APPLICATION NOTE

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

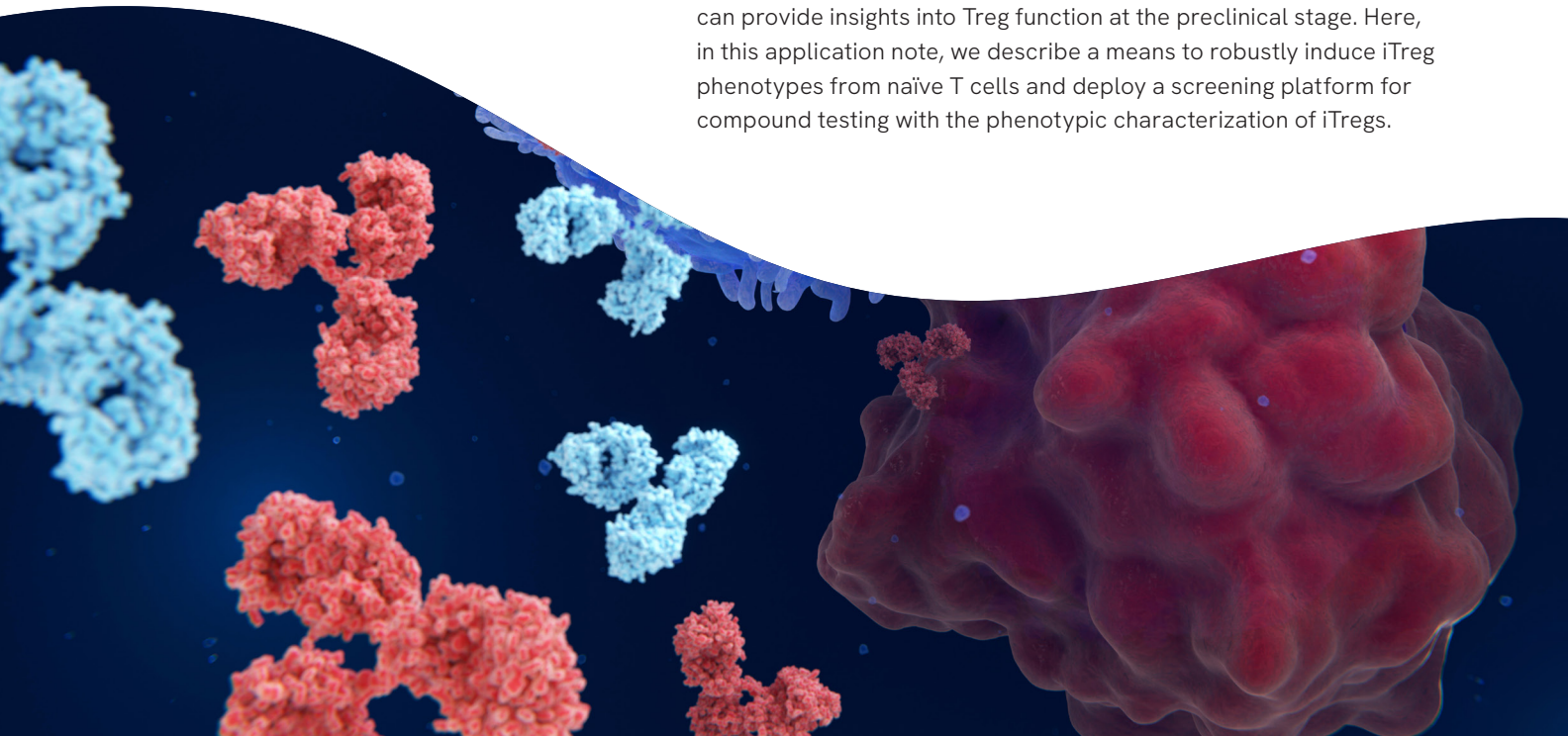
Introduction

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).

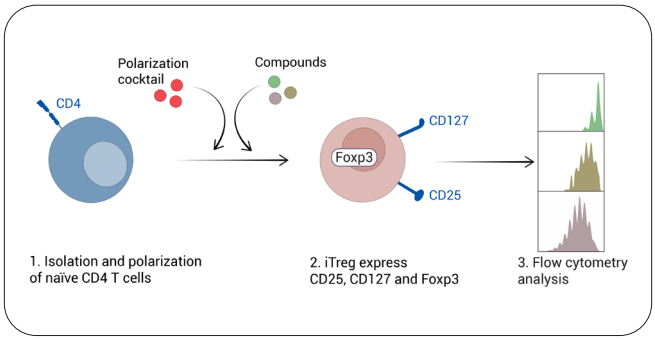


Figure 1: Standard iTreg polarization assay. Naïve CD4+ T cells isolated from peripheral blood mononuclear cells (PBMC) are stimulated with a polarization cocktail towards iTreg cells. Therapeutic candidates can be screened for their capability to influence the expression of the surface markers CD127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

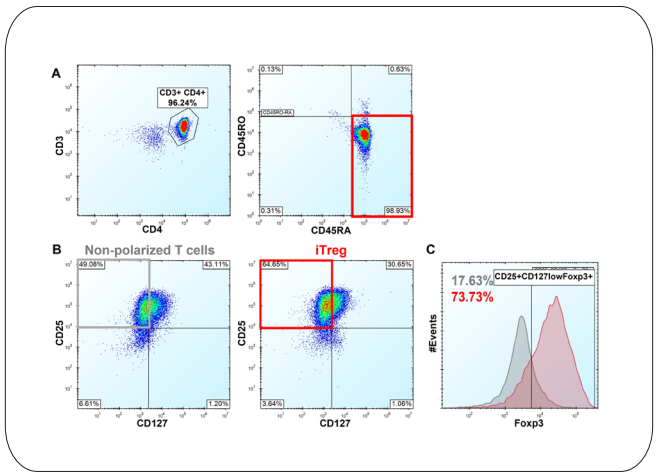


Figure 2: Gating strategies for Naïve T cells isolation and iTreg cell population. A) CD45RA+CD45RO- expression in CD3+CD4+ cells, assessed PBMC-isolated naïve CD4+ T cells purity. B) Dot plots showing CD25 and CD127 expression in non-polarized T cells (IL-2 only) and iTreg (polarized with TGF-β, IL-2, 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.

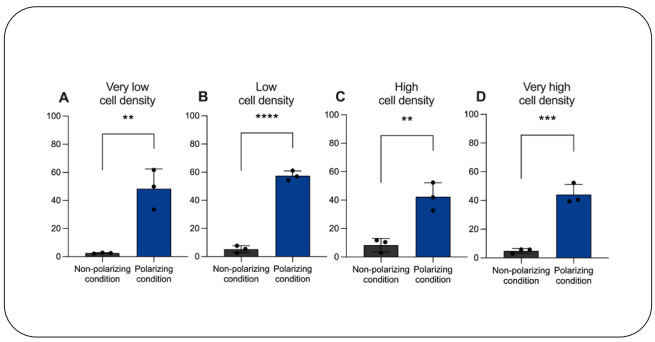


Figure 3: Optimization of naïve CD4+ T cell seeding density in the 384-well plate format. Four different cell densities for iTreg polarization were tested (A-D). Non-polarizing condition: IL-2 only; Polarizing condition: TGF-β, IL-2, rapamycin, and retinoic acid. Data represent quadruplicate technical 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.

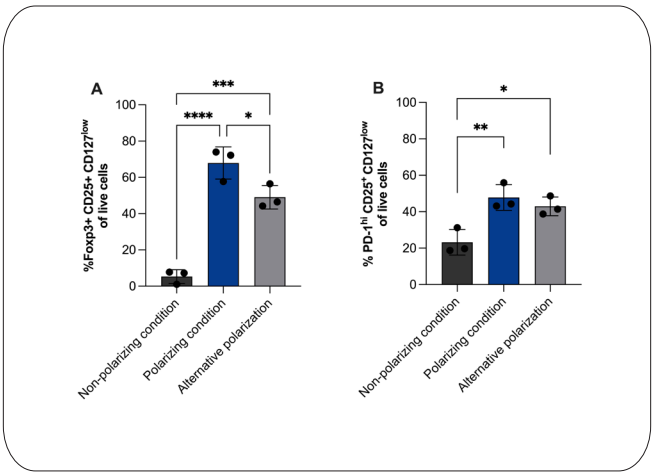


Figure 4: Expression of multiple markers for iTregs detected after polarization. (A) Foxp3 is the primary marker of iTreg phenotypes. (B) Other antigens of interest in iTregs include the surface marker PD-1. Non-polarizing condition: IL-2 only; Polarizing condition: TGF-β, IL-2, rapamycin, and retinoic 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).

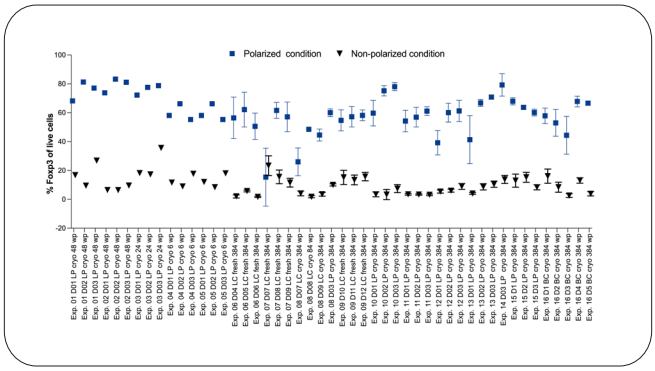


Figure 5: Foxp3 expression in polarized cells across 20 donors. Polarized: TGF-β, IL-2, rapamycin, and retinoic acid; non-polarized: IL-2 only. Assay setup from either fresh or cryopreserved (cryo) cells. Cells from 6-, 24-, 48- and 384-well plate culture vessels. Flow cytometry data obtained from 384-well 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).

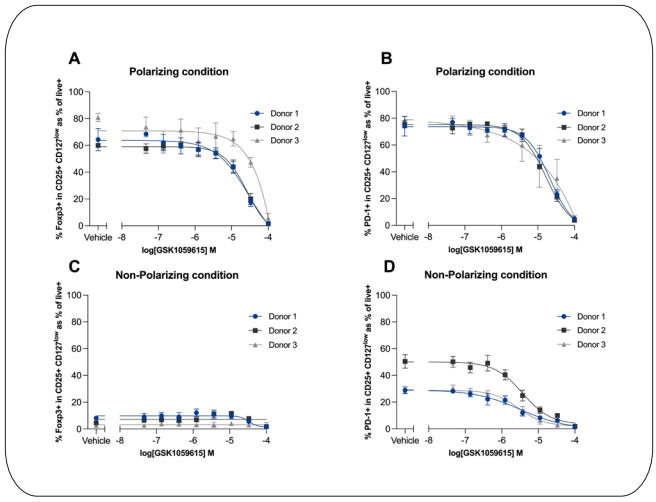


Figure 6: Drug-modulated iTreg polarization. The addition of GSK1059615 during polarization reduced the expression of Foxp3 (A) and PD-1 (B). Non-polarized cells as controls (C, D). Flow cytometry data represent naïve T cells cultured from 3 donors in quadruplicate replicates. Error bars represent SD.

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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