An efficient new column-free immunomagnetic isolation method for mouse CD4$^+$CD25$^+$ regulatory T cells

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Summary

Mouse CD4$^+$CD25$^+$ regulatory T cells (Tregs) are extremely difficult to isolate due to their rareness and the absence of a unique marker that differentiates them from other cell types. Their isolation typically requires at least two steps, often including sorting by FACS, which can be time consuming and very expensive. We thus sought to develop a simple and efficient method for Treg isolation using column-free immunomagnetic cell separation (EasySep®). The resulting two-step method begins with depletion of non-CD4$^+$ T cells, and is followed by selection on CD25 whereby both CD4$^+$CD25$^+$ and CD4$^+$CD25^{neg} T cell fractions can be isolated to a high level of purity (90.0 ± 3.1% CD4$^+$CD25$^+$, n=18). This new method proved to be highly efficient as cell output was routinely well over 10e06 Tregs per spleen. Intracellular flow cytometric analysis of FOXP3 expression clearly demonstrated that isolated CD4$^+$CD25$^+$ T cell fractions were highly enriched for FOXP3 expressing cells (n=4). In vitro culture assays also showed that isolated CD4$^+$CD25$^+$ T cells were able to strongly suppress CD4$^+$CD25^{neg} T cell proliferation responses (n=4).

Methods

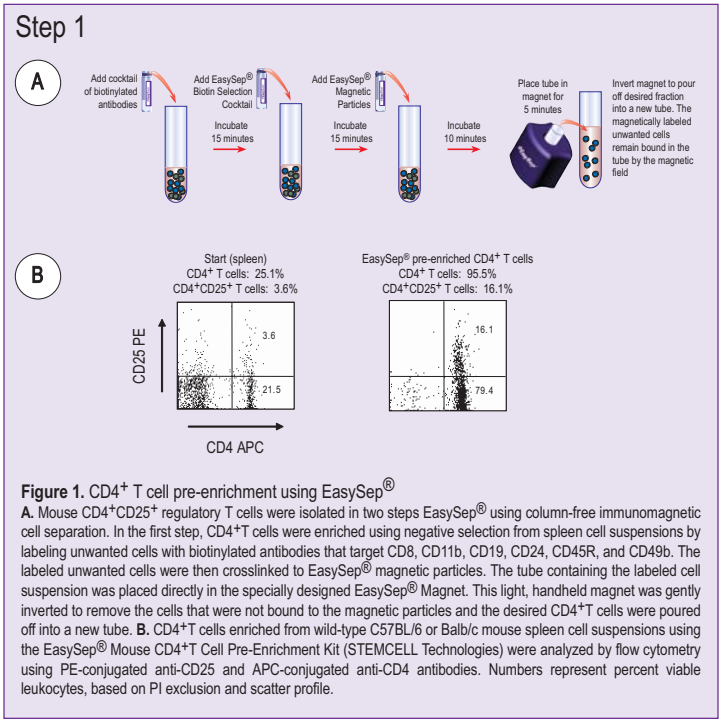

Figure 1. CD4$^+$ T cell pre-enrichment using EasySep®
A. Mouse CD4$^+$CD25$^+$ regulatory T cells were isolated in two steps EasySep® using column-free immunomagnetic cell separation. In the first step, CD4$^+$T cells were enriched using negative selection from spleen cell suspensions by labeling unwanted cells with biotinylated antibodies that target CD8, CD11b, CD19, CD24, CD45R, and CD49b. The labeled unwanted cells were then crosslinked to EasySep® magnetic particles. The tube containing the labeled cell suspension was placed directly in the specially designed EasySep® Magnet. This light, handheld magnet was gently inverted to remove the cells that were not bound to the magnetic particles and the desired CD4$^+$T cells were poured off into a new tube. **B.** CD4$^+$T cells enriched from wild-type C57BL/6 or Balb/c mouse spleen cell suspensions using the EasySep® Mouse CD4$^+$T Cell Pre-Enrichment Kit (STEMCELL Technologies) were analyzed by flow cytometry using PE-conjugated anti-CD25 and APC-conjugated anti-CD4 antibodies. Numbers represent percent viable leukocytes, based on PI exclusion and scatter profile.

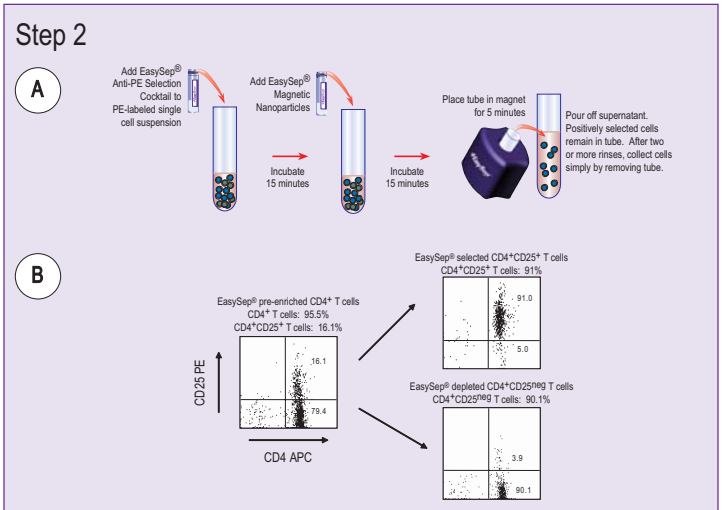

Figure 2. Selection of CD4$^+$CD25$^+$ and CD4$^+$CD25^{neg} T cells using EasySep®
A. CD25$^+$ and CD25^{neg} CD4$^+$ T cells were isolated from pre-enriched CD4$^+$T cell fractions first by labeling the cells with a PE-conjugated anti-CD25 antibody. PE-labeled cells were then cross-linked to magnetic particles. The tube containing the labeled cell suspension was placed directly in the specially designed EasySep® Magnet. The Magnet was gently inverted to remove the CD4$^+$CD25^{neg} T cells not bound to the magnetic particles, while the CD25$^+$ cells remained bound to the tube walls and were collected by removing the tube from the magnet and gently resuspending them. **B.** C4$^+$CD25$^+$ and CD4$^+$CD25^{neg} T cells isolated from pre-enriched CD4$^+$T cell fractions using the EasySep® Mouse CD25 Positive Selection Kit (STEMCELL Technologies) were analyzed by flow cytometry using PE-conjugated anti-CD25 and APC-conjugated anti-CD4 antibodies. Numbers represent percent viable leukocytes, based on PI exclusion and scatter profile.

Results

Table 1. Average purities and cell numbers obtained using the complete EasySep® Mouse Regulatory T Cell (CD4$^+$CD25$^+$) Positive Selection Kit

n=18	Step 1 % purity (CD4+T)	Step 2 % purity (CD4$^+$CD25$^+$T)	CD4$^+$CD25$^+$ T cell recovery (per spleen)
AVG ± STDEV	92.8 ± 1.3	90 ± 3.1	1.3 ± 0.3 x 10e06

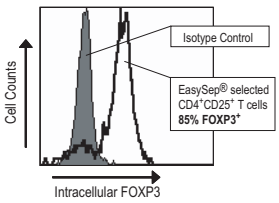

Figure 3. EasySep®-isolated CD4$^+$CD25$^+$ T cell fractions are highly enriched for FOXP3-expressing cells
Intracellular FOXP3 expression was detected in 4% parformaldehide-fixed CD4$^+$CD25$^+$ T cells using an FITC-labelled anti-FOXP3 antibody (clone FJK-16s; eBioscience). Similar results were obtained in 4 separate experiments.

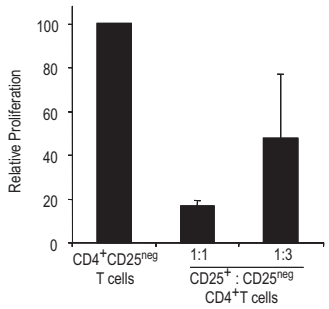

Figure 4. CD4$^+$CD25$^+$ T cells isolated using EasySep® are able to suppress T cell proliferation responses
The suppression activity of purified CD4$^+$CD25$^+$ T cells was assessed by measuring their ability to reduce the proliferative response of CD4$^+$CD25^{neg} T cells. CD4$^+$CD25^{neg} T cells were labelled with CFSE and cultured in complete media (RPMI 1640 supplemented with 10% fetal bovine serum containing penicillin, streptomycin, glutamine and β-mercaptoethanol) for up to 5 days. CD4$^+$CD25^{neg} T cell proliferation was stimulated by culturing them in the presence of 0.1 µg per mL soluble anti-CD3ε antibody combined with alloreactive EasySep®-isolated CD11c$^+$ dendritic cells (DC) at a ratio of 1 DC per 10 CD4$^+$CD25^{neg} T cells. T cell proliferation was quantified by measuring dilution of the fluorescent dye CFSE with flow cytometry. EasySep®-isolated CD4$^+$CD25$^+$T cells were co-cultured at a ratio of 1:1 and 1:3 with stimulated CD4$^+$CD25^{neg} T cells to determine their ability to suppress T cell proliferation. Results are expressed relative to the level of proliferation detected in stimulated CD4$^+$CD25^{neg} cell cultures. The bars are the means of 4 experiments with error bars indicating standard deviations.

Conclusions

- Mouse CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25^{neg} T cells can be efficiently isolated in just two steps using EasySep®
- Isolated Tregs are FOXP3$^+$ and able to suppress T cell proliferation responses