

Human regulatory T cell expansion and measurement of function

Introduction

Regulatory T cells (Tregs) are a subpopulation of T cells with the unique ability to suppress the activation of effector T cells and thereby prevent T cell function. The relative frequency of Tregs in peripheral blood is 1–2% of the total lymphocyte population, which is why *ex vivo* expansion of Tregs prior to clinical applications such as adoptive transfer is necessary. *In vitro* expansion of Tregs can be triggered by providing the primary and secondary activation signal via crosslinking of the CD3 and CD28 cell surface receptors, and the third activation signal by addition of IL-2 to the culture medium.

This application note describes an optimized protocol for activation and expansion of flow-sorted Tregs with Gibco™ CTS™ Dynabeads™ Treg Xpander. Maintenance of Treg phenotype and function is confirmed by flow cytometric analysis and coculture assay.

Materials and methods

Treg starting material

Flow-sorted Tregs from 4 donors were used as starting material. Purity of the starting material is an important factor in *ex vivo* expansion and will impact the purity of the final expanded population. The most frequently used methods to isolate Tregs are flow sorting or magnetic isolation, whereby flow sorting commonly yields a purer Treg population based on high expression of FOXP3. Note: For expansion of Treg populations with low purity, certain reagents to limit overgrowth of T effector cells can be added.

CTS Dynabeads Treg Xpander

Instructions on how to prepare the required amount of CTS Dynabeads Treg Xpander (Cat. No. 46000D) can be found in the product manual (Pub. No. MAN0018604). In short, the required amount of CTS Dynabeads Treg Xpander to allow for stimulation of Tregs at a 4:1 bead-to-cell ratio was washed once and left on a roller mixer until use. In this study, 2 separate vials of CTS Dynabeads Treg Xpander were used.

Expansion medium

CTS OpTmizer SFM (Cat. No. A10485 or A3705) are completed according to the user manual with 2.5–5% Gibco™ CTS™ Immune Cell Serum Replacement (SR) (Cat. No. A2596102). 300 IU/mL of Gibco™ CTS™ IL-2 Recombinant Human Protein (rIL-2) (Cat. No. CTP0023) was added to the medium after day 2.

Treg activation and expansion

The optimized protocol for activation and expansion of Tregs can be found in the product manual for CTS Dynabeads Treg Xpander (Pub. No. MAN0018604). To summarize, Tregs were activated with CTS Dynabeads Treg Xpander at a 4:1 bead-to-cell ratio in medium without IL-2 on day 0. Starting on day 2, the medium was supplemented with 300 IU/mL of rIL-2, and cells were kept at a density of approximately 1×10^6 cells/mL in a humidified CO₂ incubator at 37°C. Mixing of cells and beads should be avoided until day 5 to enable formation of clusters (see Results section).

Removal of Dynabeads magnetic beads

On day 9, expanded Tregs were harvested into tubes for magnetic separation. Tregs were pipetted gently but thoroughly before being exposed to the magnetic field. Magnetic capture of Dynabeads magnetic beads for 1 min allows the subsequent transfer of bead-free cells into a new tube.

Restimulation

Expanded Tregs can be restimulated to yield higher cell numbers between day 7–9. Prior to restimulation, CTS Dynabeads Treg Xpander from the initial stimulation need to be removed. For restimulation, a bead-to-cell ratio of 1:1 with CTS Dynabeads Treg Xpander is recommended. Subsequently, the cells are cultured again at 1×10^6 cells/mL in a humidified CO₂ incubator at 37°C with expansion medium containing 300 IU/mL of rIL-2.

Treg phenotyping

A fraction of the expanded Tregs were stained with antibodies against the surface and intracellular markers CD4, CD25, CD127, and FOXP3, and analyzed by flow cytometry. Staining of live and dead cells was used to exclude the signals of dead cells.

Treg suppressive function

Expanded Tregs were tested for their potential to suppress proliferation of activated T effector (Teff) cells in a Treg–Teff coculture assay. To distinguish the different cell populations, expanded Tregs were stained with Invitrogen™ CellTrace™ Far Red dye (Cat. No. C34564), and target cells were stained with Invitrogen™ CellTrace™ CFSE dye (Cat. No. C34554). Target cells were activated with Gibco™ Dynabeads™ Human T Activator CD3/CD28 (Cat. No. 11161D) at 20 target cells per bead. At the same time, expanded Tregs were added at ratios of 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 Tregs per target cell. After incubation for 4 days at 37°C, the CFSE signal, which correlates with proliferation of target cells, was assessed by flow cytometry.

Results

Cell culture

Upon activation, Tregs and CTS Dynabeads Treg Xpander form clusters (Figure 1). This clustering is necessary for optimal activation and expansion of Tregs. Throughout the culture period, cells (after day 5) will grow more confluent, and the bead–cell clusters can be resuspended during media addition and exchange.

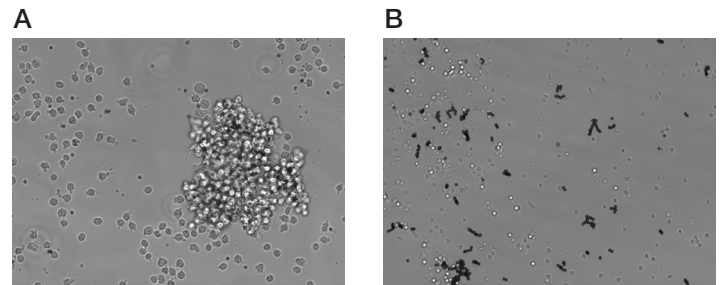


Figure 1. Visualization of bead–cell clusters using a light microscope (20x objective). (A) Activated Tregs form clusters with CTS Dynabeads Treg Xpander at a 4:1 bead-to-cell ratio. (B) Nonexpanding cells do not form clusters.

Treg fold expansion

Gibco™ CTS™ Dynabeads CD3/CD28 and CTS Dynabeads Treg Xpander are optimized for expansion of T cells and Tregs, respectively. Expansion of Tregs with CTS Dynabeads Treg Xpander yields significantly higher amounts of Tregs after a 2-week expansion period including restimulation (Figure 2).

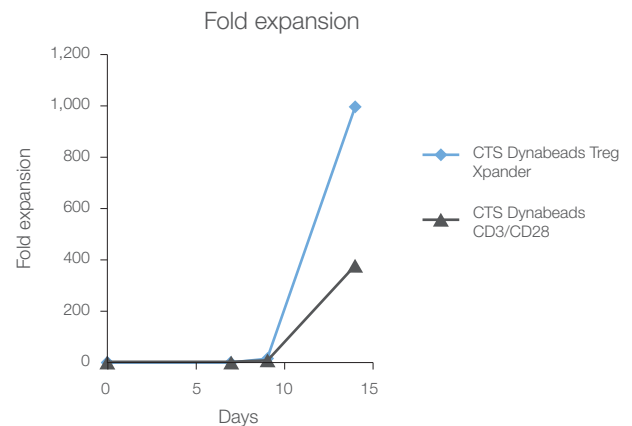


Figure 2. Comparison of Treg expansion. Superior expansion of Tregs is observed with the optimized CTS Dynabeads Treg Xpander. Tregs were expanded for 14 days using each product, with restimulation at day 9.

Treg phenotype

Flow cytometric analysis of expanded Tregs reveals over 60% FOXP3 expression in all 4 donors, with highly robust results from 2 different vials of CTS Dynabeads Treg Xpander. All cells have high viability at the end of the process (Figure 3).

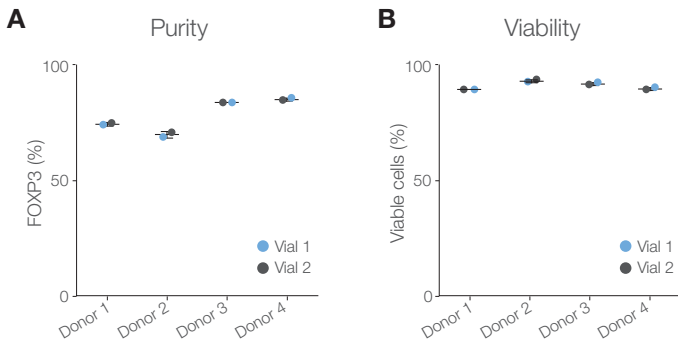


Figure 3. Assessment of Treg phenotype. (A) Purity and (B) viability of Tregs from 4 different donors are shown after expansion for 12 days with CTS Dynabeads Treg Xpander.

Conclusions

CTS Dynabeads Treg Xpander successfully expanded Tregs from 4 different donors to high numbers. Expanded Tregs are viable, express the Treg lineage-specific marker FOXP3, and suppress effector T cell proliferation in a coculture assay.

Treg suppressive function

Coculture of expanded Tregs with activated T effector target cells shows a strong suppressive potential of Tregs that are expanded with CTS Dynabeads Treg Xpander (Figure 4). Suppressive potential declines with increasing amounts of target cells.

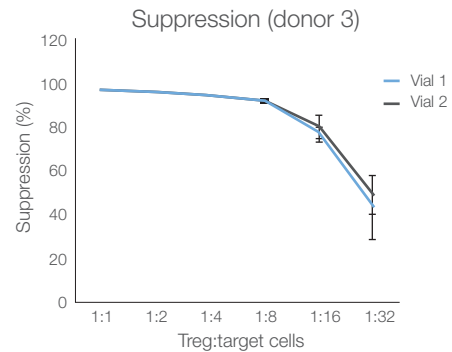


Figure 4. Strong suppression of T effector target cells by expanded Tregs. Suppressive potential declines with increasing amounts of target cells.

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