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Traf6 inhibitor boosts antitumor immunity by impeding regulatory T cell migration in Hepa1-6 tumor model

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ABSTRACT

Tumors escape immune attacks via various mechanisms, among which activation of regulatory pathways in effector immune cells and recruitment of immunosuppressive cells are usually employed. Traf6 is a member of the family of tumor necrosis factor receptor-associated factors and involved in many signaling pathways. While it plays important roles in both tumor biology and immune system, the potential therapeutic role of Traf6 in tumor immunotherapy hasn't ever been assessed. Here, we confirmed the anti-tumor effect of Traf6 inhibitor in Hepa1-6 tumor model. Flow cytometry-based analysis revealed that T cell-mediated antitumor immunity was provoked and the infiltration of Treg cells was restrained when treated with Traf6 inhibitor. Via an *in vivo* migration assay, we found that Traf6 inhibitor decreased the population of intratumor Tregs by impeding the migration of Tregs towards tumor. Finally, we demonstrated that combination of Traf6 inhibitor and PD-1 blockade could receive a better antitumor efficiency. These results implicated that Traf6 inhibitor could serve as a supplement for immune checkpoint therapy.

1. Introduction

Hepatocellular carcinoma (HCC), an inflammation-related cancer, is emerging as the fourth leading cause of cancer-related death worldwide [1,2]. As one of the major characteristics of HCC, immune evasion often accomplishes via activation of immune checkpoint signaling pathways, such as programmed death 1 (PD-1)/PD-1 ligand 1 (PD-L1) axis or through recruitment of immunosuppressive cells including $Foxp3^+$ regulatory T cells (Tregs) [1,3,4]. Recently, blockade targeting PD1–PDL1 pathway has produced favorable responses in treatment of various cancer types including HCC [5–8], and a phase 1/2 trial for HCC indicated that nivolumab treatment showed promising results in advanced HCC patients [5]. Although these immune checkpoint blockades were shown as a potentially effective treatment for patients suffering HCC, combination treatments of distinct immune checkpoint blockades or immune checkpoint blockade plus other therapies are still expected to be more efficient and safer [1,8,9].

Foxp3-expressing Treg cells play an essential role in maintaining immune homeostasis by suppressing aberrant immune responses [10]. However, the suppressive function of these cells in dampening the activation of other lymphocytes also impedes potent anti-tumor immunity [11]. Various studies have demonstrated that a great number of Treg cells infiltrated into various types of tumors in humans, such as head and neck, breast, lung, ovary, and liver [12–16]. Meanwhile, the increased ratio of $Foxp3^+$ Treg cells to tumor-infiltrating $CD8^+$ T cells is shown to correlate with poor prognosis [17]. Furthermore, the high frequency of intratumor Treg cells, especially in solid tumors, is inversely correlated with the survival of patients [18]. Therefore, numerous efforts have been made to provoke efficient antitumor immunity by removal of Treg cells in tumor setting [11].

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Traf6, a member of the family of tumor necrosis factor receptor-associated factors (Trafs), is a kind of adaptor proteins and is unique for its role in activating a cohort of signaling pathways, such as NF-κB signaling [19,20]. It has been documented that Traf6 overexpression occurred in a broad spectrum of cancers [21], such as melanoma [22] and oral squamous cell carcinoma [23]. Moreover, Sun et al. [24] revealed that Traf6 promoted the angiogenesis of lung cancer cells by upregulating HIF-1α. Li et al. [25] showed that Traf6 contributed to metastasis and deterioration of HCC via influencing cell growth and apoptosis. In the context of immune system, on another hand, Traf6 plays a critical role in the development, activation, and homeostasis of various immune cells [26]. Our recent findings demonstrated that Traf6 facilitated the nucleus-translocation of Foxp3 and therefore contributed to the *in vivo* function of Tregs [27]. However, the potential therapeutic role of Traf6 in tumor immunotherapy is still unclear.

In the present study, we found that the growth of Hepa1-6 tumor in immunocompetent mice was restrained by Traf6 inhibitor treatment, but not in immunodeficient mice. This result suggests that Traf6 may exert immune regulatory function within tumor *in vivo*. Further analysis demonstrated that Traf6 inhibitor could reduce the migration of Tregs into tumor site and therefore augment T cell-mediated antitumor immunity. In addition, combined use of Traf6 inhibitor and anti-PD1 blockade received more efficient responses in rejection of Hepa1-6 tumor. Our findings provide evidence for Traf6 as a potential therapeutic target to combat tumor.

2. Materials and methods

2.1. Mice and tumor cell line

C57BL/6 mice and NOD CRISPR Pakdc Il2r gamma (NCG) mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China), and used in immunocompetent or immunodeficient mice experiments, respectively. Animals were maintained under specific pathogen-free condition. All the mice were female and used at the ages of 6–8 weeks. Animal protocols were approved by Nanjing Medical University. The murine hepatoma cell line Hepa1-6 established from C57L/j mice was purchased from Cell Center of Shanghai Institutes for Biological Sciences (Shanghai, China). These cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin, and 100 g/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Tumor growth experiments and cell depletion

4×10^6 Hepa1-6 tumor cells suspended in 100 μl PBS were subcutaneously (s.c.) injected into the slaved side flank of the indicated strain of female mice at the ages of 6–8 weeks. When the tumor was palpable (approximately 7 days post-injection), 70 μg/mouse/injection of Traf6 inhibitor (DRQIKIWFQNRRMKWKKRKIPTEDEY) or control peptide (DRQIKIWFQNRRMKWKK) (Novus Biologicals), 200 μg/mouse/injection of anti-PD1 antibody (clone RMP1-14, BioXCell) or combination of inhibitory peptide and anti-PD1 antibody was injected intraperitoneally (i.p.) twice a week as indicated. In indicated experiments, 10 μmol/kg/injection small molecular inhibitor 6877002 (abcam) or control vehicle was injected i.p. every two days. In some experiments, cellular subsets were depleted by administering 300 μg of neutralizing antibody or control rat IgG i.p. twice weekly beginning one day prior tumor cell inoculation as indicated: CD4+ T cells with anti-CD4 (clone GK1.5, BioXCell), CD8+ T cells with anti-CD8α (clone 53–6.72, BioXCell), Treg cells with anti-CD25 (clone PC-61:5.3, BioXCell). Tumor progression was assessed by measuring changes in tumor length (L) and width (W) with electronic caliper over time. Tumor volumes were calculated using the standard formula ($V = L * W^2/2$).

2.3. Isolation of tumor-infiltrating lymphocytes

For isolation of the tumor-infiltrating lymphocytes, the tumors were excised from the mice and cut into small fragments, followed by digestion in RPMI1640 (Invitrogen) containing 2% fetal bovine serum, 0.05% collagenase type IV (gibco) and 0.01% DNase I (Invitrogen) for 1 h at 37 °C. The resulting cell suspension was smashed through a 70 μm nylon cell-strainer and then overlaid on 40%/80% Percoll (GE Healthcare) gradient and centrifuged at 450*g* for 20 min at room temperature. Lymphocytes were recovered from the interphase.

2.4. In vivo migration assay of Treg cells

For *in vivo* assessment of Tregs migration, single-cell suspensions from spleens and lymph nodes of healthy donor mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) according to the manufacturer's instruments. Then, the labeled cells were injected intravenously (3×10^7 cells per recipient mouse) into tumor-bearing recipients (mean tumor size, 1000 mm^3). These animals were treated with Traf6 inhibitor or control peptide twenty-four hours prior transfer. Sixteen hours later, tumors and spleens were resected from the recipients and subjected to FACS analysis to determinate the fraction of Treg cells within CFSE-labeling cells.

2.5. Flow cytometry and antibodies

For extracellular staining, harvested cells were washed and incubated in PBS containing 2% FBS with fluorochrome-conjugated antibodies in a U-bottom 96-well plate. For intracellular staining, cells were fixed and permeabilized using eBioscience FOXP3 Staining Kit. In experiments detecting intracellular cytokines, harvested cells were re-stimulated with PMA and Ionomycin in the presence of Golgi-Plug (BD biosciences). After five-hour incubation, the cells were fixed/permeabilized (eBioscience) and incubated with antibodies against IFN-γ or TNF-α. In the cell cycle assay, tumor cells were fixed in ice-cold 75% ethanol overnight, followed by PI (BioLegned) staining to reflect the DNA contents. Mice-specific monoclonal antibodies used for flow cytometry including CD3 (clone 17A2), CD4 (clone RM4-4), CD8 (clone 53–6.7), TNF-α (clone MP4-XT22), CCR4 (clone 2G12) and Annexin V were purchased from BioLegend. Antibodies against Foxp3 (clone FJK-16s), CD25 (clone PC61.5), CD103 (clone 2E7), Ki-67 (clone SoIA15) and INF-γ (clone XMG1.2) were purchased from eBioscience.

2.6. Western blot analysis

cells cultured in various concentration of Traf6 inhibitor were lysed with RIPA buffer (50 mM Tris–HCl, pH7.4, 1% Nonidet P-40, 0.5% Nadeoxycholate, 150 mM NaCl, 1 mM EDTA, with 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF) containing protease inhibitor cocktail (Sigma). Cell lysates were separated by standard SDS-PAGE and analyzed by immunoblot. Rabbit polyclonal antibodies against p-Akt(t308), p-Akt (s473), total Akt, Bcl-xl, cleaved caspase-3 were purchased from Cell Signaling Technology, while mouse monoclonal antibody against GAPDH was purchased from Abcom.

2.7. TUNEL assay

The Klenow-FragEL DNA Fragmentation Detection Kit (EMD Chemicals) was used to detect the DNA fragmentation characteristic of necrosis/apoptosis in formalin-fixed paraffin-embedded tumor sections. Results were scored semi-quantitatively by averaging number of apoptotic cells/microscopic field at 200× magnification. Ten fields were evaluated per sample.

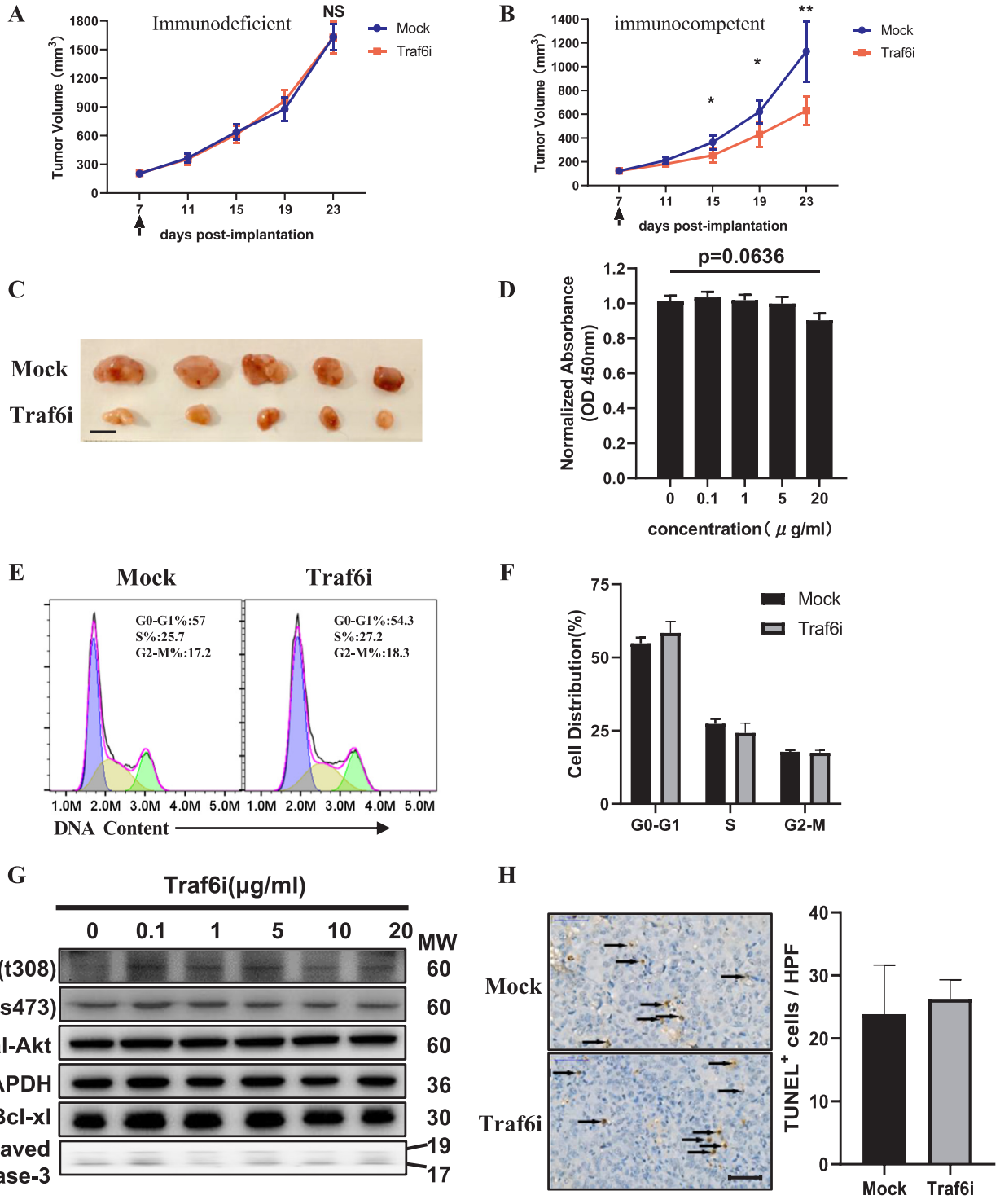

Fig. 1. Traf6 inhibitor slows Hepa1-6 tumor growth in immunocompetent mice. Immunodeficient NOD CRISPR Pakdc Il2r gamma (NCG) mice and immunocompetent C57BL/6 mice were challenged subcutaneously (s.c.) with 4×10^6 hepatocellular Hepa1-6 cells. When those tumors were palpable about 7 days after implantation, mice were administrated i.p. with Traf6 inhibitor or control peptide. The tumor dimensions were monitored every 4 days, and tumor volumes were calculated accordingly. The tumors were dissected from mice at day 23 post tumor-implantation. (A, B) Growth curves of tumors implanted in immunodeficient and immunocompetent mice respectively; NS, no significance; *p < 0.05; **p < 0.01, unpaired *t* test. (C) Representative image for tumors dissected form immunocompetent mice treated with Traf6 inhibitor or not (Scale bar: 1 cm). (D) Proliferation of cells cultured with various concentrations of Traf6 inhibitor as indicated was assessed by CCK8 assay. P = 0.0636, unpaired *t* test. (E, F) Cell cycle analysis for cells cultured in media with or without 20 μg/ml Traf6 inhibitor. Shown are representative counter dots of three independent experiments (E) and summarized values (F). (G) Western-assisted analysis of p-AKT(t308), p-Akt(s473), total-Akt, Bcl-xl, cleaved Caspase-3, Representative of three experiments. (H) Cell apoptosis by TUNEL staining in tumors implanted in NCG mice treated with Traf6i or control peptide. Results scored semi-quantitatively by averaging the number of apoptotic cells (mean ± SEM) per field at ×200 magnification (Scale bar: 50 μm). Representative of 5 mice/group.

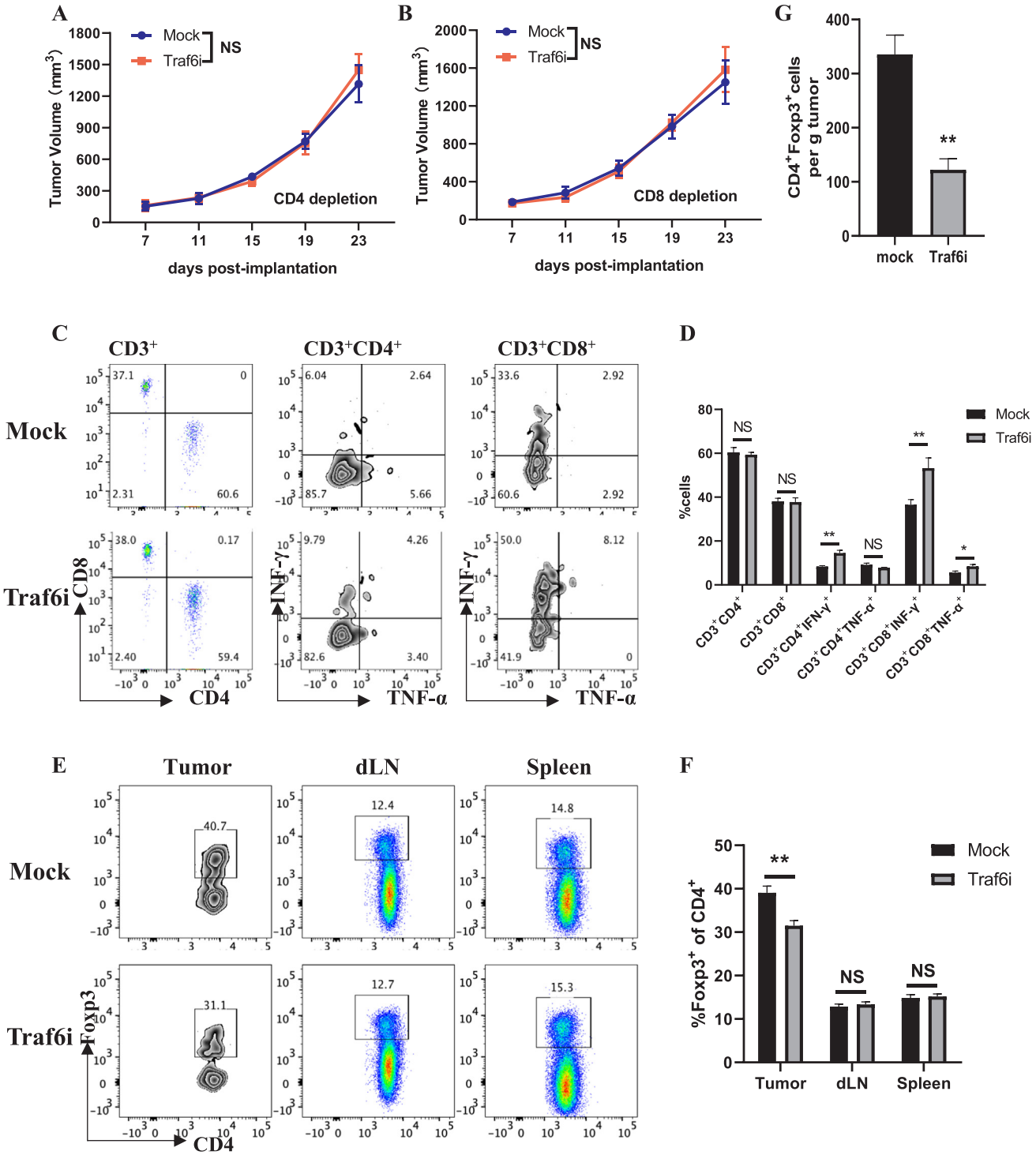

Fig. 2. Traf6 inhibitor reinforces T cell immunity by reducing tumor infiltration of Treg cells. (A) Tumor growth curves from Hepa1-6 bearing mice that were treated with or without Traf6 inhibitor in the presence of anti-CD4 mAb (GK1.5), which was administrated to deplete CD4 T cells. NS, no significance, unpaired *t* test. (B) Tumor growth curves from Hepa1-6 bearing mice that were treated with or without Traf6 inhibitor in the presence of anti-CD8 mAb (53–6.72), which was administrated to deplete CD8 T cells. NS, no significance, unpaired *t* test. (C, D) Flow cytometric characterization of tumor-infiltrating lymphocytes from mice treated with Traf6 inhibitor or control peptide (n = 3/group). Suspensions of intratumor lymphocytes recovered from indicated mice were stained for surface markers (CD3, CD4, CD8) and proinflammatory cytokines (following *ex vivo* reactivation in the presence of PMA/ionomycin and Golgi Stop, fixation, and permeabilization). Representative dot images from three independent experiments are shown in C, meanwhile the frequencies of $CD4^+$ and $CD8^+$ T cells among tumor-infiltrating lymphocytes as well as pro-inflammatory cytokines (IFNγ and TNFα) producing fractions of $CD4^+$ and $CD8^+$ T cells are summarized in D. (E. F) Frequencies of $Foxp3^+$ T cells within $CD4^+$ T cells in tumors, draining lymph nodes (dLNs) and spleens from indicated mice were measured by flow cytometry. Representative dot images from three independent experiments are shown in E, while F depicts the mean ± SEM across three independent experiments. Significances were identified by unpaired t tests, NS, no significance, *$p < 0.05$; **$p < 0.01$. (G) Absolute number of tumor-infiltrating $CD4^+Foxp3^+$ Treg cells per gram tumor was determined by flow cytometry. Shown are mean ± SEM from three independent experiments. **$p < 0.01$, unpaired *t* test.

2.8. RT-qPCR analysis

Total RNA was extracted with TRlzol (invitrogen) followed by cDNA synthesis reaction using ReverAid First Strand cDNA Synthesis Kit (ThermoScientific) in a 20 ul/well. The same amount of RNA was used in each cDNA synthesis reaction measured by OneDrop Spectrophotometer (TheroScientific). The same volume of cDNA per sample was prepared for real-time PCR analysis using SYBR Green (Vazyme) and the indicated primers to assess transcript level of each gene.

2.9. CCK8 assay

For CCK8 assay, 2×10^3 cells were plated in 96-well plates and cultured in completed DMEM medium in the presence of various concentrations of Traf6 inhibitor (0, 0.1, 1, 5, 20 μg/ml) for 4 days. Cell Counting Kit-8 (CCK8) was used to evaluate the cell proliferation. Briefly, 10 μl CCK8 solution was added to each well and cells were incubated for 2 h at 37 °C. The cell viability was revealed by the absorbance which was measured at 450 nm.

2.10. Statistical analysis

Statistical differences among multiple groups were determined using a two-way analysis of variance (ANOVA) with NewmaneKeuls Multiple Comparison Test, unless otherwise indicated. Unpaired, two-tailed student's t-tests were used for single-comparisons. For survival comparisons, Log-rank tests were used. In general, P values < 0.05 were considered significant and were indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS: not significant. GraphPad Prism7 was used to calculate P values.

3. Results

3.1. Traf6 inhibitor-mediated antitumor effect is immunity dependent

To investigate whether Traf6 inhibitor (Traf6i) could affect the tumor growth of hepatocellular cancer. Hepa1-6 tumor model was established by subcutaneous injection 4×10^6 Hepa1-6 murine liver cell line into immunodeficient NCG mice and immunocompetent syngeneic C57BL/6 mice. Tumor growth was monitored over time, and, intriguingly, we found that there were no significant volume differences

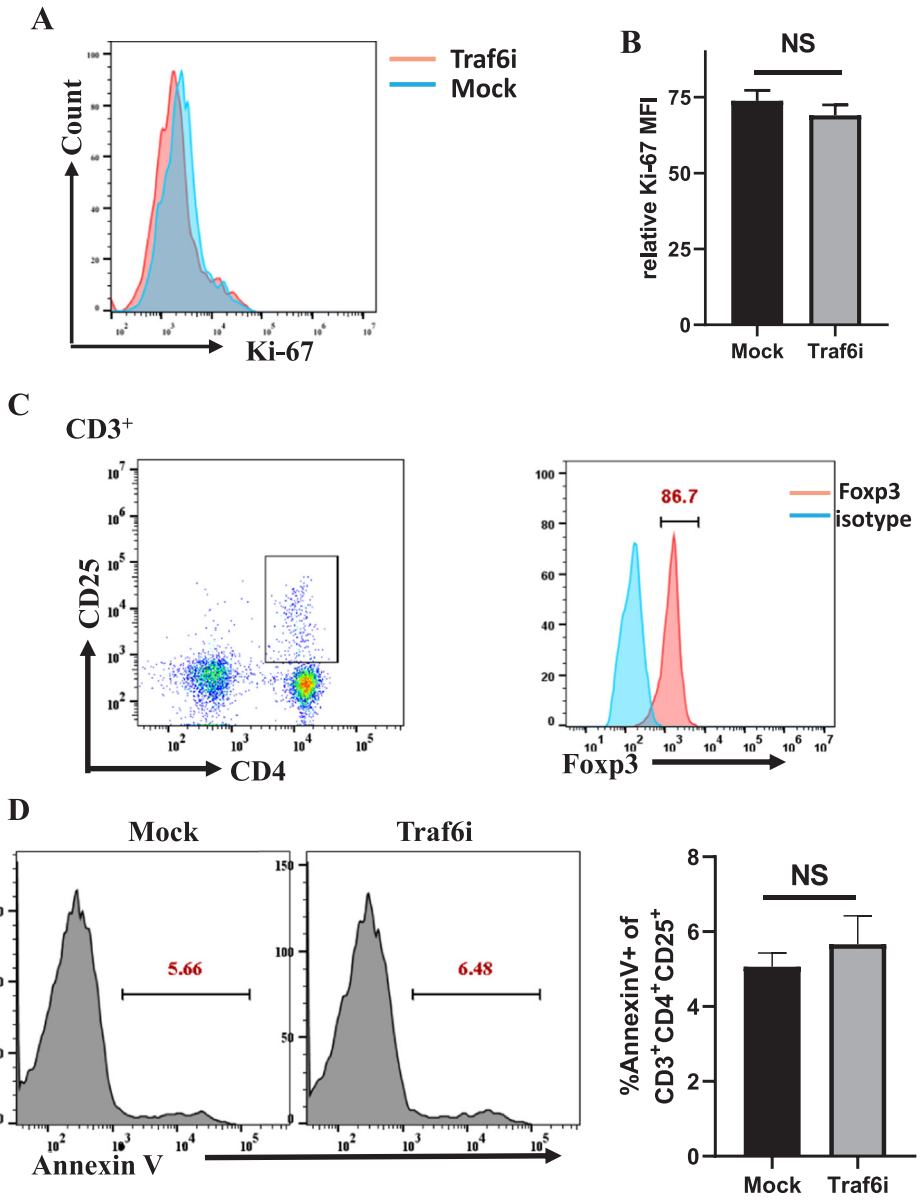

Fig. 3. Proliferation and apoptosis of intratumor Treg cells in Traf6 inhibition setting. (A, B) Flow cytometric analysis of Ki-67 expression, a cellular proliferation marker, on freshly isolated tumor-infiltrating Foxp3$^+$ Treg cells from mice treated with Traf6 inhibitor or control peptide. Shown are representative Ki-67 staining (A) and the mean value of three independent experiments ± SEM (B). NS, no significance, unpaired *t* test. (C, D) Apoptosis of intratumor CD4$^+$CD25$^+$ Treg cells was evaluated by staining the cells with Annexin V. Intratumor lymphocytes were freshly isolated from mice treated with Traf6 inhibitor or control peptide (n = 3/group) and stained with anti-CD4-Pacific blue, anti-CD25-PEcy7 and Annexin V-FITC, apoptotic intratumor Treg cells were considered as CD4$^+$CD25$^+$AnnexinV$^+$ cells by gating on CD4$^+$CD25$^+$ fraction which was almost Foxp3 positive (C). Shown are representative Annexin V staining (D, left panel) and the mean value from three independent experiments ± SEM (D, right panel). NS, no significance, unpaired *t* test.

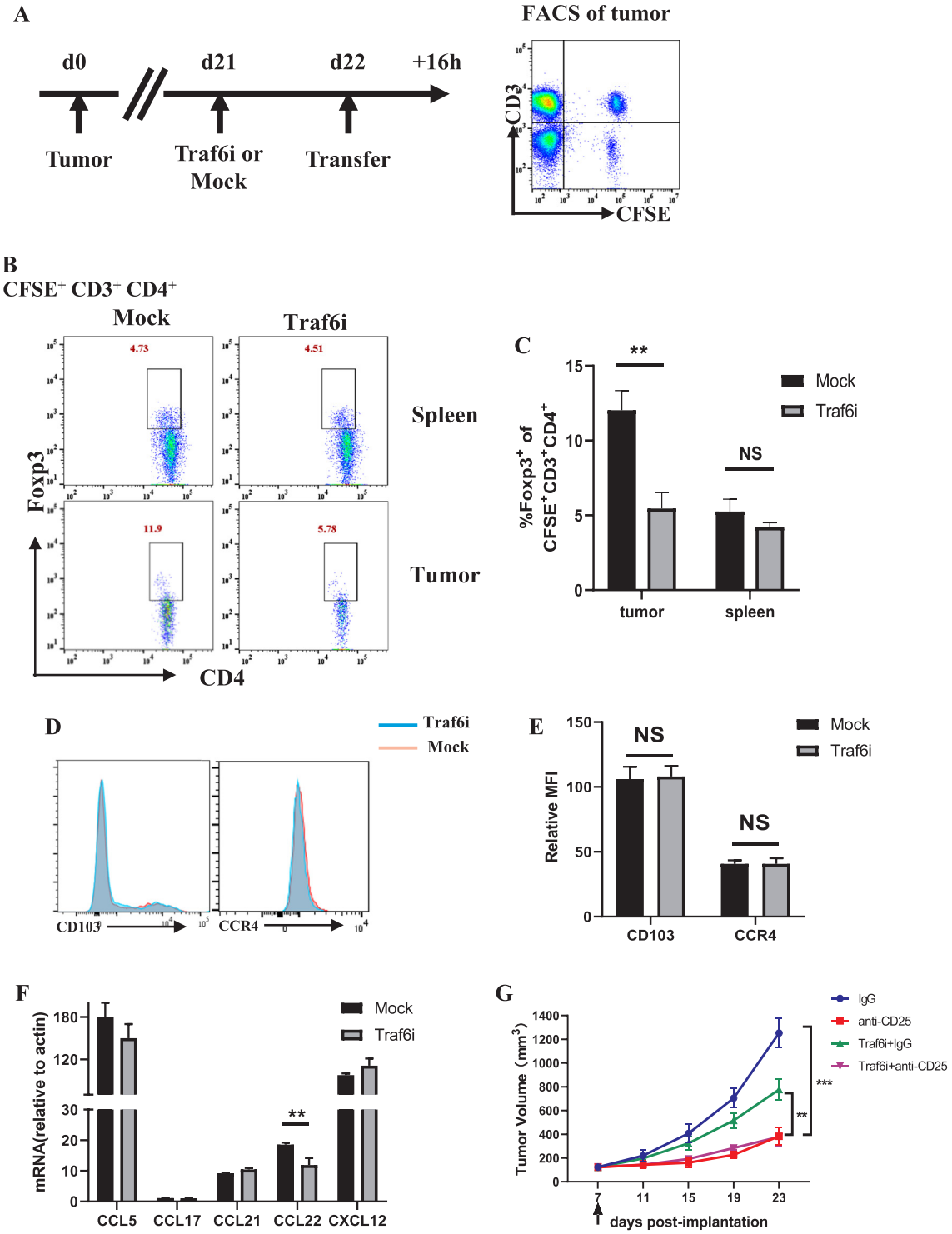

Fig. 4. Treatment with Traf6 inhibitor impedes the migration of Foxp3$^+$ cells into tumor. (A) Schema for the *in vivo* migration assay of regulatory T cells. Immunocompetent mice bearing Hepa1-6 tumors (average size, 2000 mm^3) at day 21 after tumor inoculation were treated with an intraperitoneal injection of Traf6 inhibitor or control peptide (n = 4/group). Twenty-four hours later, the mice received an intravenous transfer of CFSE-labeling cells of the spleens and lymph nodes from normal donor mice. Tumors and spleens were resected from recipients sixteen hours after transfer, followed by flow cytometric analysis of intratumor and splenic CFSE$^+$ T cells. (B, C) Proportions of Foxp3$^+$ Treg cells within CFSE$^+$ CD3$^+$ CD4$^+$cells were analyzed in tumors and spleens resected from Traf6 inhibitor or control peptide treated mice. Results are shown for one representative mouse per group (in tumor and spleen; B) and average of all mice per group (n = 4; C). **p $<$ 0.01, unpaired *t* test. (D, E) Expressions of CD103 and CCR4 on tumor-infiltrating Tregs from mice treated with Traf6 inhibitor or not were detected through FACS and overlapped by Flowjo. Shown are representative counter dots for CD103 and CCR4 (D) and mean ± SEM from three independent experiments (E). NS, no significance, unpaired *t* test. (F) Relative expressions of several migration-related chemokines within tumors were detected by RT-qPCR. Mean ± SEM are shown, **p $<$ 0.01, unpaired *t* test. (G) Tumor growth curves from Hepa1-6 bearing mice that were treated with or without Traf6 inhibitor in the presence of anti-CD25 mAb (PC-61:5.3) or an isotype control antibody. **p $<$ 0.01; ***p $<$ 0.001, unpaired *t* test.

between tumors treated with control peptide and Traf6i in the immunodeficient mice (Fig. 1A). In contrast, Traf6i did slow tumor growth in the immunocompetent mice when compared to those of control animals (Fig. 1B–C). The inhibitor we used above was a peptide proven to inhibit the binding of both Rank and CD40 to Traf6 *in vivo and in vitro* [28,29], which may raise concerns about its stability in animals. To this end, a small molecular inhibitor that binds to Traf6 and blocks the interaction between Traf6 and CD40 was also employed and achieved similar results (Supplementary Fig. 1). These data suggested that while Hepa1-6 cell line was resistant to Traf6i-mediated modulation of growth, Traf6i could exert antitumor activity through mechanisms associated with tumor immunity.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.intimp.2019.105965.

To further confirm the Traf6i-resistant characteristic of Hepa1-6 cell line, CCK8 assay and cell cycle analysis were performed. Results showed that Traf6i had no significant impact on cell proliferation (Fig. 1D) or cellular cycle distribution (Fig. 1E–F) even at a high concentration of 20 μg/ml. Furthermore, western blot analysis showed that Traf6i had litter effect on the activation of the Akt signaling pathway, which is essential for the modulation of cell survival against apoptosis (Fig. 1G). In line with above findings, TUNEL staining of sections of tumors from NCG mice showed comparable frequency of apoptotic cells between Traf6i-treated mice and the counterparts (Fig. 1H). In conclusion, these data demonstrated that Traf6i decreased tumor growth of Hepa1-6 in an immunity dependent manner.

3.2. Traf6 inhibitor promotes T cell immunity and reduces tumor infiltration of Treg cells

We speculated that T cell immunity might be involved in the Traf6i-mediated antitumor effect. Indeed, depletion of either $CD4^+$ or $CD8^+$ T cells by specific mAbs almost completely abrogated the tumor-fighting effect of Traf6i in C57BL/6 mice, supporting the notion that the antitumor activity of Traf6i is associated with both $CD4^+$ and $CD8^+$ T cells. To gain further insight into the mechanisms underlying Traf6i-mediated rejection of tumor, tumor-infiltrating lymphocytes (TILs) from Hepa1-6 tumors were separated and identified by flow cytometry. As shown in Fig. 2C–D, although the populations of $CD4^+$ and $CD8^+$ T cells were not markedly elevated in Traf6i-treated tumors, these cells produced significantly higher levels of IFN-γ and TNF-α. This phenomenon suggested that the activation of $CD4^+$ and $CD8^+$ TILs were much less restrained under the Traf6 inhibition environment. In comparison with control mice, Traf6i significantly reduced the fraction of tumor-infiltrating $CD4^+Foxp3^+$ Treg cells (Fig. 2E–G), which has been proved to suppress the activation of other lymphocytes and therefore pose a major barrier to effective antitumor immunity. However, there were no significant differences in the frequency of Treg cells in draining lymph nodes and spleens between these two groups (Fig. 2E–F). Collectively, our findings revealed that Traf6i augmented T cell-based antitumor immunity by selectively reducing the infiltration of $Foxp3^+$ Treg cells in the tumor niche.

3.3. Traf6 inhibitor shows no impact on the proliferation and apoptosis of intratumor Tregs

To determine the reason for the reduction of intratumor Treg cells, we firstly evaluated the proliferation of these cells by FACS. The expression of Ki-67, an intracellular marker for cell proliferation, in intratumor Tregs from Traf6i-treated mice was comparable to those from counterparts (Fig. 3A, B). The cellular apoptosis of tumor-infiltrating Treg cells was also analyzed by Annexin V staining in $CD4^+CD25^+$T cells fraction, which was regarded as Treg cells for the co-expression of Foxp3 (about 86%) (Fig. 3C). As shown in Fig. 3D, no significant differences were observed between these two groups, indicating that the cell apoptotic status was not altered by treatment of Traf6i. In conclusion, although Traf6 inhibitor reduced the frequency of Treg cells in tumor, the proliferation and viability of these cells remained unchanged with treatment of Traf6i.

3.4. Traf6 inhibitor abrogates the migration of regulatory T cells into tumor

To explore whether the reduced number of intratumor Treg cells stems from decreased migration of $Foxp3^+$ cells into tumor, we dissected the trafficking of adoptive transferred lymphocytes in tumor-bearing immunocompetent animals. Mice bearing established Hepa1-6 tumor received one intraperitoneal injection of Traf6i. Twenty-four hours later, CFSE-labeling cells from spleens and lymph nodes of healthy donor mice were intravenously transferred into those mice. Tumors and spleens were resected sixteen hours after cell transfer and then subjected to flow cytometry analysis (Fig. 4A). Surprisingly, in the Traf6i-treated mice, the populations of transferred Treg cells ($CFSE^+CD3^+CD4^+Foxp3^+$) within $CFSE^+CD3^+CD4^+$ cells were reduced almost 2.5-fold compared with those in control mice (Fig. 4B, C). In contrast, no differences in the fraction of transferred Tregs were observed in the spleens, which indicated that migration of regulatory T cells into the tumor site was specifically restrained.

Prior studies have documented that the peripheral migration and retention of Treg cells were majorly mediated by the interaction of specific integrins and chemokine receptors expressed on these cells [30]. To shed light on the molecular mechanisms behind the decreased migration of Tregs into the tumor site upon treatment with Traf6i, several Treg-related chemokines and chemokine receptors were analyzed. Firstly, expressions of two receptors, CCR4 and CD103, which were reported critically involved in Tregs migration and retention in tissues respectively [31,32], were detected by FACS. Results showed that treatment with Traf6i did not alter the surface expression of CCR4 or CD103 of $Foxp3^+$ cells (Fig. 4D, E). Several chemokines such as CCL5, CCL17, CCL21, CCL22, CXCL12 have been reported to direct tissue homing of Treg cells in distinct circumstances. We detected intratumor levels of all these chemokines in Hepa1-6-bearing mice through RT-qPCR. As shown in Fig. 4F, while the level of CCL5, CCL17, CCL21, CXCL12 were not apparently altered, treatment with Traf6i strikingly decreased the CCL22 level, which has been implied in the migration of Treg in several malignant tumors [33–35].

Although the abovementioned experiments established the contribution of reduced migration of Treg cells to Traf6i-mediated augmentation of antitumor immunity, the possibility that Traf6i exerts its antitumor effect through other mechanisms cannot be excluded. To this end, Treg depletion by anti-CD25 mAb was employed to further probe the mechanism of the antitumor activity of Traf6i. The efficiency of Treg depletion by intraperitoneal injection of this antibody was confirmed by staining of lymphocytes from the antibody-treated mice (data not shown). In line with previous studies, tumor growth was markedly slowed in the Treg-depleting group [36]. In contrast, combination of Traf6 inhibitor and anti-CD25 antibody showed no more effective on reduction of the tumor growth in comparison with the anti-CD25 alone group. This indicated that depletion of tumor-infiltrating $CD4^+CD25^+Foxp3^+$Treg cells could abrogate the antitumor functionality of Traf6i. All above results demonstrated that Traf6i could reduce the CCL22 level in tumor and therefore dampen the migration of Tregs into the tumor site, which contributed to Traf6i-mediated antitumor effect.

3.5. Traf6 inhibitor cooperates with PD-1 blockade to promote tumor rejection

Immune checkpoint blockades, such as anti-CTLA-4, anti-PD1/PD-L1, have become highly promising cancer therapeutic approaches to combat tumors including HCC [5,8], and combining these agents has shown synergistic effects in driving tumor rejection [9]. To check if Traf6i could work as a supplement for PD-1 blockade to fight tumors,

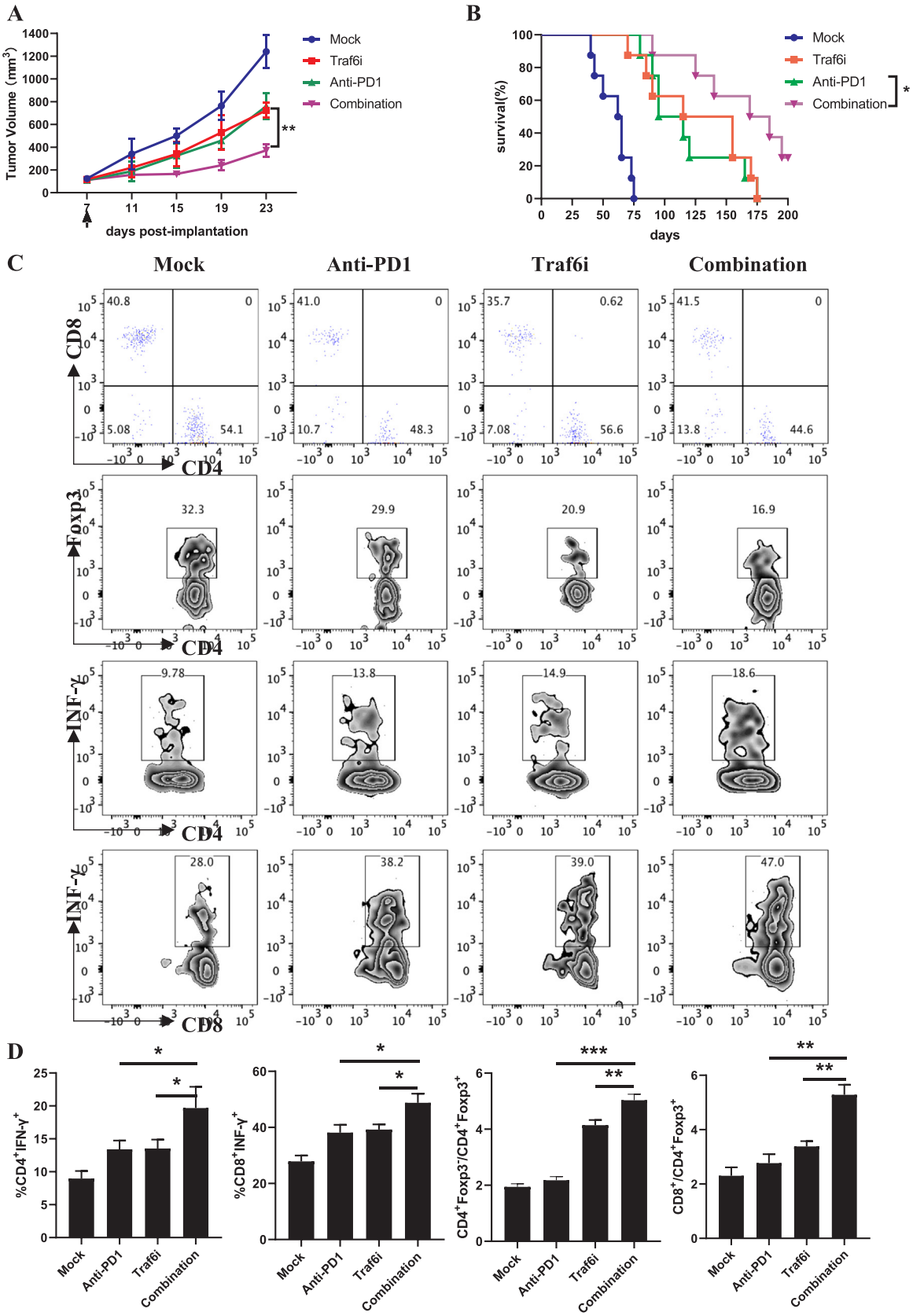

Fig. 5. Combination of Traf6 inhibitor and anti-PD1 blockade achieves a better anti-tumor efficiency. Syngenetic C57BL/6 mice were inoculated with 4×10^6 Hepa1-6 cells. When tumors were palpable at day 7, tumor-bearing mice were administrated i.p. with PBS or Traf6 inhibitor or anti-PD1 or combination of both. Tumor growth was assessed over time and recorded. Tumors were resected from mice 23 days after tumor implantation and TILs were recovered and subjected to FACS analysis. (A) Tumor growth curves of different groups (n = 5), $^{**}p < 0.01$, unpaired *t* test. (B) Survival of tumor-bearing mice from different groups (n = 8), $^{*}p < 0.05$, Log-rank test. (C) Representative dot images showing frequencies of $CD4^+$, $CD8^+$ and $CD4^+Foxp3^+$ T cells among TILs as well as IFNγ-producing fractions within $CD4^+$ and $CD8^+$ T cells from three independent experiments. (D) Summarized analysis from (C), $^{*}p < 0.05$; $^{**}p < 0.01$, unpaired *t* test.

mice bearing Hepa1-6 tumor received a combination treatment of anti-PD1 and Traf6 inhibitor, as the control anti-PD1 alone, Traf6 inhibitor alone, and PBS were utilized. Strikingly, combination treatment achieved much more potent antitumor efficacy, as shown delayed tumor growth and prolonged survival duration than single use of Traf6i or anti-PD1 (Fig. 5A, B). The decidedly enhanced anti-tumor activity seen upon was associated with the improved proportions of INFγ-producing $CD4^+$ and $CD8^+$ T cells and the reduced Tregs presence in the tumor microenvironment (Fig. 5C, D). These findings strongly suggested the potential of Traf6 inhibitor as one synergistic agent for immune checkpoint therapy.

4. Discussion

Previous researches showed that Traf6 was overexpressed in tumors and involved in carcinogenesis and various tumor biology [21]. In HCC, Traf6 recently has been proved to promote tumorigenesis by improving expression and stability of c-myc [37]. In the present study, we tested the antitumor effect of Traf6 inhibitor in the Hepa1-6 model. To our surprise, inhibition of Traf6 did not slow the tumor growth in immunodeficient NCG mice (Fig. 1A). We contributed this to cell-line specific resistance. Indeed, in *ex vivo* experiments (Fig. 1D–F), Hepa1-6 cells showed less responses even at a high concentration of Traf6 inhibitor. In addition, gene expression of Akt signaling pathway, playing crucial roles in modulation of cell survival against apoptosis, remained unchanged when treated with Traf6i, which may contribute to resistance to Traf6i of Hepa1-6 cell line (Fig. 1G). However, restraining Traf6 activity did achieve a tumor-fighting effect in immunocompetent C57BL/6 mice (Fig. 1B–C), suggesting the notion that antitumor immunity was provoked by the administration of Traf6 inhibitor.

It is well documented that Traf6 was an indispensable adaptor for the immune system [26]. Clearly demonstrating the importance of this factor, chimera mice using Traf6-deficient donor bone marrow showed to develop serious autoimmune diseases [38]. Moreover, Traf6 plays an essential role in regulatory T cells [27,39–41], whose suppressive function is always employed by tumors to escape immune attacks [11]. In our present research, inhibition of Traf6 reduced the frequency of intratumor Tregs, leading to more potent T cell-based antitumor immunity (Fig. 2C–F). There are three main reasons may account for the reduction of intratumor Treg cells: (a) downregulation of cell proliferation or increased cellular apoptosis; (b) abrogated migration of peripheral Tregs into the tumor site; (c) less induction of Treg cells from naïve T cell in tumor. Therefore, the proliferation and apoptotic status of tumor-infiltrating Tregs were assessed, but no significant differences were found (Fig. 3). An *in vivo* migration assay of Tregs by transfer of CFSE-labelling lymphocytes revealed that the migration of Tregs towards tumors was specifically limited by the administration of Traf6 inhibitor (Fig. 4A–C). In addition, in line with previous studies [27,41], Treg differentiation from naïve T cells was not altered in the presence of Traf6 inhibitor (data not shown). Furthermore, the suppressive function of Treg cells was also tested through an *in vitro* suppression assay and no obvious differences were shown (data not shown). To testify the predominant role of Tregs in Traf6-mediated antitumor effect, a depletion experiment using antibody against CD25 was carried out and results showed that depletion of Tregs virtually abolished antitumor activity of Traf6 inhibitor (Fig. 4G).

Treg cells have been found to regulate immune responses through migration and accumulation at sites where modulation is required [30]. Previous data demonstrated that the migratory capacity of Treg cells was controlled by distinct signals from chemokines/chemokine receptors and integrins/integrin ligands [42,43]. Recent data have shown that ovarian tumor-secreting CCL22 directed Tregs migration towards tumor site through the interaction with CCR4 expressed on Treg cells, which is potently suppressive and able to block tumor-specific immunity, boosts tumor growth, and predicts poor survival of patients [33]. In our study, several chemokines relative to Tregs migration and respective receptors were measured. While CD103 and CCR4 expressed on intratumor Tregs were comparable to counterparts (Fig. 4D, E), the CCL22 secreting in the tumors treated with Traf6 inhibitor was much lower than the peptide control group (Fig. 4F). Although the source of CCL22 appears to be cancer cells and tumor-associated macrophages [33–35], in-depth studies are needed to shed light on the molecular mechanisms underlying Traf6 inhibitor-mediated downregulation of CCL22 within tumor.

In the past two decades, great advances from basic science led to novel strategies for the treatment of cancers and the field of immune checkpoint therapy has appeared to be a pillar of cancer therapy [6,8]. While gratifying success of immune checkpoint therapy has been achieved in various human tumors [5–8], combinations of different immune checkpoint blockades or immune checkpoint blockade plus other therapies are rather a better strategy [1,8,9]. We also assessed the antitumor efficiency of the combination of Traf6 inhibitor and anti-PD1 antibody. Surprisingly, the combination treatment achieved a much better outcome, slowing the tumor growth and prolonging the survival of tumor-bearing mice to a greater extent in comparison with the single use of anti-PD1 blockade (Fig. 5). Given the promising results outlined above, future studies in the clinic will be needed to assess the true therapeutic potential of Traf6 inhibitor.

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Author contributions

XW, RX and LL designed the experiments; XW, RX HP, XG, XL, WY and YT performed experiments; XW, RX and XN analyzed data; HS, XMW and XHW gave suggestions and critical discussion; XW wrote the paper. XW, RX and HP revised the manuscript.

Declaration of Competing Interest

The authors have no competing interest to declare regarding this manuscript.

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