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Regulation of T cell proliferation by JMJD6 and PDGF-BB during chronic hepatitis B infection

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T cell functional exhaustion during chronic hepatitis B virus (HBV) infection may contribute to the failed viral clearance; however, the underlying molecular mechanisms remain largely unknown. Here we demonstrate that jumonji domain-containing protein 6 (JMJD6) is a potential regulator of T cell proliferation during chronic HBV infection. The expression of JMJD6 was reduced in T lymphocytes in chronic hepatitis B (CHB) patients, and this reduction in JMJD6 expression was associated with impaired T cell proliferation. Moreover, silencing JMJD6 expression in primary human T cells impaired T cell proliferation. We found that JMJD6 promotes T cell proliferation by suppressing the mRNA expression of CDKN3. Furthermore, we have identified platelet derived growth factor-BB (PDGF-BB) as a regulator of JMJD6 expression. PDGF-BB downregulates JMJD6 expression and inhibits the proliferation of human primary T cells. Importantly, the expression levels of JMJD6 and PDGF-BB in lymphocytes from CHB patients were correlated with the degree of liver damage and the outcome of chronic HBV infection treatment. Our results demonstrate that PDGF-BB and JMJD6 regulate T cell function during chronic HBV infection and may provide insights for the treatment strategies for CHB patients.

T he clearance of HBV infection depends on CD8^{+} T cell-mediated immunity[1]; however, in CHB patients, HBV-specific CD8^{+} T cells exhibit functional exhaustion as demonstrated by defective proliferation, impaired cytokine production, and increased apoptosis[2–5]. Importantly, the global CD8^{+} T cell population from CHB patients also exhibits defective proliferation[6]. Upregulated expression of the inhibitory receptors may contribute to the CD8^{+} T cell exhaustion phenotype in CHB patients[7]. Despite these findings, the molecular mechanisms regulating the functional status of CD4^{+} T cells during chronic HBV infection remain largely unexplored. CD4^{+} T cells are essential for effector and memory CD8^{+} T cell responses during viral infections including HBV infection[8,9]. CD4^{+} T cells are the master regulators of the CD8^{+} T cell response to HBV infection and are essential for HBV clearance[9,10]. Furthermore, Th1/Th2 CD4^{+} helper populations were skewed in CHB patients[11]. The peripheral CD4^{+} T cells from CHB patients exhibit a dramatically altered gene expression signature when compared to their counterparts from acute HBV patients[12]. These results suggest that CD4^{+} T cells in CHB patients may also be functionally impaired.

Jumonji domain–containing 6 protein (JMJD6) is a JmjC-containing iron- and 2-oxoglutarate–dependent dioxygenase[13]. JMJD6 serves as a histone arginine demethylase and interacts with the splicing factor U2 small nuclear RNA auxiliary factor 2 (U2AF65) to regulate alternative RNA splicing in the nucleus[14,15]. X-ray crystallographic data show that JMJD6 interacts with single-stranded RNA[16]. Elevated expression of JMJD6 in breast cancer was associated with poor outcomes and JMJD6 was shown to drive breast cancer cell proliferation[17]. A more recent study demonstrated that JMJD6 forms complex with Brd4 to function as distal enhancers regulating promoter-proximal pause release of coding genes in many cell types[18]. However, the role of JMJD6 in immune cells in healthy and diseased states has not been determined.

The PDGF family consists of five dimeric isoforms derived from four gene products: PDGF-AA, -AB, -BB, -CC, and -DD. These proteins are potent mitogens for cells of mesenchymal origin such as fibroblasts and smooth

muscle cells[19,20]. PDGF-BB has been implicated in the pathogenesis of chronic HBV infection and CHB-associated complications. Serum levels of PDGF-BB were positively correlated with the degree of liver damage, fibrosis, and hepatitis B e antigen (HBeAg) status in CHB patients[21–24]. PDGF-BB may be involved in liver pathogenesis by promoting the proliferation of hepatic stellate cells[25]. Nevertheless, it is not known whether the levels of PDGF-BB in CHB patients impact lymphocyte function and anti-HBV immunity.

In this study, we have identified the downregulation of JMJD6 as a possible cause of impaired T cell proliferation during chronic HBV infection. PDGF-BB, JMJD6, and CDKN3 may together regulate T cell proliferation during chronic HBV infection.

Results

Decreased JMJD6 expression in T lymphocytes from CHB patients. CD8^{+} T cells from chronic HBV-infected patients exhibit a global defect in proliferation[6]. We assessed the proliferation capacity of CD4^{+} and CD8^{+} T lymphocytes from a group of treatment-naïve CHB patients (Table S1). Consistent with previous findings, a fraction of CHB patients exhibited a global defect in CD8^{+} T cell proliferation (Fig. 1a and 1b). Importantly, a global defect in CD4^{+} T cell proliferation was also observed in CHB patients (Fig. 1a and 1b). In a paired comparison analysis between CHB patients and healthy donors (HDs), CD4^{+} T cells from 44% of CHB patients (14 out of 32) and CD8^{+} T cells from 19% of CHB patients (6 out of 32) exhibited defective proliferation (Fig. 1b). CHB CD8^{+} T cells exhibited impaired capability to produce interleukin 2 (IL-2) and interferon gamma (IFN-γ) but not interleukin 4 (IL-4) and tumor necrosis factor-alpha (TNFα) (Fig. 1c). In contrast, CHB CD4^{+} T cells had no obvious defect in producing any of these four cytokines (Fig. 1c). Antigen-KI 67 (Ki67)[+] proliferating CD4^{+} and CD8^{+} T cells from these patients in ex vivo analysis were also significantly reduced (Fig. 1d). CD4^{+} and CD8^{+} T cell proliferation capacities were not related to the HBeAg status in the CHB patients (Fig. 1e). When compared to CHB patients with normal T cell proliferation capacity, CHB patients with decreased CD4^{+} and CD8^{+} T cell proliferation capacity displayed more severe liver damage while there was no strong correlation between proliferation capability and HBV DNA serum levels (Fig. 1f).

To investigate the molecular mechanisms underlying the impaired T cell proliferation in CHB patients, we performed an analysis of differential gene expression in T cells from CHB patients and HDs[26]. We found that the expression of JMJD6 mRNA in the T cells of CHB patients was reduced by 66% (p = 0.009; n = 5) when compared to that in the T cells from HDs. We further confirmed this finding in lymphocytes from additional CHB patients (n = 13) and HDs (n = 9) by quantitative polymerase chain reaction (qPCR). JMJD6 mRNA expression was reduced not only in CD4^{+} and CD8^{+} T lymphocytes but also in total PBMCs (Fig. 2a). The protein expression level of JMJD6 in total PBMCs of CHB patients was decreased on average 54% when compared to that in PBMCs of HDs (Fig. 2b). As human PBMCs contain B lymphocytes and monocytes, these results suggest that host environments or factors in chronic HBV-infected patients inhibit the expression of JMJD6 in multiple populations of leukocytes.

Inhibition of CD4^{+} T cell proliferation upon JMJD6 silencing. To investigate whether JMJD6 regulates T cell function, we transfected PBMCs from HDs with siRNAs specific for JMJD6 and control siRNAs by nucleofection. Transfection with JMJD6 siRNA reduced the expression of endogenous JMJD6 by ~70%, resulting in a reduction in JMJD6 similar to that observed in T cells from CHB patients (Fig. 2c). The efficiency of JMJD6 silencing was further confirmed by Western blot analysis (Fig. 2d). The transfected cells were then stimulated with anti-CD3/CD28 for 2–4 d and examined for proliferation, activation, and apoptosis. JMJD6 silencing readily

reduced CD4^{+} T cell proliferation but only slightly impaired the proliferation of CD8^{+} T cells from eight individual HDs (Fig. 2e and 2f). JMJD6 siRNA-transfected CD4^{+} and CD8^{+} T cells did not exhibit changes in annexin V staining or in the upregulation of CD25 and CD69 expression after TCR stimulation, indicating that the silencing of JMJD6 did not affect T cell survival or activation.

JMJD6 regulates T cell proliferation by controlling CDKN3 expression. To test the possibility that JMJD6 may regulate CD4^{+} T cell proliferation by controlling the expression of genes involved in cell cycle progression, we screened the expression of a panel of 32 cell cycle-related genes (Table S2) in JMJD6-silenced CD4^{+} lymphocytes from HDs. Interestingly, the mRNA expression of two regulators of the cell cycle, cyclin-dependent kinase inhibitor 3 (CDKN3) (also known as KAP and Cdi1)[27,28] and small ubiquitin-related modifier 1 (SUMO1)[29], was significantly increased upon JMJD6 silencing (Fig. 3a), suggesting that JMJD6 is a negative regulator of these two genes. To further verify the role of JMJD6 in regulating CDKN3 and SUMO1 mRNA expression, we overexpressed JMJD6 by transfecting normal human T lymphocytes with a pMax vector containing a cyan fluorescent protein (CFP) marker and the JMJD6 gene driven by the cytomegalovirus (CMV) major immediate-early promoter by electroporation. T cells transfected with this vector readily overexpressed JMJD6 (Fig. 3b). Consistent with our observations in JMJD6 siRNA-transfected T cells, JMJD6 overexpression resulted in significant decreases in the expression of both CDKN3 and SUMO1 (Fig. 3b). This effect of JMJD6 expression on CDKN3 and SUMO1 was specific, as the expression of 30 other cell cycle-related genes was not changed in either JMJD6-silenced or JMJD6-overexpressing human T lymphocytes.

We next tested the hypothesis that T cell proliferation is impaired in JMJD6-silenced T cells due to increased expression of the cell cycle regulators CDKN3 and/or SUMO1. T lymphocytes from HDs were transfected with JMJD6 siRNA in combination with either CDKN3 siRNA or SUMO1 siRNA and then stimulated with anti-CD3/CD28 for 4 d. Co-transfection of human primary T lymphocytes with siRNA specific for CDKN3 or SUMO1 in combination with JMJD6 siRNA prevented the upregulation of CDKN3 or SUMO1 mRNA upon silencing of JMJD6 (Fig. 3c and 3d). Remarkably, silencing CDKN3 fully restored the impaired proliferation of JMJD6 siRNA-treated CD4^{+} T lymphocytes as measured by carboxyfluorescein succinimidyl ester (CFSE) or Ki67 staining (Fig. 3e, Fig. S1a and S1b). In contrast, silencing SUMO1 did not rescue the impaired proliferation of JMJD6-silenced CD4^{+} T cells (Fig. 3e). The proportion of CD4^{+} T cells able to undergo at least one division was restored from on average 25% in JMJD6 siRNA-treated T cells to 45% in T cells treated with both JMJD6 siRNA and CDKN3 siRNA (Fig. S1a). Although silencing JMJD6 and/or CDKN3 in human CD8^{+} T cells produced a similar effect to that in CD4^{+} T cells, the differences did not achieve statistic significance (Fig. 2f, Fig. S1a and S1b). Interestingly, silencing JMJD6 slightly impaired IL-2 production in CD4^{+} and CD8^{+} T cells (Fig. S1c) but had no effect on IL-2 receptor expression (Fig. S1d). To test whether JMJD6 regulates CDKN3 expression via RNA splicing, we used specific primers to amplify the two CDKN3 isoforms (NM_005192.3 and NM_001130851.1). Only the long isoform of CDKN3 (NM_005192.3) was detected in control and JMJD6 silenced T cells (Fig. S2a), suggesting that JMJD6 regulates CDKN3 expression through a mechanism other than RNA splicing. We then tested whether silencing CDKN3 in T cells from CHB patients could restore their impaired proliferation. Silencing CDKN3 in proliferation-impaired T cells from CHB patients significantly increased the proliferation of CD4^{+} but not CD8^{+} T cells (Fig. 3f). Taken together, these results suggest that JMJD6 promotes CD4^{+} T cell proliferation by suppressing CDKN3 mRNA expression and that the impaired CD4^{+} proliferation in CHB patients may be caused by a decreased JMJD6 and an increased CDKN3 expression.
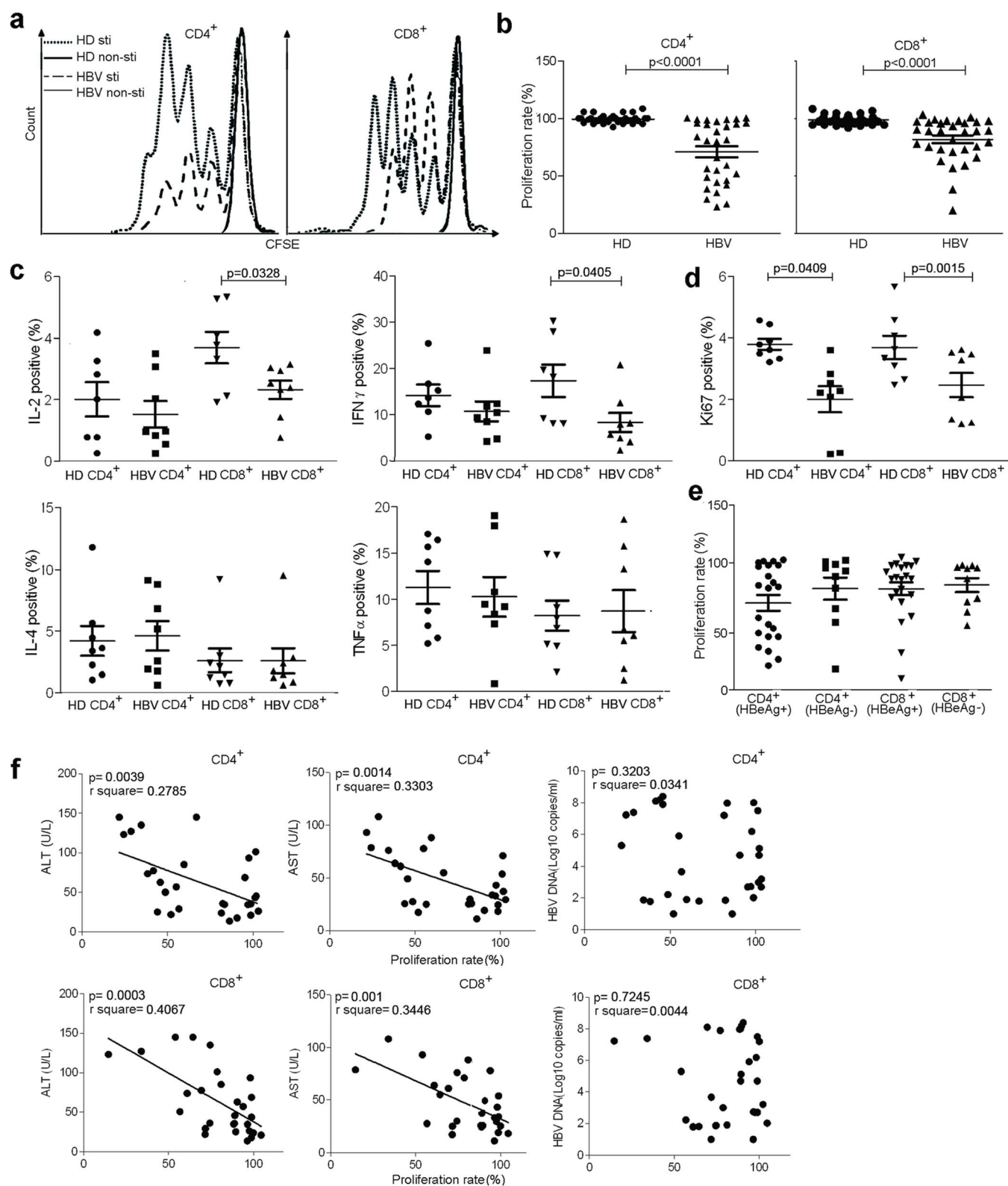
Figure 1 | Impaired T cell proliferation and reduced JMJD6 expression in CHB patients. (a) FACS profiles of T cell proliferation in CHB patients. PBMCs were stimulated with anti-CD3/CD28 for 96 h and analyzed for CFSE dilution. HD sti or HD non-sti: control cells from HDs with or without TCR stimulation; HBV sti or HBV non-sti: T cells from CHB patients with or without TCR stimulation. (b) Proliferation of T cells from CHB patients. Within each independent experiment, the degree of proliferation in one HD was defined as 100%, and the proliferation rates of patient samples were normalized to the proliferation rate of the cells from this HD. Data from 32 patients and 32 controls from >10 experiments were pooled. (c) Cytokine production in T cells from HDs and CHB patients. Shown are percent of IL-2, IFNγ, IL-4, and TNFα positive cells after stimulation as described in Methods (n = 8). (d) Proliferative T cells from HDs and CHB patients as measured by Ki67 staining. (e) Proliferative T cells from CHB patients based on their HBeAg status. (f) Correlation between the proliferative capacity of CD4+ or CD8+ T cells and ALT, AST, and HBV viral titers in CHB patients (n = 32). P values are indicated.
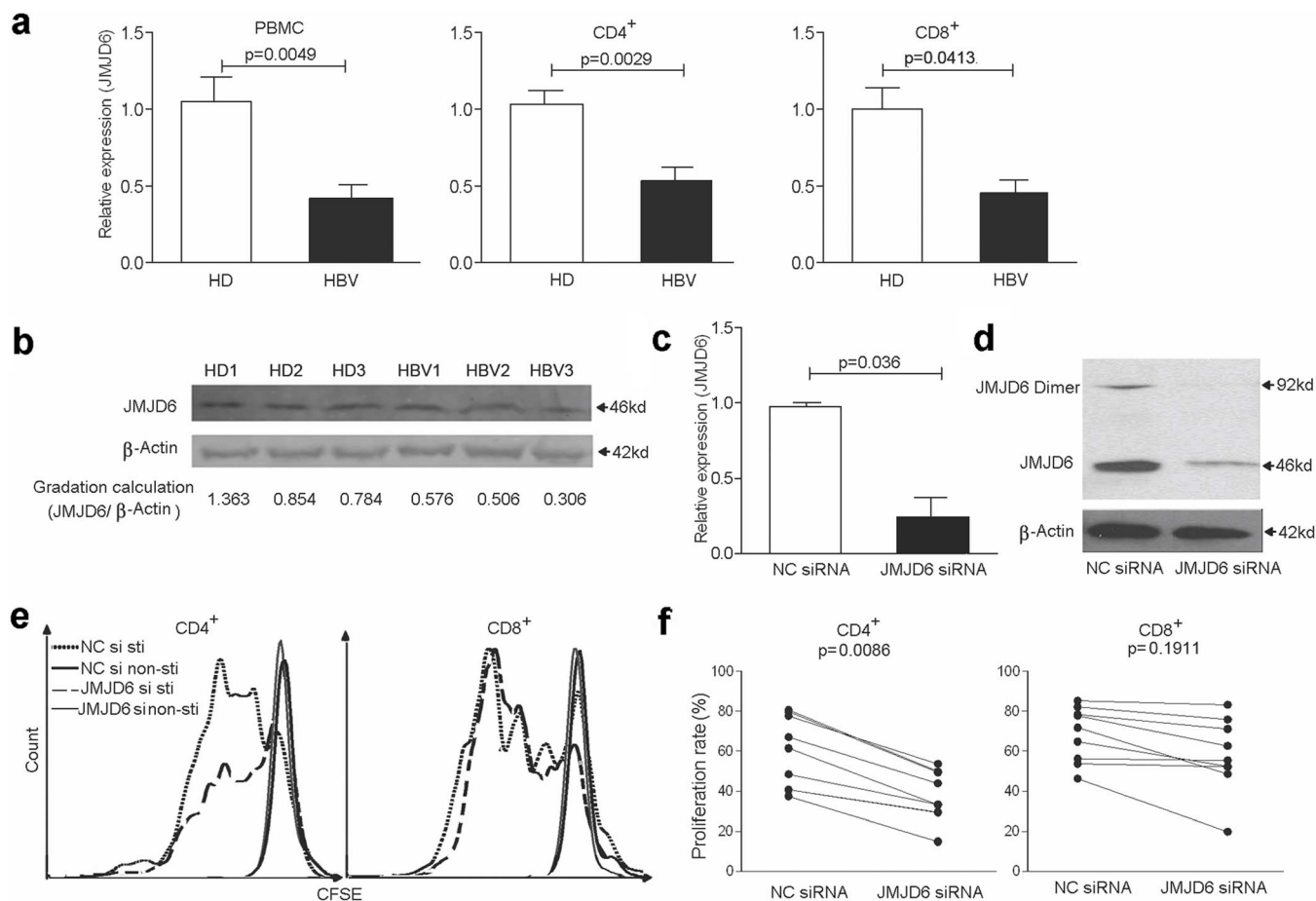
Figure 2 | siRNA-mediated silencing of JMJD6 expression inhibits CD4+ T cell proliferation. (a) JMJD6 mRNA expression in total PBMCs and purified CD4+ and CD8+ T lymphocytes from CHB (n = 13) and HDs (n = 9). (b) JMJD6 protein expression in PBMCs from HDs and CHB patients. 5×10^6 PBMCs per sample were assayed with anti-JMJD6 and β-actin antibodies in Western blot analysis. Fully uncut gel images are shown in Supplemental Figures. (c) Silencing efficiency of JMJD6 in human PBMCs. Total PBMCs from HDs were transfected by nucleofection with non-specific siRNA (NC siRNA) or JMJD6-specific siRNA (JMJD6 siRNA), and the expression of JMJD6 mRNA was measured by qPCR analysis (n = 4). (d) Western blot analysis of siRNA-transfected cells. β-actin serves as a loading control. Fully uncut gel images are shown in Supplemental Figures. (e) Representative profiles of proliferation of JMJD6-silenced T cells. Transfected PBMCs were stimulated with anti-CD3/CD28 for 96 h and then analyzed for CFSE dilution by FACS. (f) Proliferation of JMJD6-silenced T cells. Data from HDs in eight experiments were pooled. The labels in panel E are: NC si, non-specific control siRNA; JMJD6 si, JMJD6 specific siRNA; sti, stimulated; non-sti, non-stimulated.

We examined the correlation between JMJD6 expression in patients' PBMCs and the degree of liver damage. The expression of JMJD6 in the PBMCs of CHB patients was negatively correlated with the patients' levels of alanine transaminase (ALT) and aspartate transaminase (AST) (Fig. S3a). In contrast, no strong correlations between JMJD6 expression levels and HBV DNA (Fig. S3b) or total bilirubin (TBIL) or direct bilirubin (DBIL) levels (Fig. S3c) were detected, and the correlation between ALT and TBIL and between AST and TBIL were not significant (Fig. S3d).

**Expression of JMJD6, CDKN3, and PDGF-BB in CHB patients.** To determine the mechanism underlying the downregulation of JMJD6 mRNA in the T lymphocytes of CHB patients, we analyzed the relationship between the degree to which T cell proliferation was impaired in CHB patients and these patients' clinical data and cytokine profiles. We divided the patients into three groups based on their CD4+ T cell proliferation capacity: the low proliferation (low P) group consisted of 7 patients whose T cell proliferation was decreased by 50% or more when compared to that of T cells from normal donors, the middle proliferation (middle P) group consisted of 6 patients whose T cell proliferation was 51–85% of that of normal T cells, and the high proliferation (high P) group consisted of 6

patients whose T cell proliferation was 86–100% of that of normal T cells (Fig. 4a). The expression level of JMJD6 mRNA in CD4+ T cells from CHB patients was clearly associated with their T cell proliferation capacity (Fig. 4a). The patients in the low P group displayed an approximately 80% mean reduction in JMJD6 expression, and the middle P group displayed an approximately 70% mean reduction in JMJD6 expression, whereas the high P group displayed JMJD6 expression levels similar to those observed in healthy donor T cells (Fig. 4a). Consistent with the finding that CDKN3 is a target gene of JMJD6 (Fig. 3a), the expression of CDKN3 mRNA in CD4+ T cells from the low P and middle P group of CHB patients was significantly elevated when compared to that from the HDs (Fig. 4a). Furthermore, JMJD6 expression was positively correlated to CD4+ T cell proliferation capability (Fig. 4b). Patients in the low P group exhibited more active liver damage than those in the middle P and high P groups (Fig. 4c).

A soluble factor(s) induced by chronic HBV infection may down-regulate the expression of JMJD6 in lymphocytes. We performed a Luminex cytokine profile analysis in plasma samples from CHB patients and found that PDGF-BB, IL-8, and chemokine ligand 5 (RANTES) levels were all significantly elevated in the low P group when compared to those from healthy donor plasma samples
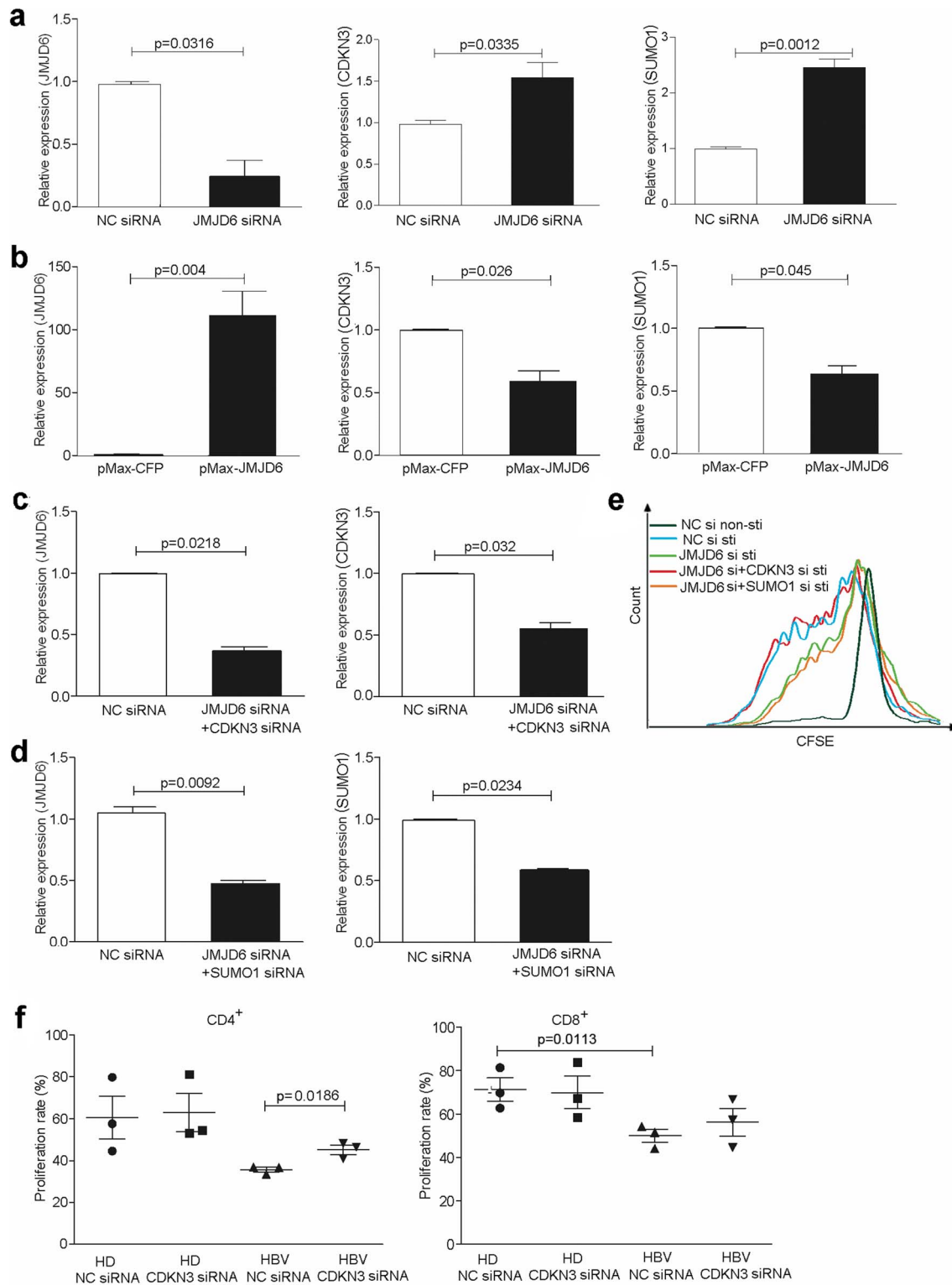
Figure 3 | JMJD6 promotes CD4+ T cell proliferation by suppressing CDKN3 mRNA expression. (a, b) Expression of CDKN3 and SUMO1 mRNA in JMJD6-silenced (a) or JMJD6-overexpressing (b) human CD4+ T cells. CD4+ T cells from PBMCs were transfected with JMJD6 siRNA and non-specific control siRNA (NC siRNA), or with a pMax vector and pMax-JMJD6. CDKN3 or SUMO1 mRNA in transfected CD4+ T cells was measured by qPCR. The level of JMJD6 expression in the transfected cells is also shown (n = 3). (c, d) siRNA-mediated inhibition of the upregulation of CDKN3 and SUMO1 mRNA in JMJD6-silenced cells. PBMCs were transfected with NC siRNA, JMJD6 siRNA, JMJD6 siRNA + CDKN3 siRNA, or JMJD6 siRNA + SUMO1 siRNA and cultured for 4 d. JMJD6, CDKN3, and SUMO1 mRNA was measured in the treated cells by qPCR (n = 3). (e) Representative profiles of restoration of the proliferation of JMJD6-silenced CD4+ T cells upon blockade of CDKN3 mRNA upregulation (n = 5, see Supporting Fig 1). siRNA-transfected CD4+ T cells were stimulated with anti-CD3/CD28 for 4 d and analyzed for CFSE dilution. Labels are: NC si, non-specific control siRNA; JMJD6 si, JMJD6-specific siRNA; CDKN3 si, CDKN3-specific siRNA; SUMO1 si, SUMO1-specific siRNA; non-sti, non-stimulated; sti, stimulated. (f) Enhanced proliferation of CD4+ T cells from CHB patients upon CDKN3 silencing. PBMCs from HDs and CHB patients were transfected with NC siRNA or CDKN3 siRNA and cultured for 4 d with anti-CD3/CD28 stimulation. Proliferation was measured by CFSE dilution. P values are indicated.
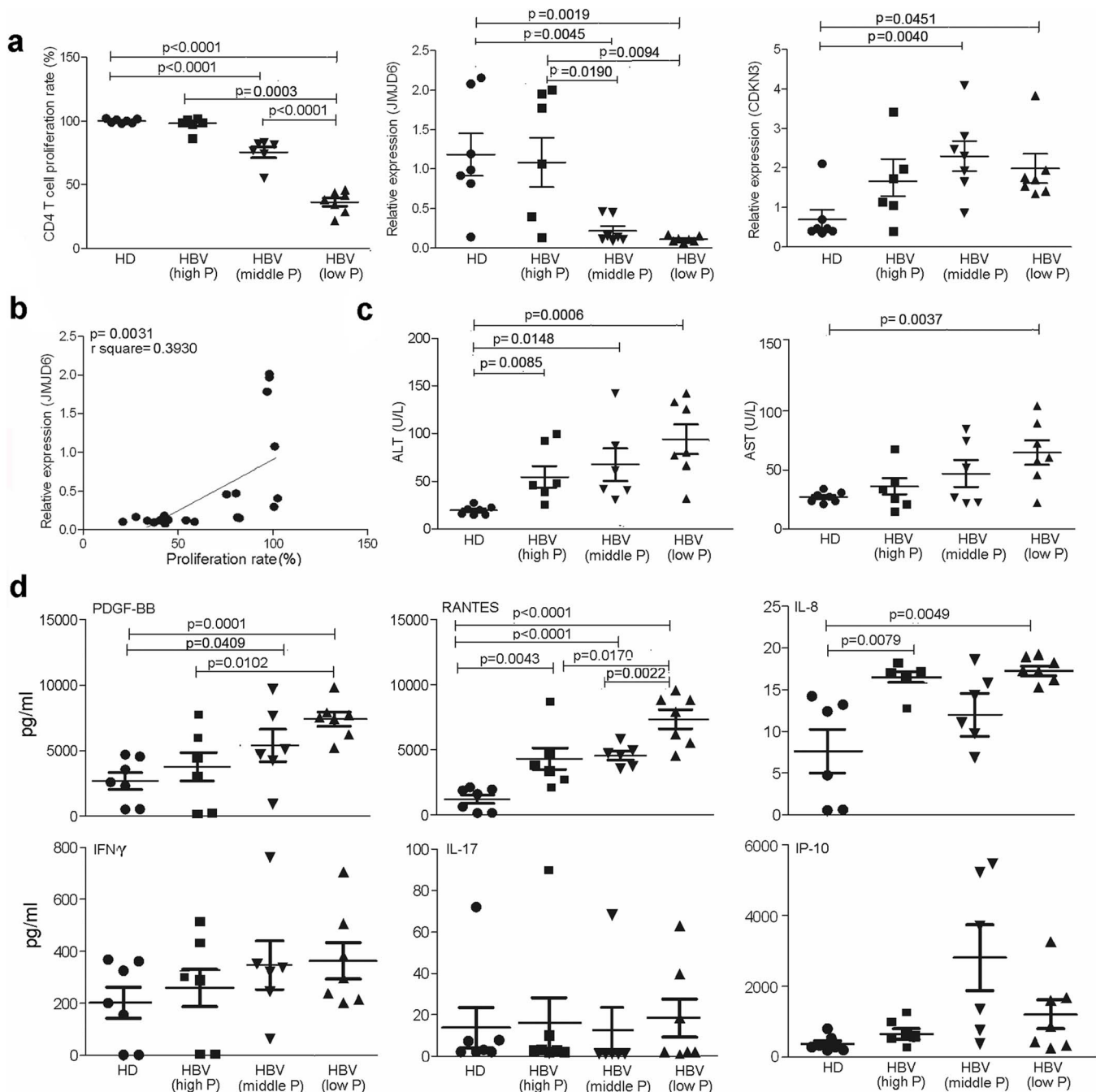
Figure 4 | CHB patients with impaired T cell proliferation exhibit reduced JMJD6 expression and enhanced CDKN3 expression in CD4+ T cells and increased plasma PDGF-BB levels. CHB patients were divided into three groups based on their CD4+ T cell proliferation capacity. The patients in the low proliferation group (low P), middle proliferation group (middle P), and high proliferation group (high P) had T cell proliferation rates <50%, 51–85%, 86–100% of those of controls, respectively. (a) The CD4+ T cell proliferation rate and JMJD6 and CDKN3 expression in CD4+ T cells from HDs and CHB patients. (b) Correlation of JMJD6 expression with proliferation rate in CD4+ T cells of CHB patients. (c) Serum ALT and AST levels in HDs and CHB patients. (d) The levels of six different cytokines in the plasma of CHB patients in HDs and CHB patients. P values are indicated in the figure.

(Fig. 4d). Other cytokines, including IFN-γ, IL-17, and chemokine ligand 10 (IP-10), were not significantly different among the four groups (Fig. 4d). These results suggest that cytokine levels, T cell proliferation capacity, and liver pathology during CHB infection may be functionally linked.

PDGF-BB induces JMJD6 downregulation and inhibits CD4+ T cell proliferation. We next examined whether the downregulation of JMJD6 expression in the PBMCs of CHB patients was mediated by HBV viral components and/or alterations in cytokine expression.

PBMCs from HDs were cultured for 20 h with 2 μg/ml HBsAg or with concentrations of cytokines equivalent to those found in the plasma of CHB patients. Culturing total PBMCs with PDGF-BB resulted in an average reduction in JMJD6 mRNA expression of ∼60% (Fig. 5a). Culturing PBMCs with IL-8 or RANTES resulted in slightly decreased expression of JMJD6 mRNA (Fig. 5a). The effect of PDGF-BB on JMJD6 expression was specific, as HBsAg and 13 other cytokines that were elevated in CHB patients[26] did not affect JMJD6 expression in human PBMCs. We next tested whether PDGF-BB, IL-8, or RANTES inhibit the proliferation of human primary T

cells. PBMCs from HDs were cultured with anti-CD3/CD28 for 96 h in the presence of PDGF-BB, IL-8, or RANTES at concentrations equivalent to those found in the plasma of CHB patients. Indeed, PDGF-BB treatment resulted in a dramatic decrease in the proliferation of CD4$^+$ T cells but not CD8$^+$ T cells from eight individual HDs (Fig. 5b and 5c), whereas IL-8 and RANTES had no effect on T cell proliferation (Fig. 5d and 5e). PDGF-BB treatment did not affect the IL-2 production (Fig. 5f) and IL-2 receptor expression (Fig. S1e) on CD4$^+$ and CD8$^+$ T cells.

We examined the clinical significance of plasma PDGF-BB levels. The PDGF-BB level in the plasma of CHB patients was negatively correlated with the proliferation capability and JMJD6 expression in CD4$^+$ T cells while weakly correlated with the proliferation capability but not JMJD6 expression in CD8$^+$ T cells (Fig. 6a and 6b). PDGF-BB level in the plasma of CHB patients was positively correlated with the levels of AST (Fig. 6c). In addition, CHB sera containing higher levels of PDGF-BB were more potent in inhibiting proliferation of CD4$^+$ but not CD8$^+$ T cells from HDs than those containing lower levels of PDGF-BB (Fig. 6d and Fig. S2b). These data suggest that liver damage during CHB infection may release PDGF-BB, which in turn induces the downregulation of JMJD6 expression, resulting in impaired T cell proliferation.

The expression of JMJD6 and PDGF-BB in CHB patients is linked to the outcome of chronic HBV infection treatment. To test whether JMJD6 or PDGF-BB expression is correlated with treatment outcome in CHB patients, PBMCs and plasma samples of 10 CHB patients who had received 13–28 months of anti-viral

treatment were collected at baseline before treatment and at the end of the treatment (EOT) (Table S3). The patients were divided into two groups based on their HBeAg seroconversion status: 5 patients who underwent HBeAg seroconversion (HBeAg$^-$) after treatment were assigned to the treatment responsive group, and 5 patients who did not undergo HBeAg seroconversion (HBeAg$^+$) after treatment were assigned to the treatment unresponsive group (Table S4). JMJD6 expression was significantly increased in CD4$^+$ T cells from the responsive group but further decreased in CD4$^+$ T cells from the unresponsive group following treatment (Fig. S4a). Remarkably, the levels of PDGF-BB in the plasma of the patients in the responsive group were significantly decreased, whereas the levels of PDGF-BB were further elevated in the plasma of 4 out of the 5 patients in the unresponsive group (Fig. S4b). These results demonstrate that the levels of JMJD6 and PDGF-BB expression are closely correlated with the disease status of CHB patients.

Discussion

Our study has provided several important insights to the molecular mechanisms underlying T cell exhaustion during CHB infection. First, similar to CD8$^+$ T cells, total CD4$^+$ T cells from a major fraction (~50%) of CHB patients exhibit impaired proliferation, and importantly, the rate of T cell proliferation is inversely correlated with the degree of liver damage in CHB patients. This is in contrast to the normal proliferation of global CD4$^+$ and CD8$^+$ T cells from a group of long-term infected (~20 years) treatment-naïve chronic HCV patients[30]. Second, the expression of JMJD6 is reduced in T lymphocytes from CHB patients, and JMJD6 expression levels are
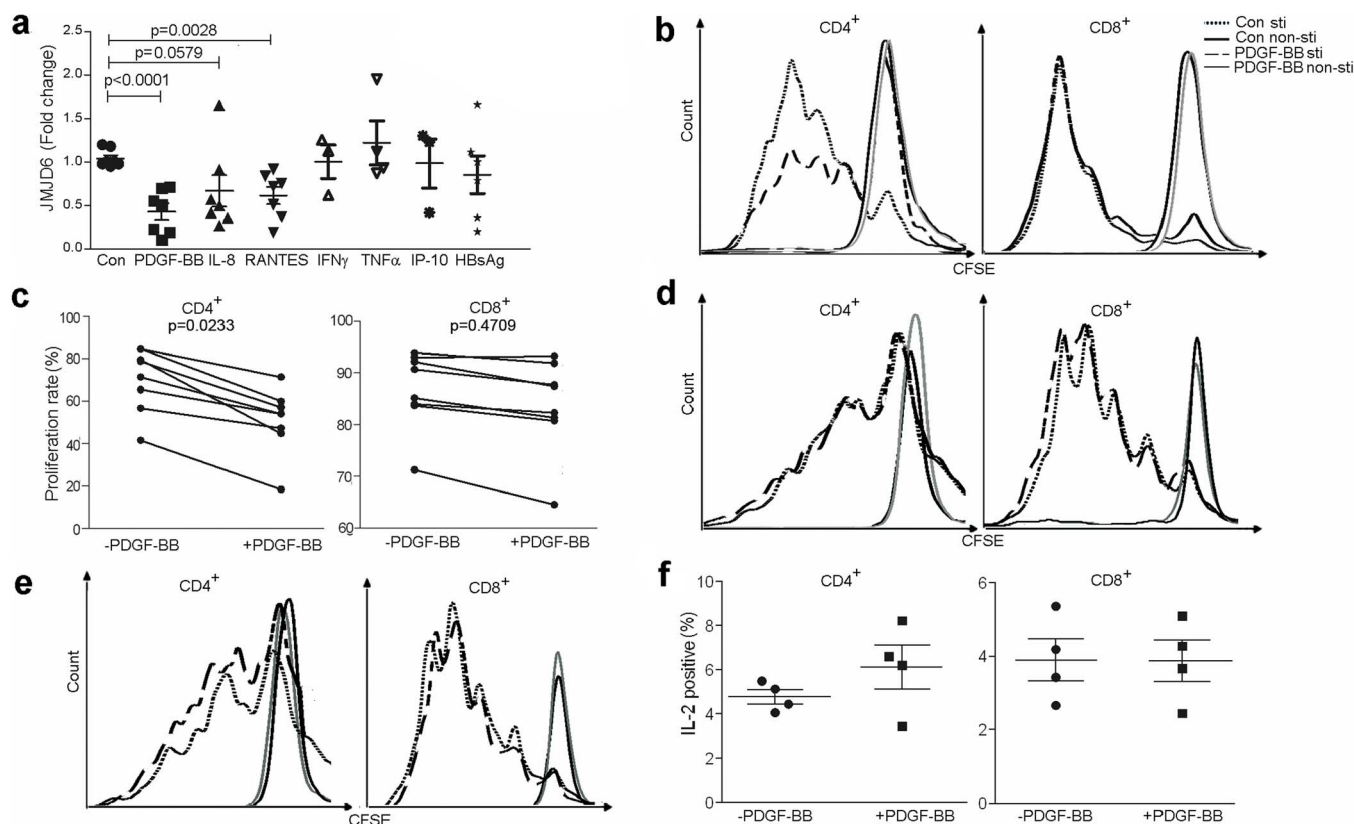
Figure 5 | PDGF-BB induces JMJD6 downregulation and inhibits CD4$^+$ T cell proliferation. (a) JMJD6 mRNA expression levels in the PBMCs of HDs were measured by qPCR. PBMCs were cultured in HBsAg (2 μg/ml), IL-8 (40 pg/ml), RANTES (30 ng/ml), IP-10 (10 ng/ml), TNFα (1 ng/ml), IFNγ (100 pg/ml), or PDGF-BB (60 ng/ml) for 20 h and collected for mRNA quantification. (b) Representative profiles of CD4$^+$ and CD8$^+$ T cell proliferation in the presence or absence of PDGF-BB (60 ng/ml). The PBMCs were labeled with CFSE and stimulated with anti-CD3/CD28 for 96 h. (c) The proliferation of PDGF-BB-treated T cells was measured as in (b). Data from HDs in eight experiments were pooled. (d, e) Representative profiles of CD4$^+$ and CD8$^+$ T cell proliferation in the presence of IL-8 (40 pg/ml) (d) or RANTES (30 ng/ml) (e). The cells were treated as in (b). (f) IL-2 production in PDGF-BB treated CD4$^+$ and CD8$^+$ T cells. The cells were treated with PDGF-BB as in (b) and intracellular IL-2 production was measured by FACS.
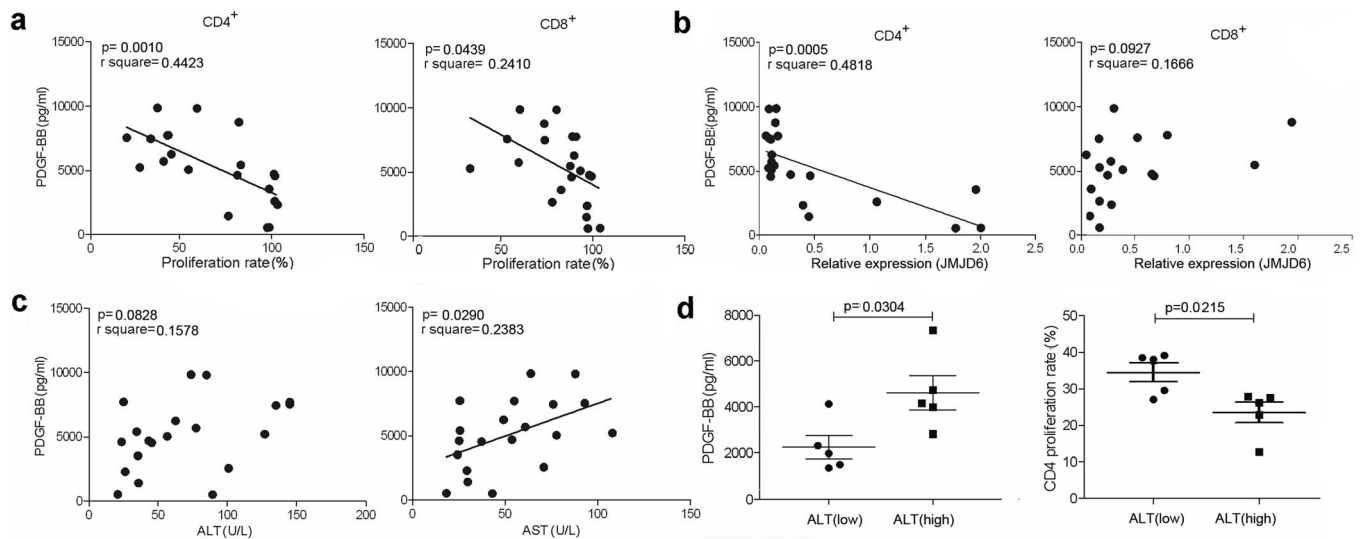
Figure 6 | Correlation between the plasma PDGF-BB levels, T cell proliferation capability and JMJ6 expression levels in CHB patients (n = 21).
(a) Correlation between the plasma PDGF-BB levels and CD4/CD8 T cell proliferation rates in CHB patients. (b) Correlation between the plasma PDGF-BB levels and JMJD6 mRNA expression in CD4/CD8 T cells of CHB patients. (c) Correlation between the plasma PDGF-BB levels and ALT or AST levels in CHB patients. (d) PDGF-BB serum levels in ALT(high) or ALT(low) CHB patients and the effect of these sera on CD4+ T cell proliferation. T cells from HDs were cultured with CHB patients' sera and measured for proliferation as described in the Method. P values and r square values are indicated in the panels.

positively correlated with T cell proliferation capacity and negatively correlated with the degree of liver damage and the treatment outcome of CHB patients. Third, JMJD6 functions as a potential regulator of CD4+ T cell proliferation by suppressing the mRNA expression of CDKN3. Fourth, PDGF-BB, a factor involved in liver fibrosis, induces the downregulation of JMJD6 in human PBMCs and inhibits CD4+ T cell proliferation. These results demonstrate that PDGF-BB, JMJD6, and CDKN3 are dysregulated in T cells from CHB patients and suggest that the altered expression of these genes may contribute to T cell functional exhaustion. It should be noted that our results are derived from studying the total populations of CD4+ and CD8+ T cells in CHB patients. JMJD6 may play a differential function in naïve, memory and activated T cells. Future studies are needed to establish its role in these different T cell subsets in CHB patients and healthy donors.

Our results suggest that JMJD6 promotes CD4+ T cell proliferation by suppressing CDKN3 mRNA expression. It is well established that CDKN3 inhibits cell cycle progression by binding to CDK2 kinase and dephosphorylating CDK2 on Thr[160] [27,28]. Here, we show that CDKN3 mRNA expression is elevated in JMJD6-silenced CD4+ T cells, and the overexpression of JMJD6 suppresses CDKN3 mRNA in primary CD4+ T cells. Importantly, the impaired proliferation of JMJD6-silenced CD4+ T cells was fully restored by preventing CDKN3 mRNA upregulation. SUMO1 also appears to be a JMJD6 target gene; however, SUMO1 may not be functionally involved in JMJD6-mediated CD4+ T cell proliferation, as its silencing had no effect on the proliferation of JMJD6-silenced CD4+ T cells. Further supporting the link between JMJD6 and CDKN3 and their functional involvement in CD4+ T cell proliferation, the expression of these two genes was altered in CD4+ T cells from the T cell low proliferation patients but not in cells from the T cell high proliferation CHB patients. Silencing CDKN3 in CD4+ T cells from CHB patients significantly improved their proliferation. Our findings are consistent with a recent report showing that the loss of JMJD6 expression in a breast cancer cell line results in suppressed proliferation[17].

A discovery emerging from our study is the inhibitory effect of PDGF-BB on human CD4+ T cell proliferation. PDGF-BB is a pleiotropic cytokine with a primary role in stimulating the proliferation of

cells of mesenchymal origin[20]. Contrary to its mitogenic effect on cells of mesenchymal origin, our data show that PDGF-BB suppresses the proliferation of primary human CD4+ T cells. PDGF-BB may mediate its inhibitory effect by downregulating JMJD6 expression in these cells. Indeed, we found that *in vitro* culture of PBMCs from HDs with PDGF-BB results in decreased JMJD6 mRNA expression. Our results are consistent with a previous study showing the effect of PDGF-BB on CD4+ T cell function using PDGF-BB-deficient mice[31]. In addition, PDGF-BB also inhibits the ability of activated T cells to produce IL-4, IL-5, and IFN-γ[32,33] and suppresses human NK cell activation[34]. PDGF-BB in CHB patients may be to inhibit the effective anti-viral immunity mediated by T lymphocytes and NK cells.

PDGF-BB is also involved in other aspects of liver pathogenesis during chronic HBV infection. Several studies have demonstrated that serum PDGF-BB levels in CHB patients are increased and are strongly correlated with the degree of liver damage, fibrosis, and HBeAg status[21–24]. PDGF-BB likely participates in the pathogenesis of liver hepatitis and cirrhosis by stimulating the proliferation of hepatic stellate cells[25,35,36]. Indeed, treatment with an antibody specific for the PDGF-B chain reduces the development of liver fibrosis[37]. Consistent with the above observations, we found that plasma PDGF-BB levels are also elevated in CHB patients. However, the increase in PDGF-BB levels was much more pronounced in the CD4+ T cell low proliferation group. Moreover, the elevated expression of plasma PDGF-BB in CHB patients is strongly correlated not only with liver damage, reduced expression of JMJD6, and enhanced expression of CDKN3 in CD4+ T cells, but also with failed CHB treatment. Our results build on previous studies to strongly suggest that targeting PDGF-BB may be an effective approach in the treatment of CHB patients. Importantly, antibody-mediated neutralization of PDGF-BB may not only prevent liver cirrhosis, but also de-repress its inhibitory effect on T lymphocytes and NK cells. Interestingly, antiplatelet therapy reduced the degree of liver inflammation and the severity of liver fibrosis in a mouse model of chronic HBV infection and prevented the development of hepatocellular carcinoma[38]. These findings suggest a promising treatment strategy for a notoriously difficult disease.

Methods

Patients. We have recruited a total of 39 treatment-naïve CHB patients and 39 healthy donors (HDs) for this study for assessing T cell proliferation and cytokine profiles. All patients were HBsAg$^+$, anti-HBc$^+$, hepatitis C virus (HCV)$^-$, and human immunodeficiency virus (HIV)$^-$. The patients were from either the YouAn Hospital (Beijing, China) or 302 Hospital (Beijing, China). The characteristics of the patients and HDs are listed in Table S1. For the treatment outcome study, a total of 10 CHB patients without previous therapy were enrolled. These patients received anti-viral treatments in YouAn Hospital (Beijing, China) for 13–23 months. The characteristics of the patients are listed in Table S3, and the treatments are listed in Table S4. All human subjects provided written informed consent. All studies were conducted according to Declaration of Helsinki guidelines and approved by the Institutional Ethics Committees for Human Studies at YouAn Hospital and 302 Hospital.

PBMC isolation, CD4$^+$ and CD8$^+$ T cell purification, and transfection. Fresh blood samples from CHB patients and HDs were diluted with phosphate-buffered saline (PBS, Hyclone, Logan, UT, USA) containing 5% fetal calf serum (FBS, Invitrogen Gibco, Carlsbad, CA, USA). Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque™ PLUS (GE Healthcare, Buckinghamshire, England). PBMCs (2×10^8) were centrifuged at $300 \times g$ for 10 min and resuspended in 80 μl PBS containing 5% FBS. The cells were mixed with 20 μl of MACS CD8 MicroBeads (130-045-201) and incubated for 15 min at 4–8°C. The cells were washed and applied to a prepared MS column (130-041-401). The column was thoroughly washed, and the CD8$^+$ cells were eluted. The effluent fraction was used to isolate CD4$^+$ T cells in 80 μl buffer with 20 μl of MACS CD4 MicroBeads (130-045-101).

PBMCs or purified CD4$^+$ or CD8$^+$ T cells were transfected using an Amaxa® Human T Cell Nucleofector® Kit (Lonza, Cologne, Germany), following the manufacturer's recommendations. The cells were cultured in a 12-well plate in 2 ml RPMI 1640 medium (Hyclone) supplemented with 10% FBS and 1–5 ng/ml human IL-7 (Biolegend, San Diego, CA, USA) per well in a humidified 37°C/5% CO$_2$ incubator for 24 h. The cells were transfected with a Nucleofector® II Device (Lonza) using program V-024. The siRNA sequences used were as follows: JMJD6, 5′-CCUGGAA-UGCCUUAGUUCA-3′; negative control, 5′-CCUACGCCACCAAUUUCGU-3′ (Bioneer, Daejeon, South Korea). siRNAs specific for CDKN3 (s2852) and SUMO1 (s14607) were purchased from ABI (Los Angeles, CA, USA).

Stimulation of PBMCs. Human PBMCs were cultured at 37°C in the presence of 5% CO$_2$ in RPMI 1640 medium supplemented with 10% FBS. Ninety six-well plates (Costar Corning, Lowell, MA, USA) were coated with 200 μl/well of PBS containing 1 μg/ml anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego, CA, USA). The PBMCs were then seeded at a density of $1–1.5 \times 10^6$ cells/ml and stimulated with anti-CD3/CD28 for 1–4 d. To assess T cell activation, the expression of CD25 and CD69 on CD4$^+$ and CD8$^+$ T lymphocytes was measured 24 h after anti-CD3/CD28 stimulation. To measure T cell proliferation, the PBMCs were suspended in a volume of 1 ml, labeled with 1 μl of 5 mM carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich, St. Louis, MO, USA), and stimulated in anti-CD3/CD28-coated plates. Cell proliferation was assessed by measuring CFSE dilution by flow cytometric analysis. To determine the effect of CHB sera on proliferation of HD T cells, 10 serum samples from CHB patients were separated into two groups by ALT level: ALT high, >78 U/L and ALT low, <40 U/L. PBMCs from HDs were seeded at a density of 1–1.5 × 10^6 cells/ml with patient's serum (10 μl serum/200 μl volume) for 6 hr and then stimulated with 1 μg/ml anti-CD3 and anti-CD28 antibodies for 3 days. CD4$^+$ T cells (left) and CD8$^+$ T cells (right) are CD3$^+$CD4$^+$CD8$^-$ or CD3$^+$CD4$^-$CD8$^+$ PBMCs. Cell proliferation was assessed by measuring CFSE dilution by flow cytometric analysis of live T cells. Apoptosis was assessed by measuring Annexin V staining. Antibodies specific for CD3, CD4, CD8, CD25, CD69, CD122, CD132, TIM3, LAG3, and PD-1 were purchased from BD Bioscience (San Jose, CA, USA) or eBioscience. All data were collected using a BD FACSCanto II flow cytometer (BD Bioscience, NJ, USA).

Intracellular staining. For intracellular staining (ICS) of cytokines, PBMCs were stimulated with 1 μg/ml anti-CD3 and anti-CD28 antibodies at 37°C for 48 h. Then 1×10^6 stimulated PBMCs or fresh PBMCs (for Ki67 staining) were cultured with phorbol myristate acetate (PMA 50 ng/ml) and ionomycin (1 mg/ml) plus 10 μg/ml Brefeldin A (Sigma-Aldrich) 6 h before ICS. PBMC suspensions stained for surface markers were washed with PBS twice and then incubated with 200 μl fixation/permeabilization solution(BD Bioscience) for 20 min. PBMC suspensions were washed with 500 μl PBS containing 0.1% saponin and 2% FBS twice. PBMCs were stained with FITC-anti-IL-2 and PE-anti-IFNγ (BD Biosciences), PE-anti-TNFα, FITC-anti-IL-4 and FITC-anti-Ki67 (BioLegend) for 30 min and then washed with PBS containing 0.1% saponin and 2% FBS twice for data acquisition.

Western blot analysis. Treated cells were washed once with PBS and lysed in lysis buffer. The lysates were subjected to SDS-PAGE, and the proteins in the gel were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA). After blocking the membranes with 5% nonfat dry milk in TBS-T for 2 h at room temperature, the PVDF membranes were incubated with the indicated primary antibody diluted in TBS-T containing 5% nonfat dry milk at 4°C overnight. The membranes were washed three times with TBS-T and probed with HRP-conjugated secondary antibodies at room temperature for 2 h. The membranes were washed four times with TBS-T, and the proteins were visualized

with the ECL system (Millipore). Anti-JMJD6 and anti-β-actin were purchased from Sigma-Aldrich.

Measurement of cytokine secretion. Plasma samples from CHB patients and HDs were collected by centrifuging fresh blood samples at $400 \times g$ for 5 min. The supernatants were collected from PBMCs 24 h after transfection and 3 d after stimulation. All cytokines were measured using a Luminex 200 system (Millipore) with a Bio-Plex Reagent Kit, Diluent Kit, and human Grp I Cytokine 27-Plex Panel (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Individual cytokines were also measured using ELISA kits (DBB00) from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Culture of PBMCs with cytokines. Freshly isolated PBMCs from healthy donors were cultured in 96-well plates with 2 μg/ml HBsAg (kindly provided by the Academy of Military Medical Sciences, Beijing) and cytokines at concentrations equivalent to the mean concentrations found in the plasma of CHB patients (IL-8: 40 pg/ml, RANTES: 30 ng/ml, PDGF-BB: 60 ng/ml, IFNγ: 1.5 ng/ml, TNFα: 100 pg/ml, and IP-10: 30 ng/ml) (PeproTech, Rocky Hill, NJ, USA) at a density of $1–1.5 \times 10^6$ cells/ml for 20 h. At the end of the culture period, the cells were collected for RNA exaction and real-time PCR.

RNA extraction and real-time quantitative PCR. Total RNA was isolated from PBMCs using an RNAqueous®-Micro Kit (Ambion, Austin, TX, USA) and used in reverse transcription PCR reactions using Superscript® III first-strand Synthesis System for RT-PCR (Invitrogen). Real-time quantitative PCR was performed on an ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix 2× (ABI) in a 20-μl reaction volume containing 0.2 μl of cDNA and 0.5 μl each of forward and reverse real time PCR primers (10 mM). Standard PCR conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and one cycle of 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s to analyze the dissociation (melt) curves and determine the primer specificity. All data were normalized to the level of the 18 s rRNA transcript. The real-time primers used were: human 18 s rRNA forward, 5′-TCAACTTTCGATGGTAGTCGCCGT-3′; and reverse, 5′-TCCTTGGATGTGGTAGCCGTTTCT-3′. The real-time primers used were: human JMJD6 rRNA forward, 5′- TTGGAAGACTACAAGGTGCC -3′; and reverse, 5′- GGTCGATGTGAATCCCAGTTC -3′. The primers for real-time PCR were: human CDKN3 forward, 5′- ACAATATCACCAGAGCAAGCC -3′, and reverse, 5′- GCAGCTAATTT GTCCCGAAAC-3′; human SUMO1 forward, 5′- GGGAAGGGAGAAGGATTTGTAA-3′, and reverse, 5′- GTCCTCAGTTG-AAGGTTTTGC-3′. The primers for CDKN3 isoforms: forward, 5′-CCGCCCA-GTTCAATACAAACAAGT-3′, and reverse, 5′- GCGATTGGATGATG ATGG-GTGATAA -3′. human GAPDH forward, 5′- ACATCGCTCAGACACCATG-3′, and reverse, 5′- TGTAGTTGAGGTCAATGAAGGG-3′. Other primer sequences are listed in Supporting Table 2.

Statistical analysis. The data were analyzed using GraphPad Prism 5.00 software and are expressed as the means ± SE. The data from various individuals were compared using the un-paired Student's t-test. Differences were considered statistically significant when $p < 0.05$. T cell proliferation rate for each patient (Fig. 1a and 1b, Fig. 2e and 2f, Fig. 3f, and Fig. 5b, 5c, 5d, and 5e) was calculated based on the Fraction-diluted of CFSE-diluted cells (fraction of cells in the final culture that divided at least once) according to the guidelines by Roederer[39].

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Acknowledgments

Author contributions

C.-F.C. and Y.-W.H. designed the experiments; C.-F.C., X.F., W.-J.J., Y.W., L.-L.G., J.-J. Liu, X.-H.Y., B.-B.Z., D.Z. and J.G. performed the experiments; X.F., H.-Y.L., J.Z., G.-F.C. and H.-P.Y. recruited the patients; Y.W. and J.Z. provided reagents/analytic tools; C.-F.C. and Y.-W.H. analyzed the data and wrote the paper.

Additional information