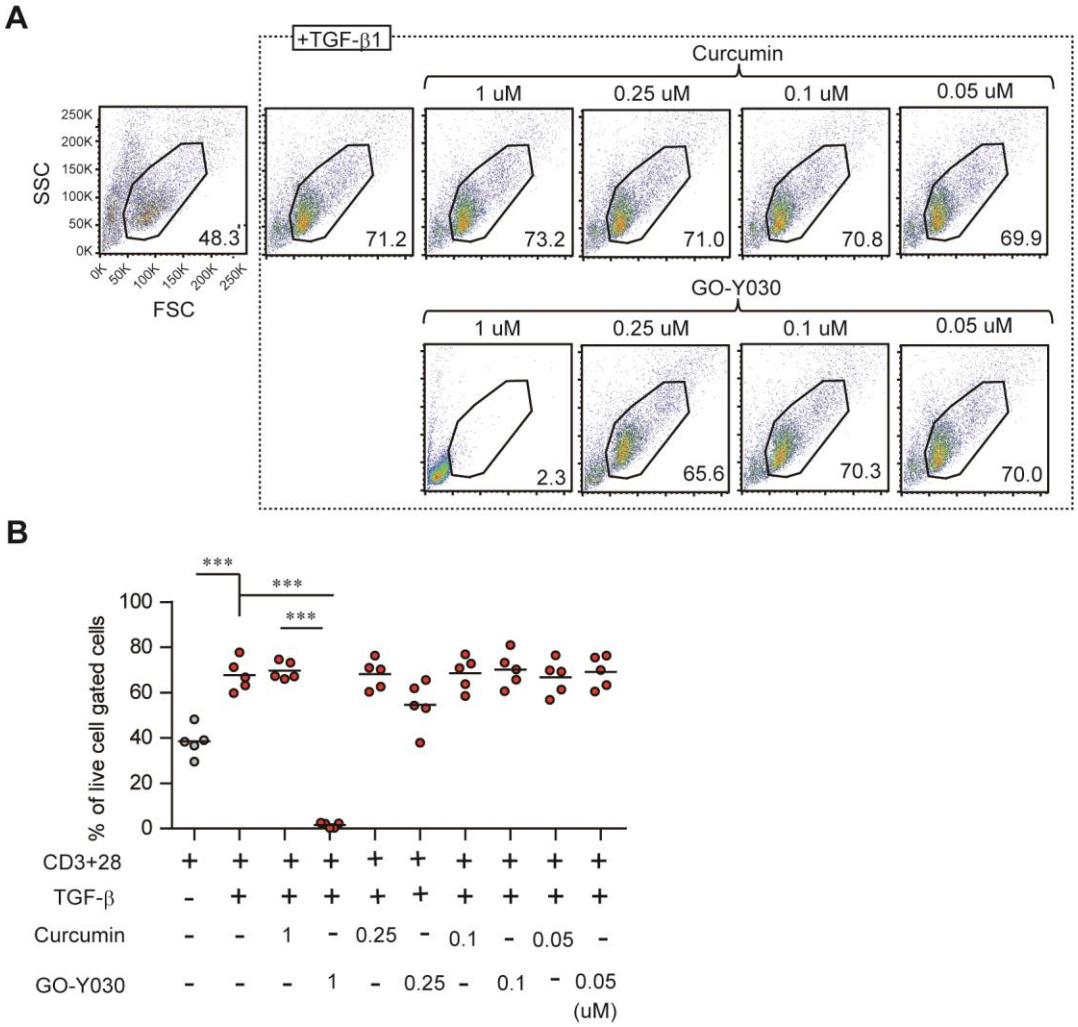
| | | |
|---|---|---|
| Interleukin-10 | Forward | 5′-CCCATTCCTCGTCACGATCTC-3′ |
| | Reverse | 5′-TCAGACTGGTTTGGGATAGGTTT-3′ |
| TGF-β1 | Forward | 5′-CTCCCGTGGCTTCTAGTGC-3′ |
| | Reverse | 5′-GCCTTAGTTTGGACAGGATCTG-3′ |
| GAPDH | Forward | 5′-CCAGGTTGTCTCCTGCGACTT-3′ |
| | Reverse | 5′-CCTGTTGCTGTAGCCGTATTCA-3′ |
| Foxp3-promoter | Forward | 5′-TTCCTCCCGCTCTCTGACTCT-3 |
| | Reverse | 5′-AAGCGCCAGTTGTGTACAAATATC-3′ |
| Foxp3-CNS1 | Forward | 5′-GTTTTGTGTTTTAAGTCTTTTGCACTTG-3′ |
| | Reverse | 5′-CAGTAAATGGAAAAAATGAAGCCATA-3′ |
| **(For Taqman)** | | **Cat#** |
| Hprt1 | | Mm00446968_m1 |
| Foxp3 | | Mm00475162_m1 |
| Tgfb1 | | Mm00441724_m1 |
| Il10 | | Mm01288386_m1 |
| Tnf | | Mm00443258_m1 |
| Tgfbr1 | | Mm00436964_m1 |
| Tgfbr2 | | Mm03024091_m1 |
| Smad6 | | Mm00484738_m1 |
| Smad7 | | Mm00484742_m1 |
| Rel | | Mm01239661_m1 |
| Tbx1 | | Mm00448949_m1 |
| Rorc | | Mm01261022_m1 |
| Nfkb1 | | Mm00446968_m1 |
| Nfkb2 | | Mm00476361_m1 |
| Ikbkb | | Mm01222247_m1 |
| Jun | | Mm00495062_s1 |
| Il2ra | | Mm01340213_m1 |
| Il2rb | | Mm00434268_m1 |
| Smad2 | | Mm00487530_m1 |
| Smad3 | | Mm00489637_m1 |
| Smad4 | | Mm03023996_m1 |
| Cd3e | | Mm01179194_m1 |
| Icos | | Mm00497600_m1 |
| Nfkbiz | | Mm00600522_m1 |
| Cd44 | | Mm01277161_m1 |
| Stat5a | | Mm03053818_s1 |
| Stat5b | | Mm0083989_m1 |
| Jak1 | | Mm00600614_m1 |
| Hdac4 | | Mm01299552_m1 |
| Ifng | | Mm01168134_m1 |
| Il2 | | Mm00434256_m1 |
| Il5 | | Mm00439646_m1 |
| Il13 | | Mm00434204_m1 |
| Inha | | Mm00439683_m1 |
| Rorc | | Mm01261022_m1 |
| Batf | | Mm00479410_m1 |
| Stat3 | | Mm00456961_m1 |

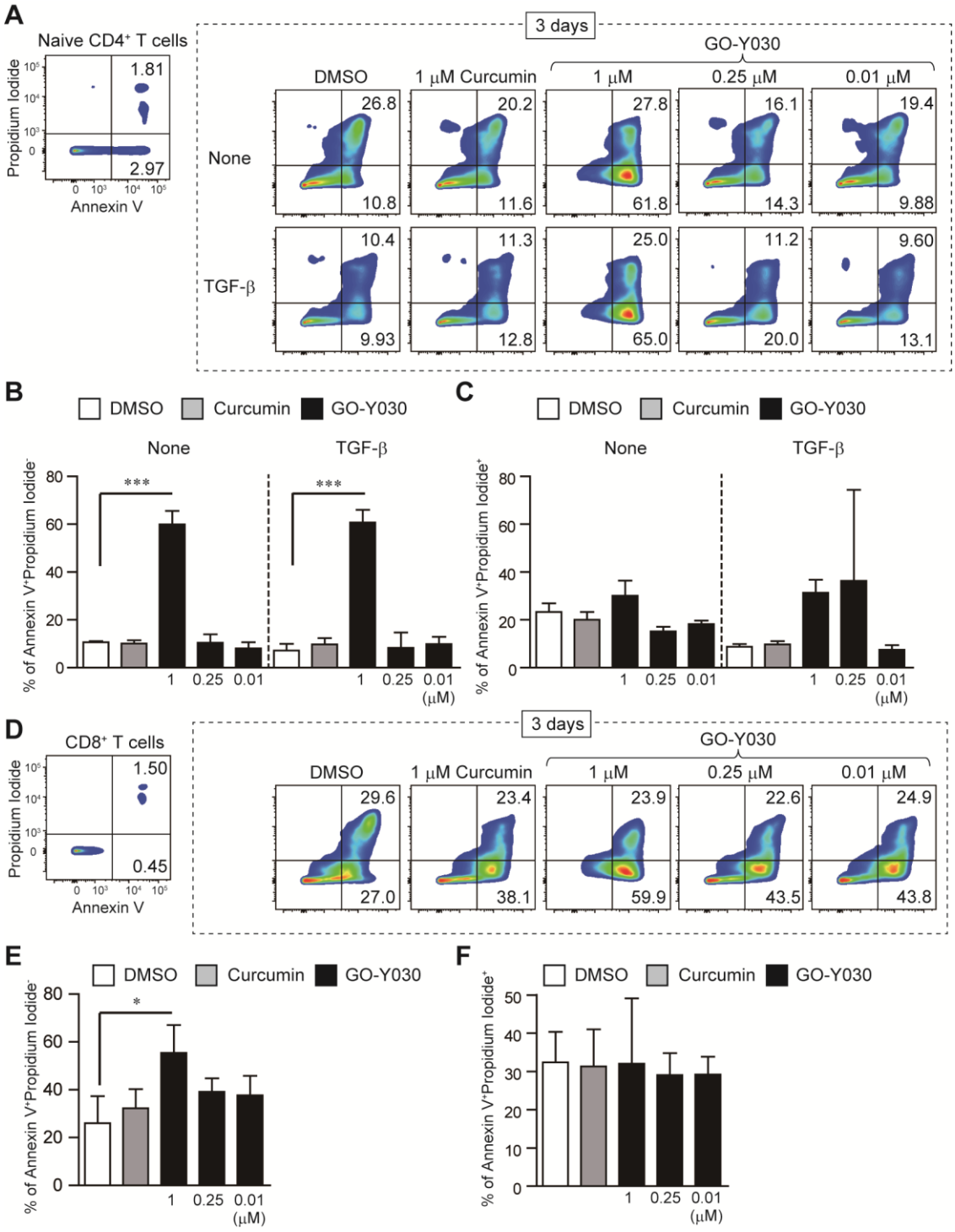
| | |
|---|---|
| Myd88 | Mm00440338_m1 |
| Myc | Mm00487803_m1 |
| Hk1 | Mm00439344_m1 |
| Hk2 | Mm00443385_m1 |
| Socs1 | Mm00782550_s1 |
| Socs3 | Mm00545913_s1 |
| Slc2a1 | Mm00441480_m1 |
| Slc2a3 | Mm00441483_m1 |
| Runx1 | Mm01213404_m1 |
| Runx3 | Mm00490666_m1 |
| Tcf12 | Mm00441699_m1 |
| Cd274 | Mm03048248_m1 |
| Rara | Mm01296312_m1 |
| Rora | Mm01173766_m1 |

**Supplementary Table 1: Primers List**




**Supplementary Figure 1: Naive CD4$^+$ T cells from C57/BL6 mice cultured in each concentration of GO-Y-030.**

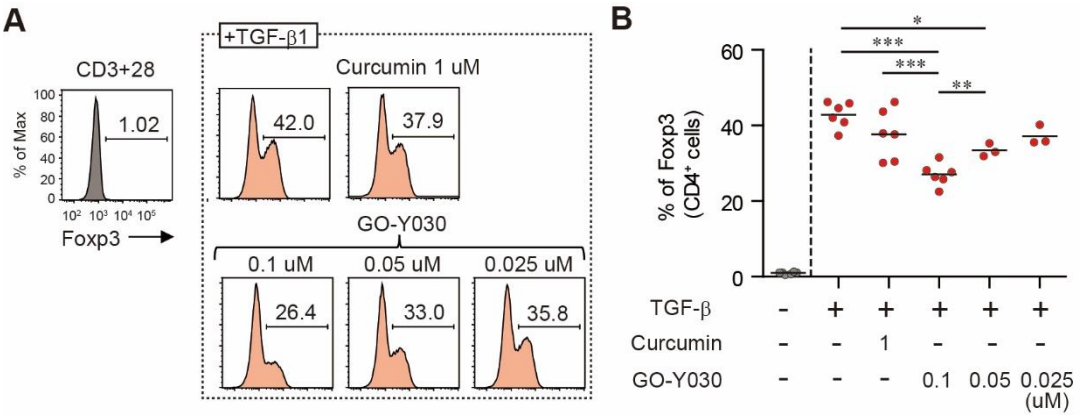
(**A**, **B**) Representative SSC and FSC FACS analysis at five independent experiments. Purified naïve CD4$^+$ T cells cultured for three days, and then gated live cell population according to SSC and FSC. Statistical analyses were performed in each concentration between Curcumin and GO-Y030, and 2 ng/mL TGF-β versus all.




**Supplementary Figure 2: Effects of GO-Y-030 in T cell viability.**

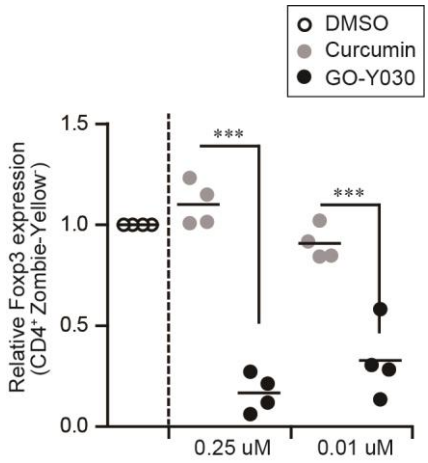
(**A**) Representative Annexin V and Propidium Iodide FACS analyses at three independent experiments. Purified naïve $CD4^+$ T cells cultured for three days, and then gated $CD4^+$ cell population. (**B**, **C**) Statistic analyses were performed in each concentration between Curcumin and GO-Y030 versus DMSO control. One-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparisons test employed. (**D**) Representative Annexin V and Propidium Iodide FACS

analyses at three independent experiments. Purified naïve $CD8^+$ T cells cultured for three days, and then gated $CD8^+$ cell population. (**E**, **F**) Statistic analyses were performed in each concentration between Curcumin and GO-Y030 versus DMSO control. One-way ANOVA with post-hoc Tukey's multiple comparisons test employed.



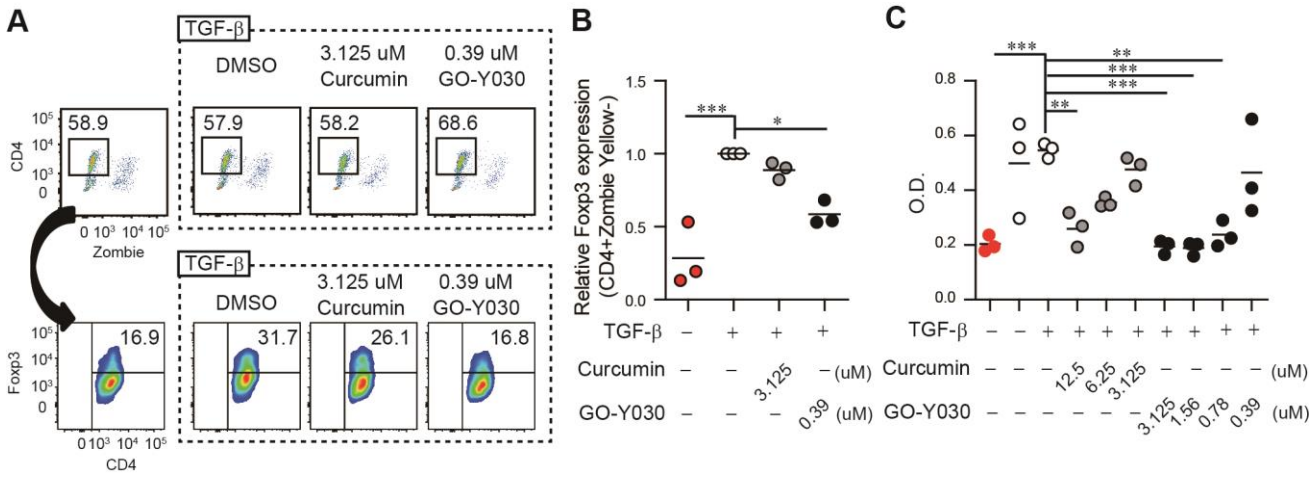

**Supplementary Figure 3: GO-Y030 prevent TGF-β induced Foxp3$^+$Tregs generation**

(**A**, **B**) Frequency of Foxp3$^+$ Tregs in total CD4$^+$ cells. Splenic naïve CD4$^+$T cells were cultured in the presence or absence of 2 ng/mL TGF-β, 1 μM curcumin, or 0.1-0.025 uM GO-Y030 for three days. One-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparisons test employed.



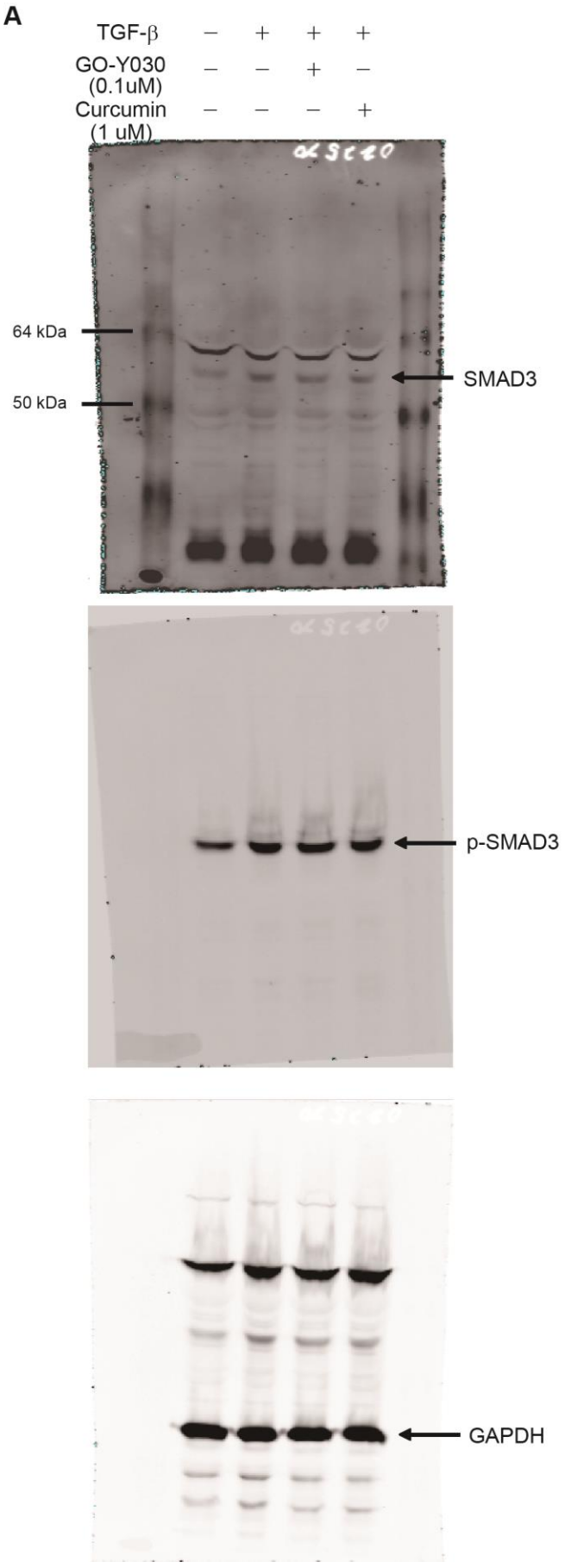

**Supplementary Figure 4: Relative Foxp3$^+$Tregs in CD4$^+$Zombie Yellow$^-$ T cells.**

Splenic naïve CD4$^+$ T cells were cultured in the presence of 2 ng/mL TGF-β with or without curcumin or GO-Y030 for three days. Percentage of TGF-β-induced Foxp3$^+$ population is as set as "1". One-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparisons test employed.



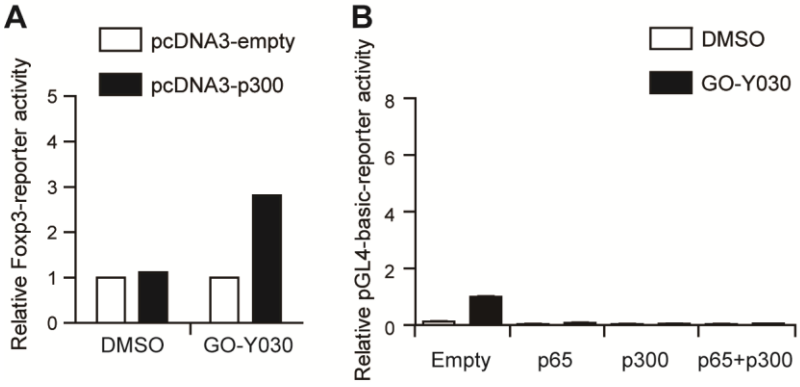

**Supplementary Figure 5: GO-Y030 prevents TGF-β-induced Foxp3$^+$Tregs in human naïve CD4$^+$ T cells.**

(**A**) Human naïve CD4$^+$T cells were cultured in the presence of 2 ng/mL TGF-β1 with or without curcumin or GO-Y030 for three days. Data are one representative at three independent experiments. (**B**) Relative Foxp3 expression in CD4$^+$ T cells. TGF-β1 stimulation only is set as "1". One-way analysis of variance (ANOVA) with post-hoc Dunnet's multiple (vs. TGF-β) comparisons test employed. (**C**) Relative live cell counts. Human naïve CD4$^+$ T cells were cultured with or without 2 ng/mL TGFβ and concentrations of Curcumin or GO-Y030 as indicated for 72 h followed by the addition of the cell counting reagent. Red: No cells (Medium alone). Data are representative at three independent experiments using different healthy donors. One-way ANOVA with post-hoc Dunnet's multiple (vs. TGF-β) comparisons test employed.


A
TGF-β – + + +
GO-Y030 (0.1uM) – – + –
Curcumin (1 uM) – – – +
64 kDa
50 kDa
SMAD3
p-SMAD3
GAPDH
B
Relative p-SMAD3 expression (p-SMAD3/GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
TGF-β – + + +
GO-Y030 (0.1uM) – – + –
Curcumin (1 uM) – – – +
Relative SMAD3 expression (SMAD3/GAPDH)
2.0
1.5
1.0
0.5
0.0
TGF-β – + + +
GO-Y030 (0.1uM) – – + –
Curcumin (1 uM) – – – +

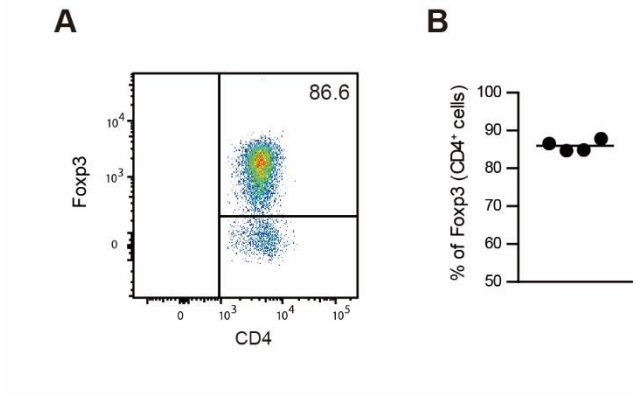
**Supplemental Figure 6: GO-Y030 does not affect TGF-β-induced SMAD pathway.**

(**A**) Representative western blotting image of SMAD3, phospho-SMAD3 and GAPDH at three independent experiments. Naive $CD4^+$ T cells were stimulated with or without TGF-β in the presence or absence of 0.1 uM GO-Y030 or 1 uM Curcumin. (**B**) Relative SMAD3 and phospho-SMAD3 expression. (n=3, Mean with standard error of the mean) Without TGF-β and GO-Y030 stimulation was set as "1". One-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparisons test employed. One-way ANOVA with post-hoc Tukey's multiple comparisons test employed.




**Supplemental Figure 7: Relative murine Foxp3-promoter activity**

(**A**) pGL4-Foxp3 promoter (-1702 to +174) activity were analyzed using the Duo-luciferase assay systems. DMSO- or 1.0 uM GO-Y030 were treated HEK293 before 24 h electroporation. pcDNA3-empty vector transfection (Firefly/Renilla) was set as "1". (**B**) pGL4-basic promoter (control) activity was analyzed using the Duoluciferase assay systems. DMSO- or 1.0 uM GO-Y030 were treated HEK293 cells before 24 h transfection. Data are one representative at two independent experiments (n=2, Mean + standard deviation).

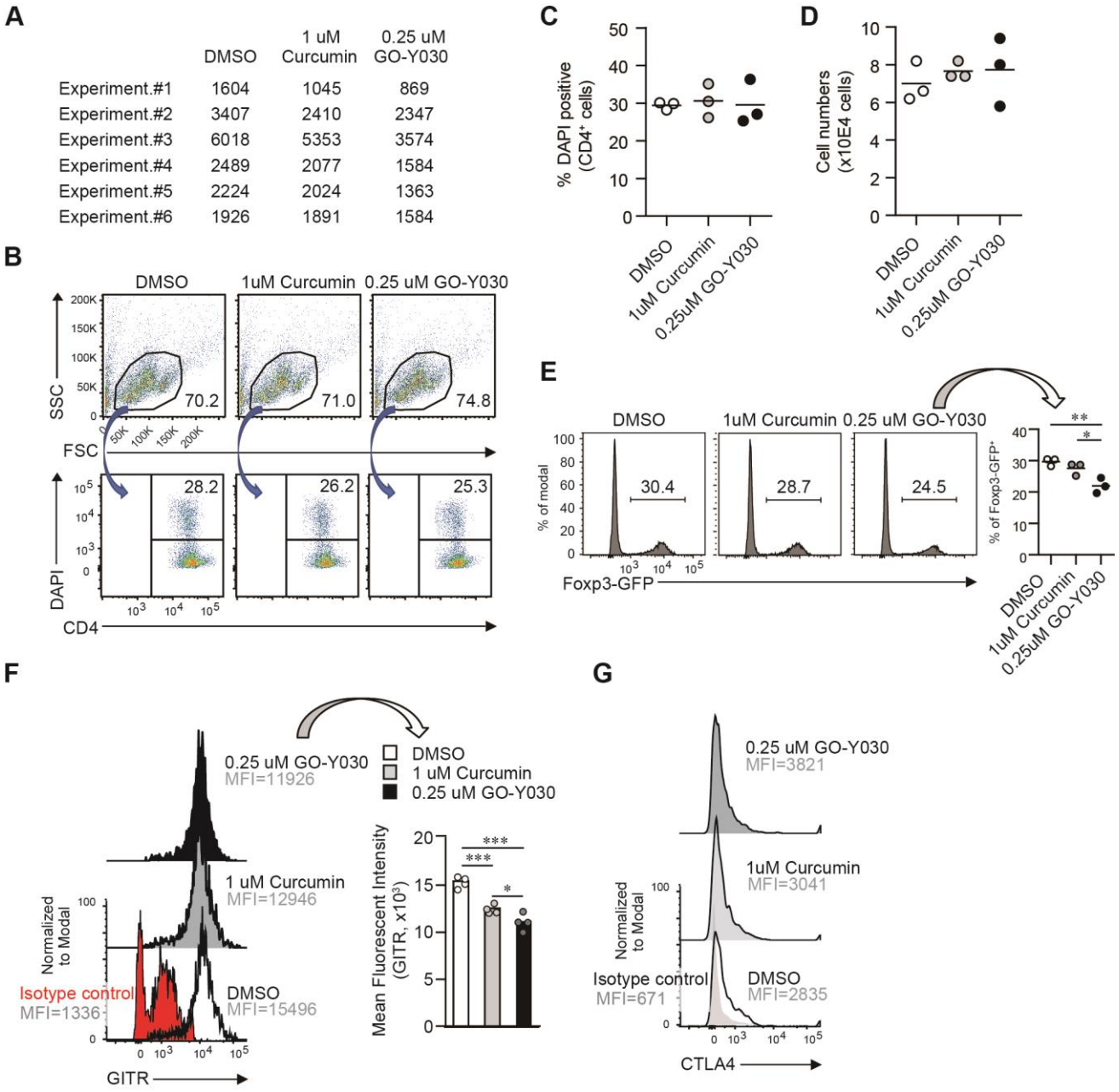



**Supplemental Figure 8: Representative Foxp3 expression in Tregs after $CD4^+CD25^+$ isolation.**

(**A**, **B**) Splenic $CD4^+CD25^+$ Tregs were isolated by using autoMACS (Milteny Biotech) and FACS analyses. Data are one representative of three independent experiments.

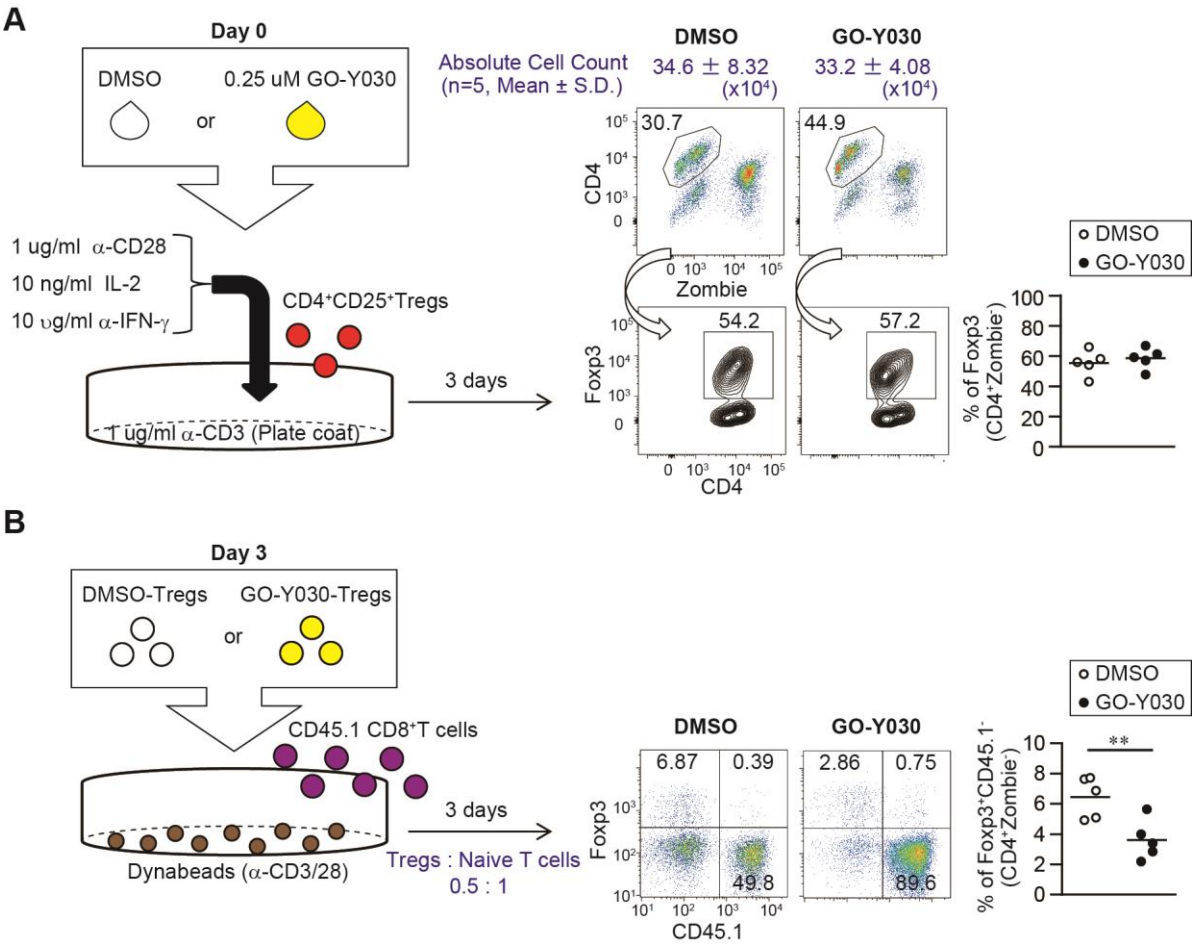
A

| | DMSO | 1 uM Curcumin | 0.25 uM GO-Y030 |
|---|---|---|---|
| Experiment.#1 | 1604 | 1045 | 869 |
| Experiment.#2 | 3407 | 2410 | 2347 |
| Experiment.#3 | 6018 | 5353 | 3574 |
| Experiment.#4 | 2489 | 2077 | 1584 |
| Experiment.#5 | 2224 | 2024 | 1363 |
| Experiment.#6 | 1926 | 1891 | 1584 |



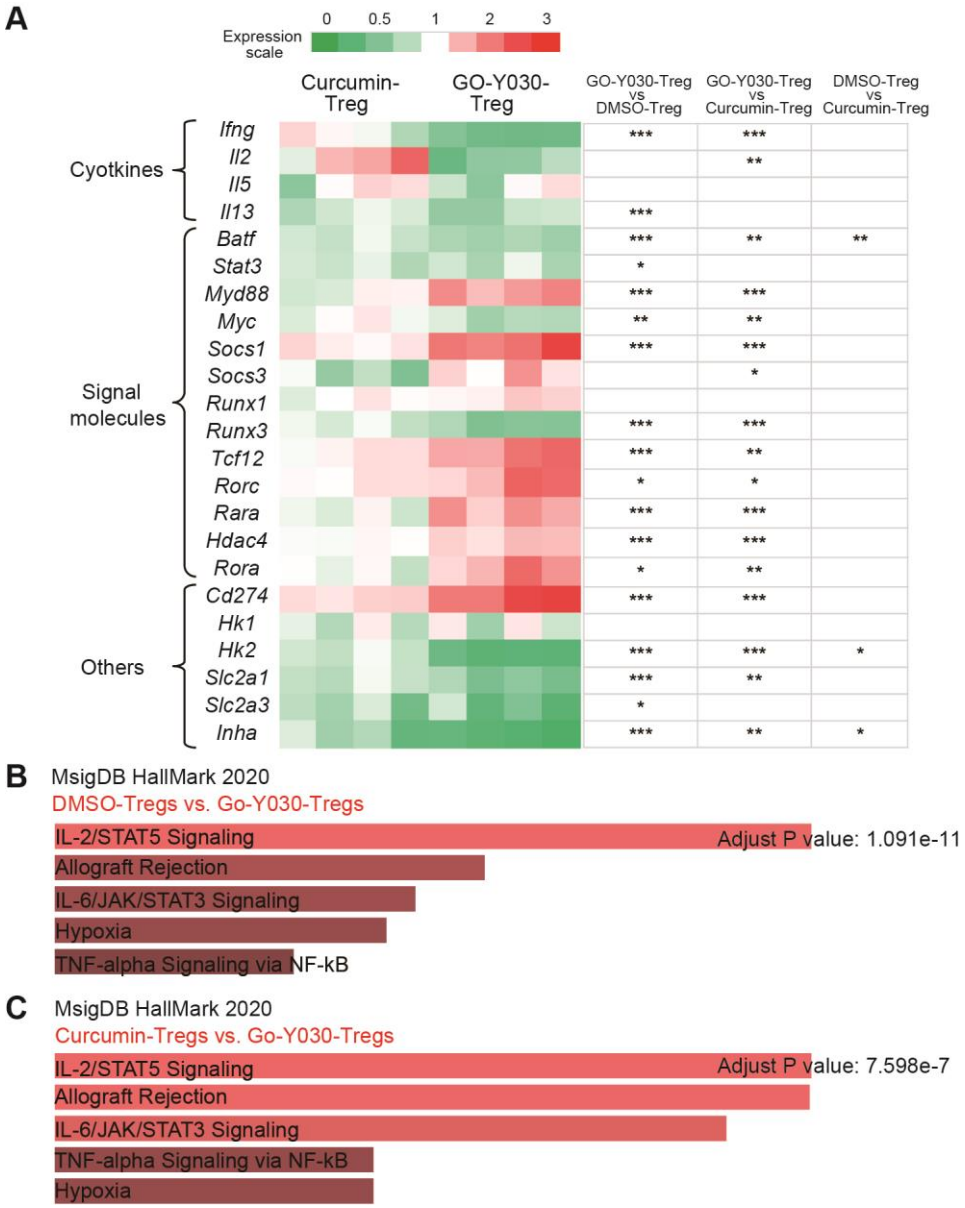

**Supplemental Figure 9: Phenotype of Cultured CD4$^+$CD25$^+$ Tregs.**

(**A**) Foxp3 Mean fluorescence Intensity in CD4$^+$Foxp3$^+$ Treg populations in Figure.3A. Data shows six independent experiments. experiments. (**B**, **C**) Relative cell survival rate after 18 h culture of CD4$^+$CD25$^+$ Tregs with or without Curcumin or GO-Y030. Data are one representative at more than three independent experiments. (**D**) Absolute number of cells after 18 h culture of CD4$^+$CD25$^+$ Tregs with or without Curcumin or GO-Y030. The starting number of cells in each wells was 1 x $10^5$ cells. (**E**) Foxp3-GFP positive cells in CD4$^+$ population. Foxp3-GFP positive cells were purified by FACS-Aria II (>95%) and cultured 18 h with CD3 + CD28. Data are one representative at three independent experiments. GITR (**F**) and CTLA4 (**G**) in CD4$^+$Foxp3$^+$ Treg populations in Figure. 5A. Gray; Isotype control, Black; GITR or CTLA4. Data are one representative at three independent experiments. One-way analysis of variance with Tukey employed for statistic difference.



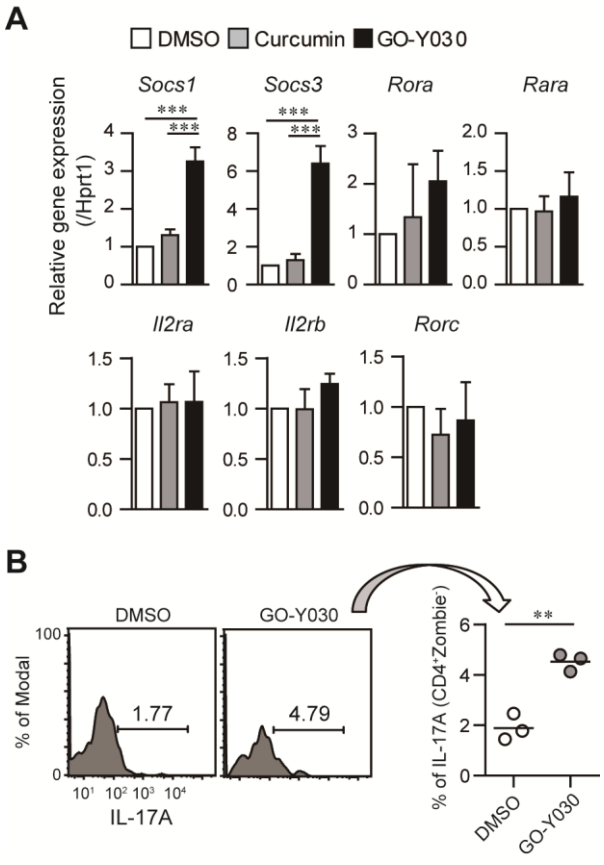

**Supplemental Figure 10: GO-Y030 controls IL-2/STAT5 axis in CD4$^+$CD25$^+$ Tregs.**

(**A**) Percentage of Foxp3 in cultured CD4$^+$CD25$^+$Tregs at day three. Data are representative at five independent experiments. Student T-test was employed. (**B**) Percentage of Foxp3 in cultured CD4$^+$CD25$^+$ Tregs in the suppression assay. DMSO- or GO-Y030-treated CD4$^+$CD25$^+$ Tregs were co-cultured with CD8$^+$ T cells (Tregs:CD8$^+$ T cells=0.5:1) for three days. Data are representative at shows five independent experiments. Student T-test was employed.



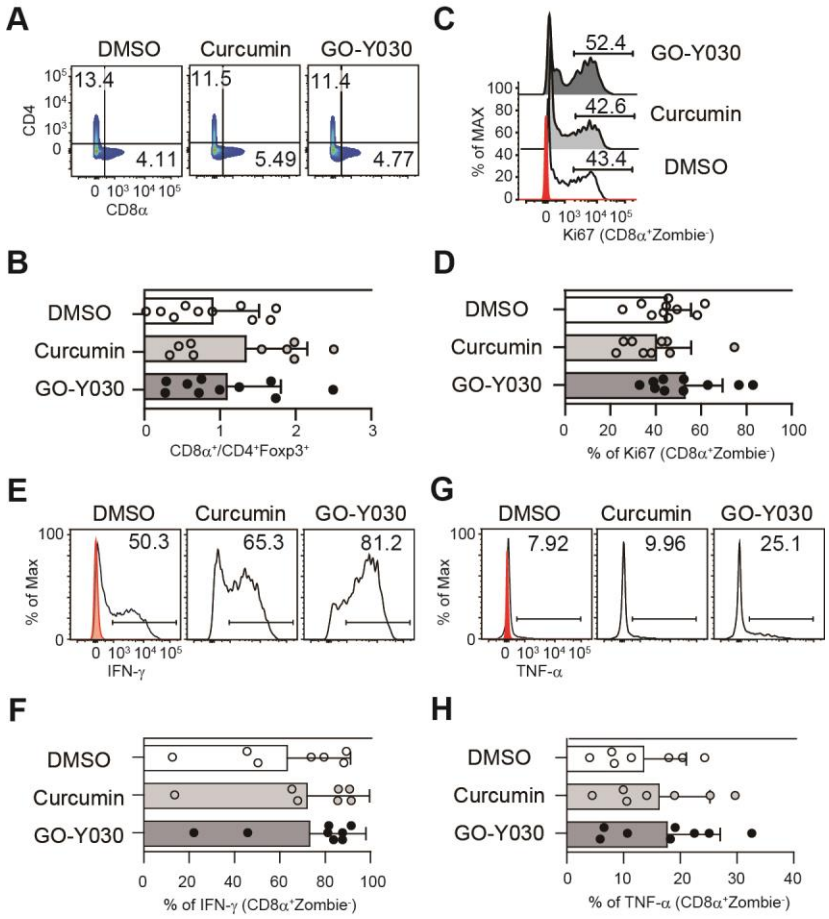

**Supplemental Figure 11: GO-Y030 controls IL-2/STAT5 axis in CD4$^{+}$CD25$^{+}$ Tregs.**

(**A**) Real time PCRs in 72 h culture of CD4$^{+}$CD25$^{+}$ Tregs with or without Curcumin or GO-Y030. The color scale is shown at the top of heat map. Each genes expression of DMSO-treated Tregs are as set as "1". Data showed four independent experiments. (**B, C**) Enrichr- was used to calculate enrichment scores of signaling pathways. We selected genes of significantly difference expression (*P<0.05*) between DMSO-Tregs and GO-Y030-Tregs (**B,** https://maayanlab.cloud/Enrichr/enrich?dataset=ce1ae4783a07360aa829bddb0fd36eb1) or Curcumin-Tregs and GO-Y030-Tregs (**C,** https://maayanlab.cloud/Enrichr/enrich?dataset=35a8b176b017fbdc7c09576aa75995cd). Statistical analyses (One-way analysis of variance with Tukey) were performed.




**Supplemental Figure 12: GO-Y030 controls IL-2/STAT5 axis in CD4$^+$Foxp3-GFP$^+$ Tregs.**

(**A**) Real time PCRs in 72 h culture of CD4$^+$Foxp3-GFP$^+$ Tregs with or without 1 μm Curcumin or 0.25 μm GO-Y030. Data pooled three independent experiments. Statistical analyses (One-way analysis of variance with Tukey) were performed. (**B**). Th17 population in cultured CD4$^+$Foxp3-GFP$^+$ Tregs with or without 0.25 μm GO-Y030. Student T-test was performed.




**Supplemental Figure 13: GO-Y030 did not prevent infiltration and activation of CD8$^+$ cells in tumor microenvironment.**

(**A**) Frequency of CD4$^+$/CD8α$^+$ cells in tumor infiltrate lymphocytes. (**B**) Ratio of tumor infiltrate CD8$^+$ cells to CD4$^+$Foxp3$^+$ Treg cells. (**C**, **D**) Ki67 expression in CD8α$^+$ cells in tumor infiltrate lymphocytes. Red; isotype control. Data are one representative of each of the two independent experiments (**A**, **C**). (**E**, **F**) IFN-γ production from CD8α$^+$ cells in tumor infiltrate lymphocytes. Red; isotype control. (**G**, **H**) TNF-α production from CD8α$^+$ cells in tumor infiltrate lymphocytes. Red; isotype control. One-way analysis of variance with post-hoc Tukey's multiple comparisons test was used (**B**, **D**, **F**, **H**). The graph shows mean and standard deviation.