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iTreg induced from CD39$^+$ naive T cells demonstrate enhanced proliferate and suppressive ability

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ABSTRACT

CD4$^+$CD25$^+$FoxP3$^+$ regulatory T (Treg) cells which consist of naturally occurring Treg (nTreg) and induced Treg (iTreg) cells are associated with the maintenance of immune homeostasis. Previous studies were focused on their potential to ameliorate graft-versus-host disease (GVHD) in human and mice. CD39 is a surface marker both expressed on CD4$^+$CD25$^-$ T cells and Treg cells. CD39$^+$ Treg cells demonstrate stronger suppressive ability compared to conventional Treg cells. However, whether the potential of CD39$^+$ naïve T cells induced Treg cells is different from conventional naïve T cells induced Treg cells in vivo and vitro remains to be inconclusive. Here we demonstrate that CD39$^+$ iTreg cells show enhanced proliferation and suppressive ability as well as lower inflammatory cytokines compared to CD39$^-$ iTreg cells. To conclude, our findings demonstrate that CD39$^+$ iTreg cells acquire high suppressive capacity in vitro and vivo, and this may provide a new insight into Treg cell therapy in GVHD clinical trials.

1. Introduction

Regulatory T (Treg) cells play an irreplaceable role in the induction of transplant tolerance and the maintenance of immune homeostasis [1]. Lack or functional alteration of Treg cells leads to many autoimmune diseases such as rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus (SLE) [2–4]. A bunch of studies were concerned with its potential to ameliorate graft-versus-host disease (GVHD) in human and mice [5–8]. Therefore, how to acquire Treg cells with excellent suppressive function for GVHD therapy remains to be explored.

CD39, an ectoenzyme that degrades ATP to AMP, is highly expressed in Treg cells [9]. CD39 could be utilized as a marker for Treg cells sorting due to CD39$^+$ Treg cells which demonstrate stronger suppressive function compared to conventional Treg cells [9,10]. However, CD39 is not the specific Treg marker like FoxP3 since it is also expressed in CD4$^+$CD25$^-$ T cells with enriched IL-2 and IFN-γ production [11,12]. To date, natural Treg (nTreg) and induced Treg (iTreg) cells are two main subsets of Treg cells well studied. Compared to nTreg, iTreg cells are more stable in the environment of pro-inflammatory cytokines [13]. Since iTreg cells could be induced from CD4$^+$CD25$^-$CD45RA$^+$ naïve T cells in the presence of IL-2, TGF-β and rapamycin [14,15], the objective of this study was to reveal the characteristic of iTreg induced from CD39$^+$ naïve T cells. We observed that CD39$^+$ naïve T cells proliferated better and became iTreg cells with higher FoxP3 levels compared to CD39$^-$ naïve T cell induced Treg cells. Furthermore, this subset of iTreg cells produced less proinflammatory cytokines and provided better survival for GVHD mice. To conclude, we provide a novel insight into iTreg generation for Treg therapy.

2. Material and methods

2.1. Isolation and generation of iTreg cells in vitro

Peripheral blood mononuclear cells (PBMCs) were prepared from healthy donors(ages from 20 to 25) by Ficoll–Hypaque density gradient centrifugation. All protocols that involved human blood donors were approved by Nanjing Medical University. CD39$^-$ and CD39$^+$ naïve T cells were isolated from PBMCs by flow cytometry. The purity of selected cells was routinely more than 95% as determined by flow cytometry. Fresh naïve T cells from PBMCs were stimulated with anti-CD3/CD28 beads (Life Technologies) at a bead/T cell ration of 1:1 in the presence of IL-2 (100 U/ml) or/and TGF-β (10 ng/mL) as well as rapamycin (100 ng/mL). All the cells were incubated at 37 °C for 7 days. IL-2 (100 U/ml) was renewed every 2 days.

2.2. Flow cytometric sorting and assays

All the cells were sorted and analyzed by flow cytometry after staining with the following antibodies (all from BD-Biosciences): anti-human-CD4, CD25, CD39, and CD127. For FoxP3 and Ki67 staining, the cells were first stained with surface antibodies, then fixed/permeabilized in cytofix/permeabilization solution (Biolegend) and stained with anti-human-FoxP3.

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2.3. Real-time PCR

Total RNA was extracted with RNA simple total RNA kit (Tiangen Biotech), and cDNA was obtained using RT-Master Mix (TaKaRa). mRNA levels were quantified with SYBR Premix Ex Taq (TaKaRa). Primer sequences were as follows (18S as internal control):

T-bet, 5′- CGGCTGCATATCGTTGAGGT -3′ and
5′- GTCCCCATTGGCATTCCTC -3′;
GATA-3, 5′- TCATTAAGCCCAAGCGAAGG -3′ and
5′- GTCCCCATTGGCATTCCTC -3′;
RORγt, 5′- GCAGCGCTCCAACATCTTCT -3′ and
5′- ACGTACTGAATGGCC TCG GT -3′;
Bcl-2, 5′- GAACTGGGGGAGGATTGTGG -3′ and
5′- GGCAGGCATGTTGACTTCAC -3′.
Bcl-xL, 5′- GCTTTGAACAGGATACTTTTGTGGA -3′ and
5′- GGGCTGCATGTAGTGGTTCT -3′.
18S, 5′-CTCTTAGCTGAGTGTCCCGC-3′ and
5′-CTGATCGTCTTCGAACCTCC-3′.

2.4. Suppressive assays of $CD4^+$ Treg cells in vitro and vivo

PBMCs were isolated as described previously and labeled with CFSE (Invitrogen). Anti-CD3 mAb-coated beads (Dynal) were added at 1:1 (bead:PBMC) and thawed/washed iTreg cells were added at ratios from 1:2 to1:32 (Treg:PBMC). Finally cultures were incubated at 37 °C. On day 4, the cells were stained with anti-CD8 APC. Data were acquired and analyzed using the proliferation platform in FlowJo, and suppression was determined from the Division Index. GvHD mice were utilized for assay in vivo after Treg injection, weight loss and the death of mice was recorded each day for survival analysis.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software . Data was presented as mean ± SEM. Evaluation of differences between two groups were evaluated using Student's t test. P value < 0.05 was considered as statistically significant difference.

3. Results

3.1. Both $CD39^+$ naïve T cells demonstrate enhanced proliferative and survival potentials

Primarily, we isolated PBMCs from healthy donors and analyzed them by flow. We gated on $CD4^+CD25^+FoxP3^+$ Treg and $CD4^+CD25^-CD45RA^+$ naïve T cells for further assay. As shown in Fig. 1A, most of the Treg cells were $CD39^+$, while naïve T cells were divided into $CD39^+$ and $CD39^-$ subsets. Ki67, as a marker of Treg proliferative ability, was also measured in this assay. Fig. 2B demonstrated that $CD39^+$ naïve T cells and Treg cells exhibited the stronger Ki67 expression compared to $CD39^-$ subsets. Next, RNA was acquired from sorted $CD39^+$ and $CD39^-$ naïve T cells. As shown in Fig. 1C, $CD39^+$ naïve T cells also showed up-regulated Bcl-2 and Bcl-xL mRNA levels, which were two important genes involved in T cell apoptosis [16,17]. To conclude, $CD39^+$ naïve T cells demonstrate better proliferative ability and survival potential.

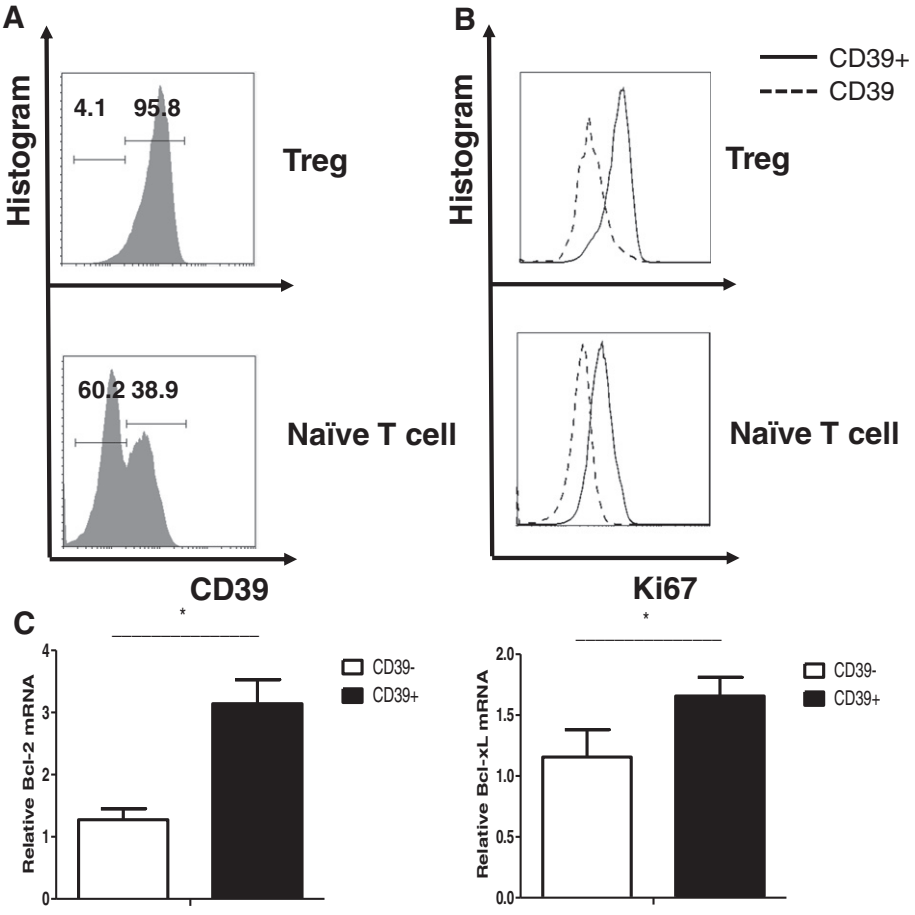

Fig. 1. Characteristics of $CD39^+$ naïve T cells. A. CD39 expression in Treg and naïve T cell. B. Ki67 expression in $CD39^+/^-$ population. C. Bcl-2 and Bcl-xL mRNA expression in $CD39^+/^-$ population. The values indicated the mean ± SEM of 3 separate experiments. $^*P < 0.05$.