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Phenotypic and functional profile of Th17 and Treg cells in allergic fungal sinusitis

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ABSTRACT

Interleukin-17 producing T helper (Th17) and regulatory T cells (Treg) cells have been identified to play a critical role in atopic inflammation. However, conflicting reports on the role of Th17/Treg cells in allergic fungal rhinosinusitis (AFRS) patients of different ethnicities has mystified its pathogenesis. To better understand the pathophysiological mechanisms involved in AFRS, we conducted a prospective, analytical, case-control study involving 40 confirmed immunocompetent AFRS patients and 20 healthy controls. The distribution of Th17 and Treg cells in PBMC, intracellular mRNA expression of retinoid orphan nuclear receptor (RORγt) in Th17 and forkhead transcription factor (FoxP3) in Treg cells, and serum cytokine levels were investigated. *Aspergillus flavus* was identified from majority (85%) of patient tissue biopsies. Total serum IgE level along with cytokines IL-17, IL-21, IL-1β and TGF-β were comparatively elevated in AFRS. Nevertheless, IL-2 and IL-10 were reduced. Higher percentages of $CD3^+CD4^+$ T cells in AFRS with increased expression of CD161 and/or IL-23R markers were observed. Though, lower percentages of $CD4^+CD25^+$ Treg cells with elevated expression of GITR were patent. Transcription factor RORγt mRNA was upregulated, whereas FoxP3 mRNA was downregulated in AFRS patients. This inclination of Th17/Treg balance towards Th17, and the proposed role of Tregs on Th1 and Th2 cells in AFRS, directed us to conclude that *Aspergillus* infestation may lead to development of atopy and immunological dysbalance inciting a Th17 driven response, thereby, promoting aggravation of nasal polyposis. The observation may provide new insight into the molecular mechanisms leading to revision of the classical paradigm.

1. Introduction

Rhinosinusitis alludes to the irritation and inflammation of nasal and paranasal sinus mucosa. Factors responsible for development of exacerbation of this allergic condition are: microbial infections, colonization, atopy, hypersensitive reaction or allergen specific immunological imbalance [1]. Fungal rhinosinusitis (FRS) is characterized as the rhinosinusitis where fungi are in charge of causing the immunopathogenesis. Because of a few speculations encompassing FRS, the comprehension of the infection is as yet advancing, however it is being perceived as a developing sickness entity.

Allergic fungal rhinosinusitis (AFRS) is a subset of chronic polypoid rhinosinusitis in which fungal antigen is responsible for its pathogenesis. It is characterized by the growth of fungi, mostly *Aspergillus* sp., in the paranasal sinuses together with the formation of nasal polyps, allergic mucin with fungal hyphae, typical computed tomography findings and increased serum total immunoglobulin E (IgE) level [2,3]. Its chronicity, inflammation of nasal and paranasal mucosa with persistence of symptoms and subsequent polypoid mucosal changes, leads patients to experiencing the consequences of recurrences and repeated failure to treatment. What enables the pathogen to sustain infection over long periods of time and modulate the immune response is still

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unclear. The pathogenic mechanism has been found to vary in Eastern and Western populations; this has been attributed to different living environments and genetic make-up. However, similar studies on Indian patients are scanty.

Studies suggest that the raised levels of IgE may contribute to a preferential activation of T- helper (Th)2 subsets of T-cells which further mediate differentiation of Th17 thereby challenging the traditional Th1/Th2 equilibrium and is considered to be the crucial mediator of the disease. Recently, it has been reported that allergic disorders can also be associated with chronic inflammation characterized by a non-Th1 and Th2-cell based mechanisms similar to the pathogenesis of inflammatory autoimmune diseases [4]. Two additional T cell subsets, i.e., interleukin (IL)-17 producing Th cells (Th17 cells) and regulatory T cells (Treg cells) involved in respiratory epithelial damage in atopic individuals have been identified to play a critical role in inflammation [4]. These cells may provide new insight into the molecular mechanisms that may lead to the revision of the classic Th1/Th2 paradigm in such settings. Moreover, conflicting reports on the role of Th17/Treg cells in patients of AFRS and healthy controls of different ethnicities has further mystified the pathogenesis [5–7].

Hence for a better understanding of the pathophysiological mechanisms involved in AFRS, the present study aimed to evaluate the distribution of Th17 and Treg cells in peripheral blood mononuclear cells (PBMCs) using flowcytometric analysis from patients with AFRS and healthy controls. The expression of retinoid orphan nuclear receptor (RORγt) and forkhead transcription factor (FoxP3) at mRNA levels, and cytokine levels were evaluated for understanding their role in pathogenesis.

2. Materials and methods

2.1. Patients

The study was a prospective, analytical, case-control study, done in a tertiary care hospital in Delhi, India from January 2015 to December 2016. Subjects enrolled for the study included 40 immunocompetent patients (21 males, 19 females) between 18 and 60 years of age, clinically diagnosed as AFRS, confirmed by subject experts through history, examination and histopathological and laboratory investigations as per Bent and Kuhn criteria [8]. Patients with AFRS had presented with symptoms of nasal obstructions predominantly with nasal discharge, irritation and sneezing. Clinical inspection included rhinoscopic examination followed by other investigations. Symptom scores were assessed for disease severity according to a visual analog scale (VAS). Preoperative CT scans of the nose and paranasal sinuses were graded according to the classification by Lund and Mackay [9]. Clinical data about patients included age, sex, and duration of disease. All the patients underwent functional endoscopic sinus surgery with clearance of paranasal sinuses, to the best extent possible. Patients were followed up and were put on systemic steroids for two weeks and intranasal steroids for a prolonged duration. Nasal washes with saline were also recommended in all cases. The patients were subsequently followed up for 6 months after surgery.

Twenty age matched healthy, adult volunteers without any history of allergy or any previous nasal or sinus surgery were enrolled as controls. The study was approved by the institutional ethics committee and informed consent was taken before collection of samples.

2.2. Sample collection and processing

Venous blood samples from all patients were obtained 1–2 days before surgery. Blood (2 ml) for serum separation and 4 ml in EDTA vials for PBMCs separation were collected from all the patients and healthy volunteers. Serum was separated and stored in aliquots at −80 °C for estimation of IgE and cytokine levels. Total serum IgE was estimated using ELISA kit from Calbiotech, USA following manufacturer's protocol. Postoperatively tissue biopsy samples, in normal saline and formalin, from nasal polyposis patients were obtained and transferred immediately to the laboratory for examinations. Tissue biopsies were subjected to direct KOH (10%) examination and culture on Sabouraud Dextrose Agar (SDA) with antibiotics (chloramphenicol- 0.5 g/L; gentamicin- 0.05 g/L; without cyclohexamide) and incubated at 25 °C. The rate of growth, surface texture and pigmentation were noted. Standard Tease Mount using Lacto Phenol Cotton Blue was prepared from the growth in culture for identification of *Aspergillus flavus* (*A. flavus*). The polyp tissue samples in 10% formalin were subjected to histopathological examination using Hematoxylin and Eosin/Gomori Methenamine Silver staining for demonstrating fungal hyphae, eosinophils, neutrophils, Charcot Layden Crystals, inflammatory cells and evidence of tissue invasion.

Table 1
Profile of allergic fungal rhinosinusitis (AFRS) patients and healthy controls.

Parameters	AFRS patients	Healthy controls
Subjects (n)	40	20
Age (years)	27.37 ± 9.58	27.4 ± 4.18
Sex (male:female)	21:19	11:09
Duration of disease (months)	9.89 ± 8.02	0
Symptom score	12.35 ± 1.76	0
CT score	8.9 ± 1.59	0

2.3. PBMCs separation and stimulation

PBMCs were isolated by standard Ficoll–Hypaque density centrifugation at 250*g* for 20 min. The cloudy layer at the interface was carefully aspirated and washed using phosphate- buffer saline (PBS). The viability of the cells was measured by a trypan blue dead cell exclusion assay. Cells were re-suspended in RPMI 1640 media (containing 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine and 10% heat-inactivated fetal calf serum) at a concentration of 1×10^6 cells/ml, and treated with phytohemagglutinin (PHA)-M at 10 μg/ml (Hi-Media Laboratories Pvt. Ltd., India) for 18 h at 37 °C in a 5% CO_2 atmosphere. PBMC were analysed for expression of various surface markers to quantify Th17 and Treg cell populations by flowcytometry.

2.4. Cell staining for flowcytometry

The expression of various cellular markers was analysed by immunostaining PBMCs with the antibodies against CD3, CD4, CD25, glucocorticoid-induced TNFR (GITR), CD161, IL-23R; (BD PharMingen). For Th17, cells were incubated with anti-human PerCP-conjugated CD3, FITC-conjugated CD4, PE-conjugated CD161 and APC-conjugated IL-23R antibodies; for Treg, cells were incubated with anti-human PerCP-conjugated CD3, FITC-conjugated CD4, PE-conjugated CD25 and APC-conjugated GITR antibodies.

After harvesting, PBMC were stained for 30 min at 4 °C in the dark with optimal dilutions of each antibody, washed, and analysed by flowcytometry (FACS, ARIA III). Fluorescence profiles were analysed using FACS diva 7.0 software (BD Biosciences). The results are expressed as a percentage of positive cells.

2.5. Real-time PCR analysis for RORγt and FoxP3 expression

The mRNA levels of the transcription factors - RORγt and FoxP3 were determined by means of real-time PCR. Total RNA was extracted from PBMCs using TRIzol (Invitrogen, USA) according to the manufacturer's instructions and was reverse-transcribed to cDNA with random hexamer primers and RNAase H-reverse transcriptase (Invitrogen, USA). Expression of mRNA was determined on LightCycler® 480 Instrument (Roche, Germany) using SYBR Green