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Immune Activation Induces Telomeric DNA Damage, Reduces Memory Precursors, and Promotes Short-lived Effector T Cell Differentiation in Chronic HCV Infection

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by

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ABSTRACT

Immune Activation Induces Telomeric DNA Damage, Reduces Memory Precursors, and Promotes Short-lived Effector T Cell Differentiation in Chronic HCV Infection

by

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Chronic hepatitis C virus (HCV) infection exhibits persistent high viral load, inducing T cells differentiation and dysfunction in chronically infected individuals. Recent longitude studies in both HCV specific- and bulk T cells reveal that chronic immune stimulation is the driving force for the impaired T cell functions, however, the underlying mechanisms remain elusive. Here, we show that peripheral $CD4^+$ T cells from chronically HCV-infected patients exhibit lymphopenia with the reduction of naïve population and expansion of effector memory T cells. $CD4^+$ T cells from HCV patient also display elevated activation markers. including HLA-DR, GLUT1, Granzyme B, and short-lived effector marker $CD127^-$ $KLRG1^+$, whereas stem cell-liked transcription factor TCF1 and telomere sheterin subunit TRF2 are significant reduced, comparing to age- and gender-matched healthy controls. Mechanistically, *ex vivo* T cell differentiation revealed that $CD4^+$ T cells from HCV patients exhibit PI3K/Akt/mTOR signaling hyperactivation upon TCR stimulation, favoring pro-inflammatory effector differentiation with TRF2 downregulation, rendering telomere dysfunction induced foci (TIFs) accumulation, resulting in telomeric DNA damage and cellular apoptosis. Importantly, exacerbation of telomere deprotection by knockdown of TRF2 expression in healthy T cells resulted in an increase in telomeric DNA damage and T cell apoptosis; whereas overexpression of TRF2 in HCV-T cells led to an alleviation of telomeric DNA damage and T cell death. Additionally, inhibition of Akt

signaling during T cell activation can preserve precursor memory population, while limiting inflammatory effector expansion, DNA damage, and cell death. Taken together, these results suggest that modulation of immune activation by inhibiting Akt signaling and protection of telomeres by enforcing TRF2 expression could open new therapeutic strategies to balance adaptive immune responses in the setting of chronic immune activation and inflammatory in vulnerable populations such as chronically viral infected individuals.

DEDICATION

To my parents Nguyen Van Ngan & Nguyen Phuong Hue who constantly support and dedicate for my education. Without them I am not who I am today.

To my younger sister and also my labmate Nguyen Ngoc Thao Lam

To my girlfriend Hoa Quach

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CHAPTER 1. INTRODUCTION

Despite highly effective direct-acting antiviral (DAA) treatment on hepatitis C virus (HCV) infection continuedly brought down HCV-related deaths in the United States from 5.03 per 100,000 population in 2013 to 3.72 in 2018, the annual rate of new HCV infection was triply increased from 0.3 per 100,000 people in 2009 to 1.2 in 2018; among those, persons from 20-29 and 29-30 years old are at the highest rate at 3.1 and 2.6 respectively, likely related to their intravenous drug uses [1]. This alarming sight of a rapid increase in new HCV cases among young individuals is complicating the efforts of eliminating HCV as a threat of public health without an effective vaccine available. T cells play a pivotal role in controlling viral infection and vaccine responses; however, the precise mechanisms underlying T cell dysfunction that leads to chronic viral infection and poor vaccine responses remain incompletely understood. Notably, the failures to successfully manage many chronic infectious diseases, and to effectively respond to vaccines in virally infected individuals, stem from our incomplete understanding of the pathogen and host interactions that dampen host immunity. Of note, HCV causes chronic infection in 70-80% of infected individuals, thus becoming a valuable model for studying the mechanisms of viral chronicity in humans [2]. Spontaneously HCV-resolved individuals show strong $CD4^+$ and $CD8^+$ T cell responses with a long-lived memory phenotype after acute infection [3, 4], indicating a critical role for T cells in controlling HCV infection. Recent longitudinal study in different infection outcomes revealed that those individuals progressed to chronic infection developed broad HCV-specific $CD4^+$ T cell responses during the early phase of viral infection; however, as the infection progresses, $CD4^+$ T cell responses waned and diminished significantly [4]. On the other hand, persistent viremia could drive T cell receptor (TCR) over-activation and upregulation of inhibitory receptors, such as PD-1, Tim-3, and CTLA-4, resulting in T cell exhaustion and

senescence [4, 5]. These observations strongly suggest that HCV-specific $CD4^+$ T cells are successfully primed but become exhausted and senescent in the setting of chronic viral infection.

Recently, we and others have found that HCV infection can accelerate T cell aging, as evidenced by overexpression of aging markers (such as KLRG-1, β-galactosidase, $p16^{ink4a}$, and $p21^{cip1}$), decline of age-associated microRNA181a, and particularly accelerated attrition of telomeres, indicating excessive cell proliferative turnover or inadequate telomeric DNA maintenance[6-13]. Telomeres are repeating hexameric sequences of DNA that are found at chromosome ends in association with a complex of proteins (shelterin) (**Figure 1.1**).

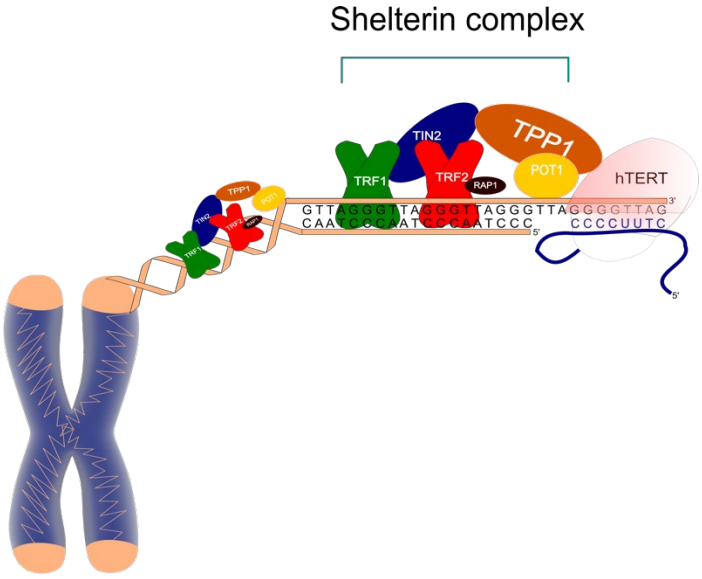


Figure 1.1. Chromosome end telomere is protected by shelterin complex composed of six subunits including telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TERF1-interacting nuclear factor 2 (TIN2), telomeric repeat-binding factor 2-interacting protein 1 (RAP1/ TERF2IP), protection of telomere 1 (POT1) and TPP1 (named from the first letter from these proteins **T**INT1, **P**IP1 and **P**TOP). Human telomerase (hTERT) (a unique human reversed transcriptase enzyme) is recruited to telomere through the interaction with TPP1 to maintain telomere length during cell division.

Telomere integrity is a key feature of the linear chromosomes that preserve genome stability and function, whereas telomere erosion is a hallmark of cell aging or senescence (a quiescent, non-replicative state) that drives cell dysfunction or apoptosis[15, 16]. While telomere length is maintained in most cases by the telomerase, shelterin is essential to protect telomeres against unwanted DNA damage response (DDR)[17, 18]. The role of shelterin is to inhibit DDR pathways at telomeres, regulate telomerase access to and activities at telomeres, prevent telomeric DNA from erosion, and thus safeguard chromosome end integrity[17] (**Table 1.1**).

Table 1.1. *Shelterin subunits and their repressions on corresponded DDR pathways (adopted from Titia de Lange, Annu Rev Genet. 2018 Nov 23;52:223-247.)*[14]

DDR pathway	Shelterin subunit(s)	Mechanism	General repressor
ATM kinase	TRF2 (TIN2)	t-loop	None
ATR kinase	POT1a (POT1b)	RPA exclusion	None
PARP1	TRF2, TIN2	Branched-DNA binding, ?	Ku70/80
c-NHEJ	TRF2	t-loop (iDDR, RAP1)	CYREN (S/G1)
Alt-NHEJ	TRF2 (TIN2, POT1a/b)	t-loop, PARP1 repression, ?	Ku70/80
HDR	POT1a or POT1b + RAP1	?, ?	Ku70/80
Hyper-resection	POT1a/b, TRF2	Repression of ATM, ATR	53BP1/rif1/rev7

Shelterin comprises six polypeptides (TRF1, TRF2, RAP1, TIN2, TPP1, and POT1), of which telomeric repeat binding factor 2 (TRF2) is a key factor that plays an essential role in maintaining telomere integrity by suppressing the ataxia telangiectasia-mutated protein (ATM)-dependent DDR and non-homologous end joining pathways[19]. TRF2 also protects chromosome ends against replicative DNA damage, particularly those that occur due to topological stress[20]. Notably, TRF2 expression is increased in a variety of human cancers; consistently, its down regulation reduces tumorigenicity[21, 22]. However, the role of TRF2 in reprogramming human T cell telomeric DNA damage and remodeling T cell senescence and apoptosis during viral infection is largely unknown. To identify factors that perturb T cell homeostasis during viral

infection, we have explored the role of TRF2 in protecting telomeres from DNA damage and T cell apoptosis in a human model of chronic HCV infection. We provide evidence revealing that TRF2 inhibition promotes telomere attrition and DNA damage in HCV infection, rendering HCV T cells more senescent and apoptotic, and thus potentially contributing to the HCV persistence and vaccine non-responsiveness.

$CD8^+$ T cell exhaustion/senescence has been well-characterized in chronic HCV and HIV infection in humans [5, 23, 24], lymphocytic choriomeningitis virus in mice [25], simian immunodeficiency virus in rhesus macaques [26], and in many cancer diseases in humans [27-31]. Exhausted $CD8^+$ T cells express high levels of inhibitory receptors, including PD-1, KLRG-1, and TIM-3, low levels of CD127, and transcription factor T cell factor 1 (TCF-1) - a stem cell-like marker for antigen-experienced T cells [32-34]. Despite the critical roles of $CD4^+$ helper T cells in orchestrating $CD8^+$ T cell and B cell responses, there were fewer studies on $CD4^+$ T cell exhaustion and senescence compared to $CD8^+$ counterpart. We and others have previously reported high levels of inhibitory receptor expression in $CD4^+$ T cells during chronic viral infections [35-38]. In particular, chronic antigen stimulation and sustained inflammation can trigger immune over-activation and rapid T cell turnover, leading to the accumulation of DNA damage, telomere erosion, and genomic stress-induced cell apoptosis [39-47].

In chronic HCV infection, bulk $CD4^+$ and $CD8^+$ T cells show an activated phenotype with significantly higher levels of PD-1 and proinflammatory cytokine expressions [48], suggesting that persistent viremia triggers activation-induced cellular exhaustion and senescence that occur not only in HCV-specific T cells but also in general T cell populations. While understanding of virus-specific T cell dysfunction attracts greater attention due to their role in the control of infection, studies of generalized T cell populations in chronic HCV infection will help to

understand the mechanisms of persistent immune activation-induced hepatic fibrosis, hepatocellular carcinoma, and extrahepatic manifestations, such as mixed cryoglobulinemia, B-cell non-Hodgkin lymphoma, chronic kidney diseases, and insulin resistance/type 2 diabetes in virally infected individuals [49, 50]. Indeed, despite DAA therapy can achieve > 95% sustained virologic response (SVR) in chronically HCV-infected individuals, persistent immune activation in circulating T cells, including $CD4^+$, $CD8^+$, and Vγ9Vδ2 T cells, are either continued or only partially recovered in virologically cured patients long after viral clearance post SVR [51-54]. In addition, dysfunctional mucosal-associated invariant T (MAIT) cells with activated phenotypes are nonreversible after HCV clearance in DAA-cured patients [55]. These observations suggest that further studies of systemic immune activation and T cell dysfunction are essential for understanding and approaching to achieve an immunologic cure and reduce comorbidities in chronic HCV infection in the era of DAA virologic cure.

In this study, we examined $CD4^+$ T cell activation-induced telomeric DNA damage and cellular dysfunctions in chronically HCV-infected individuals. We demonstrated that $CD4^+$ T cells in chronic HCV infection show highly expressed maturation and activation markers, accumulated DNA damage, and cellular apoptosis compared to gender- and age-matched healthy subjects (HS). Mechanistically, HCV-$CD4^+$ T cells exhibit stronger AKT/mTOR activation upon TCR stimulation, leading to downregulations of telomeric repeat binding factor 2 (TRF2) and memory precursor transcription factor TCF1, and biased differentiation into pro-inflammatory phenotype. Inhibition of AKT activation and/or maintaining of TRF2 expression limit immune activation and reverse these negative effects on HCV-$CD4^+$ T cells. Therefore, controlling AKT-mTOR over-activation and/or maintaining TRF2 and TCF1 expression in TCR-activated $CD4^+$ T cells could become potential therapeutic strategies to limit immune activation-induced

proinflammatory effector $CD4^+$ T cell expansion and memory precursor loss, off-balancing adaptive immune response in chronic viral infection.

Specific Aims

Aim 1: identifying factors that perturb $CD4^+$ T-cell homeostasis during chronic HCV infection.

We have explored the role of TRF2 in protecting telomeric DNA damage and T-cell apoptosis with a model of HCV infection. We provide evidence revealing that TRF2 inhibition promotes telomere attrition and DNA damage during HCV infection, rendering HCV T cells more senescent and apoptotic, thus potentially contributing to the HCV persistence and vaccine non-responsiveness.

Aim 2: examining $CD4^+$ T cell activation-induced telomeric DNA damage and cellular dysfunctions in chronically HCV-infected individuals.

We demonstrated that HCV-$CD4^+$ T cells show overexpressed activation markers, accumulated DNA damage, and cellular apoptosis. Mechanistically, HCV-$CD4^+$ T cells exhibit stronger AKT/mTOR activation upon TCR stimulation, leading to downregulations of telomeric repeat binding factor 2 (TRF2) and memory precursor transcription factor TCF1, and biased differentiation into pro-inflammatory phenotype. Inhibition of AKT signaling or maintaining of TRF2 expression reverse these negative effects on HCV-$CD4^+$ T cells, which may limit immune activation-induced proinflammatory effector $CD4^+$ T cell expansion and memory precursor loss during chronic viral infection.

CHAPTER 2. INHIBITION OF TRF2 ACCELERATES TELOMERE ATTRITION AND DNA DAMAGE IN NAÏVE $CD4^+$ T CELLS DURING HCV INFECTION

Abstract

T cells play a crucial role in viral clearance and vaccine responses; however, the mechanisms that regulate their homeostasis during viral infections remain unclear. In this study, we investigated the machineries of T-cell homeostasis and telomeric DNA damage using a human model of hepatitis C virus (HCV) infection. We found that naïve CD4 T cells in chronically HCV-infected patients (HCV T cells) were significantly reduced due to apoptosis compared with age-matched healthy subjects (HSs). These HCV T cells were not only senescent, as demonstrated by overexpression of aging markers and particularly shortened telomeres; but also DNA damaged, as evidenced by increased dysfunctional telomere-induced foci (TIF). Mechanistically, the telomere shelterin protein, in particular telomeric repeat binding factor 2 (TRF2) that functions to protect telomeres from DNA damage, was significantly inhibited posttranscriptionally via the p53-dependent Siah-1a ubiquitination. Importantly, knockdown of TRF2 in healthy T cells resulted in increases in telomeric DNA damage and T-cell apoptosis, whereas overexpression of TRF2 in HCV T cells alleviated telomeric DNA damage and T-cell apoptosis. To the best of our knowledge, this is the first report revealing that inhibition of TRF2 promotes T-cell telomere attrition and telomeric DNA damage that accelerates T-cell senescent and apoptotic programs, which contribute to naïve T-cell loss during viral infection. Thus, restoring the impaired T-cell telomeric shelterin machinery may offer a new strategy to improve immunotherapy and vaccine response against human viral diseases.

Introduction

T cells play a pivotal role in controlling viral infection and vaccine responses; however, the mechanisms underlying T-cell dysfunction that lead to chronic infection and poor vaccine response remain unclear. Hepatitis C virus (HCV) is highly efficient at establishing chronic infection, thus becoming an excellent model to study the mechanisms of T-cell dysregulation and viral persistence[56].

Recently, we and others have found that HCV infection can accelerate T-cell aging, as evidenced by overexpression of aging markers and attrition of telomeres, indicating excessive cell proliferative turnover or inadequate telomeric DNA maintenance[7-13, 57]. Telomeres are repeating hexameric DNA sequences that are 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 erosion is a hallmark of cell senescence that drives cell dysfunction or apoptosis[15, 16]. Although telomere length is maintained in most cases by the telomerase, shelterin is essential to protect telomeres against unwanted DNA damage response (DDR)[17, 18]. Shelterin comprises six polypeptides (TRF1, TRF2, RAP1, TIN2, TPP1, and POT1), of which telomeric repeat binding factor 2 (TRF2) is a key factor that plays an essential role in maintaining telomere integrity[19]. TRF2 also protects chromosome ends against replicative DNA damage, particularly those that occur due to topological stress[20]. Notably, TRF2 expression is increased in a variety of human cancers; consistently, its downregulation reduces tumorigenicity[21, 22]. The role of TRF2 in reprogramming telomeric DNA damage and remodeling T-cell homeostasis during viral infection, however, is largely unknown.

To identify factors that perturb T-cell homeostasis during viral infection, we have explored the role of TRF2 in protecting telomeric DNA damage and T-cell apoptosis with a model of HCV infection. We provide evidence revealing that TRF2 inhibition promotes telomere attrition and DNA damage during HCV infection, rendering HCV T cells more senescent and apoptotic, thus potentially contributing to the HCV persistence and vaccine non-responsiveness

Materials and Methods

Subjects. The study protocol was approved by the institutional review board (IRB) of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN). Written informed consent was obtained from each patient included in this study. The study subjects were composed of two populations: 180 chronically HCV-infected patients and 160 age-matched HS. All HCV-infected patients were positive for HCV RNA, prior to antiviral treatment. Healthy subjects (HS), obtained from Physicians Plasma Alliance (PPA), Gray, TN, were negative for HBV, HCV and HIV infection.

Cell isolation and culture. PBMCs were isolated from whole blood by Ficoll (GE Healthcare BioSciences, Pittsburgh, PA) density centrifugation. Naïve and memory $CD4^+$ T cells were isolated from PBMCs using the Naïve or Memory $CD4^+$ T Cell Isolation Kit using MidiMACS™ Separators (Miltenyi Biotec Inc., Auburn, CA). The isolated T cells were cultured in RPMI 1640 medium (Corning, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 100 IU/ml Penicillin, 100 µg/mL Streptomycin (Lonza, Walkersville, MD) and 2 mM L-glutamine (Thermo Scientific, Logan, Utah). HEK293T cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% FBS and 100 IU/ml Penicillin and 100 µg/mL Streptomycin. Cells were cultured in 5% CO2 incubator at 37°C.

Flow cytometry. For phenotypic analysis of naïve CD4 T cells, PBMCs were stained with CD3-PE, CD4-APC/-PE/-Alexa Fluor-647, CD45RA-FITC and CD28-PerCP/Cy5 antibodies or isotype controls (BioLegend, San Diego, CA). Two senescent markers CD39-PE and CD57-APC (BioLegend) were applied to access senescent status of $CD4^+$ cells and $CD4^+$ $CD45RA^+$ from total PBMCs respectively. To quantify cell apoptosis, PBMCs were stained with CD45RA-FITC, CD4-APC together with Annexin V-PE and 7-AAD from BD Pharmingen™ PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) following the manufacturer's instruction. The stained cells were analyzed on Accuri™ C6 flow cytometer (BD, Franklin Lakes, NJ), and data was analyzed by FlowJo software (Tree Star, Inc., Ashland, OR). Isotype control antibodies, single staining and FOM controls were used to determine background levels, color compensation and gating strategy respectively**.**

Flow-FISH. Telomere length was measured by Flo-FISH technique as described by Derradji H, et al[58] with modifications. In brief, PBMCs were stained with CD4-Alexa-647 antibody then fixed in Fixation buffer (BioLegend) for 20 min. Cells were then washed with flow cytometry buffer (1% BSA and 2mM EDTA in 2 μm filtered distil water) and resuspended in hybridization buffer (70% Formamide, 1% BSA, 10 mM Tris-HCl pH 7.2). Telomere probe TelC (CCCTAACCCTAACCCTAA)-FITC (PNA Bio, Newbury Park, CA) were used to hybridize cellular telomere at the concentration 0.3 μg probe/mL, cells were incubated at room temperature for 10 min in the dark then hybridized at 82°C for 10 min. Cells were kept at room temperature overnight in dark condition. In the next day, cells were washed twice with post-hybridization buffer (70% Formamide, 0.1% BSA, 10 mM Tris-HCl pH 7.2 and 0.1% Tween-20) following an addition wash with flow cytometry buffer. Samples were then stained with CD45RA-perCP/Cy5.5 and subjected for flow cytometry analysis. Isotype control antibodies,

single staining and FOM controls were used to determine background levels, color compensation and gating strategy respectively.

RNA isolation and real-time RT-PCR. Total RNA was extracted from 1.0 x 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, 10 min and then 95°C, 15s; 56°C, 60s following SYBR Green signal detection with 40 cycles. iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) was used for all quantitative PCR experiments. Gene expression was normalized to GAPDH and expressed as fold changes using the $2^{-\Delta\Delta ct}$ method. Primer sequences were shown in ***Table 2. 2***.

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, Rockford, IL). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences) which were blocked with 5% non-fat milk, 0.25% Tween-20 in Tris buffered saline (TBS) for 1 h. Membranes were incubated with anti TRF2, TPP1, RAP1, TIN2, γH2AX, phospho-$p53^{ser15}$, p21, GAPDH, Actin (Cell Signaling), POT1 (R&D System, Minneapolis, MN), TRF1 (Thermo Fisher), p53, Ub (Santa Cruz, Dallas, Texas) and Siah1 (Abcam, Cambridge, MA) primary antibodies in 1% non-fat milk-TBST overnight at 4ºC. In the next day, membranes were washed three times with TBST following horseradish peroxide-conjugated secondary antibodies (Cell Signaling) incubation for 1 h at room temperature. Membranes were then washed 5 times with TBST then developed using Amersham ECL Prime Western Blotting Detection Reagent (GE

Healthcare BioSciences) following exposure with ChemiDoc™ XRS+ System (Bio-Rad). Protein band intensity was quantitated by Image Lab software (Bio-Rad).

Table 2.1. *Primers sequences for quantitative RT-PCR*

Target gene	**Sequence 5' to 3'**
hTERT-F	CCAAGTTCCTGCACTGGCTGA
hTERT-R	TTCCCGATGCTGCCTGACC
TERF1-F	TGCTTTCAGTGGCTCTTCTG
TERF1-R	ATGGAACCCAGCAACAAGAC
TERF2-F	GGTACGGGGACTTCAGACAG
TERF2-R	CGCGACAGACACTGCATAAC
POT1-F	TTCCACTAAAGAGCAGGCAA
POT-R	TGAAGTTCTTTAAGCCCCCA
TINF2-F	CCAGAAAGGGTTCCCCATAC
TINF2-R	TTTACCAGCAGGTGAAGCAG
TERF2IP-F	TCTTCTTCAGGCAAATCTGGA
TERF2IP-R	CCTCCTCCCAGAAGCTCAA
TPP1-F	TCACCAGATCAGCCACATTC
TPP1-R	GGAAAGACTCTCGGAGCTG
TP53-F	ATGGAGGAGCCGCAGTCAGAT
TP53-R	GCAGCGCCTCACAACCTCCGTC
CDKN1A-F	CGATGGAACTTCGACTTTGTCA
CDKN1A-R	GCACAAGGGTACAAGACAGTG
CDKN2A-F	AGACTTGGGTGGAAGAGGA
CDKN2A-R	TAATCATCACAGCTGTTCGG
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG

Co-immunoprecipitation and ubiquitination assay. Naïve CD4 T cells were freshly isolated from PBMCs of HCV infected patients and age matched HS subjects as described above. Equal number of cells were lysed in ubiquitination assay buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 5% Glycerol) supplemented with 0.1% SDS. Cells were left on ice for 10 min following 5 min boiled on heat block. Protein concentration was measured by BCA protein assay kit. Equal amount of cell lysate was subjected to pull-down assay by adding TRF2 monoclonal antibody or mouse IgG control then rotated on rotator at 4ºC overnight. On the next day, 50 µl protein A/G agarose bead (Santa Cruz) was added to each sample then incubating on rotator at 4ºC for 1 and a half hour. Bead was washed 5 times with ice-cold ubiquitination assay buffer then subjected to SDS-PAGE running and western blotting as described above.

Confocal Microscopy. Naïve $CD4^+$ T cells were isolated and cultured as described above. Cells were fixed in 2% PFA 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 TRF1 antibody (Thermo Fisher) 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).

siRNA silencing. Naïve CD4 T cells from HS subjects were transfected with Smart Pools siRNA (Dharmacon, Lafayette, CO) contained 4 siRNA sequences targeted 4 different positions on TERF2 open-reading frame or scramble siRNA control, with the ratio $5x10^6$ cells/100 nM

siRNA. siRNA transfection was performed by using the Human T Nucelofector Kit and Nucleofector I Device (Lonza, Allendale, NJ) following manufacturer's instruction. Cells were harvested and subjected to flow cytometry, western blot and confocal microscopy analysis 3 days post-transfection.

Lentiviral generation and transduction. 80% confluence of HEK293T cells were transfected with 2.5 μg pMD2.G (VSV-G envelope), 7.5 μg psPAX2 (packaging) (gifts from Dr. Didier Trone (Addgene plasmid, pMD2.G # 12259 and psPAX2 # 12260)) and 10 μg pWPiR or pWPiR-TRF2 transfer plasmids which contain IRES driven GFP protein expression (generous gifts from Dr. Eric Gibson and Dr. Vincent Picco) using Transporter^{TM} 5 (Polyscience, Inc, Warrington, PA) reagent following manufacturer's instruction. Overnight post-transfection, media was changed to 6 mL DMEM with 5% FBS, cells were then cultured for 24 h and the first viral supernatants were collected. Cells were added another 6 mL DMEM, 5% FBS and cultured for 24 h to obtain the second viral supernatant. The first and second viral supernatants were mixed and filtered through 0.45 μm filter then concentrated by Lenti-X Concentrator solution (Takara Bio, Mountain View, CA) following manufacturer's instruction. The concentrated viruses were resuspended in PBS (500 μl PBS/10-mm culture dish) as viral stock and stored at 80 °C. Naïve CD4 T cells were isolated and cultured in RPMI 1640, 10% FBS supplemented with 200 IU/mL IL-2 overnight, cells were then transduced with concentrated viruses with the ratio 40 μl viral stock/1x10^6 cells in the presence of 10 μg/mL Polybrene (Santa Cruz). Cells were supplemented with 200 IU/mL IL-2 every 2 days post transduction. Cells were harvested and subjected to flow cytometry and western blot after 4 days of transduction.

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 paired T test, and multiple comparisons test/least significant difference or Tukey's procedure, depending on the ANOVA F test or by a nonparametric Mann–Whitney U test. P-values <0.05, <0.01, or <0.001 were considered to be statistically significant or very significant, respectively.

Results

T cell homeostasis and apoptosis in HCV-infected patients versus age-matched HS.

Dysregulated T cell homeostasis is a characteristic of persistent viral infection; however, the mechanisms that control T cell homeostasis and virus persistence in humans remain unclear[59]. As an initial approach to identify factors that perturb T cell homeostasis in HCV infection, we first analyzed total $CD4^+$, naive $CD4^+CD45RA^+$, and memory $CD4^+CD45RA^-$ T cell subsets in PBMCs isolated from chronically HCV-infected individuals and age-matched HS. As shown in **Fig 2.1A**, while the percentage of total $CD4^+$ T cell frequencies in peripheral blood were similar in HCV patients and HS, the compartment of naïve CD4 T cells was significantly contracted, whereas memory CD4 T cells expanded, in HCV-infected patients. To exclude the possibility that the gating of live PBMCs might include some $CD4^+$ monocytes, we gated on lymphocytes and then $CD3^+$ T cells, followed by analysis of $CD3^+CD4^+CD45RA^+$ (naïve) and $CD3^+CD4^+CD45RA^-$ (memory) T cell subsets. This generated the same results, i.e., HCV patients exhibited a significant contraction of the naïve T cell pool and expansion of memory T cells in their peripheral blood compared to HS (**Fig 2.1B**). In addition to analysis of the CD4 T cell frequencies by flow cytometry, we also examined the absolute CD4 T cell numbers in peripheral blood of HCV-infected patients versus age-matched HS by purifying T cells using the naïve $CD4^+$ T Cell Isolation Kit II and MidiMACS™ Separator (Miltenyi Biotec Inc., Auburn, CA), which can yield >95% untouched naïve CD4 T cells from human peripheral blood by this

negative selection method according to their protocol. Base on the purified PBMCs and the yield of naïve $CD4^+$ T cells, we observed a significant lower number of naïve CD4 T cells in the blood of HCV patients compared to HS (***Table 2. 2***) that is in line with the data presented in **Fig 2.1A** and **2 1B**. This finding of contraction of naïve CD4 and expansion of memory CD4 T cell subsets is consistent with previous reports by us and others[13, 60], showing reduced naïve CD4 T cell numbers that represents a state of immune activation/differentiation followed by cell exhaustion/senescence in patients with chronic HCV infection.

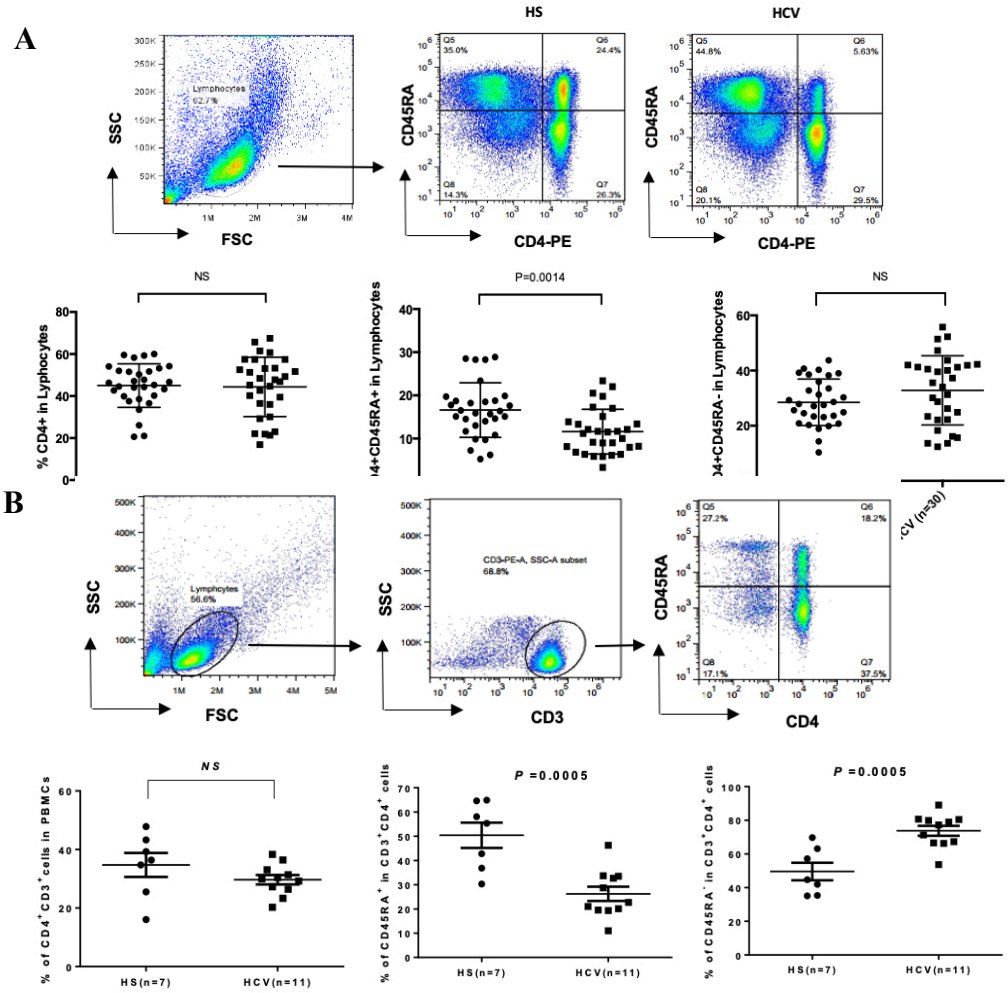
Table 2.2. *Number of isolated naive CD4 T cells from peripheral blood mononuclear cells* (PBMCs)*

	PBMCs	Naïve CD4 T cells	% Naïve $CD4^+$ T cells in PBMCs
HS1	$3.30x10^7$	$6.00x10^6$	12.12
HS2	$4.00x10^7$	$8.30x10^6$	20.70
HS3	$4.00x10^7$	$6.87x10^6$	17.18
HS4	$5.00x10^7$	$6.60x10^6$	13.20
HS5	$0.45x10^7$	$1.15x10^6$	25.56
HS6	$1.67x10^7$	$1.89x10^6$	11.28
HS7	$5.00x10^7$	$6.30x10^6$	12.60
HS8	$7.00x10^7$	$5.18x10^6$	7.40
HCV1	$3.00x10^7$	$0.89x10^6$	2.97
HCV2	$7.00x10^7$	$0.55x10^6$	0.79
HCV3	$8.00x10^7$	$0.64x10^6$	0.80
HCV4	$8.00x10^7$	$2.40x10^6$	3.00
HCV5	$8.00x10^7$	$1.26x10^6$	1.57
HCV6	$8.00x10^7$	$3.38x10^6$	4.23
HCV7	$10.0x10^7$	$5.10x10^6$	5.10
HCV8	$8.00x10^7$	$4.80x10^6$	6.00

* *Naïve $CD4^+$ T cells were isolated by Naïve $CD4^+$ T cell isolation kit II and MidiMACS™ Separator (Miltenyi Biotec Inc., Auburn, CA)*

The total size of the T cell repertoire is well-maintained by a fine balance between influx of newly generated T cells from the thymus, efflux by consumption of programmed cell death, and self-replication within the existing pool of lymphocytes[61]. With deficient influx from the thymus in adults, the immune system reacts by expanding existing T cells, leading to increased proliferative turnover, telomere shortening, cell senescence, and ultimately, cell apoptosis, which

represents a major mechanism controlling peripheral T cell homeostasis in adults[61]. To explore the contribution of apoptosis to T cell homeostasis during HCV infection, PBMCs derived from HCV-infected patients were compared with HS for the expression of Annexin V (Av) and 7-Aminoactinomycin D (7AAD). As shown in **Fig 2.1C**, Av expression in CD4 T cells showed an increased rate of cell apoptosis in HCV patients in total CD4, as well as naïve and memory CD4 T cells. Notably, naïve CD4 T cells were found to be more apoptotic than memory CD4 T cells in HCV-infected patients. This apoptotic susceptibility of T cells in HCV patients may necessitate compensatory homeostatic proliferation that can lead to telomere attrition and cell senescence.



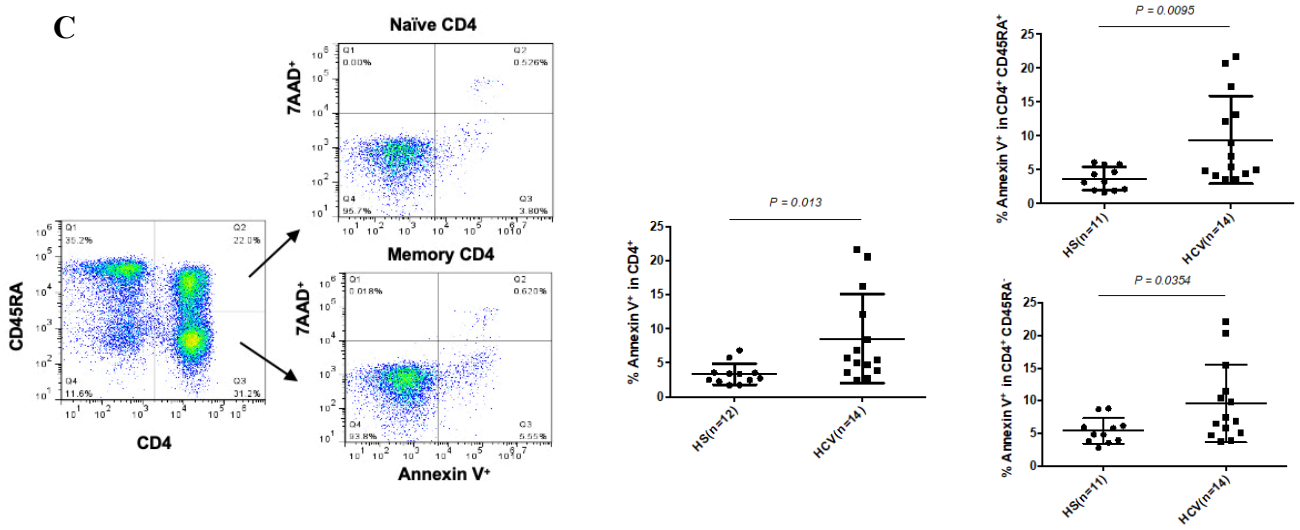


Figure 2.1. T cell homeostasis and apoptosis in HCV-infected patients versus age-matched HS . A) T cell homeostasis analysis by flow cytometry. Representative dot-plots and summary data for percentages of total $CD4^+$, $CD45RA^+CD4^+$ naïve, and $CD45RA^-CD4^+$ memory T cell frequencies within PBMCs from 24 HCV patients and 24 HS. Each symbol represents one subject. Data are expressed as mean ± SE. NS = non-significant. **B)** T cell homeostasis analysis by flow cytometry. PBMCs were first gated on lymphocytes, then $CD3^+$ T cells, and subsequently $CD4^+CD45RA^+$ naïve and $CD4^+CD45RA^-$ memory T cells from 11 HCV patients and 7 HS. **C)** PBMCs isolated from 14 HCV patients and 11 HS were analyzed for Av and 7AAD expression by flow cytometry. Representative dot plots and summary data for the percentages of cell apoptosis are shown. Given the data normality distribution, total CD4 Av is shown as median with interquartile range, and its *p* value was calculated by nonparametric test; naïve and memory T cell Av is shown as mean ± SE, p value was calculated by t-test.

T cell premature aging or senescence in HCV-infected patients versus age-matched HS. To determine the role of homeostatic proliferation in T cell senescence, we assessed the aging markers in T cells from HCV-infected patients and age-matched HS. Because loss of CD28 (a T

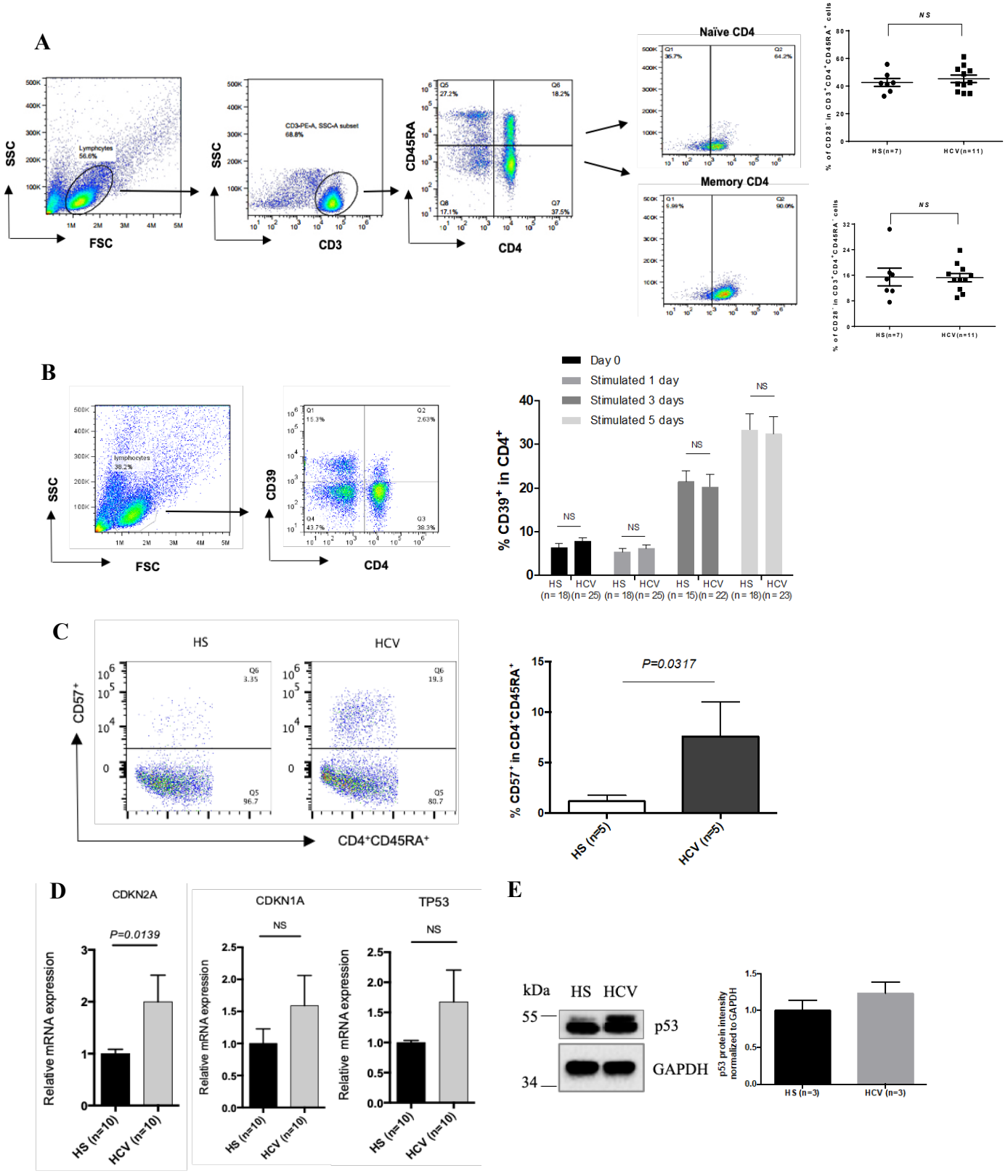
cell receptor (TCR) co-stimulatory molecule required for T cell activation and survival) has been regarded as an unequivocal marker for T cell senescence[62], we first measured CD28 expression on $CD3^+CD4^+CD45RA^+$ naïve T cells and $CD3^+CD4^+CD45RA^-$ memory T cells in PBMCs. As shown in **Fig 2.2A**, we did not observe any difference in CD28 expression on T cell subsets between HCV-infected patients and age-matched HS. We also measured the expression of CD39 (a typical cell surface-located ATPase that identifies terminally exhausted or senescent cells[63]) on CD4 T cells following anti-CD3/CD28 stimulation for 0, 1, 3, and 5 days. Again, no differences were identified in CD39 expression on either the resting or TCR-stimulated CD4 T cells in HCV-infected patients versus age-matched HS (**Fig 2.2B**). We next measured the expression levels of the glucuronyltransferase gene family member CD57 (also known as human natural killer 1, HNK1), a 110-kD glycoprotein expressed on senescent NK or T lymphocytes and considered a senescence marker for T cells[24]. Remarkably, at day 3 of TCR stimulation, CD57 expression on $CD4^+CD45RA^+$ naive T cells was significantly increased in HCV patients compared to HS (**Fig 2.2C**).

To better define the aging process in naïve ($CD4^+CD45RA^+$) T cells during viral infection, we assessed the aging-associated cell-cycle inhibitors[64] including $p16^{ink4a}$, $p21^{cip1}$, and p53. As shown in **Fig 2.2D**, HCV naïve T cells exhibited higher mRNA levels of CDKN1A (encodes $p21^{cip1}$), CDKN2A (encodes $p16^{ink4a}$), and p53, as determined by real-time RT-PCR. Although we couldn't detect $p16^{ink4a}$, $p21^{cip1}$, and $pp53^{ser15}$ protein expressions in resting naïve CD4 T cells by western blot, we observed increases in total p53 protein expression in HCV T cells compared to HS (**Fig 2.2E**). In addition, we found increases in $pp53^{ser15}$, total p53, and $p21^{cip1}$ protein expression in TCR-stimulated naïve CD4 T cells from HCV patients (**Fig 2.2F**). Notably, while we could detect significant increases in p16 mRNA (CDKN2A), we could not detect p16 protein

expression in naïve CD4 T cells, either in resting unstimulated or in TCR-stimulated state, by both western blot and flow cytometry methods (data not shown). We also examined the expression of aging makers in memory CD4 T cells at both mRNA and proteins levels by real-time RT-PCR and western blot. As showed in **Fig 2.2.G**, we did not observe any difference in mRNA expression of *TP53*, *CDKN1A* and *CDKN2A* (**Fig 2.2.G**); however, we observed increases in protein expression of p53 and cleaved PARP-1 in unstimulated memory CD4 T cells (**Fig 2.2.H**) as well as increased p21 and γH2AX in TCR-stimulated memory CD4 T cells (**Fig 2.2I**) derived from HCV patients, suggesting that memory CD4 T cells, as naïve CD4 T cells, also overexpress aging proteins during chronic HCV infection. These findings reaffirm our previous observations that aging markers, including $p27^{kip1}$, KLRG-1, β-galactosidase, and DUSP-6, are upregulated in CD4 T cells in chronic HCV infection[8-10, 12].

Previous study has shown that naïve CD4 T cells are typically resistant to death receptor/ligand (Fas/Fas-L)-mediated apoptosis, pointing toward cell internal signals as apoptosis initiators[65]. One of the internal stressors linked to cell apoptosis is damaged DNA, which is particularly important in senescent cells that have been chronically exposed to the endogenously generated ROS[66]. To determine whether ROS might be a cause of DNA damage and cell apoptosis during viral infection, we measured the ROS level in naïve CD4 T cells isolated from HCV and HS by flow cytometry using the Cellular ROS Detection Kit with the cell permeable 2',7'-dichloroflurescin diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell[67]. As shown in **Fig 2.2J**, the MFI of DCFDA was found to be significantly increased in naïve CD4 T cells derived from HCV-infected patients compared to age-matched HS. Also, we found increased level of ROS in memory CD4 T cells from HCV-

infected patients **Fig 2.2K**, indicating that ROS generated during chronic viral infection may play a role in DNA damage and cell apoptosis.



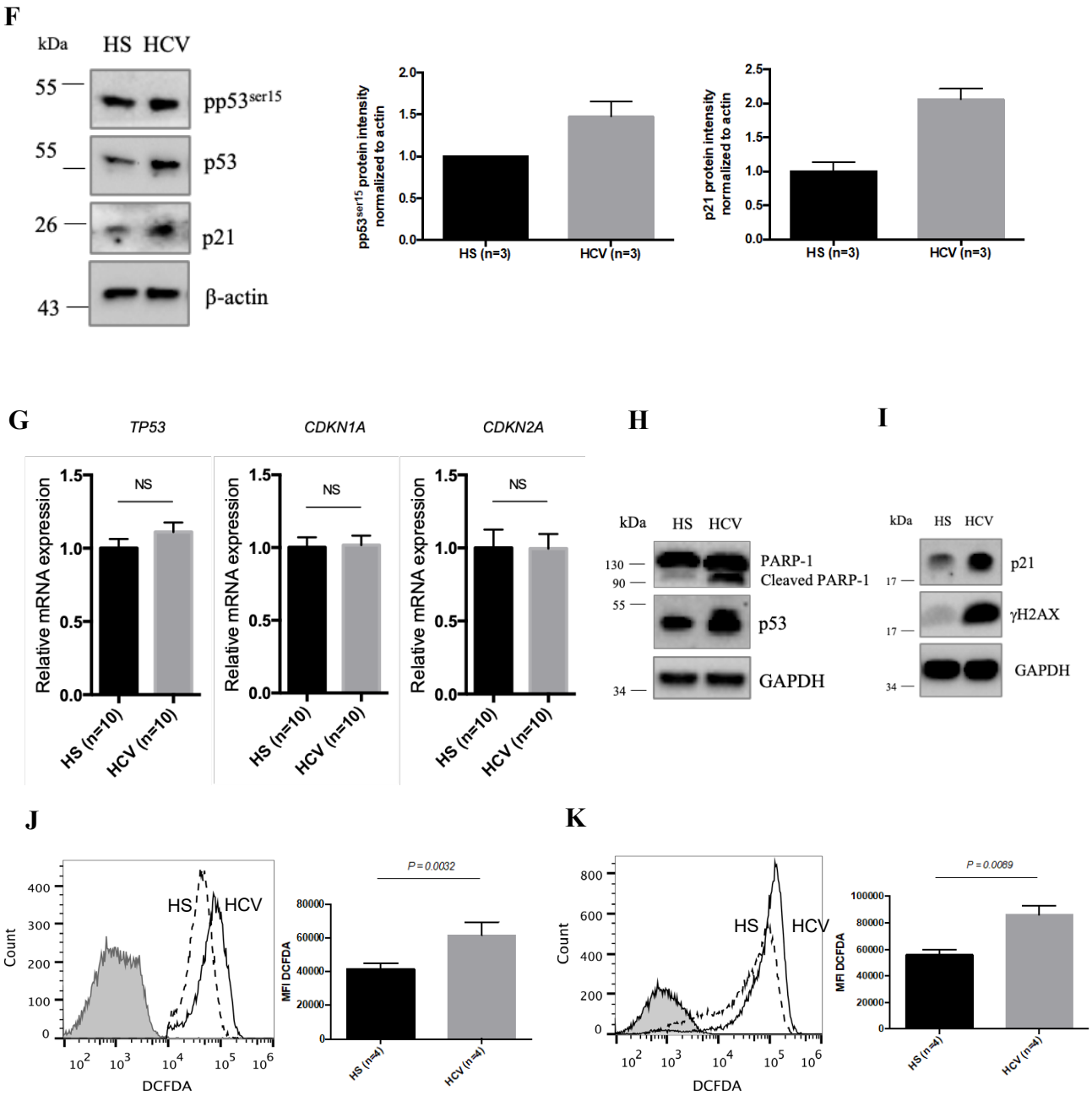


Figure 2.2. T cell senescence in HCV-infected patients versus age-matched HS. A) CD28 expression on $CD3^+CD4^+CD45RA^+$ naïve and $CD3^+CD4^+CD45RA^-$ memory T cells from 11 HCV-infected patients and 7 age-matched HS. The gating strategy and summary data of the flow cytometry analysis are shown. Each symbol represents one subject. NS = non-significant. **B)** CD39 expression in T cells from HCV patients and HS. PBMCs isolated from HCV subjects

and HS were cultured in the presence of anti-CD3/CD28 for 0, 1, 3, 5 days, followed by flow cytometric analysis for CD39 expression on $CD4^+$ T cells. Representative dot plots and summary data are shown. **C)** CD57 expression in naïve T cells from HCV patients and HS. PBMCs were stimulated with anti-CD3/CD28 for 3 days and then analyzed for CD57 expression on $CD4^+CD45RA^+$ naïve T cells isolated from 5 HCV patients and 5 HS. **D)** mRNA expression of CDKN2A (p16), CDKN1A (p21), and TP53 in naïve CD4 T cells isolated from 10 HCV patients and 10 HS. The purified $CD4^+CD45RO^-$ naïve T cells were analyzed by real-time RT-PCR for $p16^{ink4a}$, $p21^{cip1}$, and TP53 mRNA expression. Values were normalized to GAPDH expression and are presented relative to HS. **E)** Western blot analysis for p53 expression in naïve CD4 T cells isolated from HCV patient and HS. GAPDH serves as a loading control. Representative WB imaging and summary densitometry data are shown. **F)** Western blot analysis for pp53, p53, p21 expressions in TCR-stimulated naïve CD4 T cells isolated from HCV patient and HS. β-actin is used as loading control. Representative WB imaging and summary densitometry data are shown. **G)** Memory CD4 T cells were isolated from PBMCs of 10 HCV patients and 10 age-matched HS. mRNA levels of TP53, CDKN1A and CDKN2A were measured by real-time RT-PCR. HCV data were normalized to GAPDH as well as HS. **H)** Western blot analysis for PARP-1 and p53 expression in memory CD4 T cells isolated from HCV patient and HS. GAPDH served as loading control. **I)** Western blot analysis for p21 and γH2AX expression in TCR-stimulated memory CD4 T cells isolated from HCV patient and HS. GAPDH was used as loading control. **J)** Measurement of ROS level in naïve CD4 T cells. Naïve CD4 T cells were isolated from HCV patients and HS and subjected to ROS measurement using the DCFDA-based Cellular ROS Detection Kit. Representative overlaid histogram and summary data for the MFI of DCFDA level in naïve CD4 T cells from HCV patients and HS are shown (n=4). **K)** Memory

CD4 T cells isolated from HCV patients and HS were subjected to the ROS measurement by flow cytometry. Filled area = unstained cells, dashed lane = HS and solid lane = HCV. n=4.

Telomere attrition and DNA damage in T cells from HCV patients versus age-matched HS. Since telomere attrition is a hallmark of cell senescence, we further characterized T cell senescence in HCV infection by determining telomere length in total $CD4^+$, $CD4^+CD45RA^+$ naïve and $CD4^+CD45RA^-$ memory T cells by Flow-FISH. As shown in **Fig 2.3A**, telomere length was significantly shortened in HCV-derived total CD4 T cells, as well as in naïve and memory CD4 T cells, compared to age-matched HS. In addition, telomere loss was observed in activated CD4 T cells following TCR stimulation for 3 days. These data are consistent with our previous studies and published reports showing telomere attrition in T cells from chronically HCV-infected individuals[6, 7, 11, 13, 59].

Cells are equipped with DNA damage surveillance and repair machineries to prevent cellular senescence or death associated with genome instability. Since mammalian telomeres consist plenty of triple guanine repeats (TTAGGG) that are very sensitive to oxidative DNA damage, we speculate that telomere sequences in HCV 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 as well as on telomeres and acts as a docking station for other mediator or adaptor proteins to form microscopically visible nuclear foci (DNA damage foci)[68]. Thus, identifying dysfunctional telomere induced foci (TIF) is typically regarded as a hallmark of telomeric DDR. To determine telomeric DNA damage in T cells during HCV infection, we compared the number of TIFs per nucleus and the percentages of cells with > 3 TIFs[69, 70], by examining the co-localization of 53BP1/TRF1 using confocal microscopy. As shown in **Fig 2.3B**, the numbers of TIF per nucleus were significantly higher in CD4 T cells derived from

HCV patients compared to HS. The percentage of T cells with > 3 TIFs was also higher in HCV patients than HS. As we recently reported[13], the increased DNA damages were observed in both naïve and memory T cell subsets. These results suggest that telomeres in patients with chronic HCV infection are not only shortened but also DNA damaged, which may cause cell apoptosis and T cell loss, emphasizing the role of telomere integrity in securing T cell survival.

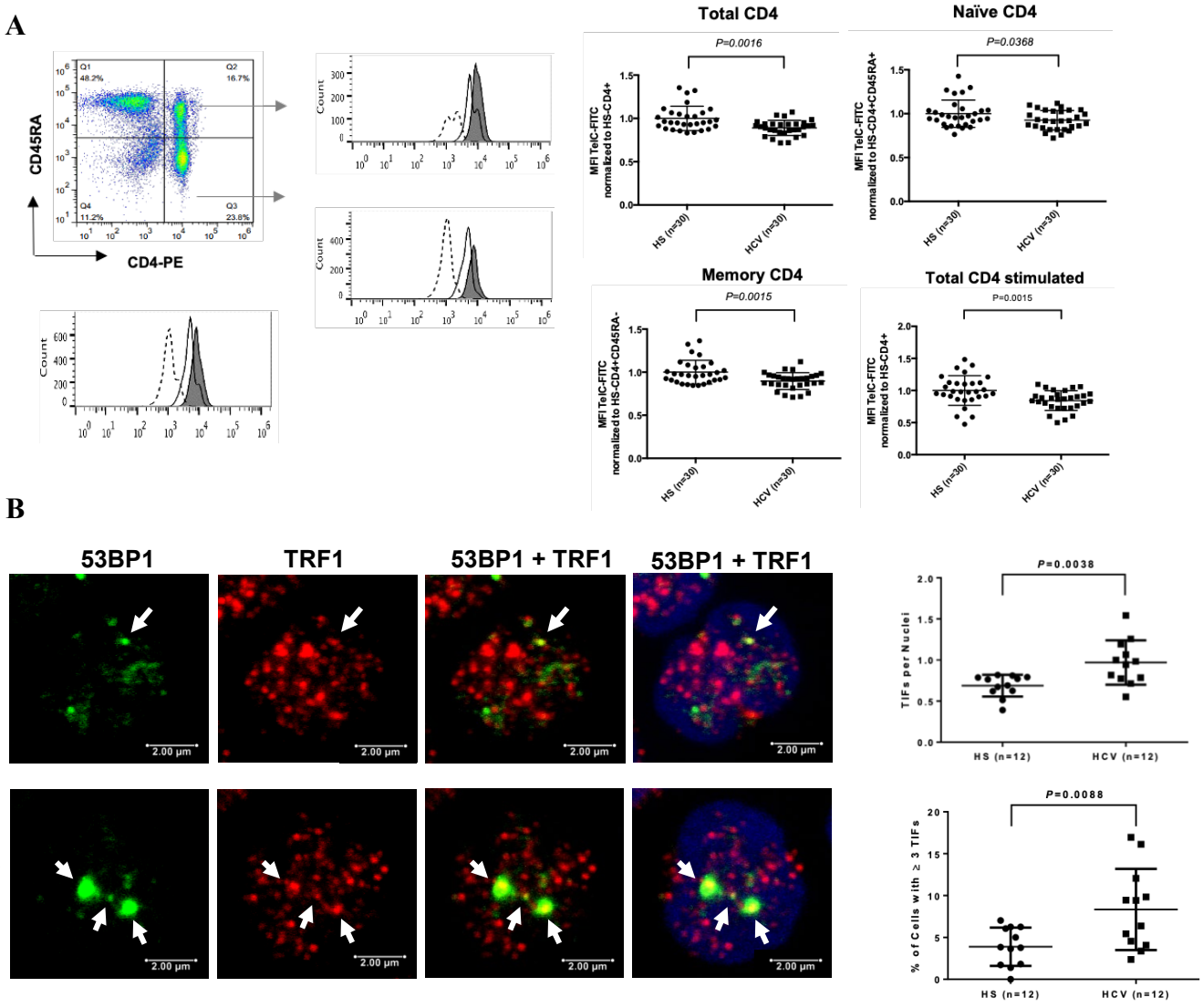


Figure 2.3. Telomere length and telomeric DNA damage in T cells from HCV patients and HS. A) Telomere attrition in CD4 T cells from HCV patients. PBMCs were isolated from 24 HCV-infected patients and 24 age-matched HS (with or without TCR

stimulation), followed by Flow-FISH analysis for telomere length in total CD4, naïve and memory CD4 T cells. The gating strategy, overlaid histograms (dashed lane = isotype control; solid lane = HCV patient; filled area = HS) and summary data of mean the fluorescence intensity (MFI) are shown. Each symbol represents one subject. The relative MFI of HCV telomere length was normalized by HS. **B)** Telomeric DNA damage in naïve CD4 T cells derived from chronically HCV-infected individuals versus HS was analyzed by confocal microscopy for colocalization of 53BP1 and TRF1. Representative imaging and summary data for the numbers of TIFs per nuclear, as well as the percentage of cells with > 3 TIFs are shown (n=12 subjects per group).

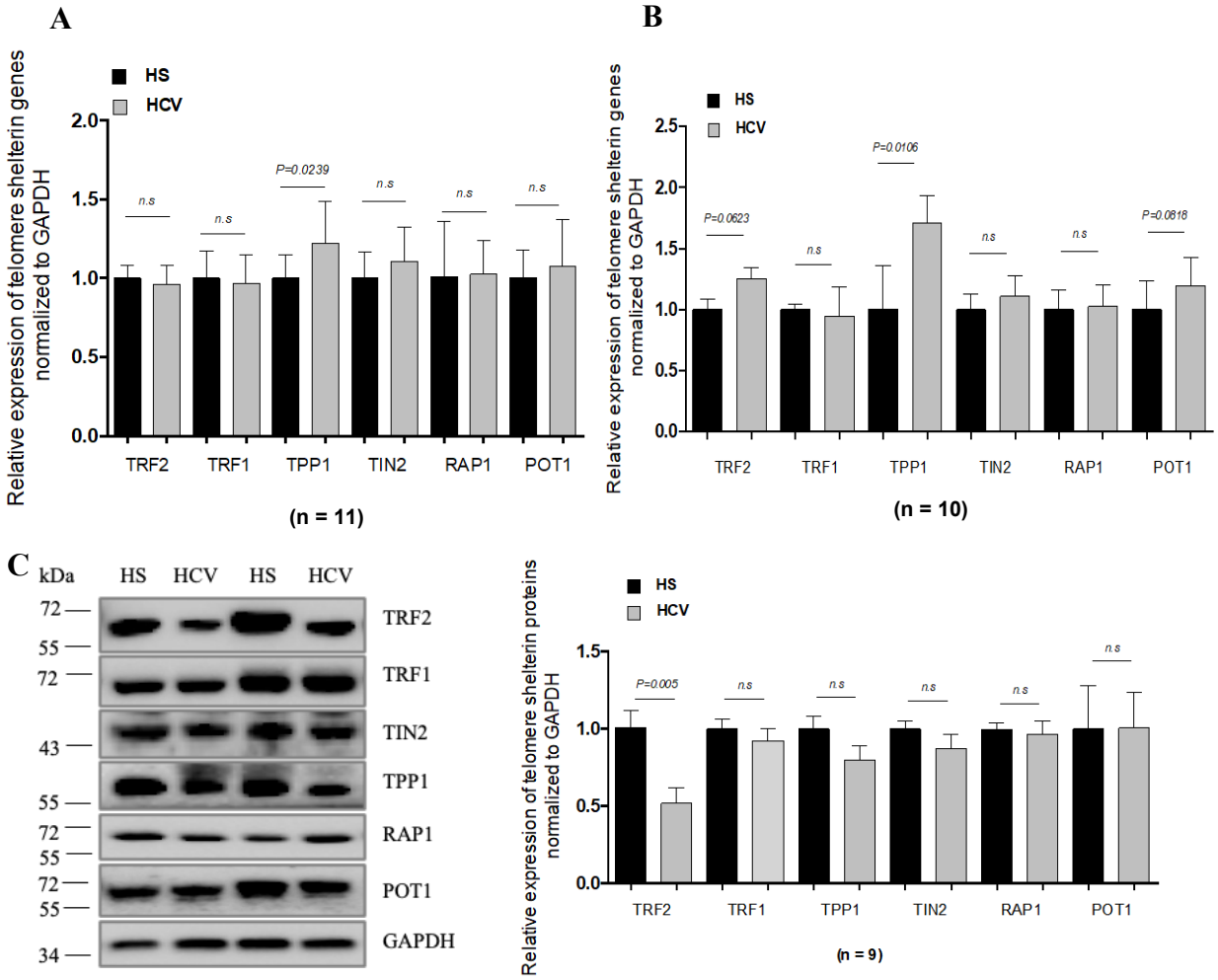
Telomeric shelterin proteins in T cells from HCV-infected patients versus age-matched HS. To determine the cause of telomere attrition in T cells during HCV infection, we next sought to examine the integrity of telomeric shelterin proteins that function to protect telomeres from unwanted DNA damage[17]. We first examined their mRNA expression, by real-time RT-PCR, in T cells isolated from HCV-infected patients and age-matched HS. As shown in **Fig 2.4A**, there were no significant difference in their mRNA levels, except TPP1 that was up-regulated in total CD4 T cells from HCV-infected patients. Notably, TPP1 was also significantly up-regulated, whereas TRF2, TIN2, and POT1 slightly elevated, in HCV-derived naïve CD4 T cells without stimulation (**Fig 2.4B**). In TCR-stimulated naïve CD4 T cells, however, all telomeric shelterin genes were slightly upregulated, with RAP1 and TIN2 significantly upregulated compared to HS (data not shown). Since mRNA expression may not necessarily linear to their protein levels within the cells, we also examined the protein levels in naïve CD4 T cells isolated from HCV patients and HS by western blot. In contrast to its mRNA transcript, the TRF2 protein level was significantly down-regulated, the TRF1, TPP1 and TIN2 proteins were slightly decreased, but

RAP1 and POT1 remained unchanged, in HCV T cells compared to HS (**Fig 2.4C**). Notably, TRF2 protein inhibition was also observed in total CD4 T cells isolated from HCV patients compared to HS (**Fig 2.4D**). These results indicate TRF2 inhibition, at the posttranscriptional level, in CD4 T cells during HCV infection.

As p53-mediated proteasomal degradation via E3 ubiquitin ligase Siah-1a is a major mechanism for regulating TRF2 protein stability in fibroblasts33, we examined these proteins by western blot in HCV- and HS-derived naïve CD4 T cells treated with or without the proteasomal inhibitor MG132. As shown in **Fig 2.4E**, p53 protein expression was significantly increased in naïve CD4 T cells from HCV patients versus HS, with or without MG132 treatment. Along with p53 upregulation, the expression of E3 ubiquitin ligase Siah-1a was also increased in both MG132-treated and untreated naïve CD4 T cells derived from HCV patients compared with HS, which was accompanied by decreases in TRF2 expression. In addition, expression of $p21^{cip1}$, a p53 downstream cell cycle inhibitor and cell senescence marker, was significantly elevated after the MG132 treatment, especially in HCV-derived naïve CD4 T cells. Concurrently, the DNA damage marker γH2AX was also markedly elevated in HCV T cells compared with HS, regardless of the MG132 treatment. Collectively, these results suggest that TRF2 inhibition is associated with increases in the p53/Siah-1a signaling in senescent, DNA damaged T cells during HCV infection.

To further address the possibility of ubiquitin degradation as a mechanism for TRF2 inhibition in T cells during HCV infection, we examined the ubiquitination of TRF2 in naïve CD4 T cells with or without MG132 treatment. We performed immunoprecipitation (IP) using a TRF2 monoclonal antibody and then probed the immunoprecipitates with both TRF2- and ubiquitin- specific antibodies. As shown in **Fig 2.4F**, without MG132 treatment, ubiquitinlated

TRF2 was significantly higher in HCV T cells, consistent with the lower level of TRF2 before the protein pull-down, compared with HS (lane 2 versus lane 1). These data suggest that ubiquitination- mediated proteasomal degradation of TRF2 occurs in HCV T cells. We also noticed an increased TRF2 ubiquitination in healthy T cells (lane 3 versus lane 1) by the MG132 treatment, but this TRF2 ubiquitination did not increase further in HCV-derived cells (lane 4 versus lane 2), suggesting a highly active TRF2 ubiquitination machinery in HCV T cells. We found a similar pattern of ubiquitin probe in naïve CD4 T cells derived from HCV and HS, with or without MG132 treatment. Taken together, these data indicate an increased proteasomal degradation of TRF2 that is associated with the activation of the p53/Siah-1a ubiquitination pathway in T cells during HCV infection.



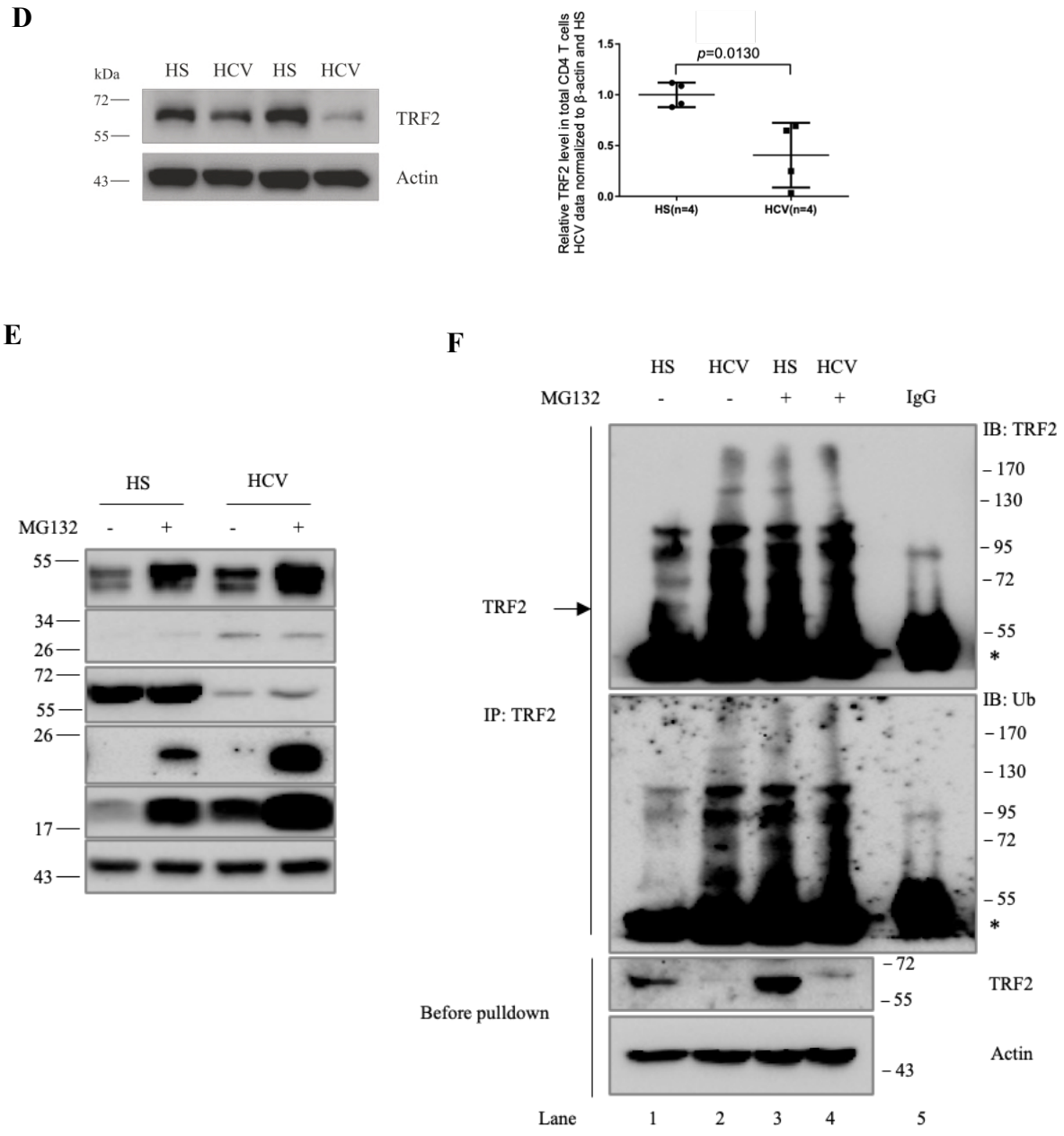


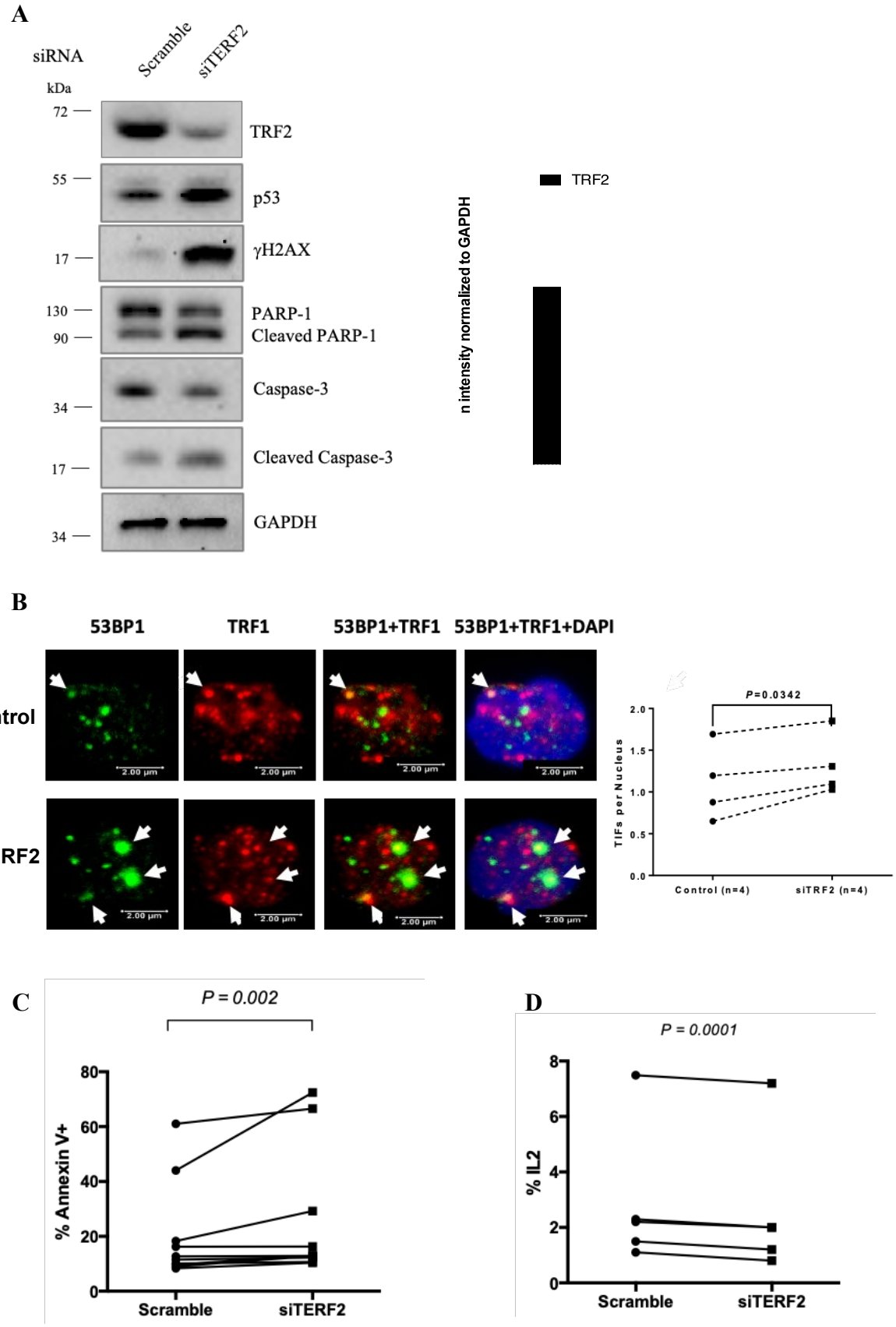
Figure 2.4. Telomere shelterin mRNA and protein levels in T cells from HCV patients and HS. A, B) mRNA expression of telomere shelterin proteins in total CD4 T cells and naïve CD4 T cells. Total CD4 T cells and CD4+CD45RO− naïve CD4 T cells were isolated from 10 HCV patients and 10 HSs. Total RNA was isolated and analyzed by real-time RT-PCR for shelterin mRNA expression. Values were normalized to GAPDH mRNA and calculated

relative to HS. **C)** Shelterin protein expressions in naïve CD4 T cells isolated from HCV patients and HS. GAPDH is used as loading control. Representative imaging and summary data for western blot densitometry are shown (n = 9). **D)** TRF2 level in total CD4 T cells from HCV patients and HSs. Representative imaging and summary data for western blot are shown (n = 4). The HCV densitometry data were first normalized to β-actin and then HS. **E)** Naïve CD4 T cells isolated from HCV and HSs were cultured for 72 h in the presence of DMSO control or proteasome inhibitor MG132 (10 μM) for the last 4 h, followed by western blot analysis for p53, Siah-1, TRF2, p21, γH2AX expressions. β-Actin serves as loading control. **F)** Proteasomal degradation of TRF2 through ubiquitin signaling pathway in T lymphocytes during HCV infection. Naïve CD4 T cells isolated from HCV (lanes 2 and 4) and HS (lanes 1 and 3) were lysed in immunoprecipitation (IP) buffer with 0.1% SDS. Protein concentrations were equalized, and small amount of cell lysates were saved before the pull-down assay (bottom panel) and used as control. The rest of cell lysates were used for IP with TRF2 antibody or IgG control (lane 5). Immunoprecipitated complexes were pulled-down by protein A/G bead and subjected to immunoblotting with the indicated antibodies.

TRF2 plays a key role in protecting telomeres from DNA damage and T-cell apoptosis. TRF2 is a key factor in telomere protection and chromosomal stability, which are critical for cell survival and function[19, 20], but its role in T-cell biology in the setting of viral infection remains unknown. To elucidate the role of TRF2 in protecting telomere integrity and T-cell survival, we knocked down TRF2 in healthy T cells and measured DNA damage, cell apoptosis, and cell function. As shown in **Fig 2.5A**, healthy naïve CD4 T cells transfected with siRNA specific to TRF2 (siTERF2) exhibited a significant decrease in TRF2 protein expression compared with cells treated with scramble siRNA. Concurrently, p53 and γH2AX expressions were remarkably

upregulated in the TRF2 knockdown T cells. Notably, the expression of caspase-3-dependent cleavage of PARP-1 (an enzyme that catalyzes the transfer of ADP-ribose onto target proteins and plays an important role in maintaining DNA chromosomal stability[71]) was decreased, whereas its cleaved form was increased in T cells after TRF2 knockdown. Correspondingly, caspase-3 was decreased but its cleaved form was increased, which is in line with the increases in apoptosis in TRF2 siRNA-treated cells that have increased p53-mediated DNA damage. Additionally, the numbers of dysfunctional TIF per nucleus (**Fig 2.5B**) and the percentages of Av+ apoptotic cells were also significantly increased (**Fig 2.5C**), and the IL-2 expression was substantially decreased in T cells after TRF2 knockdown (**Fig 2.5D**).

To determine whether reconstitution of TRF2 in T cells can alleviate the DNA damage and cell apoptosis occur- ring during HCV infection, we overexpressed TRF2 in naïve CD4 T cells derived from HCV patients using a lentiviral expression system. As shown in **Fig 2.5E, F, G**, lentivirus-mediated, IRES-driven GFP protein expression was observed in T-cell transduced with the pWPiR control vector and the pWPiR-TRF2 construct, but not in untransduced cells. Importantly, TRF2 expression was markedly increased in pWPiR-TRF2-transduced T cells and was accompanied by a decrease in p53, γH2AX, and cleaved PARP-1 levels, indicating an alleviation of p53- mediated DNA damage. In addition, Av expression was significantly reduced in HCV-derived T cells transduced by the pWPIR-TRF2. These data demonstrate that telomere uncapping, and recapping is critical for DNA damage and apoptosis, determining T-cell survival and function.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siRNA
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Caspase-3
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B
53BP1
TRF1
53BP1+TRF1
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Control
siTERF2
2.00 µm
P=0.0342
TIFs per Nucleus
Control (n=4)
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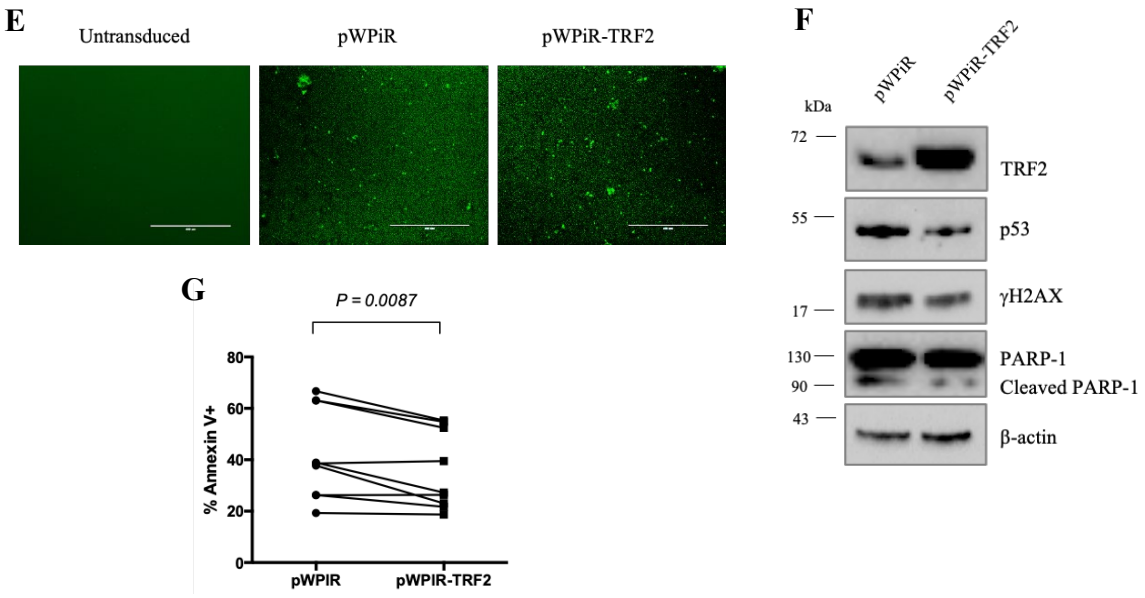


Figure 2.5. TRF2 plays a key role in protecting telomere from DNA damage and T-cell apoptosis. **A)** Representative imaging and summary data of the western blot analysis for TRF2, p53, γH2AX, PARP-1, and caspase-3 expressions in healthy naïve CD4 T cells transfected with TRF2 siRNA (siTERF2) and scrambled siRNA control. GAPDH is shown as a loading control. The experiments were performed using naïve CD4 T cells derived from HS (n = 4). **B)** Telomeric DNA damage in naïve healthy CD4 T cells. Cells were transfected with control or TRF2 siRNAs and analyzed by confocal microscopy for colocalization of 53BP1 and TRF1. Representative imaging and summary data for numbers of TIFs per nuclear, as well as percentage of cells with > 3 TIFs are shown (n = 4). **C)** Summary data for apoptotic death of healthy naïve CD4 T cells transfected with the control and TRF2 siRNA (n = 10). **D)** IL-2 expression in healthy naïve CD4 T cells transfected with the control and TRF2 siRNA. n = 5. **E)** GFP expression in lentivirus-untransduced and transduced naïve CD4 T cells derived from HCV-infected patients. **F)** Representative western blots showing TRF2, p53, γH2AX, and PARP-1 expressions in HCV-derived naïve CD4 T cells transduced with control or TRF2-expressing lentivirus. **G)** Percentage of Av+ cells in HCV T cells transduced with control or TRF2-expressing lentivirus are shown (n = 9).

Discussion

Chronic viral infections are characterized by dysfunctional T cells. Here, we show that homeostatic remodeling of the T-cell repertoire during HCV infection primarily affects the naïve T-cell compartment. Specifically, we find that naïve CD4 T cells in HCV patients are senescent, as demonstrated by the overexpression of aging markers, along with telomere attrition with telomeric DNA damage due to loss of the TRF2 protection, thus contributing to increased cell apoptosis. Healthy naïve T cells entered crisis prematurely upon knockdown of TRF2, as evidenced by increased p53 and γH2AX expression, accompanied by increases in the cleaved form of PARP-1 and caspase-3. Accordingly, TRF2 silenced T cells exhibited increased numbers of TIF and apoptosis, concomitant with decreased IL-2 production. In contrast, overexpression of TRF2 in HCV T cells reduced telomeric DNA damage and cell apoptosis. We thus conclude that TRF2 protein inhibition leads to telomere attrition and telomeric DNA damage that triggers cell apoptosis during HCV infection. Based on these novel findings, we propose a model, as depicted in **Fig.2.6**, where HCV-induced telomere deprotection by TRF2 protein inhibition causes accumulation of telomeric DNA damage and telomere erosion, thus contributing to T-cell apoptosis. Telomere attrition-mediated T-cell apoptosis may necessitate homeostatic proliferation and impose replicative stress on unprimed naïve T cells, further contributing to naïve T- cell loss. This represents a novel molecular mechanism that underlies T-cell senescence and T-cell loss.

The immune system is in constant turnover during viral infection, with high demands for lymphocyte replenishment to maintain the T-cell equilibrium. Ongoing antigenic stimulation during chronic viral infection induces continuous differentiation of naïve T cells and turnover of antigen-reactive T cells. In this regard, memory T cells would expand and thus compromise the

size and survival of naïve T-cell repertoire. Our study focused on the naïve T cells because they represent the reserves of the immune system, and their survival critically affects the outcome of immune aging. Undoubtedly, the apoptotic loss of naïve T cells determines the generation of sufficient antigen- specific T-cell clones, as well as the cellular yield of homeostatic proliferation, a process that generates new T cells upon the response to neo-antigens, including vaccines. Indeed, along with others, we have shown poor vaccine (HAV, HBV, influenza, and Pneumovax) responses in the setting of chronic viral (HCV and HIV) infection[8, 9, 72-76]. Our new findings in this study indicate that naïve helper T cells in HCV patients have aberrant abnormalities that jeopardize their ability to mount effective immune (vaccine) responses. In particular, the naïve T cells compartment is severely contracted, and naïve CD4 T cells exhibit telomere attrition with damaged DNA due to the lack of TRF2 protection. Accumulated DNA damage renders HCV T cells more prone to apoptotic death, thus imposing replicative stress and premature senescence.

A typical feature of T cells in chronic viral infection is premature aging, characterized by telomere shortening[6-11, 13, 59] compared with age-matched HS. In normal primary T cells, telomeres undergo shortening at a rate of 50–100 base pairs (bp) per cell division, and predictable loss of telomeric DNA with each cell replication allows telomeres to serve as molecular clock that controls the replicative capacity of T cells before entering cell cycle arrest, senescence, or apoptosis[77, 78]. However, telomere loss can increase up to 250 bp per cell cycle during chronic viral infection and, in compensating for this, cell cycle arrest occurs when progressive telomere loss reaches a critical point, a phenomenon known as replicative senescence[77, 78]. Based on our studies, we believe that HCV induced naïve CD4 T-cell loss is primarily driven by ROS-mediated DNA damage and telomere attrition. As depicted in **Fig.2.6**,

in healthy young subjects the telomeres are intact with shelterin complexes well formed to protect telomeres from unwanted DNA damage; thus, normal T cells can proliferate/differentiate efficiently in response to antigen stimulation. However, HCV infection can induce TRF2 protein degradation via the p53/Siah1- mediated ubiquitination, leading to telomere uncapping and telomeric DNA damage. We believe that HCV- induced TRF2 inhibition, telomere loss, cellular senescence, and apoptosis are sequential but constant events, that is, if telomeres are mildly or moderately shortening, the over-expanded cells are exhausted or senescent, and cell cycles are arrested in the G1 phase to allow for DNA damage repair. If telomeres are severely shortening to a critical point that the damaged DNAs are irreparable, then the cells will undergo suicidal apoptosis and die. This continuous depletion of naïve CD4 T cells serves as a mechanism and contributes to the high rates of HCV persistence and vaccine non-responsiveness in virally infected individuals.

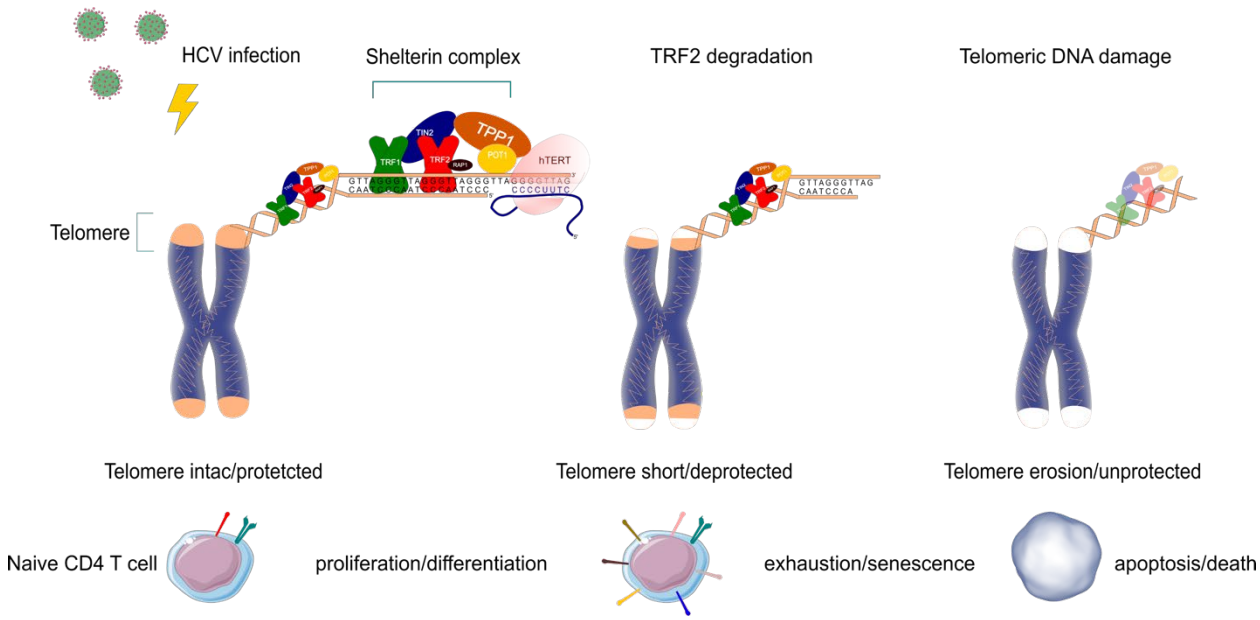


Figure 2.6. A model of HCV-induced TRF2 inhibition, leading to T-cell telomere uncapping and attrition, accelerating cell senescence, apoptosis, and naïve T-cell loss

during HCV infection. HCV infection promotes T-cell activation/proliferation/differentiation and induces TRF2 protein inhibition, which leads to telomere deprotection and triggers DNA damage and telomere erosion, thus contributing to T-cell apoptotic death. Excessive T-cell apoptosis feedback necessitates homeostatic over-expansion, then cell exhaustion/senescence, imposing replicative stress on unprimed naïve T cells and further accelerating naïve T-cell loss. This incessant regulatory cascade represents a novel molecular mechanism underlying T-cell senescence and naïve T-cell loss, which contributes to the viral persistence and vaccine non-responsiveness in human viral infection.

Several mechanisms may potentially contribute to the senescence-associated telomere attrition. First, increased T-cell proliferative turnover can cause cell division- induced telomere erosion. Typically, telomere is lost due to incomplete synthesis of the terminal DNA during cell division. The enzyme telomerase counteracts telomere loss by synthesizing telomeric repeats during cell proliferation. However, we have found that telomerase expression and its activity in CD4 T cells were unchanged or not dramatically suppressed during HCV infection (data not shown), suggesting that other mechanisms might be involved in telomere attrition. In our study, we found a significant decrease in TRF2 and a slight decrease in TRF1, TPP1, and TIN2 protein expressions in HCV T cells. Given that TPP1 and TIN2 are required to bridge the TRF1 and TRF2 complexes for by DDR and the inability of a timely repair by the ATM telomere sheltering[78], we speculate the relative lower levels of TRF2, TRF1, TPP1, and TIN2 in T cells of HCV patients could lead to poor sheltering, telomere uncapping or deprotection, and thus ROS-mediated DNA damage. Additionally, Zhong et al. reported that the oligonucleotide binding (OB)-fold domain of TPP1 recruits telomerase to telomeres through an association with Telomerase reverse transcriptase (TERT)[79]. Reconstitution of shelterin complexes reveals

unexpected stoichiometry that can enhance telomerase processivity. We thus believe that with decreased shelterin proteins, there would be insufficient recruitment of telomerase to telomeres for chromosomal end maintenance during HCV infection. This possibility is under active investigation in our lab.

Second, telomeres are highly susceptible to DNA damage. Human naïve T cells have a relatively long-life span (150–160 days) and are exposed to a multitude of genotoxic stressors, causing 1% of approximately 300 billion T cells to be replaced daily. Their telomeric DNA is particularly vulnerable to ROS-induced DDR, even more so than non-telomeric DNA. Plasmid-inserted human telomeres, for example, accumulate sevenfold higher strand breakage than control sequences[57]. In addition, the frequency of single-strand breaks is several fold higher in telomeres than in the bulk genome when cells are exposed to oxidative stress[80]. In line with these findings, we find that T cells derived from HCV patient exhibit not only shortened telomeres, but also damaged DNA, which can contribute to telomere loss.

Third, an inhibition of protective shelterin proteins may lead to deprotection of the telomeres. In our study, the expression of TRF2 is significantly inhibited at the protein level via the p53/Siah-1a-mediated ubiquitin pathway in naïve CD4 T cells during HCV infection, rendering the uncapped telomeres prone to DNA damage and cell apoptosis. T-cell replicative senescence is induced by uncapped telomeres, which activates DDR and telomere erosion. T-cell death, however, requires overriding of senescence through further telomere attrition, concomitant with loss of DNA damage checkpoints, thus causing cell apoptosis. Notably, TRF2 is a key factor that plays an essential role in maintaining telomere integrity by suppressing the ATM dependent DDR[19]. Recently, we have shown that telomere loss in HCV T cells is triggered by DDR and the inability of a timely repair by the ATM pathway[19]. Similar to our study, Guo et

al.[81, 82] reported that ATM activation in response to ROS was independent of the Mre11-Rad50-Nbs1 complex (MRN) complex. ROS-mediated ATM signaling represses mammalian target of rapamycin complex 1 (mTORC1) signaling and therefore cell growth and proliferation through activation of Tuberous sclerosis complex 2 (TSC2) (a negative regulator of mTOR) by liver kinase B1 and AMP-dependent kinases[83]. ATM engagement of the TSC2/mTORC1 signaling pathway can also regulate autophagy[84], and differential localization of ATM is correlated with activation of distinct downstream pathways[85]. We have also discovered that KML001, a telomere-targeting drug, can induce telomeric DNA damage and T-cell apoptosis by inhibiting TRF2 expression and impairing the ATM pathway[43]. Moreover, inhibition of Topoisomerase I or II by camptothecin or ICRF-193, which induces topological stress by suppressing telomere TRF2 protection, also exacerbates telomeric DNA damage and enhances T-cell death[46]. In this study, we show that TRF2 silencing amplifies telomere uncapping, triggers telomeric DNA damage, and decides cellular fate, suggesting that telomere deprotection via TRF2 inhibition is the underlying molecular mechanism that causes telomeric DNA damage and cell apoptosis in HCV infection. It should be pointed out, however, that the pathogenesis of HCV persistence is multifaceted, in that not only telomere shelterin proteins (especially TRF2) are inhibited, but other mechanisms appear impaired, including DNA repair enzymes (ATM/ ATR), telomerase access to telomeres, and DNA topoisomerases, all of which can work in concert to damage telomeres and lead to naïve T-cell loss during HCV infection. Our findings of TRF2-mediated telomere uncapping and T-cell apoptosis during HCV infection are clinically relevant and highly significant. We propose that TRF2 inhibition is the molecular mechanism that controls T- cell life span in the setting of chronic HCV infection. In line with this, we have recently found that CD4 T cells in latently HIV-infected individuals are also senescent with shortened, DNA

damaged telomeres due to TRF2 and ATM inhibition[45]. Thus, TRF2- mediated telomere attrition and cell apoptosis may represent a universal mechanism that controls T-cell homeostasis in chronic viral infections. Importantly, our results show that reconstitution of TRF2 is necessary and sufficient to protect telomeres from unwanted DNA damage and rescue HCV T cells from apoptosis, indicating the importance of TRF2 in telomere protection and T-cell survival in human infectious diseases. has been associated with tumorigenesis in checkpoint compromised cells[86], whereas exacerbation of TRF2-mediated telomere deprotection sensitizes cancer cells to telomere-targeting drugs. Similarly, bone marrow failure and related diseases are often observed in individuals with telomeropathies, which could potentially be explained by cell mitotic arrest resulting from excessive shortened and/or damaged telomeres[87, 88]. Therefore, TRF2-mediated telomere uncapping and telomere loss-driven cell cycle arrest may have broader implications through impairing diverse cellular functions. These findings might also offer a clinical opportunity for oncogenesis and anticancer treatment, as cell cycle arrest may have broader implications through impairing diverse cellular functions.

CHAPTER 3. IMMUNE ACTIVATION INDUCES TELOMERIC DNA DAMAGE, REDUCES MEMORY PRECURSORS, AND PROMOTES SHORT-LIVED EFFECTOR T CELL DIFFERENTIATION IN CHRONIC HCV INFECTION

Abstract

Hepatitis C virus (HCV) is characterized by causing a high rate of chronic infection and T cell dysfunction. While it is well-known that chronic antigenic stimulation is the driving force for the impaired T cell functions, the precise mechanisms underlying the immune activation-mediated T cell alterations remain elusive. Here, we demonstrated that circulating $CD4^+$ T cells from chronically HCV-infected patients exhibit an immune activation status, evidenced by overexpression of activation markers including HLA-DR, GLUT1, Granzyme B, and short-lived effector marker $CD127^-$ $KLRG1^+$, whereas the expressions of stem cell-liked transcription factor TCF1 and telomere shelterin subunit TRF2 are significant reduced, compared to age- and gender-matched healthy subjects. Mechanistic studies revealed that HCV-$CD4^+$ T cells exhibit PI3K/Akt/mTOR signaling hyperactivation upon T cell receptor (TCR) stimulation, favoring pro-inflammatory effector cell differentiation, telomeric DNA damage, and cellular apoptosis. Inhibition of Akt signaling during T cell activation preserves precursor memory populations and prevents inflammatory effector expansion, DNA damage, and apoptotic death. Knockdown of TRF2 reduces T cell stemness and triggers DNA damage and cellular apoptosis; whereas overexpression of TRF2 in HCV-CD4 T cells prevents telomeric DNA damage. These results suggest that modulation of immune activation by inhibiting Akt signaling and protection of telomeres by enforcing TRF2 expression may open new therapeutic strategies to finetune and balance the adaptive immune responses in the setting of persistent immune activation and inflammatory during chronical viral infection.

Introduction

Despite highly effective direct-acting antiviral (DAA) treatment on hepatitis C virus (HCV) infection continuedly brought down HCV-related deaths in the United States from 5.03 per 100,000 population in 2013 to 3.72 in 2018, the annual rate of new HCV infection was triply increased from 0.3 per 100,000 people in 2009 to 1.2 in 2018; among those, persons from 20-29 and 29-30 years old are at the highest rate at 3.1 and 2.6 respectively, likely related to their high frequencies of intravenous drug abuse [1]. This alarming sight of a rapid increase in new HCV cases among young individuals is complicating the efforts of eliminating HCV as a threat of public health without an effective vaccine available. Of note, HCV causes chronic infection in 70-80% of infected individuals, thus becoming a valuable model for studying the mechanisms of viral chronicity in humans [2]. Spontaneously HCV-resolved individuals show strong $CD4^+$ and $CD8^+$ T cell responses with a long-lived memory phenotype after acute infection [4, 89], indicating a critical role for T cells in controlling HCV infection. Recent longitudinal studies in different infection outcomes revealed that those individuals progressed to chronic infection developed broad HCV-specific $CD4^+$ T cell responses during the early phase of viral infection; however, as the infection progresses, $CD4^+$ T cell responses waned and diminished significantly [4]. In addition, persistent viremia could drive T cell receptor (TCR) over-activation and upregulation of inhibitory receptors, such as PD-1, Tim-3, and CTLA-4, resulting in T cell exhaustion and senescence [4]. These observations strongly suggest that HCV-specific $CD4^+$ T cells are successfully primed but become exhausted and senescent in the setting of chronic viral infection.

$CD8^+$ T cell exhaustion/senescence has been well-characterized in chronic HCV and HIV infection in humans [5, 23, 24], lymphocytic choriomeningitis virus in mice [25], simian immunodeficiency virus in rhesus macaques [26], and in many cancer diseases in humans [27-31].

Exhausted $CD8^+$ T cells express high levels of inhibitory receptors, including PD-1, KLRG-1, and TIM-3, low levels of CD127, and transcription factor T cell factor 1 (TCF-1) - a stem cell-like marker for antigen-experienced T cells [32-34]. Despite the critical roles of $CD4^+$ helper T cells in orchestrating $CD8^+$ T cell and B cell responses, there were fewer studies on $CD4^+$ T cell exhaustion and senescence compared to $CD8^+$ counterpart. We and others have previously reported high levels of inhibitory receptor expression in $CD4^+$ T cells during chronic viral infections [35-38]. In particular, chronic antigen stimulation and sustained inflammation can trigger immune over-activation and rapid T cell turnover, leading to the accumulation of DNA damage, telomere erosion, and genomic stress-induced cell apoptosis [39-47].

In chronic HCV infection, bulk $CD4^+$ and $CD8^+$ T cells show an activated phenotype with significantly higher levels of PD-1 and proinflammatory cytokine expressions [48], suggesting that persistent viremia triggers activation-induced cellular exhaustion and senescence that occur not only in HCV-specific T cells but also in general T cell populations. While understanding of virus-specific T cell dysfunction attracts greater attention due to their role in the control of infection, studies of generalized T cell populations in chronic HCV infection will help to understand the mechanisms of persistent immune activation-induced hepatic fibrosis, hepatocellular carcinoma, and extrahepatic manifestations, such as mixed cryoglobulinemia, B-cell non-Hodgkin lymphoma, chronic kidney diseases, and insulin resistance/type 2 diabetes in virally infected individuals [49, 50]. Indeed, despite DAA therapy can achieve > 95% sustained virologic response (SVR) in chronically HCV-infected individuals, persistent immune activation in circulating T cells, including $CD4^+$, $CD8^+$, and $V\gamma9V\delta2$ T cells, are either continued or only partially recovered in virologically cured patients long after viral clearance post SVR [51-54]. In addition, dysfunctional mucosal-associated invariant T (MAIT) cells with activated phenotypes

are nonreversible after HCV clearance in DAA-cured patients [55]. These observations suggest that further studies of systemic immune activation and T cell dysfunction are essential for understanding and approaching to achieve an immunologic cure and reduce comorbidities in chronic HCV infection in the era of DAA virologic cure.

In this study, we examined $CD4^+$ T cell activation-induced telomeric DNA damage and cellular dysfunctions in chronically HCV-infected individuals. We demonstrated that HCV-$CD4^+$ T cells show overexpressed activation markers, accumulated DNA damage, and cellular apoptosis. Mechanistically, HCV-$CD4^+$ T cells exhibit stronger AKT/mTOR activation upon TCR stimulation, leading to downregulations of telomeric repeat binding factor 2 (TRF2) and memory precursor transcription factor TCF1, and biased differentiation into pro-inflammatory phenotype. Inhibition of AKT signaling or maintaining of TRF2 expression reverse these negative effects on HCV-$CD4^+$ T cells, which may limit immune activation-induced proinflammatory effector $CD4^+$ T cell expansion and memory precursor loss during chronic viral infection.

Materials and Methods

Human subjects. The study protocol was approved by the institutional review board of East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN). Written informed consent was obtained from all patients included in this study. The study subjects were composed of two populations: 97 chronically HCV-infected patients (median viral load 1,958,744) (78 male, aged 30 - 70 years; 19 female, aged 27 - 62 years; median 50 years) and 87 age-matched healthy subjects (HSs) (69 male, aged 21 - 65 years; 18 female, aged 26 - 65 years; median 43 years), as controls. All HCV- infected patients were positive for HCV RNA, prior to antiviral treatment. HSs, obtained from Biological Specialty

Company (BSC, Gray, TN), were negative for anti-hepatitis C virus, hepatitis C virus, and human immunodeficiency virus type 1 antibodies.

Cell isolation and culture. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized whole blood by Ficoll-Paque (Cytiva) density centrifugation and cryopreserved in liquid nitrogen. Naïve and total CD4+ T cells were isolated from PBMCs using the Naïve $CD4^+$ T Cell Isolation Kit II, human and CD4+ T Cell Isolation Kit, human, respectively (Miltenyi Biotec). The isolated T cells were cultured in RPMI-1640 complete medium containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 100 IU/ml penicillin, and 2 mM L-glutamine (Thermo Fisher Scientific) at 37 °C and 5% CO_2 atmosphere. For differentiation of naïve $CD4^+$ T cell to effector $CD4^+$ T cell, Dynabeads Human T-Activator CD3/CD28 for T Cell Expansion and Activation (Thermo Fisher Scientific) were used at the ratio 1 bead:1cell for 72 hr, unless stated otherwise.

Flow cytometry. For cell surface staining, cells were stained in flow cytometry buffer (1% BSA and 2mM EDTA in 0.22 μm filtered-distilled water) in the dark at room temperature (RT) for 20 min with fluorochrome-conjugated antibodies against antigens including PerCP-CD3 (Biolegend, Clone HIT3a), FITC-CD4 (Biolegend, Clone RPA-T4), PerCP-CD8a (Biolegend, Clone RPA-T8), PE-HLA-DR (Invitrogen, Clone LN3), AF647-GLUT1 (BD Biosciences, Clone 202915), FITC-KLRG1 (Biolegend, Clone SA231A2), APC-CD127 (IL-7Rα) (Biolegend, Clone A019D5). For intracellular staining, cells stained with cell surface markers then were fixed by Fixation Buffer (Biolegend) for 20 min at RT. Cells were then were washed and processed for permeabilized with freshly prepared Foxp3/Transcription Factor Staining Buffer (Invitrogen) for 40 min in the dark at RT, following by 1 time of washing with 1X permeabilization buffer (Invitrogen) and 1 time with flow cytometry buffer. Cells were then stained in 1X

permeabilization buffer for 30 min with fluorochrome-conjugated antibodies against antigens including: PerCP/Cyanine5.5-GzmB (Biolegend, Clone QA18A28), AF647-TCF1 (Biolegend, Clone 7F11A10), FITC-γH2AX (Biolegend, Clone 2F3), AF647-phsopho Akt^{ser473} (BD Biosciences, Clone M89-61) and PerCP/Cyanine5.5-phospho-$RPS6^{ser235/236}$ (Biolegend, Clone A17020B). For intracellular cytokines staining, harvested cells were cultured with Cell Activation Cocktail (PMA, Ionomycin with Brefeldin A) for 6 hr, then following by intracellular staining as described above. Cytokines were stained using fluorochrome-conjugated antibodies: FITC-IL2 (Biolegend, Clone MQ1-17H12), PE-IFNγ (Biolegend, Clone 4S.B3), and APC-TNFα (Biolegend, Mab11). Cellular apoptosis was analyzed using PE Annexin V apoptosis detection kit I (BD Biosciences) according to the manufacturer's protocol. Flow cytometry was conducted using C6 Plus Flow Cytometer (BD Biosciences) and raw flow cytometry data were analyzed using FlowJo software (Tree Star Inc.)

Ex vivo Interferon-gamma treatment. Cryopreserved HCV patients PBMCs were thawed and cultured in supplementation of recombinant human IFNγ (carrier-free) (Biolegend) at 500 ng/mL concentration, and with or without soluble anti-CD3 (1μg/mL) and anti-CD28 (2 μg/mL) stimulation for 72 hr. Cells were harvested and subjected for flow cytometry analysis of apoptosis Annexin V or DNA damage γH2AX on $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ gated PBMCs.

Akt inhibitor treatment. Akt specific inhibitor MK-2206 2HCl (MK2206) was purchased from Selleckchem.com (Selleck Chemicals, S1078). Isolated naïve $CD4^+$ T cells from HS or from HCV patients were stimulated with Dynabeads anti-CD3/CD28 at the ratio 1 bead: 1 cell in the presence of MK2206 (1 μM) or DMSO control. Treated cells were cultured at the density of $2.5x10^6$ cells per well in a 24-well culture plate with 1 mL RPMI-1640 complete media for 72 hr. Anti-CD3/CD28 beads were removed before cells were harvested for downstream experiments.

Quantitative real-time PCR. Total RNA was extracted from 1×10^6 cells using RNeasy Mini Kit (Qiagen), and complementary DNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per the manufacturer's instruction. Quantitative real-time PCR was performed in technical triplicate using iTaq™ Universal SYBR Green Supermix (Biorad) according to the manufacturer's protocol. Gene expression was normalized to the GAPDH level and is presented as fold changes using the $2^{-\Delta\Delta ct}$ method [90]. PCR primer sequences are shown in ***Table 3.1***.

Immunoblotting. The detailed method for immunoblotting has been previously described from our group [42]. Primary antibodies were used in this study including anti-TRF2 (Cell signaling, D1Y5D), anti-phospho-Akt^{Ser473} (Cell signaling, D9E), anti-pan Akt (Cell signaling, 40D4), anti-phospho-S6$^{Ser235/236}$ ribosomal protein (Cell signaling, D57.2.2E), anti-TCF1/TCF7 (Cell signaling, C63D9), anti-PARP (Cell signaling, 46.D11), anti-phospho-histone H2A.X^{Ser139} (Cell signaling, 20E3) and anti-GAPDH (Santa Cruz Biotechnology, 0411) as housekeeping control. Secondary antibodies were used including anti-mouse IgG, HRP-linked (Cell signaling, 7076) and anti-rabbit IgG, HRP-linked (Cell signaling, 7074) antibodies. For protein degradation assay, isolated $CD4^+$ T cells from HSs were left unstimulated or stimulated with Dynabeads anti-CD3/CD28 at the ratio 1 bead: 1 cell for 2 hr. Cells were then harvested, washed with DPBS once, and reseeded in RPMI-1640 complete media with the supplementations of protein translation inhibitor cycloheximide (50 μg/mL) (Sigma) or DMSO control (Sigma) with or without proteasome inhibitor MG132 (10 μM/mL) (Sigma). Cells were harvested at 3 and 6 hr post CHX treatment and subjected for immunoblotting. Densitometric quantification for immunoblotting data was measured using ImageJ software (NIH), normalized to internal control GAPDH expression, and presented as arbitrary units (a.u.).

Table 3.1. *Primers sequences for quantitative RT-PCR*

Target gene	**Sequence 5' to 3'**
TERF2-F	GGTACGGGGACTTCAGACAG
TERF2-R	CGCGACAGACACTGCATAAC
TCF7-F	CTGGCTTCTACTCCCTGACCT
TCF7-R	ACCAGAACCTAGCATCAAGGA
Tbx21-F	GGTTGCGGAGACATGCTGA
Tbx21-R	GTAGGCGTAGGCTCCAAGG
GZMB-F	CCCTGGGAAAACACTCACACA
GZMB-R	GCACAACTCAATGGTACTGTGC
EOMES-F	GTGCCCACGTCTACCTGTG
EOMES-R	CCTGCCCTGTTTCGTAATGAT
HLA-DRB5-F	TACCTAACCCTACGGCCTCC
HLA-DRB5-R	CTTTGGAGCCAGGTGGACAG
GAPDH-F	TGCACCACCAACTGCTTAGC
GAPDH-R	GGCATGGACTGTGGTCATGAG

ImageStream flow cytometry – telomere FISH. Isolated $CD4^+$ T cells from HSs were left unstimulated and harvested at 0 hr or stimulated with Dynabeads anti-CD3/CD28 at the ratio 1 bead: 1 cell for 24 hr. Cells were fixed with fixation buffer for 15 min at RT, fixed cells were washed with and resuspended in flow cytometry buffer and stored at 4 ºC until subjected for the assay. Fixed cells were permeabilized with freshly prepared Foxp3/Transcription Factor Staining Buffer for 45 min in the dark at RT following one washing with flow cytometry buffer, cells were then stained with anti-γH2AX (1:150) in 1X permeabilization buffer at 37 ºC for 1 hr. Stained cells were washed with 1X permeabilization buffer and then fixed by fixation buffer for 15 min at RT following with one washing with flow cytometry buffer. Cells were resuspended in hybridization buffer (70% Formamide, 1% BSA, 150 mM NaCl and 20 mM Tris-HCl pH 7.2). Telomere probe TelC-(CCCTAACCCTAACCCTAA)-AF488 (PNA Bio) were used to hybridize cellular telomere at the concentration 0.5 μg probe/mL, cells were incubated at RT for 10 min in the dark then hybridized at 82 ºC for 12 min. Cells were kept at RT overnight in dark condition. The next day, cells were washed twice with post-hybridization buffer (70% Formamide, 0.1% BSA, 150 mM NaCl, 10 mM Tris-HCl pH 7.2, and 0.1% Tween-20) following an additional wash with flow cytometry buffer. Cells were then stained with secondary antibody goat anti-rabbit IgG-AF647 (1:100) (Jackson ImmunoResearch, 111-605-003) in 1X permeabilization buffer at 37 ºC for 1 hr following one washing with 1X permeabilization buffer and once with flow cytometry buffer. DAPI staining was performed in DPBS at RT for 15 min following by washing twice with flow cytometry buffer. Cells were resuspended in flow cytometry buffer and subjected for imagestream flow cytometry using Amnis ImageStream^X Mk II Imaging Flow Cytometer (Luminex) with 60x magnification. Single stained samples of PNA telomere probe TelC-AF488, goat anti-rabbit IgG-AF647 anti-γH2AX and DAPI were used for fluorochromes

compensation. 5000 cells per sample were acquired and images were analyzed using IDEAS® software version 6.2 (Luminex). Colocalization wizard was used to analyze the co-localization between telomere probe TelC-AF488 and anti-rabbit IgG-AF647 anti-γH2AX called forming dysfunctional telomere-induced foci (TIFs).

Lentiviral production and primary CD4+ T cell transduction. For lentiviral packaging, HEK293T cells at 80% confluency were transfected with 2.5μg of pMD2.G (# 12259), 7.5 μg of psPAX2 (# 12260) (both gifts from Dr. Didier Trone, Addgene), and 10μg of pWPiR or pWPiR-TRF2 plasmids (generous gifts from Dr. Eric Gibson and Dr. Vincent Picco) (for TRF2 overexpression transduction); or 10μg of pLKO.1-puro Non-Mammalian shRNA Control (Sigma, SHC002) or pLK0.1-puro-TERF2 shRNA (Sigma, SHCLNG-NM_005652, TRCN0000004811) (for TRF2 knockdown transduction) using Transporter™ 5 transfection reagent (Polyscience Inc) following the manufacturer's instruction. Lentiviruses were harvested, filtered by 0.45 μM sterile syringe filter (Sigma, SLHV004SL) and concentrated using PEG-it Virus Precipitation Solution (System Biosciences) following the manufacturer's protocol. Lentiviruses were resuspended in sterile DPBS (Lonza) and stored at -80 °C. Isolated $CD4^+$ T cells were seeded at $2.5x10^6$ cells per mL RPMI-1640 complete medium supplemented with anti-CD3/CD28 bead at the ratio 1 cell:1/2 bead for 24 hr. Cells were collected by washed-out anti-CD3/CD28 bead and subjected to lentiviral transduction of pWPiR, pWPiR-TRF2, or shRNA scramble, shRNA-TERF2 encoded viruses. TransDux™ MAX Lentivirus Transduction Reagent (System Biosciences) was used to enhance transduction efficiency following the manufacturer's protocol. Briefly, stimulated $CD4^+$ T cells were resuspended with 400 ml RPMI complete medium, 100 μl MAX Enhancer, 2 μL of TransDux™, and 4 μl of 1M HEPES buffer. Cells mixture was then seeded to a 24-well plate together with desired lentiviruses. The cell culture

plate was then centrifuged at 1,500 x g, 32ºC for 1.5 to 2 hrs. The supernatant was then discarded, and cells were resuspended with 1 mL RPMI complete media supplemented with soluble CD3/CD28 at ratio 1:2 μg/mL. Transduced cells were collected at 48 hr post-transduction and subjected for downstream experiments.

Statistics. Data was acquired from at least three independent experimental repeats and presented as mean ± SEM or median with interquartile range. Data comparisons between HS and HCV- T cells were first checked for Normality Distribution using Normality and Lognormality Tests and checked for outliers using Identify outliers the processed to unpaired two-tailed t-test: parametric test (normal distribution data) or Mann-Whitney test (compare ranks) or Kolmogorov-Smirnov test (compare cumulative distributions) (not follow normal distribution data). Comparisons between control/treatment were made using parametric paired two-tailed t-test, or nonparametric Wilcoxon matched-pairs signed-rank test. Pearson's correlation coefficient (normal distribution data) or Nonparametric Spearmen correlations will be used to assess the correlation between two variables. P-value *$p < 0.05$ is considered statistically significant and > 0.05 is nonsignificant (n.s). **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ are considered statistically very significant. GraphPad Prism version 8 was used for all data analysis.

Results

Circulating CD4+ T cells in chronic HCV patients exhibit a high activation status. We have previously reported that naïve $CD4^+$ T cells were significantly reduced, while memory $CD4^+$ T cells were markedly expanded in chronic HCV patients compared to HS [41, 42], suggesting $CD4^+$ T cells were excessively activated during chronic HCV infection. To consolidate this notion, here we examined the activation status of resting and TCR-stimulated $CD4^+$ T cells from chronically HCV-infected patients by flow cytometry. As shown in **Fig. 3.1a**, a significantly

increased frequency of human leukocyte antigen-antigen D related (HLA-DR) positive $CD4^+$ T cells was observed in HCV patients compared to gender- and age-matched HS. Also, we found an increased level of glucose transporter 1 (GLUT1), a major glucose receptor that can be upregulated upon T cell activation [91], in HCV-$CD3^+CD4^+$ T cells (**Fig. 3.1b**). Notably, the level of HLA-DR expression positively correlated to the GLUT1 expression in $CD3^+CD4^+$ T cells from these subjects (**Fig. 3.1c**), which is in line with a previous report showing highly activated $CD4^+$ T cells with elevated GLUT1 expression [92]. In addition, the $KLRG^+CD127^-$ T cells (a short-lived effector subset) were significantly upregulated in $CD4^+$ T cells from HCV patients (**Fig. 3.1d**). Moreover, HCV-$CD4^+$ T cells expressed greater Granzyme B (GrzB), a serine proteinase function in mediating cellular apoptosis, and enhances the production of pro-inflammatory cytokines (**Fig. 3.1e**).

It is well-known that $TCF1^+$ $CD8^+$ T cells exhibit stem cell-like characteristics, including self-renewal and high plasticity [93]. TCF1 is strongly expressed in naïve and memory precursor populations, while declined in more advanced differentiated T cells such as terminal effector T cells [93]. Given the critical role of TCF1 in maintaining stem cell-like properties in T cells, we evaluated the TCF1 level in $CD4^+$ T cells from HCV patients. As shown in **Fig. 3.1f**, TCF1 was significantly decreased in $CD4^+$ T cells derived from HCV patients. Consistent with our previous report, TRF2 (a subunit of telomere shelterin complex protecting chromosome ends - telomeres) from being recognized as DNA damage sites by cellular DNA damage repair machinery, was remarkedly reduced in HCV-naïve $CD4^+$ T cells (**Fig. 3.1g**). Taken together, these results indicate that HCV-$CD4^+$ T cells exhibit a more activated phenotype with less precursor marker TCF1 and telomere safeguard shelterin protein TRF2.

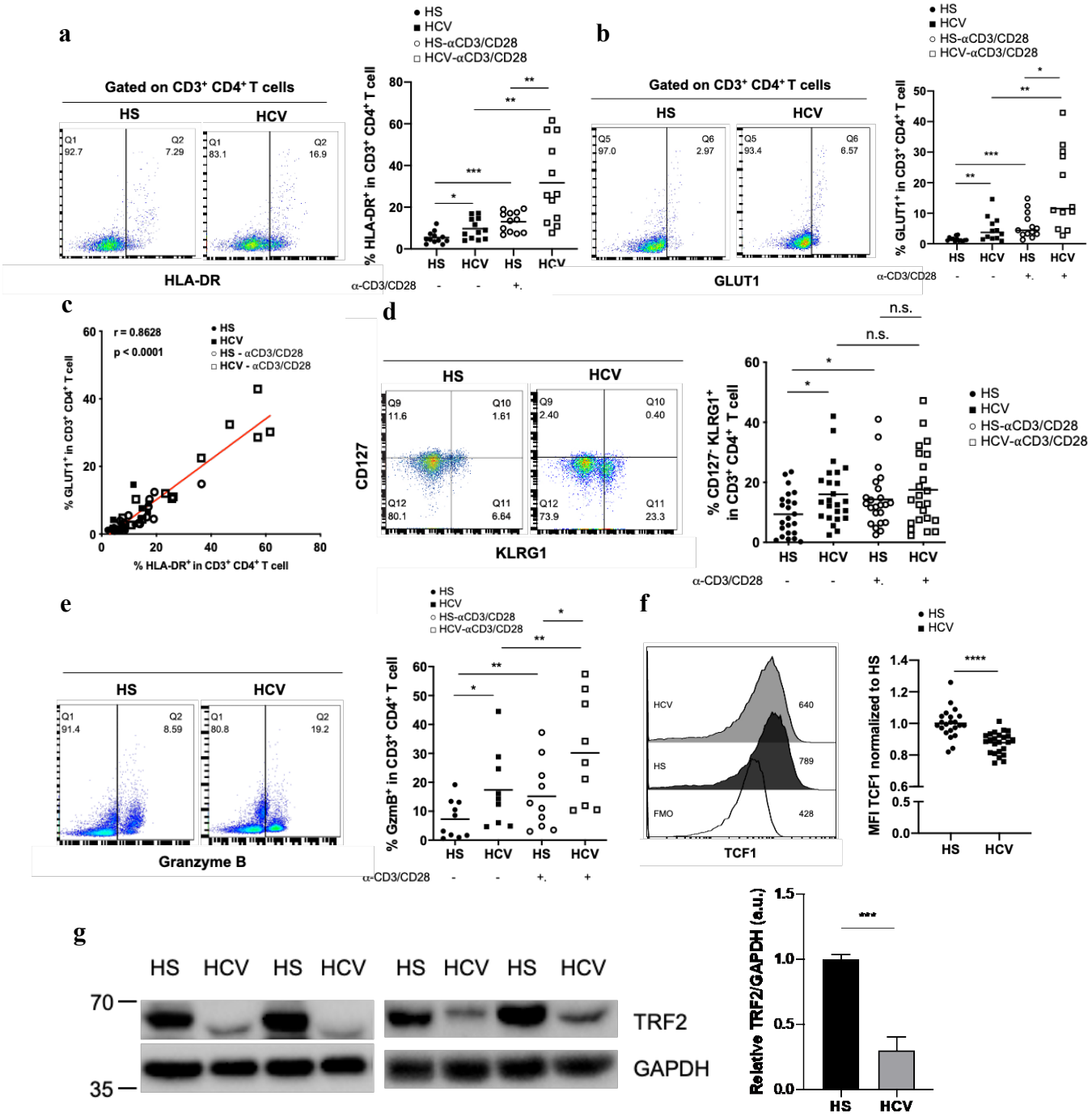


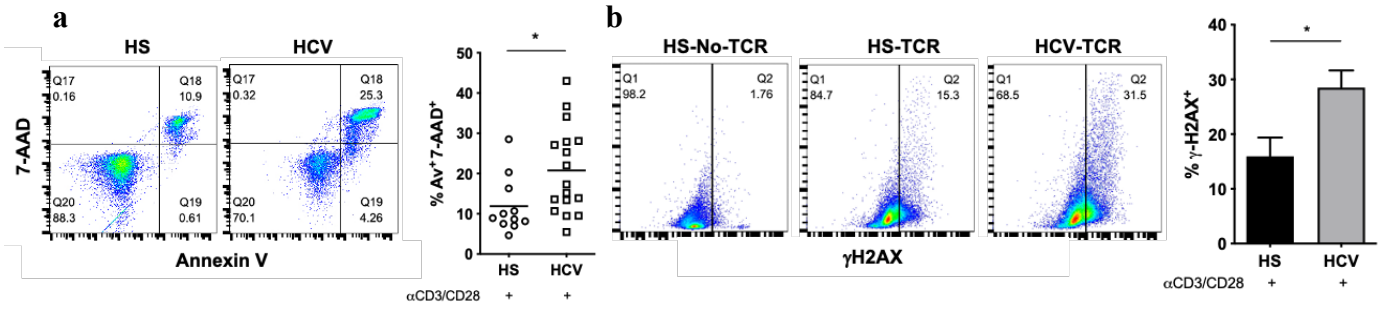
Figure 3.1. Circulating CD4$^+$ T cell activation in HCV chronically infected patients.

PBCMs from HCV chronically infected patients and age- and gender-matched HS were cultured with or without TCR stimulation with anti-CD3/CD28 bead (1 bead: 1 cell) for 2.5 hr. Cell surface and intracellular flow cytometry were performed using indicated antibodies. **a**, **b**, Representative dot plots and quantitative data for percentages of HLA-DR$^+$ and GLUT1$^+$ in

$CD3^+$ $CD4^+$ T cell from 12 HS and 12 HCV patients (unpaired and paired t-tests for HLA-DR^+ and Mann-Whitney, Wilcoxon and paired t-tests for $GLUT1^+$). **c**, Correlation of percentages of HLA-DR^+ and $GLUT1^+$ in $CD3^+$ $CD4^+$ T cell from 12 HS and 12 HCV with or without TCR stimulation (Spearman correlation, coefficients r and p-value are shown). **d**, Representative dot plots and quantitative data for percentages of $CD127^+$ $KLRG1^-$ in $CD3^+$ $CD4^+$ T cell from 23 HS and 24 HCV patients (unpaired t-test for HS vs HCV without TCR stimulation, Wilcoxon test for HS or HCV before and after TCR stimulation, Mann-Whitney test for HS vs HCV in TCR stimulation). **e**, Representative dot plots, and quantitative data for percentages of $GZMB^+$ in $CD3^+$ $CD4^+$ T cell from 10 HS and 9 HCV patients (unpaired and paired t-tests). **f**, Representative histograms and normalized TCF1 MFI in $CD3^+$ $CD4^+$ T cell from 22 HS and 24 HCV patients (unpaired t-test). **g**, Immunoblotting for TRF2 in unstimulated naïve $CD4^+$ T cells, and quantification of arbitrary unit related GAPDH control (data from 4 HS and 4 HCV patients, unpaired t-test). Mean ± s.e.m, non-significant (n.s.), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

TCR stimulation induces biased, short-lived effector memory differentiation in HCV-CD4+ T cells. $CD4^+$ T cells from HCV patients exhibit strong memory effector markers and less memory precursor transcription factor TCF1, suggesting a highly differentiated linage commitment during HCV infection. To investigate T cell differentiation during HCV infection, we isolated naïve $CD4^+$ T cells and differentiated to effector T cells by *ex vivo* stimulation with anti-CD3/CD28 for 72 hours. As shown in **Fig. 3.2a-b**, TCR-stimulated HCV-$CD4^+$ T cells showed significantly increased cellular apoptosis and DNA damage compared to HS. Also, upon TCR stimulation, HCV-$CD4^+$ T cells exhibited a significant decrease in the expression of transcription factor TCF1 but an increase in the level of phospho-ribosomal protein S6

(pS6$^{ser235/236}$) - a downstream substrate of phosphoinositol-3 kinase (PI3K)/Akt-mTOR signaling pathway (**Fig. 3.2c-d**). To confirm this finding, we performed immunoblotting in TCR-stimulated CD4$^+$ T cells from HCV patients and HS. Consistent with our flow cytometry data, TCR-stimulated HCV-CD4$^+$ T cells exhibited significantly increased levels of pAkt and pS6 but decreased TCF1 protein expression (**Fig. 3.2e-f**). Notably, there is a positive correlation between the Akt-mTOR activation marker pS6 and DNA damage marker γH2AX (**Fig. 3.2g**) as well as DNA damage marker γH2AX and cellular apoptosis markers Av/7AAD (**Fig. 3.2h**) in TCR-stimulated CD4$^+$ T cells, suggesting that stimulated HCV-CD4$^+$ T cells exhibit hyperactivation of the PI3K/Akt-mTOR signaling, resulting in activation-mediated DNA damage and thus cell death. Furthermore, our data revealed a negative correlation between the levels of pS6 and TCF1, indicating that CD4$^+$ T cells expressing a high level of pS6 exhibit lower level of TCF1, or vice versa (**Fig. 3.2i**). We also checked the transcriptional level of *TCF7* (the gene encoding TCF1) and found a significantly lower expression of *TCF7*, along with elevated levels of cytotoxic and pro-inflammatory markers, including *Tbx21* (encoding transcription factor T-bet), *Eomes* (encoding transcription factor Eomesodermin), *GZMB,* and *HLA-DRB5* in HCV-CD4$^+$ T cells (**Fig. 3.2j**). Taken together, these results suggest that HCV-CD4$^+$ T cells are biasedly differentiated toward short-lived effector phenotypes, such as pro-inflammatory and cytotoxic CD4$^+$ T cell subsets with robust DNA damage and cellular apoptosis.



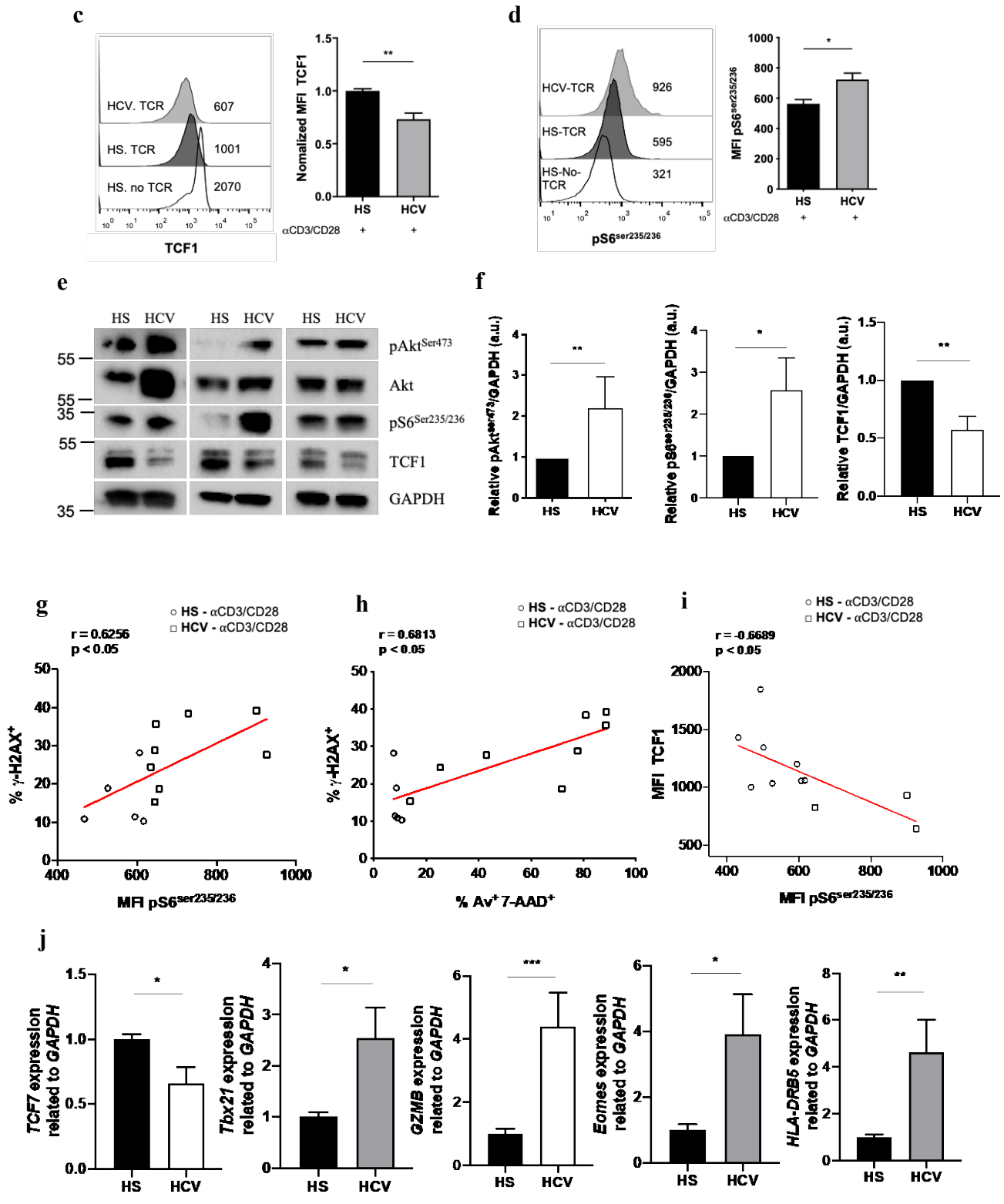
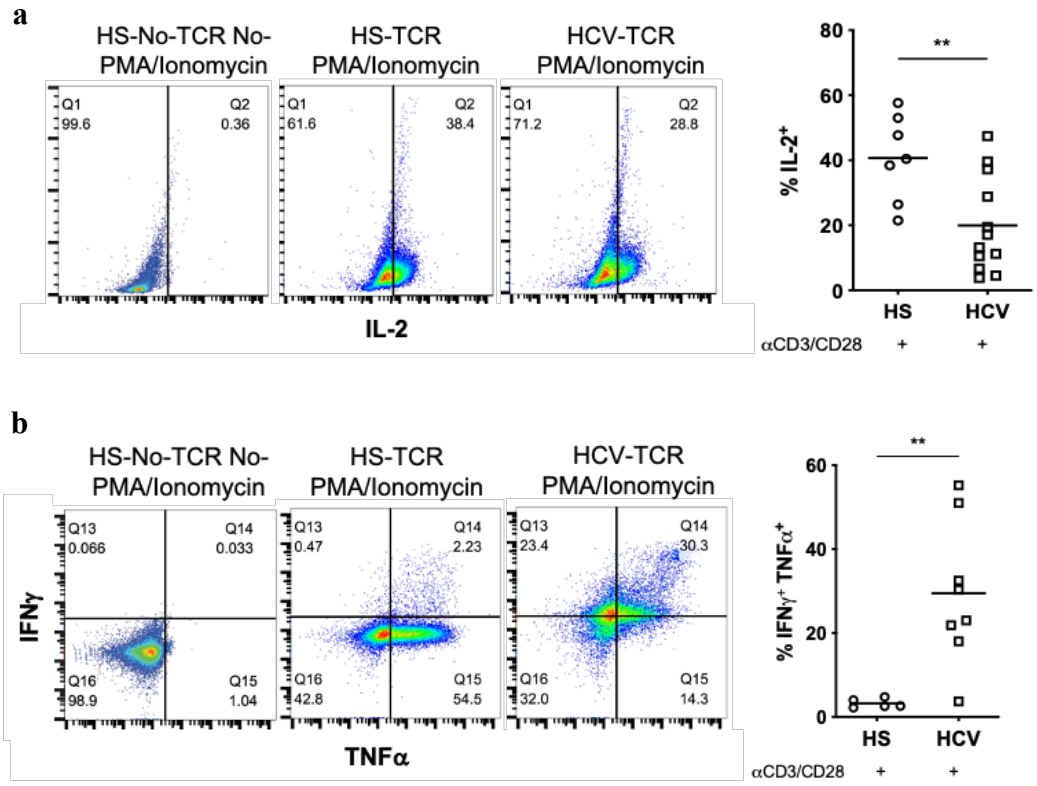


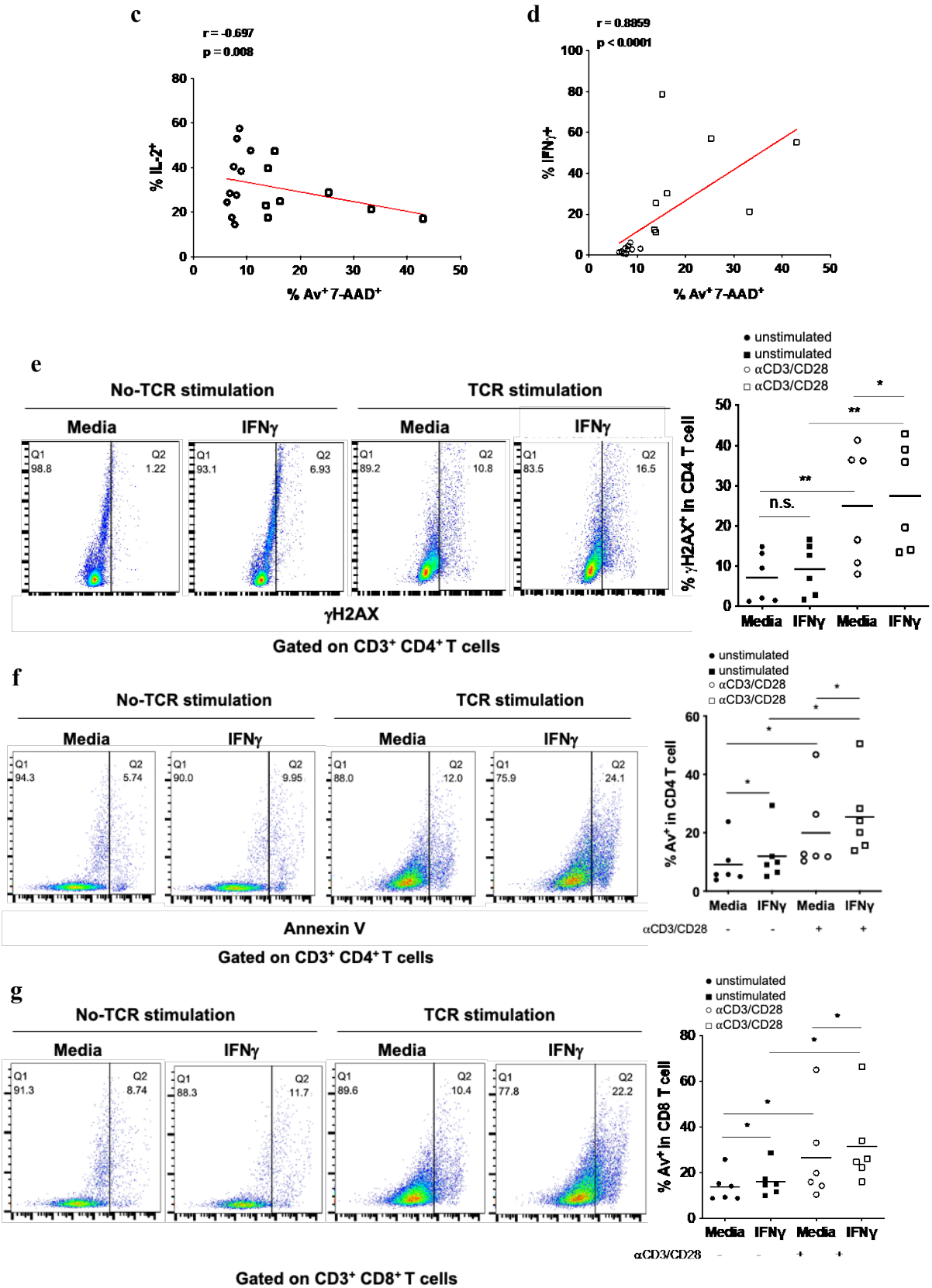
Figure 3.2. Pro-inflammatory effector differentiation in HCV patient derived naïve CD4⁺ T cell. Naïve CD4⁺ T cells from HS and HCV patients were isolated and TCR stimulated

for 72 hr. **a**, Representative dot plots and quantitative data for percentages of Annexin$^+$ 7-AAD$^+$ cells from 11 HS and 17 HCV patients (unpaired t test). **b**, **c**, **d**, Representative dot plots and quantitative data for percentages of γH2AX$^+$ cells (**b**); and representative histograms and quantitative data for TCF1 MFI (**c**) and pS6$^{ser235/236}$ MFI (**d**) from 5 HS and 8 HCV patients (unpaired t tests). **e**, **f**, Representative immunoblotting for pAkt^{ser473}, pan-Akt, TCF1, pS6$^{ser235/236}$ and GAPDH control and quantification of arbitrary unit are shown (data from 6 HS and 6 HCV patients, Wilcoxon tests). **g**, **h**, **i**, Correlation of the percentages of γH2AX$^+$ and pS6$^{ser235/236}$ MFI (HS n = 5, HCV n = 8, Pearson correlation) (**g**), percentages of γH2AX$^+$ and Annexin$^+$ 7-AAD$^+$ (HS n = 5, HCV n = 8, Spearman correlation) (**h**) and TCF1 MFI and pS6$^{ser235/236}$ MFI (HS n = 8, HCV n = 3, Pearson correlation) (**i**), coefficients r and p values are shown. **j**, Indicated genes expression were determined by quantitative real-time PCR presented by mean ± s.e.m from 8 HS and 9 HCV patients. Mean ± s.e.m, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

We next evaluated the functional cytokines production in CD4$^+$ T cells derived from HCV patients and HS and stimulated with phorbol myristate acetate (PMA), Ionomycin, and Brefeldin A for 6 hours. Intracellular flow cytometry analysis revealed that HCV-CD4$^+$ T cells produced significantly less survival cytokine IL-2, but more pro-inflammatory cytokine IFNγ and TNFα (**Fig. 3.3a-b**). Correlation analysis revealed that the percentage of IL-2$^+$ cells negatively correlated with the cellular apoptosis; however, IFNγ$^+$TNFα$^+$ cells positively correlated with CD4$^+$ T cell death (**Fig. 3.3b-d**). Although IFNγ-Th1 response plays an important role in controlling viral infection and tumor progression in humans, excessive production of IFNγ had been shown to induce cellular apoptosis in IFNγ-producing Th1 CD4$^+$ T cells [94, 95] and in tumor-infiltrating CD8$^+$ T cells [96]. We thus hypothesize that strong pro-inflammatory cytokine IFNγ production in circulating CD4$^+$ T cells could have a negative impact on T cell homeostasis

during chronic HCV infection. To test this hypothesis, here we treated HCV-CD4 T cells with or without anti-CD3/CD28 stimulation in the presence or absence of recombinant IFNγ (500 ng/mL) for 72 hours. We demonstrated that IFNγ treatment increased DNA damage in non-TCR-stimulated $CD3^+$ $CD4^+$ T cells, and this was observed at the greater extend in TCR-stimulated $CD4^+$ T cells (**Fig. 3.3e**). We further discovered that IFNγ treatment significantly induced cellular apoptosis in both unstimulated and TCR-stimulated $CD3^+CD4^+$ T cells (**Fig. 3.3f**). A similar phenomenon was also observed in $CD3^+CD8^+$ T cells (**Fig. 3.3g**) from HCV patients, suggesting that elevated IFNγ production in inflammation-differentiated effector T cells contributes to immune activation-induced DNA damage and cellular apoptosis in T cells during chronic HCV infection.

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% IL-2+
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d
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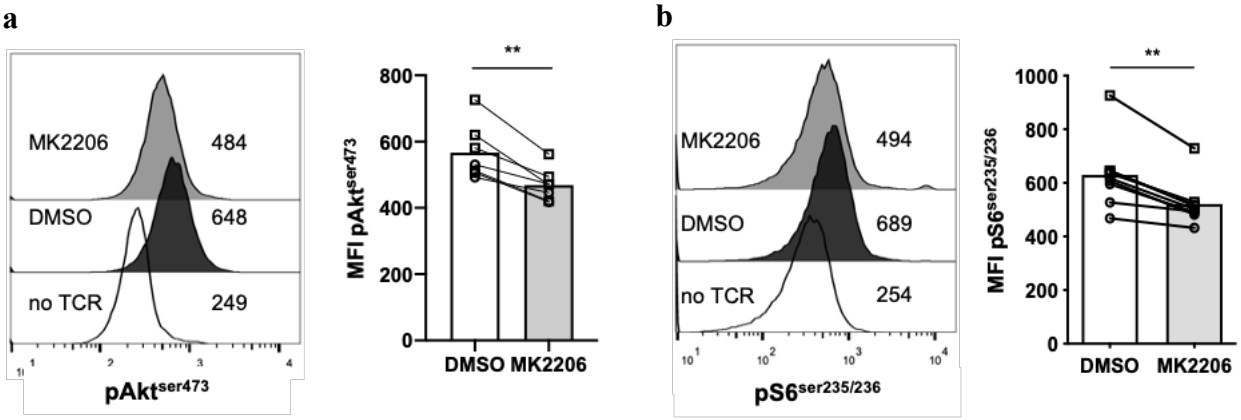
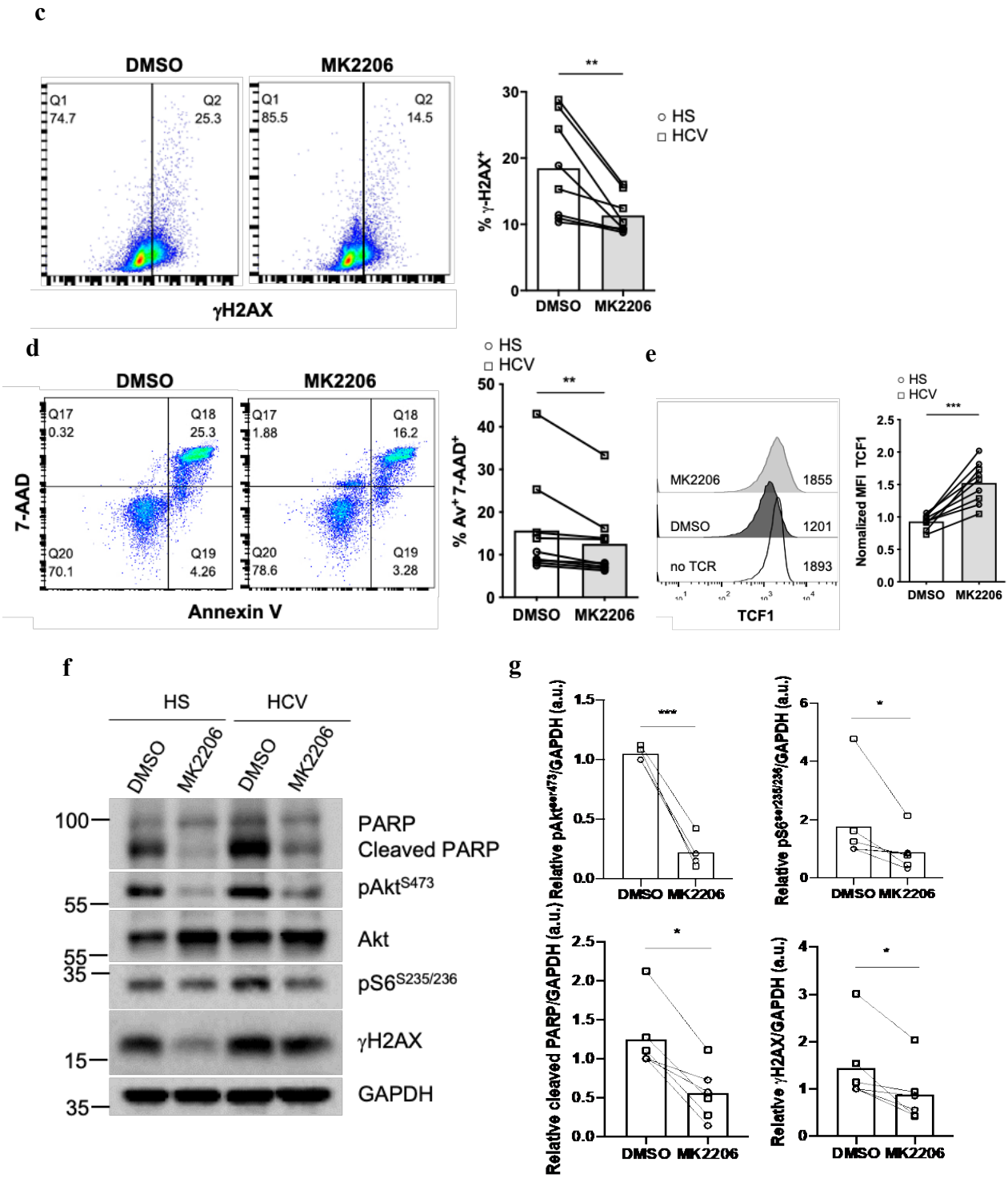
p < 0.0001
% IFNγ+
% Av+ 7-AAD+
e
No-TCR stimulation
TCR stimulation
Media
IFNγ
Q1 98.8
Q2 1.22
Q1 93.1
Q2 6.93
Q1 89.2
Q2 10.8
Q1 83.5
Q2 16.5
γH2AX
Gated on CD3+ CD4+ T cells
unstimulated
unstimulated
αCD3/CD28
αCD3/CD28
% γH2AX+ in CD4 T cell
n.s.
f
Q1 94.3
Q2 5.74
Q1 90.0
Q2 9.95
Q1 88.0
Q2 12.0
Q1 75.9
Q2 24.1
Annexin V
Gated on CD3+ CD4+ T cells
% Av+ in CD4 T cell
αCD3/CD28
g
Q1 91.3
Q2 8.74
Q1 88.3
Q2 11.7
Q1 89.6
Q2 10.4
Q1 77.8
Q2 22.2
Gated on CD3+ CD8+ T cells
% Av+ in CD8 T cell

Figure 3.3. Pro-inflammatory cytokines production induced DNA damage and cellular apoptosis in HCV patient's T cells. Naïve $CD4^+$ T cells from HS and HCV after TCR stimulation for 72 hr were harvested and stimulated with PMA/Ionomycin and Brefeldin A for 6 hr. Flow cytometry was performed to measure intracellular cytokines production. **a**, **b**, Representative dot plots and quantitative data for percentages of IL-2^+ (**a**) and TNFα^+ IFNγ^+ double-positive (**b**) from 5 HS and 8 HCV patients (unpaired t-tests). **c**, **d**, Correlation of the percentages of IL-2^+ and Annexin$^+$ 7-AAD$^+$ (**c**) and the percentages of IFNγ^+ and Annexin$^+$ 7-AAD$^+$ (**d**) from 10 HS and 8 HCV patients (Spearman correlations, coefficients r and p values are shown). **e**-**g**, PBCMs from 6 HCV chronically infected patients were stimulated or unstimulated with soluble anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) in the supplementation of human recombinant IFNγ (500 ng/ml) for 72 hr. Flow cytometry was applied to measure DNA damage marker γH2AX$^+$ and cellular apoptosis Annexin$^+$ 7-AAD$^+$. Representative dot plots and quantitative data for percentages of γH2AX$^+$ in $CD3^+$ $CD4^+$ T cell (unpaired and paired t-tests). Representative dot plots and quantitative data for percentages of Annexin$^+$ 7-AAD$^+$ in $CD3^+$ $CD4^+$ T cell (**f**) and in $CD3^+$ $CD8^+$ T cell (**g**) (unpaired and paired t-tests). Mean ± s.e.m, non-significant (n.s.), *$p < 0.05$ and **$p < 0.01$.

AKT inhibition preserves TCF1 and reduces activation-induced DNA damage and apoptosis in naïve CD4+ T cells. Since we observed a hyperactivation on the Akt-mTOR signaling pathway during HCV infection, evidenced by upregulated pS6 expression in TCR-stimulated circulating HCV-$CD4^+$ T cells. We next tested if Akt inhibition could correct the biased pro-inflammatory differentiation in HCV-$CD4^+$ T cells during TCR stimulation. To this end, naïve CD4 T cells from HCV patients and HS were TCR-stimulated for 72 hours in the presence of a specific Akt inhibitor - MK2206 (1μM), or DMSO control. In both HCV- and HS-

$CD4^+$ T cells, MK2206 significantly inhibited $pAkt^{ser473}$ and $pS6^{ser235/236}$ expressions (**Fig. 3.4a-b**). Also, Akt inhibition reduced TCR activation-induced DNA damage and cellular apoptosis (**Fig. 3.4c-d**). Remarkedly, stem cell-like transcription factor TCF1 was preserved or significantly increased by the MK2206 treatment (**Fig. 3.4e**). Consistently, immunoblotting revealed that HCV-$CD4^+$ T cells exhibited hyperactivation of Akt-mTOR markers $pAkt^{ser473}$ and $pS6^{ser235/236}$ along with upregulation of DNA damage marker γH2AX and pro-apoptotic cleaved PARP (**Fig. 3.4f -** DMSO group); however, MK2206 treatment markedly downregulated the pAkt and pS6 expressions as well as γH2AX and cleaved PARP in both HCV- and HS-$CD4^+$ T cells compared to DMSO control (**Fig. 3.4f-g**). By controlling T cell over-activation via inhibition of Akt signaling, MK2206 treatment also reduced pro-inflammatory cytokines IFNγ and TNFα (**Fig. 3.4h**) as well as IL-2 productions (**Fig. 3.4i**). Furthermore, inhibition of Akt transcriptionally preserved stem cell-like marker *TCF7* while suppressing $CD4^+$ cytotoxic T cell marker *GZMB* gene expressions (**Fig. 3.4i**). Taken together, these results suggest that attenuating TCR overactivation by targeting the PI3K-Akt-mTOR pathway can prevent antigen stimulation/immune activation-induced advanced differentiation of $CD4^+$ T cells into pro-inflammatory and cytotoxic phenotype and preserve memory precursor T cell pool, such as $TCF1^+CD4^+$ T cells.

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DMSO MK2206

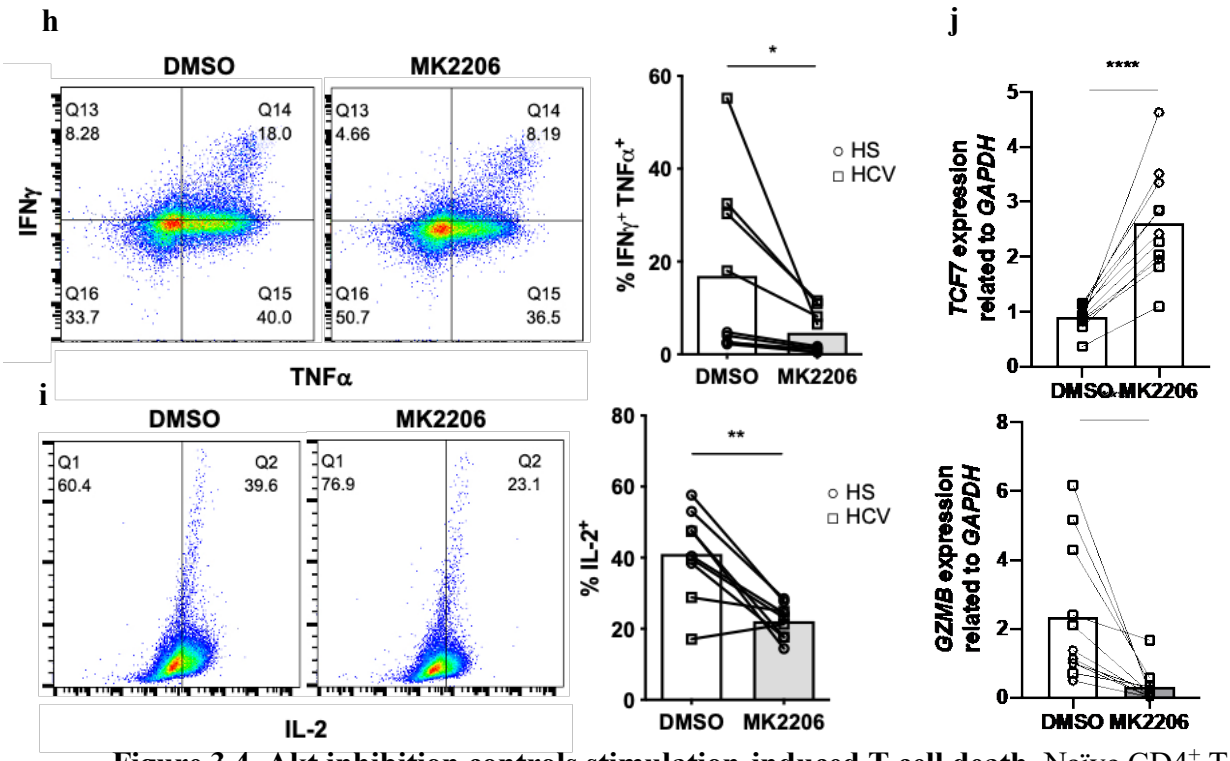


Figure 3.4. Akt inhibition controls stimulation-induced T cell death. Naïve CD4$^+$ T cells from HS and HCV patients were isolated and TCR stimulated for 72 hr in the presence of Akt inhibitor MK2206 (1μm) or DMSO control. Cell surface and intracellular flow cytometry were performed using indicated antibodies. **a**, **b**, Representative histograms and quantitative data for pAkt^{ser473} MFI (unpaired t-test) (**a**) and pS6$^{ser235/236}$ MFI (Wilcoxon test) (**b**). **c**, **d**, Representative dot plots and quantitative data for percentages of γH2AX$^+$ (paired t-test) (**c**) and Annexin$^+$ 7-AAD$^+$ (Wilcoxon test) (**d**). **e**, Representative histograms, and quantitative data for TCF1 MFI (paired t-test). **f**, Representative immunoblotting for PARP, pAkt^{ser473}, pan-Akt, TCF1, pS6$^{ser235/236}$, and GAPDH control and quantification of the arbitrary unit are shown (data from 3 HS and 3 HCV patients, Wilcoxon tests). **h**, **i**, After TCR stimulation for 72 hr, cells were harvested and stimulated with PMA/Ionomycin and Brefeldin A for 6 hr. Flow cytometry was performed to measure intracellular cytokines production. Representative dot plots and quantitative data for percentages of TNFα$^+$ IFNγ$^+$ double-positive (**h**) and IL-2$^+$ (**i**) and from 5

HS and 8 HCV patients (unpaired t-tests). **j**, Indicated genes expression were determined by quantitative real-time PCR presented by mean ± s.e.m from 6 HS and 5 HCV patients. All flow cytometry quantitative data is from 4 HS and 4 HCV patients, mean ± s.e.m, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

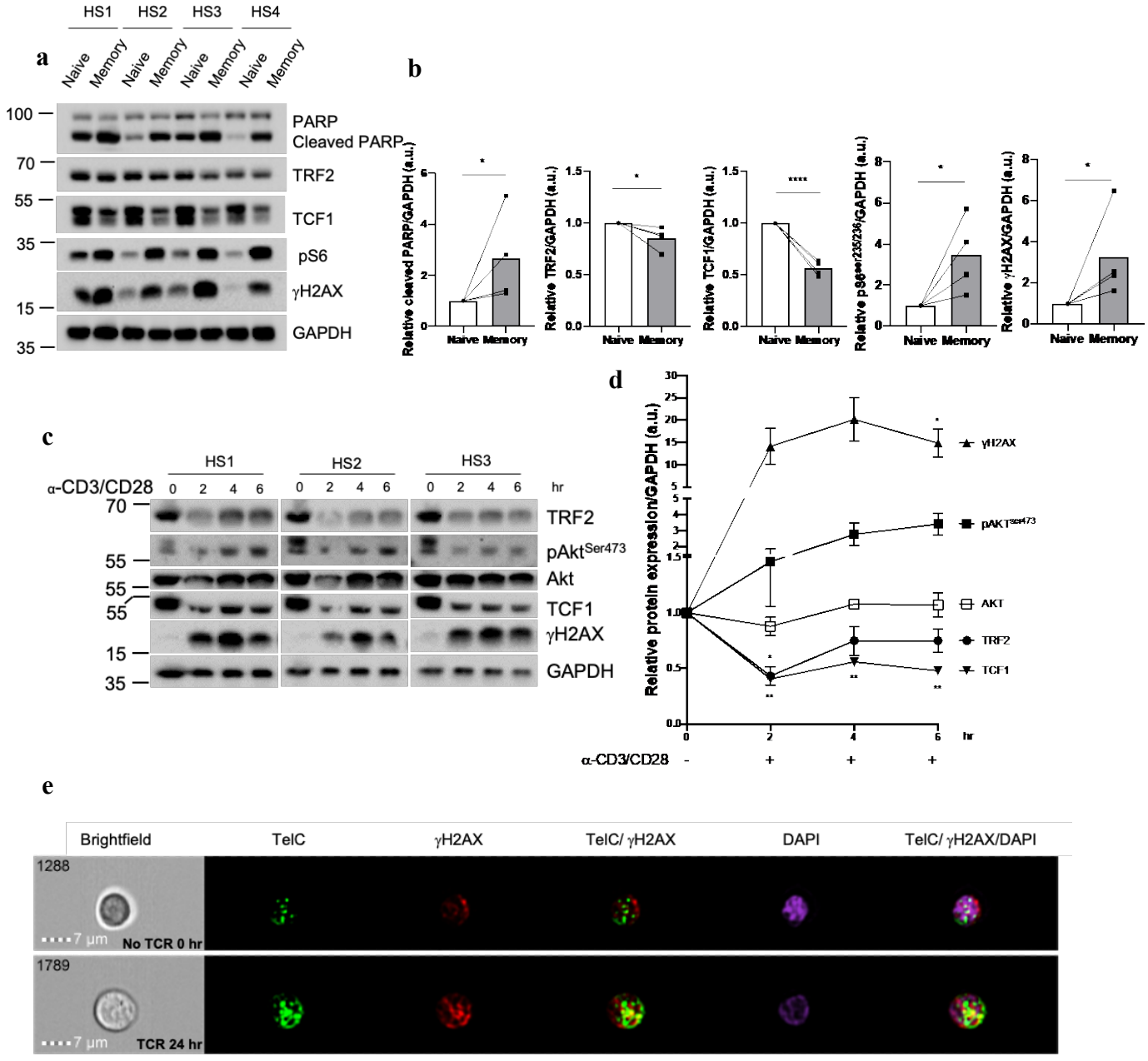
T cell activation inhibits TRF2 expression, induces telomeric DNA damage, and reduces stem cell-like memory T cells. We have previously shown $CD4^+$ T cell telomeric DNA damage and apoptosis during chronic HCV and HIV infections (25-26). Given the rapid T cell proliferation and differentiation during chronic viral infection, DNA damage accumulated in activated T cells is inevitable [47]. To investigate the role of TCR activation in telomeric DNA damage and cell fate, we isolated naïve and memory $CD4^+$ T cells from HS and compared their mTOR signaling and DNA damage markers. As shown in **Fig. 5a-b**, memory $CD4^+$ T cells exhibited hyperactivation of the mTOR signaling pathway, evidenced by a significant increase of phospho-S6 ribosomal protein compared to naïve $CD4^+$ T cell. Also, DNA damage marker γH2AX and pro-apoptotic cleaved PARP were markedly accumulated in memory $CD4^+$ T cells compared to the naïve compartment. Notably, the expressions of telomere shelterin protein TRF2 and transcription factor TCF1 were significantly reduced in memory $CD4^+$ T cells compared to their naïve counterpart. These observations indicate that advanced lineage differentiation or highly activated T cells are harbored with stronger mTOR activity, resulting in more DNA damage and pro-apoptotic phenotype.

Telomeric DNA is more vulnerable to activation-induced cellular senescence during the aging process and cellular stresses than genomic DNA [97]. We have previously reported that TRF2 downregulation in naïve CD4 T cells contributed to the telomeric DNA damage during HCV and HIV infection as well as drug treatment [41]. To examine the status of TRF2 during T

cell activation, we stimulated $CD4^+$ T cells from HS with CD3/CD28 conjugated beads for indicated time points. As shown in **Fig. 5c-d**, TCR stimulation-induced pAKT activation and DNA damage marker γH2AX accumulation; however, TRF2 was significantly decreased as early as 2 hours and remained at low levels at 4 and 6 hours following TCR stimulation. This downregulation of TRF2 could lead to telomeres to be exposed to cellular DNA damage response (DDR) since TRF2 is essential to form a T-loop structure to inhibit DDR at the telomere. Indeed, our image stream data by AMNES analysis revealed that DNA damage marker γH2AX is located at telomeres, forming dysfunctional telomere-induced foci (TIFs) in TCR-stimulated $CD4^+$ T cells (**Fig. 5e-f**), suggesting that telomeric DNA was targeted by cellular DDR during T cell activation, in part due to TRF2 deprotection.

Similar to TRF2, stem cell marker TCF1 was significantly downregulated in TCR-stimulated $CD4^+$ T cells (**Fig. 5c-d**), confirming that T cell activation suppressed TCF1 expression and reduced stem-cell-like memory T cell population. We have previously shown that TRF2 downregulation in HCV-CD4 T cells was mediated at the post-transcriptional level by proteasome degradation (25). Consistently, we observed that *TERF2* gene expression was not affected by TCR stimulation (**data not shown**). Furthermore, treatment of protein synthesis inhibitor cycloheximide (CHX) led to TRF2 protein degradation, and proteasome inhibitor MG132 partially recovered TRF2 expression in unstimulated $CD4^+$ T cell (**Fig. 5g**). In TCR-stimulated $CD4^+$ T cells, α-CD3/CD28 beads were removed 2-hour post-stimulation, then CHX was added with or without MG132 for indicated time points. Consistently, TCR stimulation-induced TRF2 degradation compared to unstimulated $CD4^+$ T cells; however, CHX treatment further downregulated TRF2 with or without proteasome inhibitor MG132 (**Fig. 5g**), suggesting that TRF2 is continuously degraded during T cell activation and protein translation is essential to

maintain TRF2 expression. Surprisingly, a similar pattern of degradation was observed for transcription factor TCF1 (**Fig. 5g**), demonstrating that TCR activation reduced stem cell plasticity in activated CD4^{+} T cells by downregulating TCF1 expression. Taken together, our data indicate that activated T cells not only exhibit telomeric DNA damage via the TRF2 degradation but also reduce the stem cell-like memory population, likely due to the downregulation of the transcription factor TCF1.



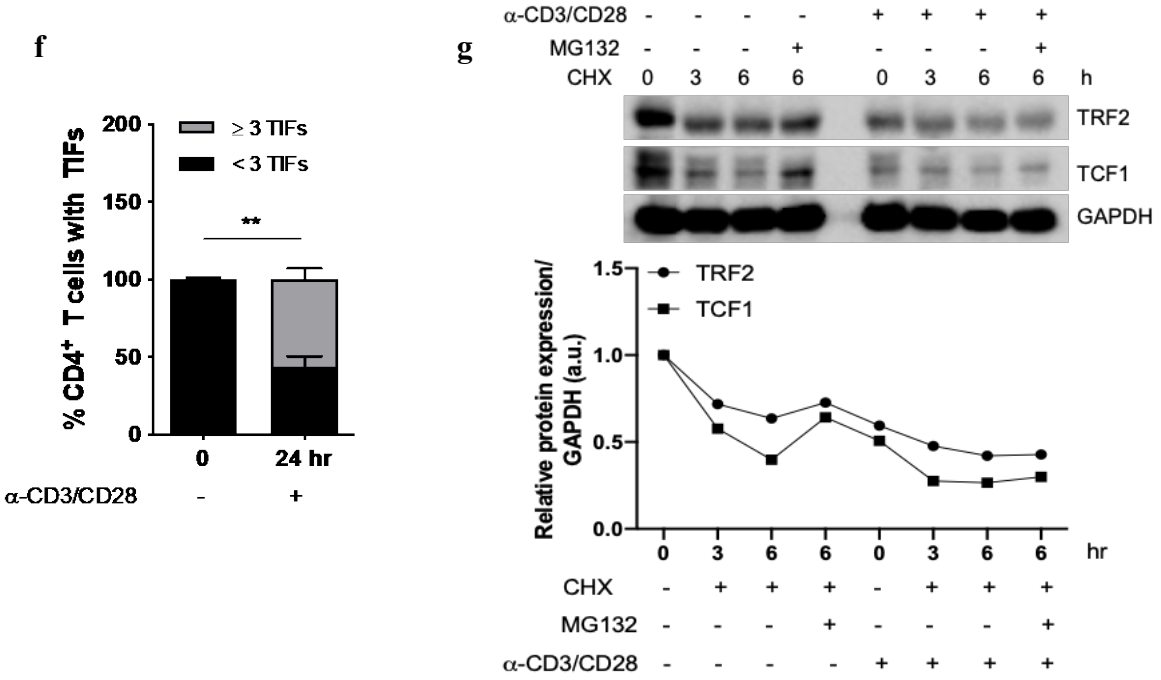
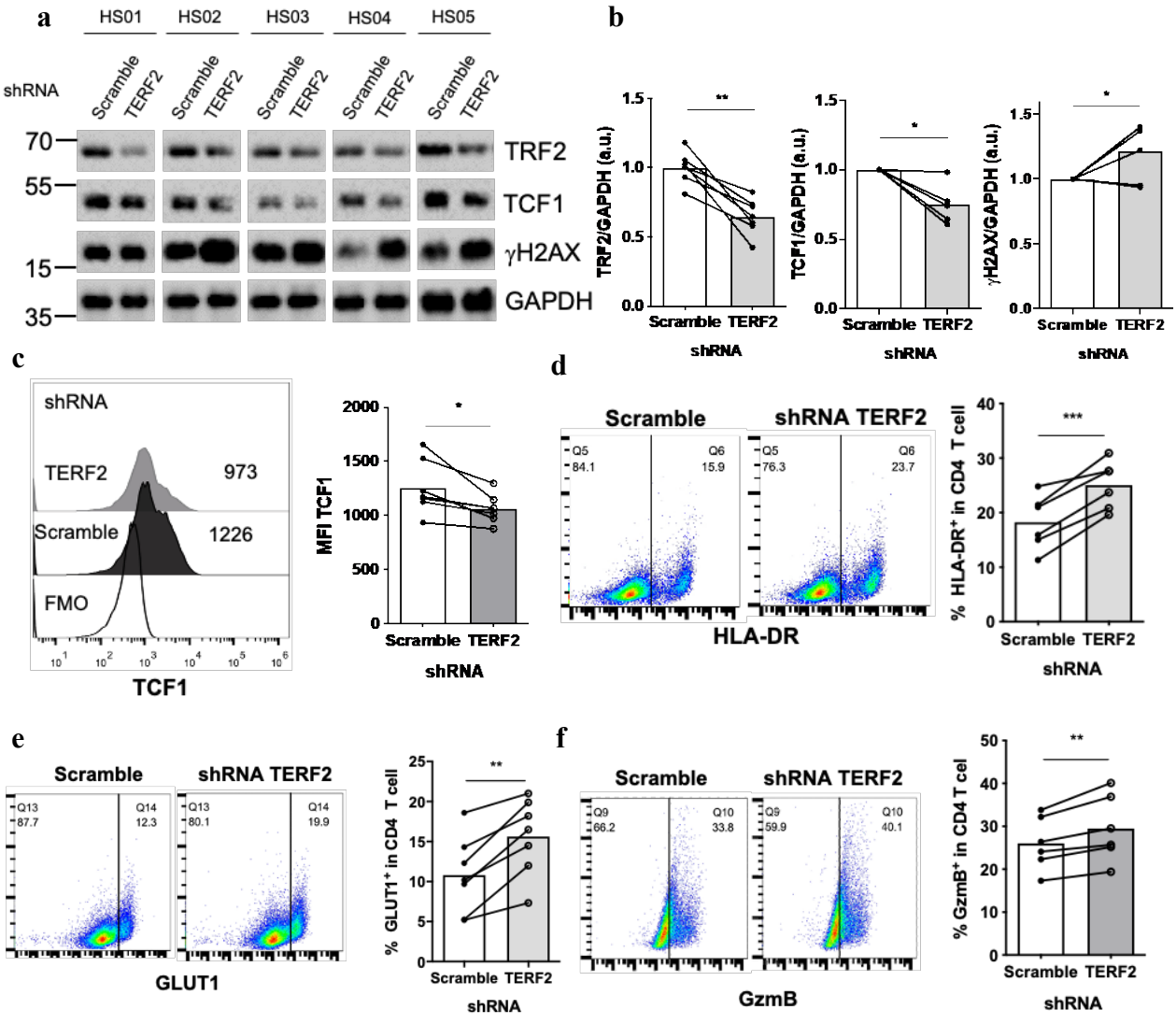


Figure 3.5. T cell activation-induced TRF2 downregulation and telomeric DNA damage. Naïve and memory CD4$^+$ T cells from the same HS individuals were isolated and subjected to immunoblotting immediately after isolation. **a**, **b**, Representative immunoblotting for PARP, TRF2, TCF1, pS6$^{ser235/236}$, γH2AX, and GAPDH control and quantification of the arbitrary unit are shown (data from 4 HS and 4 HCV patients, Kolmogorov-Smirnov tests). **c**, **d**, HS CD4$^+$ T cell was isolated and TCR stimulated with anti-CD3/CD28 bead (1 bead: 1 cell) for indicated time points. Immunoblotting for TRF2, pAkt^{ser473}, pan-Akt, TCF1, γH2AX, and GAPDH control and quantification of the arbitrary unit are shown (data from 3HS, Kolmogorov-Smirnov tests). **e**, **f**, HS CD4$^+$ T cell was left unstimulated or TCR stimulated with anti-CD3/CD28 bead (1 bead: 1 cell) for 24 hr. Cells were fixed and subjected to ImageStream flow cytometry – telomere FISH using TelC-AF488 PNA telomere probe, rabbit anti-γH2AX for the primary antibody, goat anti-rabbit IgG-AF647 for secondary antibody, and DAPI. Representative image and quantitative data from 3 independent HS, 50 cells were counted from each subject for

cells < 3 TIFs and ≥ 3 TIFs (paired t-test). **g**, HS $CD4^+$ T cell was isolated and left unstimulated or TCR stimulated with anti-CD3/CD28 bead (1 bead: 1 cell) and supplemented with protein translation inhibitor cycloheximide (50 μg/ml), proteosome inhibitor MG132 (10 μM/ml), or DMSO control for indicated time points. Immunoblotting for TRF2, TCF1, and GAPDH control and quantification of the arbitrary unit are shown. Mean ± s.e.m, *$p < 0.05$ and **$p < 0.01$.

TRF2 knockdown reduces T cell stemness and triggers DNA damage and cellular apoptosis. Our data in HCV-$CD4^+$ T cells and *ex vivo* TCR stimulation revealed that T cell activation suppresses TRF2 expression (**Fig. 3.5a, c**). We thus hypothesize that TRF2 could play an important role in T cell survival and function. To test this hypothesis, we employed lentivirus-mediated knockdown or overexpression of TRF2 in primary $CD4^+$ T cells from HS and measured T cell activation and differentiation. As shown in **Fig. 3.6a-b**, TRF2 knockdown significantly reduced TRF2 and transcription factor TCF1 but markedly increased DNA damage marker γH2AX levels. We further confirmed TCF1 downregulation in TRF2 knockdown $CD4^+$ T cells by flow cytometry (**Fig. 3.6c**), suggesting that TRF2 inhibition led to the reduction of memory precursor T cells, as evidenced by downregulating transcription factor TCF1. Similar to our observations in HCV-$CD4^+$ T cells, the expressions of activation marker HLA-DR (**Fig. 3.6d**) and GLUT1 (**Fig. 3.6e**) were upregulated in TRF2 knockdown $CD4^+$ T cells compared to scrambled control, suggesting that TRF2 inhibition facilitates T cell maturation. In line with the highly activated phenotype observed in TRF2 knockdown $CD4^+$ T cells, granzyme B was also significantly elevated compared to scrambled control (**Fig. 3.6f**). In addition, TRF2 knockdown resulted in less production of survival cytokine IL-2 and more apoptosis of these $CD4^+$ T cells with PMA/Ionomycin stimulation (**Fig. 3.6g-h**). Lastly, while TRF2 knockdown remarkedly increased inhibitory receptor KLRG1 expression and DNA damage marker γH2AX level in

$CD4^+$ T cells (**Fig. 3.6i**), overexpression of TRF2 led to an opposite effect (**Fig. 3.6j**). In essence, these data reveal TRF2 as a key factor for immune activation-induced DNA damage, T cell dysfunction, and apoptosis, therefore, modulation of immune activation and protection of telomeres by inhibiting Akt and enforcing TRF2 expression could become an important therapeutic strategy to balance adaptive immune responses in the setting of chronic immune activation and inflammatory or infectious diseases.



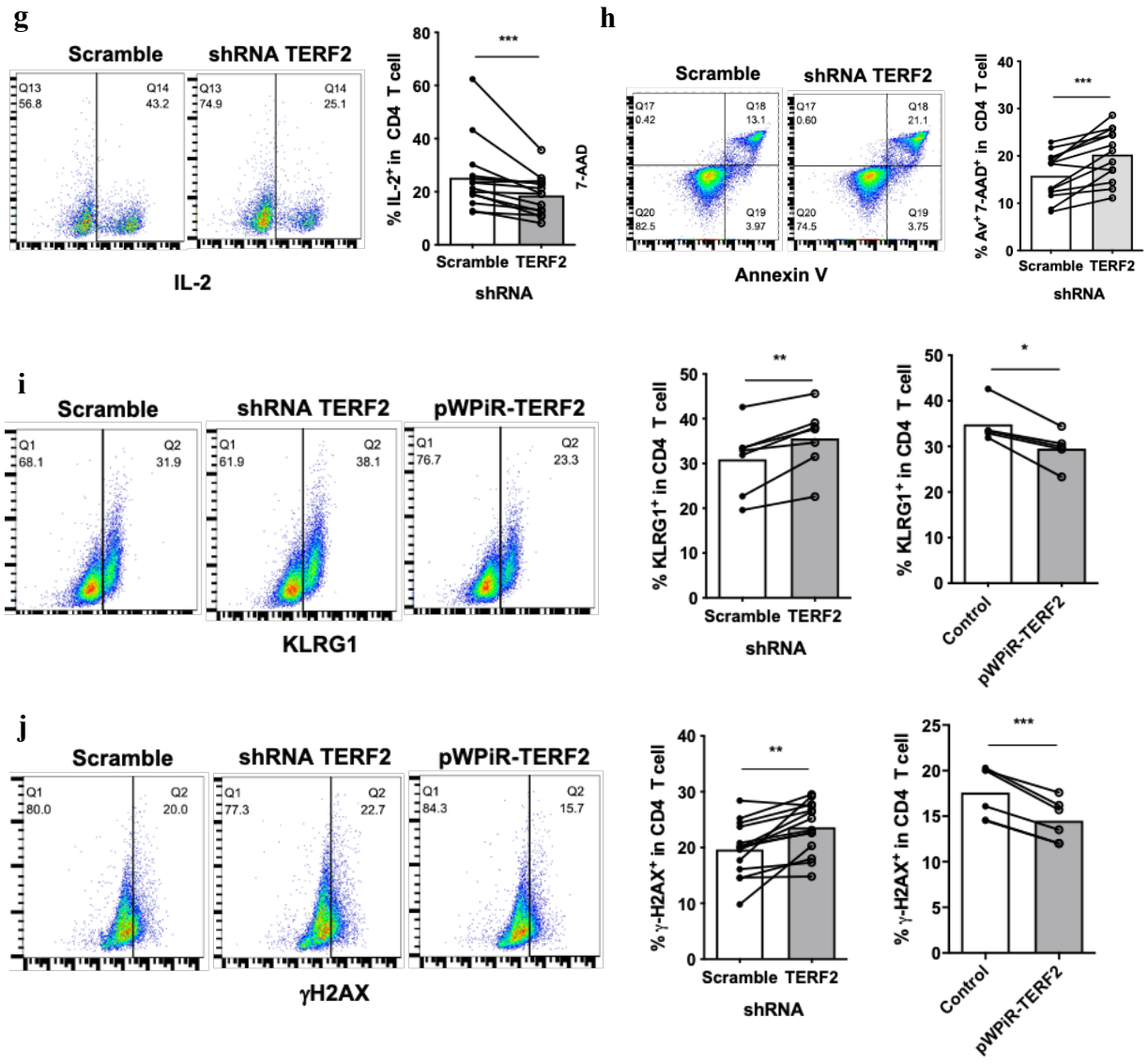


Figure 3.6. Knockdown TRF2 reduced T cell stemness and elevated T cell death. a-f, HS $CD4^+$ T cell was isolated and TCR stimulated with anti-CD3/CD28 bead (1 bead: 2 cells) for 36 hr. Cells were harvested and subjected to lentiviral transductions of TERF2 shRNA or scramble shRNA control as described in the Method. Immunoblotting for TRF2, TCF1, gH2AX, and GAPDH control (**a**) and quantification of the arbitrary unit is shown (**b**) (data from 5 HS, paired t-test). **c**, Representative histograms and quantitative data for TCF1 MFI from 7 HS (unpaired t-test). **d-f**, Representative dot plots, and quantitative data for percentages of HLA-DR^+ (**d**), $GLUT1^+$ (**e**), and $GzmB^+$ (**f**) (data from 6 HS, paired t-tests). **g**, **h**, After TRF2

knockdown, cells were harvested and stimulated with PMA/Ionomycin and Brefeldin A for 6 hr. Flow cytometry was performed to measure intracellular cytokine IL-2 production and cell apoptosis. Representative dot plots and quantitative data for percentages of IL-2^+ (Wilcoxon test) (**g**) and Annexin$^+$ 7-AAD$^+$ (paired t-test) (**h**), data from 13 HS. **i**, **j**, HS CD4$^+$ T cells was transduced with scramble shRNA or pWPiR vector control or TERF2 shRNA or pWPiR-TRF2 lentiviruses. Representative dot plots and quantitative data for percentages of KLRG1$^+$ (data from 5 HS, Wilcoxon tests) (**i**) and gH2AX$^+$ (data from 6 HS, Wilcoxon t-test) (**j**). Mean ± s.e.m, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Discussion

Globally, among estimated 1,136,000 HCV-related deaths in 2016, more than two-third (794,000) were non-liver related deaths known as extrahepatic manifestations including mixed cryoglobulinemia, chronic kidney disease, B-cell lymphoma, type 2 diabetes and major adverse cardiovascular events, *etc*. [49]. Chronic antigen exposure or immune stimulation is considered as a major factor or driving force contributed to extrahepatic manifestations reported in chronic HCV infection [98]. Thus, studying mechanisms of immune activation in chronic HCV infection could not only help to develop novel therapeutics to reduce HCV-related comorbidities but also boost basic scientific understanding of adaptive immune off-balance during chronic viral infections in humans. In this study, we examined immune activation status and cellular differentiation in peripheral CD4$^+$ T cells from chronic HCV cohort and compared to gender- and age-matched HS. We demonstrated that (**Fig. 3.7**) 1) HCV-CD4$^+$ T cells exhibited an elevated expression of cellular activation markers (HLA-DR, GLUT1, GrZB) and short-lived effector marker KLRG1$^+$CD127$^-$ compared to HS. 2) HCV-CD4$^+$ T cells showed downregulations of stem cell-liked memory T cell transcription factor TCF1 and telomere shelterin subunit TRF2. 3)

Mechanistically, HCV-CD4$^+$ T cells exhibited hyperactivation of Akt/mTOR/pS6 signaling upon TCR stimulation, inducing stronger Th1 polarization and producing high-level inflammatory cytokines IFNγ and TNFα but less survival cytokine IL-2. Strong Akt/mTOR/pS6 signaling also led to more DNA damage and cellular apoptosis, while inhibition of this signaling by Akt specific inhibitor MK2206 could reduce not only DNA damage and cell apoptosis but also slowing down precursor memory T cell loss by maintaining the expression of transcription factor TCF1 during TCR stimulation. 4) Furthermore, we identified TRF2 as a key regulator connecting T cell activation and DNA damage and cellular apoptosis. We showed that T cell activation significantly downregulated TRF2 as early as 2-hour post TCR stimulation, which leads to telomere uncapped and targeted by cellular DDR and ultimately cellular apoptotic death.

Our results on biased CD4 lineage differentiation and inflammatory (IFNγ and TNFα) cytokine production in HCV-CD4$^+$ T cells were consistent with previous reports in chronic HCV infection [48, 52, 54, 99]. Elevated proinflammatory CD4$^+$ T cell responses have been reported to be associated with chronic HCV-induced mixed cryoglobulinemia vasculitis (CV), an extrahepatic manifestation with a serious autoimmune condition [100, 101]. Although DAA therapy could benefit HCV-related CV by restoring immune tolerance, DAA therapy did not reduce IFNγ$^+$CD4$^+$ populations in chronic HCV patients at the end of treatment or long-term post-treatment (12-24 weeks) after achieving virologic cure [52, 100]. In addition, our study revealed a key role for IFNγ in activation-induced T cell death [94, 102, 103]. These results indicate that excessive production of pro-inflammatory cytokine IFNγ/TNFα during chronic HCV infection could complicate HCV-related comorbidities by inducing T cell dysfunction and apoptosis.

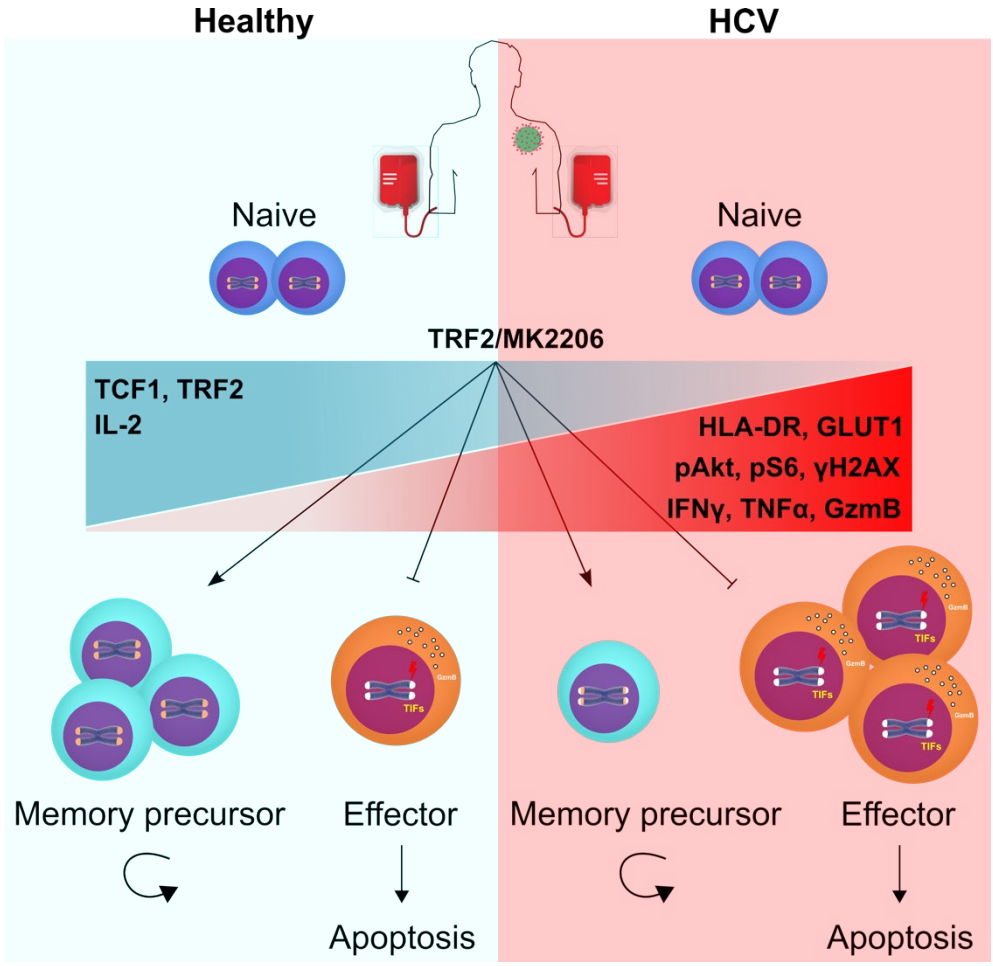


Figure 3.7. Schematic illustration of the proposed mechanism for the prone to pro-inflammatory effector memory T cell differentiation in chronic HCV infected individuals compared to age- and gender-matched HS. Naïve $CD4^+$ T cell from HCV patient exhibited elevated activation status while showed downregulation of telomere shelterin subunit TRF2 and stem cell-liked memory T cell transcription factor TCF1. *Ex vivo* TCR stimulation in HCV naïve $CD4^+$ T cell accumulating high level of PI3K/Akt/mTOR signaling, led to biased effector memory differentiation with high production pro-inflammatory cytokines (IFNγ and TNFα), telomere DNA damage accumulation, and cellular apoptosis. Mechanistically, T cell activation and accumulated PI3K/Akt/mTOR signaling induced TRF2 and stem cell-liked memory T cell transcription factor TCF1 downregulations, resulting in telomeric DNA damage accumulation inducing cellular apoptosis and losing stemness of T cell. Inhibition of Akt or re-enforcing TRF2

expression during T cell activation could promote precursor T cell differentiation and reduced activation-induced DNA damage accumulation and cell death.

The abilities of T cell sensing antigenic and inflammatory signals are critical for a successful adaptive immune response, which is critically regulated by the PI3K/Akt/mTOR signaling pathway. This pathway controlled diverse cellular processes, including T cell activation, cell cycle progression, memory and effector cell differentiation, proliferation, and metabolic cues [104-108]. Although this pathway plays an essential role in T cell immunity, many have reported that PI3K gain-of-function mutations carried individuals known as activated PI3K-δ syndrome (APDS), exhibited hyperactivation of pAkt and pS6 in T cells, led to disrupting T cell homeostasis, inducing senescent effector T cell accumulation and naïve T cell loss, resulting in failure to control infections due to T cell dysfunction [109-114]. In line with these observations, we showed here that circulating $CD4^+$ T cells from HCV patients exhibited hyperactivation of PI3K/Akt/mTOR signaling upon TCR stimulation in which T cell differentiation was skewed toward inflammatory effector phenotype with less precursor memory T cell transcription factor TCF1, along with more DNA damage and cellular apoptosis, suggesting overactivation of PI3K/Akt/mTOR signaling, at least in part, contributed to T cell dysfunction during chronic HCV infection. Indeed, inhibition of PI3K/Akt/mTOR pathway by blocking PI3K-δ, mTOR, or Akt has been shown to be successful to limit T cell defects by preserving precursor memory T cell population and improving immune dysfunction in people with APDS [110, 115]. Using Akt specific inhibitor, our results revealed a significant reduction in TCR stimulation-induced memory T cell loss, DNA damage, and apoptosis in circulating $CD4^+$ T cells derived from HCV-infected patients. The similarities in T cells lymphopenia with the reduction of naïve population and the expansion of effector memory T cells were observed in people with APDS and here in

chronic HCV infection linked to the notion that PI3K/Akt/mTOR hyperactivation is striking and harmful. Further studies are needed to elucidate the mechanisms of PI3K/Akt/mTOR hyperactivation, triggering chronic immune activation, and inducing T cell dysfunction and apoptosis during chronic viral infections in humans.

T and B lymphocytes exhibit asymmetric cell division upon stimulation to give rise to two distinct fated daughter cells of which one harbored more activated and differentiated effector prone (intense PI3K signaling and low TCF1 expression) and a sibling with more quiescent memory preserved (less PI3K activity and high TCF1 expression) phenotype [116-119]. These studies pointed out the diverse abilities of the PI3K pathway to enable lymphocytes to conduct clonal differentiation alongside self-renewal in their cell division. Thus, chronic antigen stimulation could drive PI3K hyperactivation, triggering an off-balance adaptive immune response with increasing effector functions while blunting memory precursors. In addition, studies in cancer immune therapy have shown that *ex vivo* blockade of the PI3K/Akt/mTOR pathway in adoptively transferred T cells could enhance its antitumor fitness by preventing terminal effector differentiation while preserving memory precursors in these T cells [120-123]. Consistent with these reports, we revealed here a significant negative correlation of pS6 and TCF1, indicating that TCR activation mechanistically drives memory precursor deficiency in antigen-stimulated HCV-$CD4^+$ T cells. Our data further demonstrated that using specific Akt inhibitor treatment of TCR-stimulated $CD4^+$ T cells from both HS and HCV subjects could preserve stem cell-liked memory T cell transcription factor TCF1 while reducing inflammatory cytokines production, DNA damage, and cellular apoptosis, suggesting that modulating Akt signaling could balance T cell homeostasis and downscale the immune activation/inflammation-induced DNA damage and T cell apoptosis in chronic viral infection.

Aging T cells harbored hyperactivity of the PI3K/Akt/mTOR signaling and exhibited a pro-inflammatory and short-lived effector memory phenotype that has been reported consistently in the elderly [124, 125], autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus [126, 127] and tumor-infiltrating lymphocytes in cancer [128]. These studies are in line with our results, solidifying the shreds of evidence of chronic immune activation inducing T cells dysfunction and premature aging. Additionally, given the unique ability to undergo exponentially proliferation to combat invading pathogens from limited memory precursors, T cells reportedly harbored a short cell cycle, impacting clonal progeny cells with high susceptibilities to DNA damage and cellular apoptosis [129]. Indeed, DNA damage-induced T cell apoptosis has been shown to be associated with T cell dysfunction in RA patients [130] and in chronic virus-infected individuals [41, 42, 44-46]. Similar mechanisms triggering DNA damage accumulation in T cells in RA and HIV, HCV infected patients have been reported including insufficient DNA damage repair serine-protein kinase ATM, oxidative DNA damage, and telomere shortening [39, 41, 42, 45, 65, 131]. However, direct evidence connecting immune stimulation and telomeric DNA damage accumulation in TCR-stimulated T cells remained to be elucidated. We previously reported that TRF2 was ubiquitously degraded, at least in part, regulated by p53 and E3 ubiquitin-protein ligase Siah1 axis, in both resting and TCR- stimulated naïve $CD4^+$ T cells isolated from chronic HCV infected individuals [41]. In this study, we further revealed that TRF2 was markedly suppressed in TCR-activated T cells as early as 2 hours after stimulation, while dysfunctional TIFs accumulation can be observed significantly at 24 hours post-stimulation. TRF2 plays crucial roles in repressing cellular DDR machinery by forming T-loops structures to “hide” telomere ends being recognized as DNA breaks [132] and TRF2 protein itself can directly bind to and inhibits ATM kinase activity [133] or suppresses E3 ubiquitin ligase RNF168, preventing

53BP1 accumulation at telomeres[19]. Thus, our results on TRF2 downregulation in HCV-$CD4^+$ T cells, in memory (versus naïve) $CD4^+$ T cells, and in *ex vivo*, TCR-stimulated T cells could trigger t-loops unwinding and render telomeres susceptible to cellular DDR, and with a very short cell cycle window, the DDR in activated T cells could not sufficiently repaired DNA damage, which ultimately resulted in cellular apoptosis. Importantly, we also showed that TRF2 overexpression in TCR-stimulated $CD4^+$ T cells can significantly limit DNA damage and apoptosis accumulation. Thus, in this study, we reported a significant role for TRF2 in controlling chronic immune stimulation-induced DNA damage and cell death in T cells, opening new therapeutic revenues to improve T cell function and vaccine response in vulnerable populations such as the elderly and chronically virus-infected individuals.

CHAPTER 4. SUMMARY AND FUTURE DIRECTION

Summary

Telomere integrity is essential to life, as telomere clearly preserves genomic DNA stability and cell proliferative potential. Thus, recognizing telomeric DNA damage due to TRF2 uncapping as a fundamental mechanism of cellular aging will create a new paradigm in cell aging research. Notably, chronic infection or inflammation- induced immunosenescence (i.e., inflammaging) may prove to be a key molecular process that applies to a wide range of clinical scenarios. Nevertheless, to the best of our knowledge, this is the first report showing that inhibition of shelterin protein TRF2 promotes T-cell telomere attrition and telomeric DNA damage to accelerate T-cell senescence and apoptosis in human viral infection. It should be noted that while telomere TRF2 uncapping explains both telomeric DNA damage and cell apoptosis, it may function as a double-edged sword, resulting in both an overwhelming cell death in acute infection and immune tolerance or immune suppression in chronic infection. Our findings indicate that appropriate manipulation of telomere/TRF2 shelterin machinery may restore T-cell competency and prevent premature immune senescence, thus providing a new strategy to improve immunotherapy and vaccine responses against human viral diseases.

Future Direction

The blunted HBV vaccination responses during HIV, HCV infection may be a result of profound telomere attrition and T cell aging; and a lack of TRF2-dependent telomere protection or telomerase accessibility may accelerate telomere erosion and T cell aging; thus, restoring telomere shelterin machinery may open new avenues to protecting T cells from aging and maintaining immune competence. Despite the clinical significance of T cell aging and blunted

vaccine response seen in these patients, several key questions involving telomere shelterin machinery in the setting of viral infection remain unanswered. Specifically, how telomere erosion and T cell aging are induced, and whether they promote HBV vaccine failure during HIV, HCV infection, remain unclear. This information is essential for developing novel strategies to improve vaccine efficacy in virally infected individuals. Since telomere dynamics have been implicated in determining cell aging and function, and TRF2 is a key factor involved in maintaining telomere integrity, we are going to propose an in-depth evaluation of the role and mechanisms of telomere erosion in T cell aging and HBV vaccine response during HIV, HCV infection, and whether modifying the TRF2-dependent telomere-shelterin machinery can reprogram T cell cycle progression and restore T cell survival. This research will have a major impact on our understanding as to how viral infection modulates host immune (vaccine) responses. Understanding such mechanisms, using HBV vaccine failure in HIV, HCV infection as a model, is critical for designing strategies to protect telomeres from unwanted DDR, rescue T cells from aging, and improve vaccine efficacy in virally infected hosts. As such, the proposed study is significant and timely.

1) Studying the mechanism of telomere loss on premature T cell aging in vaccine response is a novel yet unexplored area in human diseases. By examining telomeres in HBV-vaccine responder and non-responder, and the machinery of telomere-sheltering, we can better understand the mechanisms of T cell aging and dysfunction in our HIV and HCV cohort. Such a mechanistically guided approach holds promise to be more successful than broad association studies.

2) T cell exhaustion and senescence have been identified as two distinct pathways controlling T cell fate, but they often are investigated separately. It remains unclear whether

exhausted T cells are also senescent, or vice versa. Most importantly, whether blocking pathways that maintain exhaustion, senescence, or both, can boost HBV vaccine responses in HIV- or HCV-infected individuals is unknown. These studies will explore the intersection of the novel fields of telomere-TRF2 functioning with T cell exhaustion and senescence in the context of vaccine responses.

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APPENDIX

APPENDIX A: Abbreviations

HCV	Hepatitis C Virus
HLA-DR	Human Leukocyte Antigen – DR isotype
GLUT1	Glucose Transporter 1
CD127	IL-7Rα
KLRG1	Killer Cell Lectin Like Receptor G1
TCF1	T Cell Factor 1
TRF2	Telomeric Repeat binding Factor 2
PI3K	Phosphatidylinositol 3-kinase
Akt	Protein kinase B
mTOR	Mammalian Target of Rapamycin
TCR	T Cell Receptor
TIF	Telomere dysfunction induced foci
DNA	Deoxyribonucleic acid
DAA	Direct-acting antiviral
PD-1	Programmed cell death protein 1
Tim-3	T cell immunoglobulin mucin 3
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
p16	Cyclin-Dependent Kinase Inhibitor p16
p21	Cyclin-dependent kinase inhibitor 1
RNA	Ribonucleic acid
TRF1	Telomeric Repeat binding Factor 1
TIN2	TERF1-interacting nuclear factor 2
RAP1/TERF2IP	Telomeric repeat-binding factor 2-interacting protein 1
POT1	Protection of Telomere 1
TPP1	named from the first letter from these proteins **T**INT1, **P**IP1 and **P**TOP
hTERT	Human Telomerase reverse transcriptase

DDR	DNA Damage Response
ATM	Ataxia– Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related protein
PARP1	Poly (ADP-ribose) polymerase 1
c-NHEJ	Canonical Non-Homologous End Joining
Alt-NHEJ	Alternative Non-Homologous End Joining
HDR	Homology-Directed Repair
RPA	Recombinase polymerase amplification
CYREN	Cell cycle regulator of non-homologous end joining
53BP1	p53 Binding Protein 1
Rif1	Rap1-interacting factor 1
Rev7	Mitotic spindle assembly checkpoint protein MAD2B
HIV	Human Immunodeficiency Virus
MAIT	Mucosal-Associated Invariant T
SVR	Sustained Virological Response
DMEM	Dulbecco's Modification of Eagle's Medium
FBS	Fetal Bovine Serum
CO2	Carbon Dioxide
PBMCs	Peripheral Blood Mononuclear Cell
BSA	Bovine serum albumin
EDTA	Ethylenediamine tetra-acetic acid
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
PCR	Polymerase Chain Reaction
HS	Health Subject
RIPA buffer	Radioimmunoprecipitation assay buffer
BCA assay	Bicinchoninic Acid assay
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
γH2AX	Phosphorylated Histone family member X

CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
NaCl	Sodium Chloride
NP40	Nonidet P-40
SDS	Sodium Dodecyl Sulfate
IgG	Immunoglobulin G
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
DAPI	4',6-Diamidino-2-Phenylindole
siRNA	Small interfering RNA
SEM	Standard Error of Mean
ANOVA	Analysis of variance
7-AAD	7-Aminoactinomycin D
NK	Natural Killer cell
RT-PCR	Reversed Transcriptase – PCR
p27	Cyclin-dependent kinase inhibitor 1B
DUSP-6	Dual Specificity Phosphatase 6
Fas/Fas-L	FAS receptor/FAS ligand
DCFDA	2',7'-dichloroflurescin diacetate
ROS	Reactive Oxygen Species
DMSO	Dimethyl Sulfoxide
Siah-1	Siah E3 ubiquitin protein ligase 1
IRES	Internal Ribosomal Entry Site
IL-2	Interleukin 2
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
mTORC1	Mammalian Target of Rapamycin Complex 1
TSC2	Tuberous Sclerosis Complex 2
PMA	Phorbol Myristate Aacetate
HRP	Horseradish peroxidase

DPBS	Distilled Phosphate-buffered saline
CHX	Cycloheximide

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