

Specific inhibitor to KRAS^{G12C} induces tumor-specific immunity and synergizes with oncolytic virus for enhanced cancer immunotherapy

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ABSTRACT

Background Oncolytic virus (OV)-mediated immunotherapy has been shown limited efficacy. Small molecule inhibitors specific to the KRAS^{G12C} driver oncoprotein have recently been developed for cancer treatment. The combination of a potent OV with a KRAS^{G12C} inhibitor could be a potent combination strategy for treating KRAS^{G12C} cancer.

Methods We explored combination therapies using KRAS^{G12C} inhibitor and OV in cancer cells in vitro and in two KRAS^{G12C} cancer models. We employed flow cytometry to evaluate the immune cell profiles, including dendritic cells, macrophages, myeloid-derived suppressor cells, natural killer (NK), subsets of CD4⁺ and CD8⁺ T cells, and exhaustion markers (CTLA-4, PD-1, TIM-3), activation markers (granzyme B, IFN-γ and 4-1BB) as well as enzyme-linked immunospot assay to identify tumor-antigen specific T cells. The importance of CD4⁺, CD8⁺ T and NK cells in the therapeutic effects was evaluated by antibody-mediated depletion in vivo.

Results We confirmed that three inhibitors for KRAS^{G12C}, AMG510 (sotorasib), MRTX849 (adagrasib) and MRTX1257, all displayed potent cytotoxicity to cancer cells harboring KRAS^{G12C}, but not to cancer cells without this specific KRAS mutation in vitro. All three inhibitors exhibited potent antitumor activity in KRAS^{G12C} Lewis lung cancer, but not in MC38 colon cancer with wild-type KRAS. In two KRAS^{G12C} tumor models, either an IL-36γ-armed OV or orally delivered MRTX1257 inhibited tumor growth, but the combination worked much more efficiently, and efficacy was further improved with PD-1 blockade although with no statistical difference in survival, leading to complete tumor remission in a large fraction of the mice. Mechanistic studies revealed that MRTX1257, and other KRAS^{G12C} inhibitors as well, are potent inducers of antitumor immunity by themselves, and that it worked with OV to elicit potent innate and adaptive tumor-specific immunity. The combination therapeutic efficacy depended largely on increased tumor-specific CD8⁺ cytotoxic T cells, and to a smaller degree, on CD4⁺ T and NK cells.

Conclusions Small molecule inhibitors of KRAS^{G12C} are novel inducers of tumor-specific immunity, and a unique triple combination regimen is highly efficacious through elicited potent antitumor immunity for KRAS^{G12C} cancers.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Oncolytic virus (OV)-mediated cancer immunotherapy has shown limited efficacy. A specific inhibitor of KRAS^{G12C} has shown efficacy in lung cancer expressing KRAS^{G12C} oncoprotein.

WHAT THIS STUDY ADDS

⇒ The rational combination of a potent Th1-cytokine-armed OV with KRAS^{G12C} small molecule inhibitor greatly enhances antitumor immunity and leads to improved therapeutic efficacy. The addition of PD-1 blockade further improves the efficacy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our rationally designed triple combination strategy of OV, KRAS^{G12C} small molecule inhibitor and immune checkpoint blockade may provide the best efficacy for cancer driven by this particular type of KRAS mutated oncoprotein. Similar strategies could be developed for other KRAS-G12 mutated or over-expressed cancers.

INTRODUCTION

The oncoprotein KRAS has been one of the most challenging targets in cancer. Cancers driven by KRAS mutations are both common and deadly. KRAS^{G12C} is an oncogenic driver mutation in multiple cancer types, including lung, colorectal, and pancreatic cancers. About 45% of all non-small cell lung carcinoma (NSCLC) KRAS mutations in the USA are KRAS^{G12C},¹ which represents 25,000 patients with NSCLC each year.² Researchers struggled to inhibit mutated forms of KRAS—which earned a reputation as “undruggable”. Due to persistent efforts by numerous investigators over the years, a breakthrough was finally achieved a few years ago. A few small molecule inhibitors specific to KRAS^{G12C} became available for preclinical and clinical studies.^{3–5} Some of these inhibitors function



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by allosterically controlling GTP affinity and effector interactions.⁶ Two phase I clinical trials of the KRAS^{G12C} inhibitors AMG510 (sotorasib) and MRTX849 (adagrasib) have shown robust efficacy in human patients, including those with NSCLC.^{5,7} Two key features related to the current study have been noticed in the application of KRAS^{G12C} inhibitors. One is that the KRAS^{G12C} inhibitor has resulted in the downregulation of IL-36γ in the tumor.⁸ The second one is that AMG510, and possibly others as well, can induce inflammation and thus promote infiltration of immune cells into the tumor tissues.⁵ Based on the definition of immunogenic cell death (ICD), AMG510 induced a type of apoptosis that belongs to ICD.⁹ Intriguingly, the second-generation inhibitors MRTX-849 and MRTX1257 are potent, highly selective and can be delivered orally.¹⁰ Through painstaking preclinical and clinical studies, two small molecule inhibitors, sotorasib and adagrasib, have gained accelerated approval by Food and Drug Administration (FDA) to treat KRAS^{G12C}-mutant NSCLC after positive results in phase 2 trials.^{11,12}

Cancer immunotherapy is rapidly evolving, and treating cancer by activating the patient's immune system presents an attractive therapeutic strategy. Oncolytic virus (OV) is a promising regimen of immunotherapy, as showcased by T-VEC for advanced melanoma approved in the USA, Europe and Japan.^{13,14} OV selectively infects and replicates in cancer cells and/or cancer-associated stromal cells in vivo while leaving normal cells unharmed. Oncolytic vaccinia virus (OVV) and other OVs induce oncolysis of cancer cells, usually in a mode of ICD,^{15–17} leading to subsequently antitumor immunity that is associated with survival of patients with cancer.¹⁸ OVs have been shown in both preclinical studies and clinical trials to induce adaptive antitumor immunity and contribute to the overall efficacy.¹⁸ A randomized phase 3 PHOCUS trial of sequential treatment with Pexa-Vec and sorafenib in patients with advanced hepatocellular carcinoma did not achieve increased clinical benefit and fared worse compared with sorafenib alone.¹⁹ The most promising combination, an OV, T-VEC, with immune checkpoint blockade (ICB), showed highly improved efficacy in patients with advanced melanoma in early phase clinical studies yet failed in randomized, double-blinded, placebo-controlled phase III trial.²⁰ These results underscore the need to develop more rational and innovative combinations of OV-mediated cancer therapies.^{21–23}

We have focused our efforts on OVVs in the last two decades.²⁴ We and others have engineered OVVs to express tumor antigens, T-cell costimulatory molecules, and inflammatory chemokines and cytokines and studied their therapeutic efficacy and safety in preclinical tumor models.^{25–31} Evidence has been accumulated that OVVs induce ICD, with release of extracellular ATP, HMGB1, and increased cell surface exposure of calreticulin and heat shock proteins, hallmarks of danger signals from ICD.^{16,32–34} This ICD lays the foundation for subsequent elicited antitumor immunity. We previously demonstrated that a new member of the IL-1 cytokines, IL-36γ, can

modulate the tumor microenvironment (TME) and elicit potent type I antitumor immunity.^{35,36} Later we developed OVVs armed with the IL-36 gene and showed their efficacy in multiple syngeneic tumor models.³⁷ Indeed we learned that innovative combinations may be necessary to dramatically improve therapeutic efficacy in aggressive tumor models.^{22,23}

In the current study, we designed and studied a rational combination of a specific mutant KRAS-targeted inhibitor and an OV-mediated antitumor immunity for KRAS^{G12C} cancers. Because the small inhibitor to KRAS^{G12C} could downregulate IL-36γ in the tumor,⁸ we hypothesized that it is rational to combine a small molecule inhibitor to KRAS^{G12C} with an IL-36γ-expressing OV for improved antitumor immunity. We showed that this combination worked well in two KRAS^{G12C} tumor models in syngeneic mice, and the addition of ICB further improved its therapeutic efficacy. We envision that this combinatorial approach to targeting various aspects of cancer for synergistic cytotoxicity and potent antitumor immunity would lead to optimal efficacy for KRAS^{G12C} mutant cancer with the potential to become a long-term cure in human patients.

METHODS

Inhibitor compounds: The three inhibitors (AMG510, MRTX849 and MRTX1257) were obtained from commercial sources. AMG510 was purchased from AbMole BioScience (Houston, Texas, USA) while MRTX849 and MRTX1257 were obtained from Chemietek (Indianapolis, Indiana, USA). All three compounds were dissolved in DMSO solvent as stock solutions and stored at −80°C.

Mice and cell lines: Female C57BL/6J (B6) mice, aged 5–6 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and housed in specific pathogen-free conditions in the UPMC Hillman Cancer Center Animal Facility. For the animal studies conducted at China Medical University, it was also approved by the Institutional Committee.

Murine colon cancer cell line MC38, melanoma B16, and Lewis lung cancer (LLC), were originally obtained from American Type Culture Collection (ATCC; Manassas, Virginia, USA). Mouse malignant mesothelioma AE17 cancer cell line was purchased from Millipore Sigma (Burlington, Massachusetts, USA). Mouse colon cancer cell line MC38-luc was described previously.³⁸ All cell lines were tested for mycoplasma once every 3 months or so to ensure they were free of the mycoplasma contamination. The human NSCLC cell lines were originally obtained from ATCC (Manassas, Virginia, USA). H2122 cells contain the homozygous KRAS^{G12C} mutation, while A549 cancer cells contain KRAS^{G12S} mutation.

OVVs: OVVs used in this study were derived from the WR strain. The first virus vTD, previously named vSPT, is an OV with mutations of triple viral genes encoding thymidine kinase (*tk*) and antiapoptotic genes *SPI-1* and *SPI-2*.^{32,39} The second one, vTD-IL36γ, is derived from

wTD with insertion of murine IL-36 γ complementary DNA at the *tk* locus and used in our previous study.^{32 35 39} They were amplified in HeLa cells and then purified and tittered as previously described.³⁸

Cell viability assay: The viability of cancer cells infected by OV or treated with MRTX1257 in vitro was measured at 48 hours after infection using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega, Madison, Wisconsin, USA) or Cell Counting Kit-8 (Boster Bio, Pleasanton, California, USA), according to the manufacturers.

Tumor models and therapeutic treatments: For subcutaneous (s.c.) tumor models, B6 mice were subcutaneously inoculated with 5.0e5 LLC or MC38 colon cancer cells. For the s.c. AE17 tumor, 1.0e7 cancer cells were mixed with Matrigel for increased initiation and growth of the tumor. When the tumors reached the sizes 100–125 mm³, wTD, wTD-IL36 γ , or phosphate-buffered saline (PBS) was intratumorally injected at a dose of 2.0e6 pfu/tumor and mice were treated daily with oral administration of 0, 60, 100, and 200 mg/kg of MRTX1257, or with the other two inhibitors at indicated concentrations. For combination therapy, the viral dose at 2.e6 pfu/tumor and MRTX1257 at 60 mg/kg daily were used unless indicated otherwise. The primary tumor size was measured using an electric caliper in two perpendicular diameters followed by measurement once every 3 days. For long-term survival of mice, the health and survival of treated mice was closely monitored. Mice died naturally due to the disease or were sacrificed when their s.c. tumor size exceeded 20 mm in diameter.

Flow cytometry: Tumor tissues were collected from mice, then minced and incubated in RPMI 1640 medium containing 2% fetal bovine serum (FBS), 1 mg/mL collagenase IV (Sigma; #C5138), 0.1 mg hyaluronidase (Sigma; #H6254), and 200 U DNase I (Sigma; #D5025) at 37°C for 1 hour to make single cells. Single-cell samples were processed for flow cytometry as described.⁴⁰ After staining with 100 μ L Zombie Aqua Fixable Viability Kit cell dye (BioLegend, San Diego, California, USA), cells were stained in 100 μ L total stain volume (50 μ L BV stain buffer, 50 μ L 2% FBS) with antibody at a dilution of 1:200 for 30 min on ice in the dark. The sources of antibodies are listed in online supplemental table 1.

Analysis of immune responses by reverse transcription-quantitative PCR (RT-qPCR): On day 6 and day 11 post-viral treatments, tumor tissues or spleens were harvested, and then single-cell suspension was made for cell separation and further analysis of immune cells either by flow cytometry or by RT-qPCR. The primers used are listed in online supplemental table 2.

Enzyme-linked immunospot (ELISpot) assay: Collected tumor tissues were cut into pieces and incubated at 37°C in digestion buffer (Miltenyi Biotec, San Diego, California, USA) before being mashed over a 100 μ m tissue strainer. We performed the ELISpot assay as described previously.⁴⁰

Statistics

Statistical analyses were performed using unpaired Student's t test for two-group comparison. The Student's t-test was applied to compare tumor sizes between groups at the final measured time point of each experiment. For multiple group comparisons, one-way analysis of variance was used, where p value is adjusted for multiple tests by Dunnett method (GraphPad Prism V.5). Animal survival is presented using Kaplan-Meier survival curves and compared by using log-rank test (GraphPad Prism V.5). Data are presented as mean $\pm$ SD unless indicated otherwise. We considered p values of <0.05 to be statistically significant, and all p values were two-sided. In the figures, standard symbols are used: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; and NS: not significant.

RESULTS

Three inhibitors to KRAS^{G12C}, alone or in combination with OV, inhibit the growth of KRAS^{G12C} cancer cells

We have selected three small molecule KRAS^{G12C} inhibitors, with two of them approved by the FDA for cancer treatment, to confirm that they indeed inhibit growth of cancer cells harboring the specific KRAS^{G12C} mutation. For this experiment, LLC (KRAS^{G12C} mutant) and MC38 (KRAS wild type) were selected. The cancer cells were plated in 96-well plates overnight and then treated with various concentrations of the inhibitors. At 48-hour post-treatment, CCK8 assays were conducted to measure cell viability. All three inhibitors, AMG510 (sotorasib), MRTX849 (adagrasib) and MRTX1257, inhibited the growth of LLC cells in a dose-dependent manner (figure 1A). Under the same concentration, MRTX1257 showed the highest activity, followed by MRTX849, and AMG510 being the least potent. When tested in non-KRAS^{G12C} mutant MC38 cells, they showed little activity (figure 1B). These results confirmed and extended the reported results. As MRTX1257 showed most potent activity, we used MRTX1257 for most experiments carried out later but also confirmed key findings with the other two inhibitors, AMG510 and MRTX849.

We have used two OVVs, one backbone virus, wTD (without IL-36 γ gene) and the other armed with IL-36 γ (wTD-IL-36 γ) in this study (Figure 1C). To further evaluate KRAS^{G12C} inhibitors alone, we first picked MRTX1257, a covalent inhibitor specific to KRAS^{G12C} oncoprotein, and studied its cytotoxicity in a panel of human and murine cancer cell lines that harbor KRAS^{G12C} mutant protein or non-KRAS^{G12C} protein (Figure 1D). Among murine cancer cell lines, LLC (lung cancer) and AE17 (mesothelioma) are KRAS^{G12C} mutant, while MC38 (colon cancer) is not. Among human lung cancer cell lines, H2122 is KRAS^{G12C} mutant while A549 is not (in fact, KRAS^{G12S} mutant). The cancer cells were plated in 96-well plates overnight and then treated with various concentrations of MRTX1257 (figure 1D). The cancer cells were treated with MRTX1257 at doses ranging from 0.0, 0.016 up to 10,000 nM. The viable cells were measured at 48 h

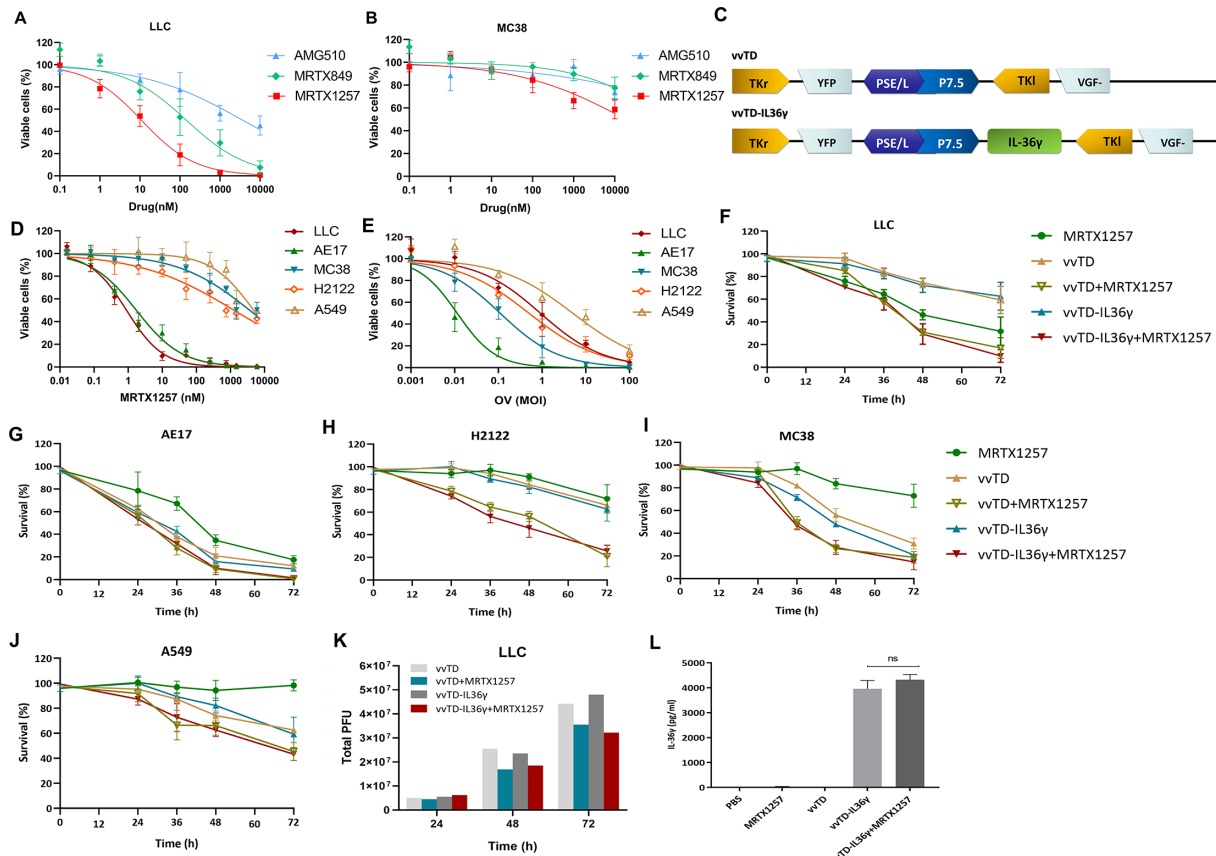


Figure 1 Cytotoxicity of three KRAS^{G12C} inhibitors and combination of MRTX1257 and OVs in KRAS^{G12C} mutant and non-mutant cancer cells in vitro. (A) Dose-dependent inhibition of LLC cancer cells harboring KRAS^{G12C} cells; (B) Effects on KRAS wild-type MC38 cancer cells. Cancer cells were plated in 96-well plates overnight and then cultured in the presence of drug at the indicated concentrations of the inhibitors for 48 hours and cell viability was assessed using CCK8 assay. (C) The schematic presentation of vVTD and vVTD-IL36γ viral constructs. TKr, the right portion of the tk gene; TKl, the left portion of the tk gene. (D) Cancer cell susceptibility towards MRTX1257. The KRAS^{G12C} mutant cancer cell lines include murine lung cancer LLC, mesothelioma AE17, and human lung cancer H2122, while non-KRAS^{G12C} mutant cancer cell lines include murine colon MC38 and human lung cancer A549. Cancer cells were plated in 96-well plates overnight and then cultured in the presence of drug at concentrations from 0, 0.016 up to 750 nM for a duration of 48 hours and cell viability was assessed using CCK8 assay. (E) Cytotoxicity induced by OVs for the KRAS^{G12C} mutant and non-mutant cancer cells. Cancer cells were infected with vVTD-IL36γ at various MOIs from 0, 0.001 to 100, and cell viability was measured at 48 hours postinfection. Data represented at least triplicates. The value for viable cells at MOI=0 was set at 100%. (F–J) Cell killing activity of vVTD and vVTD-IL36γ combined with MRTX1257. Cancer cells were treated with vVTD or vVTD-IL36γ at an MOI of 0.5 and/or 100 nmol MRTX1257 simultaneously. Cytotoxicity was measured at 24, 36, 48, and 72 hours. Data represented at least three replicates. (K) LLC cells were infected with vVTD or vVTD-IL36γ at MOI of 1 with or without 10 nM MRTX1257 simultaneously, then harvested at varying time points, as virus titers were determined by plaque assay on CV-1 cells. (L) Production and secretion of IL-36γ from infected CV1 cells. Cells were mock-infected or infected with vVTD or vVTD-IL36γ at an MOI of 1.0, with MRTX1257 alone or in combination. Then, conditioned media were harvested at 48 hours postinfection. The amount of the cytokine protein secreted into the medium was quantified using ELISA. LLC, Lewis lung cancer; MOIs, multiplicities of infection; OV, oncolytic virus; PBS, phosphate-buffered saline; tk, thymidine kinase.

post-treatment using CCK8 assay. We observed that LLC and AE17 cells (both KRAS^{G12C} mutant) are highly sensitive to MRTX1257, while MC38 is much less sensitive. The difference in sensitivity is ~1,000-fold. Human cancer cells are generally less sensitive to the inhibitor, however, H2122 cells (KRAS^{G12C} mutant) are indeed much more sensitive to the inhibitor when compared with A549 (non-KRAS^{G12S} mutant).

We also tested these cancer cells for cytotoxicity mediated by OVs. Cells were plated in 96-well plates overnight and then infected with various multiplicities of infection

(MOIs) of vVTD-IL36γ, up to 100 MOIs (figure 1E). We observed that the sensitivity of these cancer cells varied greatly, with a difference of LD50 over 1,000-fold. In this case, MC38 cells were the most sensitive, while A549 cells were the least sensitive.

Then, we examined the cytotoxicity of combinations of OV with MRTX1257 in these cancer cell lines (figure 1F–J). For straightforward comparison, we set the MOI of OV at 0.5 and MRTX1257 at 100 nM and then compared the five cancer cell lines for their susceptibility

to these two types of antitumoral agents (figure 1F-K). We made the following observations. First, we confirmed that KRAS^{G12C} mutant cancer cells (murine LLC, AE17 and human H2122) are more sensitive to MRTX1257 than non-KRAS^{G12C} mutant cancer cells (murine MC38 and human A549). Second, all cancer cell lines are sensitive to cytotoxicity induced by OV (both vTD and vTD-IL36γ), even though degrees of sensitivity were different. We also examined the effect of MRTX1257 on the viral replication of vTD and vTD-IL36γ (figure 1K). It turned out that MRTX1257 had little, if any, inhibitory effect on viral replication and accumulation. To verify, cells infected with vTD-IL36 alone or in combination with MRTX1257 led to production and secretion of high levels of IL-36γ (figure 1L). Finally, when combined, the effect was cooperative in KRAS^{G12C} cancer cells, such as LLC and H2122 (figure 1F and H).

In summary, all three KRAS^{G12C} inhibitors (AMG510, MRTX849, MRTX1257) showed significant antitumor efficacy in KRAS^{G12C} mutant cancer cells, yet little activity on KRAS^{G12C} wild-type cancer cells. The combination of OV with MRTX1257 further enhanced the killing of cancer cells. In addition, the inhibitor did not affect the replication of OV and production of the cytokine IL-36γ from vTD-IL36γ-infected cancer cells.

KRAS^{G12C} inhibitors and/or vTD-IL36γ inhibited tumor growth

We then asked if three inhibitors could inhibit tumor growth in both KRAS^{G12C} mutant and non-mutant tumors (figure 2 and online supplemental table 3). MRTX1257 was tested first. In KRAS^{G12C} mutant LLC and AE17 tumor models, MRTX1257 was very efficacious at inhibiting tumor growth and worked in a dose-dependent manner (figure 2A and B). However, the inhibitor had no effect

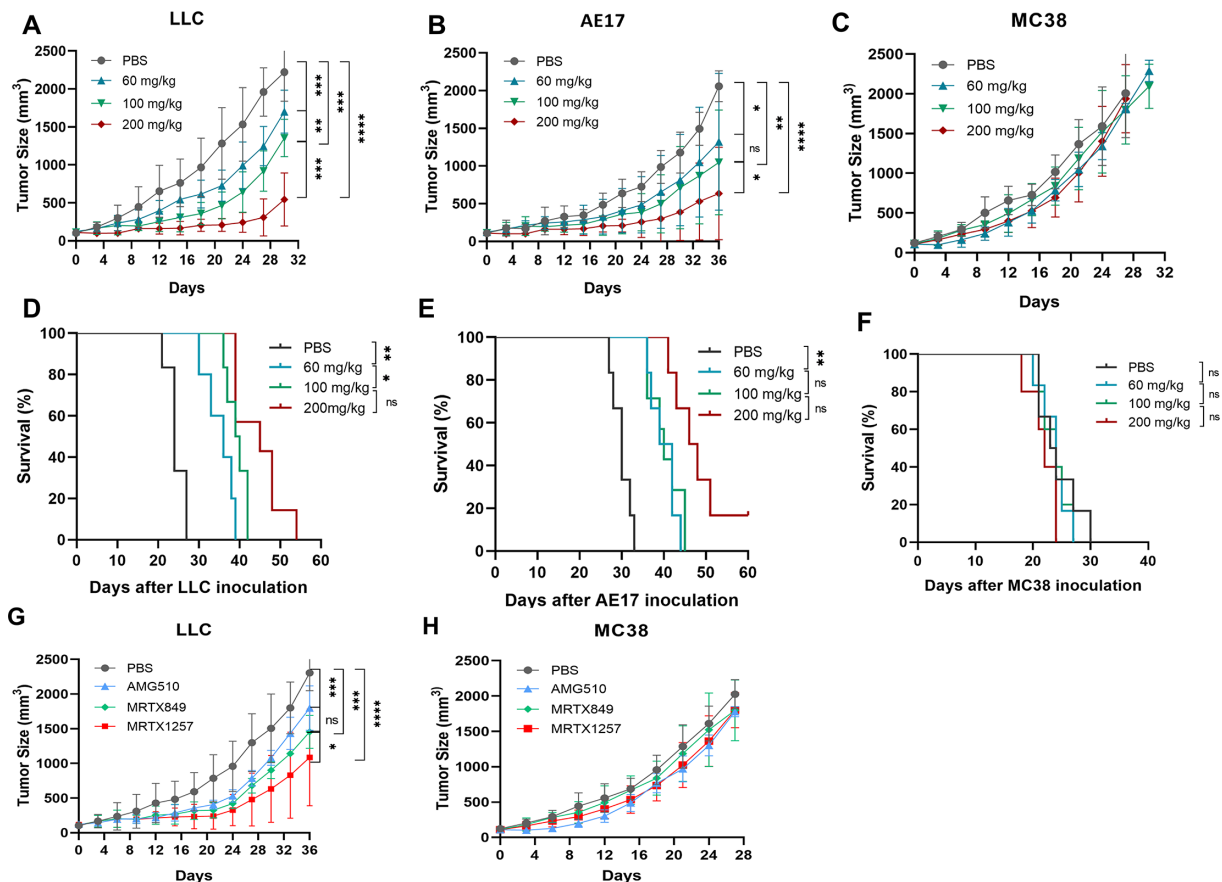


Figure 2 Antitumor efficacy of three KRAS^{G12C} inhibitors in three syngeneic subcutaneous tumor models. B6 mice were subcutaneously inoculated with LLC (1.0e6 cells), AE17 (1.0e7 cells), or MC38 (2.0e5 cells) cancer cells, respectively, on day 0 (D0). When tumor sizes reached 100–150 mm³ in size (around day 10), mice were treated daily with 0, 60, 100, and 200 mg/kg oral administration of MRTX1257. (A, D) LLC tumor; (B, E) AE17 mesothelioma; (C, F) MC38 colon cancer. LLC and AE17 are KRAS^{G12C} tumors while MC38 tumor is non-KRAS^{G12C}. (E, F) For a direct comparison, antitumor efficacy of AMG510, MRTX849 and MRTX1257 was tested in the same doses in two subcutaneous tumor models. B6 mice were subcutaneously inoculated with LLC or MC38 tumor cells on day 0 (D0). When tumor sizes reached 100–150 mm³ in size (around days 7–10), mice were treated daily with 60 mg/kg oral administration of AMG510, MRTX849 and MRTX1257. (G, H) When sizes reached 100–150 mm³ in size (around days 7–10), mice were treated daily with 60 mg/kg oral administration of AMG510, MRTX849 and MRTX1257. Student's t-test was applied to compare tumor sizes between groups at the final measured time point. (G, H) When sizes reached 100–150 mm³ in size (around days 7–10), mice were treated daily with 60 mg/kg oral administration of AMG510, MRTX849 and MRTX1257. n=6; *p<0.05, ***p<0.001. LLC, Lewis lung cancer; PBS, phosphate-buffered saline.

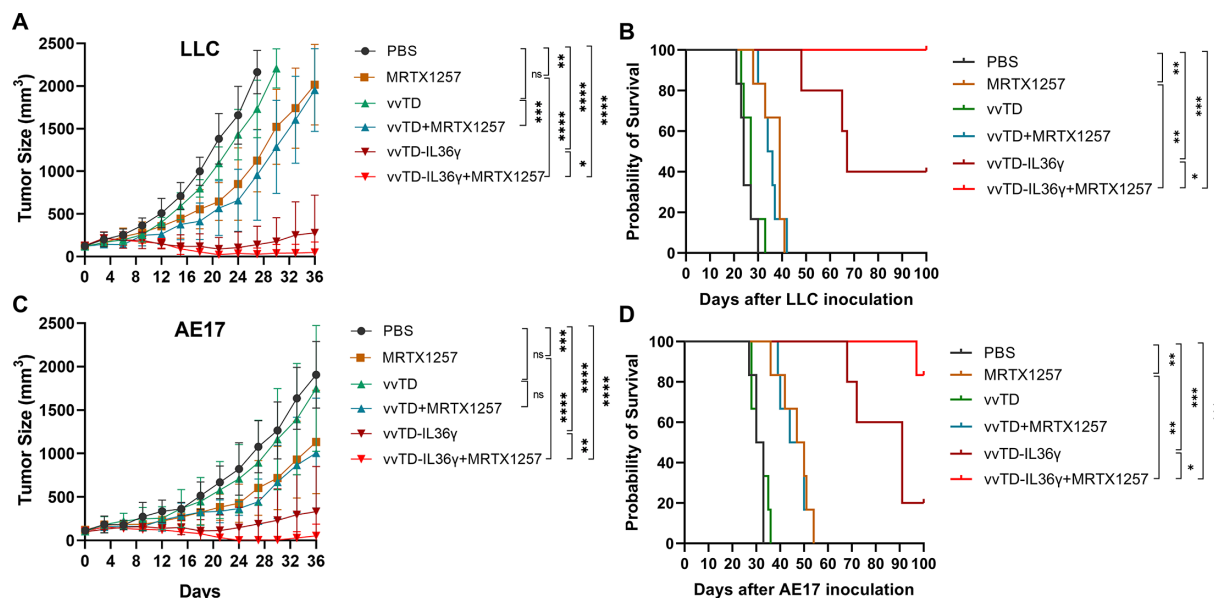


Figure 3 Antitumoral effects of an OV or in combination with MRTX1257 in murine KRAS^{G12C} tumor models. B6 mice were inoculated subcutaneously with 1.0e6 LLC or 1.0e7 AE17 cancer cells on the right flank. When the tumor reached an average of 100–150 mm³ (mostly on day 10), the mice were randomly divided into six groups (PBS; vvTD; MRTX1257; vvTD+MRTX1257; vvTD-IL36γ; vvTD-IL36γ+MRTX1257) (n=7–8). On day 11, mice were injected intratumorally with 1.0e6 PFU of vvTD, vvTD-IL36γ, or PBS, and then on day 13 were treated with the MRTX1257 (60 mg/kg, daily for 3 weeks). The growth curves of tumors and survival curves of mice are shown with LLC cancer model (A, B) and AE17 tumor model (C, D). The growth curves of tumor sizes are presented as mean±SD. Data represent two independent experiments. Student's t-test was applied to compare tumor sizes between groups at the final measured time point. n=5; *p<0.05, **p<0.01, ***p<0.001. LLC, Lewis lung cancer; ns, not significant; OV, oncolytic virus; PBS, phosphate-buffered saline.

on KRAS wild-type MC38 colon tumor model (figure 2C). The antitumoral effects were also reflected in the extension of survival of mice in a dose-dependent manner in the LLC and AE17 tumor models, but not in the MC38 tumor model (figure 2D–F). In addition, we have observed that this inhibitor displayed similar quantitative inhibitory effects over time on both LLC and AE17 tumors.

We have then directly compared the efficacy of three inhibitors under the same concentration (60 mg/kg body weight) in LLC and MC38 tumors in syngeneic B6 mice (figure 2G and H). Not surprisingly, all three inhibitors inhibited the growth of LLC, with MRTX1257 showing the largest effect and AMG510 the smallest inhibitory effect (p<0.001 for all three inhibitors compared with PBS) (figure 2G). None of the inhibitors had any inhibitory effects on the growth of MC38 tumor, which possesses wild-type KRAS gene. In summary, these results obtained from three syngeneic tumor models are consistent with the specificity and action of all three KRAS^{G12C} inhibitors.

Then we proceeded to test the potential of combining the inhibitor with a potent IL-36γ-armed OV in KRAS^{G12C} mutant tumor models (figure 3; online supplemental table 3). Looking for potential cooperation between the two agents, we used the two agents at suboptimal concentrations: MRTX1257 at 60 mg/kg body weight (oral delivery), and OVs at 2.0e6 pfu/mouse (intratumoral delivery). In the LLC tumor model (figure 3A and B), MRTX1257 showed a significant antitumoral effect (figure 3A, p<0.05 compared with PBS-treated group). vvTD, the parental OV, showed very little therapeutic

effect at this dose (not significant (ns), compared with PBS), yet IL-36γ-armed OV displayed potent antitumor efficacy (p<0.0001, when compared with PBS). The combination of vvTD with MRTX1257 did not further improve the therapeutic efficacy. In contrast, the combination of MRTX1257 and vvTD-IL36γ led to greater therapeutic efficacy than individual treatment (p<0.05 compared with vvTD-IL36γ; p<0.0001 compared with MRTX1257). Similar patterns of antitumoral effects were also observed in the AE17 tumor, another KRAS^{G12C} mutant tumor model (figure 3C). Consistently, we found that dual treatment with MRTX1257 and vvTD-IL36γ resulted in the longest overall survival in both LLC and AE17 tumor models (figure 3B and D) (p≤0.05). These data indicated that KRAS^{G12C} inhibitor and IL36γ-armed OV work cooperatively to exert their antitumoral effect in vivo.

Modulation of the tumor microenvironment by inhibitors and vvTD-IL36γ in murine tumors harboring KRAS^{G12C}

We then examined the immune profile dynamics in the tumor tissue on days 6 and 11 after treatments. We analyzed CD4⁺ and CD8⁺ T cell subsets from samples collected on days 6 and 11 post-therapy (figure 4). There was a reduction of CD4⁺ T cells in all treated groups compared with the group treated with PBS on days 6 and 11 (figure 4A,B). However, there were more CD8⁺ T cells in groups treated with either vvTD-IL36γ or MRTX1257 when compared with PBS control group (p<0.01) (figure 4A,B). In dual therapy, there was a further increase of CD8⁺ T cells compared with either one of

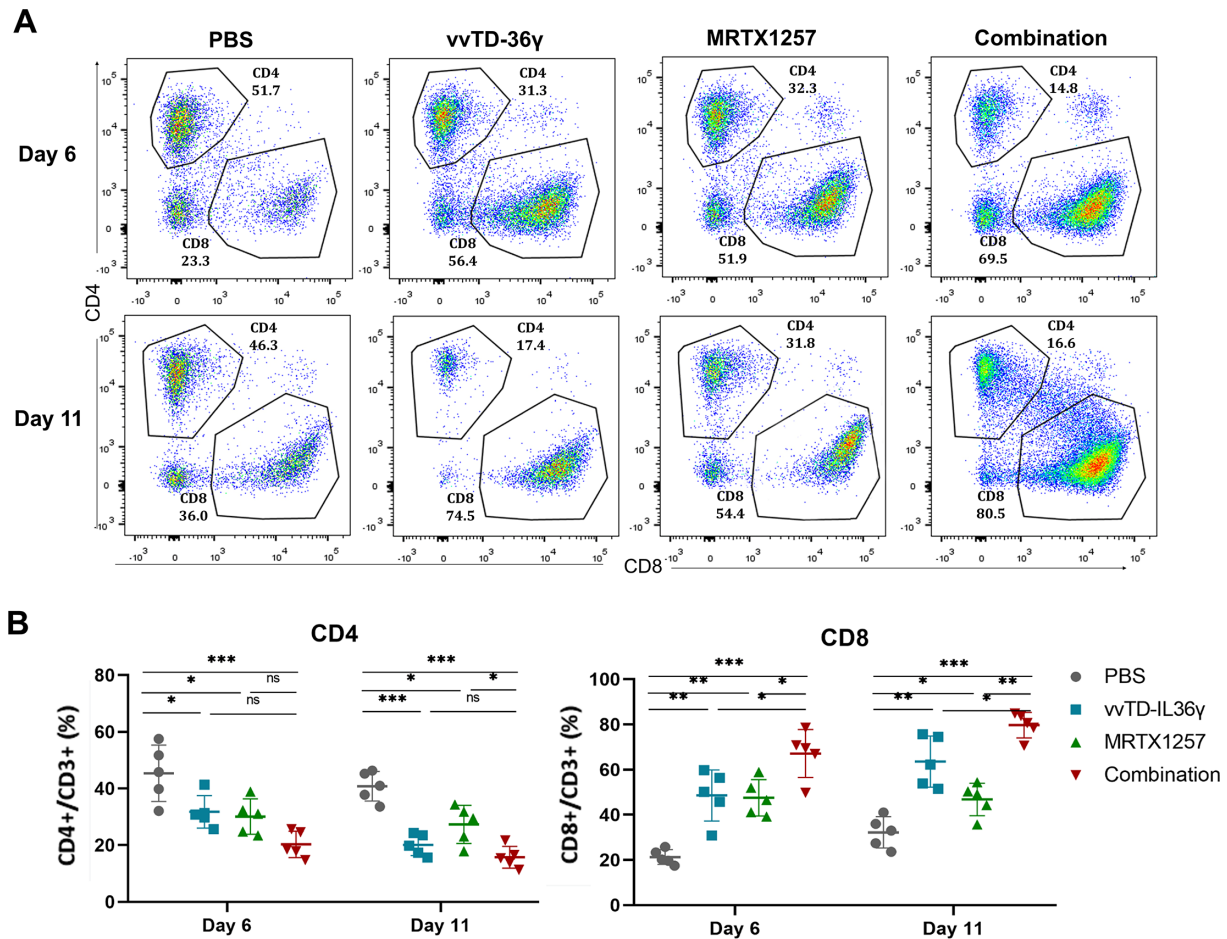


Figure 4 Changes in quantities of CD4⁺ and CD8⁺ T cell subsets and key functional molecules in the LLC tumor microenvironment after therapy with vvTD-IL36γ, MRTX1257, or the dual combination. B6 mice were inoculated s.c. with 1.0e6 LLC cells. On day 11, mice were randomly split into groups and treated with PBS, single dose of vvTD-IL36γ (2.0e6 PFU/mouse) that day, MRTX1257 (60 mg/kg; oral gavage daily for 3 weeks), or combination (vvTD-IL36γ+MRTX1257). Tumor-bearing mice were sacrificed on days 6 and 11 post-treatment, and tumor tissues were collected, and single cells were prepared and analyzed using flow cytometry (A) CD4⁺ and CD8⁺ T cells on days 6 and 11 post-therapy; (B) Four to five mice were used for each treatment group, and ANOVA (Tukey's multiple comparisons) was used for respective comparisons. *p<0.05; **p<0.01; ***p<0.001; and ****p<0.0001. ns: not significant. ANOVA, analysis of variance; LLC, Lewis lung cancer; PBS, phosphate-buffered saline; s.c., subcutaneous.

the monotherapy (p<0.05 compared with monotherapies; p<0.001 when compared with PBS). We continued to analyze the key molecular markers for the status of immunity in the TME using RT-qPCR (online supplemental figure 1 A-F). NKG2D, IFN-γ, and GzmB were all enhanced by either monotherapy, and either maintained at a high level or further increased in dual therapy (online supplemental figure 1C-E). As for IL-36γ messenger RNA expression, there were high levels of expression in vvTD-IL36γ or dual therapy on day 6 (p value: ns), and the levels were reduced by day 11 (online supplemental figure 1F).

We also performed a direct comparison of the immune profiling on the tumors treated with AMG510, MRTX849, MRTX1257 in relation to the PBS group (online supplemental figure 2). They showed very similar patterns of increases or decreases of certain subsets of immune cells. CD8⁺ T cells, natural killer (NK) cells, dendritic cell (DC), ratio of M1/M2 macrophages, IFN-γ are all increased, while regulatory T (Treg) and myeloid-derived

suppressor cell (MDSC) quantities showed a tendency to decrease although statistically insignificant. In summary, all three inhibitors elicited similar pro-antitumor immune profiles in the TME.

Then we analyzed subsets of immune cells in the tumor tissue using flow cytometry at day 6 after treatments with vvTD-IL36γ, MRTX1257 or combination (figure 5; online supplemental figure 3). The data set on day 11 are presented in online supplemental figure 4 and 5. There were NK cells and DCs in the treated groups (figure 5A,B). There were more macrophages (figure 5C), and more importantly, more M1-like macrophages in all treated groups (p<0.05 compared with PBS) (figure 5D). We then analyzed the subsets of CD8⁺ T cells (figure 5E-L). In all treated groups, both naive T cells (T_n) and central memory T cells (T_{cm}) were reduced (figure 5E,F), accompanied by a concurrent increase of effector memory T cells (T_{em}) (figure 5G). IFN-γ⁺CD8⁺ and PD-1⁺CD8⁺ T

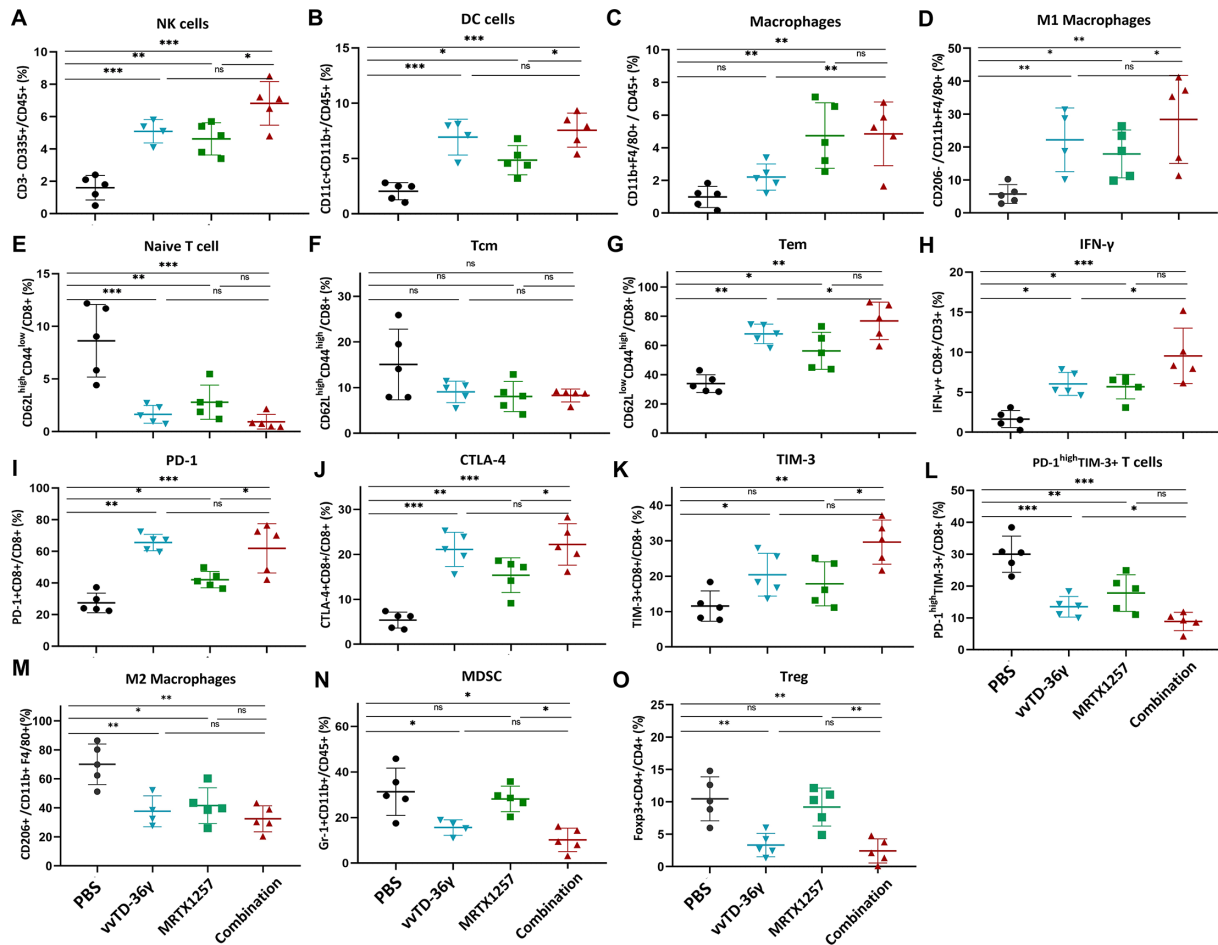


Figure 5 Changes in immune status in the tumor microenvironment after monotherapies or dual therapy with vvTD-IL36 γ and MRTX1257. B6 mice were inoculated s.c. with 1.0e6 LLC cells and treated with PBS, vvTD-IL36 γ as described (2.0e6 PFU/mouse 11 days post-tumor inoculation), MRTX1257 (60 mg/kg per mouse 13 days postinoculation using a daily schedule by oral gavage for 3 weeks), or combination (vvTD-IL36 γ +MRTX1257 scheduled as before). Tumor-bearing mice were sacrificed 6 days post-treatment and primary tumors were collected and analyzed using flow cytometry. (A) NK (NKG2D⁺ CD3⁺); (B) DC (CD11c⁺ CD11b⁺); (C) Macrophages (CD11b⁺ F4/80⁺); (D) M1-like Macrophages (CD206⁺ of CD11b⁺ F4/80⁺ cells); (E) Naive T cells (CD62L⁺ CD44^{low}); (F) Central memory T cells (CD62L⁺ CD44^{high}); (G) Effector memory T cells (CD62L^{low} CD44^{high}); (H) CD8⁺ IFN- γ ⁺ T cells; (I) PD-1⁺ CD8⁺ T cells; (J) TIM-3⁺ CD8⁺ T cells; (K) CTLA-4⁺ CD8⁺ T cells; (L) Exhausted CD8⁺ T cells (PD-1^{hi} TIM-3⁺ CD8⁺); (M) M2-like macrophages (CD206⁺ of CD11b⁺ F4/80⁺ cells); (N) Myeloid-derived suppressor cells; (O) Regulatory T cells (Foxp3⁺ among CD4⁺ cells). Four to five mice were used for each treatment group and ANOVA (Tukey's multiple comparisons) was used for respective comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. ANOVA, analysis of variance; DC, dendritic cell; LLC, Lewis lung cancer; MDSC, myeloid-derived suppressor cell; NK, natural killer; ns, not significant; PBS, phosphate-buffered saline; Tcm, central memory T cells; Tem, effector memory T cells; Treg, regulatory T cell.

cells were increased, indicating activated cytotoxic T cells (figure 5H,I). However, CTLA-4⁺ CD8⁺ T cells were increased in all treated groups (figure 5J). Tim-3⁺CD8⁺ T cells were not increased in the monotherapy groups but were enhanced in the dual therapy group (figure 5K). Interestingly, PD-1^{hi} TIM-3⁺ CD8⁺ T cells, as previously shown to be terminally exhausted,⁴¹ were reduced significantly in all treated groups ($p < 0.01$ between PBS and MRTX1257; $p < 0.001$, PBS vs vvTD-IL36 γ or dual therapy) (figure 5L). Finally, we examined three key types of immunosuppressive cells (figure 5M–O). M2 macrophages were reduced in all treated groups (figure 5M). MDSCs and Treg cells were reduced in the vvTD-IL36 γ and dual-treated groups, but not in the MRTX1257 group (figure 5N,O).

We have also analyzed the immune cell subsets and immune checkpoint markers on the cell surface in splenocytes on days 6 and 11 after treatments and observed similar patterns of changes as in TILs (online supplemental figure 6 and 7).

The efficacy of dual therapy depends largely on CD8⁺ T cells, and anti-PD-1 further enhanced the efficacy

Having demonstrated that both MRTX1257 and OV can elicit adaptive antitumor immunity, we asked if one or more types of immune cells play an essential role right after initiation of the treatments (figure 6). The schedule of the treatments, and antibodies to deplete CD4⁺, CD8⁺, and NK cells in LLC-bearing mice, is presented in figure 6A. We conducted in vivo experiments on an

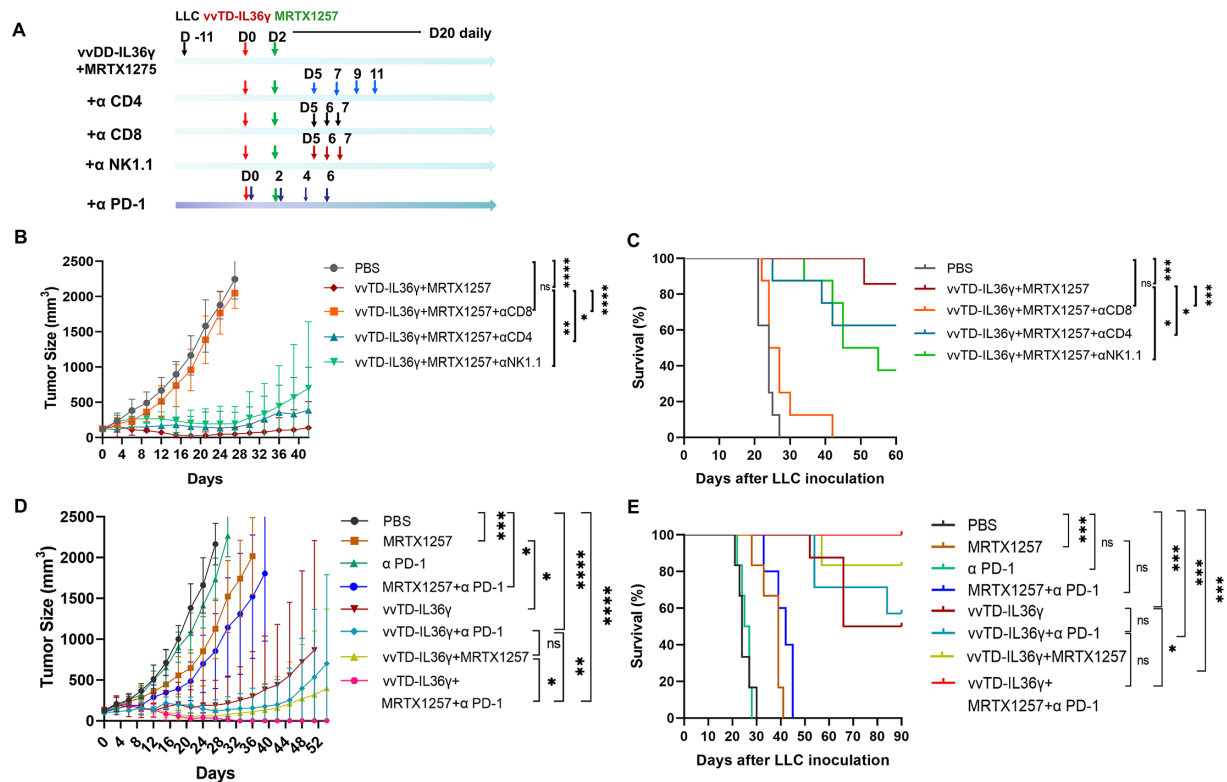


Figure 6 vVTD-IL36γ or/and MRTX 1257 stimulates CD8⁺ T cell-dependent antitumor immunity, and triple combination led to best therapeutic efficacy. B6 mice bearing subcutaneous LLC tumors, when tumors reached 100–150 mm³ in size (around D11), were injected intratumorally (i.t.) with PBS or 2.0e6 PFU vVTD-IL36γ, and/or MRTX1257 (on day 13 postinoculation) daily by oral gavage for 3 weeks. For cell depletion of CD8⁺, CD4⁺, and NK cells, anti-CD8 Ab (250 μg per injection), anti-CD4 Ab (150 μg per injection), or PK136 (300 μg per injection) were intraperitoneally injected into mice. (A) Antibody immune cell depletion was performed according to the schedule shown. (B) Tumor size progression. Student's t-test was applied to compare tumor sizes between groups at the final measured time point. (C) Long-term survival of mice. Log-rank (Mantel-Cox) test was used to compare survival rates in different groups. **p*<0.05; ***p*<0.01; ****p*<0.001; and *****p*<0.0001. (D, E) Dual therapy induced PD-1 expression in tumors and PD-1 blockade dramatically enhanced antitumor efficacy. In separate experiments, LLC tumors were established in B6 mice as described before. Mice then were injected i.t. with PBS or vVTD-IL36γ (at 2.0e6 PFU) on D11, MRTX1257 on D13 for 3 weeks, with or without 200 mg of anti-PD-1 Ab (200 μg per injection), which was given every 2 days for a total of four doses. (D) Tumor sizes and (E) survivals of mice are presented. Log-rank (Mantel-Cox) test was used to compare survival rates. **p*<0.05; ***p*<0.01; ****p*<0.001; and *****p*<0.0001. Ab, antibody; LLC, Lewis lung cancer; NK, natural killer; ns, not significant; PBS, phosphate-buffered saline.

LLC tumor model treated with dual therapy and some groups with additional infusion of antibodies to deplete CD4⁺, CD8⁺, or NK1.1⁺ cells. Tumor growth is plotted in figure 6B, and survival of the mice is plotted in figure 6C. As shown, tumor sizes in mice in the PBS group reached over 2,000 mm³ by day 24. In contrast, the group with dual treatment (vVTD-IL36γ and MRTX1257) remained at baseline on day 42, the end of the plot. When depleted with either CD4⁺ T or NK cells, there were small but significant losses of efficacy (*p*<0.05 for NK, and *p*<0.001 for CD4⁺ cells), indicating that NK and CD4⁺ T cells play some roles in therapy. When CD8 was depleted in those mice, we observed a complete loss of efficacy (*p*<0.0001), indicating that CD8⁺ T cells play absolute key roles in the therapeutic efficacy.

As we have shown earlier, dual therapy enhanced PD-1⁺ CD8⁺ T cells, especially PD-1^{hi} TIM-3⁺ CD8⁺ T cells in the TME (figure 5I,L). Therefore, it is logical to ask if PD-1 ICB would enhance the efficacy of dual therapy

(figure 6D,E). Considering all expected results from numerous control groups, we focus on the final two groups with best therapeutic effects. Indeed, the triple therapy group treated with vVTD-IL36γ+MRTX1257+αPD-1 had better results (*p*<0.05, when compared with the “vVTD-IL36γ+MRTX1257” dual therapy group). This superior tumor growth inhibition seen in the triple therapy group also translated into the best survival, with 100% of the mice surviving through the duration of the experiment (more than 90 days) (figure 6E). In the dual therapy, only 80% of mice survived, even though there was no statistical significance between the two groups.

The dual therapy elicited potent tumor-specific adaptive immunity

We examined the adaptive antitumor immunity by conducting IFN-γ ELISpot assays and analyzing a key activation marker molecule, 4-1BB, on T cells isolated from LLC tumor-bearing mice. First, we conducted the ELISpot

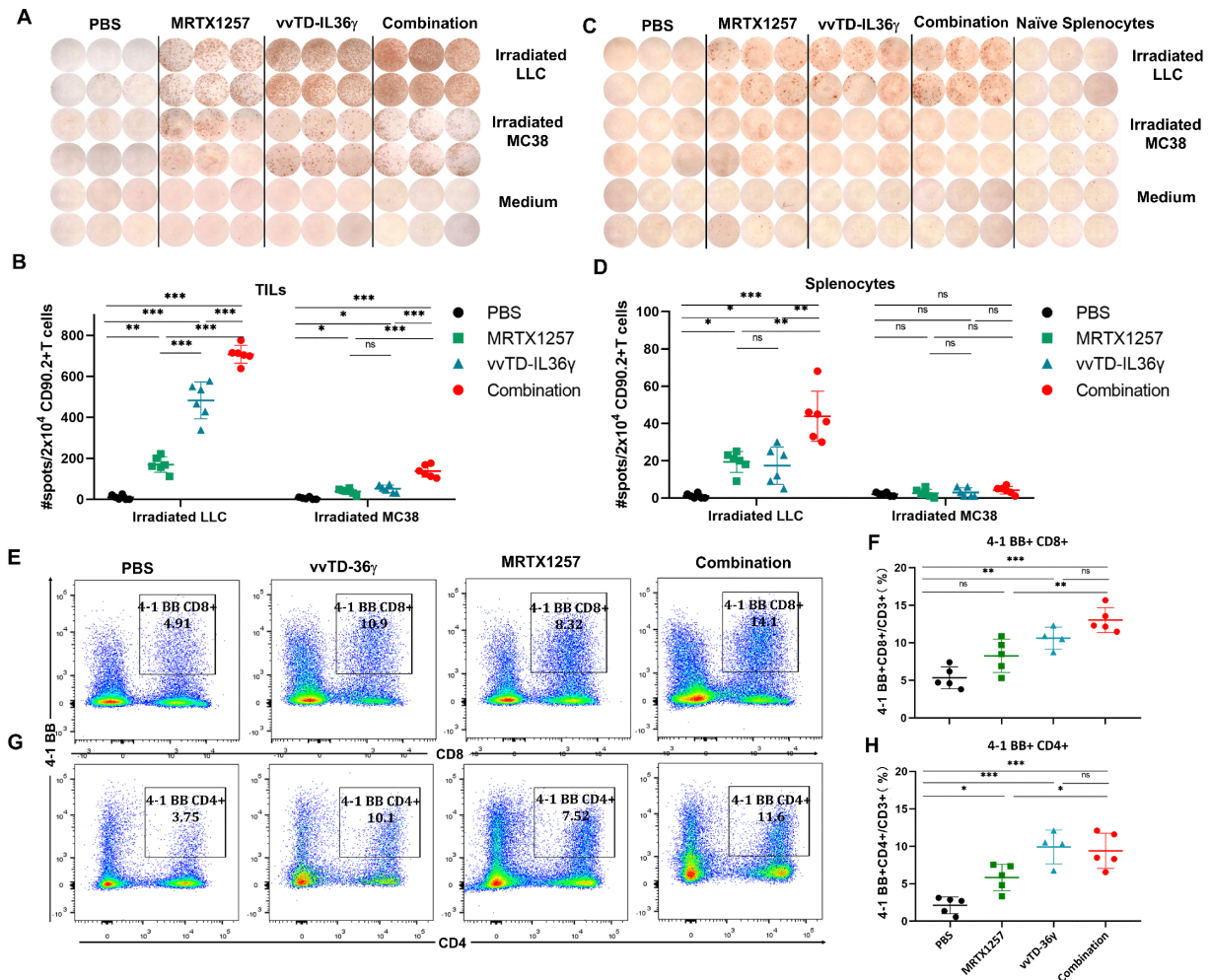


Figure 7 vTID-IL36 γ and MRTX1257 induced LLC tumor-specific CD8 $^{+}$ T cells in LLC tumor model. (A, B) Representative image and quantified data of IFN- γ ELISpot assay of 2.0×10^4 CD90.2 $^{+}$ T cells from tumor tissues (TILs) on day 7 post oncolytic virotherapy or drug treatment and co-cultured 1:1 with specific (LLC) and unspecific (MC38) target cells. (C, D) Representative image and quantified data of IFN- γ ELISpots of 1.0×10^5 CD90.2 $^{+}$ T cells isolated from the spleen on day 7 post oncolytic virotherapy or drug treatment, and co-cultured 1:1 with specific (LLC) and unspecific (MC38 tumor cells, medium, or splenocytes) target cells. (E–H) Flow cytometric analysis of percentages of 4-1BB $^{+}$ CD8 $^{+}$ and 4-1BB $^{+}$ CD4 $^{+}$ T cells in the tumor tissues at day 11 post oncolytic virus, MRTX1257 or dual therapy. (E, F) 4-1BB $^{+}$ CD8 $^{+}$ T cells. (G, H) 4-1BB $^{+}$ CD4 $^{+}$ T cells. CD137 is another name for 4-1BB. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. ELISpot, enzyme-linked immunospot; LLC, Lewis lung cancer; ns, not significant; PBS, phosphate-buffered saline; TILs, tumor-infiltrating lymphocytes.

assay using tumor-infiltrating lymphocytes (TILs) from LLC tumor tissues, and T cells from splenocytes isolated by CD90.2 affinity columns were incubated with irradiated LLC cells or control irradiated MC38 colon cancer cells (figure 7A–D). For TILs, there was a significant number of spots (~180) in TILs from the MRTX1257 group ($p < 0.01$ compared with PBS). vTID-IL36 elicited more spots (~480). In the dual therapy group, the number of spots increased further, to ~700 ($p < 0.001$, compared with either monotherapy group). There were very few spots when incubated with MC38 cancer cells, indicating tumor specificity.

We also conducted ELISpot assays with T cells isolated from splenocytes. The number of spots was at least 10-fold lower, even though the patterns looked similar (figure 7C,D). These results indicated that tumor-specific T cells were enriched in the tumor tissues.

4-1BB expressed on the surface of T cells is an activation marker for tumor-reactive T cells.⁴² Therefore, we used flow cytometry to evaluate the quantities of 4-1BB $^{+}$ CD4 $^{+}$ and 4-1BB $^{+}$ CD8 $^{+}$ T cells (figure 7E–H). We observed a statistically significant increase of 4-1BB $^{+}$ CD8 $^{+}$ T cells in both monotherapy groups and a trend to increase further in the dual therapy group ($p < 0.01$ compared with PBS). 4-1BB $^{+}$ CD4 $^{+}$ T cells also increased in all three treated groups ($p < 0.05$ compared with PBS). Together, these results strongly indicate that, just like the potent OV, MRTX1257 can also elicit potent tumor-specific antitumor immunity. The combination works synergistically to elicit more optimal tumor-specific immunity for improved therapeutic efficacy.

DISCUSSION

Cancer cells with mutant KRAS can convert a TME into an immunosuppressive state by actively recruiting immunosuppressive immune cells like MDSCs, Tregs, while simultaneously downregulating the activity of cytotoxic T cells, effectively shielding the tumor from immune attack.⁴³ Therefore, an effective cancer immunotherapy strategy would need to consider the factors of not only killing the tumor cells, but also improving the activation and proliferation of tumor-specific T cells and trafficking of those T cells to the tumor tissues. Proinflammatory cytokine-armed OV have been shown to promote not only the trafficking, but also the activation of tumor-specific T cells, thus enhancing adaptive antitumor immunity.^{37, 44} However, due to the fact that the highly immunosuppressive TME paralyzes the functions of T cells, often the OV itself is not potent enough to turn the corner, making the TME towards pro-antitumoral.⁴⁵ Therefore, it is strategic to develop combinations of OV with an antitumor agent that can target the driver oncoprotein KRAS^{G12C}.

In this study, we hypothesized that it is rational to combine a small molecule inhibitor to the driver oncoprotein KRAS^{G12C} with a potent OV for improved therapeutic efficacy on KRAS^{G12C} cancers. Two such small molecule inhibitors (AMG510 and MRTX849) have been shown recently to induce inflammation in cancer tissue.⁵ In our study, such an inhibitor by itself can induce potent innate and adaptive antitumor immunity. For innate immune cells, it increases the quantities of NK, DC, and M1 macrophages while decreasing M2 macrophages. As for the subsets of CD8⁺ T cells, it promotes the conversion from T_n, T_{cm} to T_{em} and effector T cells (T_{eff}) cells. Even though it increases the expression of CTLA-4, PD-1, and TIM-3 on CD8⁺ T cells, it reduces PD-1^{hi} TIM-3⁺ CD8⁺ T cells, the type of terminally exhausted cells, in the TME. Further analyses showed that it can increase the quantities of CD8⁺ T cells in tumor tissues, and NKG2D, granzyme B, and IFN- γ activation markers on those cytotoxic cells. Finally, ELISpot assays clearly demonstrated that MRTX1257 can induce tumor-specific T cells in the KRAS^{G12C} mutant tumor. In summary, our data showed convincingly that MRTX1257 induces tumor-specific T cells.

We previously characterized the properties of vTD-IL36 γ in other tumor models.³⁷ In the current study, we performed further studies with this OV on KRAS^{G12C} cancer. For the profile of innate immune cells, just like the small molecule inhibitors of KRAS^{G12C} protein, the OV increases the quantities of NK, DC, and M1 macrophages. More dramatically, the OV decreased M2 macrophages, MDSC, and Treg, all three major type immunoinhibitory cells in the TME. It can increase the quantities of CD8⁺ T cells in tumor tissues, and NKG2D, granzyme B, and IFN- γ activation markers on those cytotoxic cells. For CD8⁺ T cell subsets, the OV promotes the conversion from T_n, T_{cm} to T_{em} and T_{eff}. Even though it enhances CTLA-4, PD-1, and TIM-3 on CD8⁺ T cells, it reduces PD-1^{hi} TIM-3⁺ CD8⁺ T cells in the TME.

Finally, the induction of tumor-specific T cells has been shown in ELISpot assays with both TILs and splenocytes, and for 4-1BB⁺ CD4⁺ and 4-1BB⁺ CD8⁺ T cells by flow cytometry.

It is rational to combine two or more components with functions that can work synergistically or at least additively to fight cancer. As stated earlier, a specific inhibitor to KRAS^{G12C} can result in the downregulation of IL-36 γ ,⁸ thus, it is most rational to combine an inhibitor to KRAS^{G12C} with an OV expressing this cytokine to elicit potent antitumor immunity.

Indeed, the combination of the OV and MRTX1257 led to more potent proimmune properties against cancer. Yet the expression levels of PD-1, CTLA-4, and TIM-3 were mostly higher than either agent alone, even though PD-1^{hi} TIM-3⁺ CD8⁺ T cells were reduced to the lowest level. Among these three coinhibitory molecules, PD-1 was the highest. Therefore, it is rational to add anti-PD-1 blockade to the regimen for improved antitumor immunity and hopefully therapeutic efficacy. Indeed, analyses using flow cytometry and quantitative RT-PCR indicated further improved levels of innate immune cells (NK, DC, and M1 macrophages), CD8⁺ T cells, and activation markers (NKG2D, granzyme B, IFN- γ , and 4-1BB), and further reduced levels of M2 macrophages, MDSC, and Treg. These changes led to further enhanced antitumor T cells intratumorally and systemically, as shown by ELISpot assays with TILs and splenocytes. Finally, the improved antitumor potency converts to the best therapeutic efficacy, leading to complete remission of LLC tumors in the majority of mice. This therapeutic efficacy depends heavily on CD8⁺ T cells, and to a smaller degree on CD4⁺ T and NK cells.

In this study, we demonstrated that the triple combination led to complete regression of tumors in the KRAS^{G12C} mutant LLC tumor. One major issue in cancer monotherapy is the development of resistance to therapy. Cancer may possess innate or acquired resistance to immunotherapy.⁴⁶ In this case, KRAS^{G12C}-mutant cancer cells treated with KRAS^{G12C} inhibitors could quickly become either quiescent or resistant.⁸ The triple combined regimen developed in this study may overcome the resistance to each individual therapy and thus greatly enhance the overall therapeutic efficacy.

It is worth pointing out that all cancer cell lines contain multiple genetic mutations that may affect multiple signaling pathways. In addition to the KRAS^{G12C} mutation, another mutation, NRAS^{Q61H} mutation is also shared in both Lewis lung and AE17 cancers, the two models used in this study.⁴⁷ This mutation enhances NRAS's ability to bind GTP, a molecular switch that activates downstream signaling pathways involved in cell growth and survival. However, it is not clear if NRAS^{Q61H} mutation affects the sensitivity to KRAS^{G12C} inhibitors if they exist as heterodimers (KRAS–NRAS dimer), and how it affects the RAS downstream signaling pathway and biological effects. It should be interesting to further study these inhibitors alone or in combination with OV in novel murine

KRAS^{G12C} lung cancer cell line models as they are available now.⁴⁸

In summary, we found that a small molecule inhibitor to KRAS^{G12C} can elicit both innate immunity and tumor-specific T cell-mediated immunity. Moreover, the triple regimen we rationally developed acts mainly through potent antitumor immunity to exert profound antitumor activity. Recently, there have been exciting developments in new small molecule inhibitors targeting KRAS^{G12D},⁴⁹ and pan-inhibitors to KRAS.⁵⁰ We reason that this combination strategy may be applicable to a variety of cancers with either other G12 mutations or elevated KRAS activity. Our impressive efficacy and safety using this triple combination in tumor models warrant translation in clinical studies in human patients with KRAS^{G12C} cancer.

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Competing interests ZZ, HC, BL, and ZSG have filed a patent application partly based on this study. The other authors declare no conflicts of interest.

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Ethics approval Animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IBC #201700279).

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