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Association between suppressors of cytokine signalling, T-helper type 1/T-helper type 2 balance and allergic sensitization in children

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Clinical and Experimental Allergy

Summary

Background Suppressors of cytokine signalling (SOCS) family members have been shown to play an important role in the balance of cytokines that determine the onset of T-helper type 1 (Th1)- and Th2-mediated immune responses. In particular, for cytokine-induced Src-homology 2 protein (CIS), SOCS1, SOCS3 and SOCS5, a role in the regulation of T cell differentiation has been discussed. However, only few data exist so far in the human system. **Objectives** The aim of the present study was to analyse the relationship between these suppressors and Th1/Th2 regulation as well as allergic sensitizations within a population-based study.

Methods Within the Lifestyle-Immune system-Allergy plus cohort study, mRNA was prepared from blood samples of 6-year-old children for the analysis of cytokines, transcription factors for T cell regulation and SOCS molecule expression by quantitative real-time polymerase chain reaction. In addition, total and specific IgE concentrations have been measured by the Pharmacia CAP System. A complete data set from 248 children was available.

Results Among the SOCS molecules investigated, only SOCS1 showed a strong positive correlation to allergic sensitizations. In addition, an up-regulated SOCS1 expression correlated with down-regulated T-box expressed in T cells (Tbet) and higher expression levels of GATA-binding protein 3 (GATA-3) and IL-4. No association between SOCS1 and forkhead box P3 (FOXP3) was observed. For SOCS3, SOCS5 and CIS, a contradictory picture was found. The expression of these SOCS molecules was positively correlated with Tbet and FOXP3 and (with the exception of CIS) negatively with IL-4.

Conclusions Our data suggest that SOCS3, SOCS5 and CIS, which correlate with an up-regulated Th1 and regulatory T cell activity, are without relevance for the allergic status. In contrast, SOCS1 might be involved in the development of a Th2-skewed immune response and subsequent allergic sensitizations.

Keywords allergic sensitization, children, CIS, IgE, SOCS1, SOCS3, SOCS5, Th1/Th2

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Introduction

The cytokine-induced Src-homology 2 protein (CIS) and suppressors of cytokine signalling (SOCS) are members of a family of intracellular proteins that have emerged as key physiological regulators of cytokine-mediated homeostasis including innate and adaptive immunity. These proteins are engaged in the negative regulation of cytokine signalling, primarily signalling associated with the Janus kinase – signal transducer and activator of transcription (JAK – STAT) pathway, a mechanism to modulate cytokine signalling to prevent a sustained and/or an excessive action of cytokines and growth factors. So far, eight different SOCS proteins have been described: CIS and SOCS1–7 [1].

Signal down-regulation through SOCS members has been shown to play an important role in the balance of cytokines that determine the onset of T-helper type 1 (Th1) and Th2-mediated immune responses.

Th1 and Th2 cells are two distinct T cell subsets, defined by different functional abilities and cytokine profiles [2, 3]. IFN- γ is the signature cytokine of Th1 cells and IL-4 is the corresponding signature cytokine of Th2 cells. GATA-binding protein 3 (GATA-3) is a Th2-specific transcription factor and up-regulated during Th2 differentiation [4, 5]. The transcription factor T-box expressed in T cells (Tbet) controls the expression of the hallmark Th1 cytokine IFN- γ [6]. Regulatory T cells (Treg) represent a further T cell subset that actively controls or suppresses the function of other cells. The CD4⁺CD25⁺ Tregs express the forkhead box P3 (FOXP3) transcription factor [7] and these cells are more present in healthy individuals than in allergic patients [8]. Moreover, as patients who specifically lack CD25⁺ Tregs develop severe eczema, elevated IgE levels, eosinophilia and food allergy [9], a great deal more study of Treg in allergy is warranted.

In particular, for CIS, SOCS1, SOCS3 and SOCS5, a role in the regulation of T cell differentiation has been discussed. SOCS1, which is mainly expressed in Th1 cells [10], is supposed to be particularly important for limiting IFN- γ -dependent-T cell activation [11, 12]. In addition, SOCS1 regulates responses to IL-12, *in vivo* [13]. SOCS3 is

preferentially expressed in Th2 cells [10]. This SOCS molecule also represses IFN- γ signalling but less potently than SOCS1 [11, 14]. Recent analyses indicated that SOCS3 negatively regulates the IL-12 to STAT4 Th1 pathway [12, 15]. SOCS5, which was found to be expressed in Th1 cells, plays a role as a specific negative regulator for Th2 differentiation by repressing IL-4-induced signalling [16]. Although *in vitro* studies suggest that SOCS5 in T cells appeared to bias CD4⁺ cells towards Th1, there is no defect in Th differentiation in SOCS5-deficient mice [17]. CIS is the most abundant SOCS member expressed in activated T cells. Both Th1 and Th2 cells express high levels of CIS [18]. In CIS transgenic mice, helper T cells tend to differentiate into Th2 cells [19].

SOCS molecules might control the immune system function also via Treg. A down-regulation of SOCS3 supports a regulatory phenotype of T cells [20–23] whereas insufficient expression of SOCS1 results in impaired function of Treg [24–26].

Owing to their influence on Th1/Th2 differentiation, the above-mentioned SOCS molecules might also be important for allergy development. Few studies in humans have investigated the expression levels of certain SOCS molecules in allergic patients. For example, increased SOCS3 expression in T cells has been shown to correlate with the severity of asthma and atopic dermatitis [15]. Furthermore, Ekelund et al. [27] have reported that SOCS3 is consistently more highly expressed in skin from patients with atopic dermatitis than in normal control skin. In addition, specific haplotypes of the SOCS3 gene have been shown to be significantly associated with the allergic phenotype. Recently, a significant association has been found between a polymorphism in the SOCS1 promoter, which enhances the transcription of SOCS1, and the occurrence of disease symptoms in asthmatic patients [12, 28]. So far, no data are available concerning the relevance of CIS and SOCS5 in humans.

Although, there is some evidence that the expression of SOCS molecules might in fact be important for the development of an allergic reactivity, there is still a lack of knowledge in particular regarding the functional relevance of these regulatory molecules in the human system,

Footnote Continued

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which calls for further investigations. For the first time, we have used a population-based approach to study the association of SOCS molecules and the development of an allergic reactivity. Within a children's cohort study, the relationship between CIS, SOCS1, SOCS3 and SOCS5 and Th1/Th2 regulation as well as allergic sensitizations was analysed.

Methods

Study design and subjects

The Lifestyle-Immune System-Allergy (LISA) study was designed to investigate the influence of lifestyle and environmental factors on the maturation of the immune system and the atopic risk in early childhood. Three thousand ninety-seven newborns, born between December 1997 and January 1999 in the four German cities Munich, Leipzig, Wesel and Bad Honnef, were included in this population-based study. Only healthy term neonates of German descent were included. Newborn children whose mothers suffered from autoimmune disease or infectious disorders during pregnancy were excluded. The study design has been described in detail elsewhere [29]. Children from this study were followed up regularly from infancy to 6 years of age with clinical examinations and blood sampling.

At the age of 6, a clinical investigation was performed in a children's hospital. Blood samples were taken for the determination of IgE concentrations, and in the sub cohort from the city of Leipzig for further analysis of immunological parameters. Five hundred and sixty-five 6 year old children from the city of Leipzig participated in the study. Blood samples were accessible from 324 of these children.

Participation in the study was voluntary; informed consent was obtained from the parents of all children. The study was approved by the Ethics Committees of the University of Leipzig.

RNA preparation

Heparinized blood was obtained by venipuncture and RNA extraction was performed as described previously [30]. Briefly, 1 mL of heparinized blood (1 mL) was incubated with 1 mL of complete RPMI 1640 (containing 10% heat-inactivated fetal bovine serum, 0.01% penicillin, 0.0085% streptomycin, 2 mM L-glutamine, 0.35% β -mercaptoethanol) for 3 h at 37 °C and 5% CO₂. After red cell lysis twice with hypotonic buffer (0.83% NH₄Cl, 0.1% KHCO₃, 0.1 mM EDTA, pH 7.2), leucocytes were lysed with 400 μ L of MagNAPure lysis buffer containing additional 1% DTT (Roche Applied Science, Mannheim, Germany) and the sample was frozen at -80 °C.

After thawing, RNA extraction was performed with an automated standardized protocol for human whole blood

using the MagNA Pure LC system and the MagNA Pure LC mRNA Isolation Kit I (Roche Applied Science). The elution volume was set to 50 μ L. One aliquot of 8.2 μ L was reverse transcribed using AMV-RT and oligo-(dT) as the primer (First Strand cDNA synthesis Kit; Roche Applied Science) according to the manufacturer's protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was stored at -20 °C until PCR analysis.

Real-time polymerase chain reaction

Reverse transcriptase products were amplified by a real-time PCR with a LightCycler Fast Start DNA SYBR Green Kit (Roche Applied Science). Reagent kits with primers and standards for SOCS1, SOCS3, SOCS5, CIS, GATA-3, Tbet, FOXP3 and β -Actin and the amplification protocols were obtained from Search-LC (Heidelberg, Germany, www.Search-LC.com). PCR reactions were performed according to the manufacturer's instruction. Primers for IL-4 were synthesized by MWG Biotech (Ebersberg, Germany). The primer sequences for IL-4 were sense 5'-AGA AGA CTC TGT CAC CGA TTGA-3' and antisense 5'-CTC TCA TGA TCG TCT TTA GCC TTT-3'. IL-4 standard was synthesized by GenExpress (Berlin, Germany). The amplification protocol used for IL-4 primers was 5 °C, 1 s; 68 °C, 10 s; 72 °C, 16 s, with a lowered annealing temperature of 0.5 °C per cycle until reaching the final annealing temperature of 58 °C at cycle 17. Data were obtained during the exponential phase of the amplification and are therefore quantitative. Quantification of transcript concentration for the measured genes was performed by external standardization. To control the integrity of mRNA, the control gene β -actin was analysed in parallel. Data are presented as the ratio between the copy number of the target gene and the copy number of the housekeeping gene β -actin.

Total and specific immunoglobulin E

To characterize the atopic status, IgE serum concentrations were determined by the Pharmacia CAP System (Pharmacia Diagnostics, Freiburg, Germany). Sera were screened for total IgE and for specific IgE against inhalant (SX1) and food allergens (FX5) by two multi-allergen panels. SX1 (CAP FEIA) includes house dust mite (HDM) (*Dermatophagoides pteronyssinus*), cat, dog, timothy, rye, birch and mugwort; FX5 (CAP FEIA) contains the food allergens hen's egg, cow' milk, fish, wheat, peanut and soy. Samples with specific IgE concentrations >0.35 kU/L were regarded as positive. Sera with positive results in the multi-allergen panels were tested for the single allergens.

For total IgE, the cut-off was set at 180 kU/L as this value was published as the 95% value of the cumulative

frequency distribution of total IgE in the reference area Borken, Germany [31, 32].

Intracellular cytokine staining

Flow-cytometric detection of intracellular cytokines was performed as previously described [33]. Briefly, 100 µL heparinized blood in 900 µL RPMI was stimulated with 10 ng/mL of phorbol myristate acetate (Sigma Aldrich, Deisenhofen, Germany) and 1 µM of ionomycin (Calbiochem, Bad Soden, Germany) in the presence of 2.5 µM of monensin (Sigma Aldrich) for 5 h at 37 °C and 5% CO₂. Stimulated cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin.

Cells were simultaneously stained with fluorescence-labelled monoclonal antibodies (MAbs) against the T cell surface markers (CD3, CD4, CD8) and against the human cytokines IFN-γ and IL-4, all obtained from Beckman-Coulter (Krefeld, Germany). Fluorescence-labelled cells were analysed in a flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) using Cellquest software. In all samples, 10 000 cells were collected. Results are given as percentages of cytokine-producing cells of the respective cell populations.

Statistical analysis

Owing to the fact that the functional relationship between suppressors of cytokine signalling and allergic sensitization is not yet clear, SOCS expressions were categorized using quartiles (first, second, third, fourth) as cut-off values and included in the models with the lowest quartile as reference. Data for suppressors of cytokine signalling are presented as median with 25–75 percentiles. Trend tests were applied to detect dose-response relations. Multiple logistic regression models were used to determine adjusted odds ratio (OR) with 95% confidence interval (CI) for the association between suppressors of cytokine signalling expressions and allergic sensitization. OR were adjusted for family atopy history (FAH), gender, parental school education, pet keeping, exposure to environmental tobacco smoke, breastfeeding and the presence of siblings. In the same models, trend calculation was performed basing on categorized SOCS expression variables. The correlation between SOCS and T cell regulatory factors was tested using the non-parametric Spearman's rank correlation test. The Jonckheere-Terpstra test was used for trend calculation for the association between suppressors of cytokine signalling expression and IFN-γ CD4⁺-producing T cells as well as with IL-4 CD4⁺-producing T cells. Statistical analysis was performed with STATISTICA for Windows Version 7.0 (Statsoft Inc.) and StatXact version 6.2 (Cytel Inc., Cambridge, MA, USA).

Results

Characteristics of the study subjects

From the initial 565 6 year old participants from Leipzig, 324 blood samples were obtained. Complete data for IgE, mRNA, intracellular cytokine staining and all confounding variables were available from 248 children [120 girls (48.4%) and 128 boys (51.6%)]. There was a reduction of case numbers because the amount of blood was not sufficient for all participants for all analyses or parents did not give consent for genetic (DNA/RNA) analysis. The basic characteristics of the subset of the population are given in Table 1. Regarding the investigated parameter, there was no difference between the analysed subcohort ($n = 248$) and the remaining children from the LISA cohort from Leipzig ($n = 317$) (Table 1). Among the 248 investigated children, 68 (27.4%) reacted against the inhalant (SX1) and 31 (12.5%) against food allergens (Table 2).

Table 1. Characteristics of the analysed sub cohort ($n = 248$) and the remaining children of the overall LISA cohort from Leipzig (due to missing information for some characteristics, number of subjects varies between 309 and 317)

Variables	Analysed subcohort <i>n/N</i> (%)	Remaining subjects of LISA cohort <i>n/N</i> (%)	<i>P</i> -value
Breastfeeding			
Yes	114/248 (46.0)	117/312 (37.5)	0.223
No	134/248 (54.0)	195/312 (62.5)	
Gender			
Male	128/248 (51.6)	146/317 (46.1)	0.437
Female	120/248 (48.4)	171/317 (53.9)	
Parental school education			
High	5/248 (2.0)	7/309 (2.3)	0.987
Intermediate	102/248 (41.1)	128/309 (41.4)	
Low	141/248 (56.9)	174/309 (56.3)	
Parental history of atopy			
None	133/248 (53.6)	188/317 (59.3)	0.349
Single positive	83/248 (33.5)	107/317 (33.8)	
Double positive	32/248 (12.9)	22/317 (6.9)	
Presence of siblings			
None	59/248 (23.8)	73/310 (23.5)	0.310
One	147/248 (59.3)	163/310 (52.6)	
Two	37/248 (14.9)	51/310 (16.5)	
Three or more	5/248 (2.0)	23/310 (7.4)	
Pet keeping			
Yes	112/248 (45.2)	144/314 (45.9)	0.921
No	136/248 (54.8)	170/314 (54.1)	
Smoking in the dwelling			
Daily	30/248 (12.1)	37/314 (11.8)	0.996
Once per week	3/248 (1.2)	5/314 (1.6)	
Occasionally	23/248 (9.3)	30/314 (9.6)	
Never	192/248 (77.4)	242/314 (77.1)	

LISA, Lifestyle-Immune System-Allergy.

Table 2. Frequency of allergic sensitizations

Variables	Frequency n/N (%)
Total IgE*	32/248 (12.9)
Specific IgE†	79/248 (31.9)
Food allergens	31/248 (12.5)
Hen's egg	6/248 (3.0)
Cow's milk	16/248 (6.5)
Peanut	9/248 (3.6)
Soy	6/248 (2.4)
Fish	3/248 (1.2)
Wheat flour	7/248 (2.8)
Inhalative allergens	68/248 (27.4)
Der p 1	41/248 (16.5)
Cat	18/248 (7.3)
Dog	10/248 (4.0)
Thimoty	35/248 (14.1)
Rye	30/248 (12.1)
Birch	25/248 (10.1)
Mugwort	16/248 (6.5)

*The total IgE were regarded as positive at values ≥ 180 kU/L.

†A child was considered sensitized to specific allergens at specific IgE values ≥ 0.35 kU/L.

Table 3. Mean and standard deviation of raw and relative expression levels of genes determined by real-time PCR from mRNA/cDNA separated from whole blood samples without further *ex vivo* stimulation

	Median (25–75 percentile)	
	Copy number/10 μ L	Relative expression level*
β -actin	1 228 500 (217 700–3 145 000)	
SOCS1	6634 (3393–11 600)	5.225 (3.166–13.141)
SOCS3	4159 (640–12 835)	3.371 (2.011–5.144)
SOCS5	256 (29–721)	0.213 (0.053–0.327)
CIS	640 (91–2095)	0.548 (0.209–0.897)
GATA-3	6274 (1783–15 480)	5.607 (3.291–11.020)
Tbet	175 (27–937)	0.189 (0.080–0.394)
FOXP3	463 (116–1749)	0.516 (0.255–0.991)
IL-4	9238 (3748–17 953)	8.129 (3.814–21.398)

*Relative expression level = (copy number, e.g. SOCS1/copy number house-keeping gene β -actin) $\times$ 1000.

SOCS1, suppressors of cytokine signalling; GATA-3, GATA-binding protein 3; Tbet, T-box expressed in T cells; FOXP3, forkhead box P3; CIS, cytokine-induced Src-homology 2 protein.

Suppressors of cytokine signalling and T cell regulatory factors

The mean and standard deviation of raw and relative expression levels of each gene determined by quantitative real-time PCR are given in Table 3. For all analysed genes, the expression levels were above the background.

Table 4. Relation between suppressors of cytokine signalling and T cell regulatory factors, correlation coefficient from Spearman's correlation

	SOCS1	SOCS3	SOCS5	CIS
IL-4	0.49*	– 0.16	– 0.17	– 0.04
GATA-3	0.36	0.07	0.20	0.29
Tbet	– 0.22	0.34	0.48	0.30
FOXP3	0.04	0.37	0.32	0.22

*Significant correlation coefficients ($P < 0.05$) are marked bold.

Expression of T cell regulatory factors was analysed by quantitative real-time PCR from cDNA/mRNA samples prepared from heparinized whole blood samples of the study participants without further stimulation *ex vivo*.

GATA-3, GATA-binding protein 3; Tbet, T-box expressed in T cells; FOXP3, forkhead box P3; SOCS1, suppressors of cytokine signalling; CIS, cytokine-induced Src-homology 2 protein.

SOCS1 expression was positively correlated with GATA-3 and IL-4 expression and negatively correlated with Tbet. For SOCS3, reverse correlations were found as compared with SOCS1 (Table 4). SOCS3 was positively correlated with Tbet and negatively with IL-4. GATA-3 showed no association with SOCS3. As for SOCS3 and also for SOCS5, a positive correlation to Tbet and a negative correlation to IL-4 were found. CIS was positively correlated with both the Th1 regulatory factor Tbet and the Th2 regulation factor GATA-3. FOXP3, the marker for regulatory T cells, was not correlated with SOCS1 expression. In contrast, SOCS3, SOCS5 and CIS were positively correlated with FOXP3 (Table 4).

Suppressors of cytokine signalling and T-helper populations

Children with a higher SOCS1 mRNA expression had significantly lower amounts of PMA/ionomycin-stimulated IFN- γ -producing CD4⁺ T cells (P for trend = 0.03) than children with less SOCS1 mRNA expression. IL-4-producing T cells were not affected. Concerning SOCS3, the amount of IFN- γ or IL-4-producing T cells was not correlated with a higher SOCS3 mRNA expression. Amounts of IFN- γ producing Th1 cells tended to be elevated in children with a higher SOCS5 mRNA expression (P for trend = 0.06). Again, IL-4-producing T cells were not affected by SOCS5. In addition, there was a slightly positive trend between the amount of IL-4 producing T cells and a higher CIS mRNA expression (P for trend = 0.07). IFN- γ -producing T cells had no relationship with the level of CIS expression (Fig. 1).

Suppressors of cytokine signalling and allergic sensitization

The results of the multiple regression analysis for the association between SOCS1 and allergic sensitization

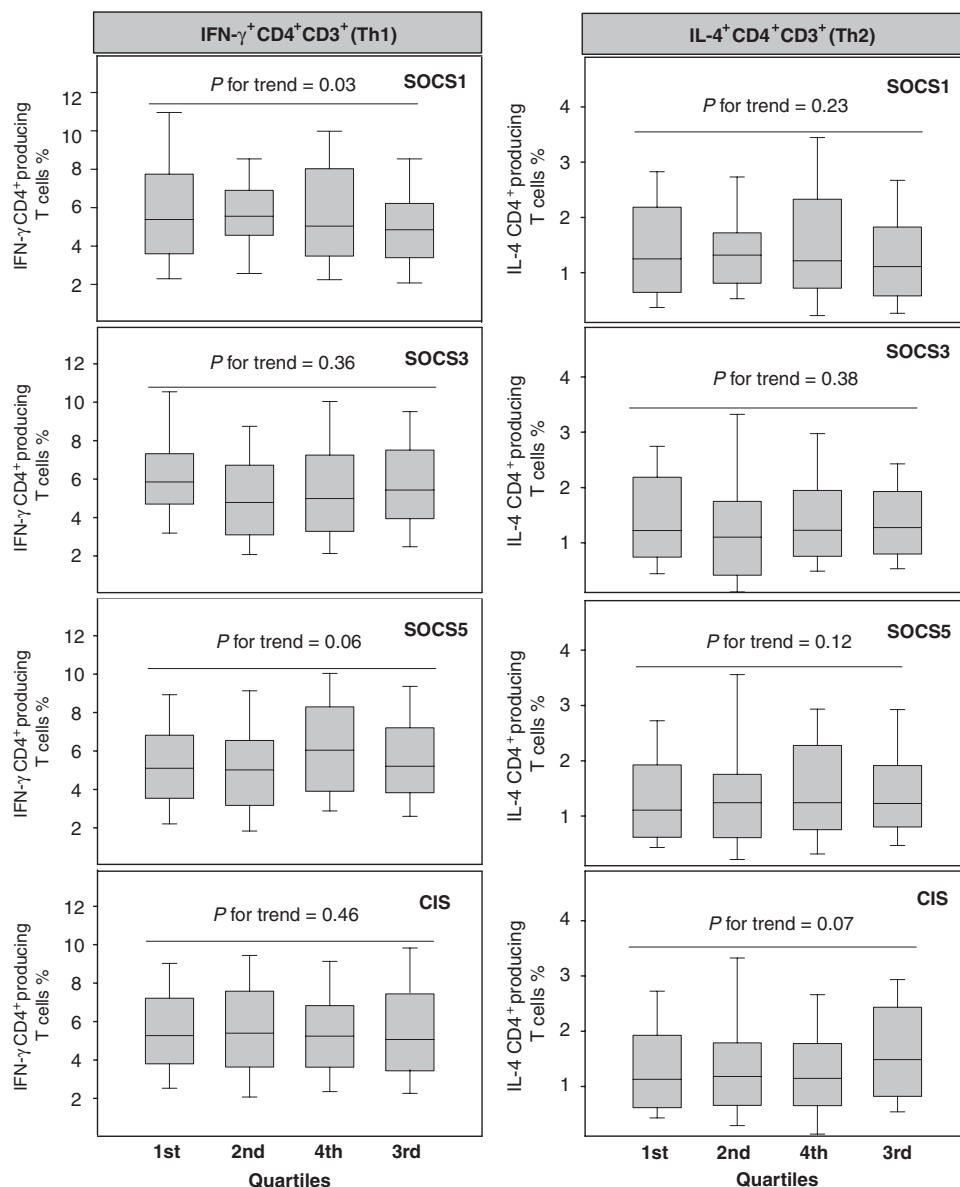


Fig. 1. Percentage of IFN- γ and IL-4 producing CD4⁺ T cells regarding SOCS1, SOCS3, SOCS5 and CIS expression. Whole blood samples of study participants were stimulated *ex vivo* for 5 h with PMA and ionomycin in the presence of monensin, subsequently stained for surface markers and intracellular cytokines and analysed by flow cytometry. SOCS1, SOCS3, SOCS5 and CIS expression levels were determined from mRNA/cDNA separated from the same blood samples by quantitative real-time PCR without further *ex vivo* stimulation. SOCS1, SOCS3, SOCS5 and CIS expression levels were categorized by using quartiles. Box plots represent medians and interquartile ranges of IFN- γ or IL-4 production in the respective quartiles, whiskers show the 10th and 90th percentile of cytokine production. *P* for trend from the Jonckheere-Terpstra test is given. SOCS1, suppressors of cytokine signalling; CIS, cytokine-induced Src-homology 2 protein.

(detectable IgE antibodies) are presented in Table 5. Although the trend test applied gives evidence of a positive correlation between SOCS1 expression and total IgE, the adjusted ORs did not show significance. However, there was a positive relationship between SOCS1 expression and the occurrence of any specific IgE (OR_{Q4} 3.13, 95% CI 1.20–8.19; *P* for trend = 0.026) as well as the detectable IgE antibodies against the food allergen mix Fx5 (OR_{Q4} 9.51, 95% CI 1.72–52.59; *P* for trend = 0.006) and the inhalative allergen mix Sx1 (OR_{Q4} 3.14, 95% CI

1.17–8.40; *P* for trend = 0.027). Among the single inhalative allergens, SOCS1 correlated with a higher risk for allergic sensitization against HDM (Der p 1, OR_{Q4} 4.65, 95% CI 1.35–16.01; *P* for trend = 0.003) and rye (OR_{Q4} 4.27, 95% CI 1.06–17.13). However, for rye the trend test was not significant (*P* for trend = 0.163). Sensitizations to timothy and birch were not significantly correlated with the SOCS1 expression.

SOCS5, SOCS3 and CIS were not associated with any allergic sensitization (Tables 6–8). Owing to the low

Table 5. Frequency [% (n/N)] and risk (adjusted* OR with 95% CI) of detectable elevated serum IgE concentrations according to SOCS1 relative expression

	SOCS1				P for trend
	First quartile (0–3.2)	Second quartile (3.2–5.3)	Third quartile (5.3–13.1)	Fourth quartile (13.1–1327.7)	
Total IgE >180 kU/L	9.7% (6/62) 1.00	8.1% (5/62) 1.00 (0.09–10.92)	14.5% (9/62) 1.55 (0.49–4.92)	19.4% (12/62) 2.85 (0.91–8.97)	0.042
Specific IgE	22.6% (14/62) 1.00	33.9% (21/62) 2.94 (1.13–7.66)	33.9% (21/62) 2.10 (0.89–4.97)	37.1% (23/62) 3.13 (1.20–8.19)	0.026
Food allergens	3.2% (2/62) 1.00	12.9% (8/62) 7.96 (1.25–50.55)	16.1% (10/62) 7.5 (1.43–39.77)	17.7% (11/62) 9.51 (1.72–52.59)	0.006
Inhalative allergens	19.4% (12/62) 1.00	29.1% (18/62) 3.06 (1.09–8.60)	27.4% (17/62) 1.81 (0.73–4.52)	33.9% (21/62) 3.14 (1.17–8.40)	0.027
Der p 1	9.7% (6/62) 1.00	9.7% (6/62) 1.30 (0.25–6.77)	21.0% (13/62) 2.61 (0.87–7.82)	25.8% (16/62) 4.65 (1.35–16.01)	0.003
Timothy	9.7% (6/62) 1.00	16.1% (10/62) 2.50 (0.72–8.66)	12.79% (8/62) 1.20 (0.36–4.09)	17.7% (11/62) 3.20 (0.93–10.97)	0.135
Rye	8.1% (5/62) 1.00	14.5% (9/62) 2.93 (0.76–11.27)	9.7% (6/62) 1.20 (0.34–4.25)	16.1% (10/62) 4.27 (1.06–17.13)	0.163
Birch	9.7% (6/62) 1.00	8.1% (5/62) 1.15 (0.29–4.57)	12.9% (8/62) 1.50 (0.45–4.96)	9.7% (6/62) 1.31 (0.35–4.95)	0.567

SOCS1 expression was analysed by quantitative real-time PCR from cDNA/mRNA samples prepared from heparinized whole blood samples without further stimulation *ex vivo*.

*Adjusted for: family atopy history, gender, siblings, parental school education, pet keeping, smoking in the dwelling and breastfeeding. Significant values are marked bold.

OR, odds ratio; CI, confidence interval; SOCS1, suppressors of cytokine signalling.

Table 6. Frequency [% (n/N)] and risk (adjusted* OR with 95% CI) of detectable elevated serum IgE concentrations according to SOCS3 relative expression

	SOCS3				P for trend
	First quartile (0–2.0)	Second quartile (2.0–3.4)	Third quartile (3.4–5.1)	Fourth quartile (5.1–315.8)	
Total IgE > 180 kU/L	11.3% (7/62) 1.00	11.3% (7/62) 1.03 (0.29–3.71)	11.3% (7/62) 1.09 (0.35–3.44)	17.7% (11/62) 3.01 (0.87–10.39)	0.247
Specific IgE	29.0% (18/62) 1.00	35.5% (22/62) 1.40 (0.60–3.25)	29.0% (18/62) 0.98 (0.42–2.29)	33.9% (21/62) 1.30 (0.51–3.33)	0.730
Food allergens	12.9% (8/62) 1.00	14.5% (9/62) 1.06 (0.33–3.44)	12.9% (8/62) 1.20 (0.36–4.03)	9.8% (6/62) 0.92 (0.23–3.57)	0.609
Inhalative allergens	24.2% (15/62) 1.00	29.0% (18/62) 1.36 (0.56–3.33)	22.6% (14/62) 0.93 (0.37–2.35)	33.9% (21/62) 1.97 (0.74–5.24)	0.261
Der p 1	14.5% (9/62) 1.00	17.7% (11/62) 1.16 (0.39–3.38)	11.3% (7/62) 0.74 (0.24–2.31)	22.6% (14/62) 1.90 (0.64–5.62)	0.395
Timothy	14.5% (9/62) 1.00	16.1% (10/62) 1.17 (0.40–3.47)	9.7% (6/62) 0.65 (0.20–2.14)	16.1% (10/62) 1.33 (0.42–4.20)	0.930
Rye	14.5% (9/62) 1.00	11.3% (7/62) 0.85 (0.27–2.71)	9.7% (6/62) 0.65 (0.20–2.14)	12.9% (8/62) 1.08 (0.32–3.61)	0.997
Birch	14.5% (9/62) 1.00	4.8% (3/62) 0.28 (0.07–1.13)	8.1% (5/62) 0.59 (0.16–2.18)	12.9% (8/62) 1.19 (0.35–4.04)	0.881

SOCS3 expression was analysed by quantitative real-time PCR from cDNA/mRNA samples prepared from heparinized whole blood samples without further stimulation *ex vivo*.

*Adjusted for: family atopy history, gender, siblings, parental school education, pet keeping, smoking in the dwelling and breastfeeding.

OR, odds ratio; CI, confidence interval; SOCS3, suppressors of cytokine signalling.

Table 7. Frequency [% (n/M)] and risk (adjusted* OR with 95% CI) of detectable elevated serum IgE concentrations according to SOCS5 relative expression

	SOCS5				<i>P</i> for trend
	First quartile (0–0.05)	Second quartile (0.05–0.21)	Third quartile (0.21–0.33)	Fourth quartile (0.33–61.84)	
Total IgE >180 kU/L	16.1% (10/62) 1.00	16.1% (10/62) 0.92 (0.32–2.62)	11.3% (7/62) 0.70 (0.23–2.14)	8.1% (5/62) 0.33 (0.09–1.21)	0.123
Specific IgE	33.9% (21/62) 1.00	37.1% (23/62) 0.82 (0.36–1.91)	29.0% (18/62) 0.81 (0.35–1.87)	27.4% (17/62) 0.52 (0.21–1.25)	0.206
Food allergens	14.5% (9/62) 1.00	14.5% (9/62) 0.76 (0.25–2.31)	8.1% (5/62) 0.57 (0.16–1.95)	12.9% (8/62) 0.77 (0.25–2.36)	0.658
Inhalative allergens	30.6% (19/62) 1.00	32.3% (20/62) 0.78 (0.32–1.91)	24.2% (15/62) 0.69 (0.28–1.70)	22.6% (14/62) 0.48 (0.19–1.21)	0.121
Der p 1	21.0% (13/62) 1.00	12.9% (8/62) 0.34 (0.11–1.08)	16.1% (10/62) 0.67 (0.23–1.90)	16.1% (10/62) 0.52 (0.19–1.46)	0.430
Timothy	12.9% (8/62) 1.00	21.0% (13/62) 1.70 (0.56–5.16)	9.7% (6/62) 0.79 (0.24–2.62)	1.6% (1/62) 0.98 (0.32–3.00)	0.442
Rye	12.9% (8/62) 1.00	17.7% (11/62) 1.68 (0.56–5.05)	6.5% (4/62) 0.52 (0.14–1.95)	11.3% (7/62) 0.88 (0.27–2.86)	0.351
Birch	16.1% (10/62) 1.00	11.3% (7/62) 0.50 (0.15–1.65)	4.8% (3/62) 0.28 (0.06–1.18)	8.1% (5/62) 0.40 (0.12–1.37)	0.079

SOCS5 expression was analysed by quantitative real-time PCR from cDNA/mRNA samples prepared from heparinized whole blood samples without further stimulation *ex vivo*.

*Adjusted for: family atopy history, gender, siblings, parental school education, pet keeping, smoking in the dwelling and breast-feeding.

OR, odds ratio; CI, confidence interval; SOCS5, suppressors of cytokine signalling.

Table 8. Frequency [% (n/M)] and risk (adjusted* OR with 95% CI) of detectable elevated serum IgE concentrations according to CIS relative expression

	CIS				<i>P</i> for trend
	First quartile (0–0.21)	Second quartile (0.21–0.55)	Third quartile (0.55–0.90)	Fourth quartile (0.90–65.46)	
Total IgE >180 kU/L	11.3% (7/62) 1.00	8.1% (5/62) 0.51 (0.12–2.23)	14.5% (9/62) 1.26 (0.40–3.98)	17.7% (11/62) 1.57 (0.52–4.68)	0.227
Specific IgE	25.8% (16/62) 1.00	38.7% (24/62) 1.66 (0.72–3.80)	30.6% (19/62) 1.14 (0.47–2.76)	32.3% (20/62) 1.15 (0.48–2.74)	0.848
Food allergens	9.7% (6/62) 1.00	17.7% (11/62) 2.15 (0.64–7.25)	9.7% (6/62) 0.93 (0.24–3.58)	12.9% (8/62) 1.32 (0.39–4.42)	0.938
Inhalative allergens	22.6% (14/62) 1.00	27.4% (17/62) 1.13 (0.45–2.83)	29.0% (18/62) 1.24 (0.49–3.15)	30.7% (19/62) 1.28 (0.52–3.14)	0.409
Der p 1	16.1% (10/62) 1.00	12.9% (8/62) 0.52 (0.16–1.67)	16.1% (10/62) 0.86 (0.29–2.55)	21.0% (13/62) 1.27 (0.47–3.41)	0.322
Timothy	8.1% (5/62) 1.00	16.1% (10/62) 2.37 (0.68–8.30)	16.1% (10/62) 2.43 (0.69–8.52)	16.1% (10/62) 2.37 (0.70–8.06)	0.269
Rye	8.1% (5/62) 1.00	14.5% (9/62) 1.98 (0.57–6.84)	12.9% (8/62) 1.90 (0.52–7.01)	12.9% (8/62) 1.79 (0.49–6.52)	0.480
Birch	11.3% (7/62) 1.00	9.7% (6/62) 0.76 (0.21–2.76)	6.5% (4/62) 0.33 (0.07–1.63)	12.9% (8/62) 1.23 (0.38–3.92)	0.944

CIS expression was analysed by quantitative real-time PCR from cDNA/mRNA samples prepared from heparinized whole blood samples without further stimulation *ex vivo*.

*Adjusted for: family atopy history, gender, siblings, parental school education, pet keeping, smoking in the dwelling and breastfeeding.

OR, odds ratio; CI, confidence interval; CIS, cytokine-induced Src-homology 2 protein.

numbers of positive cases, associations between SOCS1, SOCS3, SOCS5 and CIS expression and sensitization to the single food allergens hen's egg (3.0%), cow's milk (6.5%), peanut (3.6%), fish (1.2%), wheat (2.8%), soy (2.4%) and to the single inhalative allergens cat (7.3%), dog (4.0%) and mugwort (6.5%) could not be calculated (Table 2). Thus, these allergens are not included in the presentation of the results.

Discussion

Among the investigated SOCS molecules, only SOCS1 showed a strong correlation to the development of allergic sensitizations. This result is supported by the positive association between SOCS1 expression and the Th2 differentiation markers GATA-3 and IL-4. In addition, we observed a negative correlation between SOCS1 and Tbet, the key transcription factor for Th1 cells, and a failing association between SOCS1 and the Treg factor FOXP3. For SOCS3, SOCS5 and CIS, a contradictory picture was found. The expression of these SOCS molecules was positively correlated with Tbet and FOXP3 and (with the exception of CIS) negatively with IL-4. SOCS3, SOCS5 and CIS were of no relevance for the allergic status.

Although whole blood was used for the expression analysis and the proportions of different cell types may differ in different samples, we were able to observe correlations between the expression of SOCS molecules and T cell regulatory factors. Even if both SOCS and T cell regulatory factors are not always co-expressed in the same cell, there should be a functional association between these factors.

It has been speculated that Th1 down-regulation by SOCS1 may not only result in an accelerated Th2 response but also in Th2-related diseases like allergy [34]. Our data obtained in the human system support this hypothesis and confirm the findings from mouse models. In association with up-regulated SOCS1 expression, not only reduced numbers of IFN- γ -producing Th1 cells but also higher sensitization frequencies were found. In addition, we found that a high expression of SOCS1 correlates with high values of IL-4 and GATA-3 expression. We hypothesize that the elevated SOCS1 expression supports the development of a Th2 phenotype and subsequently allergic sensitization. But we cannot exclude the possibility that the higher SOCS1 expression level occurs as a result of Th2-polarized T cell reactivity.

Mouse studies showed a high expression of SOCS1 in regulatory T cells [25, 26]. In our study, SOCS1 was not correlated to FOXP3 expression, the marker of regulatory T cells. Because in this study SOCS1 expression was not directly assessed in Treg and Treg, represents only a minor subset of blood T cells, this result is not contradictory to previously reported data.

Overexpression studies showed that SOCS1 and SOCS3 inhibit IL-4 and IL-13 signalling [35]. These data appear to be contradictory to the positive correlation observed between SOCS1 and allergic sensitization. However, it has been clearly shown that IL-4/STAT6 signalling is repressed in SOCS1 expressing Th1 cells but not in Th2 cells that contain relatively low amounts of SOCS1 [10, 36]. Thus, high SOCS1 expression levels may lead to Th1 inhibition but do not influence the Th2-mediated IL-4 signalling.

SOCS3 is expressed in Th1 as well as Th2 cell populations. However, cells destined to become Th2 cells produce SOCS3 levels more than 20-fold higher than that produced by Th1 cells [10]. SOCS3 also represses IFN- γ signalling but less potently than SOCS1 [11, 14]. It has been postulated that SOCS3 regulates the onset and maintenance of Th2-mediated allergic diseases [15]. SOCS3-transduced dendritic cells in mice are inducers of Th2 cell differentiation. In addition, it has been shown that the absence of SOCS3 in dendritic cells results in the induction of FOXP3-positive Treg [37]. Moreover, in *in vitro* experiments it has been demonstrated that FOXP3 acts as a transcriptional suppressor for SOCS3 and this might be a mechanism by which T lymphocytes regulate SOCS3 levels to perpetuate the Treg phenotype [22]. In our study, we observed a positive correlation of SOCS3-FOXP3. This seems to be contradictory to the *in vitro* found data. Again, it might be explicable by the fact that SOCS3 is not only expressed in T cells but also in other immune cells like dendritic cells, macrophages, neutrophils, eosinophiles and natural killer T cells.

SOCS3 mRNA expression in the blood of children was negatively correlated with IL-4 mRNA expression and positively correlated with Tbet. Although SOCS3 expression levels have consistently been found to be higher in allergic patients, we observed no correlation between SOCS3 expressions and serum IgE concentrations. The negative correlation between IL-4 and SOCS3 and the positive correlation between SOCS3 and Tbet may support the failing association between SOCS3 and the allergic status observed in our study population. Data from a further study showing that SOCS3 is also expressed in normal unstimulated peripheral blood cells [38] may help to explain these obviously reverse results from our study compared with previously reported data. Children have higher amounts of naïve unstimulated cells in their blood and lower levels of Th2 cells. Thus, a higher SOCS3 expression in unstimulated peripheral blood cells may overlay the probably higher SOCS3 expression in a small Th2 population.

In contrast to SOCS3, the expression of SOCS5 is limited to Th1 cells and is undetectable in Th2 cells [16]. Therefore, SOCS5 has been suggested to be a marker of the Th1 phenotype. A cytokine environment that favours Th1 commitment may induce SOCS5 expression, leading to a concurrent suppression of IL-4-induced STAT6 activation

[16, 18]. Thus, it has been speculated that SOCS5 exerts an inhibitory function on Th2 polarization. This is consistent with our SOCS5 expression data. We found that SOCS5 was negatively correlated with IL-4 mRNA and strong positively correlated with Tbet. However, although SOCS5 expression was negatively correlated with IL-4, there was also a slightly positive correlation of SOCS5 with GATA-3. Up to now, we cannot explain this contradictory result. The *in vivo* role of SOCS5 in T-helper cell differentiation, thus, remains to be clarified.

In the present study, CIS was positively correlated with GATA-3 as well as with Tbet; this supports previous findings that CIS is expressed in both Th1 and Th2 cells [12, 18]. Moreover, CIS expression was also correlated with FOXP3, suggesting that CIS might also be expressed in regulatory T cells. In mice, CIS has been shown to skew T-helper cell development towards Th2 [18, 19]. Our results cannot confirm these observations in the human system.

However, one general remark has to be made concerning the comparability of the data presented in this study with earlier published data. It has been shown that naïve CD4 T cells express lower levels of SOCS as well as Tbet, GATA-3 and FOXP3 compared with primed Th1 or Th2 cells [10, 39, 40]. The expression analyses in our study were carried out with whole-blood samples containing a mixture of naïve, effector and memory T cell subsets. This is likely the main reason why we have controversial results compared with previous data.

To the authors' knowledge, this study analyses for the first time the relationship between SOCS factors and allergic sensitization against the background of a population-based study. Besides the opportunity to study SOCS expression in a healthy population, a further strength of our study is the high number of study subjects included. In addition, no data on SOCS regulation in children were available so far. However, as usual, in population-based studies we also have the limitation to define the cause-effect relation in our observed associations. Another limitation of this study is the low case number of children sensitized for some specific allergens. The results obtained, therefore, have to be interpreted with caution, but nevertheless they provide orientation and encourage for further studies.

In summary, our data show that SOCS1 correlates with Th2 development and a higher risk for allergic sensitizations whereas SOCS3, SOCS5 and CIS are associated with an activation of Th1 and Treg and thereby are not relevant or even protective for allergy development. Although the molecular bases of SOCS molecule activity have been extensively investigated, there are still open questions concerning the *in vivo* relevance of these molecules in particular in the human system. It has been suggested that the current cytokine environment may

influence the expression profile of SOCS molecules. Thus, in the *in vivo* situation a different microenvironment compared with the situation in experimental settings may induce other SOCS profiles. Further investigations are necessary to clarify the role of SOCS molecules in *in vivo* situations.

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