Deciphering the Cellular Signals That Promote Vγ9Vδ2⁺ T Cell Proliferation and Tumor Cell Killing By a CD33 Antigen Targeted Gamma Delta T-cell Engager In Combination with Zoledronate

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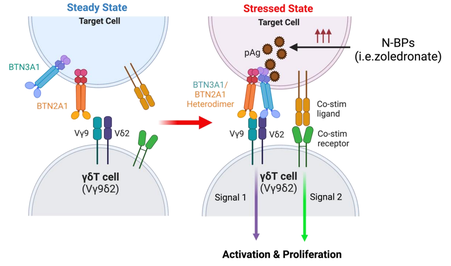
1. Introduction

- Vγ9Vδ2⁺ T cell (GDT) targeted immunotherapy is of interest to harness its MHC-independent cytotoxic potential to promote anti-tumor immunity.
- To date, clinical trials in cancer patients have failed to demonstrate monotherapy activity using a variety of GDT activating agents, including aminobisphosphonates such as zoledronic acid (ZA), Vδ2⁺ targeted T cell engagers and BTN3A1 targeted antibodies.
- It remains unclear whether GDT (1-5% of total T cells) are present in sufficient numbers, whether the agents in question are providing adequate GDT activation, and whether GDT are being directed to tumor cell targets.
- ZA is known to expand GDT by causing phosphoantigen accumulation in target cells, leading to BTN2A1 and BTN3A1 heterodimerization, which activates GDT. GADLEN, our engineered GDT engager, combines BTN domains with tumor-targeting scFvs. GADLEN has demonstrated the capacity for GDT activation, on-target tumor cell killing, and GDT proliferation.
- A CD33-targeted GADLEN (CD33-GADLEN) combined with ZA synergistically promoted GDT proliferation in human PBMCs without tumor cells, suggesting additional cellular signals exist beyond BTNs on tumor cells that promote ZA-mediated GDT proliferation.
- This study explores APC involvement and BTN-dependent and independent signals in ZA-mediated GDT proliferation.

2. Vγ9Vδ2⁺ T cell Activation by Phospho-Antigen Sensing Butyrophilins (BTN2A1/3A1)

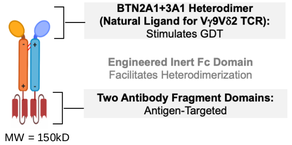


- GDT respond to transformed cells by sensing elevated phosphorylated non-peptide metabolites, or phosphoantigens (pAg) produced via the mevalonate pathway of cholesterol synthesis that becomes dysregulated in certain tumor cells.
- B7-related membrane protein BTN3A1, or CD277, expressed on target cells 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 proliferation and activation of effector functions.
- Aminobisphosphonates (N-BPs), such as zoledronate, can induce phosphoantigen accumulation in target cells or antigen-presenting cells (APCs), leading to Vγ9Vδ2⁺ T cell activation and proliferation.

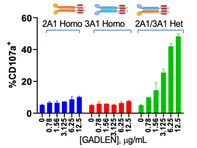
Figure adapted from Herrmann et al., 2020 and created with BioRender.com.

3. CD33-GADLEN: A BTN-Based Gamma Delta T Cell Engager Targeting the CD33 Tumor Associated Antigen

A. Design of CD33-GADLEN

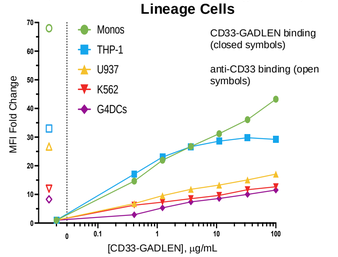


B. GADLEN Activates GDT Via BTN2A1/3A1 Heterodimers

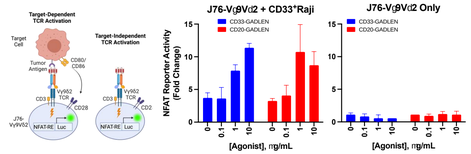


- GDTs were stimulated with plate-bound GADLEN containing BTN2A1 homodimers, BTN3A1 homodimers, or BTN2A1/3A1 heterodimers, in the presence of anti-NKG2D.
- Only the BTN2A1/3A1 engager led to GDT degranulation (%CD107a), demonstrating GADLEN's specificity in engaging Vγ9Vδ2 TCR through BTN2A1/3A1 heterodimers.

C. CD33-GADLEN Binding Correlates with CD33 Expression in Normal and Malignant Myeloid-Lineage Cells



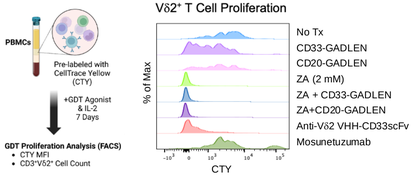
D. GADLEN Activates Upon Binding to Antigen Expressed on Target Cells

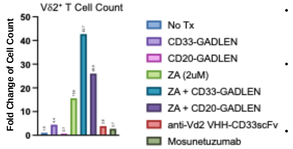


- J76-Vγ9Vδ2⁺ cells containing NFAT-Luciferase reporter were used for evaluating Vγ9Vδ2 TCR signaling. CD20⁺ Raji cells transduced with human CD33 was used as target cells to interrogate TCR activation by CD33- and CD20-targeting GADLEN.
- GADLEN promotes TCR activation only in the presence of target cells.

4. CD33-GADLEN Combined with Zoledronate (ZA) Enhances Vγ9Vδ2⁺ T cell Proliferation *in vitro* and *in vivo*

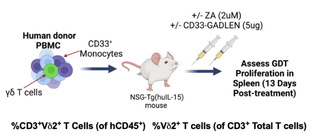
A. *In vitro* GDT Proliferation (PBMCs)

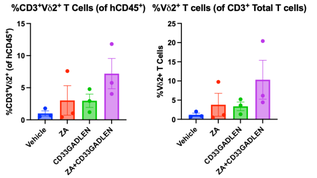




- ZA significantly enhanced GDT proliferation when combined with CD33-GADLEN.
- ZA + CD20-GADLEN also increased γδT cell proliferation, but less than CD33-GADLEN, suggesting CD33+ myeloid APCs play a role in this process.
- Anti-Vδ2 VHH-CD33scFv (high-affinity TCR binder) induced robust proliferation but didn't increase overall γδT cell counts, possibly due to activation-induced cell death.

B. *In vivo* GDT Proliferation (Human PBMC Engrafted NSG-Tg(hulL-15) mice)

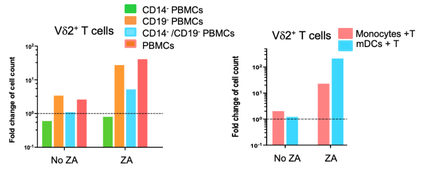




- NSG-Tg(hu-IL15) mice engrafted with human PBMCs were treated with ZA (2µM) +/- CD33-GADLEN (10 µg).
- Treatment with ZA + CD33-GADLEN resulted in increased Vγ9+Vδ2+ T cell counts in spleens of PBMC-engrafted mice.

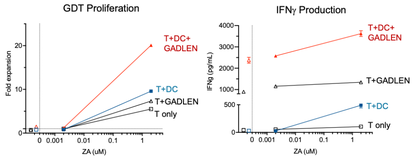
5. CD33-GADLEN Combined with Zoledronate (ZA) Promotes Robust Vγ9Vδ2⁺ T cell Proliferation via BTN-Dependent and BTN-Independent Mechanisms

A. ZA-Mediated GDT Proliferation is Dependent on Monocytes or Monocyte-Derived Dendritic Cells (mDCs)



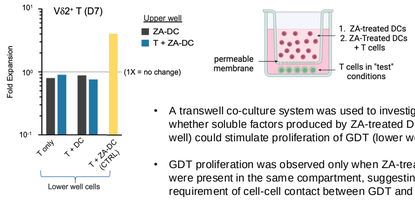
- PBMCs depleted of monocytes (CD14+) and/or B cells (CD19+) were stimulated with 2 µM Zoledronate (ZA) for 7 days to evaluate the role of APCs in GDT proliferation.
- Monocyte depletion inhibited ZA-induced GDT proliferation
- Purified T cells were co-cultured with monocytes or mDCs +/- ZA for 8 days to evaluate their role in GDT proliferation.
- Both monocytes and mDCs promoted ZA-Mediated GDT Proliferation

B. ZA Synergizes with CD33-GADLEN (BTN2A1/3A1) to Enhance GDT Proliferation but not IFNγ Production



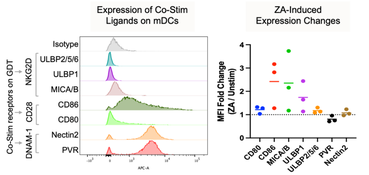
- Purified T cells +/- autologous mDCs were cultured with 5 µg/mL CD33-GADLEN and/or ZA for 6 days to evaluate GDT proliferation.
- Addition of CD33-GADLEN enhanced ZA-mediated GDT proliferation.
- IFNγ levels in 24-hour co-culture supernatants were measured by ELISA.
- CD33-GADLEN alone induced IFNγ production. Unlike cell proliferation, ZA did not further enhance cytokine production by GDT.

C. ZA-mediated GDT Proliferation Requires Cell-Cell Contact



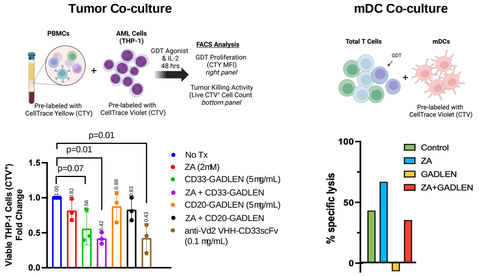
- A transwell co-culture system was used to investigate whether soluble factors produced by ZA-treated DCs (upper well) could stimulate proliferation of GDT (lower well).
- GDT proliferation was observed only when ZA-treated DCs were present in the same compartment, suggesting the requirement of cell-cell contact between GDT and APCs.

D. ZA Promotes Upregulation of Co-Stim Ligands on mDCs



- Expression of co-stimulatory molecules known to enhance GDT function via NK receptors and T-cell co-stimulatory receptors was assessed on mDCs (left panel)
- After 24 hours of stimulation, mDCs showed upregulation of CD86 and NKG2D ligands (right panel).

E. CD33-GADLEN + ZA Selectively Killed AML Cells While Preserving Viability of mDC



- PBMCs from 3 donors were co-cultured with THP-1 cells at a 10:1 ratio in the presence of GDT agonists for 48 hours.
- Zoledronate (ZA) + CD33-GADLEN promoted robust AML killing activity, despite the absence of GDT proliferation at this time point.
- Purified T cells were co-incubated with monocyte-derived dendritic cells (mDCs) at a 10:1 ratio. Cells were treated with ZA (2 µM) and/ or CD33-GADLEN (5 µg/mL) for 48 hours to evaluate potential mDC killing.
- Addition of CD33-GADLEN to ZA appeared to protect mDCs from GDT-mediated killing.

6. Conclusions

- Although both GADLEN and ZA stimulation activate GDT via heterodimeric BTNs, the combination of the two agents synergistically enhance GDT proliferation. This suggests that ZA induces additional co-stimulatory signal(s) in APCs.
- CD33-GADLEN + low-dose ZA combination demonstrates robust tumor killing and GDT expansion, showing promise for CD33⁺ AML treatment.