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MicroRNAs are implicated in the suppression of $CD4^+CD25^-$ conventional T cell proliferation by $CD4^+CD25^+$ regulatory T cells

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ABSTRACT

$CD4^+CD25^+$ regulatory T cells (Tregs) are critical for sustaining immunological homeostasis. $CD4^+CD25^-$ conventional T cells (Tcons) are the progenitors of populations including Th1, Th2, Th17, Tfh, and Treg cells. Suppression of Tcons proliferation by Tregs requires cell–cell contact and/or is mediated by immunosuppressive soluble factors. However, upon receiving suppressive signals from Tregs, the exact molecular responses in Tcons remain elusive. Here, by using microRNA (miRNA) microarray preliminary screening and quantitative RT-PCR (qRT-PCR) validation, we showed that paralleled with the suppression of the Tcons proliferation, miR-146a was induced but miR-106b and miR-21 were reduced in Tcons upon receiving suppressive signals from Tregs. Moreover, our results showed that either increase of miR-146a or decrease of miR-106b and miR-21 by using miRNA mimics or inhibitors in Tcons significantly enhanced the suppression triggered by Tregs. However, decrease of miR-146a or increase of miR-106b and miR-21 in Tcons impaired the suppression triggered by Tregs. Collectively, our findings demonstrate the roles of miR-146a, miR-106b and miR-21 in Tcons in regulating Treg-triggered immune-suppression.

1. Introduction

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) control a wide range of the immune responses. Tregs have critical roles in preventing an array of diseases including autoimmune diseases, allergies, infection-induced organ pathology, transplant rejection, and graft versus host disease. However, Tregs can also adversely dampen beneficial immune responses against pathogens and tumors (Sakaguchi, 2004; Sakaguchi et al., 2008).

The targets of Tregs can be a variety of immunocompetent cells including $CD4^+$, $CD8^+$ T cells, B cells, monocytes, and dendritic cells (Miyara and Sakaguchi, 2007). A main function of Tregs is that they suppress the activation, expansion, differentiation and/or function of $CD4^+$ T conventional cells (Tcons), the precursor of certain types of Tregs and other T helper populations including Th1, Th2, Th17, and Tfh cells. Once receiving the suppressive signals from Tregs, the activation, proliferation, differentiation and cytokine production of Tcons are inhibited (Miyara and Sakaguchi, 2007; Palomares et al., 2010). The mechanisms of action of Tregs in regard to their immuno-suppressive function have been extensively investigated. Studies showed that Tregs may suppress Tcons by secretion of suppressive soluble cytokines including IL-10 and/or TGF-β, expression high level of CD25 (IL-2 receptor α chain, IL-2Rα) to compete with Tcons for IL-2, or expression of functional molecules including TGF-β, CTLA-4 or GITR on their surface that suppress Tcons directly or indirectly by cell–cell contact (Miyara and Sakaguchi, 2007; Palomares et al., 2010; Sakaguchi and Powrie, 2007; Schmidt et al., 2012; Shevach, 2009). However, the molecular changes in Tcons that are suppressed by Tregs after receiving the suppression signals from Tregs are poorly understood.

In this study, we demonstrated that miR-146a, miR-106b, and miR-21 in Tcons are involved in regulation of the immune-suppression triggered by Tregs.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning

Abbreviations: Tregs, $CD4^+CD25^+$ regulatory T cells; Tcons, $CD4^+CD25^-$ conventional T cells; miRNA, microRNA; WT, wild-type; GFP-Tg, green fluorescent protein transgenic; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline; PI, propidium iodide; FCM, flow cytometry; TGF-βRII, TGF-β receptor II.

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Experimental Animals (1988.11.1), and all efforts were made to minimize suffering. Animal care and all procedures were carried out in accordance with the guidelines of Chinese animal protection laws and with permission from the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (Permit Number: NJMU 10-1127).

2.2. Mice

Specific pathogen-free (SPF) 8-wk-old female wild-type (WT) and green fluorescent protein transgenic (GFP-Tg) C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All mice were bred in an SPF animal facility.

2.3. Cell preparation and purification

For miRNA array or qRT-PCR analysis, single-cell suspensions of splenocytes were prepared from GFP-Tg or WT C57BL/6 mice. Then $CD4^+$ cells were enriched by magnetic separation using anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified $CD4^+$ T cells were then stained with PerCP-Cy5.5-CD3, PE-CD4, and APC-CD25 monoclonal antibodies (mAbs) (eBioscience, San Diego, CA). $CD4^+CD25^-$ Tcons from GFP-Tg mice and $CD4^+CD25^+$ Tregs from WT mice were further purified using a FACSAria cell sorter (BD Biosciences, San Jose, CA). FACS purified Tregs and GFP^+ Tcons were co-cultured and stimulated as described below. After 3 d, cells were harvested and GFP^+ Tcons were sorted by cell sorter. After each purification, an aliquot was used to analyze the purity of the isolated populations, which was >99% in all cases.

For other assays, both $CD4^+CD25^-$ Tcons from GFP-Tg or WT mice and $CD4^+CD25^+$ Tregs from WT mice were isolated by using a $CD4^+CD25^+$ regulatory T cell isolation kit for magnetic separation (Miltenyi Biotec) according to the manufacturer's instructions, achieving >92% purity as determined by FCM analysis.

APCs in all experiments were prepared from spleen cells of WT C57BL/6 mice by depletion of $CD90.2^+$ cells with anti-mouse CD90.2 microbeads and LD column (Miltenyi Biotec) and were then irradiated with 30 Gy at 2.7 Gy/min using a ^{137}Cs source (Gammacell 1000 Elite; Nordion International, Kanata, ON, Canada).

2.4. Tcon and Treg co-culture

Co-culture was performed in 96-well round-bottom plates (Corning Costar, Cambridge, MA). Purified $CD4^+CD25^-$ Tcons (1×10^5 cells/well), $CD4^+CD25^+$ Tregs (1×10^5 cells/well) or both populations were cultured with irradiated APCs (1×10^5 cells/well) in triplicate for 3 d at 37 °C in complete RPMI 1640 medium (0.2 ml/well). Cultures were stimulated with 1 μg/ml anti-CD3 (BD PharMingen, San Diego, CA).

2.5. miRNA array and data analysis

miRNA expression in Treg-suppressed or unsuppressed GFP^+ Tcons was investigated using miRCURY LNA™ microRNA Array (Exiqon, Vedbaek, Denmark). The intensity of the signal was calculated after background subtraction and replicated spots on the same slide were averaged to obtain median intensity. The median normalization method was used to acquire normalized data: (foreground – background)/median. The threshold value we used to screen up-regulation or down-regulation of miRNAs was a fold change ≥2.0 or ≤0.5 (Kong et al., 2012). The fold change was calculated by comparing the expression of miRNA in Treg-suppressed and unsuppressed Tcons.

2.6. Suppression assay

$CD4^+CD25^-$ Tcons and $CD4^+CD25^+$ Tregs were MACS purified from WT mice and co-cultured as described above. The suppressive activity of Tregs on Tcons was measured by using $[^3H]$thymidine (Amersham Bioscience, Piscataway, NJ) at 0.5 μCi/well for the final 16 h of co-culture as previously described (Hu et al., 2012).

2.7. Proliferation assay

Tcons purified from WT C57BL/6 mice were prelabeled with 10 μM CFSE (Invitrogen, Carlsbad, CA) as previously described (Hu et al., 2012), and then co-cultured and stimulated as described above. After 3 d, proliferation of Tcons was assayed by FCM with a FACSCalibur.

2.8. Analysis for cell cycle distribution

$CD4^+CD25^-$ Tcons from GFP-Tg mice and $CD4^+CD25^+$ Tregs from WT mice were co-cultured as described above. Then Cells were collected and processed for cell cycle analysis as described previously (Chu et al., 1999). Briefly, cells were resuspended in phosphate-buffered saline (PBS) containing 0.5% paraformaldehyde and incubated for 1 h at 4 °C. Cells were then washed with cold PBS, resuspended in 70% (v/v) ethanol in PBS and incubated at −20 °C overnight. Then cells were washed, resuspended in cell cycle staining solution (50 μg/mL propidium iodide (PI) (w/v), 10% Triton X-100 (v/v), 0.1 mM EDTA, 50 μg/mL RNase A (w/v) in PBS) and incubated for 0.5 h at 4 °C in dark. Cell cycle distribution of GFP^+ Tcons was detected by FCM and then analyzed using ModFit software (Verity Software House, Topsham, ME) and gated on living GFP^+ Tcons.

2.9. Apoptosis analysis

$CD4^+CD25^-$ Tcons from GFP-Tg mice and $CD4^+CD25^+$ Tregs from WT mice were MACS purified and co-cultured as described above. Evaluation of apoptosis of GFP^+ Tcons was performed using Annexin V (APC-conjugated, eBioscience) binding or PI incorporation assay. The extent of apoptosis was quantified as the percentage of annexin V^+ cells or DNA content in GFP^+ Tcons respectively.

2.10. MiRNA mimics and inhibitors transfection

MiR-146a, miR-106b or miR-21 in GFP^+ Tcons was overexpressed or knocked down by transfection with miRNA mimic or inhibitor using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Mimics, inhibitors and negative controls for mimics or inhibitors were purchased from GenePharma (GenePharma Co., Ltd., Shanghai, China). Cells were collected 24 h post-transfection for detection of miRNAs and IL-2 or TGF-βRII expression by qRT-PCR, or for detection of Tcons proliferation by ^3H incorporation.

2.11. RNA extraction and qRT-PCR analysis

Total RNA was extracted with miRNease Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcriptions were carried out using the SuperScript III First-Strand cDNA Synthesis System (Invitrogen). qRT-PCR reactions were performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the FastStart SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) and the gene-specific primers (miR-146a, miR-106b, miR-21, miR-181a-5p and small nuclear RNA U6) purchased from Ribobio (Guangzhou, China) or