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Research paper

Separation of human CD4$^+$CD39$^+$ T cells by magnetic beads reveals two phenotypically and functionally different subsets ☆

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ABSTRACT

Objective: The ectonucleotidase CD39 is an enzyme involved in adenosine production. Its surface expression on human regulatory T cells (Treg) allows for their flow-cytometry-based isolation from peripheral blood. To further develop and improve this method on a scale supporting translational studies, we introduced capture of CD39$^+$ Treg on magnetic immunobeads.

Methods: Peripheral blood mononuclear cells (PBMC) obtained from healthy donors were used for negative selection of CD4$^+$ T cells on AutoMACS using antibodies (Abs) specific for all lineage$^+$ cells. CD4$^+$CD39$^+$ Treg were captured by biotin-conjugated anti-CD39 Abs and anti-biotin Ab-coated magnetic beads. Isolated CD4$^+$CD39$^+$ T cells were phenotyped by flow cytometry for Treg-associated markers: CD39, CD73, FOXP3, CD25, CTLA-4, CCR4, CD45RO and CD121a or for the absence of CD127 and CD49d. CFSE-based proliferation assays and ATP hydrolysis were used to measure Treg functions.

Results: The purity, recovery and viability of the separated CD4$^+$CD39$^+$ T cells were satisfactory. The isolated CD4$^+$CD39$^+$ T cell population consisted of FOXP3$^+$CD25$^+$ T cells which hydrolyzed exogenous ATP and suppressed autologous CD4$^+$ T cell proliferation and of FOXP3^{neg}CD25^{neg} T cells without suppressor function. The same two subsets were detectable by flow cytometry in normal PBMC, gating on CD4$^+$CD39$^+$, CD4$^+$CD127^{neg}, CD4$^+$CD49d^{neg} or CD4$^+$CD25^{high} Treg.

Conclusion: CD4$^+$CD39$^+$ Treg capture on immunobeads led to a discovery of two CD39$^+$ subsets. Similar to CD39$^+$ Treg in the peripheral blood, half of these cells are CD25$^+$FOXP3$^+$ active suppressor cells, while the other half are CD25^{neg}FOXP3^{neg} and do not mediate suppression.

1. Introduction

The regulatory T cell (Treg) subset of human lymphocytes has attracted significant attention recently because of their key role in control of immune responses in health and disease (Sakaguchi et al., 2010). In human cancer, the frequency and suppressor activity of Treg are often upregulated, while in allergy and autoimmune diseases, they are decreased leading to over-reactive immune responses (Baecher-Allan and Hafler, 2006a; Miyara et al., 2009). Mechanisms utilized by Treg to induce suppression have been extensively examined and appear to be diverse as well as dependent on the environmental context (Sakaguchi et al., 2009). The major difficulty with Treg studies in humans, however, has been the lack of a cell marker which reliably defines Treg. While in mice, the intracellular forkhead transcription factor (FOXP3) has been widely accepted as a specific Treg marker, in humans expression of FOXP3 is not

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restricted to Treg (Gavin et al., 2006). FOXP3 may be transiently expressed in activated T cells, and its presence has also been reported in tumor cells (Ebert et al., 2008). Furthermore, FOXP3 is a nuclear protein, and its intracellular location limits its usefulness in Treg isolation, although it has often been applied to confirm the identity of Treg (Kryczek et al., 2009).

The isolation of human Treg from the peripheral blood or tissues has been largely dependent on negative immunoselection based on the lack of expression of CD127 (Peters et al., 2008; Liu et al., 2006) and CD49d on the cell surface (Kleinewietfeld et al., 2009). This type of isolation yields cells that are enriched in Treg but have low purity. We and others have used expression of CD25 to identify and isolate $CD4^+CD25^{high}$ T cells which express FOXP3 and mediate suppression (Baecher-Allan and Hafler, 2006). However, a largely arbitrary selection of high CD25 expression leads to a loss of Treg, and the number of cells obtained by this method is significantly lower than the total number of Treg present in the peripheral blood. These problems with Treg isolation contribute to existing confusion about the identity and properties of human Treg subsets.

Recently, ectonucleotidases CD39 and CD73 have been found to be expressed on the surface of murine and human Treg (Dwyer et al., 2007; Mandapathil et al., 2009). Jointly, CD39 and CD73 are responsible for ATP conversion to AMP and, ultimately, to adenosine which is a well known immunosuppressive factor (Sitkovsky et al., 2008). Thus, CD39 and CD73 are functional markers linking Treg to ATP breakdown and adenosine production (Deaglio et al., 2007). In mice, adenosine producing $CD39^+$ Treg cells promote liver metastasis and suppress NK cell functions (Sun et al., 2010) as well as contact hypersensitivity reactions (Ring et al., 2009). In humans, an increased frequency of $CD4^+CD39^+$ Treg has been reported in tuberculosis (Chiacchio et al., 2009), juvenile arthritis (Moncrieffe et al., 2010) and in tumor infiltrating T cells of lymphoma patients (Hilchey et al., 2009). A decreased frequency or function of $CD39^+$ Treg has been reported in multiple sclerosis (Fletcher et al., 2009), ryegrass allergy (Mittag et al., 2010) and vascular inflammation after transplantation (Robson et al., 2005). These findings emphasize the importance of $CD39^+$ Treg cells and their role in various pathologic conditions and a need for their isolation to provide sufficient cell numbers for potential therapies.

The relationship between the $CD4^+CD39^+$, $CD4^+CD127^{neg}$ and $CD4^+CD25^{high}$ Treg subsets has not been clear, and the overlap between these subsets contributes to the existing difficulties in a reliable identification and isolation of human Treg. As CD39 is both a surface marker and an enzyme directly involved in suppression, we considered it to be a superior candidate for the isolation of Treg. The isolation of human Treg based on CD39 expression by flow cytometry was previously described by us (Mandapathil et al., 2009). Here, we extend this method to positive immunoselection on magnetic beads of the $CD39^+$ Treg subset from PBMC on a large scale to facilitate their further characterization.

2. Materials and methods

2.1. Peripheral blood mononuclear cells

Peripheral venous blood samples used for flow cytometry studies were obtained from ten normal control (NC) subjects (University of Pittsburgh IRB approval #0403105). This cohort included 5 females and 5 males with a mean age of 50 ± 5 years (range: 39–69 years). Blood samples (30–40 mL) were drawn into heparinized tubes and were processed on Ficoll-Hypaque gradients (GE Healthcare Bioscience). For functional studies, buffy coats were purchased from the Central Blood Bank of Pittsburgh, PA. Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

2.2. Isolation of $CD4^+CD39^+$ T cells

Negative selection of $CD4^+$ cells was performed using a biotin-conjugated antibody (Miltenyi, Auburn, CA) cocktail specific for the lineage antigens (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and Glycophorin A). Next, $CD39^+$ T cells were isolated from the $CD4^+$ T cell population using biotin-conjugated anti-CD39 Abs and magnetic beads coated with anti-biotin Abs (Miltenyi). The protocol was optimized for a cell number of $5–10 \times 10^7$ PBMC using the separation programs 'depl05' (step 1) and 'posseld2' (step 2) on an AutoMACS (Miltenyi) for cell separation. An additional washing step with phosphate buffered saline (PBS) was performed before applying anti-biotin Ab-labeled magnetic beads respectively, in order to reduce the number of false positive $CD4^{neg}$ cells in the final population. Incubation times at 4 °C were 10 min for the CD4 T cell antibody cocktail as well as the biotin-conjugated anti-CD39 Ab and 15 min for the magnetic beads coated with anti-biotin.

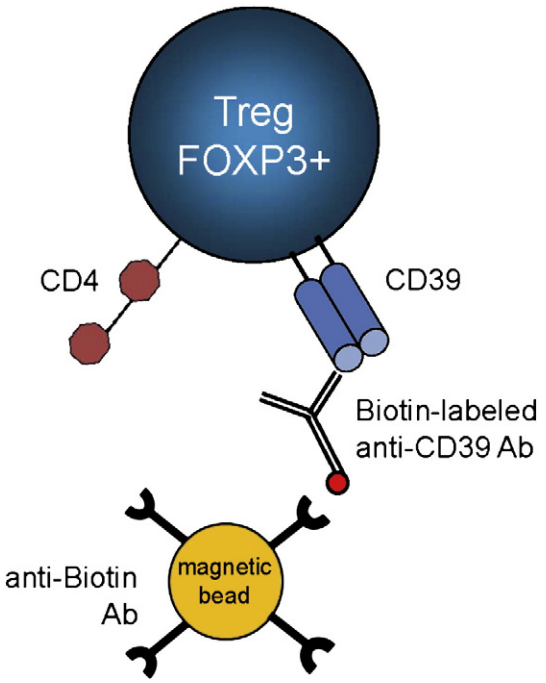

Fig. 1. Separation of CD4+CD39+ T cells by magnetic beads. Negative selection of $CD4^+$ cells was performed using a biotin-conjugated antibody cocktail specific for the lineage antigens (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and Glycophorin A). Next, $CD39^+$ T cells were isolated from the $CD4^+$ T cell population using biotin-conjugated anti-CD39 Abs and magnetic beads coated with anti-biotin Abs. The protocol was optimized for a cell number of $5–10 \times 10^7$ PBMC.