CSA INHIBITS IL-22 PRODUCTION BY MEMORY CD4+ T CELLS FROM PATIENTS WITH PSORIASIS

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Abstract - IL-22 is involved in psoriasis and exacerbates disease progression. Cyclosporine A (CsA) is an immunosuppressant drug that has been used in the treatment of psoriasis for more than 20 years. We determined IL-22 producing T cells and their phenotype, and demonstrated that IL-22 is mainly produced by CD4+ memory T cells not CD8+ T cells in peripheral blood from healthy adults. We compared Th17 and Th1 with the percentages of IL-22 producing CD4+ T cells, and demonstrated that Th1 is the majority Th subset in the blood, as the percentage of Th1 cells is significantly larger than the percentage of IL-22 producing CD4+ T cells or Th17 cells. We analyzed the correlation of IL-22, IL-17 and IFN-γ produced by CD4+ T cells from healthy adults, and confirmed that there is a subset of Th22 cells that does not produce IL-17 or IFN-γ. Furthermore, we observed that the percentage of IL-22 producing CD4+ T cells is elevated in psoriasis compared to healthy donors, and that the majority of these cells are memory CD4+ T cells. We also investigated the inhibitory effects of CsA on IL-22 production by CD4+ T cells from both healthy donors and patients with psoriasis. We observed that CsA inhibits the production of IL-22 by CD4+ T cells from healthy donors in a dose-dependent manner, and that it inhibits IL-22, IFN-γ and IL-17 production by CD4+ T cells in psoriasis. The obtained results provide critical information regarding the clinical efficacies of CsA in the treatment of psoriasis.

Key words: CD4+ memory T cells, Cyclosporine A, IL-22, psoriasis, Th22 cells

INTRODUCTION

Psoriasis is one of the most common chronic inflammatory skin diseases. It affects about 2% of the world's population, and 85%-90% of all patients with this disease present psoriasis vulgaris (Nestle et al., 2009; Lowes et al., 2007). This disease is usually characterized by a thickened epidermis, hyperproliferation and abnormal differentiation of keratinocytes, vascular hyperplasia and infiltration of inflammatory immune cells. T cells play a key role in the pathogenesis of psoriasis.
According to their functions, the secreted cytokines and transcriptional factors, CD4+ T helper cells are divided into several subsets, such as Th1, Th2 and Th17, and so on. Th1 cells produce cytokine IFN-γ, Th2 produce IL-4, and Th17 cells secrete IL-17 (Chen and O'Shea, 2008; Sallusto et al., 2012). It is thought that Th1 and Th17 cells play a predominant role in the pathogenesis of psoriasis (Austin et al., 1999; Chiricozzi et al., 2012). Recently, a new Th subset, named Th22, which produces IL-22 but does not express IL-17 or IFN-γ, was identified (Duhen et al., 2009; Trifari et al., 2009). This subset is involved in epidermal immunity and remodeling (Eyerich et al., 2009; Fujita et al., 2009). It has been reported that Th22 cells are increased in blood in psoriasis, as are IL-22 plasma levels and that this correlates with disease severity. IL-22, as one of the key, mediators, is involved in psoriasis and exacerbates disease progression (Kagami et al., 2010; Ouyang, 2010).

Cyclosporine A (CsA) is an immunosuppressant drug used in the treatment of psoriasis, transplantation, rheumatoid arthritis and other immune-mediated diseases. CsA has been used to treat psoriasis in clinics for more than 20 years; it effectively controls psoriasis, prolonging the relapse time in psoriasis patients (Zachariae and Steen Olsen, 1995; Maza et al., 2011; Colombo et al., 2010). It has been demonstrated that CsA inhibits IL-17 and IFN-γ production by CD4+ T cells from healthy donors, in Behcet’s and other diseases (Zhang et al., 2008; Chi et al., 2010; Liu et al., 2009). IL-22 and IL-22 producing CD4+ T cells are elevated in psoriasis and involved in the pathogenesis of psoriasis. In this article, we examined whether CsA inhibits IL-22 production by CD4+ T cells from healthy donors and patients with psoriasis.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of School of Basic Medicine, Guangzhou Medical University (Guangzhou, China) and Guangzhou Institute of Dermatology (Guangzhou, China). Written informed consent was obtained from all patients and healthy donors participating in this study.

Subjects

Healthy volunteers and psoriasis patients that donated blood samples for the study were recruited from Guangzhou Medical University and Guangzhou Institute of Dermatology, China. Twenty-six healthy donors and five psoriasis patients were recruited. The mean age of the healthy volunteers (14 male and 12 female) was 26.7 years (ranging from 19-47 years). The mean age of adult psoriasis patients (1 male and 4 female) was 39.8 years (ranging from 21-64 years). Exclusion criteria were local treatment with corticosteroids, phototherapy, or systemic corticosteroid therapy within 4 weeks of sample collection, patients with HIV, HBV, HCV or other autoimmune diseases.

Reagents

Purified anti-CD3 mAb, purified anti-CD28 mAb, anti-CD4 PerCP-cy5.5, anti-CD3 FITC, anti-CD45RO FITC, anti-IFN-γ FITC, anti-IFN-γ APC and isotype- matched control mAbs were purchased from BD PharMingen (San Diego, CA, USA). Anti-IL-17 APC was purchased from eBioscience (San Diego, CA, USA). Anti-IL-22 PE was purchased from R&D Systems (Abingdon, UK). PMA, ionomycin, saponin and Brefeldin A were purchased from Sigma-Aldrich (Fluka, Sigma, USA). Cyclosporine A (CsA) was obtained from Novartis (Novartis, Switzerland).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy donors and patients with psoriasis, using Ficoll-Hypaque density gradient centrifugation; the cells were washed twice in Hank’s balanced salt solution. The cells were adjusted to a final concentration of 2×10^6/ml in complete RPMI 1640 medium, supplemented with 10% FCS (Sijiqing, China), 100 U/mL penicillin, 100 mg/mL streptomycin, 50 mM 2-mercaptoethanol, and 2 mM L-glutamine (all from GIBCO, Grand Island, NY, USA).
Intracellular staining and flow cytometric analysis

PBMCs were stimulated with PMA (20 ng/ml) + ionomycin (1 µg/ml) in the presence or absence of CsA at different concentrations (0.01 µg/ml, 0.1µg/ml, 1µg/ml) for 4-6 h at 37°C in a 5% CO2 humidified atmosphere. Brefeldin A (BFA, 10 µg/ml) was added to the culture at the end of first hour of incubation. The cells were collected, washed twice in cold PBS, fixed with 4% paraformaldehyde and resuspended in permeabilization buffer (PBS containing 0.1% saponin and 0.5% BSA). After incubation at 4°C for 2 h or overnight, the cells were stained for the presence of intracellular cytokines (anti-CD4 PerCP-cy5.5, anti-CD3 FITC, anti-CD45RO FITC, anti-IL-17 APC, anti-IL-22 PE, anti-IFN-γ FITC, and isotype-matched control mAbs) at 4°C for 25-30 min. The cells were washed with PBS, resuspended in cold staining buffer and analyzed by FACSCalibur (BD Biosciences, San Jose, CA).

Statistics

The data are presented as the mean ± SD. Comparisons between two groups were performed by unpaired Student’s t-test; P < 0.05 was considered statistically significant.

RESULTS

IL-22 producing memory CD4+ T cells from health donors

First, we determined IL-22 producing CD4+ and CD8+ T cells of PBMCs from healthy donors stimulated with PMA plus ionomycin using FACS. Two subsets of CD3+ and CD4+ T cells: CD4+ T cells and CD3+ CD4+ T cells (CD8+ T cells) were gated, and analyzed for IL-22 or IL-17 production. As shown in Fig. 1A, the percentage of IL-22 producing CD4+ T cells and CD8+ T cells was 1.96% and 0.08%, respectively, of IL-17 producing CD4+ T cells (Th17 and Th17/Th1) and CD8+ T cells (Tc17) was 1.48% and 0.24%, respectively. We then compared the percentages of the IL-22 and IL-17 producing CD4+ T cells and CD8+ T cells. As shown in Fig.1B, IL-22 was mainly produced by CD4+ T cells (1.66±0.75%); very IL-22 was produced by a subset of CD8+ T cells (0.12±0.07%) (P<0.001). Similarly, IL-17 was produced mainly by CD4+ T cells (1.61±0.7%), and by a tiny subpopulation of CD8+ T cells (0.16±0.1%) (P<0.001).

We then determined the phenotypes of the IL-22 producing and IL-17 producing CD4+ T cells. As shown in Fig 2, the majority of IL-22 producing (1.75% of CD4+ T cells) and IL-17 producing cells (1.3% of CD4+ T cells) expressed CD45RO on their surface. Only small numbers of IL-22 producing (0.03% of CD4+ T cells) and IL-17 producing cells (0.03% of CD4+ T cells) did not express CD45RO. This means that IL-22 producing CD4+ T cells and Th17 cells are memory T cells.

The correlation of Th22, Th17 and Th1 from healthy donors

Th17 and Th1 cells can also produce the cytokine IL-22. We determined that cytokines IL-22, IL-17 and IFN-γ were produced by CD4+ T cells from healthy donors. Analysis of their relationship showed that there are these three cell subsets in PBMCs: IL-22 producing CD4+ T cells, Th17 cells (IL-17+ and IFN-γ-) and Th1 cells (IFN-γ+ IL-17-); there is also a small population of CD4+ T cells that produced both IL-17 and IFN-γ, referred to as Th1/Th17 cells. The percentages of IL-22 producing CD4+ T cells, Th17 cells and Th1 cells in CD4+ T cells are shown in Fig. 3B. The percentage of Th1 in CD4+ T cells is greater than the percentages of the IL-22 producing CD4+ T cells and Th17 cells in PBMCs from health donors (P<0.001). There is no significant difference between the percentages of IL-22 producing CD4+ T cells and Th17 cells (P>0.05).

We then analyzed the relationship between IL-22, IL-17 and IFN-γ. As shown in Fig. 4, some CD4+ T cells produced both IL-22 and IL-17 (0.4%) or both IL-22 and IFN-γ (1.06%); the mean values were 0.38% and 0.73%, respectively. According to IL-17 and/or IFN-γ production, the IL-22 producing CD4+ T cells were divided into four populations: IL-17+ IFN-γ; IL-17+ IFN-γ; IL-17 IFN-γ; IL-17 IFN-γ.
cells (this subset belongs to Th22 cells), with the mean percentages of the presence of these cell populations, 15.75%, 8.61%, 33.60% and 42.04%, respectively. From these results, we concluded that there are some CD4+ T cells that produce two or three cytokines IL-22, IL-17 and IFN-γ at the same time.

CsA inhibits IL-22 production by CD4+ T cells from healthy donors

Previous research has shown that CsA inhibits the immune response via inhibition of IL-17 and IFN-γ production by CD4+ T cells. We wanted to examine whether CsA inhibits IL-22 production by CD4+ T cells. PBMCs were stimulated with PMA and ionomycin in the presence or absence of different concentration of CsA, and the production of IL-22 and IFN-γ by CD4+ T cells was determined. From Fig.5 we can see that CsA inhibited IL-22 and IFN-γ production by CD4+ T cells from healthy donors in a dose-dependent manner. When the concentration of CsA was 0.01 µg/ml, the inhibitory ratio of IL-22 production was 48.2±6.1%; at 0.1 and 1 µg /ml of CsA, the inhibitory ratio was 81.1±11.5% and 87.7±8.9%, respectively. Since there was no significant difference in the inhibitory ratios of IL-22 between 0.1 and 1 µg/
Fig. 2. IL-22 produced by memory CD4⁺ T cells. PBMCs were stimulated with PMA + ionomycin for 4-6h. Cells were harvested and fixed, permeabilized, and subjected to cell surface staining for CD4 and CD45RO, and intracellular cytokine staining for IL-22, and examined by FACS. Isotype refers to control isotype staining. The numbers in the corner represent the percentage of cells in each quadrant. Data represent the result from one of eight similar results.

Fig. 3. Comparison of the percentages of IL-22⁺ CD4⁺ T cells, Th17 and Th1 cells in CD4⁺ T cells of healthy donors. A – IL-22, IL-17 or IFN-γ production by CD4⁺ T cells of PBMCs from one donor (dot plots). B – data of IL-22⁺ CD4⁺ T cells, Th17, or Th1, as described in A, are shown. Each symbol represents one value from a single donor and horizontal bars represent the mean value of all samples; **, P < 0.001; n.s, P > 0.05.
ml of CsA, we used 0.1 µg/ml of CsA in the following experiments. Similar results were also obtained for the inhibitory effect of CsA on IFN-γ production.

CsA inhibits IL-22 production by memory CD4+ T cells in psoriasis

The percentage of IL-22+ CD4+ T cells in CD4+ T cells from 5 different individuals with psoriasis were determined. The percentage of IL-22+ CD4+ T cells ranged from 1.09-5.31%. The majority of IL-22+ CD4+ T cells in psoriasis are CD45RO+ memory T cells (Fig. 6A). Compared with the percentages of IL-22+ CD4+ T cells from healthy donors, the IL-22+ CD4+ T cells were elevated in psoriasis (Fig. 6B). Next, we determined whether CsA could inhibit IL-22 production by CD4+ T cells in psoriasis. As shown in Fig. 6, CsA inhibited IL-22 production by CD4+ T cells in psoriasis, and also inhibited IL-17 and IFN-γ production by CD4+ T cells.

**DISCUSSION**

IL-22 is a member of the IL-10 family of cytokines that includes IL-19, IL-20, IL-22, IL-24 and IL-26. It signals through the IL-22 receptor (IL-22R1 and IL-10R1) whose expression is restricted to tissue epithelia such as skin and intestine but not immune cells (Ouyang, 2010; Rutz et al., 2013). IL-22 is mainly produced by CD4+ T cells, lymphoid tissue inducer cells (LTi cells), and by NKT and γδT cells involved in regulation of immunity, inflammation and tissue homeostasis at barrier surfaces (Zenevicz and Flavell, 2011; Sonnenberg et al., 2011).

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**Fig. 4.** Correlation of IL-22, IL-17 and IFN-γ produced by CD4+ T cells of healthy donors. A – the relationships between IL-22 and IL-17, IL-22 and IFN-γ and IL-17 and IFN-γ produced by CD4+ T cells in PBMCs of from one representative healthy donor. B – the mean value of A are shown from nine separate experiments.
CsA inhibits the production of IL-22 by CD4+ T cells from healthy donors. PBMCs were stimulated with PMA + ionomycin in the presence or absence of CsA at different concentrations (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml). After stimulation for 5 h, the cells were harvested, fixed, permeabilized, and subjected to cell surface and intracellular cytokine staining for IL-22 and IFN-γ, and assessed by FACS. A – the production of IL-22, IFN-γ by CD4+ T cells. Isotype refers to control isotype staining. The numbers in the corner represent the percentage of positive cells in each quadrant. B – the statistical inhibition ratio of IL-22+ CD4+ T cells and IFN-γ+ CD4+ T cells by CsA were calculated and are presented as mean ± S.D. from seven healthy donors in the bar graph; **, P < 0.001; n.s, P>0.05.

In psoriasis IL-22, as one of the key mediators, induces many of the pathogenic phenotypes and exacerbates disease progression. It has been reported that IL-22 plasma levels are strongly elevated in psoriasis, and that they correlate with disease severity (Murakami et al., 2011). Moreover, enhanced expression of IL-22 mRNA and protein has been found in skin lesions from psoriasis patients, and was involved in imiquimod-induced psoriasiform skin inflammation in mice (Van Belle et al., 2012). The major target cells for IL-22 in the skin are keratinocytes that express its receptor IL-22R. Elevated IL-22 acts on keratinocytes in psoriasis skin, inducing many histological features of psoriasis, such as acanthosis, hypogranulosis, and parakeratosis (Wolk et al., 2009; Wolk et al., 2006; Zheng et al., 2007; Wolk et al., 2009). IL-22 may serve as a therapeutic target for the treatment of psoriasis.

Recently, it was reported that T cells might be the major source of IL-22 in psoriasis. In the present study, we observed that IL-22 is mainly produced by CD4+ and not by CD8+ T cells in the peripheral blood from healthy adults, and that these CD4+ T cells are memory cells expressing CD45RO. This result is similar with to a previous study (Liu et al., 2011). CD8+ T cells could also produce IL-22 in some diseases as occurs when CD8+ T cells infiltrate damaged skin in psoriasis (Nograles et al., 2009).

It was originally assumed that IL-22 is a Th1-associated cytokine; later, IL-22 was also linked with Th17 cells. Therefore, we compared the percentages of these three subsets, and analyzed the correlation of IL-22, IL-17 and IFN-γ that were produced by CD4+ T cells from healthy adults. Our results show that the...
percentage of Th1 cells in the CD4+ T cell population was larger than the percentages of IL-22 producing CD4+ T cells and Th17 cells in PBMCs. IL-22+ IFN-γ+ and IL-22+ IL-17+ T cells exist in PBMCs from health donors. CD4+ T cells that produce IL-22 and do not produce IL-17 and/or IFN-γ are called Th22 cells. Similar to previous studies, we demonstrated that there is a subset of Th22 cells in adult blood (Duhen et al., 2009; Trifari et al., 2009).

Because IL-22 plays a key role in psoriasis progression, we determined whether CsA inhibits IL-22 production by CD4+ T cells in healthy donors and patients with psoriasis. Our results showed that CsA inhibited IL-22 production by CD4+ T cells from healthy donors in a dose-dependent manner. Circulating Th1, Th17 and IL-22 producing CD4+ T cells are increased in psoriasis (Kagami et al., 2010). Therefore, we determined whether CsA assumes an inhibitory role in psoriasis patients. Our results demonstrated that CsA inhibited IL-22, IFN-γ and IL-17 production by CD4+ T cells in psoriasis patients. A previous study showed that CsA directly inhibits IL-22 production largely through action on NF-AT in T cells (Rudloff et al., 2012), and that CsA could inhibit IFN-γ, IL-4 and IL-17 by memory T cells (Tsuda et al., 2012).

In conclusion, our study showed that CsA inhibits IL-22 production by CD4+ T cells from healthy donors and psoriasis patients. Together with previous studies, our results suggest that CsA could have an important therapeutic role in psoriasis by virtue of its ability to inhibit IL-22, IFN-γ, and IL-17 release by CD4+ T cells.

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