Supplemental data for

γ9δ2T cell diversity and the receptor interface with tumor cells

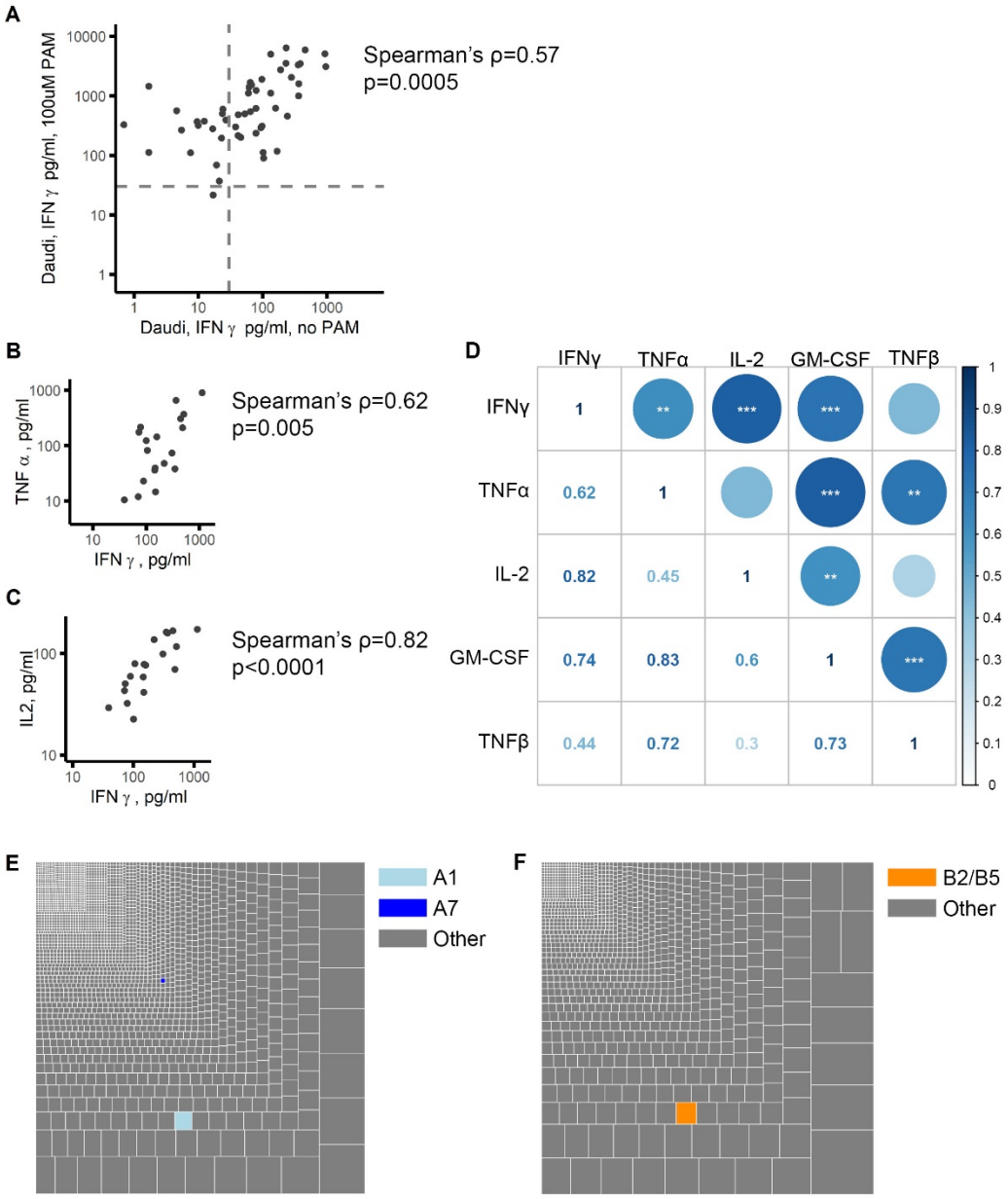
Anna Vyborova, Dennis X. Beringer, Domenico Fasci, Froso Karaiskaki, Eline van Diest, Lovro Kramer, Aram de Haas, Jasper Sanders, Anke Janssen, Trudy Straetemans, Daniel Olive, Jeanette Leusen, Lola Boutin, Steven Nedellec, Samantha L. Schwartz, Michael J. Wester, Keith A. Lidke, Emmanuel Scotet, Diane S. Lidke, Albert J. R. Heck, Zsolt Sebestyen, Jürgen Kuball

Corresponding author: Jürgen Kuball
Email: J.H.E.Kuball@umcutrecht.nl

This PDF file includes:

Supplemental Figures 1 to 9
Supplemental Table 1
Supplemental Methods
References for supplemental reference citations

Supplemental Figure 1

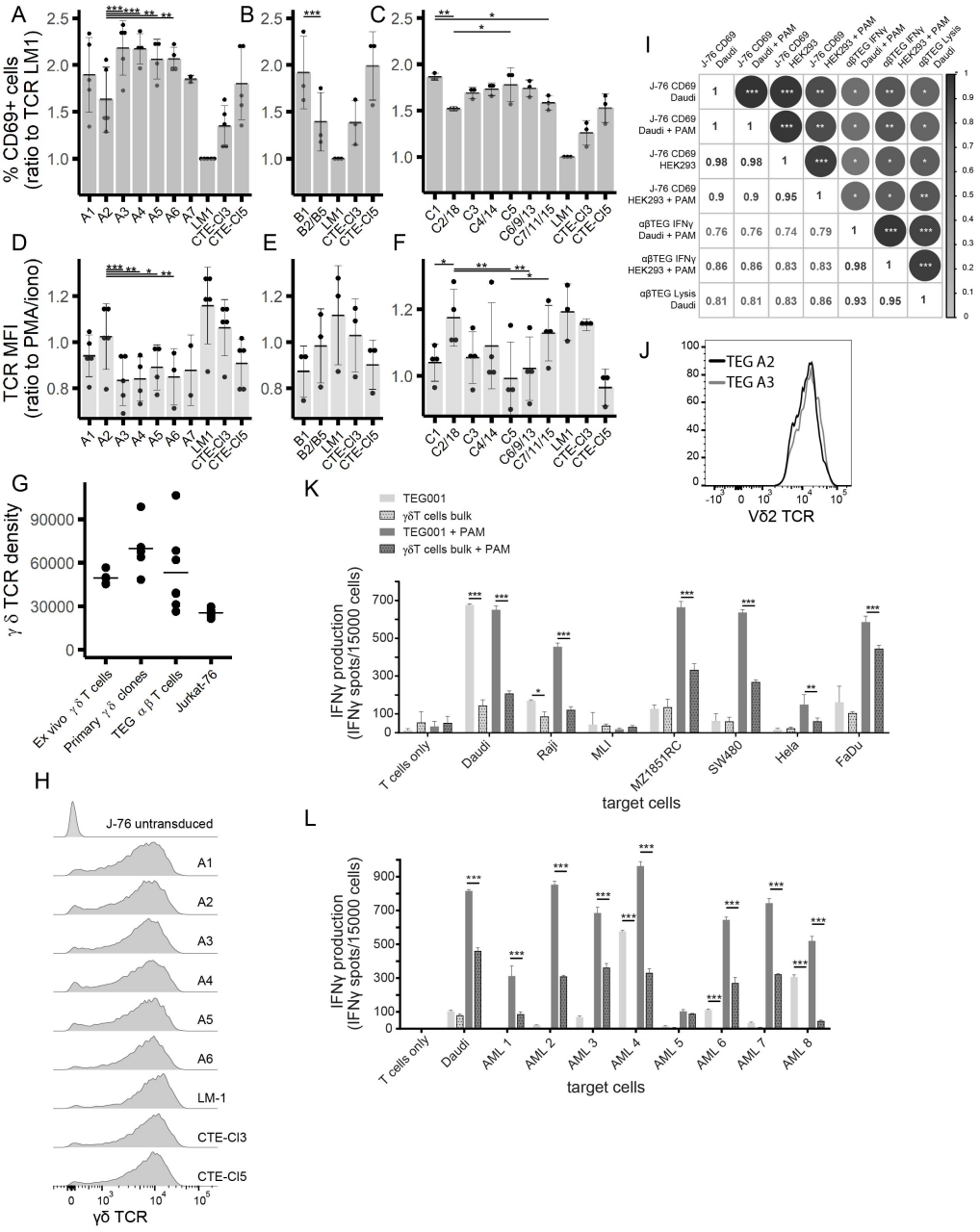


Supplemental Figure 1. Cytokine profile of the γ9δ2T cell clones and TRD repertoires of the Donors A and B (corresponding to main text Figure 1).

A. Potentiating effect of PAM treatment on IFNγ secretion by γ9δ2T cell clones (data as in Fig. 1A). Spearman rank correlation coefficient was calculated for the values above the cut-off for reliable ELISA measurements (30 pg/ml). **B-D.** Th1 cytokine profile of a subset of the investigated γ9δ2T cell clones (clones donor C, n=18). **B-C.** Examples of the scatter plots and pairwise Spearman rank correlations between IFNγ and TNFα (B), IFNγ and IL-2 (C) in response to Daudi + 100μM PAM, measured in the supernatants by Luminex. **D.** Correlogram

demonstrating all pairwise Spearman rank correlations between Th1 cytokines (IFNγ, TNFα, IL-2, GM-CSF, and lymphotoxin α (TNFβ)), measured in the supernatants by Luminex. The area and color of the circles are proportional to the absolute values of the corresponding correlation coefficients (depicted in the lower triangle of the matrix; *** $p<0.001$, ** $p<0.01$, * $p<0.05$). **E-F.** Treemaps representing the complete TRD repertoires of the donors A (E) and B (F). Each rectangle represents a unique CDR3 sequence, colored rectangles indicate TRD clonotypes belonging to the functionally tested clones from the respective donors.

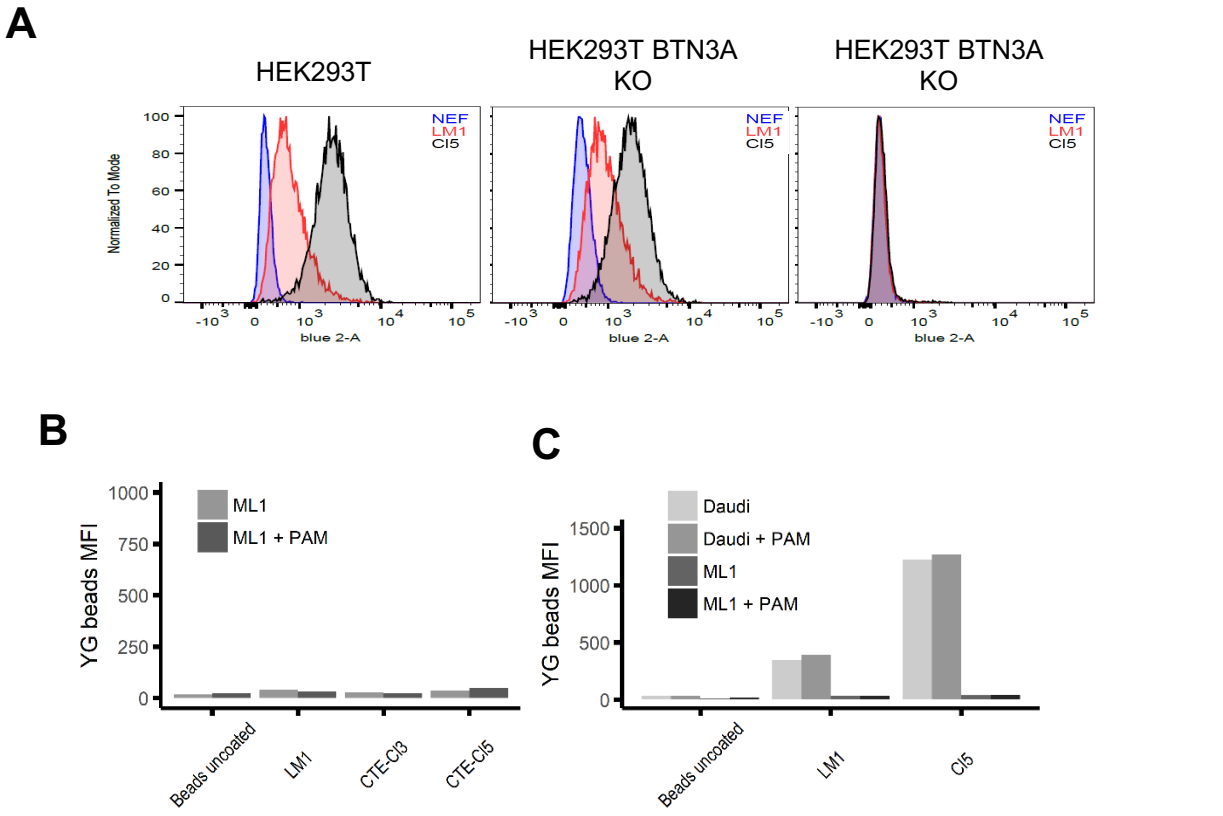
Supplemental Figure 2



Supplemental Figure 2. Functional avidity and surface expression of the isolated TCRs in the TEG format.

A-F. Functional avidity of the TCRs from Donors A, B and C, next to control TCRs, transduced into Jurkat-76 cell line (n=2-5 from at least 2 independent transductions). **A-C.** CD69 upregulation (percent CD69-positive cells) upon stimulation with the cell line Daudi in the presence of 100 µM PAM, normalized to TCR LM1 within each experiment. **D-F.** TCR downregulation measured by MFI TCRVδ2 on Jurkat-76 cells co-incubated with the cell line Daudi in the presence of 100 µM PAM, relative to MFI TCRVδ2 on the same transductants treated with PMA/ionomycin. Bars and error bars represent mean values +/- SD across experiments, significance was determined by a two-way ANOVA with Tukey's post hoc tests, a p value less than 0.05 was considered significant, *** $p<0.001$, ** $p<0.01$, * **G.** TCR surface expression density on the primary clones A1-A6 and the respective TCR transductants (A1-A6 plus control TCRs) on the background of T cell line J-76 and primary human αβ T cells. Expression density was determined using Quantum™ Simply Cellular® (QSC) anti-Mouse microspheres. **H.** Histograms showing γδTCR surface expression on the J-76 cells from one representative transduction, on the example of the TCRs from donor A and control TCRs. **I.** Correlogram demonstrating pairwise Spearman rank correlations between the functional avidity assays of the TCRs from Donor A: 1) CD69 upregulation on TCR-transduced J-76 cells stimulated with the cell line Daudi, 2) CD69 upregulation on TCR-transduced J-76 cells stimulated with Daudi+100µM PAM, 3) CD69 upregulation on TCR-transduced J-76 cells stimulated with the cell line HEK293FT, 4) CD69 upregulation on TCR-transduced J-76 cells stimulated with HEK293FT+100µM PAM, 5) IFNγ release upon TEG coincubation with the cell line Daudi+100µM PAM , 6) IFNγ release upon TEG coincubation with the cell line HEK293FT +100µM PAM, 7) specific lysis of the cell line Daudi by TEGs. **J.** 1D histograms showing γδ TCR surface expression on the transduced and expanded primary human αβ T cells (TCRs A2 and A3, corresponding to Figure 2G and Supplementary Figure 2I). **K-L.** A panel of tumor cell lines (**K**) or primary AML tumor samples (**L**) was incubated with TCR CTE-Cl5 engineered T cells or with a bulk population of primary γδ T cells, with or without 10 µM pamidronate for 20 hours. IFN-γ secretion was measured by ELISPOT. IFN-γ spots per 15000 T cells are shown as the mean of triplicates ($\pm$SD). Statistical significances were calculated by two-way ANOVA, a p value less than 0.05 was considered significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$

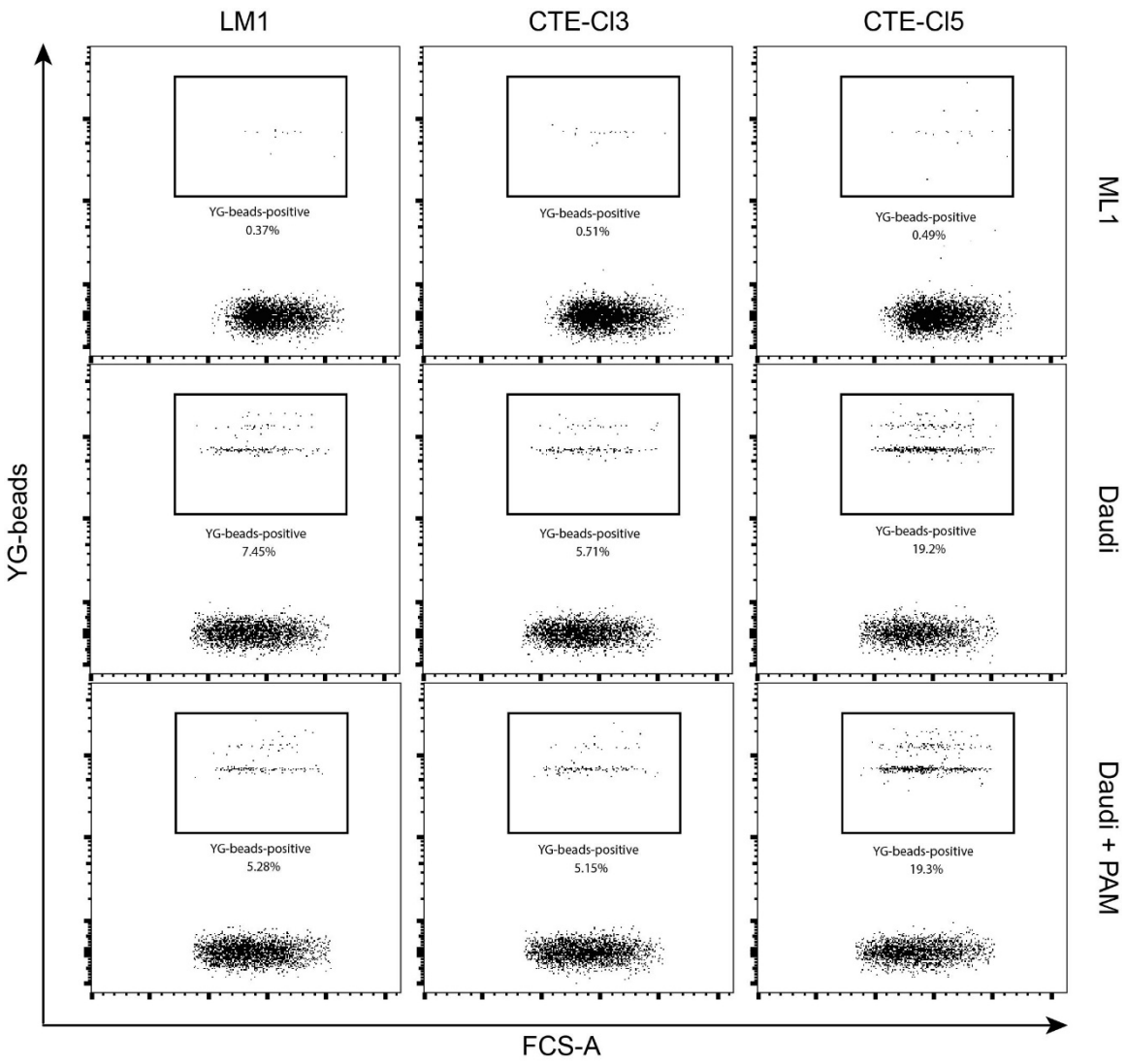
Supplemental Figure 3



Supplemental Figure 3. Staining of target cells with γδTCR multimers of increasing valency (corresponding to main text Figure 3).

A. TCR tetramer staining on HEK293T wt, BTN3A KO and BTN3A + BTN2A1 double KO cells. B. YG beads (unconjugated or conjugated with γδTCR LM1, CTE-CI3 or CTE-CI5). A representative experiment is shown (n=3), bars represent mean and SD of 3 side-by-side biological replicates. **C.** Staining of ML1 and Daudi cells using YG beads (unconjugated or conjugated with γδTCR LM1 or CI5), analyzed in the same manner as in (C). The figure corresponds to main text Figure 3, and supplemental Figure 2.

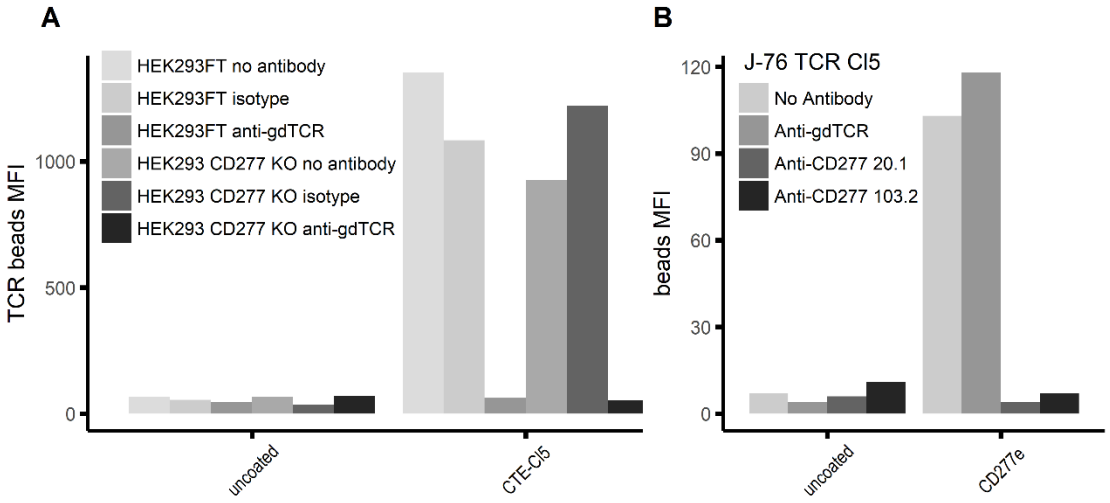
Supplemental Figure 4



Supplemental Figure 4. Staining of target cells with sTCR-conjugated beads (corresponding to main text Figure 3).

Representative flow cytometry dot plots of Daudi and ML1 cells stained with sTCR-coated YG-beads. Beads bind only a fraction of tumor cells, most likely reflecting technical limitations of the experiment rather than heterogeneity of ligand expression within the target population. The figure corresponds to main text Figure 3 and Supplemental Figure 2.

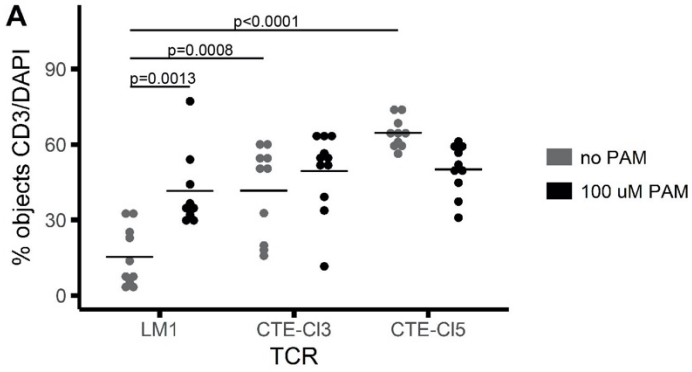
Supplemental Figure 5



Supplemental Figure 5. Antibody blocking of the TCR- and CD277e-coated bead binding (corresponding to the main text Figure 3 and supplemental Figures 2-3).

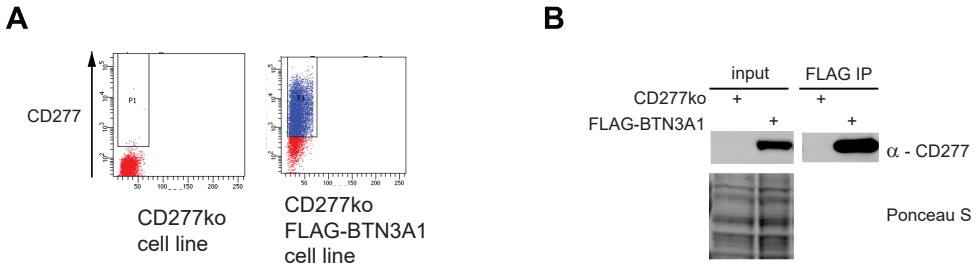
Staining of HEK293 cells (wild type or CD277 KO) with TCR-coated beads (unconjugated or conjugated with the TCR CTE-Cl5) in the presence of the anti-γδTCR vs control antibody. **B.** Staining of the Jurkat-76 cell line (transduced with the γ9δ2TCR Cl5) with CD277e-coated beads in the presence of anti-CD77 antibodies (clones 20.1 and 103.2, n=2) vs control antibody (anti-γδTCR).

Supplemental Figure 6



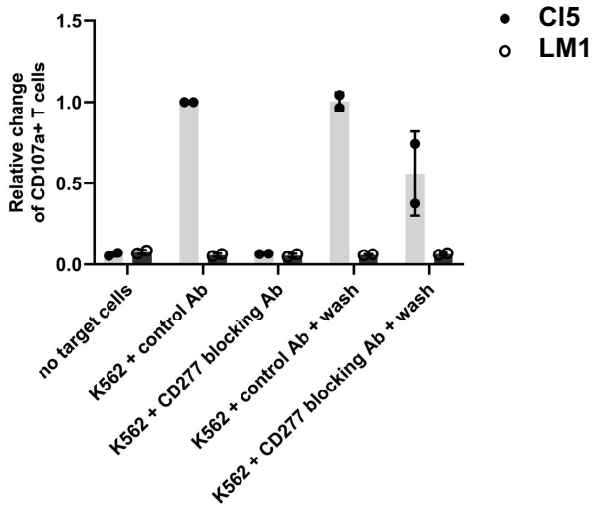
Supplemental Figure 6. TEG-tumor cell conjugation with different affinity TCRs. Conjugation was quantified as a ratio between the number of CD3-positive cells (TEGs) and the total of DAPI+ cells. Analysis was done on at least ten independent images, significance was determined by 2-way ANOVA using Mann-Whitney test, a p value less than 0.05 was considered significant. Dots represent individual images, bars represent median values.

Supplemental Figure 7



Supplemental Figure 7. Development of a FLAG-BTN3A1 stable cell line. **A.** CD277 staining of the HEK293T-CD277 knock-out parental or the FLAG-BTN3A1 stably expressing cell line. **B.** FLAG immunoprecipitation from the lysates obtained from the HEK293T-CD277 knock-out parental or the FLAG-BTN3A1 stably expressing cell line.

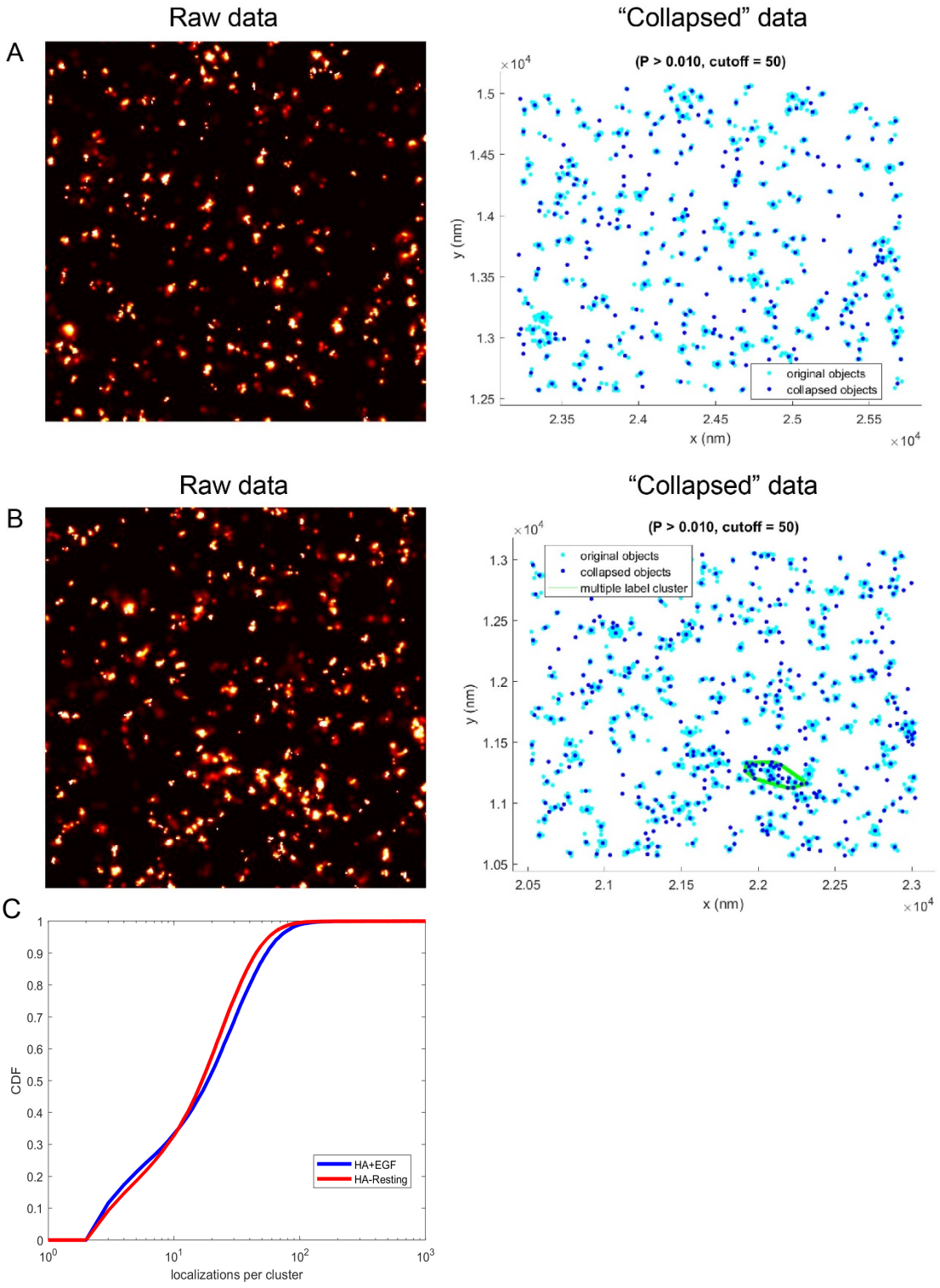
Supplemental Figure 8



Supplemental Figure 8. Evaluation of TEGs recognition after block of surface CD277. CD107a staining of TCR-Cl5 TEG or LM1 TEG cells co-cultured with K562 cells treated with 100 μM PAM, and pre-incubated for 45 minutes with a control antibody or a CD277 blocking antibody (clone 267-21f8.19) before starting the treatment or retained throughout the treatment. The dots indicate the

relative changes in CD107a positive T cells of individual FACS experiments. Bars and error bars indicate mean values +/- SD.

Supplemental Figure 9



Supplemental Figure 9. Examples of H-SET analysis of dSTORM data (corresponding to main text Figure 5D-E). Two examples of dSTORM super-resolution images from an untreated (Control) sample illustrating random distribution of molecules vs clusters. Reconstructed dSTORM images are shown on the left. “Collapsed Data” is demonstrated on the right, where light blue spots indicate original localizations from the dSTORM imaging and dark blue dots indicate the location of single emitters after H-SET collapsing of data to remove repeat localizations. A. In the majority of the data, all small clusters collapsed into single points. B. A rare example showing a cluster that persisted after collapse (green outline marks the identified cluster). Figure corresponds to main text Figure 5. C. Cumulative Distribution Function (CDF) for the Nearest Neighbor Distances (NND) for Control and EGF-treated HEK293T-EGFR cells (corresponding control to the main text Figure 5E).

C

Supplemental Table 1. Sequences of the primers for generating TCR CDR3 regions using overlap extension PCR.

TCR	Gamma	
	rev R1 5'-3'	**fwd R2 5'-3'**
A1	CCCAGCTCTTTCACCTCCCACAGGGCGCAG	GGGAGGTGAAAGAGCTGGGCAAGAAAATCAAGGTGTTCG
A2	CCAGCTCTTGCACCTCCCACAGGGCGCAG	GGGAGGTGCAAGAGCTGGGCAAGAAAATCAAGGTGTTCG
A3	CCAGCTCTTGCACCTCCCACAGGGCGCAG	GGGAGGTGCAAGAGCTGGGCAAGAAAATCAAGGTGTTCG
A4	CCCAGGCCTTCAACCTCCCACAGGGCGCAG	GGAGGTTGAAGGCCTGGGCAAGAAAATCAAGGTGTTCG
A5	AGCTCGCCGGCCTCCCACAGGGCGCAG	GAGGCCGGCGAGCTGGGCAAGAAAATCAAGGTGTTCG
A6	CAGCTCTTGCCGCACCTCCCACAGGGCGCAG	GAGGTGCGGCAAGAGCTGGGCAAGAAAATCAAGGTGTTCG
A7	CAGCTCCCGCACCTCCCACAGGGCGCAG	GGAGGTGCGGGAGCTGGGCAAGAAAATCAAGGTGTTCG
B1	CCAGTTCCACTTGAGCCTCCCACAGGGCGCAG	GGGAGGCTCAAGTGGAACTGGGCAAGAAAATCAAGGTGTTCG
B2/B5	CAGCTTCTTGTAGTATCTCACCTCCCACAGGGCGCAG	GGGAGGTGAGATACTACAAGAAGCTGTTCGGCAGC
C1	CAGCTCCCGCACCTCCCACAGGGCGCAG	GGAGGTGCGGGAGCTGGGCAAGAAAATCAAGGTGTTCG
C2	CAGCTCCCGCACCTCCCACAGGGCGCAG	GGAGGTGCGGGAGCTGGGCAAGAAAATCAAGGTGTTCG
C3	CCCAGGCCCACCTCCCACAGGGCGCAG	GGAGGTGGGCCTGGGCAAGAAAATCAAGGTGTTCG
C4/C14	CAGCTCGCCAGACACCTCCCACAGGGCGCAG	GGAGGTGTCTGGCGAGCTGGGCAAGAAAATCAAGGTGTTCG
C5	CCAGCTCTTGCACCTCCCACAGGGCGCAG	GGGAGGTGCAAGAGCTGGGCAAGAAAATCAAGGTGTTCG
C6/C9	CCCAGCTCCCTCTTCACCTCCCACAGGGCGCAG	GAGGTGAAGAGGGAGCTGGGCAAGAAAATCAAGGTGTTCG
C7/C11	CAGCTCCCGCACCTCCCACAGGGCGCAG	GGAGGTGCGGGAGCTGGGCAAGAAAATCAAGGTGTTCG
TCR	**Delta**	
	rev R1 5'-3'	**fwd R2 5'-3'**
A1	GCTGTCGCCCAGAAGCAGCAGTGTGTCGCAGGCGCAGTAGTAG	CTGCTTCTGGGCGACAGCAGCGACAAGCTGATCTTCGGCAAGG
A2	GTAGCCGCCAGGTTCAGCTGTGTCGCAGGCGCAGTAGTAG	GCTGAACCTGGCGGCTACAAGGACAAGCTGATCTTCGGCAAGG
A3	TGTGTGGCCCCAGGCGTCGCAGGCGCAGTAGTAG	GCCTGGGGCCACACAGACAAGCTGATCTTCGGCAAGG
A4	GCTGCCTGTATCGCCCAGGGCGTCGCAGGC	CTGGGCGATACAGGCAGCGACAAGCTGATCTTCGGCAAGG
A5	GTACAGCGCCCCCAGTGTGTCGCAGGCGCAGTAGTAG	CACTGGGGGCGCTGTACACCGACAAGCTGATCTTCGG
A6	GGGGTCGCCCAGCTGGTCGCAGGCGCAGTAGTAG	CAGCTGGGCGACCCCGACAAGCTGATCTTCGGCAAGG
A7	TATGCCCCAATCGGGATCGCTGTCGCAGGCGCAGTAGTAG	GCGATCCCGATTGGGGCATACTGAACACGGACAAGCTGATCTTCGGCAAGG
B1	AGTGTCCTGAGCAGCGGGAATACCCCACGGAGTGTCGCAGGCGCAGTAGTAG	ACTCCGTGGGGTATTCCCGCTGCTCAGGACACTGACAAGCTGATCTTCGGCAAGG
B2/B5	AGTAGTTCCCCAGGCTAGAAACCTTGGGTCGCAGGCGCAGTAGTAG	CCAAGGTTTCTAGCCTGGGGAACTACTGACAAGCTGATCTTCGGCAAGG
C1	CGGTATCGTGGATAGAAGGTACAGGGTCGCAGGCGCAGTAGTAG	CCTGTACCTTCTATCCACGATACCGACAAGCTGATCTTCGGC
C2	AGGGCCACCCGCTTGGTCGCAGGCGCAGTAGTAG	CAAGCGGGTGGCCCTGACAAGCTGATCTTCGGCAAGG
C3	CTGGTAGCCGCCAGACACTGTGTCGCAGGCGCAGTAGTAG	GTGTCTGGCGGCTACCAGTACACTGACAAGCTGATCTTCGGCAAGG
C4/C14	CTTGTCTGTATCTCCCAGAGCTAATGTGTCGCAGGCGCAGTAGTAG	ACATTAGCTCTGGGAGATACAGACAAGCTGATCTTCGGCAAGG
C5	GAGGTATCGCCAGGGGCAAGAAGGTCGCAGGCGCAGTAGTAG	GCCCCTGGCGATACCTCTTTCACCGACAAGCTGATCTTCGGC
C6/C9	ATCTCTGTAACCACCTGTGCCAAGCACCACTGTGTCGCAGGCGCAGTAGTAG	CTTGGCACAGGTGGTTACAGAGATGACAAGCTGATCTTCGGCAAGG
C7/C11	CCAAGAACTAGCATCACCCATGTCGCAGGCGCAGTAGTAG	CGACATGGGTGATGCTAGTTCTTGGGACACTCGCCAAATG

Supplemental Methods

Isolation of PBMCs, generation and expansion of the γ9δ2T cells clones

PBMCs were isolated using Ficoll-Paque PLUS gradient centrifugation (GE Healthcare). The cells at the interface were harvested, washed 4X with PBS (Sigma Aldrich) and further stained with monoclonal antibody (mAb) to Vδ2 (Vδ2-FITC clone B6, Biolegend Cat#2257030). The mAb-positive fraction was either sorted in bulk, expanded using rapid expansion protocol (REP, a 2-week culture protocol including irradiated feeder cells (Daudi, LCL-TM and allogeneic PBMC) and cytokines (PHA, IL-15, IL-2), as described previously)[1], and thereafter cloned by limiting dilution (donor A), or single cell-sorted using FACS sort with collection of single cells in 96 well plates (donors B, C). All FACS sorts were performed on ARIAII (BD). Eight to twelve rounds of expansion on REP preceded functional testing.

Cell lines and T cell culture

The cell lines Daudi, Raji, MZ1861RC, SW480, Hela, FaDu, LCL-TM, ML-1, K562, JurMA and Jurkat-76 were cultured in RPMI (Gibco) supplemented with 10% FCS and 1% pen/strep (Gibco). HEK293FT cell line was cultured in DMEM (Gibco) supplemented with 10%FCS and 1% pen/strep. Human primary T cell clones were cultured in RPMI supplemented with 10% pooled human serum and 1% pen/strep according to the REP protocol. Human bulk primary T cells used for retroviral transductions were cultured in RPMI, supplemented with 2,5% pooled human serum and 1% pen/strep following the REP protocol. Acute myeloid leukemia (AML) blood samples were collected from the biobank of the University Medical Center Utrecht in accordance with good clinical practice and Declaration of Helsinki regulations. All patients gave their consent prior to storage of their blood in the biobank (TCBio 16-088). For the generation of the FLAG-BTN3A1 expressing cell line, the FLAG-BTN3A1 sequence was codon optimized, custom synthesized and cloned in pBullet-IRES-puro. The retroviral particles were generated with the same procedure used to generate TEGs described below. and used to transduce the HEK293T- CD277 knock-out cell line. 48 h post-transduction the antibiotic selection started supplementing 1.5 μg/mL of puromycin to the culture medium and the selection was carried out until the control (untransduced) cell line died completely.

Functional testing of the γ9δ2T cell clones

As soon as the primary T cell clones expanded to sufficient numbers, functional testing was performed: $5*10^4$ T cells were incubated overnight, together with target cells at 1:1 E:T ratio in DMEM supplemented with 10%FCS and 1% pen/strep, without or in the presence of 100 uM Pamidronate Disodium salt (Calbiochem Cat#506600), supernatants were harvested the day after, and IFNy concentration was measured using ELISA (eBioscience Ready-Set-Go! ELISA kit, Invitrogen Cat#88731688). Data were analyzed by 4-parametric curve fitting using Microplate Manager software, version 6.3 (Biorad). Other cytokines were measured in a set

of assay supernatants using an in-house developed and validated (ISO9001 certified) multiplex immunoassay (Laboratory of Translational Immunology, University Medical Center Utrecht) based on Luminex technology (xMAP, Luminex Austin TX USA). The assay was performed as described previously[2]. In short, samples were incubated with antibody-conjugated MagPlex microspheres for one hour at room temperature with continuous shaking, followed by one hour incubation with biotinylated antibodies, and 10 min incubation with phycoerythrin-conjugated streptavidin diluted in high performance ELISA buffer (HPE, Sanquin the Netherlands). Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules USA) in combination with xPONENT software version 4.2 (Luminex). Data were analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Biorad).

TCR sequencing and vector generation

RNA was isolated from the primary T cell clones using the Qiagen RNeasy Minikit following the manufacturer's instructions. cDNA was synthesized with Superscript® II Reverse Transcriptase (Thermofisher), using a specific primer at the 3' constant region (TRDCRev TTCACCAGACAAGCGACA, TRGCRev GGGGAAACATCTGCATCA). cDNA was purified using a NucleoSpin Gel and PCR Clean-UP (Machery-Nagel). cDNA was amplified in a PCR amplification step with the same reverse primer at the constant region (TRDCRev TTCACCAGACAAGCGACA, TRGCRev GGGGAAACATCTGCATCA), and specific Vδ2 or Vγ9 forward primer (TRDV2Fw TCTCTTCTGGGCAGGAGTC, TRGV9Fw TCCTTGGGGCTCTGTGTGT), using Q5® High Fidelity DNA polymerase (New England Biolabs) on a T100 Thermal Cycler (Biorad) and the following cycling parameters: 300 s at 92°C, 30 cycles of 30 s at 92°C, 30 s at 63°C, and 45 s at 72°C, followed by 420 s at 72°C.

TCR γ and delta chains were sequenced using the same primer pairs (TRDV2Fw TCTCTTCTGGGCAGGAGTC, TRDCRev TTCACCAGACAAGCGACA, TRGV9Fw TCCTTGGGGCTCTGTGTGT, TRGCRev GGGGAAACATCTGCATCA). Sanger sequencing was performed at Macrogen.

The TCRδ and TCRγ chains of a selection of the identified γδ T cell clones were reconstructed in retroviral expression vectors using overlap extension PCR to introduce the new CDR3 sequences. In brief, a set of primers was created based on the invariant sequences flanking the CDR3 region of a codon optimized construct, encoding either the γ or δ chain of G115, the primers were extended with clone-specific nucleotides. A stretch of 15-20 bp within the overhang was designed to be a reverse complement to its pair primer (primer sequences can be found in supplemental Table 1).

In PCR "R1" the V-(D)-J part (the variable domain and part of the CDR3 region) was amplified, using forward primers binding to the template chains δ and γ TCRG115 cloned into the retroviral pBullet vector and including the restriction sites (G115δFwd: CTG*CCATGG*AGCGGATCAGC, G115γFwd: G*CCATGG*TGTCCCTGCTG, NcoI restriction site in italic), and the clone-specific reverse primer Rev R1 (see supplemental Table 1). Similarly, in PCR "R2", the (D)-J-C part of the TCR chain was amplified using a constant reverse primer (G115δRev: ATGC*GGATCC*TCACAGG, G115γRev:

TAGT*GGATCC*TCAGCTCTTCTC, BamHI restriction site in italic) and the clone-specific forward primer Fwd R2. After the gel-based size selection and purification with NucleoSpin Gel and PCR Clean-UP kit (Machery-Nagel), two of the PCR products (R1 and R2) were fused and amplified in PCR "R3" using G115δFwd/ G115δRev and G115γFwd/G115γRev primer pairs to obtain TCR chains bearing the clone-specific CDR3 regions. All reactions were performed using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) on a T100 Thermal Cycler (Biorad) and the following parameters for R1-R2: 120s at 98°C, 30 cycles of 20 s at 98°C, 20 s at 59°C, and 25 s at 72°C, followed by 600 s at 72°C; for R3: 120s at 98°C, 30 cycles of 20 s at 98°C, 20 s at 56°C, and 40 s at 72°C, followed by 600 s at 72°C.
After another gel-based size selection step, the newly synthetized TCR chains were purified and cloned into the retroviral pBullet vectors using NcoI and BamHI cloning sites. The TCRδ genes were cloned into pBullet-IRES-puromycin, the TCRγ genes were cloned into pBullet-IRES-neo[3]. Sequence identity was confirmed with Sanger sequencing (Macrogen).

Retroviral transduction of plasmids and functional testing of the γ9δ2TCRs in the TEG format

Vector pairs bearing the TCR chains of interest were transduced either into the human αβTCR-deficient T cell line Jurkat-76 or into the primary human T cells, as previously described[1]. Functional testing of the TCRs in the TEG format was performed in the same fashion as functional testing of the primary clones: overnight incubation of $5*10^4$ T cells with target cells at 1:1 E:T ratio was followed by read-out (CD69 upregulation, IFNγ production). Unstimulated effector cells or cells stimulated with 20 ng/ml PMA (Sigma-Aldrich Cat#P8139) and 1 µg/ml ionomycin (Sigma-Aldrich Cat#I0634) served as negative and positive controls, respectively. To measure CD69 upregulation, cells were stained with the following antibody mix (30' on ice): Vδ2-FITC clone B6, Biolegend Cat#2257030, CD69-APC clone FN50, Sony Biotechnology Cat#2154550, CD20-eFluor450 clone 2H7, eBioscience Cat#48-0209-42. After washing, cells were stained with LIVE/DEAD fixable aqua dead cell stain (ThermoFisher Cat#L34957) for 30 minutes on ice, fixed with 1% paraformaldehyde and analyzed on a BD FACSCanto II (BD Bioscience). Analyses of the FACS data were done using BD FACS Diva software.
To measure the target cell lysis, the target cells were first labelled with Chromium-51 radionuclide: $1*10^6$ target cells were suspended in RPMI supplemented with 32.5% FCS adding 100 µCi (3.7 MBq) ^{51}Cr Sodium Chromate in Normal Saline (PerkinElmer Cat#NEZ030002MC) for 2 hours at 37°C. After washing away the unincorporated label, $2.5*10^3$ target cells were incubated with TEG cells at 10:1 E:T ratio in complete RPMI for 4 hours at 37°C. Assay supernatant (50 µL) was transferred to the LumaPlate (PerkinElmer Cat#6006633), and radiometric detection of released Chromium (measured in counts per minute (cpm)) was performed using the MicroBeta2 Microplate Counter (PerkinElmer). The percentage of specific lysis was calculated using the formula below:

$$(cpm\ experiment - cpm\ min.release) \Big/ (cpm\ max.release - cpm\ min.release)$$

where min. release is defined as Chromium release from the target cells incubated

in complete medium with no effector cells added, and max. release as Chromium release from the target cells incubated in 3% tritonX.

High throughput sequencing (HTS) of the TCRδ chain

RNA isolation, cDNA synthesis and PCR were performed on polyclonal cells in the same way as described earlier for the expanded γ9δ2T cell clones. After purification of the PCR product with NucleoSpin Gel and PCR Clean-UP, library preparation for HTS was done with HTSgo-LibrX kit with HTSgo-IndX indices from Gendx, as recommended by the manufacturer. Cleanup of the samples was performed with HighPrep PCR beads from GC Biotech. High-throughput sequencing was performed on an Illumina MiSeq system 500 (2x250 bp) (Illumina). TCR sequence alignment, assembly and clonotype extraction were performed using the MiXCR (version-v2.1.1) program[4]. In house R scripts were used for TCRδ repertoire analysis, data were filtered to exclude clonotypes with frequency of 1 read/clonotype.

Cloning, expression and purification of soluble TCRs and CD277e

The extracellular domains of the TCR chains were amplified from synthetic DNA encoding the full length TCRs. The domain boundaries were based on those previously published for Vγ9Vδ2 TCR G115[5]. The TCRδ chains were ligated in to a modified pBullet vector containing a μ-phosphatase signal peptide at the 5'end and Fos zipper at the 3'end of the construct. The TCRγ chains were ligated in a modified pBullet vector containing a μ-phosphatase signal peptide at the 5'end, and at the 3' a Jun zipper followed by a biotin acceptor peptide (BAP) and a poly-histidine (His) tag. Synthetic DNA encoding for the bacterial biotin ligase BirA was also ligated in a pBullet vector containing a signal peptide. The extracellular domains of CD277 (BTN3A1; Uniprot code O00481; residues 30-246) were cloned in the modified pBullet vectors as was done for the extracellular domains of the TCRs using both the Fos and Jun zippers.

The expression of soluble γδTCRs was done in Freestyle 293-F cells (ThermoFisher). In short, plasmids containing TCRδ, TCRγ and BirA were mixed in a 45:45:10 ratio, combined with polyethylenimine (PEI) in a 2:3 ratio and incubated for 15' at room temperature. For the expression of CD277e a mix containing the plasmids encoding for CD277e-fos, CD277e-jun-BAP-His and BirA were mixed in a 6:3:1 ratio and combined with PEI in a 2:3 ratio. The DNA:PEI mix was added to the cells at a concentration of 1-1.5 μg plasmid DNA/10^6 cells and after 6 h the media was supplemented with Pen/Strep (Gibco) and 100 μM biotin.

5 days after transfection, the media was harvested, supplemented with phosphate buffer pH 7.5 and NaCl, at a final concentration of 20 and 300 mM respectively, and loaded on a 1 ml HisTrap Excel column (GE healthcare). A multi-step gradient, increasing the concentration of imidazole, was used to wash and elute the soluble TCR from the column. The eluted soluble TCR or CD277e was loaded on a 1 ml HiTrapQ column (GE healthcare) in 20 mM Tris pH 8.2 and 20 mM NaCl. A linear gradient was used to elute the soluble TCR or CD277e. Fractions containing the soluble TCR or CD277e were pooled and concentrated.

Tetramers and dextramers were prepared as described previously[6]. Briefly, tetramers were prepared from monomers by adding one equivalent of SA-PE (1

μM) to six equivalents of sTCR/CD277e (6 μM) in four steps over 20 min. Dextramers were prepared by preincubating SA-PE and sTCR in a molar ratio 1:3 for 15 min, and then doping the formed trimers with biotinylated dextran (MW 500 kDa, NanoCS) at a molar ratio 1:8 (dextran:SA-PE).
For bead preparation, the biotinylated soluble TCRs were mixed with streptavidin conjugated fluorescent Yellow-Green microspheres (6 μm; Polysciences, Inc.) or 5-7μm streptavidin-coated Purple beads (Spherotech) in excess, to ensure fully coated beads, 10 μg sTCR/mg microspheres.
For CD277e bead preparation ~5-7 μm streptavidin coated UV- or Purple beads (Spherotech) were used. Biotinylated CD277e was mixed in excess to ensure fully coated beads.

sTCR/CD277e multimer-cell conjugation assays
For tetramer staining, $1.0*10^5$ cells were incubated with 30 ul tetramer solution (3 μg/ml streptavidin) for 30 minutes at RT. Cells were incubated with 100 μM pamidronate (+PAM conditions) for 2 h before staining. For bead staining, $7.5*10^4$ cells were incubated with 20 μl sTCR-YG-beads/CD277e UV beads (0.33 mg beads/ml) for 30 minutes at RT. Cells were subsequently stained with fixable viability dye eFluor780 (eBioscience) for an additional 30 minutes. The mixtures were fixed by adding 40 μl 2% formaldehyde for 15 minutes. Samples were washed once with 1% formaldehyde and analyzed on a BD FACSCanto II (BD).

For the inhibition of bead staining sTCR or CD277e Purple beads were incubated for 15 minutes with 50 μg/ml of the indicated antibody, at a concentration of 0.33 mg beads/ml. After the incubation, the antibody bead mixture was used to stain cells as above, instead of fixable viability dye eFluor780, LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher Cat#L34957) was used. Antibodies used in bead blocking assay: IgG1,κ isotype (clone: MOPC-21; ab18437, AbCam), anti-γδTCR (clone B1; 331204, Biolegend), anti-CD277 (clones: 20.1 and 103.2).

Co-immunoprecipitation and Western Blotting
HEK293T-CD277ko cells were seeded at $4*10^6$ cells per T75 flasks, co-transfected the following day with 15 μg of FLAG-BTN3A1 and 15 μg of HA-BTN2A1 using FuGENE HD transfection reagent (Promega). 36 h post-transfection the cells were left untreated or treated with 100 μM pamidronate for 16 h. Subsequently the cells were collected and lysed for 30 minutes on ice in 50 mM HEPES pH 7.5, 150 mM NaCl, 1% n-Dodecyl β-D-maltoside, 10 μM MG132, Complete mini EDTA-free protease inhibitor cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche). The lysates were cleared at 20000 g for 30 minutes and quantified using the BCA assay (Thermo Fisher Scientific), and normalized across the conditions. A fraction of the lysates was saved as input and the immunoprecipitation was performed overnight at 4°C after addition of 15 μL of Anti-FLAG M2 agarose gel (Merck). The FLAG beads were washed three times in lysis buffer and the proteins eluted after supplementing with 1XSDS sample buffer and boiling. Each sample

was loaded and run onto a Criterion XT 4-12% gel (Biorad). The proteins were then transferred onto nitrocellulose, the membrane stained with Ponceau S to assess loading, then blocked for 1 hour with 5% BSA in TBS supplemented with 0.1% Tween 20 and the antibody staining performed according to the manufacturer's instructions. Each blot shown is representative of three independent experiments. The following primary antibodies were used in this study: anti-FLAG M2 (Merck), anti-HA (#H9658, Merck).

Mass spectrometry analysis – sample preparation and data analysis

Cell pellets from three independent biological samples, each corresponding to 10 million of HEK293T-CD277ko stably expressing FLAG-BTN3A1 untreated, or treated with 100 µM pamidronate for 16 hours were processed as described in the section above, and the proteins were eluted after immunoprecipitation using 3X FLAG peptide (Merck). The eluted proteins were subjected to SDS-Page until the front migrated through the gel for 1.5 cm. The gel was fixed and stained with Imperial Protein Stain (Thermo Fisher Scientific), and one band per sample was excised and subjected to in gel digestion using Trypsin (Promega). The eluted peptides were analyzed by LC-MS/MS using an Agilent 1290 Infinity System (Agilent Technologies), coupled with an Orbitrap Q-Exactive HF-X Mass Spectrometer (ThermoFisher Scientific). The peptides were then separated through reverse phase chromatography using a 100-µm inner diameter 2-cm trap column (prepared in house with ReproSil-Pur C18-AQ, 3 µm) and coupled to a 75-µm inner diameter 50 cm analytical column (prepared in house with Poroshell 120 EC-C18, 2.7 µm) (Agilent Technologies). The mass spectrometer was operated in the data-dependent acquisition mode with the following settings: Full scan MS spectra were acquired from 375-1,600 m/z at a resolution of 60000 with an automatic gain control (AGC) of 3e6. The 15 most intense precursor ions were selected for fragmentation using HCD. MS/MS spectra were acquired at a 30000 resolution with an AGC target of 1e5.

The RAW MS files were searched with the MaxQuant software (version 1.6.3.4) [7] using the standard settings, and with methionine oxidation and phospho (STY) as variable modifications, the "match between runs" option and the label-free quantification (LFQ) as the algorithm. The MS/MS spectra were searched with the Andromeda search engine against the Uniprot human protein database from April 2019. The protein and peptide spectrum matches FDR were set to 1%.

The MaxQuant output was then analyzed using the Perseus software (version 1.6.2.3) [8]. The LFQ values were log2 transformed and the matrix filtered for proteins showing 3 valid values in at least one condition (PAM treated or untreated). Missing values were then imputed using the standard Perseus settings. To identify the significantly enriched proteins, we set s0=1 and performed the Student's t test followed by a Permutation-based FDR multiple testing correction with a 1% FDR. The Gene ontology (GO) enrichment analysis was performed with the significantly enriched proteins using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8) [9], and using as background a list of all the proteins identified in the immunoprecipitation experiments, excluding the contaminants. The AP-MS data have been deposited

to the ProteomeXchange Consortium via the PRIDE artner repository with the dataset identifier PXD018055 [10].

Degranulation assays with CD277 blocking antibody

K562 cells were counted, resuspended at $4.5*10^6$ cells/mL and pre-incubated in cell culture medium, with 10 µg/mL of CD277 blocking antibody (clone 267-21f8.19), or a control antibody (clone MG2a-53, Biolegend). After 45 minutes, half of the cells were washed once in cell culture medium, and for each well 225000 target cells we mixed with 75000 endogenous Vδ2+ bulk T cells or TEG001/LM1 in the presence of anti-CD107a-PE antibody (clone H4A3, BD Biosciences) and 100 µM pamidronate. The samples were incubated 2 h in the incubator and subsequently supplemented with GolgiStop (BD Biosciences), according to the manufacturer's instructions, incubated for 4h and stained with an anti-Vδ2 antibody (clone B6, Biolegend) before FACS analysis.

Blocking of vesicle transport in tumor cells

HEK293T cells were trypsinised, washed in complete DMEM medium and transferred into 15ml tubes. Cells were treated with either 4mM monensin (BD Bioscience) or 4mM Brefeldin A (BD Bioscience), plated 50,000 cells in 96 well plates and treated for 12 hours in CO2 incubator. After pre-treatment, plates were centrifuged at 300g for 3 minutes, the medium was removed, and cells were washed 2 times with PBS. 50,000 TEGs were added to the wells and co-culture was incubated overnight in the presence of 100µM PAM. Next day, supernatant was harvested and analysed using Human INFγ ELISA Ready SET Go Kit (Affymetrix).

The CD277 stain on K562 was performed, keeping an anti-CD277-AF647 antibody (R&D, cat# FAB7136R) present throughout the treatment time (5h).

Cell-cell conjugation assays and γδTCR enrichment to the immunological synapse

HEK293FT cells were seeded in a µ-slide poly-L-lysine pre-coated chamber (Ibidi) to adhere overnight. Next day cells were treated with 100µM PAM for one hour at 37°C. TEGs were added onto HEK293FT cells and incubated at 37°C for 30-120 minutes. After co-incubation, chambers were immediately placed onto ice and fixed with 4% PFA for 30 minutes. Samples were blocked with 1% BSA/FCS and labeled with CD3ε-Alexa Fluor 647 (BD Biosciences) at 2µg/mL for two hours at room temperature, and finally fixed with 1% PFA+DAPI. Samples were imaged with Zeiss LSM-710 and analyzed with Volocity software (PerkinElmer). In order to quantify the number of conjugates between HEK293FT and TEG cells, we calculated the ratio between CD3ε positive objects and DAPI positive objects on each image taken. To analyze γ delta TCR enrichment into the immunological synapse, we calculated the ratio of CD3ε signal enrichment inside versus outside of the contact area of the target and effector cell on 63x magnified images similarly as it was described earlier[11].

Confocal imaging of BNT3A1-EmGFP molecules

Human HEK293FT cells stably expressing BTN3A1-EmGFP (HEK293FT-BTN3A1-EmGFP) were plated at low confluence on 14mm-diameter coverslips treated with human fibronectin, in 24 well plates. HEK cells were pre-treated with either 20µM PAM, 10µg/mL anti-CD277 mAbs (agonist #20.1 or antagonist #103.2) for 4h. For imaging assays, HEK cells were stained with Hoescht dye for 5 minutes, washed with PBS and then fixed with 4% PFA for 10 minutes at RT. Coverslips were mounted on microscope slides in Prolong®Diamond (Molecular Probes, Waltham, MA, USA). Imaging was performed using a Nikon A1 RS confocal microscope (60xNA 1.40 oil immersion objective). The analysis was performed with Fiji software (National Institutes of Health) using EmGFP positive object threshold and area measurement. Fluorescent events on the cell membrane were classified and counted according to their size.

Super-resolution imaging

HEK293FT cells were plated in eight-well Lab-Tek chambers to adhere and treated with 100uM pamidronate overnight. HEK293FT cells stably expressing HA-EGFR (Valley MBoC 2015) were plated overnight, were washed with PBS and then treated with 50 nM EGF for 8 min at room temperature to ensure dimerization of EGFR on the basal surface of the cell [12]. Cells were washed with PBS and immediately fixed with 4% paraformaldehyde for 2 hours. Finally, cells were washed two times with PBS and once with 10 mM Tris-HCl (pH 7.2) and stored in PBS until imaging.For super-resolution imaging, cells were washed with PBS and fixed with 4% PFA and 0.2% gluteraldehyde for 1-2 h, washed extensively with PBS and then labeled with 5µg/ml AF647-CD277 antibody clone 849203 (Novus Biologicals) at RT. Prior to super-resolution imaging, 200 µL of fresh SRB (Super-resolution buffer: 50 mM Tris, 10 mM NaCl, 10% glucose, 168.8 U/mL glucose oxidase, 1404 U/mL catalase, 10 mM cysteamine hydrochloride, pH 8.0) was added to the well.

dSTORM imaging was performed using a custom-built microscope controlled by custom-written software in MATLAB (MathWorks Inc.). A 642 nm Laser (80 mW-HL6366DG-Thorlabs) passed through 640/8 nm (LD01-640/8-12.5-Semrock) laser diode clean up filter was used as the excitation source. The objective was NA=1.49 oil immersion lens (APON 60XOTIRF-Olympus). Emission light passed through a band pass filter 708/75 nm (FF01-708/75-25-Semrock) and was collected with a sCMOS camera (C11440-22CU-Hamamatsu). An active stabilization system[13] was used to prevent drift during data collection and consisted of an 850 nm LED (M850L3, Thorlabs) illumination lamp, 835/70 nm (FF01-835/70-25-Semrock) emission filter, a camera (DMK 31AU03-imagingsource) and 3D piezo sample stage (MAX341/M, Thorlabs). Imaging was performed in a standard dSTORM imaging buffer with an enzymatic oxygen scavenging system and primary thiol: 50 mM Tris, 10 mM NaCl, 10% w/v glucose, 168.8 U/ml glucose oxidase (Sigma #G2133), 1404 U/ml catalase (Sigma #C9322), and 60 mM 2-aminoethanethiol (MEA), pH 8.0. Data was collected using a custom-built microscope consisting of a 647 nm laser excitation source (500 mW 2RU-VFL-P; MPB Communications Inc.), an sCMOS camera (C11440-22CU; Hamamatsu Photonics) a 1.35 NA silicon oil immersion objective (Olympus UPLSAPO100XS) and a 708/75-nm

emission filter (FF01-708/75-25; Semrock). A total of 60,000 frames were collected for each cell.

Super-resolution image reconstruction and data analysis

dSTORM images were analyzed and reconstructed with custom-built MATLAB functions as described previously[14,15]. For each image frame, subregions were selected based on local maximum intensity. Each subregion was then fitted to a finite pixel Gaussian intensity distribution using a maximum likelihood estimator. Fitted results were rejected based on log-likelihood ratio and the fit precision, which was estimated using the Cramér–Rao lower bound values for each parameter, as well as intensity and background cut-offs.

Analysis of dSTORM CD277 cluster data was performed using the H-SET algorithm. H-SET (Hierarchical Single Emitter Hypothesis Test) is a top-down hierarchical clustering algorithm implemented in MATLAB, that collapses clusters of observations of blinking fluorophores into single estimates of their true locations[16]. Briefly, for a cluster of observations to be collapsed into a single localization, a hypothesis test is performed with the null hypothesis that all observations come from the same fluorophore. The null hypothesis is not rejected if the p-value, calculated using a log-likelihood ratio statistic, is larger than a specified level of significance (0.01 was used here). H-SET analysis code is available as part of a package of local clustering tools (http://stmc.unm.edu). Hopkins' statistics (H) and localization nearest neighbor distances (NND) were collected for each region of interest (ROI), approximately 275 ROIs were chosen from over 10 cells per each condition. The Hopkins' statistic is defined in the interval [0, 1], with a value of 0.5 indicating that the localizations were randomly distributed in the ROI, while values near 1 indicate clustering[17]. The NND in each ROI were computed with locally written MATLAB software using “knnsearch”, producing the distance from each localization to its nearest neighbor, and so inferring an average localization density.

Cluster Analysis: Approximately 20 cells per condition (resting, PAM or mAb 20.1 treatments) were analyzed. Clustering was performed using a variation of H-SET (Hierarchical Single Emitter Hypothesis Test), a top-down hierarchical clustering algorithm implemented in MATLAB that collapses clusters of observations of blinking fluorophores into single estimates of their true locations (localizations) [Lin et al., 2016]. In this variation, no collapsing occurred; the clusters of observations were used directly for analysis. We chose a level of significance of 0.01 for the log-likelihood hypothesis test, set the minimum number of observations allowed to form a cluster to 3, and used sigma =10 nm (see Eq. 2 in [Lin et al., 2017]) in order to accommodate the potential of multiple proteins in small clusters.

Simulations: To assess the effect of small clusters on our results, we ran a series of simulations parameterized by the fraction of dimers (f = 0.01, 0.02, 0.05, 0.10, 0.20, 0.50). The simulations approximated the data in the distributions of localizations per protein and localization precision and density. The location of the proteins or dimers were randomly chosen using a homogenous spatial Poisson random distribution. Dimers were generated for a fraction *f* of the random

coordinates by introducing a new particle a random distance between 0 and 10 nm from the original coordinates. We then ran H-SET clustering on the ROIs as described above, averaging over 10 simulations per value of *f*.

Videomicroscopy

HEK293FT-BTN3A1-EmGFP cells were incubated in Ibidi chamber slides (Ibidi GmbH, Martinsried, Germany) coated with fibronectin (Merck, Darmstadt, Germany) overnight. Target cells were left to adhere on glass slides for 30 min at 37°C before addition of washed Vg9Vd2 T lymphocytes. Recording was performed using a DMI 6000B microscope (Leica Microsystems, Wetzlar, Germany). The analysis of BTN3A1-EmGFP polarization expressed by HEK293FT cells was performed using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). Two sections were manually selected and placed to equally divide HEK293FT cells, in parallel to the point of contact with Vg9Vd2 T lymphocytes. Threshold areas were generated automatically by the analysis software. BTN3A1-EmGFP fluorescence intensity was next measured and calculated into these two defined areas every 5 minutes, at indicated time points.

Statistical analysis

Correlation analysis for non-normally distributed data was performed using Spearman rank correlation. Pairwise comparisons were performed using Student's T-Test for normally distributed data, and Mann-Whitney test for non-normally distributed data. To assess differences between multiple groups, we used two-way ANOVA (correcting for the systematic effect of the assay date by including it into the linear model) with Tukey's post-hoc tests. Data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc), IBM SPSS statistics (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.), and R Software (version 3.5.3, R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

References

1. Marcu-Malina V, Heijhuurs S, van BM, et al. Redirecting alphabeta T cells against cancer cells by transfer of a broadly tumor-reactive gammadeltaT-cell receptor. *Blood.* 2011;118(1):50-59.
2. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol.* 2003;10(1):133-139.
3. Voss RH, Kuball J, Theobald M. Designing TCR for Cancer Immunotherapy. *Methods MolMed.* 2005;109:229-256.
4. Bolotin DA, Shugay M, Mamedov IZ, et al. MiTCR: software for T-cell receptor sequencing data analysis. *Nature methods.* 2013;10(9):813-814.
5. Allison TJ, Winter CC, Fournie JJ, Bonneville M, Garboczi DN. Structure of a human gammadelta T-cell antigen receptor. *Nature.* 2001;411(6839):820-824.

6. Bethune MT, Comin-Anduix B, Hwang Fu YH, Ribas A, Baltimore D. Preparation of peptide-MHC and T-cell receptor dextramers by biotinylated dextran doping. *Biotechniques.* 2017;62(3):123-130.
7. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol.* 2008;26(12):1367-1372.
8. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods.* 2016;13(9):731-740.
9. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;4(1):44-57.
10. Perez-Riverol Y, Csordas A, Bai J, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 2019;47(D1):D442-D450.
11. Roszik J, Sebestyen Z, Govers C, et al. T-cell synapse formation depends on antigen recognition but not CD3 interaction: studies with TCR:zeta, a candidate transgene for TCR gene therapy. *Eur J Immunol.* 2011;41(5):1288-1297.
12. Valley CC, Arndt-Jovin DJ, Karedla N, et al. Enhanced dimerization drives ligand-independent activity of mutant epidermal growth factor receptor in lung cancer. *Mol Biol Cell.* 2015;26(22):4087-4099.
13. McGorty R, Kamiyama D, Huang B. Active Microscope Stabilization in Three Dimensions Using Image Correlation. *Opt Nanoscopy.* 2013;2(1).
14. Huang F, Schwartz SL, Byars JM, Lidke KA. Simultaneous multiple-emitter fitting for single molecule super-resolution imaging. *Biomed Opt Express.* 2011;2(5):1377-1393.
15. Smith CS, Joseph N, Rieger B, Lidke KA. Fast, single-molecule localization that achieves theoretically minimum uncertainty. *Nature Methods.* 2010;7(5):373-U352.
16. Lin J, Wester MJ, Graus MS, Lidke KA, Neumann AK. Nanoscopic cell-wall architecture of an immunogenic ligand in Candida albicans during antifungal drug treatment. *Mol Biol Cell.* 2016;27(6):1002-1014.
17. Zhang J, Leiderman K, Pfeiffer JR, Wilson BS, Oliver JM, Steinberg SL. Characterizing the topography of membrane receptors and signaling molecules from spatial patterns obtained using nanometer-scale electron-dense probes and electron microscopy. *Micron.* 2006;37(1):14-34.