YMTHE, Volume *28*

Supplemental Information

An Optimized Full-Length FLT3/CD3 Bispecific Antibody Demonstrates Potent Anti-leukemia Activity and Reversible Hematological Toxicity

Yik Andy Yeung, Veena Krishnamoorthy, Danielle Dettling, Cesar Sommer, Kris Poulsen, Irene Ni, Amber Pham, Wei Chen, Sindy Liao-Chan, Kevin Lindquist, S. Michael Chin, Allison Given Chunyk, Wenyue Hu, Barbra Sasu, Javier Chaparro-Riggers, and Ivana Djuretic

Figure S1

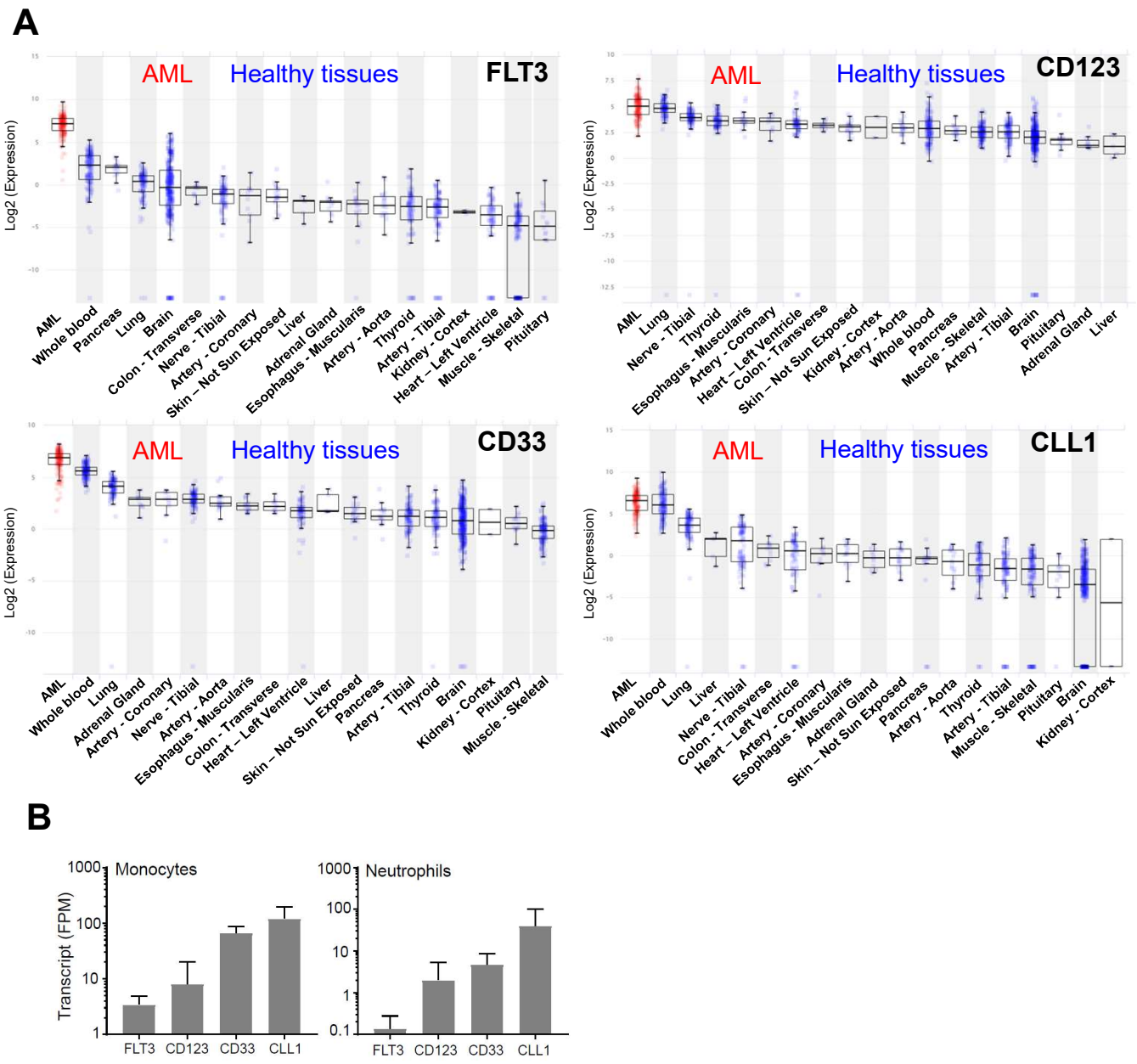


Figure S1. Gene expression profiles of FLT3, CD33, CLL1 and CD123 in AML and various major healthy tissues. (A) Comparison of gene expression (RNA-seq) for FLT3, CD123, CD33, and CLL1 from publicly available gene expression databases. AML gene expression was imported from The Cancer Genome Atlas (TCGA). Gene expression from 18 major healthy tissues was imported from Genotype-Tissue Expression data (GTEx) portal. (B) Gene expression analysis (RNA-seq) from sorted immune cells imported from the BLUEPRINT Data Analysis Portal. FLT3 expression in monocytes and neutrophils was the lowest compared to three other AML targets.

Figure S2

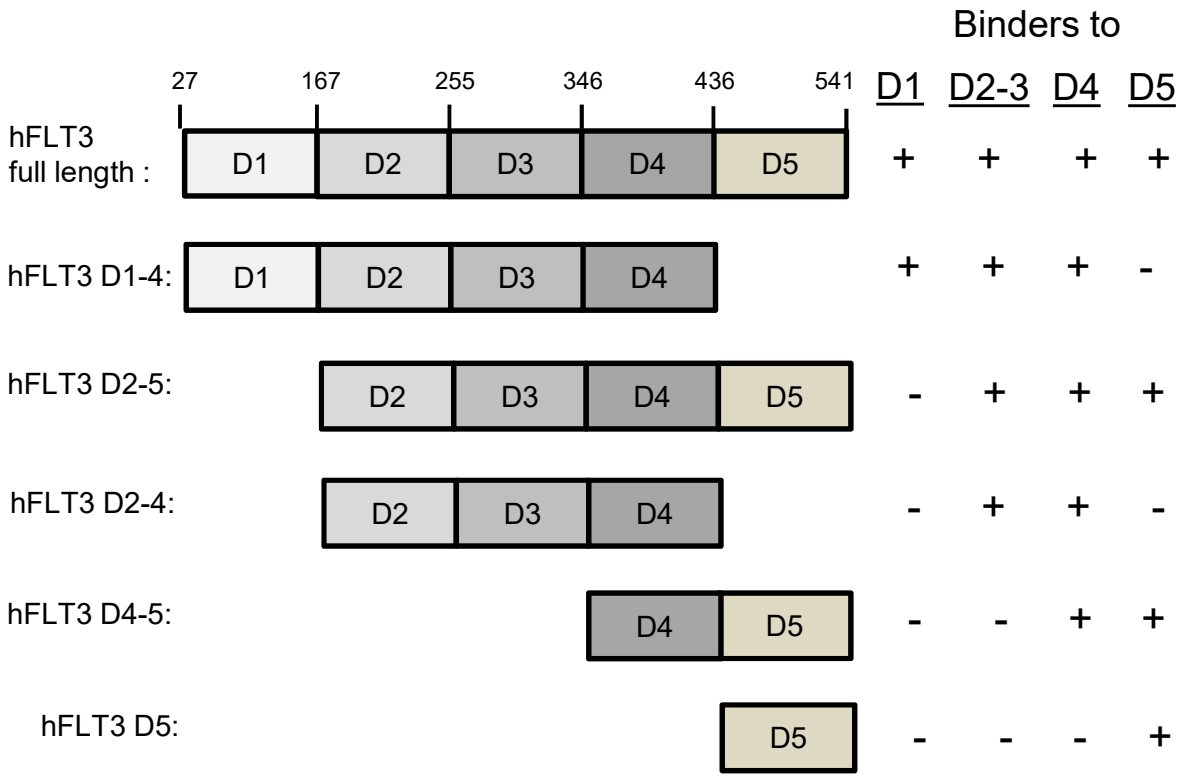
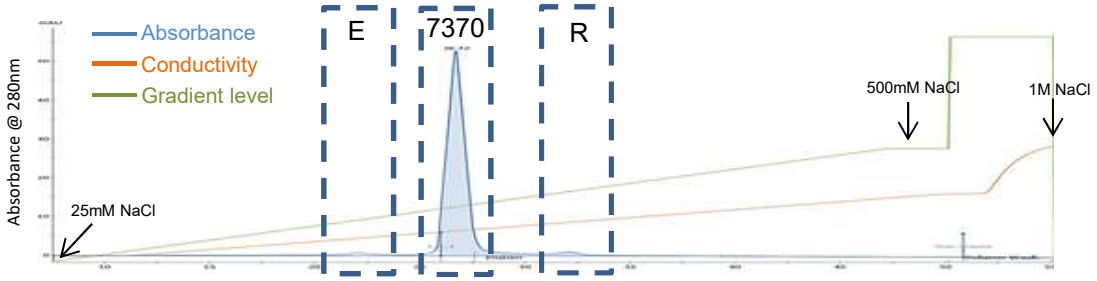


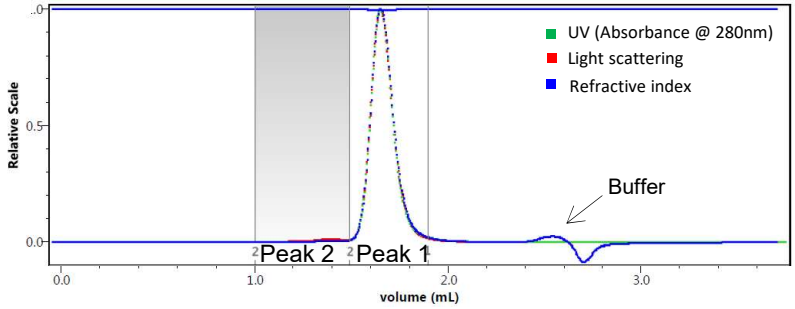
Figure S2. Schematic representation of the recombinant full length FLT3 and various truncated forms of FLT3. Recombinant his-tagged full length and truncated FLT3 proteins were expressed in HEK293 cells and purified using Nickel affinity chromatography. The recombinant proteins were used to map the binding domains of anti-FLT3 antibodies using ELISA. D1, D4 and D5 binders could be straightforwardly identified from the ELISA. Recombinant FLT3 D2 and D3 could not be expressed without the other domain, therefore binders against D2 and D3 could not be segregated into individual domain binders using these truncation variants. These clones were labeled as D2-D3 binders. “+” denotes positive binding in ELISA; “-” denotes no binding in ELISA.

Figure S3

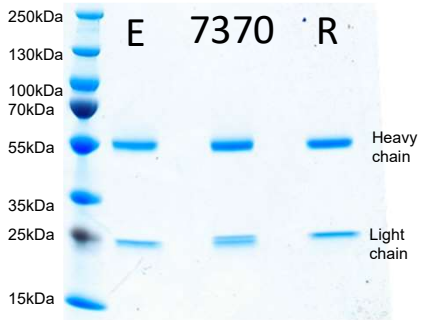
A



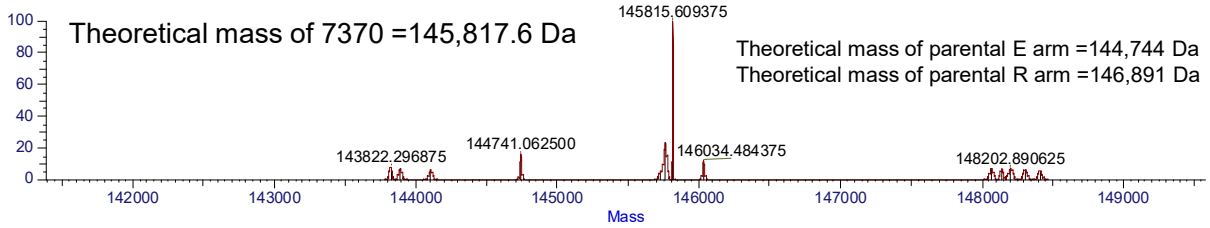
B



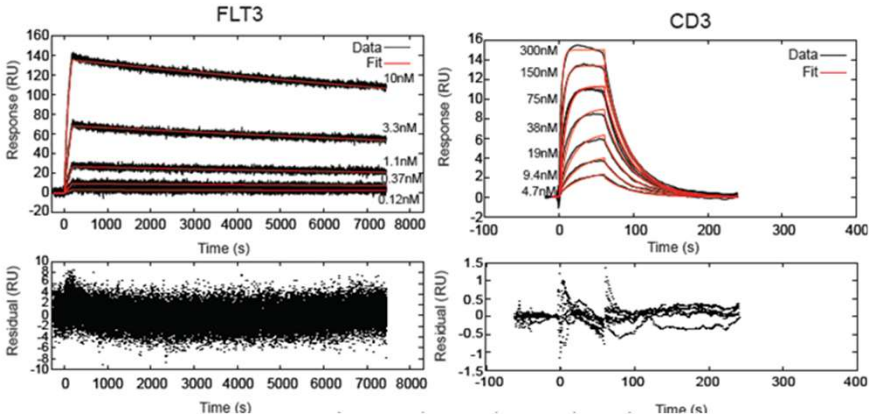
C



D



E



	k_{on} ($x10^5$ $M^{-1}s^{-1}$)	k_{off} ($x10^{-5}$ s^{-1})	K_D (nM)
Human FLT3	6.5	3.2	**0.049**
Human CD3	12	3000	**27**
Cyno CD3	9.6	2800	**29**

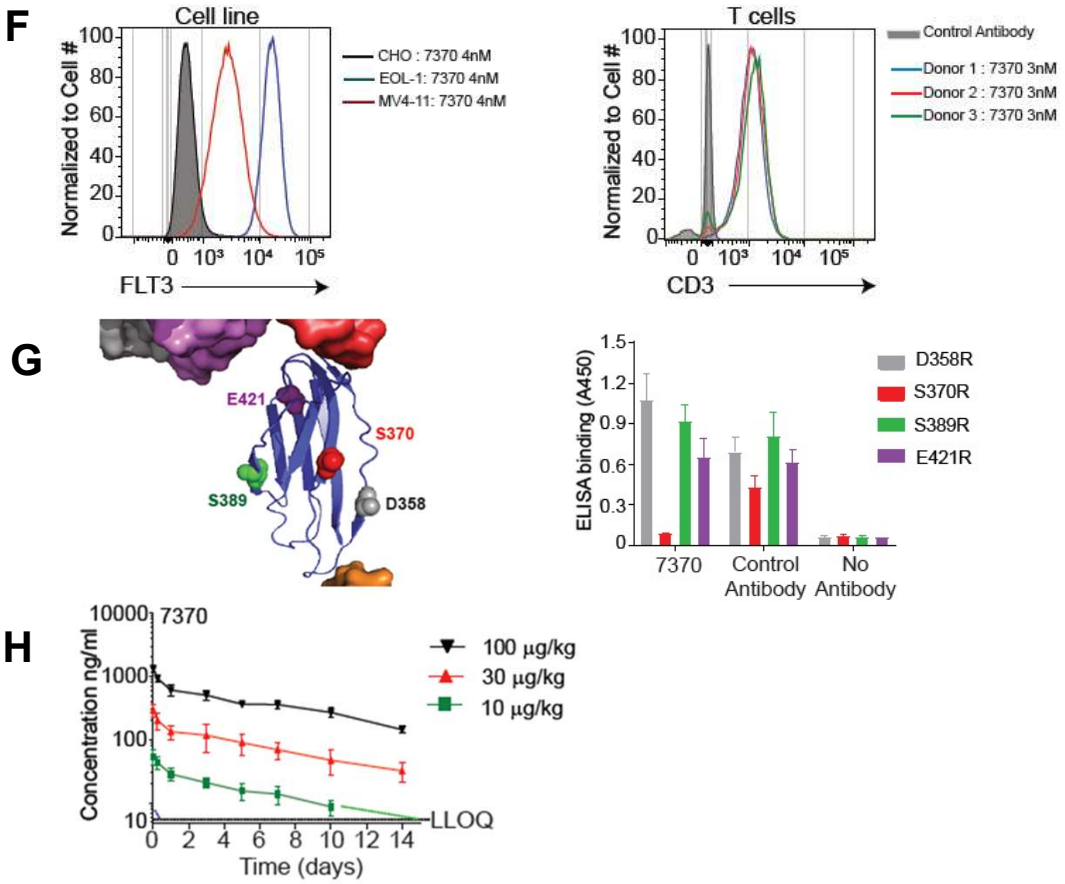


Figure S3. Properties of 7370 FLT3-CD3 bispecific IgG. (A) Analytical ion-exchange chromatographic analysis for heterodimer detection. Purified 7370 was analyzed using a MonoS column. (GE Healthcare). Running condition was 20mM MES buffer pH 5.4 with increasing NaCl concentration from 25mM to 1M. The heterodimeric bispecific antibody represent >95% of the injected materials. (B) Size-exclusion chromatographic and multi-angle light scattering (SEC-MALS) analysis. 7370 (Peak1) was determined to be 99% monomeric by SEC with an average molar mass of 1.49×10^5 (+ 0.061%) g/mol by light scattering. (C) Purity analysis of 7370 and parental E & R arms in reducing protein gel electrophoresis. (D) Intact mass analysis of 7370 by mass spectrometry. The experimental mass of 7370 matched well to the theoretical mass of 7370. (E) The dissociation constant of 7370 against recombinant human FLT3 and CD3εδ heterodimer. Binding at 37°C was determined using surface plasmon resonance. Binding sensorgram from 1 representative experiment out of 3 independent experiments was shown. Binding affinity of 7370 against recombinant cyno CD3εδ heterodimer is also summarized in the table. (F) Binding of 7370 to human T cells and AML cell lines, EOL-1 and MV4-11 by flow cytometry. CHO was used as the negative control cell line. (G) Binding of 7370 to arginine substitution variants at various human FLT3 domain 4 positions. Four residues (D358, S370, S389 and E421) at spatially distinct regions of FLT3 D4 (shown as blue ribbon) were selected to generate single-point arginine substitution variants. Binding of antibodies against FLT3 D4 arginine variants coated on plate was determined using ELISA. (H) Pharmacokinetic study of a single injection of 7370 in MOLM-13-bearing NSG mice. Three different groups of mice were injected IV with 10, 30 and 100 µg/kg of 7370. Serum concentration of 7370 was monitored over time. N=3 mice per group; LLOQ – lower limit of quantitation.

Figure S4

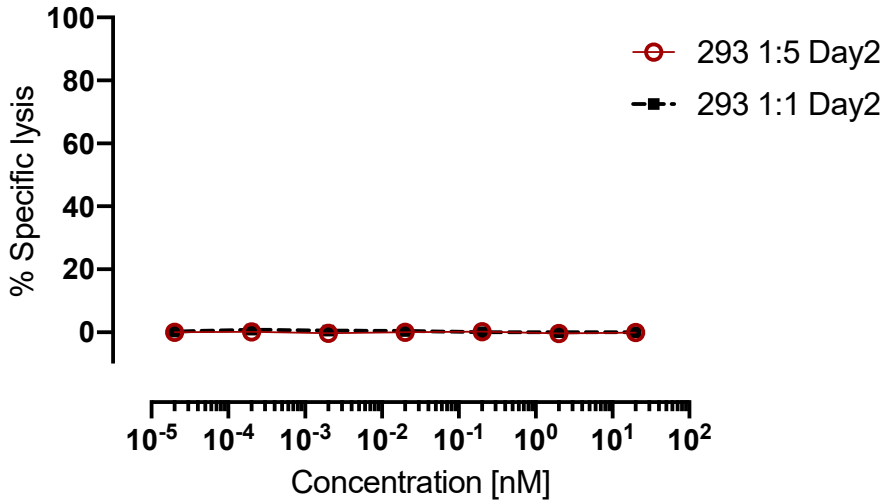


Figure S4. 7370 did not exhibit cytotoxicity against a FLT3-negative cell line FLT3-negative 293 cells were incubated with T cells isolated from one healthy human donor at E:T ratio of 1:1 and 1:5 in the presence of various concentrations of 7370. Cytotoxicity was determined after 2 days.

Figure S5

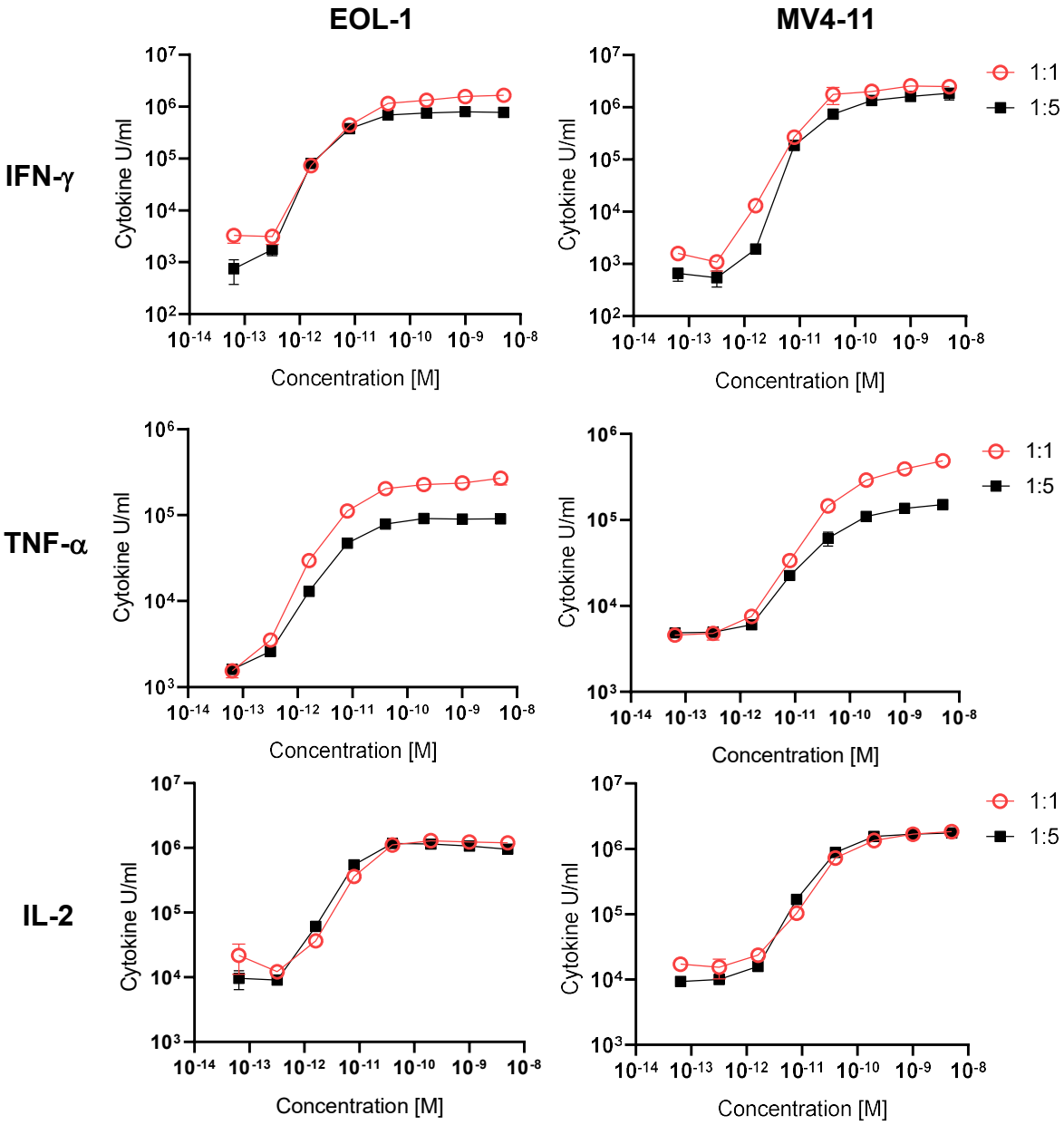


Figure S5. IFN-γ, TNF-α, and IL-2 cytokine secretion is induced by 7370. Measurement of the three cytokines in the supernatant 1 days after coculture of healthy donor human T cells with 7370 in the presence of EOL-1 (left) or MV4-11 (right) at two different E:T ratios (1:1 and 1:5). Human T cells were isolated from one human donor. Data shown is the average and error from three replicates.

Figure S6

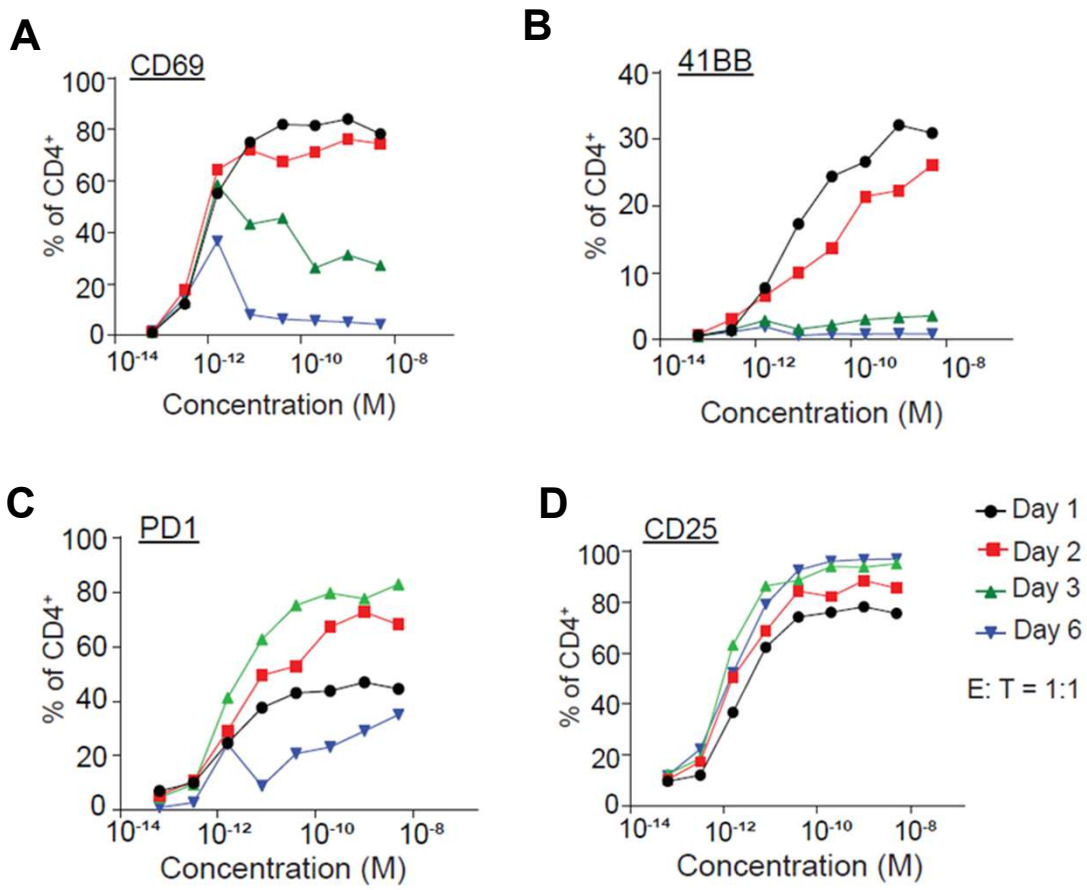


Figure S6. CD4⁺ T cells are activated following exposure to EOL-1 in the presence of 7370. Change in activation markers for CD4⁺ T cells over time**.** EOL-1 cells were co-cultured with human T cells (E:T of 1:1) and 7370 at indicated concentrations. CD4⁺ T cells were analyzed by flow cytometry for the kinetics of expression of activation markers at 1, 2, 3 and 6 following the start of co-culture. Activation markers CD69 (A), 41BB (B), PD1 (C) and CD25 (D) of CD4 T cells are shown as a function of bispecific concentration. Human T cells were isolated from one human donor. Each data point represents one measurement.

Figure S7

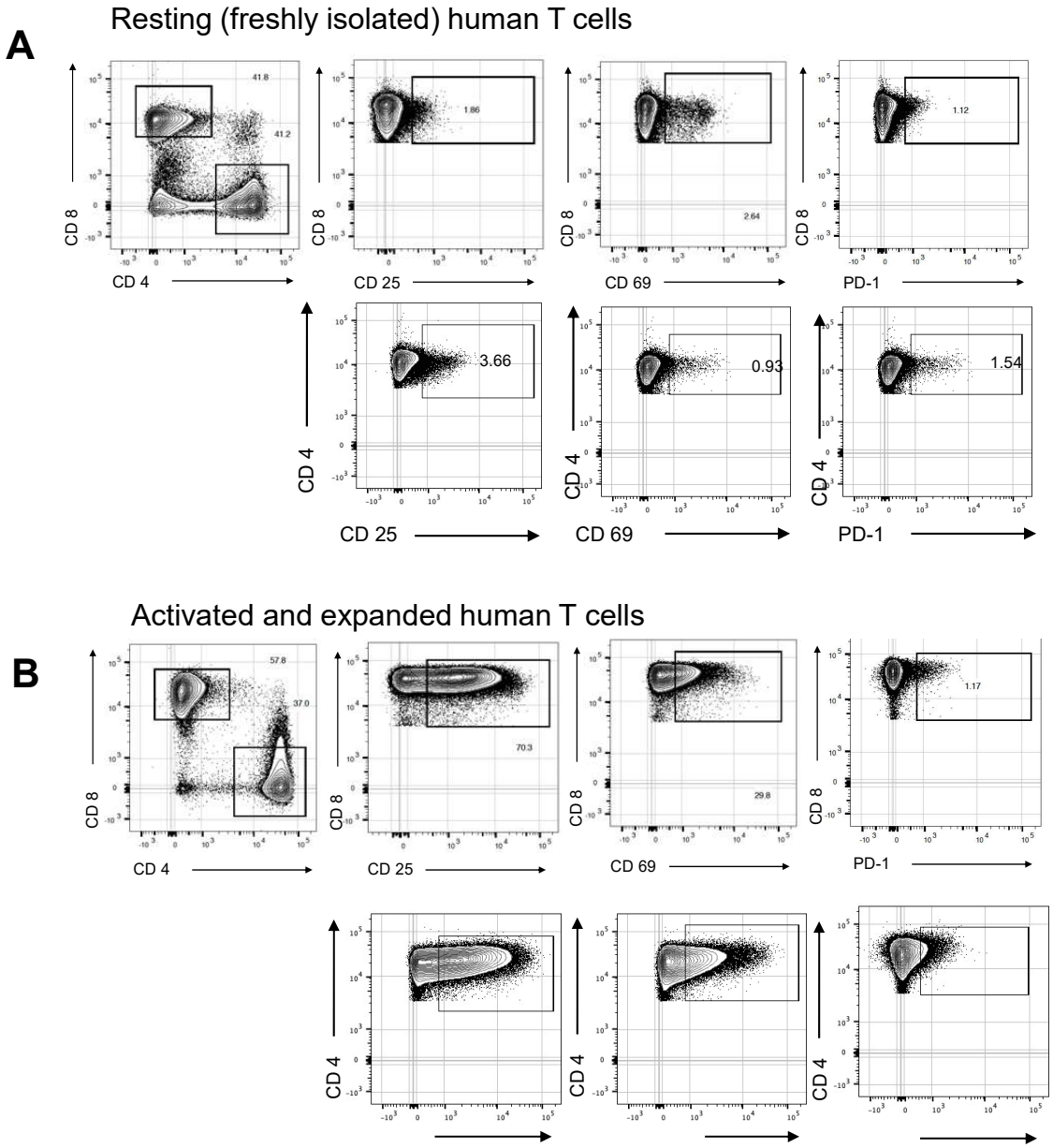


Figure S7. Immune phenotype of T cells injected into NSG mice. Cryopreserved T cells isolated from a representative human donor were thawed and analyzed for the expression of CD4, CD8, CD25, CD69, and PD1 immediately after thawing (A) or 11 days after activation with anti-CD3 and anti-CD28 antibodies and expansion in IL-2 (B).

Figure S8

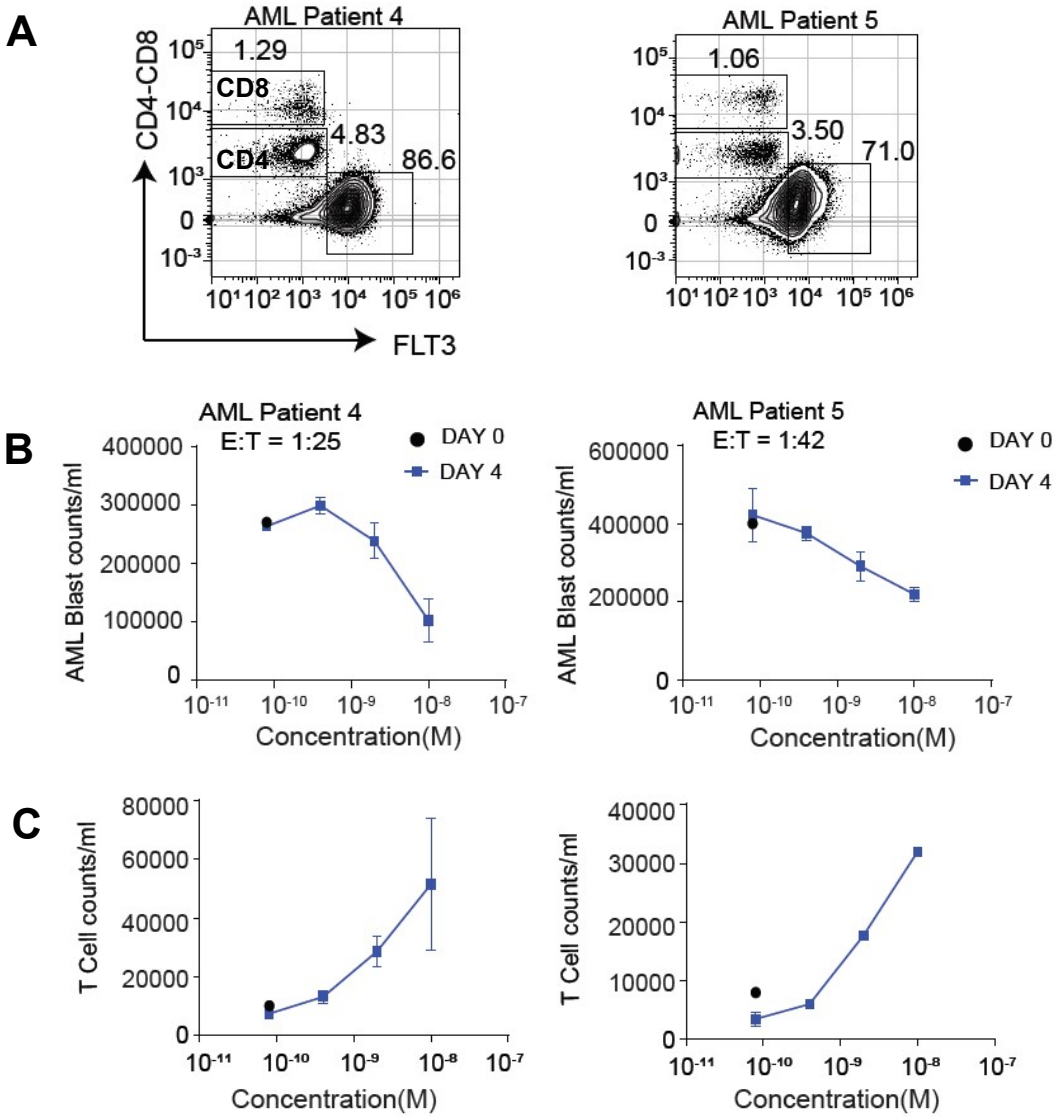


Figure S8: Autologous T cells proliferate and kill AML blasts in the presence of 7370 in a dose-dependent manner (A) Expression of FLT3 (x-axis) and CD4 and CD8 (y-axis) in PBMCs from two AML patients. Percent of CD4+ (middle left gate), CD8+ (upper left gate) and FLT3+ cells (bottom right gate) is depicted. FLT3 expression was detected only in $CD45^{low}$ blasts. (B-C) AML blast (B) and T cell counts (C) in patients 4 and 5 after 4 days of culture with 7370. Black dot on the graphs denotes the cell count at Day 0 before the addition of 7370. Each measurement is an average of three technical replicates.

Figure S9

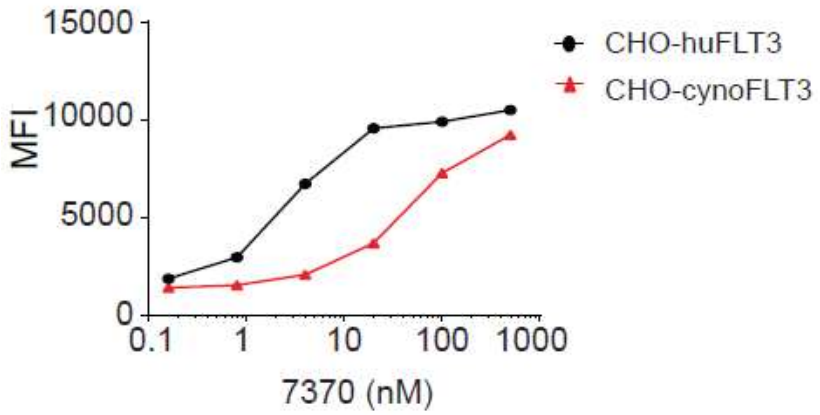


Figure S9: Binding EC_{50} of 7370 to cyno FLT3 is ~13 fold lower than that of human FLT3. Binding affinity using recombinant cynomolgus FLT3 protein could not be reliably measured due to the instability and aggregation of the purified protein (data not shown), so we tested the binding of 7370 on CHO cells expressing human (black line) or cynomolgus FLT3 (red line). Engineered cell lines were incubated with increasing concentrations of 7370 at 4°C. Binding of 7370 to the cells was analyzed by flow cytometry in two independent experiments. Each data point represents one measurement.

Figure S10

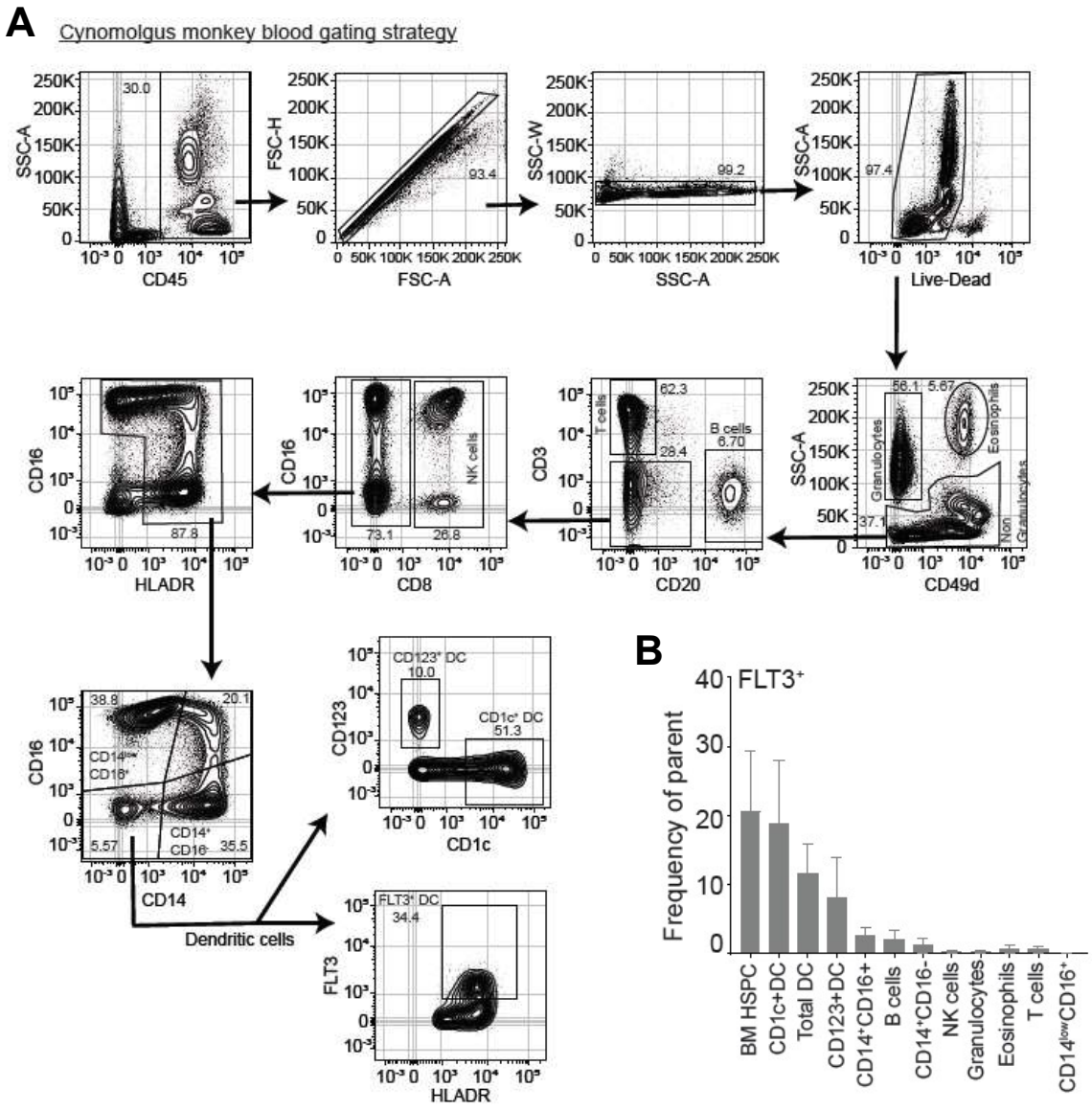


Figure S10: FLT3 expression in cynomolgus monkey blood immune cell subsets. (A) Flow cytometry gating strategy that identifies the following subsets from red blood cell-lysed whole blood is shown: eosinophils ($CD45^+CD49d^+SSC^{hi}$), granulocytes ($CD45^+CD49d^-SSC^{hi}$), T cells ($CD45^+SSC^{low}CD3^+CD20^-$), B cells ($CD45^+SSC^{low}CD3^-CD20^+$), NK cells ($CD45^+SSC^{low}CD3^-CD20^-CD16^{+/-}CD8^+$), classical monocytes ($CD45^+SSC^{low}CD3^-CD20^-CD8^-CD14^+CD16^-$), inflammatory monocytes ($CD45^+SSC^{low}CD3^-CD20^-CD8^-CD14^+CD16^+$), nonclassical monocytes ($CD45^+SSC^{low}CD3^-CD20^-CD8^-CD14^{low}CD16^+$), and dendritic cells ($CD45^+SSC^{low}CD3^-CD20^-CD8^-HLADR^+CD14^-CD16^-$). Two subsets of dendritic cells were additionally identified CD1c+ (myeloid) and CD123+ (plasmacytoid). FLT3 expression in total dendritic cells is depicted. (B) Percentage of FLT3-expressing cells in the indicated blood immune cell subsets. Bone marrow HSPCs (CD34+CD38+) are also depicted for comparison. N=6 monkeys. FLT3 was detected by BV10 antibody as 4G8 does not cross-react to cynomolgous FLT3.

Figure S11

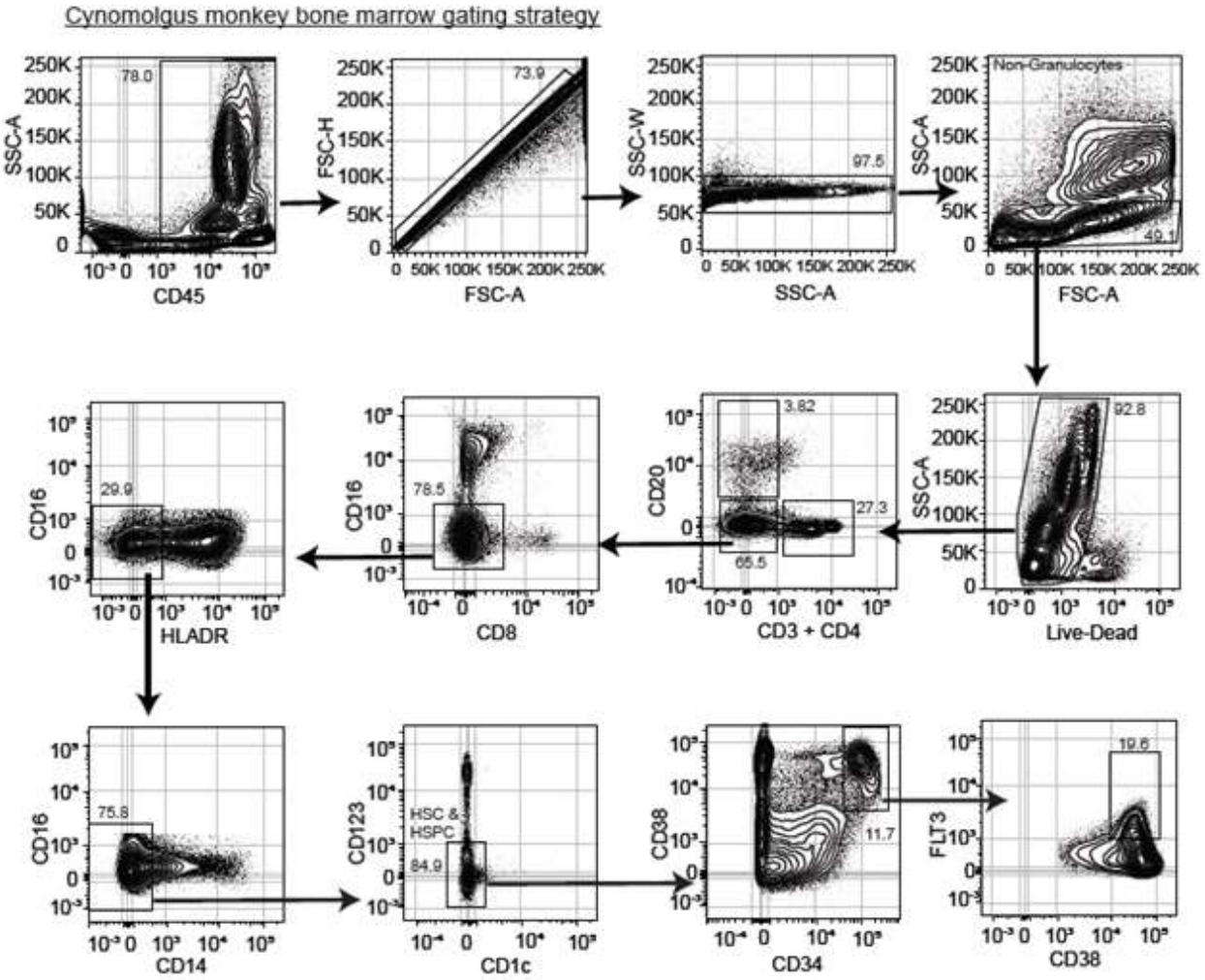


Figure S11: FLT3 expression in cynomolgus monkey bone marrow. Flow cytometry gating strategy that identifies $CD34^{+}CD38^{+}$ HSPCs from red blood cell-lysed bone marrow is shown. Identified $CD34^{+}CD38^{+}$ HSPCs were $CD45^{+}SSC^{low}$ but negative for the following lineage-specific markers: CD3, CD20, CD4, CD8, CD16, HLADR, CD14, CD123, CD1c. The expression of FLT3 in the $CD34^{+}CD38^{+}$ HSPC subset is shown. FLT3 was also expressed in bone marrow dendritic cells identified as in Supplementary Figure 6 (data not shown). Expression pattern of FLT3 in other differentiated immune subsets in the bone marrow was similar to that in the blood (data not shown).

Figure S12

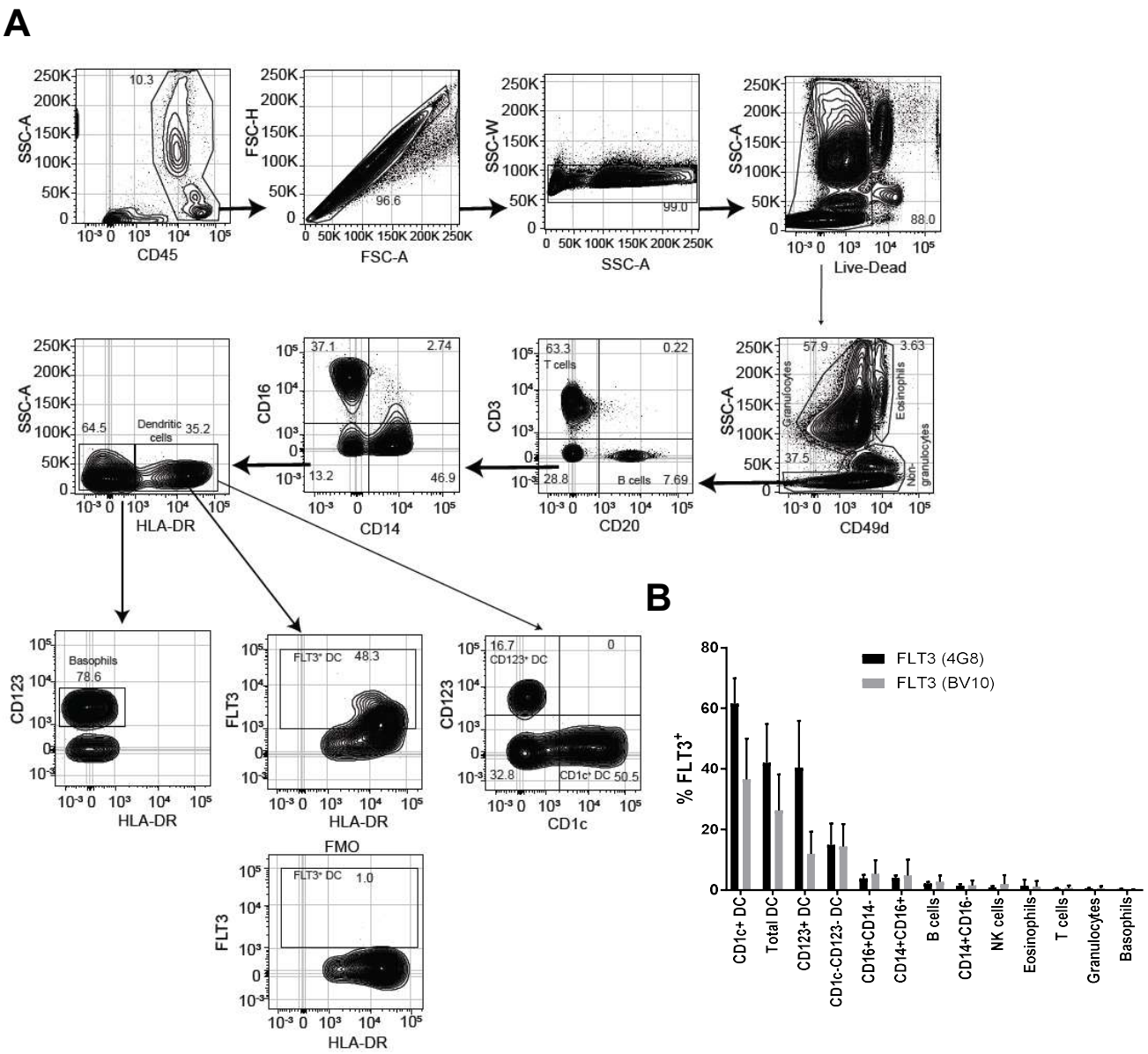


Figure S12: FLT3 expression in human blood immune cell subsets. (A) Flow cytometry gating strategy that identifies the following subsets from red blood cell-lysed whole blood is shown: eosinophils ($CD45^+CD49d^+SSChi$), granulocytes ($CD45^+CD49d^-SSChi$), T cells ($CD45^+SSC^{low}CD3^+CD20^-$), B cells ($CD45^+SSC^{low}CD3^-CD20^+$), classical monocytes ($CD45^+SSC^{low}CD3^-CD20^-CD14^+CD16^-$), inflammatory monocytes ($CD45^+SSC^{low}CD3^-CD20^-CD14^+CD16^+$), nonclassical monocytes ($CD45^+SSC^{low}CD3^-CD20^-CD14^-CD16^+HLADR^+$), NK cells ($CD45^+SSC^{low}CD3^-CD20^-CD14^-CD16^+HLADR^-$), and dendritic cells ($CD45^+SSC^{low}CD3^-CD20^-CD14^-CD16^-HLADR^+$). $CD1c^+$, $CD123^+$, and $CD1c^-CD123^-$ subsets are also indicated. FLT3 expression in total dendritic cells is shown (control with no antibody is included to show how $FLT3^+$ cells were identified). (B) Percentage of FLT3-expressing cells in the indicated blood immune cell subsets of 4 healthy donors as measured by staining with 4G8 or BV10 antibody.

Figure S13

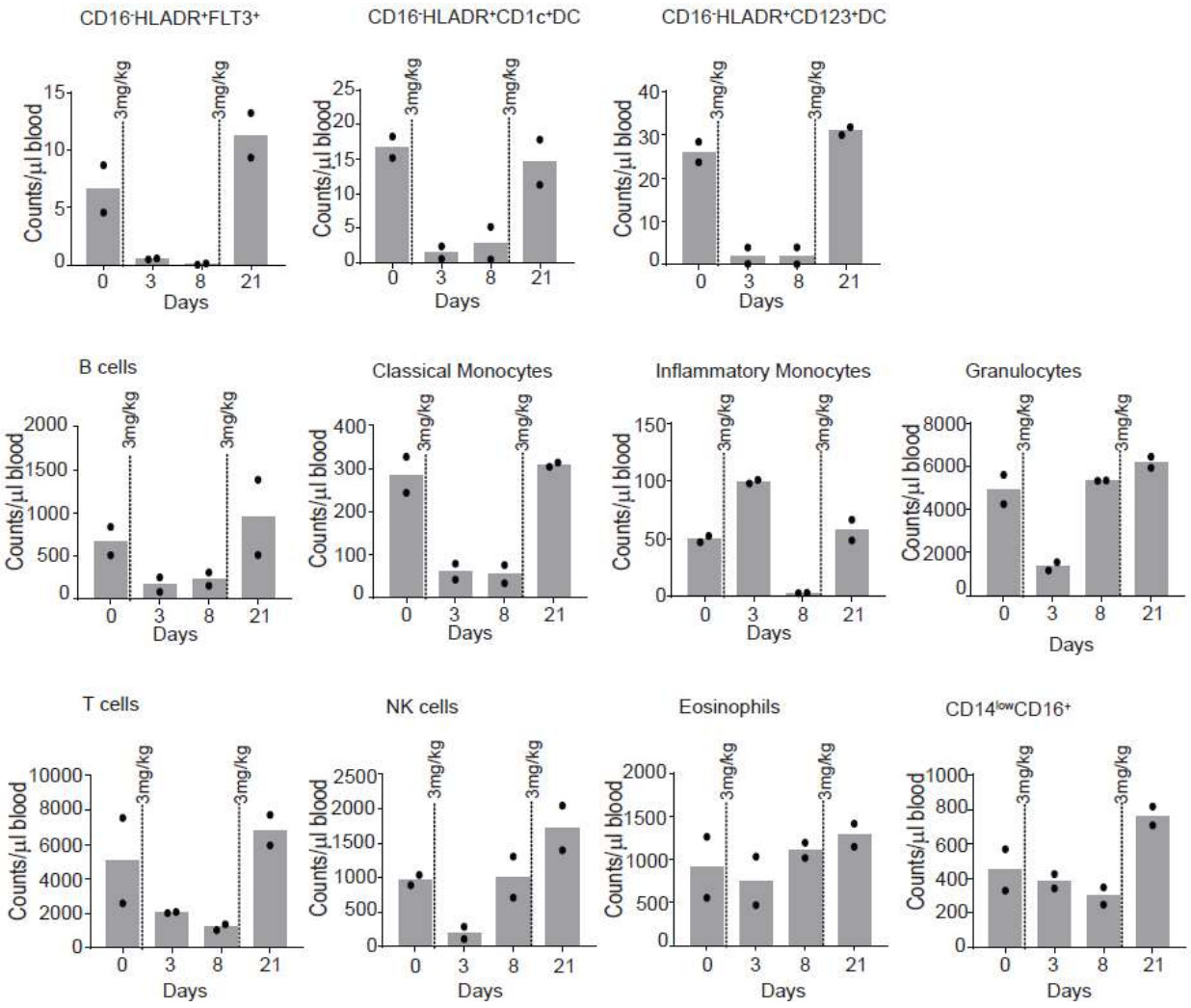


Figure S13: 7370 is well tolerated and exhibits on-target efficacy in peripheral blood of cynomolgus monkeys. Cell counts per microliter in the blood of cynomolgus monkeys treated with 7370 (monkeys “Treated 3” and “Treated 4” as depicted in Figure 5A). Immune cell subsets were identified as shown in Supplementary Figure 6. Cell counts were determined using counting beads.

Figure S14

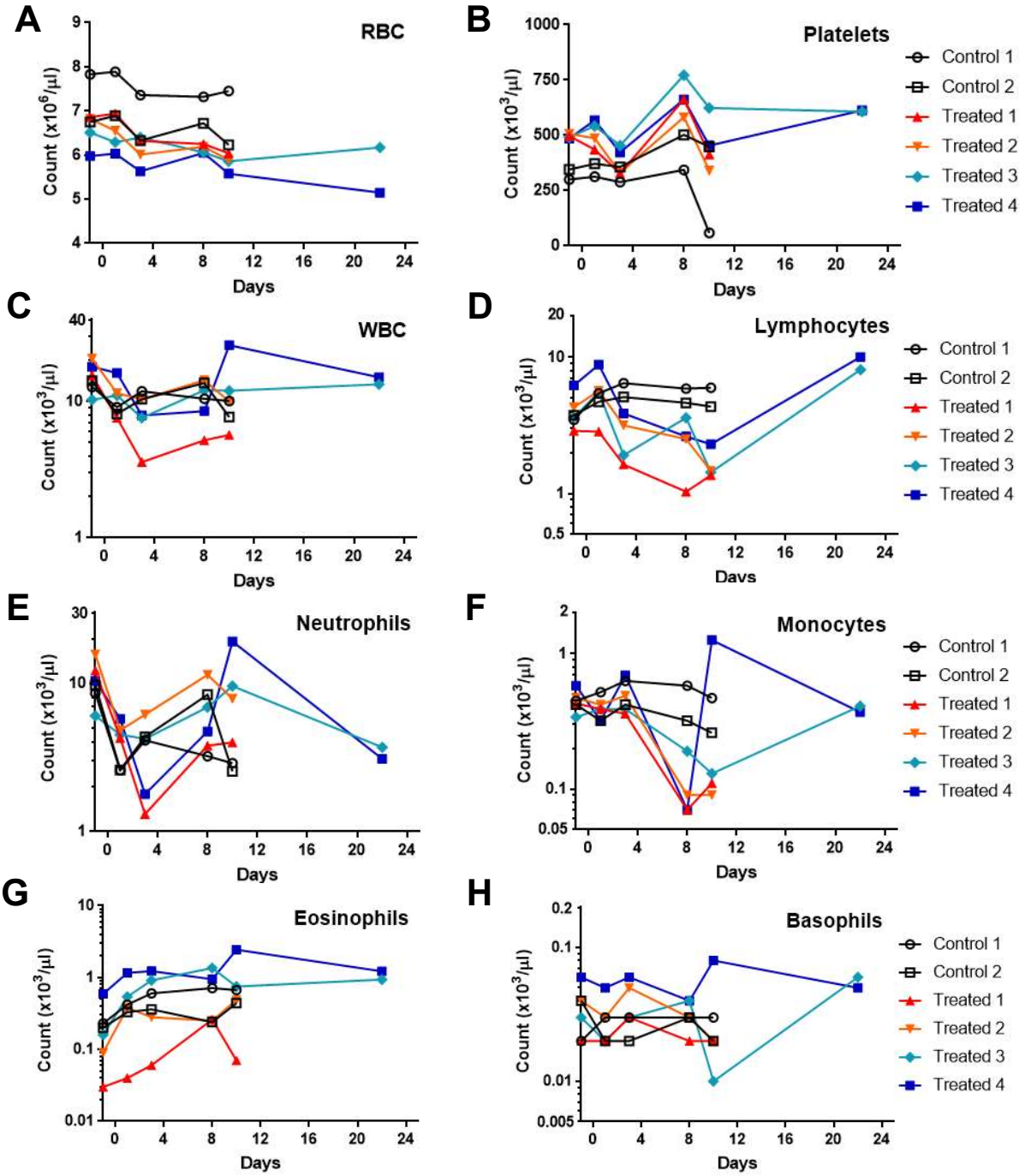


Figure S14: Peripheral immune cell populations in Cynomolgus monkeys based on hematology counts. Six monkeys were treated with PBS (Control 1 and 2) or 2 doses of 7370 at 3mg/kg at days 1 and 8 (Treated 1-4) (see Figure 5A). Standard hematology was performed at the indicated time points. Day -1 and day 1 samples were collected before the first injection of 7370. Day 8 samples were collected before the second dose of 7370. Results for (A) red blood cell (RBC), (B) platelets, (C) white blood cells (WBC), (D) lymphocytes, (E) neutrophils, (F) monocytes, (G) eosinophils and (H) basophils are shown.

Figure S15

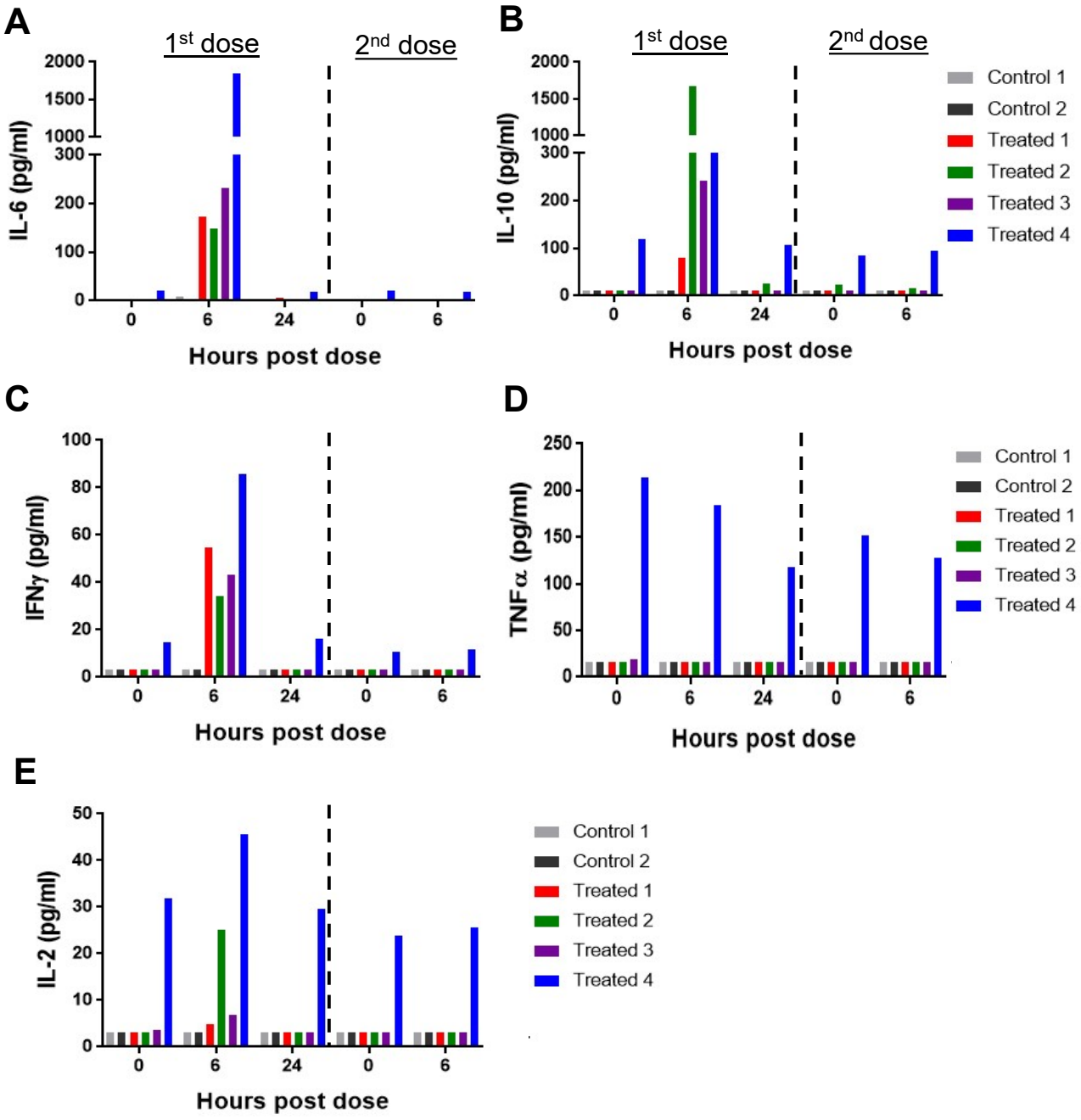


Figure S15: Serum cytokines in cynomolgus monkeys after treatment with 7370. Six monkeys were treated with PBS (Control 1 and 2) or 2 doses of 7370 at 3mg/kg at days 1 and 8 (Treated 1-4) (see Figure 5A). Serum levels for selected cytokines were monitored pre-dose (0), 6 and 24 hours post first dose, before (0) and 6 hours post second dose. Serum concentration of (A) IL-6, (B) IL-10, (C) IFNγ, (D) TNFα, and (E) IL-2 are shown. Compared to the other monkeys, treated monkey 4 had higher pre-dose levels for all evaluated cytokines, and had much higher IL-6 levels post first dose.

Supplementary methods:

Cell binding assays of 7370

FLT3-negative CHO cells, CHO cells engineered to express human or cynomolgus FLT3, EOL-1 and MV4-11 cells, and human T cells were assessed for binding to 7370. 200,000 cells were plated and incubated with 7370 at concentrations 500nM to 0.0016nM (fivefold serial dilution) for 20 minutes at 4°C. Cells were washed, stained with F_{ab} fragment of goat anti-human IgG-F_c antibody and analyzed on a LSRII with FACS Diva software (BD Biosciences).

Cytokine detection in supernatants of T cell-AML cell line co-cultures

In a 96 well U bottom plate, AML cell lines (EOL-1, and MV4-11) were co-cultured with human T cells under different concentrations of 7370 in E:T ratios of 1:1 and 1:5 for 24hrs. Supernatants of the co-cultures at 24hrs were harvested, diluted at 1:50 with the diluent provided in the MSD kit (Mesoscale discovery kit) and analyzed for cytokines (IL2, IFNγ and TNFα) on MSD plates as per manufacturers protocol.

Detection of 7370 in mouse serum

Mouse anti-hCD3 Id antibody Biotin captured onto streptavidin-coated beads on the affinity capture column of the Gyrolab Bioaffy microstructure was used to bind 7370. Bound 7370 was detected with Alexa 647-labeled goat anti-human IgG (H+L). A fluorescent signal on the column, representative of the amount of bound 7370, allows for visualization of the antibody. Response Units are read by the Gyrolab instrument at 1% Photomultiplier tube (PMT) setting. Sample concentrations are determined by interpolation from a standard curve that is fit using a 5-parameter logistic curve fit with $1/y^2$ response weighting. The standard points in 2.5% K_2 EDTA mouse plasma ranged from 0.057 ng/mL to 150 ng/mL. The range of quantitation in mouse plasma was 5.45 ng/mL to 2500 ng/mL.

Serum cytokine detection

IL-2, IL-6, IL-10, IFN-γ, and TNF-α were simultaneously quantified in serum samples using the Millipore Milliplex MAP Non-Human Primate Cytokine Magnetic Bead Panel reagent kit (cat. #PRCYTOMAG-40K). Limits of detection for the cytokine assays are 3.20 pg/mL for IL-2, IL6, and IFN-γ, 12.20 pg/mL for IL-10, and 16.00 pg/mL for TNF-α.