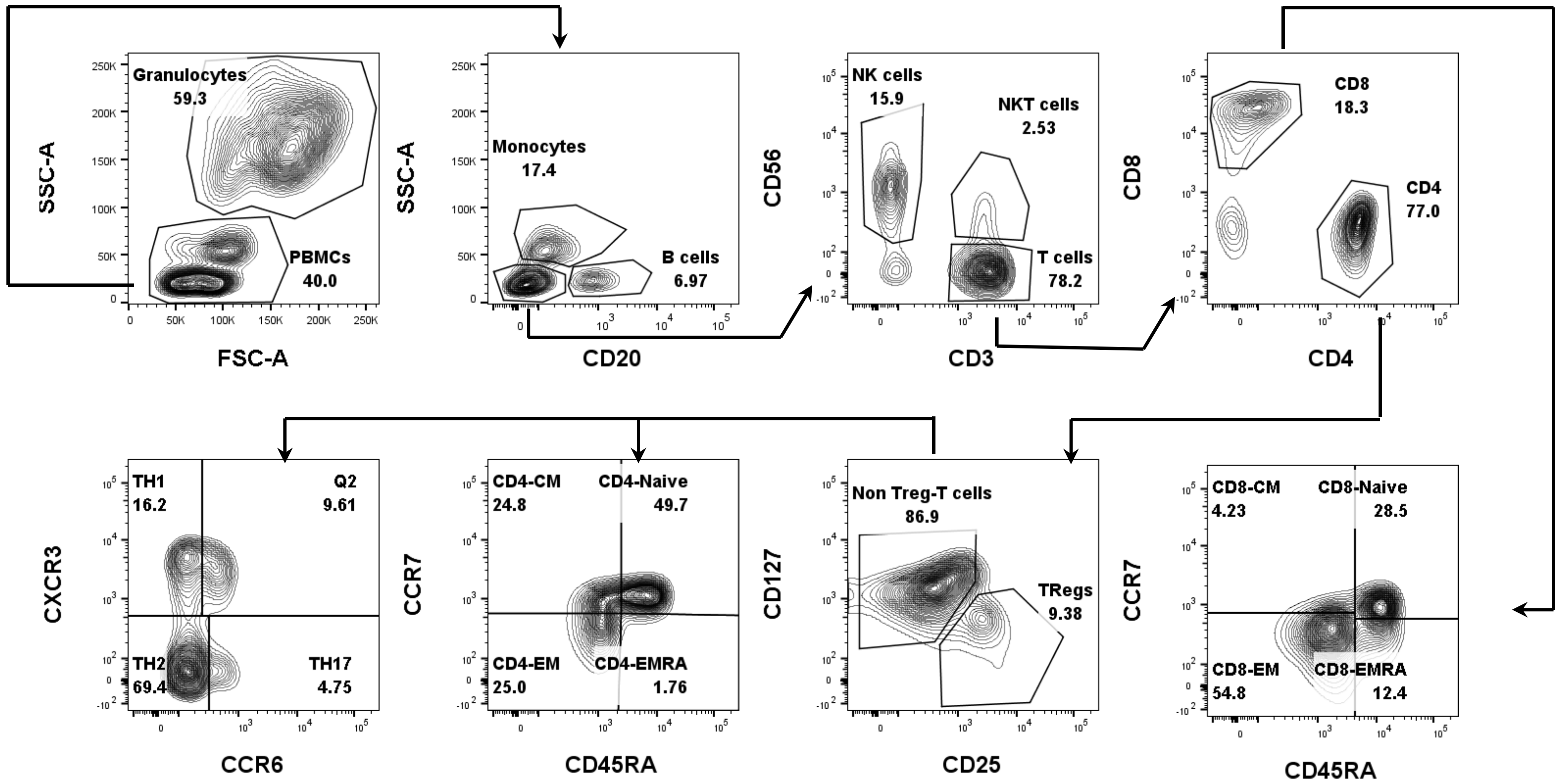
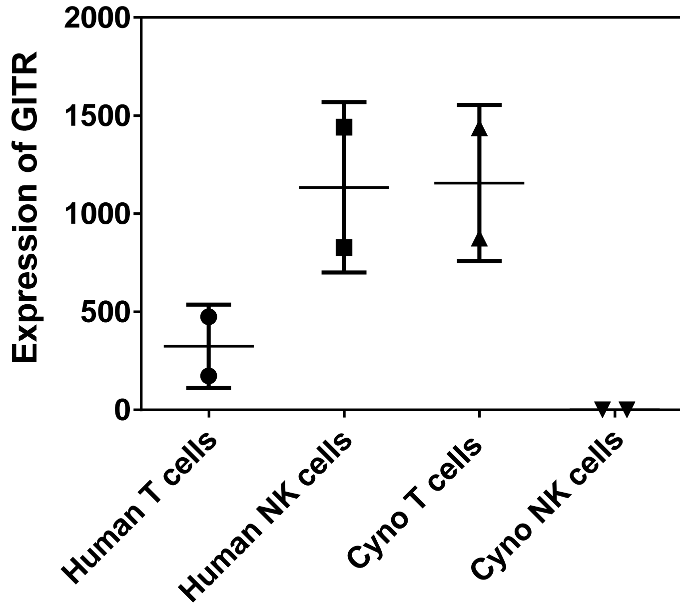
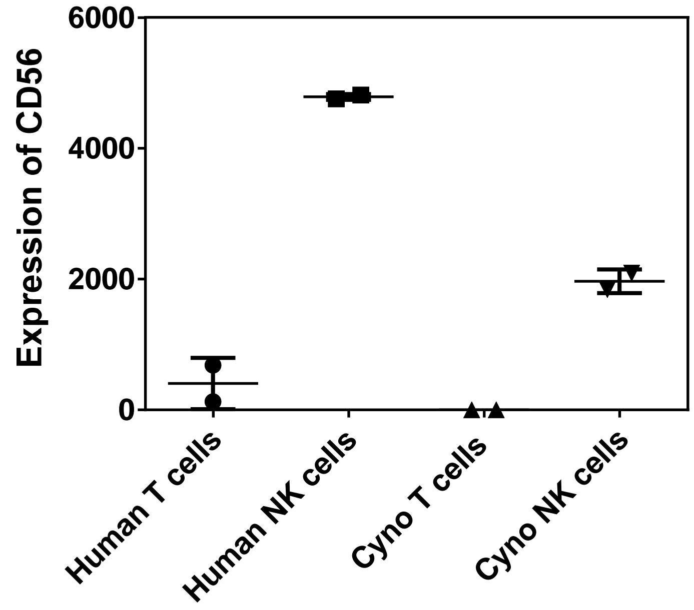
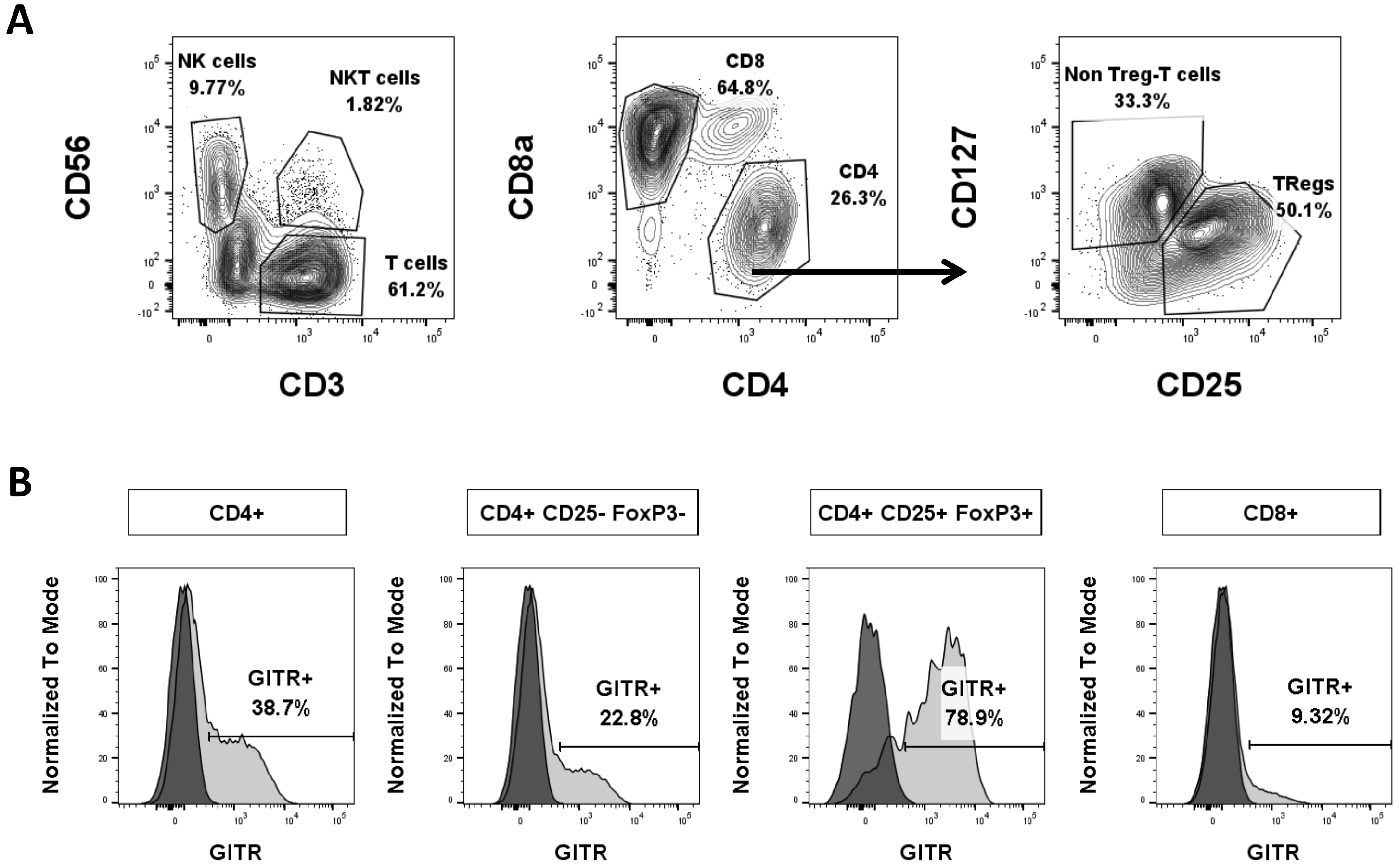


Supplementary Figure S1. Gating scheme and markers used to phenotype various T cell subsets in human blood. Flow cytometric gating scheme employed to identify monocytes, B cells, NK cells, NKT cells, CD8+ T cells, CD4+ T cells, T_{reg}, Non-T_{reg} CD4+ T cells, CD4+ Naïve, CD4+ central memory (CM), CD4+ effector memory (EM), CD4+ effector memory CD45RA+ (EMRA), TH1, TH2, TH17, CD8+ Naïve, CD8+ central memory (CM), CD8+ effector memory (EM), and CD8+ effector memory CD45RA+ (EMRA).

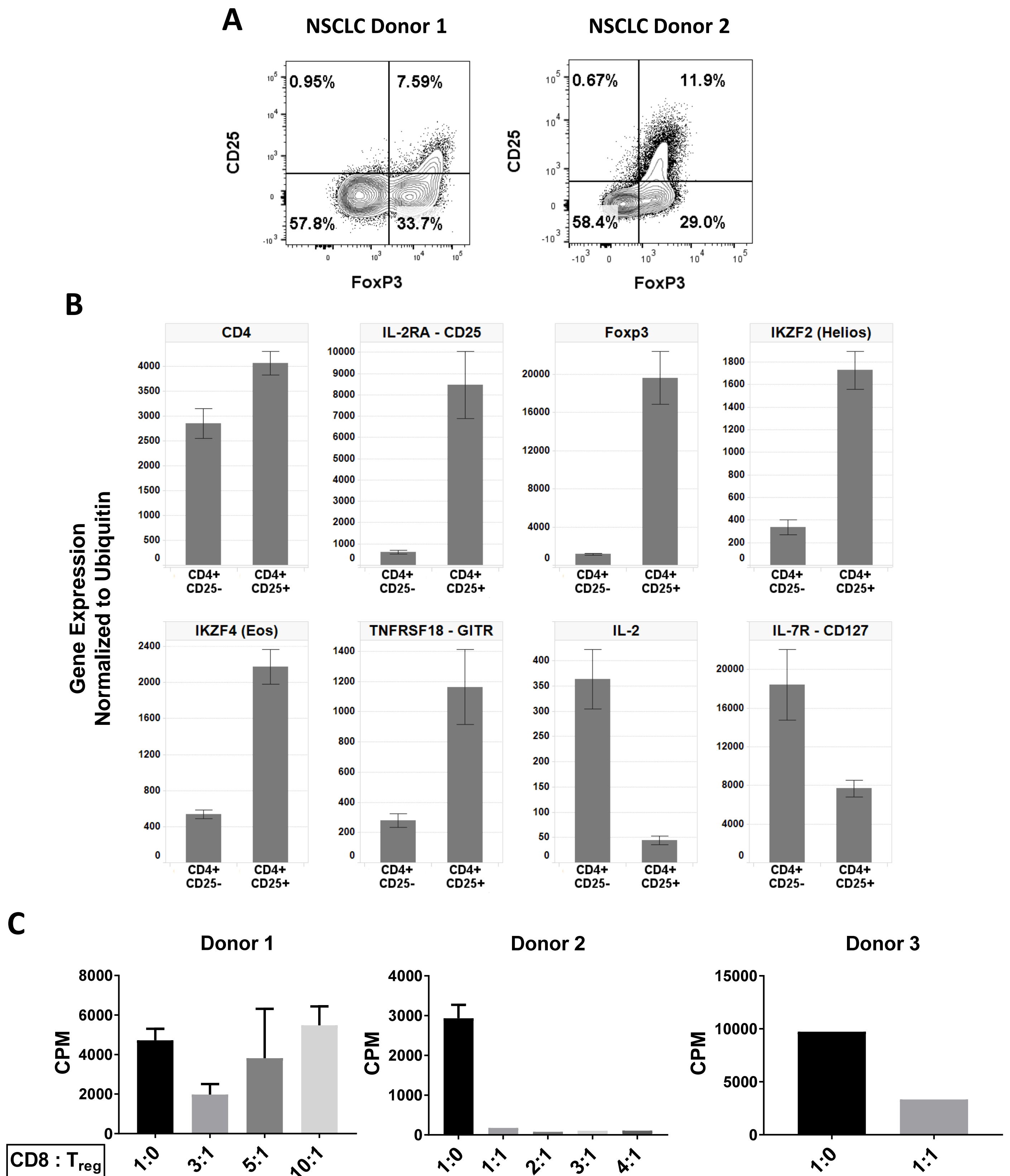




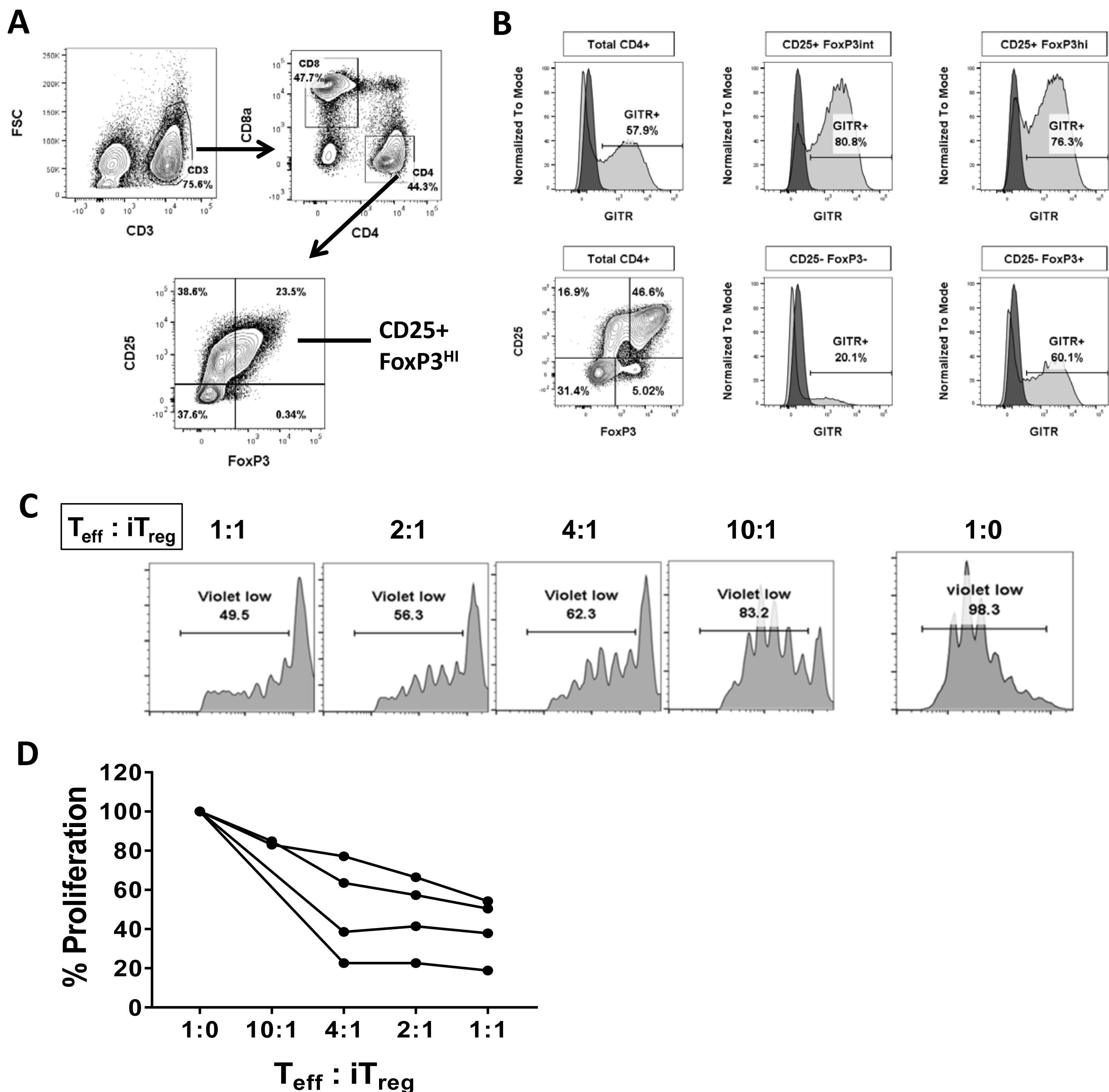
Supplementary Figure S2. Determination of GITR gene expression on human and cynomolgus monkey NK cells and T cells. T cells and NK cells were sorted from human and cynomolgus monkey peripheral blood and the expression of GITR (A) and CD56 (B) was quantified by RTqPCR. T cells were identified as CD14- CD56- CD3+ and NK cells as CD14- CD3- CD56+.



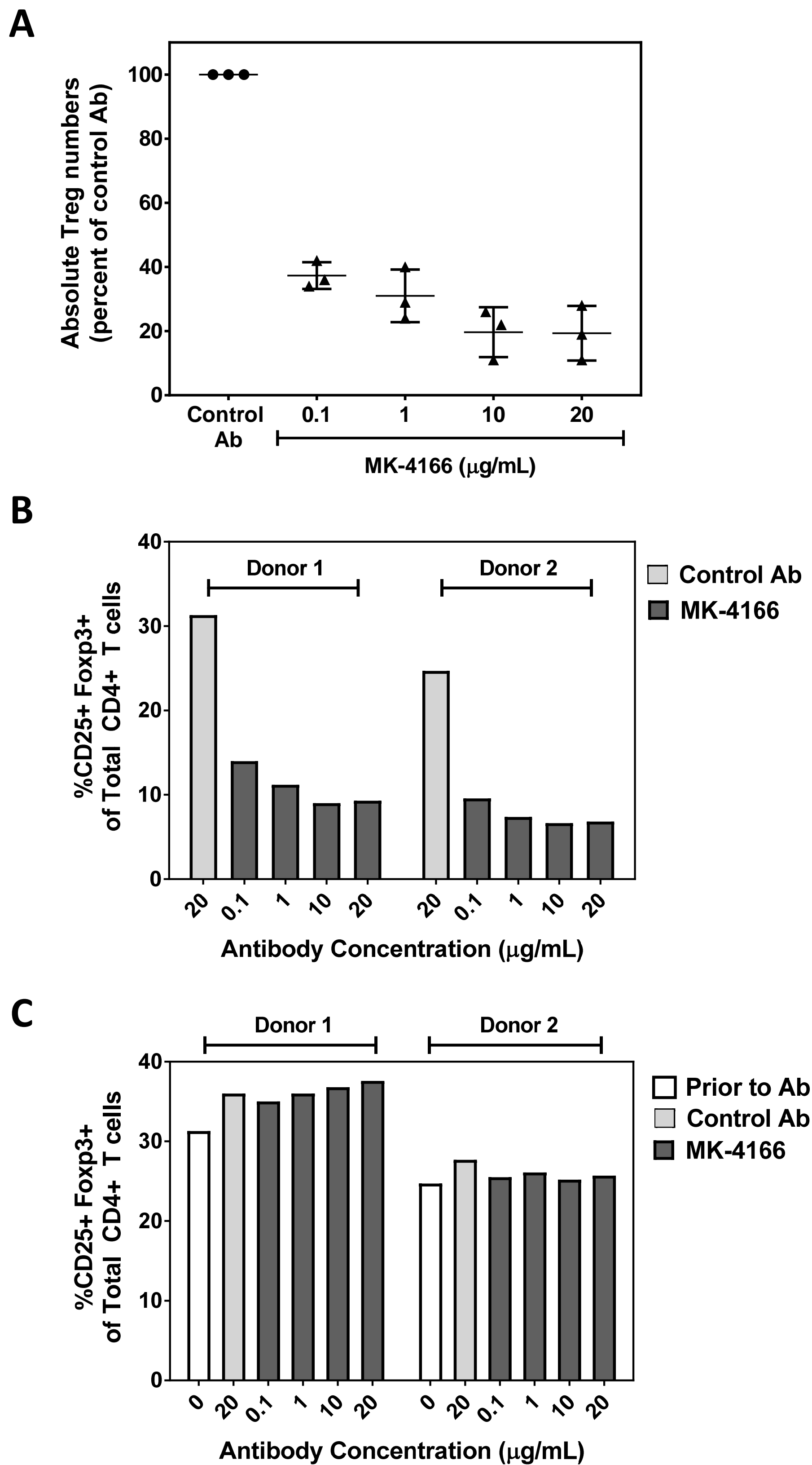
Supplementary Figure S3. Expression of GITR on various human tumor infiltrating T cell subsets. (A) The gating scheme used to identify tumor infiltrating T_{regs} and CD4+ or CD8+ T_{effs}. (B) Representative profile of GITR expression on CD4+CD25+FoxP3+ tumor-infiltrating T_{regs}, CD4+CD25-FoxP3- non-T_{reg} CD4+ T cells, and total CD4+ or CD8+ TILs from a NSCLC tumor. The dark histograms represent staining with an isotype matched control antibody whereas the gray-shaded histograms represent staining with MK-4166 in the indicated populations.



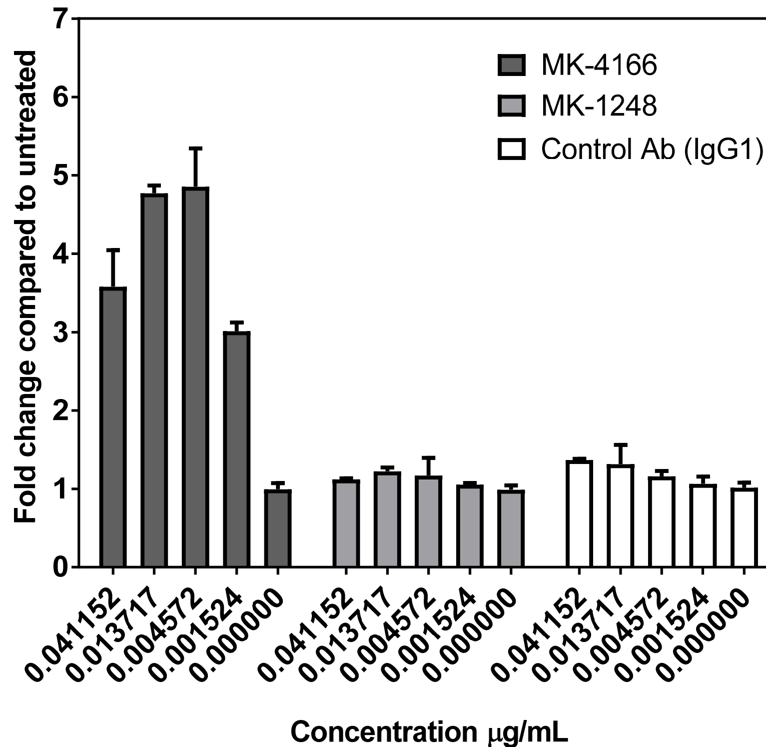
Supplementary Figure S4. Phenotypic and functional characterization of CD4+ CD25+ TILs from NSCLC tumor tissues. (A) Representative contour plots from two independent NSCLC tumors showing co-expression patterns of CD25 and FoxP3 on CD4+ TILs by flow cytometry. (B) RTqPCR expression levels of various indicated genes in CD4+ CD25- TILs and CD4+ CD25+ TILs sorted from 6 NSCLC samples. Bars represent mean ± standard error. (C) T_{regs} (CD4+ CD25+) and CD8+ cells were isolated from tumors of three independent donors by FACS and mixed in the indicated ratios. The number of CD8+ T cells was kept constant and varying amounts of T_{regs} were added to obtain the indicated ratios. Cultures were stimulated with anti-CD3 and anti-CD28 microbeads (at a bead to cell ratio of 1:2) and proliferation was measured by incorporation of ^{3}H-thymidine. Exogenous IL-2 was not added to the cultures.

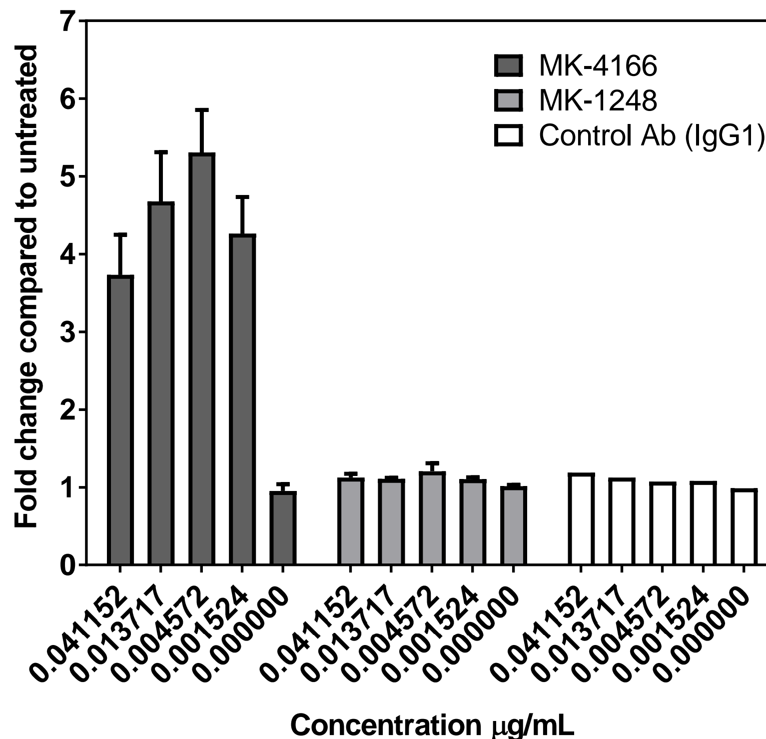


Supplementary Figure S5. Phenotypic and functional characterization of iT_{regs} generated in MLR cultures. (A) Representative contour plots showing the gating scheme used to determine the relative abundance of iT_{reg} generated in MLR cultures which were identified as CD4+ CD25+ $FoxP3^{HI}$ T lymphocytes. (B) GITR expression in the four subsets of CD4+ T cells distinguished by combinations of FoxP3 and CD25 expression from a similar MLR culture as shown in (A). The dark histograms represent staining with an isotype matched control antibody whereas the gray-shaded histogram represents staining with MK-4166 in the indicated populations. (C) Histograms showing proliferation of CellTrace violet labeled T_{effs} from one representative donor at the indicated T_{eff}:iT_{reg} ratios. iT_{regs} for these co-culture experiments were isolated from MLR cultures using CD4+CD25+ microbeads. (D) Cumulative data from 4 independent donors showing proliferation of CellTrace violet labeled T_{effs} at the indicated T_{eff}:iT_{reg} ratios normalized to proliferation of T_{effs} in the absence of iT_{regs}. The number of T_{effs} was kept constant and varying amounts of iT_{regs} were added to obtain the indicated ratios.

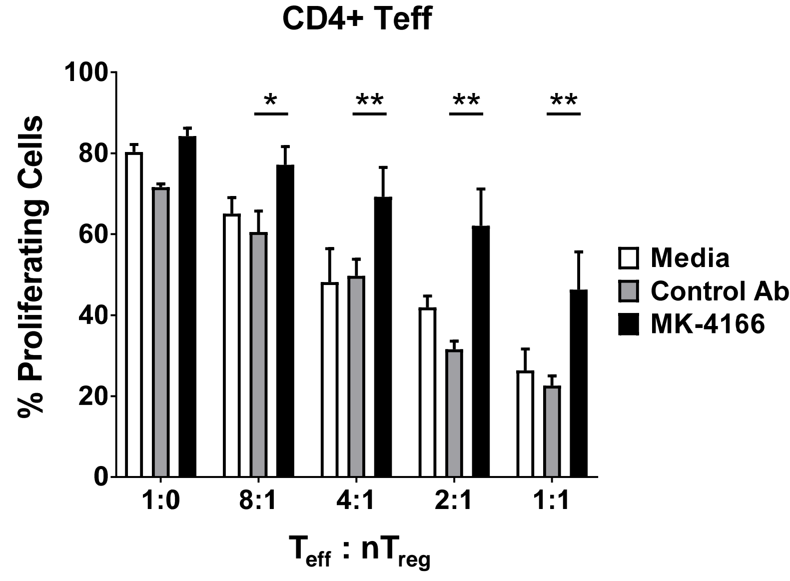


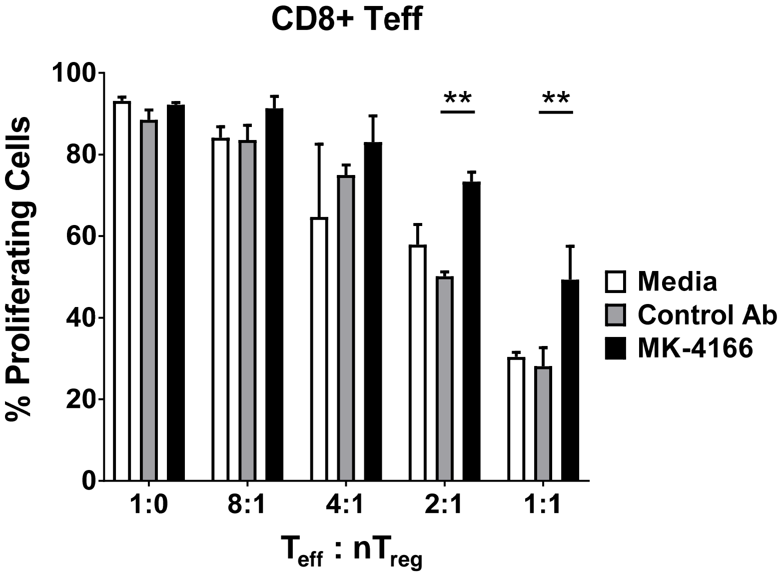
Supplementary Figure S6. Differential effects of MK-4166 when added to MLR cultures on Day 0 vs Day 7. MK-4166 or isotype matched control antibody was added at indicated concentrations to MLR cultures either at the beginning of culture on Day 0 (A, B) or after 7 days (C). The abundance of T_{regs} was determined by flow cytometry 7 days after the addition of MK-4166 and plotted as the absolute number (based on flow cytometric counting beads) normalized to control antibody (A) or as % CD25+ $FoxP3^{HI}$ of CD4+ T cells (B and C). Cultures in B and C were set up using cells from the same two donors.

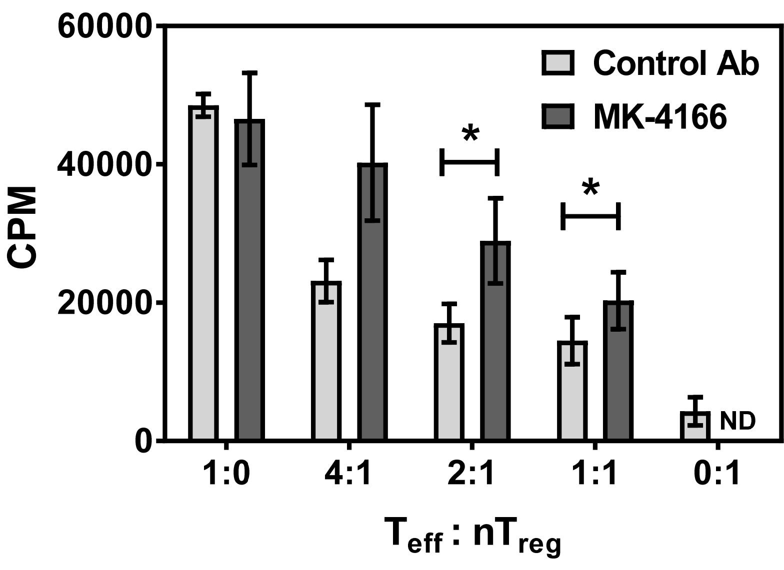




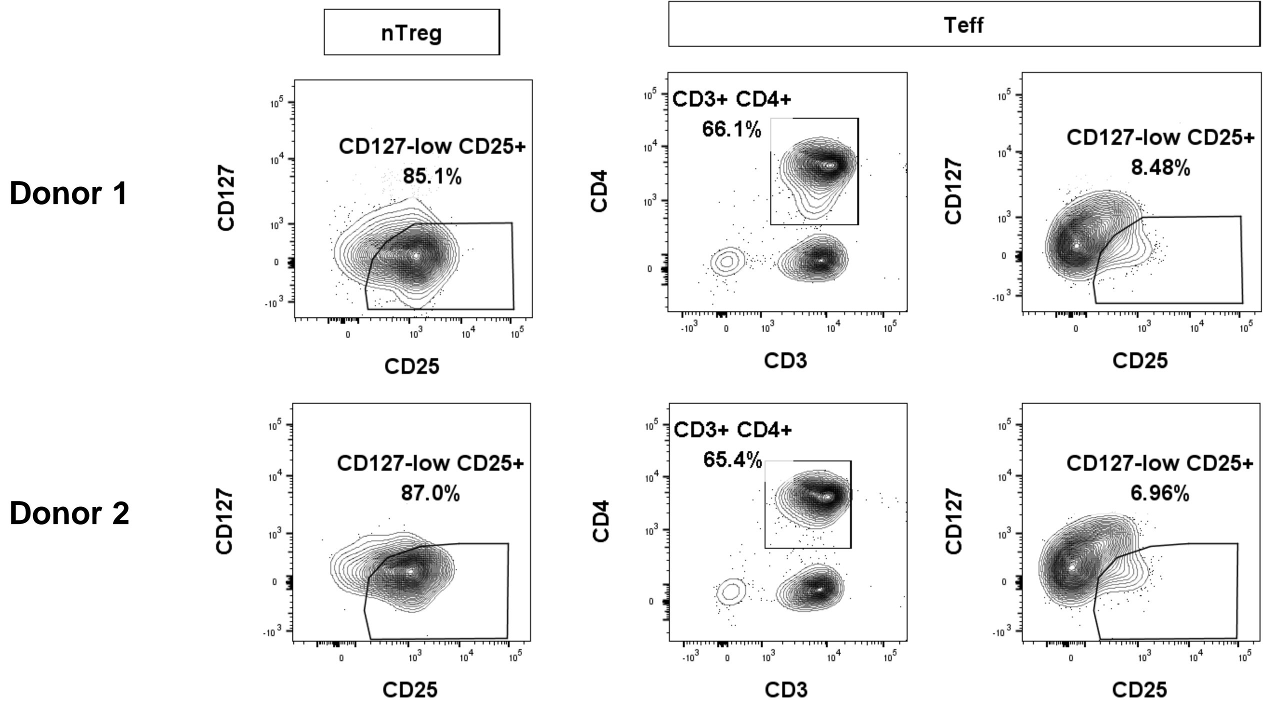
Supplementary Figure S7. MK-4166 has the potential to induce ADCC when bound to human iT$_{regs}$. The potential of MK-4166 to induce antibody dependent cell mediated cytotoxicity (ADCC) was determined using human iT$_{reg}$ as target cells and an engineered Jurkat effector cell containing FcγRIIIa (CD16) and an NFAT transcriptional reporter. MK-4166, MK-1248 or an isotype control matched control antibody was allowed to bind to the surface of human MLR-derived iT$_{regs}$ and NFAT signaling in Jurkat cells engineered to express FcγRIIIa (CD16) was determined using a luciferase reporter. Experiments were conducted with two different donors (panels A and B). iT$_{regs}$ were incubated with the Jurkat effector cells at an E/T ratio of 2.5 to 1 for 6 hours. Results are shown as fold change upon antibody treatment compared to the untreated wells.

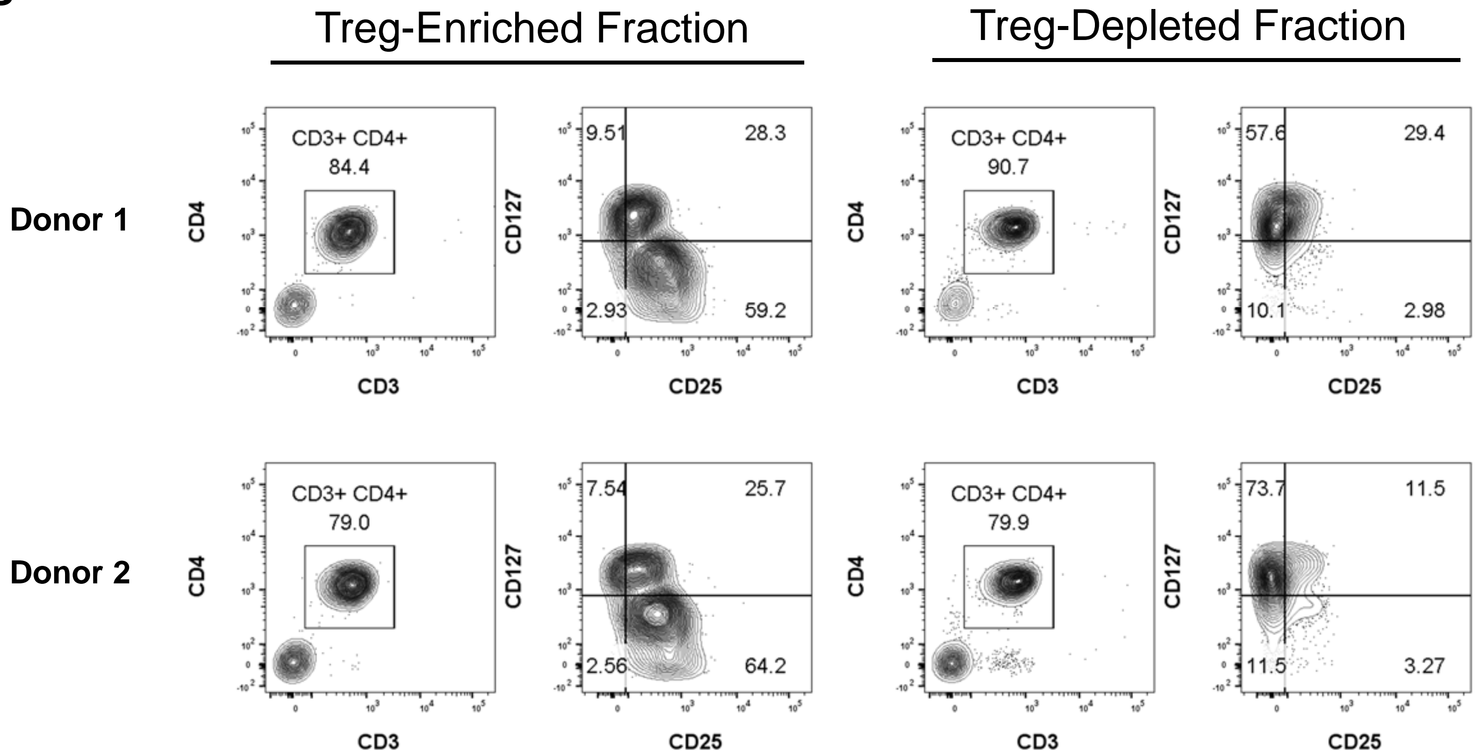






Supplementary Figure S8. MK-4166 attenuates nT_{reg} suppression of T_{eff} proliferation. (A&B) CFSE-labeled T_{effs} were stimulated with anti-CD3 and lethally irradiated autologous HLA-DR+ cells. The ability of responder matched nT_{regs} to inhibit proliferation of CD4+ (A) or CD8+ (B) T_{effs} at indicated T_{eff}:nT_{reg} ratios was measured as dilution of CFSE-label intensity in the presence of MK-4166 or isotype matched control mAb. Data shown are representative of three donors. (C) MLR cultures were initiated by the addition of allogeneic monocyte-DCs to CD4+ CD25- responder T cells (T_{eff}) and mixed with enriched donor matched nT_{reg} (CD4+ CD25+) at a ratio of 4:1. Cell proliferation in the presence of MK-4166 was measured by ^{3}H-thymidine incorporation and is plotted as fold change compared to isotype matched control mAb treated cultures. The data are aggregate of 4 donors. Bars indicate mean values +/- SEM. * and ** indicate a p-value < 0.05 and < 0.01, respectively, calculated using a paired student's t-test.



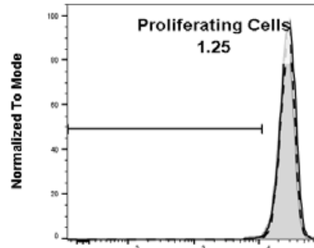


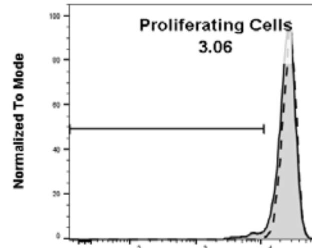
Supplementary Figure S9. Purity of enriched nT_{regs} used in T_{reg} suppression assays. (A) Representative contour plots showing the purity of nT_{regs} and T_{effs} isolated from blood using the CD4+ CD25+CD127dim/- kit (Miltenyi) and used in experiments depicted in Figure 5C&D and Figure S8A&B). (B) Contour plots from two representative donors depicting the purity of nT_{regs} and T_{effs} isolated from blood using the CD25Microbeads II kit (Miltenyi) and used in experiment depicted in Figure S8C.

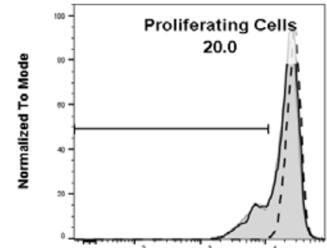
A

Bead : nT_{reg} ratio — 1:8 — 1:4 — 1:1

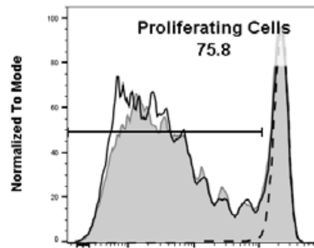
Day 3

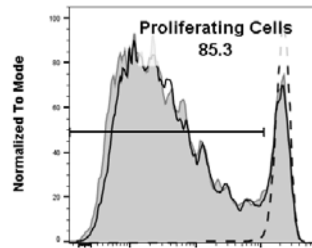


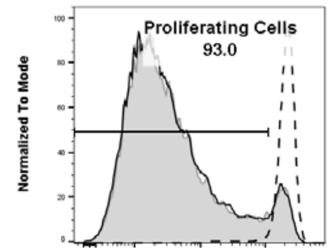




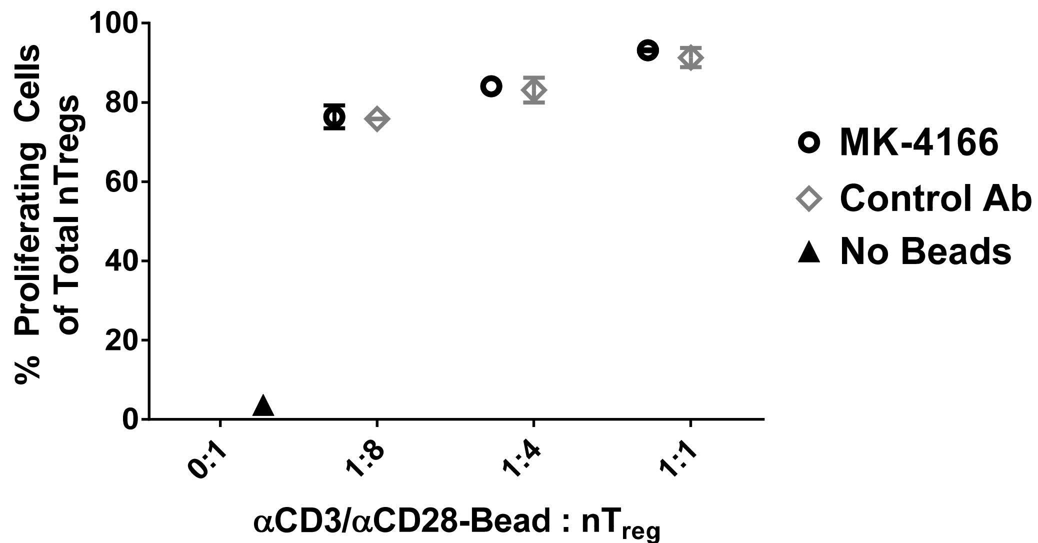
Day 6







B



Supplementary Figure S10. MK-4166 does not affect nT_{reg} proliferation. (A) Histograms showing proliferation of nT_{regs} labeled with CellTrace Violet or CellTrace Far Red after 3 or 6 days of stimulation with anti-CD3 and anti-CD28 microbeads (at the indicated bead to cell ratios) in the presence of MK-4166 (shaded gray) or isotype matched control antibody (bold line). Dashed lines in the histograms show proliferation in control wells in the absence of stimulation with microbeads. (B) Cumulative data showing proliferation of nT_{regs} from 2 independent donors after 6 days of stimulation with anti-CD3 and anti-CD28 microbeads (at the indicated bead to cell ratios) in the presence of MK-4166 or isotype matched control antibody is shown. No exogenous IL-2 was added in these cultures.