APPLICATION NOTE

Rapid screening of novel therapeutics by ImmuSignature™ T cell activation assay.

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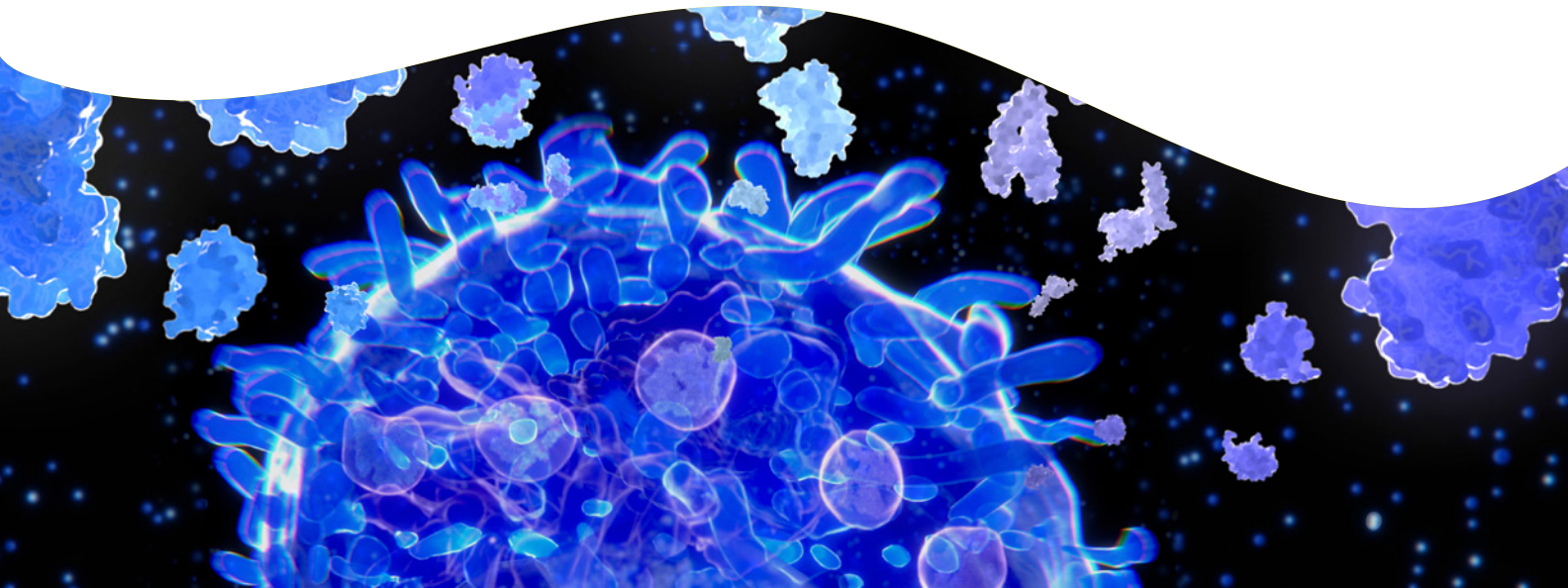
Introduction

High throughput assays are a key strategy to identify hit and lead compounds for drug discovery projects. In this application note, we describe the development of a robust T cell activation assay to aid assessing the impact of novel therapeutics on T cell function to accelerate drug discovery and development. T cells are one of the main components of the adaptive immune response with highly antigen-specific surface receptors. They initiate antigen recognition and support other cells throughout the immune response towards tumors, pathogens and allergens [1]. Moreover, T cells maintain immune memory and homeostasis. Investigating and understanding T cell function and regulation is crucial for developing immunotherapies to treat diseases such as autoimmune and infectious diseases, and cancer [2]. Recent clinical advances in cancer immunotherapy with immune checkpoint inhibitors have prompted intense interest in developing novel therapeutics capable of modulating the immune system to activate anti-tumor T cell response [3].

The specificity of the CD3 antigen for T cells and its presence at all stages of T cell development makes it an ideal surface marker for T cell isolation [4].CD3+ T cells comprise two subtypes with distinct functions: CD4+ (helper) and CD8+ (cytotoxic) T cells [5]. CD4+ T cells recognize antigens presented on MHC-II molecules on antigen-presenting cells (APCs) and are the more prevalent subtype in a total T cell population. On the other hand, cytotoxic CD8+ T cells react to antigens presented by MHC-I molecules found on all nucleated cells and are key players in the defense against infections and tumors.



In vitro, a cocktail of anti-CD3 and anti-CD28 antibodies can activate T cells and induce extensive proliferation [6]. Anti-CD3 and anti-CD28 antibodies are non-physiological agonists that bind to the cell surface ligands CD3 and CD28, thereby providing stimulatory and co-stimulatory signals, which result in robust T cell proliferation and increased CD25 expression. T cell activity can be evaluated through quantification of cell proliferation, cell surface marker expression of CD25 and effector cytokine release such as IFN-γ and TNF-α [7].

Standard T cell activation assay for compound screening

Our T cell activation assay utilizes cryopreserved T cells isolated from peripheral blood mononuclear cells (PBMCs). As illustrated in the assay outline in figure 1, purified and revived CD3+ T cells are activated by adding a cocktail of anti-CD3 and anti-CD28 antibodies and cultured in the presence of test and control compounds for four days. T cell proliferation and activation are then assessed by flow cytometry measuring CD25 surface marker expression and cell division using CellTrace Violet (CTV), a dye that tracks cell proliferation.

Since total CD3+ T cells are used in the assay, compound effects can be separately assessed for CD4+ and CD8+ subtypes through fluorophore labelling and segregation by flow cytometry.

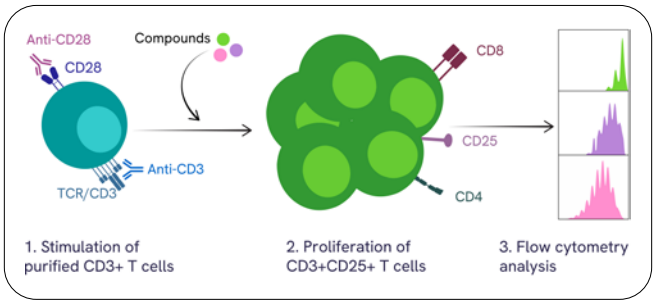


Figure 1: Schematic Representation of the Standard T cell Activation Assay. CD3+ T cells are stimulated with a cocktail of anti-CD3/CD28 antibodies and treated with test and control compounds for four days. The activation of CD4+ and CD8+ T cell subtypes is analyzed based on proliferation and the expression of CD25 by flow cytometry.

Assay development

We developed a robust and semi-automated T cell activation assay for screening of small molecules and antibody-based compounds to modulate T cell activity. T cell stimulation conditions were assessed that allow measurement of enhancers as well as inhibitors of T cell activity to be routinely screened in a 384-well format. The following section describes the assay development phases to illustrate capability and suitability of our T cell activation assay to projects aiming to assess novel therapeutics for T cell function.

1. T Cell stimulation

In order to define an optimal assay window to assess stimulatory and inhibitory effects of compounds on T cell activation we used titration of anti-CD3 and anti-CD28 antibodies to achieve a T cell activation profile of ~ 40 % proliferation (Figure 2). CD3+ T cells from three different donors were stimulated with increasing concentrations of anti-CD3 and anti-CD28 antibodies, and T cell proliferation and CD25 surface marker expression were analyzed by flow cytometry after four days in culture (Figure 2.A) (See materials and methods for details). The highest anti-CD3/CD28 cocktail concentration tested (C5) achieved ~ 80% proliferation of CD3+ T cells (Figure 2.B) and ~ 70% CD25 expressing cells (Figure 2.C) compared with ~ 1% and 2% unstimulated cells, respectively, after four days in culture.

Lowering levels of anti-CD3/CD28 resulted in reduction of T cell proliferation and decreased activation, in a dose-dependent manner (Figure 2.D). At a given concentration of anti-CD3/CD28 (C3) T cell proliferation was at ~30- 40% and CD25 expression reached 40-50% (donor-dependent), providing an optimal assay window for modulation of T cell activity by compound addition. Thus, in our T cell activation assay, T cells stimulated with C3 provide the baseline activity from which compound effects are assessed and analyzed. We selected the highest anti-CD3/CD28 concentration tested (C5) as positive assay control for T cell proliferation and CD25 expression and their corresponding isotype pair as negative controls.

2. Compound screen

In order to define an optimal assay window to assess stimulatory and inhibitory effects of compounds on T cell activation we used titration of anti-CD3 and anti-CD28 antibodies to achieve a T cell activation profile of ~ 40 % proliferation (Figure 2). CD3+ T cells from three different donors were stimulated with increasing concentrations of anti-CD3 and anti-CD28 antibodies, and T cell proliferation and CD25 surface marker expression were analyzed by flow cytometry after four days in culture (Figure 2.A) (See materials and methods for details). The highest anti-CD3/CD28 cocktail concentration tested (C5) achieved ~ 80% proliferation of CD3+ T cells (Figure 2.B) and ~ 70% CD25 expressing cells (Figure 2.C) compared with ~ 1% and 2% unstimulated cells, respectively, after four days in culture.

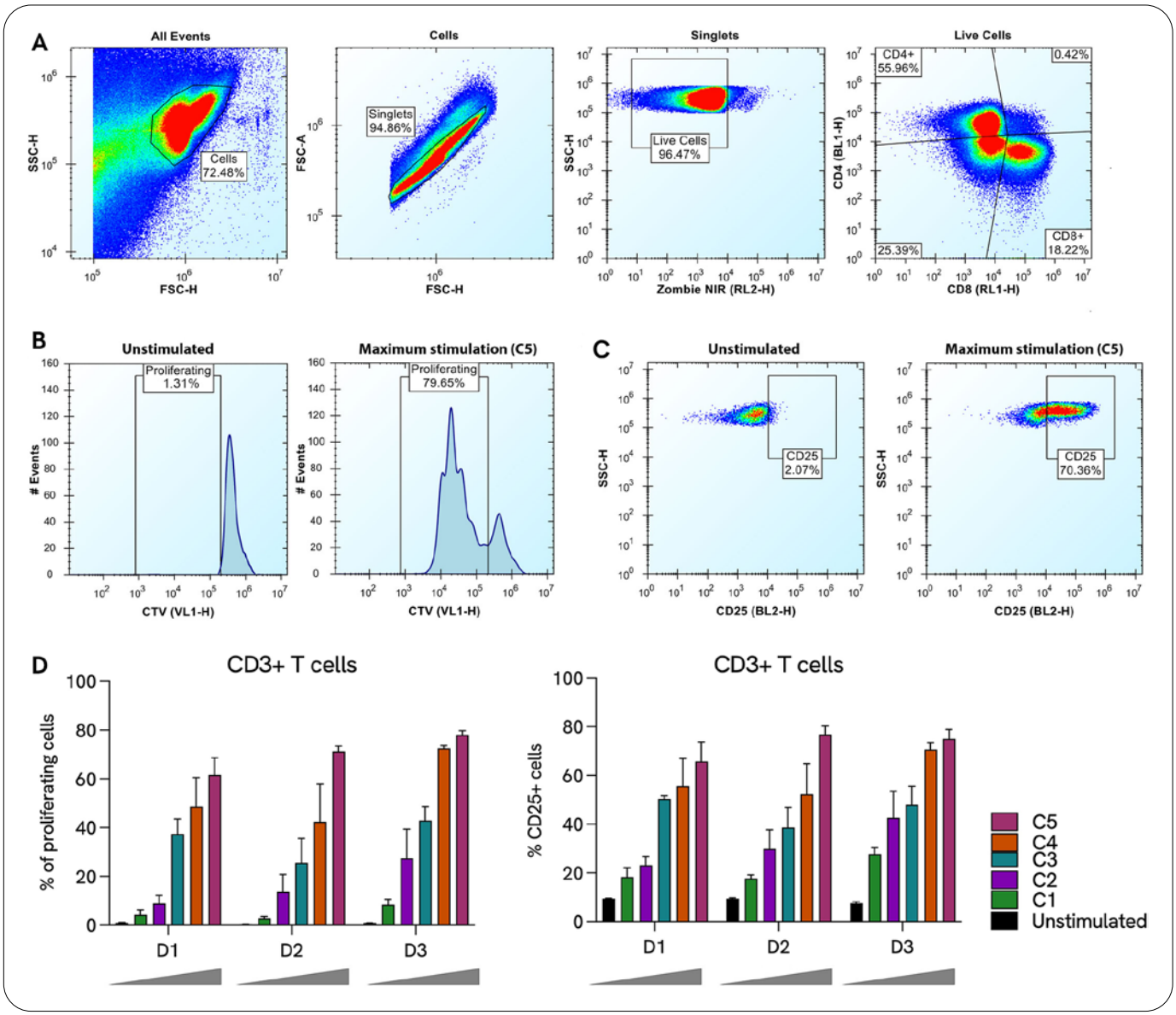


Figure 2: Proliferation and CD25 Expression in CD3+ T Cells. (A) Gating strategy for the flow cytometry analysis of CD3+ T cells. (B and C) Flow cytometry analysis of proliferation and CD25 expression of unstimulated CD3+ T cells and following treatment with the highest tested concentration of anti-CD3/CD28 antibodies. (D) Proliferation and CD25 expression in CD3+ T cells at five different concentrations (C1-C5) of anti-CD3/CD28 antibodies in three independent donors (D1-3). The data is represented as mean + standard deviation of four technical replicates per donor.

2. A.) Assay Performance

Figure 3 depicts results for our quality control on T cell proliferation (Figure 3.A) and activation (Figure 3.B) from one of the donors. As expected, T cells in vehicle condition proliferated within the 20-40% range for CD4+ and CD8+ T cells, respectively, and showed increased expression of CD25 when compared to unstimulated controls. Increasing the dose of anti-CD3/CD28 antibodies to C5 (see Figure 2) induced high levels of CD4+ T and CD8+ T cell proliferation (60-80%, respectively) and activation of CD25 (70-80%, respectively), confirming a robust and dose-dependent response to stimulation. Interestingly, CD4+ and CD8+ T cells showed different kinetics in response to anti-CD3/CD28 stimulation. CD8+ T cells proliferated faster and displayed higher levels of CD25 expression in the four-day assay window when compared to CD4+ T cells from the same CD3+ T cell population, an effect that was commonly observed across all donors tested.

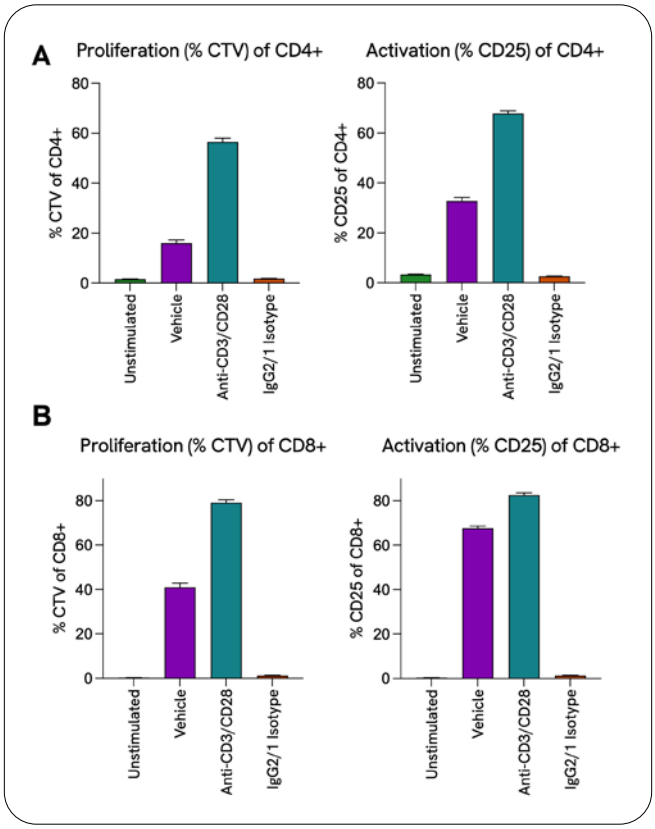


Figure 3: Assay Performance of CD4+ and CD8+ T cells. Flow cytometry evaluation of CD4+ (A) and CD8+ (B) proliferation and activation after 4 days in the indicated conditions. Data are expressed as mean + standard deviation of four technical replicates, depicting a single donor. Conditions include unstimulated, vehicle (T cells with C3 of anti-CD3/CD28), anti-CD3/CD28 (C5; positive control) and corresponding IgG2/1 isotype (negative control).

From these data we concluded that our vehicle control provided an optimal condition to assess compound modulation on T cell activity for both CD4+ and CD8+ T cells, albeit with dissimilar assay windows for both sub-populations.

2 .B.) Screen Performance

We tested a group of seven compounds for their effect on T cell activity. We detected dose responses for a range of small molecule inhibitors including GSK1059615, PCI 29732 and (5Z)-7-Oxozeaenol as well as anti-CD28 antibody and the therapeutic daclizumab (Figure 4). Cell viability was not affected by top compound concentrations and maintained at ~95% throughout the assay timeline (data not shown).

CD28 receptor engagement supports T cell stimulation, and we tested the addition of excess anti-CD28 antibodies to induce T cell activity [6]. As expected, additional anti-CD28 further enhanced T cell proliferation and CD25 expression in a dose-dependent manner, across all donors tested (Figure 4. A-B). The small molecule inhibitor GSK1059615 is a dual inhibitor of phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) [8].

Inhibition of PI3K and mTOR signaling has been shown to prevent T cell activation and induce immunosuppression [9]. Dosing T cells with GSK1059615 confirmed reduction in proliferation and decrease in CD25 expression (Figure 4.C-D). Daclizumab is an anti-CD25 monoclonal antibody that blocks IL-2 from binding to the CD25 receptor, leading to the inhibition of T cell activation [10]. Inhibition of T cell proliferation and activation was indeed detected in our assay and most prominently observed with the reduction of CD25 expression in CD4+ and CD8+ cells (Figure 4.E-F). Another small molecule inhibitor, PCI 29732, acts on Bruton's tyrosine kinase (BTK) and IL-2-inducible T cell kinase, both critical for T cell function [11]. The compound showed strong inhibitory effects on proliferation and activation in both CD4+ and CD8+ T cells, despite differences in baseline proliferation for the two subpopulations (Figure 4.G-H). Similarly, T cell response to (5Z)-7-Oxozeaenol, an inhibitor of transforming growth factor (TGF)-beta-activating kinase 1 (TAK1) which is known to play an important role in T cell development and maintenance, resulted in decreased proliferation and activation (data not shown) [12].

In summary, we treated CD3+ T cells with up to seven selected compounds to assess effects on T cell proliferation and activation. Of those seven compounds we identified four that inhibited T cell activity in a dose-responsive manner but also captured effects of increased T cell stimulation when dosing cells with anti-CD28 as stimulatory agent.

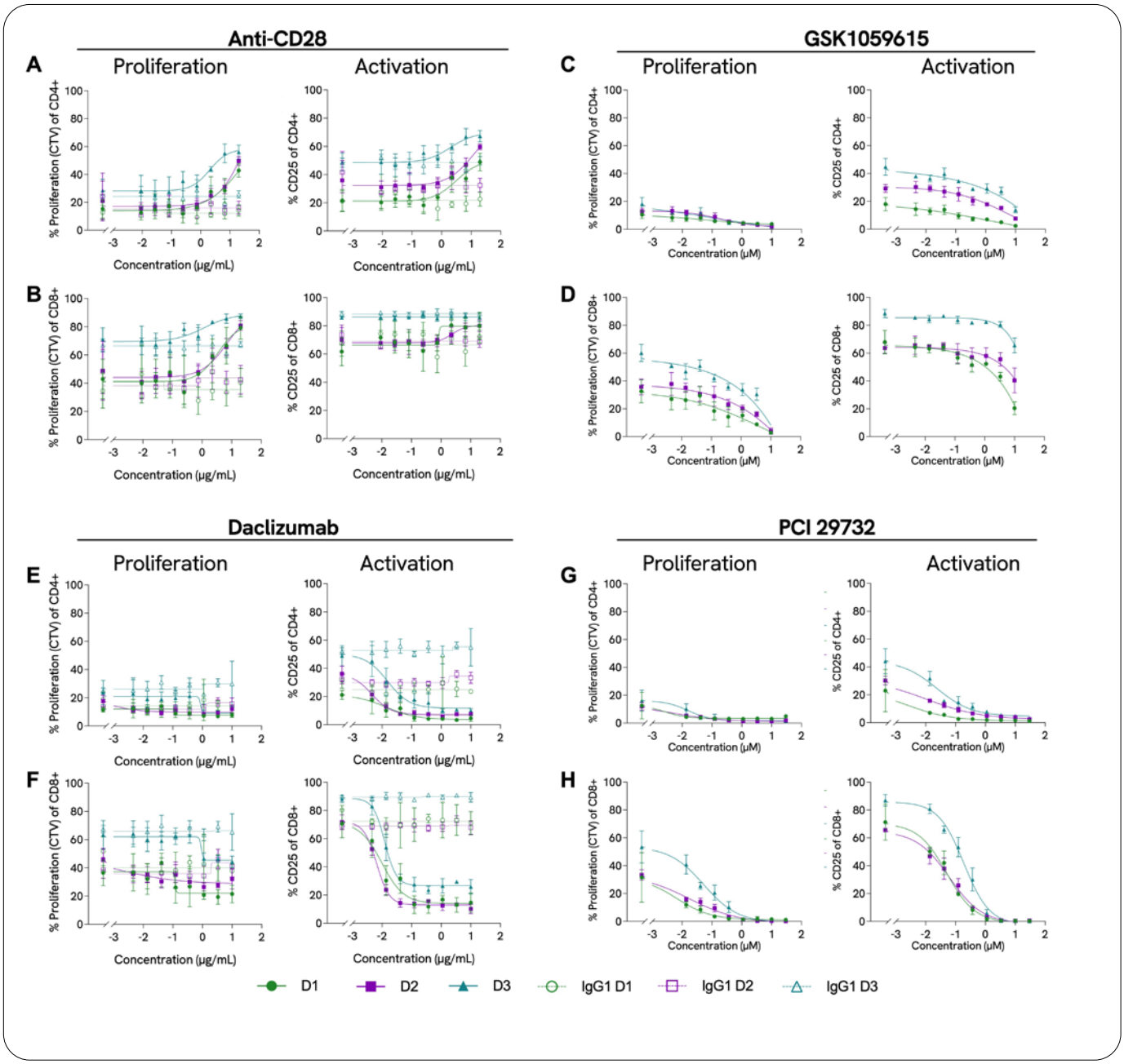


Figure 4: The effect of activating and inhibitory compounds on T cell stimulation. The effect of anti-CD28 antibody and isotype control (Panels A-B), GSK1059615 (panels C-D), daclizumab and isotype control (Panels E-F), and PCI 29732 (Panels G-H) on T cell proliferation and activation. CD4+ (Panels A, C, E and G) and CD8+ (Panels B, D, F and H) T cell proliferation was measured by Cell Trace Violet (CTV) dilution and activation through percentage of CD25 expression, both by flow cytometry. Compounds were tested in a nine-point dose range (eight doses + vehicle) at a 3-fold dilution series. Each plot represents three independent donors (D1-3). The data are plotted as mean + standard deviation of four technical replicates.

Conclusion

T cells are critical for cell-mediated immunity and their impaired function correlates with various diseases. Insufficient T cell proliferation and activation can lead to the development of cancer and infectious diseases, while over-activation can result in transplant rejections, autoimmune diseases, and allergies. Our T cell activation assay is a semi-automated screening platform offering rapid screening of novel immunotherapies to either increase or decrease T cell proliferation and activation. Our cell stimulation protocol provides a well-balanced T cell response for detecting stimulatory or inhibitory effects of tested compounds on proliferation and activation in CD4+ and CD8+ T cell subtypes. Moreover, our assay setup allows flexibility in multiplexing experimental readouts, such as applying complementary Homogenous Time-Resolved Fluorescence (HTRF) technology to detect cytokine release correlating with T cell activation.

Materials and Methods

Preparation of Activating Antibodies and Isotype Control Cocktails

Antibodies used for activation of CD3+ T cells: Ultra-LEAF™ purified anti-human CD3 antibody (Biolegend #317326); Ultra-LEAF™ purified anti-human CD28 antibody (Biolegend #302934). Isotype controls: Ultra-LEAF™ Purified Human IgG1 Isotype Control Recombinant antibody (Biolegend #403501); purified mouse IgG2a, k isotype control antibody (BioLegend #400202). Antibody dilutions and addition to assay plates were performed using liquid handling systems.

Preparation of Test and Control Compounds

Test and control compounds were prepared at a 9-point dose range, including vehicle control, in three-fold dilution steps. The top concentrations of the compounds described in this application note were as follows: PCI 29732 (30µM; Tocris #5012), anti-CD28 antibody (20µg/mL; Biolegend #302934), Daclizumab (10µg/mL; Absolute Antibody #Ab00187-10.0), GSK1059615 (10µM; SelleckChem #S1360) and (5Z)-7-Oxozeaenol (30µM; Tocris #3604). Compound dilutions and addition to assay plates were performed using liquid handling systems.

Flow Cytometry Staining

Cells were stained with a mixed antibody cocktail of Alexa Fluor® 488 anti-human CD4 antibody (BioLegend #317420), Alexa Fluor® 647 anti-human CD8a antibody (BioLegend #301022), PE anti-human CD25 antibody (BioLegend #302606) and Zombie Near IR (BioLegend #423105) for 10 min at room temperature before sample acquisition on an iQue3 Flow Cytometer assessing cell viability (Zombie Near IR), proliferation (CTV dilution) and activation (CD25 expression) in CD4+ and CD8+ T cells.

Data Analysis

iQue3 Flow Cytometer data was analyzed with Forecyt software (Version 9.0). Histograms and dose-response curves of the tested compounds were prepared using GraphPad Prism 9.1.0.

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