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Technical note

A highly relevant and efficient single step method for simultaneous depletion and isolation of human regulatory T cells in a clinical setting

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ABSTRACT

Regulatory T cells (Tregs) are pivotal in preventing autoimmunity. They play a major but still ambiguous role in cancer and viral infections.
Functional studies of human Tregs are often hampered by numerous technical difficulties arising from imperfections in isolating and depleting protocols, together with the usual low cell number available from clinical samples.
We standardized a simple procedure (Single Step Method, SSM), based on magnetic beads technology, in which both depletion and isolation of human Tregs with high purities are simultaneously achieved. SSM is suitable when using low cell numbers either fresh or frozen from both patients and healthy individuals. It allows simultaneous Tregs isolation and depletion that can be used for further functional work to monitor suppressive function of isolated Tregs (*in vitro* suppression assay) and also effector IFN-γ responses of Tregs-depleted cell fraction (OX40 assay).
To our knowledge, there is no accurate standardized method for Tregs isolation and depletion in a clinical context. SSM could thus be used and easily standardized across different laboratories.

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1. Introduction

Regulatory T cells (Tregs) are crucial in maintaining immune homeostasis. They play a pivotal role in preventing autoimmunity and their ambiguous role in cancer and viral infections is increasingly recognized (Vignali et al., 2008).

Tregs are characterized by the expression of the transcription factor Forkhead box P3 (FoxP3), as well as high levels of CD25 (alpha chain of the IL2 receptor), along with low level of CD127 expression (alpha chain of the IL7 receptor). These markers allow a reasonably precise assessment of human Tregs numbers and phenotype in peripheral blood of both patients and healthy individuals (Miyara et al., 2009; Seddiki et al., 2006).

There is a level of complexity in studying human Tregs which comes from the fact that the expression of Tregs markers overlaps significantly with the expression of the same markers by recently activated effector T cells (Teffs) (Miyara et al., 2009; Allan et al., 2007). Therefore, to fully define Tregs, it is necessary to assess their function using *in vitro* assays (McMurchy and Levings, 2012).

Two major strategies for Tregs isolation are used. The first one employs flow cytometer-based cell sorting (Seddiki et al.,

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2006) where both Tregs and Teffs are sorted based on the differential expression of CD25 and CD127 (Seddiki et al., 2006). This method is quite successful and often yields to the highest purity of isolated cells. However, it is not ideal as the long duration of the sorting often leads to increased cell mortality. Moreover, sterility requirements are harder to achieve and safety conditions are necessary when working with infectious material (especially in the context of HIV infection). Magnetic bead-based sorting method offered a convenient detour around these issues even if it usually leads to lower purities. However, the advantages are numerous: 1. the sorting is much faster when compared to flow-sorting; 2. cell viability after sorting is increased; 3. it is more convenient when working with lower cell numbers; 4. the sterility and safety requirements are easier to achieve (Miltenyi et al., 1990; Grützkau and Radbruch, 2010; Anon). It is noteworthy that when using magnetic bead-based assay, the quality of the samples is pivotal. Although this method is convenient when using freshly isolated cells, it is far from perfect when the cells are frozen. This is often the case when working with clinical samples where usually low cell numbers with poor viability are available, especially if the patients are immunodeficient. Thus the quality of the sample can deeply impact the Tregs cell isolation. For example, rapidly dying cells can block the column or bind to magnetic beads in a non-specific manner affecting the purity of isolated cells. This point is particularly relevant when two-step procedures for Tregs isolation are used (for example a first step for CD4 then followed by a second step for CD25 isolations).

Moreover, low cell number and low sample quality often influence the choice of the functional assay to be performed. Tregs function can be assessed by suppression assay using isolated Tregs or by measuring Teff responses after Tregs depletion (McMurchy and Levings, 2012). Low cell numbers are not ideal when aiming to perform both assays at the same time since the current available kits employ different experimental procedures for Tregs isolation and depletion (Miltenyi biotec product datasheets for products, 2012).

In this report we describe a simple method, named single step method (SSM) in which a one step procedure, including simultaneous depletion and isolation of Tregs, can be achieved from frozen samples with low cell numbers, instead of two (CD4 pre-enrichment then Tregs isolation or depletion), which is the current available methodology. This method could be used for isolation, depletion or simultaneous isolation/depletion of Tregs in clinical setting and is suitable for clinical trial studies that use low cell numbers and/or infectious material. Since pre-enrichment step is avoided, it allows fast and efficient isolation/depletion, preserving the maximum number of cells with functional activity.

2. Materials and methods

2.1. Blood samples

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers. Blood was collected in either heparin tubes or after cytapheresis. PBMCs were isolated from blood preparations by Ficoll density gradient centrifugation. All experiments were performed on freshly thawed cells.

Ethical committee approval and written informed consent from all subjects, in accordance with the Declaration of Helsinki, were obtained prior to study initiation. Committee and institutional review board(s) of EFS and INSERM (*REF: C CPSL UNT — No 12/EFS/079 and Convention reference number: I/DAJ/C2675*) approved our study.

2.2. Tregs isolation and depletion

The $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II (reference 130-094-775, "CD127 kit") and the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (reference 130-091-041, "CD25 kit"), both from Miltenyi Biotec, were used as indicated in product datasheet.

Briefly, the first step of the "CD127 kit" includes isolation of $CD4^+CD25^+CD127^{dim/-}$ regulatory T cells with a cocktail of biotinylated antibodies and anti-biotin beads for the depletion of non-$CD4^+$ and $CD127^{high}$ cells (CD8, CD19, CD123 and CD127 cocktail). In the second step, the flow-through fraction of pre-enriched $CD4^+CD127^{dim/-}$ T cells is labeled with anti-CD25 beads for subsequent positive selection of $CD4^+CD25^+CD127^{dim/-}$ regulatory T cells.

For the "CD25 kit", non-$CD4^+$ T cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a and anti-biotin beads. The labeled cells are subsequently depleted over a MACS Column. In the second step, the flow-through fraction of pre-enriched $CD4^+$ T cells is labeled with anti-CD25 beads for subsequent positive selection of $CD4^+$ regulatory T cells.

Of note, every step of the indicated procedures was respected except the usage of LD columns in the pre-enrichment step since they are specially designed for stringent depletion of unwanted cells. Since we needed both Tregs and Tregs-depleted fractions, we decided to use LS columns which are less stringent; this decision was made following discussions with the manufacturer.

Single step method (SSM) included only one step of labeling total PBMCs with anti-CD25 beads (reference 130-092-983) and one passage over LS columns. Ten to 20 million PBMCs were used in all experiments. Ten microliters of anti-CD25 beads was added to 10×10^6 PBMCs resuspended in 90 μL of cold MACS buffer. Cells were then incubated for 20 min at 4 °C then washed with 2–3 mL of MACS buffer before their passage through an LS column which has been placed on a manual magnetic separator. Both flow-through (Tregs-depleted) and remaining (Tregs) fractions were collected for further analysis and functional studies.

2.3. Staining and phenotyping

All staining experiments were performed at 4 °C for 30 min. Antibodies used were CD25-APC, CD127-PE, CD134-PE ((Becton Dickinson (BD) Biosciences)), CD4-Alexa Fluor 700, IFN-γ-eFluor450 (eBioscience), FoxP3-Alexa Fluor488 (BioLegend) and CD45RO-ECD (Beckman Coulter). For intracellular staining, FoxP3 buffer set (eBioscience) was used.

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