A Simple Two-Step Method for the Isolation of Human CD4$^+$CD25$^{+bright}$/FOXP3$^+$ Regulatory T Cells Directly from Whole Blood

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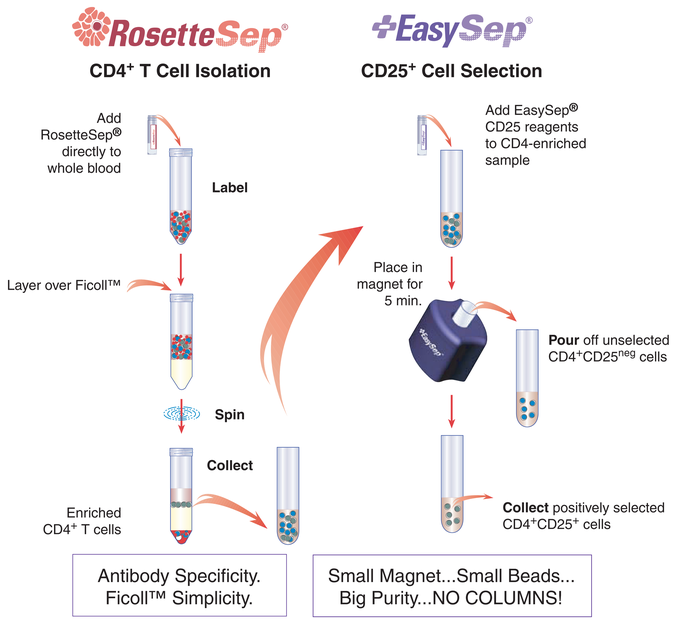
Introduction

Human CD4$^+$CD25$^+$ regulatory T cells (T_R) have the ability to suppress T cell responses and have recently been shown to play a critical role in peripheral tolerance and regulation of immune responses. CD4$^+$CD25$^+$ T_R are anergic, phenotypically CD25$^{+bright}$ and express high levels of the transcription factor FOXP3. Peripheral blood T_R are rare and must be highly enriched for their suppressor function to be detected in vitro. This, combined with the lack of a unique marker that distinguishes them from activated T cells, makes it difficult to study T_R function and evaluate their therapeutic potential. Current methods for isolating T_R are cumbersome and time-consuming, and generally require three steps: Ficoll™ density centrifugation to isolate mononuclear cells; subsequent immunomagnetic T cell enrichment; and finally FACS sorting. The objective of this study was to develop a more simple technique to isolate highly purified T_R from whole blood without using FACS sorting.

Methods

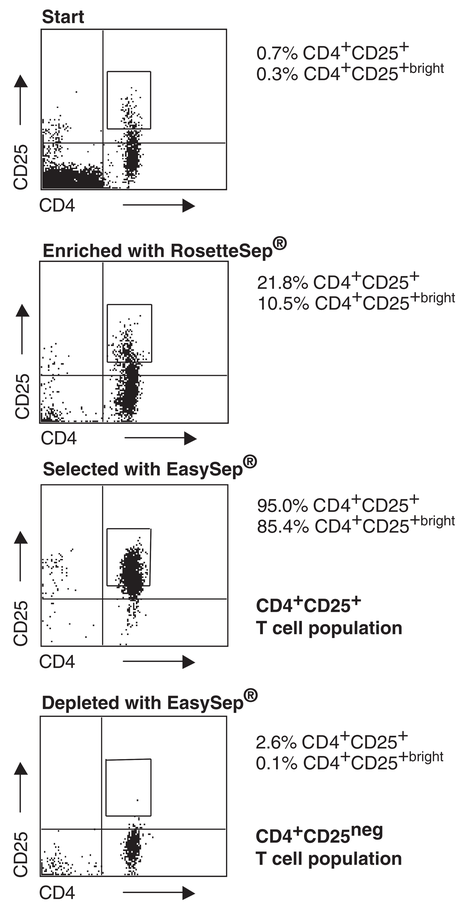
Figure 1. Isolation of CD4$^+$CD25$^+$ and CD4$^+$CD25^{neg} T cell populations from whole blood using RosetteSep® and EasySep® technologies.



Three steps were reduced to two by replacing the Ficoll™ separation and immunomagnetic T cell enrichment with a single antibody-mediated buoyant density centrifugation (RosetteSep®) to enrich CD4 T cells directly from whole blood. A cocktail of bi-specific antibodies were used to selectively bind unwanted cells to red cells causing them to pellet when centrifuged over Ficoll™. Purified CD4 T cells were recovered at the plasma-Ficoll™ interface and then separated using EasySep® column-free magnetic separation to select CD25 expressing cells. The EasySep® separation conditions were optimized for maximal CD4$^+$CD25$^{+bright}$ T cell purity and recovery.

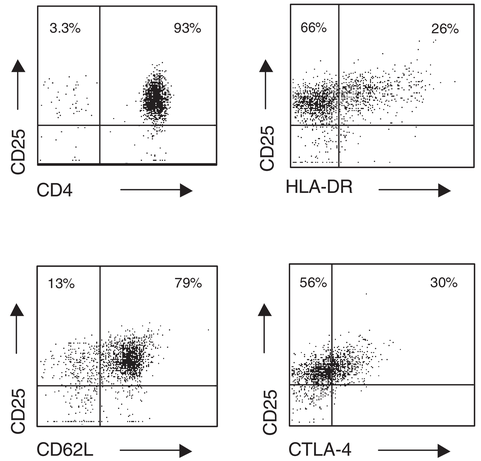
Results

Figure 2. Phenotypic characterization of cell populations isolated from whole blood.



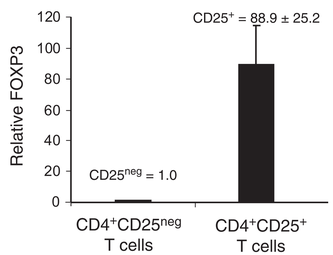
Both CD4$^+$CD25$^+$ and CD4$^+$CD25^{neg} T cell populations were isolated by combining RosetteSep® CD4 T cell enrichment and EasySep® CD25 selection. Following RosetteSep® enrichment of CD4 T cells, CD4$^+$CD25$^+$ populations were isolated with the EasySep® CD25 selection kit using the manufacturer's suggested protocol. The CD25 positively selected fraction was 94 ± 3% (n=6) CD4$^+$CD25$^+$ and 83 ± 7% (n=6) CD4$^+$CD25$^{+bright}$. The CD4$^+$CD25^{neg} T cell populations recovered in the unselected fraction were further depleted from CD25 expressing cells with the EasySep® CD25 selection kit using a depletion protocol provided by the manufacturer.

Figure 3. Phenotypic characterization of isolated CD4$^+$ CD25$^+$ T cells.



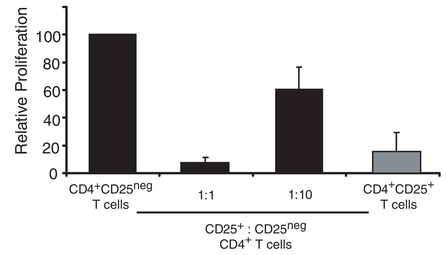
CD4$^+$CD25$^+$ T cell populations isolated as in Figure 2 were characterized by staining with fluorochrome-conjugated anti-CD4 and CD25 antibodies in combination with either anti-CD62L or anti-HLA-DR antibodies. Intracellular CTLA-4 expression was determined by staining cells with an anti-CTLA-4 antibody following fixing with 2% formaldehyde, and permeabilization with saponin. Cells were analysed by flow cytometry.

Figure 4. FOXP3 measurements in isolated CD4$^+$CD25$^+$ T cells.



FOXP3 mRNA levels in isolated CD4$^+$CD25$^+$ T cell fractions were compared to control CD4$^+$CD25^{neg} T cell fractions using quantitative PCR. For simplicity, CD4$^+$CD25^{neg} FOXP3 values were assigned the value 1, and CD4$^+$CD25$^+$ FOXP3 levels are expressed relative to 1 with the *Bar* indicating the mean of 4 donors and the *error bar* indicating standard deviation.

Figure 5. Isolated CD4$^+$CD25$^+$ T cells are anergic and suppress proliferation of CD4$^+$25^{neg} T cells.



Purified CD4$^+$CD25$^+$ T cell fractions were assessed for anergy by measuring their proliferation response to anti-CD3/CD28 coated beads (in grey). The suppression activity of purified CD4$^+$CD25$^+$ T cells was assessed by measuring their ability to reduce the proliferative response of CD4$^+$CD25^{neg} T cells to CD3/CD28 beads (in black). T cell proliferation was quantified by measuring dilution of the fluorescent dye CFSE with flow cytometry. Results are expressed as proliferation detected relative to control CD4$^+$CD25^{neg} cell populations stimulated with CD3/28 beads for 7 days in RPMI containing 5% human AB serum. The *bars* are the means of 5 experiments with *error bars* indicating standard deviations.

Conclusions

- Combining RosetteSep® CD4 T cell enrichment with EasySep® CD25 selection yields highly pure CD4$^+$CD25$^{+bright}$ / FOXP3$^+$ T_R in less time and with fewer steps than current isolation methods.
- Isolated cells display characteristics of peripheral blood CD4$^+$CD25$^+$ T_R:
  - Expression of surface and intracellular markers such as CD62L, HLA-DR, and CTLA-4
  - Expression of FOXP3 at high levels
  - Ability to suppress T cell proliferation responses