Introduction

Regulatory T (Treg) cells play a crucial role in the suppression of non-specific or excessive immune responses. Therefore, the detailed characterization of these cells and their function is of great interest. Treg cells generally express the surface markers CD4 and CD25, which are mainly used for initial identification purposes. Additional markers, including CD45RA, CD127, and in particular transcription factors such as FOXP3 and Helios, are used to distinguish subpopulations. Due to varying expression levels of these surface markers, identification of cell subsets can be very challenging. Accordingly, the accurate analysis of Treg cell subsets and the determination of cell percentages by multicolor flow cytometry can be limited by the brightness of the fluorochromes used. To overcome these limitations, new fluorescent dyes and staining protocols have been created. Vio®515/VioBright™ 515 and Vio 667 / VioBright 667 are introduced as new flow cytometry dyes, preferably conjugated to recombinantly engineered antibodies, i.e., REAfinity™ Antibodies. Rapid protocols were developed for staining hardly accessible intranuclear transcription factors. Based on comprehensive multicolor panels, these protocols allow cell surface and intracellular staining less than an hour in total, using either PBMCs, whole blood, lysed blood or even Treg cells derived from any kind of pre-enrichment.

Methods

1. Development of a multicolor panel for Treg cell phenotyping

- Flow analysis was performed on the MACS®Biotec Analyser 10
- Cells were stained with surface panels for 10 min at RT in the dark, washed, centrifuged, and resuspended in an appropriate buffer.
- 5×10⁶ cells from PBMCs or lysed blood
- Up to 100 µL whole blood
- Total fraction of enriched cells
- From M5 Column

For intracellular staining a rapid protocol was used, enabling processing of whole blood in less than 1 h and PBMCs, lysed blood, and pre-enriched cells in less than 40 min.

Panels included new VioBright Dyes for surface staining and new Vio Dyes for intracellular staining (table 1).

2. Gating strategy for phenotyping based on cell surface staining using an 8-color panel

Discrimination of Treg cell subsets was accomplished using the following gating strategy (fig. 1).
- CD45 was used for leukocyte identification.
- Gating of leukocytes via FSC-A vs. SSC-A.
- Doublets were excluded with a single gate in FSC-H vs. FSC-A.
- Exclusion of dead cells using 7AAD-negative cells.
- T cells were identified by CD3 and CD4 staining, followed by discrimination of Treg cells based on CD25 and CD127.
- Further Treg cell subsets were analyzed by CD45RA, and CD25-APC staining.
- Cell percentages, mean fluorescence intensities (MFI), and stain indices (SI) were compared for B1 and B2 VioLight Dyes (fig. 2).

3. Intranuclear staining protocols for cells from different starting materials

Three rapid protocols were established that enable intranuclear staining of PBMCs in 40 min (fig. 5A) and whole blood in 50 min (fig. 5B), as well as on-column staining in 45 min (fig. 5C). Figure 4 shows a comparison of the processing times for the rapid protocols from Miltenyi Biotec vs. four intranuclear staining protocols from other providers. Different panels were designed that comprised basic markers such as CD3, CD4, CD25 (REA770) / CD25 (REA453), and CD127 (REA464) by default. Clone 4E3 was used for staining enriched CD25² cells, thus avoiding epitope hindrance caused by the CD25 antibodies used for enrichment. Intranuclear markers included Helios, FOXP3, and Ki-67 (fig. 3).

Results

1. Eight-color panel including VioBright™ 515 and VioBright 667 for Treg cell phenotyping

Whole blood was lysed and washed after surface staining with the 8-color panel. CD4-VioGreen™ FITC vs. CD4-VioBright 515 (fig. 2A) and CD25-APC vs. CD25-VioBright 667 (fig. 2B) were compared in terms of cell percentages and MFI / SI (fig. 2C). The multicolor panel was verified for the analysis of Treg cells derived from whole blood, lysed blood, PBMCs, and pre-enriched cells (not shown).

2. Comparison of intranuclear staining using Anti-Helios clone 22F6 vs. the corresponding REA clone

A rapid intranuclear staining protocol was used to label PBMCs. The panel included CD3-VioGreen™, CD4-APC-Vio 770, CD25 (REA570)-PE, CD127-PE-Vio 770, and CD45RA-PE-Vio 615. Anti-Helios (22F6) VioBlue™ was compared to Anti-Helios (REA829)-VioBlue in the absence or presence of FcR Blocking Reagent in terms of background staining, MFI, and cell percentages (fig. 3). The multicolor panel was verified for the analysis of Treg cells derived from whole blood, lysed blood, PBMCs, and pre-enriched cells (not shown).

Conclusion

- Development of new eight-color panels, which include VioBright 515 and VioBright 667 for surface staining, and Vio 515 and Vio 667 for intracellular staining, enabling high-resolution analysis of Treg cells in PBMCs, lysed blood, and whole blood.
- Introduction of REAfinity Antibodies conjugated to the brightest Miltenyi Biotec dyes, such as the new VioBright Dyes, and also PE-Vio 615 and PE-Vio 770, avoiding non-specific binding to monocytes.
- Introduction of rapid staining protocols for detection of transcription factors such as FOXP3, Helios, Ki-67, and T-bet, either using PBMCs, lysed whole blood, or pre-enriched CD25² cells.
- Using the rapid FOXP3 staining protocol, processing time is up to twice as fast in comparison to other protocols.