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Jamie Cohen April 9th, 2025

Vasoactive Intestinal Peptide Receptor Antagonists Potentiate Activation of Human T cells and Enhance Survival Rates in Murine Leukemia Models

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Abstract

Vasoactive Intestinal Peptide Receptor Antagonists Potentiate Activation of Human T cells and Enhance Survival Rates in Murine Leukemia Models

Acute Myeloid Leukemia is an aggressive cancer of hematopoietic progenitor cells in the bone marrow that spreads into the blood. Vasoactive intestinal peptide (VIP) is an immunoregulatory peptide that promotes cancer growth in solid tumors, including lung cancer, PDAC, and breast cancer. A subset of AML clinical cases over-express VIP, and the VPAC1 receptor is upregulated in T cells from AML patients. VIP reduces T cell proliferation *in vitro*, suggesting the VIP-signaling pathway may be an immune checkpoint in AML. Previous experimentation showed that inhibiting the VIP-signaling pathway with a VIP-receptor (VIPhyb) peptide antagonist enhanced the activity of anti-leukemic T cell responses in murine models of acute myeloid leukemia (Petersen et al., 2017). AlphaFold technology identified novel peptide sequences with high predicted binding affinity *in silico* to human VPAC1 and VPAC2 VIP receptors. We tested ANT308, a peptide antagonist of the VPAC-1 and VPAC-2 receptor with a positively charged N-terminus, and additional peptide sequences, for their ability to promote T cell activation *in vitro* and potentiate *in vivo* anti-leukemia activity in mouse models. We demonstrated that ANT308 enhanced the activation of CD4+ and CD8+ T cells and down-regulated expression of the PD-1, another immune checkpoint receptor, that is

targeted in treating solid tumor malignancies. Taken together, our data indicate that novel VIP-receptor antagonists can potentiate the activation of human T cells and anti-leukemia activity in mice. These data indicate novel VIP-receptor antagonists are promising candidates for drug development in leukemia and combined with other immune check-point inhibitors for treating solid tumors such as PDAC.

Vasoactive Intestinal Peptide Receptor antagonists ANT308 & Developing Enhanced Antagonistic Peptides to Potentiate Activation of Human T cells and Enhance Survival Rates in Murine Leukemia Models

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Introduction

Background

Acute myeloid leukemia results from mutations that arise early in the differentiation of myeloid cells, impacting the formation of granulocytes, monocytes, erythrocytes, and platelets. In comparison with other cancers, AML is difficult to treat due to its aggressive nature and low mutational burden with limited frequency of neoantigens.

Acute myeloid leukemia accounts for 15-20% of leukemia in children and 33% in adolescents and young adults, with the average age at diagnosis of AML 69 years old (Creutzig, 2019). In 2025, the American Cancer Society projects that 22,010 individuals will be diagnosed with AML and 11,090 individuals will die (ACS, 2025). Initial AML treatment aims to achieve remission, defined by the presence of less than 5% of blasts among total bone marrow cells and blood. Post-remission therapy may include an allogeneic bone marrow transplant procedure, which has significant morbidity. 66% of adult patients achieve remission after the first round of chemotherapy, leaving 33% of the diagnosed population requiring salvage treatment plans, including the AML cases in children and young adults.

Treatment History and Developments

Treatment plans for acute myeloid leukemia are determined based on the cell type affected, as blood cancers don't have typical stages of cancer. A large portion of AML patients have a gain of function mutation in the *DMNT1* (DNA Methyltransferase) gene,

which induces hypermethylation of cytosine residues in tumor suppressor genes, resulting in silencing of these genes and the proliferation of cancer. Treatment options include hypomethylating agents such as Azacitidine, which function by demethylating target sequences in tumor genes, enhancing the expression of cancer-associated antigens that are targets for T cell-mediated immunotherapy, including mutated genes that are involved in cancer cell growth and metabolism (Waller et. Funk, 2024).

The *FLT3* gene mutations are the most common gene mutation found in AML patients, making up 20-30% of total AML cases. Two types of mutations are prevalent in AML *FLT3* genes, one is a point mutation located in the tyrosine kinase domain (TKD) and the other an internal tandem duplication (ITD) found in the juxta membrane domain. Both these mutations lock the FLT3 protein receptor in its active confirmation, promoting proliferation and survival of tumor blasts.

Other treatments options include chemotherapy, targeted agents for specific mutations including *FLT3*, and bone marrow transplantation. However, chemotherapy doesn't work for all patients as mentioned above, and targeted agents such as anti-PD-1 treatment are promising but aren't always effective in inducing complete remissions for all patients. Bone marrow transplantation can be efficacious as an immunotherapeutic approach, but patients often struggle with Graft vs Host disease, in which the donors' immune cells detect the hosts cells as foreign antigens, mounting an immune response leading to morbidity and death.

In AML patients, common tumor microenvironments reveal high levels of T cell exhaustion, decreased T cell function and production of cytokines. Furthermore, the number of effector T cells in comparison to regulatory T cells shrinks drastically. All these factors lead to a subdued immune response by the host and provide an ideal environment for tumors to proliferate. The microenvironment functions as a positive feedback cycle: tumors proliferate and display markers on their surface or secrete peptides that silence immune cell activity, further creating an environment for the tumors to grow, producing more silencing proteins, leading to further expansion.

Single agent treatments targeting a receptor have been developed that have offered minimal success in treating acute myeloid leukemia. One approach to other Anti-PD-1 Treatments. PD-1 (CD279) is a receptor found on the surface of B cells, NK cells, dendritic cells, macrophages, and both CD4 and CD8 T cells (Sehgal, 2016). Its corresponding ligands are PD-L1 and PD-L2, which are transmembrane proteins located on antigen presenting cells. Observed in PDAC, tumor blasts overexpress PD-L1, proving to be a method of immune evasion leading to proliferation and metastasis. Furthermore, the Regulatory T cells (Tregs) have elevated levels of PD-1 receptor expression, linking tumor proliferation with high Treg accumulation and immunosuppressive activity. PD-1 and PD-2 receptor activation induces a signaling cascade that effectively terminates the activation response of both CD4 and CD8 differentiated T cells. Previous experimentation with antagonistic peptides developed by the Waller Lab have showed similar effects to anti-PD-1 treatment to PDAC, leading to the belief that VIP antagonists can serve as an effective anti-leukemic therapy.

Aims of Study

VIP is a neuropeptide found in the intestinal organs and the brain. It is 28 amino acids long and is secreted in myeloid and lymphoid immune cells. VIP induces an anti-inflammatory environment around tumors. T cells exposed to VIP fail to become activated, noted by the reduction of key cytokines indicative of T cell proliferation, including TNF-alpha and Il-2, and Interferon-gamma (Peterson et Al., 2017).

Approximately 30% of AML patients demonstrate over-expression of VIP (Liu, 2022). Previous experimentation from the Waller lab shows that blocking VIP-R receptor signaling with VIPhyb disrupts immune evasion through augmented T cell activation levels. Although VIPhyb displayed promise as an immunotherapeutic for AML, its low potency and binding affinity in preclinical studies has led to research centered around modifying the sequences of VIP-receptor antagonist peptides to identify sequences with increased binding affinity to the VPAC1 and VPAC 2 receptors and greater potentiation T cell activation for a longer duration of time. ANT308 was developed in response to the limitations of VIPhyb, and its stronger anti-leukemic effects has spurred the study of further alterations to the amino acid sequences of antagonistic peptides to identify the peptides with higher binding affinities to the VPAC1 and VPAC2 receptors and ability to bind for longer durations.

The study tested 5 peptides (ANT1201-ANT1204 and ANT308), developed with AlphaFold *in silico* modeling by Srijon Sarkar, an undergraduate student in Dr. Waller's lab. Various doses of each peptide were tested *in vitro* to determine the most effective dose range to potentiate the magnitude and duration of T cell activation *in vitro*. The

most effective peptides are chosen were then tested *in vivo* in murine models of leukemia to determine their effect on survival rates. The data collected from these experiments identify ANT308 as the most effective VIP receptor antagonist and ANT308 advances the studies of VIP antagonists as an effective immunotherapy in acute myeloid leukemia.

Results

CD3/CD28/CD2 Activator potentiates T cell activation.

CD3/CD28 Activator is a soluble protein containing antibody to the CD3, CD28, and CD2 proteins found on T cells. The antibodies act as an antigen presenting cell, interacting with the TCR (T cell receptor) complex on both CD4 and CD8 T cells. CD3 antibodies bind to the extracellular portion of the CD3 protein complex, resulting in the phosphorylation of tyrosine residues of alpha and beta ITAMs (immunoreceptor tyrosine-based activation motifs). However, TCR activation through the CD3 complex is not strong enough to elicit a full activation response. T cells require the inclusion of CD4 or CD8 proteins to complete the MHC-TCR complex, as well as co-stimulation through CD28 to elicit an activation response. Furthermore, IL-2 (Interleukin 2) is an essential cytokine that promotes T cell development and proliferation. IL-2 is produced by lymphocyte cells and binds to the IL-2R (IL-2 Receptor) on the surface of T cells. The intracellular ITAMs of the IL-2R become phosphorylated after the extracellular domain binds to its ligand, setting off the JAK-STAT signaling cascade, concluding with upregulation of pro-inflammatory genes and activation of anti-apoptotic cell pathways (R&D Systems, 2025). Once the TCR receives effective co-stimulation from CD28,

inclusion of the CD4 or CD8 proteins, and cytokine signaling via IL-2, the CD3 ITAMs phosphorylate LCK and ZAP70, inducing consecutive stimulations of proteins that result in secondary messenger generation of calcium ions and the Protein Kinase C-RAS pathway. Secondary messenger pathways result in the activation of transcription factors NFAT, AP-1, and NFKB, generating upregulation of IL-2 mRNA, which in turn matures into IL-2 peptides, expanding the number of activated T cells. Figure 1 C and E shows that T cells treated with 1.5uL/mL of T cell activator show significantly higher levels of CD69 than the sample not receiving T cell activation.

Overexposure to CD3/CD28/CD2 Activator Cocktail leads to hyperactivation, exhaustion, and apoptosis of T cells.

An *in Vitro* T cell activation assay was performed according to the procedure below using a submaximal level of CD3/CD28/CD2 T cell activator (1.5uL/mL) and 50 IU/mL IL-2 in place of 1uL/mL and 30 IU/mL respectively. Cells that are exposed to prolonged activation signals are subjected to an environment of intense activation signals, eventually leading to T cell exhaustion. Per Figure 3, the percentage of living lymphocytes reduced drastically in samples receiving CD3/CD28/CD2 activator. Furthermore, samples treated with the combination of T cell activator and an antagonistic peptide displayed even lower survival rates. This lower overall survival in groups treated with antagonists is due to higher activation levels than groups treated with CD3/CD28/CD2 activator alone. Figure 3 depicts the effects of overabundant exposure to T cell activator as well as the effect of an increased population size of T cells in the sample. The group termed “ACT”, the samples receiving 1.5uL/mL of CD3/CD28/CD2 activator, produced a population with a smaller living percentage than

untreated cells. Furthermore, cells treated with both the activator and with a high dose of 10um of ANT308 exhibited even lower levels of live cell percentage.

Pooling donor T cells post isolation is essential for reproducible T cell activation.

In experiments testing the effects of ANT308 on T cells from individual donors, the percent level of CD69 varied considerably in comparison to experiments performed with pooled T cells from individual healthy donors isolated from donor peripheral blood mononuclear cells (PBMC). Figure 8 shows individual donors represented by different colors per treatment group. Despite receiving the same antagonistic peptide, the CD4 positive T cells of some donors expressed significantly higher concentrations of CD69 than other donors. This data shows the importance of pooling human T cells directly after isolation and prior to treatment with CD3/CD28/CD2 activator and antagonistic peptides to generate more consistent levels of activation. The effect of pooling T cells averages out the variation of innate reactivity between donors, effectively standardizing the treatments groups.

ANT308 treatment potentiates activation of healthy donor T cells.

Figure 1 depicts the results of T cell activation experiment in which healthy donor T cells displayed higher levels of activation when treated with ANT308 at a dose of 3 micromolar. T cells were isolated and separated into CD4 positive and CD8 positive groups via flow cytometry and were measured based on their production of CD69, an early transmembrane marker for T cell activation, and Ki67, a nuclear protein found in

dividing T cells. Figure 1A shows the dose dependent effects of ANT308 in terms of Ki67 expression. Furthermore, when analyzed for CD69 expression, T cells receiving no treatment at all and incubated with IL-2 for 24 hours displayed the lowest level of CD69 for both CD4 and CD8 positive T cells with 11.0% of the CD4 population and 27.2% of the CD8 population expressing the CD69 protein. When treated with a CD3/CD28/CD2 T cell activator at a dose of 1.5um/mL and incubated for 48 hours, CD4 and CD8 positive T cells activation levels jumped to 26.7% and 43.6% respectively. Treatment with ANT308 at a 3um dose along with 1.0uL/mL of CD3/CD28/CD2 T cell activator produced the highest activation levels among CD4 and CD8 positive T cells with 32.5% of CD4 and 48.3% of CD8 T cells expressing CD69 above the threshold measured by the flow cytometer. Proteins of interest, such as CD69, are tagged with antibodies conjugated to fluorescent dyes, which when passed through the flow cytometer, emit wavelengths above a threshold to signify the expression of the protein of interest. Those cells expressing the correct wavelength and at a strong magnitude are quantified through gating strategies and are represented by the population of cells found within the gated boxes (Figure 1B and 1D). The difference between CD69 production is represented in bar graphs (Figure 1C and 1E) and show a significant increase across all three treatment groups, the highest being treatment with both ANT308 and CD3/CD8 T cell activator

ANT308 inhibits VIP cell signaling pathway, maintaining T cell activation in vitro.

The VPAC1/VPAC2 cell signaling pathway involves a G coupled protein that triggers the release of cyclic AMP, which downstream phosphorylates PKA and eventually CREB,

which enters the nucleus and induces anti-inflammatory gene transcription. Samples were treated with 10um of ANT308 and incubated for 30 minutes, followed by stimulation with VIP. A western blot assay was performed and protein expression based on exposure to VIP and VIP antagonists were analyzed. Figure 2 depicts that the groups treated with 10um of ANT308 exhibited significantly decreased levels of the phosphorylated CREB protein when stimulated with 1nm VIP and 10nm VIP, signaling that ANT308 successfully inhibits the binding of VIP to VPAC receptors in this *in Vitro* experiment.

ANT308 potentiates activation and cytotoxic activity in AML donor T cells.

Figure 10 depicts the results of the T cell activation assay in which pooled AML donor PBMC was cultured with IL-2, then isolated and treated with CD3/CD28/CD3 T cell activator and various doses of ANT308. Granzyme B molecules are tagged with antibodies conjugated to fluorescent dyes, which when passed through the flow cytometer, emit wavelengths above a threshold to signify the expression of the protein of interest. Those cells expressing the correct wavelength and at a strong magnitude are quantified through gating strategies and are represented by the population of cells found within the gated boxes. Flow cytometry results show that isolated T cells previously suppressed by an AML tumor microenvironment regained cytotoxic activity when stimulated with a T cell activator and treated with 1 and 3uM of ANT308. Figures 1B and 1D show significantly elevated Perforin expression in CD8 positive T cells and Granzyme B in all T cells, demonstrative of enhanced tumor killing activity.

PEGylation of antagonist peptides enhances T cell activation; Initial Testing of Fc-fusion ANT308 resulted in minimal enhancement of T cell activation.

PEGylation is the process of adding polyethylene glycol tails to proteins of interest. In this T cell activation assay, ANT308 at 3uM and 10uM was tested against ANT1200 (SJ_1) and ANT308 PEGylated at 3um and 10um. According to figure 7A, ANT308-PEG resulted in greater activation levels via CD69 production. PEGylation of a peptide increases its solubility in human blood as chemical structure containing alcohol groups proves it hydrophilic. PEG enhances the stability of therapeutics such as ANT308 as well, as proteolytic enzyme degradation is inhibited due to the chemical structure of PEG. Stability and solubility provide ANT308-PEG with greater activation levels than naked ANT308. Figure 7B and 7C depicts the results of a T cell activation assay in which pooled donor T cells were treated with T cell activator and varied doses of ANT308 or ANT308 fused to the Fc domain of a Human IgG antibody. This modification in theory enhances the half-life through its increased molecular weight and ability to bind to the T cell Fc-gamma receptor, avoiding lysosomal degradation of the drug. T cells were stained for antibodies signifying activation and upon analysis those treated with ANT308 at higher doses (1um through 10uM) had higher expression of both CD69 and 4-1BB than those treated with ANT308 Fc-fusion. However, the dose range of .001uM-.3uM revealed that T cells treated with low doses of Fc-fusion ANT308 outperformed low dose ANT308 treated T cells to a minimal degree based on expression of CD69 surface proteins. Notably in this experiment, ANT308 10uM and Fc-fusion ANT308 10uM did not result in activation-induced cell death as seen in previous assays.

ANT308 potentiates T cell activation to the highest degree while ANT1201 induces moderate levels of T cell proliferation via CD69 expression.

The *in vitro* T cell activation assay testing the efficacy of ANT308 and experimental peptides ANT1201-1204 at a dose range of .1uM, .3uM, 1uM, 3uM, and 10uM demonstrates the superiority of ANT308 as a VIP antagonist (Figure 5). The study of ANT1201-1204 and ANT308 in that apart from ANT308, ANT1201 yields the highest level of T cell activation in reference to production of CD69. Both ANT1201 and ANT308 differ from the VIP peptide in that at their C terminus, as they contain a six amino acid long fragment of the peptide neurotensin (amino acids 6-11). At physiological pH (7.4), this fragment will have a net positive charge of +3, while the first 6 amino acids at the C terminus of the VIP peptide is neutral with a net charge of 0. The positive C terminus of ANT308 and ANT1201 account for a stronger bind effect to both the VPAC1 and VPAC2 cell receptors. ANT1201 and ANT308 function as inhibitors of the G protein-coupled receptors and maintain constant levels of T cell activation. Tumors upregulate the production of VIP, however treatment with ANT308 and ANT1201, to some degree, reverse the anti-inflammatory effects and counteract tumor evasion mechanisms. Furthermore, ANT308 displays a dose dependent response between .1uM and 3uM and at 3uM, promotes T cell activation to the highest level. 10uM of ANT308 leads to T cell exhaustion and overall cell death. ANT1201 leads to moderate T cell activation, however not to the same degree as ANT308. The results influenced the decision to test ANT308 and ANT1201 in B6 murine models with C1498 tumors.

ANT308 potentiates T cell activation to a limited degree via intracellular protein expression.

Samples cultured in IL-2 treated with either no treatment, T cell activator only, or various doses of ANT308 or AN1201 were stained with antibodies which conjugate to the intracellular proteins Interferon-gamma, Granzyme B, and Perforin. As opposed to the nascent expression of CD69 on activated T cells, these proteins exemplify a further differentiated and activated T cell. Expression of these proteins were analyzed via flow cytometry, and it was determined that ANT308 enhances T cell activation to a limited degree over non-treated T cell samples. However, ANT1201 functioned as a VIP agonist, resulting in inhibited T cell activation signals (Figure 9).

Treatment with 100ug of ANT308 enhances survival in B6 leukemic mice, 20ug of ANT308 and ANT1201 enhances T cell activation

Three treatment groups of mice were inoculated with 100 uL of 10^6 C1498 Tumor cells on day 0 via intravenous injections. On days 7-17, these mice were treated with 20mg/day of either ANT308, ANT1201, or VIP scrambled (control). Mice were monitored daily for illness and or death and survival rates were calculated for 30 days past initial tumor inoculation. On day 18, male mice from each treatment group were bled and their T cells were stained for flow cytometry analysis of surface-level proteins. Flow cytometry analysis demonstrates that ANT1201 led to the most enhanced level of 4-1BB expression while ANT308 had a moderate increase in 4-1BB expression in comparison with the VIP scrambled control group (Figure 6C). Although there is no significant increase in survival rate of mice treated with either antagonistic peptide (Figure 6B), a 100mg dose of ANT308 proves to enhance survival in leukemic mice (Wang, 2024). The

prior Waller lab leukemic murine experiments started subcutaneous injections on day 6 and had varying schedules of treatment. The enhanced survival of ANT308 treated leukemic mice paired with the increased 4-1BB expression in ANT308 and ANT1201 treatment mice demonstrate the efficacy of peptide antagonist treatment in leukemic murine models.

Discussion

Summary of Results

The results of the *in vitro* T cell activation demonstrate that ANT308 is an effective antagonistic to VIP receptors and potentiates the inflammatory responses of CD4 and CD8 positive T cells. In comparison with treatment with only a CD3/CD28/CD2 T cell activator, T cells treated with ANT308 expressed higher levels of activation via CD69, an early signal of T cell expansion and proliferation. At all doses (.1um-3uM) ANT308 induces proliferation to a greater degree than ANT1201, via higher intracellular protein expression. However, treatment with other cytokines such as IL-12, an increased dose of T cell activator, or a longer incubation period with antagonist peptides may show significant results. T cells treated with a dose of 10uM ANT308 displays lower levels of live T cells as well as lower levels of intracellular and extracellular activation proteins in comparison with ANT1201 10uM. This may be interpreted to mean that at a high enough dose, ANT308 is far more potent than ANT1201, causing activation induced T cell death. Therefore, such results suggest that ANT308 a more potent antagonist of VPAC1/2 and the VIP signaling pathway.

The inhibitory effect of VIP signaling is further supported through the results of this study. VPAC1 and VPAC2 are G protein-coupled receptors, that when bound to their ligand undergo a change in conformation and the alpha subunit dissociates from the beta-gamma trimer, eventually prompting the activation of adenyl cyclase, the production of cAMP, and the entrance of transcription factors into the nucleus that subdues an inflammatory response. T cells treated with ANT308 followed by VIP stimulation had lower levels of phosphorylated CREB protein, providing evidence that ANT308 affects signaling down-stream of the VIP receptor. This confirms that VIP is involved in regulating T cell activation and supports the potential for VIP receptor antagonists as novel Immunotherapeutics for various cancers.

Furthermore, we've determined the effects and limitations of using CD3/CD28/CD2 T cell activator to model physiologic T cell activation. *In vivo* AML models, T cells become activated through interactions with antigens presented by tumors. However, tumors aren't present in these *in vitro* assays, so CD3/CD28/CD2 T cell activator is used to mimic an MHC antigen presentation to the TCR of the T cells. Furthermore, ANT308 cannot activate T cells itself, it can only enhance the already activated T cells, justifying the need for cytokines such as IL-2 to induce T cell proliferation and CD3/CD28/CD2 to induce T cell activation.

In addition, CD3/CD28/CD2 T cell activator promotes T cell growth and proliferation at the ideal dose, however when T cells are signaled too strongly, they divide too quickly (using high doses of antagonistic peptides and CD3/CD28/CD2 T cell activator). When

these cells receive further potentiation of activation through suppression of the VIP-VPAC anti-inflammatory mechanism, exhaustion ensues, and T cells die. The procedure for future assays was modified to 1.0uL/mL of CD3/CD28 activator versus the manufacturers recommended dose of 25uL/mL to account for the high level of cell death found in this previous experiment. I interpret these data to suggest that, in this *in vitro* model system, VIP is produced by T cells as a survival response to prevent activation-induced cell death. In normal T cell physiology, T cells activated in the context of environmental infections would be similarly regulated. Once a T cell is activated, it secretes VIP into the local extracellular environment to prevent autoimmunity and activation-induced cell death in settings where inflammation has been sufficiently reduced, and the T cell response is not essential. Tumors take advantage of this negative feedback mechanism present in T cells and upregulate secretion of VIP to evade a T cell immune response.

We also studied the effects of culturing T cells with proliferation-inducing cytokines. IL-2 is added in the *in* vitro assay system as a necessary cytokine for T cell survival and activation. However, other cytokines including IL-12, IL-17, may be useful in promoting Th1/Tc1 immune polarization of T cells to express cytokines and effector molecules associated with T cell killing of tumor targets including expression of intracellular proteins (Perforin, Granzyme B, Interferon-gamma) to elicit not just T cell proliferation but cytotoxic activity as well. In the study, samples treated with only The CD3/CD28/CD2 activator and IL-2 displayed no real enhanced cytotoxic effects in comparison with non-treated samples.

Although ANT1201-ANT1204 had higher predicted binding affinities than ANT308, the *in vitro* T cell activation assays show that ANT308 is a superior VIP antagonist. It is still of interest to study altered amino acid sequences compared to ANT308 to ensure that there is no stronger peptide, however, it appears that AlphaFold technology is limited in its ability to predict *in vitro* efficacy.

Furthermore, alterations to chemical structure may prolong the *in vivo* half-life of ANT308 including PEGylation and fusions of the ANT308 peptide to the Fc fragment of IgG4. Results from the T cell activation assay comparing T cells treated with PEGylated ANT308 and ANT308 demonstrate enhanced pharmacokinetics and pharmacodynamics of the drug *in vivo* and *in vitro*. The increase in size due to additions of PEG to antagonistic peptide increase the half-life of the drugs through increasing solubility in blood as well as preventing renal clearance and enzymatic degradation. Furthermore, the addition of an Fc domain of an IgG antibody to an antagonistic peptide enhances half-life because of its increased molecular weight similarly to PEGylated peptides, however its enhanced binding capabilities to the Fc-gamma receptor on the T cell surface, promoting rescue of lysosomal degradation through the FC-gamma receptor mediated recycling mechanism (Unverdorben, 2015).

Previous research conducted in the Waller lab shows that ANT308 has a higher survival percent in leukemic mice, demonstrating the efficacy of ANT308 in an animal model (Wang, 2024). Mice treated with 100ug daily, twice a day, and every other day

demonstrated significantly increased survival rates in comparison with mice treated with 100ug twice a day VIP scrambled. However, in the *in vivo* survival experiment in which mice receive 20mg of peptide antagonists daily for 10 days (Day 7 through Day 17), neither ANT308 nor ANT1201 resulted in enhanced survival in comparison with scrambled VIP. However, when the male mice were bled on day 17, flow cytometry analysis of 4-1BB expression revealed that mice treated with 20mg of ANT308 and ANT1201 demonstrated enhanced expression of 4-1BB. In future *in vivo* leukemic mice models, it will be beneficial to use higher doses of peptide antagonists to enhance the survival rate and to start the treatment at least one day earlier. Furthermore, survival rates may be further enhanced by chemical modifications such as PEGylation and Fc-fusion.

Implications

This study demonstrates that out of the peptides tested, ANT308 remains the optimal choice for further pharmacokinetics testing and eventual clinical trials as an immunotherapeutic. AML is a difficult disease to treat as single mutations vary greatly among patients which is why single agent treatments are often ineffective as a general treatment method. Immune checkpoint therapies target specific receptors on immune cells, and peptide antagonists such as ANT308 can ideally cure a wider range of AML patients than ever seen before. The enhanced pharmacodynamics of ANT308 represent a powerful treatment against tumor evasion in AML while the enhanced pharmacokinetics of PEGylation and Fc-fusion increase the half-life of the drug, in effect improving the efficiency and safety of drug administration. Advancements in the

pharmacodynamics and pharmacokinetics through peptide sequence modification and post translational modifications model an optimistic landscape for the future of all cancer treatments, including AML.

In addition to studies demonstrating enhanced T cell activation through peptide antagonists, experiments in the Waller lab show developments in Chimeric Antigen Receptor T-cell (CAR-T) therapy with potential to treat a wide range of patients regardless of their individual mutational burden. Antigens commonly found on tumors blasts can provide valuable information in the design and development of chimeric protein receptors. Genes that code for these receptors are added to the genome of the T cells and will constituently produce receptors with high binding affinity to the tumor antigens. Techniques such as AlphaFold used in this study may prove effective in identifying high binding affinities for chimeric receptors despite its inability to identify effective receptor antagonists based on binding affinity predictions. Furthermore, T cell activation assays and murine leukemia models used in the study designed to test ANT308 and ANT1201-ANT1204 may be effective in testing CAR-T cell therapy to treat AML.

Future Directions

The small number of experiments performed and the limited replicants per treatment group exhibit the limitations of this study. Furthermore, technical problems of *in vitro* assays can induce unreliability not found as frequently as *in vivo* experimentation.

Proposals for future assays to provide increasingly significant data on the effectiveness of ANT308 as a peptide antagonist are detailed below.

Although isolated T cells through a PAN T cell isolation technique demonstrate enhanced T cell activation, it's of interest to test the effects of the newly developed antagonistic peptides on separately isolated CD4 and CD8 T cell subsets such as Tscm, Treg, and naïve T cells. This data will provide information on which T cells respond best to which peptide antagonists, and the specific proteins exhibited by CD4 versus CD8 T cells.

Co-culture assays of *in Vitro* of T cells with monocytes and/or natural killer (NK) cells may also provide additional information on advanced mechanisms of interaction between these different cell populations. Macrophages are antigen presenting cells that present the antigens obtained from phagocytosis on their MHC complex and initiate a T cell response via connection to the CD3 T cell Receptor complex. However, there may be more ligand-receptor interactions at play including VIP-VPAC that suppress immune responses to leukemia and other cancers that are targetable with VIP-Receptor antagonists. NK cells bridge the innate and adaptive immune system, functioning as cytotoxic cells as well as activators of T cells and other adaptive cell types through antigen presentation. Furthermore, in lieu of healthy donor PBMC, expanded studies testing T cell activation assays and co-cultures with monocytes and NK cells using AML patient PBMC would provide a better model for an *in vitro* assay.

Further *in vivo* assays with B6 mice in which examinations post-mortem of organs such as the spleen could provide useful information on the effects of the antagonistic peptides on various immune cells. These assays can provide information on the relative levels of naïve to differentiated or exhausted T cells circulating in the lymphatic system. Relative proportions of monocytes, T cells, NK cells, and other immune cells could display different responses to acute myeloid leukemia. Such data can lead to the study of how varying immune responses can lead to increased survival rates and which peptides and which chemical modifications to such peptides result in the strongest anti-leukemic response.

The assays described in this section will test varying peptides based on amino acid sequences as well as the chemical modifications such as PEGylation and Fc-fusions, providing information on the peptide and modifications with the highest level of pharmacokinetics and pharmacodynamics. Further Fc-fusion testing is necessary to understand the efficacy of Fc Fusion peptides at low treatment doses.

Materials and Methods

In Vitro T cell Isolation, Staining, Flow Cytometry:

PBMC cryo-samples were rinsed with PBS and rested overnight at 37°C in RPMI+10% FBS+50IU/ml IL-2. T cells were isolated from PBMC using human pan-T cell, CD4, and CD8 isolation kits, according to the manufacturer's protocol (Miltenyi Biotec, Bergisch

Gladbach, Germany, Catalog No. 130-096-535). Outside of a non-activated group serving as the negative control, Isolated T cells from healthy donors were pooled and seeded at a density of 1 × 106/mL in 100 uL media in round-bottom wells in a 96-well plate, activated with a 1uL/mL CD3/CD28/CD2 T cell activator (ImmunoCult, San Diego, CA, USA) in the presence of 30 IU interleukin 2 (IL-2). Pooled T cells were activated in the presence or absence of VIP-ANTs and cultured for 48 h. Leukocyte Activation Cocktail with Golgi Plug+PMA (BD, Franklin Lakes, NJ, USA) was added 4 hours prior to cell harvesting to assess CD69, Ki67, Granzyme B, Perforin expression in CD4+ and CD8+ T cells. Briefly, cells were stained with Fixable Aqua live/dead viability stain (1:100 dilution) for 5 min at room temperature (RT). Surface antibodies were added to the cells at the desired concentration and left to stain for 30 min at 4 °C. Following surface staining, cells were subsequently fixed and permeabilized for intracellular staining. Antibodies targeting Ki67, Granzyme B, Perforin, and Interferon-gamma were added and left to stain for 45 min at Room Temperature. List-mode files from stained samples were acquired on a five-laser Aurora cytometer (Cytek Biosciences, Inc, Fremont, CA) and analyzed using FlowJo software (Tree Star, Inc). Analysis was performed on FlowJo and Prism.

Western Blot:

T cells from healthy donors were first isolated and pooled, then cultured in complete RPMI containing 0.5% fetal bovine serum overnight. T cells were incubated at 37ºC after treatment of 10um ANT308 for 30 min followed by stimulation with VIP for 15 min. T cells were washed twice with ice-cold 1X PBS and lysed with ice-cold RIPA (R0278,

Sigma) containing 1X protease inhibitor cocktail (P8340, Millipore Sigma) and phosphatase inhibitors (P2850, Millipore Sigma). Lysates were quantified by Bradford assay (BioRad), normalized for concentration, denatured with 1XSDS sample buffer. 40 ug of protein per sample was resolved by SDS-PAGE, blotted on PVDF membrane, and probed with primary antibodies. The images were acquired using BioRad ChemiDoc™ Touch Imaging System with Image Lab™ Touch Software.

PBMC, Cell lines and Mice:

Healthy donor PBMC leukapheresis products were obtained from Stem Cell Technologies. De-identified PBMC from leukemia patients were provided by the Emory Winship Cancer Institute Cancer Tissue and Pathology Core. The C1498-myeloid leukemia cell line was obtained from ATCC. Dr. Bruce Blazar (University of Minnesota) provided the C1498-luciferase+ cell line C1498ff. Dr. Marcel van den Brink (City of Hope, Los Angeles) provided the P815 luciferase+ mastocytoma. DBA/2, C57BL/6 (CD45.2), B6 SJL (CD45.1), B6 albino (CD45.2), and B6 lucificerase+ mice (49), B6-L2G85, JAX stock #025854) were purchased from Jackson Laboratory (Bar Harbor, Maine). The mouse colonies were maintained at the Emory University Division of Animal Resources facilities. Both male and female mice were 8-10 weeks old.

Mice Leukemia Cell Injection:

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. On day 0, B6 mice (15 female and 15 male) were injected by tail vein injection (i.v.) with 200uL of 1 x 10^6 C1498 cells.

ANT308 and ANT1201 Administration:

Mice inoculated with C1498 Tumors were monitored for a week. On day 7, subcutaneous injections commenced for a duration of 10 days with daily subcutaneous injections of 20mg ANT1201, ANT308, or Fully scrambled VIP.

Conflict of Interest:

Drs. EK Waller, JM Li, Y Li, and T Passang are co-inventors of technology related to VIP antagonists licensed to Cambium Oncology, a study sponsor. Dr. Sen-Majumdar was formerly Chief Scientific Officer of Cambium Oncology. Dr. EK Waller co-founded and is chairman of Cambium Oncology. Dr. N Papadantonakis is on the scientific advisory board of Cambium Oncology. The terms of this arrangement have been reviewed and approved by Emory University in accordance with Emory University Policy 7.7, Policy for Investigators Holding a Financial Interest in Research. Resources of Winship Cancer Institute Emory University and NIH/NCI under award number P30CA138292. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Figure Legend

Figure 1

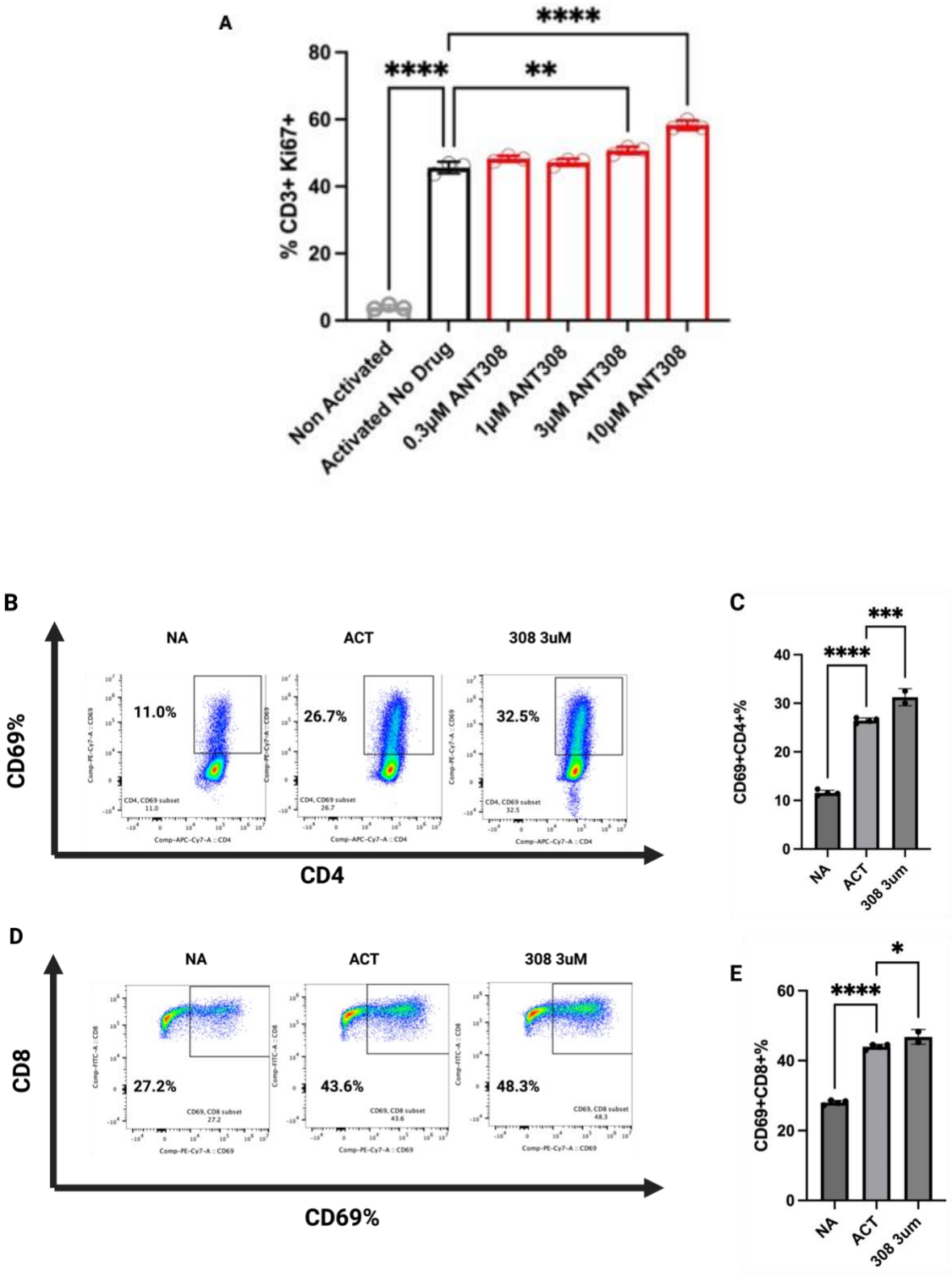
A
****
****
**
% CD3+ Ki67+
80
60
40
20
0
Non Activated
Activated No Drug
0.3μM ANT308
1μM ANT308
3μM ANT308
10μM ANT308
B
NA
ACT
308 3uM
CD69%
CD4
11.0%
26.7%
32.5%
C
***
****
CD69+CD4+%
40
30
20
10
0
NA
ACT
308 3um
D
NA
ACT
308 3uM
CD8
CD69%
27.2%
43.6%
48.3%
E
*
****
CD69+CD8+%
60
40
20
0
NA
ACT
308 3um

Figure 1: ANT308 enhances healthy donor T cell activation *in vitro* via T cell activation assay. PBMC from 4 healthy donors were isolated, pooled, then treated with varying doses of ANT308. 50IU/mL IL-2, and 1.5uL/mL CD3/CD28/CD2 T cell activator. T cell populations were separated, and relative proportions were calculated via flow cytometry. (A) Levels of Ki67 expression in T cells was quantified and compared via Prism. (B-C) Cells positive for CD4 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism. (D-E) Cells positive for CD8 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism.

Figure 2

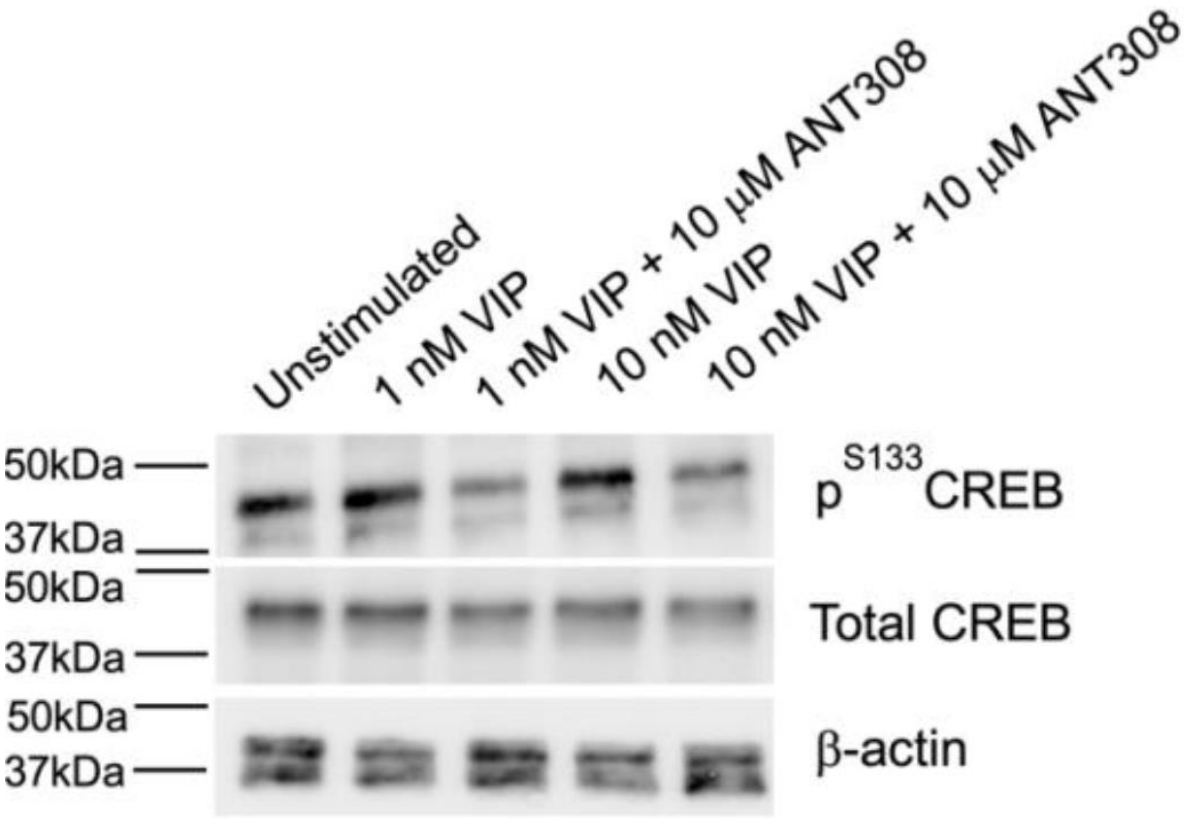

Figure 2: ANT308 enhanced *in vitro* activation, decreasing CREB phosphorylation in healthy donor human T cells via Western Blot. T cells from healthy donors were first isolated and pooled, then cultured in complete RPMI containing 0.5% fetal bovine serum overnight. T cells were incubated at 37ºC after treatment of 10um ANT308 for 30 min followed by stimulation with VIP for 15 min. T cells were washed twice with ice-cold 1X PBS and lysed with ice-cold RIPA (R0278, Sigma) containing 1X protease inhibitor cocktail (P8340, Millipore Sigma) and phosphatase inhibitors (P2850, Millipore Sigma). Lysates were quantified by Bradford assay (BioRad), normalized for concentration, denatured with 1XSDS sample buffer. 40 ug of protein per sample was resolved by SDS-PAGE, blotted on PVDF membrane, and probed with primary antibodies. Beta-actin was used as a positive control for protein expression.

Figure 3

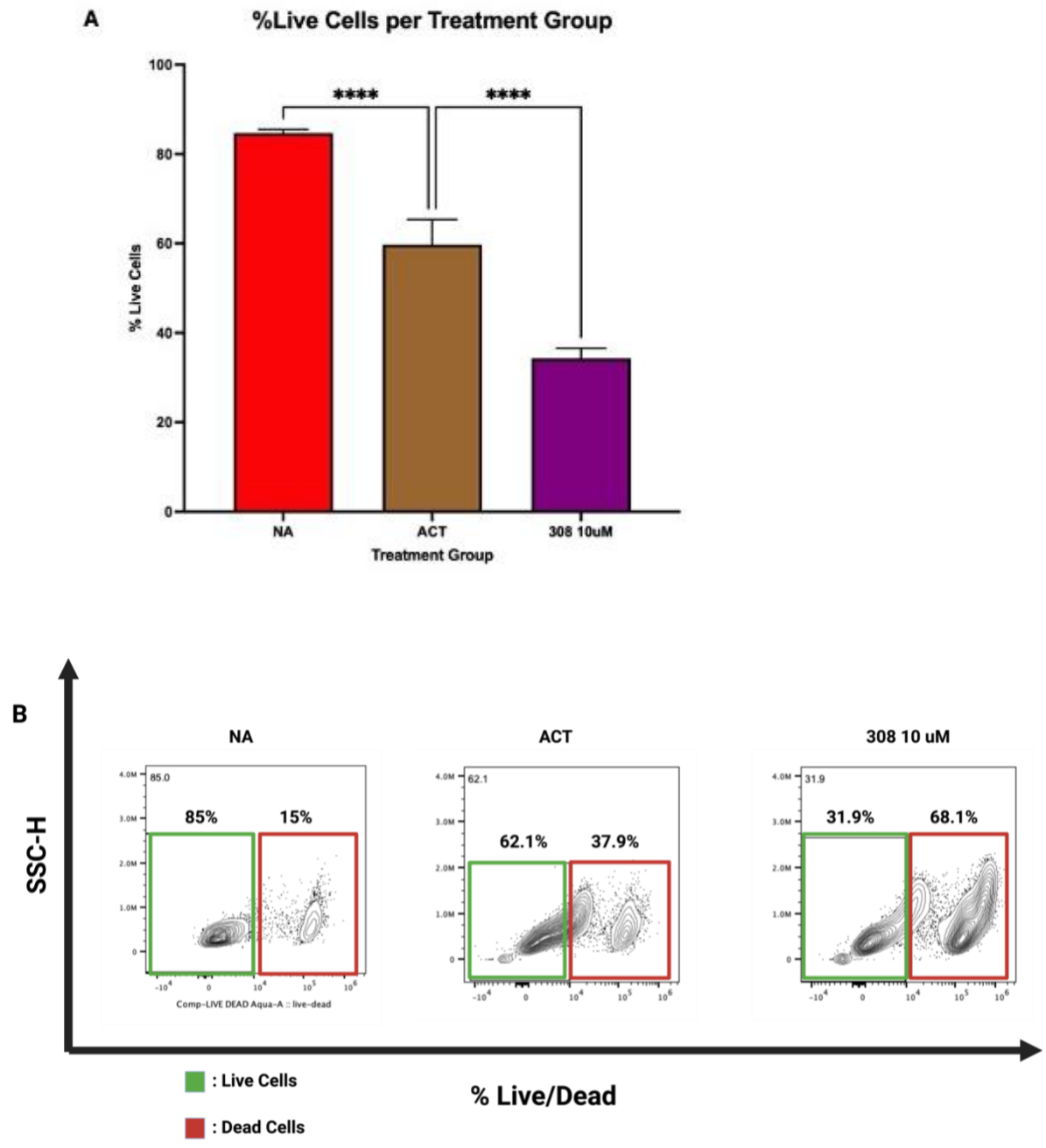

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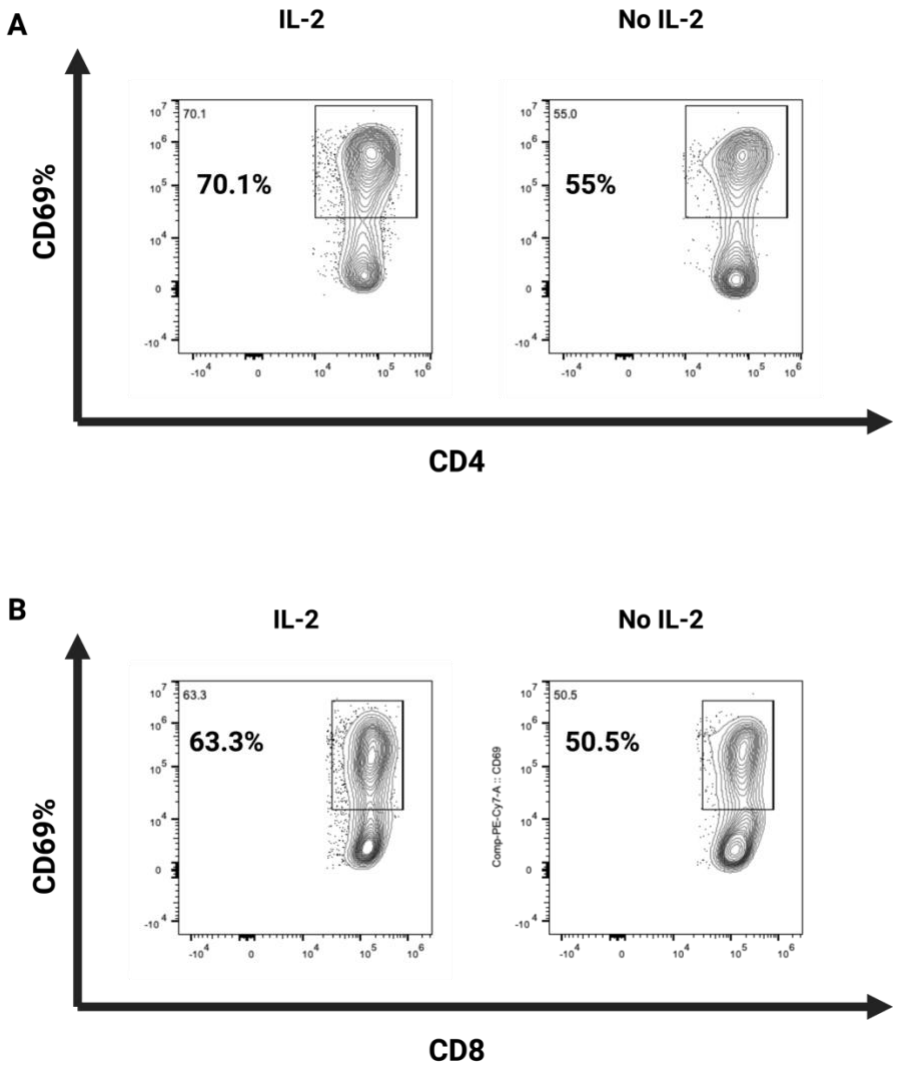
Figure 3: Overexposure to CD3/CD28 T cell Activator demonstrates T cell exhaustion and death. PBMC from 4 healthy donors were isolated, pooled, then treated with varying doses of ANT308. 50IU/mL IL-2, and 1.5uL/mL CD3/CD28/CD2 T cell activator. Cells were stained for various antibody markers including live-dead aqua. (A) The percent of living cells out of total cells per sample was determined via flow

cytometry and ANOVA statistical tests were run via Prism. (B) Comparative size and live populations of cells were quantified and compared via flow cytometry.

Figure 4



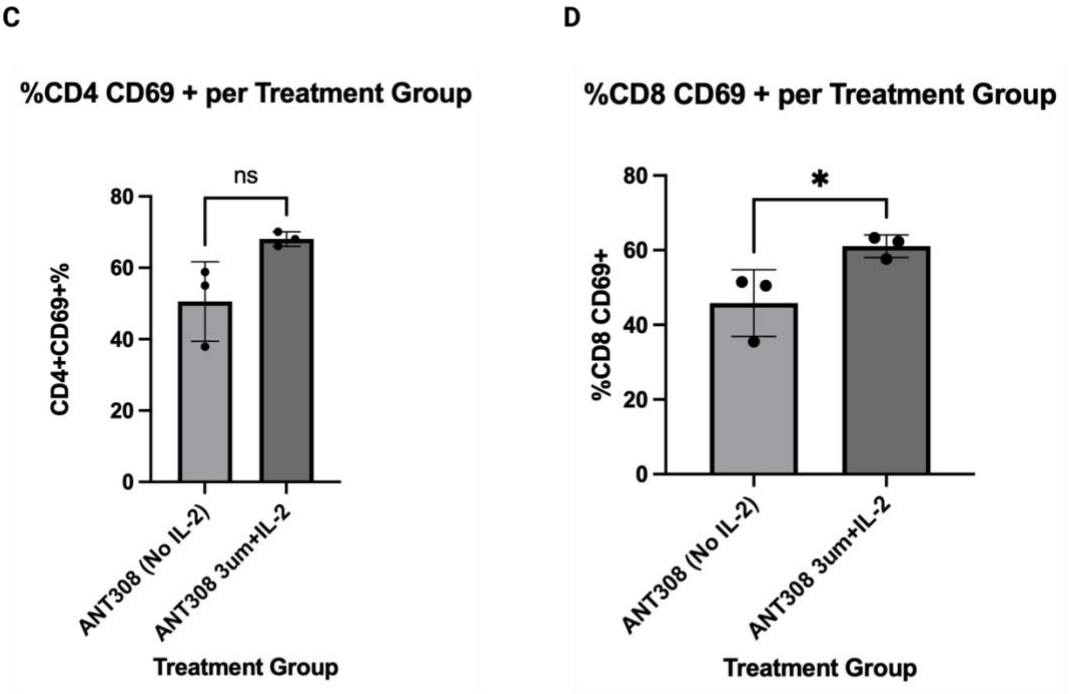


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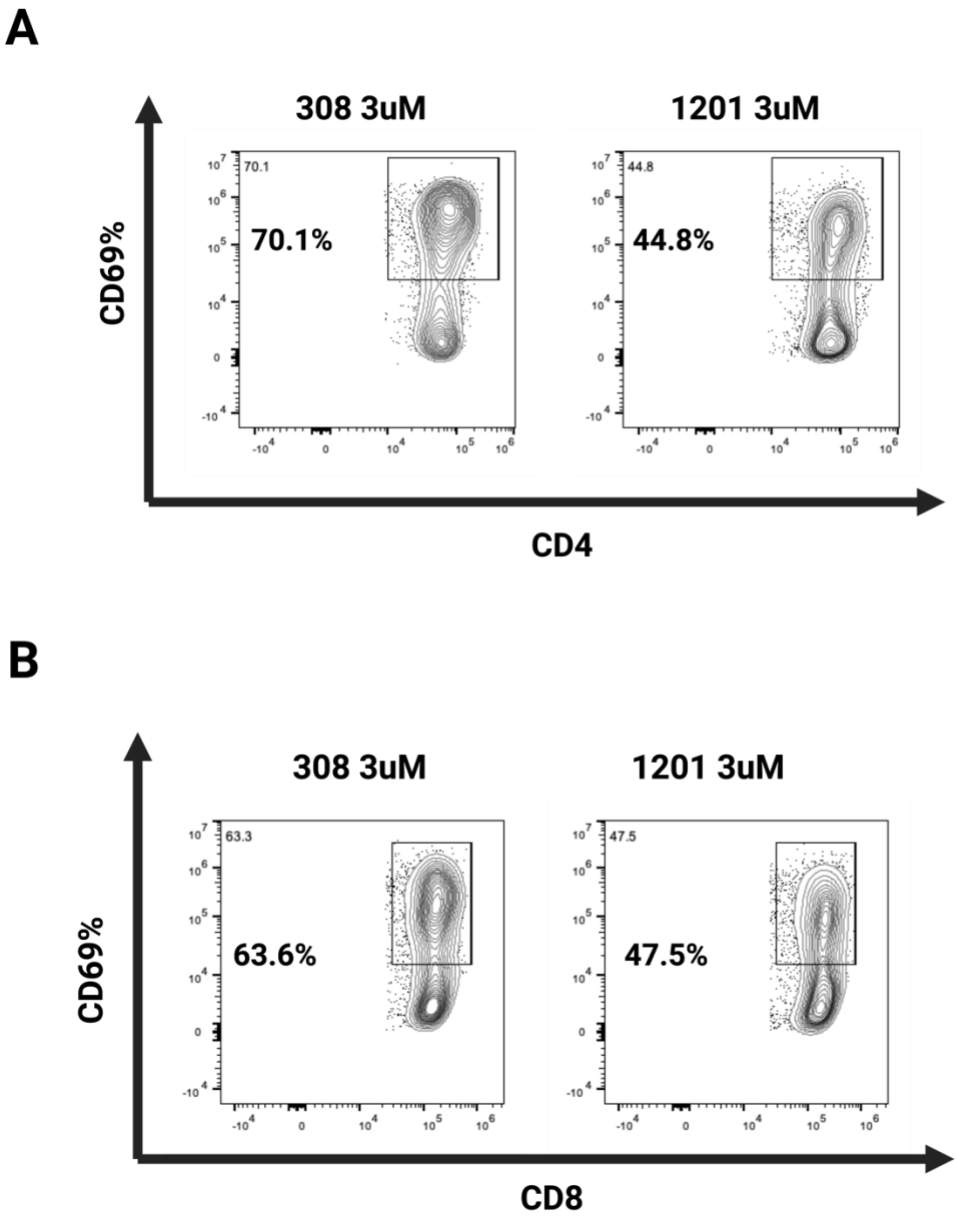
Figure 4. IL-2 induces T cell proliferation *in vitro*. PBMC from 4 healthy donors were isolated, pooled, then treated with varying doses of ANT308. and 1.5uL/mL CD3/CD28/CD2 T cell activator. Three sample of T cells receiving ANT308 3um+ T cell activator were cultured without IL-2. T cell populations were separated, and relative proportions were calculated via flow cytometry. (A, C) Cells positive for CD4 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism. (B, D) Cells positive for CD8 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism.

Figure 5


A
308 3uM
1201 3uM
70.1
70.1%
44.8
44.8%
CD69%
CD4
B
308 3uM
1201 3uM
63.3
63.6%
47.5
47.5%
CD69%
CD8

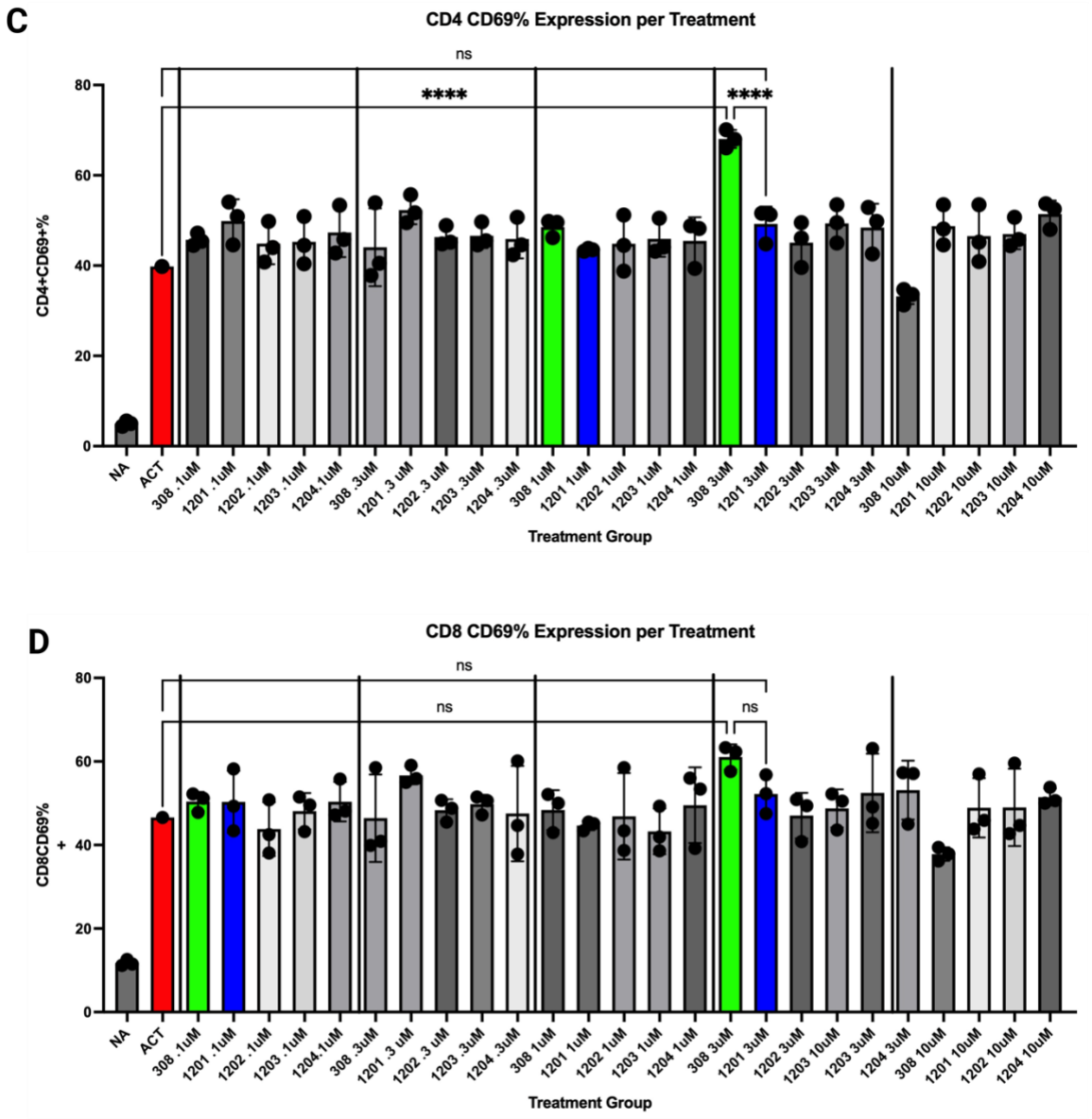


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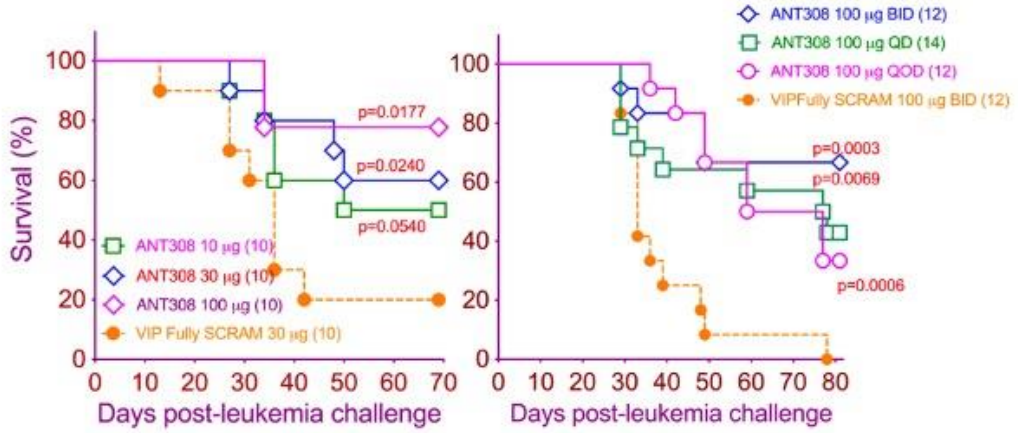
Figure 5: ANT308 potentiates T cell Activation significantly more than ANT1201, the leading experimental antagonistic peptide. PBMC from 4 healthy donors were isolated, pooled, then treated with varying doses of ANT308 and ANT1201-1204. and 1uL/mL CD3/CD28/CD2 T cell activator. T cell populations were separated, and relative proportions were calculated via flow cytometry. (A, C) Cells positive for CD4 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism. (B, D) Cells positive for CD8 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism.

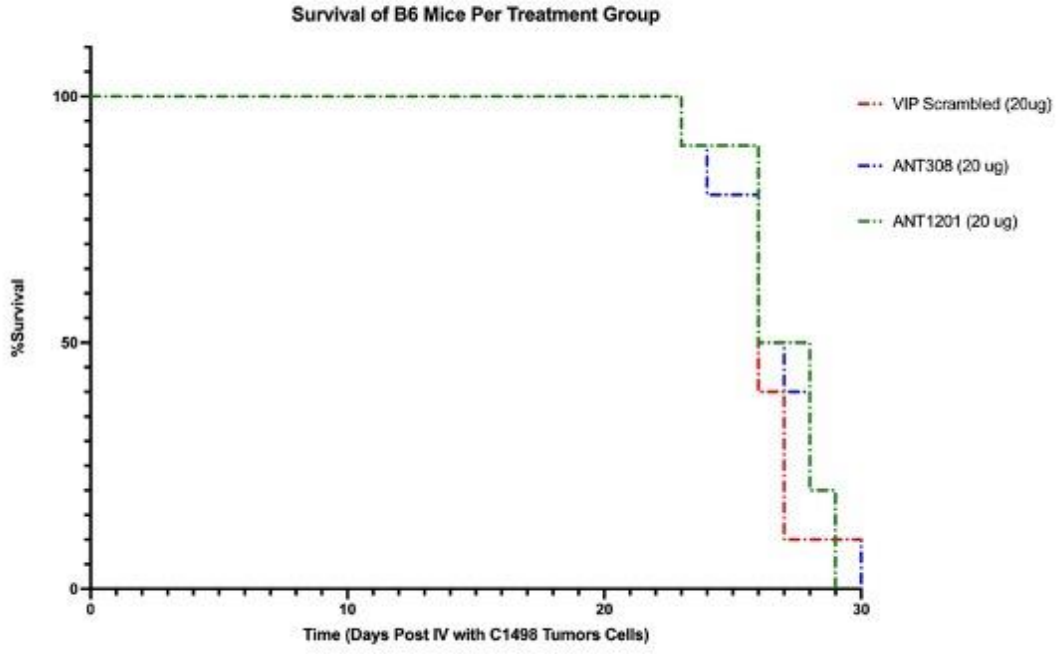
Figure 6

A



(Wang, 2024)

B



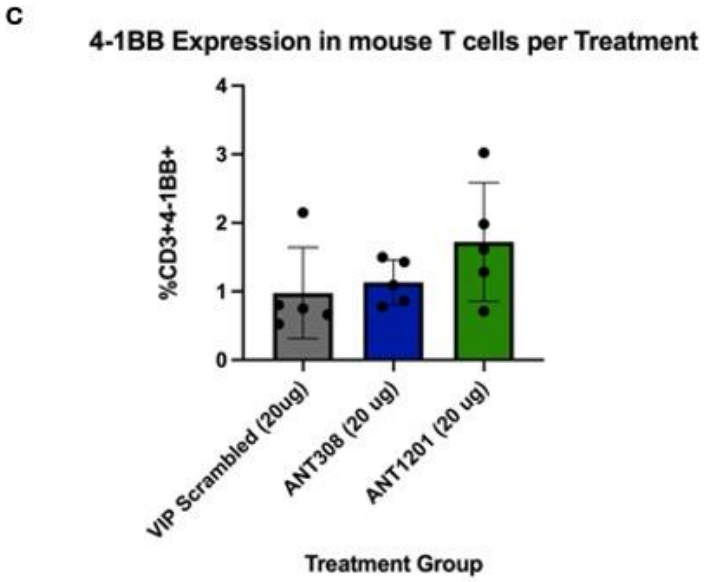
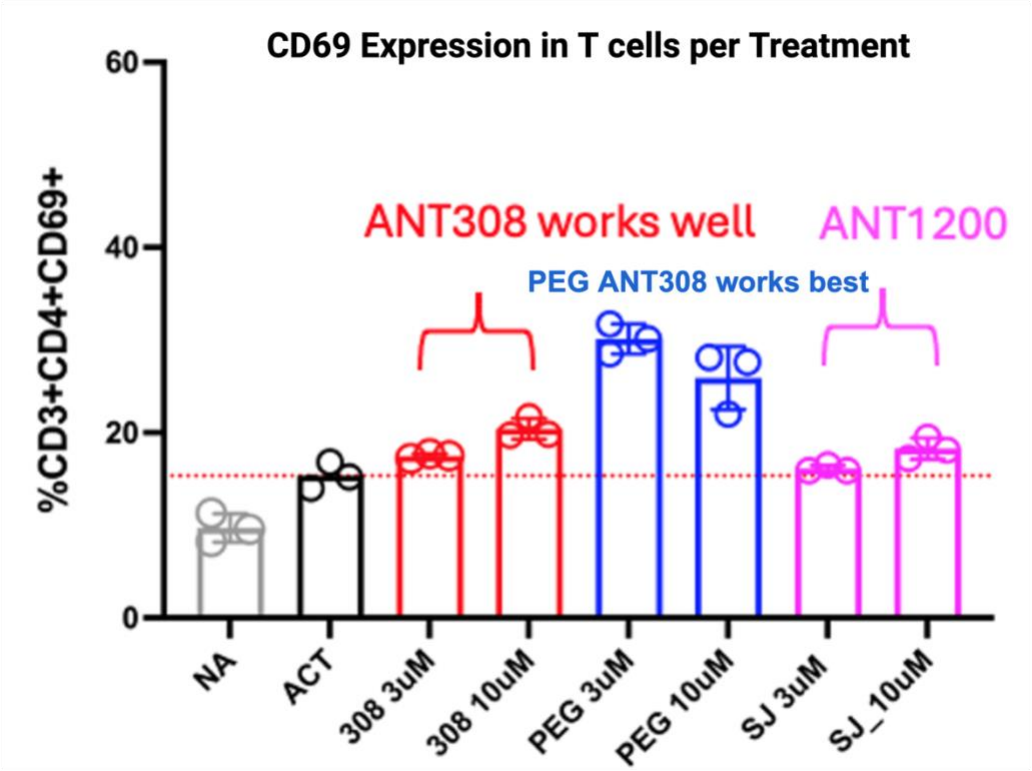


Figure 6: 100ug of ANT308 enhances survival in B6 leukemic mice, 20ug of ANT308 and ANT1201 enhances T cell activation (A) Depicts the survival curve results of prior *in vivo* B6 leukemic mice experiments. Mice receiving ANT308 twice daily (BID), once every other day (QOD), once a day (QD), and VIP scrambled twice daily all at a dose of 100ug are shown and compared for significant differences. (B, C) 30 B6 mice were inoculated with 100uL of 10^6 C1498 Tumor cells/mL on Day 0. Starting day 7 and ending day 17, mice were subcutaneously injected with either a fully scrambled VIP, ANT308, or ANT1201 at a dose of 20mg per day. A survival curve was produced (B) and the protein expression in mouse T cells were analyzed via flow cytometry and quantified via Prism (C).

Figure 7

A


CD69 Expression in T cells per Treatment
%CD3+CD4+CD69+
60
40
20
0
ANT308 works well
ANT1200
PEG ANT308 works best
NA
ACT
308 3uM
308 10uM
PEG 3uM
PEG 10uM
SJ 3uM
SJ_10uM

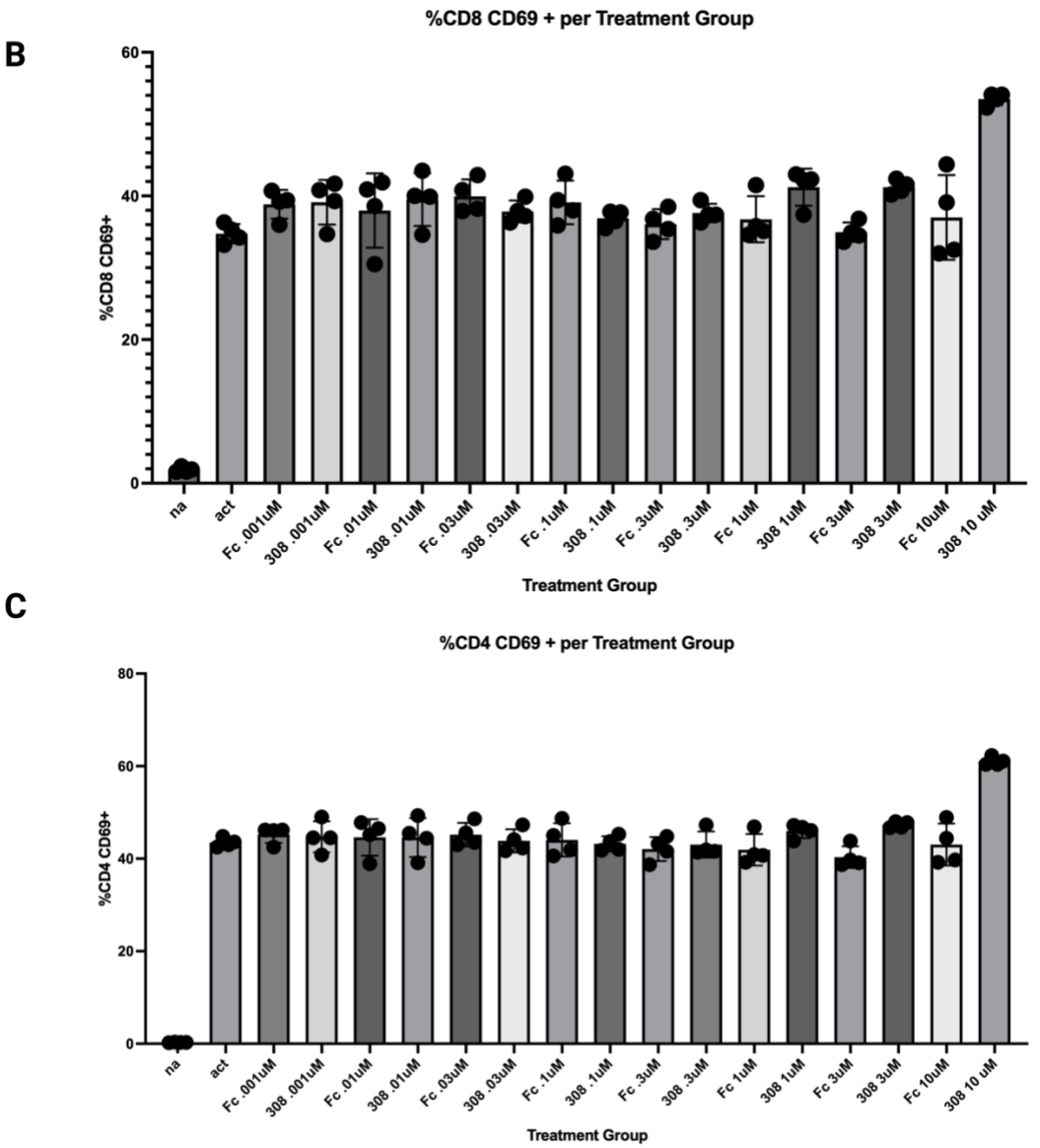


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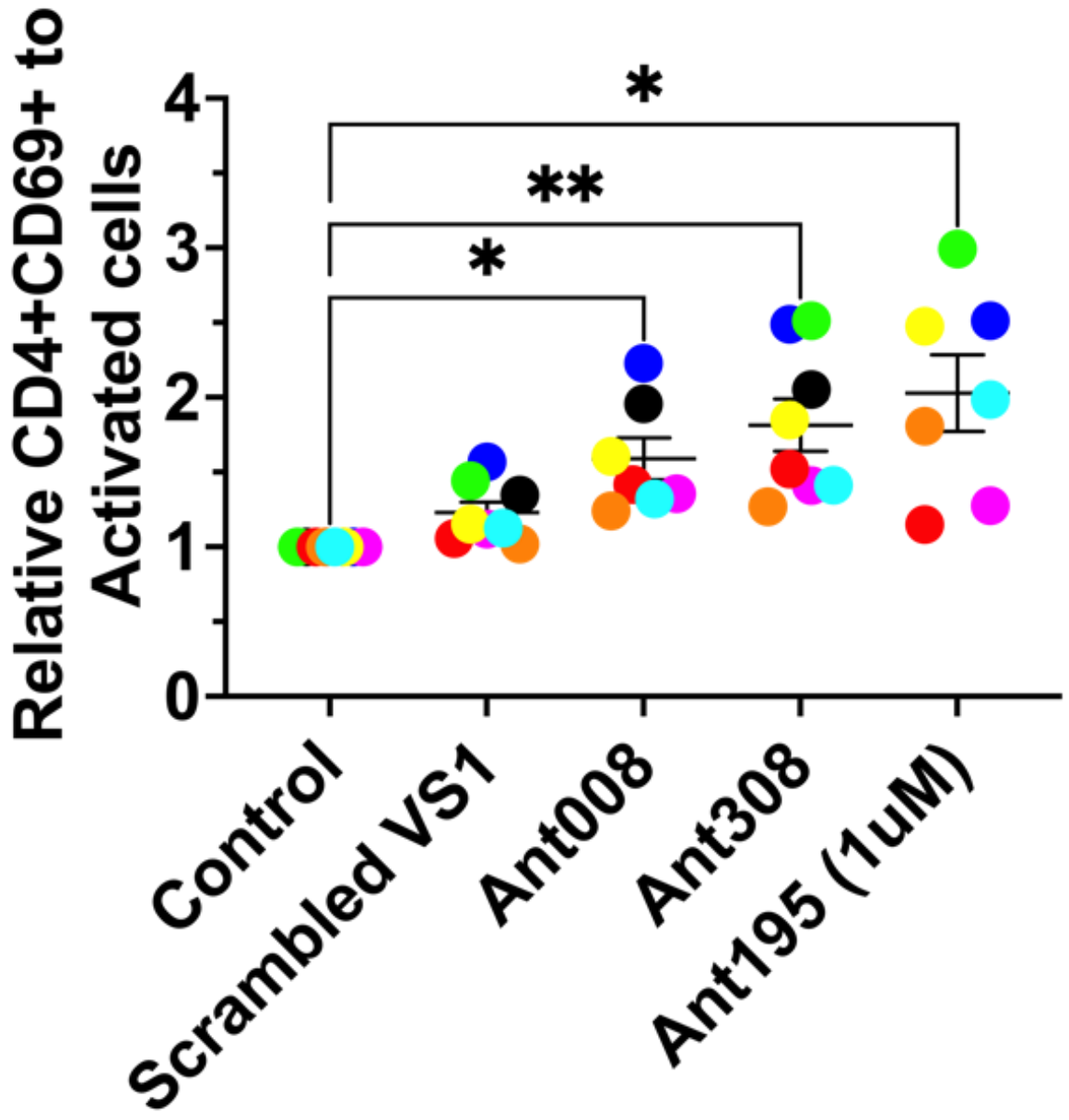
Figure 7: PEGylation of Antagonist Peptides enhances T cell Activation. (A) PBMC from 4 healthy donors were isolated, pooled, then treated with varying doses of ANT308, PEGylated ANT308, and ANT1200 (a precursor to ANT1201-1204) and 1.5uL/mL CD3/CD28/CD2 T cell activator. T cell populations were separated, and relative proportions were calculated via flow cytometry. Cells positive for CD4 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism. (C,D) PBMC from 4 healthy donors were isolated, pooled, then treated with T cell activator and varying doses of ANT308 or Fc Fusion ANT308. T cell populations were separated, and relative proportions were calculated via flow cytometry. Cells positive for CD4 and CD69 were quantified via flow cytometry and ANOVA statistical analysis was run on Prism.

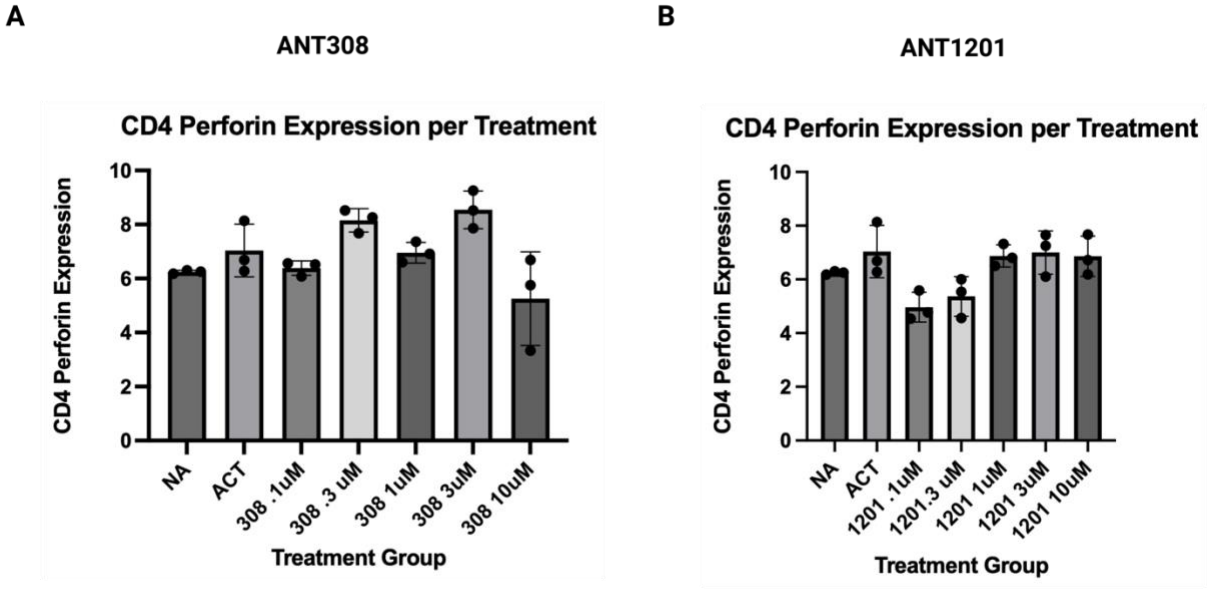
Figure 8



(Tenzin Passang Lim)

Figure 8: Individual T cell donors' express variability in VIP antagonist induced activation. Healthy donor T cells were individually isolated (Each color represents a different donor), then treated with three VIP antagonist peptides. Flow cytometry was used to quantify activation via CD69% of CD4 cells and relative levels were compared via ANOVA statistical tests on Prism.

Figure 9


A
ANT308
CD4 Perforin Expression per Treatment
CD4 Perforin Expression
10
8
6
4
2
0
NA
ACT
308 .1uM
308 .3 uM
308 1uM
308 3uM
308 10uM
Treatment Group
B
ANT1201
CD4 Perforin Expression per Treatment
CD4 Perforin Expression
10
8
6
4
2
0
NA
ACT
1201 .1uM
1201.3 uM
1201 1uM
1201 3uM
1201 10uM
Treatment Group

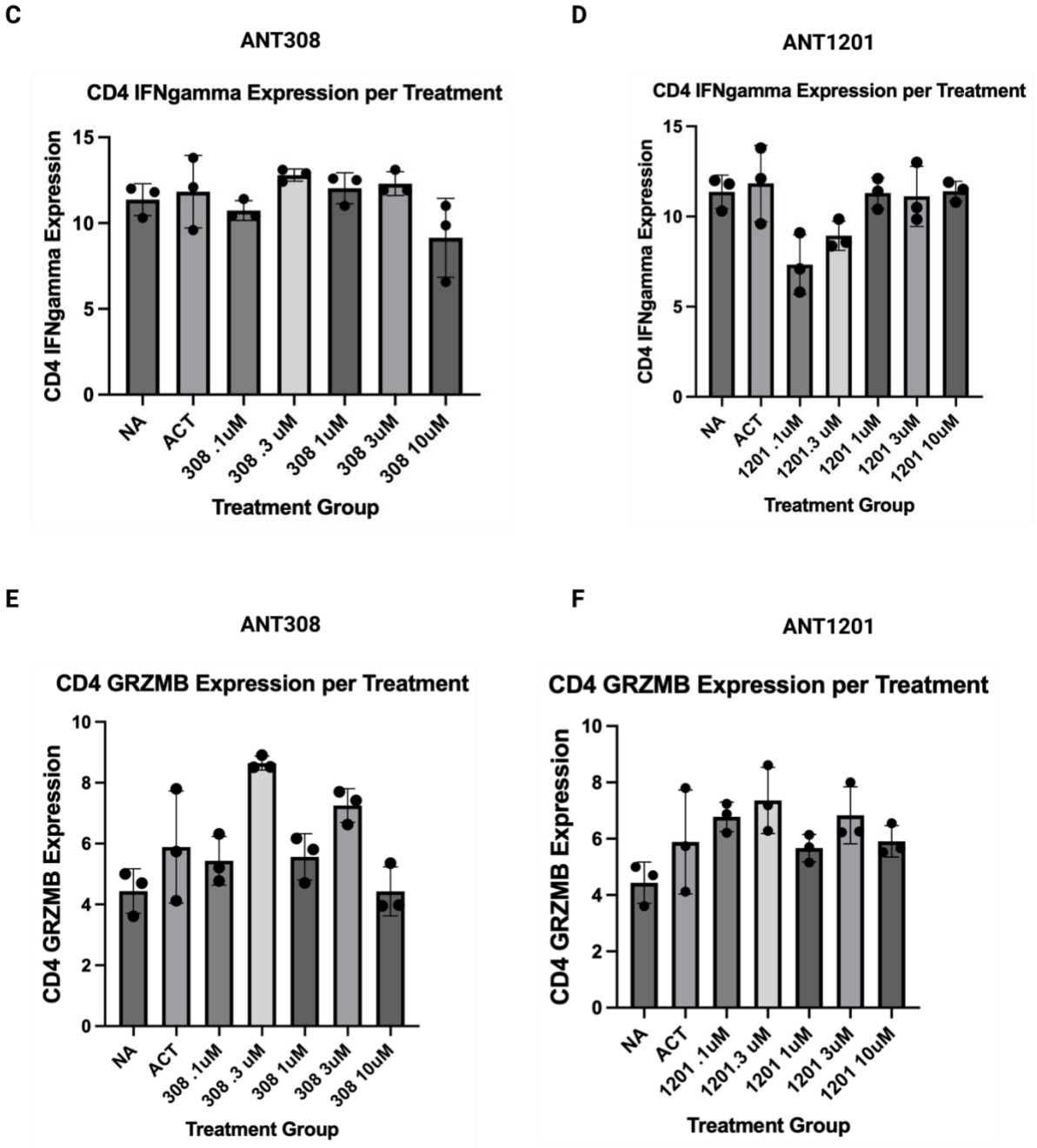


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Figure 9: ANT308 potentiates T cell activation to a limited degree via intracellular protein expression. PBMC from 4 healthy donors were isolated, pooled, then treated with varying doses of ANT308 or ANT1201, and 1ul/mL CD3/CD28/CD2 T cell activator. T cell populations were separated, and relative proportions were calculated via flow cytometry. Cells positive for intracellular proteins of interest (Perforin, Interferon-gamma, and Granzyme B) were quantified via flow cytometry and ANOVA statistical analysis was run on Prism.

Figure 10

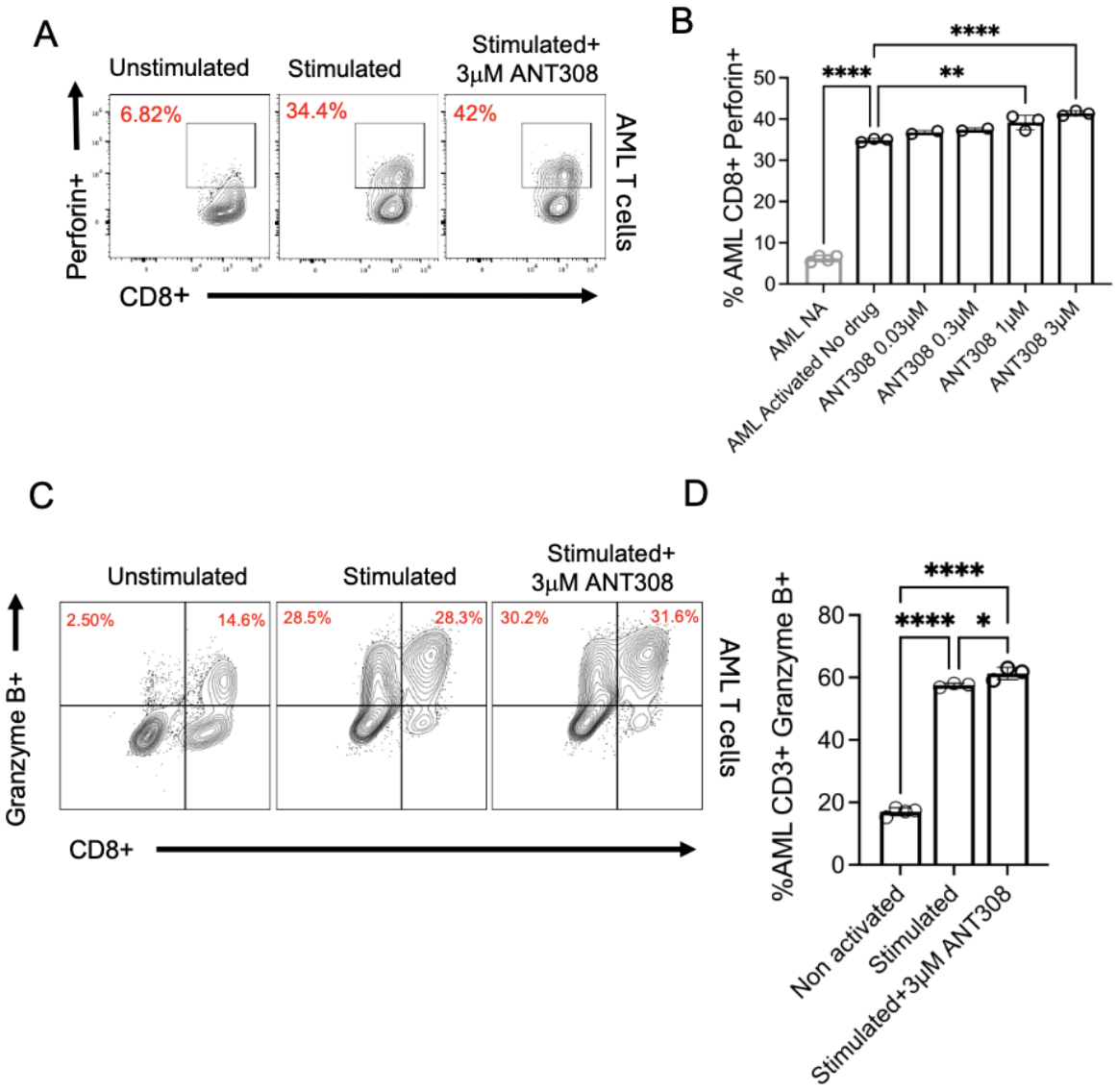

Figure 10 **ANT308 potentiates activation and cytotoxic activity in AML donor T cells.** PBMC from 4 AML patient donors were isolated, pooled, then treated with varying doses of ANT308 and 1.5ul/mL CD3/CD28/CD2 T cell activator. T cell populations were separated, and relative proportions were calculated via flow cytometry. Cells positive for intracellular proteins of interest (Perforin and Granzyme B) were quantified via flow cytometry and ANOVA statistical analysis was run on Prism. (A) Depicts the flow cytometry data of Perforin Expression in CD8+ T cells based on treatment group. (B) Shows the Statistical significance of the CD8+ T cell Perforin expression among treatments. (C) Depicts the flow cytometry data of Granzyme B Expression in CD3+ cells based on treatment group. (D) Shows the Statistical significance of the CD3+ Granzyme B expression based on treatment group.

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