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Research paper

Cyclosporin A indirectly attenuates activation of group 2 innate lymphoid cells in papain-induced lung inflammation

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ABSTRACT

Cyclosporin A (CsA) is a well-known immunosuppressant that is used against steroid-resistant asthma. Group 2 innate lymphoid cells (ILC2s) and type 2 helper T (Th2) cells produce Th2 cytokines including IL-5 and play important roles in asthma pathogenesis. Here, we studied the effects of CsA in allergen-induced lung inflammation in mice and found that CsA decreased the number of lung ILC2s and attenuated papain-induced activation of ILC2s accompanied with IL-5 expression. The ILC2 suppression mediated by CsA was not observed in culture or in lymphocyte-deficient *Rag2*$^{-/-}$ mice. Thus, we propose a new suppressive effect of CsA, i.e., administration of CsA indirectly suppresses maintenance and activation of lung ILC2s in addition to direct suppression of T-cell activation and cytokine production.

1. Introduction

Cyclosporin A (CsA) is a potent immunosuppressive drug that is used to prevent allograft rejection and to treat autoimmune diseases such as rheumatoid arthritis and psoriasis [1–3]. CsA primarily inhibits the activity of calcineurin and the calcineurin-dependent transcription factors, nuclear factor of activated T cells (NFATs) [4]. Through that inhibition, CsA blocks T cells' production of NFAT-dependent cytokines such as interleukin-2 (IL-2) and it suppresses the activation and functions of effector T cells including helper T (Th) cells [4,5].

Allergic asthma is characterized by airway hyperreactivity (AHR), pulmonary eosinophilia, and excessive mucus production. This response is induced by cytokines such as IL-4, IL-5, IL-9 and IL-13 produced by type 2 Th (Th2) cells and also by group 2 innate lymphoid cells (ILC2s) [6–8]. Administration of CsA to patients with severe steroid-resistant asthma has provided some improvement in lung function [9–11]. In addition to the suppression of T cells, recent studies have confirmed that calcineurin/NFAT inhibitors have a much broader effect and have shown that CsA targets innate immunity and the regulation of homeostasis of innate cells [5]. For example, CsA inhibits degranulation of eosinophils as well as IL-4 production by mast cells [12,13].

ILC2s produce a large amount of Th2 cytokines when stimulated with IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which is derived from damaged epithelial cells and other cells [14]. In combination with those epithelial cell-derived cytokines, IL-2 derived from activated T cells is also important for full activation and expansion of ILC2s [15,16]. Recent progress has revealed critical roles of ILC2s for progression of asthma pathogenesis [6,17]; however, the effects of CsA on ILC2 function have not been addressed in detail and are not fully understood.

In addition to cytokines described above, the activation and maintenance of ILC2s are influenced by cell-to-cell signaling through surface receptors, such as inducible T cell costimulator (ICOS) [6]. ILC2s express both ICOS and ICOS-ligand (ICOSL), and the ICOS-ICOSL interaction augments survival and function of ILC2s [18,19]. Interaction of ICOSL on ILC2s with ICOS on Treg cells suppresses ILC2s together with IL-10 and TGF-β produced by Treg cells [20]. In contrast, ILC2s promote Treg cell accumulation via ICOS-ICOSL interaction in adipose tissues [21].

In this study, we employed mice in which IL-5 expression on live cells was monitored by Venus expression [22], and we found that activation and maintenance of ILC2s were suppressed by CsA in papain-induced lung inflammation. The suppression was not a direct effect of CsA on ILC2s, and it was not observed in lymphocyte-deficient *Rag2*$^{-/-}$ mice. In spite of the decrease of Treg cells in the lungs of CsA-treated mice, IL-10 levels in BAL (bronchoalveolar lavage) fluids of CsA-treated mice were increased, suggesting that IL-10-producing cell other

Abbreviations: BAL, bronchoalveolar lavage; CsA, cyclosporin A; ICOS, inducible T cell costimulator; ICOSL, inducible T cell costimulator ligand; ILC2s, group 2 innate lymphoid cells; NFAT, nuclear factor of activated T-cells; Th2, type 2 helper T; TSLP, thymic stromal lymphopoietin

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than Treg cells might be involved in the machinery for ILC2 suppression. Our results may add a previously unrecognized mechanism by which CsA attenuates asthmatic inflammation. Thus, we suggest that CsA suppresses activation and cytokine expression not only from T cells but also simultaneously from ILC2s.

2. Materials and methods

2.1. Mice and reagents

Il5-Venus knock-in (*Il5*$^{+/Venus}$) mice [22] were crossed with *Rag2*$^{-/-}$ mice to generate *Il5*$^{+/Venus}$ *Rag2*$^{-/-}$ mice on the C57BL/6 background. They were maintained under specific pathogen-free conditions, and mice at 9–17 weeks of age were used for all experiments. Mice were handled in accordance with the Guidelines for Animal Experiments of the Research Institute, National Center for Global Health and Medicine. Cyclosporin A (CsA) and papain were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. In vivo stimulation and lung cell preparation

Mice were injected subcutaneously with CsA dissolved in olive oil or vehicle alone at a dose of 20 mg/kg, 6 times a week for 2 weeks and on days 14, 15, and 16. Mice were anesthetized intraperitoneally with an anesthetic mixture (medetomidine 0.3 mg/kg, midazolam 4 mg/kg, and butorphanol 5 mg/kg), followed by intranasal injection of papain (50 μg) in 20 μL PBS on days 14, 15, and 16, and were analyzed on day 17. Lungs were minced and digested in HBSS containing type I collagenase (1 mg/mL; Sigma-Aldrich, St. Louis, MO) and DNase I (200 U/mL; Roche Applied Science, Mannheim, Germany) for 1 h at 37 °C. Tissue samples were next mashed through a 70 μm cell strainer and washed with HBSS supplemented with 10% FBS. Red blood cells were removed by incubation for 2 min at room temperature (RT) in ammonium chloride (ACK) lysis buffer.

2.3. BAL fluid

Lungs were lavaged twice with 0.5 mL PBS and the BAL fluid was centrifuged to pellet cells and supernatants were collected. Total protein was measured by Bradford's method with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

2.4. Cytokine measurements

The levels of IL-10 in BAL fluids were determined with a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories) in a Bio-Plex 3D System (Bio-Rad Laboratories) according to the manufacturer's instructions. Briefly, undiluted culture supernatant and standards were added to 96-well microplates containing washed beads and incubated at room temperature (RT) for 1 h with shaking. After washing, biotin-labeled antibody was added to the wells. After 30 min of incubation at RT with shaking, streptavidin-phycoerythrin was added followed by 10 min of incubation with shaking. Data were collected with xPONENT for FLEXMAP 3D software, version 4.2 (Luminex Corporation, Austin, TX, USA) and the data were analyzed with Bio-Plex Manager 6.1 software (Luminex Corporation). The levels of IL-33 in BAL fluids were measured using mouse IL-33 ELISA Ready-SET-Go!® (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.5. Antibodies and flow cytometry

Single cell suspensions were preincubated with anti–CD16/32 (2.4G2, BD Biosciences) to block Fc receptors. Cells were stained with combinations of the following monoclonal antibodies conjugated with phycoerythrin: anti-CD25 (PC61; BioLegend, San Diego, CA), anti-CD127 (SB/199; eBioscience, San Diego, CA), anti-CD275 (ICOSL) (HK5.3; BioLegend), anti-ST2 (DIH9; BioLegend), anti-Siglec-F (E50-2440; BD Biosciences, San Jose, CA), anti-TCRβ (H57-597; BioLegend), and anti-Gr-1 (RB6-8C5; eBioscience). The following allophycocyanin-conjugated antibodies were used: anti-CD25 (PC61; BioLegend), anti-CD127 (A7R34; BioLegend) and anti-CD278 (ICOS) (C398.4A; BioLegend). Phycoerythrin-Cy7-conjugated antibodies included anti-Thy1.2 (30-H12; BioLegend) and anti-CD11c (HL3; BD Biosciences). We also used allophycocyanin-Cy7-conjugated anti-CD11b (M1/70; BioLegend) as well as biotin-conjugated anti-CD3e (145-2C11; BioLegend), anti-CD4 (GK1.5; BioLegend), anti-CD8 (53-6.7; BioLegend), anti-CD11b (M1/70; BioLegend), anti-CD11c (N418; BioLegend), anti-CD19 (6D5; BioLegend), anti-B220 (RA3-6B2; BioLegend), anti-NK1.1 (PK136; BioLegend), anti-TCRγδ (GL3; BD Biosciences) and anti-TER-119 (TER-119; Tonbo Biosciences, San Diego, CA) followed by allophycocyanin-Cy7-conjugated streptavidin. Dead cells were excluded by staining with 2 μg/mL 7-aminoactinomycin D (7AAD) (Sigma-Aldrich, St. Louis, MO). Identification of ILC2s was based on the expression of Thy1.2 and CD25, and the absence of lineage markers (Lin$^-$) defined below. Eosinophils were identified as CD45$^+$, Siglec-F$^+$, and CD11c$^-$ cells. ILC2 sorting was performed with a FACSAria III (BD Biosciences), and phenotypic analysis was performed using a BD FACSCanto II (BD Biosciences). Data were analyzed using FlowJo (Tree Star, Inc. San Carlos, CA).

2.6. ILC2 culture

ILC2s were sorted from lung cell suspensions and cultured for 19 h in the presence or absence of CsA (0.2 μg/mL) in complete RPMI media supplemented with 10% FBS and 50 μM β-mercaptoethanol at a density of 1.6×10^3 in 100 μL medium. Lung ILC2s were stimulated with IL-33 (10 ng/mL; R & D Systems, Minneapolis, MN) and IL-7 (10 ng/mL; PeproTech, Rocky Hill, NJ). After incubation, cells were stained with allophycocyanin-conjugated anti-CD25. Cells were washed, resuspended in 7AAD and analyzed using a BD FACSCanto II.

2.7. Quantitative RT-PCR analysis

Total lung cells were isolated and total RNA was prepared using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using TaqMan Fast Universal PCR Master Mix and primer/probe sets for the genes of interest in a StepOne Real-Time PCR System (Applied Biosystems). The primers were as follows: *Il33* (Mm00505403_m1) and *Gapdh* (Mm99999915_g1) (all Applied Biosystems). Target gene quantification was normalized to *Gapdh* expression.

2.8. Lung histology

Lungs obtained from mice were fixed by 20% (vol/vol) formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin for histological analysis [23].

2.9. T cell adoptive transfer

Splenocytes from *Il5*$^{+/Venus}$ mice were treated with biotin-labeled anti-B220 and anti-CD11b antibodies. Then, B220$^+$ and CD11b$^+$ cells were depleted to enrich T cells using anti-biotin conjugated beads and the MACS system (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's recommendations. Splenic T cells (5×10^6 cells/mouse) were adoptively transferred to *Il5*$^{Venus/Venus}$ *Rag2*$^{-/-}$ mice.