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Protection of CD4+ T cells from hepatitis C virus infection-associated senescence via ΔNp63–miR-181a–Sirt1 pathway

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ABSTRACT

T cell dysfunction has a crucial role in establishing and maintaining viral persistence. We have previously shown a decline in miR-181a, which regulates CD4+ T cell responses via DUSP6 overexpression, in individuals with hepatitis C virus (HCV) infection. Here, we describe accelerated T cell senescence in HCV-infected individuals compared with age- and sex-matched healthy subjects. Mechanistic studies revealed that up-regulation of transcription factor ΔNp63 led to the decline of miR-181a expression, resulting in an overexpression of the anti-aging protein Sirt1, in CD4+ T cells from HCV-infected individuals. Either reconstituting miR-181a or silencing ΔNp63 or Sirt1 expression in CD4+ T cells led to accelerated T cell senescence, as evidenced by an increased senescence-associated β-galactosidase (SA-β-gal) expression, shortened telomere length, and decreased EdU incorporation; this suggests that HCV-induced T cell senescence is counterregulated by the ΔNp63–miR-181a–Sirt1 pathway. An increase of IL-2 production was observed in these senescent CD4+ T cells and was driven by a markedly reduced frequency of Foxp3+ regulatory T (T_{reg}) cells and increased number of Foxp3− effector T (T_{eff}) cells upon manipulating the ΔNp63–miR-181a–Sirt1 pathway. In conclusion, these findings provide novel mechanistic insights into how HCV uses cellular senescent pathways to regulate T cell functions, revealing new targets for rejuvenating impaired T cell responses during chronic viral infection. *J. Leukoc. Biol.* 100: 1201–1211; 2016.

Introduction

HCV is a blood-born pathogen characterized by a high rate (>80%) of chronic infection. HCV has evolved numerous strategies (such as developing quasispecies and inducing T cell apoptosis or exhaustion and senescence) to evade host immunity and harness virus persistence, thus, becoming an excellent model to study the mechanisms of chronic infection in humans [1–4]. Although the use of DAAs, including sofosbuvir and ledipasvir, can completely clear HCV in most infected individuals, this therapeutic cocktail is costly and already facing new issues, such as viral mutation, relapse, and reinfection following therapy [3, 4]. The failure to successfully manage this chronic infection in specific populations and to develop an effective HCV vaccine stems from our incomplete understanding of the host immune response to HCV that permits viral persistence.

T cells have a crucial role in viral clearance or persistence; however, the precise mechanisms that control their responses during viral infection remain unclear. MicroRNAs (miRNAs or miR) are a class of small, noncoding RNAs that can regulate gene expression through posttranscriptional repression or target mRNA degradation [5]. Compelling evidence has suggested that some miRNAs are able to regulate HCV replication and its related liver diseases by directly interacting with the HCV genome or indirectly controlling virus-associated signaling pathways [6–10]. How HCV modulates the expression of miRNAs and host cellular proteins in infected individuals, particularly in T cells, for its persistence in vivo, is less understood. We have recently

Abbreviations: DUSP6 = dual specificity phosphatase 6, EdU = 5-ethynyl-2′-deoxyuridine, ETSU/VA = East Tennessee State University and James H. Quillen VA Medical Center, FISH = fluorescence in situ hybridization, HCV = hepatitis C virus, HS = healthy subject, KLRG1 = killer-cell lectin like receptor G1, MFI = mean fluorescence intensity, miR = microRNA, PD-1 = programmed death 1, PTEN = phosphatase and tensin homolog, SA-β-gal = β-galactosidase, siRNA = small interfering RNA, Sirt1 = sirtuin 1, Tim-3 = T cell immunoglobulin mucin domain protein 3, T_{eff} = effector T cell, T_{reg} = regulatory T cell

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shown that HCV impairs T cell responses via miR-181a-mediated expression of DUSP6 [10]—a signature marker for T cell aging, which raises the TCR activation threshold [11]. Interestingly, T cell proliferation is promoted by inhibiting DUSP6 but not by reconstituting miR-181a, whereas IL-2 production is improved by both manipulations. We hypothesized that miR-181a, in addition to controlling DUSP6, may also regulate other cellular proteins pertinent to T cell responses, balancing the process of immune-mediated viral clearance and inflammatory damage. Thus far, it remains unknown how HCV regulates miR-181a expression and which proteins are controlled by miR-181a to balance DUSP6-mediated T cell senescence.

Viral infections are often accompanied by accelerated, premature T cell aging from overstimulation and activation, followed by exhaustion and senescence [12–16]. Many cell cycle regulators participate in the cell senescence, with the most reliable assays for senescent cells being the telomere length or SA-β-gal [17, 18]. Notably, the transcription factor p63 has been implicated as important in regulating senescence pathways because its absence in conditional knockout mice induces premature aging [19, 20]. p63 is a tumor suppressor p53 homolog, and its major isoform ΔNp63 has been shown to control the expression of miR-181a and its target Sirt1 in keratinocyte senescence [21]. Sirt1 is a NAD^+-dependent deacetylase and protects against cell aging by deacetylating proteins that regulate cellular processes, including inflammation, infection, and malignancy [22, 23]. Silencing Sirt1 expression or inhibiting Sirt1 activity can cause telomere dysfunction, leading to cellular senescence [24, 25]. Nevertheless, these studies have not explored the role of ΔNp63, miR-181a, and Sirt1 in infection-accelerated, premature T cell aging.

In this study, we demonstrate an infection-associated acceleration of T cell aging, as evidenced by shortened telomere length and increased SA-β-gal^+ T cells, accompanied by a decline of miR-181a and overexpression of ΔNp63 or Sirt1, in individuals with chronic HCV infection when compared with age-matched, healthy subjects. We further demonstrate that HCV-induced T cell senescence is counterregulated by the ΔNp63–miR-181a–Sirt1 pathway.

MATERIALS AND METHODS

Subjects

The study protocol was approved by a joint institutional review board at East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA, Johnson City, TN, USA). Written, informed consent was obtained from all participants. The study subjects, recruited from ETSU/VA hepatitis program during 2012∼2015, comprised 2 populations: 1) 108 patients with chronic HCV infections confirmed by positive HCV RNA levels, ranging from 12,300 to ∼50,000,000 IU/ml, with 70% HCV genotype 1, 30% type 2 or 3. The clinical parameters, including infection history, risk factors, ultrasound, and transaminase levels, were also used to confirm the diagnosis, and all patients with HCV were recruited before antiviral therapy, with exclusion for other potential confounding factors, such as malignancy, transplantation, infection with HBV, HIV, or use of immunosuppressive drugs; and 2) 42 healthy subjects (HS), whose blood buffy coats were derived by Key Biologics, LLC (Memphis, TN, USA). The age and sex of HS controls were matched to HCV-infected individuals. Age-associated, comorbid conditions, mainly diabetes and hypertension, if applicable, were also matched in the study subjects. The characteristics of the subjects to be used in this study are listed in **Table 1**.

Cell isolation and culture

Human PBMCs were isolated from peripheral blood by Ficoll-density centrifugation with LymphoH (Atlanta Biologicals, Flowery Branch, GA, USA). $CD4^+$ T cells were further purified from PBMCs with a negative selection kit (purity >95%; Miltenyi Biotec, Auburn, CA, USA). Cells were cultured with RPMI 1640 containing 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) as previously described [10].

Real-time RT-PCR

miR-181a and U6 miRNA (TaqMan microRNA assays) were assayed by real-time RT-PCR, as described previously [10]. Briefly, miRNAs were isolated from $CD4^+$ T cells using RNAzol RT (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA was generated by TaqMan miRNA Reverse Transcription kit (Thermo Fisher Scientific). Real-time PCR was conducted using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). miR-181a level, normalized to the U6 small nucleolar RNA, was quantified with the relative quantification method ($2^{-\Delta\Delta Ct}$). All PCR assays were performed in triplicate, and results are represented as means ± SD.

Transfection

Purified $CD4^+$ T cells from patients with HCV infections were transfected with 30 pM of miR-181a precursors or the negative control (Thermo Fisher Scientific), or 30 pM of Sirt1 siRNA or p63 siRNA and negative control (Ambion, Austin, TX, USA; Thermo Fisher Scientific), using the human

TABLE 1. Characteristics of the subjects to be used in this study

Figure	Subject ID	Age/range (y)	Sex (M/F)	Ethnicity (C/AA/A)	Genotype (1/2/3)	Viral load (IU/ml)	ALT/AST (U/L)
Fig. 1	HCV1-22	∼50/27–66	20/2	15/7	15/3/4	12,300–26,300,000	28–226/23–372
	HS 1-22	∼46/26–57	18/4	13/9	N/A	N/A	N/A
Fig. 2	HCV23-56	∼49/28–67	31/3	25/7/2	23/6/5	15,500–50,000,000	24–200/25–172
	HS 12-34	∼46/22–65	15/7	9/5/8	N/A	N/A	N/A
Fig. 3	HCV57-68	∼52/35–61	11/1	9/3	9/2/1	399,787–3,887,432	23–154/28–121
Fig. 4	HCV69-84	∼51/31–69	15/1	13/3	11/4/1	12,354–4,946,323	41–251/28–198
	HS 35-42	∼49/26–67	7/1	6/2	N/A	N/A	N/A
Fig. 5	HCV85-108	∼51/29–68	23/1	17/7	17/3/4	14,300–4,546,279	28–210/23–236
Total	HCV1-108	∼51/27–69	100/8	79/27/2	75/18/15	12,300–50,000,000	23–251/23–372
	HS 1-42	∼47/22–67	30/12	22/12/8	N/A	N/A	N/A

A, Asian; AA, African American; ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, Caucasian; F, female; M, male; N/A, not applicable.

T lymphocyte Nucleofector kit and the Nucleofector I device (Lonza, Allendale, NJ) according to the manufacturer's instructions. After transfection, cells were cultured for 48h with 10% serum IMDM media (Lonza Inc., Allendale, NJ, USA) for further experiments.

Flow cytometry

Procedures for detection of cell surface markers and intracellular cytokine were performed as described previously [10]. Briefly, purified PBMCs or $CD4^+$ T cells were incubated with or without anti-CD3/CD28 antibodies (1 μg/ml; eBioscience, San Diego, CA, USA) as indicated in the results. For Sirt1 and IL-2 intracellular staining, 1 μg/ml Brefeldin A (BioLegend, San Diego, CA, USA) was added 4 h before cell harvesting to stop cytokine secretion. The cells were stained for surface marker expression and then fixed and permeabilized using an Inside Stain kit (Miltenyi Biotec). The following antibodies were used for immunostaining: PE-CD4 (clone RPA-T4), Alexa Fluor 488-Sirt1 (Abcam, Cambridge, MA, USA), FITC-DUSP6 (Bioss Inc., Woburn, MA, USA), APC-CD4/PE-IL-2 (clone MQ1-17H12; eBioscience), and PE-CD69 (clone FN50)/PE-CD25 (clone BC96; BioLegend). The isotype control (eBioscience) staining and the fluorescence minus one strategy were used to determine background levels and to adjust multicolor compensation for cell gating. The cells were analyzed on an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Western blot

$CD4^+$ T cells were isolated from patients with HCV or transfected and stimulated with anti-CD3/CD28 (1 μg/ml) for 48 h. The expression of ΔNp63 was measured by Western blot using anti-ΔNp63 or anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies and a HRP-conjugated secondary antibody (EMD Millipore, Temecula, CA, USA). The membranes were analyzed by Amersham ECL prime western blotting detection reagents (GE Healthcare Biosciences, Pittsburgh, PA, USA) on a Bio-Rad chemiDoc MP imaging system.

Proliferation assays

$CD4^+$ T cells were transfected as described above, stimulated with anti-CD3/CD28 for 7 d, and then incubated with 10 μM EdU for 2 h. EdU incorporation was evaluated with the Click-iT Plus EdU Alexa Fluor 488 flow cytometry assay kit (Thermo Fisher Scientific) [26], in conjunction with cell death staining using 7-aminoactinomycin D (Thermo Fisher Scientific), analyzed by an Accuri C6 flow cytometer (BD Biosciences) and FlowJo software.

SA-β-gal staining

$CD4^+$ T cells isolated from the subjects or treated with HCV core and β-gal control protein (1 μg/ml; ViroGen Corporation, Watertown, MA, USA) or transfected as described above were cultured in 12-well culture plates, washed with PBS, fixed, and stained at 37°C for 24 h using a SA-β-gal staining kit (Cell Signaling Technology, Beverly, MA, USA) per manufacturer's instructions.

Telomere length

Telomere length of $CD4^+$ T cells was measured using flow-FISH protocol as described previously [14]. Briefly, $CD4^+$ T cells from patients with HCV and HS or healthy $CD4^+$ T cells treated with HCV core and β-gal protein (1μg/ml) were stained with CD4-CY5 (SouthernBiotech, Birmingham, AL, USA). After fixation and permeabilization, the cells were incubated in hybridization buffer with 1μg/ml of FITC-PNA Tel C probe (CCCTAAC repeats) (PNA Bio Inc., Thousand Oaks, CA, USA). Samples were heated for 10 min at 85°C, rapidly cooled on ice, and hybridized at room temperature in the dark for 2 h. Samples were washed and analyzed immediately by flow cytometry and lymphocyte telomere length was shown as MFI.

Statistical analysis

Study results were summarized for each group, and results were expressed as means ± SD. Comparisons between groups were made by ANOVA using Prism 5 (GraphPad Software, La Jolla, CA, USA). A pair-wise t test was used to compare the significance of changes in siRNA and miRNA transfection assays. Values of $P < 0.05$ were considered significant; $P < 0.01$ and $P < 0.001$ were considered highly significant.

RESULTS

Chronic HCV infection is associated with an accelerated T cell senescence

It is well-established that persistent viruses (such as HCV and HIV) can lead to T cell exhaustion and/or senescence by up-regulation of PD-1, Tim-3, or KLRG1 and $p16^{ink4a}$ expression [12–16, 27–30]. Because the most reliable markers for assessing the cellular senescence are SA-β-gal expression and telomere length [17, 18], here, we examined these senescent markers in $CD4^+$ T cells from patients with chronic HCV infections vs. HS. We found that telomere length in $CD4^+$ T cells from patients chronically infected with HCV was significantly shortened when compared with age-matched HS (**Fig. 1A**). In addition, SA-β-gal expression increased in senescent $CD4^+$ T cells in HCV-infected patients compared with age-matched HS (Fig. 1B). Because patients with chronic hepatitis C often have comorbid conditions that may cause T cell senescence, we tested whether the decrease in telomere length and the increase in SA-β-gal expression were directly caused by HCV rather than other factors. Purified healthy $CD4^+$ T cells were incubated with HCV core, the protein to be expressed upon HCV infection and which has been shown to be immunosuppressive [31–33], followed by measuring the telomere length and SA-β-gal expression in $CD4^+$ T cells. Consistent with the observation in HCV-infected patients and HS in vivo, healthy $CD4^+$ T cells treated with HCV core antigen for 7 d in vitro exhibited reduced telomere length (Fig. 1C) and increased SA-β-gal^+ T cells (Fig. 1D) compared with those exposed to the control β-gal protein, although the working concentration of HCV core protein (1 μg/ml) in this in vitro experiment was rather high and not physiologic. Nevertheless, these findings suggest that HCV infection accelerates $CD4^+$ T cell senescence that may have an important role in viral persistence.

Sirt1 is involved in counterregulating the HCV infection-associated premature T cell aging

To investigate the mechanisms involved in regulating HCV-accelerated premature T cell senescence, we examined the expression levels of Sirt1 - a NAD^+-dependent deacetylase that is associated with aging and age-related diseases [22–25]. As shown in **Fig. 2A**, the protein levels of Sirt1 were significantly up-regulated in $CD4^+$ T cells from 22 HCV-infected patients compared with 22 age-matched HS. To understand the role of Sirt1 in HCV-induced T cell senescence, we silenced Sirt1 expression in $CD4^+$ T cells from HCV-infected patients by its specific siRNA, followed by measuring the markers of T cell senescence and cell proliferation. As previously reported, we could achieve an approximately 60% of transfection efficacy in

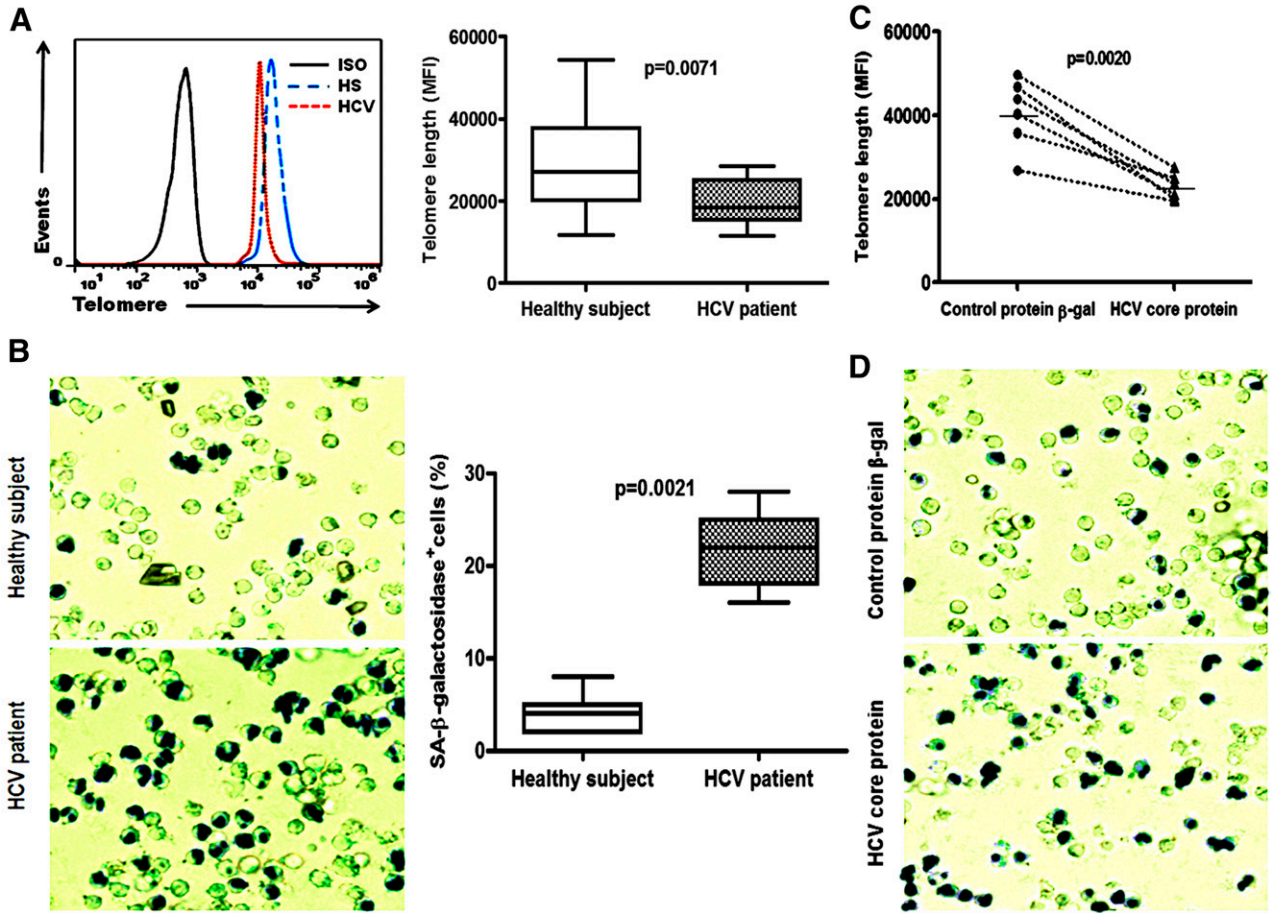

Figure 1. Chronic HCV infection is associated with an accelerated T cell senescence. (A) The telomere length of $CD4^+$ T cells is determined by flow-FISH as described in the Materials and Methods. The representative overlaid histogram and summary data show the MFI of telomere length with medians, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers, in $CD4^+$ T cells from 22 HCV-infected patients vs. 16 age-matched HS. ISO, isotype control. (B) SA-β-gal staining and quantification by blue cell counts. Values reported are means ± SD of 3 independent stains from 22 HCV-infected patients vs. 16 HS. (C) Flow-FISH analysis of telomere length in healthy $CD4^+$ T cells treated with HCV core or negative control protein β-gal for 7 d in vitro. (D) SA-β-gal staining in healthy $CD4^+$ T cells treated with HCV core or negative control protein β-gal for 7 d in vitro, as described in the Materials and Methods. The data were reproducible in repeated experiments using $CD4^+$ T cells purified from 2 HS.

human primary $CD4^+$ T cells using the Human T Lymphocyte Nucleofector Kit and the Nucleofector I Device (Lonza, Allendale, NJ) [10]. A representative histogram and summary data from 12 HCV-infected patients showed that Sirt1 expression was significantly reduced by transfecting Sirt1 siRNAs when compared to negative control siRNAs (Fig. 2B). Importantly, the telomere length decreased (Fig. 2C) and the percentage of SA-β-gal^+ T cells increased (Fig. 2D) upon Sirt1 silencing. Additionally, T cell proliferation, as assessed by the incorporation of EdU, was also decreased by the Sirt1 silencing (Fig. 2E). Taken together, these results suggest that the elevated Sirt1 plays an anti-aging effect in senescent $CD4^+$ T cells during HCV infection.

miR-181a controls HCV-accelerated, premature T cell aging by regulating Sirt1 expression

Bioinformatics analysis by TargetScanHuman database showed that the Sirt1 3′ untranslated region harbors a putative target sequence for miR-181a that is highly conserved among vertebrates [21]. To better understand the relationship between miR-181a and Sirt1 and its role in T cell senescence during HCV infection, we transfected $CD4^+$ T cells from 12 HCV-infected individuals with a miR-181a precursor or its negative control, followed by measuring the levels of Sirt1 and markers for T cell senescence and proliferation. As shown in **Fig. 3A**, transfection of miR-181a precursor, but not the negative control, led to a significant down-regulation of Sirt1 expression in $CD4^+$ T cells, supporting our hypothesis that miR-181a directly regulates Sirt1 in T lymphocytes. Moreover, miR-181a reconstitution significantly reduced the telomere length (Fig. 3B), increased SA-β-gal^+ T cell frequency (Fig. 3C), and decreased EdU incorporation (Fig. 3D), in $CD4^+$ T cells from patients with HCV. These data suggest that, although having a role in promoting HCV-induced T cell senescence through DUSP6 signaling [10], miR-181a can counterregulate HCV-accelerated, premature T cell senescence via the Sirt1 pathway. This reprogramming miR-181a–Sirt1 pathway appears to be dominant over the miR-181a–DUSP6

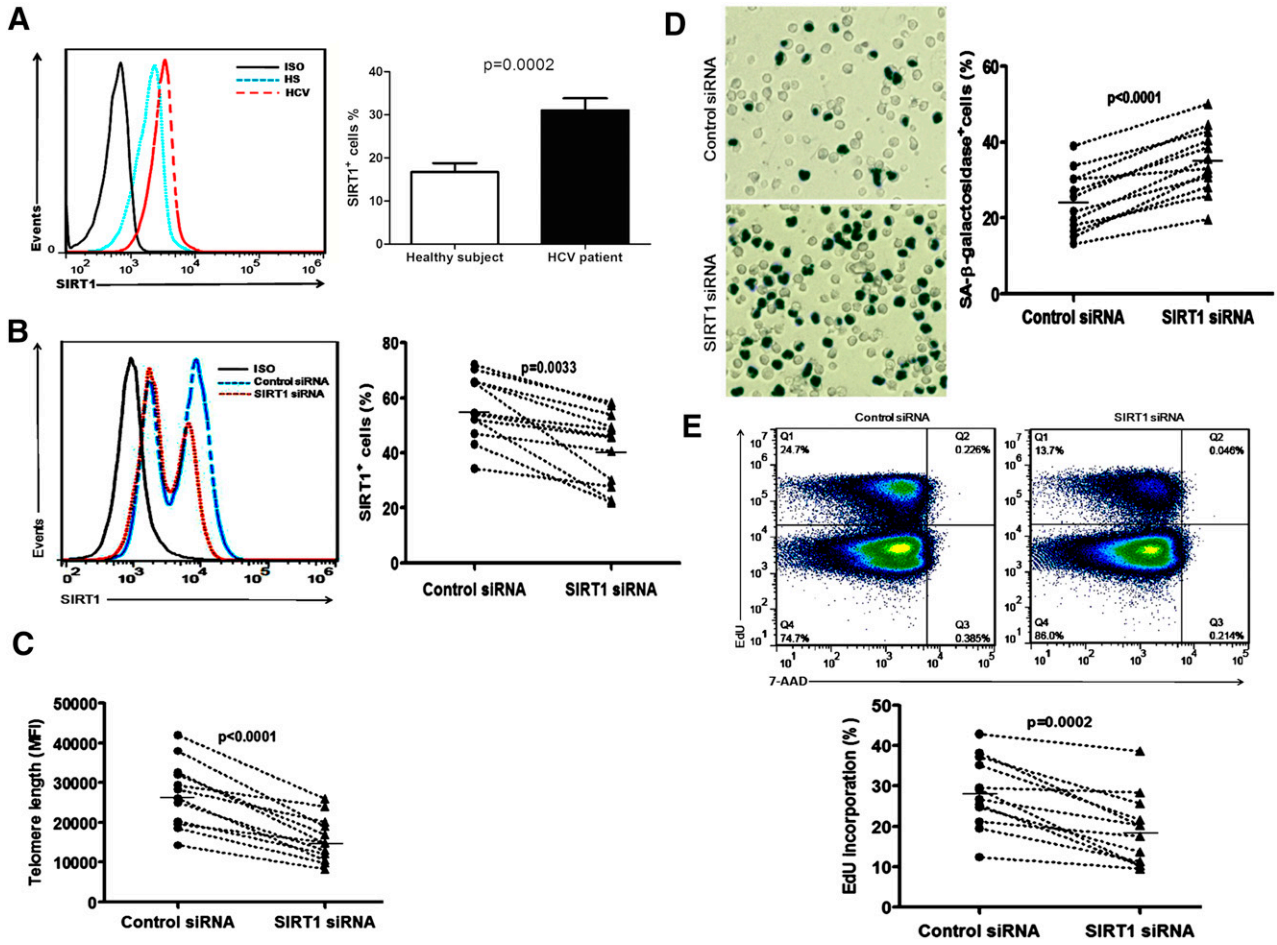

Figure 2. Sirt1 is involved in counterregulating the HCV infection-associated premature T cell aging. (A) Flow cytometric analysis of Sirt1 expression in $CD4^+$ T cells, representative overlaid histogram and summary data from 22 chronically HCV-infected individuals and 22 age-matched HS are shown. (B) Sirt1 expression in $CD4^+$ T cells transfected with Sirt1 siRNA and control siRNA, assessed by flow cytometry, are shown as representative overlaid histogram and summary data from 12 HCV-infected individuals. (C) The MFI of the telomere length, assessed by the flow-FISH at 7 d after transfection in 12 HCV $CD4^+$ T cells transfected with Sirt1 siRNA or control siRNA. (D) The percentage of SA-β-gal^+ cells in 12 HCV $CD4^+$ T cells transfected with Sirt1 siRNA or control siRNA. (E) T cell proliferation, assessed by EdU incorporation, in 12 HCV $CD4^+$ T cells transfected with Sirt1 siRNA or control siRNA. ISO, isotype control.

pathway in the setting of chronic HCV infection because reconstitution of miR-181a leads to an overall effect of greater senescent T cells.

Silencing ΔNp63 advances HCV-induced T cell senescence via the miR-181a-Sirt1 pathway

To further investigate the upstream factors that might regulate the miR-181a–Sirt1-mediated anti-T cell aging process, we chose to study ΔNp63—a transcription factor that has been shown to participate in the senescence process by repression of miR-181a expression in keratinocyte [21]. To that end, we first examined the expression levels of ΔNp63 and found significant up-regulation in $CD4^+$ T cells from HCV-infected individuals compared with HS (**Fig. 4A**). We then silenced the expression of ΔNp63 in $CD4^+$ T cells from patients with HCV and found that lower expression of ΔNp63 in T cells by transfecting its specific siRNA (Fig. 4B) led to an up-regulation of miR-181a (Fig. 4C) and a down-regulation of Sirt1 expression (Fig. 4D), strengthening the linkage of ΔNp63–miR-181a–Sirt1 signaling. As expected, ΔNp63 silencing in $CD4^+$ T cells by transfection of its specific siRNA, but not the control siRNA, resulted in a reduced MFI of telomere length (Fig. 4E), increased percentage of β-gal^+ T cells (Fig. 4F), and decreased incorporation of EdU (Fig. 4G), indicating that silenced cells undergo senescence. This behavior of ΔNp63-silenced $CD4^+$ T cells is quite similar to Sirt1 silencing or miR-181a reconstitution in the same cells, as can be seen in Figs. 2 and 3. These parallel, overlapping results suggest that the HCV-induced, premature T cell aging is counterregulated by the ΔNp63–miR-181a–Sirt1 pathway.

Manipulating the ΔNp63–miR-181a–Sirt1 pathway improves IL-2 production by reducing the $Foxp3^+$ T_{regs} in chronic HCV infection

Despite the presence of more-senescent T cells upon manipulation of the ΔNp63–miR-181a–Sirt1 pathway, TCR activation–induced IL-2 production in $CD4^+$ T cells from HCV-infected

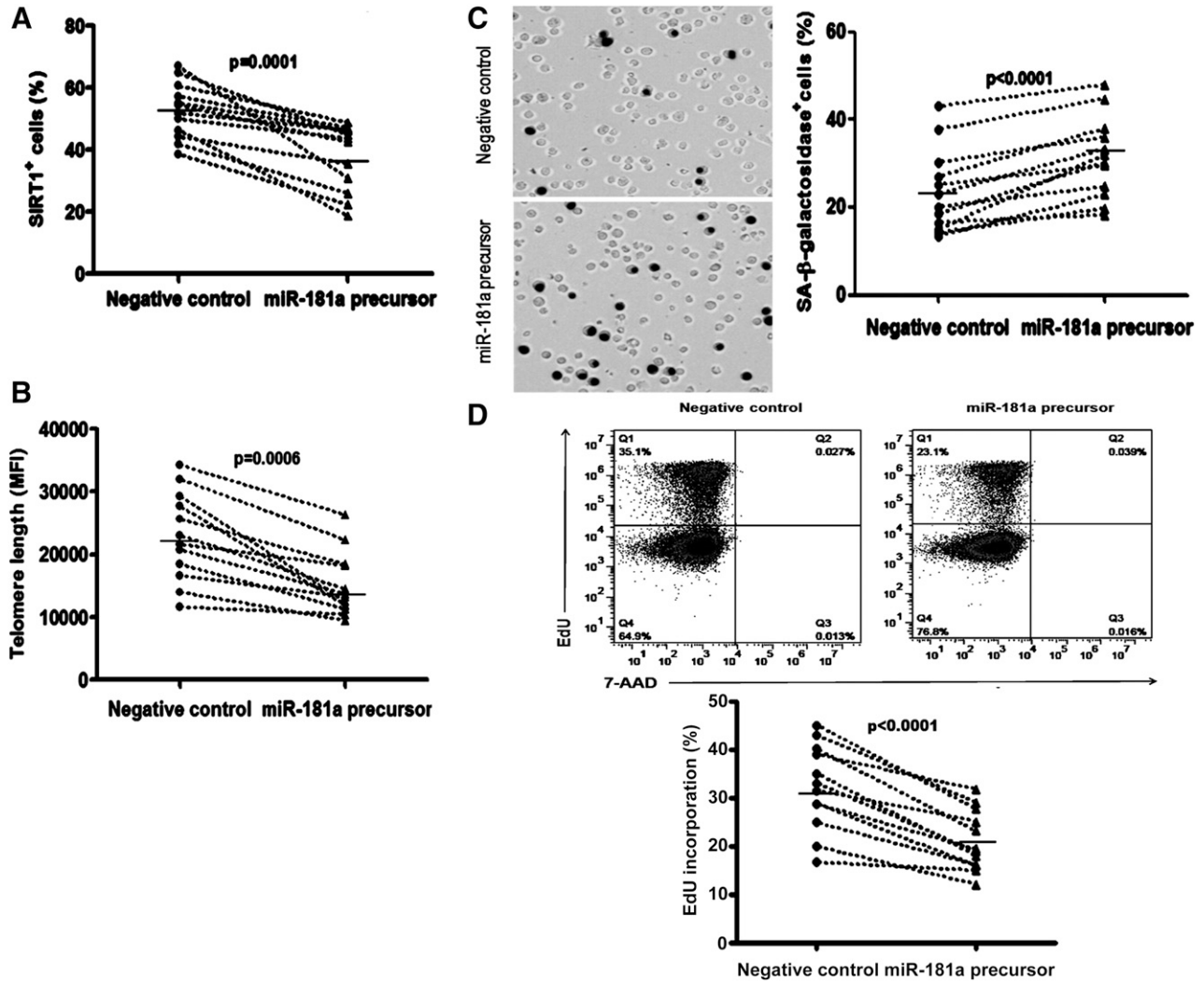

Figure 3. miR-181a controls HCV-accelerated premature T cell aging by regulating Sirt1 expression. (A) Sirt1 expression in $CD4^+$ T cells transfected with miR-181a precursor or negative control, measured by flow cytometry in 12 HCV-infected individuals. (B) The MFI of telomere length in 12 HCV $CD4^+$ T cells transfected with miR-181a precursor or negative control. (C) SA-β-gal staining in 12 HCV $CD4^+$ T cells transfected with miR-181a precursor or negative control. (D) EdU incorporation in 12 HCV $CD4^+$ T cells transfected with miR-181a precursor or negative control.

individuals was paradoxically increased following these manipulations compared with negative controls (**Fig. 5A**). This is in agreement with our previous report [10], indicating that overexpression of miR-181a or silencing of DUSP6 improves IL-2 expression in $CD4^+$ T cells but is not in line with the data as described above showing more-senescent, less-proliferating $CD4^+$ T cells following manipulation of this signaling pathway. We, thus, further sought to determine the subsets of $CD4^+$ T cells to be affected by these treatments. Of interest, the major subsets of $CD4^+$ T cells affected by manipulating the ΔNp63–miR-181a–Sirt1 pathway were primarily $Foxp3^+$ T_{regs}, as evidenced by a marked reduction of $Foxp3^+$ T_{regs} in all treatments consistently (Fig. 5B), whereas the $Foxp3^-$ T_{effs} remain unchanged by silencing Sirt1 or ΔNp63 and increased upon reconstituting miR-181a expression (Fig. 5C). In conjunction with our previous report [10], these findings suggest that the paradoxically increased IL-2 production in $CD4^+$ T cells following these treatments is likely due to an altered balance of $CD4^+$ T cell frequencies or functions in $Foxp3^+$ T_{reg}/$Foxp3^-$ T_{eff} subsets, controlled by the ΔNp63–miR-181a–Sirt1 and ΔNp63–miR-181a–DUSP6 pathways at different phases of HCV infection.

DISCUSSION

T cell dysfunction has a crucial role in establishing and maintaining viral persistence; however, the precise mechanisms that impair their responses during viral infection remain incompletely understood. We have recently shown that HCV impairs T cell responses via miR-181a-mediated overexpression of DUSP6 [10], a process similar to what has been observed in the elderly [11], suggesting that HCV infection may accelerate premature T cell aging through the miR-181a–DUSP6 signaling. In this study, we further demonstrate that the HCV infection–associated T cell

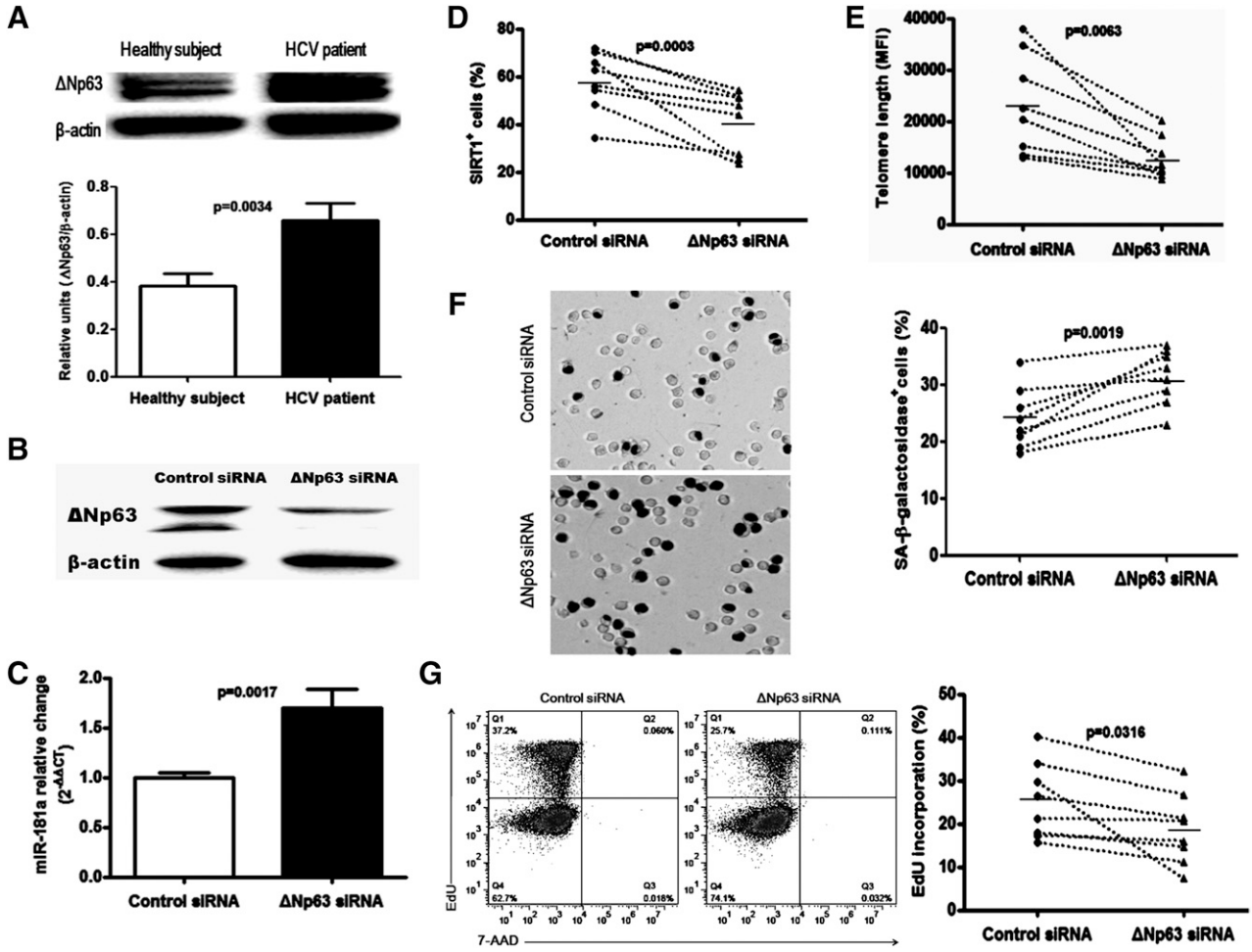

Figure 4. Silencing ΔNp63 advances HCV-induced T cell senescence through miR-181a–Sirt1 pathway. (A) Western blot analysis of ΔNp63 expression in $CD4^+$ T cells from 8 HCV-infected patients vs. 8 age-matched HS. (B) Western blot detection of ΔNp63 expression in $CD4^+$ T cells from HCV-infected patients at 48 h after transfecting ΔNp63 siRNA or control siRNA. (C) Real-time RT-PCR examination of miR-181a at 48 h after transfecting ΔNp63 siRNA or control siRNA. (D) Flow cytometry analysis of Sirt1 in 8 HCV $CD4^+$ T cells at 48 h after transfecting ΔNp63 siRNA or control siRNA. (E) The MFI of telomere length, measured after transfection of ΔNp63 siRNA for 7 d, in 8 independent pairs. (F) SA-β-gal staining in 8 HCV $CD4^+$ T cells at 48 h after transfecting ΔNp63 siRNA or control siRNA. (G) The EdU incorporation, assayed in 8 HCV $CD4^+$ T cells by flow cytometry after transfection of ΔNp63 siRNA or control siRNA and stimulated with anti-CD3/CD28 for 7 d.

senescence is counterregulated by the ΔNp63–miR-181a–Sirt1 pathway. We found that 1) chronically HCV-infected individuals exhibited signs of premature T cell aging, as evidenced by a shortened telomere length and increased SA-β-gal^+ T cells, compared with age-matched HS; 2) mechanistic studies revealed up-regulation of the transcription factor ΔNp63, which leads to a decline in miR-181a expression, resulting in overexpression of Sirt1 in $CD4^+$ T cells from HCV-infected individuals; 3) silencing ΔNp63 or Sirt1 and reconstitution of miR-181a expression in $CD4^+$ T cells from patients with HCV led to accelerated T cell senescence, as evidenced by a remarkably shortened telomere length, increased SA-β-gal^+ T cells, and decreased EdU incorporation, indicating that the HCV-induced T cell senescence may be counterregulated by the ΔNp63–miR-181a–Sirt1 pathway; and 4) IL-2 expression was paradoxically boosted in those more-aging, less-proliferating $CD4^+$ T cells, primarily because of the decreased frequency of $Foxp3^+$ T_{regs} and the increased or unchanged number of $Foxp3^-$ T_{effs} upon manipulation of the ΔNp63–miR-181a–Sirt1 pathway. Our results provide new insights into the transcriptional and translational mechanisms regarding how miR-181a-modulating proteins regulate T cell responses in the setting of chronic viral infection.

During the past 2 decades, miRNAs have emerged as crucial regulators of cell biology with a widespread impact on the etiologies of human diseases, ranging from infections to malignancies [34, 35]. Our previous work discovered that HCV-induced decline in miR-181a expression facilitates viral hijacking of the critical host DUSP6–ERK pathway during viral infection [10]. However, the precise mechanisms for how HCV and miRNA interact, in particular the upstream and downstream molecules involved in HCV-induced, miR-181a-mediated T cell regulation, remain unclear. It is the nature of miRNAs that they do not control a single gene but rather coordinate the expression of sets of genes that are often functionally related or even

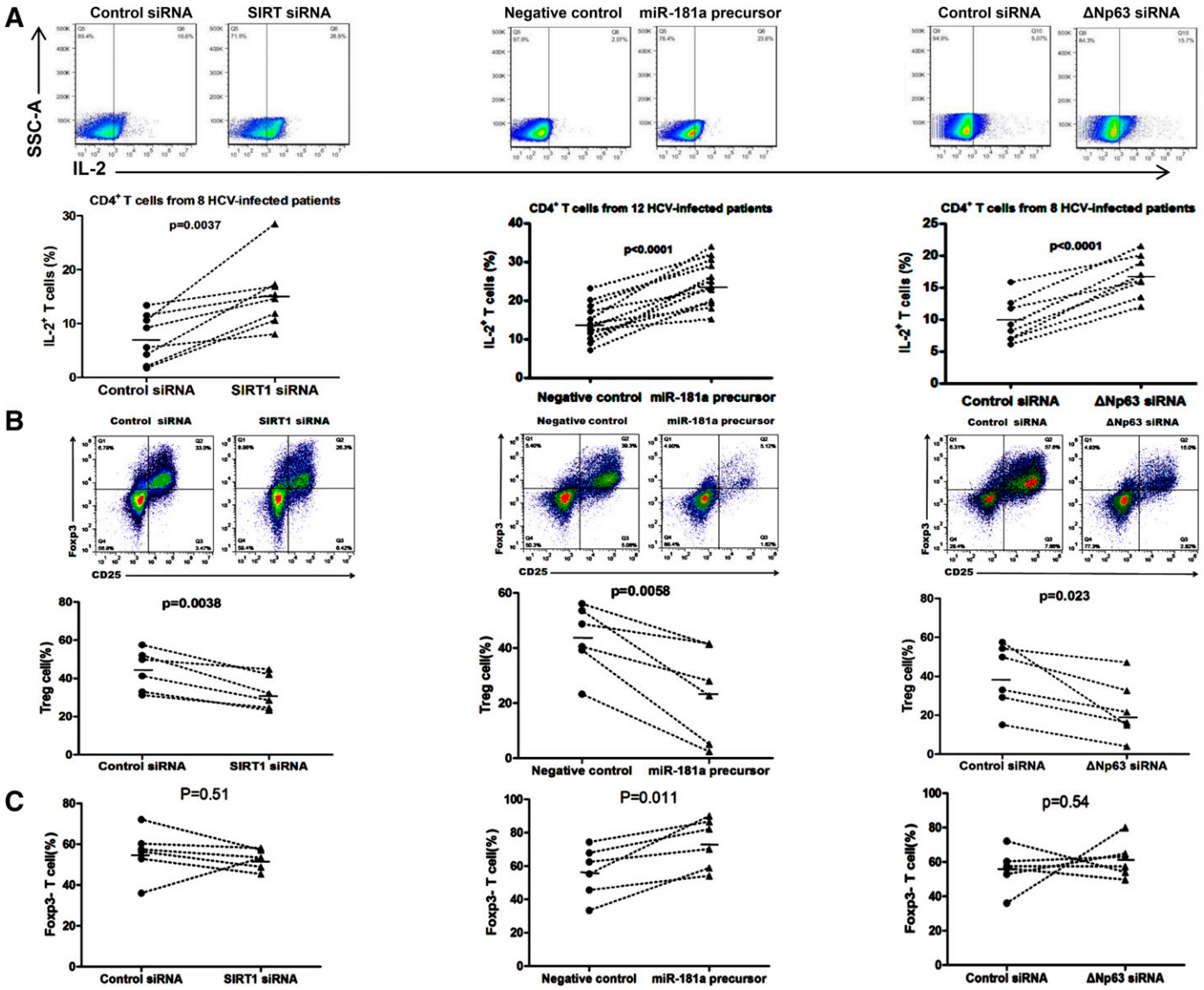

Figure 5. Manipulating ΔNp63–miR-181a–Sirt1 pathway improves IL-2 production by reducing the Foxp3$^+$ T$_{regs}$ in chronic HCV infection. (A) Flow cytometric analysis of IL-2 expression in CD4$^+$ T cells from HCV-infected individuals following transfection. (B) Foxp3$^+$ T$_{reg}$ cell analysis by flow cytometry following transfection with ΔNp63 or Sirt1 siRNA or miR-181a precursor and their negative controls. (C) Foxp3$^-$ T$_{eff}$ cell analysis by flow cytometry following transfection with ΔNp63 or Sirt1 siRNA or miR-181a precursor and their negative controls. Representative dot plots and summary data from multiple experiments are shown.

contradictory. In this study, we focused on exploring the role of miR-181a in HCV chronicity beyond the DUSP6 paradigm, and answered 2 basic but critical questions: 1) how does HCV induce the decline of miR-181a, and 2) what are the consequences of HCV-induced loss of miR-181a during chronic viral infection? Our findings indicate that HCV can suppress the expression of miR-181a via up-regulating the transcription factor ΔNp63 and that miR-181a can regulate CD4$^+$ T cell responses by targeting both a cellular senescence protein (DUSP6) and an antisenescence protein (Sirt1) during infection. On one hand, HCV infection appears to accelerate premature T cell aging via ΔNp63–miR-181a–DUSP6 signaling; on the other hand, HCV appears to protect CD4$^+$ T cells from senescence via the ΔNp63–miR-181a–Sirt1 pathway. This introduces a novel paradigm whereby miRNA (miR-181a) and its upstream transcription factor (ΔNp63) as well as downstream target protein (DUSP6) responsible for dampening T cell responses could reprogram a countermolecule (Sirt1) to ensure an appropriate balance between proviral and antiviral immunity, which is crucial in the processes of viral persistence or clearance.

Cell exhaustion and senescence following overactivation and proliferation not only contribute to the natural aging process but also diminish devastating inflammation or arrest the progression of tumors, thereby protecting the host from overwhelming infections or malignancies, suggesting cell exhaustion and senescence as anti-inflammatory or anticancer mechanisms [36, 37]. Our data indicate that HCV-induced, premature T cell senescence is counterregulated by the ΔNp63–miR-181a–Sirt1

pathway. TAp63 expression is controlled by 2 alternative promoters, giving rise to the longer transactivation isoform TAp63 and one lacking the N-terminal transactivation domain ΔNp63 [19]. The most abundant p63 isoform in the epidermis is ΔNp63 [21], and this seems also true for proliferating lymphocytes in that we can only detect ΔNp63, but not TAp63, in $CD4^+$ T cells from both HCV-infected individuals and HS (data not shown). It has been reported that overexpression of TAp63 in both human and murine fibroblasts induces cellular senescence and suppresses tumorigenesis [38], whereas ΔNp63 induces the opposite, preventing premature cell aging and protecting keratinocyte maintenance by suppressing miR-181a expression [21]. We show here that ΔNp63 is up-regulated by HCV infection to suppress miR-181a in $CD4^+$ T cells, leading to the overexpression of multiple target proteins, including Sirt1. Sirt1 is a member of the deacetylase family, known to regulate longevity in mammals and to protect against aging-related diseases [22, 39, 40]. It has been reported that Sirt1 is up-regulated in a subset of hepatocellular carcinomas, where it is essential for telomere maintenance and cancer cell growth [23, 24]. It has also been shown that Sirt1 is up-regulated in HBV-expressing cells, and gene silencing of Sirt1 significantly inhibits HBV DNA replication [25]. Here, we further demonstrated that Sirt1 is significantly up-regulated in senescent $CD4^+$ T cells from HCV-infected individuals. We show that ΔNp63 down-regulates miR-181a expression, which in turn up-regulates the expression of Sirt1 and DUSP6 [10]—2 apparently contradictory proteins that may balance the proliferation and function of $Foxp3^+$ T_{regs} and $Foxp3^-$ T_{effs} in HCV-infected individuals. These data support the notion that ΔNp63–miR-181a–Sirt1 and ΔNp63–miR-181a–DUSP6 pathways might be parallel signaling pathways that strike the balance between T cell response and senescence during viral infection, revealing a strategy used by HCV to facilitate its persistence through immune evasion, in particular $CD4^+$ T cell dysfunction [12]. Based on this study and our previous reports [10], we propose a schematic model, as depicted in **Fig. 6**, to show the mechanisms underlying HCV-induced impairment of $CD4^+$ T cell responses via senescence-related pathways in the setting of chronic HCV infection. This model incorporates novel, mechanistic insights into the HCV-mediated, premature T cell aging that may facilitate viral persistence through the ΔNp63–miR-181a-mediated Sirt1 and/or DUSP6 pathways.

It remains unclear whether these 2 signaling pathways are simultaneously or sequentially activated by HCV infection. Our current data suggest that the ΔNp63–miR-181a–Sirt1 signaling is dominant in the setting of chronic viral infection, apparently counterregulating T cell senescence, which is mediated by the ΔNp63–miR-181a–DUSP6 pathway. We speculate that the ΔNp63–miR-181a–DUSP6 pathway may be responsible for establishing T cell senescence in the early phase of viral infection, whereas the ΔNp63–miR-181a–Sirt1 pathway is activated afterward to balance or maintain the HCV-induced T cell senescence during chronic infection. Another possibility is that these 2 pathways may be differentially activated in different subsets of lymphocytes during T cell differentiation in response to viral infection. However, we do not have data to support this notion yet. For example, we have found that although memory

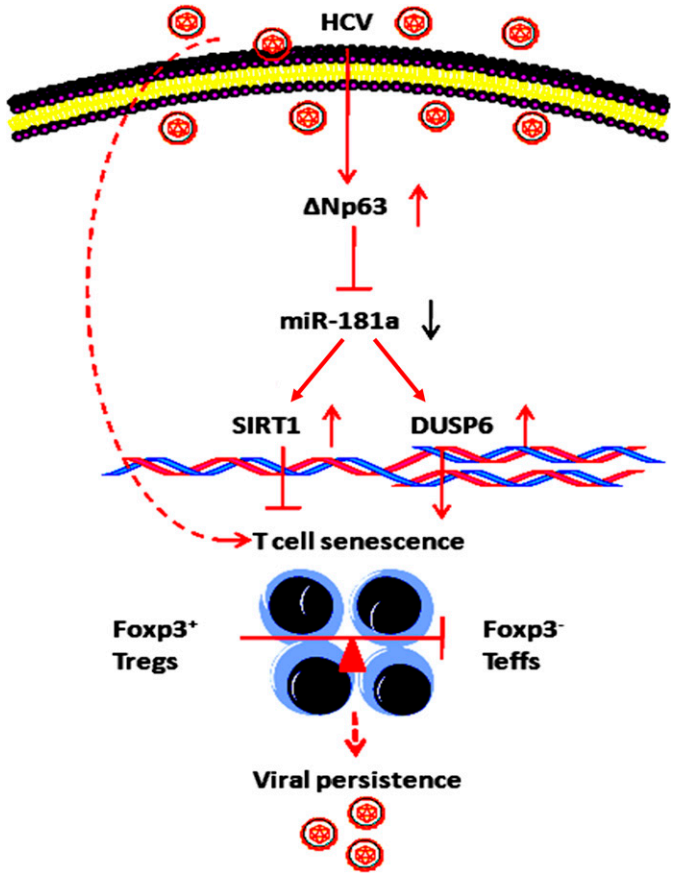

Figure 6. Schematic model for HCV-induced $CD4^+$ T cell senescence regulated by the ΔNp63–miR-181a–Sirt1 and ΔNp63–miR181a–DUSP6 signaling pathways. HCV up-regulated ΔNp63 inhibits miR-181a expression, which, in turn, increases Sirt1 and DUSP6 expression in $CD4^+$ T cells during chronic HCV infection. This 2 signaling pathways may regulate T cell senescence in different subsets of lymphocytes or at different stage of viral infection, facilitating the establishment and/or maintenance of viral persistence. Therefore, reconstitution of miR-181a and/or silencing ΔNp63, Sirt1, or DUSP6 may provide a novel approach to regulating T cell responses in virally infected individuals.

$CD4^+$ T cells are expanded, whereas naïve $CD4^+$ T cells contract, in chronically HCV-infected patients, the expression of several known miR-181a-targeting proteins, such as Sirt1, DUSP6, and PTEN but not DUSP5, are significantly increased in the naïve ($CD45RA^+$) $CD4^+$ T cell subset in HCV-infected patients compared with those in HS (data not shown). In addition, we have also speculated that DUSP6 could be dominantly expressed or activated in $Foxp3^-$ T_{effs} that are responsible for IL-2 expression, whereas Sirt1 is primarily expressed or activated in $Foxp3^+$ T_{regs} that are consume IL-2 and suppress other bystander cells. However, we recently discovered that all these miR-181a-regulating proteins (Sirt1, DUSP6, DUSP5, and PTEN) are highly expressed in $Foxp3^+$ T_{regs} rather than in $Foxp3^-$ T_{eff} cells (data not shown). Whether ΔNp63 or miR-181a has a role in controlling Foxp3 expression to affect $Foxp3^+$ T_{reg} differentiation is an interesting, yet unknown, question. Nevertheless, our data demonstrate that $Foxp3^+$ T_{regs} that expanded during chronic viral infection [29, 41, 42] are remarkably reduced, and IL-2 expression is paradoxically up-regulated in $CD4^+$

T cells by manipulating the ΔNp63–miR-181a–Sirt1 pathway. Therefore, we believe that HCV-induced activation of the ΔNp63–miR-181a–Sirt1 pathway in $CD4^+$ T cells is fundamentally important for HCV pathogenesis because it protects $Foxp3^+$ T_{regs} from senescence and thus alters the balance of $Foxp3^+$ T_{regs}/$Foxp3^-$ T_{effs}, which is key for establishing and maintaining of chronic viral infection.

In summary, this study delineated the mechanisms of HCV-induced immune senescence by manipulating senescence-associated signaling molecules in $CD4^+$ T cells and measuring markers for cell aging, proliferation, cytokine production, and differentiation in response to TCR stimulation. Our studies demonstrated that HCV-induced up-regulation of ΔNp63 leads to a decline of miR-181a, resulting in an overexpression of Sirt1 and DUSP6 [10], and thus T cell senescence and viral persistence. These findings provide novel insights into how HCV uses cellular-intrinsic senescence pathways to regulate T cell functions, revealing new targets for therapeutic rejuvenation of impaired T cell responses during chronic viral infection.

AUTHORSHIP

Y. Zhou and G.L. share senior authorship of this work. Y. Zhou, G.Y.L., J.P.R., L.W., and J.Z. planned and performed the experiments and analyzed the data. S.B.N., Y. Zhang, J.Q.L., and C.X.H. participated in the discussion. Z.S.J., J.P.M., and Z.Q.Y. conceived the study, designed the experiments, and wrote the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
T cell senescence · hepatitis C · microRNA-181a · transcription factor p63 · Sirtuin 1