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Research paper

High purity and yield of natural Tregs from cord blood using a single step selection method

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ABSTRACT

Natural regulatory T cells (Tregs), characterized as CD4 $CD25^{high}$ Foxp3+, have been described as paramount contributors in immuno-regulation and self-tolerance. CD4 and CD25 have been the main markers used for their isolation, resulting in cells with potent suppressive properties. Nevertheless, low purity and yield continue to be an issue when attempting thorough characterizations and/or up scaling to bigger models and for clinical trials. Here we present a single-step methodology optimized for cord blood CD25+ isolation, using magnetic microbeads that achieves a reproducible purity of 89% for CD4 $CD25^{high}$ $CD127^{low}$. These cells showed a more consistent suppressive effect in mixed lymphocyte cultures. In addition, the proportion of contaminating effector T cells was < 9% whilst the yield of Tregs was doubled compared to the standard protocol. Gating on CD4 $CD25^{high}$ $CD127^{low}$ populations post isolation showed better correlation with suppressive efficacy compared to CD4 CD25+ gate. These data should facilitate the clinical scale-up of this procedure to obtain consistent Tregs for clinical application and research.

1. Introduction

Regulatory T cells were first described in mice models as CD4+ CD25+ T cells, showing potent suppressive effects on their counterpart CD4+ $CD25^{neg}$ (Sakaguchi et al., 1995). A similar population was subsequently described in humans, where they constitute 4 to 10% of the total peripheral CD4+ T cells (Baecher-Allan et al., 2001; Wing et al., 2002). This specific population has been identified as paramount mediators in peripheral immuno-regulation and maintenance of self-tolerance (Wood and Sakaguchi 2003). Crucial for Tregs analysis was the identification of the transcription factor forkhead box P3 (FOXP3) as necessary for their development and functionality (Fontenot et al., 2003). Even though recent studies have shown FOXP3 expression is not entirely specific to Tregs (Wang et al., 2007), it is currently the most acceptable Treg marker. Since FOXP3 is an intranuclear marker, it cannot be used for their isolation. More recent studies (Liu et al., 2006; Seddiki et al., 2006) have shown a 90% correlation with FOXP3 expression and the $CD25^{high}CD127^{low}$ population of CD4 T cells; allowing better identification of Tregs and discrimination of effector (CD4 $CD127^{high}$) cells within the CD25 positive population *ex vivo* and *in vivo*.

Several studies using isolated CD4 CD25+ T cells, both *in vitro* and *in vivo* (Baecher-Allan et al., 2005; Godfrey et al., 2005; Lindley et al., 2005; Bresatz et al., 2007; Wing et al., 2003; Ito et al., 2008), have shown discrepant results regarding Tregs mechanism of suppression. This may not only be due to the assay system used, but also with the Tregs source and the level of purity. Tregs from adult peripheral blood have mainly a central memory phenotype, compared with cord blood (CB) Tregs, which express a naïve phenotype (Takahata et al., 2004). Secondly, most of the studies using isolated Tregs using magnetic microbeads report the purity as CD4 CD25+ cells. This is not enough to characterize true Tregs, specifically in adults, since only the $CD25^{high}$ population is considered to be the most homogenous regulatory population (Liu et al., 2006;

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Seddiki et al., 2006). Finally, most of the studies performed Treg isolation for research using a pre-step procedure of CD4 negative selection, complicating the scale-up of this procedure for clinical application.

CB has been reported (Godfrey et al., 2005) to be a good source of potent suppressive Tregs showing a well defined CD25+ population, making it easier for their isolation. Here we show an optimized protocol for CD25 isolation from CB that will enable us to achieve reproducible purity of 89% for CD4 $CD25^{high}$ $CD127^{low}$ with < 9% effectors contamination. This constitutes a very important step for further clinical scale-up of this procedure using the same technology.

2. Materials and methods

Peripheral blood for functional assays was obtained from 5 healthy controls under previous consent and regulations. Cord blood units (CBU) were obtained from the Barcelona cord blood bank with prior consent and approval by the ethical committee. Cord blood samples were collected in sterile bags containing 25ml of CPD using conventional techniques and transported to the facility in temperature between 4 and 21°C. CBU were then diluted 1/1 upon arrival with transport media: RPMI 1640 (Lonza, Belgium) supplemented with 0.6% trisodium citrate and 40nM mercaptoethanol and used within 60h after collection.

Cord blood mononuclear cells (CBMNC's) were isolated by density gradient by overlaying 35ml of cord blood onto 15ml Ficoll-Paque Premium (GE Healthcare, UK) in 50ml Falcons. A range of 1×10^8 to 3×10^8 CBMNC's, were used for each of the isolations. All isolations were made on a QuadroMACs isolation unit (Miltenyi Biotec).

2.1. CD25+ isolation protocol

CD25+ cells were isolated from complete CBMNC's following manufacturer recommendations (Miltenyi Biotec). Briefly; cells were resuspended in 90μl per 10^7 cells of chilled MACS buffer (PBS, 0.5%BSA and 2mM EDTA), and labelled with 10μl of directly conjugated anti-CD25microbeads (Miltenyi Biotec; no.120-001-596. BergishGladbach, Germany) giving a total cell density of ~ 10×10^7cells/ml (from now on this will be referred as the standard protocol). In order to maximise purity and yield, titration studies were carried to define optimum parameters for CB Treg isolation. The resulting method from these experiments will be referred as "new method"; CBMNC' were resuspended in 20μl per 10^7 cells of chilled MACs buffer, and labelled with the same directly conjugated CD25 microbeads using 2μl per 10^7 cells, with a final cell density concentration of ~ 30×10^7cells/ml. The cells from both methods were then incubated for 15min at 4°C. After washing cells were applied to an LS column (Miltenyi Biotec) and the positive fraction was obtained by applying the plunger 2 × 1ml of MACS buffer after removal of the column from the magnet. The cells were then applied to a second LS column and this time the positive fraction was only washed once with 1ml of MACS buffer.

2.2. Flow cytometry

To verify purity and characterization of each of the isolations, cells were stained for 15min at 4°C for superficial markers as standard protocol. Intranuclear FOXP3 staining was performed as recommended by the manufacturer (Ebioscience) with the recommended isotype control. Each monoclonal antibody used was previously titrated for optimal staining. Cells were stained with FITC conjugated antibodies to CD62L (BD Pharmingen; clone Dreg-56), CD31 (BD Pharmingen; clone WM59), FOXP3 (Ebioscience; clone PCH101), isotype control rat IgG2a (Ebioscience; clone eBR2a). With PE conjugated antibodies to CD25 (Miltenyi biotech; clone4E3), CD45RA (BD Pharmigen; clone HI 100), CCR7 (BD Pharmigen; clone 3D12), with Percp-Cy5 to CD127 (Ebioscience; clone eBioRDR5), and with APC conjugated antibodies to CD4 (Ebioscience; clone RPA-T4) and CD45RO (BD Pharmingen; clone UCHL1). Cells were analyzed using a four-colour FACSCalibur flow cytometer (Becton Dickinson) and analyzed with Flowjo software.

2.3. Identification of Tregs after isolation

The purity of Tregs was assessed by first gating on live lymphocytes (by forward and side scatter) and then on CD4+ cells (Fig. 1). Because of the correlation between FOXP3 expression and $CD25^{high}$ $CD127^{low}$ gated cells (Seddiki et al., 2006; Liu et al., 2006) it was decided to calculate the final Tregs yield as the proportion of CD4 $CD25^{high}$ $CD127^{low}$ cells of the starting CBMNC's count.

2.4. Suppression assay

All cultures were performed in RPMI 1640 supplemented with 10% heat-inactivated human AB serum and 1% penicillin and streptomycin.

A one way, 6day, mixed lymphocyte culture (MLC) was performed by placing a total of 5×10^4 responding complete PBMC's or $CD25^{neg}$ fractions with 1×10^5 irradiated PBMC's stimulators. All cultures were carried out on 96-well U-bottom plates. CD25+ cells were added in 1/2 and 1/5 ratios (Tregs/effectors). Experiments were set between 3 and 6 replicates. Each well was pulsed for the last 16h of cultured with ^{3}[H]-thymidine (Amersham GE). Data was gathered by adding liquid scintillation and measured using a microbeta counter. Results are expressed as counts per minute (cpm).

2.5. Statistics

Difference among isolations and functional assays were analysed using a paired two-tailed Student *t* test. Correlation studies were done using a Pearson test, for both tests, a p value of < 0.05 was considered significant. All the statistical analysis was done using Prism 5 software.

3. Results

3.1. Purity of Tregs

Nine selections were performed using the standard protocol (10μl selection Ab per 10^7 cells in 10×10^7cells/ml) and 6 with the new protocol (2μl selection Ab per 10^7 cells in 30×10^7cells/ml). As shown in Fig. 2 a higher proportion of CD4 $CD25^{high}$ $CD127^{low}$ cells was seen with the new protocol compared to the standard protocol (mean 89%