PI3Kδ/γ inhibitor BR101801 extrinsically potentiates effector CD8$^+$ T cell-dependent anti-tumor immunity and abscopal effect after local irradiation

Yi Na Yoon[1,5], Eunju Lee[1,2,5], Young-Ju Kwon[1,3], Jeong-An Gim[4], Tae-Jin Kim[1]*, and Jae-Sung Kim[1,3]*

Supplementary Figure 1. Characteristics of BR101801. (A) Chemical structure of BR101801. The chemical name of BR101801 is 4-[[(1S)-1-(4,8-Dichloro-1-oxo-2-phenyl-3-isoquinolyl)ethyl]amino]-8Hpyrido[2,3-d]pyrimidin-5-one with a molecular formula of C24H17Cl2N5O2 and a molecular weight of 478.33 g/mol. (B) *In vitro* kinase assay of recombinant PI3K subtypes with BR101801.

Supplementary Figure 2. Local irradiation inhibited tumor growth in a dose-dependent manner. 2×10^5 CT-26 cells were subcutaneously injected into the right flank of BALB/c mice. (A) Mean tumor volume following 2, 3, 5, and 7.5 Gy irradiation. (B) Percentages of tumor growth inhibition at each irradiation dose (n = 5 mice per group).

Supplementary Figure 3. Synergistic anti-tumor effect of the combined therapy in MC38 and LL/2 syngeneic mice tumor models. C57BL/6 mice bearing MC38 and LL/2 cells were orally administered (q.d.) 50 mg/kg BR101801 daily for 26 days and 24 days, respectively. The tumor mass was locally irradiated 8 days after the first dose of BR101801. (A) Mean tumor volume of subcutaneous (MC38) implants in BR101801 and irradiation treated mice (n = 10 mice per group). (B) Individual MC38 tumor growth curves (black line indicates the mean tumor volume in each group; gray line indicates individual tumor growth). (C) Mean tumor

volume of subcutaneous (LL/2) implants in BR101801 and irradiation treated mice (n ≥ 6 mice per group). (B) Individual LL/2 tumor growth curves (black line indicates the mean tumor volume in each group; gray line indicates individual tumor growth). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ using an unpaired two-tailed *t*-test.

Supplementary Figure 4. *In vitro* cytotoxicity assay of BR101801. (A) BR101801 dose dependent cell viability in CT-26 cells. CT-26 cells were treated with 40 μM to 0.625 μM BR101801 for 72 h. Cell viability was determined using a WST-8 assay. (B) Survival fraction graphs of CT-26 cells. The data are representative of three independent experiments.

Supplementary Figure 5. Antibody-mediated depletion of immune cells in BALB/c mice treated with BR101801 and/or irradiation. BALB/c mice bearing CT-26 colon cancer were orally administrated (q.d.) with 50 mg/kg BR101801 daily for 32 days. Each mouse was intraperitoneally injected with 200 μg anti-mouse CD4, CD8, or GM1 antibodies three days before irradiation and once a week (n ≥ 4 mice per group). Individual tumor growth curves of each group (the black line indicating the mean tumor volume of each group, the gray line indicating individual tumor growth).

Supplementary Figure 6. Time-dependent tumor infiltrating lymphocytes. BALB/c mice bearing CT-26 colon cancer were orally administrated (q.d.) with 50 mg/kg BR101801 daily till the sacrifice time point (Duration of BR101801 treatment; IR+1: for 9 days, IR+3: for 11 days, IR+7: for 15 days, IR+14: for 22 days). (A) Frequency of $CD3e^+$ in $CD45^+$ cells from day 1 to 14 after irradiation (n ≥ 3 mice per group). (B) Percentage of tumor-infiltrated $CD4^+$

T cells from day 1 to 14 after irradiation (n ≥ 3 mice per group). All data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 using an unpaired two-tailed *t*-test.

Supplementary Figure 7. Impact on the myeloid cell population induced by BR101801 and irradiation. BALB/c mice bearing CT-26 colon cancer were orally administrated (q.d.) with 50 mg/kg BR101801 daily till the sacrifice (IR+7: for 15 days). Flow cytometry analysis was performed on day 7 post-irradiation of the tumor. (A) Representative contour plots of I-A/I-E/CD206 prior to gating with $CD45^+/CD11b^+/F4/80^+$ (left). The percentage of I-A/I-$E^+/CD206^-$ M1-like macrophages (middle) and I-A/I-$E^-/CD206^+$ M2-like macrophages (right) at day 7 after irradiation in the tumor. (B) Representative contour plots of Gr-$1^+/CD11b^+$ cells gated with $CD45^+/F4/80^-$ (left). The percentage of $CD11b^+/Gr\text{-}1^{high}$ polymorphonuclear (PMN) MDSCs (middle) and $CD11b^+/Gr\text{-}1^{mid}$ monocytic (M) MDSCs (right) at day 7 after irradiation in the tumor. (C) Representative contour plots of I-A/I-$E^+/CD11c^+$ prior to gating with $CD45^+/F4/80^-$ (left). The percentage of I-A/I-$E^+/CD11c^+$ dendritic cells (right) at day 7 after irradiation in tumors (n = 4 mice per group). All data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 using an unpaired two-tailed *t*-test.

Supplementary Figure 8. Gene set enrichment map of NanoString data using a pathway-to-pathway network. Size indicates the enrichment score of the gene set representing each pathway. The color indicates the up/down direction of regulation: pink, up-regulation; navy, downregulation. The gene copy number of $CD45^+$ cells from CT-26 tumor was analyzed using NanoString nCounter Immunology panel 7 days after irradiation (n = 3 mice per group).

Supplementary Figure 9. Impact of PI3K subunit blockade on T cell subsets. Flow cytometry analysis was performed on day 7 post-irradiation of the CT-26 tumor. (A) The number of $CD45^+/CD3e^+/CD4^-/CD8\alpha^+$ cells. (B) The percentage of $CD45^+/CD3e^+/CD4^+/FoxP3^+$ cells in $CD45^+$ cells (n = 10 mice per group). All data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 using an unpaired two-tailed *t*-test.

Supplementary Figure 10. Heatmap analysis of *Cxcr* subunits. Heatmap of mean fold-change in gene expressions of other treatment groups versus DMSO along group for genes that are differently expressed. The gene copy number of $CD45^+$ cells from CT-26 tumor was analyzed using NanoString nCounter Immunology panel 7 days after irradiation (n = 3 mice per group). ** indicates *P*-value (*P* < 0.01) using an unpaired two-tailed *t*-test.

Supplementary Figure 11. Central memory T-cell (T_{CM}) population in TDLN. The percentage of tumor-infiltrated $CD8\alpha^+$ and $CD4^+$ T cells with the $CD44^{high}/CD62L^{high}$ phenotype at 14 days after irradiation (n = 4 mice per group). BALB/c mice bearing CT-26 colon cancer were orally administrated (q.d.) with 50 mg/kg BR101801 daily till the sacrifice (IR+14: for 22 days). All data are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 using an unpaired two-tailed *t*-test.