Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Immunother Cancer

Supplementary Figure S1. In vitro effects of IMO-2125 on pancreatic cancer cells.
A) Western Blotting of EMT markers; B) RC416, DT4313, FC1245 and FC1242 cells were treated with escalation doses of IMO-2125. Vehicle-treated cells were assigned a value of 100% and designated as control. Means and 95% CIs of three independent experiments performed in quadruplicate are shown; C) Cancer cell in vitro migration assay; D) Quantitative RT-PCR analysis of CDH1, Vimentin and TLR9 gene expression in FC1242, FC1245, DT4313 and RC416 cell lines treated or untreated with IMO-2125 (1mM, for 24 hours). Results are presented as the fold change in RNA expression between the gene of interest and β-actin. The mean values and Standard deviation from 3 independent experiments conducted in triplicates are shown.

Supplementary Figure S2. Evaluation of Microsatellite Instability of PDAC mouse models.
MMR proteins (MLH1, PMS2, MSH2 and MSH6) expression was defined as negative if staining was absent or present in less than 10% of tumor nuclei in a section. Normal epithelial cells and stromal cells were used as an internal control. Tumors were classified as MMR-deficient if one or more of the four proteins showed negative staining.

Supplementary Figure S3. Effect of ICI treatment on orthotopic and heterotpic CDG models.
Upper panel: Syngeneic orthotopic mouse models were randomly assigned (n=3 per group) to receive anti-PD1 or IgG as control (i.p. 10mg/Kg twice week for 2 weeks). Tumor size was measured with VEVO 3100 ultrasound device weekly. Mean tumor volume and standard deviation of tumor in mm^3 are shown; *Lower panel*: Syngeneic heterotopic mouse models. Data extrapolated from the experiment shown in Figure 2. (* *P*<0.05, ** *P*<0.01)

Supplementray Figure S4. Kaplan-Meier survival analysis.
A) Differences among survival duration of mice in each group were determined by log-rank test. DT4313, median survival of control 48,5 days versus anti-PD1 73 days, and undefined for IMO-2125 and combination groups; FC1242, median survival of control 44 days, versus IMO-2125 55 days, antiPD-1 41 days, combination undefined; RC416, median survival of control and anti PD-1 38 days versus IMO-2125 and combination undefined; FC1245, median survival of control 31 days versus IMO-2125 31 days, antiPD-1 31 days, combination 29 days.

Supplementary Figure S5. TLR9 agonist intratumoral administration leads to systemic inflammatory cytokines release.
Multiplex analysis of proinflammatory circulating factors. Plasma from peripheral blood was collected from each indicated group of mice (*n* = 3) treated and untreated with IMO-2125. Concentrations of cytokines (pg/mL) were calculated. Circulating factor mean values were normalized using the normalize function from the R package heatmap.

Supplementary Figure S6. RNA-sequencing analysis of differentially expressed genes from tumor tissue DT4313 and FC1242 CDG mouse models.
A) Volcano plot of genes differentially expressed between (n = 3) DT4313 and FC1242 tumors (-1.5 <fold change <1.5 and with pvalue <0.05 were in red plot);
B) Pie charts of gene ontology showing up (Fold Change >1.5) and down (Fold Change<1.5) cellular processes between (n = 3) DT4313 and non FC1242 tumors;
C) Biological processes activated in DT4313 tumor untreated and treated with IMO-2125 (i.t.) Only gene sets with an adjusted p-value < 0.05 were displayed;
D) Two most significative up-regulated (fold>1.5, P value<0.05) biological processes identified by GSEA. B) Volcano plots of differences in gene expression between (n = 3) responder (DT4313) and non responder (FC1242) tumors.

Supplementary Figure S7. RNA-sequencing analysis of differentially expressed genes from tumor tissue DT4313 and FC1245 CDG mouse models.
A) Volcano plot of genes differentially expressed between (n = 3) DT4313 and FC1245 tumors (-1.5 <fold change <1.5 and with pvalue <0.05 were in red plot);
B) Pie charts of gene ontology showing up (Fold Change >1.5) and down (Fold Change<1.5) cellular processes between (n = 3) DT4313 and non FC1242 tumors;
C) Biological processes activated in DT4313 tumor untreated and treated with IMO-2125 (i.t.) Only gene sets with an adjusted p-value < 0.05 were displayed;
D) four most significative up-regulated (fold>1.5, P value<0.05) biological processes identified by GSEA.

Supplementary Figure S8. *In vitro* **autophagy analysis.**
Western blotting analysis for the expression of basal autophagic flux measured by LC3-IIB expression in *in vitro* experiments. Immune unresponsive tumors show low levels of basal autophagic flux compared to immune-responsive pancreatic cancer models; (CHQ, 25mM, 1 hour). Each control was set = 1.

Supplementary Figure S9. DT4313 IMO-2125 responder tumors show immunopermissive characteristics.
A) RNA-Seq based PCA showing gene expression among the indicated treatment groups of anenestic DT4313 tumors (left flank);
B) Volcano plot of genes differentially expressed between (n = 3) DT4313 and FC1242 tumors (-1.5 <fold change <1.5 and with p value <0.05 were in red plot);
C) Ridge-plot s of the pre-ranked GSEA applied to the results of the differential gene expression between the indicated pairs. Only gene sets with an adjusted *p*-value < 0.05 are displayed;
D) Pie charts gene ontology showing up (Fold Change >1.5) and down (Fold Change<1.5) cellular processes between untreated and treated (comb) group (n=3) of not locally treated tumours (left flank). (CTR= control, IMO =IMO-2125, ICI=anti-PD-1, Comb= Combination).

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Immunother Cancer

Supplementary Figure S10. Immunohistochemistry analysis of immune cell subpopulation markers.
Immunohistochemistry analysis for the reported markers in tumor tissue from mice bearing (A) DT4313 and (B) FC1242 subcutaneously tumor bearing muse models. IMO-2125 untreated (Left panel) and treated (Right panel) tumors.
Quantification is provided as the average number of indicated marker positive cells per mm2. From 5 to 8 individual areas per case were examined. Statistical associations were calculated by Student's t-test. (*p < 0.05; **p < 0.01; ***p < 0.001).

Supplementary Figure S11. **Lymph node analysis.**
A) Lymph node immune counts of TDLNs (inguinal and axillary) of the both animal flanks. Lymph nodes were isolated from the both animal flanks of the indicated tumor bearing mice model. Immune cell counts were obtained from smashed lymph nodes and spectrophotometer absorbance measurement. Statistical differences were analyzed with 2-way ANOVA. All data represent average ± SEM (n = 4 per group); B) Flow cytometry analysis of the indicated immune cell populations in the spleen of FC1242 and DT4313 tumor bearing mouse models treated and untreated with IMO-2125. (*P < 0.05, ***P < 0.01).