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Sorafenib attenuated the function of natural killer cells infiltrated in HCC through inhibiting ERK1/2

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ABSTRACT

Sorafenib has been systemically utilized to therapy the advanced hepatocellular carcinoma (HCC). Natural killer cells (NKs) are important cytotoxic innate lymphocytes, which can exert effector functions especially in liver and tumor. However, how Sorafenib affects the function of NKs remains to be elucidated. Here, we utilized the subcutaneous and in situ tumor bearing mice with mouse hepatoma cell line hepa 1–6. At the endpoint, the number and function of NKs in blood, liver, TDLN, and tumor were explored using FACS, ELISA, WB, etc. To confirm the direct effects of Sorafenib on NKs, the NKs were sorted using FACS, which were then stimulated with Sorafenib to detect the functions and the relevant mechanisms using qPCR, western blot, and FACS in vitro. Finally, we found that Sorafenib led to a significant block of tumor progression, but reduced the number of NK cells through suppressing the proliferation of NK cells. This phenotype made us study the terminal function of NK cells, revealing that Sorafenib could decrease the production of effector molecules and cytokines, such as perforin, granzyme B, TNF-α, IFN-γ, etc. Besides, p-ERK1/2 in NK cells was inhibited after treatment with Sorafenib, and a similar tendency of NK cells could be achieved using ERK1/2 inhibitor. Collectively, our data suggested that Sorafenib functioned as a critical inhibitor that controlled the number and function of NK cells through inhibiting ERK1/2.

1. Background

Natural killer (NK) cells are one of the most abundant immune cell populations in liver [1–3], and are cytotoxic innate lymphocytes that are important antiviral and antitumor effector cells, which have excellent potentials for immunotherapy through ameliorating the impaired functions in tumor microenvironment [4,5]. Unlike T and B cells, NK cells do not require pre-sensitization when exerting effector functions, therefore, in contrast to T and B cells, NK cells make a rapid and robust response to tumor through two main strategies [6]. On the one hand, NK cells can directly kill tumor cells without undergoing antigen receptor rearrangement, which were first described as recognizing targets lacking major histocompatibility complex (MHC) class I, as well as producing perforin and granzyme [7]. On the other hand, NK cells can also regulate the immune response by secreting cytokines interferon gamma (IFN-γ), and by their interactions with other immune cells [8].

Sorafenib, a multi-target kinase inhibitor, mainly targets to serine/threonine kinase RAF, vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), is currently the only molecularly targeted drug that has been proven effective against the advanced liver cancer that cannot be resected surgically [9,10]. Antiangiogenic and cytotoxic effects are referred as the principle mechanisms of action of Sorafenib, approved for the treatment of hepatocellular carcinoma (HCC) [11,12]. Studies have shown that the overexpression of RAF/MEK/ERK signaling pathway was observed in HCC, which can be inhibited by Sorafenib. Furthermore, the formation of HCC and portal vein tumor thrombus is closely related to the extracellular overactivation of VEGFR and PDGFR, which can be inhibited by Sorafenib, thereby blocking the progression and metastasis. Thus, Sorafenib displays dual activities of inhibiting tumor cell proliferation and inhibiting tumor angiogenesis. However, the overall efficiency of

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Sorafenib is low, and its sensitivity is related to many factors. Therefore, to improve its efficiency, it is required to explore the immunological mechanism of Sorafenib.

Here, we show that Sorafenib can significantly inhibit the progression of HCC, but reduced the production of CD107a, granzyme B, perforin, TNF-α, and IFN-γ, some effector molecules. Taken together, these data show that Sorafenib can attenuate the effector function of NK cells through inhibiting p-ERK1/2.

2. Materials and methods

2.1. Mice

All animal protocols were approved by the Animal Care and Use Committee of Peking University. C57BL/6 mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory or Charles Rivers Laboratories. All mice were housed in the specific-pathogen-free (SPF) conditions.

2.2. Animal model

Hepa 1–6 (ATCC® CRL-1830™) and H22 from National Infrastructure of Cell Line Resource of China were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. To establish subcutaneous tumors, $1*10^6$ tumor cells were injected into C57BL/6 mice. For in situ tumor bearing mice, 10^6 tumor cells were injected into liver by surgery. After 7 days, a dose of 50 mg/kg Sorafenib (Selleck) or vehicle was administered intraperitoneal daily.

2.3. Cell purification and culture

NK cells were sorted from liver or spleen of tumor mice with HCC on a FACSAria II (Becton Dickinson). The sorted mouse NKs ($CD45^+7\text{-}AAD^-CD3^-CD49b^+$) were further treated with 50 μM of Sorafenib (Selleck) for 48 h in 96-well dishes. The supernatant of culture was collected in −80 °C for ELISA which was performed as described previously.

2.4. Flow cytometry and intracellular staining

All tissues were collected in ice-cold PBS. Blood samples were collected in tubes with heparin. Liver and tumor were chopped mechanically using a tissue chopper, and then digested for 0.5 h at 37 °C in 5 mg/ml collagenase D (Roche) and 50 mg/ml DNase I (Sigma) in serum-free DMEM medium. All single-cell suspensions were treated with ACK lysis buffer. Cells were stained with fluorescence-labeled antibodies (listed below) for 20 min in ice-cold PBS. 7-AAD (1:20; Biolegend) was added to exclude dead cells. For intracellular staining, single-cell suspensions were stimulated in DMEM containing 10% FBS with cell stimulation cocktail for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. Surface antibodies were stained first, followed by fixation and permeabilization using the Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences), then stained with intracellular antibodies according to the manual. All experiments were detected using BD celesta flow cytometer. Data analysis used FlowJo Software (TreeStar). All antibodies were purchased from Biolegend, except CD45 PE-CF594 from BD pharmagen, and CD45-eFluor 450, and Granzyme B-FITC from eBioscience. The following antibodies were used in these experiments: CD45-eFluor 450 (clone 30-F11), CD3-Brilliant Violet 510™ (clone 17A2), CD49b (pan-NK cells)-Pacific Blue™ (clone DX5), KLRG1-APC (clone 2F1/KLRG1), IFN-γ-FITC (clone XMG1.2), TIGIT-APC (clone 1G9), TNF-α-PE (clone MP6-XT22), Granzyme B-FITC (clone NGZB), Perforin-APC (clone S16009A), BrdU-APC (clone Bu20a).

2.5. Real-time PCR

RNA was extracted from the cells above, and then was converted to complementary DNA (cDNA) with PrimeScript RT Master Mix (TAKARA). cDNA was quantified by SYBR green real-time PCR with 500 nM primers using a QuantStudio™ 5 (Thermo Fisher). β-actin or GAPDH was used as reference genes. The following primer sequences were used: For mouse: m*Gzmb* forward 5′-CCACTCTCGACCCTACA TGG-3′, reverse 5′-GGCCCCCAAAGTGACATTTATT-3′; m*Klrk1* forward 5′-ACTCAGAGATGAGCAAATGCC-3′, reverse 5′-CAGGTTGACTGGTAG TTAGTGC-3′; mKLRG1 forward 5′-TTTGGGGCTTTTGACTGTGAT-3′, reverse 5′-TGTAAGGAGATGTGAGCCTTTGT-3′; m*Prf1* forward 5′-AGC ACAAGTTCGTGCCAGG-3′, reverse 5′-GCGTCTCTCATTAGGGAGTT TTT-3′; m*Tigit* forward 5′-GAATGGAACCTGAGGAGTCTCT-3′, reverse 5′-AGCAATGAAGCTCTCTAGGCT-3′; m*Lamp-1* forward 5′-CAGCACTC TTTGAGGTGAAAAAC-3′, reverse 5′-ACGATCTGAGAACCATTCGCA-3′; m*Ifng* forward 5′-ATGAACGCTACACACTGCATC-3′, reverse 5′-CCATC CTTTTGCCAGTTCCTC-3′; m*Tnfa* forward 5′-GACGTGGAACTGGCAGA AGAG-3′, reverse 5′-TTGGTGGTTTGTGAGTGTGAG-3′. RQ values (mRNA) were calculated using the formula: $2^{-\Delta\Delta CT} = 2_1^{-\Delta CT}/2_{Ctrl}^{-\Delta CT}$.

2.6. ELISA

The culture supernatants from the cultured NK cells above were subjected to detection of the concentrations of cytokines TNF-α and IFN-γ using ELISA. The assay was conducted according to the procedures recommended in the manufacturer's instructions of ELISA kits [13].

2.7. Statistical analysis

Data analysis was performed using SPSS 20.0 and GraphPad Prism 8. All data are described as the Mean ± SD. One-way ANOVA and student's *t*-test were appropriately done. $P < 0.05$ were considered statistically significant difference.

3. Results

3.1. Sorafenib could significantly interfere the progression of HCC

Here, we establish subcutaneous tumors with Hepa 1–6 administered with 50 mg/kg Sorafenib or vehicle, finding that Sorafenib could significantly provide survival benefits (Fig. 1A, B). At the endpoint, the mice were sacrificed for weighting tumor, showing that the tumor weight was decreases sharply (Fig. 1C), which was also demonstrated by living imaging (Fig. 1D, E). Therefore, all results suggested that Sorafenib provided better survival benefits.

3.2. Sorafenib could markedly decrease the number of NK cells

To a certain extent, the number of cells reflects its function. Here, we examined the number of NK cells, showing that a significantly decreased number was observed in blood (Fig. 2A). However, the difference between control and Sorafenib narrowed (Fig. 2B). Curiously, whether NK cells undergo decrease following a treatment of Sorafenib is dependent in part on the tissue specificity. Next, we attempted to explore whether NK cells would show reductive ratio following Sorafenib treatments compared with control, showing that Sorafenib-treated mice led to significant reduction of NK cells in tumor draining lymph nodes (TDLN) and tumors (Fig. 2C–E), which could be confirmed by Brdu method both in vivo and in vitro (Fig. 2F, G).

3.3. Sorafenib could markedly attenuate the function of NK cells

Usually, the decrease in quantity reflects a change in function. Here, we analyzed NK cells infiltration and activation in blood, spleen, TDLN,

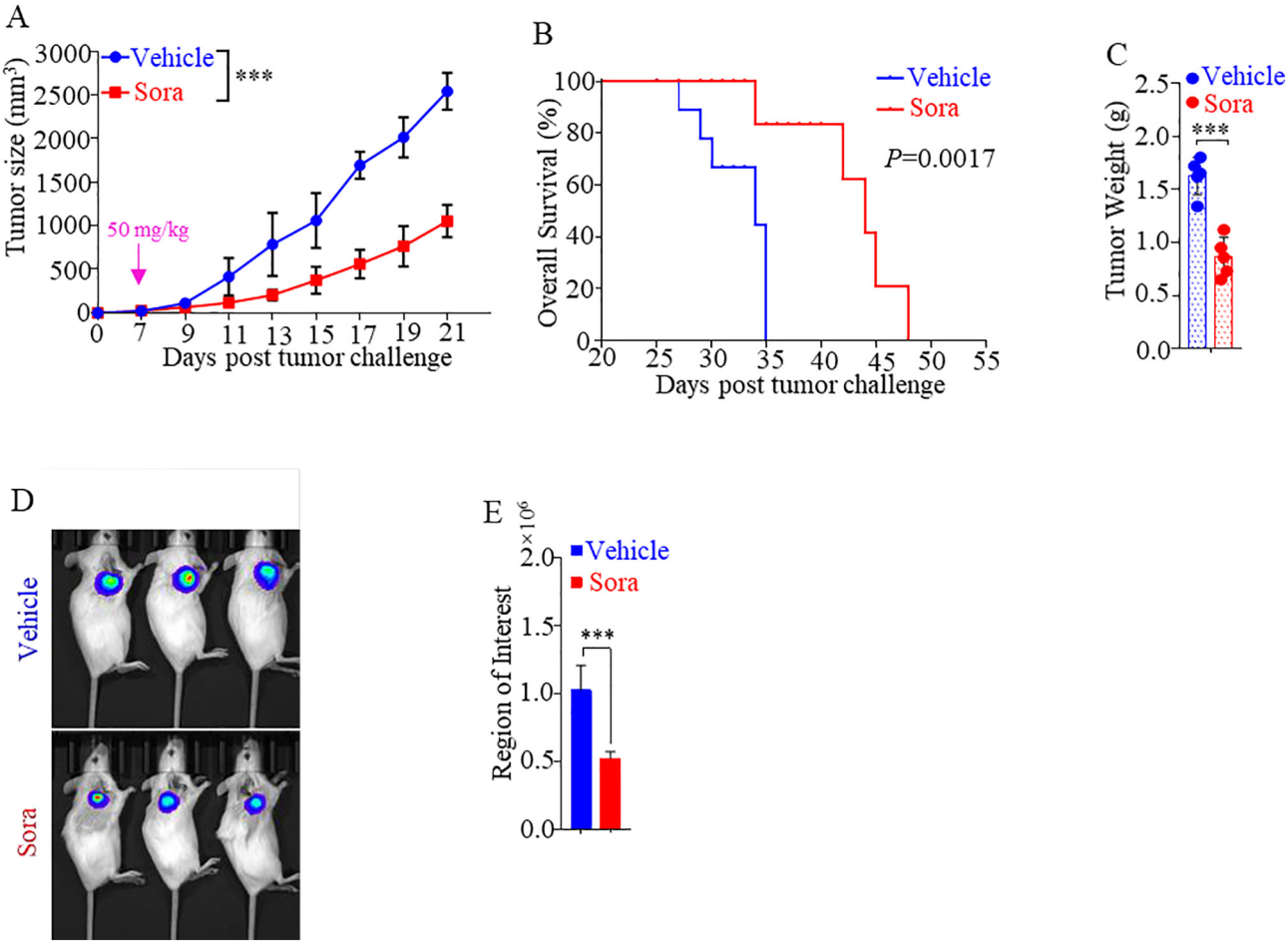

Fig. 1. Sorafenib could significantly block the progression of liver cancer.
(A) Tumor-bearing mouse model was administered with 50 mg/kg Sorafenib (Sora) intraperitoneal daily on day 7 for 14 days. Tumor size was calculated according to the formula: $V = 0.52*L*W^2$.
(B) The overall survival (OS) of was statistically analyzed using Log-rank (Mantel-Cox).
(C) At the endpoint, tumors were weighed and then analyzed.
(D, E) H22 cells were labeled using xenolight DiR, and then explored the living imaging using IVIS Spectrum (D). The ROI (region of interest) was also calculated (E). All data are represented as Mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, compared with the indicated groups.

and tumor. Sorafenib could not significantly alter the proportions of $CD49b^+TIGIT^+$ cells (TIGIT, a checkpoint). CD107a (also known as lysosomal-associated membrane protein-1, LAMP-1), a marker of NK cell degranulation, and perforin represent the effector function of NK cells, we found that Sorafenib markedly reduce the proportion of $CD49b^+CD107a^+$ and $CD49b^+perforin^+$, indicating that Sorafenib attenuated the effector function of NK cells (Fig. 3A), as well as the counterpart in spleen (Fig. 3B). We noted, in addition, a decreased proportion of $CD107a^+$ or $perforin^+$ phenotype was observed in NK cells infiltrated in TDLN and tumor (Fig. 3C, D), which also shown a lower expression of CD69 (Fig. 3E). In order to demonstrate these results, we sorted NK cells from the tumor and performed the quantitative PCR, showing a similar tendency (Fig. 3F). Besides, a weaker killing activity was observed after stimulation with Sorafenib (Fig. 3G). Together, our observations suggest that Sorafenib might suppress the function of NK cells.

3.4. Sorafenib could directly inhibit function of NK cells via ERK1/2

In order to find out whether the effect of sorafenib on the function of NK cells is direct or not, we sorted NK cells from spleen of tumor-bearing mice, and then stimulated these cells with Sorafenib to explore the direct effect. Here, we found that TNF-α and IFN-γ level in cell culture supernatant was sharply reduced, comparing with control (Fig. 4A, B), whereas a decreased proportion of $CD107a^+$, granzyme B^+, TNF-α^+, IFN-γ^+, and $perforin^+$ phenotype was observed upon treatment with Sorafenib (Fig. 4C). Furthermore, we also observed the transcriptional level of relevant genes of effector function, showing that after treatment with Sorafenib, IFN-γ, granzyme B, TNF-α, and perforin decreased significantly (Fig. 4D). Sorafenib, a multi-target kinase inhibitor, mainly targets to serine/threonine kinase, especially MEK/ERK. Here, we found that p-ERK1/2 was significantly blocked after insult with Sorafenib, which might be associated with the direct effects of Sorafenib (Fig. 4E). To confirm the guess, U0126, a selective inhibitor ERK1/2 [14], was utilized to treat the sorted NK cells, revealing that a similar phenomenon was detected, comparing with Sorafenib (Fig. 4F). Taken together, all data suggested that Sorafenib might suppress the function of NK cells via ERK1/2.

4. Discussion

NK cells, one of the most abundant immune cell populations in liver, play an important role in liver immunity and antitumor [15]. On the one hand, NK cells can directly kill target cells by producing granzyme and perforin; on the other hand, it can also secrete many kinds of cytokines to modulate immune response, such as IFN-γ and TNF-α [16].

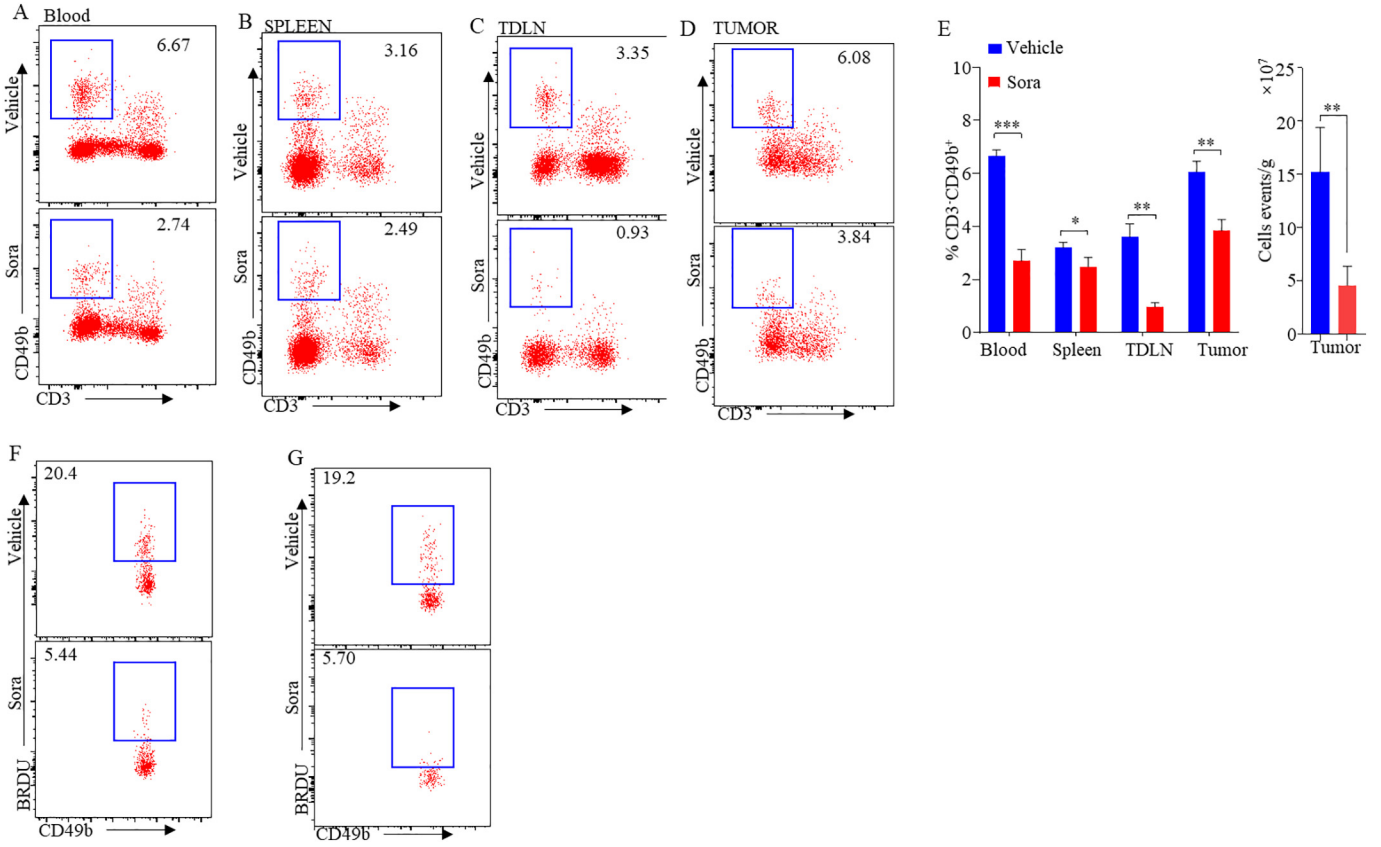

Fig. 2. Sorafenib could significantly decrease the number of NK cells.
(A–E) Representative image of flow cytometry of NKs ($CD3^-CD49b^+$) in blood, spleen, TDLN (tumor-draining lymphode), and tumor from Vehicle- or Sora-treated liver cancer mice. Statistical analysis was shown in E.
(F) The proliferative activity was examined by BrdU which was injected intraperitoneally (5 mg per mouse) 3 day before sacrifice. BrdU staining was performed according to the procedure.
(G) The proliferative activity was examined by BrdU in vitro. BrdU staining was performed according to the procedure.
Statistical data are described as Mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control.

Sorafenib is currently the only molecularly targeted drug that has been proven effective against the advanced liver cancer that cannot be resected surgically. However, the overall efficiency of Sorafenib is low. Sorafenib, as a multi-target kinase inhibitor, is bound to affect the function of immune cells, especially NK cells, as important cytotoxic cells in liver [17]. Therefore, to find ways to improve efficiency, it is necessary to explore the effect of Sorafenib on immune cells. In present study, we found that Sorafenib could significantly inhibit the progression of HCC, but reduced the production of CD107a, granzyme B [18,19], perforin, TNF-α, and IFN-γ, some effector molecules, suggesting that Sorafenib can attenuate the effector function of NK cells [20].

The number of immune cells often reflects its function [21]. We, therefore, first focused on the number of NK cells before exploring the direct roles of Sorafenib in affecting the function. As reported, Sorafenib could significantly slow tumor growth of HCC, while it seemed to reduce the number of NK cells. To confirm it, the proliferation of NK cells was detected using BrdU method, a classic gold standard of detecting proliferation in vivo. BrdU-positive cells represented the new population of proliferated cells. As speculated, a significantly low proliferation rate was obtained after administration of Sorafenib, which might account for the low number of NK cells infiltrated in tumor. Once activated, NK cells could highly express CD107a, as a marker of degranulation following stimulation. CD107a expression correlates with both cytokine secretion and NK cell-mediated lysis of target cells [22]. Some inhibitory receptors were also expressed on NK cells, such as TIGIT and KLRG1 [23]. In addition, NK cells can produce some effector molecules and immunomodulating cytokines, including granzyme B, perforin, TNF-α, IFN-γ, etc. In present study, we found no significant change in TIGIT and KLRG1 in blood, spleen, TDLN or tumor, but observed that both CD107a and perforin were reduced markedly, suggesting that Sorafenib might attenuate the effector function of NK cells. To demonstrate this, we sorted NK cells, and stimulated it using Sorafenib in vitro, showing a phenomenon similar to that in vivo. Again, we confirmed that Sorafenib did attenuate the effector of NK cells. To explore the mechanism, the pharmacological inhibitor U0126 was also utilized to verify our finding that ERK1/2 participate this process [24], a result similar to Sorafenib which can also inhibit U0126. In conclusion, through our research, it is found that Sorafenib not only suppresses tumor cells, but also attenuates anti-tumor immunity to a certain extent. Therefore, how to improve the anti-tumor immunity in this process will be an important strategy to improve the efficiency of Sorafenib in future.

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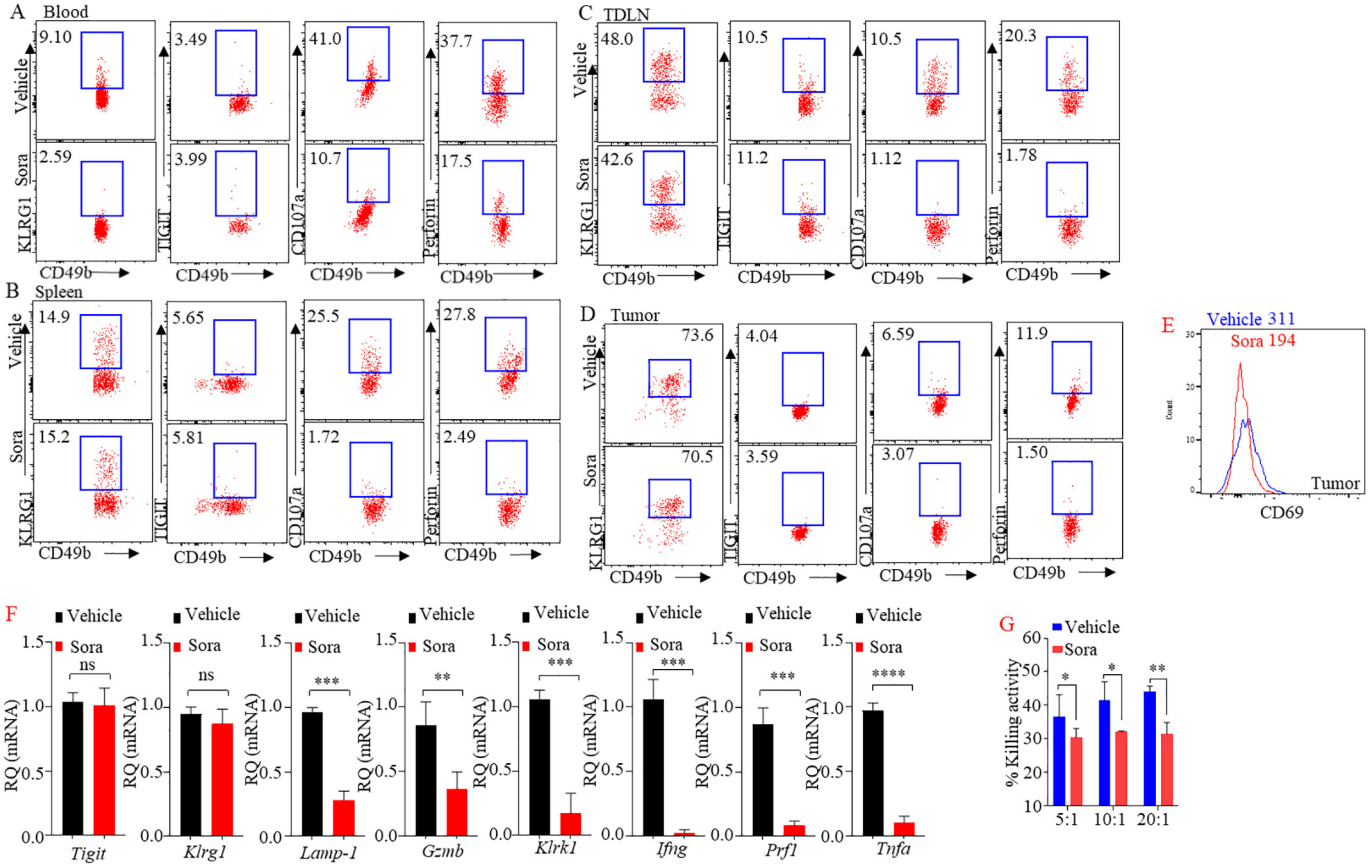

Fig. 3. Sorafenib could markedly attenuate the function of NK cells.
(A–D) Representative image of flow cytometry of NK ($CD3^-CD49b^+$) in blood, spleen, TDLN (tumor-draining lymphode), and tumor from Vehicle- or Sora-treated liver cancer mice.
(E) The mean fluorescence intensity (MFI) of CD69 on NK cells infiltrated in tumor were exhibited in histogram.
(F) NK cells were sorted from tumor of Vehicle- or Sora-treated liver cancer mice, and then performed qPCR.
(G) NK cells killed the target cells Yac-1 by 5:1, 10:1 and 20:1 with or without Sorafenib.
Statistical data are described as Mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control.

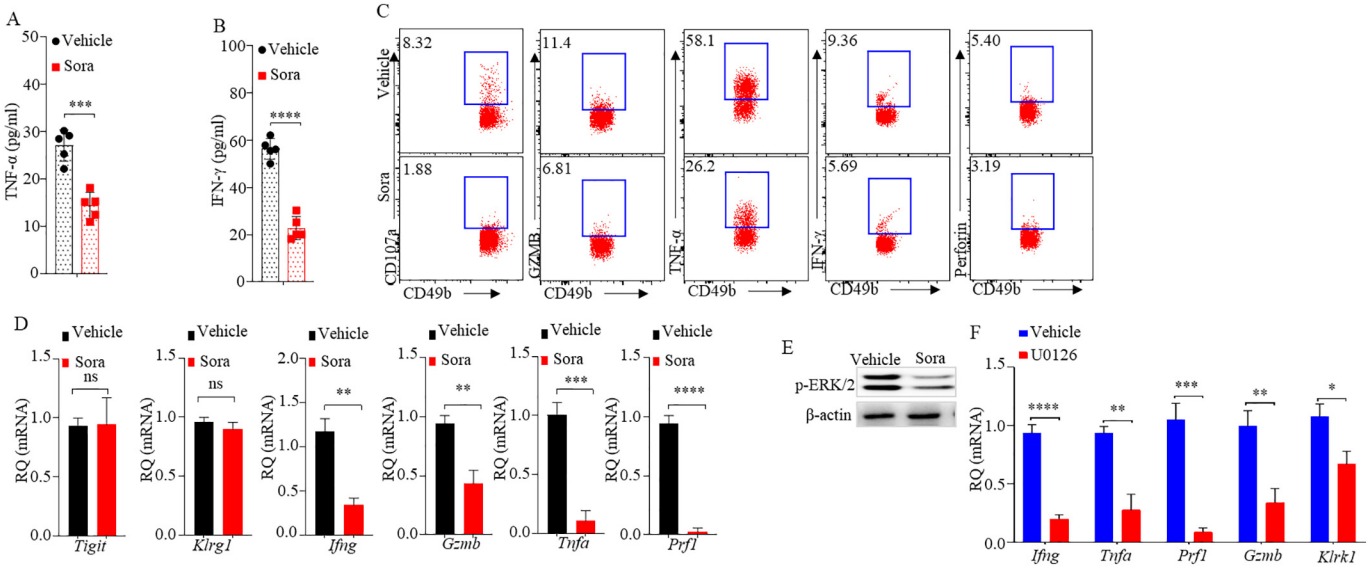

Fig. 4. Sorafenib could directly inhibit function of NK cells via ERK1/2.
(A) NK cells were sorted from tumor, and then stimulated with 50 μM of Sorafenib for 48 h. The supernatant were collected for detecting cytokines using ELISA;
(B–E) After treatment with Sorafenib, NK cells were gathered for FACS, qPCR, and immunoblotting.
(F) NK cells were sorted from tumor, and then stimulated with 10 μM of U0126 for 48 h, and next performed qPCR.
Data are described as Mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control.

Authors' contributions

C. Li designed and conducted these experiments with cells and mice, analyzed data, provided funds support, and wrote the manuscript; S. Wei established animal model, cellular staining and cell culture; X. Xu provided technical supports; L. Xue provided platforms services of central lab, Y. Jiang and P. Jiang collected the samples, and J. Wang developed the concept and provided funds support and overall direction.

Declaration of competing interest

The authors declare no competing interests.

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