APPLICATION NOTE

Induced regulatory T cell suppression assay for compound screening.

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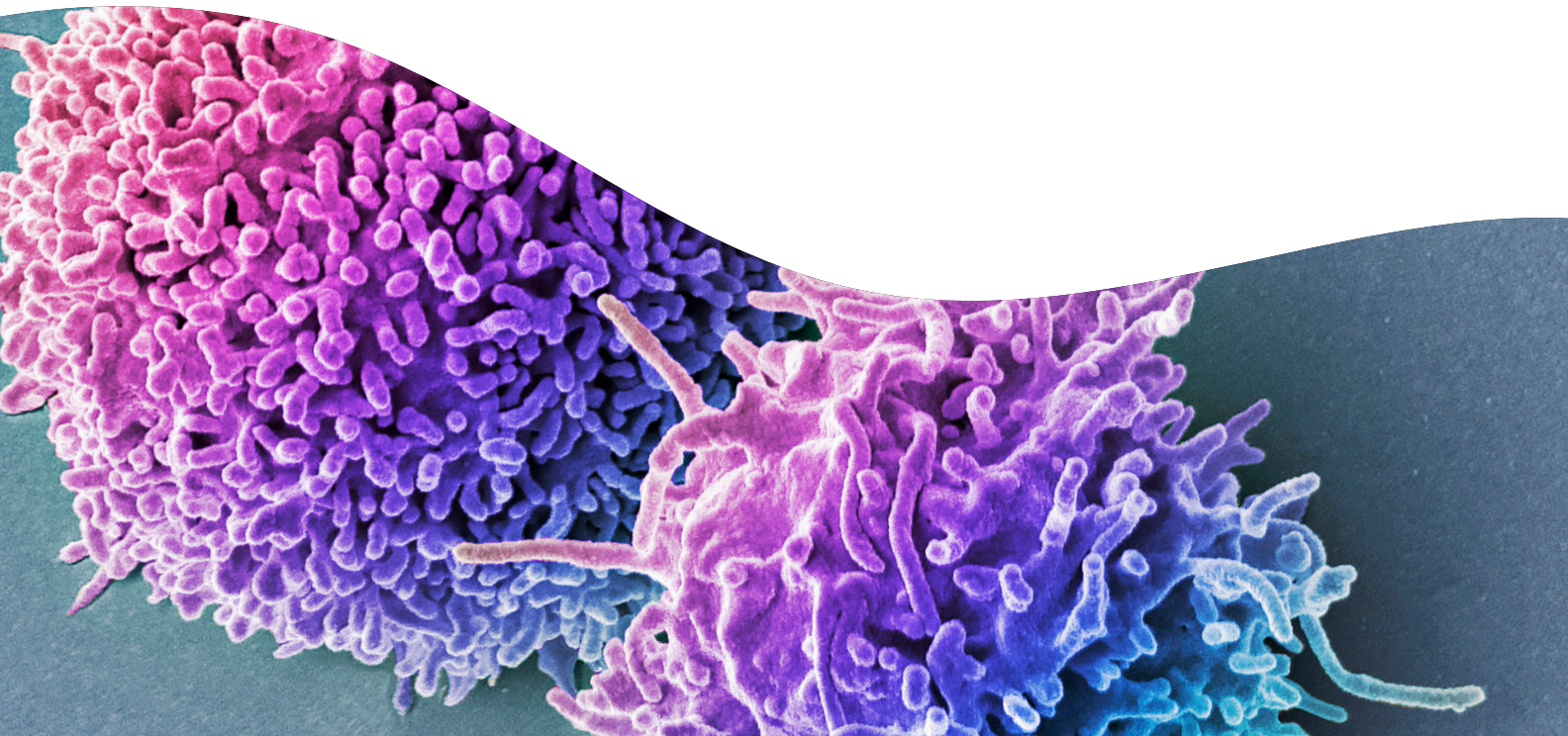
Introduction

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.

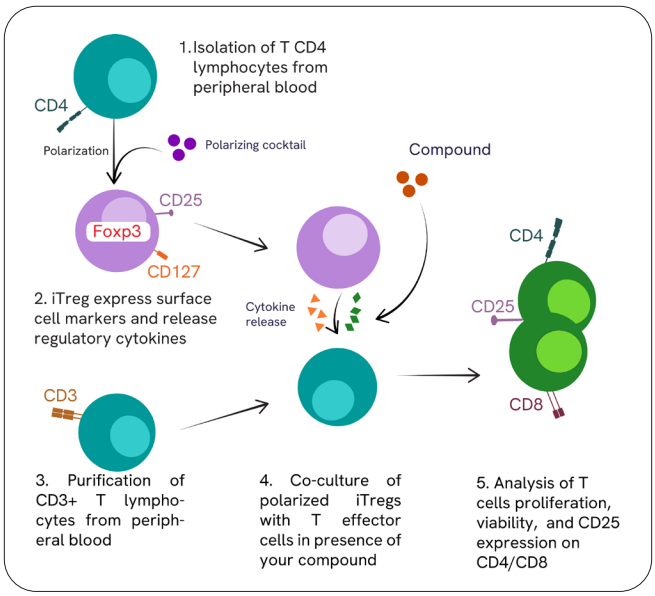


Figure 1: iTreg Suppression Assay setup. Polarized iTreg cells are co-cultured with T effector cells in the presence of therapeutic candidates. T effector cells proliferation and activation are measured by flow cytometry in total CD3+ T cells and CD4+ and CD8+ T cell subsets.

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].

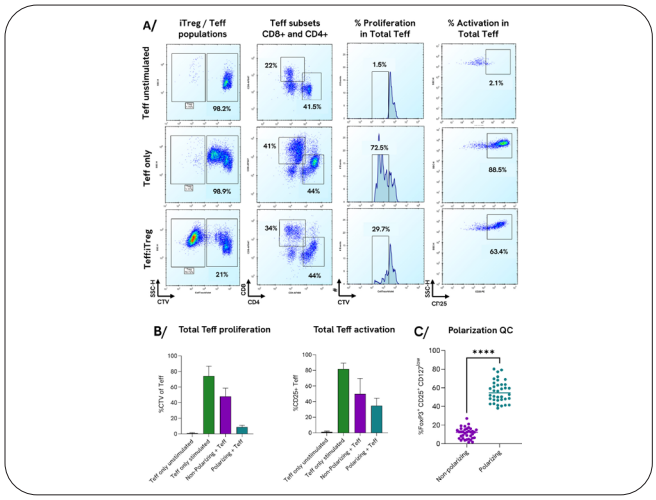


Figure 2: iTreg Suppression Assay overview and gating strategy. A/ After three days of co-culture with Teff cells at a ratio of 1:1, the following gating strategy is applied: iTreg and Teff are isolated by gating on the CTV+ Teff population (first column). They can be further separated by antibody-staining 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).

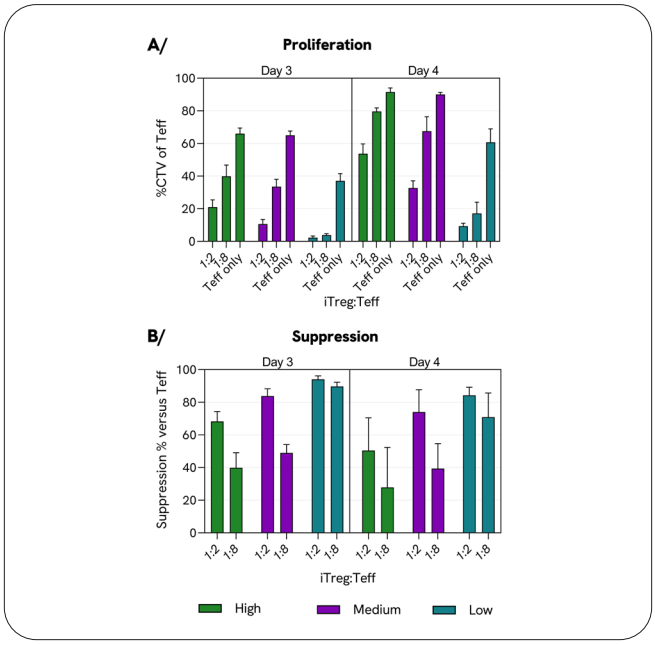


Figure 3: Intensity of stimulation is conversely correlated to suppression efficacy: Teff cells are co-cultured for 3 or 4 days with iTreg cells at 1:2 and 1:8 iTreg:Teff ratios, with Teff cells, kept constant. Results are expressed in % of proliferation (CTV) of Teff (A) or % of suppression (B). Data 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).

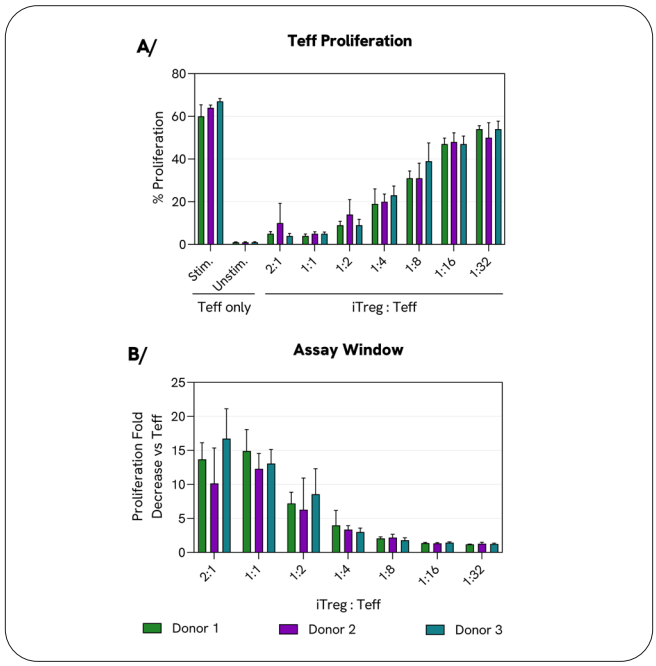


Figure 4: iTreg Suppression of Teff proliferation is correlated to iTreg number. Teff cells were incubated for three days with three different iTreg donors at seven different ratios (iTreg:Teff, with Teff cell number remaining fixed). Results are expressed in % of the proliferation of Total CD3+ Teff 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

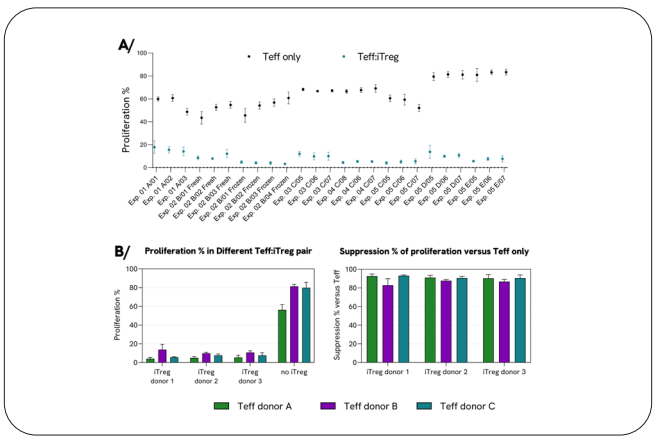


Figure 5: Assessing proliferation in multiple allogeneic iTreg/Teff donor pairs. A/ Freshly isolated or cryopreserved naïve CD4+ T cells from either leukopaks, leukocytes cones, or buffy coat are polarized into iTreg cells and co-cultured with CD3+ T effector cells (Teff) at a 1:1 ratio to assess suppression 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.

for iTreg and Teff cells was consistently over 80% for all pairs tested (Figure 5B, right).

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).

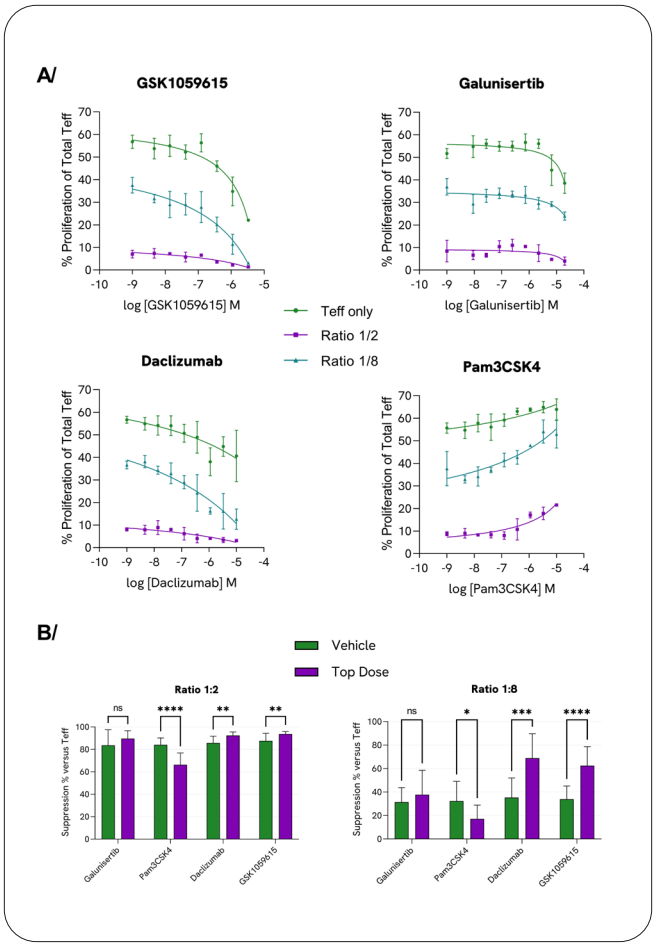


Figure 6: Compound screen in suppression assay. A. Compound dose-response curves in iTreg/Teff cocultures and T effector cells only. Three donors for iTreg and one separate donor for T effector cells were tested. Results are expressed as the mean of four technical replicates of Total Teff cells ± SD 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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