

ISOLATION AND EXPANSION OF CD4⁺CD25⁺ REGULATORY T CELLS BY USE OF DYNABEADS[®] MAGNETIC SEPARATION TECHNOLOGY

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BACKGROUND: Regulatory CD4⁺CD25⁺ T cells are a specialized subpopulation of T cells that act to maintain homeostasis within the immune system. Recent advances in the characterization of this cell population have firmly established their existence and their critical role in regulating the immune response. Interest in regulatory T cells has been accelerated by evidence from experimental mouse and human models demonstrating that the immunosuppressive potential of these cells can be utilized in the treatment of various diseases such as autoimmunity, infectious diseases and cancer.

Materials and methods:

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy individuals. **Isolation of effector and regulatory T cells:** CD25⁺ regulatory T cells were isolated using the Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit. To avoid that the IL-2 α chain (CD25) is occupied by antibodies after cell isolation we have developed an isolation strategy that allows the release of both the bead and the anti-CD25 antibody. This offers a unique positive isolation method that eliminates the risk of any unwanted effects that the antibody may have by binding the IL-2 receptor. This kit also allows the isolation of both T effector (CD4⁺, CD25⁻) and T regulatory (CD4⁺, CD25⁺) populations. **Expansion of the CD25⁺ regulatory T cell population:** Dynabeads[®] Treg Expander (3 beads/cell) was added to 1x10⁶ cells/ml for 14 days. The cultures were supplemented with 500U/ml of IL-2. **Suppression Assays:** CD4⁺CD25⁻ effector T cells were stained with CFSE and mixed with CD4⁺CD25⁺ regulatory T cells. Dynabeads[®] CD3 (1 bead/cell) were added to activate the effector CD25⁻ T cells and the suppression was measured 4 days later using CFSE staining.

Overview

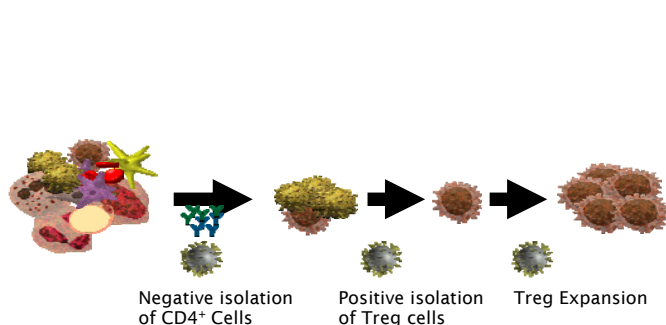


Figure 1. Simplified overview of isolation and expansion protocol of human Treg cells.

The Three Step Isolation Procedure

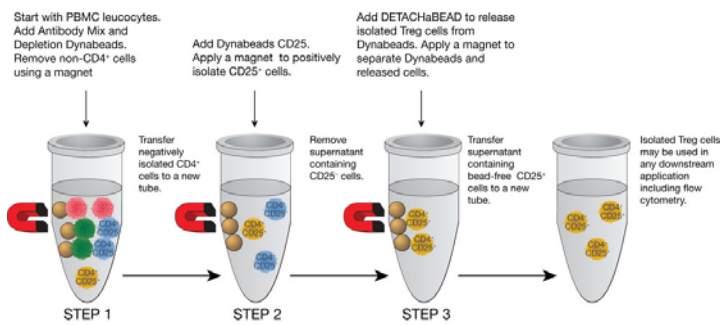


Figure 2. CD4⁺CD25⁺ regulatory T cells are isolated from PBMC with Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit by negative isolation of CD4⁺ T cells followed by positive isolation with Dynabeads[®] CD25 and bead detachment with DETACHaBEAD[®]. Isolated CD4⁺CD25⁺ regulatory T cells are bead- and antibody-free.

Expansion of Treg Cells

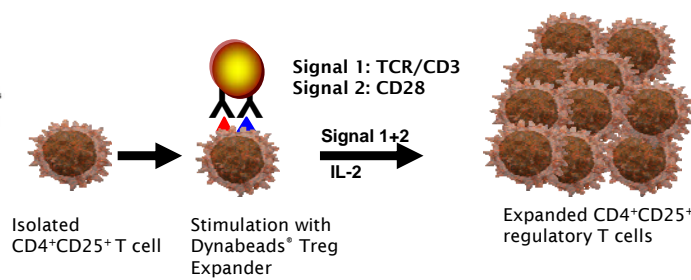


Figure 3. Expansion of CD4⁺CD25⁺ Treg cells using Dynabeads[®] Treg Expander.

AIM: Develop isolation and expansion protocols for human CD4⁺CD25⁺ regulatory T cells with the characteristic phenotype and suppressive capacity.

Results:

Highly pure ($\geq 95\%$) regulatory CD4⁺CD25⁺ T cells were isolated from healthy blood donors using Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit (Fig. 4). A large majority of these cells expressed the transcription factor Foxp3 (avg. $\geq 80\%$). Comparison of the Dynabeads[®] technology with a column-based isolation technology revealed that the tube-based isolation strategy from Invitrogen Dynal resulted in a significantly higher number of CD25⁺ T cells as well as Foxp3⁺ cells (Fig 5). Treg cells isolated with Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit suppressed the proliferation of CD4⁺CD25⁻ effector T cells in the presence of CD3 activation by up to 96% (Fig 8), showing that the isolated regulatory T cells retain their normal function. Low number of regulatory T cells can be a road block for scientists to perform functional and/or adoptive cell transfer experiments. As shown in Fig 9 there is robust expansion (up to 100-fold) of Treg cells using the Dynabeads[®] CD3/CD28 approach. For clinical research, Treg cells can be generated by expanding CD4⁺ T cells with Dynabeads[®] CD3/CD28 in the presence of rapamycin (Fig 11 and 12).

Purity of CD4⁺CD25⁺Foxp3⁺ cells

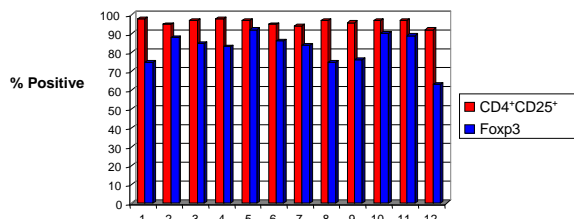


Figure 4. Treg cells were isolated from 12 different donors using Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit, and analyzed for purity as identified by expression of CD25 and Foxp3.

Higher number of Foxp3⁺ T cells after isolation using Dynabeads compared to a column-based method

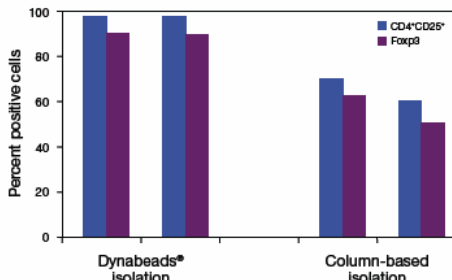


Figure 5. Treg cells were isolated from 2 different donors using Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit or a column-based method. Purity was assessed by flow cytometric analysis with anti-CD25 and anti-Foxp3 antibodies

Expansion of isolated regulatory T cells

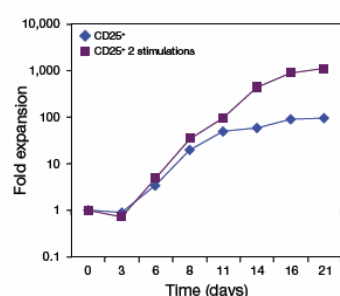


Figure 9. Treg cells isolated with Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit can be subsequently activated with Dynabeads[®] Treg Expander and expanded 100-fold, while retaining their Treg phenotype (Figure 10). A higher expansion number can be obtained through re-stimulation at day 8 with $>97\%$ pure CD4⁺CD25⁺ Treg cells to avoid overgrowth of non-Treg cells.

Generation of Treg cells using rapamycin and Dynabeads[®] CD3/CD28

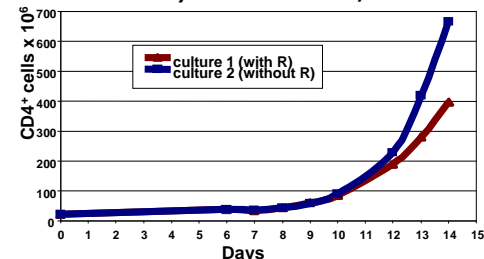


Figure 11. CD4⁺ T cells negatively isolated with Dynal[®] CD4 Negative Isolation Kit were expanded with Dynabeads[®] CD3/CD28 with and without rapamycin for 2 weeks. A lower fold expansion was achieved in culture with rapamycin, indicating the selective suppression of effector cells by rapamycin.

Suppressive capacity of rapamycin-generated Treg cells

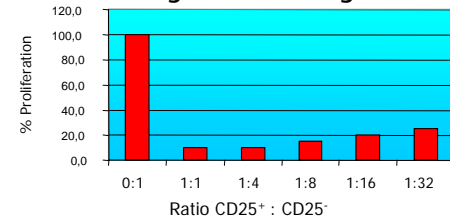


Figure 12. CD4⁺ T cells expanded with rapamycin were co-cultured with cells expanded without rapamycin in order to evaluate the rapamycin-expanded cells' suppressive capacity. At day 4 proliferation was analyzed in a thymidin incorporation assay. 80-90% suppression could be maintained with a 1:32 CD25⁺ : CD25⁻ ratio.

Presence and percentage of CD4⁺ CD25⁺ cells during the three step isolation process

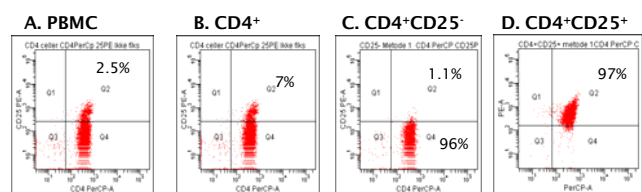


Figure 6. Isolation of Treg cells with Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit (A) In the PBMC sample, 2.5% of the cells are CD4⁺CD25⁺. (B) After negative isolation of CD4⁺ cells, 7% of the CD4⁺ cells express CD25. (C) After positive isolation of CD25⁺ cells, 1% of the low expressing CD25⁺ cells remain in the CD4⁺CD25⁻ fraction. (D) The isolated CD4⁺CD25⁺ cells are 97% pure.

Treg phenotype after isolation

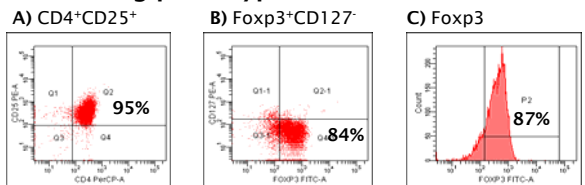


Figure 7. Isolation of Treg cells with Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit (A) The isolated Treg cells are 95% pure. (B) Within the CD4⁺CD25⁺ Treg cell population, 84% express Foxp3 and are negative for CD127. (C) In total, 87% of the CD4⁺CD25⁺ Treg cells express Foxp3.

Suppressive capacity of isolated Treg cells

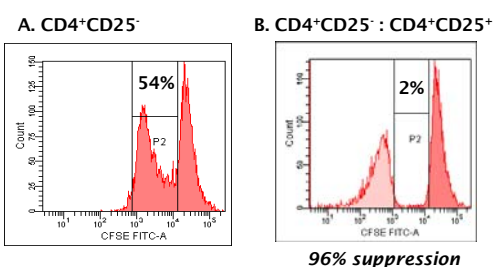


Figure 8. A) CD4⁺CD25⁻ cells were CFSE stained and stimulated with Dynabeads[®] CD3 (1 bead/cell) for 4 days. On day 4, 54% of the cells were dividing as identified by flow cytometry (B) CD4⁺CD25⁻ cells stained with CFSE were stimulated with Dynabeads[®] CD3 in the presence of CD4⁺CD25⁺ Treg cells in a 1:1 ratio. After 4 days, only 2% of the CD4⁺CD25⁻ cells were dividing and 96% suppression of cell division was achieved in the presence of CD4⁺CD25⁺ Treg cells (unstained CD4⁺CD25⁻ cells shown in light red).

CONCLUSIONS

- Dynabeads[®] Regulatory CD4⁺CD25⁺ T Cell Kit can be used to isolate $\geq 95\%$ pure CD4⁺CD25⁺ T cells; more than 80% of the isolated CD25⁺ cells express the transcription factor Foxp3.
- Dynabeads[®] Treg Expander expand human CD4⁺CD25⁺ regulatory T cells up to 100-fold during 2-3 weeks of culture while retaining their suppressive phenotype and expression of CD25 and Foxp3.
- Such expansion will facilitate further characterization of Treg cells as well as the evaluation of their potential in clinical applications.

Ordering information

Product	Cat.no	Application
Dynabeads [®] Regulatory CD4 ⁺ CD25 ⁺ T Cell Kit	113.63D	Treg cell isolation
Dynabeads [®] Human Treg Expander	111.29D	Treg expansion
Dynabeads [®] CD3	111.51D	Short-term stimulation/suppression assays
Dynabeads [®] CD3/CD28	111.41D	T cell expansion in pre-clinical research
Dynabeads [®] ClinExVivo™ CD3/CD28	402.03D	T cell expansion in clinical research
Dynal [®] CD4 Negative Isolation Kit	113.17D	CD4 ⁺ T cell isolation