

Inhibition of MBTPS1 enhances antitumor immunity and potentiates anti-PD-1 immunotherapy

Corresponding Author: Professor Rui-Hua Xu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The researchers conducted a customized in vivo CRISPR-Cas9 screen targeting genes encoding membrane and secreted proteins in colorectal cancer (CRC) mouse models. They discovered that the membrane-bound transcription factor site-1 protease (MBTPS1) in tumor cells competed with USP13 for binding to STAT1, thus disrupting USP13-dependent deubiquitination and stabilization of STAT1. As a result, MBTPS1 regulated Th1-type chemokines, such as CXCL9 and CXCL10, through STAT1. Their findings were further linked to patients with colorectal cancer and the efficacy of immunotherapy. While the research is potentially significant, several major shortcomings were identified:

1. Tumor-Intrinsic Mechanisms Controlling Th1-Type Chemokines in Cancer: Various key genetic and epigenetic mechanisms, such as EZH2, ARID1A, and LSD1, can regulate Th1-type chemokines and cancer immunotherapy (PMCID: PMC4779053; PMC7190935; PMC6063761). The authors should contextualize their work within this relevant literature. Is the MBTPS1-mediated regulation of Th1-type chemokines related to these mechanisms?
2. Clinical Significance: The authors made a correlative observation in a cohort from SYSUCC, but the pathological and other relevant information are missing. It is unclear if the survival impact of MBTPS1 is independent. The quantification of STAT1 is unclear, and the role of STAT1 in promoting tumors has been reported. The functional importance lies in the activation of STAT1, not just its expression.
3. Quality of IHC Data: The immunohistochemistry (IHC) data presented is of poor quality, making it technically challenging to provide a convincing quantitative assessment.
4. Additional Experimental Controls: The experiments regarding USP13-dependent deubiquitination and stabilization of STAT1 require additional controls. STAT1 can be regulated at various post-translational modification (PTM) levels, and the importance of STAT1-PTM is not demonstrated in the in vivo model.
5. MSI Status Overlooked: Given the focus on colorectal cancer and immunotherapy, the status of microsatellite instability (MSI) in their model and IHC studies is not addressed. The correlative studies extend to other cancer types, which also needs to be clarified in terms of mechanisms and potential translation.

Reviewer #2

(Remarks to the Author)

This study investigates the role of membrane-bound transcription factor site-1 protease (MBTPS1) in colorectal cancer (CRC) and its potential as a target for enhancing anti-tumor immunity. The researchers found that inhibition of MBTPS1 in tumor cells increases CD8+ T cell infiltration by stabilizing STAT1, leading to elevated chemokine expression of CXCL10, which in turn enhances the efficacy of anti-PD-1 immunotherapy. These findings suggest MBTPS1 as a novel target for improving immunotherapy outcomes in CRC patients. Overall, the conclusion of this study is based on very solid data, and the authors explored in-depth of the molecular mechanisms by which MBTPS1 interacts with STAT1 to decrease T cell infiltration. Meanwhile, the following concerns need to be addressed by the authors.

1. In Figure 1 the authors used CRISPR screen to identify MBTPS1. However, I am a little confused about this. Why did the authors compared the tumor volume of C57 and Rag-/- mice? The data of MBTPS1 deficiency in C57 and Rag-/- mice is lacking. Also, among the top 10 genes, why did the authors chose MBTPS1, since other candidates such as NUP93 also has high expression in different cancer types. The author should provide more clear data to highlight the value of MBTPS1.
2. The authors focus on the impact of MBTPS1 in CD8+ T cells. However, although they provided data that there is no

change of CD4⁺ T cell number in MBTPS1 KD or Vec tumors, the possible change of CD4 functional markers such as T-bet, GATA3, are unknown.

3. The color label of Figure 4l is missing.

4. The IHC images in Figure 7n among the three markers is not from the same layer, please explain.

Reviewer #3

(Remarks to the Author)

Reviewer's comments

In this manuscript, the authors investigated the role of MBTPS1 in tumor progression using both in vivo and vitro experiments. They observed a negative correlation between the proportion of tumor-infiltrating lymphocytes (TILs) and the expression of MBTPS1. Utilizing a subcutaneous injection model with MBTPS1-knockdown cancer cells, the authors demonstrated that MBTPS1 loss in the tumor epithelium suppressed tumor growth by enhancing T cell-mediated anti-tumor immunity and increasing the treatment efficacy of anti-PD-1 antibody through the promotion of CD8⁺ T cell infiltration. Additionally, they identified that MBTPS1 suppressed the expression of certain chemoattractants essential for recruiting CD8⁺ T cells by enhancing the ubiquitination of STAT1, through the disruption of USP13' ability to bind to STAT1.

While the data are intriguing and suggestive, several concerns must be addressed by the authors before the manuscript can be considered for publication in Nature Communications. The raised concerns are outlined below.

Major comments

1. In Figure 1, the authors conducted an in vivo CRISPR screen focused on membrane and secreted proteins using xenograft models with MC38 cells, identifying MBTPS1 as a potential target for immunotherapy. They identified MBTPS1 by examining genes downregulated in immunocompetent mice compared to Rag1 KO mice. This suggests that MBTPS1 may be downregulated by T or B cells, which reasonably explains the negative correlation between TIL proportion and MBTPS1 expression (Figure 1g). However, the authors proposed MBTPS1 as a potential target for immunotherapy based on these data, but the rationale for this proposal is not clearly demonstrated. To support their hypothesis, the authors should investigate whether there is a correlation between MBTPS1 expression and the MSI-high status of tumors. Additionally, the source of MBTPS1 in human tumors was not identified. The authors should clarify which cell types express MBTPS1 in human tumors.

2. In Figure 2, the authors showed that loss of MBTPS1 in the tumor epithelium suppressed tumor growth and enhanced the efficacy of anti-PD-1 therapy in xenograft models using MC38, CT26 and E0771 cells, where MBTPS1 was knocked down (KD). Firstly, the authors should provide data confirming MBTPS1 expression in those murine tumors. Secondly, it is recommended to conduct a cell growth assay with MBTPS1-overexpressed cells. Additionally, the authors performed subcutaneous co-injections with MBTPS1-intact and MBTPS1-KD tumor cells, demonstrating that MBTPS1-KD cells decreased in tumors compared to MBTPS1-intact cells (Figure 2f-g and Supplementary Figure 2j), despite equivalent growth and survival rates in vitro. To clarify this, the authors should compare both the rate of tumor cell growth and death between these two cells in vivo (e.g., by immunohistochemistry for Ki67 and cleaved-cas3). It is also recommended to perform this co-injection experiment in immunodeficient mice to confirm whether immune cells contribute to this phenotype. If immune cells are involved, the authors should analyze the changes in immunogenicity between MBTPS1-intact and -KD cells. Moreover, while shMBTPS1-tumors treated with anti-PD-1 showed extremely low tumor burden, some mice died within 60 days post injection. What was the cause of death of these mice? Additionally, the authors demonstrated that the combination of Mbtps1 KD and anti-PD-1 treatment suppressed tumor growth, while neither treatment alone affected tumor growth in KPC and B16 models. The authors need to elucidate the mechanism of this or at least discuss about this.

3. In Figure 3, the authors demonstrated that loss of MBTPS1 in cancer cells increased tumor-infiltrating CD8⁺ T cells by analyses of scRNAseq and FACS. However, the changes in the proportion of each cellular cluster were not thoroughly analyzed. For example, the proportions of Mono/Mph/DC cells, B cells, and Tregs appeared to decrease in shMbtps1-tumors, but there were no quantitative analyses. The authors should provide more detailed analyses. Furthermore, while scRNA-seq analysis showed decreased CD4⁺ T cells and Mono/Mph/DC cells in shMBTPS1-tumors, FACS analysis showed equivalent levels compared to controls. The authors need to provide an explanation about this discrepancy. Additionally, to support their assertion, it is recommended to analyze M1 and M2 signatures in the scRNA-seq data and examine the proportion of exhausted CD8⁺ T cells by FACS. The authors should also provide data on which cells express MBTPS1 using scRNA-seq.

4. In Figure 4, the authors showed that MBTPS1 suppressed anti-tumor immunity by regulating chemoattractants for recruiting CD8⁺ T cells in xenograft models. Firstly, please examine the expression levels of both MBTPS1 and MBTPS2 in those tumors. Since intracellular cholesterol metabolism may not be affected by a high cholesterol diet in some cases, to support their assertion that the tumor-suppressive phenotype observed with MBTPS1 loss is independent of SREBP-1, SREBP-2, or AFT6-a, the authors should perform subcutaneous injections using Srebf1-, Srebf2-, or Atf-alfa-overexpressing shMBTPS1 cells. Secondly, to confirm the source of Cxcl9, Cxcl10 and Cxcl11 upregulated in shMBTPS1 tumors, the authors should analyze the expression of each population in the scRNA-seq data from control and shMBTPS1 tumors, as these chemokines are typically expressed in myeloid cells. Thirdly, the T cell cytotoxicity assay showed that CD8⁺ T cells were more aggressive toward shMBTPS1 cells compared to controls, indicating increased immunogenicity in shMBTPS1 tumors. The authors should clarify the mechanism underlying this cytotoxic change. Lastly, while Cxcl9, Cxcl10, and Cxcl11 were all upregulated in shMBTPS1 tumors, only shCxcl10 cells were analyzed to elucidate the shMBTPS1 phenotype mechanism. The authors should either explain why Cxcl10 was selected or provide additional data.

5. In Figure 6, the authors showed that MBTPS1 decreased STAT1 stability by disrupting USP12 binding to STAT1 via co-IP experiment. Based on co-IP data showing that both MBTPS1 and USP13 can bind to the SH2 domain of STAT1, the

authors suggested that MBTPS1 may compete with USP13 for binding to STAT1. Is there a possibility of direct interaction between USP13 and MBTPS1? To support their assertion, it is recommended to perform subcutaneous injections using shMBTPS1-shUSP13 tumor cells. Additionally, the authors should examine USP13-expressing cells in vivo.

6. In Figure 7, the authors investigated the impact of MBTPS1 loss in tumor development using the AOM/DSS model in Villin-creERT2;Mbtps1f/f mice and identified MBTPS1 as a marker of poor prognosis in TCGA and SYSUCC cohort datasets. However, AOM/DSS model is a widely used representative murine model of colitis-associated colorectal cancer. Given that tumorigenesis via inducing mutation in Apc is a more common CRC model, the authors should consider performing a conditional intestinal-specific knockout of Mbtps1 under Apc loss.

7. The authors asserted that MBTPS1 inhibition converted immune-cold tumors into inflamed tumors (lines 542-544). However, since MC38, the most used cancer cell line in the manuscript, is widely known to develop MSI-high-like tumors with abundant TILs, the presented data do not support this assertion. Additionally, the authors demonstrated that MBTPS1 loss increased CXCL10 expression via increased STAT1 protein levels. Since the activation of STAT1 is commonly regulated by IFN γ , the concluded mechanism of tumor suppression by MBTPS1 loss would require IFN γ signaling. Given that IFN γ is primarily produced by T cells, sufficient tumor-infiltrating T cells would be necessary for this mechanism to work. Additionally, given that MBTPS1 is downregulated in immunocompetent tumors compared to immunodeficient tumors (Figure 1), the existence of TIL (especially activated TIL) would downregulate MBTPS1 to enhance T cell-mediated anti-tumor immunity. The authors need to clarify or discuss these regulatory mechanisms.

Minor comments

1. Please label which side represents shMbtps1 and the control in the graphs presented in Figure 4d and Supplementary Figure 4b, 5d and e.
2. It is better to analyze the number of activated CD8+ T cells in Figure 5p.
3. It is also recommended to analyze the expression of phospho-STAT1 in murine CRC model and human CRC samples in Figure 7.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed previous comments.

Reviewer #2

(Remarks to the Author)

Thanks for the revision and I have no more questions.

Reviewer #3

(Remarks to the Author)

Reviewer's comments

In the revised manuscript, the authors performed substantial amounts of experiments to address the concerns raised by this reviewer. However, there are still several points that need to be addressed. Details are highlighted below.

Major comments

1. (Related to previous comments 1 and 3) The response data indicated that the primary sources of MBTPS1 are tumor cells and fibroblasts. The authors demonstrated a negative correlation between MBTPS1 expression and tumor-infiltrating T cells. Since immune-cold tumors are often enriched with abundant stroma, like in example pancreatic cancers (including KPC tumors), it is possible that MBTPS1-high tumors may have enriched stroma including a lot of fibroblasts. Given this, MBTPS1 in fibroblasts might play a role in shaping the immune activity within these tumors. The authors should provide additional data, or at the very least, discuss about this in the manuscript.

2. (Related to previous comment 2) The response Fig. 10j demonstrated that knockdown of Mbtps1 increased the expression of MHC class I in tumor cells. This suggests the possibility that the vulnerability of tumor cells for the cytotoxicity of T cells is enhanced by Mbtps1 loss. This finding is important, as it provides a potential explanation for the decreased abundance of Mbtps1-targeted cells in immunocompetent tumors during the first CRISPR-screening. Does the underlying mechanism for the increased expression of MHC class I involve an increase in STAT1 levels? To confirm this, the authors are recommended to evaluate MHC class I expression also in shMbtps1-shStat1 tumors as well.

3. (Related to previous comment 4) It appears that the authors have not addressed the previous comment from this reviewer: "To confirm the source of Cxcl9, Cxcl10, and Cxcl11 upregulated in MBTPS1 KD tumors, the authors should analyze the expression of each population in the scRNA-seq data from control and shMBTPS1 tumors." The authors should provide a clear response, as the effect of MBTPS1 loss on other cell populations is crucial for elucidating the mechanism underlying the shMBTPS1 phenotype.

4. Please include data shown in response Figs. 9c and 10a-k and 11i,l and 13a to the main manuscript (not just in response figures).

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

Authors have adequately addressed all the concerns I previously raised.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Manuscript ID: NCOMMS-24-39566

Title: Inhibition of MBTPS1 enhances antitumor immunity and potentiates anti-PD-1 immunotherapy

Detailed point-by-point responses to the reviewers' comments:

Note to reviewers: We thank the reviewers for dedicating the time to evaluate our manuscript and for offering insightful comments that have significantly contributed to its improvement. In this response letter, we have addressed each comment raised by the reviewers with detailed, point-by-point responses. To facilitate a comprehensive review, we have incorporated all new data into figures within this letter and have cross-referenced the corresponding figures and text in our revised manuscript. Additionally, we have highlighted all corresponding changes in our revised manuscript using colored text to ensure clarity.

Reviewer #1 (Remarks to the Author):

The researchers conducted a customized in vivo CRISPR-Cas9 screen targeting genes encoding membrane and secreted proteins in colorectal cancer (CRC) mouse models. They discovered that the membrane-bound transcription factor site-1 protease (MBTPS1) in tumor cells competed with USP13 for binding to STAT1, thus disrupting USP13-dependent deubiquitination and stabilization of STAT1. As a result, MBTPS1 regulated Th1-type chemokines, such as CXCL9 and CXCL10, through STAT1. Their findings were further linked to patients with colorectal cancer and the efficacy of immunotherapy. While the research is potentially significant, several major shortcomings were identified.

Response: We sincerely thank you for reviewing our manuscript. We are deeply grateful to you for recognizing the potential significance of our findings. Additionally, we greatly appreciate the constructive suggestions outlined below,

which have been extremely helpful in enhancing our work.

1. Tumor-Intrinsic Mechanisms Controlling Th1-Type Chemokines in Cancer: Various key genetic and epigenetic mechanisms, such as EZH2, ARID1A, and LSD1, can regulate Th1-type chemokines and cancer immunotherapy (PMCID: PMC4779053; PMC7190935; PMC6063761). The authors should contextualize their work within this relevant literature. Is the MBTPS1-mediated regulation of Th1-type chemokines related to these mechanisms?

Response: We thank you for your valuable suggestions. The epigenetic mechanisms indeed play pivotal roles in regulating the expression of relevant chemokines and anti-tumor immune modulation. We have added the necessary background information and related discussions in the revised manuscript (line 344-348 in revised manuscript). Furthermore, we have examined the expression of common histone methylation modifications that affect the expression of Th1-type chemokines, including H3K27me3, H3K4me2, and H3K79me2^{1, 2, 3}. Our results indicated that the knockdown of *Mbtps1* did not lead to alterations in these relevant methylation modifications (**response Fig. 1**; Supplementary Fig. 5a).

Response Fig. 1

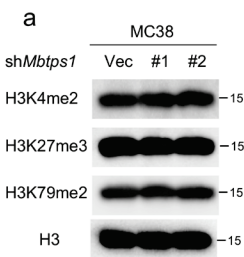


Figure legend 1.

a IB detection of H3K4me2, H3K27me3 and H3K79me2 expression in MC38 cells after *Mbtps1* knockdown. H3 was included as a loading control.

2. Clinical Significance: The authors made a correlative observation in a cohort

from SYSUCC, but the pathological and other relevant information are missing. It is unclear if the survival impact of MBTPS1 is independent. The quantification of STAT1 is unclear, and the role of STAT1 in promoting tumors has been reported. The functional importance lies in the activation of STAT1, not just its expression.

Response: We thank you for your valuable suggestions. In Supplementary Tables 1-2, we have summarized the correlation analysis of clinical information such as patient age, gender, clinical stage, degree of differentiation, and depth of invasion in the SYSUCC cohort. Our findings revealed that MBTPS1 can predict patient recurrence, metastatic status, and survival status, independently of the aforementioned clinical factors. Therefore, after excluding confounding factors, we found that MBTPS1 is an independent prognostic factor.

Upon stimulation by interferons, STAT1 is phosphorylated and activated by JAK, subsequently entering the nucleus to regulate downstream gene transcription⁴ (line 640-642 in revised manuscript). Therefore, we investigated the impact of MBTPS1 expression on STAT1 phosphorylation in response to interferon stimulation. In addition, we investigated the effect of MBTPS1 expression on the level of STAT1 in the nucleus through nuclear-cytoplasmic separation, and supplemented information on the expression of phosphorylated STAT1 (p-STAT1) in the AOM/DSS mouse model and SYSUCC CRC cohort samples. Our results showed that *shMbtps1* increased the expression of phosphorylated STAT1 under interferon stimulation (**response Fig. 2a**; Fig. 5j in the revised manuscript), while overexpression of *Mbtps1* (*OEMbtps1*) inhibited STAT1 phosphorylation under the same conditions (**response Fig. 2b**; Fig. 5k in the revised manuscript). Nuclear-cytoplasmic separation experiments demonstrated that MBTPS1 expression also negatively regulated the amount of STAT1 in the nucleus (**response Fig. 2c**; Supplementary Fig. 5j in the revised manuscript). Immunohistochemical analysis further revealed that

Intestinal-specific knockout of *Mbtps1* upregulated the expression of p-STAT1 in intestinal tumors of AOM/DSS mice (**response Fig. 2d**; Fig. 7c in the revised manuscript) and MBTPS1 expression negatively correlated with both STAT1 expression and the expression of p-STAT1 in SYSUCC CRC cohort (**response Fig. 2e-g**; Fig. 7o, p and Supplementary Fig. 7k in the revised manuscript).

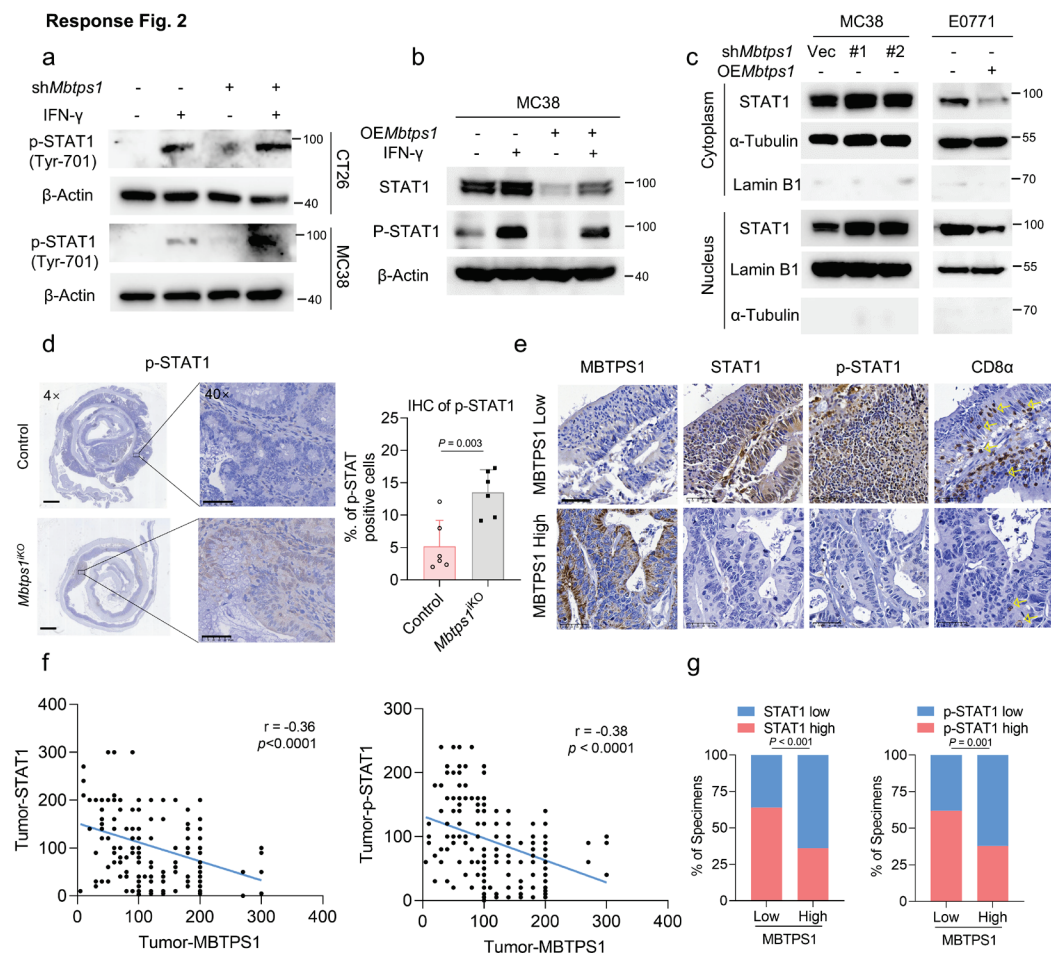


Figure legend 2.

a, b IB detection of STAT1 and phospho-STAT1 (Ser701) expression in CT26 and MC38 cells with *Mbtps1* knockdown (**a**) or overexpression (**b**) followed by IFN- γ (10 ng/ml, 24 h) stimulation. β -Actin was included as a loading control.

c The cytoplasmic and nuclear components were isolated from MC38 and E0771 cell lysates for IB detection of STAT1. α -Tubulin was included as a loading control for cytoplasmic extraction and Lamin B1 was included as a loading control for nuclear extraction.

d Representative IHC images of STAT1 staining (left) and p-STAT1 (right) in

intestinal spontaneous tumor tissues of control and *Mbtps1*^{iKO} mice. Scale bar: 1 mm (4×); 50 μm (40×). | Statistical analyses of the number of the intestinal spontaneous tumors in WT-*Apc*^{Min/+} and *Mbtps1*^{iKO}-*Apc*^{Min/+} mice.

e Representative IHC staining of the indicated markers in CRC tumor tissues from patients with high or low MBTPS1 levels. Scale bar: 50 μm.

f Correlation between tumoral STAT1 (left) or p-STAT1 (right) and MBTPS1 in the colorectal cancer cohort of SYSUCC.

g Percentages of tumor tissues exhibiting high or low expression of STAT1 (left) and p-STAT1 (right) presented for the group with low or high MBTPS1 expression.

3. Quality of IHC Data: The immunohistochemistry (IHC) data presented is of poor quality, making it technically challenging to provide a convincing quantitative assessment.

Response: We apologize for the oversight in our previous submission. We noticed that our images were compressed during the export process, resulting in blurred immunohistochemical images. To address this issue, we have increased the resolution of the images and have added arrows to some of the representative images to indicate the location of the positive cells in our revised figures.

4. Additional Experimental Controls: The experiments regarding USP13-dependent deubiquitination and stabilization of STAT1 require additional controls. STAT1 can be regulated at various post-translational modification (PTM) levels, and the importance of STAT1-PTM is not demonstrated in the in vivo model.

Response: We thank you for your valuable suggestions. To further confirm the USP13-dependent deubiquitination and stabilization of STAT1, we knocked

down or overexpressed *Usp13* and found that the expression of USP13 negatively regulates the ubiquitination level of STAT1 (response Fig. 3a; Supplementary Fig. 6d in the revised manuscript). Additionally, *shUsp13* rescued the increased stability of STAT1 caused by *Mbtps1* knockdown, while *OEUsp13* reversed the decreased stability of STAT1 induced by *Mbtps1* overexpression (response Fig. 3b, c; Fig. 6i and Supplementary Fig. 6k in the revised manuscript). To validate the importance of ubiquitination modification of STAT1 *in vivo*, we constructed *OEStat1-shUsp13* MC38 cells and performed subcutaneous injection in mice. We found that overexpression of *Stat1* inhibited tumor growth *in vivo*, while the effect was abolished after the knockdown of *Usp13* (response Fig. 3d-f; Supplementary Fig. 6e-g in the revised manuscript).

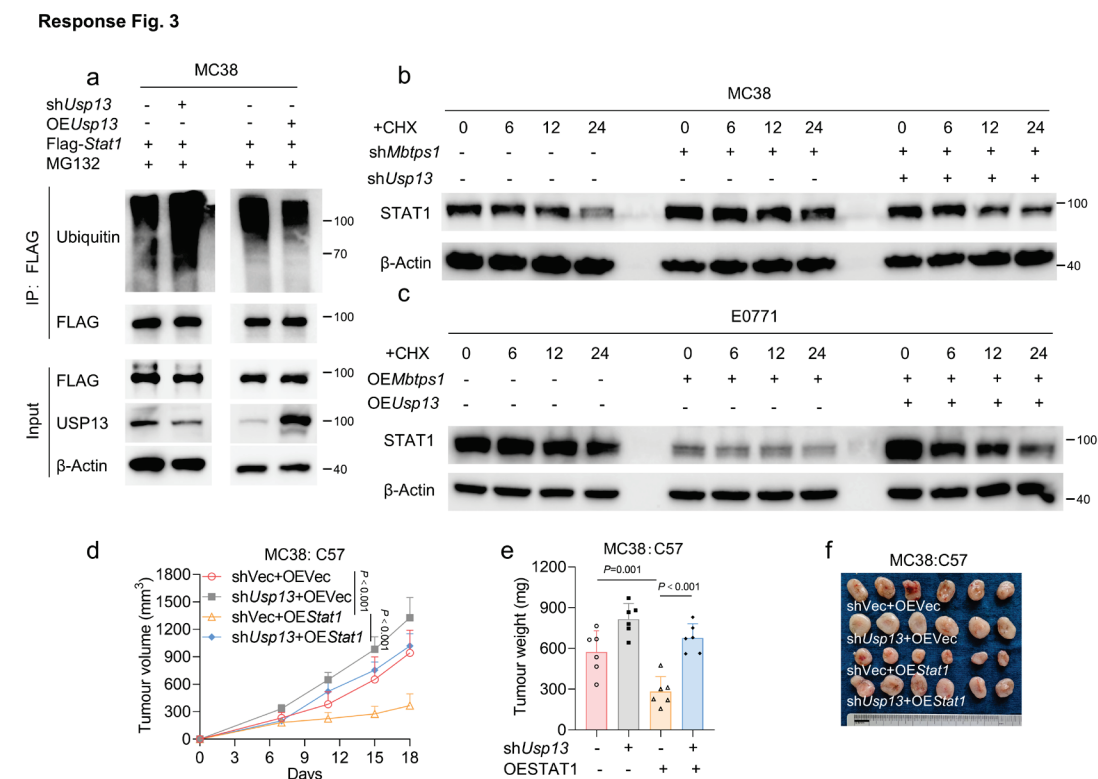


Figure legend 3.

a Co-IP analysis of the interaction between FLAG-STAT1 and endogenous ubiquitin in MC38 cells with *Usp13* knockdown or overexpression.

b IB detection of STAT1 expression in MC38 cells after knockdown of *Mbtps1*

alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*, followed by treatment with CHX (5 µg/ml).

c IB detection of STAT1 expression in MC38 cells after knockdown of *Mbtps1* alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*, followed by treatment with CHX (5 µg/ml). β -Actin was included as a loading control.

d-f Growth curves (d), weight analysis (e) and representative image (f) of MC38 tumors under the conditions of knockdown of *Usp13* alone, overexpression of *Stat1* alone, and simultaneous knockdown of *Usp13* with overexpression of *Stat1*.

5. MSI Status Overlooked: Given the focus on colorectal cancer and immunotherapy, the status of microsatellite instability (MSI) in their model and IHC studies is not addressed. The correlative studies extend to other cancer types, which also needs to be clarified in terms of mechanisms and potential translation.

Response: Thanks for your suggestion. We assessed the MMR status of patients in SYSUCC cohort through immunohistochemical analysis, which was scored and evaluated by a pathologist. Our results indicated that there is no significant correlation between MBTPS1 expression and the MMR status of the patients (**response Fig. 4a**; Fig. 7q in revised manuscript). Additionally, analysis of the TCGA database also demonstrated that MBTPS1 expression did not show a significant correlation with MSI value across more than 30 types of tumors, including colorectal cancer⁵ (**response Fig. 4b**). Meanwhile, we also analyzed the correlation between MBTPS1 expression and tumor-infiltrating T cells as well as Tide scores, which is a recognized method for predicting the efficacy of immunotherapy⁶, across multiple tumor types using the TCGA database (**response Fig 4c, d**; Fig. 1h and Supplementary Fig. 7j). The results indicated that MBTPS1 expression is negatively correlated with tumor-

infiltrating T cells in various tumors and positively correlated with Tide scores.

Response Fig. 4

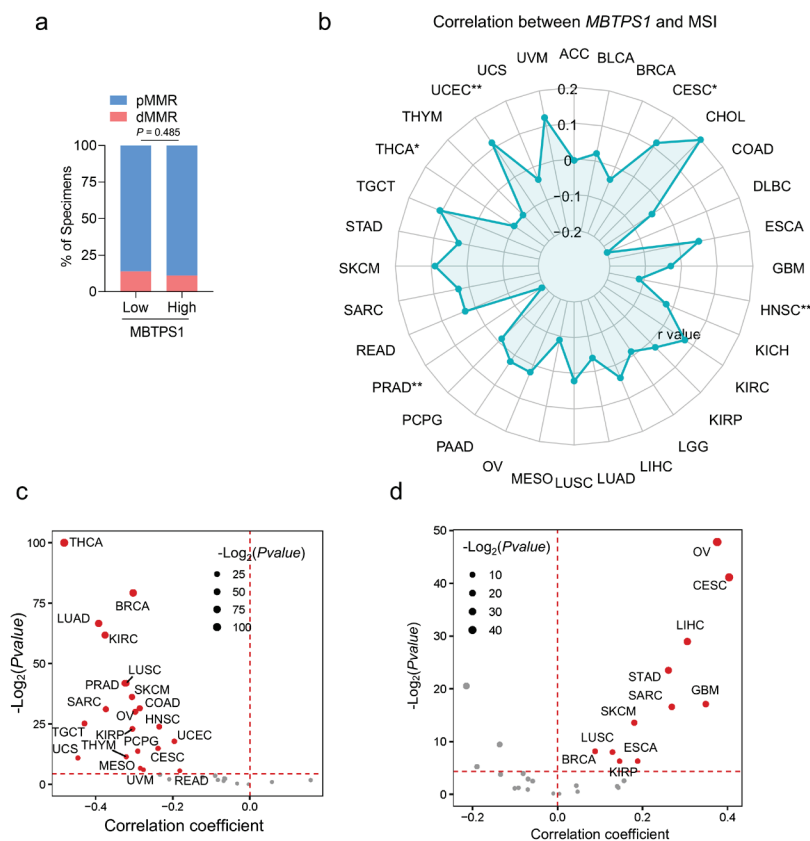


Figure legend 4.

a Percentage of tumor tissues exhibiting either pMMR (proficient mismatch repair) or dMMR (defective mismatch repair) was presented for the groups with low and high MBTPS1 expression, respectively.

b Correlation analysis between MBTPS1 and MSI value utilizing TCGA database.

c Pearson correlation between tumor-infiltrating lymphocytes (TILs) proportion and MBTPS1 expression across 33 cancer types from TCGA.

d Pearson correlation between TIDE prediction scores and MBTPS1 expression across 33 cancer types from TCGA.

References:

1. Peng D, *et al.* Epigenetic silencing of TH1-type chemokines shapes

- tumour immunity and immunotherapy. *Nature* **527**, 249-253 (2015).
2. Li J, *et al.* Epigenetic driver mutations in ARID1A shape cancer immune phenotype and immunotherapy. *J Clin Invest* **130**, 2712-2726 (2020).
 3. Sheng W, *et al.* LSD1 Ablation Stimulates Anti-tumor Immunity and Enables Checkpoint Blockade. *Cell* **174**, 549-563.e519 (2018).
 4. Agashe RP, Lippman SM, Kurzrock R. JAK: Not Just Another Kinase. *Mol Cancer Ther* **21**, 1757-1764 (2022).
 5. Ramos M, *et al.* Multiomic Integration of Public Oncology Databases in Bioconductor. *JCO Clin Cancer Inform* **4**, 958-971 (2020).
 6. Jiang P, *et al.* Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nature medicine* **24**, 1550-1558 (2018).

Reviewer #2 (Remarks to the Author):

This study investigates the role of membrane-bound transcription factor site-1 protease (MBTPS1) in colorectal cancer (CRC) and its potential as a target for enhancing anti-tumor immunity. The researchers found that inhibition of MBTPS1 in tumor cells increases CD8+ T cell infiltration by stabilizing STAT1, leading to elevated chemokine expression of CXCL10, which in turn enhances the efficacy of anti-PD-1 immunotherapy. These findings suggest MBTPS1 as a novel target for improving immunotherapy outcomes in CRC patients. Overall, the conclusion of this study is based on very solid data, and the authors explored in-depth of the molecular mechanisms by which MBTPS1 interacts with STAT1 to decrease T cell infiltration. Meanwhile, the following concerns need to be addressed by the authors.

Response: We sincerely thank you for acknowledging the solid foundation of our study's conclusions. Your appreciation of our in-depth exploration of the molecular mechanisms involving MBTPS1 and STAT1, and their role in

reducing T cell infiltration, underscores the significance of our findings. We deeply appreciate your insightful comments and have taken careful steps to address each of the critiques you raised in our revised manuscript. We are hopeful that our efforts have adequately responded to your concerns.

1. In Figure 1 the authors used CRISPR screen to indentify MBTPS1. However, I am a little confused about this. Why did the authors compared the tumor volume of C57 and Rag^{-/-} mice? The data of MBTPS1 deficiency in C57 and Rag^{-/-} mice is lacking. Also, among the top 10 genes, why did the authors chose MBTPS1, since other candidates such as NUP93 also has high expression in different cancer types. The author should provide more clear data to highlight the value of MBTPS1.

Response: Thanks for your precious suggestions. To demonstrate the presence of selective pressure in our CRISPR screening strategy, we compared the tumor volumes of C57 and *Rag1*^{-/-} mice, showing that the presence of the immune system acts as the screening pressure¹, which resulted in smaller tumor volumes in immunocompetent mice compared to immunodeficient ones. Additionally, we have supplemented the tumor growth data for MC38-sh*Mbtps1* and MC38-shVec in both C57 and *Rag1*^{-/-} mice, revealing that the expression of MBTPS1 only influenced tumor growth in C57 mice but not in *Rag1*^{-/-} mice (**response Fig. 5a-c**; Fig. 2f in revised manuscript). This emphasized the dependence of the function of MBTPS1 on the immune system.

Among the top 10 candidate genes, based on multiple omics data, combined with the expression status in tumor tissues, prognosis, and tumor-infiltrating lymphocytes (TILs) status, we comprehensively assessed and found that only MBTPS1 met the criteria of high expression in tumors. High expression of MBTPS1 is a poor prognostic factor for colorectal cancer, and its expression

negatively correlates with TILs in cancers such as colorectal cancer (response Fig. 5d-f; Fig. 1f, g and Supplementary Fig. 1d in the revised manuscript; line 134-143 in the revised manuscript).

Response Fig. 5

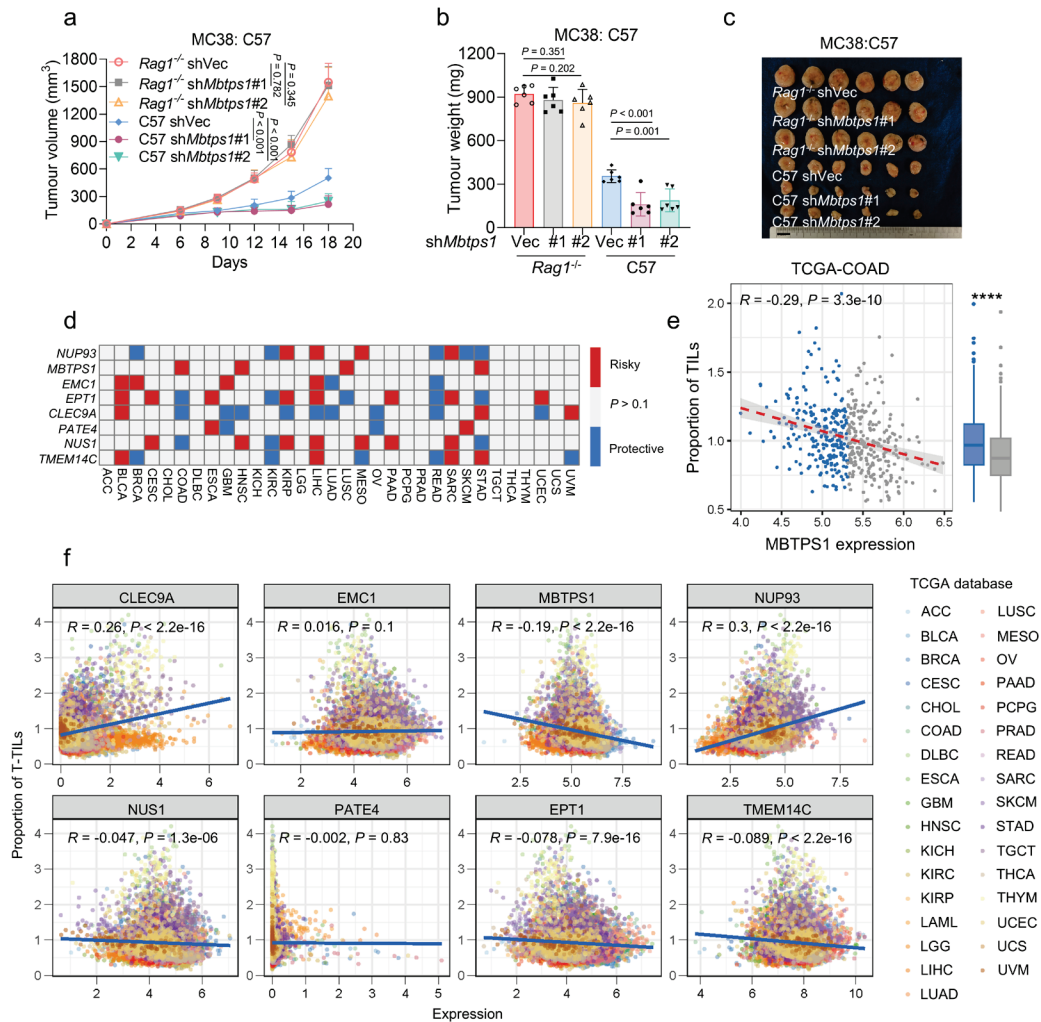


Figure legend 5.

a-c Growth curves (**a**), Tumor weight analysis (**b**), and representative image (**c**) of MC38 tumors with knockdown of *Mbtps1* in C57 and *Rag1*^{-/-} mice, respectively.

d Overall survival analysis based on the high or low expression of the top 10 genes across 32 cancer types from TCGA.

e Correlation between MBTPS1 expression and tumor-infiltrating lymphocytes (TILs) in TCGA-COAD dataset.

f Pearson correlation between T-TILs proportion and the expression of the top

10 genes across 33 cancer types from TCGA.

2. The authors focus on the impact of MBTPS1 in CD8+ T cells. However, although they provided data that there is no change of CD4+ T cell number in MBTPS1 KD or Vec tumors, the possible change of CD4 functional markers such as T-bet, GATA3, are unknown.

Response: Thanks for your suggestion. We have performed flow cytometry to analyze the CD4 functional markers, including T-bet and GATA3, and found that the knockdown of *Mbtps1* did not affect the expression of these markers (**response Fig. 6a-c**; Supplementary Fig. 3g in the revised manuscript).

Response Fig. 6

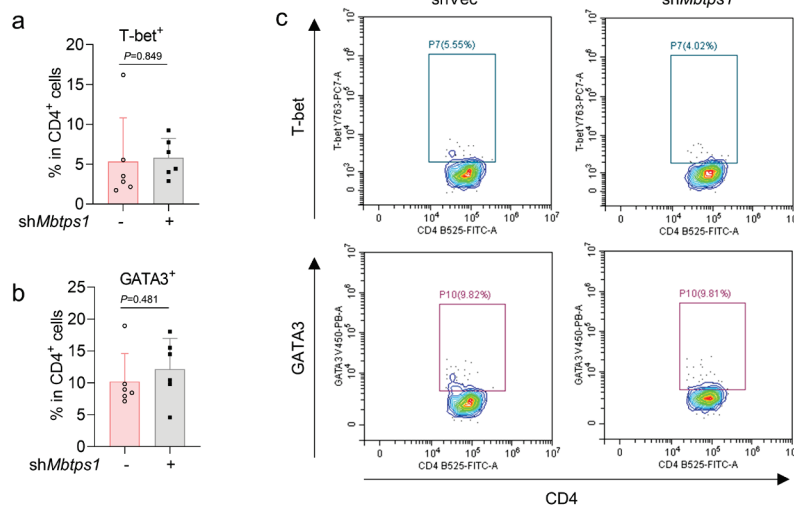


Figure legend 6.

a, b Percentages of T-bet⁺ CD4⁺ T cells (**a**) and GATA3⁺ CD4⁺ T (**b**) in shVec and shMbtps1 MC38 tumors analyzed by flow cytometry (n = 6).

c Schematic diagram of flow cytometry in **a** and **b**.

3. The color label of Figure 4l is missing.

Response: We apologize for missing the color label. We have added the color label to Fig. 4m in the revised manuscript (**response Fig. 7a**).

Response Fig. 7

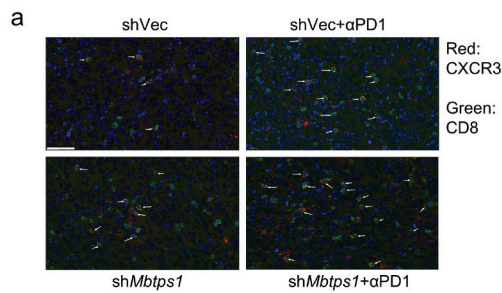


Figure legend 7.

a Representative images of immunofluorescence assay showing the staining of CXCR3 (red), CD8 (green) and nuclei (blue).

4. The IHC images in Figure 7n among the three markers is not from the same layer, please explain.

Response: The MBTPS1-high and MBTPS1-low samples in Fig. 7n are derived from the same tumor tissue of the same patient. The morphological differences observed between the sections may be attributed to several factors, including sample loss during sectioning (given that each section has a thickness of 3-5 micrometers) and alterations in tissue morphology due to the preparation of multiple consecutive sections. To address this concern, we have provided the complete, uncropped IHC images of the tissue microarray for this patient (**response Fig. 8a, b**; Fig. 7o in the revised manuscript).

Response Fig. 8

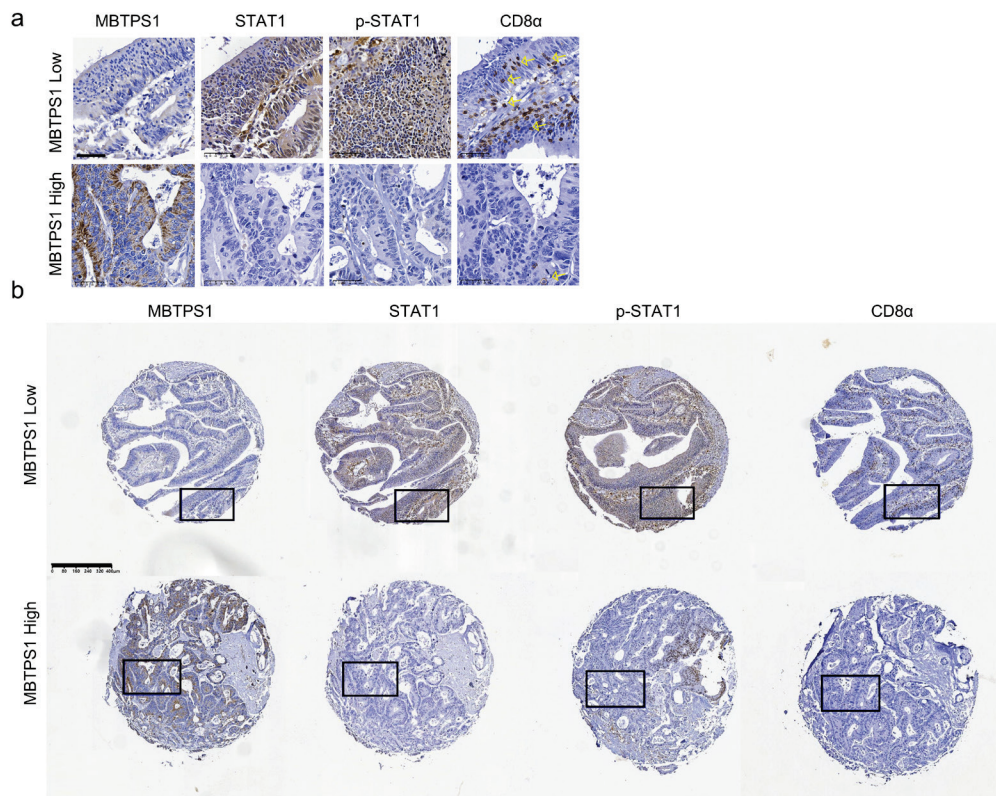


Figure legend 8.

a Representative IHC staining of the indicated markers in CRC tumor tissues from patients with high or low MBTPS1 levels. Scale bar: 50 μ m.

b Complete, uncropped IHC images of the tissue microarray in **a**.

References:

1. Martin TD, *et al.* The adaptive immune system is a major driver of selection for tumor suppressor gene inactivation. *Science* **373**, 1327-1335 (2021).

Reviewer #3 (Remarks to the Author):

Reviewer's comments

In this manuscript, the authors investigated the role of MBTPS1 in tumor

progression using both in vivo and vitro experiments. They observed a negative correlation between the proportion of tumor-infiltrating lymphocytes (TILs) and the expression of MBTPS1. Utilizing a subcutaneous injection model with MBTPS1-knockdown cancer cells, the authors demonstrated that MBTPS1 loss in the tumor epithelium suppressed tumor growth by enhancing T cell-mediated anti-tumor immunity and increasing the treatment efficacy of anti-PD-1 antibody through the promotion of CD8+ T cell infiltration. Additionally, they identified that MBTPS1 suppressed the expression of certain chemoattractants essential for recruiting CD8+ T cells by enhancing the ubiquitination of STAT1, through the disruption of USP13' ability to bind to STAT1.

While the data are intriguing and suggestive, several concerns must be addressed by the authors before the manuscript can be considered for publication in Nature Communications. The raised concerns are outlined below.

Response: Thank you very much for your thorough evaluation of our manuscript. We are truly grateful for your positive assessment of our data as intriguing and suggestive. We appreciate your insightful comments and the constructive feedback you have provided. We have taken your comments into careful consideration and worked diligently to provide additional data and clarified any uncertainties.

Major comments

1. In Figure1, the authors conducted an in vivo CRISPR screen focused on membrane and secreted proteins using xenograft models with MC38 cells, identifying MBTPS1 as a potential target for immunotherapy. They identified MBTPS1 by examining genes downregulated in immunocompetent mice compared to *Rag1* KO mice. This suggests that MBTPS1 may be downregulated by T or B cells, which reasonably explains the negative

correlation between TIL proportion and MBTPS1 expression (Figure 1g). However, the authors proposed MBTPS1 as a potential target for immunotherapy based on these data, but the rationale for this proposal is not clearly demonstrated. To support their hypothesis, the authors should investigate whether there is a correlation between MBTPS1 expression and the MSI-high status of tumors. Additionally, the source of MBTPS1 in human tumors was not identified. The authors should clarify which cell types express MBTPS1 in human tumors.

Response: Thanks for your suggestions. We assessed the MMR status of patients in SYSUCC cohort through immunohistochemical analysis, which was scored and evaluated by a pathologist. Our findings indicated no significant correlation between MBTPS1 expression and the MMR status of the patients (**response Fig. 9a**; Fig. 7q in the revised manuscript). Furthermore, an analysis of the TCGA database, encompassing over thirty types of tumors including colorectal cancer, also revealed no clear correlation between MBTPS1 expression and MSI value¹ (**response Fig. 9b**).

To address the question of which cell types express MBTPS1 in human tumors, we have examined a comprehensive single-cell atlas of human colorectal cancer from a previous study that integrated multiple single-cell datasets and established the largest colorectal cancer cohort study, encompassing samples of normal tissue and tumor tissue from patients with CRC². The results demonstrated that MBTPS1 was highly expressed in malignant cells and fibroblasts, among other cell populations (**response Fig. 9c**).

Response Fig. 9

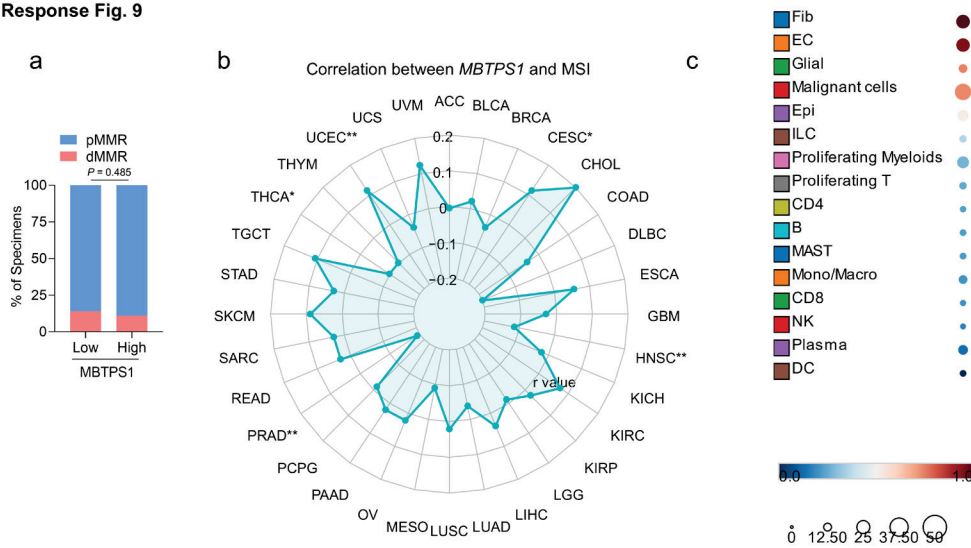


Figure legend 9.

a Percentage of tumor tissues exhibiting either pMMR (proficient mismatch repair) or dMMR (defective mismatch repair) was presented for the groups with low and high MBTPS1 expression, respectively.

b Correlation analysis between MBTPS1 and MSI value utilizing TCGA database.

c Schematic diagram of the expression level of MBTPS1 in different cell types in patients with CRC analyzed by scRNA-seq data².

2. In Figure 2, the authors showed that loss of MBTPS1 in the tumor epithelium suppressed tumor growth and enhanced the efficacy of anti-PD-1 therapy in xenograft models using MC38, CT26 and E0771 cells, where MBTPS1 was knocked down (KD). Firstly, the authors should provide data confirming MBTPS1 expression in those murine tumors. Secondly, it is recommended to conduct a cell growth assay with MBTPS1-overexpressed cells. Additionally, the authors performed subcutaneous co-injections with MBTPS1-intact and MBTPS1-KD tumor cells, demonstrating that MBTPS1-KD cells decreased in tumors compared to MBTPS1-intact cells (Figure 2f-g and Supplementary Figure 2j), despite equivalent growth and survival rates in vitro. To clarify this, the authors should compare both the rate of tumor cell growth and death

between these two cells *in vivo* (e.g., by immunohistochemistry for Ki67 and cleaved-cas3). It is also recommended to perform this co-injection experiment in immunodeficient mice to confirm whether immune cells contribute to this phenotype. If immune cells are involved, the authors should analyze the changes in immunogenicity between MBTPS1-intact and -KD cells. Moreover, while shMBTPS1-tumors treated with anti-PD-1 showed extremely low tumor burden, some mice died within 60 days post injection. What was the cause of death of these mice? Additionally, the authors demonstrated that the combination of *Mbtps1* KD and anti-PD-1 treatment suppressed tumor growth, while neither treatment alone affected tumor growth in KPC and B16 models. The authors need to elucidate the mechanism of this or at least discuss about this.

Response: Thanks for your suggestions. Firstly, to confirm MBTPS1 expression in MC38, E0771, and CT26 tumors, we conducted western blotting and verified the knockdown efficiency of *Mbtps1* in these tumors (**response Fig. 10a-c**). Secondly, growth assays with MC38 and E0771 cells overexpressing MBTPS1 revealed no significant impact on cell growth rates *in vitro* (**response Fig. 10d, e**).

To compare the proliferation and death rates of shVec and sh*Mbtps1* cells *in vivo*, we performed IHC staining for Ki67 and cleaved caspase-3 on tumor tissues from *Rag1*^{-/-} and C57 mice. As shown in **response Fig. 10f-h**, MBTPS1 inhibition had no significant effect on the proliferation rate (% of Ki67 positive cells) of tumor cells but increased their death rate (% of cleaved caspase-3 positive cells) in C57 mice.

An *in vivo* competition assay with co-injection of shVec and sh*Mbtps1* cells in immunodeficient NCG mice demonstrated similar proportions of both cell types, indicating that the integrity of the immune system is necessary for the MBTPS1

phenotype (**response Fig. 10i**; Supplementary Fig. 2m in the revised manuscript).

To analyze the changes in immunogenicity between MBTPS1-intact and -KD cells, we used flow cytometry to detect the expression of the MHC class I molecule H-2K^b on the surface of MC38 cells. Knockdown of *Mbtps1* increased the expression of H-2K^b, thereby enhancing the antigen-presenting capacity and immunogenicity of the cells. Given previous reports that STAT1 can upregulate MHC class I expression^{3, 4}, we speculated that MBTPS1, as a regulator of STAT1, may also regulate MHC class I expression through this mechanism (**response Fig. 10j, k**).

We define the observation endpoint for mice as the ethical endpoint when their tumor reaches a measured volume of 2000 mm³, which is also the approach adopted by many current studies, rather than waiting for the physiological death of the mice. We have provided the tumor volumes of mice at various time points in Source Data 1.

In KPC and B16 tumors, neither *Mbtps1* knockdown nor PD-1 treatment alone affected tumor growth, with tumor inhibition only observed when both treatments were combined. We hypothesized that this difference might arise from the heterogeneity in treatment response among different tumor types and cell populations, as well as the varying susceptibility of different cells to T cell-mediated killing. To support this, we isolated CD8⁺ T cells from mouse spleens and conducted killing assays, which showed that B16 and KPC cells were more resistant to killing by the same number of CD8⁺ T cells compared to MC38 or E0771 cells (**response Fig. 10l, m**). This may explain why, in the KPC and B16 models, tumor inhibition was only observed when MBTPS1 inhibition was combined with PD-1 antibody treatment, resulting in the highest infiltration level

and strongest killing ability of CD8⁺ T cells (line 238-240 in revised manuscript).

Response Fig. 10

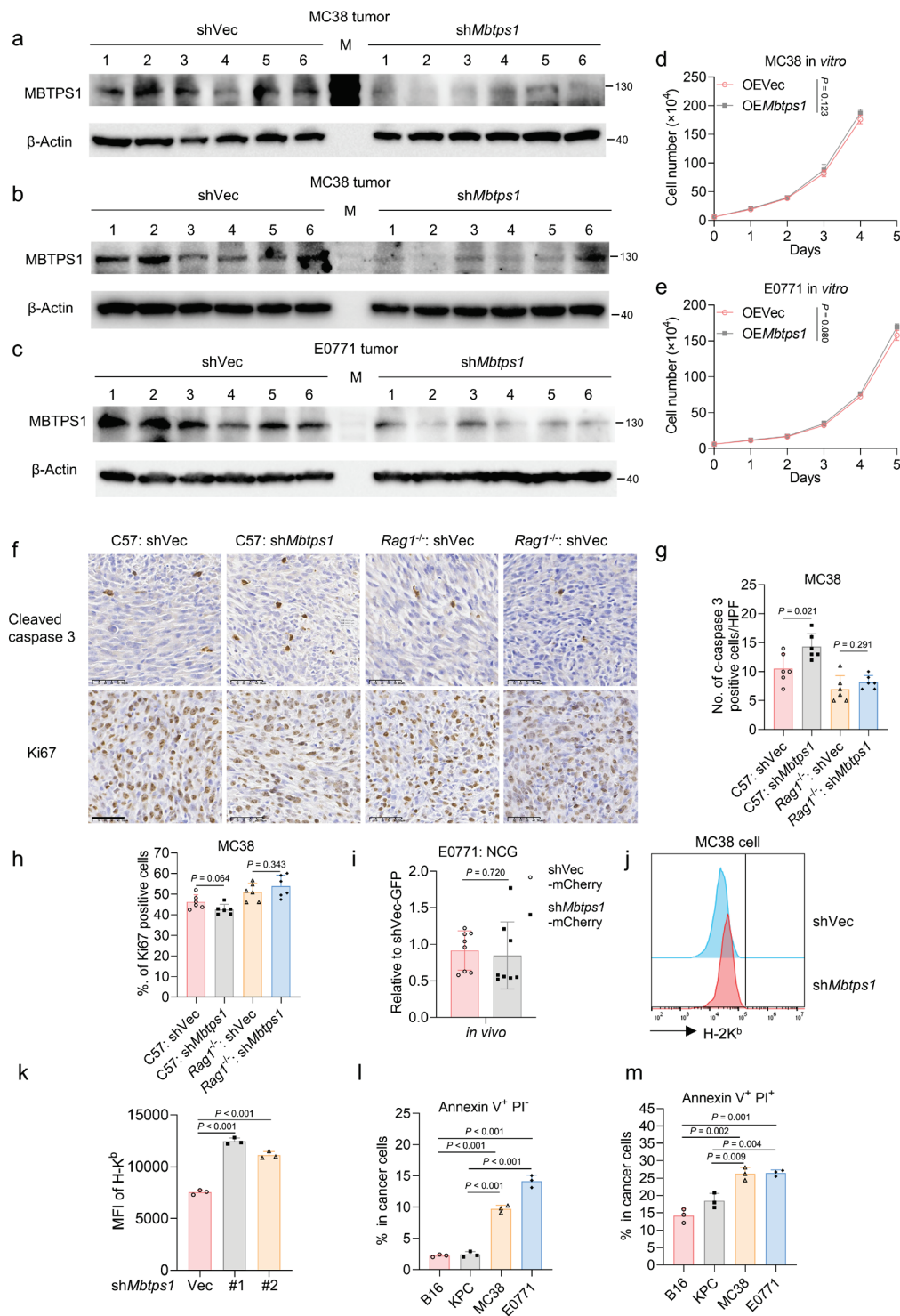


Figure legend 10.

a-c IB detection of MBTPS1 expression in MC38 (**a**), CT26 (**b**), and E0771 (**c**) tumor with *Mbtps1* knockdown, respectively. β-Actin was included as a loading

control.

d, e Growth curves of OEVec and OEMbtps1 MC38 (**d**) or E0771 (**e**) cells *in vitro*.

f Representative IHC images of cleaved caspase-3 and Ki67 staining in MC38 tumors from *Rag1*^{-/-} and C57 mice. Scale bar: 50 μm.

g, h Percentages of cleaved caspase-3 positive cells (**g**) and Ki67 positive cells (**h**) in MC38 tumors from *Rag1*^{-/-} and C57 mice analyzed by IHC staining.

i Change in the ratios of shVec: shVec and shVec: sh**Mbtps1** E0771 cells in NCG mice (n = 6).

j, k Expression level of H-2K^b on the cell surface after *Mbtps1* knockdown analyzed by flow cytometry.

l m Ratio of Annexin V⁺ PI⁻ and Annexin V⁺ PI⁺ in B16, KPC, MC38, and E0771 cells under coculture-induced killing treatment with CD8⁺ T cells, respectively.

3. In Figure 3, the authors demonstrated that loss of MBTPS1 in cancer cells increased tumor-infiltrating CD8⁺ T cells by analyses of scRNAseq and FACS. However, the changes in the proportion of each cellular cluster were not thoroughly analyzed. For example, the proportions of Mono/Mph/DC cells, B cells, and Tregs appeared to decrease in sh**Mbtps1**-tumors, but there were no quantitative analyses. The authors should provide more detailed analyses. Furthermore, while scRNA-seq analysis showed decreased CD4⁺ T cells and Mono/Mph/DC cells in shMBTPS1-tumors, FACS analysis showed equivalent levels compared to controls. The authors need to provide an explanation about this discrepancy. Additionally, to support their assertion, it is recommended to analyze M1 and M2 signatures in the scRNA-seq data and examine the proportion of exhausted CD8⁺ T cells by FACS. The authors should also provide data on which cells express MBTPS1 using scRNA-seq.

Response: Thank you for your valuable comments. In response to your feedback, we have further divided the CD45⁺ cells into eight cellular clusters: T

cells, NK cells, B cells, monocytes, macrophages, dendritic cells (DCs), neutrophils, and mast cells, and indicated the proportions of each subpopulation (**response Fig. 11a**; Fig. 3a in the revised manuscript). Besides, we divided myeloid cells into ten cellular clusters (**response Fig. 11b**). The results demonstrated that T cells were the most significantly altered cell population upon *Mbtps1* knockdown. Additionally, we conducted further quantitative analysis of various immune cell populations in sh*Mbtps1* tumors using flow cytometry. The results showed no significant changes in the proportions of NK cells, B cells, DCs, macrophages, M1/M2 macrophages, or neutrophils upon *Mbtps1* knockdown (**response Fig. 11c-g**; Supplementary Fig. 3h-I in the revised manuscript). Moreover, we observed a slight decrease in Treg cells (**response Fig. 11h**).

Regarding the single-cell RNA sequencing (scRNA-seq), our primary aim was to initially analyze the major impact of MBTPS1 expression on immune cell populations and to preliminarily infer the immune cell populations that play a pivotal role in MBTPS1-mediated phenotypes. Taking into account the cost considerations, we conducted single-cell sequencing using a 1 versus 1 sample comparison, following the approach of some previous studies^{5, 6}, which may have introduced inter-sample heterogeneity. To further validate the results of single-cell sequencing, after observing that T cells, particularly CD8⁺ T cells, were the most significantly altered population based on scRNA-seq data, we also conducted multicolor flow cytometry experiments using multiple samples to analyze various immune cell populations and similarly observed a significant change in CD8⁺ T cells (n=6, the experiment was independently repeated three times). Furthermore, we performed CD8⁺ T cell depletion experiments to confirm that CD8⁺ T cells are the immune cells responsible for mediating the effects of MBTPS1.

To analyze the M1 and M2 signatures in the scRNA-seq data, we scored and

classified macrophages using classical gene sets for M1 and M2 types. The results showed that *Mbtps1* knockdown did not affect the subclassification of macrophages (**response Fig. 11i**). Flow cytometry analysis of the proportions of TIM-3⁺ and PD-1⁺ cells within CD8⁺ T cells revealed that *Mbtps1* knockdown reduced the proportion of TIM-3⁺ exhausted T cells but had no significant effect on the proportion of PD-1⁺ cells (**response Fig. 11j, k**). The scRNA-seq data indicated that MBTPS1 was mainly expressed in tumor cells and fibroblast populations (**response Fig. 11l**), which is consistent with human colorectal cancer scRNA-seq data (**response Fig. 9c**).

Response Fig. 11

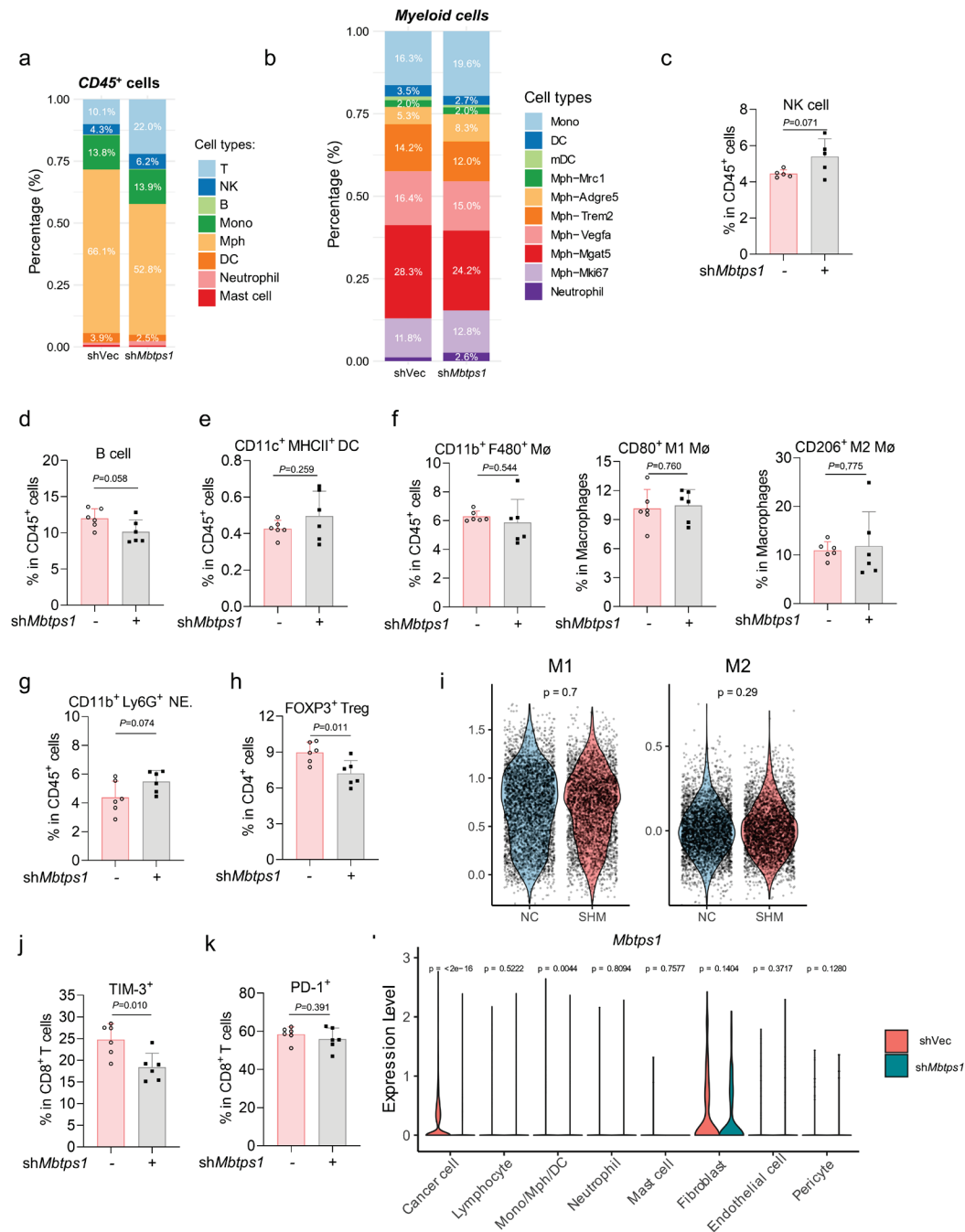


Figure legend 11.

a Bar plots indicating the proportion of major cell lineages in each group within CD45⁺ cells.

a Bar plots indicating the proportion of major cell lineages in each group within myeloid cells.

c-h Percentages of NK cells (b), DC cells (c), macrophages (d), M1-type

macrophages (e), M2-type macrophages (f), B cells (g), and Treg cells (h) in shVec and sh*Mbtps1* MC38 tumors analyzed by flow cytometry (n = 6).

i M1 and M2 module scores of macrophages calculated by AddModuleScore function of Seurat package, using the classical M1 and M2 gene lists.

j, k Percentages of TIM-3-positive and PD-1-positive cells in shVec and sh*Mbtps1* MC38 tumors analyzed by flow cytometry (n = 6).

l Expression levels of *Mbtps1* in various cell types from the scRNA-seq data.

4. In Figure 4, the authors showed that MBTPS1 suppressed anti-tumor immunity by regulating chemoattractants for recruiting CD8⁺ T cells in xenograft models. Firstly, please examine the expression levels of both MBTPS1 and MBTPS2 in those tumors. Since intracellular cholesterol metabolism may not be affected by a high cholesterol diet in some cases, to support their assertion that the tumor-suppressive phenotype observed with MBTPS1 loss is independent of SREBP-1, SREBP-2, or AFT6-a, the authors should perform subcutaneous injections using Srebf1-, Srebf2-, or Atf-alfa-overexpressing shMBTPS1 cells. Secondly, to confirm the source of Cxcl9, Cxcl10 and Cxcl11 upregulated in shMBTPS1 tumors, the authors should analyze the expression of each population in the scRNA-seq data from control and shMBTPS1 tumors, as these chemokines are typically expressed in myeloid cells. Thirdly, the T cell cytotoxicity assay showed that CD8⁺ T cells were more aggressive toward shMBTPS1 cells compared to controls, indicating increased immunogenicity in shMBTPS1 tumors. The authors should clarify the mechanism underlying this cytotoxic change. Lastly, while Cxcl9, Cxcl10, and Cxcl11 were all upregulated in shMBTPS1 tumors, only shCxcl10 cells were analyzed to elucidate the shMBTPS1 phenotype mechanism. The authors should either explain why Cxcl10 was selected or provide additional data.

Response: Thank you for providing these valuable suggestions. Firstly, we

examined the expression levels of both MBTPS1 and MBTPS2 in the tumors using Western Blotting, confirming the knockdown efficiency in the tumors (**response Fig. 10a, response Fig. 12a**).

Previously, we investigated the effects of individual knockdown of *Srebf1*, *Srebf2*, and *Atf6* on tumor growth in vivo and found that knockdown of *Srebf1* and *Atf6* did not affect tumor growth (**response Fig. 12b**; Fig. 4b in the revised manuscript). Therefore, we believe that the tumor-suppressive phenotype observed with MBTPS1 loss is not mediated through SREBP1 and ATF6. To further validate whether the tumor suppression is independent of SREBP-2, we constructed sh*Mbtps1*-OE*Srebf2* cells and found that overexpression of *Srebf2* did not affect the tumor suppressive effect caused by *Mbtps1* knockdown (**response Fig. 12c and d**; Fig. 4d in the revised manuscript).

Given that our initial screening focused on identifying tumor cell-intrinsic immune regulatory genes, we focused on the changes in the immune microenvironment resulting from alterations in tumor cell-derived factors after MBTPS1 expression was inhibited in tumor cells. We confirmed the upregulation of these cytokines in the supernatant of tumor cells after *Mbtps1* knockdown using qPCR and Elisa. Therefore, we believe that the changes in cancer cell-derived chemokines are the initial perturbations leading to alterations in the tumor microenvironment.

As shown in **response Fig. 10j and k**, knockdown of *Mbtps1* increased the expression of H-2K^b molecules on the surface of tumor cells (consistent with previous reports that STAT1 can increase MHC class I expression^{3, 4}), which may have led to an increase in the immunogenicity of the tumor cells. Additionally, the increased secretion of tumor cell-derived chemokines may have increased the opportunities for CD8⁺ T cells to interact with tumor cells, resulting in the positive outcome of the CD8⁺ T cell cytotoxicity assay.

CXCL9/10/11 share similar functions as they are common downstream target genes of STAT1 transcriptional regulation and all act as ligands for CXCR3-positive T cells, chemotaxing these cells^{7, 8, 9, 10, 11}. We used ELISA to detect the expression of CXCL9/10/11 in the supernatants of MC38 cell cultures and found that CXCL10 was the most significantly upregulated chemokine after knocking down *Mbtps1* (response Fig. 12f-h; Fig 4h in revised manuscript). Therefore, we selected *Cxcl10*, which showed the most pronounced upregulation, for the rescue experiment.

Response Fig. 12

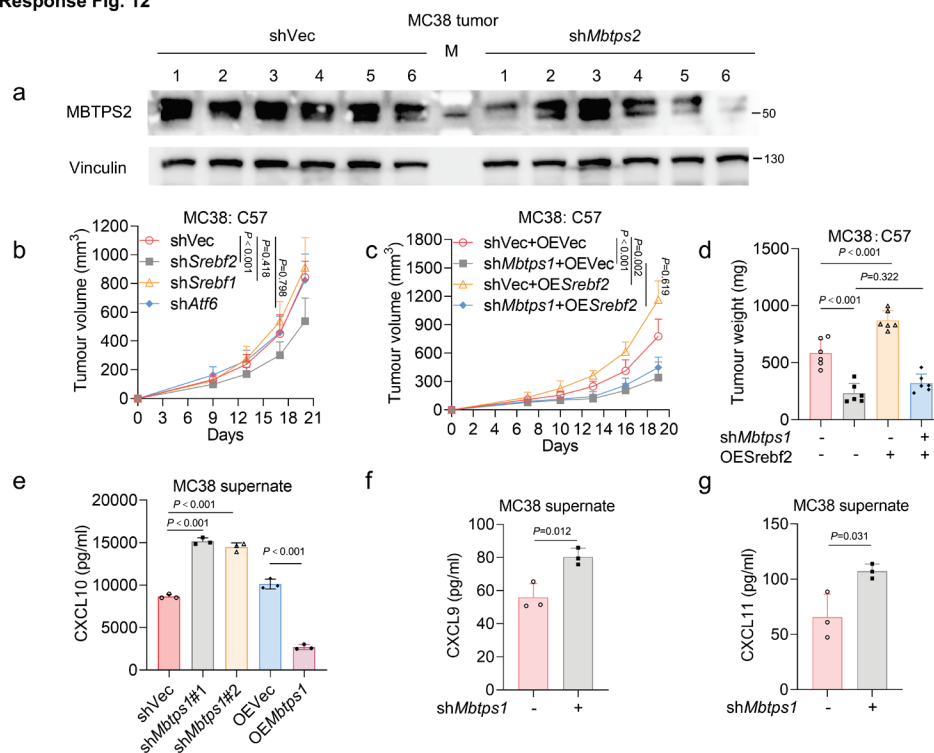


Figure legend 12.

a IB detection of MBTPS2 expression in MC38 cells with *Mbtps2* knockdown. Vinculin was included as a loading control.

b Growth curves of shVec, shSrebf1, shSrebf2 and shAtf6 MC38 tumors transplanted into C57 mice.

c Growth curves of shVec-OEvec, shMbtps1-OEvec, shVec-OESrebf2 and shMbtps1-OESrebf2 MC38 tumors transplanted into C57 mice.

d Weight analysis of shVec-OEvec, sh*Mbtps1*-OEvec, shVec-OE*Srebf2* and sh*Mbtps1*-OE*Srebf2* MC38 tumors transplanted into C57 mice.

e ELISA detection of CXCL10 secreted into the culture medium by MC38 cells with *Mbtps1* knockdown or overexpression.

f, g ELISA detection of CXCL9 (**f**) and CXCL11 (**g**) secreted into the culture medium by MC38 cells with *Mbtps1* knockdown.

5. In Figure 6, the authors showed that MBTPS1 decreased STAT1 stability by disrupting USP13 binding to STAT1 via co-IP experiment. Based on co-IP data showing that both MBTPS1 and USP13 can bind to the SH2 domain of STAT1, the authors suggested that MBTPS1 may compete with USP13 for binding to STAT1. Is there a possibility of direct interaction between USP13 and MBTPS1? To support their assertion, it is recommended to perform subcutaneous injections using shMBTPS1-shUSP13 tumor cells. Additionally, the authors should examine USP13-expressing cells in vivo.

Response: Thank you for your constructive comments. In response to your suggestion, we conducted a Co-IP assay to investigate the potential direct interaction between MBTPS1 and USP13. Our results indicated that there was no direct interaction between these two proteins (**response Fig. 13a**). Furthermore, we generated sh*Mbtps1*-sh*Usp13* cells and performed subcutaneous injections in mice. Our findings revealed that inhibition of USP13 abrogated the tumor growth suppression observed with sh*Mbtps1* (**response Fig. 13b-e**; Fig. 6m and Supplementary Fig. 6j, l in the revised manuscript). As shown in **response Fig. 13f**, the expression of USP13 was confirmed using Western Blotting. The results showed that the expression of USP13 was significantly reduced in the tumors of the knockdown group.

Response Fig. 13

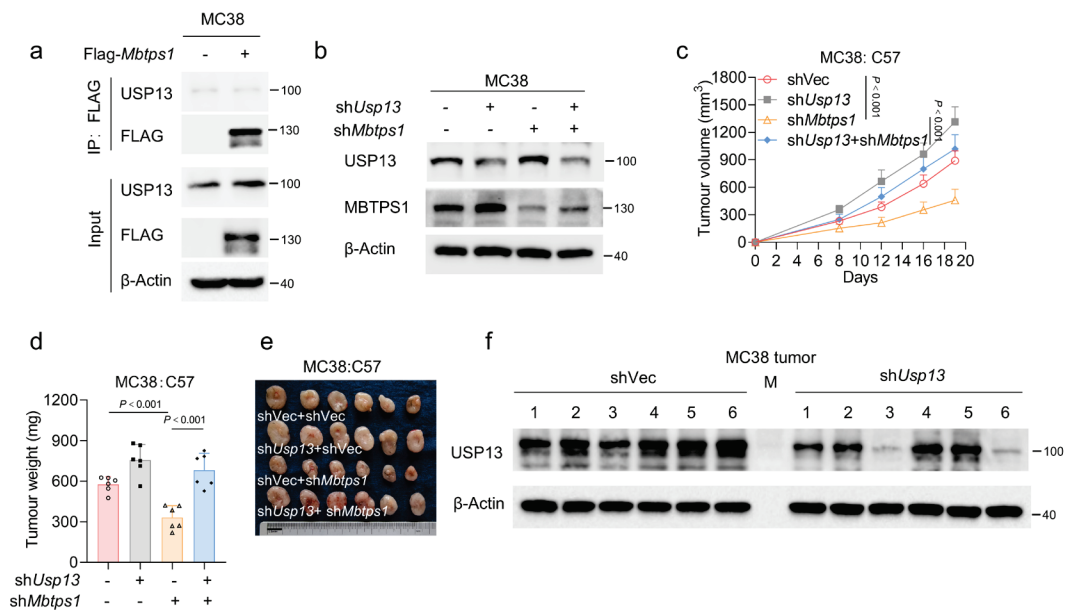


Figure legend 13.

a Co-IP analysis of the interaction between MBTPS1 and USP13 in MC38 cells.

b IB detection of STAT1 expression in MC38 cells after knockdown of *Mbtps1* alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*, followed by treatment with CHX (5 μ g/ml). β -Actin was included as a loading control.

c Growth curves of MC38 tumors with knockdown of *Mbtps1* alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*.

d, e Weight analysis (**d**) and representative image (**e**) of MC38 tumors with knockdown of *Mbtps1* alone, knockdown of *Usp13* alone, and simultaneous knockdown of both *Mbtps1* and *Usp13*.

f IB detection of USP13 expression in MC38 cells after knockdown of *Usp13*. β -Actin was included as a loading control.

6. In Figure 7, the authors investigated the impact of MBTPS1 loss in tumor development using the AOM/DSS model in Villin-creERT2; *Mbtps1*^{f/f} mice and identified MBTPS1 as a marker of poor prognosis in TCGA and SYSUCC cohort datasets. However, AOM/DSS model is a widely used representative murine

model of colitis-associated colorectal cancer. Given that tumorigenesis via inducing mutation in *Apc* is a more common CRC model, the authors should consider performing a conditional intestinal-specific knockout of *Mbtps1* under *Apc* loss.

Response: Thanks for your suggestions. In addition to utilizing the AOM/DSS model to investigate the impact of MBTPS1 loss on tumor development, we have also considered employing the *Apc*^{Min/+} model based on previous CRC studies^{12, 13}. Consequently, we generated WT-*Apc*^{Min/+} and *Mbtps1*^{iKO}-*Apc*^{Min/+} mice by crossing *Mbtps1*^{iKO} mice with *Apc*^{Min/+} mice (**response Fig. 14a**). Upon reaching the tumorigenic endpoint at 17 weeks of age, we dissected the intestines of these mice and counted the adenomas. Our findings revealed that specific knockout of *Mbtps1* in intestinal epithelia suppressed adenoma formation in the *Apc*^{Min/+} background (**response Fig. 14b, c**; Fig. 7I in the revised manuscript). These results align with those obtained from other mouse models we previously employed.

Response Fig. 14

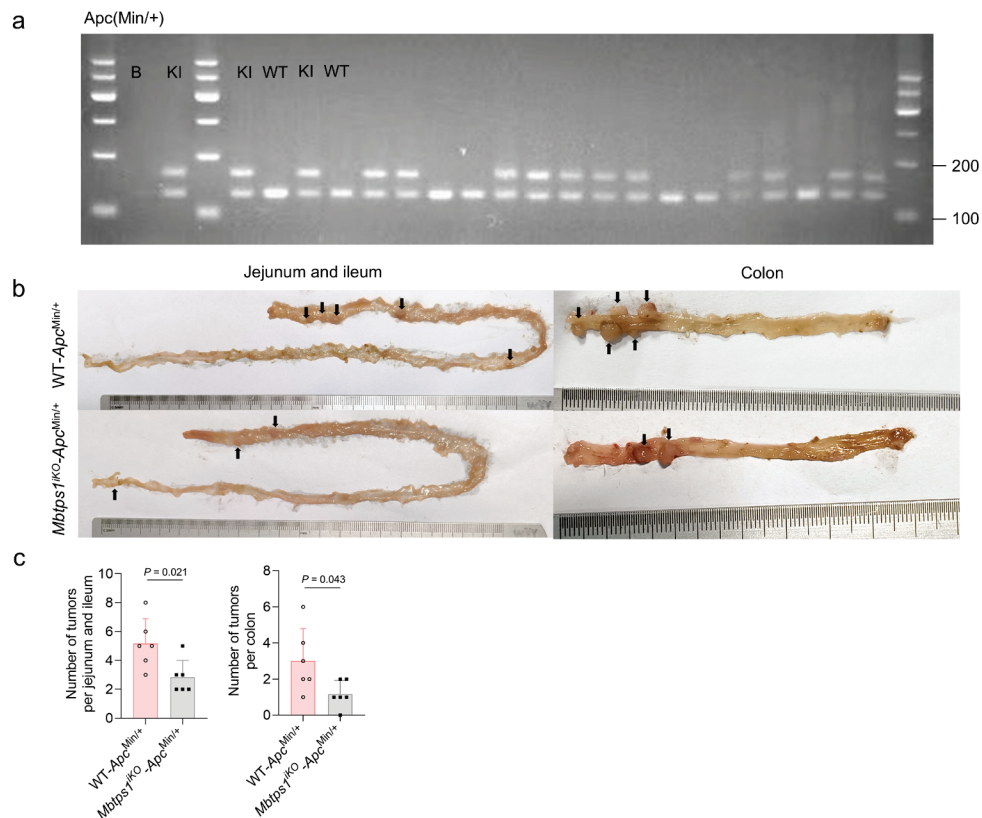


Figure legend 14.

a Representative result image of PCR genotyping for *Apc*^{Min/+} mice using mouse tail DNA.

b Representative images of the intestinal spontaneous tumors in WT-*Apc*^{Min/+} and *Mbtps1*^{iKO}-*Apc*^{Min/+} mice.

c Statistical analyses of the number of the intestinal spontaneous tumors in WT-*Apc*^{Min/+} and *Mbtps1*^{iKO}-*Apc*^{Min/+} mice.

7. The authors asserted that MBTPS1 inhibition converted immune-cold tumors into inflamed tumors (lines 542-544). However, since MC38, the most used cancer cell line in the manuscript, is widely known to develop MSI-high-like tumors with abundant TILs, the presented data do not support this assertion. Additionally, the authors demonstrated that MBTPS1 loss increased CXCL10 expression via increased STAT1 protein levels. Since the activation of STAT1 is commonly regulated by IFN γ , the concluded mechanism of tumor

suppression by MBTPS1 loss would require IFN γ signaling. Given that IFN γ is primarily produced by T cells, sufficient tumor-infiltrating T cells would be necessary for this mechanism to work. Additionally, given that MBTPS1 is downregulated in immunocompetent tumors compared to immunodeficient tumors (Figure 1), the existence of TIL (especially activated TIL) would downregulate MBTPS1 to enhance T cell-mediated anti-tumor immunity. The authors need to clarify or discuss these regulatory mechanisms.

Response: We apologize for not discussing this issue appropriately and causing confusion for you. Therefore, we have updated the expression with more accuracy: “The loss of MBTPS1 increases the infiltration of CD8⁺ T cells, thereby enhancing anti-tumor immunity and the efficacy of immunotherapy” (line 583-585). Furthermore, we have previously used the CT26 cell line to validate the tumor growth inhibition caused by *Mbtps1* knockdown in BALB/c mice (**response Fig. 15a**; Fig. 2b in the revised manuscript). This was accompanied by an increase in the number and proportion of CD8⁺ T cells (**response Fig. 15b, c**; Fig. 3e in the revised manuscript) and an increase in the proportion of IFN- γ ⁺ CD8⁺ T cells (**response Fig. 15d**; Fig. 3g in the revised manuscript). Additionally, we also detected an increase in STAT1 protein levels following *Mbtps1* knockdown (**response Fig. 15e**) and an increase in the proportion and number of CXCR3⁺ CD8⁺ T cells in the tumor microenvironment using CT26 cell line (**response Fig. 15f, g**; Fig. 4l and Supplementary Fig. 4l in the revised manuscript).

Additionally, we acknowledge that the mechanism by which MBTPS1 loss leads to tumor suppression via increased CXCL10 expression indeed involves the regulation of STAT1 activation, which is commonly modulated by IFN- γ ^{8, 14}. Our data suggest that MBTPS1 loss results in an increase in immune infiltration, particularly of CD8⁺ T cells, within the tumor microenvironment. This increase in immune cell infiltration likely leads to a heightened production of IFN- γ ,

primarily by the infiltrating T cells. The elevated levels of IFN- γ can then positively feedback to activate the STAT signaling pathway, creating a cascade amplification effect. This, in turn, further enhances the antitumor immune response and the efficacy of immunotherapy. In essence, MBTPS1 loss triggers a complex interplay between immune infiltration, IFN- γ production, and STAT signaling, ultimately culminating in the suppression of tumor growth.

In response to your question regarding the downregulation of MBTPS1 in immunocompetent tumors compared to immunodeficient tumors, we would like to provide the following explanation.

Firstly, CRISPR screening is a powerful gene editing technique that utilizes CRISPR-Cas9 complexes with specific sgRNAs (single-guide RNAs) to precisely cut target gene DNA sequences, enabling gene knockout or mutation¹⁵. In our study, we constructed a CRISPR library encompassing a large number of membrane and secreted protein genes and introduced these sgRNAs into immunocompetent and immunodeficient tumor models. By comparing the enrichment of sgRNAs under different immune response states we screened for genes that significantly impact tumor immune responses.

Regarding MBTPS1, our study did not directly observe its expression differences between the two tumor models. Instead, through CRISPR screening, we found that sgRNAs targeting the *Mbtps1* gene were negatively enriched in the immunocompetent mouse model, suggesting that the knockout of this gene may enhance T-cell-mediated antitumor immunity in immunocompetent conditions.

Response Fig. 15

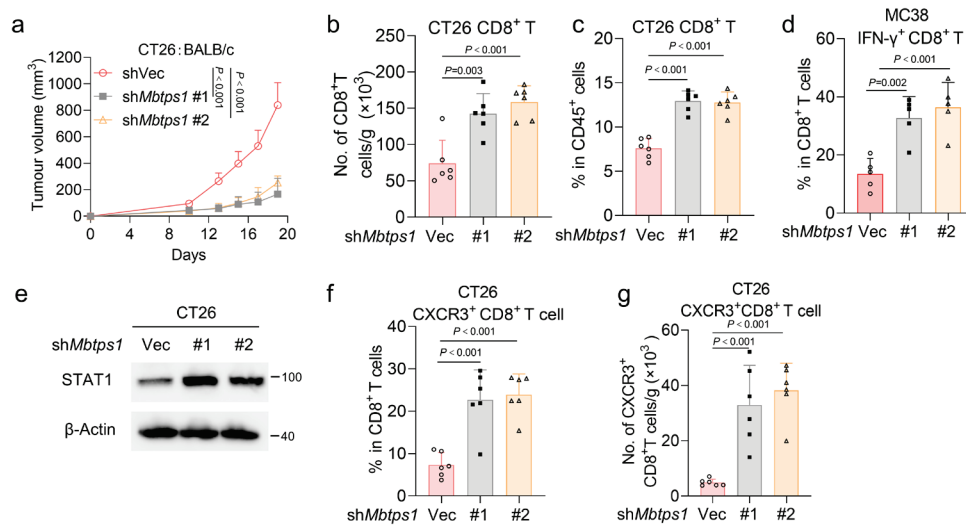


Figure legend 15.

a Growth curves of shVec or shMbtps1 CT26 tumors transplanted into C57 mice.

b, c The number (**b**) and percentage (**c**) of CD8⁺ T cells in shVec or shMbtps1 CT26 tumors analyzed by flow cytometry.

d The percentage of IFN-γ⁺ CD8⁺ T cells in shVec or shMbtps1 CT26 tumors analyzed by flow cytometry (n = 5 or 6).

e IB detection of STAT1 in CT26 cells with Mbtps1 knockdown.

f, g The percentage (**f**) and number (**g**) of CXCR3⁺ cells in CD8⁺ T cells in CT26 tumors with Mbtps1-knockdown analyzed by flow cytometry (n = 6).

Minor comments

1. Please label which side represents shMbtps1 and the control in the graphs presented in Figure 4d and Supplementary Figure 4b, 5d and e.

Response: We apologize for overlooking this detail. We have addressed the issue by labeling the groups used for comparing in the corresponding GSEA analysis figures, including Fig. 4d (**response Fig. 16a**; Fig. 4e in the revised manuscript) and Supplementary Fig. 4b, 5d, and 5e (**response Fig. 16b-d**; Supplementary Fig. 5e).

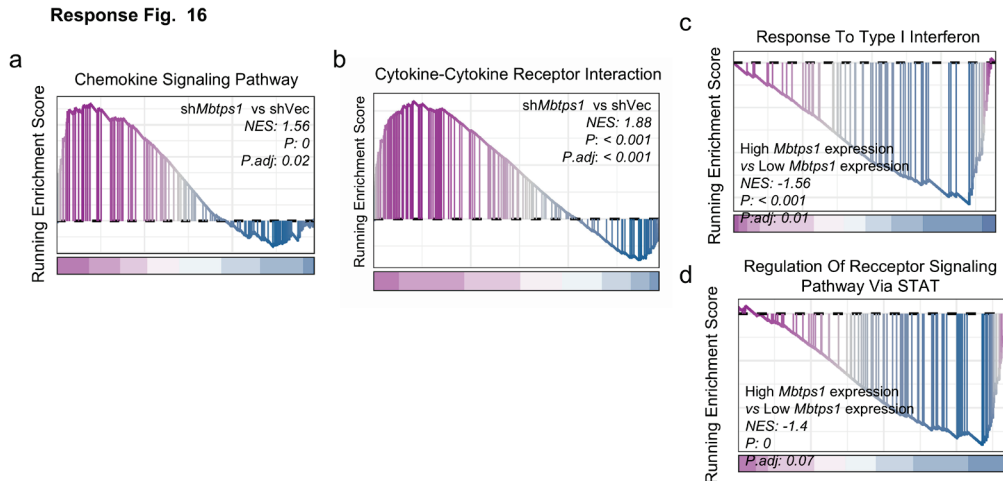


Figure legend 16.

a GSEA enrichment plot for the chemokine signaling pathway comparing shMbtps1 and shVec MC38 tumors.

b GSEA enrichment plot of the cytokine-cytokine receptor interaction signaling pathway in MC38 tumors, indicating significant enrichment in shMbtps1 tumors.

c, d GSEA of the response to type I interferon pathway (**c**) and the regulation of receptor signaling pathway via STAT pathway (**d**), utilizing the TCGA-COAD dataset, demonstrating a comparison between high and low expression levels of Mbtps1.

2. It is better to analyze the number of activated CD8⁺ T cells in Figure 5p.

Response: We thank you for providing this valuable suggestion. We have analyzed the number of activated CD8⁺ T cells in Fig. 5p by flow cytometry, examining the proportions of IFN-γ and GZMB positive CD8⁺ T cells in tumors from the indicated groups in Figure 5p. Our findings indicated that knockdown of *Stat1* reversed the increase in CD8⁺ T cell numbers caused by *Mbtps1* knockdown (**response Fig. 17a**; Supplementary Fig. 5l in the revised manuscript), as well as the elevated proportions and numbers of IFN-γ and GZMB expressing CD8⁺ T cells (**response Fig. 17b-e**; Fig. 5q and Supplementary Fig. 5m, n in the revised manuscript).

Response Fig. 17

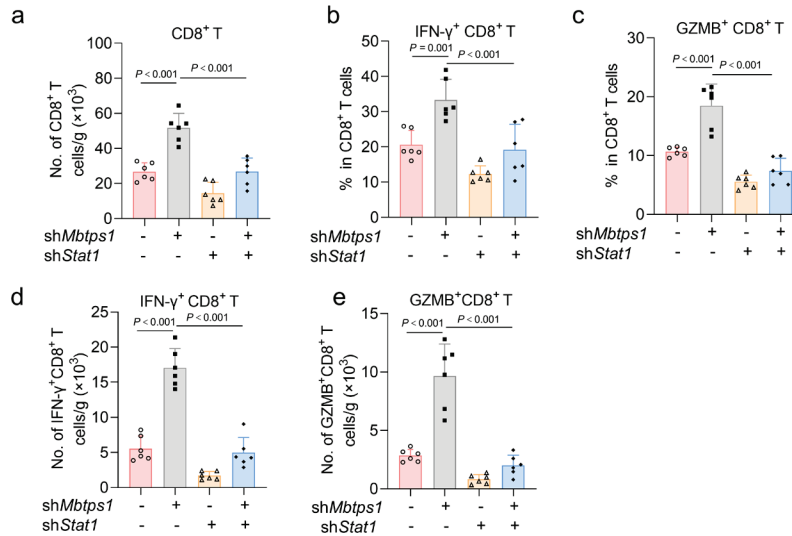


Figure legend 17.

a Flow cytometric analysis of the numbers of CD8⁺ T cells in mice from indicated groups.

b, c Flow cytometric analysis of the percentages of IFN- γ ⁺ CD8⁺ T cells (**b**) and GZMB⁺ CD8⁺ T cells (**c**) in mice from indicated groups.

d, e Flow cytometric analysis of the numbers of IFN- γ ⁺ CD8⁺ T cells (**d**) and GZMB⁺ CD8⁺ T cells (**e**) in mice from indicated groups.

3. It is also recommended to analyze the expression of phospho-STAT1 in murine CRC model and human CRC samples in Figure7.

Response: Thank you for providing this valuable suggestion. We conducted IHC staining of phospho-STAT1 using the AOM/DSS-induced CRC model and patient samples from the SYSUCC CRC cohort. The results demonstrated that intestinal epithelium-specific knockout of *Mbtps1* upregulated the expression of phospho-STAT1 in AOM/DSS-induced intestinal adenomas (**response Fig. 18a, b**; Fig. 7c in the revised manuscript). Additionally, in the SYSUCC cohort, we found that the expression of MBTPS1 was negatively correlated with the expression of phospho-STAT1 (**response Fig. 18c-e**; Fig. 7o and

Supplementary Fig. 7I in the revised manuscript).

Response Fig. 18

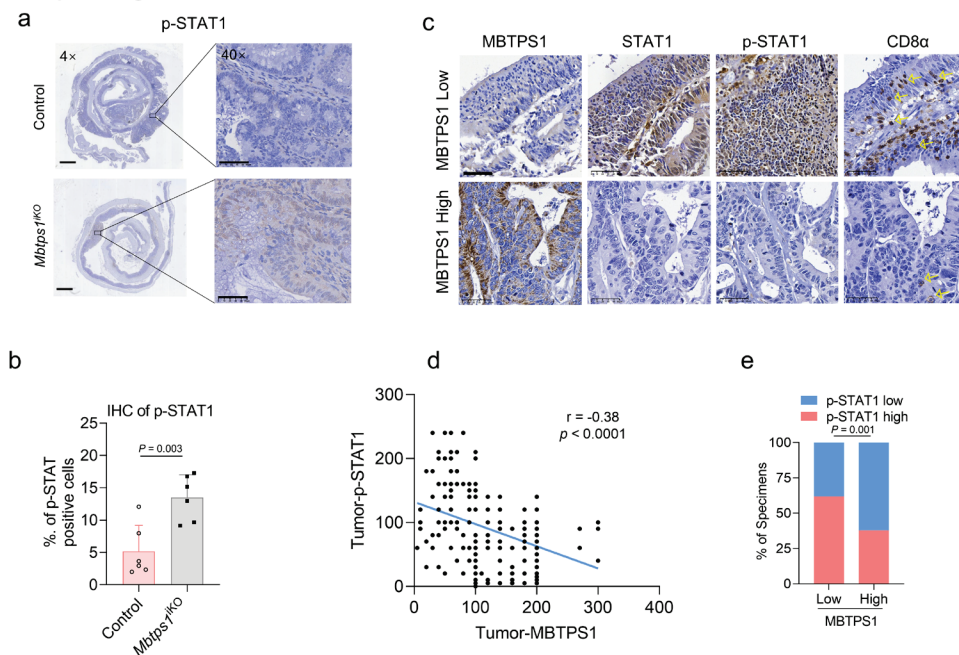


Figure legend 18.

- a** Representative IHC images of p-STAT1 staining in intestinal spontaneous tumor tissues of control and *Mbtps1*^{ikO} mice. Scale bar: 1 mm (4×); 50 μm (40×).
- b** Percentages of p-STAT-positive cells in control and *Mbtps1*^{ikO} MC38 tumors of AOM/DSS-induced CRC mice analyzed by IHC staining (n = 6).
- c** Representative IHC staining of the indicated markers in CRC tumor tissues from patients with high or low MBTPS1 levels. Scale bar: 50 μm.
- d** Correlation between tumoral p-STAT1 and MBTPS1 in the colorectal cancer cohort of SYSUCC.
- e** Percentage of tumor tissues exhibiting high or low expression of p-STAT1 presented for the group with low or high MBTPS1 expression.

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Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you very much for co-reviewing our manuscript. We will carefully consider all the comments and suggestions provided and will make necessary revisions to enhance the quality of our work.

1 **Manuscript ID: NCOMMS-24-39566B**

2 **Title:** Inhibition of MBTPS1 enhances antitumor immunity and potentiates anti-
3 PD-1 immunotherapy

4 **Detailed point-by-point responses to the reviewers' comments:**

5
6 **Reviewer #1 (Remarks to the Author):**

7 The authors have addressed previous comments.

8 **Response:**

9 Thank you for acknowledging our efforts in addressing your earlier
10 feedback. Your insights have been truly helpful.

11
12 **Reviewer #2 (Remarks to the Author):**

13 Thanks for the revision and I have no more questions.

14 **Response:**

15 Thank you for your confirmation of our revisions and for finding them
16 satisfactory. We deeply appreciate the constructive comments and feedback
17 you provided throughout the process, as they have been invaluable to us.

18
19 **Reviewer #3 (Remarks to the Author):**

20 Reviewer's comments

21 In the revised manuscript, the authors performed substantial amounts of
22 experiments to address the concerns raised by this reviewer. However, there
23 are still several points that need to be addressed. Details are highlighted below.

24 **Response:**

25 Thank you for acknowledging the efforts we have made in our previous
26 revision. We greatly value your thorough review and the constructive feedback
27 you have provided. We are deeply grateful for your insightful comments and
28 have taken careful steps to address the points you raised in our newly revised
29 manuscript. We sincerely hope that our response can address your concerns.

Major comments

1. (Related to previous comments 1 and 3) The response data indicated that the primary sources of MBTPS1 are tumor cells and fibroblasts. The authors demonstrated a negative correlation between MBTPS1 expression and tumor-infiltrating T cells. Since immune-cold tumors are often enriched with abundant stroma, like in example pancreatic cancers (including KPC tumors), it is possible that MBTPS1-high tumors may have enriched stroma including a lot of fibroblasts. Given this, MBTPS1 in fibroblasts might play a role in shaping the immune activity within these tumors. The authors should provide additional data, or at the very least, discuss about this in the manuscript.

Response:

We deeply appreciate your insightful comments and acknowledge the significance of investigating the potential role of MBTPS1 expression in fibroblasts within the tumor microenvironment. In response to your suggestion, we have incorporated a new section in the manuscript to discuss the potential impact of MBTPS1 expression in fibroblasts on immune cells within tumors (lines 700-713 in revised manuscript).

Specifically, fibroblasts, which are one of the components of the tumor stroma, can transform into cancer-associated fibroblasts (CAFs) when influenced by the tumor microenvironment (TME)¹. These CAFs affect tumor progression, anti-tumor immunity, and therapeutic resistance². CAFs play a pivotal role in reshaping the immune microenvironment through interactions with other cells within the TME and the secretion of various cytokines³. Furthermore, CAFs can be classified into multiple subtypes based on the differential expression of surface markers⁴. Different subtypes may exhibit diverse effects on immune microenvironment remodeling⁵. CAFs have been shown to exert immunoregulatory effects by secreting immunosuppressive factors, pro-inflammatory cytokines, chemokines, and by remodeling the extracellular matrix⁶. CAFs produce immunosuppressive factors such as CCL2, IL-6, and TGF- β to suppress anti-tumor immunity^{7, 8, 9}. On the other hand,

studies have also found that CD248 expression can inhibit the expression and secretion of CXCL9/10 in CAFs, thereby suppressing CD8⁺ T cell infiltration¹⁰. Based on this, we hypothesize that MBTPS1 in certain populations of fibroblasts may exert immunosuppressive effects by inhibiting the STAT1-CXCL10 signaling axis, which is similar to its role in tumor cells. However, given the numerous differences in the biological characteristics between tumor cells and fibroblasts, MBTPS1 may have distinct functions and roles in fibroblasts, which require further exploration. Future studies are needed to explore the function of MBTPS1 in fibroblasts (for example, by generating mice with conditional *Mbtps1* knockout in fibroblasts, etc.). We eagerly anticipate that future research will continue to unravel the complex interactions within the tumor microenvironment and how MBTPS1 might contribute to these processes. Your suggestions have been highly enlightening for our further research, and we are grateful for your guidance.

2. (Related to previous comment 2) The response Fig. 10j demonstrated that knockdown of *Mbtps1* increased the expression of MHC class I in tumor cells. This suggests the possibility that the vulnerability of tumor cells for the cytotoxicity of T cells is enhanced by *Mbtps1* loss. This finding is important, as it provides a potential explanation for the decreased abundance of *Mbtps1*-targeted cells in immunocompetent tumors during the first CRISPR-screening. Does the underlying mechanism for the increased expression of MHC class I involve an increase in STAT1 levels? To confirm this, the authors are recommended to evaluate MHC class I expression also in sh*Mbtps1*-shStat1 tumors as well.

Response:

Thank you for your valuable suggestion. To verify whether the increase in MHC class I molecules induced by *Mbtps1* knockdown is correlated with the elevated expression of STAT1, we generated MC38 cells with double knockdown of *Mbtps1* and *Stat1* and examined the expression of MHC class I

molecules by flow cytometry. We found that knockdown of *Stat1* reversed the increased expression of H-2K^b caused by *Mbtps1* knockdown (response Fig. 19a and b; Supplementary Fig. 6n in revised manuscript). This finding is consistent with previous studies reporting that MHC class I molecules are one of the downstream targets of STAT1^{11, 12} and provided an explanation for the increased MHC class I expression after *Mbtps1* knockdown.

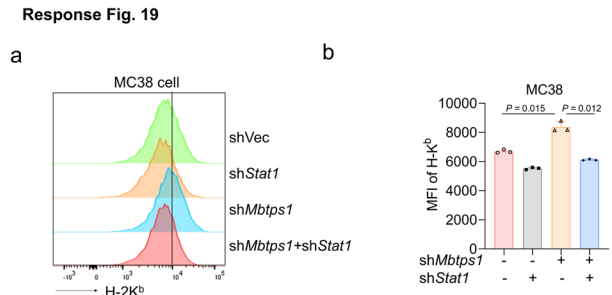


Figure legend 19.

a, b Representative image (a) and mean fluorescence intensity (MFI) (b) of H-2K^b on the cell surface after *Mbtps1* and *Stat1* knockdown analyzed by flow cytometry. Data are presented as means $\pm$ SDs. n = 3 biologically independent samples per group, representative of at least two independent experiments. *P* values were determined by one-way ANOVA.

3. (Related to previous comment 4) It appears that the authors have not addressed the previous comment from this reviewer: "To confirm the source of Cxcl9, Cxcl10, and Cxcl11 upregulated in MBTPS1 KD tumors, the authors should analyze the expression of each population in the scRNA-seq data from control and shMBTPS1 tumors." The authors should provide a clear response, as the effect of MBTPS1 loss on other cell populations is crucial for elucidating the mechanism underlying the shMBTPS1 phenotype.

Response:

Thank you for bringing this oversight to our attention. Your suggestion has been very helpful in improving the clarity of the logic of our article. We apologize for the oversight and have addressed it as follows.

We analyzed the expression of *Cxcl9*, *Cxcl10*, and *Cxcl11* across various cell populations in the single-cell RNA sequencing (scRNA-seq) results of *shMbtps1* and control tumors (**response Fig. 20a**; Supplementary Fig. 5e in revised manuscript). Notably, in tumor cells, we observed a remarkable increase in *Cxcl10* expression (**response Fig. 20a, upper**), which is consistent with the results of the *in vitro* ELISA (**response Fig. b**; Fig. 5g and h in revised manuscript) and Luminex assay (Supplementary Fig. 5g and h in revised manuscript), showing a significant elevation of CXCL10 in the supernatants of tumor cells after *Mbtps1* knockdown. In addition, the bulk RNA-seq data of *shMbtps1* and control MC38 cells *in vitro* also revealed an increase in *Cxcl10* level after *Mbtps1* knockdown (**response Fig. 20c**; Supplementary Fig. 5f in revised manuscript).

Regarding *Cxcl9*, no significant difference was observed in tumor cells analyzed by scRNA-seq data, potentially because the expression of *Cxcl9* in most tumor cells were at very low levels (**response Fig. 20a, lower**). In most scRNA-seq studies, due to the limitations of sequencing depth and detection efficiency, only a small fraction of transcripts in each cell is sequenced, resulting in a very small number of reads allocated per cell and low efficiency¹³. This leads to increased randomness in the measurement and reduced statistical efficiency for lowly expressed genes¹³. In addition, *Cxcl11* has not been detected by either single-cell or bulk RNA-seq due to factors such as the detection efficiency of sequencing and low gene expression levels. However, we have previously conducted Elisa assays and detected an elevation of CXCL9 and CXCL11 expression in the supernatants of tumor cell cultures *in vitro* following knockdown of *Mbtps1* (**response Fig. 20d, e**; Supplementary Fig. 5k in revised manuscript).

As reported, CXCL9 and CXCL10 can be produced by several cell types including tumor cells and antigen-presenting cells (dendritic cells or macrophages)¹⁴. Additionally, upregulation of CXCL10 was observed not only in tumor cells but also in some other cell types such as macrophages and

neutrophils analyzed by scRNA-seq data. We speculate that the elevated CXCL10 levels in these cells may be part of an IFN- γ -induced positive feedback mechanism. More specifically, the inhibition of *Mbtps1* in tumor cells triggers the recruitment of CD8⁺ T cells, which secrete IFN- γ ¹⁵. The IFN γ functions through autocrine and paracrine mechanisms to activate the IFN γ -STAT1 signaling pathway in various cellular constituents within the TME^{16, 17}, which in turn increases the production of chemokines including CXCL9 and CXCL10 by these cells, forming a positive feedback loop that amplifies immune activation and enhances antitumor responses. To further support this, we note the results from rescue animal experiments involving double knockdown of *Mbtps1/Cxcl10* in tumor cells, where the tumor growth inhibition observed upon *Mbtps1* knockdown was reversed. This underscores the crucial role played by the intrinsic secretion of CXCL10 by tumor cells in initiating and driving the alterations within the immune microenvironment. While the elevation of CXCL10 in other cell types may be secondary to the increase in tumor cells, it nevertheless contributes to the enhanced antitumor immunity following *Mbtps1* knockdown, as demonstrated in studies highlighting the importance of macrophage-derived CXCL9 and CXCL10 for antitumor immune responses¹⁸. Thus, the increased CXCL10 expression across other cell types may reflect comprehensive immune activation in the immune microenvironment. The discussion in this section has been integrated into **lines 668-673** of the revised manuscript.

What's more, by integrating the bulk RNA-seq data from tumor cells *in vitro*, Elisa assay and Luminex assay of tumor cell culture supernatants, as well as the results from rescue animal experiments involving double knockdown of *Mbtps1/Stat1* and *Mbtps1/Cxcl10* in tumor cells, we have determined that the initial disturbance resulting in enhanced anti-tumor immunity is the activation of the STAT1 signaling pathway and subsequent increased chemokine expression in tumor cells. In light of your suggestion and taking into account the ELISA results of cell supernatants *in vitro*, which confirmed CXCL10 as the most

significantly upregulated chemokines associated with CD8⁺ T cell infiltration following *Mbtps1* knockdown, we have emphasized the importance of CXCL10 in the revised manuscript (with the relevant modifications highlighted).

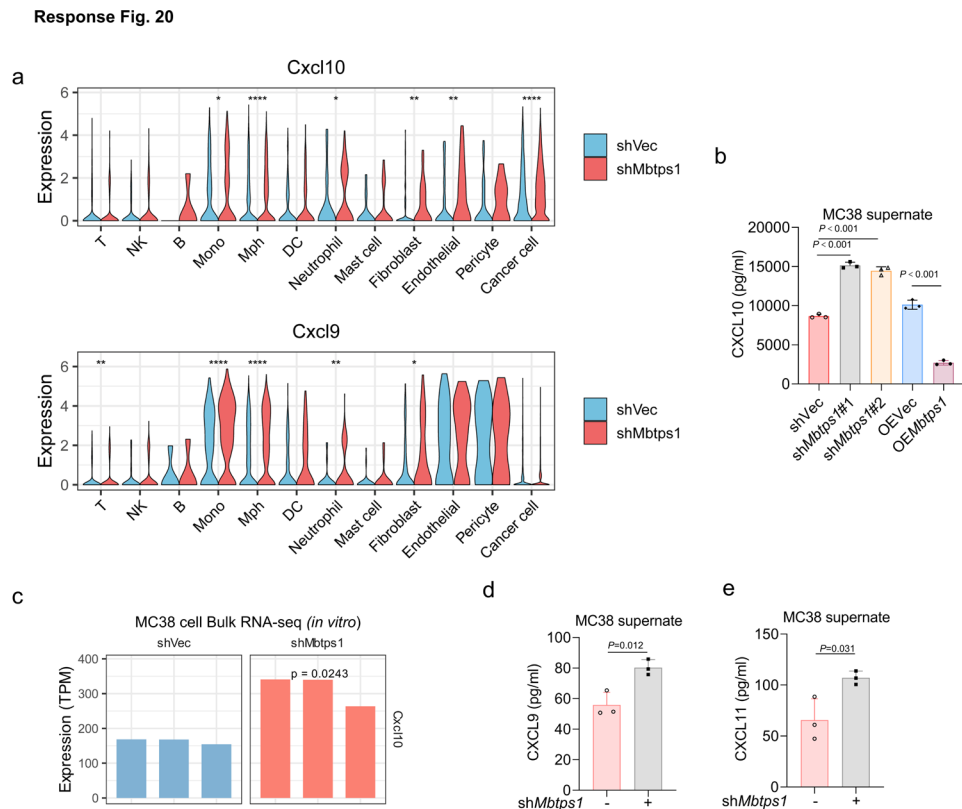


Figure legend 20.

a Violin plots showing the *Cxcl10* mRNA expression in various cell types analyzed using bulk scRNA-seq data.

b ELISA detection of CXCL10 secreted into the culture medium by MC38 cells with *Mbtps1* knockdown or overexpression.

c Bar graph showing the *Cxcl10* mRNA expression in MC38 cells analyzed using bulk RNA-seq data.

d, e ELISA detection of CXCL9 (**d**) and CXCL11 (**e**) secreted into the culture medium by MC38 cells with *Mbtps1* knockdown.

Data are presented as means $\pm$ SDs. $n = 3$ biologically independent samples per group, representative of at least two independent experiments in (b, d and e). P values were determined by two-tailed unpaired Student's t test.

4. Please include data shown in response Figs. 9c and 10a-k and 11i, l and 13a to the main manuscript (not just in response figures).

Response:

Thank you for your insightful advice. Incorporating these data into the main manuscript has significantly enhanced the completeness of the study. We have now supplemented these data and the revised portions of the manuscript have been highlighted.

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251 dependent effects on glioma blood vessels and tumor cells. *Clin Cancer*

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253
254 **Reviewer #4 (Remarks to the Author):**

255 I co-reviewed this manuscript with one of the reviewers who provided the listed
256 reports. This is part of the Nature Communications initiative to facilitate training
257 in peer review and to provide appropriate recognition for Early Career
258 Researchers who co-review manuscripts.

259 **Response:**

260 Thank you very much for co-reviewing our manuscript. We will carefully
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262 enhance the quality of our work.