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Immune cell compound screening compendium.

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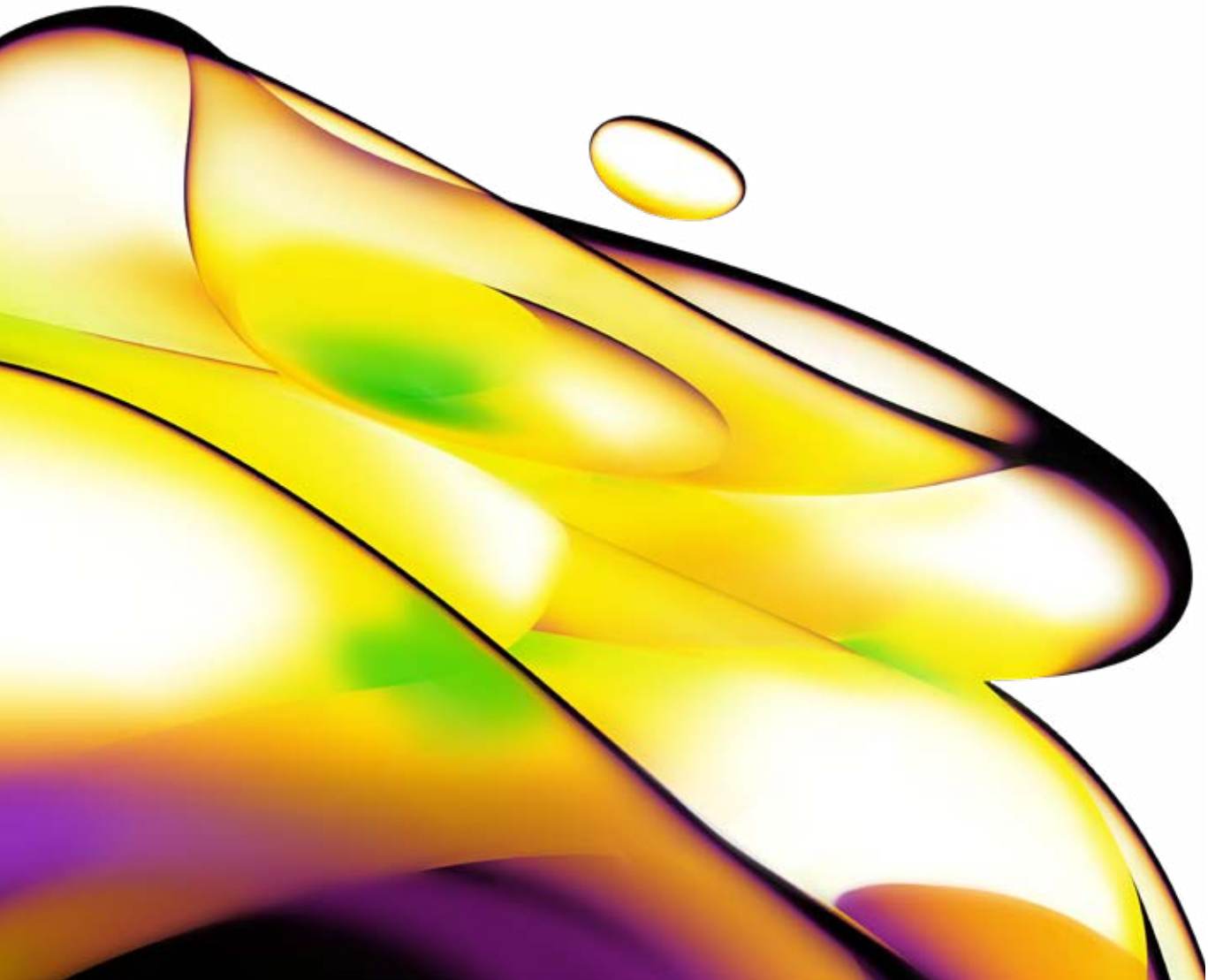

Rapidly advancing you towards clinical trials

The phase of therapeutic lead optimization is a pivotal and intricate step in drug development, especially when dealing with primary immune cells. Assessing the impact on the patient's immune system is paramount in laying the foundation for clinical trials during therapeutic design. For this reason, a heightened level of scrutiny becomes essential for drug developers focused on addressing conditions related to the immune system.

Analyzing the behavior of primary immune cells via screening assays is a time-intensive process. Yet, it yields invaluable insights into the interactions of compounds within an in vitro simulated immune microenvironment. This expedites pinpointing top-tier therapeutic candidates, making screening over immune cells vital within the drug development pipeline, especially when treatments are engineered to engage with the immune system. Notably, the pace and precision of data acquisition play a pivotal role in advancing within the competitive landscape of funding and scientific exploration.

Introducing our ImmuSignature™ suite of immune cell screening standardized assays, a product of meticulous development and rigorous validation. These assays yield a wealth of comprehensive data for your compound screening needs, all within a matter of weeks. Partnering with our immunology experts ensures unwavering consistency, relevance, and the delivery of robust high-throughput outputs. This collaborative approach streamlines your research and empowers you with the time-saving tools and confidence required to pioneer therapeutic exploration.

Closer to clinic, faster to market

ImmuSignature™ Platform

The ImmuSignature™ assays is a suite of validated services centered on T cells, designed to deliver high-throughput functional data on the effects of therapeutic candidates in an immune cell in vitro model. They encompass four interconnected approaches for studying therapeutic impacts on T cell physiology and collectively provide in-depth insights to support drug development.

Each assay has utilized the advancement in automation technologies and improved primary immune cell sourcing procedures that have facilitated the establishment of in vitro models to emulate the immune system's behavior. These primary immune cell models enhance physiological relevance in vitro by directly sourcing cells from human donors, thereby capturing a broader spectrum of diversity and genetic variability.

Investigating therapeutics' effects on T cell biology through this platform provides a comprehensive understanding of how compounds interact with the immune system. This knowledge advances your drug development, enabling the identification of potential immunotherapies, evaluating safety, and ultimately contributing to more effective treatments for a range of conditions, from immuno-oncology to autoimmune diseases.

Key advantages

- Rapid semi-automated and reproducible 384-well high throughput assay
- Applicable for drug safety, immuno-oncology, and autoimmunity applications
- Providing concise and robust quantitative data in as little as three weeks
- Highly enriched human primary cells from multiple donors to address donor-to-donor variability.
- Leveraging HTRF® Homogeneous Time-Resolved Fluorescence technology.

ImmuSignature™ Assays

T Cell Activation (TCA) Assay: By assessing the impact of therapeutics on CD4+ and CD8+ T cell proliferation and activation, this assay provides rapid insights into potential compound effects. Understanding how compounds modulate T lymphocyte stimulation and inhibition helps gauge their potential immunotherapeutic applications. This in-depth exploration aids in predicting immune response alterations, a crucial step in drug development.

Mixed Lymphocyte Reaction (MLR) Assay: The MLR assay is a robust platform to evaluate interactions between antigen-presenting cells and T cells, offering a glimpse into potential therapeutic impacts on immune dynamics. By deciphering how compounds influence the activation, deactivation, or repolarization of lymphocytic responses, the MLR assay contributes significantly to comprehending potential immunomodulatory effects.

Treg Polarization (TRP) Assay: Regulating the immune system is pivotal, and this assay helps investigate how therapeutics affect the generation of T regulatory cells. Understanding the impact on inducible T lymphocyte regulatory cells is vital in controlling immune responses and maintaining self-tolerance. The TRP assay provides insights into how compounds influence the polarization of regulatory phenotypes, offering avenues for potential anti-inflammatory treatments.

Treg Suppression (TRS) Assay: Evaluating the ability of compounds to regulate T effector cell suppression is crucial for understanding potential therapeutic impacts. This assay aids in uncovering how therapeutics could potentially influence immune regulation, providing valuable data to support clinical applications. The TRS assay offers a comprehensive view of immunomodulation potential by co-culturing polarized T regulatory cells with effector cells and analyzing compound effects.

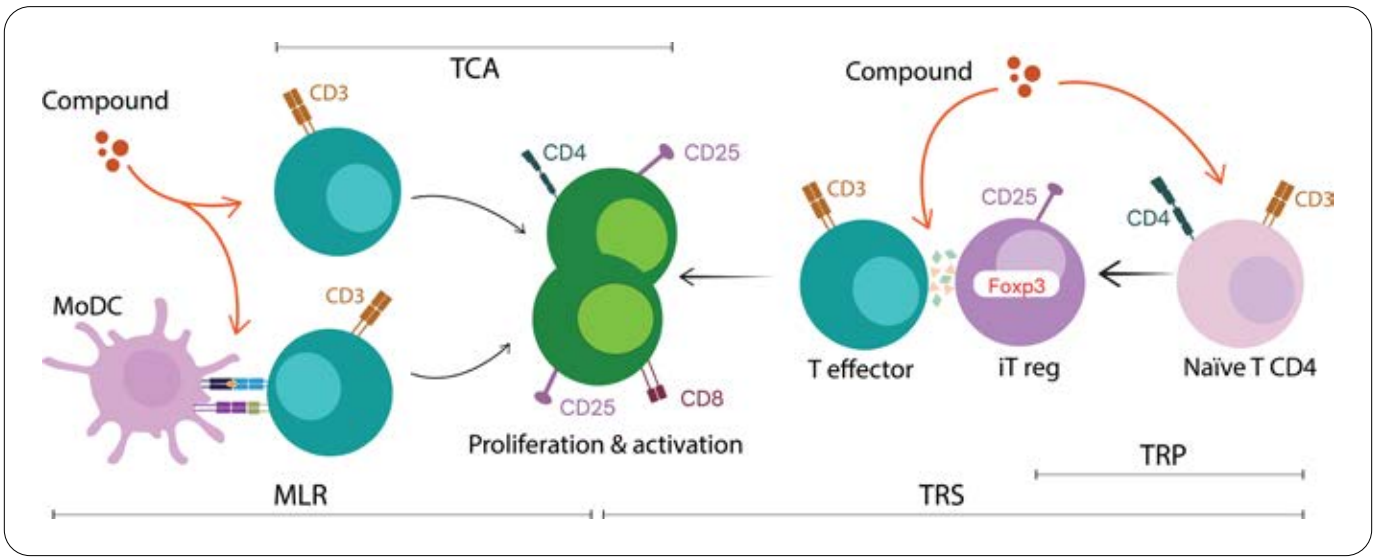

Diagram of ImmuSignature assays. It comprises four complementary models of therapeutic analysis over T cell physiology, centered on quantifying the proliferation and activation of T CD4+ and TCD8+ cells. The T cell activation assay (TCA) is a simple dose-response layout of compounds over isolated T cells, while the mixed lymphocyte reaction (MLR) analyses the compound effect over a co-culture of an antigen-presenting cell (MoDc) and T lymphocytes. On the other hand, compounds can also be investigated in their effect on the polarization of naïve T cells to a regulatory phenotype, an iTreg polarization (TRP) assay. Furthermore, compounds can also be challenged in their influence of the iTregs to modulate the regulatory function over T effector cells in a suppression assay (TRS).

APPLICATION NOTE

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

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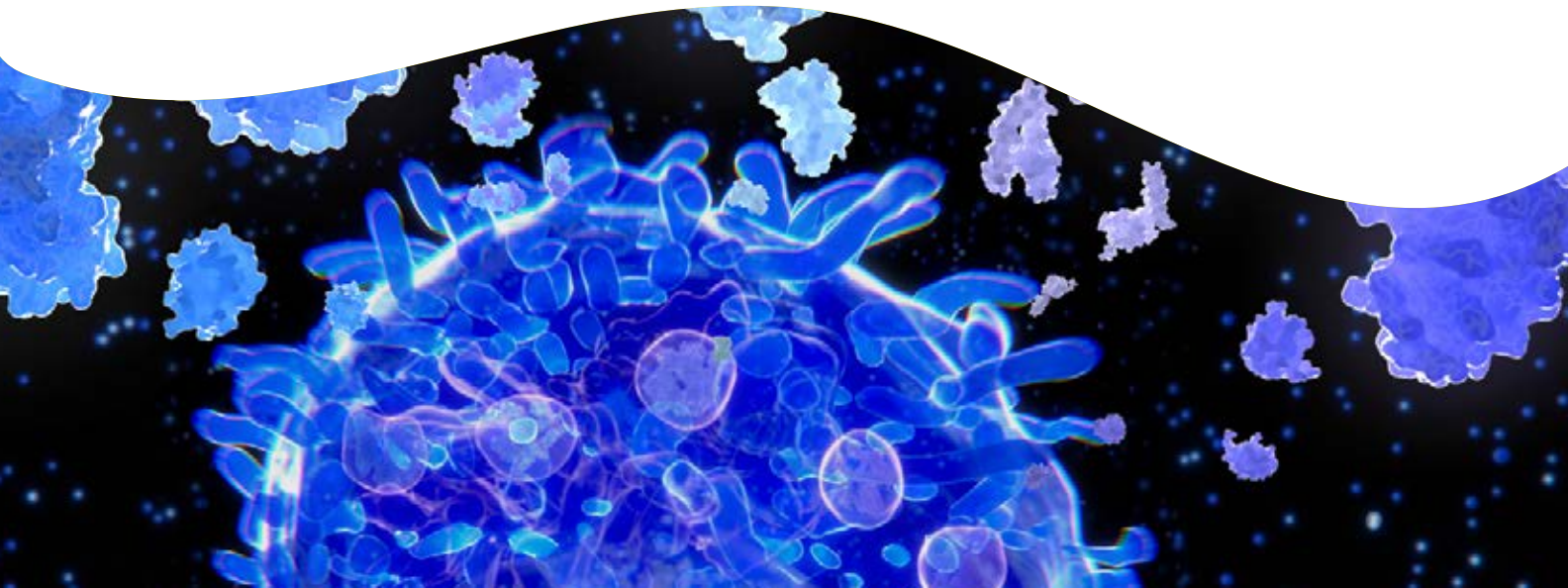
Introduction

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.

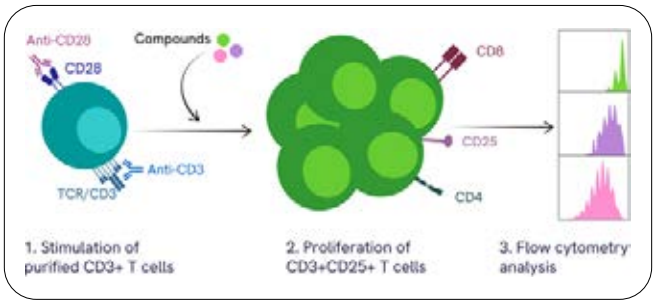


Figure 1: Schematic Representation of the Standard T cell Activation Assay. CD3+ T cells are stimulated with a cocktail of anti-CD3/CD28 antibodies and treated with test and control compounds for four days. The activation of CD4+ and CD8+ T cell subtypes is analyzed based on proliferation and the expression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

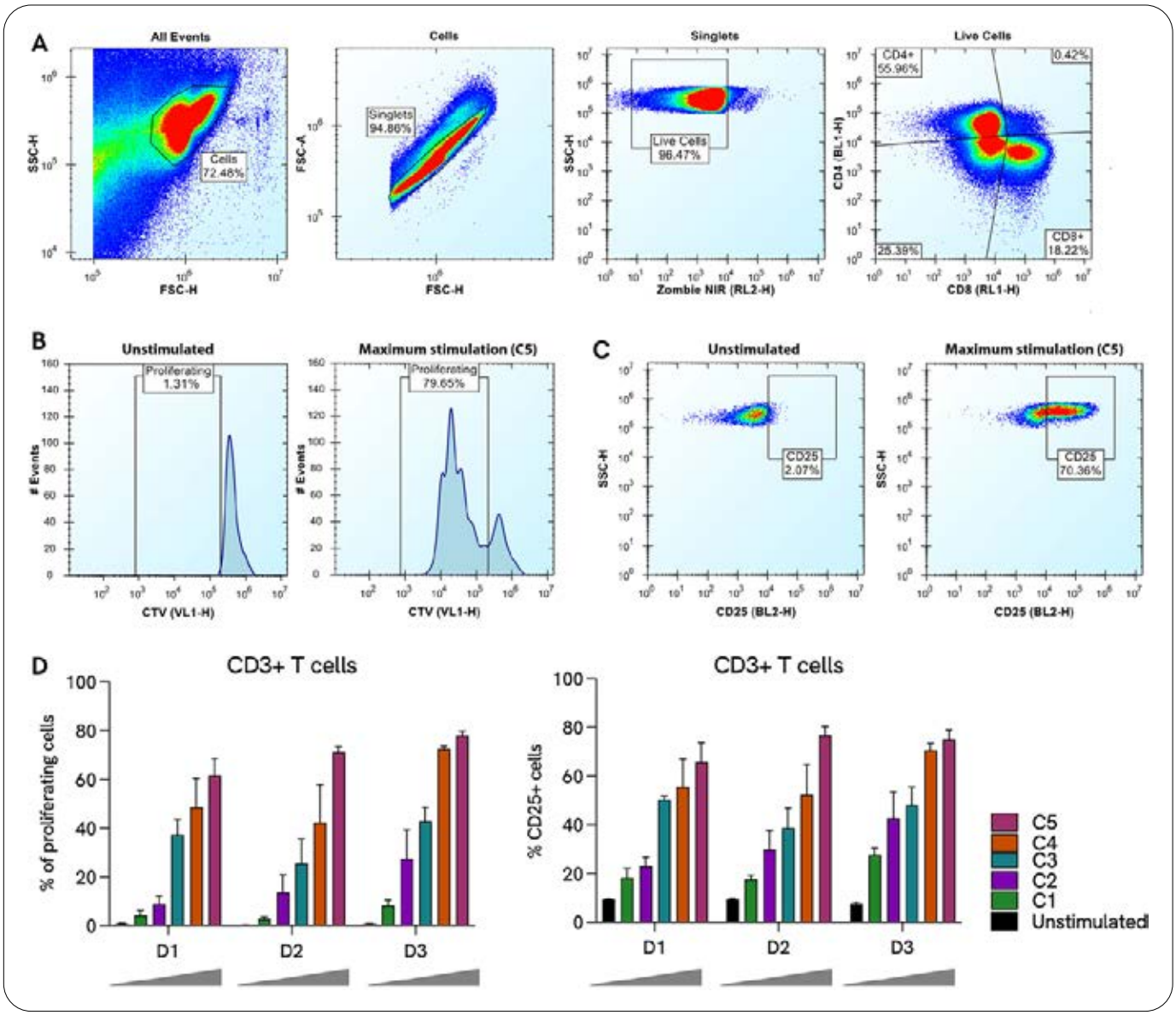


Figure 2: Proliferation and CD25 Expression in CD3+ T Cells. (A) Gating strategy for the flow cytometry analysis of CD3+ T cells. (B and C) Flow cytometry analysis of proliferation and CD25 expression of unstimulated CD3+ T cells and following treatment with the highest tested concentration of anti-CD3/CD28 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.

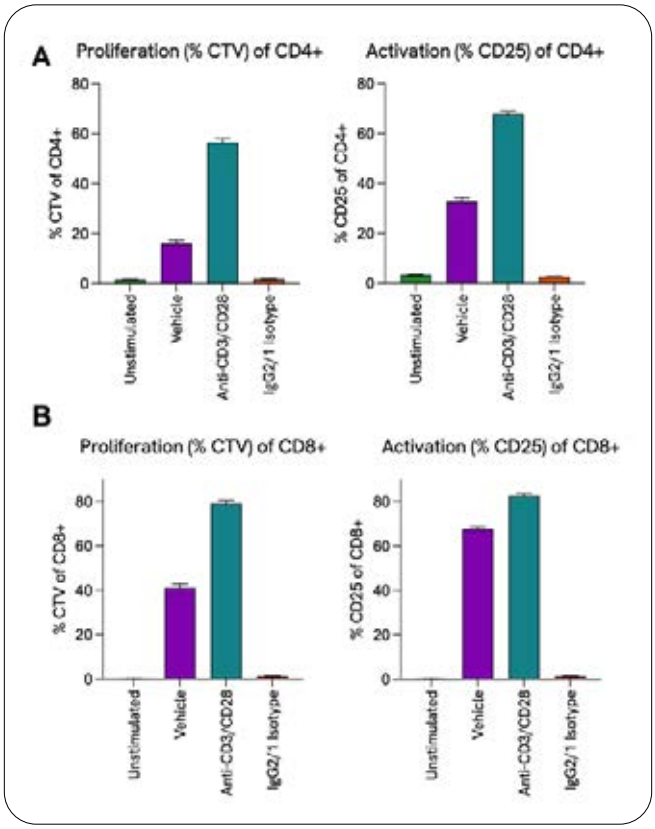


Figure 3: Assay Performance of CD4+ and CD8+ T cells. Flow cytometry evaluation of CD4+ (A) and CD8+ (B) proliferation and activation after 4 days in the indicated conditions. Data are expressed as mean + standard deviation of four technical replicates, depicting a single donor. Conditions include unstimulated, 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.

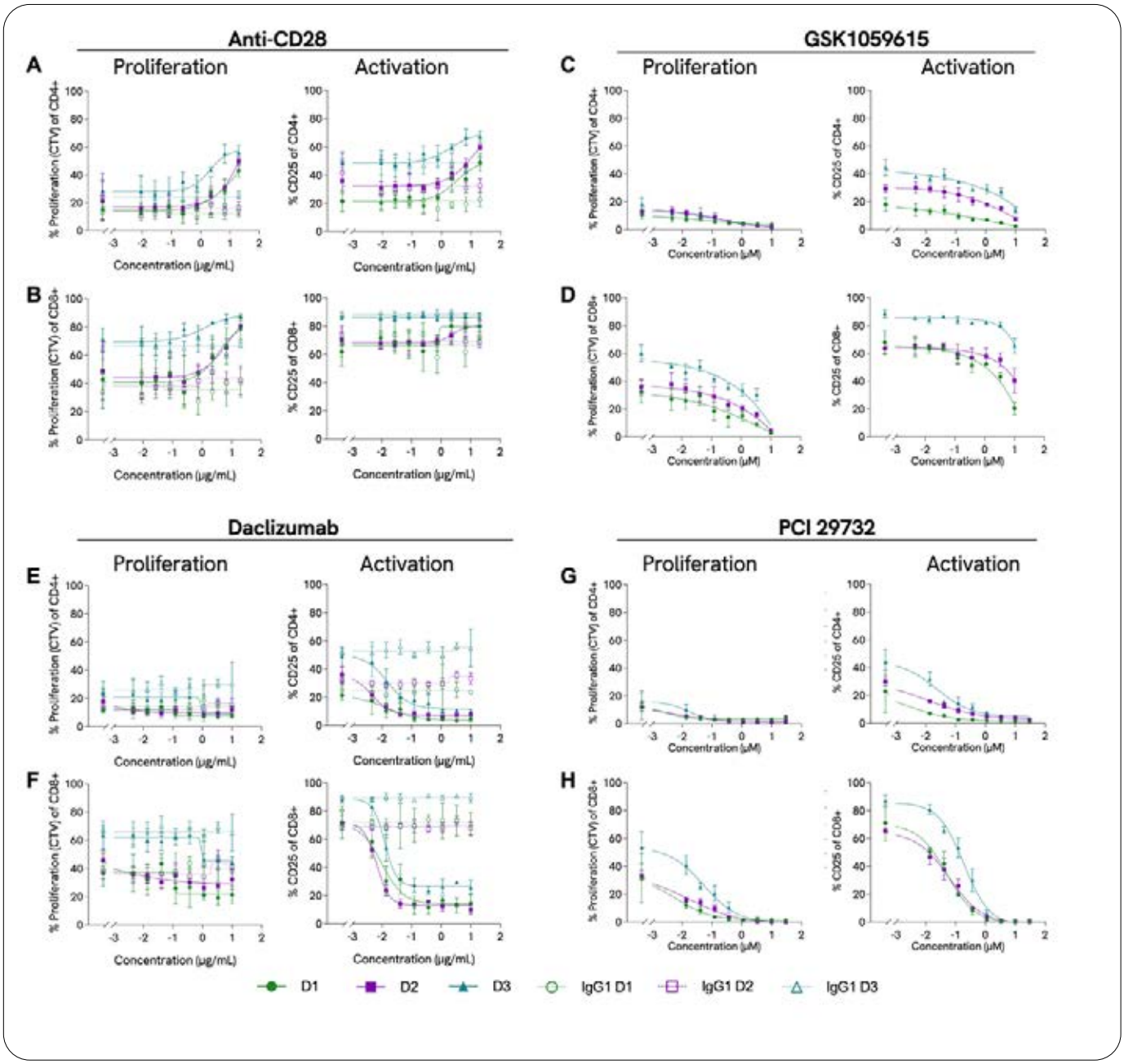


Figure 4: The effect of activating and inhibitory compounds on T cell stimulation. The effect of anti-CD28 antibody and isotype control (Panels A-B), GSK1059615 (panels C-D), daclizumab and isotype control (Panels E-F), and PCI 29732 (Panels G-H) on T cell proliferation and activation. CD4+ (Panels A, 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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APPLICATION NOTE

ImmuSignature™ MLR: Rapid high-throughput assessment of therapeutic immunogenicity

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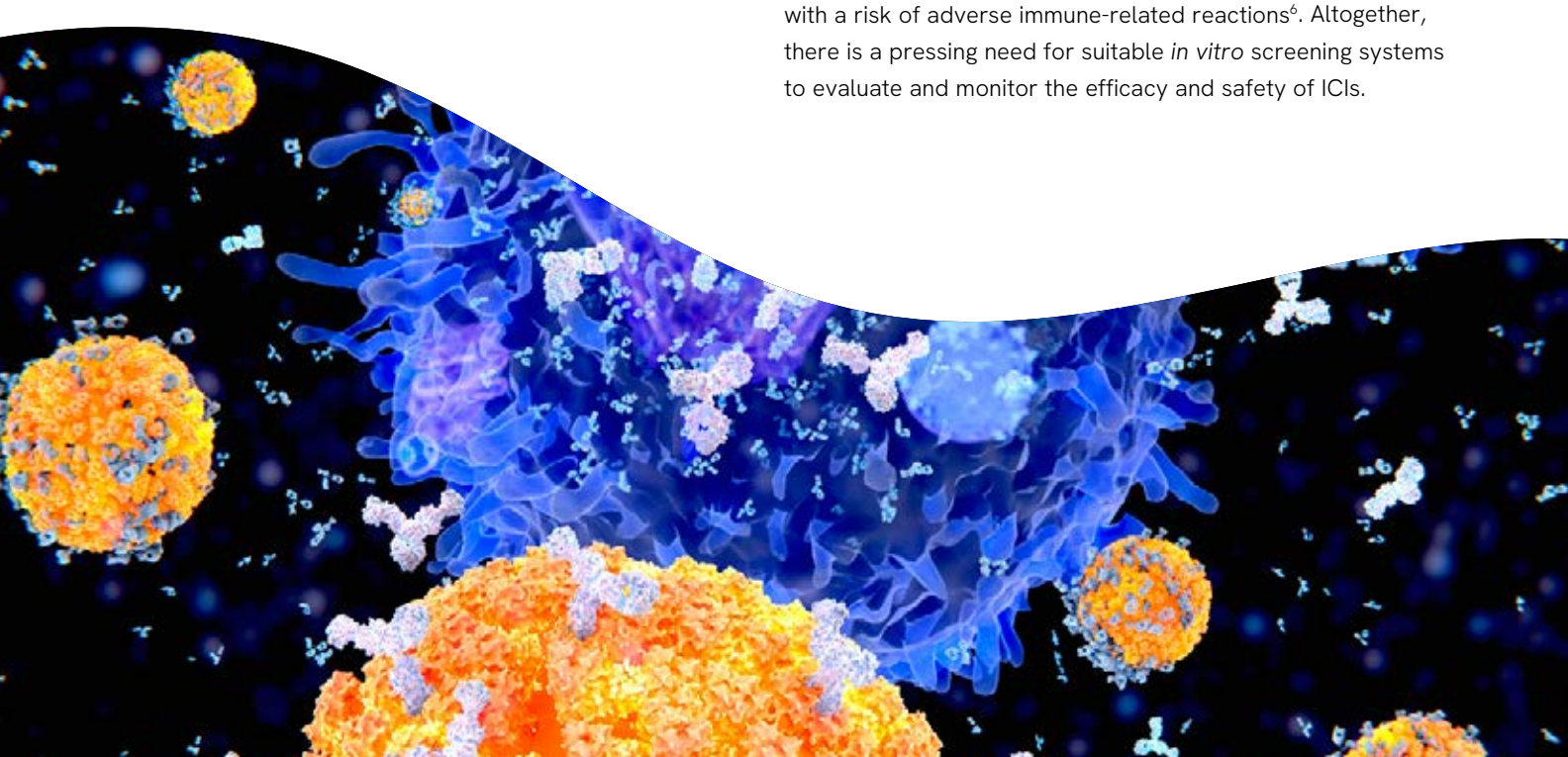

Introduction

Immune checkpoints are regulatory signals that act as 'on and off switches' for T cells[1], a mechanism tumor cells commonly deploy to evade immunosurveillance[2]. Immune checkpoints are engaged when a T cell binds to partner proteins on an antigen-presenting cell (APC) or tumor cell. Interaction of these receptor and ligand pairs results in dysfunction and/or exhaustion characterized by impaired effector function such as reduced cytotoxicity or cytokine production, lack of response to stimuli, and altered transcriptional and epigenetic states[3]. Immune checkpoint inhibitors (ICIs) target this co-inhibitory synapse between T cells and APCs/tumor cells to elicit an anti-tumor response[4-5]. These therapies aim not to kill cancer cells directly but to release blocks that shield tumor cells from immune destruction.

Immunomodulators are now used as single agents or combination therapies for various cancer types and represent about two thirds (~3000) of active clinical oncology trials[1]. With the success of existing ICI therapies and substantial clinical progress in treating certain aggressive cancers, ICIs continue to be an area of focus for intensive research and development for bringing newer therapies to market.

Given the nature of these drugs, characterizing their mechanism of action requires complex assays reliant upon the interaction between primary T cells and APCs. Additionally, although ICIs show immense promise, they have historically been associated with a risk of adverse immune-related reactions[6]. Altogether, there is a pressing need for suitable *in vitro* screening systems to evaluate and monitor the efficacy and safety of ICIs.

This application note focuses on the properties of our mixed lymphocyte reaction (MLR) assay as a rapid, relevant, and reliable platform to characterize the functionality of molecules of interest and address safety considerations.

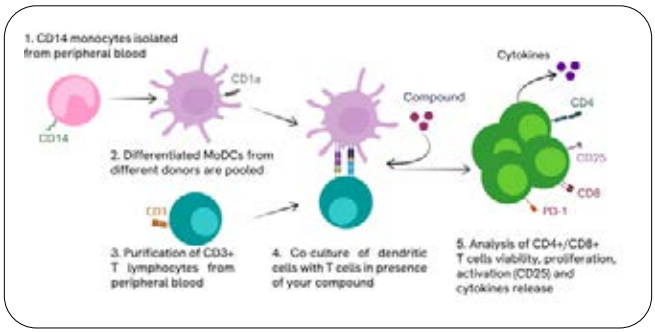

Figure 1. **One-way MLR assay setup**. MoDCs derived from CD14+ monocytes are co-cultured with CD3+ T cells over several days. Effect of compounds addition to the coculture is assessed by a multiplexed readout that measures the activation and proliferation of CD3+ T cells in response to the MoDC donor mismatch. Flow cytometry measures CD25 expression as a marker of activation and proliferation in the total CD3+ T cell fraction and CD4+ and CD8+ T cell subsets. Activation of T cells is determined by cytokine release using HTRF (Homogenous Time-Resolved Fluorescence) technology.

Mixed lymphocyte reaction

The interaction between T cells and APCs is critical in mounting an effective immune response, with dendritic cells (DCs) widely recognized as the most efficient class of APCs[7]. The mixed lymphocyte reaction (MLR) mimics this immunological synapse and is helpful as an *in vitro* model to explore the modulatory effects of drugs and facilitate drug discovery processes. In an MLR assay, T cells (responders) are co-cultured with APCs (stimulators) in an allogeneic manner. In the *in vitro* microenvironment, the T cells scan the surface of the stimulator cells. The recognition of 'non-self' antigen resulting from Human Leukocyte Antigen (HLA) donor mismatch leads to potent T cell activation that can be evaluated through increased production and cytokine production. Conventionally, MLR assays can be conducted as either one-way or two-way reactions. In a one-way MLR, T cells from one donor are cultured with APCs from an alternative donor. For a two-way MLR, Peripheral Blood Mononuclear Cells (PBMCs) isolated from two distinct donors are co-cultured with a bidirectional activation of T cells within both populations. Due to the unidirectional nature of stimulation, a one-way MLR is advantageous as a cleaner system for measuring T cell activation in a background-free model. Screening of compounds and lead identification can be a lengthy investment process.

Our standard MLR assay, described in Figure 1, offers a rapid solution for screening agents with a multiplexed readout, with data delivery in as little as four weeks. A miniaturized 384-well format combined with a semi-automated approach enables the screening of up to 18 compounds in three independent T cell donors in a single run, including dose-response controls. Additionally, to further shorten the process, we utilize cryopreserved cells in our assay with ample evidence through optimizations and comparisons that cryopreserved cells perform equally well as freshly isolated cells.

Our MLR assay is a robust standard offering several quality control (QC) assessment stages. First, isolated and differentiated cells undergo a thorough purity check before cryopreservation and assay set-up. Second, assay performance is a prerequisite to further data analyses, evaluated through our resolute MLR bioinformatic pipeline and the use of relevant positive and negative controls. This assay performance QC also informs on the existence of plate-to-plate variations, if any. Figure 2 overviews our assay performance QC criteria and the use of internal assay controls (CD4+ T cells shown as representative). The top panel reflects proliferation in T cells as measured by CellTrace™ Violet (CTV), and the bottom panel indicates CD25 expression as a surface marker of T cell activation. CTV is a proliferation tracker dye that diminishes in intensity upon cell division, with each division representing a distinct peak. CD3+ T cells cultured alone do not proliferate and are not activated, as evidenced by a single CTV peak (no loss in CTV intensity) and lack of CD25 staining. In comparison, CD3+ T cells co-cultured with MoDCs (medium) undergo cell division and express high levels of CD25 in an allogeneic reaction.

As internal assay performance controls, we use a single dose of recombinant cytotoxic T-lymphocyte antigen 4 (CTLA4) or anti-CD3/28 antibodies as negative and positive controls, respectively. Interactions of APC ligands with CTLA4 result in T cell anergy/serve to inhibit T-cell responses[8]. True to this, CTLA4 protein (CTLA4-Ig) acts as a negative regulator of T cell proliferation and activation in our assay (Figure 2). Anti-CD3/28 antibodies, on the other hand, serve as a positive control and provide a co-stimulatory signal that engages the T cell receptor resulting in robust proliferation and increased CD25 expression.

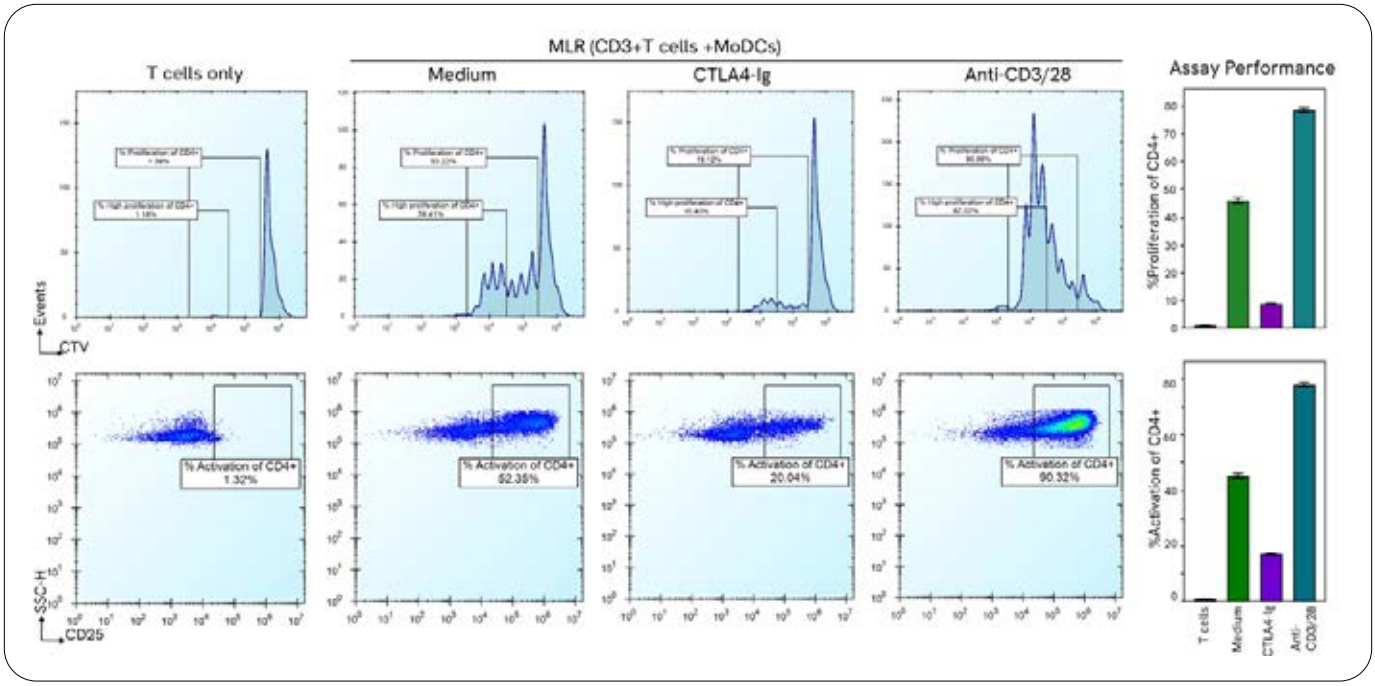

Figure 2. MLR assay performance QC measure using internal assay controls. Representative flow cytometric measure of proliferation (top panel) and activation (bottom panel) in CD4+ T cell fraction of CD3+ T cells cultured alone or in the presence of MoDCs. Co-culture conditions comprise no treatment (medium) or treatment with a single dose of CTLA4-Ig (negative control) or anti-CD3/28 (positive control). The reduction of the fluorescent tracker dye CellTrace™ Violet (top panel) shows proliferation levels and CD25 expression as a T cell activation surface marker (bottom panel). Cumulative assay performance data obtained from quadruplicates and standard deviation of a single donor data are depicted in the graphs on the right for proliferation (top panel) and activation (bottom panel), respectively.

Each condition is run in quadruplicate (per plate), and the overall assay performance is depicted in the graphs on the far right in Figure 2 for both proliferation (top panel) and activation (bottom panel).

Robust assay window and multiplexed readout for screening activators and inhibitors

T cells play a critical role in shaping the immune landscape in the context of the tumor microenvironment and in autoimmune and inflammatory diseases. In this context, the MLR assay makes for a highly relevant *in vitro* model to understand the effects of biologics and/or small molecules that modify the interaction between APCs and T cells to activate, deactivate or repolarize the lymphocyte response. It can also provide critical information for undesired immunological responsiveness from a drug safety perspective. The assay window becomes critical to address the impact of test agents, keeping all these considerations in mind.

Achieving proliferation and activation of T cells in the 40-60% range is ideal for a reliable assay window for screening activators and inhibitors. Furthermore, this assay window must be consistently reproducible using primary immune cells and potential donor variability as a by-product. To control donor dependencies without impacting physiological relevance, our MLR assay considers several factors: 1) To maximize the chance of 'non-self' recognition, we utilize MoDCs pooled from multiple individual donors. These are then co-cultured in a fixed ratio to the number of T cells in the assay to achieve consequent T cell activation and proliferation in a controlled manner. 2) A minimum of three independent T cell donors are assessed in the assay to account for both donor reproducibility and variability. 3) The semi-automated process controls the overall assay variability and reduces manual error.

Our optimized MLR offers a reliable assay window that assesses outcomes linked to either activation or inhibition of T cell function. To assess the suitability of our assay window as fit for purpose, we conducted a 20-compound alpha screen that included a mix of biologics and small molecules. This panel included the monoclonal antibodies, nivolumab and daclizumab, and their respective isotype controls, depicted in Figure 3 as representative examples. Nivolumab is a clinically approved humanized IgG4 antibody that blocks

the immune checkpoint PD-1 and can restore antitumoral response by abrogating PD-1 pathway-mediated T-cell inhibition[9]. On the other hand, daclizumab is a humanized IgG1 antibody that inhibits effector T cell activation by blocking CD25, a critical subunit of the interleukin-2 receptor (IL-2R) required for T cell maintenance[10].

A nine-point dose range (eight doses plus vehicle control) was tested using a three-fold semi-logarithmic serial dilution series and the highest final concentration of 10 µg/mL. Using a multiplexed readout, we assessed T cell activation, proliferation, and viability through flow cytometry and IFN-g production by HTRF. Beyond total CD3+ T cells, the flow cytometric readout also provides granularity on CD4+ and CD8+T cell subsets. Donor reproducibility is essential when screening test agents for their ability to modulate responses. To this end, we observed in Figures 3A-B and E-F the respective inhibitory and activating effects of daclizumab and nivolumab on the activation and proliferation of these T cell subsets in all three donors. While panels 3A and 3E show the impact of these compounds on CD25 expression (activation) and CellTrace Violet staining (proliferation) in CD4+ helper T cells, panels 3B and 3F show their equivalent in cytotoxic CD8+ T cells. Figures 3C and G depict the effect of these test agents on T cell viability. As anticipated, daclizumab targets IL-2R critical for T cell maintenance and thus affects the viability of all three donors (Figure 3C). On the contrary, viability upon nivolumab treatment did not have significant changes (Figure 3G).

In summary, this data reiterates the strength and robustness of our MLR assay in terms of an assay window that allows for the screening of inhibitors/activators and its ability to capture donor reproducibility and variability, including T cell subset granularity.

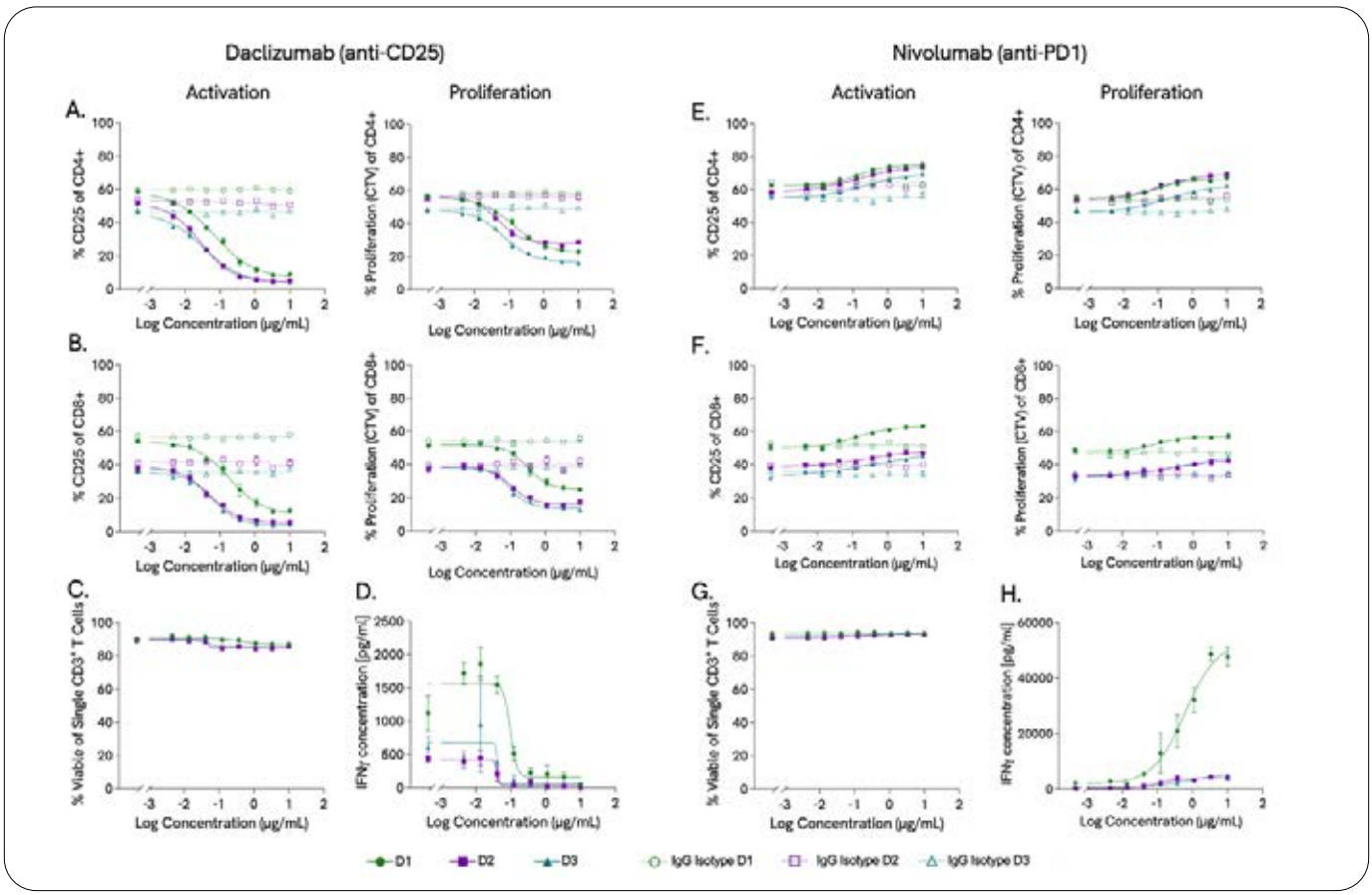

Figure 3. **Screening of inhibitory and activating immunomodulators using the MLR assay multiplexed readout**. Treatment of CD3+ T cell and MoDC co-cultures with anti-CD25 monoclonal antibody daclizumab (Panels A-D) and anti-PD1 immunotherapy nivolumab (Panels E-H) and the relevant isotype controls. Nine-point dose range (eight points + vehicle) and 3-fold semi-logarithmic dilution. A & E. Flow cytometric measure of activation (CD25) and proliferation (CTV) in CD4+ T cells. B & F. CD25 expression (activation) and proliferation (CTV) in CD8+ T cells as measured by flow cytometry. C & G. Flow cytometric assessment of total CD3+ T cell fraction percentage viability upon drug treatment. D & H Quantification of IFN-γ production by T cells using HTRF technology. D1-3 represent three independent CD3+ T cell donors. Data plotted as mean with standard deviation.

Capturing subset-specific effects

As detailed above, one of the advantages of multiparameter flow cytometry is that it allows the distinction of different subsets within a cell population, enabling the teasing apart of subset-specific effects of compounds that may otherwise get masked. Another outcome from our alpha screen was basiliximab (biosimilar)[10], a chimeric mouse-human monoclonal antibody that, like daclizumab, targets the α chain (CD25) of the IL-2R of T cells. Although the basiliximab biosimilar results in a reduction in CD25 expression in both CD4+ and CD8+ T cells in line with its target (Figure 4, A and C), interestingly, the outcome on the proliferation of these cells is converse (Figure 4, B and D).

While CD4+ T cells exhibit a reduction in proliferating cells (as measured by the percentage of cells positive for CTV), the co-culture in the presence of basiliximab increases the percentage of proliferating CD8+ T cells. CD4+ T cells comprise ~70% of total CD3+ T cells and are the dominant population in culture. In the absence of this subset granularity, the impact of this biologic on CD8+ T cell proliferation would have been disguised, with potentially significant implications *in vivo*. Overall, this data emphasizes the importance of utilizing an assay that screens for subset specific and/or potentially undesirable effects in the early stages of drug discovery and would offer end-users a good indication of the research focus.

A platform for biologics and small molecules

To test the applicability and relevance of our MLR assay in a greater context, we included a variety of compounds in our 20-compound alpha screen. Adalimumab is a monoclonal TNF-a blocker currently administered for several autoimmune conditions such as rheumatoid arthritis and Crohn's disease to dampen the immune response[11]. Our assay in Figures 5A and 5C reflects the resultant inhibition of proliferation in CD4+ and CD8+ T cells upon treatment with an adalimumab biosimilar in all three donors (identical impact on activation; data not shown). Emerging evidence suggests that small molecule targeted inhibitors can promote an antitumoral immune response in combination with immunotherapy. Counterintuitive to their immunosuppressive properties, mechanistic target of rapamycin (mTOR) inhibitors can promote or repress the immune response. Based on this reasoning, we also included the dual mTOR1/2 inhibitor KU-0063794 in our alpha screen to support small molecule drug discovery. Data from Figures 5B and 5D exhibit the negative impact of KU-0063794 on CD4+ and CD8+ T cell proliferation. Once again, this effect is consistent across three independent T cell donors. Altogether, these data showcase the suitability of our MLR assay for screening different types of compounds ranging from biologics to small molecules in various contexts.

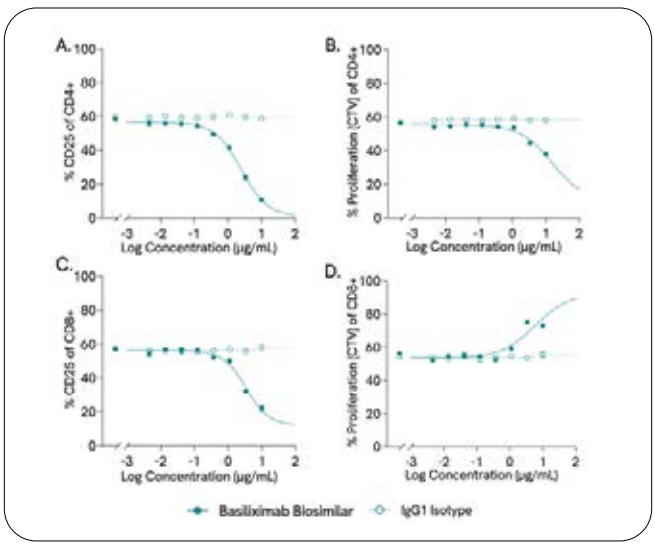

Figure 4. **Subset specific effects in T cells upon treatment with basiliximab biosimilar.** Flow cytometric measure of CD25 expression (activation) and proliferation (CTV intensity) in CD4+ (A and B) and CD8+ T cells (C and D). CD3+ T cell and MoDC co-cultures were treated with either a basiliximab biosimilar or IgG1 isotype control. Single donor depicted for representative purposes; data plotted as mean with standard deviation.

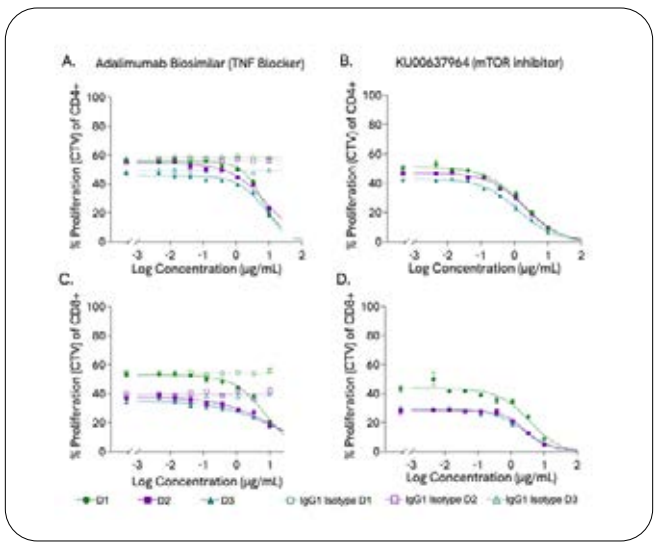

Figure 5. **Suitability of the MLR assay for screening biologics and small molecules**. CD3+ T cell and MoDC co-cultures were treated with either A&C. Adalimumab biosimilar or IgG1 isotype control or B&D. the small molecule mTOR inhibitor KU00637964. Percentage proliferation (CTV intensity) in CD4+ (A-B) and CD8+ T cells (C-D) as measured by flow cytometry. D1-3 represent three independent CD3+ T cell donors. Data plotted as mean with standard deviation.

Decipher compound effect with additional readouts

In addition to IFN-γ, other cytokines can be quantified in the supernatant of MLR assay, comprising TNF-α and IL-2. TNF-α is a major pro-inflammatory cytokine produced in response to toll-like receptor stimulation, and IL-2 is an early-produced cytokine that promotes T cell growth. The release of those three cytokines in the supernatant has been assessed by HTRF every day, for all duration of the assay, with or without the addition of control compounds (Figure 6A).

As expected, quantities of the three cytokines are not significantly modulated over time in the assay supernatant without compound treatment (medium condition). The addition of Anti CD3/28 TCR activators induced strong production of the three tested cytokines compared to baseline but with different kinetics. IFN-γ and TNF-α release in the supernatant increases over time and is maximal after 3-4 days. On the other hand, anti-CD3/CD28 leads to potent induction of IL-2 as soon as day 1, but that decreases over time, probably because of T cells activation and proliferation that consumes the IL-2 present in the medium.

The addition of daclizumab induces a slight increase of IL-2 in the supernatant over time. This observation can be explained by the fact that this antibody targets a subunit of the IL-2 receptor and therefore blocks IL-2 uptake by T cells[12]. Nivolumab antibody induces T cell activation in MLR assay, like anti-CD3/28, but with slower kinetics for IFN-γ and TNF-α, and the effect on IL-2 cannot be seen. Early cytokines assessment does not impact final readouts (data not shown), showing our MLR assay robustness and customizable readouts.

In addition to secreted protein, other markers can be induced by MoDC-induced T cell activation, like the immune checkpoint PD-1 (Figure 6B). PD-1 plays a critical role in the induction and maintenance of immune tolerance and is a target for cancer immunotherapies. Therefore, we added its detection as an optional readout in our flow cytometry panel.

As shown in Figure 6C, adding nivolumab, an anti-PD1 antibody, leads to decreased PD-1 detection in our MLR assay, as expected. This readout allows us to detect the compound effect on this specific immune checkpoint, and it can be used to verify the efficiency of antibodies targeting this receptor at the surface of T cells.

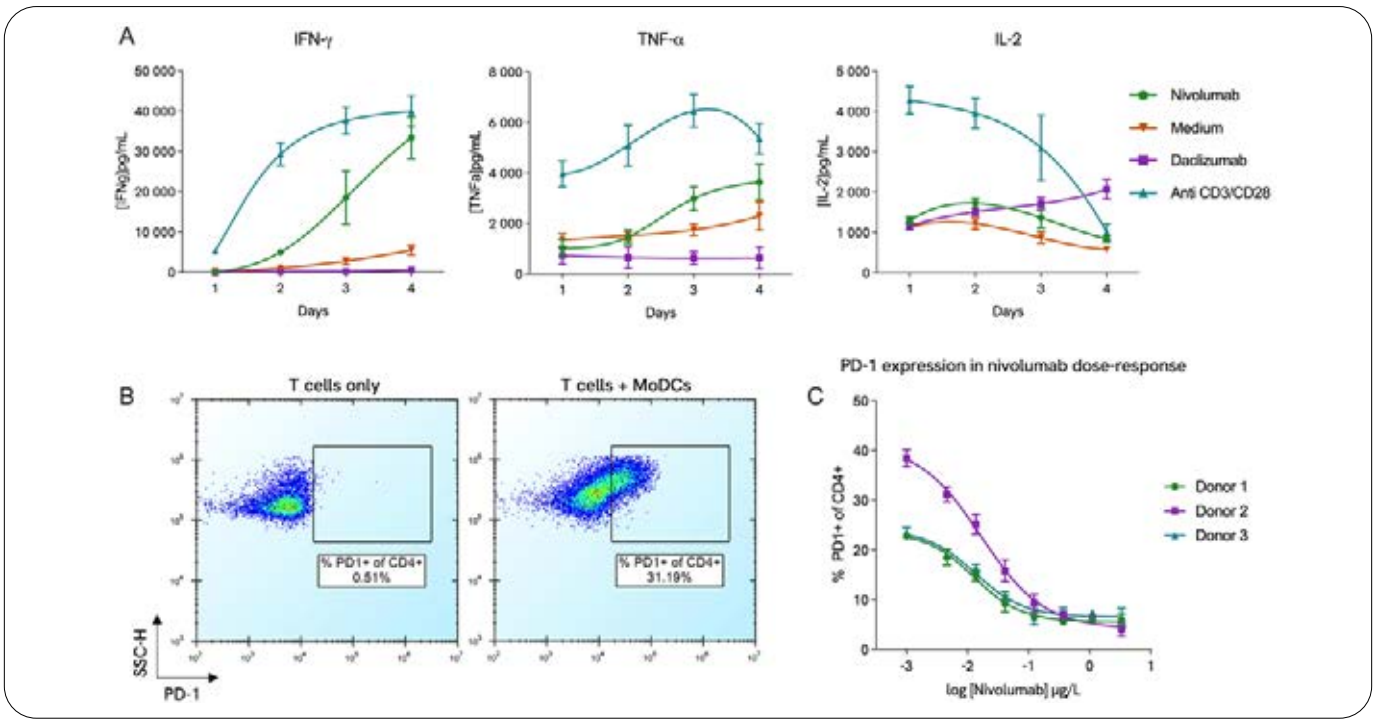

Figure 6. **Optional readout available in MLR assay**. A. CD3+ T cells from 3 different donors, in co-culture with pooled MoDCs, were treated with different compounds at day 0, and the supernatant was analyzed every day for cytokines release. Graphs show results in 1 representative CD3+ T cells donor as the mean of 4 replicates ± CV% for each time point. B. Flow cytometry representative dot plot for PD-1 expression in T cells only or cocultured with MoDcs. C. PD-1 blockade assessment by nivolumab in MLR assay. Data are plotted as the mean 4 replicates of the CD4+ T cells subset results with standard deviation, in three different T cell donors.

Summary

Boosting or suppressing the immune system to overcome disease has revolutionized therapy. Testing the efficacy and safety of potential therapeutics in a relevant and timely manner comes with challenges. With a semi-automated workflow and rapid turnaround time, we demonstrate how our standard MLR assay offers a powerful approach to furthering biological research applications and drug discovery processes. This application note exhibits its suitability, scalability, and flexibility for screening biologics and small molecules. Our multifunctional MLR assay is a highly relevant primary immune co-culture *in vitro* model to assess drugs for various conditions. It enables the understanding and monitoring of any significant immunomodulatory effects of drug candidates, not only for oncology but also for autoimmunity, inflammation, and host/graft rejection applications.

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APPLICATION NOTE

Polarization of naïve CD4+ T cells to induced regulatory T cells for compound screening.

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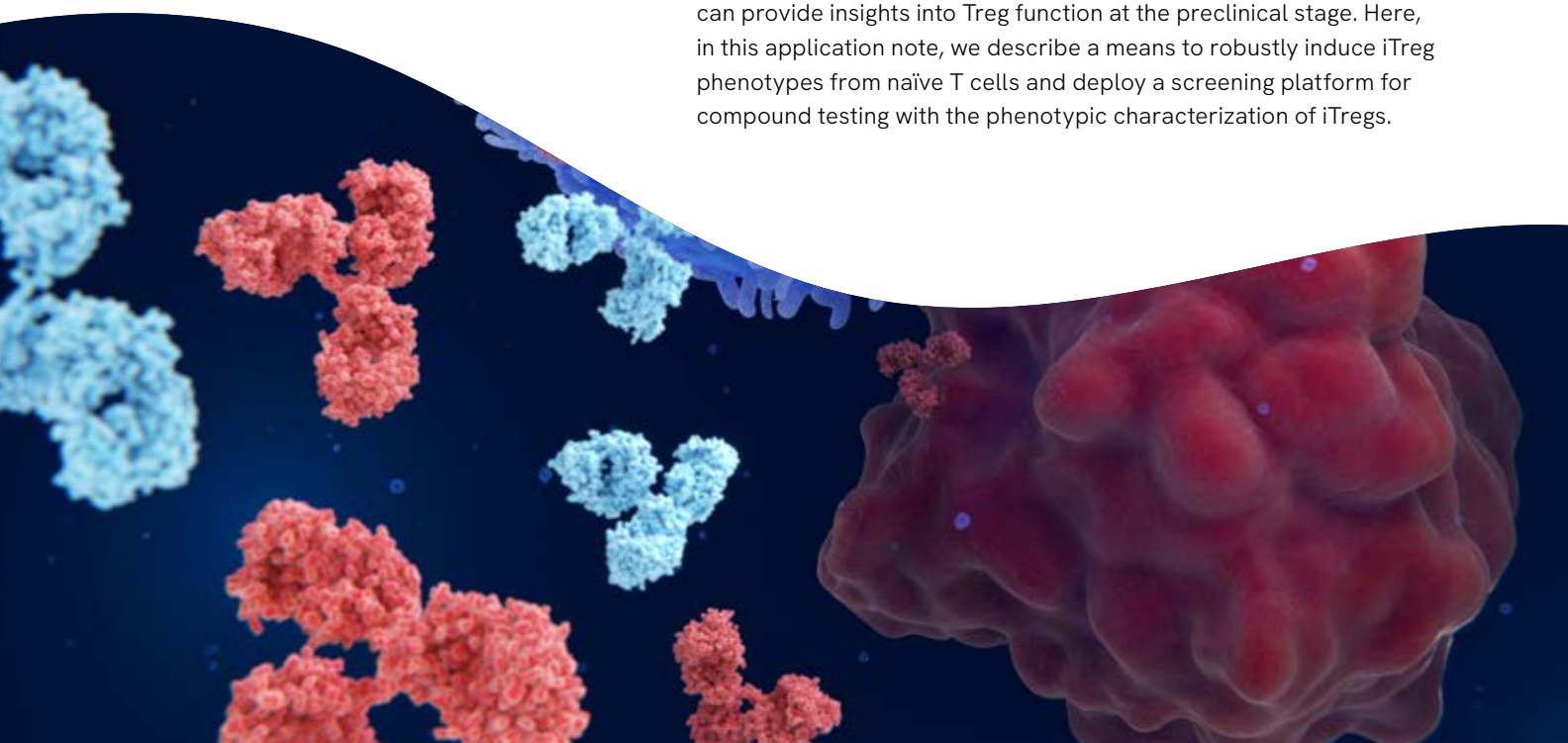
Introduction

Identifying novel pharmaceutical compounds that modulate the regulatory T lymphocytes function is critical for applying novel molecular and cell therapies to unlock the inhibition of immune responses against tumors or promote their role in the case of treating autoimmune diseases. For this, multi-analyte compound screening can deliver the immunological profiles of these drug candidates over regulatory lymphocyte subpopulations. Here we applied a rapid high-throughput in vitro functional assay to assess the effect of drugs on the capabilities to polarize a regulatory T lymphocyte phenotype from naive CD4+ T cells.

Regulatory T cells (Tregs) are a unique subpopulation of CD4+ T cells whose function is to down-regulate the immune response. Tregs are central in preventing autoimmunity, being pivotal in diseases such as type I diabetes (1), playing a significant role in chronic inflammatory diseases such as asthma and inflammatory bowel diseases (2, 3), and limiting anti-tumor immunity (4). Due to their functional diversity and significance in human diseases, Tregs are an attractive target in the immunological landscape for therapeutic interventions.

The Tregs suppressive functions derive from the production of anti-inflammatory cytokines, such as TGF-β and IL-10, and suppression by other means, such as cytolysis and metabolic disruption (5). Mediators of Treg suppressive function through direct cell-to-cell interactions of most interest include CTLA4, LAG3, and PD-1 receptors (6). To help elucidate their biological role, Tregs can be obtained in vitro by induction from peripheral naïve T cells into a regulatory phenotype (iTregs) using defined culture conditions (7).

To predict clinical applications, the in vitro analysis of iTreg polarization can provide insights into Treg function at the preclinical stage. Here, in this application note, we describe a means to robustly induce iTreg phenotypes from naïve T cells and deploy a screening platform for compound testing with the phenotypic characterization of iTregs.



Assay overview

We developed an assay that led to the polarization of iTregs through in vitro culture of naïve CD4+ T cells using a combination of TCR activators and TGF–β, IL-2, rapamycin, and retinoic acid (8,9). Naïve CD4+ T cells utilized were isolated from peripheral blood mononuclear cells and cryopreserved before polarization assay setup (Figure 1).

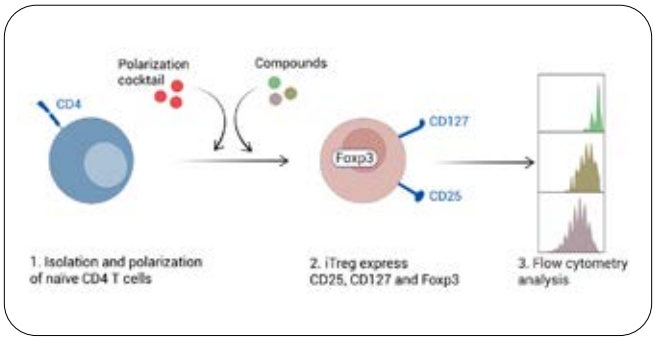


Figure 1: Standard iTreg polarization assay. Naïve CD4+ T cells isolated from peripheral blood mononuclear cells (PBMC) are stimulated with a polarization cocktail towards iTreg cells. Therapeutic candidates can be screened for their capability to influence the expression of the surface markers CD127 and CD25 and the transcription factor Foxp3.

The assay setup incorporates liquid handling automation for compound serial dilution, media addition, compound dispensing, and cell seeding steps, increasing the accuracy and reproducibility of assay results. Assay readouts include flow cytometric phenotypic analysis of Foxp3 expression as the lineage-defining marker for identifying iTregs. Since Foxp3 expression is not exclusive to T cells with regulatory phenotypes (10), our flow cytometry analysis also includes the detection of cell surface receptors CD25 and CD127 (10, 11), used to define the iTreg population CD25+CD127^{low}Foxp3+. With this approach, our multiplexed flow cytometry method facilitates efficient and rapid analyses of multiple donors with the opportunity to assess further analytes of interest based on assay requests.

Assay development

We developed a robust and semi-automated iTreg polarization assay for screening small molecules and antibody-based compounds to modulate the polarization potential of iTregs in vitro. iTreg polarization conditions described here provided an assay window to evaluate inhibitors of T-cell polarization, illustrated by decreased iTreg marker expression of Foxp3. The following section describes the assay development phases to demonstrate the capability and suitability of our iTreg cell polarization assay to projects aiming to assess novel therapeutics for T cell function.

1. iTreg identification criteria

Due to the limitation in cell numbers of nTregs from PBMCs, we developed an assay to generate iTregs in vitro. Using a commercially available naïve T cell isolation kit, we isolated CD3+CD4+CD45RA+CD45RO- cells from the PBMCs of multiple donors. Our QC analysis of isolated naïve CD4 T cells demonstrated >90% purity, as determined by high levels of CD45RA and low levels of CD45RO expression (Figure 2A). As T cells mature and undergo antigen exposure, they downregulate CD45RA and increase CD45RO expression (12). As well as this shift in CD45 expression, during activation, iTregs typically increase levels of CD25 (IL-2R) and downregulate CD127 (IL-7R) (11). We, therefore, considered iTregs cells expressing the phenotype CD25+CD127^{low}Foxp3+. Figure 2B and 2C shows a typical gating strategy, comparing iTregs with non-polarized activated T cells. The iTregs generated effectively suppressed T-cell activity (data not shown).

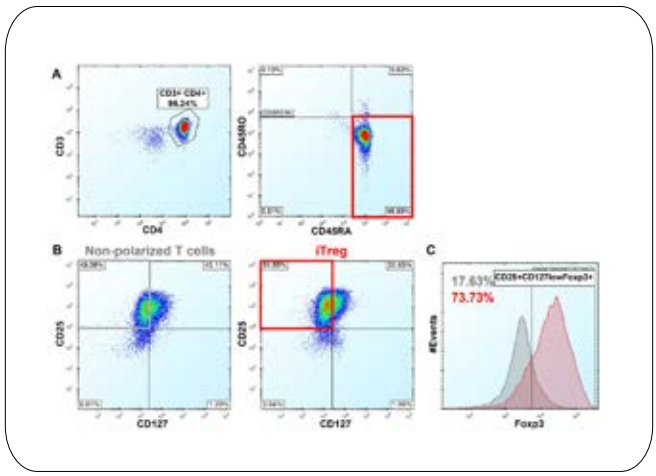


Figure 2: Gating strategies for Naïve T cells isolation and iTreg cell population. A) CD45RA+CD45RO- expression in CD3+CD4+ cells, assessed PBMC-isolated naïve CD4+ T cells purity. B) Dot plots showing CD25 and CD127 expression in non-polarized T cells (IL-2 only) and iTreg (polarized with TGF-β, IL-2, rapamycin, and retinoic acid) after six days in culture. C) Histogram of Foxp3 expression in CD25+CD127^{low} population. Control cells: grey distribution (17.63%); polarized cells: red distribution (73.73%).

2. Culture condition optimization

Using isolated naïve CD4+ T cells, we tested four different cell seeding densities for iTreg polarization in a 384-well plate assay format (Figure 3). All cells received activation through anti-CD3/CD28 antibody stimulation and were co-cultured with IL-2 to maintain T-cell viability. Cells in the polarizing condition received TGF-β supplemented with rapamycin and retinoic acid, whereas non-polarized control cells received no further media supplements. After six days of culture, we analyzed the cells for expression of iTreg markers by flow cytometry using the gating strategy outlined in Figure 2. In all seeding densities tested, we observed the induction of an iTreg phenotype in polarizing conditions compared to non-polarized cells. Cells seeded at relatively low density (Figure 3B) showed the most robust and reliable CD25+CD127^{low}Foxp3+ phenotype, allowing further analysis of other markers, such as PD-1, during the polarization process.

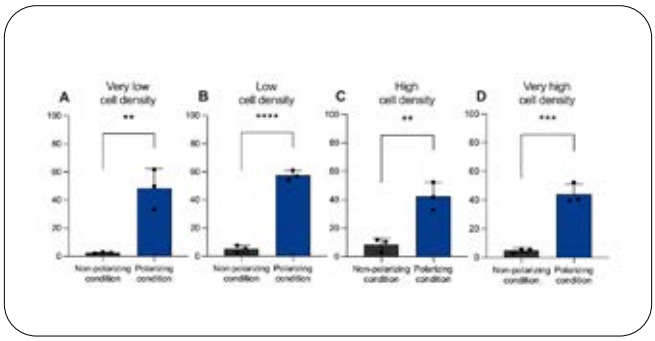


Figure 3: Optimization of naïve CD4+ T cell seeding density in the 384-well plate format. Four different cell densities for iTreg polarization were tested (A-D). Non-polarizing condition: IL-2 only; Polarizing condition: TGF-β, IL-2, rapamycin, and retinoic acid. Data represent quadruplicate technical repeats of Naïve CD4+ T cells cultured from three donors. Unpaired parametric t-test was applied. Error bars represent SD.

3. Expanding the functional phenotype analysis

Using the optimized cell number for polarization assay setup in a 384-well plate format, we added a second polarization condition with TGF-β and IL-2 only (alternative polarization) to analyze the expression of Foxp3 alongside PD-1, a key mediator of Treg function (13). TGF-β/IL-2 alone induced Foxp3 significantly over control cells, yet the addition of rapamycin and retinoic acid augmented Foxp3 expression further (Figure 4A), substantiating observations from our previous experiment (Figure 3B). Similarly to Foxp3, PD-1 was upregulated in CD25+CD127^{low} cells after six days in culture in both polarization conditions (Figure 4B), providing a second readout for the functional characterization of iTregs. Thus, our assay offers flexibility in readout options and culture conditions for the generation of iTreg cells—depending upon the expected mode of action of compounds to screen.

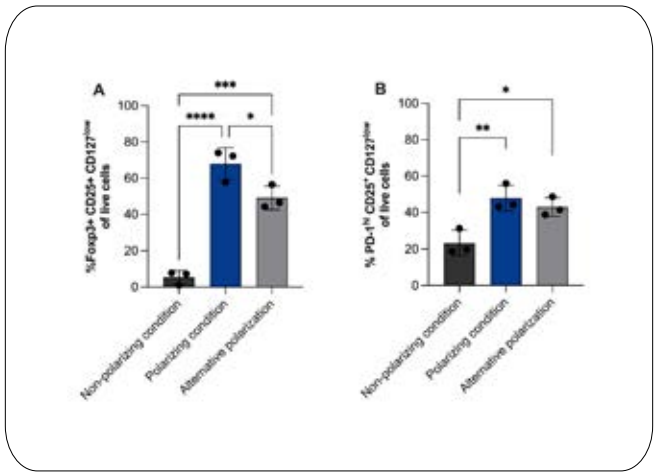


Figure 4: Expression of multiple markers for iTregs detected after polarization. (A) Foxp3 is the primary marker of iTreg phenotypes. (B) Other antigens of interest in iTregs include the surface marker PD-1. Non-polarizing condition: IL-2 only; Polarizing condition: TGF-β, IL-2, rapamycin, and retinoic acid; Alternative polarization: TGF-β, IL-2. Flow cytometry data represent naïve T cells cultured from three donors in quadruplicate. Ordinary one-way ANOVA with Tukey's post-test was applied. Error bars represent SD.

4. Assay Reproducibility

We assessed the reproducibility of the assay over several months with cells isolated from 17 donors from either leukopak (#3), leukocyte cone (#9), or buffy coat fractions (#5). We also tested polarizations in cell different culture vessels, including 6-, 24-, 48- and 384-well plate formats (Figure 5). We analyzed the expression of Foxp3 in multiple independent assays using our optimized culture condition with TGF-β, IL-2, retinoic acid, and rapamycin and compared it to non-polarized cells (IL-2 only). Polarization provided reliable induction of Foxp3 in >90% of donors tested, using cells from fresh and cryopreserved cells for assay setup. On average, our iTreg assay generated ~60% Foxp3 expressing cells in the polarizing condition, providing an optimal assay window to determine additional positive or negative effects on iTreg polarization using therapeutic compounds. The proportion of Foxp3 expressing cells did, on average, increase three-fold in the polarizing conditions

when compared to non-polarized cells (Figure 5), which can be used as a benchmark to test the effects of small molecules or biologics on the induction of Foxp3 when added to naïve CD4 T cells in the assay.

In summary, our iTreg polarization assay provides several options for compound assessment:

1) Assess the capacity of compounds to induce an iTreg phenotype by addition to naïve CD4 T cells and comparing Foxp3 expression to our positive control condition achieving ~60% Foxp3 expressing cells.

2) Assess the capacity of compounds to modulate Foxp3 expression during the polarization process in the presence of:

a. TGF-β, IL-2, retinoic acid, and rapamycin.

b. TGF-β and IL-2 (alternative polarization).

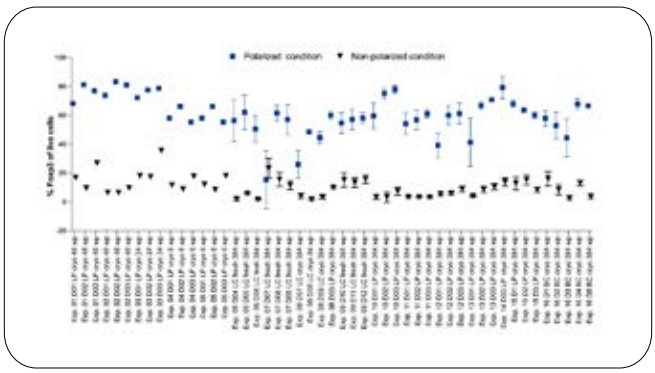


Figure 5: Foxp3 expression in polarized cells across 20 donors. Polarized: TGF-β, IL-2, rapamycin, and retinoic acid; non-polarized: IL-2 only. Assay setup from either fresh or cryopreserved (cryo) cells. Cells from 6-, 24-, 48- and 384-well plate culture vessels. Flow cytometry data obtained from 384-well plate culture in quadruplicate technical repeats. LC: Leucocyte cone, LP: Leukopack, BC: Buffy Coat. Error bars represent SD.

5. Compound dosing

We tested the effect of an mTOR and PI3Kinase inhibitor during the polarization process by adding GSK1059615 at a nine-point dose range to cells in polarizing and non-polarizing conditions on day 1. We assessed Foxp3 and PD-1 expression in both conditions on day 6 (Figure 6). Interestingly, GSK1059615 blocked Foxp3 induction and reduced expression of PD-1, suggesting negative regulation of the iTreg phenotype by this compound. Cell viability was not affected by GSK1059615 (data not shown).

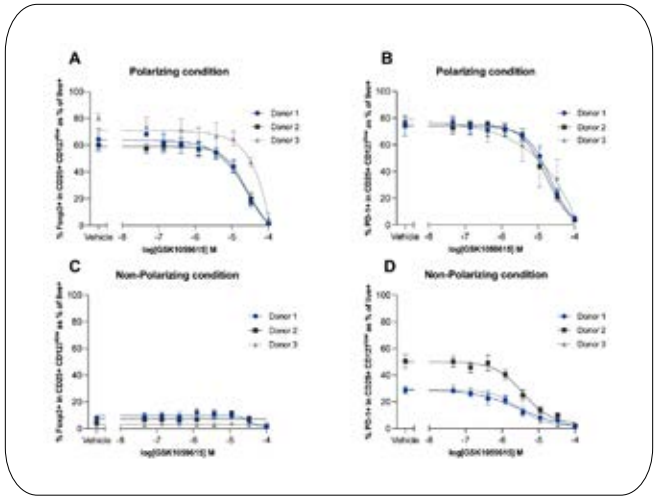


Figure 6: Drug-modulated iTreg polarization. The addition of GSK1059615 during polarization reduced the expression of Foxp3 (A) and PD-1 (B). Non-polarized cells as controls (C, D). Flow cytometry data represent naïve T cells cultured from 3 donors in quadruplicate replicates. Error bars represent SD.

Summary

This application note describes a robust and effective method for the polarization of naïve T cells into iTregs performed on a semi-automated miniaturized platform. The assay allows for the testing of compounds and biologics to induce or modulate iTreg polarization as measured by intracellular and extracellular phenotypic analysis by flow cytometry.

Furthermore, this iTreg polarization assay serves as a platform for CRISPR-based editing of iTreg cells to assess the functionality of genes in the polarization process or maintenance of the suppressive state by entering the cells into a suppression assay.

Materials and methods

Naïve CD4+ T cell isolation

Peripheral Blood Mononuclear Cells (PBMCs) from different donors were isolated from leukocyte cones, leukopaks, or buffy coats using Human Naïve CD4+ T cell Isolation Kit as per the manufacturer's instructions. Purity QC was performed by flow cytometry using Zombie Near IR (#B334686, Biolegend) with antibodies anti-CD3 PE (#300408, Biolegend), anti-CD4 APC (#317416, Biolegend), anti-CD45RO AF488 (#304212, Biolegend) and anti-CD45RA BV785 (#304140, Biolegend).

Preparation of polarization and control media

Naïve CD4 T cells were cultured in Immunocult-XF T cell Expansion Medium supplemented with human serum, Pen/Strep, and IL-2. In the polarizing condition, cells received additional TGF-β (Biolegend, #781802), Rapamycin (Selleckchem, S1039), and all-trans Retinoic Acid (Sigma, R-2625). In the alternative polarizing condition, cells received only TGF-β in addition. All cells received one dose of Immunocult human CD3/CD28 T cell Activator Mix on the day of assay setup. Fresh media containing all relevant cytokines and compounds replaced half of the media on day three, excluding the CD3/CD28 activator mix.

Preparation of the control compound

GSK1059615 (SelleckChem #S1360) was prepared at a 9-point dose range, including vehicle control, in three-fold dilution steps. The highest concentration of GSK1059615 was 10µM. Compound dilutions and addition to assay plates were performed using liquid handling systems.

Flow cytometry assessment of polarization of Naïve CD4+ T cells to iTregs

On day six post-polarization, analysis of polarization markers was performed using anti-CD25 PE (#302606, Biolegend), anti-CD127 BV421 (#351310, Biolegend), anti-human PD-1 PE/Cyanine7 (Biolegend #621616) and anti-Foxp3 AF647 (#320114, Biolegend). The viability was assessed with Zombie Near IR (#B334686, Biolegend).

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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APPLICATION NOTE

Induced regulatory T cell suppression assay for compound screening.

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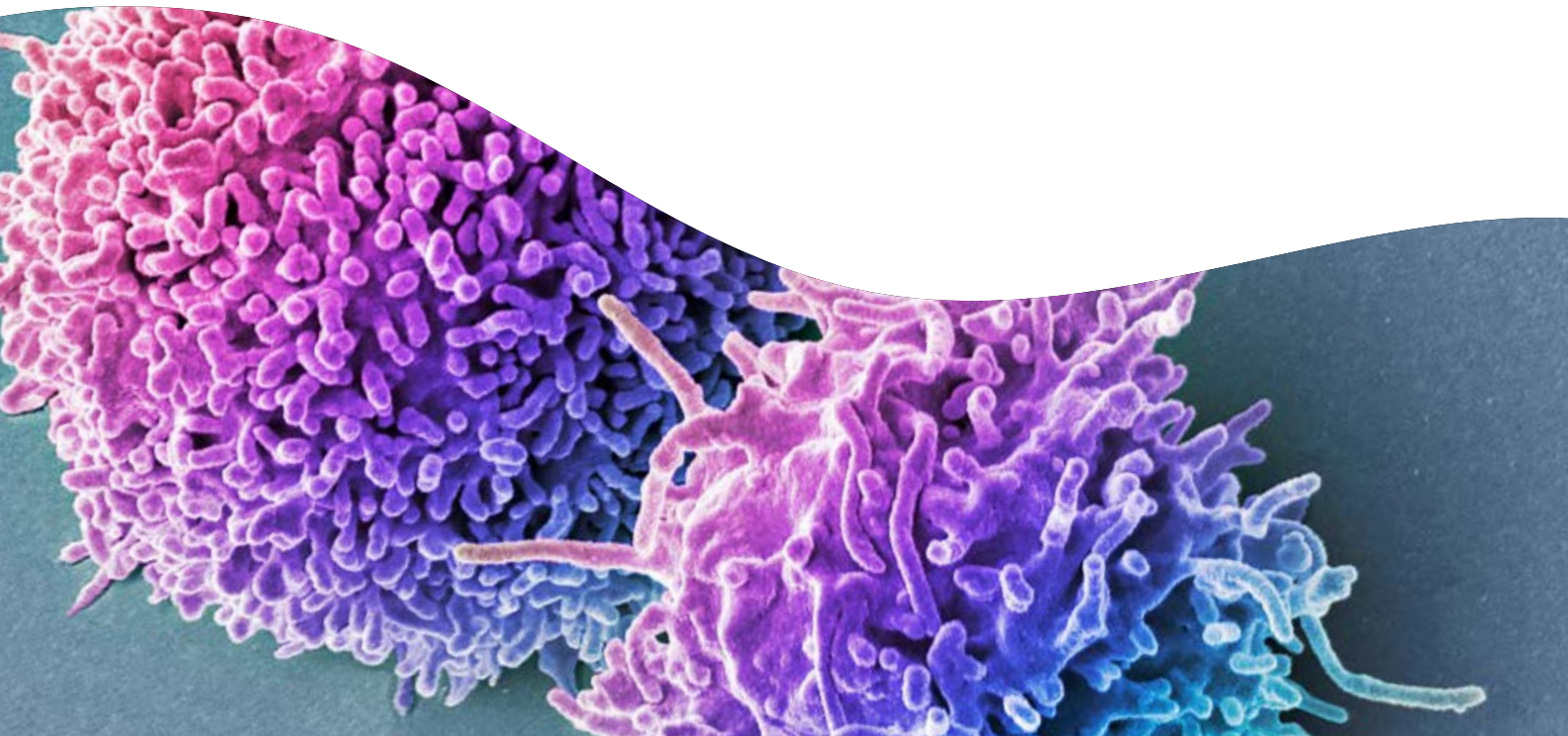
Introduction

Regulatory T cells (Treg) are crucial to maintaining immune homeostasis by suppressing excessive T cell responses to self and non-self-antigens[1]. There are two major subsets of Treg cells, "natural" Treg (nTreg) cells that develop in the thymus and "induced" Treg (iTreg) cells that arise in the periphery from CD4+ Foxp3- conventional T cells. These iTreg cells can be induced in vitro from peripheral naive T cells using a combination of T-cell Receptor (TCR) activators and cytokines[2]. New immunomodulators for disease treatment are of great interest due to aberrant Treg function, which can be found in autoimmune diseases like allergy, as well as in transplant rejection and immuno-oncology[3]. To investigate immunomodulators on iTreg function, compound screening can be a valuable tool to help decipher the immunological profile of drug target candidates.

Here, we developed a rapid high-throughput *in vitro* functional assay to assess the effect of compounds on iTreg function to either enhance or inhibit their suppressive activity on T cells. This assay can be run complementary to our previously developed iTreg polarization assay to elucidate the overall activity of compounds on iTreg biology[4].



Assay overview

Our optimized iTreg suppression assay is illustrated in Figure 1. Naïve CD4+ T cells are isolated from PBMCs and cryopreserved. On day 0 (step 1), cells are revived and cultured with a polarization cocktail, committing them towards a CD25+CD127^{low}Foxp3+ iTreg suppressive phenotype (step 2). On day 7, this polarized iTreg population is co-cultured with total CD3+ T effector cells (Teff) in a TCR activator cocktail, providing a T cell stimulation signal (step 3). At this stage, compounds can be added to the co-culture for three days to assess their effect on the suppressive activity of the iTreg cells (step 4). The proliferation and activation of Teff cells in response to iTreg activity are then measured by flow cytometry (step 5). Data are obtained for total Teff cells and CD4+ and CD8+ T cell subsets by staining with anti-CD4 and anti-CD8 antibodies, respectively. Activation is assessed by measuring CD25 marker expression and proliferation by CellTrace™ Violet (CTV) stain, a proliferation tracker dye that diminishes in intensity upon cell division. To increase the accuracy and reproducibility of assay results, the assay set-up incorporates liquid handling automation for steps 2-5.

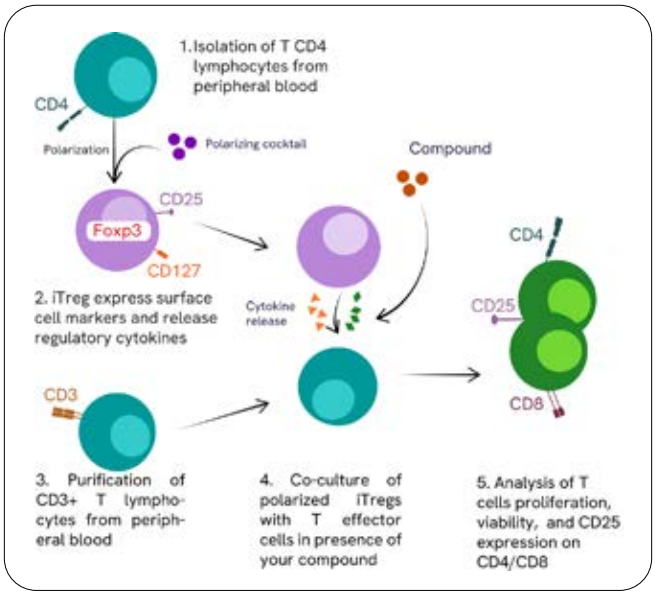


Figure 1: iTreg Suppression Assay setup. Polarized iTreg cells are co-cultured with T effector cells in the presence of therapeutic candidates. T effector cells proliferation and activation are measured by flow cytometry in total CD3+ T cells and CD4+ and CD8+ T cell subsets.

Our iTreg suppression assay shows that naïve CD4 T cells treated with a polarization medium effectively suppress Teff cells (Figure 2). Polarized iTreg cells (Figure 2C) were co-cultured with Teff cells for three days at a 1:1 iTreg:Teff ratio before assay endpoint assessment (Figures 2A, 2B). We observed a substantial reduction in proliferation of approximately 65% and a 2-fold decrease in activation in our co-culture conditions compared to Teff cells only (Figure 2B). Non-polarized cells also had a negligible effect on suppressing proliferation and activation. As previously shown, culturing naïve CD4 T cells in only IL-2 and TCR activator mix can lead to low expression levels of Foxp3 and, consequently, a minor suppressive effect[2].

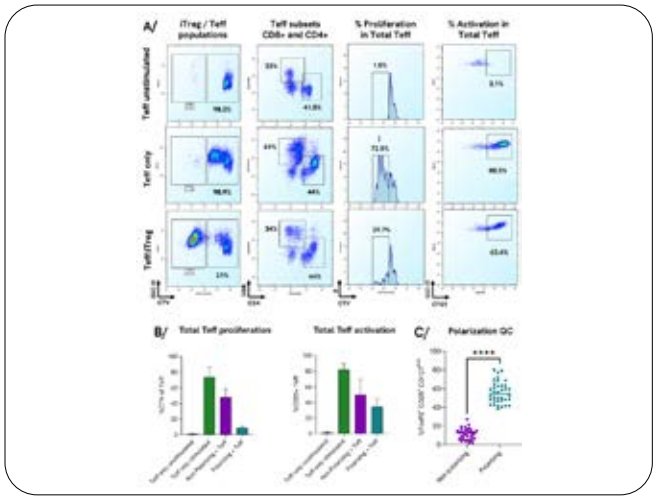


Figure 2: iTreg Suppression Assay overview and gating strategy. A/ After three days of co-culture with Teff cells at a ratio of 1:1, the following gating strategy is applied: iTreg and Teff are isolated by gating on the CTV+ Teff population (first column). They can be further separated by antibody-staining into CD4+ and CD8+ T cell fractions (second column). Proliferation is assessed based on the dilution of the CTV signal through cell division (third column), and activation is measured by CD25 expression (fourth column). Proliferation (CTV) and activation (CD25) are assessed in total Teff cells and CD4+ and CD8+ T cell subpopulations of stimulated and non-stimulated cells. B/ Graphs summarize the results of 3 independent experiments using three iTreg donors with three heterologous donors of CD3+ T effector cells at a 1:1 ratio (4 replicates per donor). These are shown as means of all results ± SD in total Teff cells. C/ Polarization QC on day 7 of polarization with TCR activators and cytokines (polarizing condition) or with TCR activators and IL-2 only (non-polarizing condition). iTreg cells from 9 donors are quantified by flow cytometry for FoxP3+, CD25+, and CD127low markers expression. The graph shows the results of each replicate for all donors with median, analyzed with an unpaired t-test with Welch correction (P<0.001).

Our results from three independent experiments showed that similar suppression levels of iTreg cells could be observed across multiple individual donors, confirming the consistency and reproducibility of the assay. Our assay set-up incorporates liquid handling automation for compound serial dilution, media and compound dispensing, and cell seeding. Using this approach, our assay facilitates efficient and rapid analyses of multiple donors with the opportunity to assess further analytes of interest based on assay requests.

Establishing key assay parameters allowed us to capture both iTreg activity-enhancing and inhibiting compound properties. The section below offers a comprehensive analysis of testing different assay conditions that helped define optimal assay windows for compound screening projects investigating iTreg biology.

Assay development

1. Culture condition optimization

Monitoring iTreg suppression requires that T effector cells be stimulated in culture. To find the right balance between the strength of iTreg suppression, T effector cell stimulation, and assay duration, we tested two ratios of iTreg:Teff with three different stimulation conditions at two incubation time points (Figure 3).

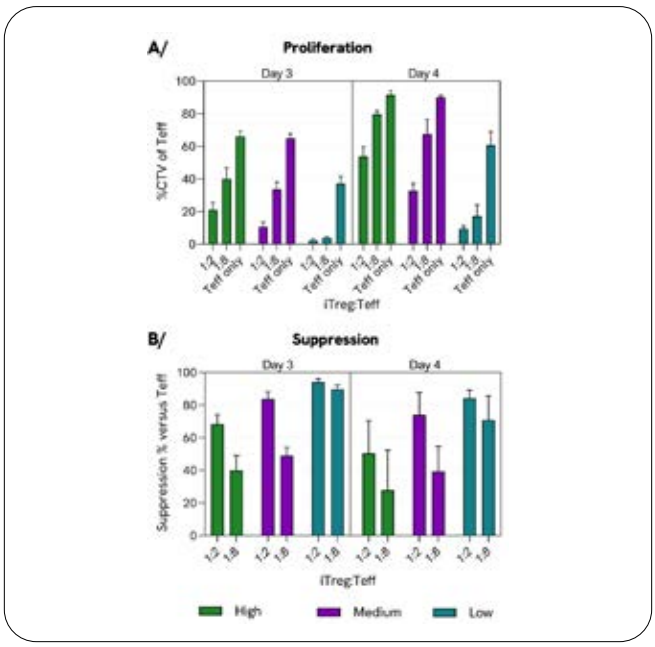


Figure 3: Intensity of stimulation is conversely correlated to suppression efficacy: Teff cells are co-cultured for 3 or 4 days with iTreg cells at 1:2 and 1:8 iTreg:Teff ratios, with Teff cells, kept constant. Results are expressed in % of proliferation (CTV) of Teff (A) or % of suppression (B). Data represent the results in 3 different iTreg donors as the mean of four replicates of all conditions ± SD.

Under the same stimulation conditions (high, medium, low), Teff cells alone (Teff only, Figure 3A) showed increased proliferation (60% - 92% from low to high stimulation) on day 4 compared to day 3 (40% and 67%), as expected. We calculated the percentage of Teff cell suppression by comparing the proliferation rate of each ratio to Teff cells only (Figure 3B). The four days of stimulation decreased the percentage of suppression across all ratios and conditions tested. For example, with high stimulation, suppression decreased from 68% on day 3 to 50% on day 4 in the 1:2 co-culture. Since the high stimulation condition, corresponding to the manufacturer's recommended concentration of TCR stimulating reagents, showed equivalent results to the medium condition for Teff-only proliferation and led to a higher suppression efficiency, we selected the medium concentration for all subsequent assay development steps.

Furthermore, the 4-day assay window introduced a lower suppression percentage and higher assay variability across the donors (Figure 3B), suggesting that a 3-day assay setup improves assay robustness. As expected, the low stimulation condition allowed a lower basal proliferation of Teff cells but high suppression rates at both ratios used in the assay. The low stimulation condition could thereforein the assay. The low stimulation condition could therefore be attractive in investigating inhibitory compounds of iTreg suppression but could impair the detection of activators.

We considered the interplay between stimulation rate (high, medium, low) and assay time using three different iTreg:Teff cell ratios. We concluded that the medium concentration of the TCR activator and a 3-day co-culture assay setup provided the best compromise between T effector stimulation and assay duration.

2. The co-culture ratio in the suppression assay

The suppressive effects of iTregs can vary from one donor to another. The co-culture ratio between Teff and iTreg is a crucial parameter to find an optimal balance between suppression and assay window for compound assessment. We, therefore, tested up to 7 different ratios using three iTreg donors in our established co-culture conditions and analyzed their proliferation and suppression profiles (Figure 4).

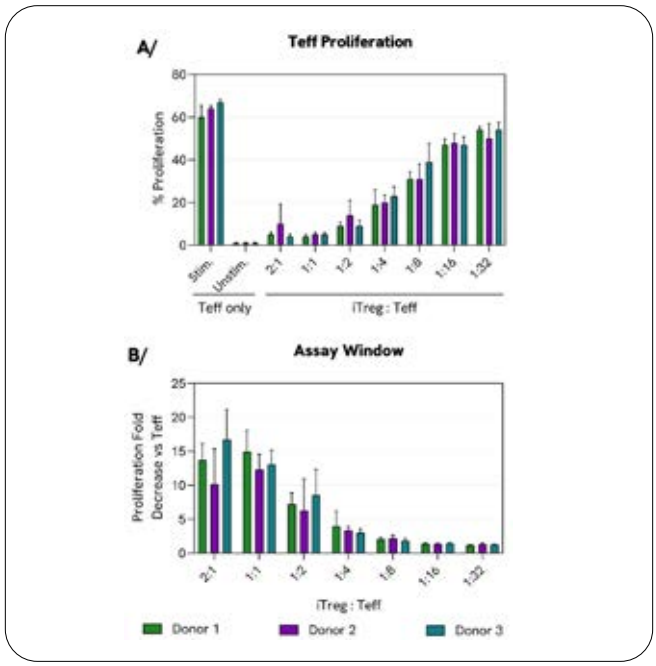


Figure 4: iTreg Suppression of Teff proliferation is correlated to iTreg number. Teff cells were incubated for three days with three different iTreg donors at seven different ratios (iTreg:Teff, with Teff cell number remaining fixed). Results are expressed in % of the proliferation of Total CD3+ Teff cells (**A**) or proliferation fold decrease (**B**). Results are represented as the mean of 4 technical replicates ± SD.

Using one donor for T effector cells, the basal proliferation of Teff's alone was around 60% (Figure 4A). The addition of iTreg downregulated Teff proliferation from 1.2-fold for the lowest ratio (1:32) to about 15-fold for the highest

ratio at 2:1 (iTreg:Teff) (Figure 4B). These results indicate that increasing the number of iTreg cells in the assay while keeping the number of T effector cells constant positively correlates to the level of suppression observed. Therefore, depending on a compound's mode of action to be tested in the suppression assay, choosing one ratio over the other might be advantageous to detect iTreg inhibitory or activating effects. Alternatively, at least two ratios could be selected to capture both effects on one screen.

3. Donor variability assessment

We then assessed assay reproducibility across different donors of Teff and naïve CD4+ T cells. Cells were isolated from various blood supplies, such as leukopaks, leucocyte cones, or buffy coat fractions, and set up in the assay from fresh or cryopreserved samples (Figure 5A). We observed an expected donor variability in proliferation rates of Teff-only cells in the five donors tested, ranging from 43% to more than 80%. However, despite the range in proliferation capacity of Teff cells, suppression with iTreg cells at a ratio of 1:1 was relatively uniform at less than 20% Teff proliferation, irrespective of blood supply or assay set-up using fresh or frozen cells (Figure 5A). Since assay set-up from frozen cells provides greater flexibility when performing more extensive studies to assess the compound impact on iTreg suppression, we investigated assay performance across several donors for naïve CD4+ T cells and T effector cells; this time, all from cryopreserved (Figure 5B). We, once more, observed that despite donor-donor variability in Teff cell proliferation (Figure 5B, left), overall suppression using three different heterologous pairs for iTreg and Teff cells was consistently over 80% for all pairs tested (Figure 5B, right).

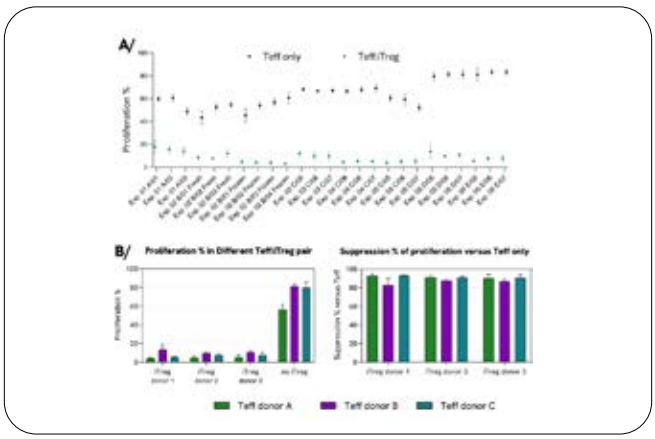


Figure 5: Assessing proliferation in multiple allogeneic iTreg/Teff donor pairs. A/ Freshly isolated or cryopreserved naïve CD4+ T cells from either leukopaks, leukocytes cones, or buffy coat are polarized into iTreg cells and co-cultured with CD3+ T effector cells (Teff) at a 1:1 ratio to assess suppression of Teff proliferation through CTV dye dilution by flow cytometry. Letters indicate individual Teff donors. Numbers indicate individual iTreg donors. Results are represented as the mean of four technical replicates ± SDs. **B/**. Frozen Naïve CD4+ T cells from 3 donors (1, 2, 3) are polarized into iTreg for seven days before assessing their suppressive activity by coculturing with three separate donors for Teff cells (A, B, C) at a 1:1 ratio. The proliferation of Teff cells is evaluated by flow cytometry (proliferation %), and the Suppression % about corresponding Teff-only controls is calculated for each Teff:Treg pair (suppression %). Results are represented as the mean of four replicates ± SDs.

In conclusion, these results demonstrate that our assay is suitable for a wide range of T cell donors and can reproducibly achieve suppression of iTreg cells. Having established key assay parameters, we next set out to test published and commercially available compounds in our suppression assay.

4. Compound dosing

We selected four compounds using two ratios of 1:2 and 1:8 (iTreg:Teff) and three iTreg donors. The first ratio (1:2) was chosen for having a robust suppressive effect, which is optimal for identifying inhibitors of suppression by leading to an increase in the proliferation of effector cells. The second ratio (1:8) was chosen to achieve a lower degree of suppression, allowing an optimal assay window for observing the activation of iTreg cells. We also added the compounds to Teff cells alone to discriminate between the impact of compounds on effector cells and influencing the suppression capacity of iTreg cells.

The tested compounds were selected based on information from the literature and a known mode of action. However, identifying compounds that specifically affect Treg biology is challenging. Therefore, our standardized iTreg suppression assay is designed to screen multiple compounds to determine drug-target specificity for Treg cells.

The mTOR signaling pathway has been shown to control Treg function[5]. We selected a small molecule, GSK1059615, a dual inhibitor of PI3K and mTOR, that has been tested in our polarization assay and showed inhibition of iTreg polarization. Another compound, Galunisertib, is a potent TGFβ receptor 1 inhibitor used in several clinical trials to treat cancer[6]. TGFβ is described as critical in driving suppression; therefore, inhibition of TGFβ receptor 1 signaling could lower suppression effects. Daclizumab, an antibody anti-IL-2 receptor targeting the CD25 receptor subunit, can deplete Treg in vivo[7]. Finally, we tested Pam3CSK4, a synthetic peptide binding to Toll-like Receptor (TLR) 1 and 2. This is one of the few compounds described as modulating suppression[7].

We tested all these compounds on our iTreg assay platform (Figure 6). GSK1059615, Galunisertib, and Daclizumab inhibited the proliferation of Teff cells, whereas Pam3CSK4 increased the T effector number in a dose-dependent and cell ratio-dependent manner (Figure 6A). To determine target specificity, we calculated suppression for each compound concentration and compared it to the Teff-only condition for the same dose (see Materials and Methods section for more information).

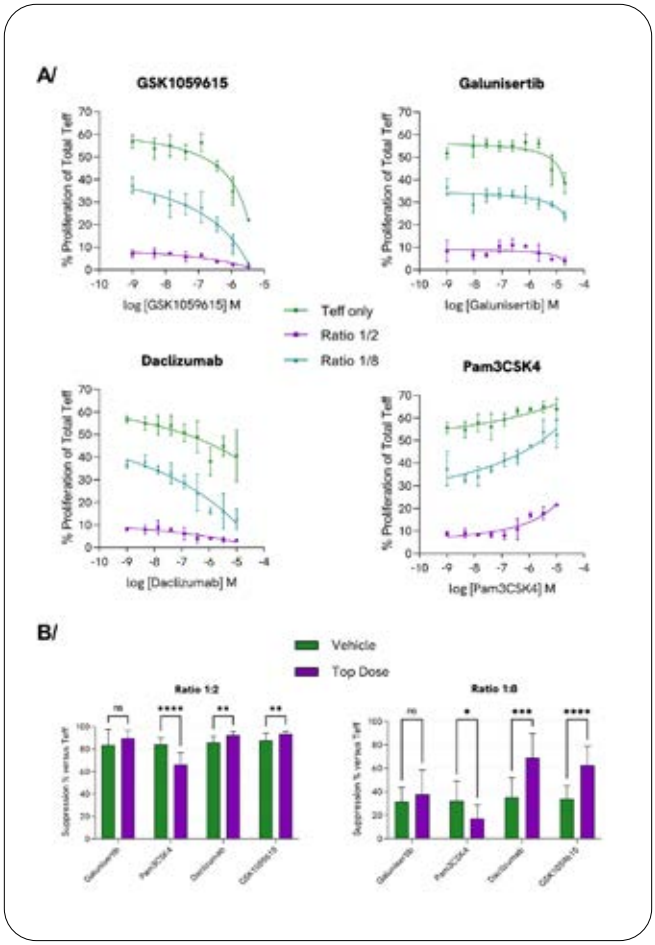


Figure 6: Compound screen in suppression assay. A. Compound dose-response curves in iTreg/Teff cocultures and T effector cells only. Three donors for iTreg and one separate donor for T effector cells were tested. Results are expressed as the mean of four technical replicates of Total Teff cells ± SD of the three tested donors. **(B)** The percentage of suppression is calculated on both vehicle and compound maximum dose for each replicate compared to the mean of the four replicates of the corresponding Teff-only condition. Data are represented as the mean of suppression % of all tested donors (12 replicates per condition). Statistical analysis was performed by using GraphPad Prism unpaired t-test.

Figure 6B represents the % point suppression for the top compound concentrations. This representation allowed the classification of the compounds as activators or inhibitors of iTreg suppression. Galunisertib displayed no significant effect between vehicle and the dose-response highest concentration at both tested ratios, indicating a non-specific mode of action on T cell proliferation. On the other hand, GSK1059615 and Daclizumab had a significant and specific effect on suppression (Figure 6A). Both compounds showed a ~2-fold increase in suppression at the highest concentration tested, suggesting a dose-dependent rise in iTreg activity (Figure 6B). Interestingly, this phenotype was more pronounced at the lower ratio of iTreg to T effector cells, highlighting the importance of ratio titration to find an optimal assay window to observe compound activity fully. The addition of Pam3CSK4 resulted in the opposite effect. We measured a significant loss of suppression in the co-culture as indicated by a rapid dose-dependent increase in T effector cell proliferation compared to T effector cells alone (Figure 6B).

The results of the compound screen suggest that the assay allows deciphering both activating and inhibitory effects of the compound on iTreg activity. Through careful titration of iTreg:Teff cell ratios, we can achieve adequate suppression levels to observe both phenotypes with the same assay set-up.

Summary

This application note describes a robust and effective method to routinely assess iTreg suppressive effects on T effector cells performed on a semi-automated and miniaturized platform. The assay allows testing multiple compounds and biologics for their activity to modulate iTreg suppression of T cells, as measured by proliferation and the expression of activation markers by flow cytometry.

Materials and methods

Cell isolation

Peripheral Blood Mononuclear Cells (PBMCs) from different donors were isolated from leukocyte cones, leukopaks, or buffy coats using CD3+ T cell or Human Naïve CD4+ T cell Isolation Kits as per the manufacturer's instructions. Purity QC was performed by flow cytometry using DAPI, anti-CD3 PE (#300408), anti-CD4 APC (#317416), anti-CD45RO AF488 (#304212), and anti-CD45RA BV785 (#304140), all from BioLegend.

iTreg suppression assay setup

Naïve CD4+ T cells are polarized as described previously[4]. On day 7, iTreg cells are collected and counted to be adjusted to an appropriate cell density depending on the Teff:iTreg ratio selected. CD3+ T effector cells are labeled with CellTrace Violet reagent and co-cultured with allogeneic iTreg at different ratios in assay media (ImmunoCult-XF medium supplemented with 5% human serum). The TCR activator mix (Immunocult human CD3/CD28 T cell Activator Mix) is added to T cells for 3 or 4 days. Unstimulated Teff-only and stimulated Teff-only controls were included. Proliferation and activation of CD4+ and CD8+ Teff cells are assessed by flow cytometry using antibodies supplied by Biolegend: anti-CD4 AF488 (#317420), anti-CD8 AF647 (#301022), CD25 PE (#302606) and Zombie Near IR stain (#B334686) for viability.

Data analysis

iQue3 Flow Cytometer data were analyzed with Forecyt software. Histograms and dose-response curves were prepared using GraphPad Prism 9.5.0. The suppression percentage is calculated for each replicate using the formula:

$$\text{Supp. \%} = \frac{(a - b)}{a} \times 100$$

Where a is the mean of the four replicates of proliferation % in Teff only, and b is the proliferation % of the replicate in co-culture. The fold of suppression is calculated by dividing the Proliferation % in Teff only by the Proliferation % in the coculture condition.

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