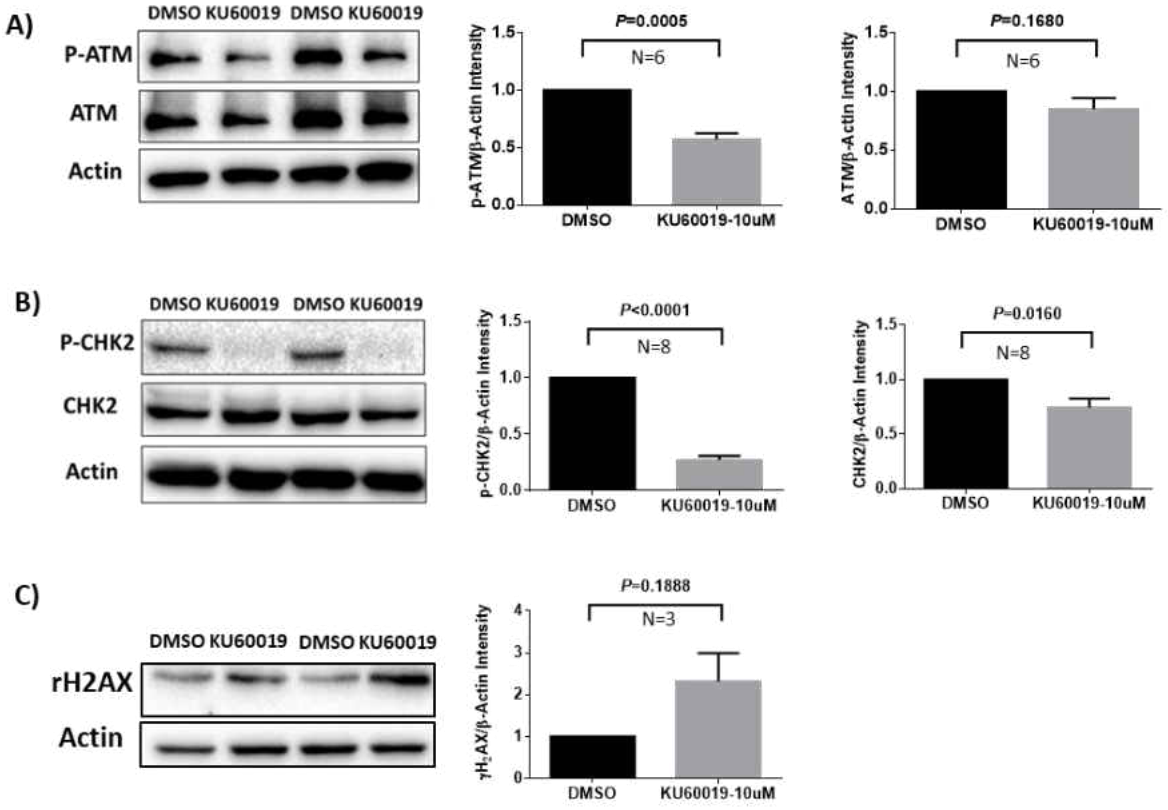
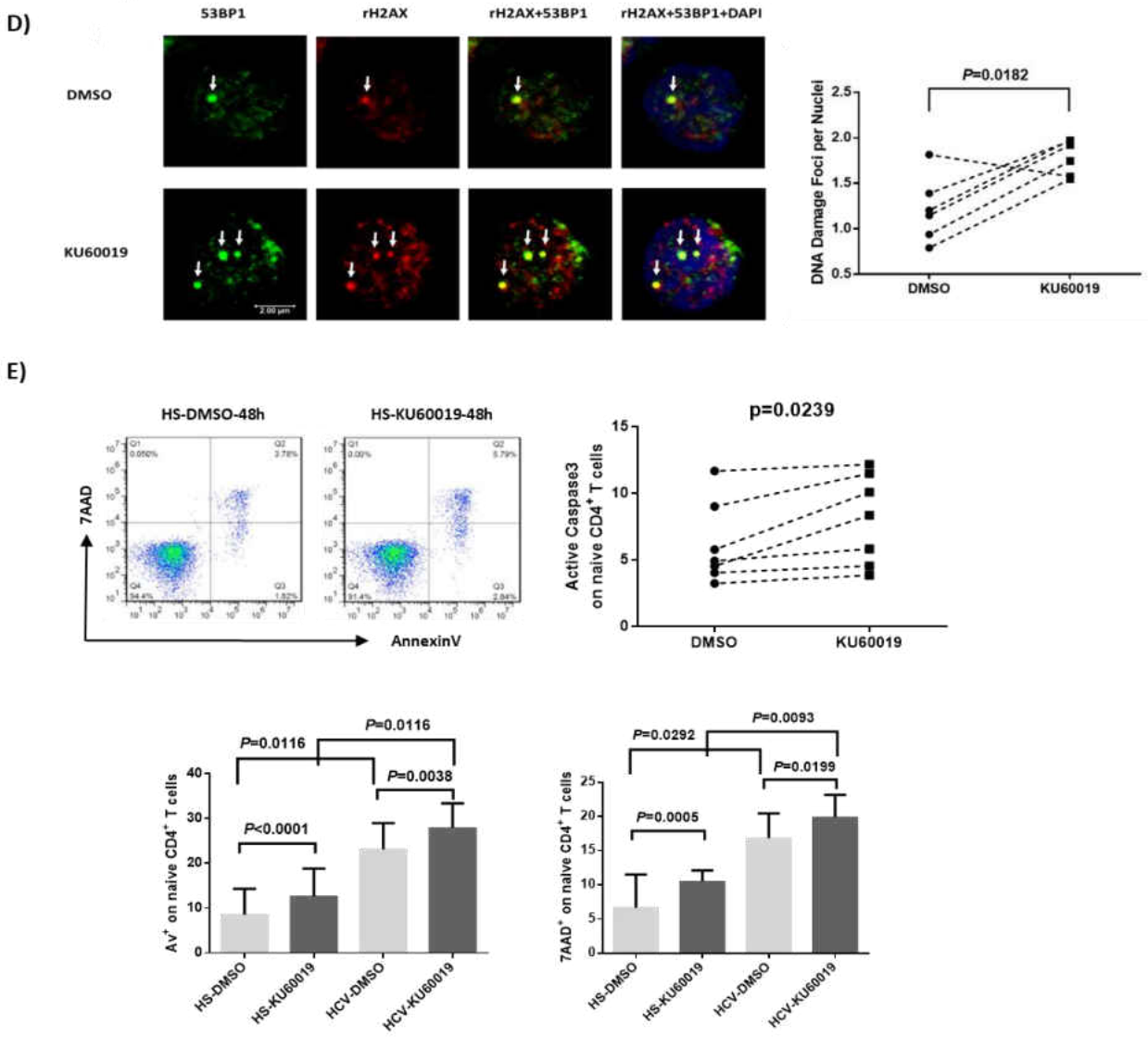


Fig.2.5. KU60019, an ATM inhibitor, induces DNA damage and cell apoptosis via inhibiting ATM pathway. A) KU60019 inhibits ATM/pATM expressions in naive CD4 T cells. Representative imaging and summary data show Western blot analysis for ATM/pATM expressions in naïve CD4 T cells treated with 10 μM KU60019 or DMSO for 48 h. **B)** KU60019 inhibits CHK2/pCHK2 expressions in naïve CD4 T cells. **C)** KU60019 enhances γH2AX expression in naïve CD4 T cells. **(continued on the next page). D)** KU60019 increases DNA damage foci (γH2AX/53BP1 co-localization) in naïve CD4 T cells. **E)** KU60019 increases T cell apoptosis in naïve CD4 T cells. Av, 7AAD, and active caspase-3 expressions are shown in naïve CD4 T cells treated with KU60019 vs. DMSO control at 48 h.

Fig.2.5. (Continued)



Reconstitution of ATM in HCV CD4 T Cells Repairs DNA Damage, Survival Defect, and Cellular Functions

Given the critical role of ATM in repairing DNA-DSBs, we hypothesized that reconstitution of ATM could protect HCV-derived T cells from DNA damage and restore the signaling network required for repairing DNA breaks. To test this, we transfected CD4 T cells

derived from HCV-infected individuals with Flag-His-ATM constructs or controls, including an empty vector without ATM insert (Mock), and an ATM mutant (ATM-S1981A) in which the serine (S) phosphorylation site at residue number 1981 was substituted by alanine (A), using the Lonza transfection system. Despite high transfection efficiency (70%) with GFP transfection by this system, fluorescence-activated cell sorting analysis revealed intracellular His-ATM expression in only 20-40% of the T cells transfected with Flag-His-ATM or control constructs (**Fig.2.6A**). Western blotting confirmed an increase in ATM expression in the wild-type, as well as mutant ATM-S1981A-transfected T cells (**Fig.2.6B**). Although ectopic overexpression of ATM had broad biological consequences, we focused our investigation on the DNA repair, cell survival, and cell function, by assessing γH2AX, caspase-3, and IL-2 expression levels as readout. As shown in **Fig.2.6C**, compared to mock transfection, ATM reconstitution reduced γH2AX expression after 48 h, whereas transfection of the non-phosphorylated form with an ATM-S1981A mutant was unable to restore the level of DNA repair, indicating the importance of ATM phosphorylation at serine 1981 in protecting cells from excessive DNA breaks. In parallel, transfection of wildtype ATM significantly reduced T cell apoptosis, whereas mock- or ATM-S1981A-transfected cells exhibited relatively higher levels of active caspase-3 expression (**Fig.2.6D**), suggesting that ectopic ATM expression exerts immediate effects in determining T cell fate by securing cell survival. Most importantly, implementing ATM significantly improved T cell function, as shown by the increase in IL-2 expression in ATM-transfected cells, whereas ATMS1981A transfection could not induce such an effect (**Fig.2.6E**). Taken together, these results suggest that restoring an adequate ATM level in naïve CD4 T cells from chronic HCV infection is sufficient to ameliorate DNA damage, survival defects, and cell dysfunctions.

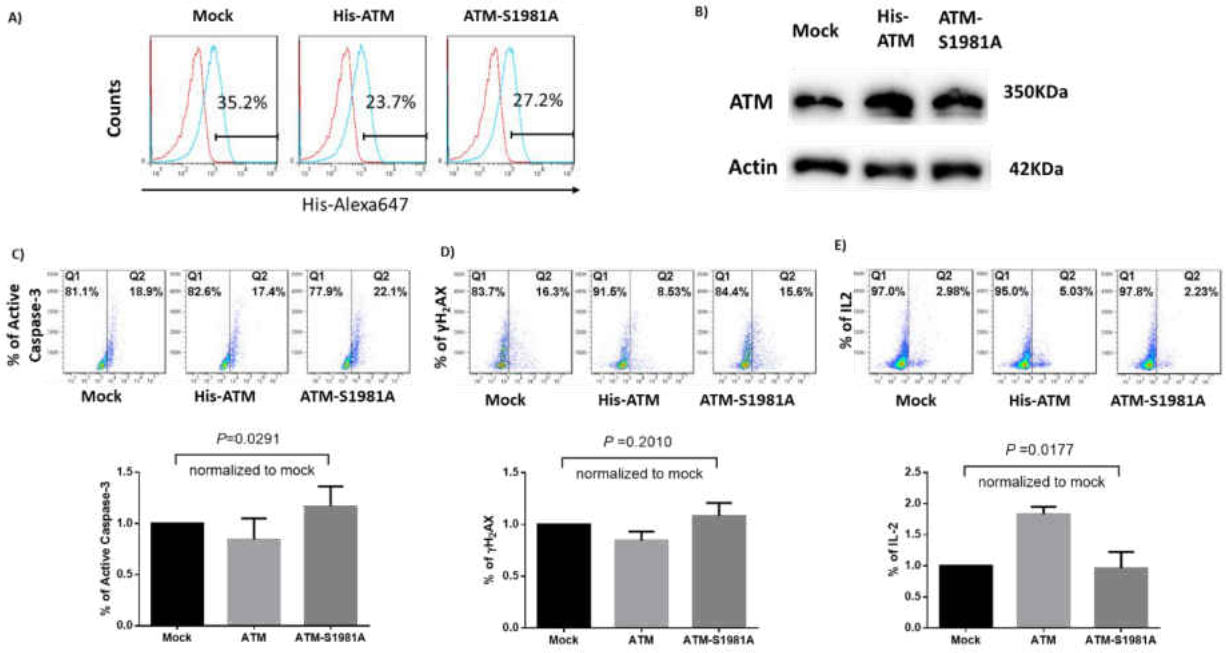


Fig.2.6. Ectopic ATM expression repairs DNA damage, cell apoptosis, and cellular dysfunctions from HCV infection. A) Representative histogram of His Expression (Blue) in Mock-, ATM, and ATM-S1981A-transfected naïve CD4 T cells derived from HCV-infected patients. Isotype control (Red) was used for gating the His^+ cells in flow cytometry analysis. **B)** ATM expression in Mock, ATM, and ATM-S1981A-transfected naïve CD4 T cells from HCV-infected patients, measured by western blot. **C)** Representative dot plots and summary data of flow cytometric analysis for the expression of γH2AX (n=10), active caspase-3 (n=9), and IL-2 (n=3) in Mock, ATM, and ATM-S1981A-transfected naïve CD4 T cells derived from HCV-infected patients.

Discussion

We and others have previously shown that T cells derived from patients with chronic viral infections prematurely reach senescence, characterized by the shortening of telomeres and expression of aging markers [2-6]. In this study, we further demonstrate that homeostatic remodeling of the T cell repertoire during HCV infection primarily affects the naïve T cell compartment, characterized by an accumulation of DNA damage (owing to insufficient

activation) of the DNA repair ATM enzyme, which leads to naive T-cell apoptosis and loss. Indeed, phosphorylation of ATM in T cells exposed to HCV (core protein) is inhibited through dampening the PI3K pathway, resulting in an increase in DNA damage and cell apoptosis. Moreover, pharmacological inhibition of ATM phosphorylation leads to more DNA damage and apoptosis in naive T cells. Most importantly, reconstitution of ATM repairs the DNA damage, cell apoptosis, and functional defects in naïve CD4 T cells derived from HCV-infected patients. Based on these novel findings, we propose a model, depicted in **Fig.2.7**, where HCV-induced ATM deficiency leads to accumulation of DNA damage and cell apoptosis. The excessive T cell loss necessitates high homeostatic proliferation and imposes replicative stress on unprimed naïve T cells; this represents a novel molecular mechanism underlying T cell senescence in the setting of chronic viral infection. Importantly, ectopic overexpression of ATM is necessary and sufficient to repair the DNA damage, survival defect, and cell dysfunctions in HCV-derived T cells, thus providing a new strategy to improve immunotherapy and vaccine responses against human viral diseases.

We and others have observed poor vaccine responses in the setting of chronic viral (HCV, HIV) infections [2, 3, 15-19], but the underlying mechanisms for vaccine failure in virally-infected individuals remain unclear. Data presented in this study indicate that naïve helper T cells in chronically HCV-infected patients have abnormalities that jeopardize their ability to mount effective immune (vaccine) responses. Specifically, we demonstrate that naïve CD4 T cells have accumulation of damaged DNA and fail to repair their DNA-DSB owing to deficiency of the ATM pathway. Accumulated DNA damage renders HCV-derived T cells prone to apoptotic death, imposing replicative stress and premature aging on naïve T cell pools. These

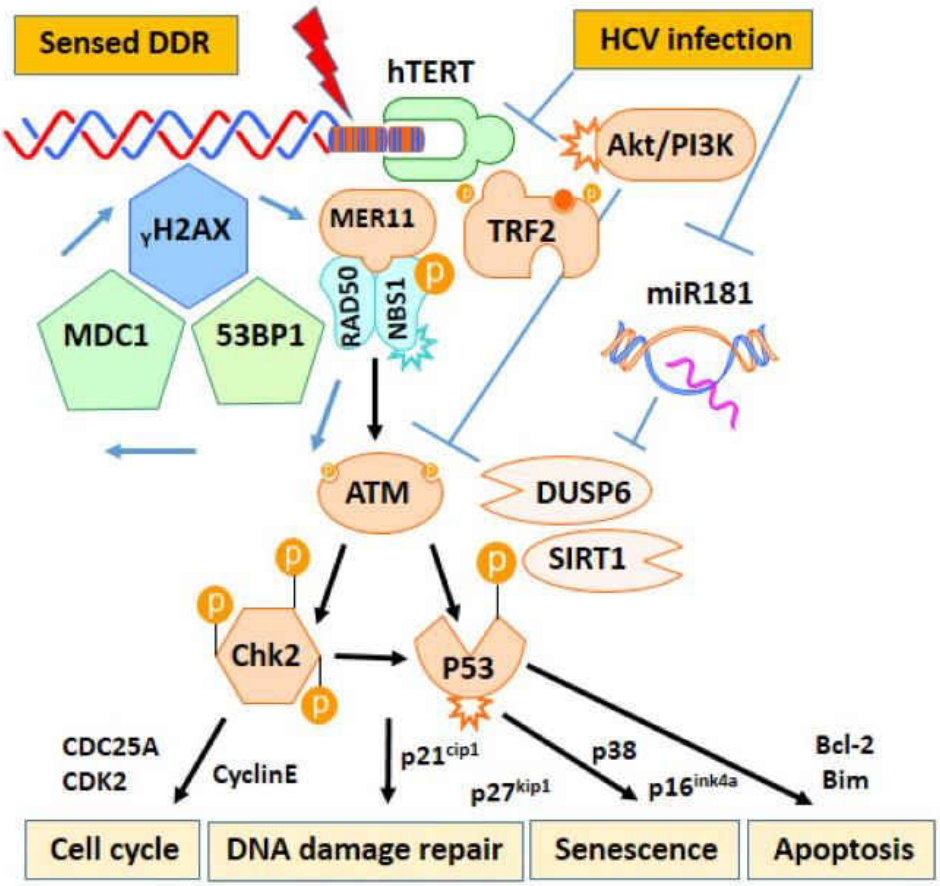


Fig.2.7. A novel model of HCV-induced ATM deficiency in T cell cycle arrest, DNA damage repair, cell senescence, and apoptosis. HCV infection triggers a DNA damage response (DDR) in the early phase via activation of MRN-ATM-CHK2 and P53 signaling pathways in naive CD4 T cells, prompting cell cycle arrest and allowing for DNA damage repair; or, if the infection is overwhelming and causes unrepairable DNA damage, the cell will commit suicide and initiates apoptosis. Persistent antigenic and inflammatory stimulation, however, drives ATM exhaustion and insufficiency, leading to impaired DNA damage repair and accumulation of DNA DSB, which results in constant cell apoptosis and naïve T cell loss. Excessive T cell loss necessitates high homeostatic proliferation and imposes replicative stress on unprimed naïve T cells, emerging as a novel molecular mechanism underlying T cell senescence in the setting chronic viral infection. Importantly, ectopic overexpression of ATM is necessary and sufficient to repair the DNA damage, survival deficit, and cellular dysfunction in HCV-derived T cells, providing a new strategy to improve immunotherapy and vaccine responses against human viral diseases.

findings are important because naïve T cells represent the reserve pool of the immune system, and their survival critically determines the cellular yield of homeostatic proliferation, a process that generates new T cells in response to neo-antigens, including vaccines.

Insufficient activation of ATM would be expected to affect both unprimed and primed T cells. Indeed, we observed unrepaired DNA damage and cell apoptosis in both naive and memory T-cell populations. However, ongoing antigenic stimulation during chronic viral infection could drive naive T-cell differentiation and turnover of antigen-reactive T cells. In this regard, memory T cells would expand and compromise the size and survival of naive T cells. Eventually, the entire T-cell pool would be comprised by antigen-reactive T cells at the expense of naive T cells. In the setting of chronic viral infection, however, memory T cells could be functionally biased as a result of chronic antigenic stimulation; as such, we focused our studies on the T-cell population that has yet to be recruited for immune responses. With the decrease in newly generated naive thymic T cells in adults, chronic infection or inflammation might force the immune system to restore equilibrium by replicating the available or existing naive T cells, thereby driving telomere shortening and senescence in naive T-cell populations. Thus, the ability to generate immune response to new antigens, such as HBV vaccine, could be compromised.

ATM has a unique role in lymphocyte biology, as programmed DNA damage repair is part of the gene rearrangement necessary for formation of a highly diverse T cell receptor repertoire. Under non-stress conditions, ATM is inactive and exists in the form of a dimer-(like other PI3Ks). It requires a signal for activation (usually a DNA damage signal), signaling through intermolecular autophosphorylation at ATM residue S1981, and resulting in the dimer dissociation into monomers [31]. In our study, a mutant of S1981A rendered ATM a dominant-negative protein, triggering more severe DNA damage (γH2AX expression), cell apoptotic death

(Av/7AAD expression), and cellular dysfunction (IL-2 inhibition) - underscoring the importance of ATM phosphorylation for its biological functions. ATM is predominantly localized in the nucleus, and undergoes activation once the MRN complex senses and binds to DNA-DSB ends, providing a platform for ATM recruitment and autophosphorylation [32, 33]. Phosphorylation of S1981 also stabilizes ATM at the damaged DNA sites and recruits more downstream effector proteins to participate in the DDR [34]. Among the multiple substrates phosphorylated by ATM is the checkpoint kinase 2 (CHK2), which is phosphorylated at residue T62 following DSB formation and prevents cells from progressing from G1 to S phase or, alternatively, leads to cell apoptosis. The discovery of these DNA damage response proteins has shed light on the cellular machinery that contributes to DNA repair and cell homeostasis.

Recently, Li et al. [35] identified prematurely aged T cells with damaged telomeres in patients with rheumatoid arthritis resulting from defective activity of the DNA break sensor MRE11A. In patients with chronic HCV infection, however, we find that the DNA damage sensor MRN complex is intact in naïve T cells. Rather, the DNA damage repair enzyme ATM is inhibited at a posttranscriptional level by HCV infection. Interestingly, Guo et al. [36, 37] reported that ATM activation in response to ROS was independent of the MRN complex. ROS mediated ATM signaling represses mTORC1 signaling and therefore cell growth and proliferation through activation of TSC2 (a negative regulator of mTOR) by liver kinase B1 (also known as STK11) and AMP-dependent protein kinases[38]. ATM engagement of the TSC2/ mTORC1 signaling pathway can also regulate autophagy[39], and differential localization of ATM is correlated with activation of distinct downstream signaling pathways[40]. We have previously reported that T cells treated with HCV core protein exhibit G1/S cell cycle arrest, which was associated with the dysregulation of cell cycle regulatory proteins CDKs/Cyclins and $P27^{kip1}$ [41]. Here, we

demonstrate that concomitant with the insufficiency of ATM activation, the phosphorylation of CHK2 is defective in naïve T cells from HCV-infected patients. Moreover, pharmacological inhibition of ATM in healthy T cells also leads to a CHK2 defect, accompanied by a marked increase in DNA damage and cell apoptosis - resembling the biological effects characteristic of HCV-derived naive T cells. These results establish that in human T cells, CHK2 is targeted by ATM and that the overall defect of this pathway can be attributed to ATM insufficiency due to chronic HCV infection.

A typical feature of CD4 T cells in chronically HCV-infected patients is the shortening of telomeres compared with age-matched healthy controls [5, 6]. Several mechanisms may contribute to this age associated loss of telomeres. Increased proliferative turnover can cause cell division-induced telomeres shortening. In addition, telomeric DNA is highly susceptible to DNA damage, even more so than non-telomeric DNA. Plasmid-inserted human telomeres accumulate sevenfold higher strand breakage than control sequences [42]. Also, the frequency of single-strand breaks is several-fold higher in telomeres than in the bulk genome when cells are treated with alkylating agents or exposed to oxidative stress [43]. In line with these findings, we have recently found that the expression of the telomere shelterin TRF2 is significantly inhibited at the protein level in naïve CD4 T cells derived from HCV-infected individuals, which renders the uncapped telomeres prone to DNA damage (unpublished observations). Thus, telomere loss in HCV T cells is triggered by DDR and the inability of timely repair by the ATM signaling pathway. In addition, we have also discovered that KML001, a telomere-targeting drug, can induce telomeric DNA damage and T cell apoptosis by impairing the ATM pathway (unpublished observations). Notably, ATM is widely expressed in human T cells at an extremely high level to ensure integrity of the genomic DNA in replicating lymphocytes. ATM activation represents the

initiation of DDR, but its inhibition in persistently stimulated T cells indicates insufficiency of this DNA repair enzyme and cell exhaustion and senescence in the setting of chronic viral infection. This notion is supported by our observation in an *in vitro* stimulated T cell system that ATM phosphorylation is increased in the early phase of KML001-treated T cells (3~6 h) and decreased in persistently treated cells (24~48 h), along with increases in DNA damage, cell apoptosis and functional impairment (unpublished observations).

In summary, accumulation of DNA damage and failure to repair the DNA-DSBs owing to deficiency of the ATM-dependent DNA repair machinery during chronic viral infection may have broader implications through impairing diverse cellular functions. As interferon (IFN)-mediated T-cell apoptotic death has been well-studied in persistent viral infections [44-47], this virus-induced DNA damage-mediated T-cell loss represents a new mechanism of immune evasion. How HCV induces DNA-DSBs, and its relationship to the IFN-signaling pathway, are under further investigation. As counteracting ATM deficiency may restore T-cell competency during viral infection and prevent premature immune aging, these studies may provide new strategies to improve immunotherapy and vaccine responses against human viral diseases.

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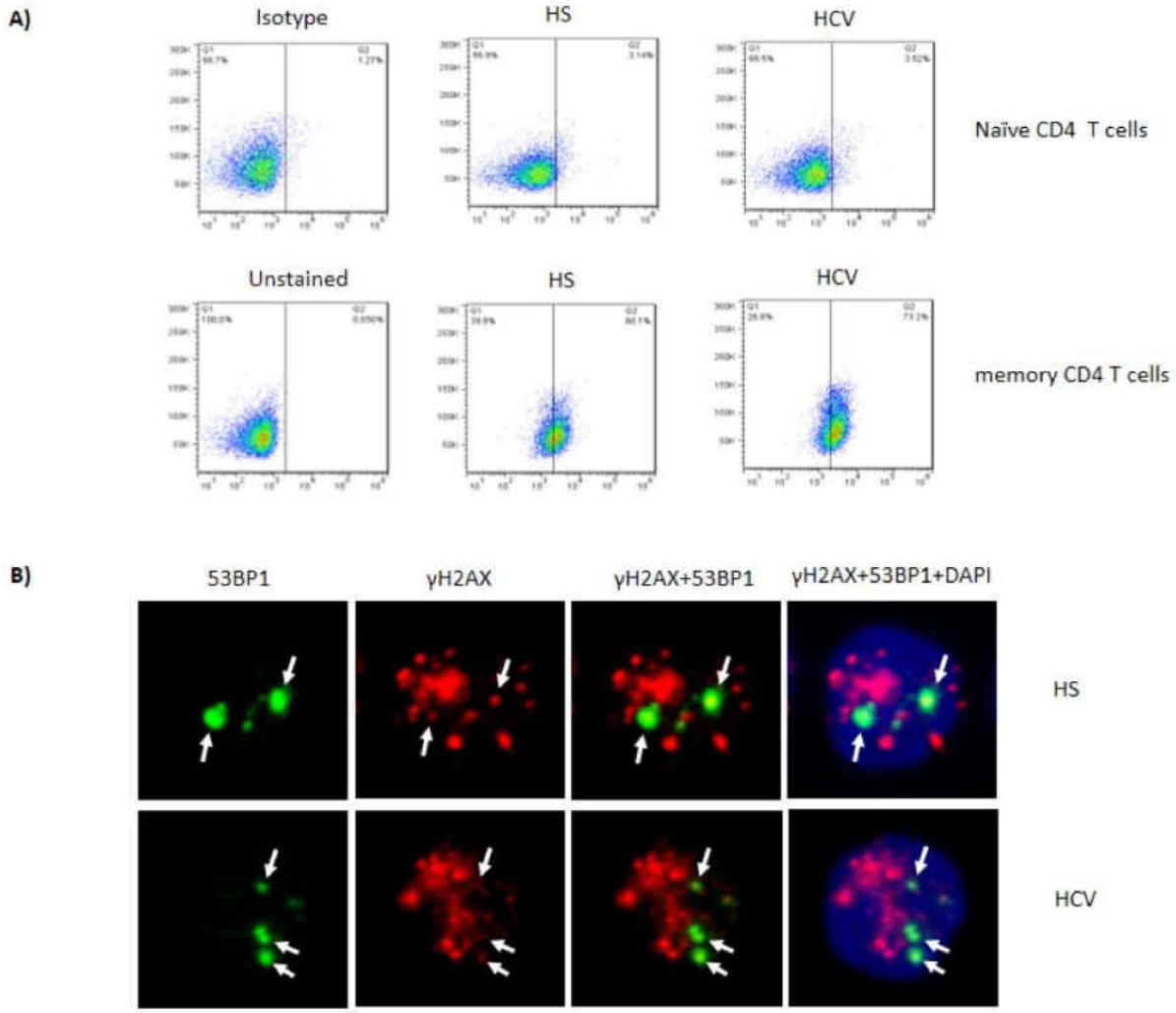
Supplement Data



Supplementary Figure S2.1 A) Flow cytometric analysis of Fas receptor expression on resting naïve and memory CD4 T cells in PBMCs derived from HCV patients and HS. **B)** Confocal microscopic analysis of 53BP1/γH2AX co-localization in memory CD4 T cells isolated from HCV-infected patient and HS. Data were reproducible in cells derived from multiple subjects.

CHAPTER 3

ATM DEFICIENCY ACCELERATES DNA DAMAGE, TELOMERE EROSION AND PREMATURE T CELL AGING IN VIRUS-SUPPRESSSED, LATENT HIV INFECTION

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Running title: ATM deficiency leads to CD4 T cell loss in HIV infection

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Abstract

HIV infection leads to a phenomenon of inflammaging, in which chronic inflammation induces an immune aged phenotype. Here, we investigated T cell homeostasis, telomeric DNA damage and repair machineries in patients with virus-suppressed, latent HIV infection at risk for inflammaging. We found a significant depletion in CD4 T cells, which was correlated with apoptosis in latently HIV-infected individuals compared to age-matched healthy subjects (HS). In addition, HIV CD4 T cells were prone to DNA damage that extended to chromosome ends (telomeres), leading to accelerated telomere erosion - a hallmark of cell senescence. Mechanistically, the DNA double-strand break (DSB) sensor MRE11, RDA50, and NBS1 (MRN) remained intact, but the DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) and its downstream checkpoint kinase 2 (CHK2) were significantly suppressed in HIV T cells. Consistently, ATM/CHK2 activation, DNA repair, and cellular functions were also impaired in primary CD4 T cells following ATM knockdown or exposure to the ATM inhibitor KU60019, as well as in a CD4 T cell line infected with HIV-1 in the presence of an integrase inhibitor - raltegravir, which recapitulates the biological effects observed in HIV T cells *in vivo*. Importantly, ectopic expression of ATM was essential and sufficient to reduce the DNA damage, survival deficit, and cellular dysfunction in T cells from HIV-infected subjects. These results demonstrate that failure of DSB repair due to ATM deficiency leads to increased DNA damage and renders HIV T cells prone to senescence and apoptotic death, thus contributing to CD4 T cell loss/dysfunction during latent HIV infection. Our study reveals a novel mechanism by which ATM deficiency promotes T cell apoptosis/senescence, and provides a new therapeutic target for inflammaging-induced immune dysfunction during chronic viral infection.

Introduction

Aging is associated with, and may be caused by, low-grade inflammation - a phenomenon known as inflammaging, which is a common denominator in the majority of age-related diseases[1-3]. Inflammaging is a major driver of increased susceptibility to infections, reduced responses to vaccines, increased incidences of cancers, cardiovascular diseases, and neurodegeneration, all of which contribute to comorbidity and mortality in the elderly [1-3]. In human immune system, CD4 T cells are long-lived, and thus are exposed to extensive genomic insults and replication pressure, making them vulnerable to aging-associated abnormalities. Notably, the progressive loss of T cell proliferative capacity during the aging process directly correlates with a gradually shortening of telomeres - a hallmark of cell senescence. Therefore, telomere length has been deemed as a biological clock controlling cell aging, survival or function, whereas telomere attrition has been considered as a faithful readout of inflammaging[1-3].

Growing evidence suggests that this aging phenotype is recapitulated in chronic viral infections [4], particularly in human immunodeficiency virus (HIV) infection, which is characterized by disrupting CD4 T cell homeostasis and accelerating premature immune aging. Many of the alterations that affect the immune system in HIV-infected individuals are reminiscent of the course of inflammaging in the elderly [5, 6]. Notably, antiretroviral therapy (ART) does not always result in complete CD4 T cell recovery [7-10]. Despite successful control of HIV replication, residual inflammaging persists, leading to significant immune dysfunction. Even with reasonable CD4 T cell numbers, ART-controlled individuals often experience a profound inflammaging, characterized by extremely short telomeres, low IL-2 production, poor cellular proliferation, and blunted vaccine responses [5-16]. The inflammaging imposed by HIV

exposes the immune system to unique challenges that lead to T cell exhaustion and senescence, similar to that observed in the elderly. These alterations accelerate the decline of CD4 T cell competency and thus define the overall immune dysfunctions during HIV latency. Therefore, the virus-suppressed, latent HIV infection can be deemed as an excellent model of inflammaging in humans, and it is thus important to elucidate the mechanisms underlying T cell senescence and inflammaging that is characterized by telomere erosion in individuals with latent HIV infection.

Because DNA damage can cause telomere erosion, leading to premature cell senescence and/or apoptosis [17, 18], we hypothesized that progressive CD4 T cell loss and failure of immune recovery during latent HIV-1 infection could be, at least in part, due to a deficiency in the DNA repair that alters telomere integrity. Indeed, we have previously shown that patients with chronic hepatitis C virus (HCV) infection exhibit premature T cell aging, evidenced by overexpressed aging markers and, particularly, shortened telomeres - indicative of excessive proliferative turnover or inadequate telomeric maintenance [19, 20]. Notably, in healthy primary T cells, telomeres undergo shortening at a rate of 50-100 base pairs (bp) per cell division, and this predictable loss of telomeric DNA with cell replication allows telomeres to serve as molecular clock that controls the replicative capacity of human T cells before entering cell cycle arrest, senescence, or apoptosis [21, 22]. However, telomere loss can increase up to 250 bp per cell division during viral infection and, in compensating for this, cell cycle arrest occurs when progressive telomere loss reaches a critical point - known as senescence (a quiescent, non-replicative state with decline in functional activities) [21, 22]. Thus far, the precise mechanisms controlling telomeric DNA damage, T cell homeostasis, inflammaging and immune senescence, however, remain unclear.

In this study, we investigated the molecular mechanisms that perturb T cell homeostasis leading to inflammaging using a model of latent HIV infection in individuals on ART with undetectable viremia, the state in which the majority of our HIV patients exist. We demonstrate that deficiency of DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) promotes DNA damage, telomere erosion, T cell senescence and apoptosis in virus-suppressed, latent HIV infection. Specifically, we show that latent HIV infection is characterized by telomeric DNA damage that remains unrepaired due to the ATM deficiency, leading to telomere attrition, CD4 T cell senescence and apoptosis, a novel mechanism that controls T cell homeostasis and premature immune aging. Thus, restoring the impaired ATM machinery may provide a new strategy to improve T cell survival and functions during human viral diseases.

Materials and Methods

Ethics Statement

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). All participants were adults and signed the informed consent form.

Subjects

The study subjects were composed of two populations: 150 latently HIV-infected individuals and 166 age-matched HS. HIV subjects were virologically suppressed for HIV replication with ART, as evidenced by undetectable level of HIV-RNA. Healthy subjects

supplied by Physicians Plasma Alliance (Gray, TN) were negative for HBV, HCV, and HIV infection.

Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density centrifugation (GE Heathcare, Piscataway, NJ). $CD4^+$ T cells were isolated from PBMCs using the $CD4^+$ T Cell Negative Selection Kit (Miltenyi Biotec Inc., Auburn, CA). T cells were cultured in RPMI 1640 medium containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 100 IU/ml penicillin and 2 mM L-glutamine (Thermo Scientific, Logan, Utah) at 37°C and 5% CO_2 atmosphere. Cells were harvested at day 1 or day 4 in culture for detection of apoptosis and DNA damage. To test the role of ATM activation in repairing DNA damage and apoptosis, 10 μM ATM inhibitor (KU60019, Abcam, Cambridge, MA) or DMSO were added to the cultures at day 1, and the cells were collected after 48 h for measuring apoptosis and DNA damage. To determine the role of the PI3K pathway in ATM activation, purified CD4 T cells were cultured in the presence or absence of 20 μM PI3K inhibitor (LY294002, Sigma) for 48 h, followed by flow cytometry or western blot analysis.

Flow Cytometry

For phenotypic analysis of CD4 T cells, PBMCs were stained with CD4-FITC, CD45RA-PerCP710, CD57-APC (BioLegend, San Diego, CA), CD28-PE (Invitrogen, Carlsbad, CA), PD1-FITC (eBioscience, San Diego, CA), or isotype control antibodies. To quantify cell apoptosis, PBMCs were stained with CD4-A647 and CD45RA-FITC for naïve or memory cell

populations. In some experiments, CD4 T cells were cultured for the indicated times, and then stained with Annexin V-PE and 7-AAD using BD Pharmingen™ PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). $CD4^+$ T cells were also stained for caspase-3 expression following the protocol from CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Invitrogen). Levels of reactive oxygen species (ROS) in CD4 T cells were measured using the DCFDA-based Cellular ROS Detection Kit (Abcam, Cambridge, MA) or CellROX Green ROS Detection kit (ThermoFisher Scietific, Waltham, MA) according to manufacturer's protocol. For intracellular staining, the cells were fixed and permeabilized with Foxp3 Transcription Factor Staining Buffer Set (eBioscience), and stained with pATM (Ser1981)-PE antibody (BioLegend), pCHK2 (Thr68)-PE antibody, γH_2AX-PE (eBioscience), IL-2-PE , IFN-γ-PE (Invitrogen), or KC57-FITC for HIV p24 (NIH-AIDS Reagent Program). Flow cytometry was carried out as described previously [19, 20].

Flow-FISH

Telomere length was measured by a modified Flow-FISH method as described previously [20]. Briefly, PBMCs were stained with CD4-A647 and fixed with Cell Fixation buffer (BioLegend) for 20 min. The cells were incubated with telomere probe TelC (CCCTAACCCTAACCCTAA)-FITC (0.3µg probe/mL, PNA Bio,Newbury Park, CA) at room temperature for 10 min in the dark, and then at 82 °C for 10 min. The cells were washed with post-hybridization buffer, followed by flow cytometry buffer, and then stained with CD45RA-perCP/Cy5.5 and analyzed by flow cytometry.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted from 1 x 10^6 cells with PureLink RNA Mini Kit (Invitrogen), and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Bio systems, Fostercity, CA) per the manufacturer's instruction. Quantitative real-time PCR was performed in triplicates using the following conditions: 95 °C 10 min and then 95°C, 15s; 60°C, 60s with 40 cycles. Gene expression was normalized to GAPDH and values are expressed as fold changes using the $2^{-\Delta\Delta ct}$ method. Primer sequences are shown in Table 3.1.

Western Blotting

Western blot was performed as described previously [19, 20], using pATM (Ser1981) (D6H9), MRE11, RAD50, NBS1, PARP-1, pAKT, pCHK2 (Thr68) (C13C1), γH2AX (Cell Signaling, Danvers, MA), p24 (NIH-AIDS Reagent Program), and β-Actin (8H10D10) antibodies (Cell Signaling). Appropriate horseradish peroxide-conjugated secondary antibodies (Cell Signaling) were used, and proteins were detected with the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA). The blotted membranes were stripped and re-probed with ATM (D2E2), AKT and CHK2 (D9C6) antibodies (Cell Signaling). Protein bands were captured and quantified by Chemi DocTM MP Imaging System (Bio-Rad System).

Confocal Microscopy

$CD4^+$ T cells were isolated and cultured as described above, followed by immunofluorescence staining using a method described previously [19, 20]. Primary antibodies

Table 3.1 Primer sequences to be used in the chapter 3

Genes to be amplified	Primer sequences
ATM	F: 5'-TGGATCCAGCTATTTGGTTTGA-3'
	R: 5'-CCAAGTATGTAACCAACAATAGAAGAAGTAG-3'
MRE11A	F: 5'-CTTGTACGACTGCGAGTGGA-3'
	R: 5'-TTCACCCATCCCTCTTTCTG-3'
RAD50	F: 5'-CTTGGATATGCGAGGACGAT-3'
	R: 5'-CCAGAAGCTGGAAGTTACGC-3'
NBN	F: 5'-TTGGTTGCATGCTCTTCTTG-3'
	R: 5'-GGCTGCTTCTTGGACTCAAC-3'
CHEK2	F: 5'-CCCAAGGCTCCTCCTCACA-3'
	R: 5'-AGTGAGAGGACTGGCTGGAGTT-3'
TP53	F: 5'-TCAACAAGATGTTTTGCCAACTG-3'
	R: 5'-ATGTGCTGTGACTGCTTGTAGATG-3'
GAPDH	F: 5'-TGCACCACCAACTGCCTTAGC-3'
	R: 5'- GGCATGGACTGTGGTCATGAG-3'
HIV-1 specific primers	F: 5'-CAGATCCTGCATATAAGCAGCTG-3'
	R: 5'- TTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC-3'

included: rabbit anti-53BP1, anti-RAD51, anti-KU70 (Cell Signaling) and mouse anti-γH_2AX (Ser139) (Biolegend), or mouse anti-TRF1 (Thermo Fisher). Secondary antibodies included: anti-rabbit IgG-Alexa Fluor 488 and anti-mouse IgG- Alexa Fluor 555 (Invitrogen). The cells

were mounted with DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL), and images were acquired using a confocal laser-scanning inverted microscope (Leica Confocal, Model TCS sp8, Germany).

HIV-1 Plasmid Transfection and Virus Infection

The pNL4-3 plasmid, which contains a full-length HIV-1 viral DNA inserted in pUC18 vector, was obtained from NIH AIDS Reagent Program (originated from Dr. Malcolm Martin) [23]. The NL4-3 mCherry Luciferase plasmid was a gift from Warner Greene (Addgene plasmid # 44965) [24], and was used as a positive control for transfection. About 20 µg of the HIV-1 and HIV-1 mCherry plasmid were used for transfection of Human Embryonic Kidney 293 cell line transformed with SV40 large T antigen (HEK293T) using the polyethylenimine (PEI) method [25]. Transfection efficiency and HIV-1 protein expression were confirmed by fluorescent microscopy examination of mCherry, real-time RT-PCR detection of HIV-RNA, and ELISA (Abcam kit #ab218268), or western blotting of p24. The supernatants of HIV-1-transfected 293T cells were used to infect human Sup-T1 cells (NIH AIDS Reagent Program, cat# 100; originally from Dr. Dharam Ablashi and was isolated from a 13-year old individual with Non-Hodgkin's T cell lymphoma) using the spinoculation method [25]. Briefly, 1.5 x 10^6 Sup-T1 cells were infected with supernatant containing 1~5 x 10^6 HIV-1 in culture plates using centrifugation at 1620 x g in a 37°C incubator. After 2 h of spinoculation, the supernatants were removed, to discard the unattached viruses. Reltegravir (RAL, 66 mM, from NIH AIDS Reagent Program) was added to some cultures as an antiretroviral drug. The media were changed every 3 days, and approximately 1.5 x 10^6 were maintained at each passage with or without adding RAL. Cells were harvested at days 3, 6, 9, 12 and 15 for flow cytometry, RT-PCR, and western blot analysis.

ATM Silencing

Approximately, 1x 10^7 CD4 T cells were isolated from HS and transfected with 100 pmol of ATM-specific SMARTpool siGENOME siRNAs or scramble siRNAs (Dharmacon, Lafayette, CO) using the Human T Nucelofector Kit and Nucleofector I Device with program U-14 (Lonza, Allendale, NJ). After 48 h, the cells were harvested and analyzed by flow cytometry, western blot, and confocal microscopy.

ATM Overexpression

Purified $CD4^+$ T cells from HIV patients were transfected with 2.5 µg of pcDNA3.1, or pcDNA3.1(+)Flag-His-ATM wt, or human mutant ATM (S1981A; Addgene plasmids), using the Human T Nucleofector Kit and Nucleofector I Device (Lonza, Allendale, NJ). After 48 h, transfection efficiency was monitored by flow cytometry to measure the frequency of His^+ cells. Ectopic ATM expression was detected by western blot. Active caspase-3, γH_2AX, and IL-2 expressions were assessed in the His-positive cells by Flow Cytometry.

Statistical Analysis

The data were analyzed using Prism 7 software and are presented as mean ± SEM or median with interquartile range. Differences between two groups were analyzed by independent Student's t test or Mann Whitney test, paired T test or Wilcoxon matched-pairs signed rank test. Multiple groups were analyzed by one-way ANOVA test. P-values <0.05, <0.01, or <0.001 were considered statistically significant or very significant, respectively.

Results

CD4 T Cell Homeostasis and Apoptotic Susceptibility in Latently HIV-Infected Individuals on ART

Dysregulated T cell homeostasis is a characteristic of chronic viral infections; however, the precise mechanisms that control T cell homeostasis and virus persistence in humans remain unclear [26]. As an initial approach to identify factors that perturb T cell homeostasis in latent HIV infection, we analyzed total ($CD4^+$), naive ($CD4^+CD45RA^+$), and memory ($CD4^+CD45RA^-$) T helper cell frequencies within PBMCs isolated from latently HIV-infected individuals on ART with undetectable viremia (n=24) and age-matched HS (n=27). As shown in **Fig.3.1A** (representative dot plots and summary data of flow cytometry), total $CD4^+$ T cells ($p<0.0001$) and naïve CD4 ($p=0.0043$), particularly memory CD4 T cell pools ($p<0.0001$), were significantly contracted in latently HIV-infected individuals compared to HS. To determine which subsets of memory CD4 T cells were most affected in latent HIV infection, we analyzed the central memory ($CD4^+CD45RA^-CCR7^+CD28^+$), effector memory ($CD4^+CD45RA^-CCR7^-CD28^{+/-}$), and terminally differentiated memory ($CD4^+CD45RA^+CCR7^-CD28^-$) T cells for this patient group. We found that central memory CD4 T cell subsets were the most significantly contracted during HIV latency (data not shown). This contraction of CD4 T cells, particularly central memory CD4 T cell subsets, is consistent with previous studies [27, 28], which indicate a state of immune activation followed by T cell exhaustion and senescence in patients with latent HIV-1 infection.

The T cell repertoire in peripheral blood is well-maintained by a fine balance between influx of newly generated T cells from the thymus, efflux by consumption via programmed cell

death, and self-replication within the existing pool of lymphocytes. With deficient influx from the thymus in adults, the immune system responds to challenges by expanding existing T cells, leading to increased proliferative turnover, telomere erosion, and ultimately, cell apoptosis, which represents a major mechanism for controlling peripheral T cell homeostasis in adults [29]. To explore how apoptosis contribute to T cell homeostasis during HIV latency, we compared the Annexin V (Av) and 7-Aminoactinomycin D (7AAD) expressions in PBMCs derived from latently HIV-infected individuals (n=8) and age-matched HS (n=8). As shown in **Fig.3.1B** (representative dot plots and summary data of flow cytometry), Av/7AAD staining of CD4 T cells showed increases in the rate of early apoptotic cell death in latently HIV-infected individuals within the total CD4 (p=0.0050), as well as in naïve (p=0.0013) and, particularly, memory CD4 T cells (p=0.0006). Importantly, apoptosis-prone CD4 T cells, particularly memory CD4 T cells, appeared to be negatively correlated with their cell frequencies, analyzed by Pearson Correlation (**Fig.3.1C**; n=8 per group; r=-0.4981, p=0.0496). Also, the flow cytometry analysis of CD4 T cell frequencies faithfully correlated with the actual count of CD4 T cell numbers (**Fig.3.1D**; n=20; r=0.6039, p=0.0048), suggesting that CD4 T cell apoptosis closely correlates with CD4 T cell loss during HIV infection. These results also indicate that the apoptotic susceptibility of CD4 T cells in virus-suppressed, HIV-infected individuals may necessitate compensatory homeostatic proliferation, leading to CD4 T cell exhaustion and senescence.

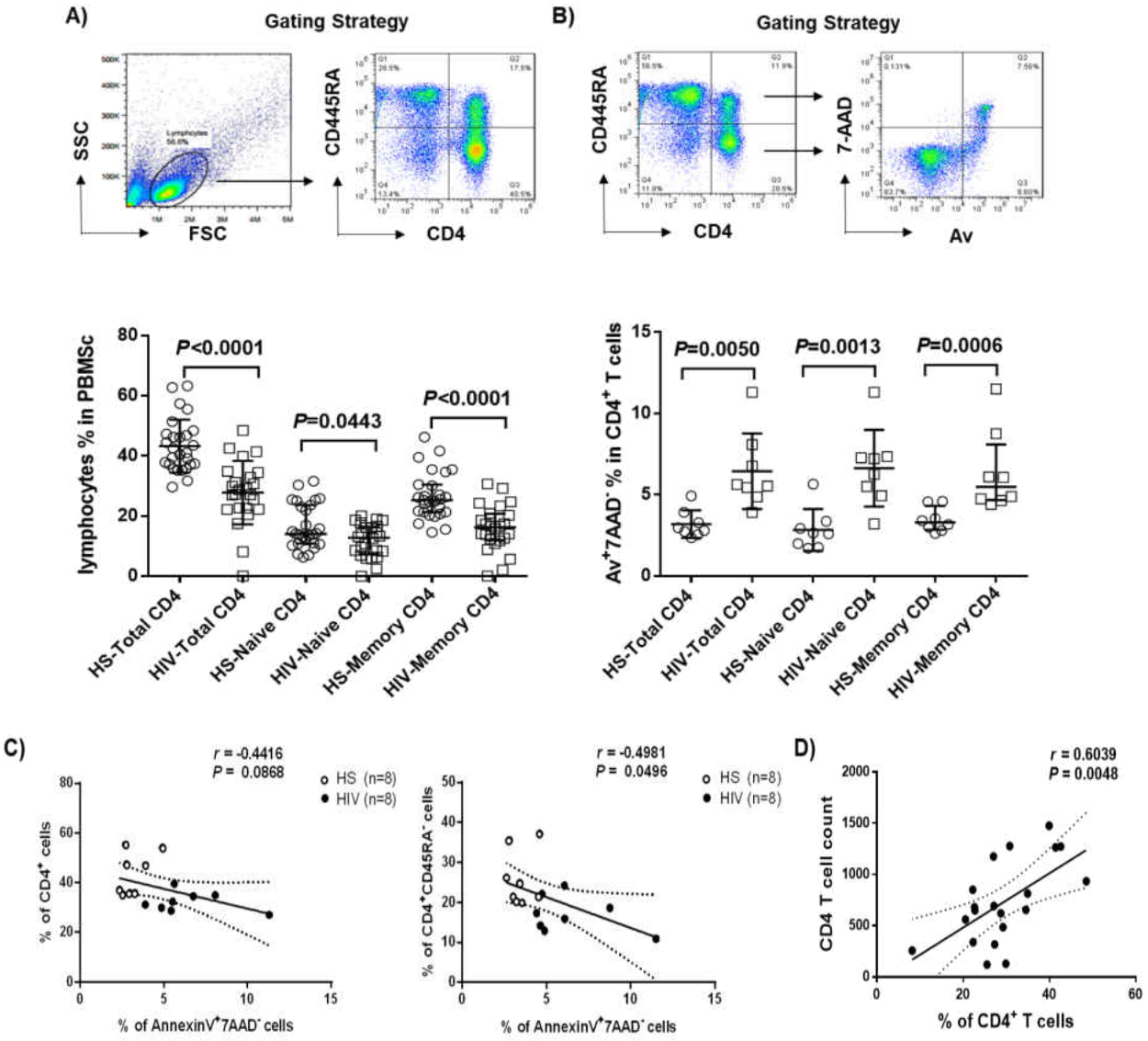


Fig.3.1. T cell homeostasis and apoptosis in HIV-infected patients versus age-matched HS. **A)** PBMCs were isolated from 24 HIV-infected patients and 27 HS and analyzed for T cell homeostasis by flow cytometry. Representative dot plots and summary data for percentages of total $CD4^+$, $CD45RA^+CD4^+$ (naïve), and $CD45RA^-CD4^+$ (memory) T cell frequencies are shown. Each symbol represents an individual subject. The mean ± SE and p value are shown. **B)** PBMCs isolated from 8 HIV-infected patients and 8 HS were analyzed for T cell apoptosis by flow cytometry. Representative dot plots and summary data for percentages of Av and 7AAD expression in total $CD4^+$, $CD45RA^+CD4^+$ (naïve), and $CD45RA^-CD4^+$ (memory) T cells are shown. Each symbol represents an individual subject. The mean ± SE and p value are shown. **C)** Pearson analysis of the correlation between total or memory CD4 T cell frequencies and early apoptotic cells in PBMCs isolated from HIV patients and HS. **D)** Pearson analysis of the correlation between CD4 T cell frequencies and the actual CD4 T cell counts in HIV-infected individuals.

CD4 T Cell Exhaustion and Senescence in Latent HIV Infection

To determine the role of homeostatic proliferation in T cell exhaustion and senescence, we first compared relevant markers on CD4 T cells isolated from latently HIV-infected individuals versus age-matched HS. As shown in **Fig.3.2A**, the expression of programmed death-1 (PD-1), a marker for cell exhaustion, was significantly upregulated on lymphocytes (p=0.0055), as well as on $CD4^+$ T cells (p=0.0231) in HIV patients compared to HS, though the frequencies of PD-1^+ $CD4^+$ T cells in resting state were extremely low, whereas PD-1^+ $CD8^+$ T cells were relatively high (data not shown), in the peripheral blood of both HIV and HS.

Since loss of CD28, a T cell receptor (TCR) co-stimulatory molecule required for T cell activation and survival, is regarded as an unequivocal marker of T cell senescence [30], we examined the $CD28^-$ populations within $CD4^+$ T cells. As recently reported by the AGEhIV Study Group [9], we observed a slight increase in the $CD28^-$ $CD4^+$ T cells in latently HIV-infected individuals; however, these increases were not significantly different (p=0.2829) compared to the age-matched HS in our cohort (**Fig.3.2B**).

We have previously reported that CD57, also known as human natural killer 1 (HNK1) that is expressed on the senescent NK or T lymphocytes [31], is highly expressed on CD4 T cells derived from chronically HCV-infected individuals [20]. We found that the percentage of $CD57^+$ $CD4^+$ T cell subsets was significantly increased in HIV patients versus HS (**Fig.3.2C;** p=0.0059). This significant upregulation of CD57 expression was also observed in CD4 T cells isolated from HIV and HS that were cultured *in vitro* for 3 days with or without TCR stimulation (n=12 per group; p=0.0003 and p=0.0002, respectively), suggesting that HIV CD4 T cells are senescent.

Telomere Attrition and Telomeric DNA Damage in CD4 T Cells During Latent HIV Infection

Telomeres are repeating hexameric sequences of DNA found at chromosome ends in association with a complex of shelterin proteins. Telomere integrity is a key feature of linear chromosomes that preserve genome stability and function, whereas telomere attrition is a hallmark of cell aging or senescence that drives cell dysfunction or apoptosis [17, 18]. Given the importance of telomere attrition in cell senescence, we further investigated T cell aging in HIV latency by measuring telomere length in total $CD4^+$, $CD4^+CD45RA^+$ naïve and $CD4^+CD45RA^-$ memory T cells by Flow-FISH. As shown in **Fig.3.2D** (representative plots for gating strategy and pooled data of flow cytometry), telomere length was significantly shortened in HIV-derived total CD4 T cells (p=0.0423) and, particularly, memory CD4 T cells (p=0.026), compared to age-matched HS (n=24 per group). Most importantly, telomere length appeared to be inversely correlated with the cell apoptotic rate in total, naïve and memory CD4 T cells from HIV and HS, as determined by Pearson correlation (**Fig.3.2D** lower panel; n=8 per group; r=-0.3318, p=0.0212), indicating that telomere erosion is associated with apoptosis of CD4 T cells in HIV latency.

Since HIV replication is well-controlled by ART in our cohort, an important question is: what drives telomere erosion and T cell apoptosis during latent HIV infection? We and others have previously shown that naïve CD4 T cells are typically resistant to death receptor/ligand (Fas/Fas-L)-mediated apoptosis [19, 20, 32, 33]. Indeed, resting CD4 T cells typically do not express Fas on their cell surface, and blocking the exogenous death pathways such as Fas-Fas ligand, TNFα-TNF receptor, and TRAIL-TRAIL receptor interactions in CD4 T cells did not affect the

KML001 (NaAsO2, an arsenic telomere targeting drug)-induced cell apoptosis (unpublished observations), which points to cell internal signals as apoptosis initiators. Notably, one internal stressor linked to cell apoptosis is damaged DNA, which is particularly prominent in senescent T lymphocytes that have been chronically exposed to oxidative stress, such as endogenously generated ROS [34].

To determine whether ROS might be an offender causing DNA damage and cell apoptosis during latent HIV infection, CD4 T cells were isolated from latently HIV-infected patients and HS, and cultured *in vitro* without stimulation for 1~4 days (to generate endogenous ROS). Then ROS levels were measured by flow cytometry using the Cellular ROS Detection Kit based on the absorption of cell permeable 2',7'-dichloroflurescin diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell [35]. As shown in **Fig.3.2E**, the MFI of DCFDA was significantly increased in CD4 T cells derived from HIV-infected patients compared to age-matched HS (n=10 per group; p=0.0939 at day 1 and p=0.0035 at day 4 in culture, respectively). Interestingly, when these cells were cultured *in vitro* without stimulation for 1~4 days, the MFI of $DCFDA^{high}$ cells remained high in HIV T cells, whereas the percentage of $DCFDA^{high}$ cells decreased along with an increase in Av^{+} apoptotic cells in HIV versus HS (data not shown). Because cell apoptosis can affect the DCFDA measurement, we used another fluorescence-based method (CellROX Green) to measure ROS production and assessed their relationship with apoptosis in cultured CD4 T cells derived from HIV and HS for 4 days, and we found the same phenomena. As shown in **Fig.3.2F**, depending on the levels of ROS and Av, CD4 T cells from both HIV and HS were gated into two major populations: Av^{+} ROS^{low} and Av^{-} ROS^{high}. Notably, in both HIV and HS, apoptotic dying cells

(Av^+) produced less ROS (MFI ROS^{low}), and live cells (Av^-) produced more ROS (MFI ROS^{high}). While the MFI of both Av^- ROS^{high} and Av^+ ROS^{low} subsets remained higher in HIV than HS, the percentage (%) of Av^- ROS^{high} cells was lower, whereas the % of Av^+ ROS^{low} cells was much higher, in HIV than HS. These data suggest that the intracellular ROS generated during HIV infection may play an important role in triggering cell apoptosis, and the apoptotic, dying cells produce less ROS.

Cells are equipped with DNA damage surveillance and repair machineries to prevent cell death associated with genomic instability. Since mammalian telomeres consist of triple guanine repeats (TTAGGG) that are very sensitive to oxidative DNA damage, particularly vulnerable to ROS, we hypothesized that telomere sequences in HIV-derived T cells are not only shortened, but more importantly, DNA damaged. Notably, following genotoxic insult, 53BP1 is recruited to the DNA damage site on chromosomes (including telomeres), and acts as a docking site for other mediator, transducer and DNA repair proteins to form microscopically visible nuclear foci (DNA damage foci) [36]. Thus, identifying damaged telomere induced foci (TIF) is typically considered as a hallmark of telomeric DNA damage response (DDR) [37]. To determine telomeric DDR in T cells during latent HIV infection, we compared the number of TIFs per nucleus, as well as the percentage of cells with > 3 TIFs, by examining co-localization of 53BP1/TRF1 using confocal microscopy. As shown in **Fig.3.2F** (representative imaging and summary data), the number of TIF per nucleus (p=0.0209) and the percentage of T cells with > 3 TIFs (p=0.0340) were significantly higher in CD4 T cells derived from HIV patients compared to HS. Moreover, the percentage (p=0.0383) as well as the mean fluorescence intensity (MFI) of phosphorylated H2AX (γH2AX), a marker of DNA damage, were significantly upregulated in total (p=0.0192),

naïve (p=0.0087) and memory (p=0.0148) CD4 T cells from HIV patients compared to HS, as determined by flow cytometry (**Fig.3.2G**). These results suggest that telomeric DNA damage may cause cell apoptosis and T cell loss in patients with latent HIV infection, emphasizing the role of telomere integrity in securing T cell survival.

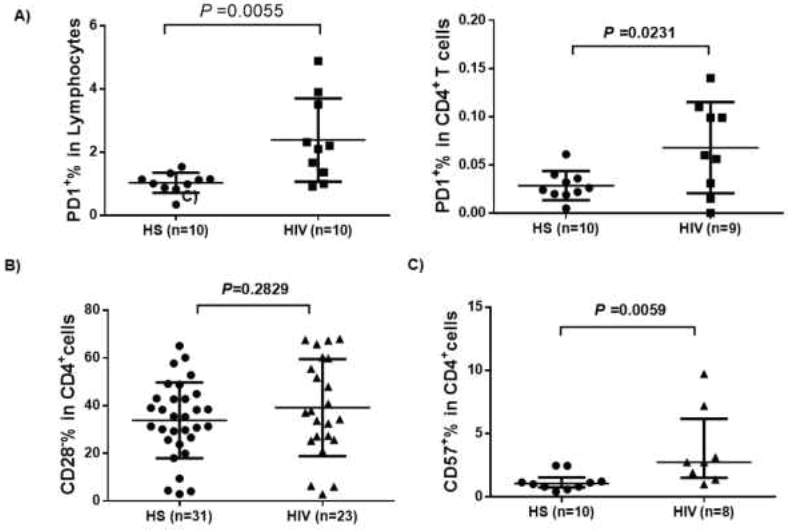
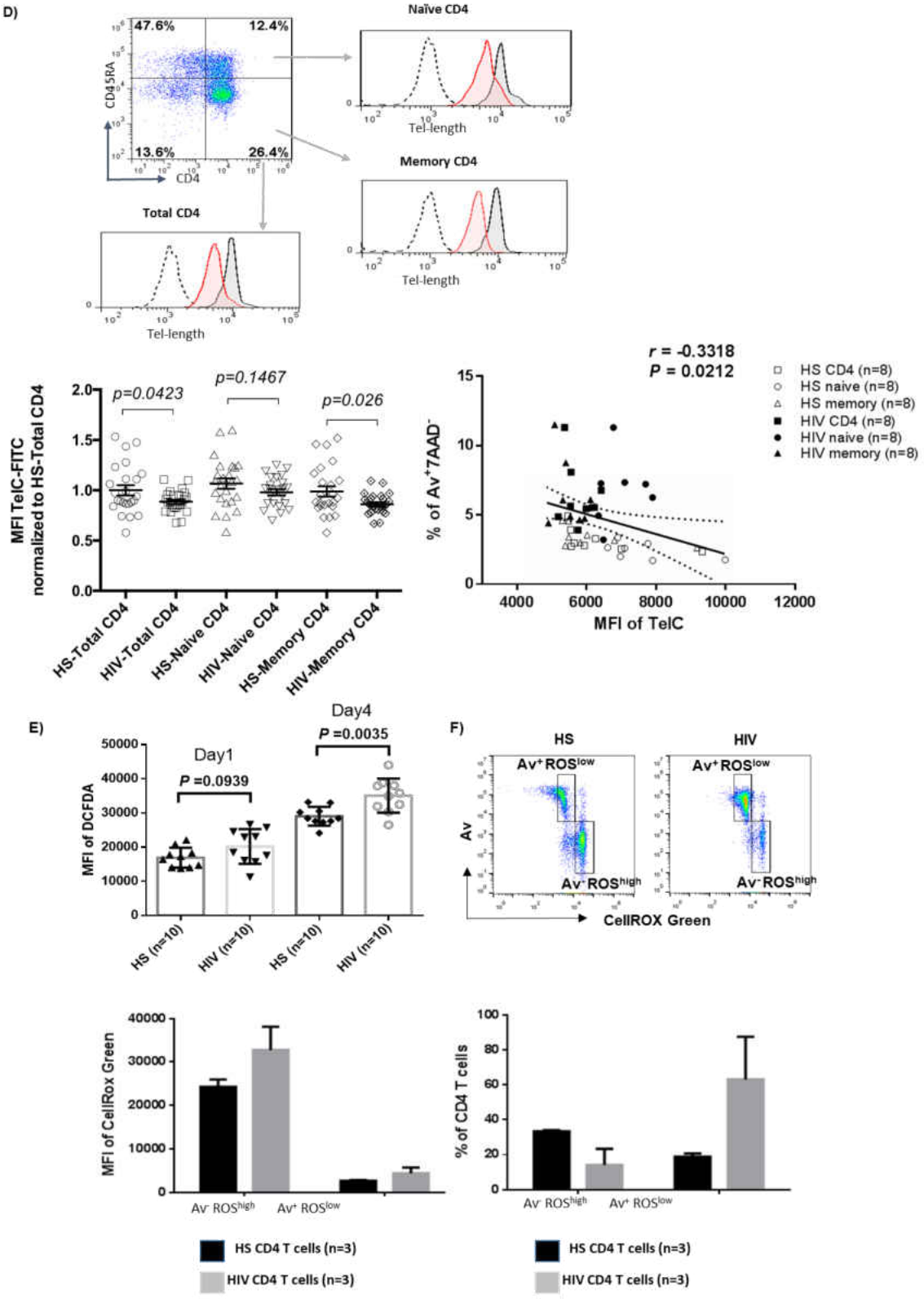


Fig.3.2. CD4 T cell exhaustion, senescence, telomere attrition and DNA damage in latent HIV infection. **A)** Percentage of PD-1 expression on total lymphocytes (left panel) and CD4 T cells isolated from HIV patients versus HS, as determined by flow cytometry (n=number of subjects). **B)** Percentage of $CD28^-$ population in CD4 T cells from HIV patients versus HS. Number of subjects examined (n) and p value are shown. **C)** Percentage of CD57 expression on CD4 T cells from HIV patients versus HS. **(continued on page 91)** **D)** Representative dot plots, histogram and summary data from 24 HIV patients and 24 HS by Flow-FISH analysis of telomere length in total, naïve and memory CD4 T cells. Pearson correlation analysis of the telomere length (MFI) and the percentage of early apoptotic cells (Av^+7AAD^-) are shown below. . **E)** Flow cytometry analysis of the mean fluorescence intensity (MFI) of DCFDA level in CD4 T cells isolated from PBMCs of 10 HIV patients and 10 HS, and cultured *in vitro* without stimulation for 1~4 days. **F)** Flow cytometry analysis for the percentage (%) and MFI of CellROX Green as well Av levels in cultured CD4 T cells derived from HIV and HS for 4 days ex vivo. Representative dot plots and summary data of the MFI as well as % of the two gated cell populations (AV^- ROX^{high} and Av^+ ROX^{low}) from three subjects in each group are shown. **(continued on page 92)** **G)** Confocal microscopy analysis of 53BP1 and TRF1 co-localization in the nuclei of CD4 T cells isolated from HIV patients versus HS. 100 cells were counted per subjects (n=number of subjects examined). Summary data of dysfunctional telomere-induced foci (TIF) number per nuclei, and the percentage of cells with $\geq$ 3 TIFs in the two groups are shown. **H)** Representative dot plots and summary data of the percentage of γH2AX expression in CD4 T cells (n=16 per group), as well as the MFI in total, naïve and memory CD4 T cells from HIV patient versus HS (n=8 per group).

Fig.3.2 (Continued)



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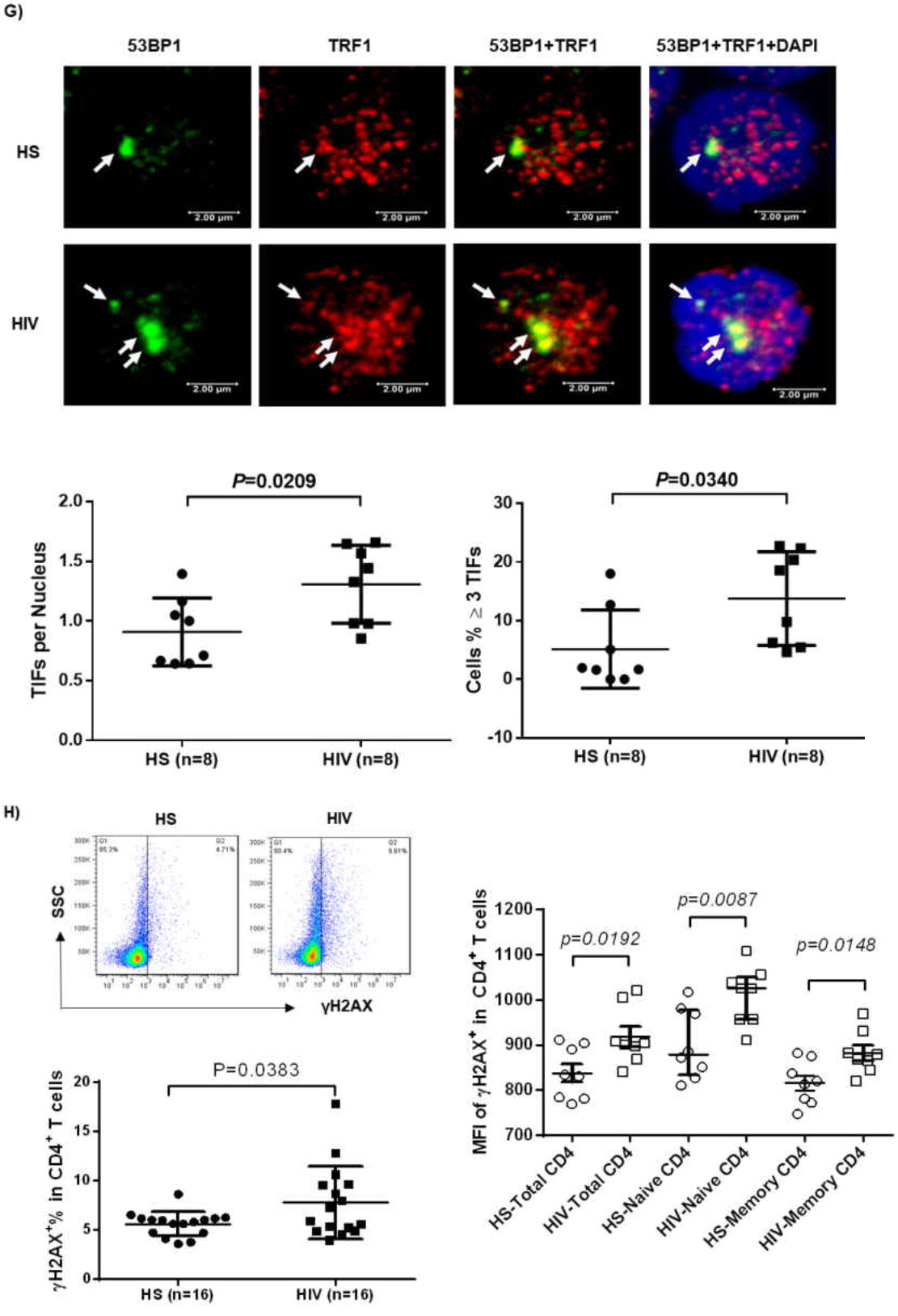


ATM Expression and Activity Are Inhibited in CD4 T Cells During Latent HIV Infection

Accumulation of damaged DNA in CD4 T cells indicates that the DNA damage sensing and repairing machinery are impaired during HIV infection. Essential components of this machinery include DNA damage sensors, such as the MRE11, RAD50, and NBS1 (MRN) complex, which recruits and mediates activation of DNA damage checkpoint kinase ATM that can phosphorylate several downstream checkpoint proteins, including checkpoint kinase 2 (CHK2) and p53 [38-40]. To investigate the cellular machineries that promote the DNA damage during HIV infection, we examined mRNA transcripts and protein expression of these DDR proteins in CD4 T cells. As shown in **Fig.3.3A**, real-time RT-PCR analysis of the MRN complex transcripts showed no alterations in the level of MRE11 (p=0.1830), RAD50 (p=0.7124), and NBS1 (p=0.7920). In parallel, the protein levels of these DNA damage sensors in CD4 T cells remained unchanged in HIV patients and HS, detected by western blotting (**Fig.3.3B**). Intriguingly, while the mRNA level of ATM was unchanged (p=0.2103, **Fig.3.3C**), ATM protein level (p=0.0107) and phosphorylation on Ser1981 (p=0.0031), an indicative of its activity, were significantly reduced and inhibited, respectively, in CD4 T cells from HIV patients compared to HS (**Fig.3.3D**; representative imaging and summary data of western blotting). The inhibition of ATM protein phosphorylation was confirmed by flow cytometry in total (p=0.006), naïve (p=0.0254), and memory (p=0.0091) CD4 T cells from the studied subjects (**Fig.3.3E**). Concurrently, while the mRNA levels of the ATM downstream signaling molecule CHK2 and p53 remained unchanged (**Fig.3.3F**), phosphorylation of CHK2 (pCHK2), measured by flow cytometry, was significantly inhibited in HIV T cells (**Fig.3.3G**). CHK2 inhibition, but not p53 expression, was also detected by western blot in HIV T cells compared to HS (**Fig.3.3H**). Moreover, in line with the increased DNA damage and cell apoptosis in HIV T cells, the total

level of Poly ADP-Ribose Polymerase 1 (PARP-1, an apoptosis marker) [41] was decreased, whereas the caspase 3-dependent cleaved PARP-1 was increased in HIV T cells. Taken together, these results indicate that the DNA damage sensing machinery remains intact, but the protein expression and activation of ATM/CHK2, two key components critically involved in repairing DSB during DDR for cell survival, are significantly suppressed, at the post-transcriptional level, during latent HIV infection.

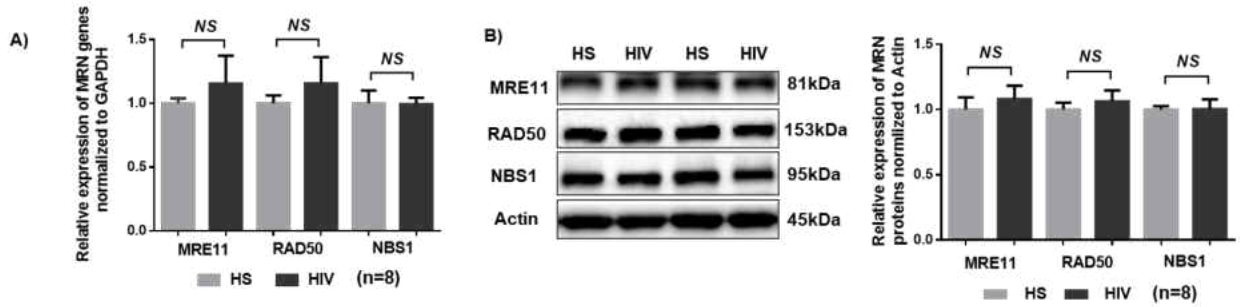
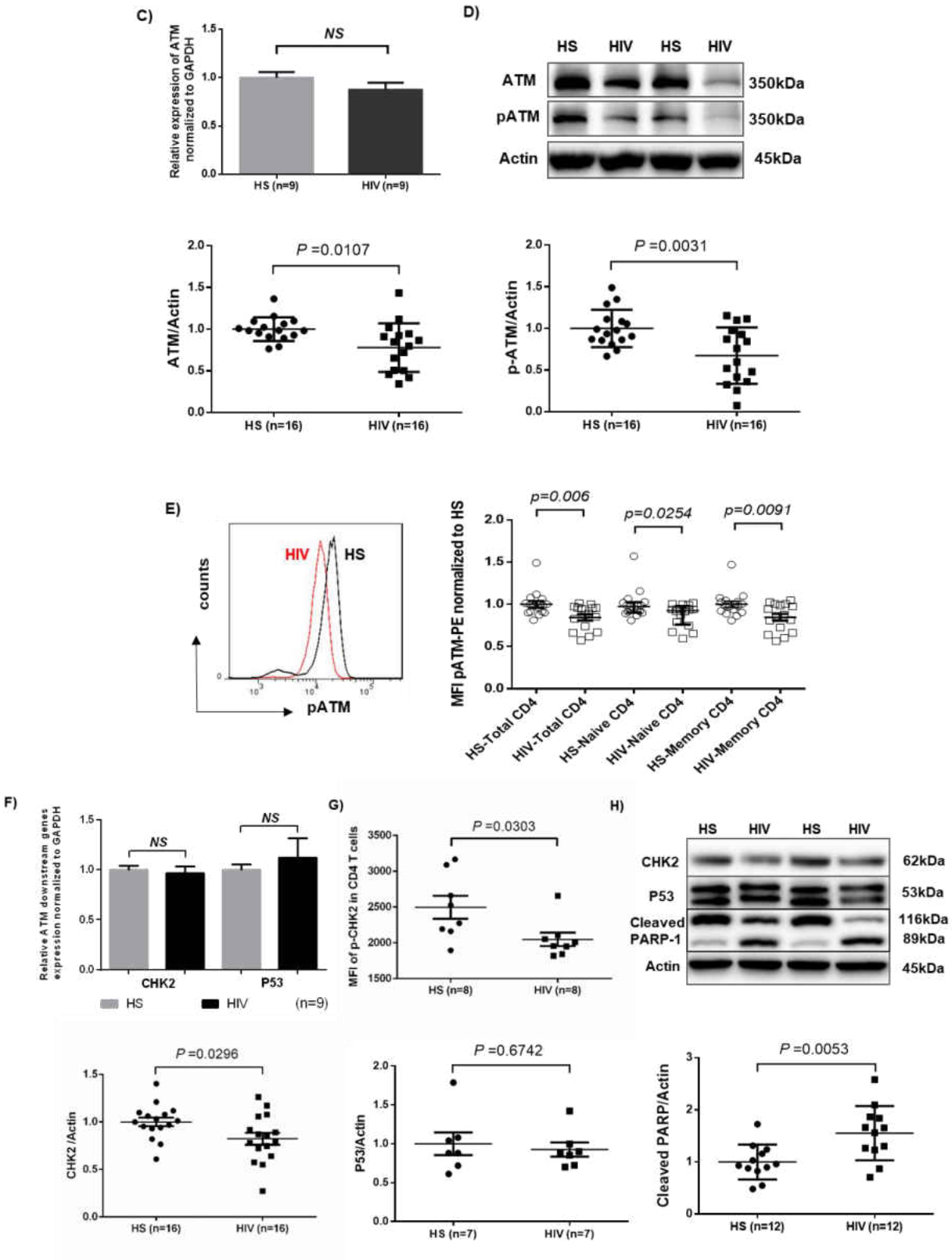


Fig.3.3. DNA damage repair ATM signaling pathway in T cells of HIV-infected patients versus HS.
A) MRE11, RAD50, and NBS1 mRNA expression, measured by real-time RT-PCR, in CD4 T cells isolated from HIV-infected patients and age-matched HS (n=number of subjects to be examined). B) Western blot showing MRN protein expression in CD4 T cells derived from HIV patients versus HS. Representative imaging and summary data from 8 subjects in each group are shown. **(continued on the next page)** **C)** ATM mRNA expression, measured by RT-PCR, in CD4 T cells isolated from the study subjects (n=8 in each group). **D)** Western blot analysis of ATM protein expression and phosphorylation in CD4 T cells derived from HIV patients versus HS. Representative imaging and summary data from 16 subjects in each group are shown. **E)** Flow cytometry analysis of pATM expression in total, naïve and memory CD4 T cells from 8 HIV patients versus 8 HS. **F)** CHK2 and p53 mRNA expressions, measured by RT-PCR, in CD4 T cells from 9 HIV patients and 9 HS. **G)** Flow cytometry analysis of pCHK2 expression in CD4 T cells from 8 HIV patients versus 8 HS. **H)** Western blot analysis of CHK2, p53, and cleaved PARP-1 protein expressions in CD4 T cells derived from HIV patients versus HS. Representative imaging and summary data from the indicated subjects in each group are shown.

Fig.3.3 (Continued)



ATM Protects T Cells from DNA Damage, Cell Apoptosis and Dysfunction via the PI3K Pathway

The ATM signaling pathway is pivotal to the maintenance of genome integrity and cell survival [42]. To determine its role in T cell function and survival, we knocked down ATM expression in healthy CD4 T cells using specific siRNAs, and measured cell apoptosis and cytokine expression. As shown in **Fig.3.4A**, healthy CD4 T cells transfected with siRNA specific to ATM (siATM) exhibited a significant decrease in ATM and pATM protein levels compared to those treated with control siRNAs (siCON). Concurrently, CHK2 and pCHK2 expressions were also diminished in ATM-silenced T cells. In addition, the frequencies of Av^+ and caspase-3^+ apoptotic cells were markedly increased, whereas the IFN-γ-producing CD4 T cells was significantly decreased (**Fig.3.4B**) with the ATM knockdown.

To further investigate the role of ATM in maintaining T cell survival and normal function, primary CD4 T cells isolated from HS were treated with a specific ATM inhibitor (ATMi, KU60019) for 48 h, followed by measuring expression and activation of the ATM/CHK2 pathway and cell apoptosis. As shown in **Fig.3.4C**, healthy CD4 T cells exposed to KU60019 showed a marked decrease in the expression of ATM, in particular pATM, compared to the DMSO-treated control. Concurrently, the levels of ATM downstream signaling molecule CHK2, and particularly pCHK2, were significantly suppressed by this treatment. In addition, T cells exposed to the ATMi exhibited an elevated level of cleaved PARP-1, suggesting increased caspase 3-dependent, DNA damage-mediated cell apoptosis.

Since ATM belongs to the phosphoinositide 3-kinase (PI3K) family, which plays a key role in cell survival and function [42, 43], we examined whether the expression and activation of

AKT could be suppressed by the ATM inhibition. As shown in **Fig.3.4C**, both AKT and pAKT were inhibited in CD4 T cells exposed to the KU60019 treatment. Conversely, we also examined whether ATM expression and activation could be blocked by the PI3K inhibitor (PI3Ki). To this end, we incubated CD4 T cells with or without the PI3Ki (LY294002) for 48 h, followed by examination of ATM/pATM, CHK2/pCHK2, AKT/pAKT, as well as cleaved PARP-1 expressions by western blot. As shown in **Fig.3.4D**, compared to the DMSO control, T cells treated with 20 μM of LY294002 exhibited a significant inhibition of ATM/pATM and CHK2/pCHK2 expressions, along with suppression of AKT/pAKT levels, and an increase in the level of cleaved PARP-1. These results indicate that ATM deficiency is mediated via inhibition of the PI3K pathway.

The NHEJ Pathway Is Involved in the ATM-Mediated DNA Repair and Telomere Maintenance

We next examined the role of ATM in protecting T cells from DNA damage, and found that T cells treated with the ATMi exhibited an increase in the expression of γH2AX, a marker for DNA damage (**Fig.3.4E**). These T cells also had increases in DNA damage foci (γH2AX/53BP1 co-localization), measured by confocal microscopy (**Fig.3.4F**). It is well-known that Ku70 and Ku80 make up the Ku heterodimer, which bind to DSB ends and is required for the non-homologous end joining (NHEJ) pathway of DNA repair. It is also required for V(D)J recombination, which utilizes the NHEJ pathway to promote antigen diversity in the mammalian immune system. In addition to its role in NHEJ, Ku is involved in telomere maintenance [44]. It has been reported that mutant mice deficient Ku70 exhibit premature aging [45], suggesting that Ku70

plays an important role in longevity assurance and that reduced ability to repair DSB or maintain telomere integrity causes early aging. To determine the role of ATM in Ku70-mediated telomeric DNA damage repair, we measured the Ku70-dependent, damaged telomere induced foci (TIF) in CD4 T cells exposed to ATMi for 48 h by confocal microscopy. As shown in **Fig.3.4G**, Ku70/TRF1-formed TIFs were clearly found increased in cells treated with the ATMi compared to the DMSO control. We also examined possible involvement of the ATM pathway in homologous recombination (HR) of DNA during DSB repair. Since RAD51 is involved in the search for homology and strand pairing process [46, 47], we used confocal microscopy analysis for the RAD51/TRF1-dependent TIF in CD4 T cells exposed to ATMi or DMSO for 48 h, but found no significant difference between the two treatments (**Fig3.4H**). These results suggest that the NHEJ, rather than the HR, pathway appear to be involved in the ATM-mediated telomeric DNA repair.

Moreover, we examined the role of ATM in protecting T cells from cell apoptosis and cellular dysfunction. We demonstrated that ATM inhibition in CD4 T cells increased T cell apoptotic death, and HIV T cells appeared to be more vulnerable to the ATMi-mediated apoptosis, as evidenced by the higher baseline and the slope of increase in Av/7ADD (**Fig.3.4I**), as well as caspase-3 expressions (**Fig.3.4J**) in CD4 T cells derived from HIV patients versus HS. Meanwhile, these ATMi-treated CD4 T cells exhibited diminished IL-2 (**Fig.3.4K**) and IFN-γ (**Fig.3.4L**) expressions compared to the controls. Taken together, these results demonstrate that ATM inhibition can lead to increased DNA damage, cell apoptosis, and cell dysfunction, which potentially contribute to the CD4 T cell loss and poor immune responses observed in latent HIV infection.

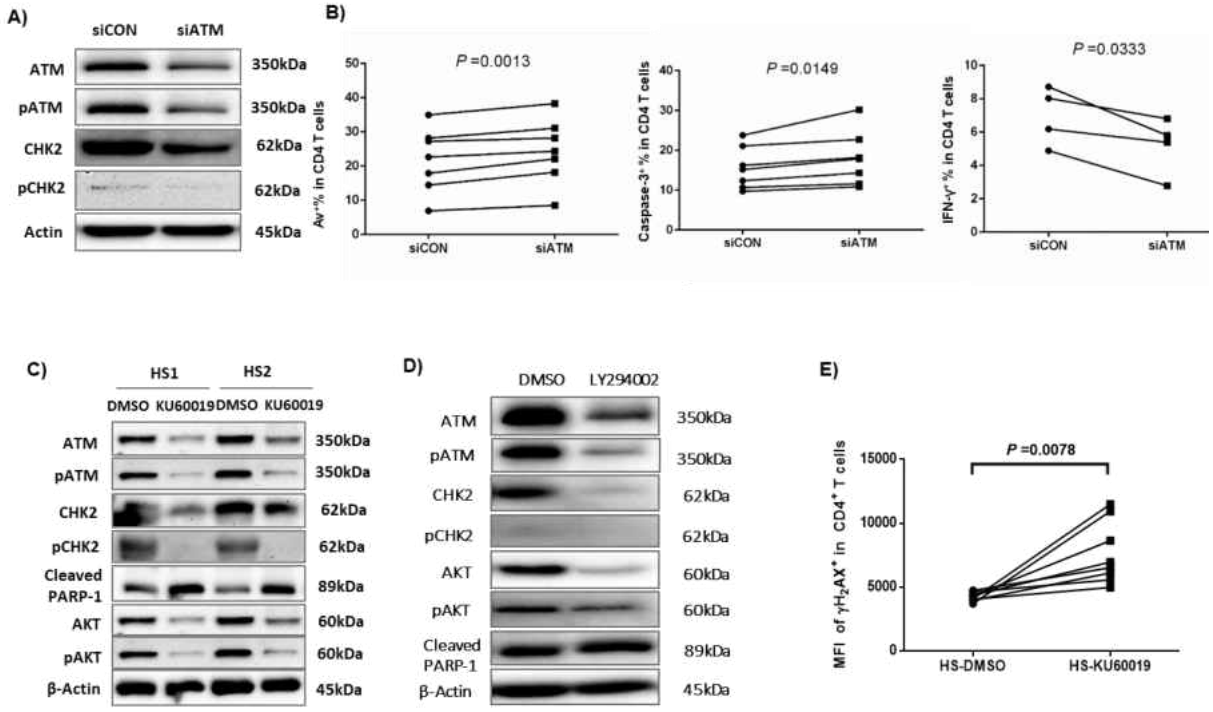
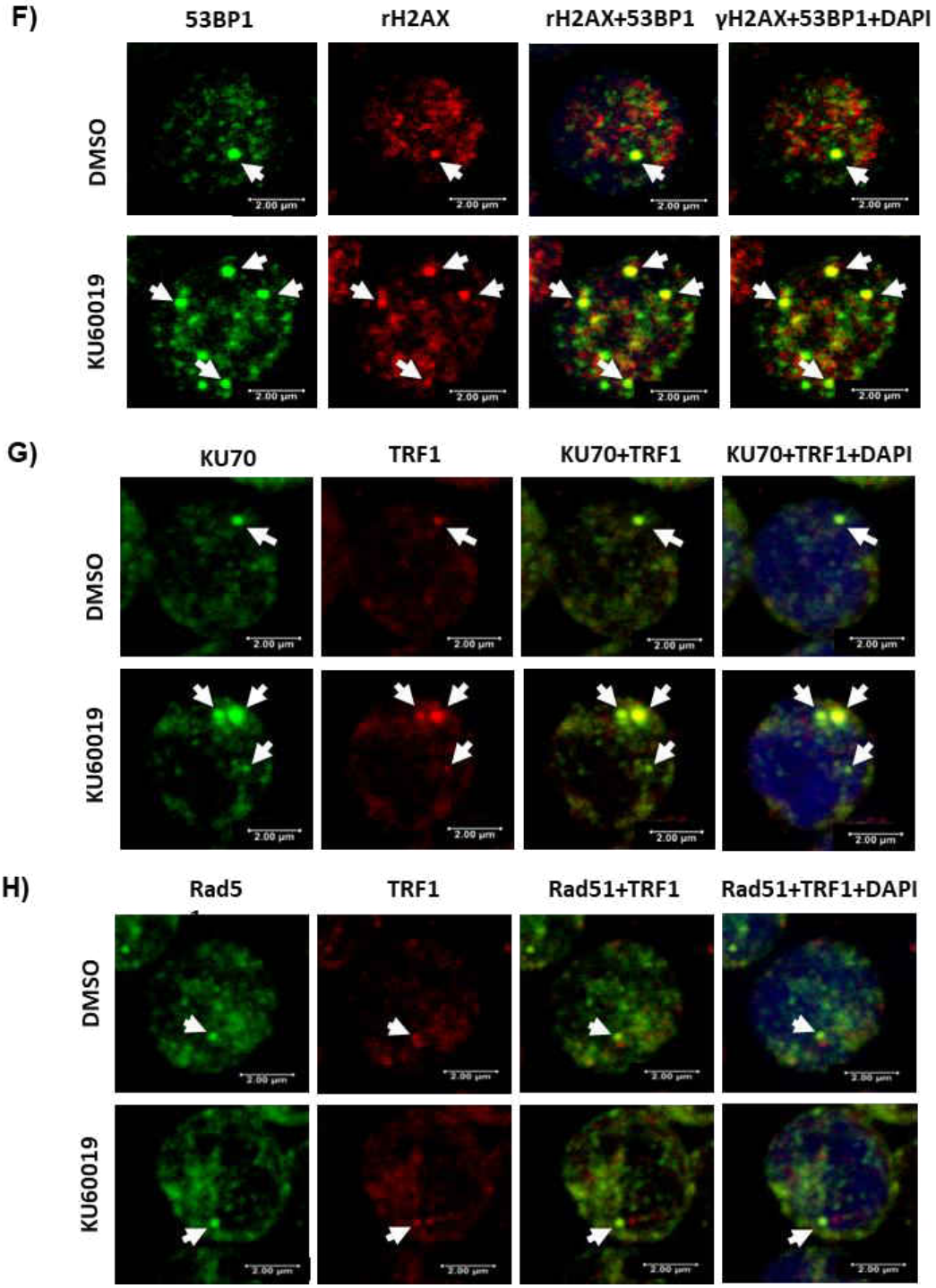


Fig.3.4. ATM plays a key role in protecting T cells from DNA damage, cell apoptosis and cellular dysfunction. A) Western blot analysis of ATM, pATM, CHK2, and pCHK2 expressions in CD4 T cells transfected with ATM siRNA (siATM) or control siRNA (siCON). Representative imaging from repeated experiments is shown. **B)** Apoptosis and functional assays of CD4 T cells with ATM knockdown. Representative dot plots and summary of the percentage of Av (n=7), caspase-3 (n=7), and IFN-γ (n=4) expressions in CD4 T cells transfected with siATM or siCON. **C)** Western blot analysis of ATM, pATM, CHK2, pCHK2, cleaved PARP-1, AKT, and pAKT expressions in CD4 T cells treated with the ATM inhibitor (KU60019) or DMSO control for 48 h. **D)** Western blot analysis of ATM, pATM, CHK2, pCHK2, AKT, pAKT, and cleaved PARP-1 expressions in CD4 T cells treated with the PI3K inhibitor (LY294002) or DMSO control for 48 h. Representative imaging from repeated experiments is shown. **E)** Flow cytometry analysis for the MFI of γH2AX expression in CD4 T cells treated with KU60019 or DMSO for 48 h. **(continued on page 100)** **F-H)** Representative imaging of confocal microscopy examination of co-localization of 53BP1 and γH2AX, Ku70 and TRF1, RAD51 and TRF1, in CD4 T cells treated with KU60019 or DMSO for 48 h (n=6). **I-J)** Flow cytometry analysis of Av% and caspase-3% in CD4 T cells from HIV and HS exposed to KU60019 or DMSO for 48 h. **(continued on page 101)** **I-J)** Flow cytometry analysis of Av% and caspase-3% in CD4 T cells from HIV and HS exposed to KU60019 or DMSO for 48 h. **K-L)** Representative dot plots and summary data of IL-2 and IFN-γ productions in healthy CD4 T cells stimulated with PMA and inomycin in the presence of KU60019 or DMSO for 48 h.

Fig.3.4 (Continued)



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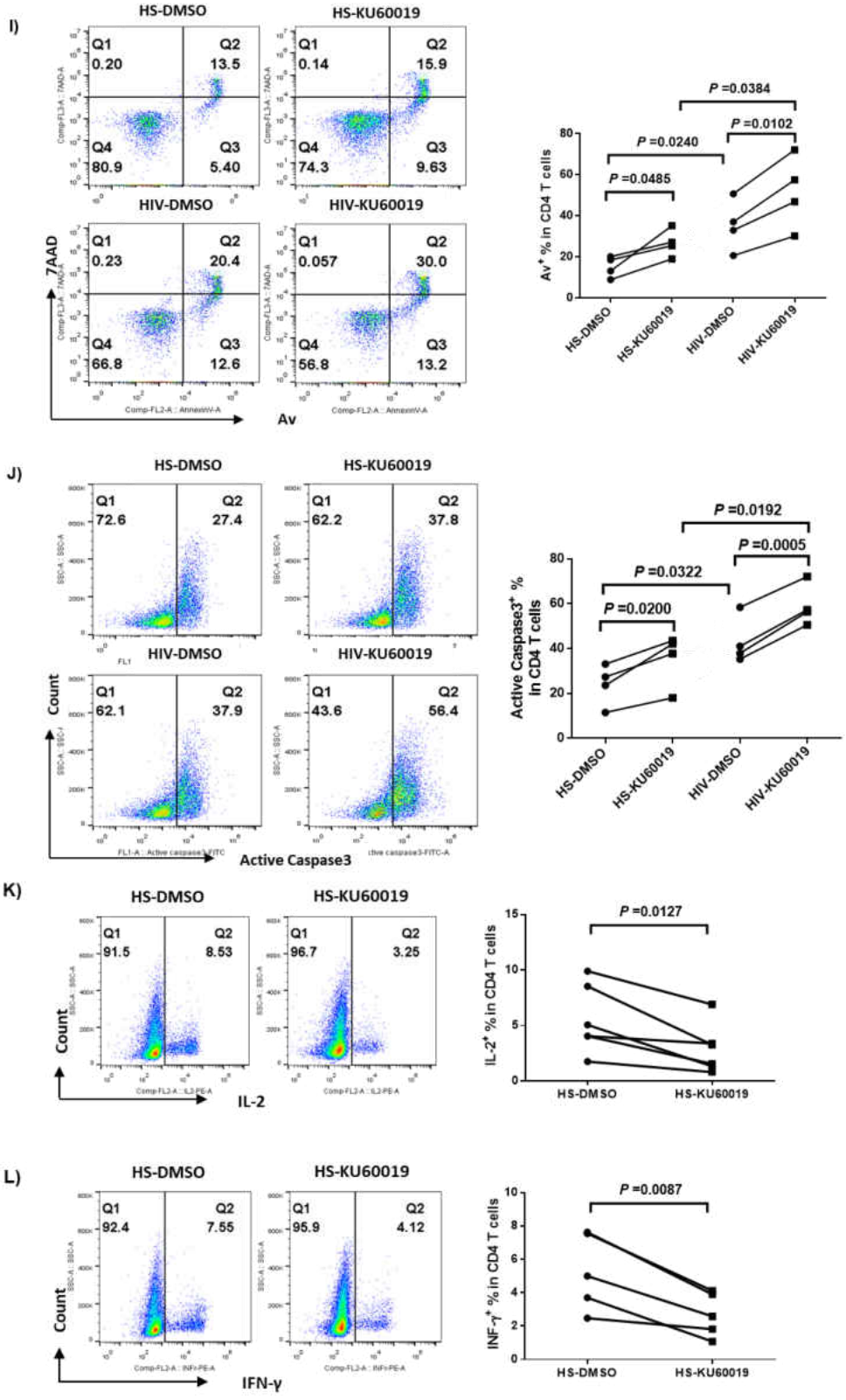

HIV Infection of CD4 T Cells *in Vitro* Promotes Telomere Loss and Cell Apoptosis

Because chronically HIV-infected patients often have co-morbidities that may contribute to immune dysregulation, we sought to examine the role of HIV infection in promoting telomere loss and CD4 T cell apoptosis without cofounding factors. To this end, we established an *in vitro* HIV-1 cell culture system by employing a pNL4-3 plasmid that contains a full-length HIV-1 viral genome [23]. A NL4-3 mCherry Luciferase plasmid was used as a positive control for cell transfection [24]. Successful transfection of HEK293T cells was confirmed by fluorescence microscopy to examine HIV-1 mCherry cassette in red color, whereas mock transfected cells show negative fluorescence signal (**Fig.3.5A**). HIV-RNA and p24 antigen were detected in the supernatant of transfected 293T cells by real-time RT-PCR and ELISA (data not shown). Since 0.1 ng/ml p24 protein is equivalent to 1 x 10^6 HIV-1 virus particles [48], culture supernatants containing 1~5 x 10^6 HIV-1 virions were employed to infect 1.5 x 10^6 human Sup-T1 CD4 T cells using the spinoculation method [25]. As shown in **Fig.3.5B**, p24 antigen was detected in HIV-1-infected Sup-T1 cells by flow cytometry, with $p24^+$ cells increase in a time-dependent manner. Also, HIV-RNA and p24 protein were found in the supernatants of HIV-1-infected Sup-T1 cells, but not in the supernatants of uninfected cells, measured by RT-PCR and ELISA (data not shown). To mimic the clinical settings of HIV-infected patients on ART, we added 66 nM of RAL, an integrase inhibitor, after 2 h spinoculation infection. As shown in **Fig.3.5C**, HIV-RNA detection remained negative in uninfected Sup-T1 cells, but was positive in HIV-1-infected cells (lane 2, 6 at day 3, 6 after infection), and was undetectable in HIV-1-infected cells treated with RAL, as well as in uninfected cells in the presence of RAL. These results demonstrate that Sup-T1 cells can be infected by HIV-1 and that RAL potently inhibits HIV-1 replication. Thus this

cell culture system represents an *in vitro* model mimicking the active and suppressed HIV infection on ART *in vivo*.

We then asked whether HIV infection *in vitro,* with or without RAL treatment, can cause T cell apoptotic death. Interestingly, T cell apoptosis, measured by the Av/7AAD expression, was not observed in the early phase (1~3 days) of HIV infection, and apoptotic death was measured at 6 days after HIV-1 infection (representative dot plots of Av and 7AAD expression in Sup-T1 cells, with or without HIV-1 infection and RAL treatment; and summary data of Av^+ $7AAD^-$ early apoptotic cells as well as Av^+ $7AAD^+$ late apoptotic cells at day 3, day 6, and day 9 after infection were shown in **Fig.3.5D**). Notably, uninfected Sup-T1 cells cultured for 6 days showed baseline levels of early apoptotic (Av^+7AAD^-, 10.0%) and late apoptotic (Av^+7AAD^+, 2.6%) cells. Low (1 x 10^6 virions) ~ high (5 x 10^6 virions) concentrations of HIV-1 infection increased the number of early apoptotic (Av^+7AAD^-, 23.4 ~ 36.5%) and late apoptotic (AV^+7AAD^+, 9.1 ~ 23.3%) cells in an infection dose-dependent manner. The presence of RAL had no effect on apoptotic death in uninfected cells (early and late apoptotic cells were 9.5% and 2.9%, respectively). However, compared to the infected cells without RAL, RAL treatment significantly reduced HIV-1-induced cell apoptotic death, as evidenced by remarkable decreases in the Av^+7AAD^+ late apoptotic cells from 9.1 ~ 23.3% to 3.3 ~ 2.2%, and the Av^+7AAD^- early apoptotic cells reduced from 23.4 ~ 36.5% to 9.9 ~ 17.5% at day 6 after infection. We also observed the same trend at day 9 after HIV-1 infection, i.e., RAL treatment significantly reduced the Av^+7AAD^+ late apoptotic cells from 43.2 ~ 47.1% to 2.4 ~ 1.9%, but the Av^+7AAD^- early apoptotic cells remained high (39.9 ~ 46.3% and 36.0 ~ 42.7%, respectively) in an HIV-1 dose-dependent manner in the absence or presence of RAL treatment.

In parallel, we also counted the actual cell numbers with or without HIV-1 infection and/or RAL treatment. To this end, Sup-T1 cells were cultured with 1.5 x 10^6 cells/3 ml on day 0 of HIV-1 infection. The cells were counted every 3 days and then seeded with 1.5 x 10^6 cells/3 ml per well at each passage. As shown in **Fig.3.5E**, the number of Sup-T1 cells increased rapidly in the uninfected group, and HIV-1 infection clearly reduced the cell proliferation and killed the cells in a virus dose- and time-dependent manner. However, no such effects were observed in the RAL-treated cells. These data support the Av/7AAD assay results showing that RAL can reduce the cell death of HIV-1 infected cells, although they remain in an early apoptotic state (**Fig.3.5D**). These results are also in line with a previous report showing that HIV-1 can induce formation of syncytia that kill CD4 lymphocytes [49].

We next asked whether HIV-1-induced T cell apoptosis is related to the DNA damage at telomeres. We used Flow-FISH to measure the telomere length in Sup-T1 cells with or without HIV-1 infection. As shown in **Fig.3.5F** (representative overlaid histogram and summary data from 3 independent experiments), T cells infected with HIV-1 at 3 days, and particularly, at 6 days showed a significantly shortened telomere length compared to uninfected cells (P=0.0148 and P=0.0288, respectively). These results are in line with the observations of shortened telomere in CD4 T cells derived from HIV-infected patients (**Fig.3.2D**), indicating that telomere erosion is associated with cell apoptosis in HIV infection.

ATM Is Dysregulated in HIV-Infected T cells *in Vitro*, which Promotes DDR and Cell Apoptosis.

To determine whether telomeric DNA damage and cell apoptosis is associated with the

ATM alteration induced by HIV-1 infection, we measured ATM, CHK2, and AKT expressions, along with the DNA damage marker γH2AX and apoptosis marker PARP-1, in Sup-T1 cells with varying concentrations of HIV-1 or without infection for 3~6 days. As shown in **Fig.3.5G**, kinetic measuring of p24 expression in Sup-T1 cells by western blot showed HIV-1 infection in a virus dose- and time-dependent manner. Interestingly, the amount of ATM and its downstream signaling kinase CHK2 were increased in the early phase (day 3) and then decreased in the late phase (day 6) of HIV-1 infection. Also, the level of PI3K/AKT showed the same trend as the ATM/CHK2 alteration. Correspondingly, the γH2AX and caspase 3-dependent cleavage of PARP-1, which plays an important role in DNA damage-mediated cell apoptosis [39], was increased at day 6, but not at day 3 that has no apoptosis detectable yet, after HIV-1 infection.

Based on these observations, we chose the day 6 time-point after HIV-1 infection, with or without RAL treatment, to get more insight into the role of the ATM pathway that is critically involved in the DDR and DSB repair during viral infection. As shown in **Fig.3.5H**, p24 was only detected in HIV-1-infected Sup-T1 cells, but not in uninfected or HIV-1-infected + treated with RAL. Intriguingly, compared to the uninfected control (lane 1), HIV-infected cells (lane 2) exhibited a slightly inhibited ATM, but pATM was increased, likely reflecting HIV-1-induced DDR. In parallel, CHK2 and AKT were inhibited, but pCHK2 and pAKT were significantly upregulated by the HIV-1 infection, along with upregulation of DDR-mediated apoptotic markers, such as γH2AX and PARP-1. Interestingly, RAL treatment alone (lane 3) did not significantly alter the ATM, pATM, CHK2, pCHK2, AKT, pAKT, γH2AX, and PARP-1 levels in Sup-T1 cells when compared to the uninfected cells without RAL treatment (lane 1). However, RAL treatment of HIV-1-infected cells (lane 4) inhibited p24 expression and resulted

in lower expression levels of ATM/pATM, CHK2/pCHK2, and AKT/pAKT. We also found similar inhibition of ATM/pATM, CHK2/pCHK2, and AKT/pAKT expressions in Sup-T1 cells at day 12 after HIV-1 infection and RAL treatment (data not shown), which recapitulated the findings in patients with latent HIV-1 infection on ART. Taken together, these results suggest that the ATM signaling pathway is dysregulated in CD4 T cells by HIV-1 infection that is associated with the DDR-mediated cell apoptosis.

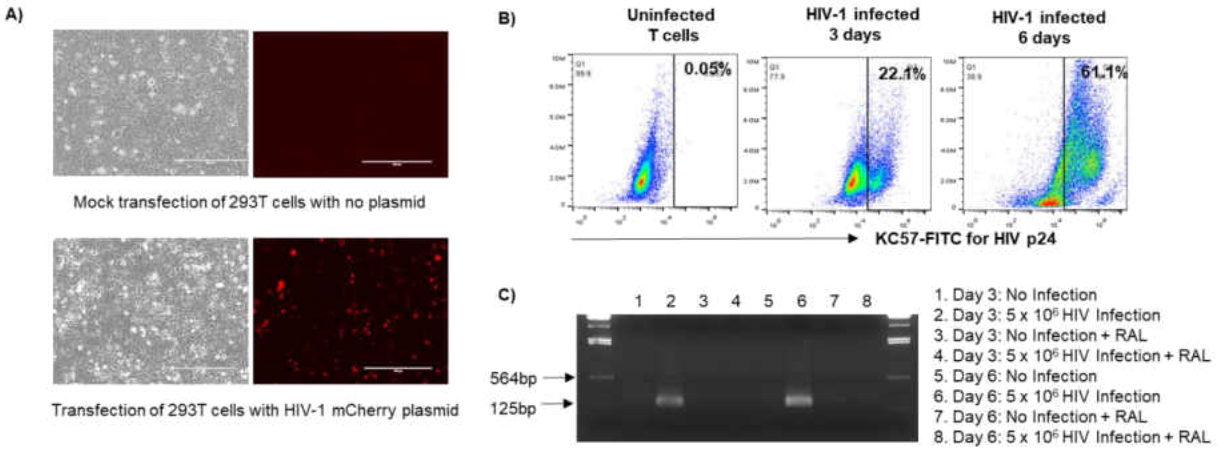

Fig.3.5. Mechanisms involved in inhibition of ATM expression and activation. A) Fluorescence microscopy examination of 293T cells transfected with no plasmid (mock) or HIV-1 mCherry plasmid for 48 h. **B)** Representative dot plots of flow cytometry analysis of HIV-1 p24 antigen expression in Sup-T1 cells (CD4 T lymphoblast isolated from a 13 year old individual with Non-Hodgkin's T cell lymphoma) with or without HIV-1 infection for 3 and 6 days. **C)** RT-PCR detection of HIV-RNA in Sup-T1 cells with or without HIV-1 infection and RAL treatment for 3 and 6 days. **(continued on the next page)** **D)** Flow cytometry analysis of apoptosis in Sup-T1 cells with escalating concentrations of HIV-1 inoculation dose. Representative dot plots and summary data of early ($Av^+ 7AAD^-$) as well as late ($Av^+ 7AAD^+$) apoptotic cells at different time point (3, 6, 9 days) after HIV-1 infection with or without RAL treatment are shown. **E)** Cell number counts of Sup-T1 cells infected with HIV-1 and treated with or without RAL for different time points (0, 3, 6, 9 days after HIV-1 infection). **F)** Representative overlaid histogram and summary data of Flow-FISH analysis for telomere length in Sup-T1 cells infected with or without HIV-1 at 3 and 6 days. The data are summarized from three repeated experiments, and telomere length (MFI) in HIV-infected cells are normalized by those in uninfected cells. **G)** Western blot analysis of p24, ATM, CHK2, AKT, γH2AX, and PARP-1 expressions in Sup-T1 cells infected with escalating concentrations of HIV-1 for different time points (3 and 6 days). **H)** Western blot analysis of p24, ATM, pATM, CHK2, pCHK2, AKT, pAKT, γH2AX, and cleaved PARP-1 expressions in Sup-T1 cells with or without HIV-1 infection and RAL treatment for 6 days.

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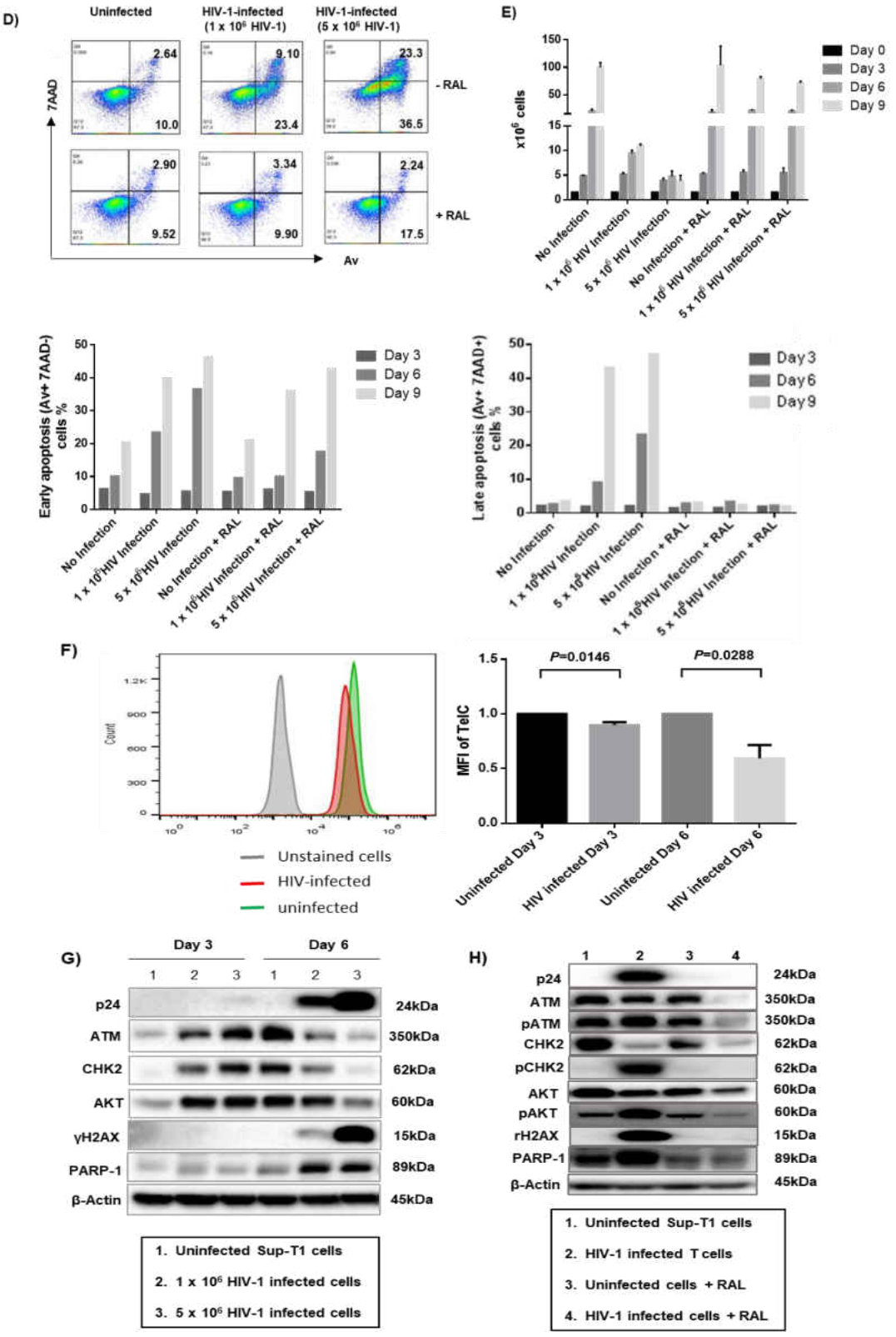

Reconstitution of ATM in HIV CD4 T Cells Reduces DNA Damage, Survival Defects, and Cellular Dysfunctions

Given the critical role of ATM in repairing DNA damage, we hypothesized that reconstitution of ATM could recover HIV T cells from DNA damage and restore the signaling network required for repairing DNA-DSB. To test this, we transfected CD4 T cells derived from HIV-infected, ART-treated individuals with Flag-His-ATM expression construct or control plasmids, an empty vector without ATM insert (Mock) and an ATM kinase-dead mutant (ATM-S1981A) in which the serine residue at 1981 was substituted by an alanine residue. As shown in **Fig.3.6A**, western blot analysis confirmed the increases in ATM and pATM expressions in the ATM-transfected T cells compared to the control. Additionally, protein expression and phosphorylation (pCHK2) of the ATM downstream signaling molecule CHK2 were increased in ATM-transfected cells (**Fig.3.6B**). Although ectopic overexpression of ATM could have broad biological consequences, we focused our investigation on DNA repair, cell survival, and cell function by assessing PARP-1, caspase-3, γH2AX, IL-2, and IFN-γ expressions as readouts. Specifically, the caspase-3-dependent cleavage of PARP-1 was decreased in T cells transfected with the wild-type ATM, but not the ATM kinase-dead mutant (**Fig.3.6C**). Concurrently, level of the active form of caspase-3 was reduced in T cells with ATM reconstitution, but not with the ATM-S1981A transfection, though such decrease was not significant (data not shown). Compared to the mock transfection, overexpression of ATM significantly reduced γH2AX level in transfected (His^+) CD4 T cells, whereas transfection of the ATM-S1981A mutant did not elicit such an effect, indicating the importance of ATM kinase activity in protecting cells from excessive DNA damage **(Fig.3.6D)**. These results are in line with the data showing that transfection of wild-type ATM significantly reduced cleaved PARP-1, whereas mock- or ATM-

S1981A-transfected cells exhibited relatively higher levels of cleaved PARP-1 (**Fig.3.6C**). Thus, these results suggest that ectopic ATM expression exerts immediate effects on repairing DNA damage and regulating T cell fate to secure cell survival. Importantly, reconstituting ATM eventually restored T cell function, as demonstrated by the significant increase in IL-2 (**Fig.3.6E**) and the partial recovery of IFN-γ expression (**Fig.3.6F**) in ATM-transfected cells, whereas ATM-S1981A transfection could not induce such an effect. Taken together, these results demonstrate that restoring an adequate wild-type ATM level in CD4 T cells from latent HIV infection is necessary and sufficient to reduce the DNA damage, survival defects, and cell dysfunctions.

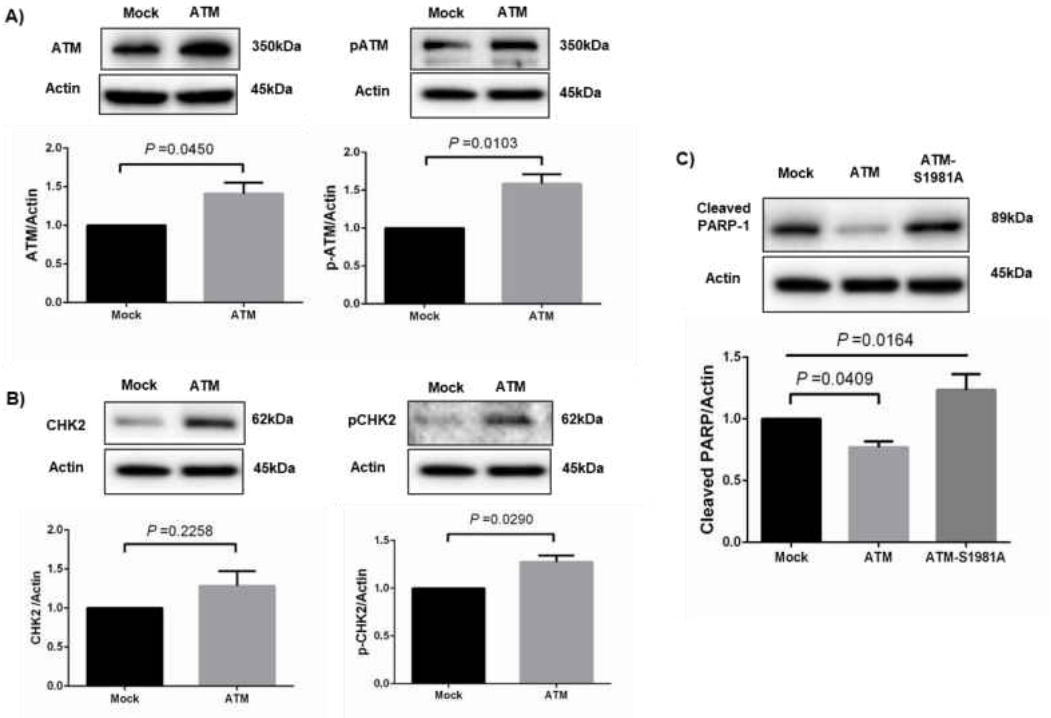

Fig.3.6. Ectopic expression of ATM reduces DNA damage, cell apoptosis and dysfunction of CD4 T cells from HIV patients. A-C) Western blot analysis of ATM/pATM, CHK2/pCHK2, and cleaved PARP-1 expressions in CD4 T cells derived from HIV-infected patients with or without ATM overexpression (n=9). **(continued on the next page) D~F)** Representative dot plots and summary data of flow cytometry analysis of the expression of γH2AX (n=4), IL-2 (n=5) and IFN-γ (n=5) in Mock, ATM, and ATM-S1981A-transfected (His^+) CD4 T cells derived from HIV-infected patients. *P* values between each group are shown.

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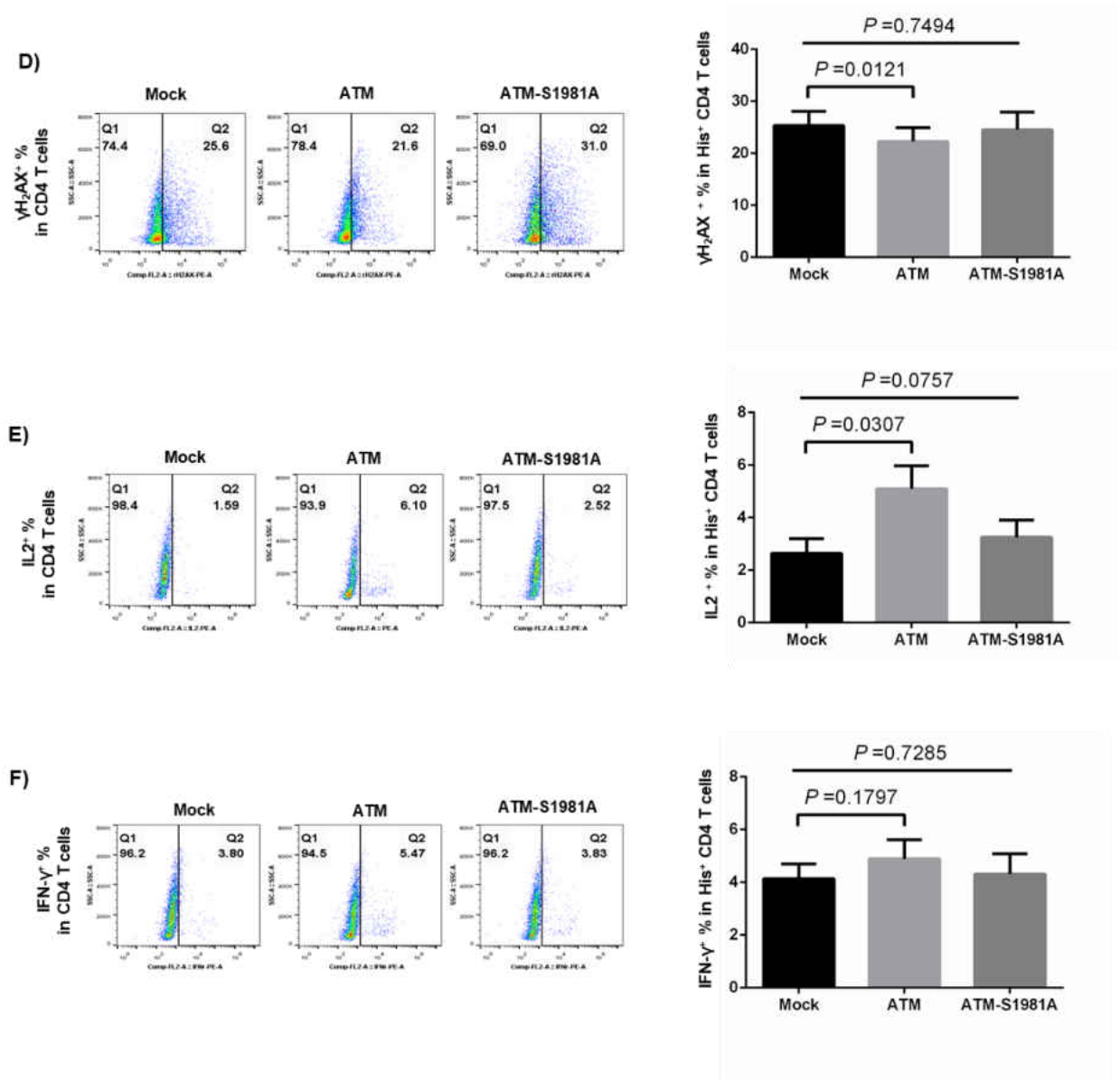

Discussion

T cells play a pivotal role in controlling viral infection and vaccine responses. However, the mechanisms that dysregulate T cell functions during viral infections, particularly in latent phase, remain incompletely understood. In this study, we investigated the mechanisms that

perturb T cell homeostasis, apoptosis, DNA damage and repair machineries in CD4 T cells from patients with virus-suppressed, latent HIV infection. We found a significant depletion of CD4 T cells, which was inversely correlated with cell apoptosis in HIV-infected individuals. Additionally, HIV-derived T cells exhibited accumulation of DNA damage that extended to telomeres, leading to accelerated telomere attrition. Mechanistically, the DNA damage sensor MRN complex remained intact, but the expression and activation of PI3K family member ATM and its downstream checkpoint kinase CHK2 were significantly suppressed in HIV T cells. Consistently, ATM/CHK2 activation and cellular functions were impaired in primary healthy CD4 T cells following ATM silencing, or exposure to an ATM inhibitor, and also in a CD4 T cell line infected with HIV-1 in the presence of RAL, which recapitulates the biological effects we observed *in vivo* in HIV-infected patients on ART. Importantly, ectopic expression of ATM was essential and sufficient to reduce the DNA damage, survival deficit, and cell dysfunctions in HIV-derived T cells. These results demonstrate that ATM deficiency may lead to accumulation of telomeric DNA damage, rendering CD4 T cells prone to apoptosis in the setting of latent HIV infection. Based on these novel findings, we propose a model, as depicted in **Fig.3.7**, in which HIV infection-induced ATM deficiency drives accumulation of telomeric DNA damage, premature T cell senescence, and apoptosis. This constant, excessive CD4 T cell depletion would necessitate high homeostatic proliferation and impose replicative stress on naïve and particularly memory CD4 T cells, leading to further cell senescence or apoptosis - a malicious cycle for CD4 T cell dysregulation during chronic HIV infection.

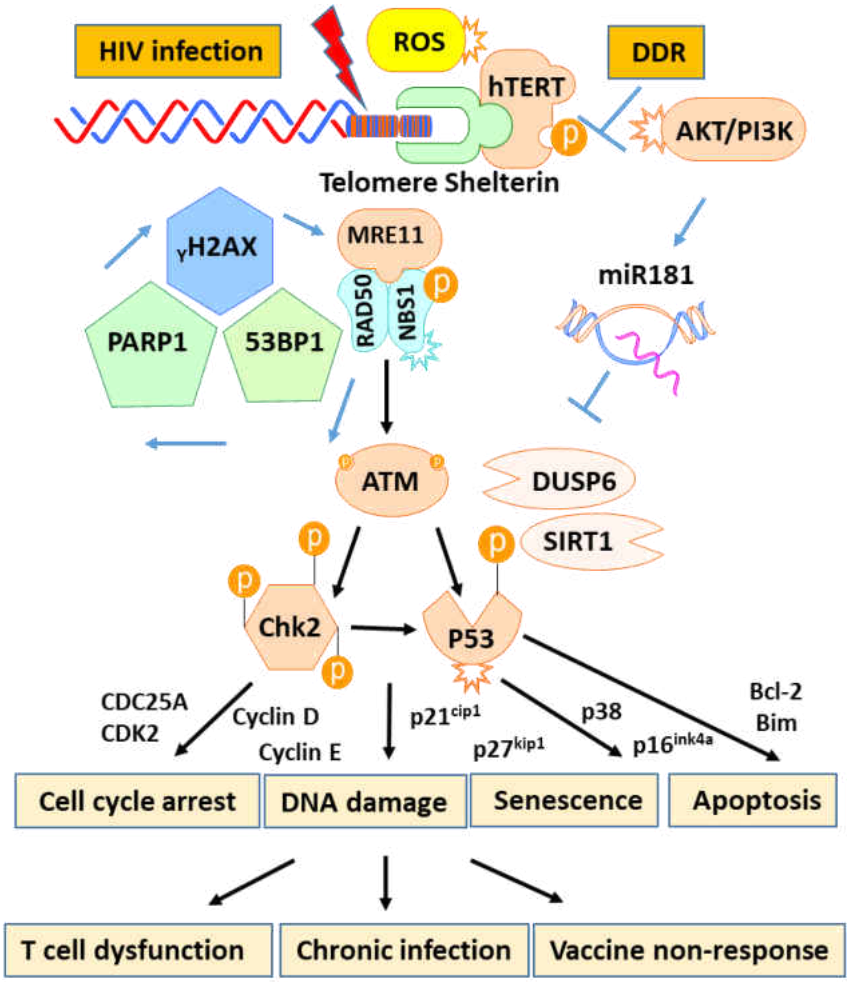

Fig.3.7. A working model depicting the role of HIV-induced ATM deficiency in T cell cycle arrest, telomeric DNA damage, cell senescence, and apoptosis. HIV infection triggers a DNA damage response (DDR) during the early phase of infection via the activation of MRN-ATM-CHK2/P53 signaling pathways in CD4 T cells, prompting cell cycle arrest (through the p21/p27-regulated CDK/Cyclins) and allowing DNA damage repair. If the infection persists and causes unrepairable DNA damage, the cell will commit suicide and undergo apoptosis. Persistent antigenic and inflammatory or ROS stimulation in the setting of latent HIV-1 infection in individuals on ART, however, drives T cell exhaustion (PD-1) and senescence (telomere attrition) by inducing ATM (PI3K-like kinase) deficiency (low protein expression and phosphorylation, likely via a miRNA181-mediated DUSP6/SIRT1 dysregulation), leading to inflammaging and impaired DNA damage repair, which results in constant DNA damage (γH2AX, 53BP1 accumulation), cell apoptosis (caspase-3-dependent cleavage of PARP-1), and thus CD4 T cell loss. Excessive CD4 T cell loss necessitates high homeostatic proliferation and imposes replicative stress on reactive memory CD4 T cells and unprimed naïve T cells, leading to accelerated, premature T cell aging or senescence. This represents a novel molecular mechanism underlying the T cell dysfunction and immune (vaccine) non-responsiveness seen in the setting of latent HIV infection.

The mechanisms underlying inflammaging development in ART-controlled, latent HIV infection remain elusive, but multiple factors may be involved. Specifically, HIV latency in the era of ART is characterized by the existence of viral reservoirs that prevent HIV-1 eradication and likely drive inflammaging [50, 51]. Thus, HIV-mediated inflammaging could result from a myriad of insults, such as viral particles or viral proteins released from the reservoirs, cell-secreted pro-inflammatory mediators, HIV-enhanced gut permeability and altered gut microbiota or dysbiosis, frequent cytomegalovirus (CMV), Epstein–Barr virus (EBV), and HCV coinfections, ART regimens, as well as other closely associated comorbidities including malignancies, or even social, personal stresses and environmental factors [52-55]. As demonstrated in this study, ROS generated within the cellular milieu during viral infection might also drive inflammaging.

This residual chronic, low-grade inflammation-mediated inflammaging during latent HIV infection appears to trigger immune activation that may increase the risk of developing comorbidities, similar to those observed in the elderly. For example, individuals living with HIV have accentuated risks for age-associated comorbidities; a 2-fold higher risk of cardiovascular disease, a 3-fold increased risk of fracture, and a risk of developing kidney disease that is comparable to those with diabetes [8]. Some comorbidities may present at younger ages compared to the general population, suggesting an accelerated, premature aging in the setting of well-controlled but latent HIV infection [56].

An important question is why and how do CD4 T cells accumulate DNA damage and fail to repair telomere defects during HIV infection? Human CD4 T cells have a relatively long life span (~150~160 days) and thus are exposed to a multitude of genotoxic stresses, leading to ~1%

out of a pool of 300 billion T cells to be replaced daily [29]. To maintain genomic stability and cell survival, cells continuously recognize and respond to DNA damage by either activation of DNA damage checkpoints to arrest cell cycle progression and allow for repair, or, if the damaged DNA is beyond repair, to undergo apoptosis [38]. Notably, a major sensor of DSB is the MRN complex, which subsequently recruits the protein kinase ATM, an enzyme critically involved in repairing DNA damage for cell survival [39, 40]. Our results revealed that while the MRN complex was intact, ATM was dramatically affected by HIV even in the setting of a robust viral suppression in both *in vitro* and *in vivo* models, and was associated with significant DNA damage at telomeres. Accumulation of damaged DNA activates the ATM cascades, along with ataxia telangiectasia Rad3-related (ATR) and DNA-dependent protein kinase catalytic subunit c (DNA-PKc), which all belong to the PI3K-related kinase (PIKK) family and are important for DNA reprogramming and T cell rearrangement [42, 43].

ATM was originally identified in individuals with ataxia telangiectasis (AT), an autosomal recessive disorder that manifests with progressive ataxia, telangiectasia, immunodeficiency, genomic instability, and cancer predisposition [57]. It is a key kinase within the DDR signaling cascade [42, 43], and plays a unique role in lymphocyte biology, as programmed DDR participates in the gene remodeling process necessary for formation of a highly diverse TCR repertoire. ATM requires a signal for activation (usually a DNA damage signal), which promotes intermolecular auto-phosphorylation at ATM residue at serine 1981, resulting in its inactive dimer dissociation into active monomers [58]. In the current study, a mutant of S1981A rendered ATM a dominant-negative protein and triggered distinct DNA damage-mediated cell apoptosis and cellular dysfunction (IL-2 inhibition), which underscored the importance of ATM

phosphorylation for its biological functions. ATM is predominantly localized in the nucleus, and is activated once the MRN complex senses and binds to DSB ends, thus providing a platform for ATM recruitment and auto-phosphorylation [59, 60]. Phosphorylation of S1981 also stabilizes ATM at the damaged DNA sites and recruits more downstream effector proteins to participate in the DDR [61]. Among the multiple substrates phosphorylated by ATM is CHK2, which is phosphorylated at residue T62 following DSB formation and prevents cells from progressing from G1 to S phase or, alternatively, leads to cell apoptosis.

Our results demonstrated that, concomitant with the ATM deficiency, the phosphorylation of CHK2 was defective in CD4 T cells during HIV infection. Moreover, siRNA-mediated silencing or pharmacological inhibition of ATM in healthy T cells also led to the CHK2 defect, which was accompanied by dramatic DNA damage and cell apoptosis - mimicking the biological defects we observed in HIV-derived CD4 T cells. These results establish that in human T cells, CHK2 is targeted by ATM and that the overall defect of this signaling pathway can be attributed to ATM insufficiency, driven by chronic HIV infection. Further investigations of the molecular mechanisms regulating the posttranscriptional expression and phosphorylation/dephosphorylation of these DDR kinases will shed new lights onto the cellular machinery that contributes to DNA repair and cell homeostasis.

Recently, Li et al. [62] identified prematurely aged T cells with damaged telomeres in patients with rheumatoid arthritis resulting from defective activity of the DNA break sensor MRE11A. In patients with latent HIV infection, however, we found an intact DNA damage sensor MRN complex in CD4 T cells. However, ATM was inhibited at the posttranscriptional level in these cells, which produced considerable amounts of ROS. Interestingly, Guo et al. [63, 64]

similarly reported that ATM activation in response to ROS was independent of the MRN complexes. ROS-mediated ATM signaling represses mTORC1 signaling, and therefore cell growth and proliferation through the activation of TSC2 (a negative regulator of mTOR) by liver kinase B1 (LKB1) and AMP-dependent protein kinases (AMPKs) [65]. ATM engagement of the TSC2/mTORC1 signaling node can also regulate autophagy [66], and differential localization of ATM correlates with activation of distinct downstream signaling pathways [67]. We have recently reported that T cells derived from chronic HCV infection also exhibit pronounced telomeric DNA damage due to remarkably elevated ROS, with intact DNA damage sensors MRN complex but impaired ATM/CHK2 signaling pathway [19, 20]. Investigation is underway in our laboratory to detail the mechanisms of ROS-mediated ATM deficiency during latent HIV infection.

It should be pointed out that ATM, as a key kinase in DDR, can be activated in the early phase of DNA damage, as observed in our *in vitro* model, in which pATM, pCHK2, and pAKT were upregulated, whereas their total protein levels were downregulated in CD4 T cells following active HIV infection. These changes led to a global loss of ATM, CHK2, and AKT, which suggests an activation and a subsequent exhaustion or senescence process. Notably, ATM is widely expressed in human T cells at an extremely high level to ensure integrity of genomic DNA in replicating lymphocytes. Its activation may represent the initiation of DDR process, but its inhibition and insufficiency in persistently activated T cells indicate deficiency in DNA repair that can promote cell exhaustion and senescence in the setting of chronic viral infection. This notion is also supported by our observation in the *in vitro* stimulated T cell system in which ATM phosphorylation was elevated in the early phase of KML001 (a telomere targeting drug)-treated T cells (3~6 h), but decreased with the treatment for longer periods (24~48 h), and

associated with increases in DNA damage, cell apoptosis and functional impairment (unpublished observations).

As noted above, our data demonstrated that both ATM and CHK2 expression and activation were ultimately diminished in the setting of latent HIV infection on ART. This was present in T cells from our *in vivo* population of HIV-infected subjects on ART and matched our *in vitro* T cells infected with HIV in the presence of an integrase inhibitor, RAL. Interestingly, this drug was added very early (2 h) after HIV was introduced into the cells, but led to dramatic reduction in ATM, CHK2 and their phosphorylated moieties when compared to uninfected T cells exposed to RAL. These data beg the question of whether it is even feasible to avoid disruption of telomeric DDR signaling and cellular senescence/apoptosis in the HIV-infected host without actually targeting the repair machinery itself. Consistent with our findings, Rossiello and Fumagalli et al. [68, 69] reported that telomeric DNA damage in mammalian cells is irrepairable and occurs preferentially at telomeres in linear genomes. Their studies suggested a paradigm in which cellular senescence and organismal aging resulting from persistent DNA damage signaling and accumulated, irrepairable telomeric DNA damage could be permanent via a shared causative mechanism; i.e., once damaged, they remain damaged. Other data suggested that persistent DDRs may trigger senescence-associated inflammatory cytokine secretion, facilitating communication of a compromised state to the surrounding cells [70]. Similarly, a bystander effect in which senescent cells induce DDR via cell-cell contact and generate ROS to proximate cells within the milieu, a senescence-induced senescence [71]. These could certainly occur in our model, given that we observed DDR effects after a short period despite the treatment with ART. Importantly, latently HIV-infected cells are more susceptible to DDR-

inducing agents, and thus are vulnerable to DDR-induced cell apoptosis [72]. Sensitivity to DDR highlights a unique vulnerability of latently infected cells, a new feature that could potentially be exploited in developing therapeutics to eliminate HIV-1 reservoirs. Further studies to explore the DDR triggers are underway in our laboratory.

We and others have shown poor vaccine (HAV, HBV, Influenza, and Pneumovax) responses in the setting of chronic viral (HCV, HIV) infections [11-16, 73-79], but the underlying mechanisms for vaccine failure in virally-infected individuals remain elusive. Our new findings herein indicate that CD4 helper T cells in chronically HIV-infected patients have abnormalities that jeopardize their ability to mount effective immune (vaccine) responses. The immune system is in constant turnover during viral infection, with high demands for lymphocyte replenishment to maintain the equilibrium of the T cell compartment. In this regard, insufficient activation of ATM would be expected to affect both unprimed and primed T cells, but memory T cells would over-expand rapidly with greater DNA damage and cell apoptosis, which could compromise the size and survival rate of the naïve T cell repertoire. Indeed, ongoing antigenic and/or inflammatory stimulation during chronic viral infection induces continuous differentiation of the naïve T cells and turnover of antigen-reactive T cells, resulting in telomere erosion and cell senescence or apoptosis, which we observed more prominently in the memory T cells. Eventually, the majority of the T cell pool would be comprised of antigen-expanded T cells at the expense of naïve T cells. With the decrease in newly generated naïve thymic T cells in adults, chronic inflammation or infection might force the immune system to restore equilibrium by replicating the existing naïve T cells, thereby driving telomere shortening and senescence in

naïve T cell populations in the peripheral blood. Thus, the ability to generate immune response to new antigens, such as vaccines, would be compromised.

In summary, accumulation of DNA damage and failure of repair due to deficiency of the ATM-dependent DNA repair machinery during chronic viral infection may broadly impair diverse cellular functions. Counteracting ATM deficiency may restore T cell homeostasis and competency during chronic viral infection and prevent premature immune senescence. This study reveals a novel molecular mechanism underlying T cell senescence, and provides a new strategy to correct an aberrant immunopathology, to avoid the untoward consequences of immunosenescence, and to improve immunotherapy and vaccine responses against human viral diseases.

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CHAPTER 4

SUMMARY AND FUTURE DIRECTION

Summary

T cells play a crucial role in viral persistence and vaccine responses; however, the mechanisms regulating their responses to viral infection or vaccinations remain elusive. We and others have previously shown that T cells derived from patients with chronic viral infections prematurely reach senescence, characterized by the overexpression of aging markers, such as $p16^{ink4a}$, $p21^{cip1}$, CD57, and KLRG1, differential regulation of aging-associated microRNA-21/-181a, and particularly, shortened telomeres. In this study, we investigated T cell homeostasis, apoptosis, DNA damage and repair machineries in a large cohort of subjects with chronic HCV or HIV infection. we demonstrate that:

1) Homeostatic remodeling of CD4 T cells during HCV infection primarily affects the naïve T cell compartment, whereas HIV infection mainly depletes memory CD4 T cell pool, characterized by an accumulation of DNA damage due to insufficient activation of the DNA repair kinase ATM, leading to CD4 T cell apoptosis and loss.

2) Phosphorylation (activation) of ATM in T cells exposed to HCV or HIV is inhibited through dampening the PI3K pathway, resulting in an increase in unrepaired DNA damage and cell apoptosis.

3) Silencing ATM expression or pharmacological inhibition of ATM phosphorylation leads to more DNA damage and apoptosis in CD4 T cells. Conversely, reconstitution of ATM repairs the DNA damage, cell apoptosis, and functional defects in CD4 T cells derived from both HCV- and HIV-infected individuals.

Based on these novel findings, we propose a model, as depicted in **Fig.2.7 and Fig 3.7**, where HCV- or HIV-induced ATM deficiency leads to accumulation of DNA damage and cell apoptosis. The excessive naïve and/or memory CD4 T cell loss necessitates high homeostatic proliferation and imposes replicative stress on unprimed naïve T cells, further accelerates premature T cell aging and cellular dysfunction. This study reveals a novel mechanism by which ATM deficiency promote T cell apoptosis and senescence, and provides a new therapeutic target for virus-induced immune dysfunction during chronic infection.

Future direction

Mechanisms Involved in ATM Inhibition in T Cells During Viral Infection

While we have demonstrated ATM deficiency in T cells from HCV and HIV infection, how ATM expression and activity are inhibited by the viral infection is yet elucidated. We will define which specific proteins from HCV and HIV virus are responsible for suppressing ATM expression and activation. In addition, since ATM inhibition is primarily found at the posttranscriptional level, the role of microRNA-21/-181s, which had been shown to be differentially regulated in T cells from individuals with HCV or HIV infection, in translational suppression of ATM protein expression will be investigated in future. Also, the role of protein ubiquitin degradation, the role of AKT/PI3K in phosphorylation, and the role of DUSP6/PP2A in dephosphorylation of ATM in senescent T cells, will be further studied in the setting of chronic viral infections.

Mitochondrial Dysfunction and ROS Production in T Cells During Viral Infection

An emerging paradigm in human immunology indicates that cellular metabolic reprogramming is tightly linked with the immune activation and function. Specifically, T cell activation results in an increase in mitochondrial respiration, which is associated with an increase in the production of ROS during chronic viral infection. Overproduction of ROS may directly decrease mitochondrial membrane potential and lead to a lower ATP supply, causing mitochondrial network fragmentation and subsequent, cell apoptosis, or cell senescence. Thus in future, we will determine ROS metabolism in dysregulated T cells during chronic HCV or latent HIV-1 infection. Specifically, we will compare the ROS levels in naïve and memory CD4 T cells derived from virally infected patients versus HS. We will also characterize the mechanisms of ROS production by determining the non-enzymatic antioxidant GSH and enzymatic antioxidants SOD, NAPDH and Catalase levels during chronic viral infections.

The Interplay between ROS and ATM in TV Cells During HIV-1 Infection

Besides functioning as a master regulator of the DDR, ATM also responds directly to the oxidative stress and regulates multiple cellular signaling pathways. For example, ATM kinase has been shown to negatively regulate ROS production. In addition, blocking ATM-mediated DDR during oxidative stress resulted in deficiencies in mitochondrial function and autophagy. The interplay between ROS and ATM during chronic viral infection has not been studied yet. Understanding how ROS contribute to the telomeric DDR and promote premature T cell aging via dysregulating the ATM pathway, and whether ATM can feedback

regulate ROS metabolism, may raise the possibility of developing novel therapeutic targets for inflammaging-induced immune dysfunction during chronic viral infection.

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APPENDIX

ABBREVIATIONS

53BP1	p53-binding protein 1
7AAD	7-Aminoactinomycin D
8-oxoG	8-oxogunine
AIDS	Acquired immune deficiency syndrome
AKT	Protein Kinase B
AMPKs	AMP-dependent protein kinases
ART	Antiretroviral therapy
AT	Ataxia telangiectasis
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia Rad3-related
Av	Annexin V
bp	Base pairs
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CMV	Cytomegalovirus
DAA	Direct acting antiviral
DAPI	4′,6-diamidino-2-phenylindole
DCFDA	Dichloroflurescin diacetate
DDR	DNA damage response
DMSO	Dimethyl sulfoxide

DNA-PKc	Protein kinase catalytic subunit c
DSB	Double strain breaks
DUSP6	Dual specificity phosphatase 6
EBV	Epstein-Barr virus
FMO	Fluorescence minus one
GFP	Green fluorescent protein
GSH	Glutathione
H_2AX	Histone family member X
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HNK1	Human natural killer 1
HS	Healthy subjects
IL-2	Interleukin-2
INF-γ	Interferon gamma
IRB	Institutional review board
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LKB1	Liver kinase B1
MFI	Mean florescence intensity
miR	MicroRNA
MRE11	Double-strand break repair protein
MRN	MRE11, RAD50 and NBS1

mTOR	mammalian target of rapamycin
NAPDH	Nicotinamide adenine dinucleotide phosphate hydrogen
NHEJ	Non-homologous end joining
NS	No significance
PARP-1	Poly ADP-Ribose Polymerase 1
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PEI	Polyethylenimine
PIKK	Phosphatidylinositol 3-kinase-related kinase
PI3K	Phosphatidylinositol 3-kinase
RAL	Reltegravir
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SOD	Superoxide dismutase
STK1	Serine/threonine kinase 11
TBS	Tris buffered saline
TCR	T cell receptor
TIF	Telomere induced foci
Tim-3	T-cell immunoglobulin and mucin-domain containing-3
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
TSC2	Tuberous sclerosis complex 2

VITA

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