# An efficient workflow for functional reversal of Treg-mediated suppression for drug testing

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## Introduction

Regulatory T cells (thymically and peripherally derived Treg) have critical roles in maintaining immune homeostasis and controlling aberrant/excessive responses to self and non-self antigens. Typically defined as $CD4^+$ T cells with high expression of CD25 and FoxP3, these cells mediate tolerance to autoantigens, microflora, and food antigens. While there is considerable interest in harnessing Treg activity therapeutically in autoimmune and tissue transplant settings, their activity has garnered significant interest in the immuno-oncology field[1]. Tregs are enriched in the tumor microenvironment (TME) and in this context are tumor-permissive, facilitating immune evasion and tumor progression. Tregs are able to suppress conventional $CD4^+$ and $CD8^+$ T cell responses, preventing their efficient targeting of cancer cells, and are mediating their suppressive function by a variety of mechanisms[2], including: secretion of inhibitory cytokines (TGF-β, IL-10, and IL-35); suppression by metabolic interference (by sequestration of IL-2 by means of high CD25 expression or by inhibition via the adenosine pathway); suppression by cytolysis (e.g. granzyme A/B production); and modulation of dendritic cell (DC) function (via CTLA-4/ CD80/CD86 or LAG3/MHC class II interactions with DCs) (fig. 1). These mechanisms are multifaceted, temporally and spatially, impacting both priming and effector T cell responses in lymphoid and TME settings respectively, in addition to the conditioning of antigen-presenting cells (APCs) and innate immune cells to dampen anti-tumor activity.

Preclinical studies demonstrating that disruption of intratumoral Treg activity is associated with reduced tumor burden, and approval of ipilimumab (Yervoy, an anti-CTLA-4 antibody) for treatment of metastatic melanoma[3], set a clear precedent in targeting this cell population. Several therapeutic strategies are now underway to identify different ways in which Tregs may be leveraged for treatment of various tumor types. Numerous Treg targets and approaches are under investigation: CTLA-4, CD25, FoxP3, ubiquitin, GITR, CD39, CCR4, CCR8, IL-10, and TGF-β to name a few. Consequently, now more than ever, there is a demand for defined, robust, *in vitro* Treg assays to test candidate immunotherapies for their ability to modulate Treg phenotype and, importantly, functional activity.

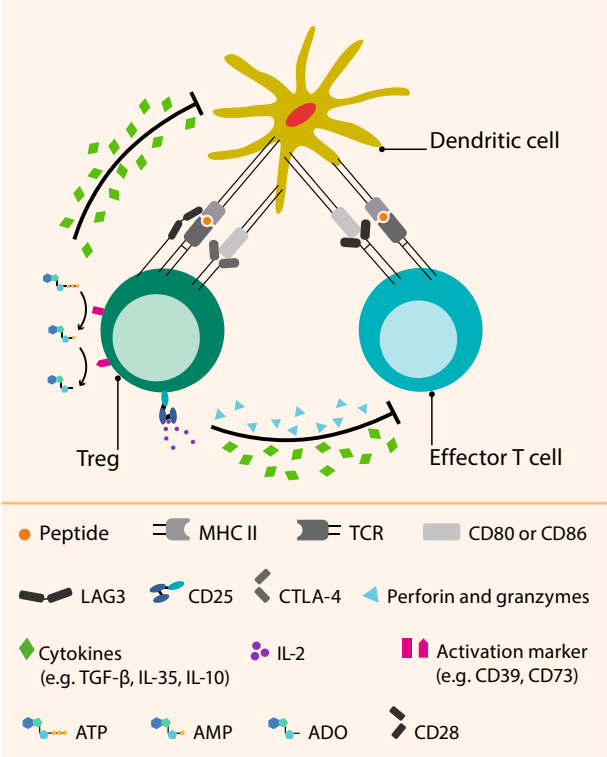


**Figure 1:** Tregs mediate immunosuppression, regulate inflammatory processes, prevent autoimmunity, and contribute to poor tumor immunity. These mechanisms can also impact APCs, such as DCs, dampening their activation and ability to effectively stimulate adaptive immune responses.

In this article, we describe how scientists from Antibody Analytics have employed Miltenyi Biotec products to establish a Treg suppression assay workflow. The assay includes isolation of natural Treg cells from peripheral blood mononuclear cells (PBMCs) and coculture with anti-CD3–stimulated autologous PBMCs at different Treg-to-PBMC ratios to demonstrate functional suppression of the responder (conventional or $T_{conv}$) T cell population. This is coupled with Treg immunophenotyping, cytokine release, and Treg-specific reversal of suppressive activity with a novel immunotherapy.

## Materials and methods

Cryopreserved human PBMCs from three healthy donors were used to perform the study, allowing the setup of autologous Treg/PBMC target cocultures. An overview of the experimental setup can be seen in figure 2.

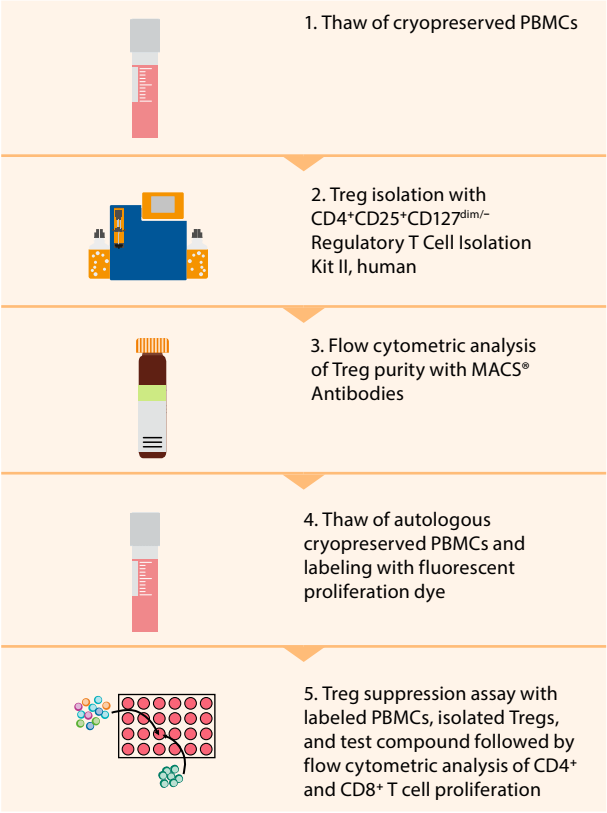


**Figure 2:** Schematic representation of experimental workflow.

### Immunomagnetic isolation of regulatory T cells

Tregs were isolated from PBMCs using the two-step procedure outlined in the CD4+CD25+CD127dim/– Regulatory T Cell Isolation Kit II (Miltenyi Biotec).

### Flow cytometric analysis of Treg cell purity

The purity of the isolated T cell population was determined by immunophenotyping by flow cytometry, staining CD4, CD25, and FoxP3 antibodies, and utilizing the FoxP3 intracellular staining kit (all from Miltenyi Biotec). Typically, purity of the isolated CD4+ cell population ranged from 87% to 94% CD25+FoxP3+ (fig. 3A).

### Treg suppression assay

Target (responder) PBMCs were labeled with the eFluor670 proliferation dye and treated with a fixed dose of anti-CD3 antibody to stimulate T cell responses. Isolated Tregs were titrated into cultures at various doses to give Treg:PBMC ratios of 1:1, 1:2, 1:4, and 1:8. Controls included unstimulated and anti-CD3–stimulated PBMCs in the absence of Tregs. Cocultures were performed for five days, in the presence or absence of exogenous test articles or vehicle control, and proliferation was assessed by flow cytometry. Target cell proliferation was determined by proliferation model fitting to calculate the frequency divided (also referred to as precursor frequency, PI, in other analysis software packages). The percentage of suppression was calculated as a function of the proliferation seen in the PBMC positive control group:
$100 - (100 \times (\text{Frequency Divided}_{\text{Suppressed group}}/\text{Frequency Divided}_{\text{Stimulated PBMC control group}})$. Supernatants were also collected to measure IFN-γ release by ELISA. A small molecule inhibitor of Casitas B-lineage lymphoma proto-oncogene-b (Cbl-b) was employed as a test article to investigate potential reversal of Treg activity. The Cbl-b inhibitor was utilized at two doses (one high and one low) in the coculture assay, or as a single high dose in the Treg pre-treatment setup. In the former, the test article remained present throughout the five day coculture period. In the latter, freshly isolated Tregs were incubated in the presence of the inhibitor for three hours and were then washed and centrifuged prior to coculture with autologous PBMCs and anti-CD3.

## Results

### Treg titration and reversal of suppression

Tregs were isolated from PBMC donor material and incubated with stimulated autologous PBMCs at varying ratios. While unstimulated PBMCs from each of the donors demonstrated no dilution of the proliferation dye, anti-CD3 stimulation of PBMCs in the absence of Tregs drove robust proliferative responses by both CD4+ and CD8+ T cells. In each case, both CD4+ and CD8+ T cell proliferation was inhibited by the presence of Tregs in the coculture (fig. 3B red histograms), with 1:1 Treg:PBMC exhibiting the lowest levels of T cell proliferation/greatest suppression, with the effect on CD4+ T cells being more pronounced.

We next sought to determine whether the functional suppression observed in the *in vitro* assay could be reversed. Here, we employed an inhibitor of the E3 ligase, Cbl-b, a target of interest in emerging strategies to enhance anti-cancer immunity. Cbl-b has been shown to be a negative regulator of T cell receptor signaling and crucial for maintaining T cell tolerance[4]. In this context, it was considered that inhibition of Cbl-b may be able to counteract Treg suppressive mechanisms, allowing restoration of responder $T_{conv}$ cell proliferation. Tregs and PBMCs were cocultured at a 1:1 ratio in the presence or absence of low/high dose Cbl-b inhibitor. Treg suppressive activity was confirmed in cocultures where media or vehicle only were present, proliferation of $T_{conv}$ cells in the PBMC population was almost completely inhibited (fig. 3C, D). While a low dose of the Cbl-b inhibitor was able to partially restore proliferation of both CD4 and CD8 $T_{conv}$ cells, a high dose restored proliferative responses to comparable levels with stimulated PBMCs in the absence of Tregs. The degree of restoration of proliferation, or reversal of suppression, was similar in both CD4 and CD8 $T_{conv}$ populations (fig. 3C, D).

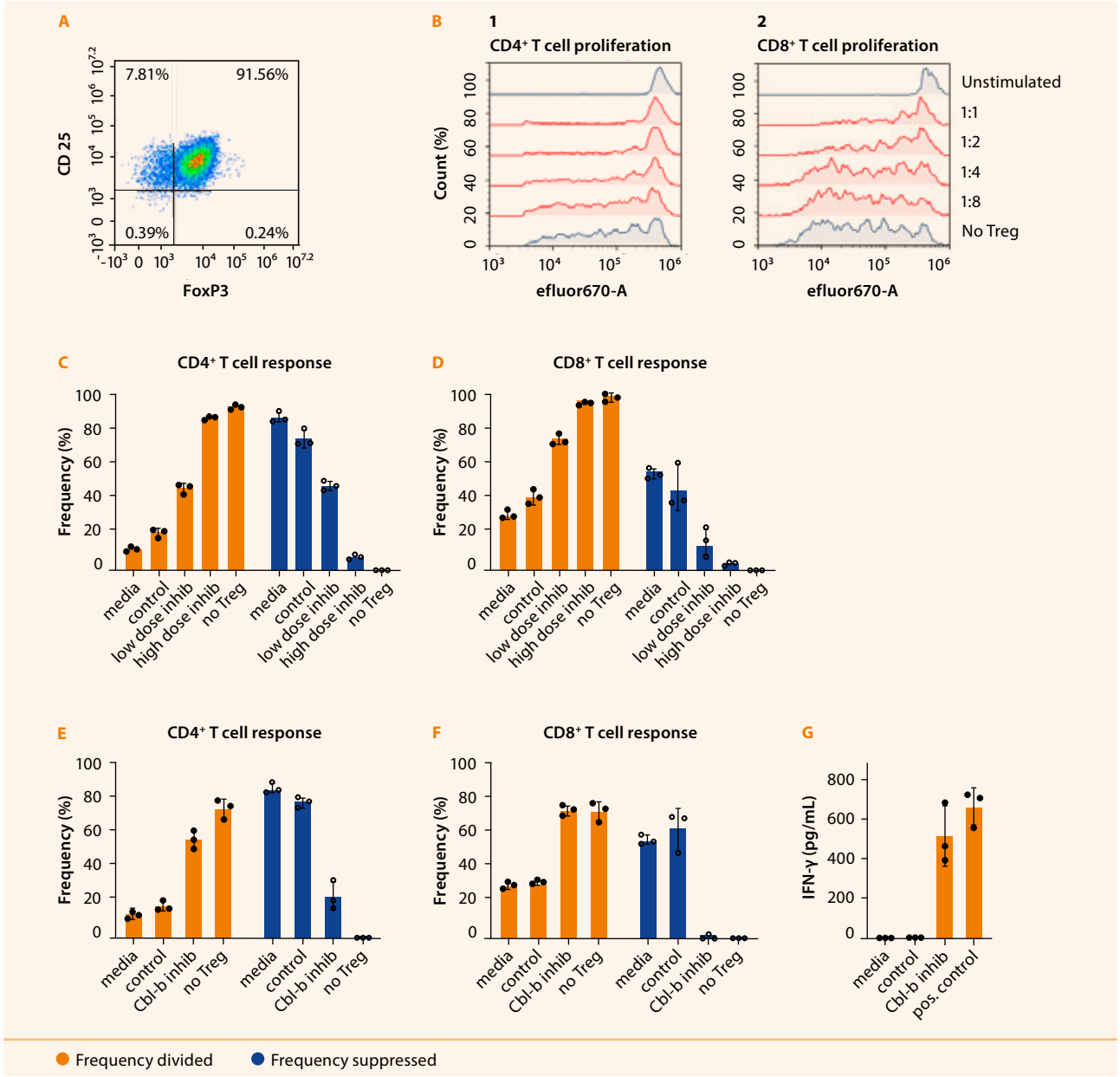


**Figure 3: Treg assay demonstrates effectiveness of Cbl-b antagonism in reversal of T cell suppression.** Treg purity assessed by flow cytometry staining for CD4, CD25, and FoxP3: CD4 purity was consistently above 91%, and 87 to 94% CD25$^+$FoxP3$^+$ (A). Titration of Treg into anti-CD3/PBMC cultures demonstrated dose-dependent suppression of CD4 and CD8 T cell proliferation assessed by efluor670 proliferation dye dilution (B). Direct addition of a Cbl-b inhibitor to the assay at low and high doses enhanced CD4$^+$ and CD8$^+$ T cell division/reduced suppression (C, D). Pre-treatment of Tregs with a Cbl-b inhibitor prior to their use in the assay abrogated their ability to suppress CD4$^+$ and CD8$^+$ cell proliferation (E, F), as well as secretion of IFN-γ (G).

**Pre-treatment of the Treg population**

As the drug target Cbl-b is expressed in both $T_{conv}$ cells and regulatory T cells, we modified the assay setup to delineate effects of the inhibitor on the intrinsic ability of Tregs to mediate suppression versus $T_{conv}$ being rendered refractory to regulation. As such, a pre-treatment of isolated Tregs with a high dose Cbl-b inhibitor was performed prior to inclusion in the suppression assay. Again, a single ratio of 1:1 Tregs:PBMCs was employed. Stimulated PBMCs in the absence of Tregs demonstrated robust proliferation, while Tregs pre-treated in media or in the presence of the vehicle control exhibited significant inhibition of $T_{conv}$ proliferation (fig. 3E, F).

Interestingly, Tregs that were pre-treated with the Cbl-b inhibitor were incapable of suppressing either CD4$^+$ or CD8$^+$ $T_{conv}$ proliferation (fig. 3E, F). Furthermore, levels of secreted IFN-γ were undetectable by ELISA in control Treg: PBMC cocultures but were equivalent to the positive control (stimulated PBMCs) in Cbl-b pre-treated Treg cocultures (fig. 3G). While modification of the assay in this fashion allowed demonstration that direct targeting of a pathway in the Treg population can abrogate their suppressive capacity, the assay would be equally amenable to pre-treatment of the PBMC fraction facilitating assessment of $T_{conv}$ susceptibility to suppression.

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## Conclusion

- Together, these data demonstrate a robust *in vitro* Treg suppression assay utilizing human primary immune cells, facilitating interrogation of therapeutic intervention on active regulation of $T_{conv}$ responses and delineation of effects intrinsic to the Treg cells themselves.
- Use of PBMCs, rather than isolated $T_{conv}$ cells as the responding population has the advantage that both direct and indirect mechanisms of suppression can be investigated, by relying on supply of additional costimulatory signals from APCs present in the PBMC fraction.
- Use of Miltenyi Biotec products allows reproducible isolation and phenotyping of human $CD4^+CD25^+FoxP3^+$ Treg cells, flow cytometric analysis of responding PBMC populations in the Treg assay, ultimately enabling robust *in vitro* testing of candidate immunotherapies for their ability to modulate immune responses, Treg phenotype and, most importantly, functional activity.

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