Bi38-3 is a novel CD38/CD3 bispecific T-cell engager with low toxicity for the treatment of multiple myeloma

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Legends for Supplementary figures

Supplementary figure S1: Structural and functional characterizations of Bi38-3. (A) Schematic of Bi38-3. The anti-human CD38 scFv and the anti-human CD3ε scFv domains are represented in red and blue respectively. Plain lines represent 15-amino-acid glycine–serine spacers. Myc-tag and His-tag are indicated by green and brown boxes respectively. (B) (Left) Western blot analysis of HEK293 cells transfected with Bi38-3 expression vector or an empty vector. Bi38-3 was detected with an anti-Myc antibody. β-actin was used as a loading control. (Right) Analysis of purified Bi38-3 by gel electrophoresis and Coomassie blue staining. (C) Binding of Bi38-3 to MM cell lines. (Left panel) FACS analysis of NCI-H929, KMS11, MM1.S and CD38-deficient MM1.S (MM1.S-KO) cells stained with PE-anti-CD38 (red) or isotype control (blue). (Right panel) Cells were stained with Bi38-3 (red) or left untreated (blue) and revealed with APC-anti-Fab' antibody. The percentage of cells falling in the gate are indicated in the histogram. Representative of 3 independent experiments. (D) Binding of Bi38-3 to $CD3^+$ Jurkat cells deficient or not for CD38 (KO). (First and second panels) FACS analysis of Jurkat and Jurkat-KO cells stained with, respectively, APC-anti-CD3/PE-anti-CD38 (red) or isotype control (blue). (Third panel) Cells were stained with Bi38-3 (red) or left untreated (blue) and revealed with APC-anti-Fab' antibody. (E and F) Proliferation assessed by violet CellTrace dye dilution. Purified T cells, isolated from healthy donors (n=4), were stained with CellTrace violet dye and co-cultured with (or without) MM1.S target cells or CD38 deficient MM1.S (MM1.SKO) at an E:T ratio of 1:1 with fixed concentration of Bi38-3 (10^1 ng/mL) for 96 hours. T cell expansion index was calculated after a 96-hours culture in medium alone (M), CD3/CD28 beads (3/28) or Bi38-3 (10^1 ng/mL). Histograms show the average expansion indexes of T cells in 4 independent experiments with 4 different donors. Standard deviations are shown and P values were determined by two-sided Mann–Whitney *U*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (G) Activation markers CD25 and CD69 on CD4-positive and CD8-positive T cells was determined by FACS analysis. T cells were grown in medium (M) or stimulated with Bi38-3 at

10^0 or 10^2 ng/mL, with (or without) MM1.S or CD38 deficient MM1.S (MM1.S-KO) at an E:T ratio of 5:1. Histograms show average percentages of CD25+ and CD69+ on CD4-positive and CD8 positive T cells and error bars indicate the SD. (H) Production of Interferon gamma (IFNγ), Interleukin 2 (IL-2) and Tumor necrosis factor (TNFα) in MM1.S/T cell co-cultures at an E:T ratio of 5:1. Data represent the average of 4 independent experiments with 4 different donors.

The normality of populations was established with the Shapiro-Wilk normality test and P-values were determined by the unpaired Student's *t test* (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

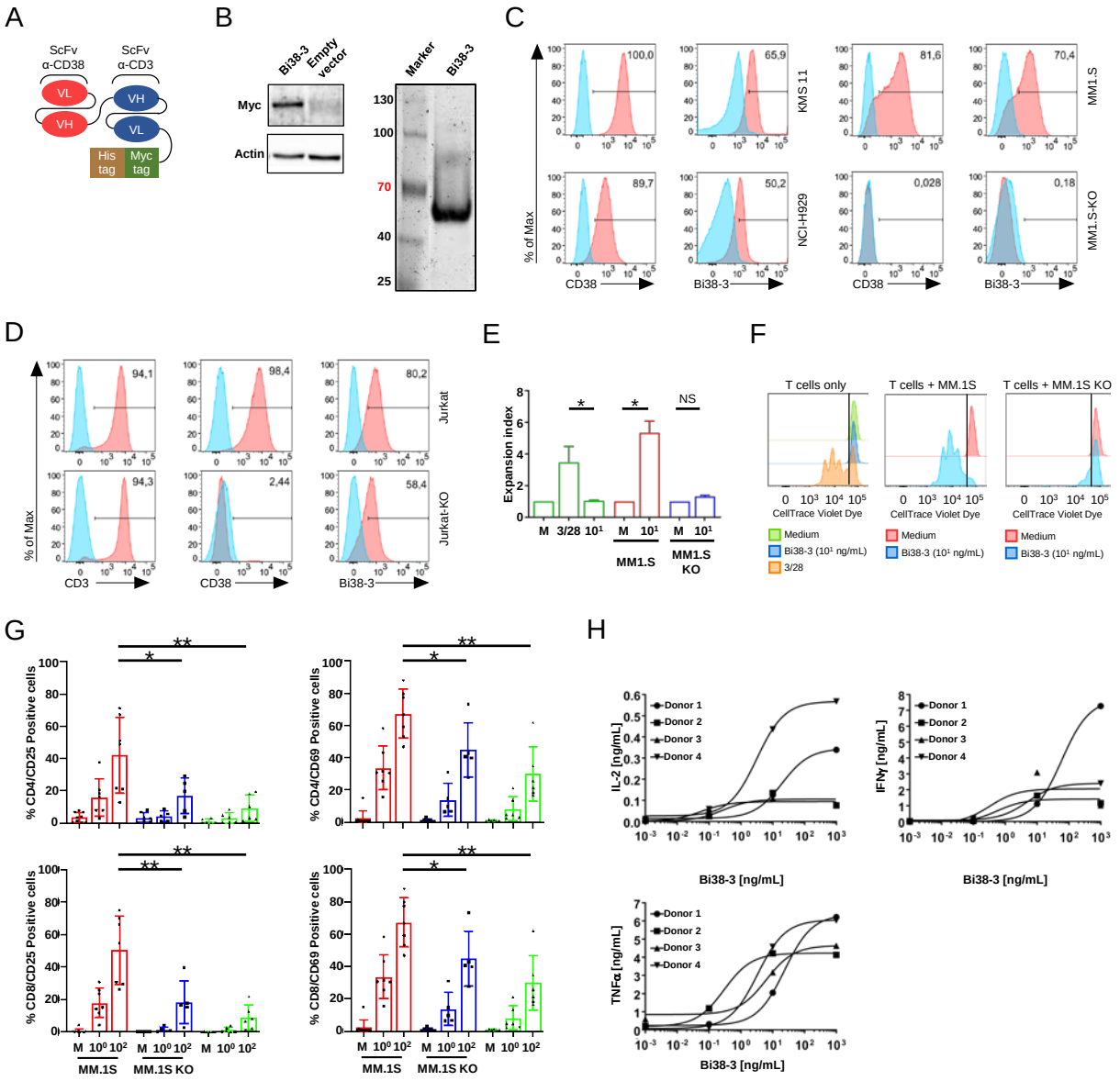
Supplementary Figure S2: Shown are the gating strategies to determine Bi38-3 mediated autologous T cell cytotoxicity on fresh tumor plasma cells used in Figure 1B. Purified bone marrow $CD138^+$ plasma cells from patients were co-cultured with autologous PBMC $CD3^+$ T cells at an E:T cell ratio of 5:1 for 24 hours. Cultures were analyzed by FACS to monitor the number of $CD138^+$ cells falling into the live gate as measured with counting beads. Culture conditions are indicated above each dot plot pair: FSC/SSC (top) and SSC/CD138 (bottom). Representative of 4 different patients at diagnosis and 3 at relapse.

Supplementary Figure S3: Shown are the gating strategies to determine Bi38-3 mediated autologous T cell cytotoxicity on PBMC cell subsets cells used in Figure 2A. PBMC from healthy donors (n=3) were cultured with various concentrations of Bi38-3 for 24 hours and the percentages of T, myeloid, B, NK cells were determined by FACS. Dot plots depict the percentages of live $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, and $CD56^+$ NK cells in the culture. Culture conditions are indicated on the left. The percentages of cells falling into the gates are shown is each dot plot. Representative of 3 independent experiments.

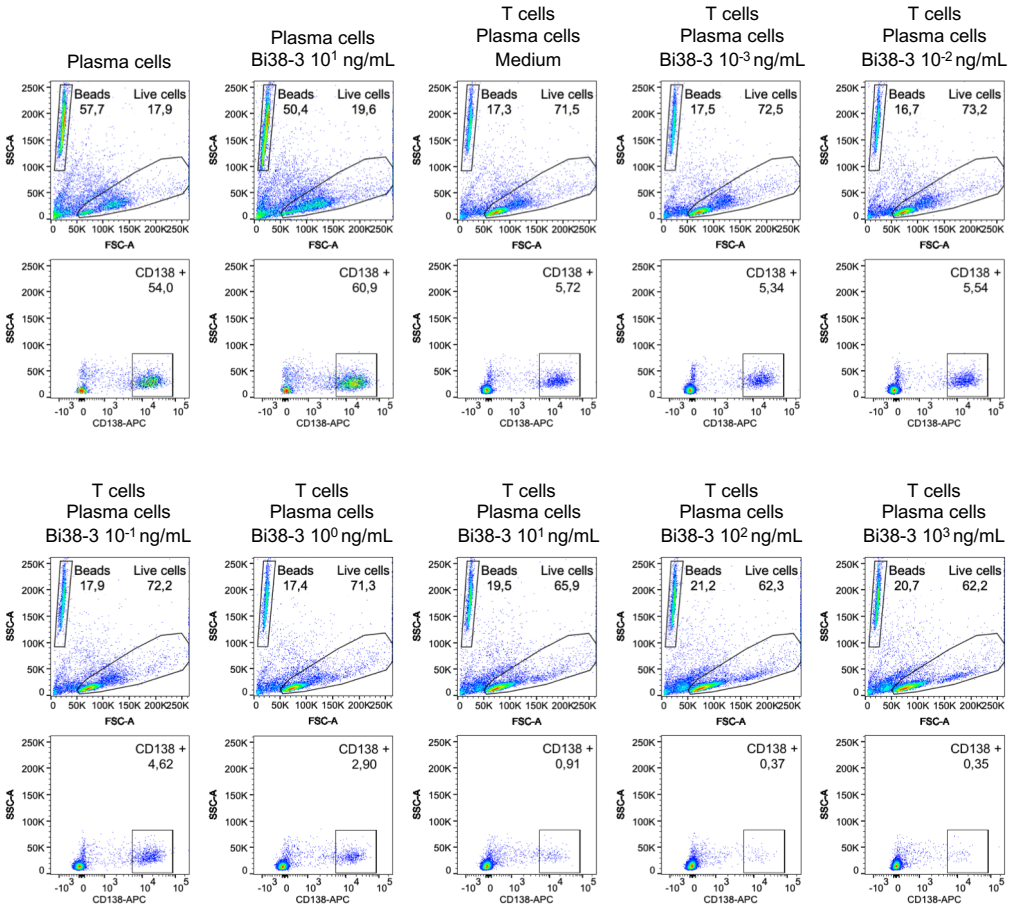
Supplementary Figure S4: (A) Shown are the gating strategies to determine Bi38-3 mediated T cell cytotoxicity on MM1.S and autologous B cells co-culture used in Figure 2C. Purified paired T and B cells from healthy donors (n=5) were co-cultured with MM1.S cells at an E:T cell ratio of 5:1 for 24 hours. (B) Shown are the gating strategies to determine Bi38-3 mediated T cell cytotoxicity on MM1.S and FoxP3$^+$ Treg cells co-culture used in Figure 2D. Purified T cells from donors were co-cultured with MM1.S cells at an E:T cell ratio of 5:1 for 24 hours. (C) Shown are the gating strategies to determine Bi38-3 mediated T cell cytotoxicity on MM1.S and CD34$^+$ bone marrow hematopoietic progenitors cells co-culture used in Figure 2E. Purified paired CD34$^+$ hematopoietic progenitors and T cells from bone marrow samples from healthy donors (from hip surgery) (n=4) were co-cultured with MM1.S cells at an E:T cell ratio of 5:1 for 24 hours.

Cultures were analyzed by FACS to monitor, respectively, the number of CD138$^+$ cells (MM1.S), B cells (CD20$^+$), Treg cells (FoxP3$^+$) and hematopoietic progenitors (CD34$^+$) falling into the live gate as measured with counting beads. Culture conditions are indicated above each dot plot pair: FSC/SSC (top) and CD20/CD138 (bottom A), FoxP3/CD138 (bottom B) or CD34/CD138 (bottom C). Data are representative of 5 (A), 3 (B) and 4 (C) independent donors.

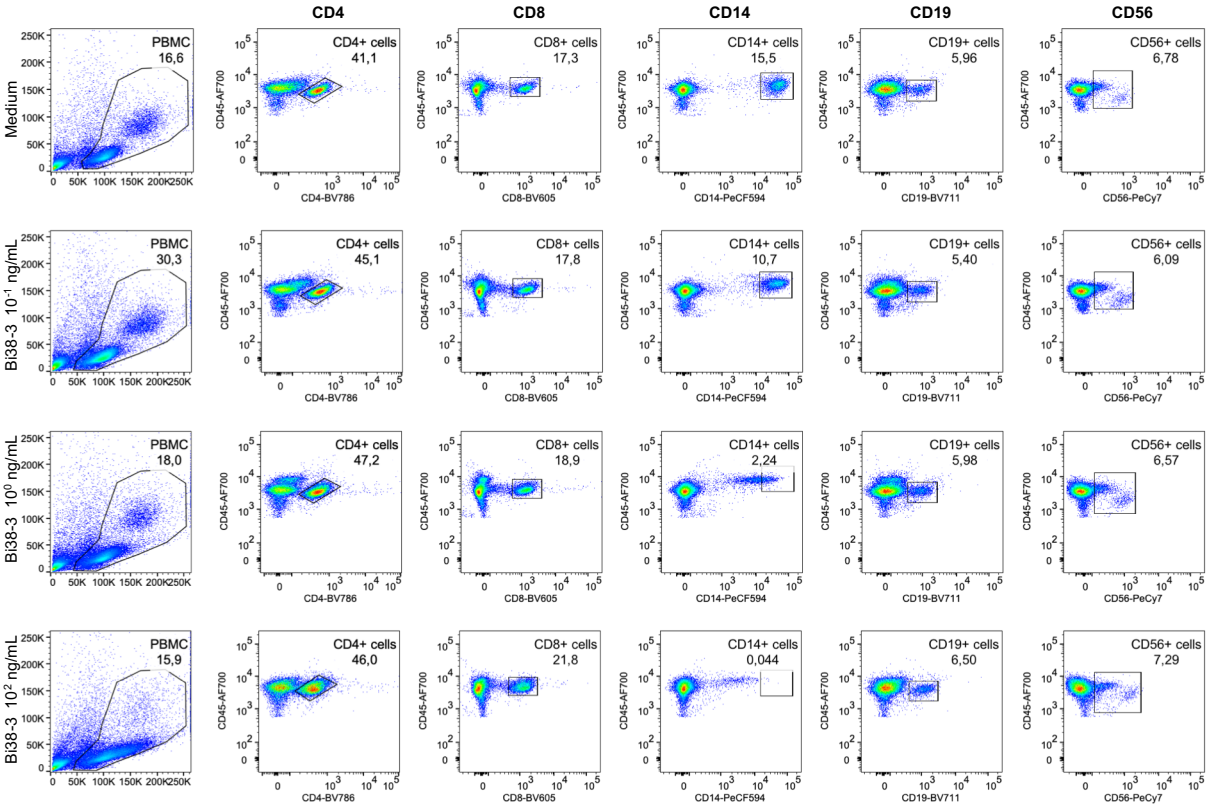
SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4