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Protocol

Isolation, propagation and characterization of cord blood derived CD4+ CD25+ regulatory T cells

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Abstract

Regulatory T cells (Treg) have recently come to the fore in studies of immune regulation, particularly in autoimmune disease and cancer. While there appear to be several distinct subsets of T cells with regulatory function, a population described as natural Treg and characterized by expression of the transcription factor FOXP3 has attracted particular interest. These cells can be enriched using the surface markers CD4 and CD25, and cord blood is a convenient source of CD25+ Treg. We present detailed protocols for the enrichment of Treg from cord blood using CD25 and a magnetic bead procedure, yielding populations >80% positive for CD25 and 50–65% FOXP3 positive. This enrichment can be followed by a second magnetic bead or a flow sorting step, yielding >95% CD25 and >65% FOXP3 positive populations. Protocols are presented for propagation of these cells in culture (yielding >80% FOXP3 positive cells) and for their phenotypic and functional characterization.

Keywords: Treg; Cord blood; MACS; FoxP3; CD25+; Suppressor function

1. Introduction

The ability of CD4+ CD25+ regulatory T cells to suppress activated T cells has been demonstrated clearly in both mouse (Sakaguchi et al., 1995; Shevach, 2000) and human (Stephens et al., 2001; Levings et al., 2001). These cells are perhaps the best studied of the regulatory T cell populations, but it is clear that in mouse at least, there are several cell subsets with regulatory capacity, in both the CD4 and CD8 lineages. A rare X linked lymphoproliferative disorder with gross autoimmune defects has been characterized in both man (IPEX) (Bennett and Ochs, 2001) and mouse (SCURFY) (Smyk-Pearson et al., 2003; Khattri et al., 2001), and in both cases the CD4+ CD25+ Treg are absent, suggesting that these cells serve the same function across species *in vivo*. The identification of the forkhead winged helix transcription factor FoxP3 as the genetic lesion in both the scurfy mouse (Brunkow et al., 2001) and the human IPEX patients (Bennett et al., 2001) has established a direct link between the CD4+ CD25+ Treg cells and suppression of auto reactive T cells. The correlation between FoxP3 expression and suppressor function has been well established for the mouse CD4+ CD25+ Treg (Fontenot et al., 2003; Kasprowicz et al., 2003) and to a lesser degree the human counterpart (Peng et al., 2004; Kuniyasu et al., 2000).

Abbreviations: CB, Cord blood; FACS, Fluorescence-activated cell sorting; MNC, Mononuclear cells; Treg, Regulatory T cell.

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Treg are relatively rare in human blood, ranging from 1–5% of T cells. Studies designed to characterize the phenotype and function of Treg requires access to substantial numbers of enriched Treg. Cord blood is a good source of Treg because it is available in large quantity, most of which is generally disposed of. The proportion of regulatory cells in this compartment is approximately the same as in adult blood (Baecher-Allan et al., 2004; Godfrey et al., 2005; Li et al., 2005; Takahata et al., 2004). The correlation between FoxP3 expression, CD4+ CD25+ expression and suppressor function has been established for cord blood cells (Godfrey et al, 2005; Li et al., 2005). These naive Treg cells may represent a pool that can be expanded *in vitro* for cell based therapies, either alone or in consort with dendritic cells.

We describe here a protocol for isolation of CD4+ CD25+ Treg cells from cord blood and the expansion of Treg numbers by cell culture.

2. Time required

i) Isolation of CD25+ Treg from cord blood cells by single-step magnetic bead procedure: 3 h from receipt of blood
ii) Purification of CD25+ Treg from cord blood cells by two-step magnetic bead/flow sorting purification: (add 30 min for a second MACS step or 2 h for flow sorting)
iii) Expansion of CD25+ cord cells: Washing Treg expander beads and adding to freshly isolated CD25+ Tregs: 15 min. Culture of Tregs with expander beads: 2–3 weeks.

3. Materials

Cord blood: cord blood was obtained with informed maternal consent as approved by the Children's, Youth and Women's Health Service Research Ethics Committee.
Anticoagulant: CPDA (#FBR7110 Baxter Healthcare, USA)
Ficoll–paqueÔplus: (GE Healthcare, Amersham UK)
Phosphate-buffered saline
Culture medium: RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, penicillin and streptomycin (SAFC Biosciences, Melbourne, Australia)
Recombinant human IL-2 (rhIL-2) (eBioscience, San Diego, CA)
Miltenyi MACS beads, column (#130-042–201) and anti-PE beads (#130-048-801) (www.MiltenyiBiotec.com)
MACS buffer (PBS + 0.5% BSA + 2 mM EDTA)
Dynabeads Human Treg Expander (magnetic beads precoated with CD3 and CD28), Invitrogen, Carlsbad, CA
Conjugated mAb: PE conjugated CD25 (M-A251) and matched isotype control (MOPC-21) (BD Bioscience San Jose, Ca); CD4-PC5 (13B8.2) and isotype control (679.1Mc7) (Beckman Coulter, http://www.beckmancoulter.com); FITC-conjugated Foxp3 (PCH101) and isotype control (eBR2a) (eBioscience San Diego, Ca)
FACS wash buffer: PBS, 0.5% FCS and 0.2% sodium azide
Permeabilization buffer: comes with FoxP3 antibody kit
FACS fix buffer: PBS, 2% glucose, 0.02% sodium azide and 1% formaldehyde

4. Detailed procedure

4.1. Collection of cord blood and isolation of cord blood mononuclear cell (CBMC) fraction

1. Collect 50-60 ml venous blood directly from the cord just post partum into pre-weighed Fenwell single collection blood bags containing anticoagulant.
2. Dilute cord blood 1/3 with sterile PBS using a 60 ml syringe and sterile canula, which is inserted into the tube feeding into the blood bag.
3. Remove the diluted cord blood from the collection bag using a 60 ml syringe and sterile canula.
4. Overlay 35 ml cord blood onto 15 ml Ficoll–paqueÔplus in 50 ml Falcon tubes.
5. Centrifuge at 400 ×*g*, 30 min continuously.
6. Collect the interphase layer and combine in 50 ml Falcon tubes, top up tubes with sterile PBS.
7. Centrifuge for 5 min at 290 ×*g*.
8. Resuspend the cells in 50 ml PBS.
9. Centrifuge for 5 min at 290 ×*g*.
10. Count cells.

If the interphase layer is contaminated with red blood cells, resuspend the cells in 5 ml PBS and overlay onto 3 ml Ficoll-paque. Repeat steps 5–10.

4.2. Treg enrichment by positive selection of CD25+ cells on MACS beads

1. Resuspend CBMNC cells (10^8) in 250 µl cold MACS buffer.
2. Add 25 µl anti-human CD25-PE per 10^8 CBMNC.
3. Incubate for 30 min on ice.
4. Wash by adding 10 ml cold MACS buffer.
5. Spin for 10 min at 290 ×*g*.