In Vivo Expansion of Gamma Delta (γδ) T cells by a CD19-Targeted Butyrophilin Heterodimer Leads to Elimination of Peripheral B cells

Suresh de Silva, George Fromm, Louis Gonzalez, Arpita Patel, Kyung Jin Yoo, Zachary Opheim, Robert Farmer, Dean Chamberlain, Anne Lai & Taylor H. Schreiber

Shattuck Labs, Inc. Austin, TX & Durham, NC

#688

Abstract

Background: 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, e.g., BTN2A1) directly binds the γδ TCR but is only activated if certain molecular patterns (e.g., phosphoantigens) facilitate recruitment of a second BTN (e.g., BTN3A1) into a complex to form a BTN2A1/3A1 heterodimer. The BTN2A1/3A1 complex specifically activates the predominant γδ T cell population in the peripheral blood, comprising the γ9δ2 T cell receptor (TCR), but does not activate the primary γδ T cell population in mucosal tissues, comprising the γ4 TCR. 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, with a lower risk of off-target activation common with CD3-directed T cell engagers.

Methods: Mouse BTNL1/6-Fc-CD19scFv and human BTN2A1/3A1-Fc-CD19scFv heterodimeric fusion proteins were purified and binding to CD19 or the respective γδ TCRs was assessed by ELISA, Octet and flow cytometry using γδ T cells isolated from human peripheral blood and mouse intestinal tissue. The functionality of the constructs to activate γδ T cells and mediate killing of tumor cells was assessed using live cell imaging *in vitro* as well as a murine B-cell lymphoma model *in vivo*.

Results and Conclusions: The CD19-targeting scFv domains of the BTN heterodimer fusion proteins bound to human and mouse CD19 with low nanomolar affinity. The BTN2A1/3A1-Fc-CD19scFv compound specifically bound to the Vγ9δ2 TCR on human γδ T cells while the mouse BTNL1/6-Fc-CD19scFv bound to γδ TCR on mouse T cells. Both compounds were able to activate γδ T cells in a co-culture assay resulting in degranulation and increased surface expression of CD107a and increased apoptosis of CD19+ tumor cells. Intraperitoneal administration of the mouse BTNL1/6-Fc-CD19scFv led to anti-tumor effects in A20 tumor bearing BALB/c mice. Phenotyping from BTNL1/6-Fc-CD19scFv treated mice revealed profound and rapid expansion of the endogenous γδ T cells in the circulation and tumor, with concomitant depletion of peripheral CD19+ B-cells, confirming the mechanism of action of the heterodimer as a γδ T cell specific engager. These results provide proof of mechanism for *in vivo* manipulation of γδ T cells using antigen-targeted butyrophilin heterodimeric fusion proteins for the treatment of cancer.

Gamma Delta T Cell Engagers / Mechanism of Action

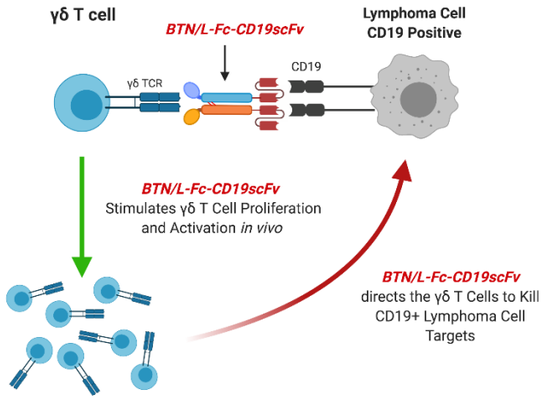

Figure 1. Mechanism of Action of Gamma Delta T Cell Engagers. The extracellular domains of butyrophilin (BTN) and butyrophilin-like (BTNL) proteins can be paired within the framework of a heterodimeric construct to enable activation of γδ T cells through the engagement of the TCR. The CD19-specific scFv domain of the engager allows for targeting CD19+ lymphoma cells for killing by the activated γδ T cell.

In Vitro Characterization of γδ T Cell Engagers

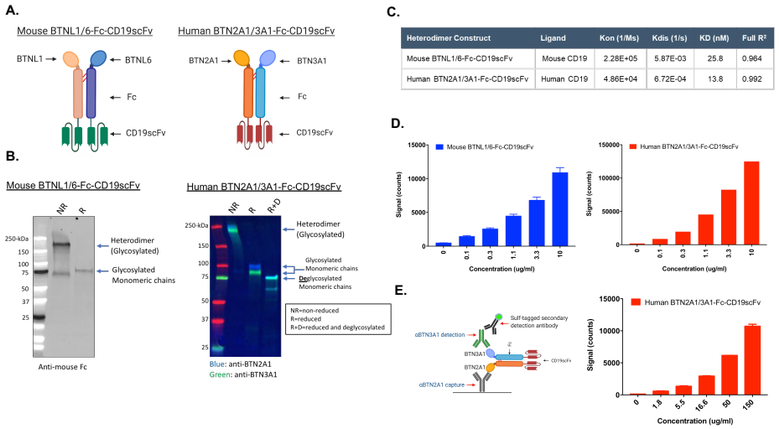

Figure 2. Characterization of Mouse and Human γδ T cell Engager Constructs. (A) Schematic representation of the heterodimeric (HD) fusion proteins showing the specific pairings of the extracellular domains of the BTN and BTN-like proteins and the CD19-specific single chain variable fragment (scFv) domain. (B) Single color and dual color Western blot analysis of the heterodimeric protein constructs. (C) Binding kinetics of the HD proteins to recombinant mouse and human CD19. (D) Meso Scale Discovery (MSD)-based ELISA methods were used to confirm binding of the HD constructs to recombinant CD19. The mouse HD was detected using an anti-mFc antibody and the human HD with a BTN3A1 antibody. (E) A sandwich-based MSD-based ELISA method was used to detect the presence of a heterodimer in the human construct by capturing with a BTN2A1 antibody and detecting with a BTN3A1 antibody.

Binding to Target Cell Receptors – CD19 and γδ TCR

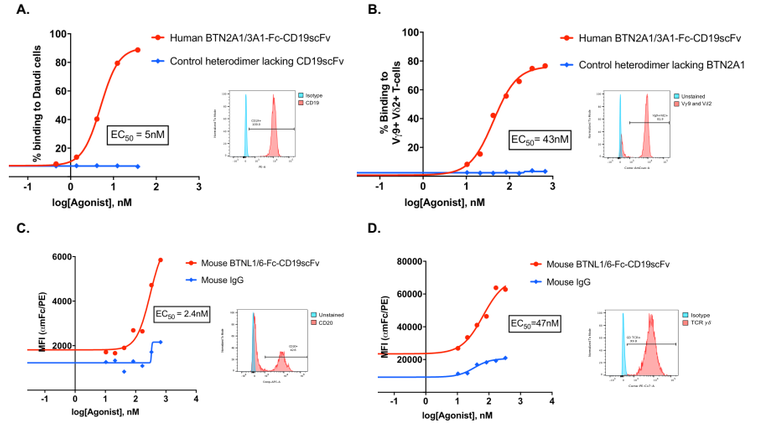

Figure 3. Binding of Human and Mouse γδ T Cell Engagers to Cellular Target Receptors. Flow cytometry analysis of, (A) Human BTN2A1/3A1-Fc-CD19scFv binding to CD19+ lymphoma (Daudi) cells; (B) Human BTN2A1/3A1-Fc-CD19scFv binding to human γδ T cells expressing the Vγ9Vδ2 TCR; (C) Mouse BTN2A1/3A1-Fc-CD19scFv binding to CD20+ B cells from splenocytes; and (D) Mouse BTNL1/6-Fc-CD19scFv binding to mouse γδ T cells expressing the γδ TCR. Human γδ T cells were isolated from healthy donor PBMCs and expanded for 14 days prior to use in the experiment. Mouse γδ T cells were isolated from the intraepithelial layer of BALB/c mouse intestines and immediately used in the experiment.

Cell Based Functional Assays (Mouse)

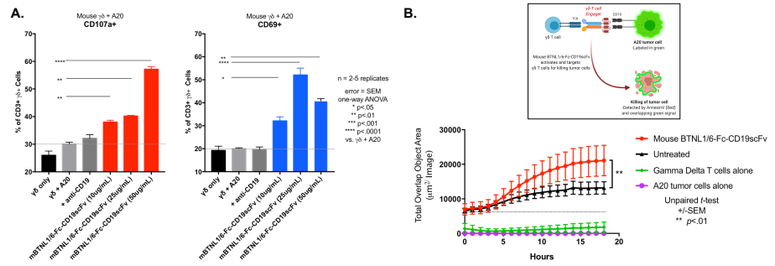

Figure 4. Mouse BTNL1/6-Fc-CD19scFv Activates and Degranulates γδ T Cells and Leads to Tumor Cell Killing. (A) Flow cytometry analysis of mouse γδ T cells following a 6 hour co-culture with mouse A20 lymphoma cells in the presence of increasing concentrations of the mouse BTNL1/6-Fc-CD19scFv. Degranulation and activation of mouse γδ T cells was detected using a CD107a and CD69 antibodies, respectively. (B) Incucyte based killing assay detects increased killing of A20 tumor cell by activated γδ T cells upon incubation with mouse BTNL1/6-Fc-CD19scFv.

In Vivo Assays (Mouse)

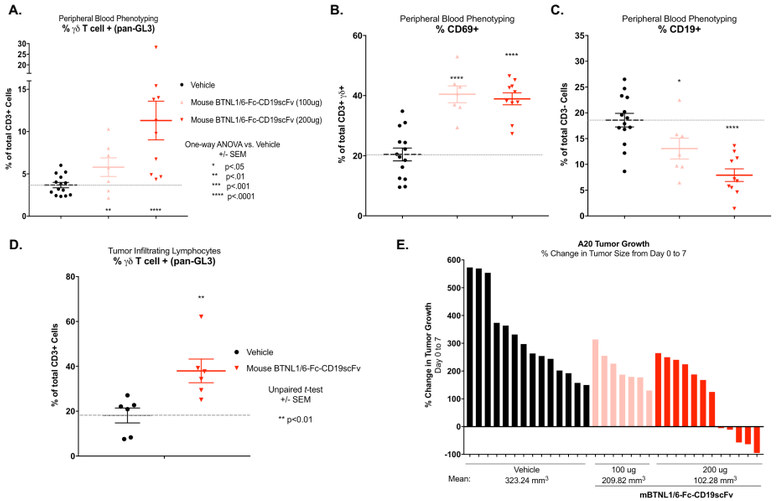

Figure 5. *In Vivo* Activity of Mouse BTNL1/6-Fc-CD19scFv. A20 tumor bearing syngeneic BALB/c mice were treated with three doses of either 100 or 200ug of mouse BTNL1/6-Fc-CD19scFv on days 0, 3 and 6, where day 0 was when the average tumor volume was ~80mm³. Peripheral blood from the treated mice was analyzed by flow cytometry on day 4, which was 24 hours following the 2nd dose, to detect (A) Peripheral expansion of γδ T cells; (B) Activation status of the γδ T cells; and (C) Levels of CD19 B cells. (D) Tumors were harvested on day 7 to assess the levels of γδ T cells among TILs. (E) All treatment groups were assessed for tumor growth inhibition on day 7 post treatment.

Conclusions

- Heterodimerization of the extracellular domains of mouse BTNL1 and BTNL6 within the framework of a fusion protein scaffold that includes a CD19-targeting scFv can activate mouse γδ T cells *in vitro* and *in vivo* to target the killing of lymphoma cells.
- The human heterodimeric fusion protein BTN2A1/3A1-Fc-CD19scFv, has also demonstrated its ability to bind both the Vγ9Vδ2 TCR and CD19.
- These data provide proof-of-concept for the further development and expansion of butyrophilin-based heterodimer Gamma Delta T cell engagers for the treatment of cancer.