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ORIGINAL ARTICLE

Down-regulation of PR/SET domain 10 underlies natural killer cell dysfunction in hepatocellular carcinoma

Jiantao Han[1] | **Chao Ke**[2] | **Bin Jiang**[2] | **Hongjian Zhou**[2] | **Hanbin Xu**[2] | **Xingwang Xie**[1]

[1]The Department of Hepatobiliary and Pancreatic Surgery, Tongren Hospital of Wuhan University, Wuhan, Hubei Province, China

[2]The Department of Gastrointestinal, Hernia and Abdominal Wall Surgery, Tongren Hospital of Wuhan University, Wuhan, Hubei Province, China

Correspondence
Xingwang Xie, The Department of Hepatobiliary and Pancreatic Surgery, Tongren Hospital of Wuhan University, 241 Pengliuyang Road, Wuchang District, Wuhan, Hubei Province, China, 430060.
Email: xiexingwang6304@hotmail.com

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Abstract

Hepatocellular carcinoma (HCC) is the world's leading cause of tumor-related mortalities. Natural killer (NK) cells play a critical role at the first immunological defense line against HCC initiation and progression. NK cell dysfunction is therefore an important mechanism for immune evasion of HCC cells. In the present study using a murine HCC model, we revealed the down-regulation of PR/SET Domain 10 (PRDM10) in hepatic NK cells that were phenotypically and functionally exhausted. PRDM10 silencing diminished the expression of natural killer group 2 member D (NKG2D) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), augmented T cell immunoglobulin and ITIM domain (TIGIT) expression, and decreased the expression of interferon (IFN)-γ, perforin and granzyme B in normal hepatic NK cells in vitro. Consistently, PRDM10-deficient NK cells exhibited impaired cytotoxicity on target cells. In contrast, PRDM10 over-expression promoted NKG2D and Fas ligand (FasL) expression, reduced CD96 expression and enhanced transcripts of IFN-γ, perforin and granzyme B in NK cells *in vivo*. Moreover, PRDM10 silencing and PRDM10 over-expression down-regulated and up-regulated Eomesodermin (Eomes) expression, respectively. In summary, this study reveals PRDM10 down-regulation as a novel mechanism underlying NK cell dysfunction and identifies PRDM10 as a supporting factor of NK cell function.

KEYWORDS

eomesodermin, hepatocellular carcinoma, natural killer cells, PR/SET Domain 10, tumor immunity

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and fatal malignancies across the globe [1]. Immunotherapeutic strategies exploiting natural killer cells (NK cells) have been regarded as a promising treatment against multiple tumors, including HCC. NK cells produce cytotoxic mediators to kill target cells such as malignant cells. The prominent effect of NK cells in immune responses against HCC has been reported previously [2–4]. Nonetheless, convincing studies also demonstrated down-regulated NK cell frequency and NK cell dysfunction in HCC, such as abnormal cytolytic activity, reduced production of tumor necrosis factor (TNF)-α and interferon (IFN)-γ [5–10]. Defects in NK cell functions, which might be caused by functional exhaustion, anergy or senescence, are considered crucial mechanisms for immune evasion of HCC cells [11]. NK cell dysfunction is linked to immunosuppressive regulatory T cells, myeloid suppressor cells and fibroblasts in tumor sites [8,9,12]. However, the intracellular molecules/signaling essential for NK cell dysfunction during HCC initiation and progression remain less clear.

Members of the PR/SET domain-containing (PRDM) family feature an N-terminal PR domain relating to the SET methyltransferase domain and multiple zinc fingers essential for DNA binding and protein–protein interplays [13]. PRDM10 is a highly conserved PRDM family member and is expressed in multiple organs and tissues [14]. However, its function remains largely unidentified. The gene infusion of CITED2–PRDM10 or MED12–PRDM10 has been mentioned in undifferentiated pleomorphic sarcoma [15,16]. Recent research suggests its important role as a sequence-specific transcription factor in preimplantation embryos and embryonic stem cells [14]. To our knowledge, the significance of PRDM10 to HCC immunity is completely unknown.

In the present project, using a chemically induced HCC mouse model, we identified the down-regulation of PRDM10 in hepatic NK cells and its role in NK cell dysfunction and exhaustion. Both *in-vitro* and *in-vivo* experiments suggest that PRDM10 attenuates NK cell dysfunction and maintains NK cell function possibly through promoting Eomesodermin (Eomes) expression. Therefore, this study reveals a novel molecular mechanism underlying NK cell dysfunction in HCC.

MATERIALS AND METHODS

Animals

The animal study was approved by Wuhan University Animal Care and Use Committee and conducted following the Wuhan University Animal Use Guidelines. Eight-week-old male C57BL/6J mice were purchased from the Center for Animal Experiment at Renmin Hospital of Wuhan University.

HCC model

To induce HCC, N-nitrosodiethylamine (60 μg/g body weight; Weikeqi Biotech Co., Ltd, Wuhan, China) was administered into the peritoneal cavity of each mouse. Three days later, 10% carbon tetrachloride (10 μl/g body weight; Thermo Fisher, Waltham, Massachusetts, USA) was intraperitoneally administered twice every week for 30 days. Another single dose of 60 μg/g body weight N-nitrosodiethylamine was then administered the same manner. After that, 10 μl/g body weight 10% carbon tetrachloride was intraperitoneally injected once every week for a consecutive 14 weeks. Livers with initiation foci, hyperplastic nodules and culminating appearance indicated successful HCC induction. Mice with confirmed HCC induction were used for the study.

Enrichment of splenic and hepatic immune cells

After HCC induction, mice were euthanized and spleens were ground on cell strainers (Corning, Inc., Corning, New York, USA) to prepare splenocyte suspensions. Mouse livers were ground on steel mesh filters and liver cells were suspended in 30% Percoll (Sigma-Aldrich), followed by overlaying onto an equal volume of 70% Percoll. Liver cell suspensions were centrifuged at 500 relative centrifugal force (rcf) for 20 min and mononuclear cells were harvested in the Percoll interface. Red blood cells were removed by incubation in the red blood cell lysis buffer (Beyotime Biotech, Shanghai, China).

Fluorescence-activated cell sorting (FACS)

Antibodies were obtained from Biolegend (San Diego, California, USA): allophycocyanin–cyanin 7 (APC-Cy7) anti-NK1.1 (PK136), peridinin chlorophyll (PerCP)-Cy5.5 anti-CD3 (17A2), APC anti-CD49a [hamsters monoclonal (HM)α1], phycoerythrin (PE)-Cy7 anti-CD49b (HMα2), PE anti-CD27 (LG.3A10), fluorescein isothiocyanate (FITC) anti-CD11b (M1/70), APC anti-interferon (IFN)-γ [interferon gamma antibody (XMG1.2)], Alexa Fluor® 647 anti-granzyme B (GB11), APC anti-perforin (S16009B), PE anti-NKG2D (CX5), PE anti-tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (N2B2), PE anti-Fas ligand (FasL) (Kay-10), PE anti-CD96 (3.3), PE anti-T cell immunoglobulin and ITIM domain (TIGIT) (1G9) and PE anti-Ki67 (11F6). For NK receptor detection, 1×10^5 or 1×10^6/ml cells were incubated with 5 μg/ml of each antibody in phosphate-buffered saline on ice for 20 min. Dead cells were excluded by staining with the LIVE/DEAD™ fixable violet dead cell stain kit (L34963; Thermo Fisher), following the supplier's brochure. To measure cytotoxic mediators and Ki67, cells were fixed with 2% paraformaldehyde for 15 min, incubated in 90% methanol on ice for 30 min and incubated with 5 μg/ml of antibodies at room temperature for 1 h. Apoptosis was quantified using the annexin V-APC assay kit (Abcam, Cambridge, UK), following the supplier's instructions. An LSR II flow cytometer was used for analysis and a FACSAria sorter was used for sorting (BD Biosciences, Franklin Lakes, New Jersey, USA).

Reverse transcription and real-time polymerase chain reaction (RT–PCR)

cDNAs were synthesized using the PrimeScript first-strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan). The SYBR green quantitative RT–PCR kit (Sigma-Aldrich)

was used for quantitative PCR on a CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, California, USA) following the standard protocol. The amounts of target transcripts were normalized to β-actin transcript levels and computed by the $2^{-\Delta\Delta Ct}$ formula. Primer sequences are provided in Supporting information, Table S1.

NK cell culture and lentiviral infection

Hepatic NK cells were sorted by flow cytometry and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) in the presence of 1000 U/ml mouse interleukin (IL)-2 (R&D Systems, Minneapolis, Minnesota, USA). The PRDM10 RNAi lentivector (cat. co. 375670940495) and PRDM10 lentiviral vector (cat. no. 375670640395) were obtained from Applied Biological Materials Inc. (Richmond, British Columbia, Canada). Lentivirus preparation and titration were conducted by the Biofavor Biotech Co., Ltd (Wuhan, China). To infect NK cells, 1×10^6/ml NK cells were incubated with the corresponding lentivirus at a multiplicity of infection of 40 with 8 μg/ml of polybrene (Thermo Fisher). The cell suspension was centrifuged at 800 *g* for 1 h at 32°C. The supernatant was replaced by fresh medium supplemented with 1000 U/ml of rmIL-2 (R&D Systems, Minneapolis, MN, USA) for 2 consecutive days.

Cytotoxicity assay

The mouse lymphoma cell line YAC-1 was authenticated and purchased from Procell Life Science and Technology (Wuhan, China); 1×10^4 YAC-1 cells were co-cultured with 1×10^5 lentivirus-infected NK cells for 6 h in a 96-well microplate (Corning). YAC-1 cells, which were NK1.1-negative, were then analyzed for apoptosis using the annexin V-APC assay kit (Abcam). In some experiments the supernatants of the co-cultured cells were collected, and YAC-1 cell lysis was measured by determining the lactate dehydrogenase activity in the supernatants using the LDH assay kit (ab65393; Abcam). To determine the expression of cytotoxic mediators in co-cultured NK cells, 5 μg/ml brefeldin A and 5 μg/ml monensin (Sigma-Aldrich) were added into co-cultured cells 2 h before the end of co-culture. The cells were then stained with the APC-Cy7 anti-NK1.1 antibody (PK136) followed by intracellular staining of cytotoxic mediators as described above.

Western blot

Cellular proteins were extracted using the RIPA buffer (Thermo Fisher) supplemented with protease inhibitors (Sigma-Aldrich). The anti-PRDM10 monoclonal antibody (48AT1224.90.46), anti-Eomes monoclonal antibody (21Mags8) and anti-β-actin monoclonal antibody (mAb-GEa) were purchased from Thermo Fisher.

Adoptive transfer

After HCC was induced, 2×10^6 lentivirus-infected NK cells in 100 μl of saline were infused into each HCC-bearing mouse through retro-orbital injection. The injection was repeated 1 day later. Seven days after the second injection, donor-derived GFP^+ NK cells were sorted from the livers of the recipients.

Statistics

Each experiment was independently performed two or three times. The data were shown as mean ± standard deviation. Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was used for statistical analysis. A p-value < 0.05 was regarded as significant.

RESULTS

Hepatic NK cells exhibit exhaustion phenotype in HCC

After HCC induction, we evaluated the phenotype of splenic and hepatic NK cells in HCC-bearing mice. $CD3^-NK1.1^+$ cells were first gated within live mononuclear cells isolated from mouse spleens and livers (Figure 1a and Supporting information, Figure S1), followed by discrimination of $CD49a^+CD49b^-$ type 1 innate lymphoid cells (ILC1) and $CD49a^-CD49b^+$ NK cells within $CD3^-NK1.1^+$ cells (Figure 1b). The proportion of ILC1 in hepatic mononuclear cells was not significantly changed (Figure 1c), whereas the proportion of NK cells in hepatic mononuclear cells was reduced (Figure 1d). No significant changes in the proportions of splenic ILC1 and NK cells were observed, respectively (Figure 1c,d).

We then sorted splenic and hepatic $CD3^-NK1.1^+CD49a^-CD49b^+$ NK cells and determined the expression of an array of activating receptors and inhibitory receptors. As shown in Figure 2a–g, hepatic NK cells of HCC-bearing mice expressed fewer mRNAs of NKG2D, TRAIL and FasL compared with their counterparts in healthy mice. Interestingly, they expressed more mRNAs of CD96 and TIGIT than their counterparts in healthy mice. The expression of these receptors was not remarkably changed in splenic NK cells of HCC-bearing

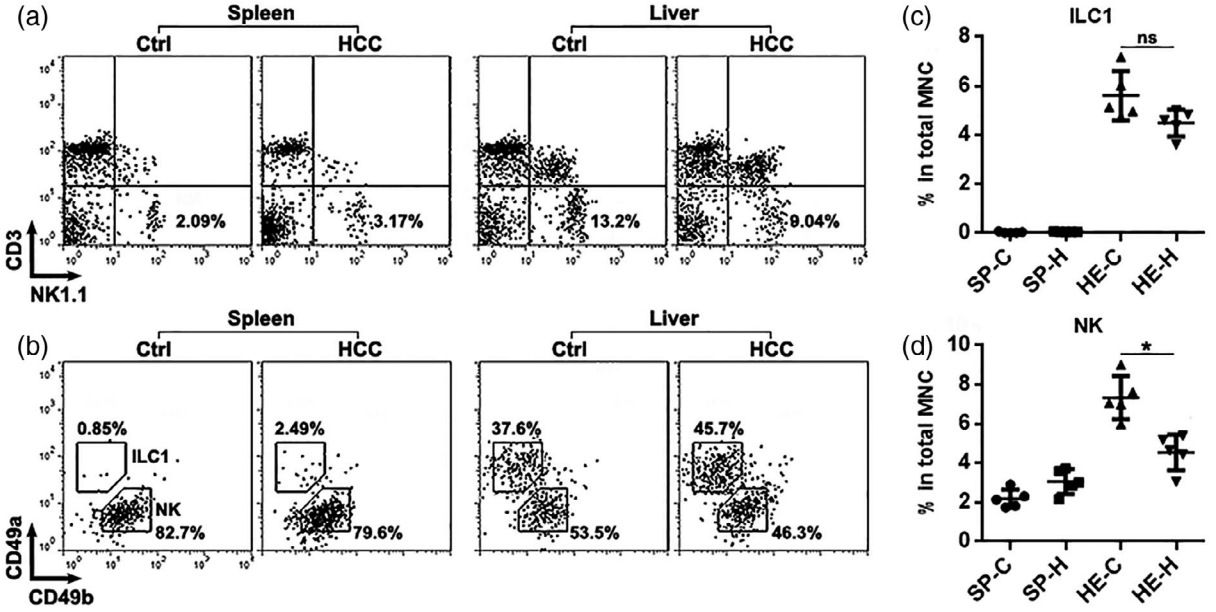

FIGURE 1 Detection of splenic and hepatic natural killer (NK) cells. (a) Detection of $CD3^{-}NK1.1^{+}$ cells in mouse spleens and livers. Ctrl = healthy control mice; HCC = hepatocellular carcinoma-bearing mice. (b) Detection of $CD49a^{+}CD49b^{-}$ innate lymphoid cells (ILC1) and $CD49a^{-}CD49b^{+}$ NK cells within $CD3^{-}NK1.1^{+}$ cells. (c,d) The percentages of ILC1 (c) and NK cells (d) in splenic or hepatic mononuclear cells. SP-C = spleens of control mice; SP-H = spleens of HCC-bearing mice; HE-C = livers of control mice; HE-H = livers of HCC-bearing mice; $n = 5$ mice. Student's t-test. NS; = not significant; $^{*}p < 0.05$

mice in comparison to their counterparts in healthy mice except for FasL, which was up-regulated in splenic NK cells after HCC induction (Figure 2a–g). Programmed cell death 1 (PD-1) mRNA levels were comparable between splenic and hepatic NK cells in either healthy mice or HCC-bearing mice (Figure 2h). The alterations of NKG2D, TRAIL, FasL, CD96 and TIGIT were substantiated by FACS (Figure 2i). Hepatic NK cells of HCC-bearing mice induced fewer YAC-1 cell deaths than their counterparts of healthy mice, suggesting their weaker cytotoxicity (Figure 2j and Supporting information, Figure S3). Therefore, hepatic NK cells were dysfunctional in HCC.

Hepatic NK cells down-regulate Eomes and PRDM10 in HCC

In our unpublished pilot study conducting transcriptome sequencing of hepatic NK cells in HCC-bearing mice we noticed profound decreases in Eomes and PRDM10 (data not shown). Therefore, we checked the protein levels of Eomes and PRDM10 in sorted splenic and hepatic $CD3^{-}NK1.1^{+}CD49a^{-}CD49b^{+}$ NK cells. As shown in Figure 3a, there was a mild increase of Eomes in splenic NK cells after HCC induction. Interestingly, hepatic NK cells expressed higher Eomes than splenic NK cells in healthy mice. No change in T-bet was observed (Supporting information, Figure S2). However, after HCC induction, Eomes was markedly decreased in hepatic NK cells (Figure 3a). PRDM10 expression showed a similar pattern to Eomes, featuring significant down-regulation in hepatic NK cells after HCC induction (Figure 3b). As the effect of PRDM10 on NK cells has not been reported, we focused upon this molecule in the following experiments. We then dissected hepatic NK cells into three subsets according to CD27 and CCD11b: $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$ and $CD27^{-}CD11b^{+}$ cells (Figure 3c). Previous studies show that these subpopulations differ in maturation stages, proliferative activity and cytotoxic capacity, with $CD27^{-}$ NK cells having lower cytotoxic potential and producing lower cytokines than $CD27^{+}$ NK cells [17,18]. After HCC induction, PRDM10 mRNAs were decreased in all three subsets, with the most profound decrease in the $CD27^{+}CD11b^{-}$ subset (Figure 3d).

PRDM10 silencing induces NK cell exhaustion

To specifically evaluate the role of PRDM10, we infected hepatic $CD3^{-}NK1.1^{+}CD49a^{-}CD49b^{+}$ NK cells with lentiviral particles encoding *PRDM10* shRNA and GFP. The infection rate was approximately 40%, as seen by GFP expression (Figure 4a, Supporting information, Figure S4a). *PRDM10* was successfully silenced 2 days after infection (Figure 4b), and the survival of NK cells was not impacted (Figure 4c, Supporting information, Figure S4b). The

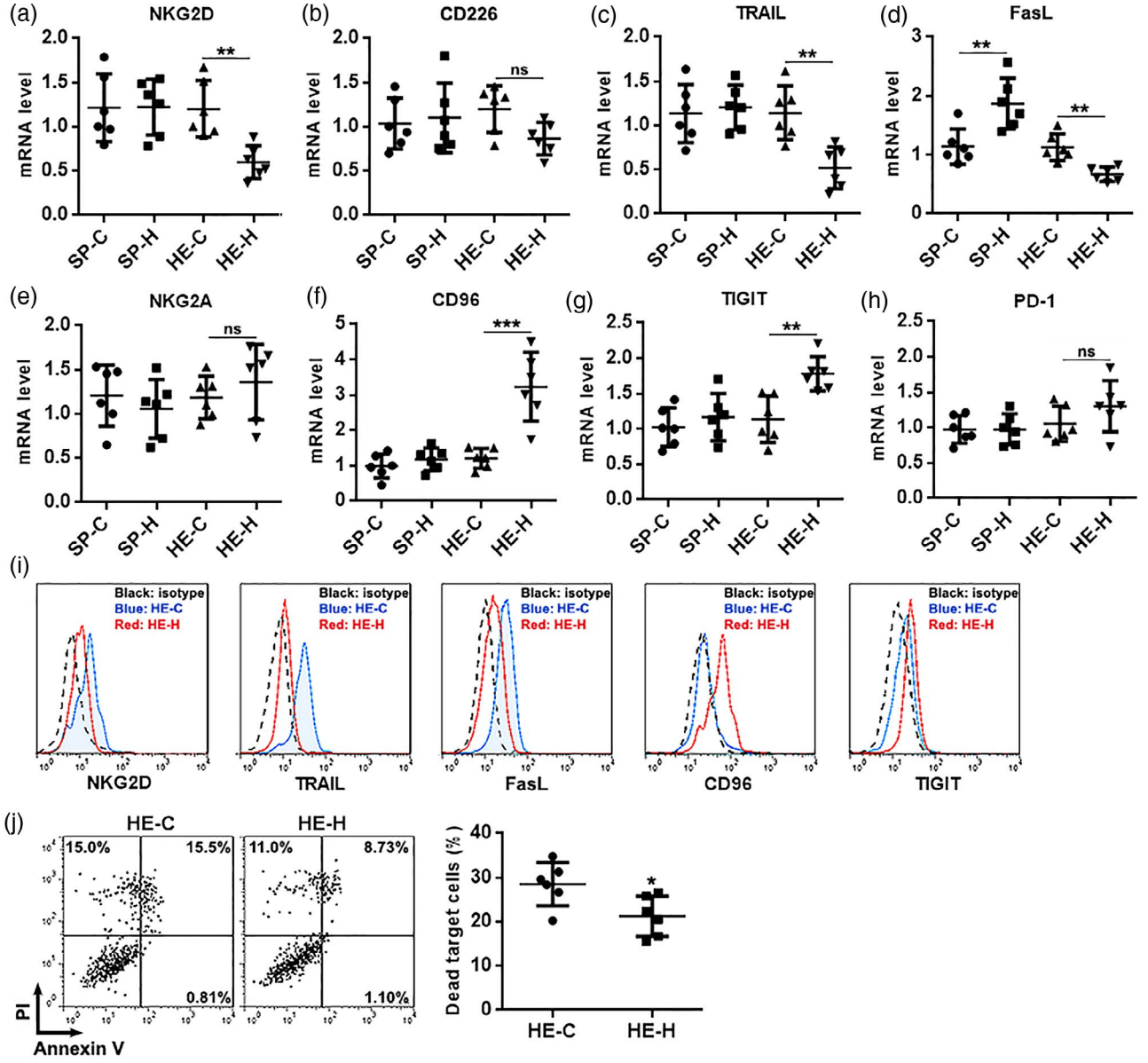

FIGURE 2 Natural killer (NK) cell phenotype. (a–h) mRNA levels of indicated receptors in splenic and hepatic NK cells. SP-C = splenic NK cells of control mice; SP-H = splenic NK cells of hepatocellular carcinoma (HCC)-bearing mice; HE-C = hepatic NK cells of control mice; HE-H = hepatic NK cells of HCC-bearing mice. One-way analysis of variance (ANOVA). (i) Fluorescence activated cell sorter (FACS) histograms showing the expression of indicated receptors on hepatic NK cells. HE-C = hepatic NK cells of control mice; HE-H = hepatic NK cells of HCC-bearing mice. (j) Apoptosis of YAC-1 cells. HE-C = YAC-1 co-cultured with hepatic NK cells of control mice; HE-H = YAC-1 co-cultured with hepatic NK cells of HCC-bearing mice. Left panel: representative dot plots. Right panel: statistics of the frequencies of dead YAC-1 cells; $n = 6$ mice. Student's t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.01$

expression of NKG2D and TRAIL was remarkably down-regulated while TIGIT was up-regulated after PRDM10 silencing (Figure 4d–f). No significant changes in FasL and CD96 were observed (Figure 4d–f). PD-1 mRNA levels were equivalent in control NK cells and PRDM10-deficient NK cells (Figure 4g). We then co-cultured NK cells with YAC-1 cells to activate NK cell cytotoxicity. After co-culture, control NK cells produced abundant IFN-γ, perforin and granzyme B (Figure 5a–c). However, PRDM10 silencing caused significant decreases in these cytotoxic mediators, with the most profound decrease in IFN-γ (Figure 5a–c). Compared with control NK cells, PRDM10-deficient NK cells were less capable of inducing the apoptosis of YAC-1 cells (Figure 5d). Moreover, PRDM10-deficient NK cells were less cytotoxic to YAC-1 cells than control NK cells (Supporting information, Figure S5). Therefore, PRDM10 silencing caused NK cell dysfunction.

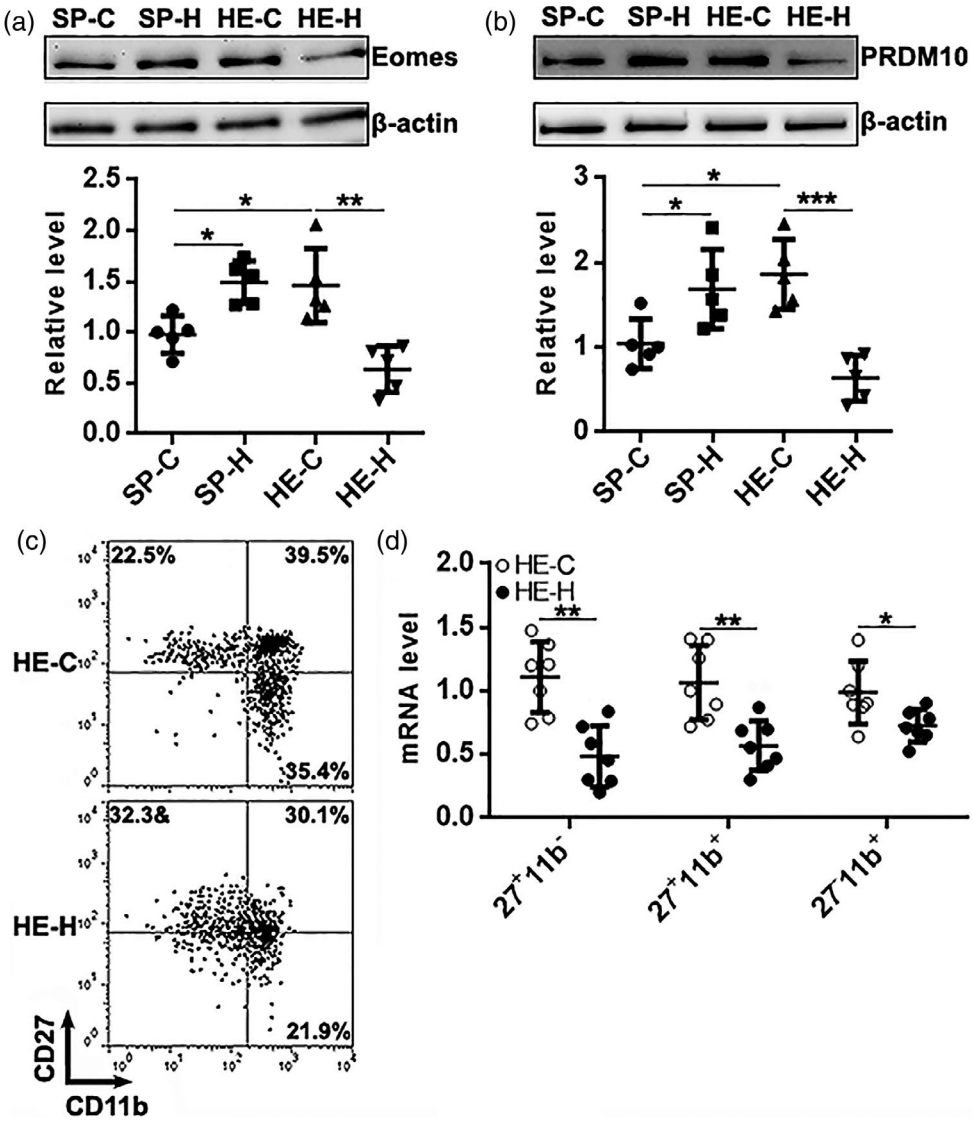

FIGURE 3 PR/SET Domain 10 (PRDM10) expression in natural killer (NK) cells. (a,b) Protein levels of Eomes (a) and PRDM10 (b) in NK cells. Upper panels: representative western blot images. Lower panels: Statistics. SP-C = splenic NK cells of control mice; SP-H = splenic NK cells of hepatocellular carcinoma (HCC)-bearing mice; HE-C = hepatic NK cells of control mice; HE-H = hepatic NK cells of HCC-bearing mice; $n = 5$ mice. One-way analysis of variance (ANOVA). (c) Hepatic NK subsets based on CD27 and CD11b. (d) mRNA levels of PRDM10 in hepatic NK subsets; $27^{+}11b^{-}$ = $CD27^{+}CD11b^{-}$ NK; $27^{+}11b^{+}$ = $CD27^{+}CD11b^{+}$ NK; $27^{-}11b^{+}$ = $CD27^{-}CD11b^{+}$ NK; $n = 7$ mice. Student's *t*-test. $p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.01$

PRDM10 regulates Eomes expression

PRDM10 is a transcription factor and could potentially bind to the promoter/enhancer region of Eomes. To investigate whether PRDM10 impacts Eomes expression, we first quantified Eomes mRNA and found a significant reduction of Eomes in PRDM10-deficient NK cells relative to control NK cells (Figure 6a). This change was confirmed by western blot, demonstrating lower Eomes protein in PRDM10-deficient NK cells (Figure 6b). To further confirm the effect of PRDM10, we infected naive hepatic NK cells with lentivirus encoding PRDM10 and GFP to induce PRDM10 over-expression (Figure 6c, Supporting information, Figure S6). As expected, PRDM10 over-expressing NK cells produced more PRDM10 and Eomes than control NK cells (Figure 6d–f). Therefore, PRDM10 is a positive regulator of Eomes. However, PRDM10 over-expression did not promote NK cell cytotoxicity *in vitro* (Supporting information, Figure S7). Consistently, PRDM10 over-expression had no remarkable impact on the expression of IFN-γ, perforin, granzyme B, NKG2D, TRAIL and FasL (Figure 6g), suggesting the redundancy of exogenous PRDM10 when endogenous PRDM10 is already sufficient to support NK cell function.

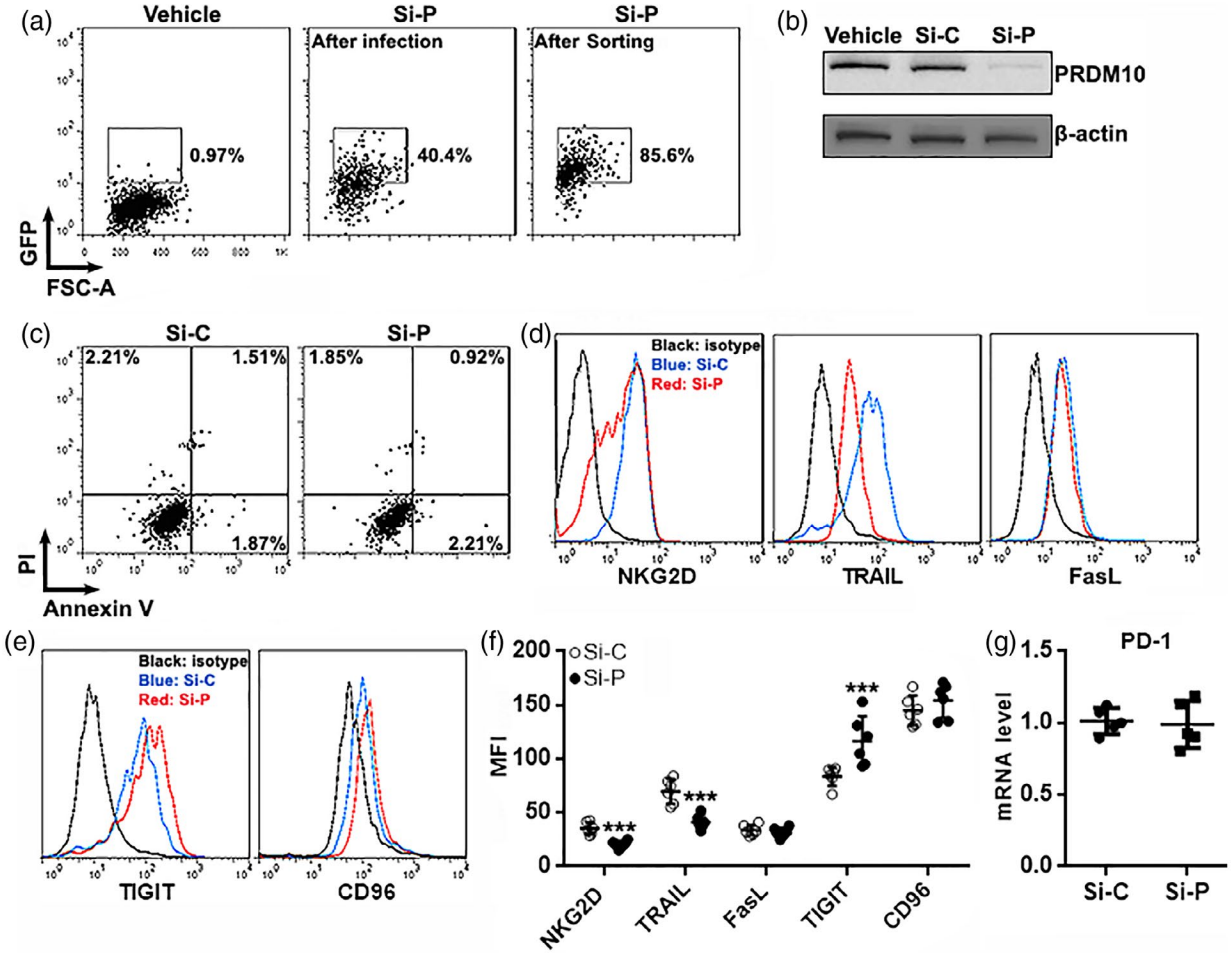

FIGURE 4 Natural killer (NK) cell phenotype after PR/SET Domain 10 (PRDM10) silencing *in vitro*. (a) Green fluorescent protein (GFP) expression after lentiviral infection. Vehicle: infection reagents only. Si-P = infection with lentivirus encoding PRDM10 shRNA. After infection: day 2 after infection. After sorting: after fluorescence activated cell sorting (FACS). The transfection rate of control lentivirus was similar (data not shown). (b) PRDM10 protein in sorted NK cells on day 2 after infection. Si-C = infection with control lentivirus encoding scramble shRNA; Si-P = infection with lentivirus encoding PRDM10 shRNA. The image represents two independent experiments. (c) NK cell apoptosis on day 2 after infection. (d,e) Representative histograms of indicated receptors on infected NK cells. (f) Statistics of the mean fluorescence intensities (MFI) of indicated receptors on infected NK cells. (g) mRNA levels of PD-1 in lentivirus-infected NK cells

PRDM10 over-expression strengthens NK cell function *in vivo*

To evaluate the effect of PRDM10 *in vivo*, we adoptively infused control NK cells and PRDM10 over-expressing NK cells into HCC-bearing mice. Seven days after infusion, GFP^+ donor-derived NK cells were found in the livers of recipients (Figure 7a). The proportions of control NK cells and PRDM10 over-expressing NK cells were equivalent, suggesting that PRDM10 did not affect NK cell homing to livers (Figure 7a, Supporting information, Figure S8). We then sorted these donor-derived NK cells to quantify Eomes mRNA and found higher Eomes expression in PRDM10 over-expressing NK cells (Figure 7b). Analysis of mRNAs of IFN-γ, perforin and granzyme B indicated higher expression of these cytotoxic mediators in PRDM10 over-expressing NK cells *in vivo* (Figure 7c). Furthermore, PRDM10 over-expressing NK cells exhibited higher expression of NKG2D, TRAIL and FasL than control NK cells in recipients' livers (Figure 7d–i), whereas TIGIT expression was not altered and CD96 expression was lower in PRDM10 over-expressing NK cells (Figure 7g–i). Therefore, PRDM10 over-expression maintained NK cell activation and inhibits NK cell exhaustion *in vivo*. However, the progression of HCC and mouse survival were not improved by the infusion of PRDM10 over-expressing NK cells (data not shown).

DISCUSSION

The current study suggests that PRDM10 could support NK cell function. PRDM10 protein consists of an

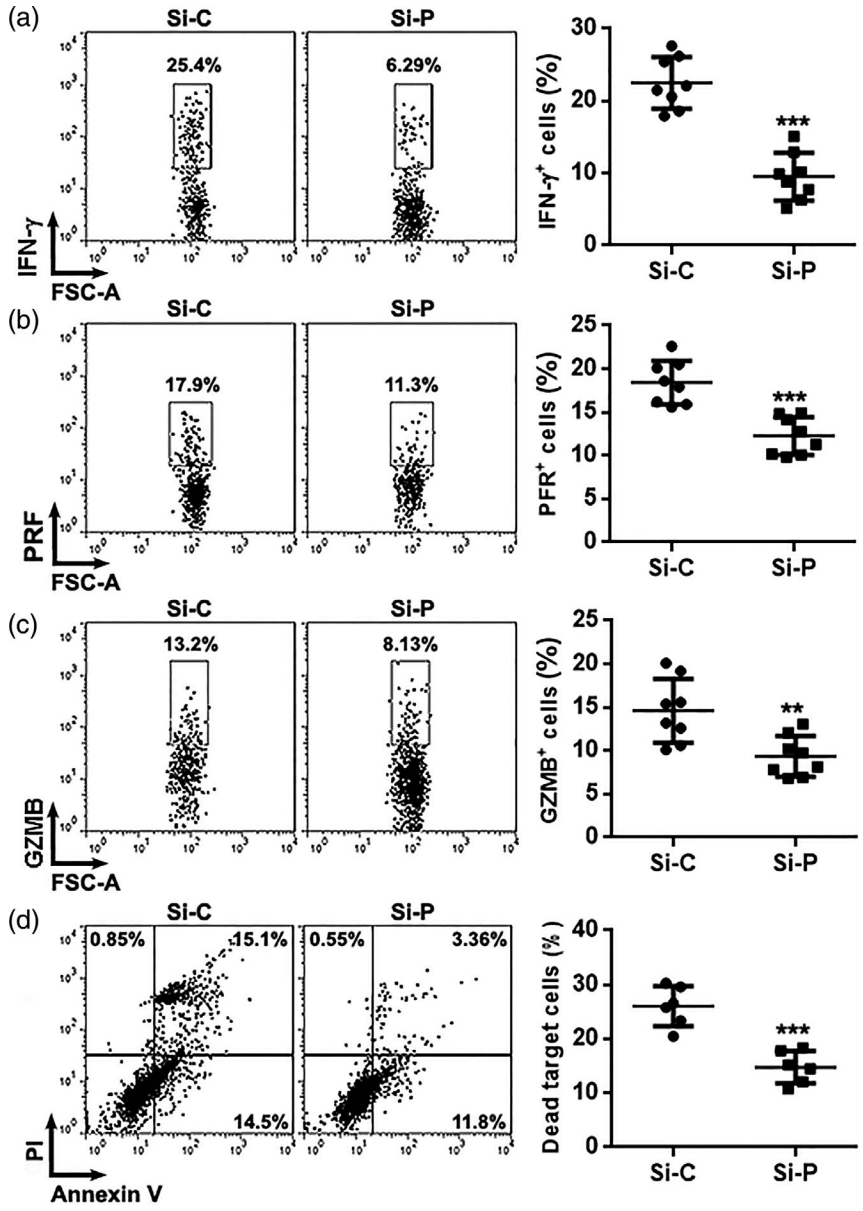

FIGURE 5 Natural killer (NK) cell function after PR/SET Domain 10 (PRDM10) silencing *in vitro*. (a–c) Expression of indicated cytotoxic mediators in infected NK cells after contact with target cells. Si-C = infection with control lentivirus; Si-P = infection with lentivirus encoding PRDM10 shRNA; PRF = perforin; GZMB = granzyme B. Left panels: fluorescence activated cell sorter (FACS) dot-plots. Right panels: statistics; $n = 8$ samples in three experiments. (d) YAC-1 cell death after co-culture with NK cells; Si-C = YAC-1 co-cultured with NK cells infected with control lentivirus. Si-P = AC-1 co-cultured with NK cells infected with lentivirus encoding PRDM10 shRNA; $n = 6$ samples in three experiments. Student's *t*-test. $^{**}p < 0.01$; $^{***}p < 0.01$

N-terminal PR domain, 10 C2H2 zinc fingers and a C-terminal glutamine (Q)-rich transactivation domain [14]. PRDM10 maintains global translation in early embryonic development through regulating the expression of eukaryotic translation initiation factor 3 [14]. However, its role in modulating adult cell function has never been elucidated. Thus, to our knowledge, our work offers insights into this field for the first time.

Tumor-induced NK cell exhaustion is characterized by a marked decrease of the T-box transcription factors Eomes and T-bet [19], as well as down-regulation of activating receptors (NKG2D, CD226, TRAIL, FasL) and persistent expression of inhibitory receptors such as NK group 2 member A (NKG2A), CD96 and/or TIGIT [20]. In HCC, NK cell dysfunction is attributed to the decline of hepatic NK cell frequency [21], NKG2D down-regulation [22], stimulation of inhibitory receptors [23] and immunosuppressive cell-mediated functional inhibition [24]. These mechanisms trigger defective recognition of tumor cells and suppressive intracellular signaling to impair NK

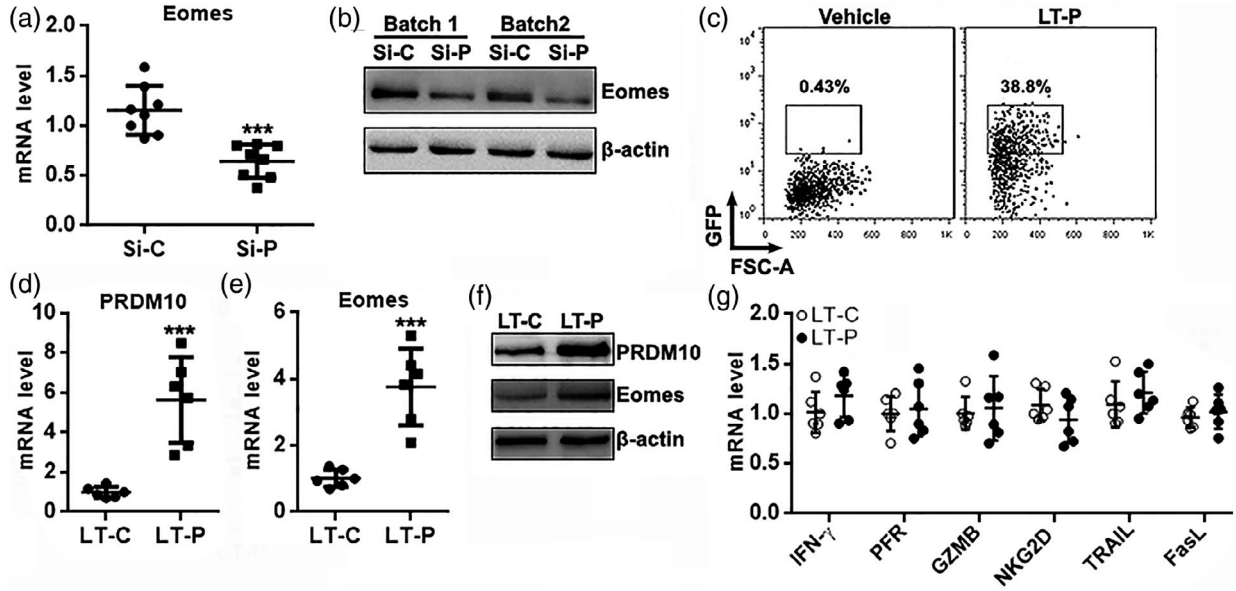

FIGURE 6 Eomes expression in PR/SET Domain 10 (PRDM10)-deficient or PRDM10-over-expressing natural killer (NK) cells. (a) Eomes mRNA levels in NK cells. Si-C = infection with control lentivirus; Si-P = infection with lentivirus encoding PRDM10 shRNA; $n = 8$ samples in three experiments. (b) Eomes protein in NK cells. Batch 1: infection batch 1; batch 2: infection batch 2. (c) Green fluorescent protein (GFP) expression after lentiviral infection. Vehicle: infection reagents only. LT-P = infection with lentivirus encoding PRDM10. (d,e) mRNA levels of PRDM10 (d) and Eomes (e) in GFP^+ NK cells on day 2 after infection. LT-C = infection with control lentivirus without PRDM10 sequence; LT-P = infection with lentivirus encoding PRDM10. (f) PRDM10 and Eomes protein in GFP^+ NK cells. The image represents two independent experiments. (g) mRNA levels of indicated NK cell function markers; $n = 6$ samples in three experiments. Student's *t*-test. $^{***}p < 0.01$

cell activation and cytotoxicity. Our data are consistent with some of the above-mentioned alterations such as attenuation of NKG2D, TRAIL and FasL, together with high expression of CD96 and TIGIT.

We found a moderate increase of PRDM10 in splenic NK cells and a decrease of PRDM10 in hepatic NK cells in HCC-bearing mice. Interestingly, the expression pattern of Eomes was highly similar to that of PRDM10, strongly suggesting the correlation between the two molecules. Eomes is regarded to be essential to NK cell development, maturation and function both in infants and adults [25–27]. Particularly, Eomes expression correlates positively with IFN-γ production *in vitro* in mice and humans [19,28], and Eomes down-regulation is strongly linked to NK cell exhaustion and impaired anti-tumor activity [19,20]. Furthermore, Eomes drives the differentiation and cytotoxic function of NK cells and effector $CD8^+$ T cells [27,29,30]. Therefore, it is likely that PRDM10 modulates the cytolytic function of NK cells through controlling Eomes expression. However, whether PRDM10 directly influences the transcription of Eomes is unknown. Our team plans to carry out a luciferase assay to check if PRDM10 binds to the promoter/enhancer region of Eomes and subsequently alters Eomes transcription. It is also noteworthy that PRDM10 over-expression promoted NK cell cytotoxicity *in vivo* but not *in vitro*. Perhaps this is because the PRDM10–Eomes axis inhibits NK exhaustion to maintain NK cell cytotoxicity in the HCC microenvironment, but could not further enhance NK cell activity during acute activation upon *in-vitro* contacting target cells. Future investigations inducing NK cell exhaustion after chronic exposure to IL-15 might answer whether PRDM10 over-expression suppresses NK cell exhaustion *in vitro*. Also, it would be valuable to exploit PRDM10 knock-out mice to study the role of this molecule in the modulation of NK cells and other innate lymphoid cells.

However, the mechanisms by which PRDM10 affects the expression of NK receptors remain unidentified. To our knowledge, no hard evidence has shown the direct effect of PRDM10 or Eomes on NK receptors such as NKG2D. If future investigations fail to identify the direct binding of PRDM10 or Eomes to the genes of these receptors, other mechanisms would be considered. NKG2D expression in NK cells is transcriptionally regulated by signal transducer and activator of transcription (STAT)-3 and [31,32]. Multiple pathways, including STAT-1, nuclear factor of activated T cells (NFAT), forkhead box transcription factors, O subfamily (FoxO) and NF-κB, have been reported to modulate TRAIL gene transcription [33]. TIGIT is up-regulated by B lymphocyte-induced maturation protein-1 (Blimp-1) [34]. Therefore, it would be necessary to determine if PRDM10 influences the

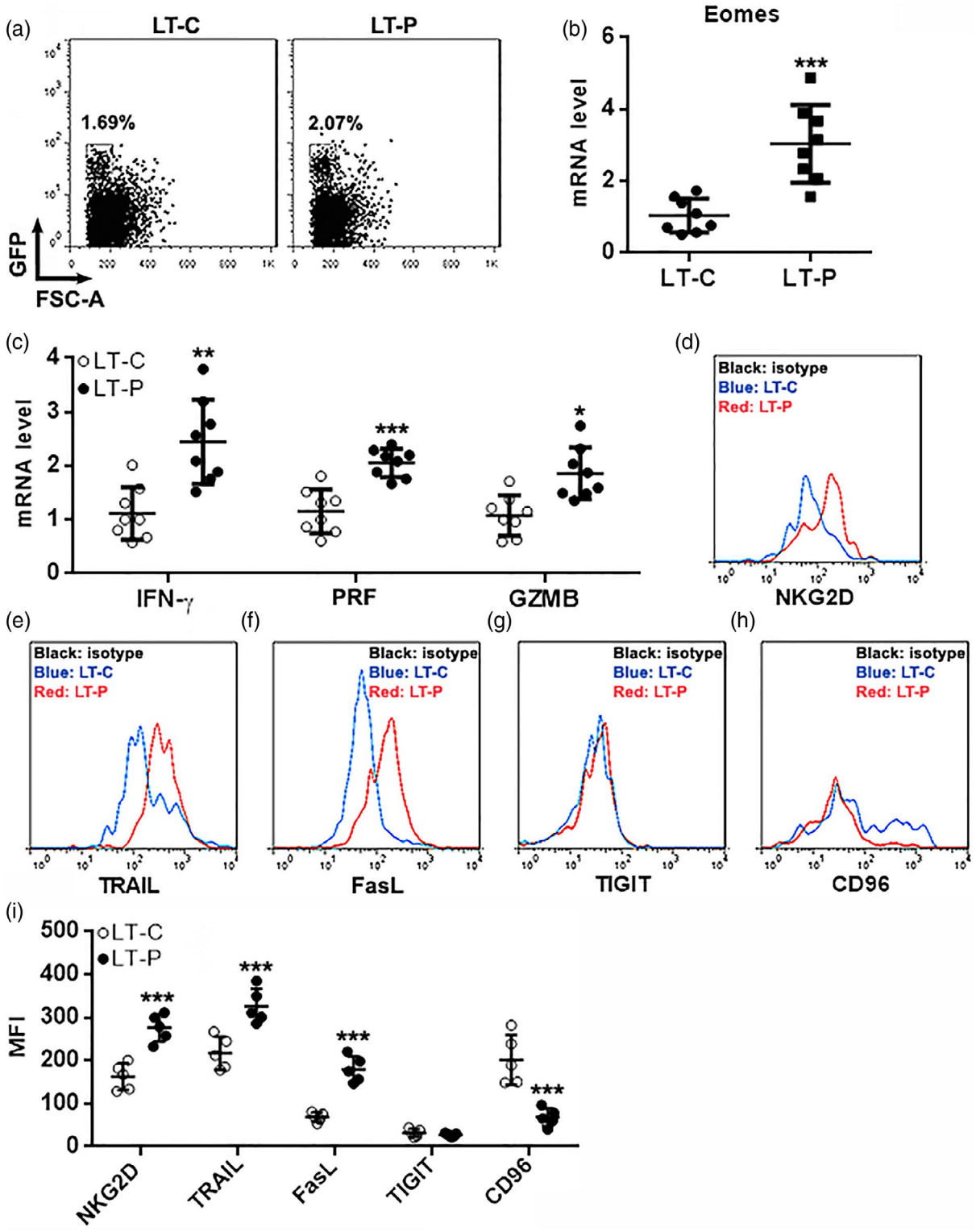

FIGURE 7 Phenotype and function of PR/SET Domain 10 (PRDM10)-over-expressing natural killer (NK) cells *in vivo*. (a) Detection of donor-derived green fluorescent protein $(GFP)^+$ NK cells in recipients' hepatic mononuclear cells. LT-C = NK cells infected with control lentivirus; LT-P = NK cells infected with lentivirus encoding PRDM10. (b) Eomes mRNA levels in donor-derived NK cells. (c) mRNA levels of cytotoxic mediators in donor-derived NK cells. (d–h) Fluorescence activated cell sorter (FACS) histograms showing the expression of indicated receptors on the surface of donor-derived NK cells. (i) Statistics of the mean fluorescence intensities of receptors in (d) to (h); $n = 5$ or 8 mice. Student's *t*-test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.01$

abundances of these signaling molecules or the activation of associated pathways.

Although we observed PRDM10 down-regulation in hepatic NK cells, the factor(s) responsible for this change is unidentified. PRDM2, another member of the PRDM family, is reported to controls PRDM10 in mouse C2C12 myoblasts [35]. However, our pilot transcriptome sequencing data did not list PRDM2 in the differentially expressed genes; therefore, some other factors were involved. We are still analyzing the transcriptome sequencing data to look for transcription factors that potentially bind to the promoter region of the *PRDM10* gene. Also, the temporal PRDM10 expression pattern in hepatic NK cells is unknown. In the future, hepatic NK cells at the early and late stages of HCC should be enriched for PRDM10 quantification and cytotoxicity analysis.

In summary, this study reveals a novel mechanism underlying NK cell exhaustion in HCC livers. Manipulation of PRDM10 expression might be a new therapeutic strategy for strengthening NK cell-mediated immunotherapy.

CONCLUSIONS

- PRDM10 is down-regulated in hepatic NK cells in HCC.
- PRDM10 down-regulation is associated with hepatic NK cell dysfunction.
- PRDM10 over-expression promotes hepatic NK cell cytotoxicity on tumor cells.
- PRDM10 regulates Eomes expression in hepatic NK cells.
- PRDM10 is a supporting factor of NK cell function.

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflicts of interest.

AUTHOR CONTRIBUTIONS

X.X. designed the study, inspected the data, and composed the manuscript. J.H. performed most experiments. C.K. and B.J. established the animal model and adoptive transfer. H.Z. conducted lentiviral infection. H.X. performed statistical analysis. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data are available from the correspondence author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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