Bispecific γδ T Cell Engagers Containing Butyrophilin 2A1/3A1 Heterodimeric Fusion Protein Efficiently Activate Vγ9Vδ2⁺ T Cells and Promote Tumor Cell Killing

#3514

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Abstract

A primary mechanism of cancer immunotherapy resistance involves downregulation of specific antigens or major histocompatibility complex (MHC) based antigen presentation, which renders tumor cells invisible to αβ T cells, but not γδ T cells. Recently, a two-step model of γδ T cell activation has emerged, wherein one butyrophilin (BTN, i.e., BTN2A1) directly binds the γδ TCR but is only activated if certain molecular patterns (e.g., phosphoantigens) facilitate recruitment of a second BTN (i.e., BTN3A1) into a complex to form a BTN2A1/3A1 heterodimer. The BTN2A1/3A1 complex specifically activates the Vγ9Vδ2⁺ T cells found in the peripheral blood in response to phosphoantigen build up in tumor cells. The unique mechanism of action and specificity of γδ TCR/BTN interactions suggests that therapeutic proteins comprising specific BTN heterodimers could be used to target specific γδ T cell populations to tumor cells which lack expression of high affinity neoantigens or MHC class I molecules.

In this study, we generated bispecific γδ T cell engagers (GADLEN) containing heterodimeric BTN2A1 and BTN3A1 extracellular domains (ECD) fused via inert Fc linkers to scFv domains targeting a tumor-antigen (CD19 or CD20) to test their ability to modulate Vγ9Vδ2⁺ T cells and to promote tumor cell killing in co-culture assays. GADLEN induced proliferation, degranulation and cytokine production including IFNγ and TNFα in Vγ9Vδ2⁺ T cells but requires co-stimulation of a natural cytotoxicity receptor (via anti-NKG2D) or T cell co-stimulatory receptor (via anti-CD28), highlighting parallels of signal 1 and signal 2 requirements of TCR activation in αβ and γδ T cells. In Vγ9Vδ2⁺ T cells and tumor co-culture assays, the addition of GADLEN alone enhanced killing of CD19⁺/CD20⁺ lymphoma cells such as Daudi and Raji, expressing known ligands for CD28 (e.g., CD80 and CD86). Similarly, GADLEN enhanced Vγ9Vδ2⁺ T cell mediated killing of K562 cells engineered to express CD19, which express MICA/B (ligands for NKG2D) on the cell surface.

These results highlight the ability of GADLENs in promoting targeted killing of tumor cells by providing the "active" heterodimeric BTN2A1 and BTN3A1 that is critical to enhance cytotoxic killing by Vγ9Vδ2⁺ T cells. Furthermore, this study provides mechanistic insights into BTN-mediated activation of Vγ9δ2⁺ T cells and offers proof of concept for using antigen-targeted butyrophilin heterodimeric fusion proteins for the treatment of cancer.

γδ T Cell Engager Platform (GADLEN)

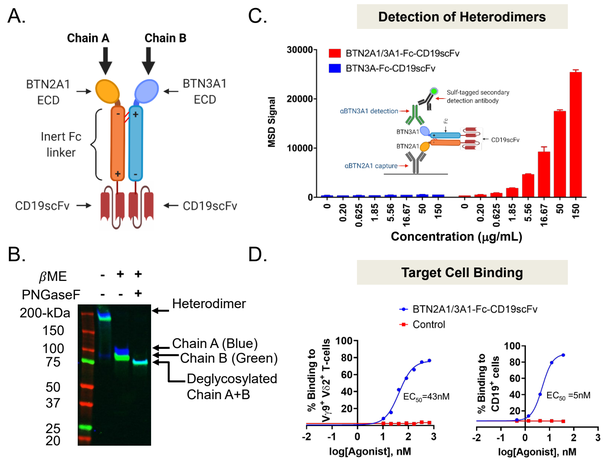

Figure 2. Characterization of the BTN2A1/3A1-Fc-CD19scFv GADLEN construct. (A) Schematic representation of the heterodimeric BTN2A1/3A1-Fc-CD19scFv construct comprising of two polypeptide chains (A and B) brought together by interchain disulfide bonds and charge polarized linkers. **(B)** Western blot analysis of the purified BTN2A1/3A1-Fc-CD19scFv molecule under non-reduced, reduced, and deglycosylated conditions. Chain A and chain B of the construct were detected anti-2A1 and anti-3A1 conjugated antibodies. **(C)** A dual, antibody-based MSD method detects the formation of a heterodimeric fusion protein construct. **(D)** The binding of BTN2A1/3A1-Fc-CD19scFv to CD19 expressed on Daudi cells and Vγ9Vδ2⁺ T cells was confirmed using flow cytometry. A heterodimer lacking the CD19scFv sequence or BTN3A1 homodimer construct was used as negative control, respectively.

GADLEN Activates Vγ9Vδ2⁺ T Cells

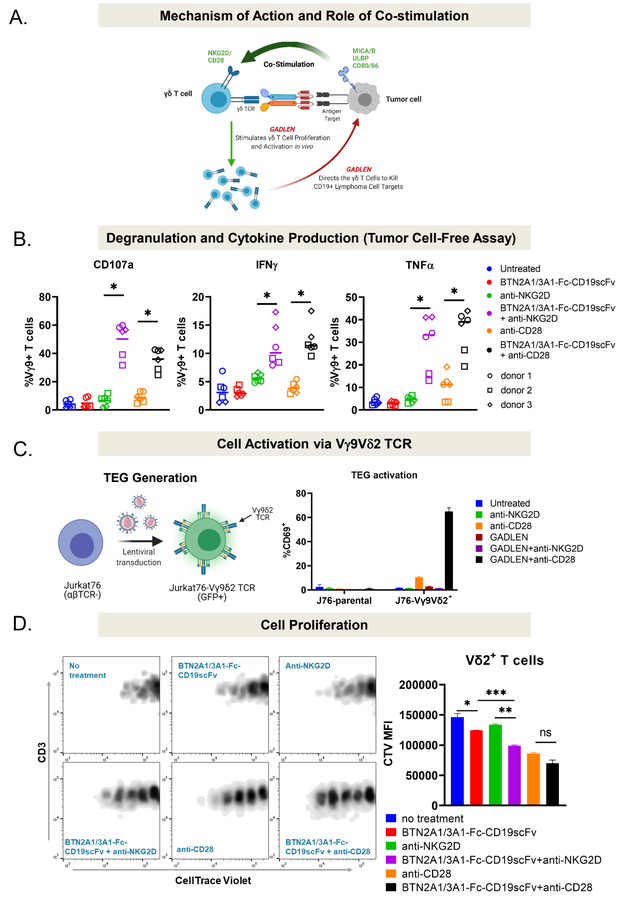

Figure 4. GADLEN activates Vγ9Vδ2⁺ T cells in the presence of NKG2D or CD28 co-stimulation (A) Mechanism of action of the GADLEN depicting the role of co-stimulation via NCRs and costimulatory receptors. **(B)** Vγ9Vδ2⁺ T cells derived from three different donors were stimulated with plate-bound BTN2A1/3A1-Fc-CD19scFv with and without anti-NKG2D or anti-CD28 for 4 hours. Proportion of cells expressing CD107a, IFNγ, and TNFα were determined by flow cytometry. Median level of two technical replicates from three different donors shown. * p<0.05 by Wilcoxon matched-pairs signed rank test. **(C)** GADLEN only activates T cells expressing functional Vγ9Vδ2 TCR. Jurkat76-Vγ9Vδ2⁺ or parental Jurkat76 (TCR-) were stimulated with plate-bound BTN2A1/3A1-Fc-CD19scFv with and without anti-NKG2D or anti-CD28 for 24 hours. Proportion of cells expressing CD69 was analyzed. Mean ± SD is shown. Only CD28 but not NKG2D is expressed on Jurkat76. **(D)** GADLEN in combination with anti-NKG2D enhanced proliferation of Vδ2+ T cells. Total γδ T cells were purified from PBMCs and labeled with CellTrace™ Violet (CTV) before stimulated with plate-bound BTN2A1/3A1-Fc-CD19scFv with and without anti-NKG2D or anti-CD28 for 96 hours. CTV Mean fluorescence intensity (MFI) was analyzed in CD3⁺Vδ2⁺ T cell populations by flow cytometry. Increased proliferation is indicated by decrease in MFI.

GADLEN Enhances Tumor Killing Activity

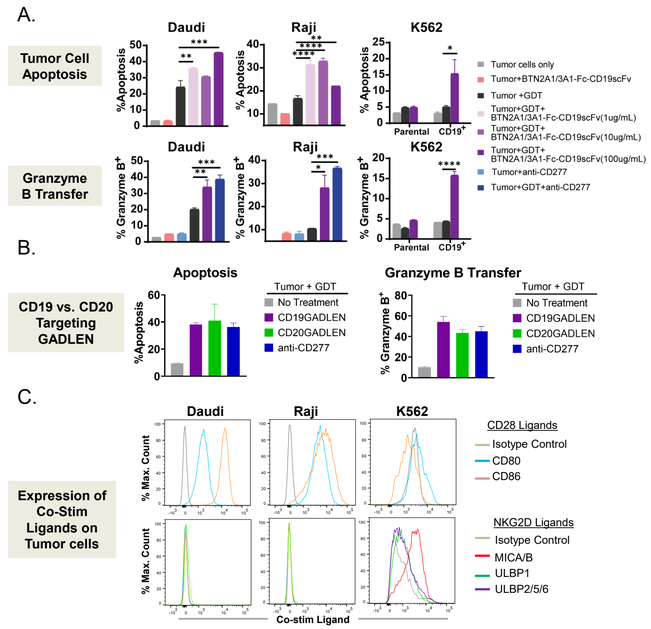

Figure 5. GADLEN enhances Vγ9Vδ2⁺ T cell killing of tumor cells expressing ligands for CD28 or NKG2D . (A) Vγ9Vδ2⁺ T cells (GDT) were co-cultured with Daudi or Raji (CD19⁺CD20⁺ lymphoma cell lines cells), or K562 leukemic cell line ectopically expressing CD19, at 1:1 ratio in the presence of BTN2A1/3A1-Fc-CD19scFv or anti-CD277 (anti-BTN3A1, clone 20.1) as positive control. The proportion of apoptotic tumor cells were detected by Apotracker⁺ cells in CD3⁻CD20⁺ cells (Daudi and Raji) or CD3⁻ cells (K562) after 4 hours by flow cytometry. **p<0.01, ***p<0.001, ****p<0.0001 by Student's t-test. The proportion of tumor cells with active granzyme activity analyzed using the GranToxiLux assay kit (OncoImmunin), as determined as %GranToxiLux⁺ cells in fluorescently labeled tumor cells. *p<0.05, **p<0.01, and ***p<0.001 by Student's t-test. **(B)** Both BTN2A1/3A1-Fc-CD19scFv (CD19GADLEN) and BTN2A1/3A1-Fc-CD20scFv (CD20GADLEN) similarly enhanced Vγ9Vδ2⁺ T cell killing of Daudi lymphoma cells, which express both CD19 and CD20 on cell surface. **(C)** Ligands for CD28 or NKG2D are expressed on tumor cells, suggesting the co-stim requirement for GADLEN activity can be provided by tumor cells.

Butyrophilins in Vγ9Vδ2⁺ T cell Activation

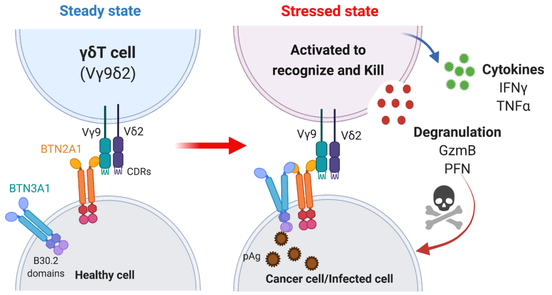

Figure 1. Heterodimerization of BTN2A1 and BTN3A1 results in high affinity engagement with TCR leading to Vγ9Vδ2⁺ T cell activation. Vγ9Vδ2⁺ T cells are the predominant circulating γδ T cells. They respond to transformed cells by sensing elevated phosphorylated non-peptide metabolites, or phosphoantigens (pAg) produced via the mevalonate pathway of cholesterol synthesis that becomes dysregulated in certain tumor cells. B7-related membrane protein BTN3A1, or CD277, expressed on target cells is responsible for direct pAg sensing through its cytoplasmic B30.2 domain. pAg binding to BTN3A1 initiates a conformational change in its extracellular domain, which facilitates interaction with BTN2A1 that can in turn engage with Vγ9Vδ2 TCR, leading to activation of effector functions. Figure adapted from Herrmann et al., 2020 and Created with Biorender.com.

2A1/3A1 GADLEN Specifically Targets Vγ9Vδ2⁺ T Cells

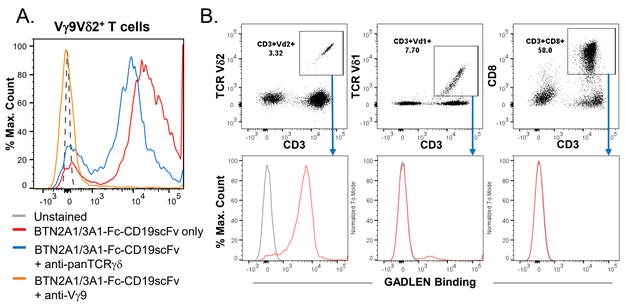

Figure 3. Specificity of GADLEN to Vγ9Vδ2 T Cell Receptor. (A) Binding of BTN2A1/3A1-Fc-CD19scFv to Vγ9Vδ2⁺ T cells was blocked by anti-pan TCRγδ and anti-TCRVγ9 antibodies, indicating specificity to Vγ9 subunit of TCR. Vγ9Vδ2⁺ T cells were co-incubated with BTN2A1/3A1-Fc-CD19scFv and mouse anti-pan TCRγδ or mouse anti-TCRVγ9, followed by staining with APC-anti-human Fc for detection of BTN2A1/3A1-Fc-CD19scFv binding by flow cytometry. **(B)** Binding of BTN2A1/3A1-Fc-CD19scFv to T cell subsets was assessed by flow cytometry. PBMCs were incubated with BTN2A1/3A1-Fc-CD19scFv, followed by staining with antibodies against CD3, CD8, TCRVδ1, TCRVδ2 (all mouse monoclonal antibodies), and APC-anti-human Fc for detection of BTN2A1/3A1-Fc-CD19scFv binding. Only CD3⁺Vδ2⁺, but not CD3⁺Vδ1⁺(predominately Vγ9⁻) or CD3⁺CD8⁺ T cells bound to BTN2A1/3A1-Fc-CD19scFv.

Conclusions

Functional characterization of GADLEN demonstrates the ability of heterodimeric BTN2A1/3A1 in a bispecific engager format can promote targeted activation of Vγ9Vδ2 T cells and enhances their anti-tumor function

- Mimics the active conformation of endogenous BTNs in engaging Vγ9Vδ2 TCR
- Target specificity prevents systemic activation of all T cells
- Co-stimulation requirement enables targeted-killing of tumor cells, while preventing systemic activation of Vγ9Vδ2⁺ T cells and activation-induced cell death
- Protein engineering of the GADLEN platform allows for targeting of tumor antigens expressed on solid tumors.