Harly et *al.*

Supplemental data

Supplemental Materials and Methods

Expression of full-length, truncated and chimeric BTN3 proteins. Full-length cDNAs for human BTN3A1 (LIFESEQ3294566), BTN3A2 (LIFESEQ6701037), and BTN3A3 (BC015815) were from Open Biosystems and subcloned in pEGFP-1 (Clontech), or pSP72 (Promega). 5'-phosphorylated oligonucleotides 5'-TCTGCTACTTTCAAGATGGTGACTTCTATGA-3' and 5'-GATATTTGCCACTGTCAGAGGCTGTGACGT-3' were used for PCR mutagenesis of the cDNAs to mutate the whole sequence targeted by shRNA#284 (mutation of 5'-GGAAAGTACTTGTGTTATTTC-3' to 5'-GGCAAATATCTCTGCTACTTT-3'). Mutated cDNAs were subcloned in a modified pIRES1hyg (Clontech). Following hygromycin selection, stable transfectants were checked for the selective re-expression of mutated BTN3A1, BTN3A2 or BTN3A3 by flow cytometry. Carboxy-terminus EmGFP- or mCherry-tagged BTN3A1 and BTN3A2 molecules were obtained by subcloning full-length cDNAs in frame with the sequences encoding for the fluorescent tag from, respectively, the pcDNA6.2/C-EmGFP-GW/TOPO (Invitrogen) or the pmCherry-N1 (Clontech) vectors. When specified, helix-forming peptide linkers A(EAAAK)4A were introduced in frame to separate BTN3 and fluorescent domains.33 Plasmids were used for transient or stable transfections in either CD277 kd HEK293FT cells (sh#284; clone#30) or wild-type HEK293 cells. For truncated BTN3A1 proteins lacking the B30.2 intracellular domain, the following oligonucleotides: 5'-ACCATGAAAATGGCAAGTTTCCTGGCC-3' and 5'–GATGGGGTTTGCTGTTTTTG-3' were used for PCR. Chimeric BTN3A3-BTN3A1 B30.2 proteins were generated by using the oligonucleotides 5'-CGCAGGCTTGAAGAGGGCCATTTTCCAT-3' and 5'-TGAAGAGGGCCATTTTCCATTCATGATAGGCCA-3'.

Knockdown of BTN3 isoforms in HEK293FT cells. Short hairpin RNA (shRNA) sequences (sh#240, sh#284) were designed for targeting fully conserved RNA sequences encoding for the extracellular region of BTN3A1, BTN3A2 and BTN3A3 isoforms. Oligonucleotides 5'-ACCGGAAGGCTGCTCTCCGAATACCGAAGTATTCGGAGAGCAGCCTTCCTTTTTG-3', 5'-TCGACAAAAAGGAAGGCTGCTCTCCAA TACTTCGGTATTCGGAGAGCA

GCCTTC-3' (sh#240); 5'-ACCGGAAAGTACTTGTGTT ATTTCCGAAGAAATAACAC

AAGTACTTTCCTTTTTG-3, 5'-TCGACAAAAAGGAAA GTACTTGTGTTATTTCTT

CGGAAATAACACAAGTACTTTC-3' (sh#284); and irrelevant sequence (shControl) were annealed and cloned downstream from the human U6 snRNA gene promoter before introduction of the sequence-verified U6-shRNA cassette into the lentiviral vector FG12.34 Lentiviral particles were produced by transient transfection of HEK293FT cells. Virus particules contained in cell supernatants were next concentrated by ultracentrifugation, titrated, and used to infect HEK293FT target cells at a multiplicity of infection of 10. The expression of CD277 in transduced cells was checked by flow cytometry and cells were sorted using a FACSAriaIII cell sorter (BD Biosciences), then cloned by limiting dilution.

Cell division. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Invitrogen. Freshly isolated PBMCs (2 x 10^6 cells) were labeled with CFSE (1.5 μM in PBS) for 10 min at 37°C, washed and maintained for 15 min at 37°C in complete medium to allow the release of dye excess. Labeled cells were activated, in the presence of IL-2 (300 IU/ml). After 4 days, cells were harvested, stained for γδ TCR surface expression and analyzed by flow cytometry. Peaks of cell division and frequencies were calculated by using the FlowJo analysis software.

Antibody-dependent cell cytotoxicity. Anti-human CD20 mAb (Rituximab) and human αβ $CD8^{pos}$ T cells (CMVpp65/A2) expressing or not CD16 FcγRIII lentiviral transduction, were kindly provided by B. Clémenceau and H. Vié (INSERM UMR892).

Quantification of endogenous PAg. The detection and quantification of endogenous IPP and ApppI was performed as described (Monkkonen et al., 2007).

Microscopy

HEK293FT cells (1x10^5) expressing carboxy-terminus Emerald GFP (EmGFP)-tagged CD277 molecules were laid on BD Cell-Tak pre-coated slides (BD Biosciences). Vγ9Vδ2 T cells (1x10^5) were added and left to conjugate to HEK293FT cells for 30 min at 37°C, pretreated or not with anti-CD277 mAb. After addition of primary anti-CD3 mAb (#OKT3, 10 μg/ml) and incubation for 10 min at 37°C, paraformaldehyde-fixed samples were stained with Alexa 568-labeled anti-mouse IgG2a (2 μg/ml) for 10 min at 37°C and washed. The slides were mounted by using Fluoromount reagent (Southern Biotechnology) and analyzed using a Nikon A1 RS confocal microscope (60xNA 1.40 oil immersion objective). Images were analyzed with Metamorph 7.5 (Molecular Devices, Universal Imaging) and NIS (Nikon) imaging softwares. Measurement of the intracellular Ca^{2+} levels were performed within T cells loaded with 1 μM Fura-2 AM (Molecular Probes) as described (Nedellec et al., 2010). For FRAP analysis, HEK293 cells expressing either EmGFP or mCherry-fused CD277 were laid on μ-slides (Ibidi) and analyzed using a Nikon A1 RS confocal microscope (60xNA 1.40 oil immersion objective). Selected rectangular areas were photobleached for 500 ms by using full power of laser intensity (> 90% of loss of fluorescence). Images were collected every 5 s, before (30 s) and after (120 s) bleaching using low laser intensity. The resulting curves were fitted using one-phase exponential equations.

Supplemental reference

Monkkonen H., Ottewell P.D., Kuokkanen J., Monkkonen J., Auriola S., Holen I. Zoledronic acid-induced IPP/ApppI production in vivo. *Life Sci.* 2007; 81(13):1066-1070.

Nedellec S, Sabourin C, Bonneville M, Scotet E. NKG2D costimulates human Vgamma9Vdelta2 T cell antitumor cytotoxicity through protein kinase Ctheta-dependent modulation of early TCR-induced calcium and transduction signals. *J Immunol*. 2010;185(1):55-63.

SUPPLEMENTAL FIGURES

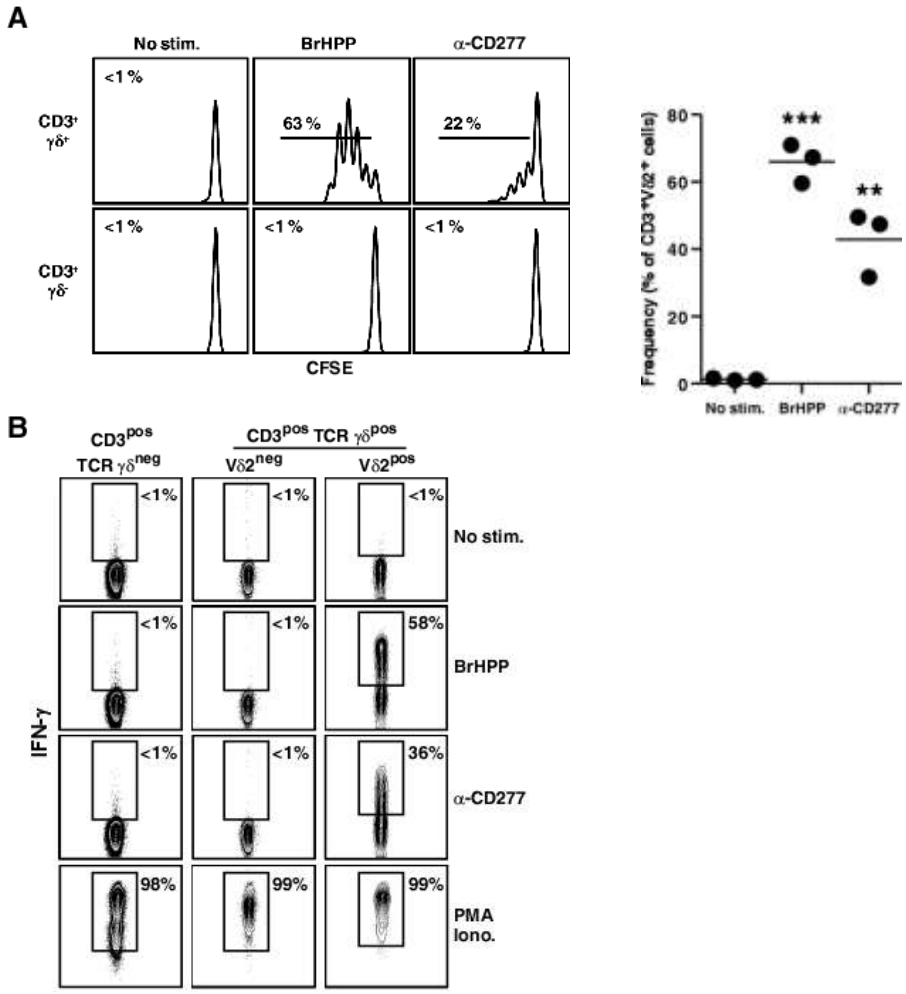

Figure S1. Anti-CD277 mAbs induce activation of ex vivo human Vγ9Vδ2 T cells. (A) *Left*, CFSE dilution measured within fresh ex vivo human PBMC-CD3^{pos} TCR γδ^{pos} or TCR γδ^{neg} T cell subsets at day 4 following an initial stimulation with either PAg (BrHPP; 3 μM) or anti-CD277 mAb (#20.1; 10 μg/ml) in the presence of recombinant human IL-2. The values for the percentage of divided T cells are indicated. *Right*, frequencies of CD3^{pos}Vδ2^{pos} cells within fresh human PBMCs (n=3 healthy donors) at day 10 following an initial stimulation with either PAg (BrHPP; 3 μM) or anti-CD277 mAb (#20.1; 10 μg/ml) in the presence of recombinant human IL-2. Bar, mean value; ** $p < 0.005$, *** $p < 0.0005$. (B) Intracellular staining of IFN-γ in freshly isolated CD3^{pos} PBL subsets after treament for 5 h with anti-CD277 mAb (#20.1; 10 μg/ml), soluble PAg (BrHPP; 3 μM) or PMA and ionomycin (PMA/Iono.). Numbers adjacent to outlined areas indicate the percentage of IFN-γ^{pos} T cells. The data presented in this panel are representative of independent experiments performed with PBL from distinct healthy donors (n>3).

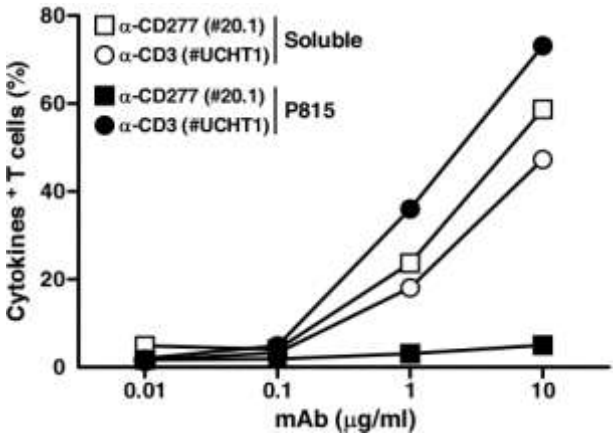

Figure S2. Lack of Vγ9Vδ2 T cell activation by anti-CD277 mAbs, when immobilized on FcRI^{pos} murine P815 cells. Cytokine response of human Vγ9Vδ2 T cells (clone GR4) was measured following coculture with murine P815 cells, loaded with grading doses of either anti-CD277 (#20.1, mouse IgG1) or anti-CD3 (#UCHT1, mouse IgG1) mAbs (filled symbols). Controls: no preloading of P815 cells, cocultures performed in the presence of soluble mAbs (open symbols). Intracellular stainings of TNF-α and IFN-γ were performed and analyzed by flow cytometry. Data are presented in the graph as the percentage of IFN-γ^{pos} and TNF-α^{pos} γδ T cells and are representative of at least 3 independent experiments.

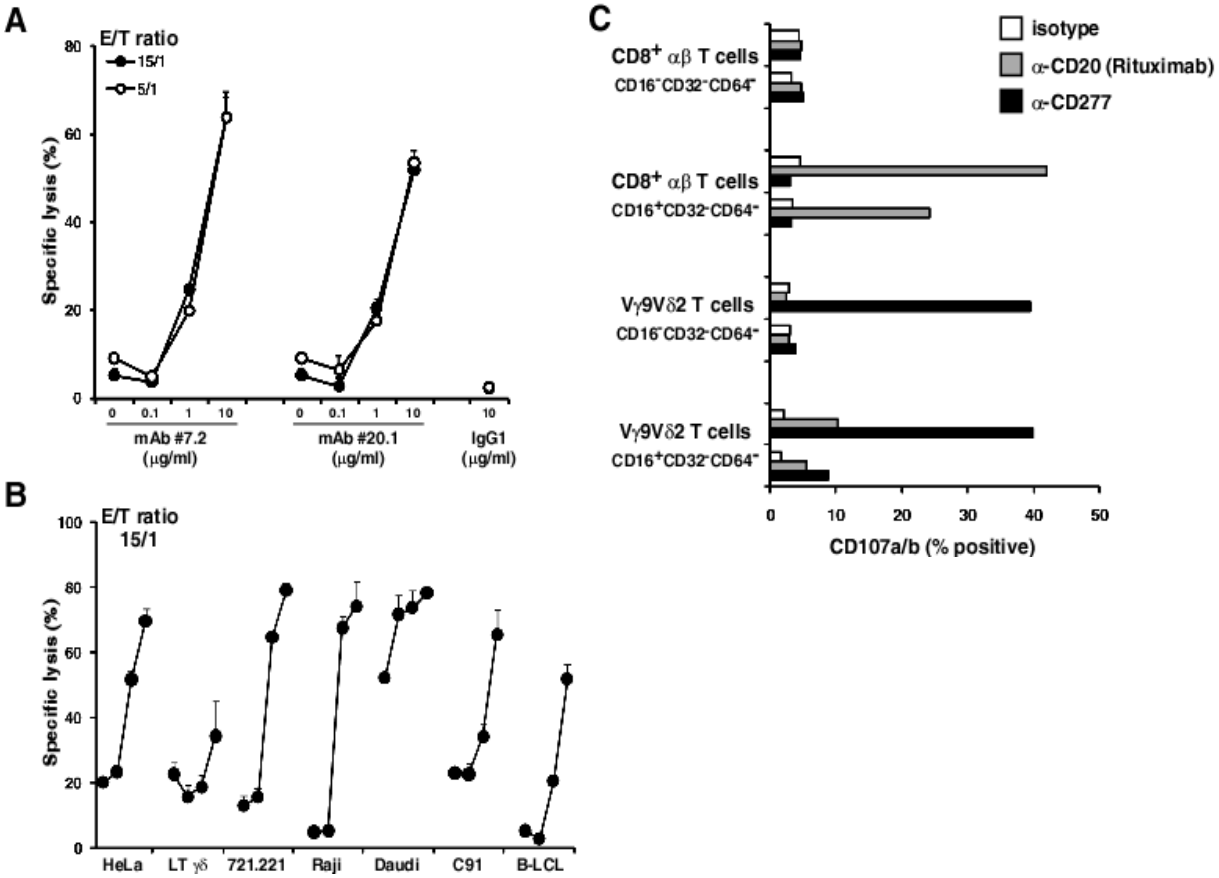

Figure S3. Anti-CD277 mAbs trigger cytolytic activity of Vγ9Vδ2 T cells against various human target cells. (A) Cytolytic responses of human Vγ9Vδ2 T cells (clone GR4) against Raji target cells (effector to target ratios: 15/1 and 5/1), pretreated for 2 h with grading doses of two clones of anti-CD277 mAbs (#7.2 and #20.1). IgG1, isotype control. (B) Cytolytic responses of Vγ9Vδ2 T cells (clone GR4) against various human target cells (effector to target ratios: 15/1), pretreated for 2 h by grading doses (0, 0.1, 1 and 10 μg/ml) of anti-CD277 mAb (#7.2). Data are presented as the mean value ± s.d. of triplicate samples. (C) Expression of CD107a/b on human αβ $CD8^{pos}$ and Vγ9Vδ2 T cells, expressing or not FcγR molecules following coculture with human B-LCL ($CD20^{pos}CD277^{pos}$) pretreated for 2 h with either anti-CD20 (Rituximab) or anti-CD277 (#20.1) mAbs. The surface expression of CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI) molecules on effector αβ (pp65 CMV/HLA-A2-specific line αβ $CD8^{pos}$ T cell transduced, or not, for CD16 expression) and γδ ($CD16^{neg}$, clone GR4; $CD16^{pos}$, line GUI) T cells was checked by flow cytometry. The values for the percentage of $CD107a/b^{pos}$ T cells are indicated and each antibody was used at 0.3 μg/ml and 10 μg/ml.

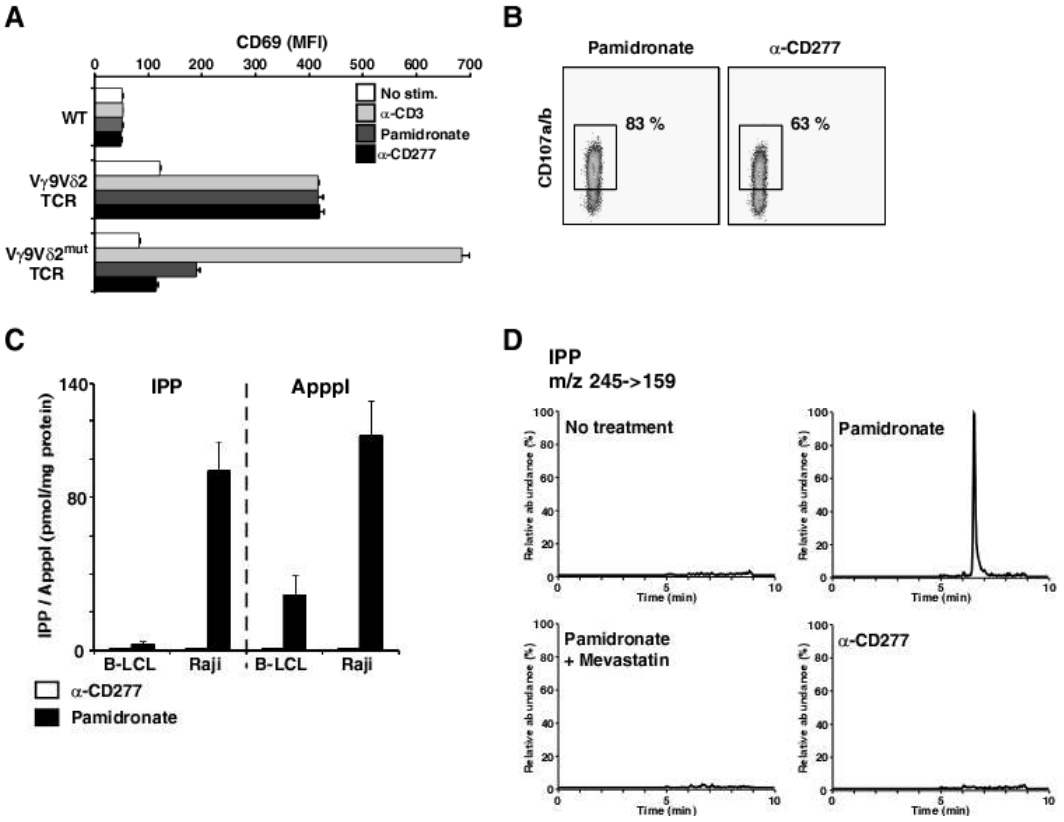

Figure S4. Anti-CD277 mAbs trigger a TCR-mediated activation process similar to PAg but do not induce upregulation of PAg production and/or accumulation in target cells. (A) Expression of CD69 on Vγ9Vδ2 TCR Jurkat T cell transductants following coculture with B-LCL target cells (line 721.221), pretreated for 2 h with anti-CD277 mAb (#20.1; 10 μg/ml) or NBP (Pamidronate, 250 μM). WT, control Jurkat T cells without TCR; Vγ9Vδ2 TCR, Jurkat T cells expressing a wild-type Vγ9Vδ2 TCR, Vγ9Vδ2^{mut} TCR, Jurkat cells expressing a Vγ9Vδ2 TCR carrying a mutation for the Vδ2 chain L97 residue. Anti-CD3 (#OKT3; 10 μg/ml). Data are presented as the mean value of geometric mean of fluorescence intensity (MFI) ± s.d. of triplicate samples and are representative of at least three independent experiments. (B) CD107a/b surface expression on Vγ9Vδ2 T cells following coculture with Raji target cells pretreated for 2 h with either NBP (Pamidronate, 250 μM) or anti-CD277 mAb (#20.1; 10 μg/ml). The values for the percentage of CD107a/b^{pos} T cells are indicated. (C) IPP and ApppI formation in human B-LCL or Raji cells treated overnight with NBP (Pamidronate, 250 μM) or for 5 h with anti-CD277 mAb (#20.1; 10 μg/ml). The molar amounts of IPP and ApppI were determined in cell extracts by using high performance liquid chromatography–electrospray tandem mass spectrometry (detection limit: 30 fmole). The molar amounts of IPP and ApppI in extracts prepared from untreated B-LCL or Raji cells were below the detection limit. Data are presented as the mean value ± s.d. (n>3). (D) Selective reaction monitoring chromatograms of extracts from Raji cells: untreated (-), treated overnight with NBP (Pamidronate, 250 μM), pretreated first with mevastatin (25 μM) for 6 h and next incubated overnight with NBP (Pamidronate, 250 μM), and treated for 5 h with anti-CD277 mAb (#20.1; 10 μg/ml). The chromatograms are drawn on the same scale.

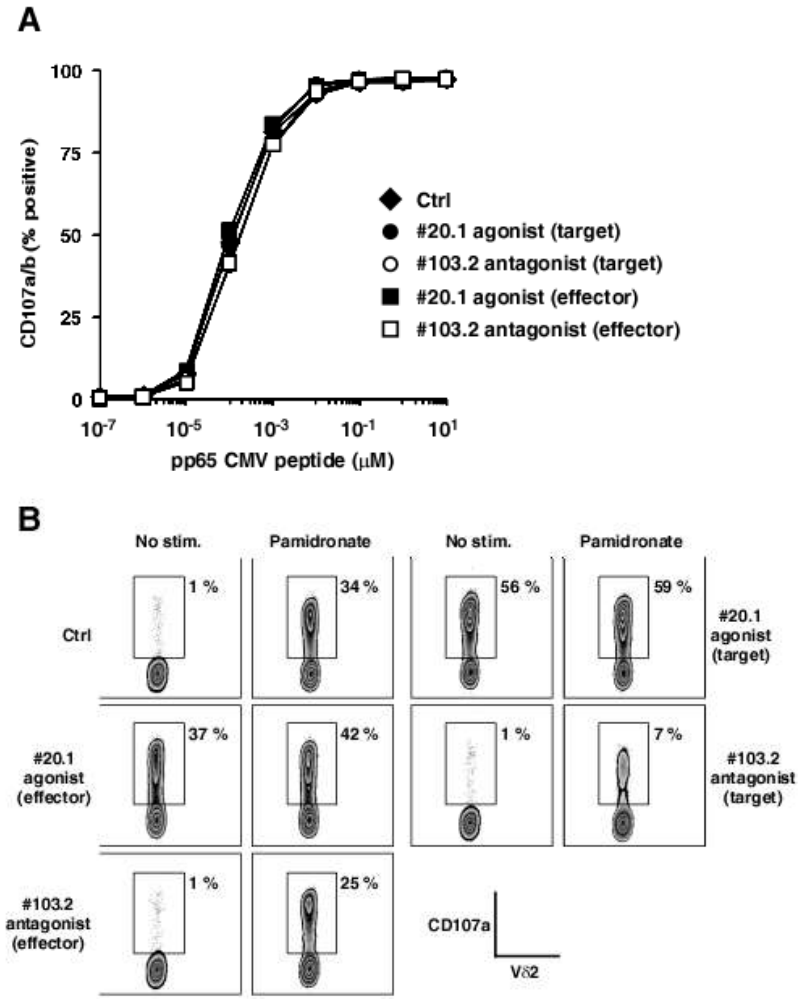

Figure S5. Anti-CD277 mAbs do not affect activation of CD8^{pos} αβ T cell clones by anti-CD3 mAb or a specific antigenic peptide. (A) CD107a expression on human αβ CD8^{pos} T cells (polyclonal line) following coculture with HLA-A2^{pos} human B-LCL (line HEN) cells loaded with increasing concentrations of relevant peptide (CMVpp65/HLA-A2). (B) CD107a expression on human Vγ9Vδ2 T cells (line GUI) following coculture with Raji target cells, pretreated with NBP (Pamidronate, 100 μM). In both (A) and (B) experiments, effector T or target cells were treated, or not (Ctrl), with either agonist (#20.1; 10 μg/ml) or antagonist (#103.2; 10 μg/ml) anti-CD277 mAbs. The values for the percentage of CD107a^{pos} Vδ2^{pos} T cells are indicated. The data presented in this figure are representative of 3 independent experiments.

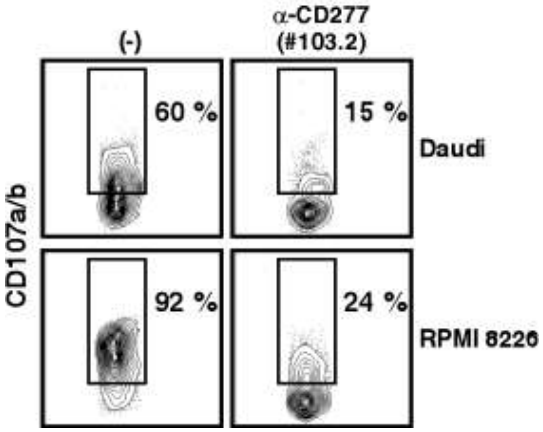

Figure S6. CD277 plays a key role in Vγ9Vδ2 T cell responses against susceptible tumor and infected human cells. Expression of CD107a/b on Vγ9Vδ2 T cells (clone GR4) following coculture with Daudi or RMPI 8226 tumor cells, in the presence or in the absence of #103.2 anti-CD277 mAb (10 μg/ml). Numbers adjacent to outlined areas indicate the percentage of CD107a/b^{pos} γδ T cells. The data presented in this figure are representative of >3 independent experiments performed with different human tumor cell lines.

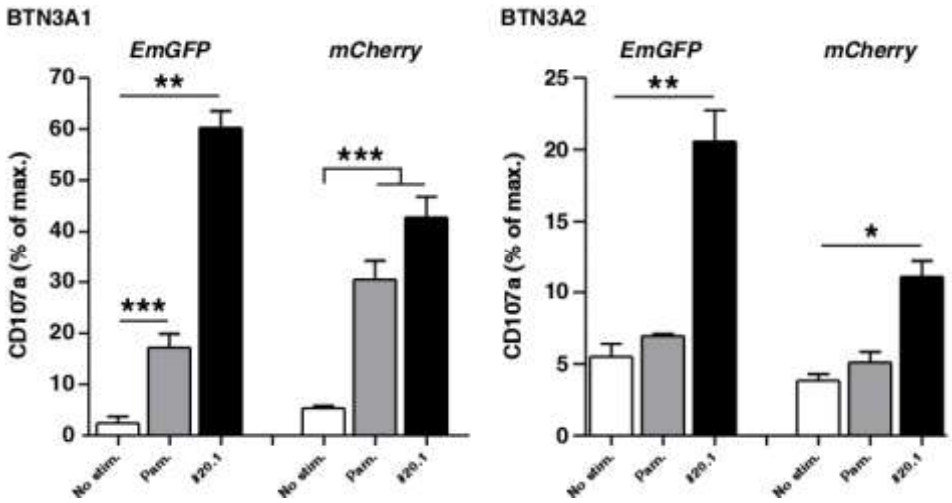

Figure S7. Functionality of fluorescent BTN3A1 and BTN3A2 chimeras. CD107a expression on human Vγ9Vδ2 T cells (polyclonal line) following coculture with shRNA#284 transduced-HEK293FT cells and transiently transfected for the expression of carboxyterminal EmGFP or mCherry-BTN3A1 (*left*) and -BTN3A2 (*right*) molecules. Cells were pretreated for 2 h with either anti-CD277 mAb (#20.1; 10 μg/ml) or NBP (Pam., Pamidronate, 250 μM). The values for the relative percentage of $CD107a^{pos}$ $V\delta2^{pos}$ T cells calculated by using the maximal response obtained, within each experimental test, by transfecting wild-type BTN3A1 molecules are indicated. * $p < 0.05$, ** $p < 0.005$ and **** $p < 0.0005$ (Student's *t*-test).

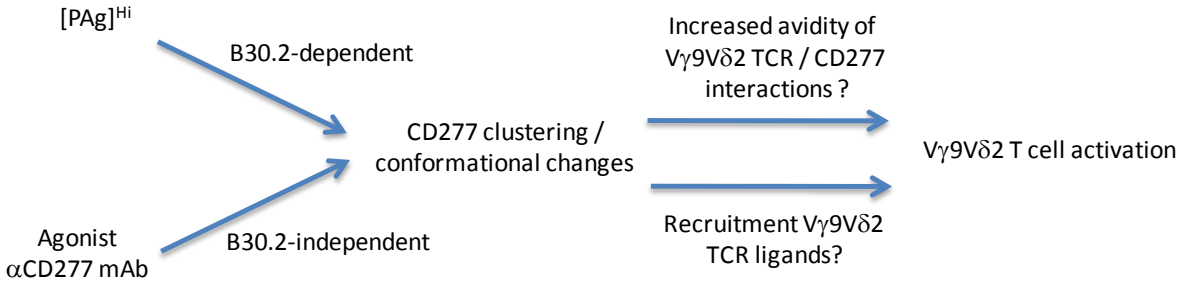

Figure S8. An hypothetical model for CD277-dependent activation of Vγ9Vδ2 T cells. Activation of Vγ9Vδ2 T cells requires prior conformational modification and/or clustering of CD277 molecules, that can be induced either by intracellular accumulation of PAg or by agonist anti-CD277 mAb. Generation of Vγ9Vδ2 T cell-stimulating CD277 complexes by PAg requires the B30.2 domain of BTN3A1, whereas generation of such complexes by agonist anti-CD277 mAb is B30.2 domain-independent. The topological changes of CD277 could either increase the affinity or avidity of Vγ9Vδ2 TCR/CD277 interactions, or recruit additional receptors (eg F1-ATPase) recognized by Vγ9Vδ2 TCRs, resulting in both cases to Vγ9Vδ2 T cell activation.