Supplementary Material and Methods

Cells, Cell lines, and Primary Material.

PBMCS were isolated by Ficoll-Paque (GE Healthcare, Eindhoven, The Netherlands) from buffy coats obtained from Sanquin Blood Bank (Amsterdam, The Netherlands). αβT cells were expanded from PBMCs using CD3/CD28 dynabeads (Thermo Fisher scientific, United States) and (1.7×10^3 IU/ml of MACS GMP Recombinant Human interleukin (IL)-7 (Miltenyi Biotec, Germany), and 1.5×10^2 IU/ml MACS GMP Recombinant Human IL-15 (Milteny Biotec, Germany). CD4+ and CD8+ αβT cells were selected using MACS isolation with CD4- and CD8- microbeads respectively (Miltenyi Biotec, Germany). γδ T cells were first selected from PBMCs by MACS isolation using TCR γδ+ isolation kit (Miltenyi Biotec, Germany), after which the Vδ2+ T cells were isolated via FACS sort based on positive staining for Vδ2-FITC (clone B6, Biolegend). Vδ2+ cells were expanded using the previously described rapid expansion protocol(1). RPMI 8226 stably expressing GFP-luciferase was generated by a previously described retroviral transduction protocol(2). The plasmid containing the GFP and luciferine transgenes was kindly provided by Jeanette Leusen (UMC Utrecht, Utrecht, Netherlands). The following cell lines were obtained from ATCC between 2010 and 2018, HL60 (CCL-240), ML-1 (CVCL_0436), MDA-MB231 (HTB-26), RPMI 8226 (CCL-155), Saos-2 (HTB-85), SCC9 (CRL-1629), HEK293T (CRL-3216). HEKBTN3 knock-out was a gift from Erin Adams (Chicago, United States). BV173 (ACC 20) was obtained from DSMZ. MZ1851RC was a kind gift from Barbara Seliger (University Halle, Germany). MDA-MB157 was kindly provided in 2016 by Thordur Oskarsson (Deutschen Krebsforschungszentrum, Heidelberg, Germany). Freestyle 293-F cells (R790-07) were obtained from Invitrogen (United States). HL60, RPMI

8226 ML-1 and BV173 were cultured in RPMI (Gibco, United states), 10% FCS (Bodinco, Alkmaar, The Netherlands), 1% Pen/Strep (Invitrogen, United States). Freestyle 293-F in Freestyle expression medium (Gibco). All other cell lines in DMEM, 10% FCS, 1% Pen/Strep. RPMI 8226 B2M knockout was created using Alt-R Crispr-CAS9 system (IDT, United States) according to the manufacturers protocol, with guide RNA sequence AAGTCAACTTCAATGTCGGA. Transfection was done with Neon Transfection system (Thermo Fisher Scientific) using the following settings: pulse voltage 1050 V, pulse width 20, 3 pulses.

Primary Material

Primary acute myeloid leukemia and multiple myeloma blasts were obtained from the biobank of the University Medical Center, Utrecht in accordance with good clinical practice and the Declaration of Helsinki regulations. All patients gave their consent prior to storage in the biobank (TCBio 16-088). B cells and monocytes were isolated from PBMCs by MACS-separation using CD19 and CD14-microbeads (Miltenyi Biotec, Germany) respectively, according to the manufacturers' protocol. Fibroblasts were a kind gift from Marieke Griffioen (Leiden University Medical Centre, Leiden, The Netherlands) and cultured in DMEM medium containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin**.** Multipotent mesenchymal stromal cells (MSCs) were isolated from healthy bone marrow (Hematology Department, UMC Utrecht, The Netherlands) by adherence to tissue culture flasks, and cultured in MSC-medium; α-minimal essential media (Gibco, USA) containing 0.2 mM L-ascorbic acid 2-phosphate, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin**.** Endothelial progenitor cells (EPCs) were isolated from healthy umbilical cord blood by density-gradient centrifugation using Ficoll-paque. The isolated MNCs were seeded on

collagen I-coated tissue flasks and expanded in EGM-2 medium (Lonza, Switzerland) containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin CD34+ were isolated from human umbilical cordblood using magnetic bead selection (Milteny Biotec, Germany). Umbilical cordblood was obtained after informed consent and approval by the ethics comittee of the University Medical Center Utrecht (TC-bio 15-345). To induce stress, cells were irradiated with 3500 cGy, or treated with 5mM cyclophosphamide (Sigma Aldrich, Germany) and 20 µM fludarabine (Sigma Aldrich) Activation was done with huCD40LT (Milteny Biotec, Germany) 400 ng/ml for 72 hours prior to assay (CD19+), IFNy (R&D systems, Canada) 1000IU/ml 16 hours prior to assay (CD34+, Fibroblasts), LPS 100ng/ml (Invitrogen, United States) added during the assay (CD14+).

Flow cytometry

0.2*10^6 T cells were incubated with GAB (10 µg/ml if not indicated differently) in 20 µl FACS buffer (PBS, 1% BSA (Sigma Aldrich, Germany), 0.01 % sodium azide (Severn Biotech Ltd, United Kingdom) for 30 minutes at room temperature. Cells were washed once in FACS buffer and incubated with the appropriate secondary antibody mix for 30 minutes at room temperature. Cells were washed 2 times in FACS buffer and fixed in 1% paraformaldehyde (Merck, Germany) in PBS. Data acquisition was done on FACS Canto and analyzed using FACS Diva software (BD, United States) or FlowJo. Antibodies that were used are pan-γδTCR-PE (Beckman Coulter, United States, clone IMMU510, 1:10), pan-γδTCR APC (BD Pharmigen, United States, clone B1,1:5), Vδ2-FITC (Biolegend, United States, clone B6, 1:10) and Vγ9-PE (BD pharmingen, clone B3, 1:25).

IFNγ Elispot

15.000 effector cells and 50.000 target cells were incubated together, with or without GAB (10 µg/ml if not indicated differently) for 16 hours at 37 °C 5% CO_2. In PAM conditions, 100 µM PAM (Calbiochem, United States) was added to the cells. The co-culture was done in nitrocellulose-bottomed 96-well plates (Millipore, United States) precoated with α-IFNγ antibody (clone 1-D1K) (Mabtech, Sweden). After 16 hours, the plates were washed with PBS and incubated with mAb7-B6-1 (II; Mabtech, Sweden) followed by Streptavadin-HRP (Mabtech, Sweden) IFNγ spots were visualized with TMB substrate (Mabtech, Sweden) and analyzed using A.EL.VIS ELISPOT Scanner and analysis software (A.EL.VIS, Germany).

CD107 degranulation assay

300.000 target cells were incubated with 100.000 T cells, GAB (10 µg/ml) and 100 µM PAM (Calbiochem, United States) in the presence of CD107a-PE (BD, United States, clone AB4, 1:200) for 7 hours, after 2 hours Golgistop (BD, United States) was added (1:1500) After 7 hours, cells were washed in FACs buffer and stained with aCD3-eFluor450 (eBioscience, United states, clone OKT3, 1:50) and aCD8-PerCP-Cy5.5 (Biolegend, United States, clone RPA-T8, 1:1000). Cells were washed 2 times in FACS buffer and fixed in 1% paraformaldehyde (Merck, Germany) in PBS. Data acquisition was done on FACS Canto and analyzed using FACS Diva software (BD)

Luciferase based cytotoxicity

5000 RPMI 8226 target cells stably expressing luciferase were incubated with T cells at different E:T ratios (1:1 to 1:30), with or without 10 µg/ml GAB in the presence of 0.1 mM PAM (calbiochem, United States). After 16 hours, beetle luciferin (Promega,

United States) was added to the wells (125 µg/ml) and bioluminescence was measured on SoftMax Pro plate reader. The signal in treatment wells was normalized to the signal measured for untreated targets, which was assumed to represent 100% living cells.

Animal model

NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Experiments were conducted under institutional guidelines after permission from the local Ethical Committee and in accordance with the current Dutch laws on Animal Experimentation. Mice were housed in sterile conditions using an individually ventilated cage (IVC) system and fed with sterile food and water. Irradiated mice were given sterile water with antibiotic ciproxin for the duration of the experiment. Adult female mice (16 weeks old) received sublethal total body irradiation (1.75 Gy) on Day -1. For the iv model, mice received a subcutaneous injection of 10 x 10^6 RPMI 8226-luc cells in PBS on day 0. Ten days later, mice were randomized into two groups of 4 or 5 mice and one group was intravenously injected with 10 x 10^6 huPBMCs. Tumor growth was measured once a week by bioluminescence imaging (BLI). For the subcutaneous model, mice received a subcutaneous injection of 10 x 10^6 RPMI 8226-luc B2M knockout cells in PBS, on day 0. One week later, mice were randomized based on tumor volume into two groups of 10 mice and intravenously injected with 10 x 10^6 huPBMCs. Next, mice received 7 consecutive injections of either CL5 GAB or LM1 GAB (2,7 mg/kg body weight). Pamidronate (10 mg/kg body weight) was injected together with the GABs on days 9 and 12. Moreover, an extra group (n=4) that received tumor and huPBMCs but no GABs was included as additional control. Tumor volume was measured three times a

week as primary outcome measure. Percent change in tumor volume was calculated using the formula: (Vf-V0)/V0*100, where V0 = volume at the beginning of the treatment, and Vf = final volume. Mouse peripheral blood samples were obtained via cheek vein (maximum 50–80 µl/mouse) once a week. Red blood cell lysis was performed for blood samples using 1× RBC lysis buffer (Biolegend) before cell staining. Blood samples were stained with γδTCR-PE (clone RPA-T4,Biolegend), αβTCR-FITC (Clone IP26, Invitrogen), huCD45-PB (Clone HI30, Sony). The persistence of GABs bound to αβ T cells was measured in peripheral blood by quantifying the absolute αβTCR positive and αβTCR^{+}/γδTCR double positive cell number by flow cytometry using using Flow-count Fluorospheres (Beckman Coulter).

1. Marcu-Malina V, Heijhuurs S, van Buuren M, Hartkamp L, Strand S, Sebestyen Z*, et al.* Redirecting αβ T cells against cancer cells by transfer of a broadly tumor-reactive γδT-cell receptor. Blood **2011**;118:50-9
2. Grunder C, van DS, Hol S, Drent E, Straetemans T, Heijhuurs S*, et al.* gamma9 and delta2CDR3 domains regulate functional avidity of T cells harboring gamma9delta2TCRs. Blood **2012**;120:5153-62

S1

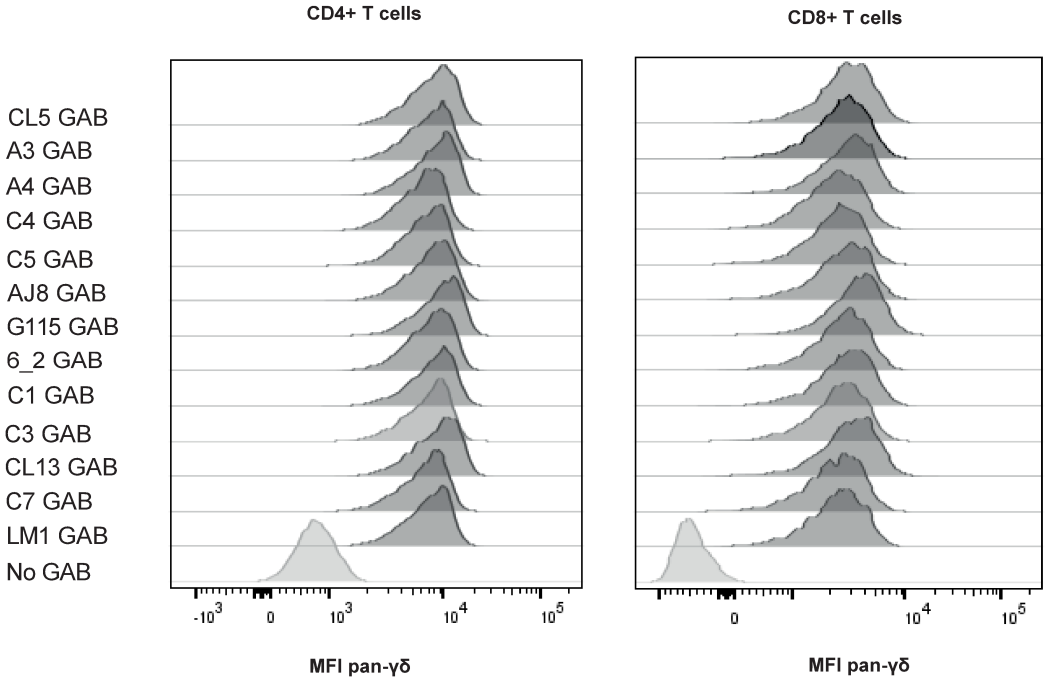
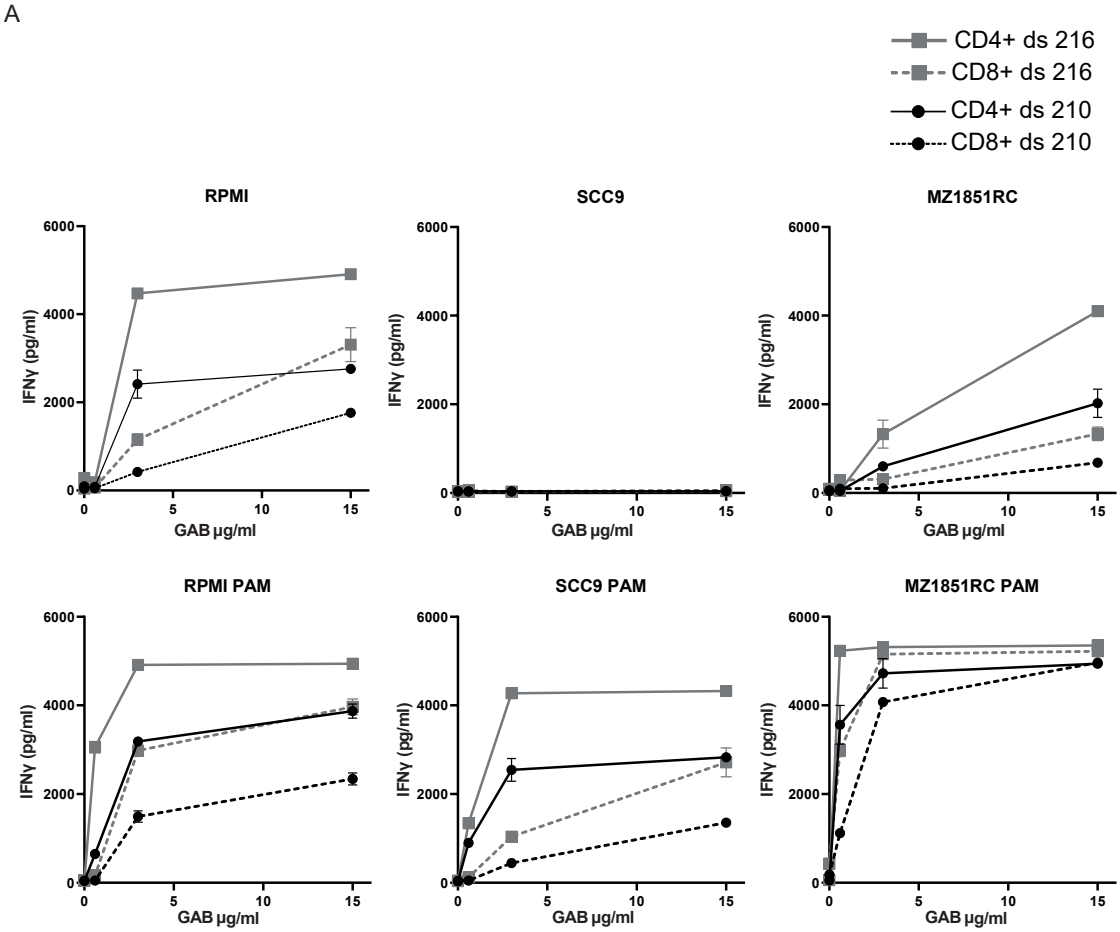


Figure S1. GAB coating of CD4 and CD8+ αβT cells. Coating of αβT cells with GAB (90 µg/ml), followed by staining with fluorochrome labeled anti pan γδ, CD4 and CD8 antibodies. MFI was measured by flow cytometry, histrograms represent MFI for γδ for CD4+ and CD8+ T lymphocytes.

S2
A



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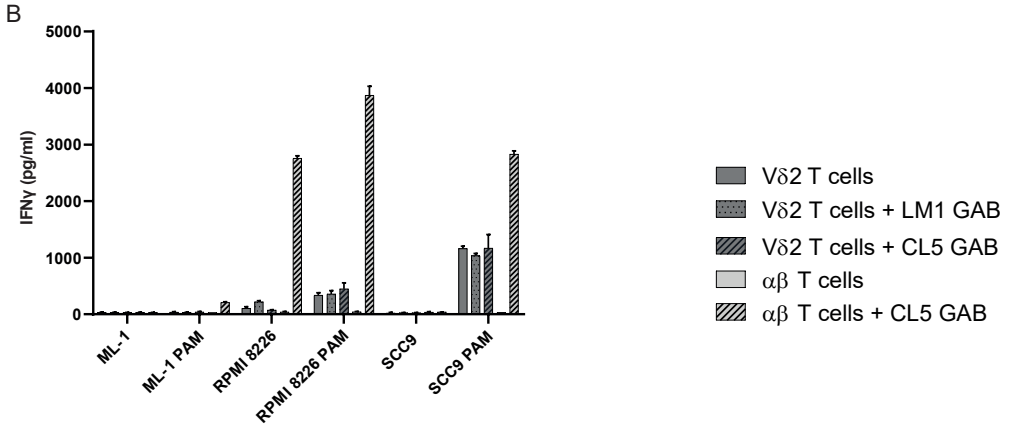

Figure S2. Differential GAB mediated activation of CD4+, CD8+ αβ T cells and Vδ2+ T cells. IFNγ release was measured using ELISA after a co-culture of A) MACS seperated CD4 and CD8 αβ T cells from 2 diiferent T cells donors with 3 different target cells in the presence of different concentraiton of CL5 GAB, with and without PAM (100 µM). B) bulk αβ T cells or bulk Vδ2+ T cells with 2 recognized (RPMI 8226 + SCC9) and 1 unrecognized cell line (ML-1) with and without LM1/CL5 GAB (15 µg/ml) and PAM (100 µM). N=1 error bars represent SD from technical duplicates.

S3

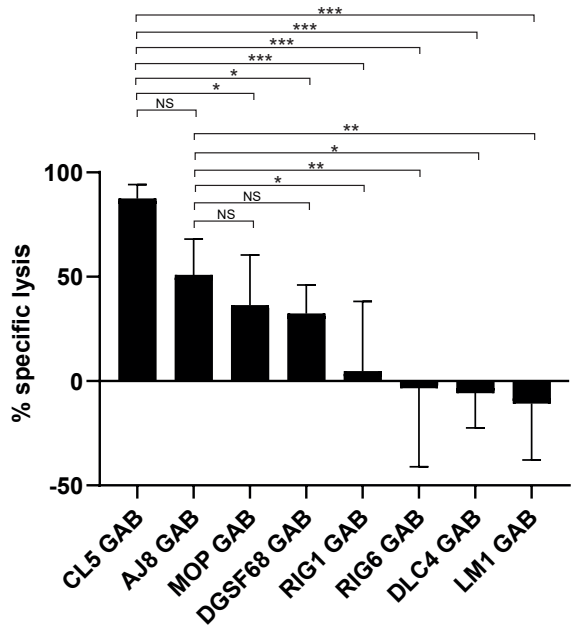

Figure S3. Higher activity CL5 GAB compared to GABs derived from publicly available γ9δ2 TCRs. A) T effector and luciferase transduced RPMI 8226 cells were coincubated for 16 hours in the presence of GABs (10 µg/ml) derived from different Vγ9Vδ2 TCRs and PAM (30 µM) at 10:1 E:T ratio. Percentage specific lysis was determined by comparing luminescence signal to untreated target cells, representing 100% viability. N=2, with technical duplo's error bars represent SD, significance was calculated using a one-way ANOVA; * P<0.05, ** P<0.001, ***P<0.0001

S4

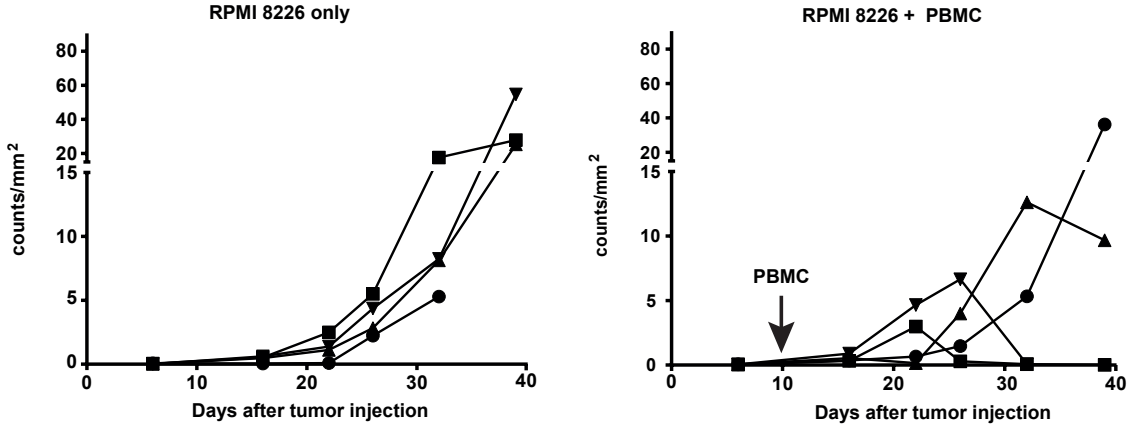

Figure S4. Poor outgrowth of IV injected RPMI 8226 in NSG mice when co-engrafted with huPBMCs. NSG mice were irradiated at day -1, and injected intra venous (i.v) with 10*10^6 RPMI 8226 tumor cells one day later, after 10 days the mice were randomized over two groups. One group was injected i.v with 10*10^6 huPBMCs (n=5 right panel) while the other group recieved no further treatment (n=4 left panel). Tumor growth was monitered by bioluminescence imaging (BLI) once a week and plotted overtime, each line represents one mouse.