Published September 12, 2022; doi:10.4049/jimmunol.2200185

The Journal of Immunology

Cutting Edge: Bispecific γδ T Cell Engager Containing Heterodimeric BTN2A1 and BTN3A1 Promotes Targeted Activation of Vγ9Vδ2$^+$ T Cells in the Presence of Costimulation by CD28 or NKG2D

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Vγ9Vδ2$^+$ T cell–targeted immunotherapy is of interest to harness its MHC-independent cytotoxic potential against a variety of cancers. Recent studies have identified heterodimeric butyrophilin (BTN) 2A1 and BTN3A1 as the molecular entity providing "signal 1" to the Vγ9Vδ2 TCR, but "signal 2" costimulatory requirements remain unclear. Using a tumor cell–free assay, we demonstrated that a BTN2A1/3A1 heterodimeric fusion protein activated human Vγ9Vδ2$^+$ T cells, but only in the presence of costimulatory signal via CD28 or NK group 2 member D. Nonetheless, addition of a bispecific γδ T cell engager BTN2A1/3A1-Fc-CD19scFv alone enhanced granzyme B–mediated killing of human CD19$^+$ lymphoma cells when cocultured with Vγ9Vδ2$^+$ T cells, suggesting expression of costimulatory ligand(s) on tumor cells is sufficient to satisfy the "signal 2" requirement. These results highlight the parallels of signal 1 and signal 2 requirements in αβ and γδ T cell activation and demonstrate the utility of heterodimeric BTNs to promote targeted activation of γδ T cells. *The Journal of Immunology*, **2022, 209: 1–6.**

γδ T cells represent a minority subset (1–10%) of circulating T lymphocytes, but they play conserved roles in immune surveillance against microbial pathogens and malignant neoplasms (1). Overall, γδ T cells exhibit properties of both the innate and adaptive immune systems, and their transcriptional program overlaps with the profiles of CD8$^+$ T cells and NK cells (2). Target recognition by γδ T cells via NK receptors (NKRs), as well as TCR, has been demonstrated in a variety of experimental settings (3). In addition to their robust cytotoxic potential, the presence of tumor-infiltrating γδ T cells represents a strong favorable prognostic marker for overall survival in multiple solid and hematological cancer types (4), validating the development of γδ T cell–targeted therapies to promote antitumor immunity.

The majority of γδ T cells in the peripheral blood of humans express a TCR composed of Vγ9 and Vδ2 chains. In the context of antitumor immunity, Vγ9Vδ2$^+$ T cells respond to transformed cells by sensing elevated phosphorylated nonpeptide metabolites or phosphoantigens (pAgs), such as isopentenyl pyrophosphate, produced via the mevalonate pathway of cholesterol synthesis that becomes dysregulated in certain tumor cells (5). pAg sensing by Vγ9Vδ2$^+$ T cells is TCR dependent and requires engagement with B7-related membrane proteins butyrophilin (BTN) 2A1 and BTN3A1 on tumor cells. In tumor cells, pAg binding to BTN3A1 initiates a conformational change in its extracellular domain (ECD) (6), which facilitates association with BTN2A1 that can in turn engage with Vγ9Vδ2 TCR (7–9). Consistent with this model, treatment of tumor cells with agonistic anti-BTN3A1/CD277 enhances γδ T cell–mediated killing, but the enhanced effect is abrogated in the absence of BTN2A1 (10, 11).

Although the role of BTN2A1/3A1 in providing "signal 1" to engage Vγ9Vδ2 TCR is characterized, it remains unclear whether additional costimulation is required that parallels the signal 1 and signal 2 requirements of αβ T cell activation. T cell costimulatory receptors, including CD28, CD27, and 4-1BB, have been shown to synergize with TCR signaling in Vγ9Vδ2$^+$ T cells to promote effector function, proliferation, and survival (12–14). In addition, activating NKRs also have the potential to costimulate Vγ9Vδ2$^+$ T cells. NK group 2 member D (NKG2D) is constitutively expressed on γδ T cells and recognizes stress-induced ligands, including MHC class I–related molecules A or B and UL16-binding protein molecules on infected or transformed cells (15). Engagement of NKG2D

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Received for publication March 28, 2022. Accepted for publication August 13, 2022.

This work was supported by Shattuck Labs, Inc.

A.P., F.B., K.E., K.J., K.J.Y., and L.G. performed experiments. A.Y.L., G.F., K.W., T.H.S., and S.d.S. designed, executed, and interpreted experiments. A.Y.L. and S.d.S. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BTN, butyrophilin; DNAM-1, DNAX accessory molecule-1; J76, Jurkat 76; NKG2D, NK group 2 member D; NKR, NK receptor; pAg, phosphoantigen.

has been shown to amplify TCR signaling (16), as well as stimulate tumor-killing activity in TCR-dependent (17) and -independent (18, 19) manners in Vγ9Vδ2$^+$ T cells. Similarly, activation of DNAX accessory molecule-1 (DNAM-1), another activating NKR, has demonstrated involvement in Vγ9Vδ2$^+$ T cell–mediated cytotoxicity against acute myeloid leukemia and hepatocellular carcinoma cells (20, 21). The interplay and hierarchy between TCR and NKR in tumor target recognition and activation of Vγ9Vδ2$^+$ T cells remain incompletely understood, because loss-of-function studies indicate varying degrees of contribution from each component depending on the tumor cell line/type interrogated (22).

In this study, we generated a bispecific γδ T cell engager containing heterodimeric BTN2A1 and BTN3A1 ECDs fused via inert Fc linkers to scFv domains targeting tumor-antigen CD19 (BTN2A1/3A1-Fc-CD19scFv), to test its ability to activate Vγ9Vδ2$^+$ T cells and to promote targeted killing of B cell lymphoma cells in a pAg-independent manner. Our results showcase the feasibility of recombinant BTN2A1/3A1 heterodimers in promoting targeted activation of Vγ9Vδ2$^+$ T cells and demonstrate the requirement of a signal 2 via either a canonical T cell costimulatory receptor or NKR to fully activate BTN-mediated cytotoxicity in Vγ9Vδ2$^+$ T cells.

Materials and Methods

Cells

Daudi, Raji, and K562 were obtained from ATCC. Jurkat 76 (J76) was obtained from Dr. Heemskerk, Leiden University Medical Center (23). Human Vγ9Vδ2$^+$ T cells were expanded by zoledronate and cultured as previously described (24) from healthy donor leukopaks (StemCell Technologies).

Construct generation and protein production

The sequences for BTN2A1-Fc-CD19scFv and BTN3A1-Fc-CD19scFv were codon optimized and directionally cloned into mammalian expression vectors. Vectors were transiently cotransfected into CHOS cells or stably transfected into CHO cells, and the resulting heterodimeric fusion protein was purified using affinity chromatography. BTN2A1-Fc and BTN3A1-Fc homodimers were produced in a similar manner.

Cell-binding assays

10^5 Vγ9Vδ2$^+$ T cells or CD19$^+$ lymphoma cells were incubated with Fc receptor blocking reagent (BioLegend), followed by incubation with BTN fusion proteins for 1 h in serum-free media at 4°C. Cells were washed and incubated with allophycocyanin–anti-human Fc (Jackson ImmunoResearch) in Dulbecco's PBS containing 1% BSA, 0.02% sodium azide, and 2mM EDTA for 30 min at 4°C. Cells were washed before analysis by flow cytometry. EC_{50} was determined using built-in nonlinear regression analysis in GraphPad Prism.

In vitro Vγ9Vδ2$^+$ T cell activation

Recombinant BTNs or Abs were incubated overnight at 4°C in high-binding 96-well plates (Corning) before adding 10^5 Vγ9Vδ2$^+$ T cells or J76-Vγ9Vδ2 in the presence of CD107a–allophycocyanin, GolgiStop, and GolgiPlug (BD Biosciences) at concentrations specified by the manufacturer. Vγ9Vδ2$^+$ T cell cultures were incubated at 37°C for 4 h and stained for cell surface and intracellular markers for analysis by flow cytometry: anti-CD3ε (clone UCHT1), anti-Vγ9 (clone B3), anti–IFN-γ (clone 4S.B3), anti–TNF-α (clone Mab11), all purchased from BioLegend. J76-Vγ9Vδ2 cultures were incubated overnight for analysis of CD69 (clone FN50; BioLegend) expression by flow cytometry. Data analysis was performed using FlowJo v10.8.0.

Vγ9Vδ2$^+$ T and tumor cell coculture

A total of 10^5 target cells were prebound with BTN2A1/3A1-Fc-CD19scFv or anti-CD277 (clone 20.1; Thermo Fisher) for 30 min at 4°C. A total of 10^5 Vγ9Vδ2$^+$ T cells were added to target cells and cultured for 4 or 1 h for detection of apoptotic or granzyme B$^+$ tumor cells, respectively. Apoptracker Green (BioLegend) was used for detection of apoptotic tumor cells, and granzyme activity in tumor cells was analyzed using the GranToxiLux assay kit (OncoImmunin).

Statistical analysis

Graphing and statistical analysis were performed using GraphPad Prism. Unless noted otherwise, values plotted represent the mean triplicates, and error bars denote SD. Statistical significance (p value) was determined using unpaired Student t test. Significant p values are labeled with one or more asterisks, denoting $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Results and Discussion

Vγ9Vδ2$^+$ T cell activation by recombinant BTNs requires a costimulatory signal via NKR or T cell costimulatory receptor

The ability of recombinant BTNs to promote degranulation and cytokine production in Vγ9Vδ2$^+$ T cells was first assessed using homodimeric BTN2A1-Fc and/or BTN3A1-Fc chimera proteins. Although both anti-CD3 and anti–pan-TCRγδ potently activated Vγ9Vδ2$^+$ T cells in vitro, neither BTN2A1, BTN3A1, nor BTN2A1+BTN3A1 combination (1:1 ratio) led to degranulation or production of cytokines IFN-γ and TNF-α (Supplemental Fig. 1A). The lack of Vγ9Vδ2$^+$ T cell activation by BTN2A1 and BTN3A1 suggested that either recombinant BTNs were not in an "active" conformation to engage with TCR, or that a second costimulatory signal was needed to induce Vγ9Vδ2$^+$ T cell activation. Expression of known NKRs and T cell costimulatory receptors was analyzed on both ex vivo and in vitro expanded Vγ9Vδ2$^+$ T cells to identify possible costimulatory receptors that are constitutively expressed. Both sources of Vγ9Vδ2$^+$ T cells expressed high levels of NKG2D and DNAM-1 but did not express significant amounts of natural cytotoxicity receptors NKp30 or NKp44 (Supplemental Fig. 1B). Vγ9Vδ2$^+$ T cells also constitutively expressed T cell costimulatory receptors CD28 and CD27, but only upregulated OX40 and 4-1BB on in vitro expansion via pAg stimulation (Supplemental Fig. 1B). To test the ability of NKR or T cell costimulatory receptors to provide "signal 2," we tested agonistic anti-NKG2D and anti-CD28 alone or in combination with recombinant BTNs to activate Vγ9Vδ2$^+$ T cells. While BTNs, anti-NKG2D, or anti-CD28 treatment alone led to background or low levels of degranulation and cytokine production, stimulation of Vγ9Vδ2$^+$ T cells with BTN2A1+BTN3A1 in combination with anti-NKG2D or anti-CD28 resulted in increased degranulation, IFN-γ, and TNF-α production (Fig. 1A, Supplemental Fig. 2A). To further demonstrate that recombinant BTNs activated Vγ9Vδ2$^+$ T cells via TCR activation, we generated a T cell line expressing γδ TCR (TEG) by introducing Vγ9 and Vδ2 TCR chains in J76 cells that lack endogenous TCR expression (23, 25). Although the parental J76 did not express any components of the TCR complex, J76-Vγ9Vδ2$^+$ expressed TCRVγ9, TCRVδ2, and CD3 on the cell surface (Supplemental Fig. 2B). Consistent with primary Vγ9Vδ2$^+$ T cell activation, BTN2A1+BTN3A1 activated J76-Vγ9Vδ2$^+$ TEG in the presence of anti-CD28, as indicated by CD69 upregulation (Supplemental Fig. 2D). Because NKG2D is not expressed on J76-Vγ9Vδ2$^+$ (Supplemental Fig. 2C), BTNs+

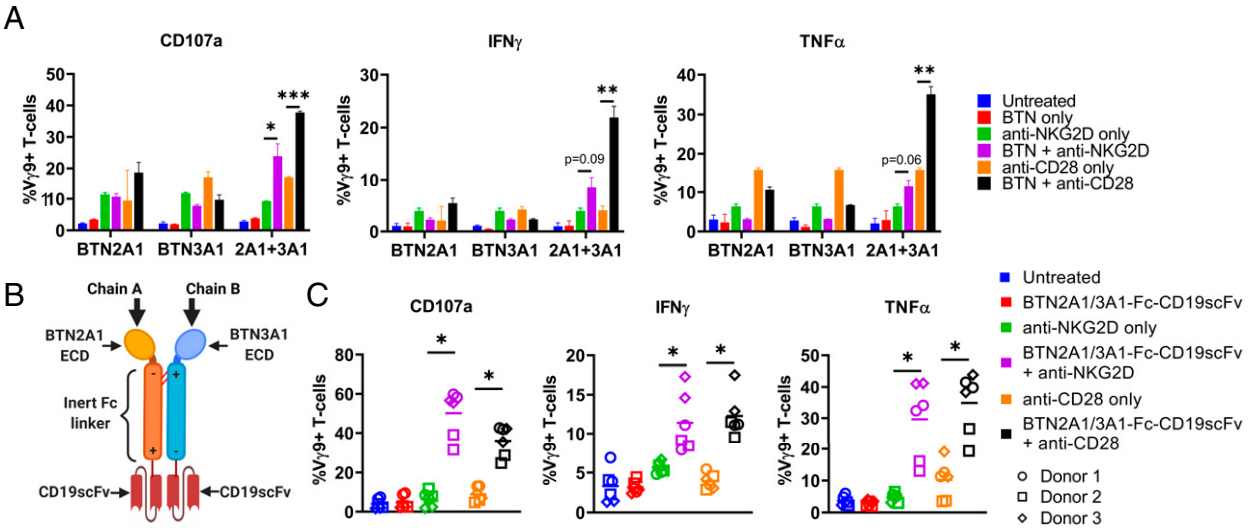

FIGURE 1. $V\gamma9V\delta2^+$ T cell activation by recombinant BTNs in combination with a costimulatory signal via NKR or T cell costimulatory receptor. (**A**) $V\gamma9V\delta2^+$ T cells were stimulated with plate-bound BTN2A1-Fc (5 μg/ml), BTN3A1-Fc (5 μg/ml), or BTN2A1+BTN3A1 (1:1 ratio, 5 μg/mL) with and without anti-NKG2D (1 μg/ml) and anti-CD28 (2.5 μg/ml). The proportion of $CD3^+V\gamma9^+$ cells expressing CD107a (left panel), IFN-γ (middle panel), and TNF-α (right panel) were analyzed. Mean ± SD from three biological replicates is shown. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by Student *t* test. Data are representative of at least five independent experiments. (**B**) Schematic representation of the heterodimeric BTN2A1/3A1-Fc-CD19scFv engager construct comprising two polypeptide chains (A and B) brought together by interchain disulfide bonds and charge polarized linkers. Created with BioRender.com. (**C**) $V\gamma9V\delta2^+$ T cells derived from three different human donors were stimulated with plate-bound BTN fusion proteins (10 μg/ml) containing BTN2A1/BTN3A1 heterodimers with and without anti-NKG2D (1 μg/ml) or anti-CD28 (2.5 μg/ml). The proportion of $CD3^+V\gamma9^+$ cells expressing CD107a (left panel), IFN-γ (middle panel), and TNF-α (right panel) was determined. Horizontal bars denote mean of two biological replicates from three different donors. $^*p < 0.05$ by Wilcoxon matched-pairs signed rank test. Data are representative of at least five independent experiments.

anti-NKG2D did not lead to TEG activation. Although BTN2A1+ anti-CD28 also upregulated CD69 in J76-$V\gamma9V\delta2^+$ TEG (Supplemental Fig. 2D), cytokine production was observed only when $V\gamma9V\delta2^+$ T cells were stimulated by both BTN2A1 and BTN3A1 (Fig. 1A). These results confirm the involvement of BTN2A1 and BTN3A1 in TCR-dependent activation of $V\gamma9V\delta2^+$ T cells and demonstrate the requirement of a "signal 2" for BTN-mediated activation of $V\gamma9V\delta2^+$ T cells. Furthermore, these results suggest that close proximity of plate-bound BTN2A1 and BTN3A1 homodimers was sufficient to mimic the active form of BTNs to engage with Vγ9Vδ2 TCR.

Heterodimeric BTN2A1/BTN3A1 activates $V\gamma9V\delta2^+$ T cells in the presence of a costimulatory signal

We generated a bispecific γδ T cell engager containing heterodimeric BTN2A1 and BTN3A1 ECDs fused via inert Fc linkers to scFv domains specific for CD19 (Fig. 1B) to test its ability to modulate $V\gamma9V\delta2^+$ T cells and promote killing of CD19-expressing tumor cells. The presence of BTN2A1 and BTN3A1 ECDs on the two polypeptide chains on the BTN2A1/3A1-Fc-CD19scFv molecule was confirmed by Western blot under nonreduced, reduced, and deglycosylated conditions using specific Abs (Supplemental Fig. 3A). The formation of a BTN2A1/BTN3A1 heterodimer was confirmed by a dual-binding immunoassay using capture and detection Abs that bind to the individual BTN domains (Supplemental Fig. 3B). Only the BTN2A1/3A1-Fc-CD19scFv construct containing CD19scFv, but not an unrelated scFv, bound to a $CD19^+$ lymphoma cell line (Supplemental Fig. 3C, left panel). Furthermore, BTN2A1/3A1-Fc-CD19scFv (but not a control construct containing a BTN3A1 homodimer) bound to $V\gamma9V\delta2^+$ T cells, but not $V\delta1^+$ (predominately $V\gamma9^-$) or $CD8^+$ T cells in PBMCs (Supplemental Fig. 3C, right panel, and Supplemental Fig. 3E). BTN2A1/3A1-Fc-CD19scFv binding to $V\gamma9V\delta2^+$ T cells was partially inhibited by the presence of anti–pan-TCRγδ and completely blocked by anti-Vγ9 (Supplemental Fig. 3D), confirming the specificity of heterodimeric BTN2A1 and BTN3A1 to the Vγ9 subunit of the TCR complex.

Consistent with cell-binding specificity, stimulation of J76-$V\gamma9V\delta2^+$ TEG, but not parental J76, with BTN2A1/3A1-Fc-CD19scFv led to robust upregulation of CD69, but only in the presence of CD28 costimulation (Supplemental Fig. 3F). Similarly, BTN2A1/3A1-Fc-CD19scFv induced $V\gamma9V\delta2^+$ T cell degranulation and cytokine production in the presence of NKG2D or CD28 costimulation across multiple donor-derived in vitro expanded and naive $V\gamma9V\delta2^+$ T cells (Fig. 1C, Supplemental Fig. 4C). Furthermore, while anti-CD28 stimulation alone led to proliferation-naive $V\delta2^+$ T cells, BTN2A1/3A1-Fc-CD19scFv in combination with anti-NKG2D enhanced proliferation of $V\delta2^+$ T cells (Supplemental Fig. 4A, 4B). Although stimulation with BTN2A1 or BTN3A1 homodimers alone did not elicit activation of $V\gamma9V\delta2^+$ T cells (Fig. 1A), a plate-bound mixture of BTN2A1 and BTN3A1 homodimers provided costimulation-dependent activation of $V\gamma9V\delta2^+$ T cells similar to the BTN2A1/3A1 heterodimeric engager (Fig. 1A, Supplemental Fig. 4C, 4D). Taken together, these results suggest that activation of $V\gamma9V\delta2^+$ T cells requires the simultaneous presence of BTN2A1, BTN3A1, and costimulation via NKR or potentially other costimulatory receptors to fully activate the cytotoxic properties of $V\gamma9V\delta2^+$ T cells. The comparison of the BTN2A1/3A1 heterodimer with the mixture of plate-bound BTN2A1/3A1 homodimers raises an important question of whether the $V\gamma9V\delta2^+$ TCR is optimally activated by closely approximated BTN2A1/3A1 domains with or without an associated heterodimerization-driven conformational change in the ECD, or whether both BTN2A1 and 3A1 simply both need to be present within the immune synapse to provide "signal 1" to the $V\gamma9V\delta2^+$ TCR, and these questions should be the subject of further inquiry.

Addition of BTN2A1/3A1-Fc-CD19scFv enhances Vγ9Vδ2$^+$ T cell cytotoxicity against B cell lymphoma

To evaluate the ability of BTN2A1/3A1-Fc-CD19scFv to enhance Vγ9Vδ2$^+$ T cell–mediated killing of tumor cells, we cultured CD19$^+$ Daudi and Raji cells (Supplemental Fig. 4E) with Vγ9Vδ2$^+$ T cells in the presence of BTN2A1/3A1-Fc-CD19scFv. Addition of BTN2A1/3A1-Fc-CD19scFv to the coculture resulted in an increased proportion of apoptotic tumor cells, as evidenced by detection of translocated phosphatidylserine residues on the cell surface (Fig. 2A). Similar levels of tumor killing were induced by 1–100 μg/ml (6.7–670 nM) BTN2A1/3A1-Fc-CD19scFv, suggesting a concentration at or less than EC_{50} for tumor cell binding by BTN2A1/3A1-Fc-CD19scFv (Supplemental Fig. 3C) can efficiently induce cytotoxicity in Vγ9Vδ2$^+$ T cells. BTN2A1/3A1-Fc-CD19scFv–mediated Vγ9Vδ2$^+$ T cell cytotoxicity was additionally investigated using a cell-based fluorogenic cytotoxicity assay designed to measure granzyme B activity in live target cells after the successful transfer of granzyme B by cytotoxic lymphocytes. In agreement with tumor cell apoptosis, the proportion of tumor cells exhibiting granzyme B activity (Fig. 2B), as well as secreted levels of cytokines such as IFN-γ and TNF-α (Fig. 2C), significantly increased when BTN2A1/3A1-Fc-CD19scFv was added to Vγ9Vδ2$^+$ T and Daudi or Raji cell coculture, but not to tumor cells alone, confirming that the mechanism of action for BTN2A1/3A1-Fc-CD19scFv is through enhancement of Vγ9Vδ2$^+$ T cell cytotoxicity. The frequency of granzyme B$^+$ tumor cells was comparable when BTN2A1/3A1-Fc-CD19scFv or a saturating dose of agonistic anti-CD277/BTN3A1 (20.1) was added to the Vγ9Vδ2$^+$ T cells and tumor coculture. This further demonstrates that recombinant heterodimeric BTN2A1/3A1 can provide an activating signal to Vγ9Vδ2 TCR in a similar fashion as endogenous BTN2A1 in combination with an active conformation of BTN3A1 induced by the 20.1 Ab. Phenotypic analysis of CD19$^+$ lymphoma cell lines indicated the presence of CD28 ligands CD80 and CD86 (Supplemental Fig. 4F), suggesting the costimulatory signal needed for BTN2A1/3A1-Fc-CD19scFv activity was provided by tumor cells. To assess the ability of BTN2A1/3A1-Fc-CD19scFv to enhance Vγ9Vδ2$^+$ T cell–mediated killing of tumor cells expressing NKG2D ligands, we generated a CD19-expressing K562 cell line with endogenous MHC class I–related molecules A or B on the cell surface (Supplemental Fig. 4G). Whereas the addition of BTN2A1/3A1-Fc-CD19scFv to Vγ9Vδ2$^+$ T cell + parental K562 coculture did not enhance tumor cell killing, BTN2A1/3A1-Fc-CD19scFv significantly enhanced Vγ9Vδ2$^+$ T cell–mediated cytotoxicity of CD19-expressing K562 cells (Fig. 2A, 2B, right panels). These results highlight the ability of BTN2A1/3A1-Fc-CD19scFv in promoting targeted killing of tumor cells expressing the select tumor Ag, as well as ligands for CD28 and/or NKG2D to deliver "signal 2" to activate Vγ9Vδ2$^+$ T cells. Curiously, the maximal killing of Daudi cells occurred

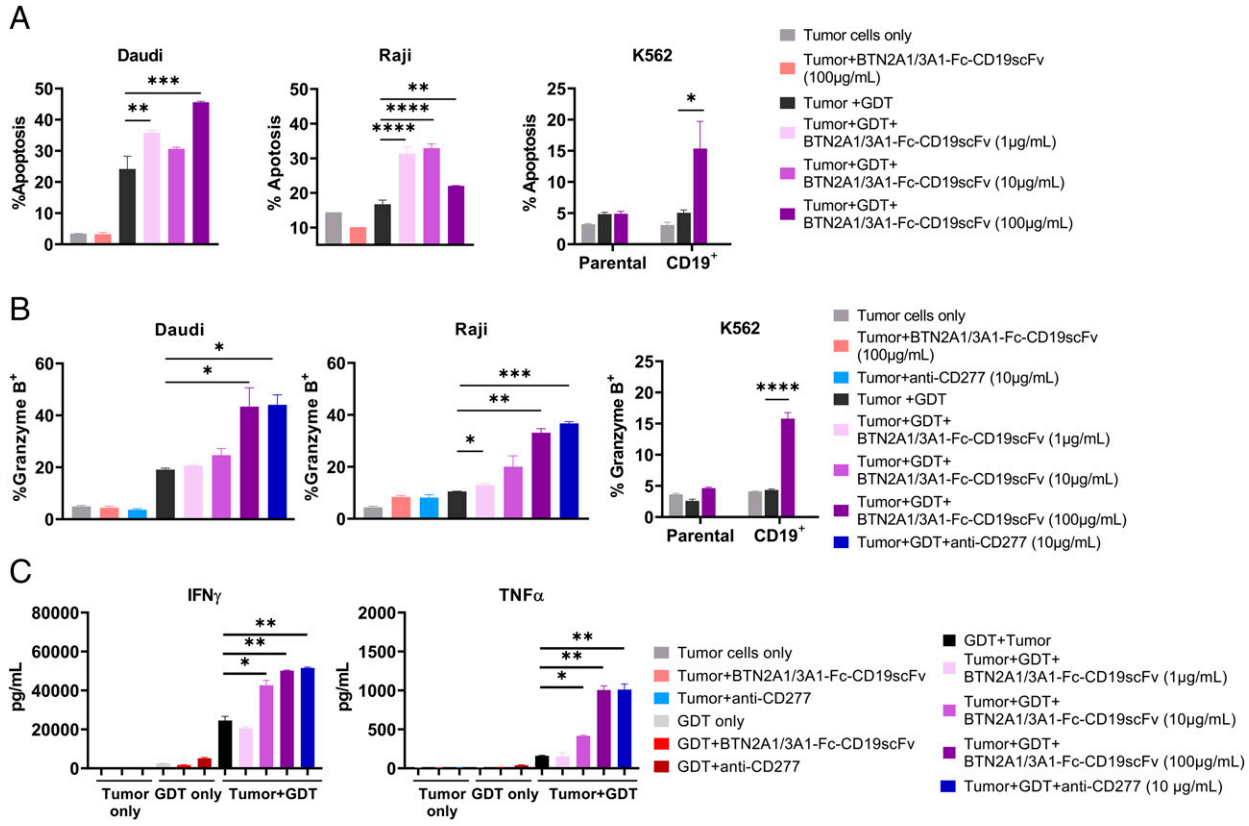

FIGURE 2. BTN2A1/3A1-Fc-CD19scFv enhanced tumor killing in vitro as a single agent. (**A**) Vγ9Vδ2$^+$ T cells (GDT) were cocultured with the indicated tumor cells at 1:1 ratio in the presence of BTN2A1/3A1-Fc-CD19scFv. The proportion of apoptotic tumor cells was detected by Apotracker-Green$^+$ cells in CD3$^-$CD20$^+$ cells (Daudi and Raji) or CD3$^-$ cells (K562). Mean ± SD from three biological replicates is shown. Data are representative of at least three independent experiments from three different Vγ9Vδ2$^+$ T cell donors. (**B**) Vγ9Vδ2$^+$ T cells were cocultured with the indicated tumor cells at 1:1 ratio in the presence of BTN2A1/3A1-Fc-CD19scFv or anti-CD277/BTN3A1. The proportion of tumor cells with active granzyme activity was detected by flow cytometry, as determined as %Granzyme B$^+$ cells in fluorescently labeled tumor cells. Mean ± SD from three biological replicates is shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student *t* test. Data are representative of at least three independent experiments from three different Vγ9Vδ2$^+$ T cell donors. (**C**) IFN-γ (left) and TNF-α (right) levels in supernatant from Vγ9Vδ2$^+$ T and Raji coculture in (A) were quantified by human U-PLEX T-Cell Combo immunoassay (MSD). Mean ± SD from three biological replicates is shown. Data are representative of at least three independent experiments from three different Vγ9Vδ2$^+$ T cell donors. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by Student *t* test.

in the presence of 100 μg/ml BTN2A1/3A1-Fc-CD19scFv, while the same was achieved at 1 or 10 μg/ml for Raji cocultures (Fig. 2A). Because Raji expressed a slightly lower level of CD19 (Supplemental Fig. 4E), we speculate that when adding a high concentration of BTN2A1/31A-Fc-CD19scFv, a higher level of unbound fusion proteins remained in the culture after the initial tumor prebinding step. These "free" BTN2A1/3A1-Fc-CD19scFv may occupy the TCRs on Vγ9Vδ2$^+$ T cells and prevent the direct cell–cell interaction that is needed to mediate tumor killing. In addition, Raji expressed a higher level of CD80 (Supplemental Fig. 4F) that likely enabled stronger CD28 costimulation on Vγ9Vδ2$^+$ T cells, and therefore a lower level of BTN2A1/3A1 may be needed to trigger cytotoxic functions. Nonetheless, while the dose response varied between the two lymphoma cell lines for the apoptosis readout, the proportion of granzyme B transferred into tumor cell lines was highest at 100 μg/ml for both lymphoma cell lines. We speculate that there might also be additional mechanisms used by Vγ9Vδ2$^+$ T cells to mediate cytotoxicity against lymphoma cells (i.e., via Fas or TRAIL) that can account for the differences seen in the Raji and Daudi cocultures.

Collectively, results from this study have demonstrated the feasibility of using recombinant heterodimeric BTN2A1 and BTN3A1 in a bispecific engager format to enhance antitumor activity of Vγ9Vδ2$^+$ T cells. While in a tumor-free culture system we demonstrated the need for the presence of a costimulatory signal to activate Vγ9Vδ2$^+$ T cells by BTNs, BTN2A1/3A1-Fc-CD19scFv as a single agent was sufficient to promote tumor killing, indicating the delivery of costimulatory signal(s) by ligands natively expressed by tumor cells. We identified NKG2D and CD28 as two costimulatory receptors for Vγ9Vδ2$^+$ T cells and confirmed expression of their corresponding ligands on tumor cells. Phenotypic analysis suggests additional costimulatory receptors are present on Vγ9Vδ2$^+$ T cells, including DNAM-1 and CD27 (Supplemental Fig. 1B), which likely contribute to Vγ9Vδ2$^+$ T cell activation. Likewise, multiple stress-induced molecules or ligands for NKRs are likely expressed on infected or transformed cells to promote BTN-mediated killing by Vγ9Vδ2$^+$ T cells. Comprehensive investigation into additional costimulatory/coinhibitory receptors on Vγ9Vδ2$^+$ T cells and regulation of cell-surface expression of the corresponding ligands in different cancer cell types will be invaluable to further our understanding of Vγ9Vδ2$^+$ T cell biology. In addition, these biological insights will also facilitate translation of effective γδ T cell–targeted therapies and ultimately selecting cancer patients most likely to respond to this emerging class of therapy.

While Abs against the γδ TCR components (i.e., CD3, TCRγδ, and TCR Vδ2) can induce robust Vγ9Vδ2$^+$ T cell activation without the need for "signal 2" (Supplemental Fig. 1A) (26), the weaker TCR signal delivered by its natural ligand in the format of BTN heterodimers has the potential to provide a more targeted approach to exert cytotoxicity in cancer cells, at the same time preventing systemic activation of T cells in the clinical setting. Preliminary data comparing antitumor activity of Vγ9Vδ2$^+$ T cells in the presence of different agonists suggests comparable levels of IFN-γ and TNF-α produced by engagers containing heterodimeric BTN2A1/3A1 or anti-Vδ2. More thorough understanding of the qualitative and quantitative differences in Vγ9Vδ2$^+$ T cells on stimulation by different agonists is warranted, to determine the optimal TCR signaling strength necessary to promote cell proliferation and antitumor effector functions, while preventing detrimental effects such as immunosuppression, activation-induced cell death, and anergy in Vγ9Vδ2$^+$ T cells.

Acknowledgments

We thank Dr. Jenny Gumperz (University of Wisconsin-Madison) for scientific advice and review of the manuscript.

Disclosures

All authors are employees of or consultants for Shattuck Labs and hold an equity interest in the company.

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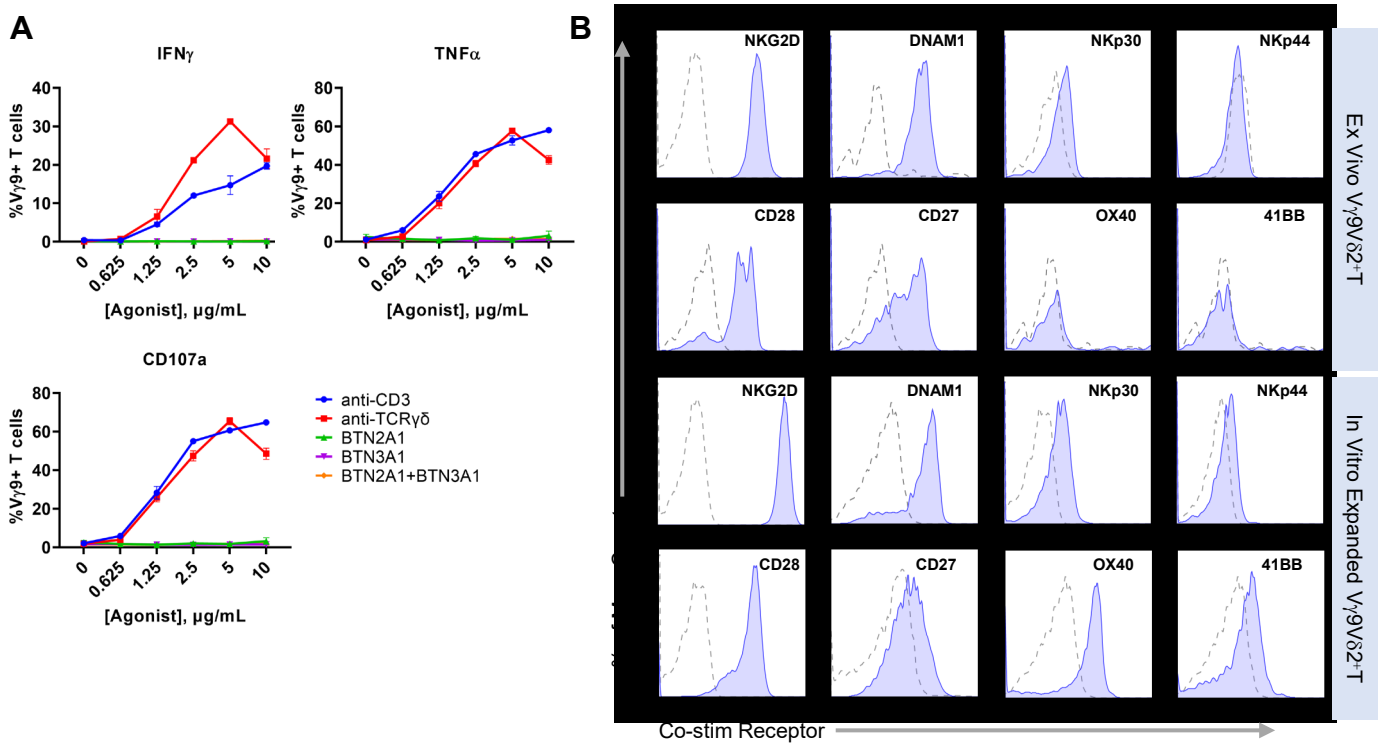
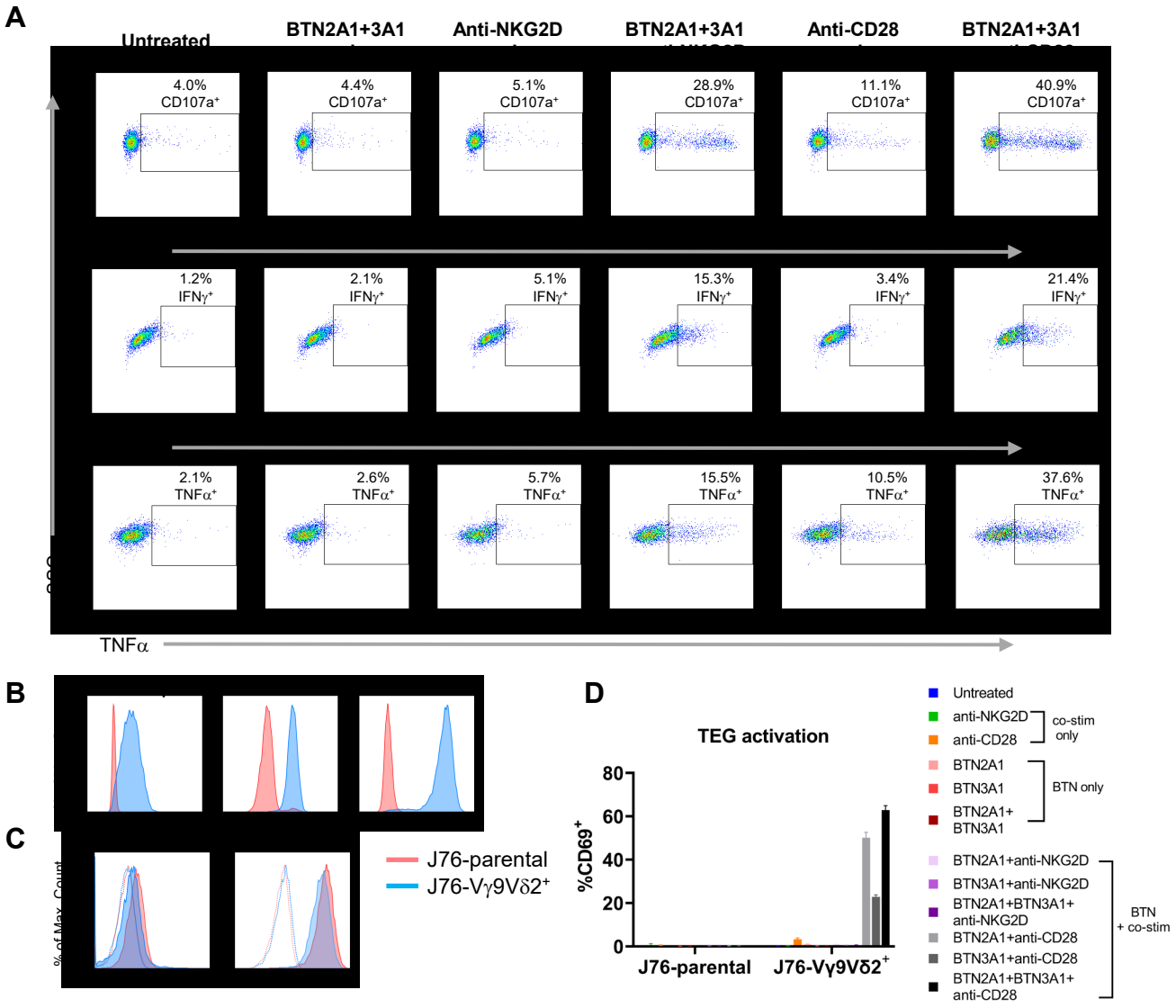


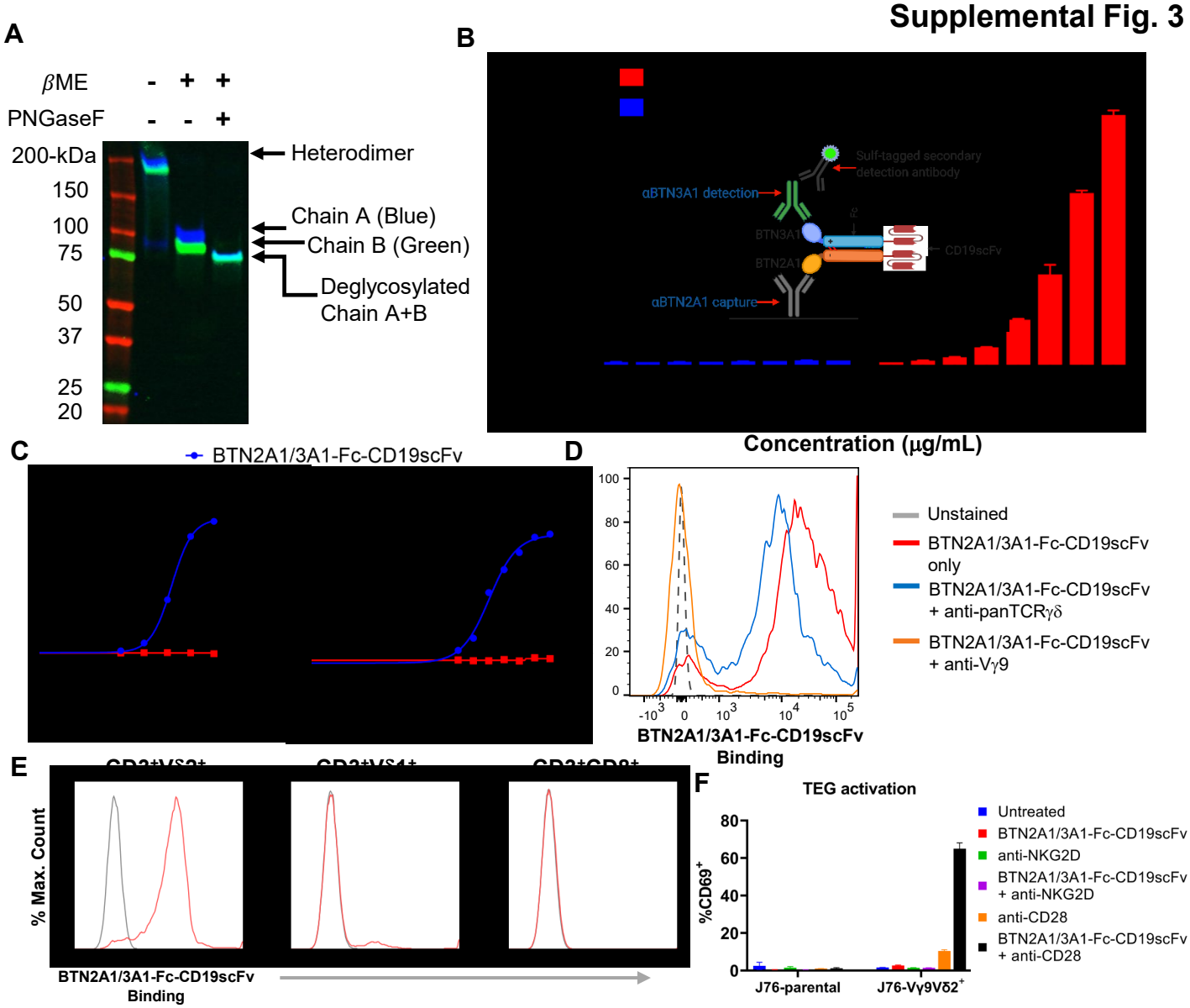
Supplemental Fig. 1. Phenotypic analysis of Vγ9Vδ2$^+$ T-cells

(A) In vitro expanded Vγ9Vδ2$^+$ T-cells were stimulated with varying concentrations of plate-bound anti-CD3, anti-TCRγδ, and recombinant BTN2A1-Fc and/or BTN3A1-Fc for 4 hours. Proportion of cells expressing CD107a, IFNγ, and TNFα were detected by flow cytometry. Mean ± SD from three biological replicates is shown. Data is representative of at least 3 independent experiments. **(B)** Phenotypic analysis of NK receptors and T-cell co-stimulatory receptors on Vγ9Vδ2$^+$ T-cells by flow cytometry. Ex vivo Vγ9Vδ2$^+$ T-cells in PBMC (top two panels) and in vitro expanded Vγ9Vδ2$^+$ T-cells (bottom two panels) were analyzed. Data is representative of Vγ9Vδ2$^+$ T-cells from three different donors in three independent experiments.



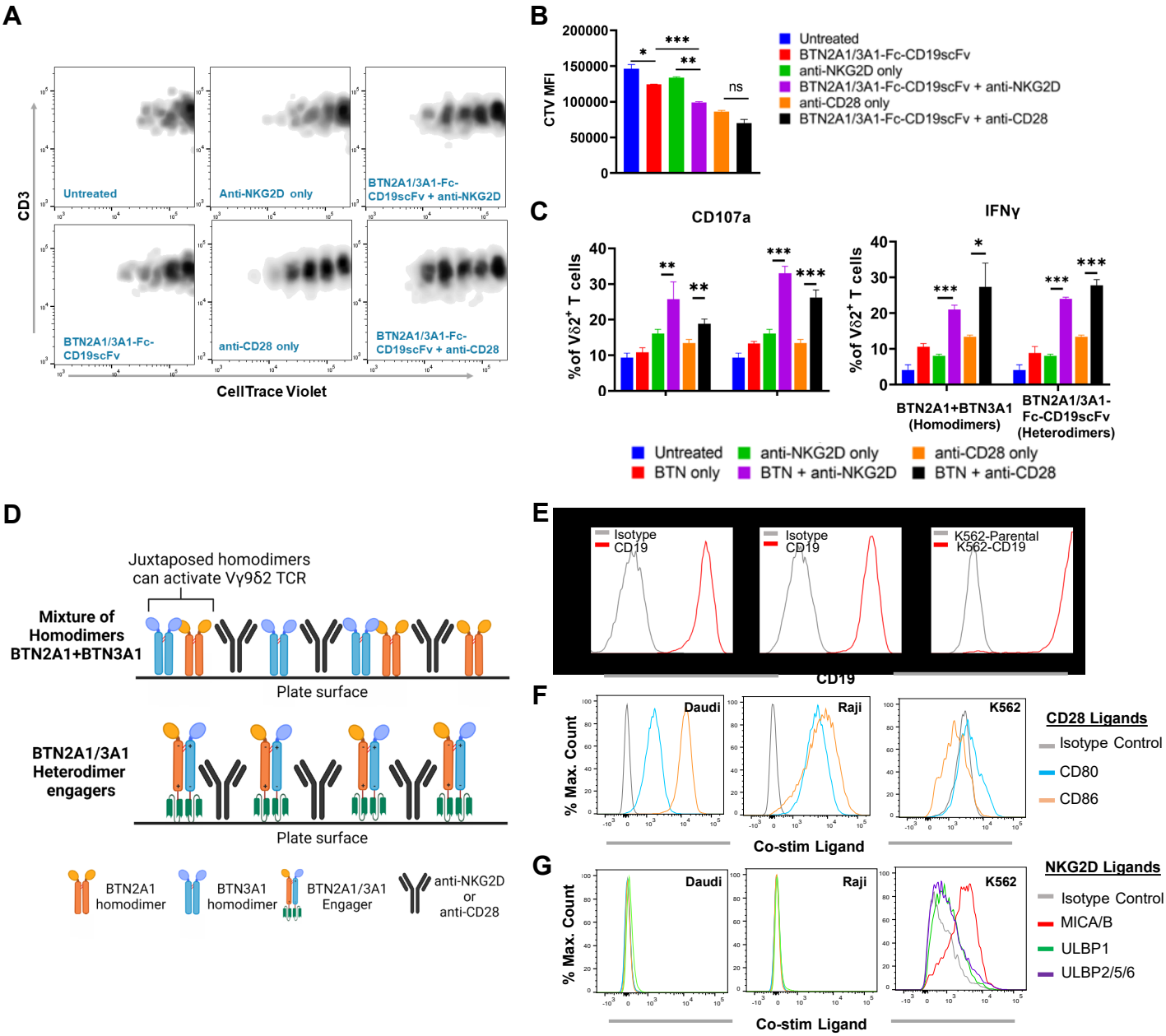
Supplemental Fig. 2. Activation of Vγ9Vδ2⁺ T-cells by recombinant BTN-Fc proteins

(A) In vitro expanded Vγ9Vδ2⁺ T-cells were stimulated with plate-bound BTN2A1+BTN3A1 (1:1 ratio, 5 μg/mL) with and without anti-NKG2D (1 μg/mL) and anti-CD28 (2.5 μg/mL) for 4 hours. Proportion of cells expressing CD107a (top), IFNγ (middle), and TNFα (bottom) were analyzed by flow cytometry. Data is representative of at least 5 independent experiments. **(B)** Vγ9Vδ2⁺ TEG was generated by transducing Jurkat(J)76 cells with pLenti-EF1a-IRES-GFP lentiviral construct containing full length TCR Vγ9 and TCR Vδ2 linked by sequence encoding the P2A self-cleaving peptide. The sequence of TCRVγ9 and Vδ2 was derived from Vγ9Vδ2 T-cell clone A3 as previously published by Vyborova et al. Expression of TCRVγ9, TCRVδ2, and CD3 was confirmed on a single-cell clone of J76 transduced with Vγ9Vδ2 TCR lentiviral construct but not parental J76. Data is representative of three independent experiments. **(C)** Expression of CD28 but not NKG2D on parental J76 and J76-Vγ9Vδ2⁺. Dotted lines indicate staining with isotype control antibody. Data is representative of three independent experiments. **(D)** TEG (J76-Vγ9Vδ2⁺) or parental J76 were stimulated with plate-bound BTN2A1-Fc (5 μg/mL), BTN3A1-Fc (5 μg/mL), or BTN2A1+BTN3A1 (1:1 ratio, 5 μg/mL) with and without anti-NKG2D (1 μg/mL) and anti-CD28 (2.5 μg/mL). Proportion of cells expressing CD69 was determined. Mean ± SD is shown from three biological replicates. Data is representative of at least 3 independent experiments.



Supplemental Fig. 3. Heterodimeric BTN2A1/3A1 binds and targets Vγ9Vδ2$^+$ but not Vδ1$^+$ T-cells

(A) Western blot analysis of the purified BTN2A1/3A1-Fc-CD19scFv under non-reduced (βME$^-$PNGaseF$^-$), reduced (βME$^+$PNGaseF$^-$), and deglycosylated (βME$^+$PNGaseF$^+$) conditions. Chain A and chain B of the construct were detected as indicated. Data is representaive of three independent production of recombinant protein. **(B)** A dual, antibody-based MSD method was used to confirm the formation of a heterodimeric fusion protein, capturing using anti-BTN2A1 and detected via anti-BTN3A1 in combination with a sulfo-tagged anti-species specific secondary (see inset). Mean of triplicate for each recombinant protein concentration is shown. Data is representative of three independent production of recombinant protein. **(C)** Binding of BTN2A1/3A1-Fc-CD19scFv to Vγ9Vδ2$^+$ T-cells and CD19$^+$ Daudi cells. A control homodimer containing BTN3A1 ECD sequence only or a control heterodimer lacking the CD19scFv sequence was used as negative controls, respectively. Mean of two replicates for each protein concentration is shown. Data is representative of three independent production of recombinant protein. **(D)** Binding of BTN2A1/3A1-Fc-CD19scFv to Vγ9Vδ2$^+$ T-cells was blocked by anti-pan TCRγδ or anti-TCRVγ9. Vγ9Vδ2$^+$ T cells were co-incubated with 100 μg/mL BTN2A1/3A1-Fc-CD19scFv and a saturating concentration of anti-pan TCRγδ or anti-Vγ9, followed by APC-anti-human Fc for detection of BTN2A1/3A1-Fc-CD19scFv binding. Data is representative of three independent experiments. **(E)** Binding of BTN2A1/3A1-Fc-CD19scFv to T cell subsets by flow cytometry. PBMCs were incubated with 100 μg/mL BTN2A1/3A1-Fc-CD19scFv, followed by staining with antibodies against CD3, CD8, TCRVδ1, TCRVδ2, and APC-anti-human Fc for detection of BTN2A1/3A1-Fc-CD19scFv binding. Data is representative of PBMCs from three different donors. Grey histogram represents APC-anti-Fc staining only, red histogram represent BTN2A1/3A1-Fc-CD19scFv + APC-anti-Fc **(F)** TEG (J76-Vγ9Vδ2$^+$) or parental J76 were stimulated with plate-bound BTN2A1/3A1-Fc-CD19scFv (10 μg/mL) with and without anti-NKG2D (1 μg/mL) or anti-CD28 (2.5 μg/mL) for 24 hours. Proportion of cells expressing CD69 was analyzed. Mean ± SD from three biological replicates is shown. Data is representative of at least three independent experiments.



Supplemental Fig. 4. BTN2A1/3A1-Fc-CD19scFv promotes activation of Vγ9Vδ2⁺ T-cells in the presence of co-stimulation **(A)** Total γδ T-cells purified from PBMCs were labeled with CellTrace™ Violet (CTV) before stimulated with plate-bound BTN2A1/3A1-Fc-CD19scFv (10 μg/mL) ± anti-NKG2D (1 μg/mL) or anti-CD28 (2.5 μg/mL) for 96 hours. Mean fluorescent intensity (MFI) of CTV was analyzed in $CD3^+V\delta2^+$ T-cell population to evaluate cell proliferation. Quantification of CTV MFI is shown in **(B)**. Mean ± SD is shown from three biological replicates. *p<0.05, **p<0.01, and ***p<0.001 by Student's t-test. Data is representative of PBMCs from five different donors in three independent experiments. **(C)** Total γδ T-cells were stimulated with plate-bound BTN2A1-Fc + BTN3A1-Fc homodimers (1:1 ratio, 5 μg/mL) or BTN2A1/3A1-Fc-CD19scFv heterodimer (5 μg/mL) ± anti-NKG2D (1 μg/mL) or anti-CD28 (2.5 μg/mL) for 48 hours. Anti-CD107a, GolgiStop, and GolgiPlug were added to the culture 6 hours prior to analysis of degranulation ($CD107a^+$, left panel), or IFNγ production (right panel) in $CD3^+V\delta2^+$ T cells. Data is representative of PBMCs from three different donors across two experiments. **(D)** Diagram comparing $V\gamma9V\delta2^+$ T cell activation by BTN2A1 + BTN3A1 homodimers vs. engager containing BTN2A1/3A1 heterodimer, created with BioRender.com **(E)** Confirmation of CD19 expression on Daudi, Raji, and K562 transduced with CD19 lentivirus by flow cytometry. Data is representative of three independent experiments. **(F)** Analysis of CD80 or CD86 (ligands for CD28) by flow cytometry on Daudi, Raji, and on K562 cell lines. Data is representative of three independent experiments. **(G)** Analysis of MICA/B, ULBP1, and ULBP2/5/6 (ligands for NKG2D) by flow cytometry on Daudi, Raji, and on K562 cell lines. Data is representative of three independent experiments.