Antigen-Specific Targeting of Tissue-Resident Gamma Delta T Cells with Recombinant Butyrophilin Heterodimeric Fusion Proteins

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Abstract

A major mechanism of acquired resistance to immune checkpoint inhibition involves downregulation of antigen presentation, including the major histocompatibility complex (MHC I) complex itself. Downregulation of antigen presentation on MHC I can render tumor cells invisible to alpha/beta T cell ($\alpha\beta$T) T cell directed therapies. Gamma/delta T cells ($\gamma\delta$T) are a small subset of the overall T cell compartment but are characterized by increased cytolytic capacity relative to $\alpha\beta$T cells. Rather than via MHC I, $\gamma\delta$T recognize target cells via a complex of heterodimerized butyrophilin (BTN) proteins. Thus, display of BTN heterodimers on the surface of tumor cells may enhance immunity to tumors that have downregulated MHC I, or which express low abundance or low affinity antigens.

We have previously reported the generation of a heterodimeric BTN protein targeting the CD19 antigen, referred to as BTN2A1/3A1-Fc-CD19scFv. While the BTN2A1/3A1 complex is a potent activator of $\gamma\delta$T expressing the $\gamma9\delta2$ T cell receptor (TCR), which is the major $\gamma\delta$T population in human peripheral blood, $\gamma9\delta2$ are not the predominant $\gamma\delta$T in many tissues. The murine equivalent of BTN2A1/3A1-Fc-CD19scFv, BTNL1/6-Fc-CD19scFv, stimulated specific proliferation and increased the cytolytic capacity of peripheral blood $\gamma\delta$T in mice, but not other tissue-restricted $\gamma\delta$T populations. These data suggested that distinct BTN heterodimers may preferentially activate tissue-restricted subsets of $\gamma\delta$T.

To characterize potential differences between peripheral blood and tissue-restricted $\gamma\delta$T, we performed a multi-layered analysis, including single-cell sequencing of $\gamma\delta$T TCR and CDR3 analysis from paired peripheral blood and tumor tissues from human cancer patients with melanoma, prostate, and colon cancer. These data identified tissue-specific enrichment of individual $\gamma\delta$T TCRs, with corresponding tissue-specific preferences for individual BTN proteins. Based on this information, we generated a panel of distinct heterodimeric BTN proteins, and show that specific $\gamma\delta$T populations are preferentially activated by specific BTN heterodimers in a lock-and-key fashion. These data are a prerequisite for designing $\gamma\delta$T specific therapeutics that may target both immune neglected and acquired resistant tumors that have limited response to $\alpha\beta$T cell directed approaches.

Methodology for Identifying $\gamma\delta$ TCR Sequences in Patient Samples

I. *Analysis of $\gamma\delta$ TCRs in TCGA tumors using the MiXCR framework*

TCR identification was assessed using MiXCR version 2.1.6 with custom R scripts (Bolotin 2015). IMGT TCR repertoire sequence reference imgt.201918-4.sv5.json from http://www.imgt.org/IMGTrepertoire was used to facilitate alignment of germline TCR γ and TCR δ genes and assembly of repertoires (Lefranc 2011). Bulk RNA-seq fastq files for four TCGA tumor types (COAD, DLBC, SARC and STAD) were used as input. To maximize sensitivity of capturing TCR γ and TCR δ alignments, the *align* function in MiXCR was run with the *assemblePartial* and *extend* options. Quantification of gene expression was generated using Salmon's quasi-alignment method (Patro 2017). Gencode GRCH38 v27 CHR transcripts were used to build the index used for Salmon (Frankish 2018).

II. *Single cell sorting and sequencing of $\gamma\delta$ TCRs from dissociated tumors*

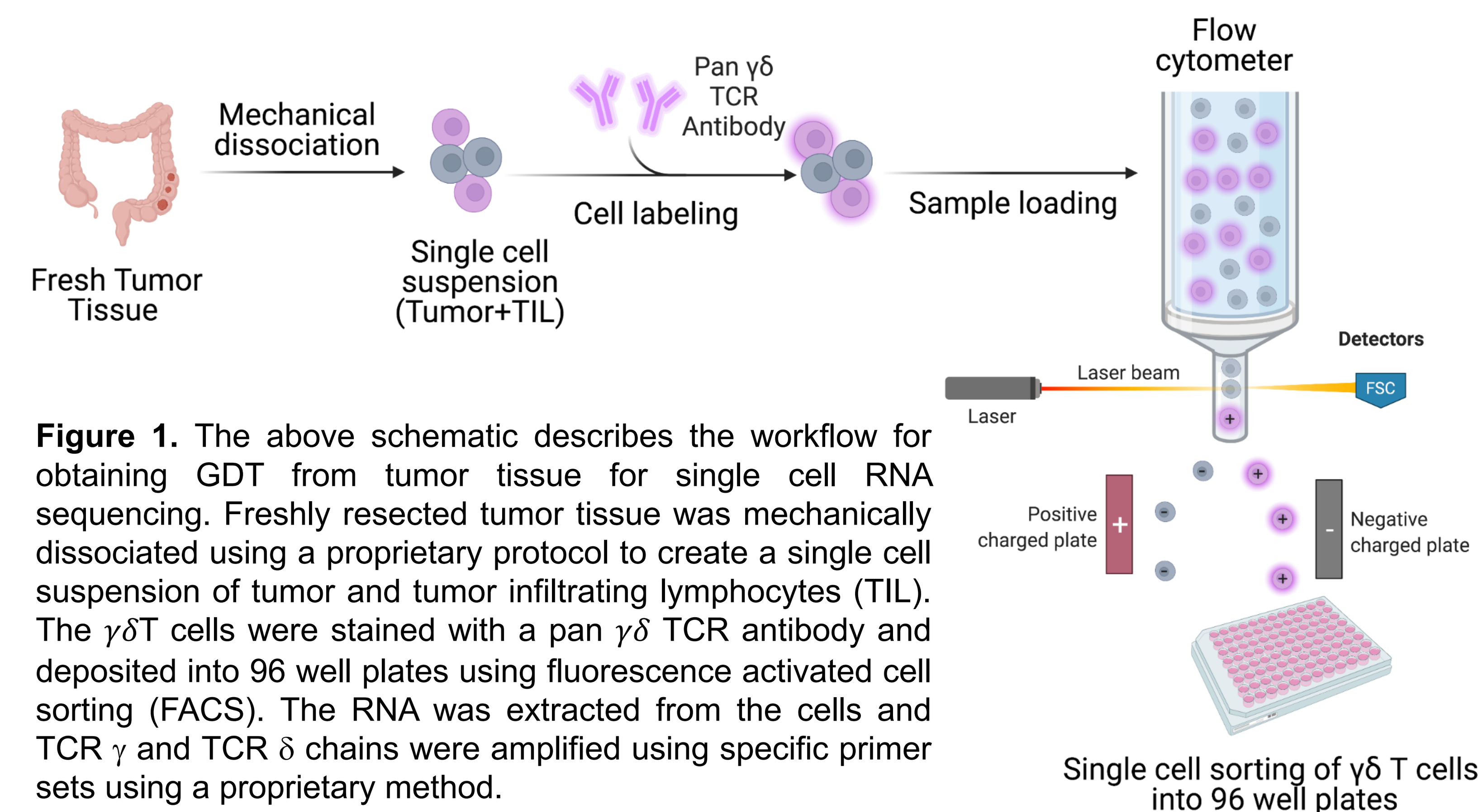


Figure 1. The above schematic describes the workflow for obtaining GDT from tumor tissue for single cell RNA sequencing. Freshly resected tumor tissue was mechanically dissociated using a proprietary protocol to create a single cell suspension of tumor and tumor infiltrating lymphocytes (TIL). The $\gamma\delta$T cells were stained with a pan $\gamma\delta$ TCR antibody and deposited into 96 well plates using fluorescence activated cell sorting (FACS). The RNA was extracted from the cells and TCR γ and TCR δ chains were amplified using specific primer sets using a proprietary method.

$\gamma\delta$ TCR (TRGV and TRDV) Composition in Select TCGA Tumor Types

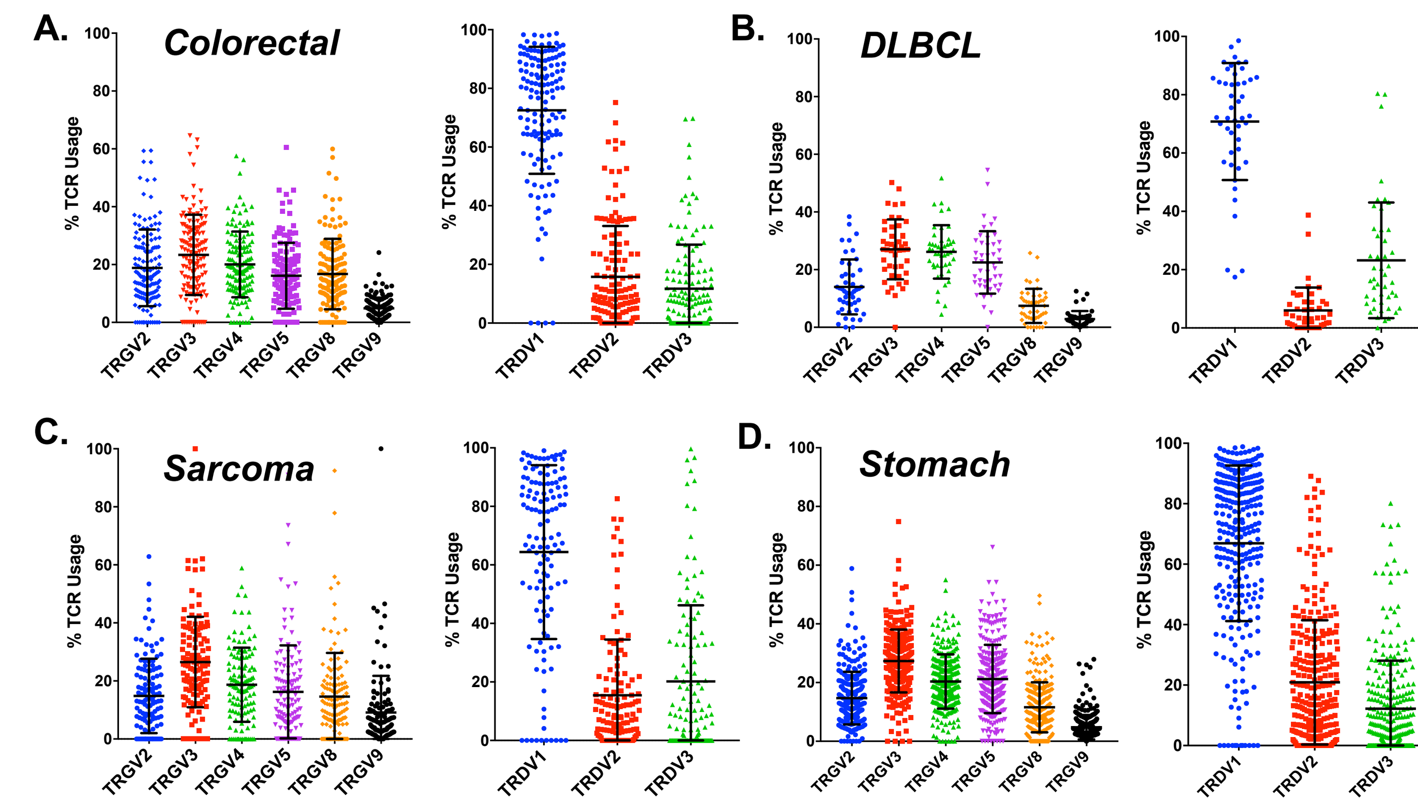


Figure 2. TCGA data sets for (**A**) Colorectal adeno-carcinoma, (**B**) Diffuse large B cell lymphoma (DLBCL), (**C**) Sarcoma and (**D**) Stomach adeno-carcinoma were analyzed for $\gamma\delta$ TCR usage using the MiXCR framework. Results revealed that all tumors expressed multiple Vγ chains with broad usage while Vδ chains were restricted to TRDV1, 2 and 3 with Vδ1 being the most prevalent across the four tumor types.

$\gamma\delta$ TCR Usage and CDR3 Diversity in Matched Tumor and Peripheral Blood in Colorectal Adenocarcinoma Patients Detected by Single Cell RNA Sequencing

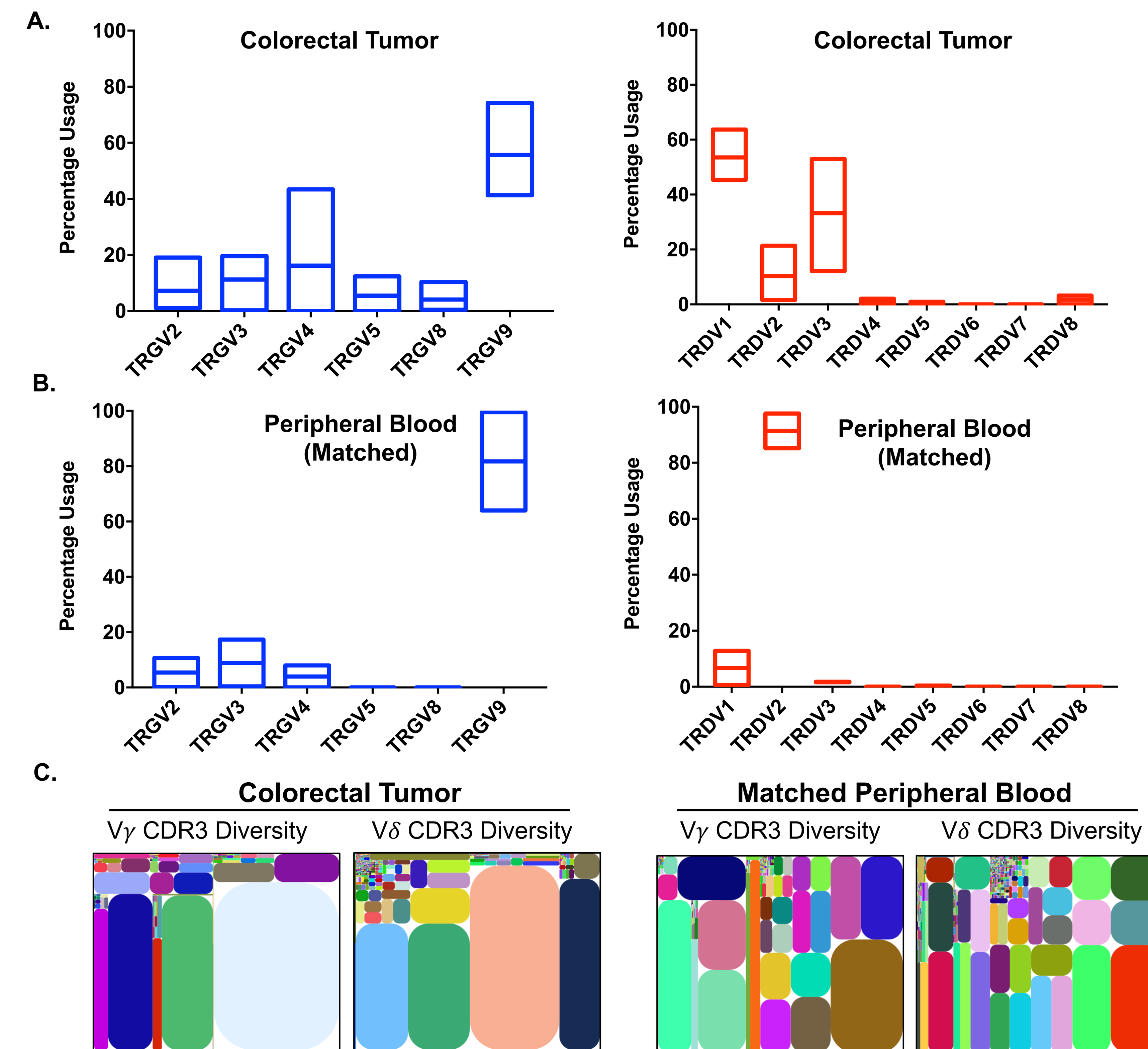


Figure 3. Single cell RNA sequencing of $\gamma\delta$ TCRs from (**A**) Colorectal tumors and (**B**) matched peripheral blood revealed a distinct difference in usage between the two compartments with tumor-derived $\gamma\delta$ T cells expressing multiple γ chains with TRGV9 and TRGV4 being most abundant. Colorectal tumor derived $\gamma\delta$ T cells also expressed more TRDV1 compared to peripheral blood $\gamma\delta$ T cells that predominantly express TRDV2. (**C**) CDR3 sequence analysis revealed a greater diversity in both the γ and δ chains in the peripheral blood compared to the tumor as depicted by the representative CDR3 tree maps from a single patient. Clonal expansion of $\gamma\delta$ T cells was observed in colorectal tumors when compared to peripheral blood.

Correlation of BTN/L and $\gamma\delta$ TCR Expression in Colorectal Adenocarcinoma

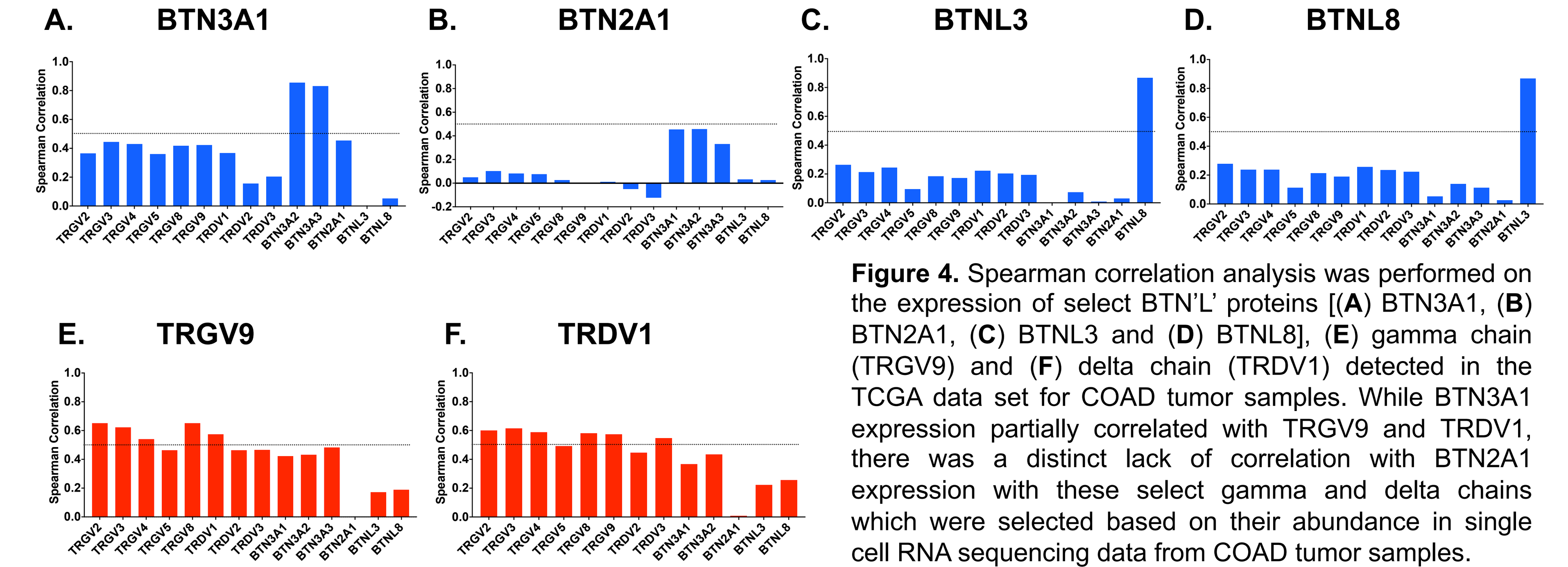


Figure 4. Spearman correlation analysis was performed on the expression of select BTN'L' proteins [(**A**) BTN3A1, (**B**) BTN2A1, (**C**) BTNL3 and (**D**) BTNL8], (**E**) gamma chain (TRGV9) and (**F**) delta chain (TRDV1) detected in the TCGA data set for COAD tumor samples. While BTN3A1 expression partially correlated with TRGV9 and TRDV1, there was a distinct lack of correlation with BTN2A1 expression with these select gamma and delta chains which were selected based on their abundance in single cell RNA sequencing data from COAD tumor samples.

Development of a Jurkat-76 Cell Line-Based In Vitro Assay to Screen BTN/L Pairs that Activate Specific $\gamma\delta$ TCRs Detected Using Single Cell RNA Sequencing

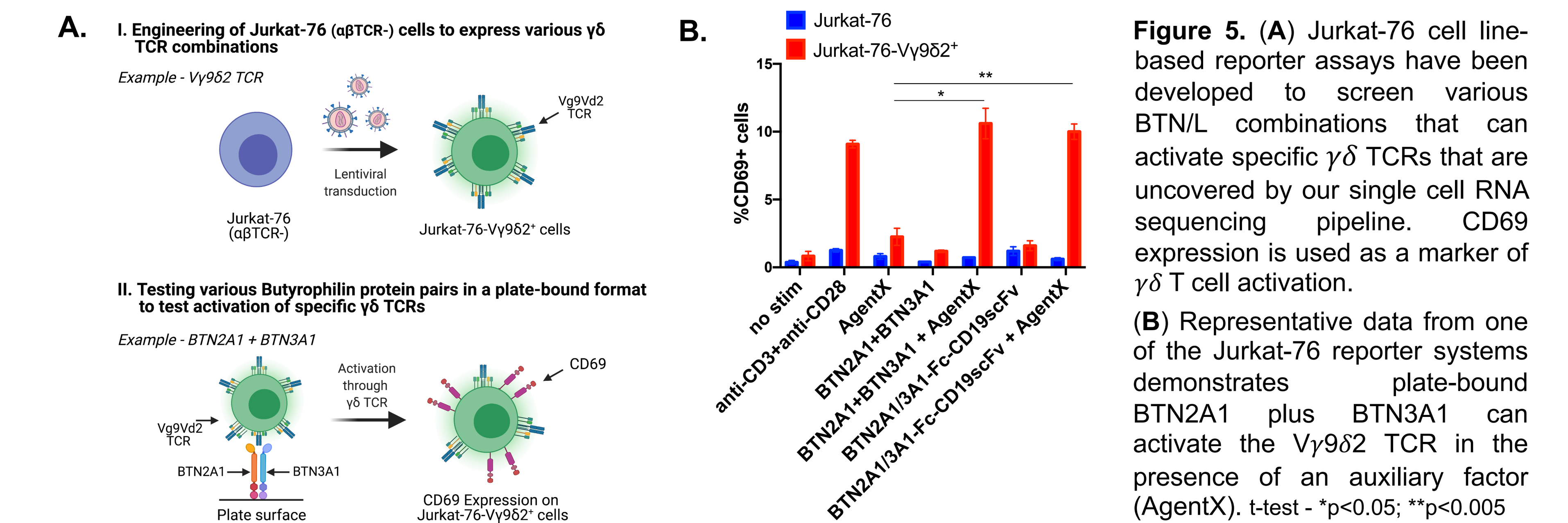
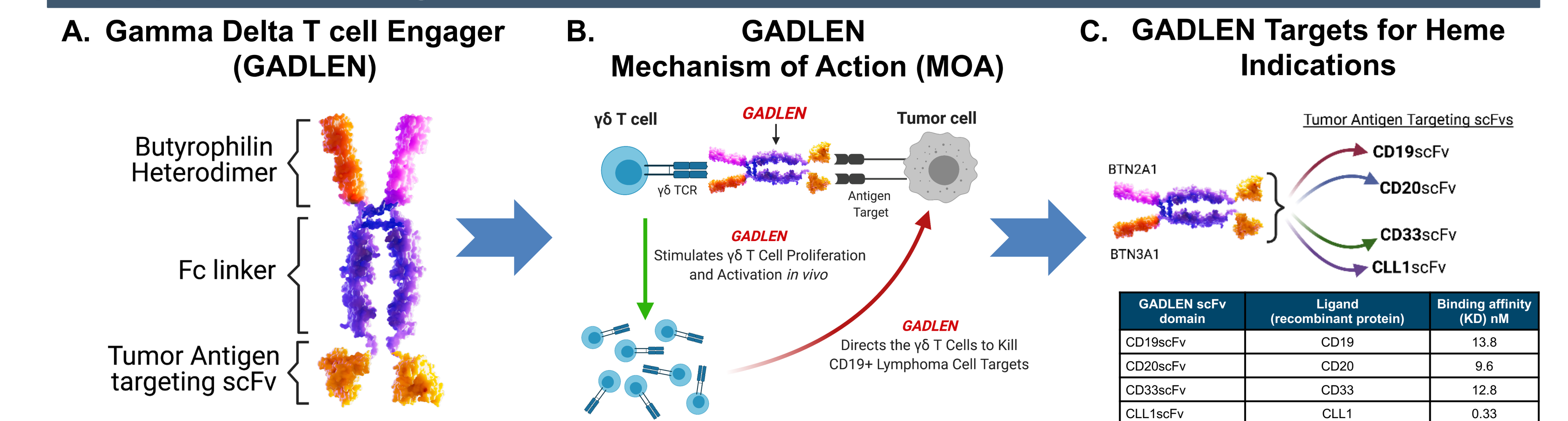


Figure 5. (**A**) Jurkat-76 cell line-based reporter assays have been developed to screen various BTN/L combinations that can activate specific $\gamma\delta$ TCRs that are uncovered by our single cell RNA sequencing pipeline. CD69 expression is used as a marker of $\gamma\delta$ T cell activation.

(**B**) Representative data from one of the Jurkat-76 reporter systems demonstrates that plate-bound BTN2A1 plus BTN3A1 can activate the V$\gamma9\delta2$ TCR in the presence of an auxiliary factor (AgentX). t-test - $^{*}p<0.05$; $^{**}p<0.005$

The GADLEN™ Platform Allows for the Generation of Therapeutics that Target Multiple Tumor Antigens



GADLEN scFv domain	Ligand (recombinant protein)	Binding affinity (KD) nM
CD19scFv	CD19	13.8
CD20scFv	CD20	9.6
CD33scFv	CD33	12.8
CLL1scFv	CLL1	0.33

Figure 6. (**A**) The GADLEN design consists of a heterodimer of butyrophilin (BTN'L') extracellular domains fused to a tumor antigen targeting scFv domain via an Fc linker. (**B**) The MOA of the GADLEN platform involves activation of the $\gamma\delta$ TCR through the BTN/L heterodimer domain and targeted killing of a tumor cell via the binding of a surface tumor antigen by the scFv domain. (**C**) Multiple GADLENs have been generated and are currently in preclinical development that target specific antigens expressed on lymphoma and leukemia cells (CD19, CD20, CD33, and CLL1). Binding of the select scFvs to their recombinant targets was confirmed by MSD-based ELISA.

Summary of Findings

- A combined approach of TCGA analysis using the MiXCR framework and single cell RNA sequencing was employed to better understand the V gamma chain (TRGV) and V delta chain (TRDV) usage, diversity, and the butyrophilin expression profile in solid tumors and lymphoma.
- This information is being used to screen specific BTN/L combinations and their ability to activate various $\gamma\delta$ TCR subtypes that are resident in specific tumor tissues, which will ultimately guide the design of targeted GADLEN therapeutics.
- We are currently developing multiple GADLEN therapeutics that include scFv domains that target specific tumor antigens that are highly expressed in hematologic malignancies and solid tumors.