Supplemental Figure legends

Supplemental Fig. 1 Gating strategy for isolation of regulatory T cells. (A) A gate surrounding CD3-positive cells of spleen cells was utilized for the isolation of T cells from TILs. The isolated populations were separated from doublets by gating for FSC-A and FSC-H, and further isolated according to FSC and SSC. The populations were further enriched as $CD4^+$T cells. The spleen and TILs were recovered from MethA-bearing mice on day 7 after the tumor challenge. (B) $CD4^+CD25^+$T cells were identified from the populations of (A) and further examined for the expression of Foxp3.

Supplemental Fig. 2 Met-induced reduction of $CD4^+CD25^+$Treg cells in TILs but not in the spleen is caused by increased apoptosis induction. (A) On days 10 and 13 (or day14), spleen cells and TILs were recovered from tumor (MCA or RLmale1)-bearing mice, which were receiving (+) or not receiving (−) Met, and the $CD4^+CD25^+$Treg population was examined by FACS analysis. (B) Annexin V binding to the $CD4^+CD25^+$Treg population in (A) was examined. Increased annexin V binding to the Treg cells was only observed in TILs but not in the spleen of the mice receiving Met. Pooled cells from each group (N = 5) were used for the FACS analysis. The results are representative of four independent experiments.

Supplemental Fig. 3 Met-induced increase in cleaved caspase-3 in $CD4^+Foxp3^+$Treg cells in TILs but not in the spleen. (A) On day 13, TILs were recovered from tumor (MethA)-bearing mice, treated with (+) or without (−) Met, and the $CD4^+Foxp3^+$Treg and the $CD4^+Foxp3^-$ conventional T cell population was examined by FACS analysis for the expression of cleaved caspase-3. Increased cleaved caspase-3 was observed in $CD4^+Foxp3^+$Treg population in Met-treated mice (N=3). (B) On day 13, spleen cells and TILs were recovered from tumor (MethA)-bearing mice, treated with (+) or without (−) Met, and the $CD4^+Foxp3^+$Treg population was examined by FACS analysis for the

expression of cleaved caspase-3. Increased cleaved caspase-3 was observed in TILs but not spleens of mice receiving Met. Pooled cells from each group (N = 3) were used for FACS analysis. The individual results are plotted as a bar graph (N = 3). The results are representative of two independent experiments. *$P < 0.05$

Supplemental Fig. 4 Viable Treg cells are reduced in TILs of mice receiving Met compared with those of mice not taking Met. B6 mice inoculated with MCA tumor cells received Met dissolved in free drinking water at a concentration of 5 mg/mL from day 6 after the tumor challenge (+). On days 6, 10, and 13, TILs were recovered from each mouse and stained with antibodies to CD3, CD4, CD8, and CD25 to identify Treg cells. Annexin V binding to these Treg populations was evaluated by FACS analysis. N = 10 for each group. (A, B, and C) The ratios of $CD4^+CD25^+/CD4^+$ (A), $CD4^+CD25^+$ annexin V^+/ $CD4^+CD25^+$ (B), $CD4^+CD25^+$ annexin $V^-/CD4^+$ (C) in each group were plotted as bar graphs. The ratios of $CD4^+CD25^+$ annexin $V^-/CD4^+$ on days 10 and 13 were dramatically reduced in the mice receiving Met compared with those in the mice not taking Met. (D, E) Absolute numbers of $CD4^+CD25^+$ annexin V^- T cells (D) and $CD8^+$ annexin V^- T cells (E) per tumor volume (mm^3) are shown. (F) The ratios of $CD4^+CD25^+$annexin $V^-/CD8^+$ annexin V^- T cells are shown. (G) The tumor (MCA) growth curve was monitored in the mice receiving (+) or not receiving (−) Met. N = 10 for each group. The results are representative of four independent experiments. *$P <$ 0.05, **$P < 0.01$.

Supplemental Fig. 5 No Foxp3 expression changed in Met-pretreated nTreg cells in response to TCR stimulation.

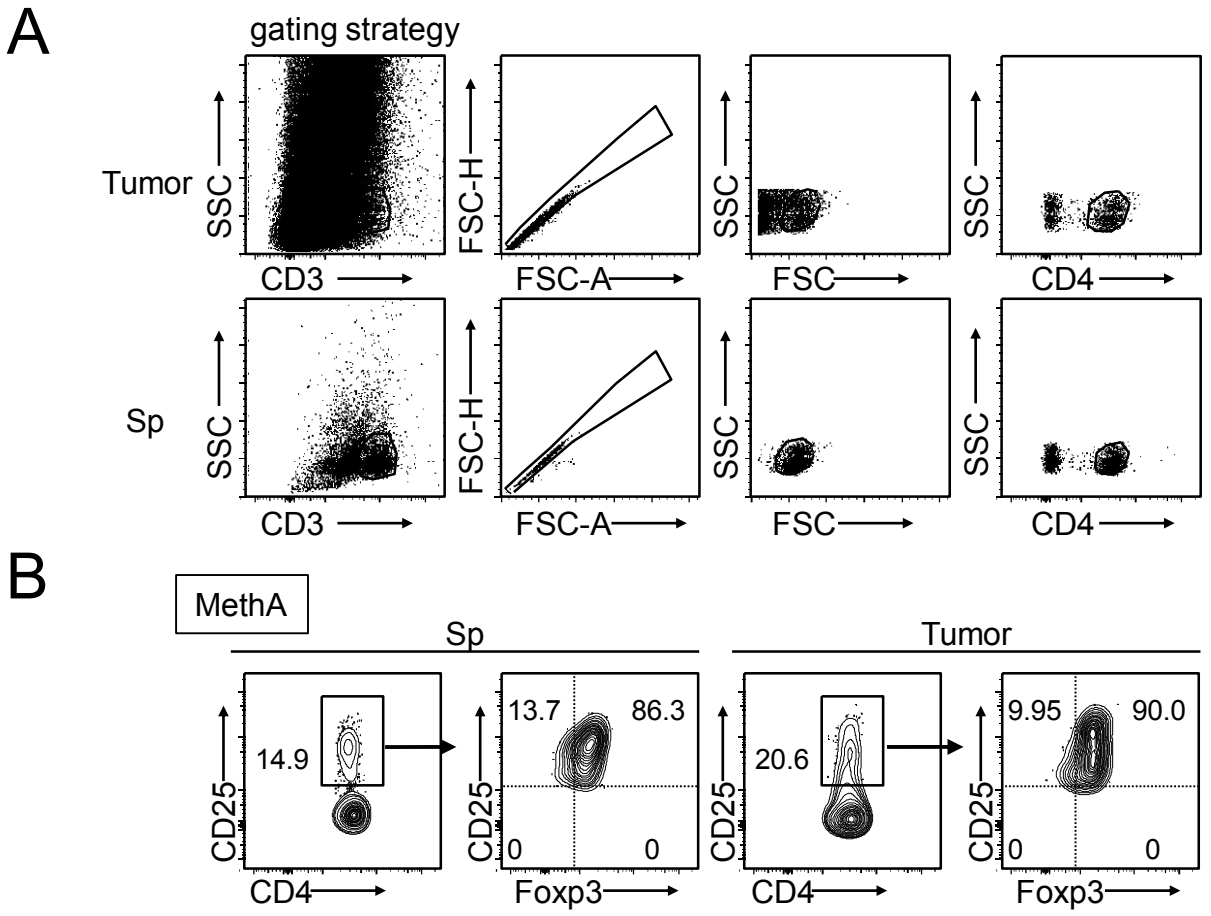
nTreg cells were isolated from spleen cells and pretreated with Met (+) or not (-) along with or without inhibitors, rapamaycin (RA) or compound C (CC), and were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of TGFβ. No effect was observed in the nTreg cells. The results are representative of three independent experiments.

Supplemental Fig.6 Relationship between $CD4^+$ $CD25^{high}$ Ti-Treg cells and $CD103^+KLRG1^+$Ti-Treg cells. The same gating strategy was used for the isolation of Ti-Treg cells as in Supplemental Fig. 1A. The isolated populations were further determined to be $CD4^+CD25^+$ cells (24.7%) and $CD4^+$ $CD25^{high}$ cells (3.8%), and expression of CD103 and KLRG1 in the two distinct Treg populations was shown as indicated. Note that $CD25^{high}$ population includes a larger $CD103^+KLRG1^+$ population and that phosphorylated mTOR (p-mTOR) levels are significantly higher in the population. The results are representative of three independent experiments.

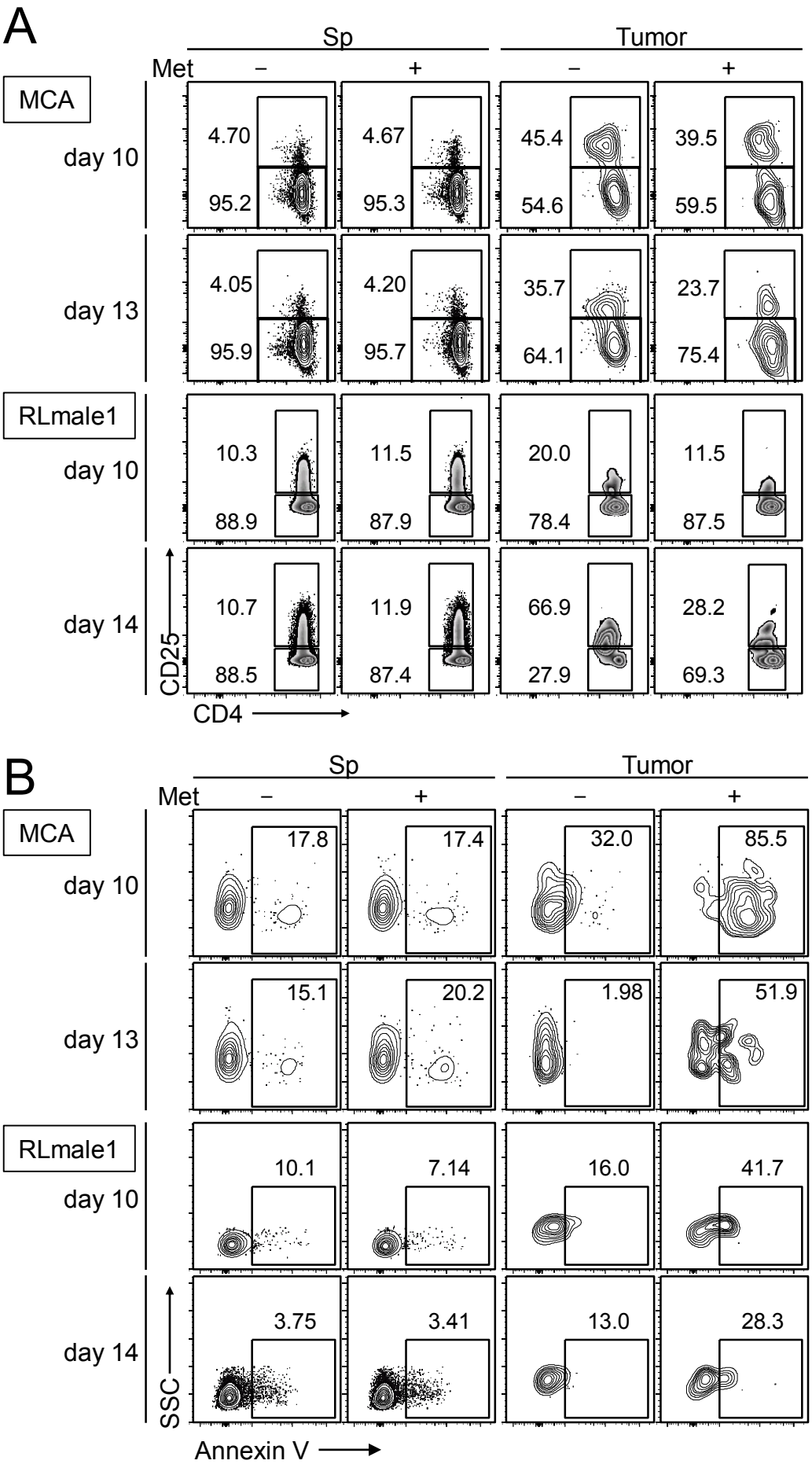
Supplemental Fig.7 Only Met-pretreatment of naïve CD4T cells before TCR stimulation increased glycolysis. (A) Pre-treatment assay: $CD4^+CD25^-$ T cells were treated with Met for 6 hours and washed before TCR stimulation. Post-treatment assay: $CD4^+CD25^-$ T cells were treated with culture medium for 6 hours and then treated with Met during TCR stimulation. Three days after TCR stimulation, the Foxp3 expressions were examined. The histogram results are representative of three independent experiments, and the individual data are plotted as a bar graph.
(B) The cells on day 3 culture in (A) were subjected to metabolic analysis. The OCR and ECAR were assayed using a Seahorse XF96 analyzer by the addition of anti-mitochondrial reagents, oligomycin, FCCP, rotenone (R), antimycin A, and 2-deoxyglucose (2-DG). Data in the upper two panels are representative of three independent experiments. The lower panels show the basal levels of OCR and ECAR a under each cell culture condition. The ECAR was assayed in Dulbecco's modified Eagle's medium without glutamate. The ratio of OCR/ECAR is plotted as a bar graph. Notably, only Met pretreatment decreased OCR/ECAR in iTregs. The results are representative of three independent experiments. $*P < 0.05$; $**P < 0.01$

Supplemental Fig.8 16S rRNA sequence of the fecal microbiota of tumor-bearing mice treated or not treated with Met. Feces were collected from MO5-bearing mice 1 week after the Met administration. 16S rRNA was PCR-amplified and sequenced by MiSeq. Phylum (A), class (B), order (C), family (D), and genus (E) levels are shown.

Supplemental Fig. 1



Supplemental Fig. 2



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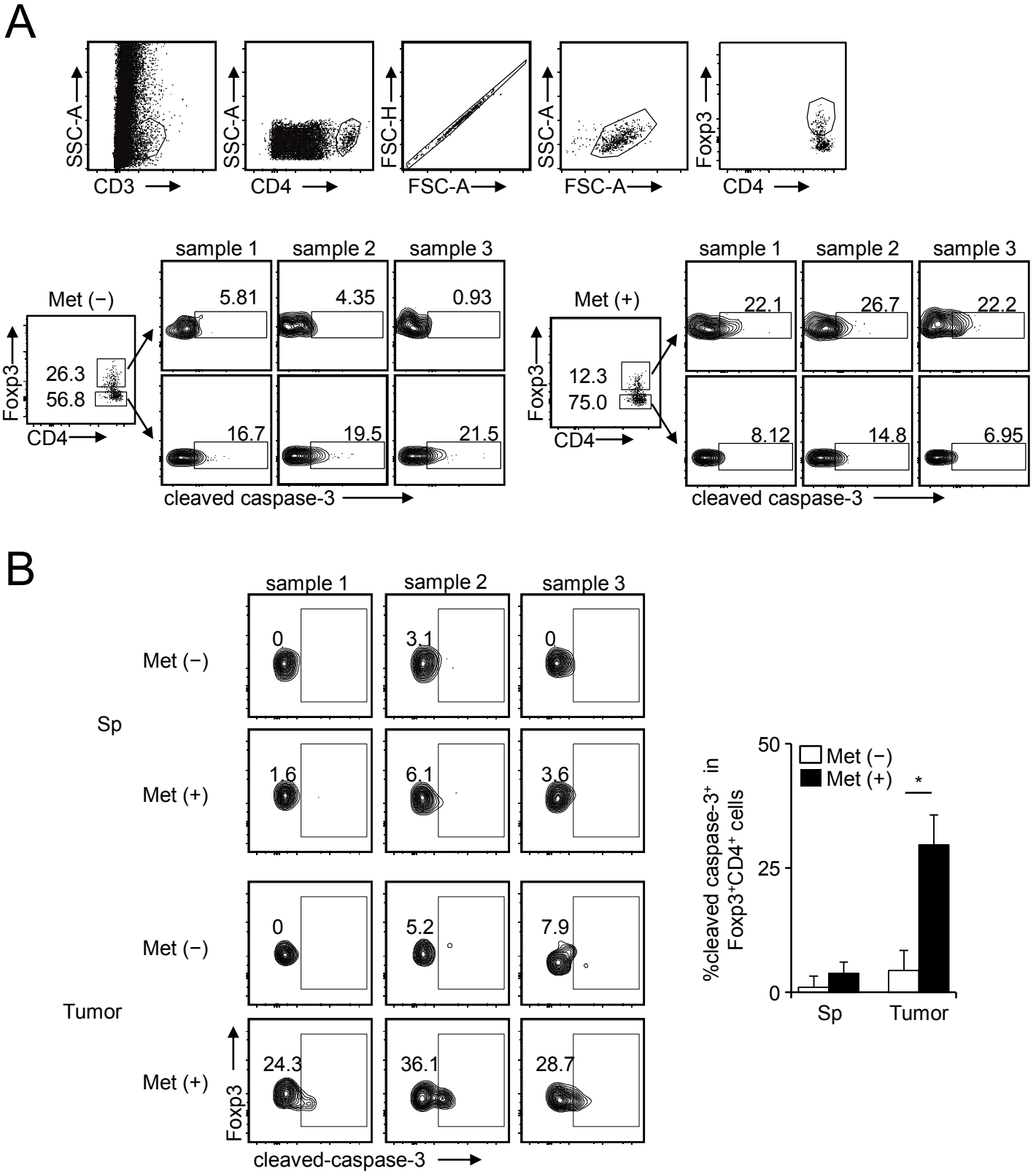

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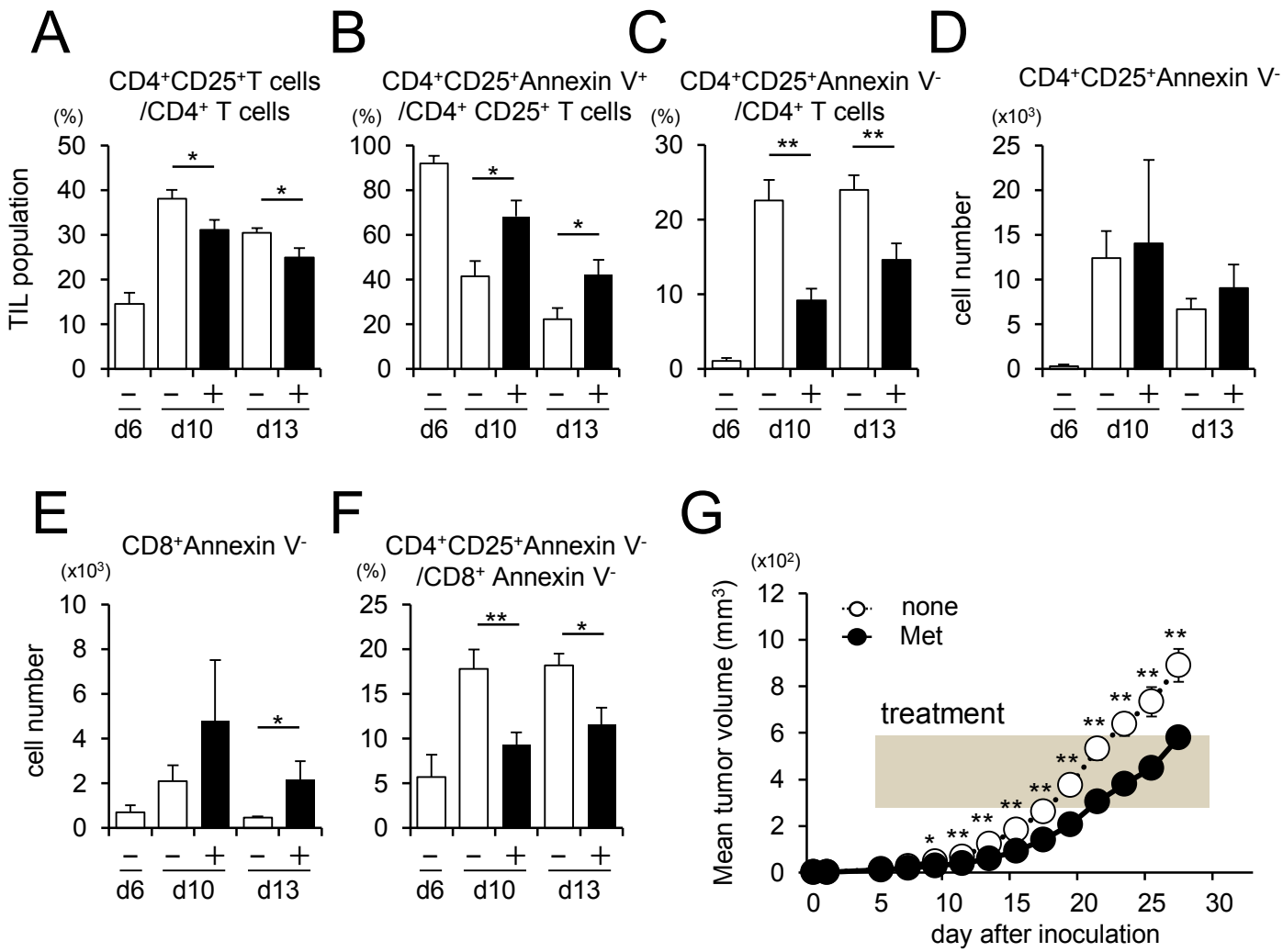

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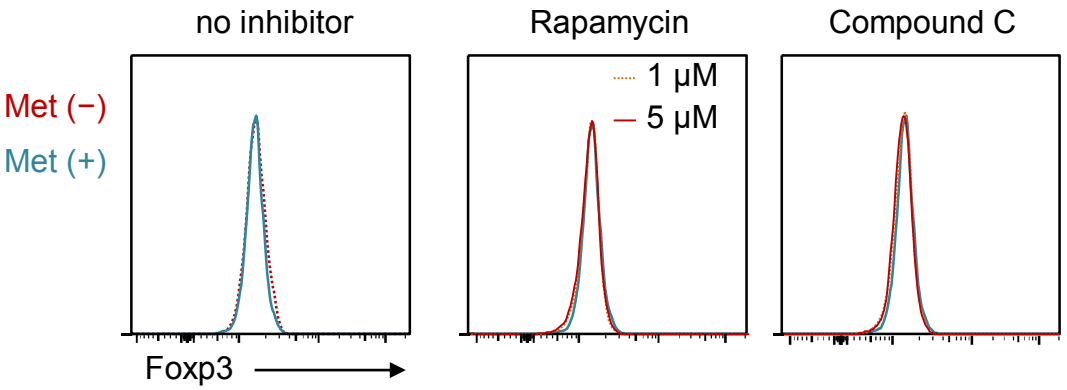

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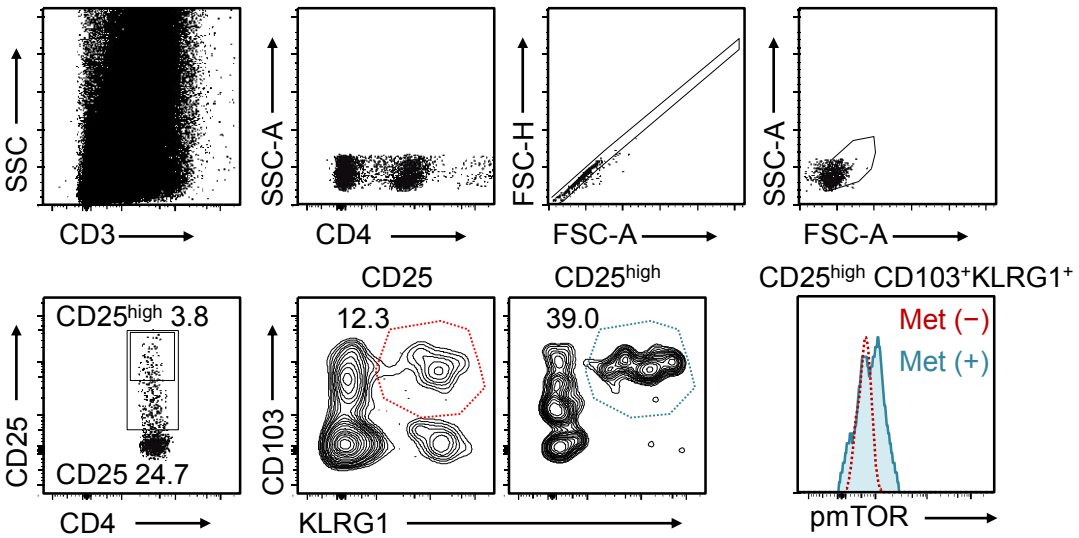
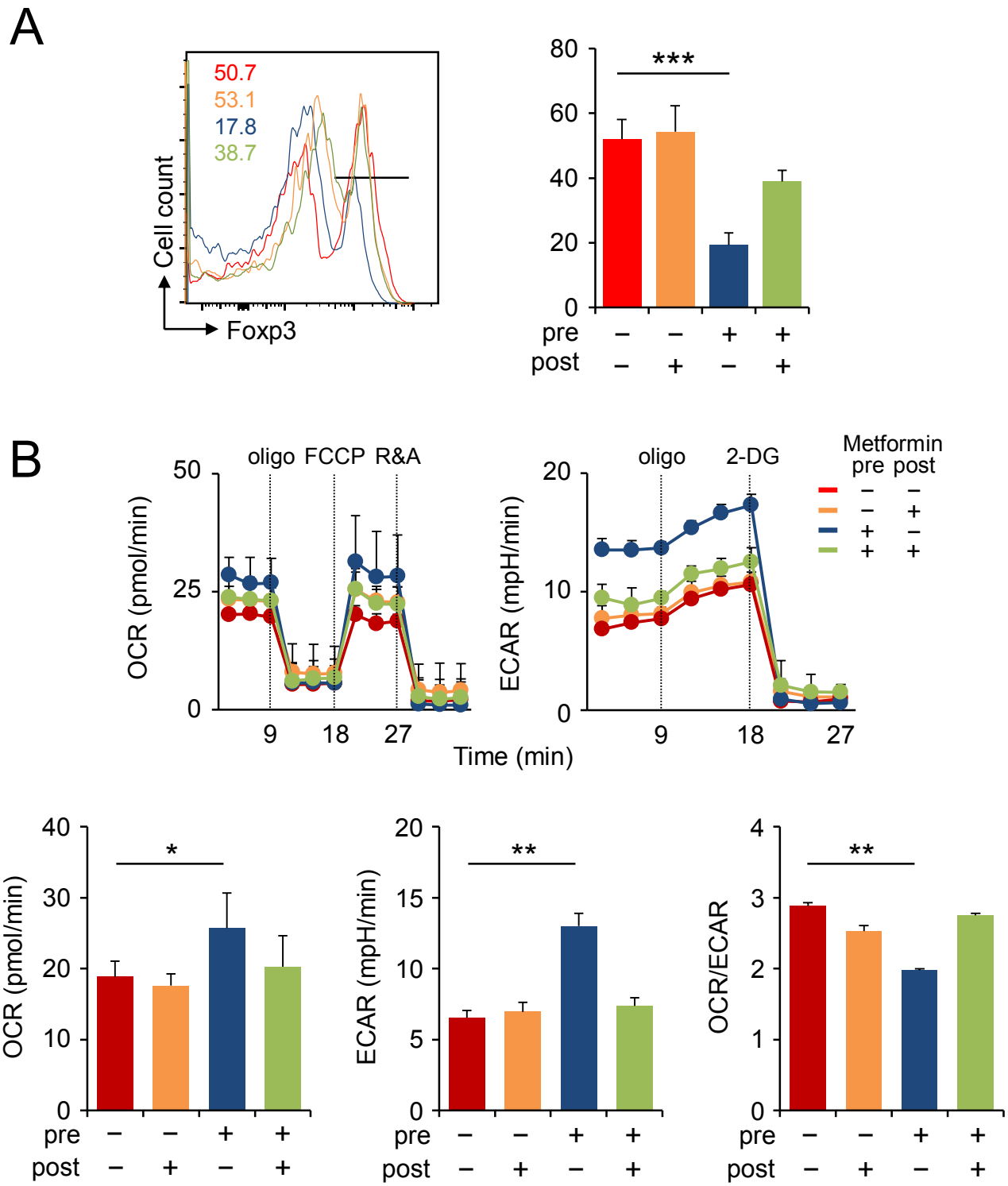


Supplemental Fig. 7



Supplemental Fig. 8