

Misregulation of suppressors of cytokine signaling in eosinophilic esophagitis

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Abstract

Background Several findings suggest that eosinophilic esophagitis (EoE) is strongly associated with atopy and allergen-driven, Th2-type immune responses, indicating the association of EoE with immune dysregulation. The objective of this study is to ascertain the molecular mechanism involved in EoE disease development a Th2 condition.

Methods 25 patients with diagnosis of EoE and 17 non-EoE controls were recruited by the gastroenterology and allergy departments from three different hospitals. Transcription analysis of suppressors of cytokine signaling 1, 3, 5 (SOCS), interleukin-5 (IL), IL-13, eotaxin (CCL26),

eotaxin receptor (CCR3), and mitogen-activated protein kinase 1 (MAPK1) was performed in esophageal biopsies by real time PCR. Western blot of ERK esophageal protein and additional measures of IL-5 and VEGF levels in serum were performed.

Results The esophagus of EoE patients expresses and synthesizes high levels of SOCS1 and SOCS3 proteins ($P < 0.05$), and these expression correlated with levels of IL-5, IL-13, CCL26, CCR3, and MAPK1 genes. In addition, we demonstrate the implication of the ERK pathway ($P < 0.001$).

Conclusions SOCS proteins probably contribute to EoE pathogenesis by directly or indirectly inducing the Th2 profile, as well as by promoting the production of Th2 cytokines. All these findings further enhance our understanding of the mechanism of EoE, and accumulating evidence suggests that EoE pathogenesis is likely to be due to misregulation of immunological pathways.

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Introduction

Eosinophilic esophagitis (EoE) is a disease of increasing recognition and prevalence in both pediatric and adult populations [1–3]. EoE is characterized by the accumulation of eosinophils in the esophageal mucosa [4]. Recently, a consensus definition has been accepted and published [5] which states that EoE represents a chronic, immune/antigen-mediated esophageal disease characterized clinically by symptoms related to esophageal dysfunction and histologically by eosinophil-predominant inflammation. Histologically, diagnosis requires an eosinophil peak esophageal count that is greater than 15 eosinophils per high-power field

(hpf) in the upper, middle, and lower esophagus. Clinical symptoms including dysphagia, vomiting, and abdominal pain are common.

Several findings suggest that EoE is strongly associated with atopy and allergen-driven, Th2-type immune responses [6–8], although cases of patients with EoE but with no detectable sensitization to food or inhalant allergens have been described, thus indicating the association of EoE with immune dysregulation.

Eosinophilic esophagitis has a complex pathogenesis in which multiple etiologic factors interact. Environmental factors and genetic background certainly play a role in the onset, persistence, and pathogenesis of EoE. Like most Th2 diseases, EoE is a disorder with multiple factors that strongly contribute to disease expression. Inflammatory mediators and cytokines play an essential role in controlling immune systems; not only do they act as growth factors, but they also regulate the differentiation, maintenance, and activation of naïve effectors and the memory state of immune cells [9]. The Th1/Th2 balance determines the nature of the immune response [10]; however, the mechanism by which Th1 and Th2 cytokines cross-regulate the immune response remains unclear.

In both physiological and pathological conditions, cytokine function is strictly controlled to prevent inappropriately sustained activation of downstream responses. Cytokine signaling pathways are negatively regulated by the so-called suppressor of cytokine signaling (SOCS) family of proteins, in which there are eight members [11]. Several reports have indicated that SOCS proteins are necessary for regulation of normal immune responses [12]. SOCS proteins not only act as simple negative-feedback regulators, but are also involved in fine-tuning the immune response and in the crosstalk of complicated cytokine signal networks. Since cytokines are constantly present in the microenvironment of immune cells, signal regulation by SOCS-family proteins may be important for the proper progress, remission, and relapse of an immune response. Therefore, SOCS1, SOCS3, and SOCS5 participate in CD4⁺ Th-cell differentiation and in Th1/Th2-cell balance [13]. SOCS3 is predominantly expressed in Th2 cells and inhibits Th1 differentiation [14–16]. Conversely, SOCS5 is predominantly expressed in Th1 cells and inhibits Th2 differentiation [12, 17]. The fact that these SOCS proteins (SOCS1, 3 and 5) are involved in the regulation of Th1/Th2 balance suggests the SOCS implication in EoE pathogenesis, for these reasons, we studied SOCS expression in EoE and Th2-relating cytokines.

Thus, although several advances have been made in the understanding of EoE pathogenesis, the objective of this study is to ascertain the molecular mechanism involved in EoE disease development as a Th2 condition.

Methods

Subjects

Patients with a previous diagnosis of EoE as defined consensus guidelines [1] were proposed for inclusion to this prospective study with a cross-sectional design. Recruitment was performed in Madrid in the allergy departments of Fundación Jiménez Díaz, Hospital Niño Jesús, and Hospital La Paz from January, 2009 to May, 2011. All individuals were selected regardless of age, atopic status, or gender. EoE diagnosis was performed using established criteria based on clinical and histopathologic features [1]: clinical symptoms of esophageal dysfunction; >15 eosinophils/hpf; and GERD was excluded. Initially, forty-one subjects fulfilled the inclusion criteria and went through all study tests. Sixteen patients were excluded for the following reasons: 10 patients with prior EoE diagnosis were determined to have fewer than 5 eosinophils/hpf according to current esophageal biopsies; in esophageal samples from 3 patients, not enough RNA could be extracted for analysis; one patient reported having received active treatment with oral corticosteroids in the 2 weeks before the endoscopy was performed; two patient voluntarily abandoned the study. A final number of 25 EoE patients were included for analysis.

Most of the non-EoE controls were recruited by the Gastroenterology Department based on the following criteria: complaints of abdominal pain along with other clinical features compatible with gastro-esophageal reflux disease (GERD); no prior diagnosis of EoE; and esophageal biopsies showing an eosinophil count below 5 with no basal layer expansion. A final number of 17 control subjects were included for analysis.

All study procedures were approved by the Ethics Committee, and informed written consent was provided by all subjects recruited. The study was conducted according to the principles set forth in the Declaration of Helsinki.

Skin prick tests

Skin prick tests (SPT) were performed following the recommendations of the European Academy of Allergy and Clinical Immunology [18] using a large spectrum of common aeroallergens within our region. All subjects ($n = 42$) underwent SPT to aeroallergens (ALK-Abello, Madrid, Spain) with house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), molds (*Aspergillus* spp, *Alternaria alternata*), animal danders (cat and dog), tree pollens (*Cupressus sempervirens*, *Olea europaea*, *Platanus acerifolia*), grass pollens (*Phleum pratense*) and weed pollens (*Parietaria judaica*, *Artemisia vulgaris*, and *Salsola kali*). The following food allergens were also

tested: wheat, rice, maize, oat, egg white, cow's milk casein, peach, apple, melon, chicken, beef, shrimp, lentil, chickpea, pea, soy, squid, megrim, hake, tuna, swordfish, peanut, walnut, garlic, potato, and *Anisakis simplex*.

Skin prick tests were performed on the volar face of the forearm using a standard 1-mm-tip lancet (ALK-Abello, Madrid, Spain). Histamine hydrochloride (10 mg/ml) and saline were used as positive and negative controls, respectively. Readings were taken after 15 min, and reactions were expressed as the mean of the major and minor wheal diameters. A wheal diameter of 3 mm or more compared to saline control was considered positive [19].

Total IgE and serum specific IgE

Blood samples, which were collected on the same day the endoscopy was performed or over the following 7 days, were centrifuged at 4000 rpm for 15 min to obtain serum. Total and specific IgE to the same panel of aeroallergens and foods tested in SPT were determined in serum, using the ImmunoCAP immunoassay system and the ISAC platform (Phadia, Uppsala, Sweden).

PBMC and eosinophil purification

Peripheral blood mononuclear cells (PBMCs) and eosinophils obtained from patients' and controls' blood were purified using a two-step procedure. First, the polymorphonuclear and mononuclear cell fractions were obtained by density gradient centrifugation using Lymphoprep (Rafer, Spain), followed by lysis of the red cells with an ammonium chloride solution (155 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM EDTA). The second step involved removal of residual cells from the polymorphonuclear cell fraction. For eosinophil purification, CD2, CD3, CD14, CD16, CD19, CD20, CD36, CD56, CD123, and glycophorin A positive cells were discarded by a magnetic bead separation technique, as described in the manufacturer's procedure (EasySep[®]; StemCell Technologies, Vancouver, Canada). Eosinophils are defined as $\text{CCR3}^+\text{CD16}^-$ cells, so we stained the cells with fluorochrome-conjugated anti-CCR3 (CCR3-FITC) and anti-CD16 (CD16-PE) antibodies. The purity obtained was routinely >98 % as measured by flow cytometry. 1×10^6 eosinophils were lysed in Trizol reagent (Invitrogen Life Technologies, CA, USA). PBMCs were obtained from the mononuclear fraction and were washed twice with saline solution and stained with trypan blue to check for viability.

Endoscopies and esophageal biopsies

Before endoscopy, the patients were required to have a minimum wash-out period of 2 weeks during which they

abstained from inhaled and swallowed corticosteroids. All patients underwent endoscopies and a minimum of two biopsies of the upper, medium and from the lower esophageal mucosa. A sample from each site was stained with hematoxylin/eosin and evaluated in each participating center for eosinophil count and histologic analysis. A peak number of intraepithelial eosinophils >15 EoE per high-power field (0.158 mm^2) ($\times 400$) in the upper, middle, and lower esophagus was considered an EoE diagnosis criterion if accompanied by endoscopic findings and/or clinical manifestations suggestive of EoE. The second biopsy from the upper, medium and lower site was immersed in Trizol[®] reagent and kept frozen at -80°C for further analyses.

Reverse transcription PCR

Total RNA was isolated from peripheral blood eosinophils, lymphocytes, and esophageal biopsies by Trizol[®] reagent (Invitrogen, Carlsbad, CA, USA) and was treated with DNase I (Promega, Wisconsin, USA). Esophageal biopsies were homogenized previously. RNA was measured by spectrophotometry, and 1 μg of RNA was reverse-transcribed to cDNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK).

Gene expression on cells and esophageal biopsies

Real-time quantitative PCR of peripheral blood eosinophils, lymphocytes, and biopsies was conducted for several genes.

Quantitative real-time PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems, Warrington, UK). Taqman PCR was performed using a 20 μl final reaction volume containing 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA), 1 μl of 20X Assays-on-Demand Gene Expression Assay Mix, and 9 μl of cDNA diluted in RNase-free water. Each assay was performed in triplicate. The PCR conditions used in all reactions were: 2 min at 50°C and 10 min at 95°C , repeated over 40 two-step cycles (95°C for 15 s and 60°C for 60 s). Assays-on-Demand Gene Expression primers specific for SOCS1, SOCS3, SOCS5, IL-5, IL-13, CCL26, CCR3, MAPK1, and rRNA 18S (used as an endogen) were obtained from Applied Biosystems (<http://www.appliedbiosystems.com/>).

Messenger RNA was calculated for each sample using the cycle threshold (C_t) value. The number of amplification steps required to reach an arbitrary intensity C_t was computed. The relative gene expression was calculated as follows: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{\text{target gene}} - \Delta C_{18s}$. The fold-change for the treatment was defined as the relative expression compared with the corresponding control, and was calculated as follows: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{\text{patient}} - \Delta C_{\text{healthy}}$ [20].

Immunohistochemistry

Paraffin-embedded sections of proximal esophageal biopsies were studied by immunohistochemistry. Briefly, after antigen retrieval and blockade of endogenous peroxidase, proximal esophageal biopsies sections were incubated with primary antibodies overnight at 4 °C, rabbit anti-SOCS3, anti-SOCS1, anti-pERK antibody (Santa Cruz Biotech, CA, USA) or mouse anti-IL-13 (eBiosciences, CA, USA). Antibody binding was detected with a rabbit EnVision HRP System (Dako, Golstrup, Denmark) or an anti-mouse HRP (eBioscience), and the distribution of the peroxidase was visualized with diaminobenzidine. Sections were counterstained with Carazzi's hematoxylin, dehydrated, and mounted. The specificity was checked by omission of primary antibodies and use of non-immune sera. The esophageal section for each patient was evaluated in a blinded manner by 2 investigators who assessed the localization of SOCS protein.

Western blot analysis

Protein extracts from esophageal biopsies (10 µg total protein) were resolved on SDS-PAGE and analyzed by western blotting using a 1:500 dilution of phospho-ERK antibody (BioLegend, San Diego, CA, USA). The secondary antibody, HRP-conjugated goat anti-rabbit IgG (Millipore, Billerica, MA, USA), was diluted 1:10000. Membranes were also probed with anti-ERK (Calbiochem, La Jolla, CA, USA) as control of protein loading.

Chemiluminescent protein bands were detected by an ECL detection system (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. The protein concentration was estimated according to the Bradford method [21].

Cytokine measurement

The human cytokine array panel A was used, employing serum pools from EoE patients and control groups according to the manufacturer's protocol (R&D Systems). These are the selected captured antibodies spotted in duplicate on the nitrocellulose membrane: C5a, CD40 ligand, G-CSF, GM-CSF, CXCL1, CCL1, CD54, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, CXCL10, CXCL11, CCL2, MIF, CCL3, CCL4, Serpin E1, RANTES, CXCL12, and TNF-α.

Moreover, serum IL-5 concentrations from EoE and control subjects were determined by enzyme-linked immunosorbent assay (ELISA) purchased from Bender MedSystems, Austria, following the manufacturer's procedure. Fifty microliters of each serum sample was used,

and the assay was performed in duplicate. The optical density was read at 450 nm and wavelength correction was set to 620 nm using a microplate reader (TECAM).

The normalization of protein levels in serum was performed using total albumin measuring.

Statistics

The subject characteristics were described using descriptive statistics and expressed as geometric mean, standard deviation (SD), median, and range.

Data comparisons between the groups were performed using the Mann–Whitney test (two tailed) and Fisher's exact test (two sided). Normality was analyzed with the Kolmogorov–Smirnov test. Correlation coefficients and statistical significance were determined using Spearman rank correlation coefficient. Statistical significance was recognized at $P \leq 0.05$. Statistical analyses were performed using GraphPad InStat3 (GraphPad Software Inc., San Diego, CA, USA).

Results

Patients' baseline characteristics

Forty-two subjects were included for analysis, including 25 diagnosed with EoE and 17 controls. Median age at inclusion was 28.9 (range 22–35) and 38.6 (range 30–47) years in EoE and non-EoE patients, respectively, with no significant differences ($P > 0.05$). Five EoE patients and 1 control were 14 years of age or younger. There were no differences in gender distribution between the groups, although there was a higher number of males in both groups (88 % among EoE patients and 59 % in non-EoE subjects). All subjects were Caucasian.

When personal history of atopy (defined by one or more sensitizations) was compared in the study subjects, 20 out of 25 (80 %) EoE patients displayed at least one sensitization to an aeroallergen compared to 4/17 (23.5 %) in non-EoE subjects ($P < 0.001$). Twenty (80 %) EoE patients had allergic rhinoconjunctivitis compared to 5 (29.41 %) in controls ($P < 0.005$); and 11 (44 %) had IgE-mediated food allergy versus 3 (18 %) in controls ($P > 0.05$). A summary of epidemiologic and clinical features of all the patients studied is shown in Table 1.

Eosinophil peripheral blood count, SPT and sIgE

Differences in peripheral blood eosinophil count (cell/mm³) were observed between EoE and non-EoE subjects (502.2 ± 331 vs. 286.4 ± 188.4 , $P < 0.05$; Table 1). Total IgE (kU_A/L) in patients with EoE was higher than in those

Table 1 Epidemiologic, clinical, and endoscopic features of subjects studied

	Controls subjects (non EoE) (n = 17)	Eosinophilic esophagitis patients (EoE) (n = 25)	P value ^{1,2}
Age (mean, range)	38.69 (13–68)	28.9 (9–49)	NS
Male (%)	58.8 %	88 %	NS
Allergic rhinoconjunctivitis (%)	29.41 %	80 %	<0.005 ²
Food allergy (%)	18 %	44 %	NS
Total serum IgE, kU/L (mean ± SD)	271 ± 538	713 ± 1127	<0.005 ²
Positive specific IgE to food (%)	17.64 %	84 %	<0.001 ²
Positive specific IgE to pollen (%)	23.52 %	80 %	<0.001 ²
Peripheral blood eosinophilia (mean ± SD)	286.42 ± 188	502.2 ± 331	<0.05 ¹
Endoscopy description (n, %)			
Rings and strictures	1 (5.9)	15 (60)	<0.001 ²
Longitudinal furrows	0 (0)	7 (28)	<0.05 ²
Trachealization	1 (5.9)	8 (32)	NS
Lacerations	0 (0)	2 (8)	NS
Whitish exudates	1 (5.9)	5 (20)	<0.005 ²
Hiatal hernia	2 (11.8)	1 (4)	NS
Normal	11 (64.7)	3 (12)	<0.001 ²
Peak eosinophil count in biopsy (mean, range)	<5 eos/hpf	35.8 (24–100)	<0.001 ¹

NS not significant

¹ Two tailed P (Mann–Whitney test)² Two-sided P value (Fisher's exact test)**Table 2** Sensitization profile: skin prick tests and serum specific IgE

	Control subjects (non-EoE) (n = 17)	Eosinophilic esophagitis patients (EoE) (n = 25)	P value ¹
Pollen SPT (%)	4 (24 %)	20 (80 %)	<0.001
Animal danders SPT (%)	2 (12 %)	16 (64 %)	<0.005
Molds SPT (%)	0 (0 %)	7 (28 %)	<0.05
Dust mites SPT (%)	1 (6 %)	5 (20 %)	NS
sIgE to fish (%)	0 (0 %)	4 (16 %)	NS
sIgE to cereals (%)	0 (0 %)	16 (64 %)	<0.001
sIgE to potato (%)	1 (6 %)	13 (52 %)	<0.005
sIgE to legumes (%)	0 (0 %)	13 (52 %)	<0.001
sIgE to chicken (%)	0 (0 %)	6 (24 %)	NS
sIgE to egg yolk (%)	0 (0 %)	6 (24 %)	NS
sIgE to garlic (%)	1 (6 %)	13 (52 %)	<0.005

NS not significant

¹ Two-sided P value (Fisher's exact test)

without EoE (712 ± 1125 , and 271 ± 538.9 , $P < 0.005$; Table 1).

Most patients with EoE were sensitized to pollens (80 %, $P < 0.001$) and animal dander (64 %, $P < 0.005$) as well as food allergens. Table 2 describes in detail different sensitization profiles.

Endoscopy

Detailed macroscopic changes in endoscopy are described in Table 1. Rings, strictures ($P < 0.001$), whitish exudates ($P < 0.005$), and longitudinal furrows ($P < 0.05$) were the most important changes in EoE patients compared to

controls, whereas the finding of a normal esophagus was found more frequently in non-EoE patients ($P < 0.001$). Although the pediatric EoE population was too small for a consistent statistical analysis, it is noteworthy that trachealization was found in 80 % of the children compared to 20 % of the EoE adults; also notable are the whitish exudates found in 60 % of children and 10 % of adults and the finding of longitudinal furrows in 60 % of children vs. 20 % of adults. None of the pediatric patients showed rings or strictures.

Biopsies of the upper, middle, and lower esophagus showed that all EoE patients had an eosinophil count that was greater than 15/hpf, while none of the controls showed

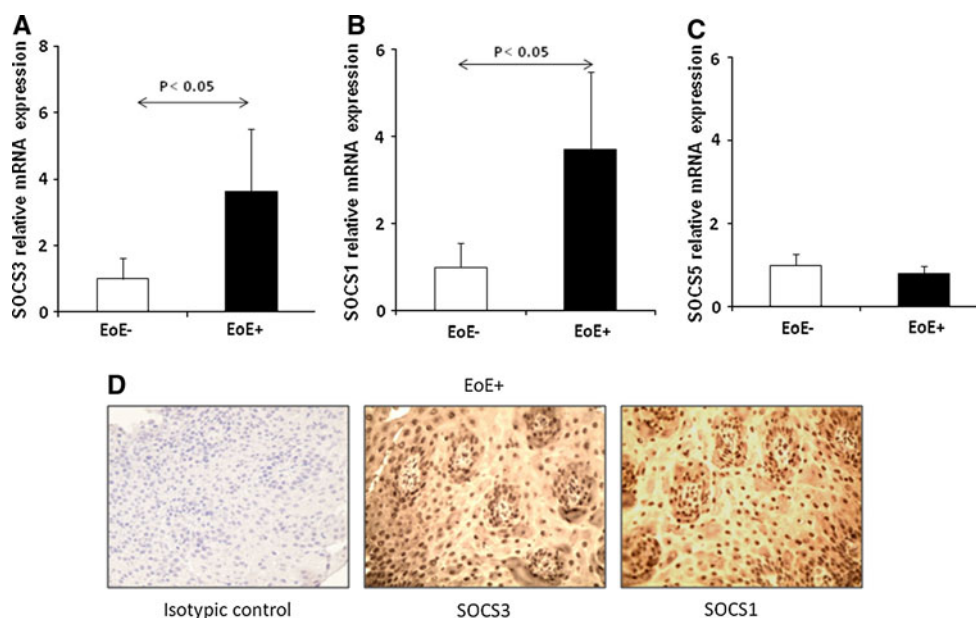


Fig. 1 SOCS 1, 3, and 5 gene expressions in proximal esophageal biopsies from EoE patients and control subjects. Relative SOCSs mRNA levels from EoE patients and control subjects were determined by real-time quantitative PCR. *White bars* represent the control group (mean $\pm$ SD, $n = 17$), and *black bars* the EoE group (mean $\pm$ SD, $n = 25$). The results show relative gene expressions as determined by the $\Delta\Delta C_t$ method, and higher differences in SOCS3 (**a**) and SOCS1

(**b**) expression levels were obtained for the EoE group versus the control group, $P < 0.05$. No differences were achieved when SOCS5 gene expression was assessed ($P > 0.05$, **c**). **d** A representative immunohistochemical staining of SOCS1 and SOCS3 in the esophageal biopsies from EoE patients. Negative control (*left side panel*): isotypic antibody was substituted for primary antibody. *Brown colour* indicates positive staining for all primary antibodies ($n = 5$; $\times 400$)

a peak eosinophil count over 15 in any region of the esophagus. A summary of endoscopic features of all the patients studied is shown in Table 1.

SOCS1 and SOCS3 gene expression, but not SOCS5 expression, is up-regulated in proximal esophageal biopsies from EoE patients

We evaluated transcripts for SOCS1, SOCS3, and SOCS5 in proximal, esophageal biopsies from 25 EoE patients and 17 controls. We observed up-regulation of SOCS3 and SOCS1 mRNA levels in biopsies from EoE patients when compared with those of the control group (Fig. 1a, b) ($P < 0.05$). In contrast, esophageal biopsies revealed no difference in the mRNA expression of SOCS5 of EoE patients and control subjects based on the results of real-time qPCR (Fig. 1c).

SOCS expression was also determined in distal and medium esophageal biopsies displaying similar results (data not shown).

To support these findings as well as to locate the expression, we performed SOCS3 and SOCS1 immunohistochemistry in proximal esophageal sections from EoE patients. As displayed in Fig. 1d, positive SOCS3 and SOCS1 stained cells were found in proximal esophageal sections from EoE patients, this positivity was mainly noted in epithelial and inflammatory cells. Numerous positive clusters were observed on esophageal rings.

IL-5 and the CCL26-CCR3 axis are involved in EoE

Our results show an elevation in gene expression in EoE patients relative to controls for all genes tested: IL-5, CCL26, and CCR3 (89.7-fold; 86.5-fold; and 5.9-fold respectively, $P < 0.05$) (Fig. 2), thus corroborating previous findings in proximal biopsies.

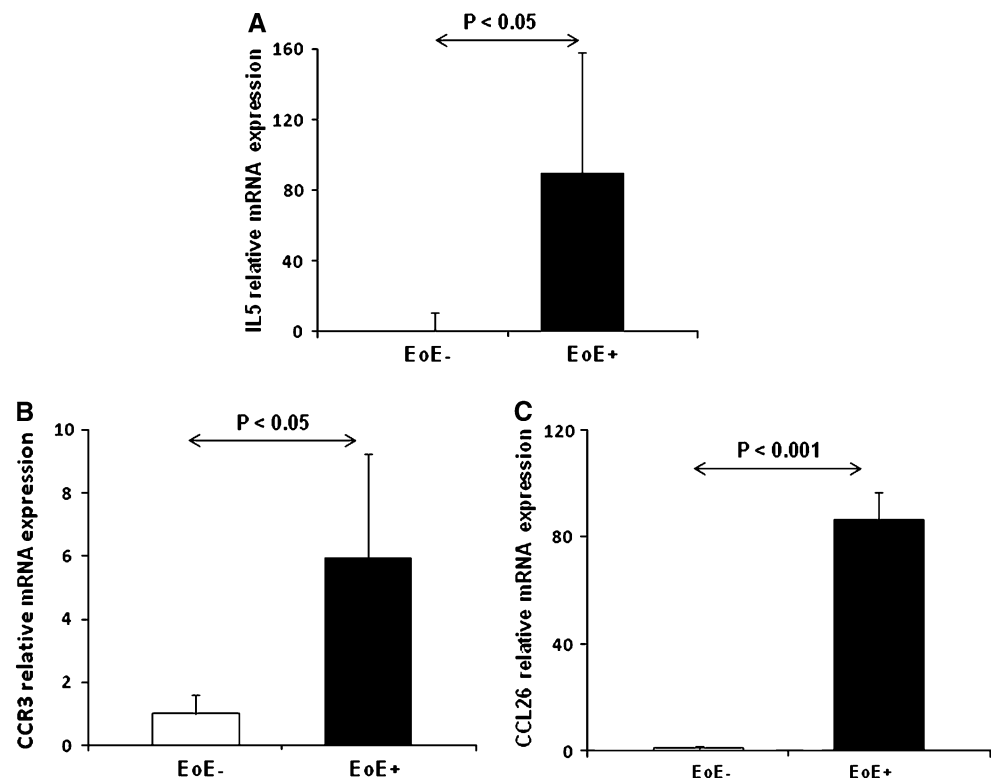
IL-13 detection in proximal esophageal biopsies from EoE patients

We also found an up-regulation of IL-13 mRNA levels (10.5-fold) in proximal esophageal biopsies from EoE patients compared with those of the control group measured by real-time quantitative PCR (Fig. 3a). Additionally, IL-13 immunostaining was performed in proximal esophageal biopsies from EoE patients to locate this interleukin. Positive IL-13 cells were observed at the inflammatory site and also in epithelial cells (Fig. 3b).

Implication of the ERK pathway in EoE disease

CCL26 acts through the ERK pathway; therefore, we analyzed MAPK 1 mRNA levels by RT-PCR. Accordingly, in proximal esophageal biopsies from EoE patients, ERK gene expression was demonstrated to be stronger than in the biopsies from the control group (Fig. 4a). To ascertain

Fig. 2 Augmented IL5, CCL26, and CCR3 gene expression in proximal esophageal biopsies of EoE patients. Real-time quantitative PCR to IL5 (a), CCR3 (b), and CCL26 (c) were performed from esophageal biopsies. Significant differences in gene expression of IL-5 ($P < 0.05$), CCL26 ($P < 0.001$), and CCR3 ($P < 0.05$) were found between the two groups of study. Data represent mean $\pm$ SD, control group ($n = 17$), and EoE group ($n = 25$)



whether the same effect was observable at the protein expression level, a western blot analysis was performed on the esophageal biopsies. As depicted in Fig. 4b, higher phospho-ERK (p-ERK) protein expression was detected in biopsies from EoE patients (3.3-fold higher, $P < 0.001$, Fig. 4c). The location of p-ERK in the esophageal tissue was performed by immunohistochemistry (Fig. 4d). Positive p-ERK stained cells were observed in the esophagus of EoE patients.

Correlation of SOCS1 and SOCS3 with the genes analyzed

Transcript levels of SOCS1 and SOCS3 correlated with esophageal and peripheral eosinophilia in EoE patients ($r = 0.41$, $P < 0.05$ and $r = 0.46$, $P < 0.005$ respectively; Spearman rank correlation). We were also interested in testing that gene expression levels observed would correlate with each other. Indeed, we found significant Spearman correlations between SOCS1, SOCS3 and the most of genes measured (IL-5, IL-13 CCL26, CCR3, and MAPK1). A summary of correlation analysis of all parameters studied is shown in Table 3.

Cytokine profile and SOCS3 expression displayed no alterations at the systemic level in EoE subjects

Determination of SOCS3 mRNA relative levels in blood PBMCs (Fig. 5a) and eosinophils (Fig. 5b) exhibited no

differences ($P > 0.05$) between EoE and non-EoE patients. Therefore, a cytokine array panel was performed to analyze an extensive pattern of mediators implicated in inflammation. Measurement was carried out in two separate pools of serum samples: patients with EoE and controls. Similar patterns of cytokine expression were observed in the two groups (Fig. 5c). To corroborate these results, we determined IL-5 by ELISA in sera from all study subjects, and again, no differences between controls and EoE patients were evident (Fig. 5d).

Discussion

In this study, we identify a set of genes that exhibited differential expression in the esophagi of EoE patients compared with non-EoE patients. Here, we present a novel finding related to the Th2 condition, a consistent characteristic in a set of adult and children patients with EoE. We describe for the first time that, in patients with EoE, the esophagus expresses and synthesizes high levels of SOCS1 and SOCS3 proteins. In addition, we demonstrate the implication of the ERK pathway, suggesting a potential mechanistic link between SOCS regulation and the Th2 phenotype in patients with EoE. IL-5, IL-13 and the CCL26-CCR3 axis were also upregulated at the mRNA levels in the esophagus.

Regarding population characteristics, we have found a higher ratio of males/females than described in the

Fig. 3 Elevated IL-13 expression in esophageal biopsies from EoE patients. Relative IL-13 mRNA levels were determined by real-time quantitative PCR (**a**). Statistical differences were observed between EoE patients and the control group ($P < 0.05$). Data represent mean $\pm$ SD, control group ($n = 17$), and EoE group ($n = 25$). **b** A representative IL-13 immunohistochemistry from biopsies of EoE patients reveals positive staining as indicates the brown colour (central and right panel, $\times 200$ and $\times 400$ magnification, respectively). Negative control (left side panel): isotypic antibody was substituted for primary antibody, $n = 5$

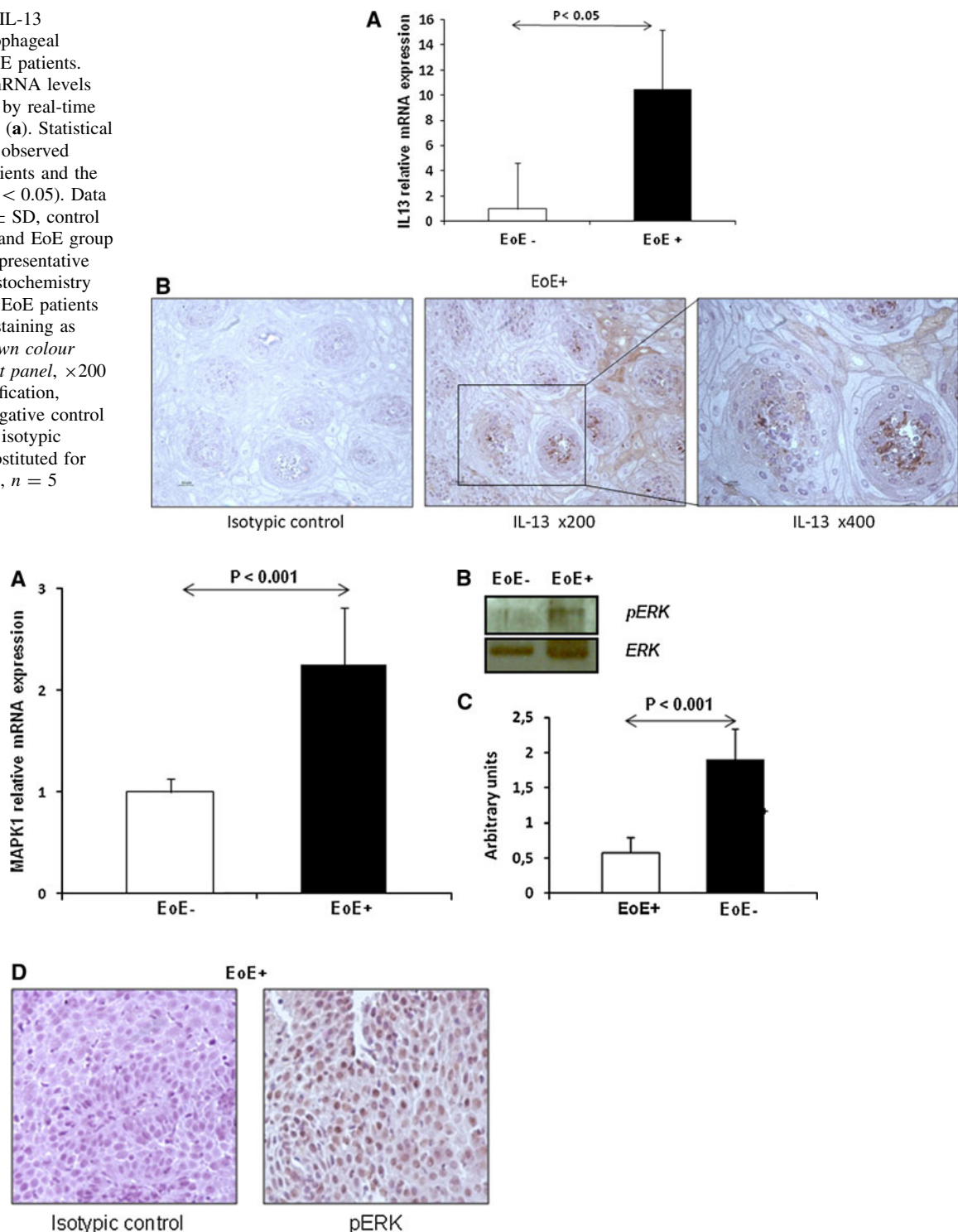


Fig. 4 Implication of ERK pathway in EoE disease. **a** Real-time quantitative PCR was performed from proximal esophageal biopsies to analyze MAPK1 gene expression. White bars represent the control group (mean $\pm$ SD, $n = 17$), and black bars the EoE group (mean $\pm$ SD, $n = 25$, $P < 0.001$). **b** Western blot analysis of the extract of esophagus biopsies was achieved using antibody against ERK and phospho-ERK. Lane 1 biopsy lysate from non EoE patient; lane 2 biopsy lysate from EoE patients. The picture is a representative

example of 5 individuals, all displaying similar results. **c** pERK bands were quantified by densitometry. Data are expressed as the geometric mean $\pm$ SD, $n = 5$, $*P < 0.05$. As a loading control, the total amounts of ERK were determined. **d** p-ERK in esophagus biopsies from EoE patients was detected by immunohistochemistry. An isotypic antibody was tested as control of the technique. Brown colour indicates positive staining for pERK antibody ($n = 5$; $\times 400$)

literature, with almost a 9:1 ratio compared to the 3:1 reported in other studies [22]. As in other studies [23], most of our population was highly atopic, with up to 80 % of our EoE patients suffering from another allergic disease. Supporting the claim of age-dependant evolution in endoscopic findings suggested by Spergel et al. [24], none of the pediatric patients showed esophageal strictures, which are a disease severity marker.

The present study further analyzed SOCS gene expression and its functional role in the EoE and in the mechanistic induction of eosinophils by selective cytokine and chemokines in the esophagus of EoE patients. Using quantitative real-time PCR analysis, we demonstrated that SOCS1 and

SOCS3 mRNA levels were significantly increased in the esophagi of EoE patients but not in peripheral cells such as eosinophils and PBMCs. Notably, transcript levels of SOCS1 and SOCS3 correlated with esophageal and peripheral eosinophilia in EoE patients ($r = 0.41$, $P < 0.05$ and $r = 0.46$, $P < 0.005$, respectively; Spearman rank correlation). Moreover, a strong correlation was found between eosinophils and eotaxin-3 ($r = 0.56$, $P < 0.0002$) or eosinophils and CCR3 ($r = 0.44$, $P = 0.006$) (data not shown).

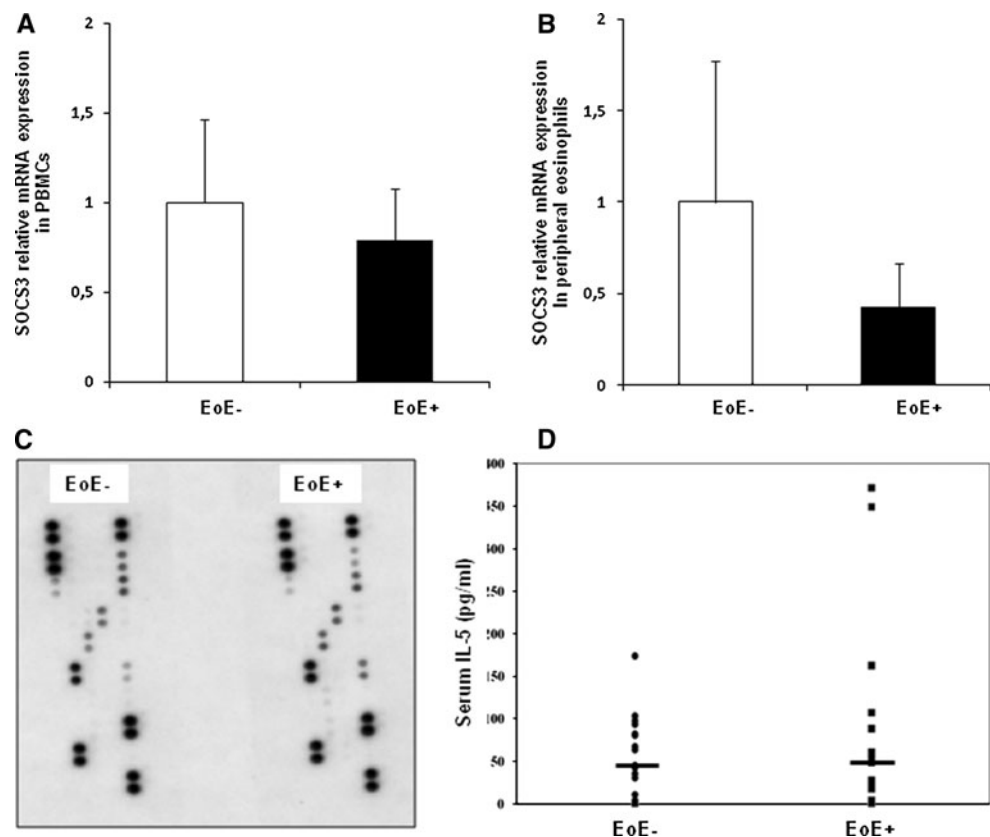
Eosinophils, which are a plentiful source of SOCS1 and SOCS3 [25], play a main role in EoE pathogenesis by producing different mediators that can activate, regulate, and act as effector molecules in different processes. This finding point at the SOCS protein, produced by eosinophils and by other cells, is a potential regulator implicated in EoE pathogenesis. These data is corroborated by our correlation studies, in which we found significant Spearman correlations between SOCS1, SOCS3 and the rest of genes measured (IL-5, CCL26, CCR3 and IL-13). A recent report showed that IL-13 was overproduced in the esophageal tissue of EoE patients and there was a correlation between IL-13 mRNA and eotaxin expression [26], in our study we also obtained a marked increase of IL-13 mRNA (10.5-fold) in esophageal biopsies from EoE patients compared with those from healthy individuals, and moreover we found a positive correlation between IL-13 and SOCS1.

Table 3 Correlation between SOCS1, SOCS3 and genes studied

	SOCS1	SOCS3
SOCS3	<0.0001 ($r = 0.9$)	
IL-5	<0.0001 ($r = 0.80$)	0.0004 ($r = 0.70$)
CCL26	<0.0001 ($r = 0.60$)	0.0007 ($r = 0.50$)
CCR3	<0.0001 ($r = 0.59$)	<0.0001 ($r = 0.68$)
IL-13	<0.01 ($r = 0.36$)	NS ($r = 0.25$)
MAPK1	<0.0001 ($r = 0.70$)	<0.0001 ($r = 0.63$)

¹ Two-tailed P value (r). Spearman correlation r and P values were calculated to test correlation between the ranked genes levels (nonparametric)

Fig. 5 Similar inflammatory cytokines and SOCS3 levels found at the systemic level between the EoE and the control group. SOCS3 real-time quantitative PCR from PBMCs (a) and eosinophils (b) purified from blood samples of EoE patients and control subjects. White bars represent the control group (mean $\pm$ SD, $n = 17$), and black bars the EoE group (mean $\pm$ SD, $n = 25$). c Human cytokine array panel was performed with a pool of serum samples of EoE patients and the control group. IL-5 (d) levels were measured by ELISA in serum samples of 25 EoE patients and 17 control subjects



The present study also identified ERK as an important director of EoE regulation, probably through the SOCS route. Several findings support this conclusion, thus in different cells a link has been demonstrated between ERK activation and SOCS3 accumulation [27–29].

SOCS may stimulate esophageal cells to induce eosinophil-selective chemoattractants in the esophagus of EoE patients. Eosinophils in turn produce more SOCS protein, and thus the up-regulation loop is maintained in the esophagus. One of these chemoattractants is eotaxin-3 (CCL26), which plays an important role in eosinophil migration [30, 31]. Eotaxin-3 is, together with IL-5, one of the most highly expressed genes in human EoE, and epithelial cells are the major source of eotaxin-3 in the human esophagus [32]. It has been described that IL-5 plays a pivotal role in regulating esophageal eosinophilia [33–35] and also in esophagus remodeling [2]. In our study, we obtained elevated levels of IL-5, IL-13, eotaxin-3, and CCR3 mRNA expression in the esophagus of EoE patients. Recently, Blanchard et al. [36], have performed a study of esophageal cytokine expression profile in eosinophilic esophagitis and they have reached similar results.

Eotaxin-3 mediates its biological effect through the ERK pathway [37, 38]. Our data show an increased expression of pERK in esophageal tissue and a strong correlation was found between MAPK1 and SOCS1 and 3 ($P < 0.0001$), and it is possible that, together with other cytokines and chemokines (IL-5, eotaxin-3), it enhances activation of eosinophils in EoE. In fact, activation of ERK may induce both degranulation and chemotaxis of eosinophils [38]. In conclusion, we have demonstrated that the esophageal biopsies of EoE patients exhibit increased expression of SOCS1 and SOCS3 mRNA and protein. SOCS proteins probably contribute to EoE pathogenesis by directly or indirectly inducing Th2 profile, as well as by promoting the production of Th2 cytokines.

Better understanding of the mechanism by which eosinophils promote tissue injury in the esophagus should contribute to the development of novel therapeutic approaches for blocking eosinophil recruitment in the esophagus and/or reversing the harmful consequences of eosinophil recruitment. The identification of SOCS in the pathogenesis of EoE suggests that targeting this molecule may hold promise for the treatment of EoE.

All these findings further enhance our understanding of the mechanism of EoE, and accumulating evidence suggests that EoE pathogenesis is likely to be due to misregulation of immunological pathways.

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