Altasciences, 6605 Merrill Creek Pkwy, Everett, WA 98203
Altasciences.com | contact@altasciences.com

Detection of Peripheral Blood Foxp3+ Regulatory T Cell Population in a Preclinical Setting

Milan Patel, Andrea Wong, and Tina Rogers

Altasciences, Seattle, WA, United States

ABSTRACT

Research focused on T regulatory cells (Tregs) has seen an increase for immunotherapy treatments designed to treat various malignancies. For instance, studies in humans and animal models have shown that by suppressing tumor immunity, Tregs can prevent the host immune system from detecting and controlling tumor growth. As a result, interest in targeted depletion of Tregs has also increased in preclinical and clinical settings. However, it is difficult to quantify and characterize the naturally occurring Treg population characterized as CD3+CD4+CD25+Foxp3+ using flow cytometry in peripheral blood. This is due to difficulties involved in Foxp3 staining and relatively rare presence of Tregs in peripheral blood. To alleviate these concerns, we developed a method that can reproducibly detect a large number of Tregs in 250µL of whole peripheral blood of cynomolgus monkeys; this allows effective evaluation of therapies designed to characterize or deplete Tregs. In animals analyzed using our developed method, the Foxp3+ Treg population ranged from 2.1% to as low as 0.2% of the total lymphocyte population. Nonetheless, our method was reliably able to detect up to 21,500 Foxp3+ Tregs in peripheral blood. The results were confirmed using an extracellular method of detecting the Treg population characterized as CD3+CD4+CD25+127^{low}. Our method provides a reproducible way of detecting a Foxp3+ Treg cell population in cynomolgus monkeys and presents a unique opportunity to study modulation of the Treg cell population *in vivo*.

INTRODUCTION

Helper T cells represent a key component of the adaptive immune system involved in clearance of pathogens and tumors. These cells are characterized by expression of cellular markers such as CD3 and CD4. However, this immune response also has a possibility of going awry and attacking host cells, resulting in autoimmune diseases. Thus, a population of CD4+ T cells known as Tregs are involved in regulating activation of the immune response. These cells are characterized by expression of the Foxp3+ transcription factor. They also have lower CD127 expression compared to helper T cells. The regulatory T cell population is often the target of immunotherapy for treatment of tumors since their regulatory function can interfere with immune-mediated clearance of tumors. Since Foxp3+ Tregs represent a very small fraction of T cells, they are often difficult to detect and characterize. In this presentation, we describe two robust methods for detecting a large number of Foxp3+ Tregs in a preclinical setting.

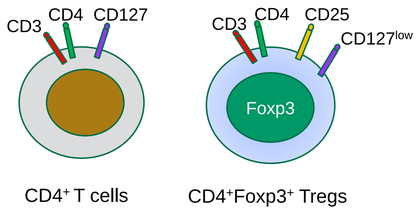

Figure 1. Overview of Helper T cells Vs. regulatory T cells.

METHODS

Two methods were used to detect the regulatory T cell population in whole blood of Cynomolgus monkeys.

Method 1 utilized extracellular staining to characterize the CD3+CD4+CD25+CD127^{low} population. Whole blood (1.4mL) from six animals was lysed with RBC lysis buffer for 5 to 12 minutes at ambient temperature (AT). Cells were centrifuged and washed with PBS. Live cells were manually counted on a hemocytometer with trypan blue exclusion. Ten million cells were stained with antibodies to CD3, CD4, CD8, CD25, and CD127 for 15 to 20 minutes at AT in the dark. Cells were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes at AT in the dark and washed again with PBS. The fixed cells were analyzed using BD FACSCanto II cytometer. Data was analyzed using FACSDiva™.

Method 2 used a more definitive identification of Tregs by staining intracellularly for Foxp3. Whole blood (250µl) from 6 animals was stained with antibodies to CD3, CD4, CD8, and CD25 for 15 to 20 minutes at AT in the dark. Without additional washing, RBC was lysed with RBC lysis buffer for 5 to 12 minutes at AT in the dark. Cells were washed with flow cytometry staining buffer and incubated with 1x Fix/Perm buffer for 45 to 60 minutes at 2 to 8 ºC in the dark. Cells were washed once with 1x Permeabilization buffer. The cells were then stained with anti-Foxp3 antibodies in 1x Permeabilization buffer for 40 to 50 minutes at 2 to 8 ºC in the dark. Cells were washed once 1x Permeabilization buffer, resuspended in flow cytometry buffer, and analyzed using a BD FACSCanto II cytometer.

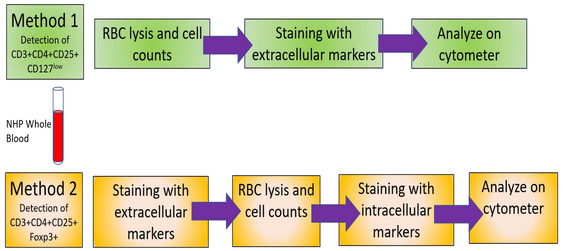

Figure 2. Schematic of methods used for detection of regulatory T cell populations.

Data collected on cytometer was analyzed using FACSDiva™. Relative percentages of Tregs were calculated as percent of CD3+CD4+ T cells. Total number of Tregs analyzed was obtained from the events count value present in FACSDiva™ statistics table. For Table 1, the relative % values were used to determine Tregs counts/mL of whole blood using lymphocytes counts obtained from Advia-180 hematology analyzer.

RESULTS

1. Identification of Treg population

Data collected using flow cytometer was gated to identify the Treg populations as shown below.

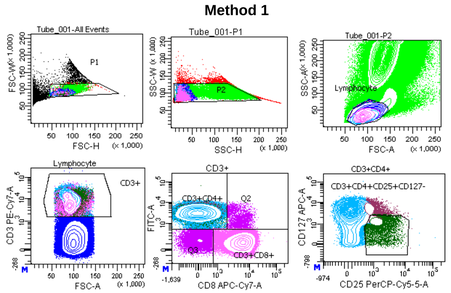

Figure 3. Gating strategy for detection of Tregs characterized as CD3+CD4+CD25+CD127^{low}. The top two panels to the left show gating of single cells using FSC and SSC parameters. Top right panel shows gating on lymphocyte populations. Bottom left panel is for gating on CD3+ total T cell population out of lymphocytes. Bottom middle panel shows gating on CD4+ and CD8+ T cells. Bottom right panel is gated on CD4+CD25+CD127^{low} cells.

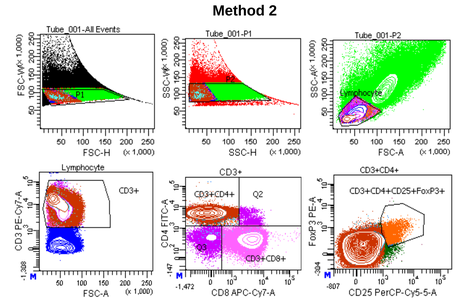

Figure 4. Gating strategy for detection of Tregs characterized as CD3+CD4+CD25+Foxp3+. The top two panels to the left show gating of single cells using FSC and SSC parameters. Top right panel shows gating on lymphocyte populations. Bottom left panel is for gating on CD3+ total T cell population out of lymphocytes. Bottom middle panel shows gating on CD4+ and CD8+ T cells. Bottom right panel is gated on CD4+CD25+Foxp3+ cells.

2. Total number of Tregs detected

Results indicated that both methods were able to detect relatively high numbers of Tregs (minimum 6,000 cells) per animal.

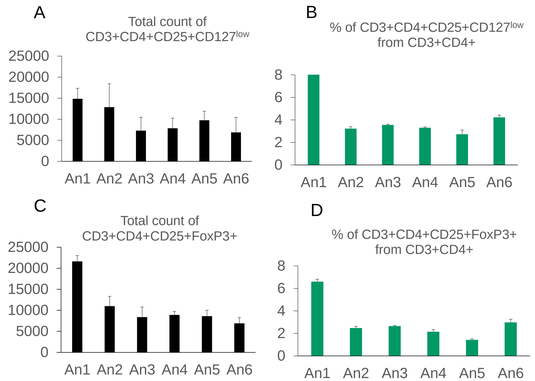

Figure 5. Tregs detected using A) method 1, total number of CD3+CD4+CD25+CD127^{low} cells B) method 1, Relative % of CD3+CD4+CD25+CD127^{low} population within CD4+ T cells C) method 2, total number of CD3+CD4+CD25+CD127^{low} cells detected in whole blood D) method 2, relative % of CD3+CD4+CD25+CD127^{low} population within CD4+ T cells. Six animals with 4 replicates per animal were analyzed.

3. Data compilation from multiple animals (n=15)

Expected "normal" Treg counts and relative % of CD3+CD4+CD25+Foxp3+ T cells in cynomolgus monkeys were determined from data obtained using Method 2.

Total CD3+CD4+CD25+Foxp3+ cell count/mL of whole blood (n=15)	CD3+CD4+CD25+Foxp3+ cells as % of CD3+CD4+ population (n=15)
$1.46x10^4 \pm 1.75x10^4$	2.5 ± 1.33

Table 1. Compiled statistics for absolute counts and relative percentages of Foxp3+Tregs in cynomolgus monkeys.

CONCLUSIONS

Two robust methods for detecting a difficult to detect population of Foxp3+ Tregs were developed at Altasciences for use in preclinical testing with whole blood from cynomolgus monkeys. The starting blood volumes were increased to ensure over 6,000 Tregs were detected per animal. Both methods were able to detect comparable percentages of CD25+CD127^{low} and CD25+Foxp3+ cells out of CD4+ T cells. These methods present an opportunity to assess treatments designed to modify Treg numbers or function.

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