Targeting metabotropic glutamate receptor 4 for cancer immunotherapy

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In this study, we report a novel role of metabotropic glutamate receptor 4 (GRM4) in suppressing antitumor immunity. We revealed in three murine syngeneic tumor models (B16, MC38, and 3LL) that either genetic knockout (Grm4−/−) or pharmacological inhibition led to significant delay in tumor growth. Mechanistically, perturbation of GRM4 resulted in a strong antitumor immunity by promoting natural killer (NK), CD4+ T cells into interleukin-17 (IL-17)–producing T helper 17 (TH17) cells, which leads to the EAE progression (5). Mice lacking Grm4 were shown to be highly susceptible to experimental autoimmune encephalomyelitis (EAE; a mouse model of multiple sclerosis). It appears that knockout (KO) of Grm4 in dendritic cells (DCs) drives the activation and polarization of naïve CD4+ T cells into interleukin-17 (IL-17)–producing T helper 17 (T17) cells, which leads to the EAE progression (5).

The studies on the roles of GRM4 in cancers are very limited and controversial. While the expression of GRM4 is associated with poor prognosis in several types of cancers (malignant gliomas, colorectal cancer, and rhabdomyosarcoma) (7) and drug resistance in colon cancer (8), GRM4 agonists are shown to inhibit the proliferation of cultured cancer cells of non-CNS origin including bladder cancer cells (9) and breast cancer cells (10). A role of GRM4 in myeloid cells in regulating tumorigenesis was recently reported by Kansara et al. (6): Genetic KO of Grm4 promoted the initiation and progression of irradiation-induced osteosarcoma through increased production of IL-23.

We report herewith that either genetic KO (Grm4−/−) or pharmacological inhibition of GRM4 led to significant delay of tumor growth in male mice in three syngeneic tumor models (B16, MC38, and 3LL) through activating antitumor immunity. To unravel the complex Grm4 depletion–mediated changes in the immune system, changes in the phenotypic landscape of tumor-infiltrating immune populations were characterized by flow cytometry, followed by single-cell RNA sequencing (scRNA-seq) and single-cell T-cell receptor (scTCR) sequencing profiling. The effect of Grm4 deficiency on naïve immune cells and the underlying mechanism were also studied. We further explored the therapeutic potential of GRM4.
inhibitor, alone or in combination with anti–PD-1 or anti–CTLA-4 antibody. Our study suggests that GRM4 is a negative regulator of antitumor immunity and targeting of GRM4 may represent a novel strategy to improve cancer immunotherapy.

RESULTS
Grm4−/− mice have significantly delayed tumor growth and skewed TME toward an immunogenic phenotype

To investigate whether GRM4 has a tumor-suppressive or promoting effect, we performed a series of experiments using three syngeneic tumor models. We first investigated the growth of B16 melanoma cells subcutaneously inoculated into Grm4−/− mice and their wild-type (WT) littermates in a C57BL/6 background (Fig. 1A). No significant difference was observed in tumor growth in the female littermates (Fig. S1). However, in comparison to their Grm4+/+ littermates, the male Grm4−/− mice demonstrated markedly delayed tumor growth (Fig. 1B), which was also correlated with a significantly prolonged survival (Fig. 1C). Similar results were observed in murine colon adenocarcinoma (MC38) and Lewis lung carcinoma (3LL) models (Fig. 1, D to F). Collectively, our observations indicated that global loss of Grm4 markedly inhibited the tumor growth in male mice in multiple murine tumor models. All subsequent studies were performed in male mice.

To explore the underlying mechanism for the Grm4−/−-mediated antitumor response, we performed RNA-seq on B16 tumor tissues harvested from either the Grm4+/+ or their WT littermates (Fig. 1G). This analysis identified 290 significantly up-regulated and 260 significantly down-regulated genes in tumors from Grm4−/− mice (>1.3-fold, P < 0.05) (Fig. 1H). Gene set enrichment analysis (GSEA) revealed significant down-regulation of several signaling pathways that promote tumor growth, including tricarboxylic acid (TCA) cycle, ribosome, oxidative phosphorylation, Myc, butanoate metabolism, glycolysis, proteasome, and spliceosome signaling pathways (Fig. 1I and table S1), which may partially explain our observed phenotype in tumor growth. Nonetheless, the most notable changes in Grm4−/− mice were related to the immune pathways. Ten of the top 15 up-regulated pathways revealed in RNA-seq were immune-related, suggesting that loss of Grm4 plays important roles in modulating the tumor immune microenvironment. Among these pathways, the most significantly enriched ones are related to inflammatory response, adaptive immune response, interferon-γ (IFN-γ) response, natural killer (NK) cell–mediated cytotoxicity, TCR signaling, T cell activation, IFN-α response, myeloid cell activation, Janus kinase signal transducers and activators of transcription (JAK STAT) signaling, Toll-like receptor signaling, and innate immune response (Fig. 2 and table S1). In addition, we observed significant up-regulation in antigen presentation and processing, major histocompatibility complex I (MHC I) protein binding, and MHC II biosynthetic process, along with down-regulation of tryptophan metabolism. Further analysis of the above data clearly suggests a more active tumor immune microenvironment in Grm4−/− mice as evident from increased transcriptional expression of various genes involved in antitumor activity including (i) TSH1 polarizing cytokine IL-12b; (ii) T cell and NK cell cytokine and cytokine genes Prf1 and Ifng; (iii) T cell costimulatory molecules Tnfsf10 and Tnfsf14; (iv) T cell activation transcription factors Stat1, Irf3, Irf4, and Irf9; and (v) proinflammatory cytokines and cytokine receptors Ccl3, Ccl22, Il1b, Il23a, and Il18r1 (Fig. 1I). These results suggest that loss of Grm4 leads to enhanced immune responses in TME, which likely plays an important role in controlling the tumor growth.

Grm4 is expressed in tumor-infiltrating immune cells

GRM4 has been shown to be expressed in naïve DCs and CD4+ T cells isolated from mouse splenocytes (5). Moreover, its expression was identified in the tumor-infiltrating DCs (CD45+ MHC II, and CD11c) in a mouse model of irradiation-induced osteosarcoma. However, only few CD4+ T cells were detectable to characterize GRM4 expression in this bone cancer model (6). These results demonstrated that the expression of GRM4 could be context dependent in the population of immune cells. To date, few studies regarding the expression profile and functionality of GRM4 in immune cells in the context of cancer have been reported.

To determine the expression pattern of GRM4 in human cancer TME, we analyzed both the single-cell assay for transposase-accessible chromatin with high-throughput sequencing (scATAC-seq) and scRNA-seq data. By analyzing the chromatin accessibility profile in GRM4 promoter regions in basal cell carcinoma (BCC) tumors (Fig. 1K), we have revealed that the GRM4 promoter regions are generally active in various immune populations, including naïve CD4+ T cells, regulatory T (Treg) cells, naïve CD8+ T cells, memory CD8+ T cells, effector CD8+ T cells, and NK cells. We have also analyzed four additional scRNA-seq data from patients with hepatocellular carcinoma (HCC), non–small cell lung cancer (NSCLC), and skin cutaneous melanoma (SKCM). As shown in fig. S2, the expression of GRM4 was found in several types of immune cells analyzed including CD4+ T cells, CD8+ T cells, NK cells, DCs, monocytes, and macrophages. We further examined the expression of GRM4 in the tumor-infiltrating immune cells (CD45+) isolated from B16 tumor– or MC38 tumor–bearing Grm4+/+ mice. Consistent with the study of Thomas and colleagues (6), GRM4 was expressed in the tumor-infiltrating CD45+ as confirmed by both RNA-seq (Fig. 1L) and flow cytometry (Fig. 1M). More specifically, our data have demonstrated that GRM4 was expressed in tumor-infiltrating NK, CD4+ T, and CD8+ T cells, suggesting those cells as potential targets of GRM4-mediated effects (Fig. 1M). There were no significant differences between male and female Grm4−/− mice in the levels of GRM4 expression in several immune cell subpopulations in the spleen (NK, CD4+ T, and CD8+ T cells) as assessed by flow cytometry (fig. S3A). In addition, analysis of Genotype-Tissue Expression (GTEx) Portal database revealed no differences between males and females in the expression levels of Grm4 in splenocytes (fig. S3B).

Grm4−/− reshapes the landscape of tumor-infiltrating immune cells and the related signaling

The above data suggest that whole-body Grm4−/− led to a proinflammatory TME and that GRM4 was expressed in several major immune cell subpopulations. To further investigate how the Grm4−/− shaped the TME and the antitumor response, we characterized tumor-infiltrating leukocytes (TILs) in B16 tumor model by flow cytometry. As shown in Fig. 2A, both the percentage and the total number of CD45+ leukocytes were significantly increased in B16 tumors of Grm4−/− mice versus WT control. To more clearly define the global changes in immune profile after Grm4−/−, we further performed RNA-seq on fluorescence-activated cell sorting (FACS)–sorted tumor-infiltrating CD45+ cells from the Grm4−/− and the WT littermates (Fig. 2B). There are distinct differences...
Fig. 1. Global loss of Grm4 led to delayed tumor growth in multiple tumor models through modulating TME. (A to F) Male Grm4−/− or WT mice were subcutaneously inoculated with equal numbers of B16, MC38, and 3LL tumor cells, respectively, and tumor growth was followed every 2 to 3 days. Tumors were weighed at the completion of the experiment (B, D, and F). (C and E) Kaplan-Meier survival curves for mice (n = 9 to 12 per group). (G to I) RNA-seq was applied to the B16 tumor tissues. (H) Volcano plot showing all differentially expressed genes (DEGs) between tumor samples. (J) Gene set enrichment analysis (GSEA) analysis showing the top enriched gene sets that were significantly altered in Grm4−/− mice. (K) Histograms showing the fold changes in the expression levels. (L) Genome tracks of aggregate single-cell assay for transposase-accessible chromatin with high-throughput sequencing (scATAC-seq) data in patients with basal cell carcinoma (BCC), peaks indicating the accessible regions (highlighted in yellow) in the promoter regions of GRM4 gene locus. (M) Reanalysis of RNA-seq data reveals expression of GRM4 in CD45+ cells. (M) Flow cytometry analysis of the expression of GRM4 in tumor-infiltrating lymphocytes. Bar graphs represent data summarized as means ± SEM. **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Fig. 2. Global loss of Grm4 led to delayed tumor growth in multiple tumor models through conditioning NK, CD4+ T, and CD8+ T cells toward a highly proliferative and activated phenotype. (A) Representative plots and the quantification of tumor-infiltrating CD45+ cells in B16 tumors. (B) RNA-seq was applied to sorted CD45+ cells. (C) Volcano plot showing genes differentially expressed. (D) GSEA analysis of the top enriched gene sets that were up-regulated in CD45+ cells from Grm4−/− mice. (E) Representative plots, quantification of NK1.1+ cells, and GSEA analysis of up-regulated NK cytotoxicity pathway. (F) Representative plots and the quantification of CD4+ and CD8+ T cells in the tumor tissues, and GSEA analysis of up-regulation of TCR signaling pathway. (G) Flow cytometric plots and strip plots depicting the percentages of Ki-67+–positive cells. (H) Bar graphs indicating the median fluorescence intensity (MFI) of CD44+ cells. (I) Flow cytometric plots and bar graphs depicting the percentages of IFN-γ–producing cells. (J) Grm4−/− and WT mice inoculated with B16 cells were injected intraperitoneally with anti-CD8 or anti-NK1.1 antibody or isotype-matched control immunoglobulin G (IgG). Tumor sizes were measured every 2 to 3 days (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. (anti-CD8 or anti-NK1.1 versus control IgG).
between the gene expression profiles of the tumor-infiltrating CD45+ cells from Grm4+/− and WT mice with significant up-regulation of 290 genes and significant repression of 247 genes (>1.3-fold, P < 0.05) (Fig. 2C). GSEA showed that CD45+ cells from the Grm4+/− mice have a transcriptional program that is enriched with pathways that promote antitumor immunity, which is consistent with the enhanced antitumor immunity in tumor tissues (RNA-seq) (Fig. 2D and table S2).

As shown in Fig. 2E, the percentage of NK cells in the tumors was increased notably in Grm4+/− mice. The absolute numbers of NK cells in the tumors were also significantly higher in the Grm4+/− mice compared to those in WT mice. GSEA plots showed that the signaling of NK cell–mediated cytotoxicity was significantly enriched in Grm4+/− mice compared to WT mice [normalized enrichment score (NES) = 1.544, false discovery rate (FDR) = 0.026]. There were no significant differences in the percentages of CD4+ T or CD8+ T cells in B16 tumors between WT and Grm4+/− mice. However, because of a large increase in CD4+ T cells overall, the total numbers of CD4+ and CD8+ T cells were significantly increased (Fig. 2F). This is likely due to increases in both recruitment and local proliferation of CD4+ and CD8+ T cells. Along with increases in the numbers of CD4+ and CD8+ T cells, GSEA plots showed that TCR signaling was significantly enriched in Grm4+/− mice (NES = 1.772, FDR = 0.018).

Various classes of cell types derived from the mononuclear phagocyte system (MPS), such as tumor-infiltrating macrophages, monocytes, and myeloid-derived suppressor cells (MDSCs), have been implicated in promoting tumor development and metastasis while inhibiting a productive immune response by T cells and NK cells (11). FACS analysis revealed no differences in the monocyte (fig. S4A) and macrophage (fig. S4B) populations between WT and Grm4+/− mice. However, the percentage of tumoral MDSCs (CD11b+ Gr1+) was significantly decreased in Grm4+/− mice (fig. S4C). Along with this change, GSEA plots further showed that MDSC signaling pathway was significantly down-regulated in Grm4+/− mice (NES = -1.226, FDR = 0.066).

DCs, another cell type derived from the MPS, are the predominant antigen-presenting cells (APCs) for T cells in lymphoid organs and in tissues. As shown in fig. S4D, the number of DCs was significantly increased in Grm4+/− mice. GSEA plots showed that antigen processing and presentation were significantly up-regulated in tumors isolated from Grm4+/− mice (NES = 1.784, FDR = 0.016). The level of MHC II molecules was also significantly increased (fig. S4E). GSEA plots showed that MHC II protein synthesis was notably enriched in Grm4+/− mice (NES = 1.548, P < 0.05). The higher levels of MHC II suggest that the APCs cells are most likely to be effective for incoming effector T cells (12).

The above data suggest that Grm4+/− promoted an immunostimulatory TME, which was favorable for antitumor immune responses in B16 tumor model. A similar study was also conducted in MC38 syngeneic tumor model, which is a more immunogenic model than B16 (13). Compared to B16, MC38 model showed similar trends of changes in CD45+, NK cells, CD4+ T cells, CD8+ T cells, monocytes, macrophages, MDSCs, and DCs after Grm4+/− (fig. S5, A to J). In addition, the changes in T cells were more marked in the MC38 tumor–bearing mice. Although we observed an increase in the total number of CD45+ cells in female B16 tumor–bearing Grm4+/− mice compared to female WT mice, there were no significant differences in other immune populations including CD8+ T cells and IFN-γ–producing CD8+ T cells (fig. S6, A to F). Together, these data suggested a pivotal role of immune cells in Grm4+/−–mediated antitumor response.

**Grm4+/− conditions NK, CD4+ T, and CD8+ T cells toward a highly proliferative and activated phenotype**

Our studies so far have shown significant changes in the numbers of immune cell populations and the associated gene expression profiles after loss of Grm4. Further studies showed that the most marked changes in the proinflammatory cytokine profiles were associated with NK, CD4+, and CD8+ T cells (RNA-seq from isolated CD45+) (Fig. 2D and table S2). We went to further characterize the functionality of the three immune cell subpopulations via flow cytometry. Ki-67 is a marker of cell proliferation, and CD44 is a cell activation marker. Figure 2G shows that the expression levels of Ki-67 were significantly up-regulated in Grm4+/− NK cells, CD4+ T, and CD8+ T cells in comparison to WT controls. GSEA analysis indicates that T cell proliferation pathway was significantly up-regulated (NES = 2.069, FDR = 0.000) (fig. S7A). Similar results were obtained for the expression profile of CD44 in all three immune cell subpopulations (Fig. 2H and fig. S7B). GSEA analysis further confirmed the activation of NK (NES = 1.858, FDR = 0.003) (fig. S7C) and T cells (NES = 2.174, FDR = 0.001) (fig. S7D) at transcriptional level. Upon activation and clonal expansion, these immune cells gain effector functions and can produce large quantities of effector cytokines such as IFN-γ and granzyme B (GZB). IFN-γ is produced predominantly by NK cells, CD4+, and CD8+ T cells as part of the innate and adaptive immune responses, respectively. Figure 2I shows that Grm4 deficiency led to significant increases in the expression levels of IFN-γ among all three immune cell subpopulations, indicative of their heightened effector cytokine production. GSEA plots show that IFN-γ signaling pathway was significantly enriched (NES = 2.362, FDR = 0.000) (Fig. 2D, fig. S7E, and table S2) and ranked top among the cytokine pathways.

The above data showed marked changes of immune landscape in the TME of Grm4+/− mice. A preliminary study was also conducted to examine changes in the spleen to evaluate a role of GRM4 in mediating systemic antitumor immunity. No significant differences were observed in the IFN-γ–producing NK cells in mouse spleen (fig. S7F). However, the expression of IFN-γ was significantly up-regulated in CD4+ and CD8+ T cells in comparison to WT controls, suggesting that GRM4 regulates the same sets of genes in the tumor tissues and secondary lymphoid system (14). There were no statisti- cal differences in the percentages of GZB–producing NK and CD8+ T cells (fig. S7, H and I) in both tumors and spleens, despite that there were trends of increased GZB–producing CD8+ T cells. Together, our data suggest that Grm4 deficiency promotes TIL activation, proliferation, and IFN-γ production.

**Grm4+/−–mediated tumor regression is dependent on NK and CD8+ T cells**

NK cells play an important role in cancer innate immunity, while CD8+ T cells are critically involved in the adaptive immune response against cancer. In addition, these two populations can directly engage with and kill the tumor cells in comparison to many other immune cells. In the Grm4+/− TME, NK and CD8+ T cells were not only increased in numbers but also conditioned to be more active, proliferative, and functional. We sought to characterize the role of NK and CD8+ T cells in the significant tumor inhibition
in Grm4−/− mice. NK and CD8+ T cell neutralizing monoclonal antibodies (mAbs) were used for depletion of NK and CD8+ T cells, respectively, and the tumor growth was similarly monitored as described before. Consistent with the data of an early study (Fig. 1B), B16 tumor grew significantly slower in Grm4−/− mice compared to WT mice when they were treated with an isotype-matched control immunoglobulin G (IgG). Injection of either anti-NK or anti-CD8 mAb significantly abolished the tumor growth inhibition in the Grm4−/− mice (Fig. 2J). This implies that both NK cells and cytotoxic CD8+ T cells contributed significantly to the antitumor immunity in Grm4−/− mice.

Grm4−/−-triggered immune activation is associated with induction of immune resistance and synergizes with treatment with immune checkpoint inhibitors

Although Grm4−/− led to an immune-active TME, it also triggered concurrent induction of immune checkpoint molecules. The RNA-seq data of either the tumor tissues or isolated CD45+ cells revealed significant up-regulation of PD-1 and CTLA-4 signaling (Fig. 3A). In consistent with RNA-seq data, FACS showed significant up-regulation of PD-1 expression in NK cells, CD4+ T cells, and CD8+ T cells (Fig. 3B) in tumors from Grm4−/− mice. In addition, we observed higher percentages of Forkhead box protein P3 (FoxP3) CD4+ Treg cells in tumor tissues and splenocytes of Grm4−/− mice (Fig. 3C). CTLA-4 is constitutively expressed in mouse Treg cells (15), in part because FoxP3 directs CTLA-4 transcription (16). This is in consistent with the up-regulated CTLA-4 signaling as described above.

The induction of PD-1/CTLA-4 signaling is likely attributed to a feedback mechanism following immune activation (17), which may limit the overall antitumor T cell responses. We hypothesize that the tumor growth inhibition in Grm4−/− mice can be further improved through combination with anti–PD-1 or anti–CTLA-4–based immune checkpoint blockade (ICB). Treatment with anti–PD-1 antibody led to further growth inhibition of B16 tumors in Grm4−/− mice compared to Grm4−/− mice treated with control IgG (Fig. 3D). Similar results were seen in Grm4−/− mice treated with anti–CTLA-4 antibody (Fig. 3D), which is largely attributed to the inhibition of the increases in Treg infiltration and/or function (17).

High-throughput scRNA-seq identifies the reshaped landscape of tumor-infiltrating CD8+ T cells

Studies conducted so far were focused on the impact of Grm4−/− on the overall changes in NK or CD8+ T cells at transcriptional or protein level. Both innate and adaptive immune responses seemingly work together to constitute Grm4−/−-mediated antitumor immunity, but the adaptive immune system plays a pivotal role in mediating robust and highly specific immune responses against tumors. Therefore, we went to further investigate how Grm4−/− modulated the global landscape of tumor-infiltrating CD8+ T cells. To address the intrinsic heterogeneity and complex changes within CD8+ T cells from Grm4−/− and WT mice in an unbiased approach, we used combination approaches of scRNA-seq (10x Genomics) and TCR sequencing (Fig. 4A).

We analyzed 3216 single CD8+ T cells (1672 WT and 1544 KO) and performed unsupervised clustering of all sequenced CD8+ T cells. The CD8+ T cells revealed eight distinct clusters based on the gene expression signatures using t-distributed stochastic neighbor embedding (t-SNE) analysis (Fig. 4B). According to the differentially expressed genes (DEGs), T cell subclusters were designated as CD8-C0-Sell (naïve), CD8-C1-Xcl1 (stem-like) (18), CD8-C2-Cd5 (tissue-resident memory), CD8-C3-Tigit (exhausted), CD8-C4-Hmgb2 (cycling), CD8-C5-Cdc7a (memory), CD8-C6-Prf1 (cytotoxic), and CD8-C7-Itgb1 (superior cytotoxic), with Grm4+/+ and Grm4−/− CD8+ T cells showing a distinct distribution across the clusters (Fig. 4, C and D, and fig. S8A). The CD8+ T cell marker genes were coded in color for eradicating noise signals (fig. S8B). The proportions of each cluster in either WT or Grm4−/− CD8+ T cells among either the total CD8+ T cells (Fig. 4E) or within the total cells in each cluster (Fig. 4E) were quantitated.

Notably, ~27% CD8+ T cells from the Grm4+/+ mice were distributed in the cluster of CD8-C0-Sell in comparison with ~19% in Grm4−/− mice, indicating that Grm4−/− TME potentially primes the differentiation of the naïve CD8+ T cells into functional states (Fig. 4E). It has been previously recognized that T cells within the TME are prone to exhaustion, thus preventing such CD8+ T cells from eliciting sufficient T cell-mediated killing of tumor cells. Focusing our analysis on tumor-infiltrating CD8+ T cells in the context of B16 melanoma, we observed fewer effector CD8+ T cells but a large proportion of T cells with exhausted phenotypes in the TME. As shown in Fig. 4 (E and F), the exhausted subcluster CD8-C3-Tigit occupied ~17% CD8+ T cells, while only ~4.6% and ~3% of CD8+ T cells were in the clusters of CD8-C6-Prf1 and CD8-C7-Itgb1 in WT mice, respectively. It seems that Grm4−/− might maintain the cytotoxic status and prevent CD8+ T cells from entering into an exhausted state because a significant reduction of CD8-C3-Tigit (~9%) was observed in Grm4−/− mice. The data from scRNA-seq further defined that Grm4−/− selectively increased the cytotoxic CD8+ T cells. More than 60% of all cells in the clusters of CD8-C6-Prf1 and CD8-C7-Itgb1 were Grm4−/− CD8+ T cells (Fig. 4F).

As a cytotoxic marker of CD8+ T cells, Prf1 is predominately accumulated in the cluster of CD8-C6-Prf1 and partially in CD8-C7-Itgb1. There was a significant increase in the percentage of the subcluster of CD8-C6-Prf1 in Grm4−/− mice (~8%) compared to the WT mice (~4.6%) (Fig. 4E). Itgb1 (CD29) identifies IFN-γ-producing and marks superior cytotoxic CD8+ T cells (19). As shown in Fig. 4G and fig. S8A, IFN-γ is majorly accumulated in the cluster of CD8-C7-Itgb1. Here, there were approximately 6.5% CD8-C7-Itgb1 in Grm4−/− mice versus about 3% in WT mice, indicative of significantly increased (>2-fold) IFN-γ-producing cells after KO of Grm4. In addition, this phenotype is consistent with the increased expression of MKi67 (Ki-67), Cd44, and Tnfsf10 (costimulatory molecules) (Fig. 4G) (20). Our data from the early study suggest that Grm4−/−-conditioned CD8+ T cells toward an activated and proliferative phenotype at the overall transcriptional and protein levels (Fig. 2, D and G to I). Here, scRNA-seq data defined that Grm4−/− preferentially increased the proliferative and activated functionality in the subcluster of CD8-C7-Itgb1.

Elevated expression of transcripts encoding PD-1 (Pdcd1) and CTLA-4 (Ctla4), the corresponding T cell inhibitory receptors, was also observed in effector CD8+ subsets particularly in CD8-C7-Itgb1 (fig. S8C), which is consistent with previous research (21). These results might be due to the feedback mechanisms: (i) The released IFN-γ can trigger the inducible expression of PD-L1 and CTLA-4 (22); (ii) the proliferating subsets are more likely to express receptors PD-1 and CTLA-4 (23). In melanoma, a higher proportion of Ki-67+ CD8+ T cells was associated with improved response to checkpoint inhibitors, which is consistent with the enhanced tumor
growth inhibition in \textit{Grm4}^{-/-} mice treated with immune checkpoint inhibitors (Fig. 3D).

We also found increased percentages of CD8-C1-Xcl1, CD8-C2-Ccl5, and CD8-C4-Hmbg2 subsets in \textit{Grm4}^{-/-} mice (Fig. 4, E and F). CD8-C1-Xcl1 (stem-like) subset may also maintain cytotoxic function with increases in the capacity of proliferation and the expression of Gzmc and Gzmb (Fig. 4G). Expression of Gzma is selectively accumulated in CD8-C2-Ccl5 subset (tissue-resident memory) (Fig. 4G). These results indicate that tissue-resident memory CD8\(^{+}\) T cells can also maintain their cytotoxic effect. No significant changes were observed in CD8-C5-Cdca7 (memory) subset between WT and \textit{Grm4}^{-/-} mice (Fig. 4, E and F). Together, \textit{Grm4}^{-/-} also increased the stem-like and cycling CD8\(^{+}\) T cells in the TME, which also supports the antitumor immunity.

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**Fig. 3.** \textit{Grm4} depletion induced adaptive immune resistance and synergized with immune checkpoint inhibitor–based treatments. (A) GSEA analysis of the PD-1 and CTLA-4 signaling pathways in either B16 tumor tissues or sorted CD45\(^{+}\) cells from \textit{Grm4}^{-/-} mice in comparison to WT mice. (B) Representative flow cytometric plots and the quantification of the percentages of PD-1\(^{+}\) cells within the gated NK1.1\(^{+}\), CD4\(^{+}\), and CD8\(^{+}\) T populations in B16 tumors. (C) Representative flow cytometric plots and the percentages of FoxP3\(^{+}\) cells within the gated CD4\(^{+}\) T population in B16 tumors and spleens. (D) Treatment regimen of anti–PD-1 and CTLA-4 antibodies, and the tumor growth curves in WT and \textit{Grm4}^{-/-} mice. Data shown are representative of three to five experiments. Graphs shown represent data summarized as means ± SEM and were analyzed by unpaired two-tailed unpaired Student’s t test. Two-way analysis of variance (ANOVA) was used to determine statistical significance for time points when all mice were viable for tumor measurement. \(*P < 0.05\), \(**P < 0.01\), and \(***P < 0.001\).
**Fig. 4.** High-throughput single-cell RNA-seq of tumor-infiltrating CD8\(^+\) T cells revealed distinct transcriptional signatures between WT and Grm4\(^{-/-}\) mice. (A) Scheme of the overall study design. scRNA-seq was applied to CD8\(^+\) T cells obtained from B16 tumor tissues, and the output data were used for gene expression analysis and TCR profiling. (B and C) Eight clusters defined from the \(t\)-distributed stochastic neighbor embedding (\(t\)-SNE) projection of the total 3216 single T cells from both WT and Grm4\(^{-/-}\) mice (1672 WT and 1544 Grm4\(^{-/-}\)) (B) and the CD8\(^+\) T cells from each strain of mice (C). (D) Heatmap of the top DEGs within the eight clusters. (E and F) Percentages of WT and Grm4\(^{-/-}\) CD8\(^+\) T cells among the total CD8\(^+\) T cells (E) and within each cluster (F). (G) Expression of selected genes across the \(t\)-SNE plot. The color keys indicate the gene expression values for maturation markers, activating receptors, cytotoxic effector molecules, and effector cytokines, respectively. (H) Projection of the top 10 frequent TCRs (red dots) to clusters of CD8\(^+\) T cells. (I and J) Quantification of total highly expanded TCR (I) and in each cluster (J). (K) Projection of identical clone types (shown in this panel are two representative clone types in either WT or Grm4\(^{-/-}\) mice). (L and M) Ordering of CD8\(^+\) T cells along pseudo-time in a three-dimensional state space defined by Monocle 3. Cell orders are inferred from the expression of the most dispersed genes across CD8\(^+\) T cell populations. Each point corresponds to a single cell, and each color represents a CD8\(^+\) T cell subcluster.
tumor-infiltrating CD8+ T cells in the early developmental stage. To test whether CD8+ T cells can be directly modulated by GRM4, we sought to model antigen stimulation in vitro. CD8+ T cells were isolated from the spleen and lymph nodes of Grm4−/− and WT naïve mice and activated with plate-bound anti-CD3 plus anti-CD28 (Fig. 5A), followed by culturing in the presence of IL-2–containing RPMI 1640 for 5 days (31). Then, the medium of cultured CD8+ T cells was replaced with stimulation cocktail, and the IFN-γ production was measured by flow cytometry. Grm4−/− CD8+ T cells produced significantly more IFN-γ compared to the WT CD8+ T cells (Fig. 5B). In addition, we adoptively transferred Grm4−/− NK cells into the RAG-1–deficient mice that lack mature T cells and B cells, before B16 inoculation (32). This led to reduced tumor growth compared with mice receiving CD8+ T cells isolated from WT mice (Fig. 5C), indicative of a cell-intrinsic role of GRM4 in regulating the antitumor activity of CD8+ T cells. We then similarly cultured isolated NK cells in the presence of IL-2 for 7 days (fig. S10A). Again, Grm4−/− NK cells produced significantly more IFN-γ compared to the WT NK cells (fig. S10B). These data suggest that GRM4 is capable of directly modulating the function of NK and CD8+ T cells and KO of Grm4 led to enhanced function of NK and CD8+ T cells.

The mechanism for the enhanced function of NK and CD8+ T cells after Grm4−/− is unclear. GRM4 has been shown to negatively regulate intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) levels in a G protein–dependent fashion in presynaptic nerve terminals and microglia as well as in DCs (5, 33). RNA-seq results demonstrated significantly up-regulated cAMP signaling pathway after Grm4−/− in the isolated CD45+ cells (fig. S10C). As an initial step to gain the mechanistic insights into how GRM4 regulates cAMP signaling, we measured the cAMP concentration in naïve CD8+ T cells isolated from the spleen and lymph nodes of Grm4−/− and WT mice. There was an increasing trend in the basal levels of cAMP in Grm4−/− CD8+ T cells compared to WT cells. However, in the presence of forskolin (an activator of adenylyl cyclase), the intracellular concentration of cAMP was significantly higher in Grm4−/− CD8+ T cells than that in WT CD8+ T cells (Fig. 5D),

In our early study, we also observed an increased recruitment of tumor-infiltrating CD4+ T cells and their activated phenotype (Fig. 2, F to I), which shall indirectly contribute to the antitumor immunity. We performed scRNA-seq for isolated CD4+ T cells as well (fig. S9) and identified five CD4+ T cell clusters (fig. S9A). Cells from the largest cluster, CD4-C0-Itgb1, expressed Th1 marker genes such as Cxcr3, Ifng, and Ccl5 (fig. S9B). In addition, we found that C4-C1-Cxcl7 had higher expression of naïve/stem T cell marker genes such as Ccr7 and Sell (fig. S9B). Overall, there were fewer naïve CD4+ T cells and more CD4-C0-Itgb1 (fig. S9, C and D). It is noted that Cd44, Ifng, and MkI67 were selectively accumulated in CD4-C0-Itgb1, indicating that Grm4−/− also selectively increased Th1 cells (fig. S9E). However, these changes are less marked in comparison to CD8+ T cells. Overall, there were fewer genes affected and smaller changes in the levels of gene expression in the scRNA-seq of CD4+ T cells (fig. S9F), which is different from a more marked changes in the Th1 response in Grm4−/− mice in an EAE model (5). This suggests a complex and context-dependent role of GRM4 in immune modulation.
Fig. 5. The enhanced IFN-γ production and antitumor activity of stimulated Grm4−/− CD8+ T cells was correlated with activation of cAMP/CREB immune pathway.

(A) Experimental scheme for isolation, in vitro culture, and in vitro stimulation of CD8+ T cells and the subsequent flow cytometry analysis. (B) Representative flow cytometric plots and the quantitative data showing the IFN-γ production in CD8+ T cells isolated with from WT or Grm4−/− mice. (C) Growth of subcutaneous B16 tumors in RAG-1−/− mice receiving CD8+ T cells isolated from either WT (n = 5) or Grm4−/− mice (n = 5). (D) Measurements of intracellular levels of adenosine 3’,5’-monophosphate (cAMP) in WT and Grm4−/− CD8+ T cells stimulated with forskolin. (E and F) Flow cytometric analysis of the expression levels of phosphorylated Creb (pCreb) in cultured WT and Grm4−/− CD8+ T cells. (G) Representative histogram and a quantitative bar graph showing the expression of IFNGR1 in the CD8+ T cells isolated from WT or Grm4−/− mice subjected to stimulation. The expression of IFNGR1 was analyzed by flow cytometry and presented as geometric MFI (gMFI). (H) GSEA plots of up-regulated cAMP/cAMP response element binding protein (CREB) pathway from single RNA-seq results of CD8+ T cells isolated from Grm4−/− mice in comparison to WT mice. (I) Correlation of Creb1 with ifngr1 and ifng in either WT or Grm4 KO tumor-infiltrating CD8+ T cells. (J) Proposed mechanism of GRM4-mediated cAMP/CREB pathway. Data are representative of two independent experiments and presented as means ± SEM. Graphs shown represent data summarized as means ± SEM and were analyzed by unpaired two-tailed unpaired Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
sustaining a role of GRM4 in controlling the intracellular cAMP concentration.

The cAMP response element binding protein (CREB) is one of the transcriptional factors that positively regulate the expression of IFN-γ (34, 35). Figure 5 (E and F) shows that the levels of phosphorylated CREB (pCREB) in Grm4−/− CD8+ T cells were significantly increased. Along with these changes, we also observed a significant increase in the expression level of IFN-γR1 in Grm4−/− mice (Fig. 5G), which is very important for the initiation of IFN-γ signaling.

scRNA-seq revealed enhanced cAMP-dependent protein kinase (PKA)/CREB signaling in Grm4−/− CD8+ T cells (Fig. 5H). In addition, the expression of Creb1 (encoding CREB) is positively correlated with those of Ifng (encoding IFN-γ) and Ifngr1 in either WT or Grm4−/− tumor-infiltrating CD8+ T cells (Fig. 5I). It is worthwhile to mention that a better correlation was seen in Grm4−/− CD8+ T cells. Together, we propose that GRM4/cAMP/CREB pathway may play a role in the direct activation of CD8+ T cells (Fig. 5J).

**Pharmacological inhibition of GRM4 significantly delays tumor growth**

Our data so far have clearly demonstrated that genetic KO of Grm4 led to an immune-active TME, which plays an important role in inhibiting the growth of transplanted tumors. We then went to study whether similar outcome could be achieved via pharmacological inhibition using MSOP, a GRM4 antagonist (36). C57BL/6 mice bearing B16, MC38, or 3LL tumors (∼50 mm3) received daily intravenous injection of MSOP [(RS)-α-Methylserine-O-phosphate] (10 mg/kg) for 10 days, and tumor growth was monitored once every 3 days (Fig. 6A). Similar to what was observed in the genetic model, there was no significant difference in the growth of B16 or 3LL tumor between MSOP treatment group and control group (saline) in female mice (Fig. S11, A to D). On the other hand, MSOP treatment led to significant inhibition of tumor growth in male mice for all three tumor models (Fig. 6, B to D, and fig. S11, E to G).

In addition, the inhibitory effect of MSOP on B16 tumor was abolished in Grm4−/− mice (Fig. 6E and fig. S11H), suggesting that the MSOP-mediated tumor growth inhibition is dependent on targeting of GRM4.

Pharmacological inhibition of GRM1 has been shown to cause inhibition of tumor cell proliferation both in vitro and in vivo (37). To evaluate whether targeting of GRM4 by MSOP has a direct impact on cancer cells, we treated cultured B16, MC38, or 3 LL tumor cells with various concentrations of MSOP and examined the cytotoxicity by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Riluzole, a GRM1 antagonist, was used as a positive control. In consistent with previous studies (38), riluzole inhibited the proliferation of B16 melanoma cells in a concentration dependent manner (Fig. 6F and fig. S11). However, MSOP did not show obvious inhibitory effect at all concentrations used, even at a concentration as high as 500 μM. Similar results were observed in two other murine cancer cell lines MC38 (Fig. 6G and fig. S11) and 3LL (Fig. 6H and fig. S11K). Lack of effect on the proliferation of the three cancer cell lines was also shown with MAP-4 [(S)-2-Amino-2-methyl-4-phosphonobutanoic acid], another GRM4 antagonist (8). These data, together with the data from the genetic model, suggest that MSOP inhibits the tumor growth through modulating the antitumor immunity rather a direct inhibitory effect on tumor cells.

Figure 6I shows that the numbers of tumor-infiltrating CD45+ cells were significantly increased following MSOP treatment in B16 tumor model. In particular, MSOP treatment led to significant increases in the numbers of NK cells, CD4+ T cells, and CD8+ T cells (Fig. 6J). To define the immune effector molecules involved in the MSOP-mediated antitumor activity, we conducted a preliminary study to examine the therapeutic effect of MSOP in IFN-γ−/− mice. As shown in Fig. 6K, the inhibitory effect of MSOP on tumor growth was significantly attenuated in IFN-γ−/− mice compared to WT mice, suggesting that IFN-γ is critically involved in the MSOP-mediated antitumor immunity.

We also observed increased number of FoxP3+ CD4+ T cells in the spleen and up-regulated expression of PD-1 in NK and CD8+ T cells in the TME (Fig. 6, L and M). We then went to investigate whether MSOP could synergize with ICB to improve the therapeutic efficacy (Fig. 6N). Figure 6 (O to Q) shows that anti–PD-1 or anti–CTLA-4 monotherapy had only moderate effect on B16 tumor, which was consistent with literature (39, 40). In contrast, combination of MSOP with either anti–PD-1 (Fig. 6, O and Q) or anti–CTLA-4 (Fig. 6, P and Q) led to marked inhibition of tumor growth. Overall, our data with pharmacological inhibition of GRM4 are consistent with the data from the genetic KO model, further establishing a role of GRM4 in immune cells in modulating the antitumor immunity. It also suggests a new therapy based on the use of GRM4 antagonist, alone or in combination with ICB.

**MSOP calcium phosphate nanoparticles significantly improve the antitumor effect**

Despite the demonstrated antitumor activity of MSOP, frequent dosing is needed because of its rapid elimination following systemic administration. As a highly polar compound, oral dosing is not effective because of its limited bioavailability (41). Systemic delivery of MSOP using nanoparticles (NPs) can not only increase its half-life in blood but also facilitate enhanced delivery to tumors. In addition, the non-specific uptake of NPs by reticuloendothelial system (RES) shall benefit the delivery to immune cell–enriched spleen. As MSOP bears a phosphate group (ionic group), calcium phosphate NPs represent a simple and highly effective system to improve its in vivo delivery.

Figure 6R and fig. S12 show the scheme for the preparation of MSOP NPs. Mixing of calcium chloride with MSOP led to the formation of MSOP nanocrystals with slightly excess negative charges that facilitate the next step of coating of a lipid layer through interaction with positively charged 3,4-dihydroxyphenylalanine (dopa). Further incorporation of additional lipids including dioleoylphosphatidylcholine and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine–polyethylene glycol (DSPE-PEG) led to the formation of MSOP NPs. The MSOP NPs were homogenous and spherical in shape, as shown in the transmission electron microscope (TEM) micrograph (Fig. 6S). The average size of the MSOP NPs was ~150 nm (Fig. 6S), and the surface zeta potential was ~2.8 mV (Fig. 6S).

Figure 6T shows that intravenous injection of MSOP NPs three times every once 3 days led to a significant inhibition of tumor growth in a B16 tumor model. In contrast, injection of free MSOP following the same treatment regimen was not effective. The mice were well tolerated at the dose used without any obvious changes in body weights (fig. S13). In addition, treatments with MSOP NPs had no effect on bone marrow, liver, and kidney functions as evident from little changes in red blood cell and white blood cell counts in peripheral blood and serum levels of transaminases and creatinine (fig. S14, A to F). Figure S15 shows that incorporation of
Fig. 6. Pharmacological inhibition of GRM4 with MSOP significantly delayed tumor growth. (A to E) In vivo antitumor activity of MSOP was tested in three cancer types (B16, MC38, and 3LL). MSOP was given to WT (B to D) and Grm4−/− mice (E) intravenous daily for 10 days at a dosage of 10 mg/kg, and the tumor growth was followed every 2 days. (F to H) Cytotoxicity of riluzole (GRM1 antagonist), MSOP, and MAP-4 (GRM4 antagonist) against cultured B16, MC38, and 3LL cancer cells (n = 5). (I and J) Flow cytometry analysis and quantification after MSOP treatment (n = 3). (K) Antitumor activity of MSOP in WT and IFN-γ−/− mice (n = 5). (L and M) Flow cytometry analysis and the quantification of PD-1+ cells and Treg cells after MSOP treatment (n = 3). (N to Q) Treatment regimen for the combination of MSOP with either anti–PD-1 (O) or anti–CTLA-4 (P) antibody in B16-bearing mice. Individual tumor growth trajectories were presented (Q). (R) Schematic presentation of the design and fabrication of MSOP-loaded CaP nanoparticles (NPs). (S) Representative transmission electron microscope (TEM) image, size, and zeta potential of MSOP CaP NPs. (T) Treatment schedule and average tumor growth curves of B16-bearing male mice treated with free MSOP and MSOP NPs, respectively (n = 5). PDI, polydispersity index. N.S., not significant. *P < 0.05, **P < 0.01, and ***P < 0.001.
MSOP into the NPs was not associated with any increase in cytotoxicity toward either tumor cells or splenocytes. These preliminary data clearly demonstrate that intravenous delivery of MSOP using NPs is safe and enhances the antitumor activity largely through improved delivery to target tissues/cells.

**NK^high^-GRM4^low^ and CD8^high^-GRM4^low^ signatures predict improved patient survival**

Our data so far have clearly pointed GRM4 as a negative regulator of antitumor immunity and a potential therapeutic target to improve cancer immunotherapy. Considering an inhibitory role of GRM4 in regulating the recruitment and activation of both NK and CD8^+^ T cells, we sought to analyze whether a signature of NK^high^-GRM4^low^ and/or CD8^high^-GRM4^low^ in human tumor samples would be of clinical significance in predicting the prognosis of patients with cancer.

Three independent cancer patient cohorts were analyzed, including (i) 103 patients with primary SKCM from The Cancer Genome Atlas (TCGA) datasets, (ii) 34 patients with acral melanoma (42), and (iii) 110 patients with metastatic melanoma (43). In addition to analyzing the overall correlation between NK-GRM4 (or CD8-GRM4) signatures and patient survival, the impact of gender on the correlation in each cohort was also examined. The data were presented as comparing patient survival between patients with NK (or CD8)^high^-GRM4^low^ versus NK (or CD8)^low^-GRM4^high^ signatures (see Materials and Methods) (Fig. 7 and fig. S16). The results clearly indicate that patients with NK^high^-GRM4^low^ signature show a trend of better survival in two independent datasets. Similarly, CD8^high^-GRM4^low^ signature was also significantly correlated with the better patient survival in three independent datasets (Fig. 7 and fig. S16). These data suggest that the NK^high^-GRM4^low^ and/or CD8^high^-GRM4^low^ signatures may serve as biomarkers for predicting the prognosis of patients with melanoma. Similar to what was found in mouse study, the correlation of NK (or CD8)-GRM4 expression signature with clinical prognosis was of higher predictive values in male patients compared to female patients (Fig. 7 and fig. S16). More studies with larger sample size are needed to further define the clinical significance of these biomarkers.

**DISCUSSION**

We have shown in this study that GRM4 in immune cells plays an important role in negatively modulating the antitumor immunity. Global Grm4 KO or pharmacological inhibition of GRM4 led to significant improvement in tumor immune microenvironment and significant inhibition of tumor growth in several syngeneic tumor models.

The information about the role of GRM4 in cancer so far is limited and controversial. A number of studies suggest a negative correlation between the expression of GRM4 in tumor tissues and the patients’ prognosis in several types of cancer, including colorectal cancer, pediatric CNS tumors, rhabdomyosarcoma, and multiple myeloma (7, 44). However, GRM4 agonists are also shown to inhibit the proliferation of human breast and bladder cancer cells (9, 10). These studies focus on the direct effect of GRM4 agonists on cancer cells, and only limited antitumor activity is shown in a human tumor xenograft model (9). A recent study from Kansara et al. (6) shows that Grm4 gene–targeted mice have accelerated tumor development in an irradiation-induced osteosarcoma mouse model. It was hypothesized that increased production of IL-23 from Grm4^−/−^ myeloid cells plays a role based on the protective effect of IL-23 on the animal model and a negative correlation of IL23 expression with the prognosis of patients with osteosarcoma. In addition, they have further shown that PHCCC [N-Phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxamide], a GRM4 agonist, significantly inhibits the growth of transplanted osteosarcoma cells (OS18) in Grm4^−/−^ mice (10). However, the direct impact of GRM4 agonist on the proliferation of OS18 cells is not known, and it is unclear whether the inhibitory effect of PHCCC on OS18 tumor is maintained in Grm4^−/−^ mice. So far, there is hardly any information of how genetic KO or pharmacological inhibition of GRM4 affects the innate and adaptive immunity in the context of established tumor models.

Using Grm4^−/−^ mice and the control Grm4^+/−^ littermates, we first showed that genetic KO of Grm4 led to significant inhibition of tumor growth in three syngeneic tumor models (B16, 3LL, and MC38). Pharmacological inhibition of GRM4 with MSOP led to similar results in the three tumor models in Grm4^+/+^ mice. In addition, the tumor inhibitory effect of MSOP was abolished in Grm4^−/−^ mice, suggesting a role of GRM4 in MSOP-mediated antitumor activity. These data strongly suggest that the tumor growth inhibition following GRM4 disruption may be largely mediated via an immune mechanism rather than direct effect on tumor cells.

Genetic KO of Grm4 in B16 tumor model led to significant changes in gene expression profile, and GSEA of the tumor bulk RNA-seq data revealed significant down-regulation of several tumor-promoting signaling pathways. In addition, significant enrichment of numerous immune-related pathways was found in B16 tumor grown in Grm4^−/−^ mice: 10 of the top 15 up-regulated pathways revealed in RNA-seq were immune-related. Note that both NK cells and CD8^+^ T cells in tumor tissues were significantly increased in Grm4^−/−^ mice and these immune cells are of highly active and proliferative phenotype. scRNA-seq suggests that Grm4^−/−^ prevented CD8^+^ T cells from entering into an exhausted state and preferentially increased the proliferative and activated functionality in the subcluster of CD8-C7-Itgb1 that is highly active in producing IFN-γ. scTCR profiling suggests that CD8^+^ T cells of different clusters are not completely independent but might undergo extensive state transitions. The most significant differences in clonal expansion of CD8^+^ T cells between WT and Grm4^−/−^ mice were seen in the clusters of CD8-C1-Xcl1, CD8-C6-Prf1, and CD8-C7-Itgb1. There were more frequent TCRs in the clusters of CD8-C1-Xcl1, CD8-C6-Prf1, and CD8-C7-Itgb1 in Grm4^−/−^ mice compared to WT control. Significant clonal expansion was also seen in WT mice but was selectively concentrated within the subcluster of CD8-C3-Tigit. The underlying mechanism is unknown at present, but, clearly, KO of Grm4 redirects the transition of expanded CD8^+^ T cells to the subclusters of CD8-C6-Prf1 and CD8-C7-Itgb1.

Both NK and CD8^+^ T cells are critically involved in the antitumor activity in Grm4^−/−^ mice as neutralizing antibody for each subpopulation markedly attenuated the tumor inhibitory effect. The NK cells may be associated with the tumor growth suppression at early phase after tumor inoculation, while the cellular immunity plays an important role in controlling the tumor growth at a late stage. Similar changes in immune subpopulations were observed in B16-bearing Grm4^−/−^ mice treated with MSOP, further establishing a role of GRM4 in modulating antitumor immunity.

The role of GRM4 in immune modulation was first reported by Fallarino et al. (5) in an EAE model. They hypothesized that disruption of GRM4 in DCs led to differentiation of naïve CD4^+^ T cells.
T cells into Th17 cells through increased production of IL-23 and IL-6 in DCs, which contributed to the initiation and progression of EAE. Similar to the study by Fallarino et al. (5) and the work by Kansara et al. (6) in an osteosarcoma model, we also saw increased expression of IL-23 in Grm4<sup>−/−</sup> mice in our B16 model. However, only small changes were found in the expression levels of IL-17 from both bulk and scRNA-seq in our study. The overall changes in tumor-infiltrating Grm4<sup>−/−</sup> CD4<sup>+</sup> T cells were much less marked compared to those in Grm4<sup>−/−</sup> CD8<sup>+</sup> T cells (Fig. 4 and fig. S7). Naïve NK or CD8<sup>+</sup> T cells isolated from Grm4<sup>−/−</sup> mice are more active as shown by the increased production of IFN-γ and higher level of antitumor activity in an adoptive T cell transfer study. Therefore, while a role of the impact of Grm4<sup>−/−</sup> on DCs and CD4 cells cannot be ruled out in Grm4<sup>−/−</sup>-mediated antitumor activity, our data strongly suggest that Grm4<sup>−/−</sup> can directly activate CD8<sup>+</sup> T cells and possibly NK cells as well.

Fig. 7. NK (CD8)<sup>high</sup>–GRM4<sub>low</sub> signature is associated with improved patient survival in male patients with melanoma. Patients with melanoma were grouped based on NK (CD8)–GRM4 signature. If gender information was included, then patients were first separated on the basis of gender and then grouped on the basis of the NK (CD8)–GRM4 signature. Statistical significance for patient survival between NK (CD8)<sup>high</sup>–GRM4<sub>low</sub> and NK (CD8)<sup>low</sup>–GRM4<sub>high</sub> groups was calculated by Log-rank (Mantel-Cox) test. (A and B) TGEN-AM indicates an acral melanoma dataset from the Translational Genomics Research Institute (see Materials and Methods). DFS, disease-free survival. (C) TCGA-SKCM indicates an SKCM dataset from TCGA; PFI, progression-free interval.
Mechanistically, GRM4 has been shown to negatively regulate intracellular cAMP levels in a G_{i} protein–dependent fashion in presynaptic nerve terminals and microglia as well as in DCs (5, 33). cAMP has long been known to exert suppressive effects on the immune system. Accordingly, bacterial toxins and chemical agents that cause a sustained elevation of cAMP are immunosuppressive (45). However, recent studies have highlighted a positive role of cAMP in immune cell function (35–48). The differential effects of cAMP on cellular functions can be accounted for by a number of factors such as different cell types, the amount of cAMP produced, and the cAMP target genes involved. Our data showed that the basal level of intracellular cAMP was slightly increased in Grm4−/− CD8+ T cells but became significantly higher in the presence of forskolin. Along with these changes, we observed increased levels of pCREB and IFNGR1 in Grm4−/− CD8+ T cells. scRNA-seq revealed enhanced PKA/CREB immune signaling in Grm4−/− CD8+ T cells and strong correlation of Creb1 expression with those of Ifng and Ifngr1. Together, our data suggest a likely role of GRM4/cAMP/CREB pathway in direct activation of CD8+ T cells following Grm4−/−. In addition to cAMP/CREB, other signaling pathways are also regulated by GRM4 (49, 50). Whether and how each pathway is modulated by GRM4 could be affected by the cell type, structure, and concentration of the ligand involved. More studies are needed to better define the underlying mechanism for GRM4-mediated antitumor immunity.

The immune-active TME following Grm4 KO was also associated with induction of adaptive immune resistance characterized by up-regulation of PD-1 and CTLA-4 signaling. Up-regulation of PD-1 and CTLA-4 was similarly observed following MSOP treatment. The induction of adaptive immune resistance is likely attributed, at least partially, to the enhanced IFN-γ response, which was further confirmed by scRNA-seq results. The elevated expression of transcripts encoding PD-1 (Pdcd1) and CTLA-4 (Ctit4a), the corresponding T cell inhibitory receptors, was observed in effector CD8+ subsets particularly in the cluster of CD8+−C7−Igb1 (IFN-γ-producing subpopulation) instead of CD8−C3−Tigit (exhausted). Nonetheless, this suggests an opportunity of further improving the antitumor activity through combining the inhibition of GRM4 signaling with anti–PD-1 or anti–CTLA-4–based ICB. Treatment with anti–PD-1 or anti–CTLA-4 antibody led to more marked inhibition of the growth of B16 tumor in Grm4−/− mice. The improvement in antitumor response was similarly achieved in Grm4−/− mice through combination of a pharmacological inhibitor (MSOP) with anti–PD-1 or CTLA-4 mAb, suggesting a potential of translation into clinic.

One drawback of MSOP is its short half-life in blood that necessitates frequent dosing and limits its translation into clinic. NPs are known to improve the half-life of the formulated drugs in blood and enhance their delivery to tumors (51). One common concern over NPs is the non-selective uptake by RES. However, this “undesired feature” may work favorably for in vivo application of MSOP through enhanced delivery to immune cell–enriched organs such as spleen. Lipid-coated calcium phosphate (LCP) NP has been developed as a versatile platform for delivery of various therapeutics including gene, protein/peptide, and chemotherapeutic agents due to its simplicity and biocompatibility (52). LCP is particularly suitable for delivery of MSOP due to the presence of a phosphate group, which facilitates the formation of a CaP core. Our preliminary data showed that this approach is both simple and effective in improving the in vivo therapeutic efficacy of MSOP.

The studies on the clinical relevance of GRM4 in patients with cancer are limited so far, and conflicting results have been reported. Considering our appealing data that are suggestive of a role of immune cell–derived GRM4 in anticancer immunity, we investigated whether a signature of NK^{high}.GRM4^{low} and/or CD8^{high}.GRM4^{low} would be of clinical significance in predicting the prognosis of patients with melanoma. Analysis of three independent datasets shows a positive correlation of the NK^{high}.GRM4^{low} and/or CD8^{high}.GRM4^{low} signature with a better prognosis of patients with cancer. More studies with larger sample sizes are needed to further define the clinical value of these signatures as biomarkers in cancer care.

One unexpected observation in this study is that the significant tumor growth inhibition with both genetic and pharmacological perturbation of Grm4 was only seen in male mice and not in female mice. In addition, the correlation of the signature of NK^{high}.GRM4^{low} and/or CD8^{high}.GRM4^{low} with cancer patient survival was of better predictive values in male patients than in female patients, despite the relatively small sample sizes of the three cohorts analyzed (Fig. 7). The underlying mechanism is unclear at present. Differences in the levels of GRM4 expression in immune cells between males and females do not appear to play a major role (fig. S3, A and B) although more studies are needed to further establish this notion. Nonetheless, unlike what was seen in male mice (Fig. 2), KO of Grm4 resulted in minimal changes in the numbers of IFN-γ–producing tumor-infiltrating CD8+ cells in female B16 tumor–bearing mice (fig. S6), suggesting that the differences in immune response likely play a role. Sex has been shown to have a major impact on both innate and adaptive immunity as well as numerous immune pathways (53). More studies are needed to understand how sex differences affect the GRM4–mediated antitumor immunity. Our data also suggest caution in considering the gender factor in future studies on the immunomodulating function of GRM4.

In summary, we have unveiled a novel role of immune cell–derived GRM4 in regulating antitumor response. Inhibition of GRM4 signaling led to transformation of TME into a more immunogenic phenotype that is associated with significant inhibition of tumor growth. Combination of a pharmacological inhibitor (e.g., MSOP) with ICB using anti–PD-1 or anti–CTLA-4 led to a further improvement in antitumor activity. We have also demonstrated that the therapeutic potential of GRM4 inhibitor can be further enhanced via improved delivery using NPs. Our data suggest that targeting of GRM4, alone or in combination with ICB, may represent a new therapeutic strategy to improve cancer immunotherapy.

**MATERIALS AND METHODS**

**Mice**

Heterozygous Grm4+/− mice, on a C57/BL6 background, were purchased from the Jackson Laboratory. Mice were bred and maintained at the animal facility of University of Pittsburgh, under specific pathogen-free conditions in accordance with all standards of animal care. Littermates (Grm4+/− or Grm4−/−) were from Grm4+/−/Grm4−/− breeding offspring; Grm4+/− mice were referred to as WT controls and Grm4−/− as Grm4 KO. All mice used in these studies were genotyped by polymerase chain reaction of DNA isolated from tail clippings. Ifng−/− mice and WT C57/BL6 mice were purchased from the Jackson Laboratory. Experiments were conducted with age-matched (6 to 12 weeks old) and gender-matched (male and female) mice. All experiments were conducted following the guidelines of the Institutional Animal Care and Use Committees of University of Pittsburgh.
Tumor cell lines
The mouse B16 melanoma cells, 3LL Lewis lung carcinoma cells, and MC38 colon adenocarcinoma cells were purchased from American Type Culture Collection. B16 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). MC38 and 3LL cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin (100 U/ml). The cells were all cultured in the humidified incubator at 37°C and 5% CO₂.

Tumor model
B16, MC38, and 3LL tumor cells were subcutaneously injected into C57BL6, Grm4+/−, or Grm4−/− mice, respectively, and the sizes of tumor were monitored every 2 to 3 days. Tumor volume was calculated by the following formula: tumor volume = 0.5 × length × width². To study the contribution of NK and CD8⁺ T cells to the antitumor effect, five mice in each group were intraperitoneally injected with 200 µg of anti-CD8α (clone 2.43, Bio X Cell) or anti-mouse NK1.1 (clone PK156, Bio X Cell) antibody or rat IgG2a isotype control (clone 2A3, Bio X Cell) four times before and after tumor inoculation (days −2, 1, 7, and 14). To test the therapeutic effect of combinational blockade of GRM4 and either PD-1 or CTLA-4, control antibody, anti–PD-1 (clone RMP1-14, Bio X Cell), or anti–CTLA-4 (clone 9D9, Bio X Cell) was administered at 200 µg per dose via intraperitoneal MSOP and was intravenously injected at a dosage of 10 mg/kg for either single or combination therapy.

Analysis of tumor-infiltrating lymphocytes and MDSCs
Flow cytometry experiments were all performed with the instrument LSRII (BD Biosciences) Aurora (Cytex Biosciences) and analyzed by FlowJo (BD Biosciences). Single-cell suspensions were prepared from mouse spleens or tumors as previously described (54). Briefly, tumors were dissected and transferred into RPMI 1640. Tumors were disrupted mechanically using scissors, digested with a mixture of deoxyribonuclease I (0.25 mg/ml; Roche) in serum-free RPMI 1640 for 30 min, and dispersed through a 70-µm cell strainer (BD Biosciences).

After red blood cell lysis, Live/Dead cell discrimination was performed using Ghost Dye Violet 510 (Tonbo Biosciences) or Zombie Aqua Fixable Viability Kit (BioLegend). Surface staining was performed at 4°C for 30 min in FACS staining buffer (1× phosphate-buffered saline/0.5% FBS/0.05% sodium azide) containing designated antibody cocktails. For transcription factor (Ki-67) and intracellular proteins staining, cells were fixed and permeabilized using Foxp3 Transcription Factor Buffer Set (eBioscience), following the manufacturer’s instructions. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/ml) and ionomycin (500 ng/ml) for 5 hours in the presence of Monensin. Cells were fixed/permeabilized using the BD Cytofix/Cytoperm kit before cell staining. More detailed information can be found in the Resource table in the Supplementary Materials.

Mouse NK and CD8⁺ T cells isolation and culture
NK cells were isolated by negative selection from WT mouse spleen and lymph nodes with the NK Cell Isolation Kit (Miltenyi) and cultured for 7 days in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, penicillin/streptomycin (100 U/ml), 50 µM β-mercaptoethanol, 1% minimum essential medium nonessential amino acids, 1 mM sodium pyruvate, and recombinant human IL-2 (rhIL-12; 1700 U/ml). For stimulation, the cultured NK cells were treated with recombinant mouse IL-12 (1 ng/ml) and recombinant mouse IL-18 (10 ng/ml) (MBL) or Cell Activation Cocktail.

CD8⁺ T cells were isolated from the mouse spleen and lymph nodes by negative selection according to the manufacturer’s protocol. They were initially plated at 1 × 10⁶ per well in a 24-well plate, in the presence of plated-coated anti-CD3 (5 µg/ml) and anti-CD28 (2 µg/ml) and cultured in RPMI 1640 supplemented with rhIL-2 (20 U/ml) and 10% FBS. After 5 days of culture, CD8⁺ T cells were treated with Cell Activation Cocktail.

MTT assay
The in vitro cytotoxicity of MSOP and MAP-4 (GRM4 antagonist), as well as riluzole (GRM1 antagonist and glutamate transporter inhibitor) against B16, MC38, and 3LL was evaluated and compared by MTT assay. Cells were seeded into a 96-well plate at a density of 1500 cells per well (B16 and MC38) or 1800 cells per well (3LL) and incubated in 100 µl of DMEM or RPMI 1640 containing 10% FBS. After 12 hours of incubation, cell culture medium was removed, and a series of concentrations of MSOP, MAP-4, and riluzole were added into the cells in the presence of 2% FBS DMEM or RPMI 1640. After 48 or 72 hours of incubation, 50 µl of MTT solution (2 mg/ml) were added to each well, and the cells were incubated for another 4 hours. After gently removing the medium, 200 µl of dimethyl sulfoxide was added into each well so as to dissolve the MTT formazan crystals. The optical density was measured using a microplate reader, and the cell viability was calculated using untreated cells as a control.

Preparation and characterization of MSOP CaP NPs
LCP NPs were prepared and characterized in vitro and in vivo. At first, LCP cores were prepared by water-in-oil microemulsions in the oil phase containing Igepal CO-520/cyclohexane solution (29/72, v/v) (52). One hundred and eighty microliters of 20 mM MSOP was well mixed with 12.5 mM Na₂HPO₄ (pH 9.0) to a total volume of 600 µl before adding 20 ml of oil phase with continuous stirring. The calcium microemulsion was prepared by adding 600 µl of 2.5 M CaCl₂ to a separate 20 ml of oil phase. Four hundred microliters of 20 mM dopa in chloroform was added to the phosphate phase, and then the two separate microemulsions were mixed. After stirring for 5 min, another 400 µl of 20 mM dopa was added into the emulsion. The emulsion was stirred continuously for 25 min before adding 40 ml of absolute ethanol. The ethanol emulsion mixture was centrifuged at 9000g for 30 min to pellet the LCP core. The LCP core was washed twice with absolute ethanol and dried in the presence of nitrogen. The LCP core pellets were dissolved in 2 ml of chloroform and stored in a glass vial at −20°C.

To prepare the MSOP-loaded LCPs (MSOP-LCP-PEG) with an outer lipid coating, 330-µl LCP core in chloroform was mixed with 38.7 µl of cholesterol (10 mg/ml), 28 µl of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP; 25 mg/ml), and 96 µl of DSPE-PEG (25 mg/ml). The final molar ratio for cholesterol, DOTAP, and DSPE-PEG was 7:7:6. After evaporation of the chloroform, the residual lipids were dissolved in 30 µl of tetrahydrofuran (THF), followed by addition of 50 µl of absolute ethanol and 160 µl of water. After brief sonication, the solution was dialyzed against distilled water to remove THF and ethanol. The particle size and zeta potential of the formulated MSOP NPs were determined by dynamic light scattering using a Malvern ZetaSizer Nano series. MSOP encapsulation efficiency was measured by ultra-high performance
liquid chromatography–quadrupole time-of-flight mass spectrometry analysis. TEM images of MSOP-loaded NPs were acquired through the use of JEOL 100CX II TEM (Tokyo, Japan).

**Bulk RNA-seq analysis**

RNA-seq was performed by the Health Sciences Sequencing Core at Children’s Hospital of Pittsburgh. RNA-seq libraries were sequenced as 75-base pair paired-end reads at a depth of ~73 to 77 million reads per sample. Reads were mapped to the mouse genome (GRCh38) using STAR Aligner 2.6.1a (55). Gene expression quantification and differential expression analysis between Grm4−/− and WT were performed using Cuffdiff of Cufflinks 2.2.1 (56). Volcano plots were generated to show the overall differential expression, where the x axis indicates the log2(fold change) (log2FC) between KO and WT and the y axis indicates the corresponding −log10(P value).

To interpret the function of regulated genes in CD45+ cells per tumor cells in Grm4−/− mice in comparison with those in WT mice, the GSEA (57) was performed on the basis of the gene list ranked by the log2FC between KO and WT, using “gseapy” Python package. The gene sets we used, including Hallmark gene sets, Kyoto Encyclopedia of Genes and Genomes gene sets, gene ontology gene sets, and other topic related gene sets, were collected and downloaded from Molecular Signatures Database (57).

**Analysis of cancer patient data**

Survival analysis was performed on the basis of gene expression data and patient clinical information. For TCGA data, gene expression data were downloaded from TCGA Pan-Cancer project (Data Freeze 1.3). Clinical information of TCGA patients was collected from TCGA Pan-Cancer Clinical Data Resource (58). The other two melanoma datasets were downloaded from cBioPortal (www.cbioportal.org/), including an acral melanoma dataset from the Translational Genomics Research Institute (42) and a metastatic melanoma dataset from the Dana-Farber Cancer Institute (43). Because of the availability of gene expression data and clinical follow-up data of the patients, 33 of 44 patients in the acral melanoma data set and 40 of 110 patients in the metastatic melanoma data were used to perform the survival analysis, respectively.

The NK signature consists of the NK-associated genes NCR1, NCR3, KLRB1, CD160, and PRF1 (59). The CD8 signature consists of the CD8-associated genes CD8A and CD8B. The PKA/Creb immune pathway was summarized from the study of Yao et al. (47), including PRKACA, PRKACB, PRKACG, PRKAR1A, PRKAR1B, PRKAR2A, PRKAR2B, CRTC2, CREB1, IL12RB2, IFNGR1, and IL2RB.

To study the correlation between NK (or CD8)–GRM4 signature and patient survival, we first ranked the patients based on NK (or CD8) signature level. We also obtained a reversed rank of the patients based on GRM4 expression. Then, we used the average rank of the two metrics to divide the patients into three groups: The top one-third was classified as NK (or CD8) high–GRM4 low, and the low one-third was classified as NK (or CD8) low–GRM4 high, while the remaining patients were classified as intermediate. When gender information was included, patients were first separated by gender and then grouped on the basis of the NK (or CD8)–GRM4 signature. Log-rank test was used to compare the survival distribution of NK (CD8)high–GRM4low and NK (CD8)low–GRM4high group.

**scTCR sequencing**

The scTCR data were processed by Cell Ranger (version 4.0.0, 10x Genomics) pipeline. The TCR data were first filtered with the following criteria: (i) A barcode must have a contig that aligns to a V segment to be identified as a targeted cell, and (ii) there must also be at least three filtered UMIs with at least two read pairs each. The filtered data were analyzed using the R package scRepertoire (V 1.1.2). Clonotypes were removed if any cell barcode had more than two immune receptor chains. scTCR data were merged with scRNA-seq data of the T cell clusters only based on the cell barcodes. TCR clones that matched barcodes of cells that were not located in T cell clusters (based on our previous annotations using Seurat) were removed. The top 10 most frequent TCRs were labeled and projected in the t-SNE map associated with cell barcode and CD3 gamma amino acid sequences. Last, we calculated the overlap of CD3 gamma amino acid sequences from each CD8 clusters in either WT or KO group.

**Reanalysis of single-cell RNA-seq and ATAC-seq data**

To examine the GRM4 expression in different immune cell populations, we analyzed publicly available scRNA-seq data of HCC (GSE140228), NSCLC (GSE99254 and GSE127465), and SKCM (GSE72056), and RNA-seq data of CD45+ cells from B16 and MC38 tumor models (GSE132748). “N/A” indicates that the specific immune cell populations were not available in a dataset. To examine the chromatin accessibility of GRM4 promoter in different cell identities, scATAC-seq data (61) generated from the TME of BCC were visualized in the WashU Epigenome Browser.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software. Values are reported as means ± SEM. When comparing two groups,
$P$ values were calculated using two-tailed Student’s $t$ tests. For time to event and survival analysis, $P$ values for the Kaplan-Meier survival curves were calculated with a log-rank (Mantel-Cox) test. Significance was conventionally accepted at $P \leq 0.05$. For multiple treatment group comparisons, significance was determined by one-way analysis of variance (ANOVA), followed by the Tukey’s post hoc multiple comparisons test, where $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$, and $^{****} P < 0.0001$.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abj4226

**REFERENCES AND NOTES**

1. D. S. Chen, J. Millman, Elements of cancer immunity and the cancer–immune set point. Nature 541, 321–330 (2017).
2. D. O. Khair, H. J. Bax, S. Crescioli, G. Pellizzari, A. Khiabany, M. Nakamura, R. J. Harris, E. French, R. M. Hoffman, J. P. Williams, A. Cheung, B. Thair, T. C. Beales, E. Touzour, A. W. Signell, N. L. Tasnova, J. F. Spicer, D. H. Josephs, J. L. Geh, A. MacKenzie Ross, C. Healy, S. Papa, K. E. Lacey, S. N. Karagiannis, Combining immune checkpoint inhibitors: Established and emerging targets and strategies to improve outcomes in melanoma. Front. Immunol. 10, 453 (2019).
3. P. Baral, B. D. Umans, L. Li, A. Wallrapp, M. Bist, T. Kirschbaum, Y. Wei, Y. Zhou, V. K. Kuchroo, P. R. Burkett, B. G. Yipp, S. D. Libeliers, I. M. Chiou, Nociceptor sensory neurons suppress neutrophil and γδ T cell responses in bacterial lung infections and lethal pneumonia. Nat. Med. 24, 417–426 (2018).
4. M. Julio-Pieper, P. J. Flor, T. G. Dinan, J. F. Cryan, Exciting times beyond the brain: Metabotropic glutamate receptors in peripheral and non-neural tissues. Pharmacol. Rev. 63, 53–88 (2011).
5. F. Fallarino, C. Volpi, F. Fazio, S. Notartomaso, C. Vacca, B. Bicciato, G. Battaglia, V. Bruno, P. Puccetti, M. C. Fioretti, F. Nocitelli, U. Grohmann, R. D. Marco, Metabotropic glutamate receptor-4 modulates adaptive immunity and restrains neuroinflammation. Nat. Med. 16, 897–902 (2010).
6. M. Kansara, K. Thomson, P. Pang, A. Dutour, L. Mirabello, F. Acher, J.-P. Pin, E. G. Demicco, J. Yan, M. W. Teng, M. J. Smyth, D. M. Thomas, Infiltrating myeloid cells drive osteosarcoma progression via GRM4 regulation of IL23. Cancer Res. 79, 1511–1519 (2019).
7. H. J. Chang, B. C. Yoo, S.-B. Lim, S.-Y. Jeong, W. H. Kim, J.-G. Park, Metabotropic glutamate receptor 4 expression in colorectal carcinoma and its prognostic significance. Clin. Cancer Res. 11, 3288–3295 (2005).
8. B. C. Yoo, E. Jeon, S.-H. Hong, Y.-K. Shin, H. J. Chang, J.-G. Park, Metabotropic glutamate receptor 4-mediated 5-fluorouracil resistance in a human colon cancer cell line. Clin. Cancer Res. 10, 4176–4184 (2004).
9. Z. Zhang, Y. Liu, K. Wang, K. Zhu, X. Zheng, L. Wang, Y. Luan, X. Wang, H. Lu, K. Wu, X. Chen, D. He, Y. Liu, Activation of type 4 metabotropic glutamate receptor promotes cell apoptosis and inhibits proliferation in bladder cancer. J. Cell. Physiol. 234, 2741–2755 (2019).
10. B. Xiao, D. Chen, Q. Zhou, J. Hang, W. Zhang, Z. Kuang, Z. Sun, L. Li, Glutamate metabotropic receptor 4 (GRM4) inhibits cell proliferation, migration and invasion in breast cancer and is regulated by mirt326-3p and mirt370-3p. BMC Cancer 19, 891 (2019).
11. M. Chittezhath, M. K. Dhillon, J. Y. Lim, D. Laiou, I. N. Shalova, Y.-L. Teo, J. Chen, R. Kamaraj, L. Raman, J. Liu, T. P. Thamboo, E. Chiong, F. Zolezzi, H. Yang, J. A. Van Ginderachter, J. A. Seidel, A. Otsuka, K. Kabashima, Anti-PD-1 and anti-CTLA-4 therapies in cancer: Mechanisms of action, efficacy, and limitations. Nat. Immunol. 14, 1173–1182 (2013).
12. A. R. Syage, H. A. Kiz, D. D. Skinner, C. Stone, R. M. O’Connell, T. E. Lane, Single-cell RNA sequencing reveals the diversity of the immunological landscape following central nervous system infection by a murine coronavirus. Viriol. J. 94, e01295-20 (2020).
13. X. B. Wang, C. Y. Zheng, R. Giscombe, A. Lefvert, Regulation of surface and intracellular expression of CTLA-4 on human peripheral T cells. Scand. J. Immunol. 54, 453–458 (2001).
14. X. Chen, D. He, Y. Liu, Activation of type 4 metabotropic glutamate receptor promotes osteosarcoma progression via GRM4 regulation of IL23. Cancer Res. 79, 1511–1519 (2019).
15. J. A. Seidel, A. Otsuka, K. Kabashima, Anti-PD-1 and anti-CTLA-4 therapies in cancer: Mechanisms of action, efficacy, and limitations. Front. Oncol. 8, 86 (2018).
16. S. I. Im, S.-J. Ha, Re-defining T-cell exhaustion: Subset, function, and regulation. Immune Netw. 20, e2 (2020).
17. R. Shah, S. J. Singh, K. Eddy, F. V. Filipp, S. Chen, Concurrent targeting of glutaminolysis and metabotropic glutamate receptor 1 (GRM1) reduces glutamate bioavailability in GRM1−/− melanoma. Cancer Res. 79, 1799–1809 (2019).
18. J. M. Mehnhert, A. W. Silk, J. H. Lee, D. Dudek, B.-S. Jeong, L. Ji, M. Schenkel, E. Sadimin, M. Kan, H. Lin, W. I. Shih, A. Zloza, S. Chen, J. S. Goydos, A phase II trial of niluzole, an antagonist of metabotropic glutamate receptor 1 (GRM1) signaling, in patients with advanced melanoma. Pigment Cell Melanoma Res. 31, 534–540 (2018).
19. T. Takahashi, T. Tagami, S. Yamaizaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, S. Sakaguchi, Immunologic self-tolerance maintained by CD25+ CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte–associated antigen 4. J. Exp. Med. 192, 303–310 (2000).
20. A. L. Doedens, A. T. Phan, M. H. Stradner, J. K. Fujimoto, J. V. Nguyen, E. Yang, R. S. Johnson, A. W. Goldrath, Hypoxia-inducible factors enhance the effector responses of CD8+ T cells to persistent antigen. Nat. Immunol. 14, 1173–1182 (2013).
21. T. Takahashi, T. Tagami, S. Yamaizaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, S. Sakaguchi, Immunologic self-tolerance maintained by CD25+ CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte–associated antigen 4. J. Exp. Med. 192, 303–310 (2000).
39. Y. Wang, J.-J. Li, H.-J. Ba, K.-F. Wang, X.-Z. Wen, D.-D. Li, X.-F. Zhu, X.-S. Zhang. Down regulation of c-FLIPL enhances PD-1 blockade efficacy in B16 melanoma. *Front. Oncol.* **9**, 857 (2019).

40. D. De Henau, M. Rausch, D. Winkler, L. F. Campesato, C. Liu, D. H. Cymerman, S. Budhu, A. Ghosh, M. Pink, J. Tchaicha, M. Douglas, T. Tibbitts, N. Kosmider, J. Wang, L. Wang, Y. Yin, P. G. Giresi, A. L. S. Chang, G. X. Y. Zheng, W. J. Greenleaf, E. Sahai, S. Zelenay, C. R. e Sousa. NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control. *Cell* **172**, 1022–1037. e14 (2018).

41. A. Butler, P. Hoffman, P. Smibert, E. Satija, Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* **36**, 411–420 (2018).

42. A. T. Satpathy, J. M. Granja, K. E. Yost, Y. Qi, F. Meschi, G. P. McDermott, B. N. Olsen, M. R. Mumbach, S. E. Pierce, M. R. Corces, P. Shah, J. C. Bell, D. J. Hunter, N. C. Rogers, E. Bonavita, P. Chakravarty, H. Blees, M. Cabeza-Cabrerizo, S. Sammicheli, N. C. Rogers, E. Bonavita, P. Chakravarty, H. Blees, M. Cabeza-Cabrerizo, S. Sammicheli, P. Shah, J. C. Bell, D. J. Hunter, N. C. Rogers, E. Bonavita, P. Chakravarty, H. Blees, M. Cabeza-Cabrerizo, S. Sammicheli.