TECHNICAL NOTE
HUMAN REGULATORY T CELLS

Benoit Guilbault, PhD, Field Application Scientist • **Andy Kokaji**, PhD, Scientist

Introduction

Regulatory T cells (Tregs) are a specialized subset of lymphocytes that play a critical role in maintaining immune homeostasis and peripheral tolerance to self antigens.[1] Since their rediscovery, there has been exponential growth in basic and applied research investigations of their development, function and potential clinical application.[2-4] The regulatory ability of Tregs holds great promise for the treatment of autoimmune disease, allergic responses and transplant rejection.[5] Furthermore, as Tregs can reduce anti-tumor responses, circumventing their suppressive function has the potential to augment immune responses against weakly immunogenic tumor antigens.[6, 7]

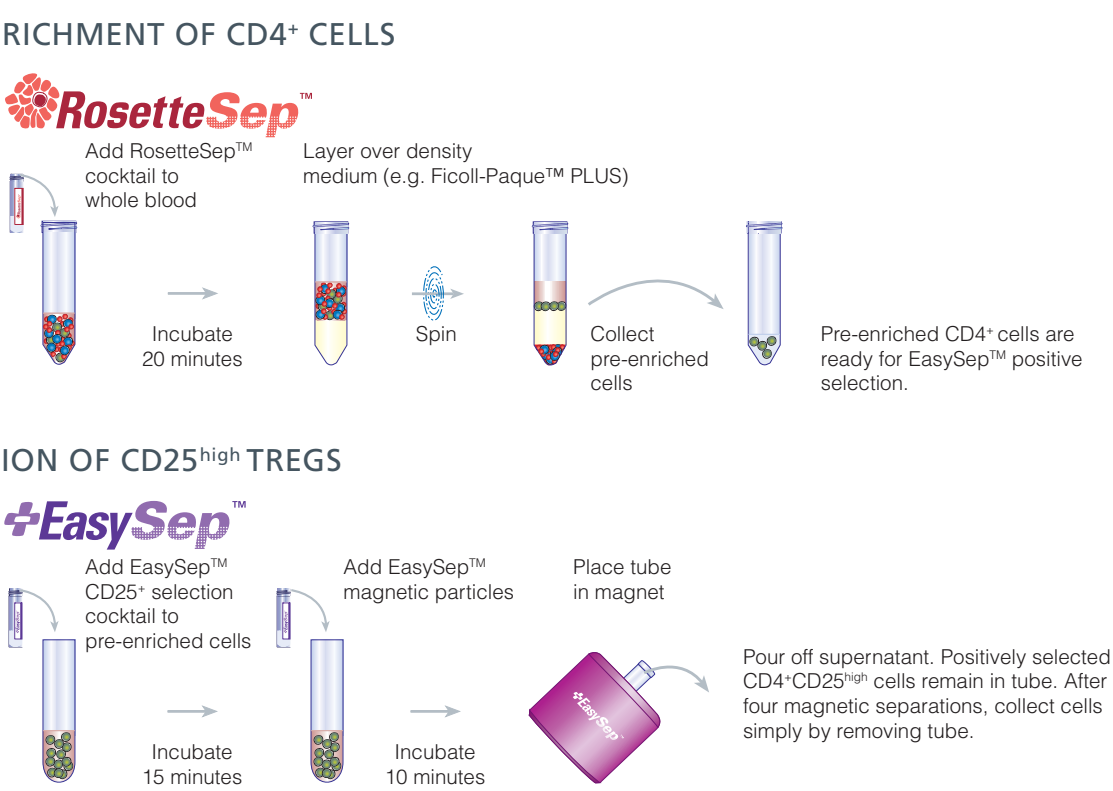
Tregs comprise only 2 - 3% of the total circulating leukocyte population. Their low frequency, combined with a lack of known unique cell surface markers has made isolation of highly purified, functional Tregs challenging. Both human and mouse Tregs were initially identified as $CD4^+$ T cells that constitutively expressed the alpha chain of the IL-2 receptor (CD25).[8-11] The discovery that Tregs specifically express the transcription factor FoxP3 has helped to further identify and characterize this unique lymphocyte population.[12-14] FoxP3 remains the best marker for the identification of bona fide Tregs, but its intracellular localization currently precludes its use for the isolation of viable Tregs. To overcome these technical difficulties, researchers have exploited the expression of other surface markers, either alone or in combination, in order to enrich for distinct Treg populations.

Typically, the expression of CD127 (IL-7 receptor alpha chain) inversely correlates with FoxP3 expression, while CD49d, the α-chain of the integrin VLA-4 (α4ß1), is expressed on a majority of pro-inflammatory effector cells but is absent on Treg cells.[15-17] Accordingly, removal of $CD127^{high}$ cells from the $CD4^+CD25^{high}$ T cell population enriches for a subset of Tregs with high levels of FoxP3 expression. Additional depletion of CD49d expressing cells, further removes contaminating IFN-gamma and interleukin 17 secreting cells. Together this strategy permits selective enrichment of highly suppressive Tregs.

The first step towards cutting-edge Treg research is the isolation of highly purified, functional Tregs. With unique cell isolation platforms, a team of dedicated and knowledgeable scientists, and a collaborative network of academic researchers, STEMCELL Technologies has developed a full range of products for the rapid and efficient isolation of highly functional Tregs, which are immediately ready for downstream use. Our Treg isolation kits provide a fast and easy way to isolate various subtypes of Tregs directly from human whole blood, buffy coat, PBMC or from mouse spleen with high purity and recovery.

TOLL-FREE T. 1 800 667 0322 • T. +1 604 877 0713 • TOLL-FREE F. 1 800 567 2899 • F. +1 604 877 0704
ORDERS@STEMCELL.COM • INFO@STEMCELL.COM • FOR FULL CONTACT DETAILS WORLDWIDE VISIT OUR WEBSITE

FIGURE 1. Two-step Isolation Procedure for the Enrichment of $CD4^+CD25^{high}$Tregs



Isolation of Treg Populations

The Treg isolation kits offered by STEMCELL Technologies fall approximately into three categories. These kits can save up to 2 hours over column-based procedures.

Classic Tregs

Isolation of $CD4^+CD25^{high}$ T cells enables study of the broadest phenotypic range of Tregs. Starting from whole blood, the Complete Kit for Human $CD4^+CD25^+$ T Cells (Catalog #15862) isolates classic Tregs in two steps – first, negative selection of $CD4^+$ T cells from whole blood; second, positive selection of $CD25^{high}$ cells from the pre-enriched population (see Figure 1).

TABLE 1. Summary of Purities Obtained by Combining RosetteSep™ $CD4^+$ T Cell Enrichment and RoboSep™ CD25 Positive Selection

CELLULAR PHENOTYPE	$CD4^+CD25^+$	$CD25^{high}$
Average Purity (%)	94.0 ± 2.4	83.7 ± 7.6

Results were obtained using fresh whole blood samples. n =14 (7 individual donors).

Highest Purity Tregs

Removal of $CD127^{high}$ and $CD49d^+$ cells from $CD4^+CD25^+$ T cells further enriches for highly suppressive Tregs, generating the highest purity population of Tregs for downstream analysis without the need for additional flow sorting. Starting from whole blood, the Complete Kit for Human $CD4^+CD127^{low}CD49d^-CD25^+$ Regulatory T Cells (Catalog #15864) isolates the highest purity FoxP3+ T regs in two steps – first, negative selection of $CD4^+CD127^{low}CD49d^-$ cells from whole blood; second, positive selection of $CD25^{high}$ cells from the pre-enriched population.

TABLE 2. Summary of Purities Obtained by EasySep™ and RoboSep™ $CD4^+CD127^{low}$ T Cell Enrichment

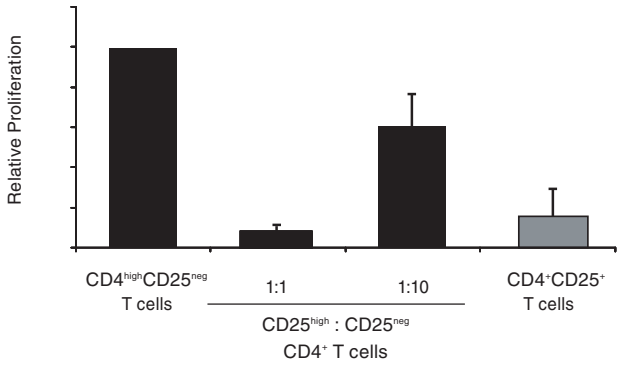
CELLULAR PHENOTYPE	$CD4^+CD127^{low}$	
Cell Separation Platform	EasySep™	RoboSep™
Average Purity (%)	92.4 ± 2.2	92.8 ± 3.1

n=6 (EasySep™) and n=4 (RoboSep™).

TABLE 3. Summary of Purities Obtained by EasySep™ $CD4^+CD127^{low}CD49d^-$ T Cell Enrichment

CELLULAR PHENOTYPE	$CD4^+$	$CD25^+FOXP3^+$
Average Purity (%)	94.3 ± 5.5	69.1 ± 9.1

FIGURE 2. Isolated CD4$^+$CD25^{high} T cells are anergic and suppress proliferation of CD4$^+$CD25^{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/CD28 beads for 7 days in RPMI media containing 5% human AB serum. The bars are the means of 5 experiments with error bars indicating standard deviations.

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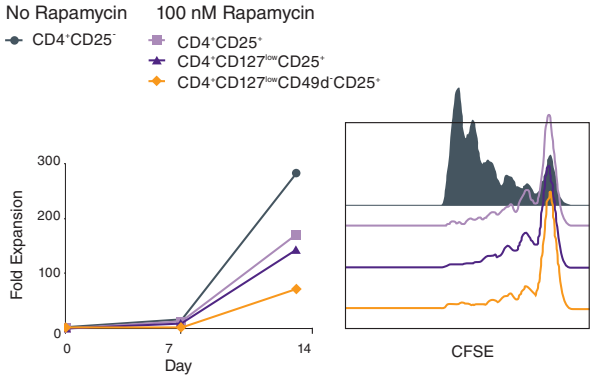
FIGURE 3. Expanded Tregs Suppress Proliferation of CD4$^+$CD25$^-$ Responder Cells



Left: Freshly isolated Tregs were stimulated with anti-CD3/CD28 coated beads in the presence of exogenous IL-2 and 100 nm rapamycin. On day 7 the cells were restimulated and the cultures continued until day 14. The CD4$^+$CD25$^-$-depleted cells in grey, were cultured in the absence of rapamycin for the duration of culture.

Right: Following the 14 days of culture, the expanded Tregs were co-cultured with autologous CD4$^+$CD25-depleted responder T cells labelled with CFSE at a 1 Treg : 2 responders ratio.

Untouched Tregs

For researchers who prefer a gentle method of isolating untouched Tregs, STEMCELL Technologies offers kits that enrich Tregs without the need for positive selection. Starting with PBMC, our new EasySep™ CD4$^+$CD127^{low}CD49d$^-$ Kit (Catalog #19232) isolates highly functional Tregs as little as 45 minutes.

Functional Analysis of Isolated Tregs

Tregs isolated using cell separation kits from STEMCELL Technologies are highly functional with a suppressor phenotype. Freshly isolated Tregs and Tregs expanded ex vivo are able to suppress proliferation of CD4$^+$CD25^{neg} T cells.

Concluding Remarks

Advances in the field of Treg biology are both dependent upon and necessary for progress in Treg isolation methods. Recent evidence suggests that Tregs are a heterogeneous cell population, comprising resting and activated subtypes.[18,19] Both resting (CD45RA$^+$FoxP3^{low}) and activated (CD45RA$^-$FoxP3^{high}) Tregs can be distinguished by their relative expression of CD45RA and FoxP3, markers that additionally separate them from a known population of non-suppressive CD45RA$^-$FoxP3^{low} CD4$^+$ T cells. Effective discrimination between resting and activated Tregs would thus not only enable investigations into Treg differentiation dynamics, but also facilitate the purification of Tregs from other T cell subsets. Kits for the isolation of CD45RA$^+$ and CD45RA$^-$ Treg subpopulations are thus currently under development at STEMCELL Technologies. These new products will add to the array of powerful yet convenient tools that STEMCELL Technologies offers for the functional analysis of Tregs, and should contribute to the development of novel therapeutic approaches for treating complex diseases.

References

1. Sakaguchi S, et al.; Annu Rev Immunol 22: 531-562, 2004
2. Sakaguchi S, et al.; Cell 133: 775-787, 2008
3. Wood KJ, et al.; Nat Rev Immunol 3: 199-210, 2003
4. Kang SM, et al.; Am J Transplant 7: 1457-1463, 2007
5. Riley JL, et al.; Immunity 30: 656-665, 2009
6. Pentcheva-Hoang T, et al.; Immunol Rev 229: 67-87, 2009
7. Dougan M & Dranoff G, Annu Rev Immunol 27: 83-117, 2009
8. Sakaguchi S, et al.; J Immunol 155: 1151-1164, 1995
9. Asano M, et al.; J Exp Med 184: 387-396, 1996
10. Levings MK, et al.; J Exp Med 193: 1295-1302, 2001
11. Baecher-Allan C, et al.; J Immunol 167: 1245-1253, 2001
12. Fontenot JD, et al.; Nat Immunol 4: 330-336, 2003
13. Hori S, et al.; Science 299: 1057-1061, 2003
14. Khattri R, et al.; Nat Immunol 4: 337-342, 2003
15. Liu W, et al.; J Exp Med 203: 1701-1711, 2006
16. Seddiki N, et al.; J Exp Med 203: 1693-1700, 2006
17. Kleinewietfeld M, et al.; Blood 113: 827-836, 2009
18. Miyara M, et al.; Immunity 30: 899-911, 2009
19. Sakaguchi S, et al.; Nat Rev Immunol 10: 490-500, 2010