# Supplementary methods

## Binding to CD20- and CD3-expressing cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from the Blutspendezentrum Zürich, Switzerland. Briefly, blood was diluted 1:3 with phosphate-buffered saline (PBS; Gibco/Lubioscience) and separated by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, USA). PBMCs were collected, erythrocytes depleted and the PBMCs washed three times in PBS. Cynomolgus monkey PBMCs were isolated from fresh cynomolgus monkey blood taken from animals at Roche Basel, Switzerland. For isolation, 20 mL/tube of 90% room-temperature Histopaque-1077 plus 10% PBS were added to 50 mL falcon tubes. Fresh blood was diluted 1:1 in PBS, added to Histopaque tubes and then centrifuged (520 x g for 30 min, w/o break, at room temperature). Following centrifugation, the band containing the cells was collected and transferred into 50 mL tubes. These tubes were filled with PBS up to 50 mL and centrifuged (400 x g for 10 min at 4°C). The supernatant was removed and the pellet resuspended in PBS. To remove platelets, one low-speed centrifugation was performed at 150 x g for 15 min at 4°C. Erythrocytes were lysed using 1 mL of a classical ammonium-chloride-potassium (ACK) lysis buffer (0.155 M NH4Cl + 10 mM KHCO3 + 0.1 mM EDTA pH7.3 in ddH2O) (5 min at room temperature) and 10 mL of cell culture medium (e.g. RPMI1640 or DMEM + 10 % FCS + 1 % Glutamax) was added prior to centrifugation (300 x g for 10 min at room temperature). The binding assay was performed with 100 µL/well of human or cynomolgus monkey PBMCs (380 000 per well) in 96-U bottom plates. Antibody dilutions were prepared in FACS Buffer (PBS + 2 % FCS + 5 mM EDTA + 0.25 % sodium azide) and 50 µL of the pre-diluted TCBs or FACS Buffer were added to the cells. After 30 min incubation at 4°C in the fridge, the cells were washed with FACS Buffer and stained with CD19-AF647, CD4-PerCPCy5.5 and CD8-APCCy7. The cells were then incubated for 30 min at 4°C in the dark. Thereafter, cells were washed twice with FACS Buffer before addition of 150 µL/well freshly prepared BD FACS Lysing solution (BD Biosciences, Franklin Lakes, USA). Samples were analyzed on a BD FACS Canto II device (BD Biosciences).

CD20+ tumor targets (Z-138) and CD3+ T-cell lines (Jurkat [human T-cell line] and HSC-F [cynomolgus monkey T-cell line]) were re-suspended at 0.75 x 106/mL in FACS Buffer and 100 µL/well (75 000/well) were seeded into 96-U-bottom plates. Antibody dilutions were prepared in FACS Buffer and 50 µL of the pre-diluted TCBs or PBS were added after centrifugation to the cell pellets. After 30 min incubation at 4°C in the fridge, cells were washed with FACS Buffer and stained with prepared PE-conjugated AffiniPure F(ab’)2 Fragment goat anti-human (anti-hu) IgG (Jackson ImmunoResearch Laboratories, Inc West Grove, USA). The samples were incubated for 30 minutes at 4°C in the dark followed by washing with FACS Buffer. Thereafter, 2% PFA 100 µL per well was added to the plates. Samples were measured by flow cytometry.

## Assessment of T-cell activation in tumor lysis experiments *in vitro*

PBMCs were recovered from tumor lysis experiments at indicated time points, washed with FACS buffer (PBS + 2% FCS + 5 mM EDTA + 0.25% sodium azide) and stained with different FACS antibody cocktails containing anti-hu CD4 BV605 (Biolegend, San Diego, USA via Lucerna-Chem, Luzern, Switzerland), anti-hu CD8 BV711 (BioLegend), anti-hu CD19 BV510 (BD Biosciences), anti-hu CD25 PE-Dazzle 594 (BioLegend), anti-hu Ox40 FITC or APC-Cy7 (Biolegend), anti-hu ICOS PE-Cy7 (Biolegend), anti-hu LAG3 PE or APC (R&D Systems or Biolegend), anti-4-1BB A700 (Biolegend) and anti-human PD-1 BV421 or BUV737 (BioLegend or BD Biosciences) for 20 min at RT or 30 min at 4°C. Cells were then washed and fixed (BD FACS Lysing solution) prior to analysis by flow cytometry (MACSQuant; Miltenyi Biotec, Bergisch Gladbach, Germany).

## Quantification of cytokine release *in vitro*

Supernatants derived from 20−24 h incubated tumor cell lysis assays with Z-138 and isolated pan T-cells (Miltenyi Biotec) (effector:target 3:1) were analyzed by Cytometric Bead Array (CBA, BD Biosciences) for interleukin (IL)-2, IL-6, IL-10, IFNγ, tumor necrosis factor (TNF), and Granzyme B according to the manufacturer instructions. Samples were analyzed using a BD FACS Canto II device.

## T-cell proliferation *in vitro*

PBMCs derived from blood of healthy donors were labeled with 200 nM CFSE (Sigma Aldrich) and incubated with WSU-DLCL2 (CD20 high), SU-DHL-5 (CD20 medium) or Toledo (CD20 low) (effector:target ratio 10:1) in the presence of CD20-TCB for 6 days at 37°C and 5% CO2 in a humidified incubator (all samples in triplicate). Cells were then washed twice with PBS and labeled with Fixable Viability Stain 660 for 30 min at 4°C before staining with anti-hu CD4 PerCPCy5.5 (BD Biosciences), anti-hu CD8 APCCy7 (BioLegend), anti-hu CD25 PECy7 (BioLegend) and anti-hu CD69 BV421 (BioLegend) for 30 min at 4°C followed by fixation (BD FACS Lysing solution) and analysis by flow cytometry.

## T-cell adhesion to endothelial cells *in vitro*

PBMCs were isolated from buffy coats obtained from the Blutspendezentrum Zürich, Switzerland as described above and incubated with Z138 cells (ratio 5:1) and either 1 nM CD20-TCB or untargeted TCB for 24 h at 37°C in the incubator. Supernatants of these cultures have been collected and used to activate HUVEC cells and to analyze cytokines by CBA (BD Biosciences). In addition, pan T-cells have been isolated from the cultures (Human Pan T-cell Isolation Kit, Miltenyi Biotec) to test adhesion on non-activated and conditioned media-stimulated HUVEC. Briefly, HUVEC were grown to confluency in a 96 well plate. After overnight starvation in low serum, conditioned media were added for 6 h. T-cells were labeled with Calcein AM (Invitrogen), washed and 25,000 T-cells per well were left to adhere to the endothelial monolayer for 1 h in PBS containing 0.2% BSA, 2 mM MgCl2 and 2 mM CaCl2. After extensive washes, fluorescence was measured with a microplate reader (Perkin Elmer).

## Endothelial activation assay *in vitro*

HUVEC (Lonza) were grown to confluency in 96 well plates in complete media (EGM-2, Lonza). After overnight starvation in low serum media (EBM-2, Lonza, supplemented with 0.2% FBS), endothelial monolayers were stimulated for 6 h with conditioned media, prepared as described above, in the presence or absence of 10 μg/mL cytokine neutralizing antibodies (anti human TNFα mIgG1, anti-human IL-1β mIgG1, anti-human IL-6 mIgG2b, anti-human IFN-γ mIgG2a, all from R&D systems). Alternatively, HUVEC monolayers were stimulated with pooled sera collected from mice 24 h after treatment with CD20-TCB (0.5 mg/kg), obinutuzumab (Gazyva10 mg/kg) or vehicle and diluted 1:1 in endothelial starving media. Stimulated endothelial cells were harvested with Accutase (Gibco), washed 2x with PBS and stained with anti-human ICAM BV421 (BD Biosciences), anti-human VCAM PE (Biolegend), anti-human E-selectin APC (Biolegend) fluorescently labeled antibodies for 30 min at 4°C. Cells were washed twice with FACS buffer and analyzed by flow cytometry using a BD LSR-II.

## LFA-1 and VLA-4 staining *in vitro*

PBMCs were isolated from 5x buffy coats obtained from the Blutspendezentrum Zürich, Switzerland as described above and incubated with Z138 cells (ratio 5:1) and either 1 nM or 100 nM CD20-TCB or untargeted TCB for 24 h at 37°C in the incubator. For VLA-4 staining, cells were harvested, washed 2x with FACS Buffer and stained with anti-CD4 APC (Biolegend), anti-CD8 PE (BD Biosciences) and anti-VLA-4 BV421 (Biolegend) or corresponding isotype control mIgG1 BV421 (Biolegend) for 30 min at 4°C. For LFA-1 staining, cells were harvested, washed 2x with assay medium (RPMI1640 + 10 % FCS + 1 % Glutamax) and stained with anti-LFA-1 (Abcam) or corresponding isotype control mIgG1 for 30 min at 4°C. Unbound antibodies were removed by washing before incubation of cells with anti-mouse IgG-FITC (Jackson Immunoresearch) for 30 min at 4°C. Again, unbound antibodies were removed by washing before staining of the cells using anti-CD4 APC and anti-CD8 PE (30 min, 4°C). After washing, cells were fixed using FACS Lysing solution (BD Biosciences) before analyzing the cells using a BD FACS CantoII.

## Generation of humanized mice

NSG female mice (The Jackson Laboratory), aged 5 weeks at study initiation, were maintained under specific-pathogen-free conditions with daily cycles of 12 h light /12 h darkness according to committed guidelines (GV-Solas; Felasa; TierschG). The experimental study protocol was reviewed and approved by local government authorities (2011-128). After arrival, animals were maintained for one week to get accustomed to the new environment and for observation. Continuous health monitoring was carried out on a daily basis. For humanization, mice were injected with Busulfan (15 mg/kg) followed by an injection of 100 000 human CD34+ cord blood cells (STEMCELL Technologies) 24 h later. At 15 weeks post-injection, humanized mice were screened for human T-cell frequencies by flow cytometry (Supplemental Figure 7) and randomized into different treatment groups. Only humanized mice that revealed a humanization rate greater than 25% (defined as 25% human immune cells within all leucocytes) were used in the efficacy studies.

## *Ex vivo* analysis of murine peripheral blood by flow cytometry

Blood samples were lysed for erythrocytes using lysis buffer (BD Biosciences). Cells were re-suspended in FACS buffer (PBC, 2% FCS, 2mM EDTA) and stained with anti-human CD45 mAb, anti-human CD3 mAb, anti-human CD4 mAb, anti-human CD8 mAb and anti-human PD1 mAb (BioLegend) for 20 min at 4°C in a total volume of 100 uL of FACS buffer. After a washing step, the cell suspension was stained with DAPI for dead cell exclusion and analyzed at the LSR Fortessa (BD Biosciences). Serum was collected at the indicated time points after therapy injections and analyzed for different cytokines/chemokines by Bioplex analysis (Bio-Plex Pro Human Cytokine 17-plex Assay, Bio-Rad). Serum samples were diluted 1:10 in assay buffer prior to Bioplex analysis.

## Single-dose pharmacokinetic studies and ELISA for CD20-TCB in serum

A single dose of 0.5 mg/kg of CD20-TCB was injected into non-humanized and humanized NSG. All mice were injected i.v. with 200 µL of the appropriate solution. To obtain the proper compound concentration per 200 µL, the stock solution was diluted with histidine buffer. Three mice per time point were bled at 10 min, 1 h, 3 h, 6 h, 24 h, 48 h, 72 h, 96 h, and 7 days. The injected compound was analyzed in serum samples by ELISA. Biotinylated anti-huCD3-CDR antibody, test sample, Digoxigenin labelled anti-huFc antibody and anti-Digoxigenin detection antibody (POD) are added stepwise to a 96-well streptavidin-coated microtiter plate and incubated after every step for 1 h at room temperature. The plate was washed three times after each step to remove unbound substances. Finally, the peroxidase-bound complex is visualized by adding ABTS substrate solution to form a colored reaction product. The reaction product intensity, which is photometrically determined at 405 nm (with reference wavelength at 490 nm), was proportional to the analyte concentration in the serum.

## Immunohistochemistry

Tumors and organs were fixed in PFA 4% and processed for formalin-fixed paraffin-embedded tissue (Leica TP1020 tissue processor, Germany). Paraffin sections (4 µm) were prepared using microtome (Leica RM2235 Microtome, Germany). Haematoxylin and eosin staining was performed using standard protocol. CD19, CD20, CD3, CD8 and CD4 immunohistochemistry was performed in paraffin sections with anti-human antibodies (CD3: SP7 Thermo Fisher; CD4: SP35 Cell Marque; CD8: SP16 Cell Marque; and CD20: SP32 Cell Marque) in the Leica Autostainer platform following the manufacturer’s protocols (Leica Biosystems, Germany). Images were obtained with Olympus scanner VS120 Virtual Slide Microscope (Olympus, Germany) and analyzed with Definiens software for cell quantification.

## Routine care of mice and Cynomolgus monkeys

Mice were maintained for one week after arrival to get accustomed to the new environment and for observation. Daily continuous health monitoring was conducted, and food and water were provided ad libitum. Mice were controlled daily for clinical symptoms and detection of adverse events. Tumor volume was measured by caliper every second day. Study exclusion criteria for the mice were described and approved in the corresponding local veterinary license (ZH193/2014).

Cynomolgus monkeys were kept at 18°C to 29°C and 30% to 70% humidity under conditions of 12 hours light and 12 hours dark (except during designated procedures) with ten or more air changes per hour with 100% fresh air (no recirculation). Municipal tap water after treatment by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system (except during designated procedures). Periodic analyses of the water were performed, and results of these analyses are on file at the Testing Facility. PMI Nutrition International Certified Primate Chow No. 5048 was provided daily in amounts appropriate for the size and age of the animals. The diet was supplemented with fruit or vegetables at least 2-3 times weekly. Each animal was subjected to a general physical examination by a qualified member of the veterinary staff to ensure normal health status. Veterinary care was available throughout the course of the study and animals were examined by the veterinary staff as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments were documented in the study records. The animals were acclimated to laboratory housing before the initiation of dosing.