Antigen targeted butyrophilin heterodimer-based bispecific engagers induce Vγ9δ2⁺ T cell-mediated anti-tumor activity

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Abstract

Background: Decreased antigen expression and antigen presentation via major histocompatibility complexes (MHCs) evades αβ T cell recognition. γδ T cells recognize stressed cells in an MHC-independent manner, and consequently, may be exploited to overcome immunotherapy resistance. The butyrophilin (BTN) 2A1/3A1 heterodimer specifically activates Vγ9Vδ2⁺ T cells, the predominant subtype in peripheral blood. BTN2A1 directly binds to the Vγ9 chain of the γδ T cell receptor (TCR), but only activates the γδ T cell if phosphoantigen-sensing BTN3A1 forms a heterodimer complex with BTN2A1. To mimic BTN-mediated activation of γδ T cells, we generated bispecific γδ T-cell engagers (GADLEN) containing heterodimeric BTN2A1 and BTN3A1 extracellular domains (ECDs) fused via inert Fc linkers to scFv domains targeting a tumor antigen. We previously reported that in the presence of costimulatory signals from either a cytotoxicity receptor (NKG2D) or T-cell co-stimulatory receptor (CD28), GADLEN compounds activated Vγ9Vδ2⁺ T cells to facilitate antigen-dependent tumor cell killing. The specificity of γδ TCR/BTN interactions and dependence upon a secondary costimulatory signal suggests that GADLENs could be used to redirect Vγ9Vδ2⁺ T cells against hematologic and solid tumors, with a lower risk of off-target activation common with other bispecific engagers. Here, we report the functional characterization of CD20- and B7H3-targeting GADLEN compounds (BTN2A1/3A1-Fc-B7H3scFv) for targeting heme malignancies and solid tumors, respectively.

Methods: Specificity of CD20- and B7H3-targeting GADLENs were evaluated using MSD/ELISA and cell-based assays by flow cytometry. The functionality of the compounds to activate Vγ9Vδ2⁺ T cells and mediate killing of tumor cells was assessed *in vitro* in tumor coculture assays using flow cytometry and live cell imaging.

Results and Conclusions: CD20- and B7H3-targeting GADLENs bound to human cells expressing CD20 or B7H3 and to Vγ9Vδ2⁺ T cells with nanomolar affinity. GADLEN compounds activated Vγ9Vδ2⁺ T cells in *in vitro* coculture assays resulting in degranulation and apoptosis of CD20⁺ or B7H3⁺ tumor cells, respectively. Importantly, GADLEN treatments induced the secretion of pro-inflammatory cytokines suggesting the potential of both direct and indirect tumor killing mechanisms via additional immune cell subset activation and recruitment. Introduction of CD20-GADLEN into NSG-Tg(huIL15) mice engrafted with human PBMCs efficiently depleted human CD20⁺ B cells in the blood and spleen. These results provide proof of concept for *in vivo* manipulation of γδ T cells using antigen targeted GADLENs for the treatment of hematologic and solid tumor malignancies.

GADLEN Design & Mechanism of Action

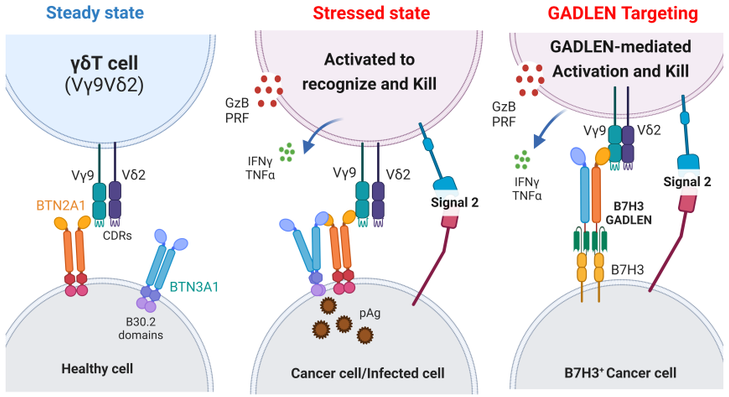

Figure 1. Heterodimerization of BTN2A1/BTN3A1 facilitates Vγ9Vδ2⁺ TCR engagement & T cell Activation.

- Vγ9Vδ2⁺ T cells are the predominant circulating γδ T cells, which respond to transformed cells by sensing elevated phosphorylated non-peptide metabolites, or phosphoantigens (pAg) produced by tumor cells
- B7-related membrane protein BTN3A1 (CD277) is responsible for direct pAg sensing and binding to initiate a conformational change in its extracellular domain and BTN2A1 interaction
- BTN3A1-associated BTN2A1 engages the Vγ9Vδ2⁺ TCR, leading to activation of effector functions
- Heterodimeric BTN2A1 & BTN3A1 ECDs fused with an Fc domain and B7H3-specific single chain variable fragments (scFv) form the gamma delta engager (B7H3-GADLEN) capable of activating Vγ9Vδ2⁺ T cells in the presence of co-stimulation (Signal 2)[1]
- In the presence of B7H3⁺ cancer cells, GADLEN molecules mimic natural BTN biology to bind and activate Vγ9Vδ2⁺ T cells leading to tumor cell killing mediated by degranulation and cytokine production
- Figure adapted from Herrmann et al., 2020 and Created with Biorender.com

[1] *J Immunol*. 2022 Oct 15;209(8):1475-1480.

In Vitro Characterization of B7H3-GADLEN

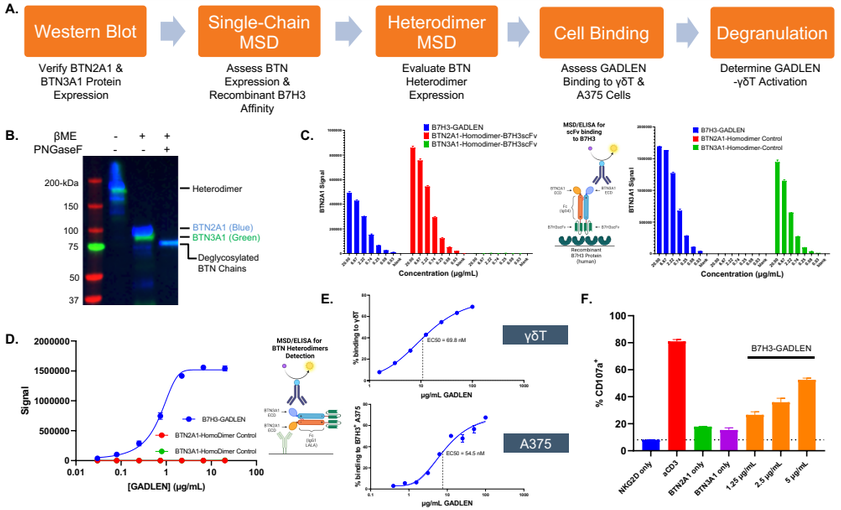

Figure 2. Characterization of the B7H3-targeting GADLEN Construct. (A) Schematic representing the work-flow and goals of GADLEN validation experiments **(B)** Western blot analysis of purified BTN2A1/3A1-Fc-B7H3scFv (B7H3-GADLEN) molecules **(C)** BTN-specific antibody-based assessments of recombinant B7H3 binding by GADLEN fusion proteins show BTN2A1 & BTN3A1 expression along with B7H3 binding **(D)** A dual antibody-based MSD method detects the formation of heterodimeric GADLEN fusion proteins **(E)** Binding of B7H3-GADLEN to B7H3⁺ expressed on A375 melanoma cells and Vγ9Vδ2⁺ T cells using flow cytometry **(F)** Vγ9Vδ2⁺ T cells were stimulated by plate-bound B7H3-GADLEN with anti-NKG2D for 4 hours. Proportion of cells expressing CD107a were determined by flow cytometry.

B7H3-GADLEN Facilitates Tumor Killing by γδT Cells *In Vitro*

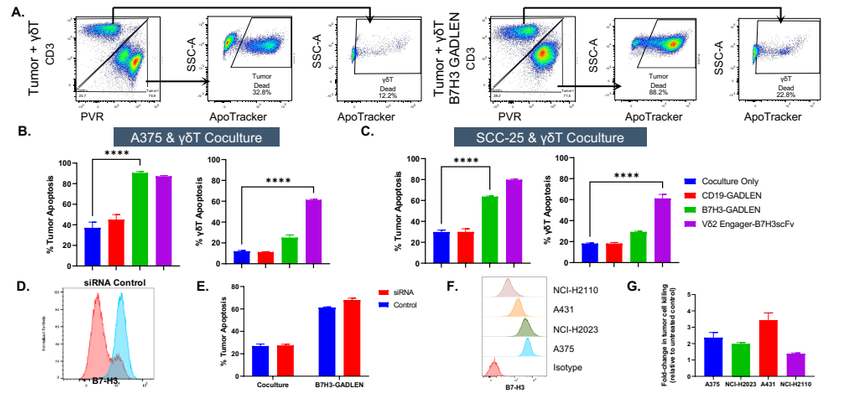

Figure 3. B7H3-GADLEN Activates *γδ* T Cells and Leads to Tumor Cell Killing. (A) Flow cytometry analysis of γδ T cell and A375 melanoma cell cocultures after 72h **(B)** Flow cytometry analysis of apoptotic A375 melanoma cells with CD19-GADLEN (Non-targeting control), B7H3-GADLEN, or B7H3-Vδ2 Engager for 72h **(C)** Flow cytometry analysis of apoptotic cells from *γδ* T cell and SCC-25 HNSCC cell cocultures treated as in (B) **(D)** B7H3 expression 48h after transfection with siRNA against B7H3 **(E)** Flow cytometry analysis of γδ T cell and A375 melanoma cell cocultures after 72h with or without siB7H3 pretreatment **(F)** A375, NCI-H2023, A431, & NCI-H2110 express varying levels of B7H3 **(G)** B7H3-GADLEN mediated killing across varying expression levels of B7H3

B7H3-GADLEN Induces Cytotoxic & Inflammatory Cytokine Production

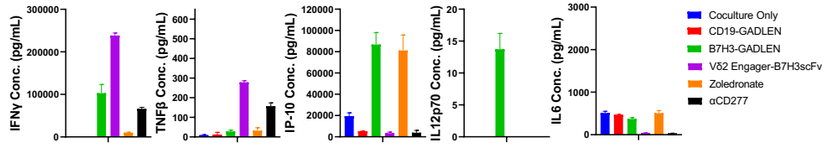

Figure 4. *In vitro* Cytokine Production from *γδ* T Cell & Tumor Cell Cocultures. *Ex vivo* expanded Vγ9Vδ2⁺ T cells were introduced to A375 melanoma tumor cells along with various *γδ* T cell-targeted molecules for 24h. Supernatants were harvested and analyzed for cytokine concentrations by MSD/ELISA.

CD20-GADLEN Enhances Lymphoma and B Cell Depletion by γδT Cells

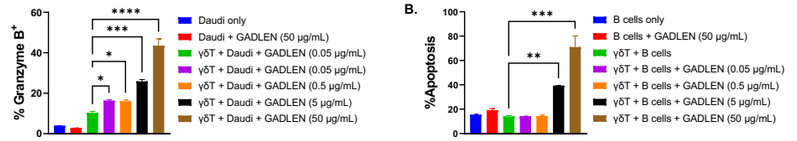

Figure 5. *In vitro* Killing of Tumor and Healthy B Cells by CD20-GADLEN. (A) Vγ9Vδ2⁺ T cells were cocultured with Daudi at a 1:1 ratio in the presence or absence of CD20-GADLEN for 1 hour prior to flow cytometry analysis of granzyme activity analyzed using the GranToxiLux assay kit (OncoImmunin) **(B)** Vγ9Vδ2⁺ T cells were cocultured with Daudi at a 2:1 ratio in the presence or absence of CD20-GADLEN for 24 hours prior to flow cytometry analysis of apoptotic cells by ApoTracker dye

CD20-GADLEN Drives B Cell Depletion *In Vivo* Using Humanized Mice

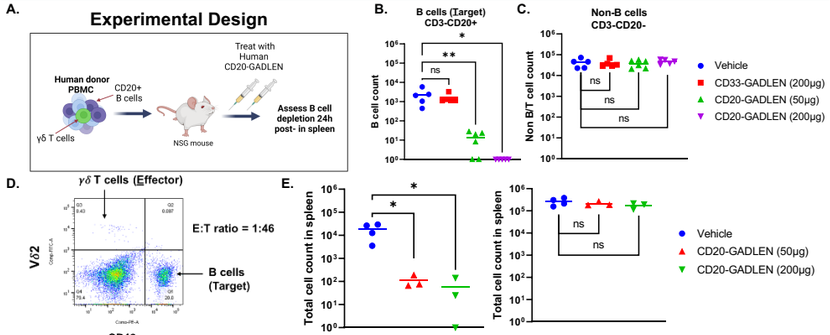

Figure 6. *In Vivo* Depletion of B cells by CD20-GADLEN. (A) Schematic representation of healthy donor PBMCs being introduced into NSG mice constitutively expressing hIL-15 (NSG-Tg(huIL15)) prior to GADLEN treatment by retroorbital injection **(B-C)** 5 days after PBMC engraftment in the presence or absence of CD33- or CD20-GADLEN treatments, spleens were harvested and analyzed for B and T cell numbers using TruCount beads and flow cytometry. The initial E:T ratio of γδT to B cells for this donor was 1:2 **(D-E)** Similar levels of B cell depletion were observed in a separate donor with an initial E:T ratio of γδT to B cells at 1:46

Conclusions

- Heterodimerization of BTN2A1 and BTN3A1 ECDs within the framework of a fusion protein scaffold including an antigen-targeting scFv activates γδ T cells *in vitro* and *in vivo* to kill target antigen positive cells including CD20 and B7H3
- The heterodimeric fusion proteins CD20-GADLEN & B7H3-GADLEN bind both the Vγ9Vδ2 TCR and CD20/B7H3 positive cell lines to facilitate *in vitro* cell killing and unique cytokine production compared to alternative mechanisms of Vγ9Vδ2+ T cell activation in coculture assays
- B7H3-GADLEN induced target cell killing is observed across varied levels of antigen expression in coculture assays across varied cell lines and siRNA treatment
- These data provide proof of concept for the further development and expansion of butyrophilin-based heterodimer γδ T cell engagers for the treatment of cancer and B-cell mediated inflammatory diseases