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Ex-vivo iTreg differentiation revisited: Convenient alternatives to existing strategies

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Abstract

Ex-vivo differentiation of regulatory T cells (Tregs) from naïve CD4⁺ T-cells has been widely used in immunological research. Isolation of a highly pure naïve T cell population is the key factor that determines the efficiency of subsequent Treg differentiation. Currently, this step relies mostly on FACS sorting, which is often costly, time consuming, and inconvenient. Alternatively, magnetic separation of T-cells can be performed; yet, available protocols fail to reach sort level purity and consequently result in low Treg differentiation efficiency. Here, we present the results of a comprehensive side-by-side comparison of various magnetic separation strategies and FACS sorting in multiple levels. Additionally, we propose a novel optimized custom made magnetic separation protocol, which not only yields sort level purity and Treg differentiation but also lowers the reagent costs up to 75% compared to the commercially available purification kits. The highly pure naïve CD4⁺ T-cell population obtained by this versatile method can also be used for differentiation of other T-cell subsets; therefore this protocol may have broad applications in T-cell research.

Keywords

FACS sorting; Magnetic separation; Regulatory T cells; Foxp3; Suppression assay

1. Introduction

Regulatory T-cells (Tregs) are among the main controllers of immune response. Due to their critical roles in suppressing effector T-cell activation, they have been extensively studied in the recent years. Both rodent and human studies largely depend on obtaining sufficient numbers of Tregs, which is often challenging due to the low abundance of thymic-derived (tTregs) or peripherally-induced (pTregs) Tregs (Karlsson et al., 2011; Shevach and

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Conflict of interest

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Thornton, 2014). Therefore, alternative strategies including in-vitro differentiation and expansion of Tregs have been proposed (Chen et al., 2003; Fantini et al., 2007). Tregs that are generated by in-vitro stimulation of non-Treg CD4⁺ T-cell populations (iTregs) share most of the functional characteristics of Tregs and are used in many experimental models (Shevach and Thornton, 2014). The one key factor determining the efficiency of iTreg generation is the purity of the starting CD4⁺ CD25⁻ Foxp3⁻ non-Treg T-cell population. Contamination of the starting population with memory T cells producing T effector cell cytokines markedly inhibits iTreg generation in vitro (Quintana et al., 2010). Several strategies employing FACS sorting and/or magnetic enrichment of CD4⁺ T-cell populations have been published in the recent years. Currently, FACS sorting of non-Treg CD4⁺ T-cells is considered as the gold standard technique for obtaining pure starting populations while magnetic separation techniques have been shown to generate variable success rates which are almost always lower than sorting (Karlsson et al., 2011; Flaherty and Reynolds, 2015). However, FACS sorting requires access to advanced sorting facilities that are costly and often require scheduling in advance. Additionally FACS sorting, compared to magnetic separation, requires significantly longer time periods during which the cells are maintained under suboptimal conditions. Finally, the potential harmful effects of subjecting cells to high-pressure fluidics of the FACS sorter is another drawback. Here, we compare the current non-Treg T-cell enrichment strategies in a comprehensive way and propose novel improved magnetic separation strategies, which offer cost-effective alternatives to sorting without compromising the purity and subsequent iTreg yields.

2. Materials and methods

2.1. Animals and reagents

B10.A and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Moth Cytochrome C (MCC) specific TCR transgenic 5CC7-FoxP3 GFP Rag2^{-/-} and WT FoxP3 GFP mice were purchased from Taconic Farms (Hudson, NY, USA). All mice were maintained in National Institutes of Health animal facilities in compliance with Animal Care and Use Committee standards.

Cells were cultured in sterile complete RPMI media (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 µM streptomycin, 1 mM sodium pyruvate 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 µM 2-mercaptoethanol and 10 mM HEPES) (ThermoFisher; Waltham, MA, USA). For magnetic separations, cells were maintained in filtered and degassed MACS buffer: PBS (Lonza; Allendale, NJ, USA) supplemented with 0.5% BSA (Sigma-Aldrich, St. Louis, MO, USA), 2 mM EDTA (Sigma-Aldrich). Samples were stained for flow cytometry using FACS buffer: PBS or HBSS supplemented with 2% FBS, 1% HEPES and 10 mM Sodium Azide (Sigma-Aldrich). The antibodies used for cell separation, stimulation and staining purposes are listed in Table 1.

2.2. Purification of CD4⁺ T-cells

6–8 weeks old male mice were euthanized by CO₂ asphyxiation and spleens were collected into sterile complete RPMI. Single cell suspensions were obtained by mashing the spleen

and passing cells through 70 μ m cell strainer (BD, Franklin Lakes NJ, USA). Cells were washed with MACS buffer and resuspended in 5–10 ml ACK buffer (ThermoFisher) to remove red blood cells. After 5 min of incubation at RT, cells were washed and then re-filtered to remove any clumps. In order to sort for naïve CD4⁺ T-cells, splenocytes from Foxp3-GFP mice were incubated with fluorochrome labeled (Fl-Ab) anti-CD4 (Clone RM4–5) and anti-CD44 (Clone IM7) antibodies in PBS for 20 min at 4 °C in dark. Cells within the CD4⁺ CD44^{low} Foxp3[–] gate were collected as the naïve fraction.

For positive magnetic separation, splenocytes were incubated with L3T4 Microbeads (Miltenyi Biotec, San Diego, CA, USA) for 15 min at 4 °C. For negative and naïve magnetic separations, cells were treated with CD4⁺ T-cell Biotin-Antibody Cocktail (Miltenyi) and Naïve CD4⁺ T-cell Biotin-Antibody Cocktail (Miltenyi) respectively, for 5 min at 4 °C. This was followed by a 10 min incubation of Anti-Biotin Microbeads (Miltenyi) for negative and naïve separations with an addition of CD44 Microbeads (Miltenyi) for the naïve separation. The amount of antibodies and beads were determined based on the initial cell count according to manufacturer's protocol. Cells were maintained in pre-chilled MACS buffer throughout the incubations. For all magnetic separations, cells were applied to LS Columns (Miltenyi) and AutoMACS following manufacturer's instructions. In positive selection, labeled cells were collected as the positive fraction (autoMACS Possel program), in negative and naïve selections, unlabeled cells were collected as the negative fraction (autoMACS Depletes program).

For custom isolation of naïve CD4⁺ T-cells, 8×10^7 – 1×10^8 splenocytes were resuspended in 1 ml PBS and mixed with equal amount of antibody cocktail containing biotinylated antibodies against CD8a, CD11b, CD11c, CD19, CD25, CD44, CD45R (B220), CD49b (DX5), CD105, CD24, Anti-MHC Class II, Ter-119, TCR γ/δ in PBS. Incubation was performed for 20 min at 4 °C. After antibody treatment, cells were washed twice with pre-chilled MACS buffer. Cell pellets were resuspended with MACS buffer (90 μ l buffer/ 10^7 cells). Streptavidin Microbeads (Miltenyi) were added (10 μ l beads/ 10^7 cells) to the suspension and cells were incubated for 15 min at 4 °C. Cells were then washed once with MACS buffer and applied to either LS column or AutoMACS (Depletes Program). Unlabeled cells were collected in the negative fraction.

2.3. iTreg differentiation

24 well sterile tissue culture plates (Corning, NY, USA) were coated with 4 μ g/ml anti-CD3e and 4 μ g/ml anti-CD28 in 0.5 ml/well PBS at 37 °C for 2 h.

Purified CD4⁺ T-cells were washed using complete RPMI medium twice to eliminate the MACS buffer and then resuspended in complete RPMI media supplemented with 100 IU/ml recombinant human IL-2 (Peprotech; Rocky Hill, NJ, USA) and 5 ng/ml recombinant human TGF- β (Peprotech). 3×10^5 cells were added to the wells at a volume of 1 ml/well. Cells were cultured at 37 °C 5% CO₂ for 3 days.

2.4. T-cell suppression assays

For polyclonal suppression assays, naïve CD4⁺ T responder cells (harvested from C57BL/6 mice) and in-vitro differentiated iTregs were labeled with e450 and e670 Cell Proliferation

Dyes (eBioscience San Diego, CA, USA) respectively. T-cell depleted and irradiated splenocytes (from C57BL/6 mice) were prepared as previously described (Thornton et al., 2004) and used as accessory cells. Naïve T responder cells were resuspended in complete RPMI medium and cultured in flat bottom 96-well plates at a density of 5×10^4 cells/well together with 5×10^4 cells/well accessory cells in the presence of 1 µg/ml soluble anti-CD3. iTregs were added into the culture at 1:2, 1:4, 1:8, 1:16 and 1:32 (Treg:T responder) ratios. Complete media was added as needed to adjust the culture volume to 250 µl/well.

For antigen specific suppression assays, naïve CD4⁺ T responder cells were isolated from the spleens of 6–8 weeks old 5CC7-Rag2^{−/−} mice. CD11c⁺ dendritic cells were purified from spleens of WT B10A mice as previously described (Chattopadhyay and Shevach, 2013) and incubated in 2 µM Moth Cytochrome C peptide containing complete RPMI medium at 37 °C for 30 min. 5CC7 naïve T responders and iTregs that were labeled as above were added into flat bottom 96 well plates at 1:10, 1:5 and 1:2.5 (Treg:T responder) ratios. T responder cells and dendritic cells were seeded at 2×10^5 /well and 4×10^4 /well respectively. All co-cultures were incubated at 37 °C 5% CO₂ for 3 days.

Proliferation status of T responder cells was determined using e450 proliferation dye (eBioscience) according to manufacturer's guidelines. Percent suppression of T responder proliferation was calculated using the formula: $100 \times [(\text{proliferation \% of T responder cells alone}) - (\text{proliferation \% of T responder cells co-cultured with Treg})] / (\text{proliferation \% of T responder cells alone})$ (Collison and Vignali, 2011).

2.5. Flow cytometry

Flow cytometry experiments were carried out using BD Fortessa and BD LSR-II cytometers. Data were analyzed in FlowJo software.

2.6. Statistical analysis

Statistical analyses were done using Graphpad Prism software. Results are representative of at least three independent experiments each of which was done in triplicates. For each graph, bars and error bars represent mean and standard deviation respectively. Students' *t*-test was carried out for determining the significance of the differences between sort and other strategies. ($P > 0.05 = \text{ns}$; $0.01 < P \leq 0.05 = *$; $0.001 < P \leq 0.01 = **$; $0.0001 < P \leq 0.001 = ***$; $P \leq 0.0001 = ****$)

3. Results and discussion

To compare the efficiencies of various cell isolation techniques, splenocytes harvested from Foxp3-GFP reporter mouse were either stained with fluorochrome-labeled anti-CD4, and anti-CD44 antibodies in order to purify CD4⁺ CD44^{low} GFP[−] population by FACS sorting, or processed using magnetic separation strategies. For magnetic separation, Positive (L3T4 Microbeads) or Negative (Untouched) CD4⁺ T-cell Isolation Kits and Naïve CD4⁺ T-cell Isolation Kit were used. Cells were then purified using conventional columns (LS:Manual separation) or autoMACS® (AM: Automated separation). Viability and purity of each strategy were tested using flow cytometry. Stepwise gating of naïve CD4⁺ (GFP[−] CD62L^{high} CD44^{low}) population revealed that FACS sorting and the Naïve Kit generated comparable

purities of naïve CD4⁺ T-cells (>97.5%) whereas Positive and Negative Total CD4⁺ T-cell Kits yielded lower naïve CD4⁺ T-cell purity (<50%) with significant Treg (GFP⁺) and memory/activated T-cell (CD44^{high} CD62L^{low}) contaminations (Fig. 1A–D). Similarly, due to more efficient purification by FACS sorting and the Naïve Kit, final cell numbers were significantly lower in these groups (Fig. 1B). While Negative, Naïve and FACS-sorted cells uniformly yielded (>95%) viable cells, Positive Kit purification resulted in consistently lower viability which may be due to non-specific binding of anti-CD4 antibodies to apoptotic cells (Fig. 1C).

FACS sorting is the only strategy that can clearly gate out the doublets and the dead cells. However, magnetic separation did also achieve sort level viability without significant amount of doublets owing to quick processing of cells and keeping them in pre-chilled solutions containing EDTA (to prevent cell aggregation) throughout the process. Nevertheless, checking the purity and viability of the magnetically-sorted T cells by flow cytometry is recommended especially when optimizing the isolation strategy in different mouse models.

Although total CD4⁺ T-cell isolation kits failed to produce pure naïve CD4⁺ T-cells using WT mouse models, they may still be used efficiently to isolate pure naïve CD4⁺ T-cells when TCR transgenic Rag knock-out mouse models are used. To test this hypothesis, splenocytes harvested from Moth Cytochrome C (MCC) specific TCR transgenic (5CC7-FoxP3 GFP-Rag2^{-/-}) mice (Chattopadhyay and Shevach, 2013) were either FACS sorted for naïve CD4⁺ T-cells or processed using Positive, Negative and Naïve CD4⁺ T-cell isolation kits. As expected, all isolation strategies uniformly yielded naïve CD4⁺ T-cells (Fig. 1E–F).

After demonstrating the efficiency of the Naïve CD4⁺ T Cell Isolation Kit as an alternative to FACS sorting of naïve CD4⁺ T-cells, we compared the performance of this kit with a custom depletion strategy that uses a biotinylated antibody cocktail (Table 1) and streptavidin Microbeads. All tested dilutions of the antibody cocktail (1:100–1:600) yielded approximately equal levels of naïve CD4⁺ T-cell purity using both LS and AM elution systems providing a low cost alternative to commercial naïve CD4⁺ T-cell isolation kits (Fig. 1G–H)

Cells that were purified using the strategies above, were seeded in anti-CD3 and anti-CD28 coated plates and cultured for three days in complete media supplemented with TGF- β and IL-2 in order to facilitate iTreg differentiation. In the FoxP3-GFP WT mouse model, FACS sorted and naïve kit isolated CD4⁺ T-cells generated >95% iTregs, whereas the methods that provided low naïve CD4⁺ T-cell purity resulted in <50% iTreg conversion. This confirms the importance of naïve CD4⁺ T-cells as the starter population for iTreg differentiation (Fig. 2A–C). Similarly, the yields obtained in 5CC7-Foxp3 GFP-Rag2^{-/-} mouse model were uniformly high, reflecting the similarity in the efficiency of purification for all strategies in this model (Fig. 2D–E). Additionally, naïve CD4⁺ T-cells purified using naïve kit or biotinylated antibody cocktail yielded similar iTreg percentages (Fig. 2F–G).

To test the possible effects of different isolation strategies on the functionality of Tregs, iTregs generated from FoxP3-GFP mice were co-cultured with naïve CD4⁺ T-cells and

irradiated T-cell depleted splenocytes in the presence of soluble anti-CD3 antibody. Decrease in the proliferation rate of naïve CD4⁺ T-cells was used as an indicator for the suppressive ability of iTregs. As expected, iTregs differentiated from naïve CD4⁺ T-cells via the Naïve kit, performed similar to those generated via FACS sorting at all Trespender/Treg ratios (Fig. 2H).

iTregs generated from 5CC7-Foxp3 GFP-Rag2^{-/-} cells were also tested in an antigen specific suppression assay in which they were co-cultured with 5CC7 naïve CD4⁺ T-cells in the presence of MCC-pulsed DCs. The similarity in the initial naïve CD4⁺ T-cell purities as well as the subsequent iTreg yields resulted in approximately identical suppressive capacities which validates the use of positive and negative CD4⁺ T-cell separation kits as equally efficient alternatives for isolating naïve CD4⁺ T-cells from TCR transgenic Rag knock-out mouse models (Fig. 2I).

In conclusion, our observations clearly demonstrate the link between the initial naïve CD4⁺ T-cell purity and the subsequent iTreg yield/function. The magnetic naïve T-cell isolation strategy, we propose here as an alternative to FACS sorting, not only depletes the Tregs but also eliminates the CD62L^{low} CD44^{high} activated/memory CD4⁺ T-cell populations (Akkaya et al., 2016). In this aspect, our method offers a superior yield compared to previously-described magnetic separation strategies that overlook the activated/memory T-cell contamination and therefore fail to achieve sort level performance. Finally, we have been able to optimize an antibody cocktail which can be used as an alternative to the commercial naïve CD4⁺ T-cell separation kits saving researchers up to 75% of the reagent costs.

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Fig. 1.

Comparison of mouse CD4⁺ T-cell isolation strategies. A–D) CD4⁺ T-cells were isolated from Foxp3-GFP mouse spleens using FACS sorting or different magnetic isolation strategies. Flow cytometry plots demonstrating the naïve CD4⁺ T-cell percentages (A), as well as bar graphs representing the total number (B), viability (C) and naïve CD4⁺ T-cell percentage (D) of isolated cells are shown. E–F) CD4⁺ T-cells were isolated from 5CC7-Foxp3 GFP-Rag2^{-/-} mouse spleen. The flow cytometry plots (E) and bar graphs (F) representing the percentage of naïve CD4⁺ T-cells are shown. G–H) T-cells were isolated

from Foxp3 GFP WT mouse spleen using either Naïve CD4⁺ T-cell Isolation Kit or different dilutions of depleting antibody cocktail. Flow cytometry plots (G) and bar graphs (H) demonstrating the naïve CD4⁺ T-cell purities are shown.

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Fig. 2.

Comparison of iTreg yields and suppressive activities based on different initial CD4⁺ T-cell isolation strategies. A–C) T-cells isolated as described in Fig. 1A were stimulated for three days in order to generate iTregs. Flow cytometry plots (A) and bar graphs (B) representing the percentage of iTregs as well as bar graphs demonstrating the total number of iTregs (C) are shown. D–E) T-cells isolated as described in Fig. 1E were used to generate iTregs. iTreg purities are shown as flow cytometry plots (D) and as bar graphs (E). F–G) T cells isolated as described in Fig. 1G were cultured to generate iTregs. Flow cytometry plots (F) and bar

graphs (G) represent the purity of iTregs. H–I) iTregs generated from T-cells obtained from WT FoxP3 GFP mice (H) and 5CC7-Foxp3 GFP-Rag2^{-/-} mice (I) were tested using polyclonal and antigen specific suppression assays respectively. Percentage of suppression is shown as bar graphs.

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Table 1

	Conjugate	Clone	Catalog Number	Vendor
Cell separation^(*)				
Anti-CD8a	Biotin	53-6.7	100704	Biolegend
Anti-CD11b	Biotin	M1/70	101204	Biolegend
Anti-CD11c	Biotin	N418	117304	Biolegend
Anti-CD19	Biotin	6D5	115504	Biolegend
Anti-CD25	Biotin	PC61	102004	Biolegend
Anti-CD44	Biotin	IM7	103004	Biolegend
Anti-CD45R (B220)	Biotin	RA3-6B2	103204	Biolegend
Anti-CD49b	Biotin	DX5	108904	Biolegend
Anti-CD105	Biotin	MJ7/18	120404	Biolegend
Anti-CD24	Biotin	M1/69	101804	Biolegend
Anti-MHCII	Biotin	M5/114.15.2	107604	Biolegend
Anti-Ter119	Biotin	TER-119	116204	Biolegend
Anti-TCR γ/δ	Biotin	GL3	118103	Biolegend
Flow Cytometry				
Anti-CD4	PE-CF594	RM4-5	562285	BD Biosciences
Anti-CD4	e450	RM4-5	48-0042-82	eBioscience
Anti-CD44	AF700	IM7	103026	Biolegend
Anti-CD44	APC	IM7	103012	Biolegend
Anti-CD62L	PE	MEL-14	104408	Biolegend
Cell stimulation/ Differentiation				
Anti-CD3e	Purified	2C11	100331	Biolegend
Anti-CD28	Purified	37.51	102112	Biolegend

^(*) Stock concentration of the depleting antibodies is 0.5 mg/ml.

Biolegend; San Diego, CA, USA.

BD Biosciences; San Jose, CA, USA.

eBioscience; San Diego, CA, USA.