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A SOCS-1 Promoter Variant Is Associated with Total Serum IgE Levels

Justin Mostecky,^{*,1} Suzanne L. Cassel,^{†,1} Walter T. Klimecki,[‡] Debra A. Stern,[‡] Judit Knisz,[†] Sachiyo Iwashita,[†] Penelope Graves,[‡] Rachel L. Miller,[§] Maartje van Peer,^{*} Marilyn Halonen,[‡] Fernando D. Martinez,[‡] Donata Vercelli,[‡] and Paul B. Rothman[†]

SOCS-1 is a critical regulator of multiple signaling pathways, including those activated by cytokines that regulate Ig H chain class switching to IgE. Analysis of mice with mutations in the *SOCS-1* gene demonstrated that IgE levels increase with loss of *SOCS-1* alleles. This suggested that overall SOCS-1 acts as an inhibitor of IgE expression in vivo. A genetic association study was performed in 474 children enrolled in the Tucson Children's Respiratory Study to determine if genetic variation in the SOCS-1 locus correlates with altered levels of IgE. Carriers of the C-allele for a novel, 3' genomic single nucleotide polymorphism (SNP) in the *SOCS-1* gene (*SOCS1*+1125G > C; rs33932899) were found to have significantly lower levels of serum IgE compared with those of homozygotes for the G-allele. Analysis demonstrated that the *SOCS1*+1125G > C SNP was in complete linkage disequilibrium with an SNP at position *SOCS1*-820G > T (rs33977706) of the SOCS-1 promoter. Carriers of the T-allele at the *SOCS1*-820G > T were also found to be associated with the decreased IgE. The promoter SNP increased transcriptional activity of the SOCS-1 promoter in reporter assays and human B cells. Consistent with this observation, the presence of this polymorphism within the promoter abolished binding of yin yang-1, which is identified as a negative regulator of *SOCS-1* transcriptional activity. These data suggest that genetic variation in the *SOCS-1* promoter may affect IgE production. *The Journal of Immunology*, 2011, 187: 000–000.

Asthma and other allergic diseases are increasingly prevalent in both developing and Westernized nations (1). The immunological hallmark of allergic diseases is elevated total and allergen-specific IgE levels along with inflammation. The immune response underlying the inflammation found in allergic states is extremely complex, involving interplay between cytokines, chemokines, and cells within the innate and adaptive immune system. Current models invoke activation of Th2-type memory/effector CD4⁺ T cells by allergen as a major initiator of allergic inflammation (reviewed in Ref. 2). The ensuing production of the Th2 cytokines IL-4, IL-5, and IL-13 by activated CD4⁺ T cells promotes the migration and activation of inflammatory cell types such as basophils, eosinophils, and mast cells within the affected tissue. These cells in turn release soluble factors that promote and exacerbate the inflammatory state. In addition to cells within the immune system, IL-4 and IL-13 also interact with receptors expressed on non-hematopoietic cells, thus contributing to allergic inflammation by inducing functional

responses in these populations. In addition to elevated levels of Th2 cytokines, atopic individuals can exhibit increased "baseline" IgE levels and IgE-reactive cells even prior to an allergic reaction. As with allergic inflammation, IL-4 and IL-13 are inducers of class switching to IgE by B cells (3). Conversely, IFN- γ can suppress class switching to IgE (4, 5).

One of the essential properties of cytokines is their limited duration of action. This property leads to the effective curtailment of immune responses once an Ag or allergen is removed from the responding organ. Recent studies have demonstrated that cytokine signaling is limited by several mechanisms. A family of proteins, termed SOCS, appears to be essential for the normal control of cytokine action in vivo. The eight members of the SOCS gene family are characterized by a C-terminal SOCS-Box region and a central SH2 domain (6). SOCS-1, also called JAK binding protein-1 or STAT-induced STAT inhibitor-1, is an important regulator of many cytokine signaling pathways including those activated by cytokines that regulate IgE class switching (7–9). The SOCS-Box region is important for the recruitment of the ubiquitin transferase apparatus that targets SOCS-1-interacting proteins for proteasome-mediated degradation (10, 11). SOCS-1 has been shown to interact with all four JAK kinases through its SH2 domain and inhibit their tyrosine kinase activity in vitro (8, 9, 12). Thus, SOCS-1 negatively regulates the action of IL-4, which promotes IgE production, as well as IFN- γ , which suppresses it.

SOCS-1^{-/-} mice, although normal at birth, display stunted growth with a multiorgan disease that is characterized by lymphopenia, fatty acid degeneration of the liver, and macrophage infiltration of various tissues, followed by death prior to 3 wk of age (13). Lethality can be significantly delayed on the *RAG2*^{-/-}, *IFN- γ* ^{-/-}, *STAT1*^{-/-}, and *STAT6*^{-/-} backgrounds, thus implicating SOCS-1 as a critical regulator of both the IFN- γ and IL-4 signaling pathways (14, 15). Many of the phenotypes associated with SOCS-1 deficiency can be reconstituted by the transfer of

*Department of Microbiology, Columbia University, New York, NY 10032;

[†]Department of Medicine, University of Iowa, Iowa City, IA 52242; [‡]Arizona Respiratory Center, University of Arizona, Tucson, AZ 85724; and [§]Department of Medicine, Columbia University, New York, NY 10032

¹J.M. and S.L.C. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Paul B. Rothman, University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242. E-mail address: paul-rothman@uiowa.edu

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Abbreviations used in this article: AA, African ancestry; EA, European ancestry; LD, linkage disequilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism; YY1, Yin Yang-1.

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SOCS-1^{-/-} bone marrow into JAK3^{-/-} mice, suggesting that the pathology is mediated by hematopoietic cells (15). However, specific deletion of *SOCS-1* in the thymocyte/T/NKT cell compartment is not sufficient to result in lethal multiorgan disease, although it leads to abnormalities including both elevated levels of CD8⁺ T cells and increased sensitivity to common γ -chain cytokines (16). Consistent with this observation, *SOCS-1*^{-/-} dendritic cells induced increased IFN- γ - and IL-4-mediated responses, suggesting an important role for SOCS-1 in non-lymphoid cell function as well (17). Furthermore, altered transcriptional activity of *SOCS-1* may have important ramifications for the cytokine unresponsiveness demonstrated by many tumors, as the gene has been shown to be silenced by CpG methylation in hepatocellular carcinoma (18) and in multiple myeloma (19).

Cytokines and their downstream signaling pathways are critical regulators of the immune response. Human genetic studies have demonstrated that polymorphisms affecting genes encoding cytokines or components of cytokine signaling pathways are strongly associated with allergic diseases phenotypes (reviewed in Ref. 20). One of the most replicated findings is the linkage of atopy with polymorphisms within the human chromosome 5q31-33, which contains genes encoding the cytokines IL-4, IL-5, and IL-13 (21–23). In addition, polymorphisms in the signaling molecule STAT6, which is activated downstream of IL-4 and IL-13, have also been associated with an increased risk of asthma (24–28). Considered together with functional studies establishing that IL-4 and IL-13 are central mediators of allergic inflammation, these data argue that the IL-4 and IL-13 signaling pathways have a critical and perhaps predominant role in atopic disease development. Our analysis showed that the loss of one copy of SOCS-1 results in increased total and Ag-specific IgE production in mice. This suggests that alteration in the SOCS-1 locus may alter IgE levels in human as well. Indeed, our study identified a single nucleotide polymorphism (SNP) within the SOCS-1 locus (*SOCS1*+1125G > C; rs33932899) that is associated with lower levels of total serum IgE. An additional SNP (*SOCS1*–820G > T; rs33977706) is in strong linkage disequilibrium with the *SOCS1*+1125G > C SNP, also associated with lower levels of IgE, and increases the transcriptional activity of the *SOCS-1* promoter in reporter assays and in human CD19⁺ cells. Further, the *SOCS1*–820T SNP decreases the ability of Yin Yang-1 (YY1), a negative regulator of *SOCS-1* transcriptional activity, to bind *SOCS-1* promoter sequences in vitro. These data suggest that genetic variation in the *SOCS-1* locus may affect IgE levels in humans through loss of negative regulation of SOCS-1 with resultant increased SOCS-1 expression blocking the IL-4-mediated class switch to IgE.

Materials and Methods

Tucson Children's Respiratory Study subjects

Anonymous DNA samples from healthy individuals of self-reported ancestry were obtained from the Coriell Cell Repositories (Camden, NJ). Twenty-three samples from individuals of European ancestry (EA) and 24 samples from individuals of African ancestry (AA) were studied. Repository numbers are as follows. African-American DNA: NA17101, NA17102, NA17103, NA17104, NA17105, NA17106, NA17107, NA17108, NA17109, NA17110, NA17111, NA17112, NA17113, NA17114, NA17115, NA17116, NA17133, NA17134, NA17135, NA17136, NA17137, NA17138, NA17139, and NA17140. CEPH DNA: NA12560, NA12547, NA10845, NA10853, NA10860, NA10830, NA10842, NA10851, NA07349, NA10857, NA10858, NA10848, NA12548, NA10844, NA10854, NA10861, NA10831, NA10843, NA10850, NA07348, NA10852, NA06990, and NA07019.

The characteristics of the children enrolled in the Tucson Children's Respiratory Study, comparisons of participants with and without available DNA samples, and methods used to assess total serum IgE levels have been described in detail elsewhere (29).

Tucson Children's Respiratory Study subjects PCR

Genomic sequences for *SOCS-1* were accessed from the University of California at Santa Cruz Human Genome Browser, November 2002 freeze. PCR amplicons were designed completely to traverse the gene, such that each amplicon was ~900 bp in length, and consecutive amplicons overlapped each other by ~200 bp. PCR reactions contained 20 ng genomic DNA, 1 pmol of each primer, 0.2 U Platinum *Taq* polymerase (Invitrogen), and 0.1 μ M dNTPs in a total volume of 10 μ l. Specific reaction conditions including primer sequences are available from the authors.

Tucson Children's Respiratory Study subjects direct PCR sequencing

PCR amplicons were prepared for cycle sequencing by diluting them with water using a dilution range of 1:3 to 1:6, depending on the reaction yield as determined by agarose gel electrophoresis. Cycle sequencing reactions were assembled using 0.4 μ l of cycle sequencing premix (BigDye V3.0), 1 pmol sequencing primer, 1.8 μ l 5 $\times$ sequencing dilution buffer, and 5 μ l PCR product in a final volume of 10 μ l. Cycle sequencing reactions were purified using DNA-affinity magnetic beads (Agencourt Biosciences). Purified sequencing reactions were electrophoretically analyzed using a DNA Analyzer 3730 (Applied Biosystems).

Tucson Children's Respiratory Study subjects polymorphism identification and analysis

Sequence chromatograms were processed for base calling and assembly using the Phred, Phrap, and Consed suite of software programs (30, 31). Initial polymorphism tagging was performed using Polyphred, with a minimum sequence quality of phred 25 (32). Potential polymorphic sites that were initially identified by Polyphred were individually confirmed by visual inspection of sequence traces. A criterion of this visual inspection—confirmation was that the polymorphism must be observed in multiple chromatograms from singleton polymorphisms (polymorphisms occurring in only one subject), or in multiple subjects. For these confirmed polymorphic sites, each genotype for each subject was also confirmed by visual inspection of chromatograms. Polymorphic sites and associated subject-identified genotypes were automatically output to a relational database for further analysis, which included the automated generation of ethnicity-specific genotype frequencies, allele frequencies, and goodness of fit tests for Hardy-Weinberg equilibrium. Haplotypes were inferred using a Gibbs-sampling algorithm as implemented in the Phase software program (33). Because the accuracy of statistically inferred haplotypes has been shown to increase with increasing haplotype frequency, we used polymorphisms with a minor allele frequency (MAF) ≥ 0.10 to define relatively common haplotypes (34). Pairwise linkage disequilibrium was calculated as r^2 , a measure of the product-moment correlation coefficient (35).

Tucson Children's Respiratory Study subjects gene context of polymorphisms

Each gene was annotated graphically using the Artemis software program (36). Annotations were based on mapping cDNA GenBank accession number AB000734 to the human genomic sequence. This produced a gene structure for SOCS1 containing two exons, the first of which is untranslated, followed by a 552-bp intron. The translation initiation site is 49 bp from the 5' aspect of exon 2.

Human B cells

Whole-blood samples from anonymous human donors were obtained from the DeGowin Blood Center (University of Iowa Hospitals and Clinics). Samples were diluted with an equal volume of sterile PBS and mononuclear cells isolated using Histopaque 1077 (Sigma) following the manufacturer's instructions. CD19⁺ cells were positively selected using CD19 microbeads (Miltenyi Biotec) following the manufacturer's instructions. Cells were cultured at 4×10^6 cells/ml for 24 h, after which cells were harvested and genomic DNA and RNA isolated.

Human B cells real-time PCR

RNA was isolated from cultured CD19⁺ cells using TRIzol (Invitrogen) following the manufacturer's protocol. mRNA was transcribed to cDNA using Superscript III 1st Strand Synthesis Supermix (Invitrogen) following the manufacturer's protocol. Real-time PCR was performed with SYBR Green and SOCS1 primers (forward 5'-GAA CTG CTT TTT CGC CCT TA-3', reverse 5'-CTC GAA GAG GCA GTC GAA G-3'), and β -Actin primers (forward 5'-GGC ATC CTC ACC CTG AAG TA-3', reverse 5'-

AGG TGT GGT GCC AGA TTT TC-3') according to the manufacturer's instructions (Applied Biosystems). PCR was performed using Applied Biosystems Model 7000 and analyzed with Applied Biosystems Sequence detection software version 1.2.3.

Human B cells SNP genotyping

Genomic DNA was isolated from human CD19⁺ cells using Qiagen DNA Blood mini kit (Qiagen) according to the manufacturer's protocol. Genotype at the -820 SNP was determined using TaqMan SNP Genotyping Assays ID C60261090_10 and C3189845_20 according to the manufacturer's protocol. End-point analysis was performed using Applied Biosystems Model 7000 and analyzed with Applied Biosystems Sequence detection software version 1.2.3.

Plasmids

Regions of the SOCS-1 promoter were cloned into the BglIII and MluI sites of the pGL3 Basic Vector (Promega) using primers with the appropriate restriction sites. The common sequence was cloned from the NA12547 DNA sample, and the sequence containing a T at the -820 position was cloned from the NA17135 DNA sample. All plasmids were confirmed by sequencing. The pRL-TK *Renilla* luciferase plasmid was purchased from Promega.

Cell lines and transfection

The RAW/FPR.10 clone derived from the RAW 264.7 murine macrophage cell line was obtained from Dr. Steve Greenberg (Columbia University) (37). Transient transfections were performed using the Lipofectamine reagent (Invitrogen) as per the manufacturer's instructions with 1 μ g of reporter plasmid and 100 ng of *Renilla* luciferase control plasmid (Promega) for 4 h. After this period, the transfection mixture was removed and replaced with media with or without 100 ng/ml LPS for 20 h. Cell extracts were subsequently prepared and assayed using the Dual Luciferase Kit (Promega) per the manufacturer's instructions.

Mice

The B6.129S7-Ifng^{tm1Ts}/J mice were purchased from The Jackson Laboratory, and the *SOCS-1*^{-/-} mice were provided by Dr. James Ihle (St. Jude Children's Research Hospital).

Assay of serum Ig levels and allergen-specific responses

Total serum Ig levels were assessed by ELISA using the anti-Ig Abs (BD Biosciences) per the manufacturer's protocol. Ag-specific responses were assessed by injecting mice i.p. with 50 μ g OVA and 2 mg alum at 4 wk of age and measuring OVA-specific IgE serum levels at 6 wk of age. Levels of OVA-specific IgE were determined by ELISA, precoating plates with 50 μ g/ml OVA instead of the anti-IgE capture Ab. An anti-OVA IgE mAb standard (2C6) (38) was provided by Dr. Lester Kobzik (Brigham and Women's Hospital).

EMSA

Preparation of nuclear extracts has been described previously (39). Binding reactions were performed for 15 min at room temperature in 20 μ l buffer containing 5 mM HEPES pH 7.9, 20% glycerol, 2.5 mM MgCl₂, 2.5 mM DTT, 500 ng dIdC, and 40 μ g BSA. Competitions and supershift experiments included a 15-min room temperature incubation step with unlabeled competitor oligonucleotides (10-, 50-, and 100-fold molar excess) or Ab (0.8 μ g), respectively, prior to the addition of the labeled probe. Supershift analyses were performed using anti-YY1 mAb (Active Motif). Probe sequences are as follows: *SOCS-1*-820G (-827/-813): 5'-gatcAAAAGTGGAGCTGGG-3' and 5'-gatcCCAGCTCCACTTTT-3'. *SOCS-1*-820T (-827/-813): 5'-gatcAAAAGTGTAGCTGGG-3' and 5'-gatcCCAGCTCACTTTT-3'. The polymorphic -820 position is underlined.

Results

IgE levels increase as levels of *SOCS-1* decrease in mice

Given the importance of SOCS-1 in regulating both IL-4 and IFN- γ signaling, it is difficult to predict how alterations in SOCS-1 expression may influence IgE levels. To determine the importance of SOCS-1 in regulating Ig production, two animal model systems (*SOCS-1*^{+/-} versus *SOCS-1*^{-/-} and *IFN- γ* ^{-/-} mice, *SOCS-1*^{+/-} versus *IFN- γ* ^{-/-}, *SOCS-1*^{-/-} mice) were compared. A comparison between *SOCS-1*^{+/-} and *SOCS-1*^{-/-} mice was not performed

because the *SOCS-1*^{-/-} mice suffer from a severe multiorgan pathology due most significantly to a failure properly to regulate IFN- γ signaling. Instead, we examined the levels of Igs in *SOCS-1*^{+/-} mice, which do not demonstrate any of these severe pathologies (13, 40). In this effort, we were encouraged by a previous study showing elevated IgE production in *SOCS-1*^{+/-} but not *SOCS-1*^{+/+} control mice after challenge with *Nocardia brasiliensis*, a pathogen that elicits Th2 responses (41). This suggests that the lack of one copy of *SOCS-1* can alter Ab responses in vivo. We examined levels of IgM, IgG_{2a}, IgG1, and IgE in wild-type and *SOCS-1*^{+/-} mice. Fig. 1A shows that basal levels of IgE were 2.5-fold higher ($p = 0.023$) among *SOCS-1*^{+/-} mice than those in *SOCS-1*^{+/+} littermates. In contrast, the levels of the other Ig isotypes were not different. These data demonstrate an important role for SOCS-1 in the regulation of basal IgE levels. In order to examine mice that lack both copies of *SOCS-1*, *SOCS-1*-deficient mice were crossed onto the *IFN- γ* ^{-/-} background (129SV), and Ig production was assessed (Fig. 1B). Consistent with the findings discussed above, basal levels of serum IgE were 11.5-fold higher among *IFN- γ* ^{-/-}, *SOCS-1*^{-/-} mice compared with those of the *IFN- γ* ^{-/-}, *SOCS-1*^{+/+} littermate controls ($p = 0.002$). The IgE levels in the *IFN- γ* ^{-/-}, *SOCS-1*^{+/-} littermates were intermediate and significantly different from both *IFN- γ* ^{-/-}, *SOCS-1*^{+/+} ($p = 0.001$) and *IFN- γ* ^{-/-}, *SOCS-1*^{-/-} ($p = 0.007$) mice, corroborating the importance of *SOCS-1* gene dosage in IgE regulation. No other significant alterations in Ig levels were observed for IgM, IgG1, or IgG_{2a} in these mice, implying that the effects of SOCS-1 dysregulation were specific to IgE (Fig. 1A, 1B).

To determine whether SOCS-1 is important for the regulation of Ag-specific IgE responses, serum levels of OVA-specific IgE were assessed 2 wk after immunization with a single OVA-alum i.p. injection (Fig. 1C). The *IFN- γ* ^{-/-}, *SOCS-1*^{+/-} mice demonstrated a 2.1-fold increase in mean OVA-specific IgE levels in comparison with those of *IFN- γ* ^{-/-}, *SOCS-1*^{+/+} controls ($p = 0.029$), confirming that SOCS-1 is an important regulator of Ag-specific IgE responses in vivo. The *IFN- γ* ^{-/-}, *SOCS-1*^{-/-} mice showed an even higher (4.1-fold) increase in mean anti-OVA IgE relative to the *IFN- γ* ^{-/-}, *SOCS-1*^{+/+} mice ($p = 0.045$), whereas OVA-specific IgE levels in *IFN- γ* ^{-/-}, *SOCS-1*^{+/-} mice were not significantly different. Collectively, these data demonstrate that SOCS-1 is an important regulator of IgE levels in vivo, and deletion of a single SOCS-1 allele is sufficient to significantly alter both basal and Ag-specific IgE responses.

Genetic variants and haplotypic structure in the human *SOCS-1* locus

Our results from the *SOCS-1*-deficient mice prompted us to assess whether variants within the *SOCS-1* locus might be associated with altered IgE levels. To this purpose, we started by analyzing genetic variation in the *SOCS-1* genomic locus. Sequencing of DNA samples of both AA and EA identified 21 polymorphisms, which were observed in the ~3.5-kb region that contains *SOCS-1* (Fig. 2A). Of these polymorphisms, 20 were observed in AA samples and 9 in EA samples (Fig. 2B). Although one polymorphism was unique to Europeans, and 12 SNPs were observed only in AA samples, most high-frequency polymorphisms were shared by the two ethnic groups. Notably, although overall nucleotide variability was high, no polymorphic sites were found within the *SOCS-1* coding region. Given the relatively small genomic region resequenced in this study, the full extent of linkage disequilibrium (LD) among high-frequency polymorphic sites (in this article arbitrarily defined as having a MAF in excess of 10%) could not be determined.

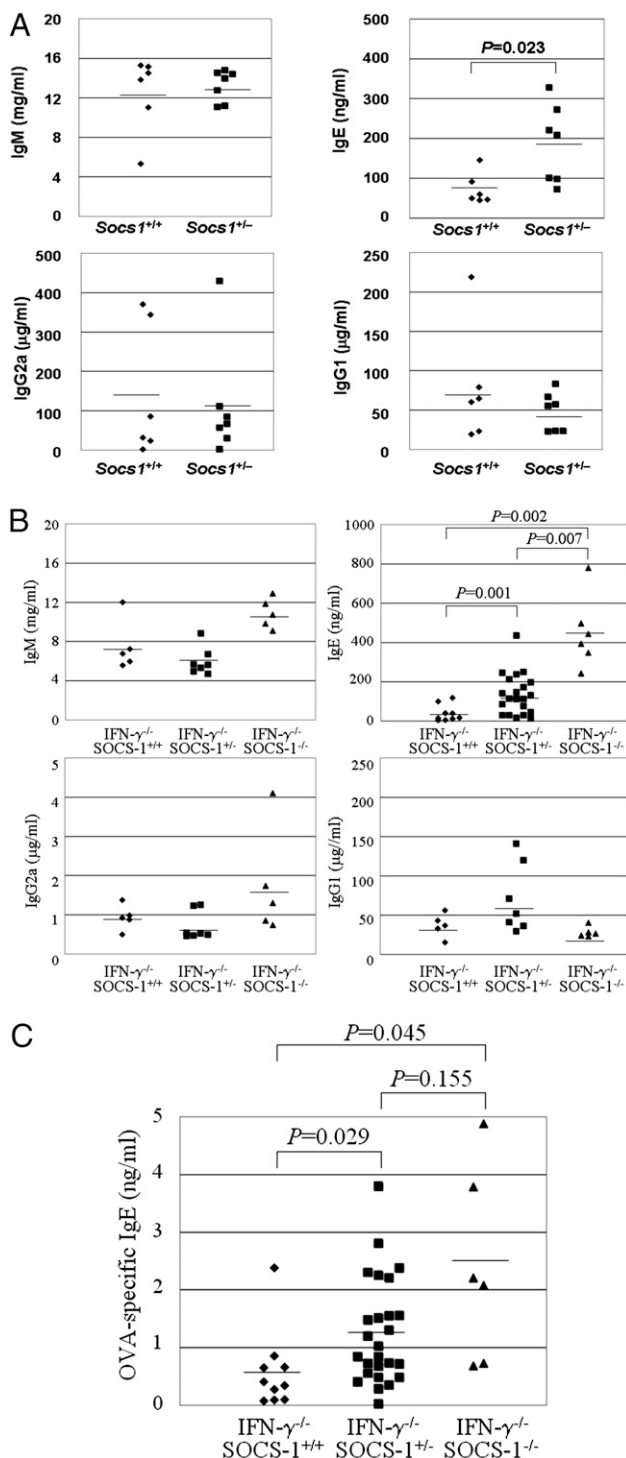


FIGURE 1. Elevated IgE levels in mice lacking *SOCS-1*. **A**, The total baseline unstimulated IgM, IgE, and IgG1 serum concentrations in 4-wk-old *SOCS-1*^{+/+} mice ($n = 7$) and *SOCS-1*^{-/-} littermate controls ($n = 6$) were assessed by ELISA. **B**, The total baseline unstimulated Ig serum concentrations were compared in 6-wk-old *IFN-γ*^{-/-}, *SOCS-1*^{+/+} ($n = 5$), *IFN-γ*^{-/-}, *SOCS-1*^{+/-} ($n = 7$), and *IFN-γ*^{-/-}, *SOCS-1*^{-/-} ($n = 5$) littermate mice by ELISA. The total baseline unstimulated IgE serum concentrations were compared in *IFN-γ*^{-/-}, *SOCS-1*^{+/+} ($n = 9$), *IFN-γ*^{-/-}, *SOCS-1*^{+/-} ($n = 22$), and *IFN-γ*^{-/-}, *SOCS-1*^{-/-} ($n = 6$) littermate mice by ELISA. **C**, *IFN-γ*^{-/-}, *SOCS-1*^{+/+} ($n = 10$), *IFN-γ*^{-/-}, *SOCS-1*^{+/-} ($n = 26$), and *IFN-γ*^{-/-}, *SOCS-1*^{-/-} ($n = 6$) littermate mice were injected at 4 wk of age with OVA (50 μg)/alum (2 mg), and OVA-specific IgE serum levels were determined at 14 d postinjection by ELISA. Each shape indicates the concentration from one mouse with the mean noted by a line. The statistical significance was determined using a Student *t* test.

Decreased levels of serum IgE in children with the *SOCS1*+1125C SNP

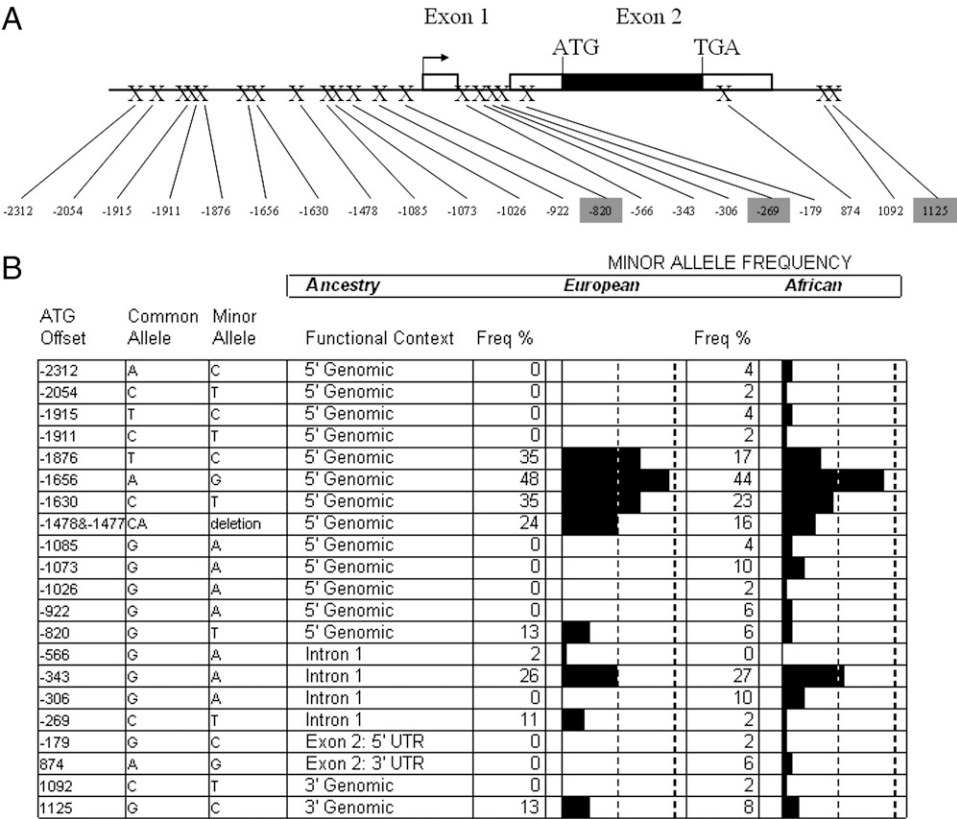
Three SNPs (*SOCS1*-1630C > T, *SOCS1*-343G > A, and *SOCS1*+1125G > C), which tagged the three bins most frequent in Europeans, were genotyped in 488 unselected participants in the Tucson Children's Respiratory Study who had serum IgE levels measured at 11 y of age. Sample sizes varied slightly for each SNP, and all four SNPs were in Hardy-Weinberg equilibrium (data not shown). There was no association between total serum IgE and *SOCS1*-1630C > T or *SOCS1*-343G > A (data not shown). In contrast, genotypes for *SOCS1*+1125 were significantly associated by total serum IgE [ANOVA with 2 df, $p = 0.035$ (Fig. 3); no genotype could be obtained in 19 subjects]. Inspection of the results showed that carriers of the *SOCS1*+1125CC and *SOCS1*+1125CG genotypes had significantly lower total serum IgE levels than *SOCS1*+1125GG homozygotes, indicating a dominant effect of the C-allele, and the corresponding test (CC+CG versus GG) was highly significant ($p = 0.009$). Results remained significant after adjusting for ethnic background and limiting the analysis to white, non-Hispanic children ($p = 0.04$). There was no significant association between any *SOCS-1* polymorphism and specific IgE to common aeroallergens (data not shown).

A *SOCS-1* promoter with a T at the -820 position demonstrates increased activity

SOCS1+1125G > C is in near complete LD with *SOCS1*-820G > T (noted by the solid arrow in Fig. 4), an SNP located within a region of the *SOCS-1* gene known to be important for the regulation of *SOCS-1* promoter activity. The *SOCS1*-820G > T region is adjacent to an IFN regulatory factor-binding element that regulates the *SOCS-1* promoter in response to LPS (42). This suggested the possibility that an alteration in the ability of LPS to regulate *SOCS-1* due to the *SOCS1*-820T SNP might factor into the lower levels of IgE observed in the population study. Additionally, there is an Sp1/Sp3-binding GC-Box located next to the *SOCS1*-820 region (Fig. 4). Alignment of the human and murine *SOCS-1* promoter demonstrates that both of these sites are conserved (data not shown). The proximity to these two functional elements suggested the possibility that *SOCS1*-820G > T might influence the transcriptional activity of the *SOCS-1* promoter.

To confirm the association of the *SOCS1*-820G > T SNP with IgE levels as implied by its linkage analysis with *SOCS1*+1125G > C, the genotypes at *SOCS1*-820 were determined for the patient samples analyzed earlier and compared with serum IgE levels. As shown in Supplemental Fig. 1, significantly lower total serum IgE levels were associated with a dominant effect of the T-allele for *SOCS1*-820. However, the relation was not statistically significant after adjusting for ethnic background ($p = 0.063$) and limiting the analysis to white, non-Hispanic children ($p = 0.12$), though the effect size for both was similar to that seen in the whole population. The decreased significance observed for the *SOCS1*-820/IgE association likely resulted from still unexplained phenotypic fluctuations within the population. Even though *SOCS1*+1125G > C and *SOCS1*-820G > T were in high linkage disequilibrium, a small group of children ($n = 17$) with a geometric mean total serum IgE of 42.4 IU/ml were GG homozygous at *SOCS1*-820 and heterozygous at *SOCS1*+1125G > C. IgE levels placed these subjects in the low-IgE group for *SOCS1*+1125 but in the high-IgE group for *SOCS1*-820. Therefore, data from these 17 children appeared to contribute to the decreased significance observed for the relationship between IgE levels and *SOCS1*-820. Indeed, excluding this group from the analysis, carriers of *SOCS1*-820 GT and TT had significantly

FIGURE 2. The *SOCS-1* genomic locus and SNP LD groups. **A**, The *SOCS-1* genomic locus is shown with exons depicted as boxed regions. The shaded coding region is entirely within the second exon; the translational start and stop codons are illustrated above. The transcriptional start site as defined by the cDNA sequence of *SOCS-1* (GenBank accession number NM_003745) is denoted with the bent arrow. SNP locations are shown with an “X” and numbered relative to the A(+1) of the translation ATG start codon (GenBank accession number DQ086801). **B**, SNPs from the sequencing of 23 EA and 24 AA patients are listed with the MAF shown on the right.



lower total serum IgE levels than those of *SOCS1*–820GG homozygotes ($p = 0.018$). To test the possibility that *SOCS1*–820G > T could modify the activity of the *SOCS-1* promoter, *SOCS-1* promoter reporter constructs carrying either a G or T at the –820 position were generated and transiently transfected into the RAW 264.7 macrophage cell line. Previous experimental evidence demonstrated that LPS is capable of inducing endogenous *SOCS-1* expression in the RAW 264.7 cell line (43), and *SOCS-1* overexpression inhibits

LPS signaling in these cells (44). Previous experimental evidence showed that the –882/–659 region of *SOCS-1* contains important *cis*-acting elements within the *SOCS-1* promoter and is sufficient to drive maximal basal and LPS-induced transcriptional activity (42). These promoter constructs contain an IFN regulatory factor-binding element (position –839/–824) and an early growth response-1 binding site (–849/–841) that are required for the full LPS and IFN- γ response of the promoter (42). In an average of three experiments, the –820T (–882/–659) promoter construct containing the minor –820T allele demonstrated a 2.4-fold increase in transcriptional activity in comparison with that of the –820G (–882/–659) construct (Fig. 5A). Notably, the basal promoter activity of the –820T construct was also increased compared with the basal activity of –820G –882/–659 and –945/–659 constructs. Furthermore, a shorter construct (–819/–659) lacking the –820 site exhibited the lowest increase among all constructs tested in both basal and LPS-induced transcriptional activity. These data suggest that the *SOCS1*–820 site has a crucial role in regulating the *SOCS1* promoter and the presence of the *SOCS1*–820T allele in the *SOCS-1* promoter increases *SOCS-1* transcription. To confirm the relevance of these in vitro findings, the expression of *SOCS-1* in human cells was analyzed. The association of the genotype with modifications in IgE levels suggested the effect of the SNP would be found in B cells. Human CD19⁺ cells were purified and cultured in vitro. Expression of *SOCS-1* was measured via real-time PCR and found to be, in accordance with our in vitro promoter analysis, significantly higher in samples heterozygous or homozygous for the –820T SNP (Fig. 5B).

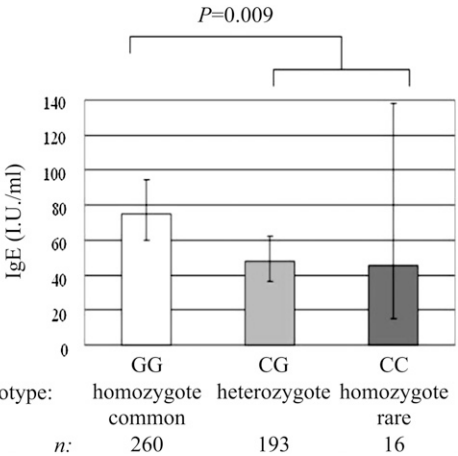


FIGURE 3. Decreased levels of IgE in non-selected children with the *SOCS1*+1125C allele. Total serum IgE levels were measured at 11 y of age in the Tucson Children’s Respiratory Study. IgE levels for the three *SOCS1*+1125 genotypes are shown along with the number of subjects with each genotype (n). Number of subjects, geometric means (in I.U.), and 95% confidence intervals were 260, 75.0 (59.7–94.2); 193, 47.7 (36.4–62.5); and 16, 45.4 (15.0–138.0) for the GG, CG, and CC genotypes, respectively. Genotype could not be determined in 19 patients.

The SOCS1–820T allele abolishes the ability of YY1 to bind to the SOCS-1 promoter

To determine the *trans*-acting factor(s) responsible for the altered transcriptional activity demonstrated by the *SOCS1*–820T

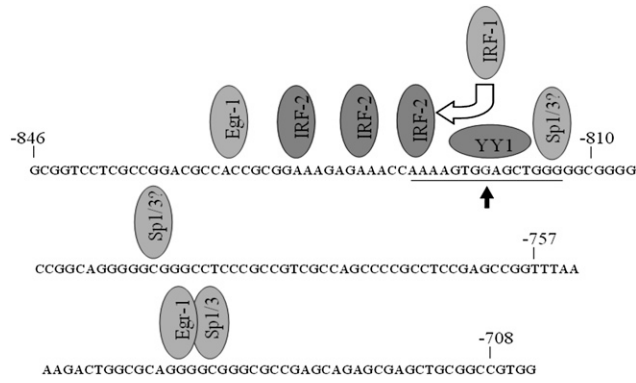


FIGURE 4. Structure of the human *SOCS-1* promoter region. The promoter sequence is numbered relative to the translation ATG start codon. The region that is important for the regulation of the *SOCS-1* promoter is underlined. The *SOCS-1*-820G > T SNP is marked by the solid arrow.

promoter, DNA-protein interactions occurring at the *SOCS-1* promoter were studied by EMSA using probes containing either a G (Fig. 6, lanes 3–15) or a T (Fig. 6, lanes 1 and 2) at the –820

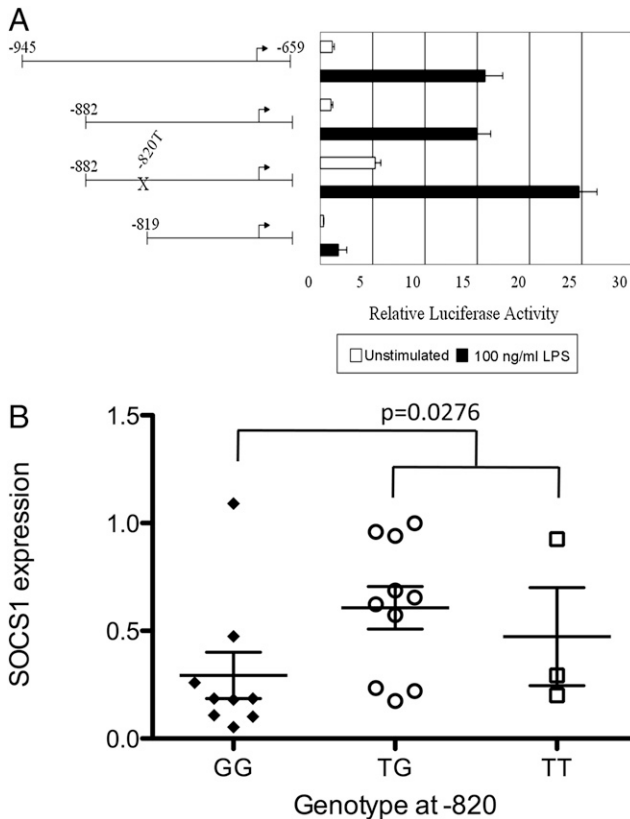


FIGURE 5. Definition of human *SOCS-1* promoter using luciferase reporters and the expression of *SOCS-1* in human CD19⁺ cells. **A**, The luciferase activities were tested with pGL3 basic promoter constructs containing the –882/–659 [relative to the A (+1) of the ATG start codon] region of the *SOCS-1* promoter with either a G or T at the –820 position as indicated. Additionally, a longer construct (–945/–659) containing G at the –820 position and a shorter construct (–819/–659) lacking the –820 site were tested as well for reporter activity. RAW cells were transfected for 4 h, after which time the transfection solution was removed and replaced with medium for 20 h. Luciferase activities were first normalized to the *Renilla* transfection control and then subsequently graphed relative to the value of the –882/–659 region. Values are given as the mean of three independent experiments ± SD. **B**, Expression of *SOCS-1* mRNA in human CD19⁺ cells by genotype at *SOCS-1*–820. Each point represents a single sample; bars represent mean ± SEM.

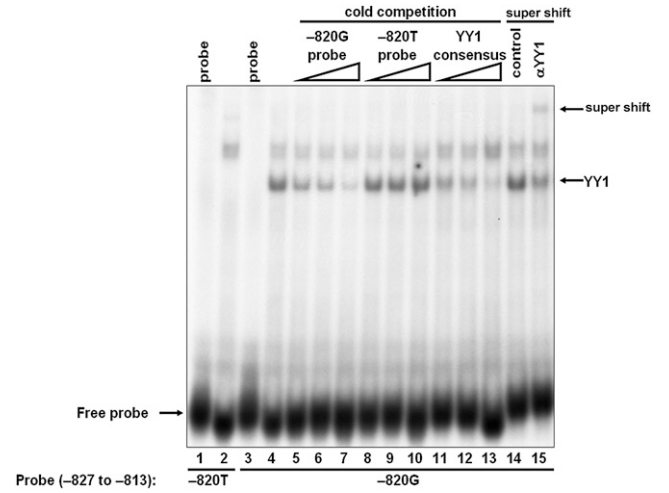


FIGURE 6. A T at the –820 position abolishes the ability of YY1 to bind the *SOCS-1* promoter by EMSA. Nuclear extracts from BALB/c thymocytes were used in EMSA with sequences from the *SOCS-1* promoter. Abs (0.8 μg) for the supershift assay and excess unlabeled probe for the cold competition were added for a 10-min room temperature incubation period prior to the addition of labeled probe. The –820G (–827/–813) or –820T (–827/–813) labeled probes were incubated for 15 min at room temperature. The cold competitions included a 10-, 50-, and 100-fold molar excess of the indicated unlabeled probe. The control unlabeled probe includes sequence from the YY1 binding site in the α-actin promoter.

position (Fig. 6). A specific complex (complex 1) was observed with thymocyte nuclear extracts and a labeled –827/–813 oligonucleotide carrying the common –820G allele (Fig. 6, lane 4). This complex was not observed using the –820T variant of the same probe (Fig. 6, lane 2). Cold competition assays demonstrated that 10-, 50-, and 100-fold molar excess of unlabeled –827/–813 oligonucleotide carrying the –820T sequence failed to compete complex 1, indicating that the G/T substitution is sufficient to abolish binding activity (Fig. 6, lanes 8–10). In contrast, a 100-fold molar excess of the –820G competitor was sufficient virtually to abrogate complex 1 formation. (Fig. 6, lanes 5–7). These results, combined with the data from reporter assays, suggest that the *SOCS-1*–820G allele of the *SOCS-1* promoter binds a transcriptional repressor, whereas the *SOCS-1*–820T SNP prevents this interaction, thereby increasing *SOCS-1* transcriptional activity.

An analysis of the *SOCS-1* promoter sequence surrounding *SOCS-1*–820G > T was performed to determine the identity of the protein(s) present within this complex. The –820/–830 sequence of the *SOCS-1* promoter (5'-CCACTTTTGGT-3') resembled the YY1 consensus binding motif for repressor sites: 5'-CCATNT^T_A NNN^A/T-3' (45). Further, the –818/–826 region of *SOCS-1* (5'-CTCCACTTT-3') bears considerable homology with the adeno-associated virus P5 initiator sequence (5'-CTCCATTTT-3') that makes base contacts with the DNA binding domain of YY1 in the cocrystal structure (46). YY1 is a constitutively expressed member of the GLI-Krüppel family of zinc finger transcription factors that has been shown to function as a transcriptional repressor, activator, and initiator of various viral and cellular promoters (45, 47). Addition of an anti-YY1 Ab generated a supershift of the –820G sequence-specific complex, identifying the unknown binding factor as YY1 (Fig. 6, lane 15). The slower migrating complexes that bind to both the –820T and –820G probes were not affected by the addition of the anti-YY1 Ab. An unlabeled probe that corresponds with the YY1 consensus sequence within the α-actin

promoter competed with the formation of this complex, further supporting the identification of YY1 as the factor binding the *SOCS-1* promoter (Fig. 6, lanes 11–13). This complex was observed with nuclear extracts from thymocytes and monocytic cell lines, consistent with the ubiquitous expression pattern of YY1 (data not shown). Together, these data suggest that *SOCS1*–820T inhibits the binding of YY1 to the *SOCS-1* promoter, thereby altering promoter activity.

A T at the –820 position reduces the ability of overexpressed YY1 to repress the SOCS-1 promoter

The interaction of YY1 with the *SOCS-1* promoter sequence and the lack of binding to the promoter sequence containing the –820T alteration suggest that YY1 may act as a transcriptional repressor of *SOCS-1*. Cotransfection of 10 ng of YY1 expression plasmid effectively repressed the –820G (–882/–659) promoter construct that demonstrated only 0.28 relative activity to the empty vector control (1.0) (Fig. 7). The –820T construct showed slightly higher levels of basal transcription (1.28) and was not as effectively repressed by YY1 overexpression, demonstrating a relative activity of 0.57. These data indicate that the overexpression of YY1 inhibits *SOCS-1* promoter activity and that a T at the –820 position reduces the ability of YY1 to inhibit this activity.

Discussion

A genetic predisposition for the increased production of IgE has been established in patients with atopic conditions (48). The importance of the regulation of cytokines that mediate the balance between Th1 and Th2 development made *SOCS-1* an attractive candidate gene for study with atopy and asthma. Our studies demonstrate that *SOCS-1* is an important regulator of IgE levels in vivo. The loss of one allele of *SOCS-1* in mice increased IgE levels, suggesting that *SOCS-1* has important functional roles in maintaining both the normal basal IgE levels and limiting Ag-specific IgE responses. Another group also described poly-

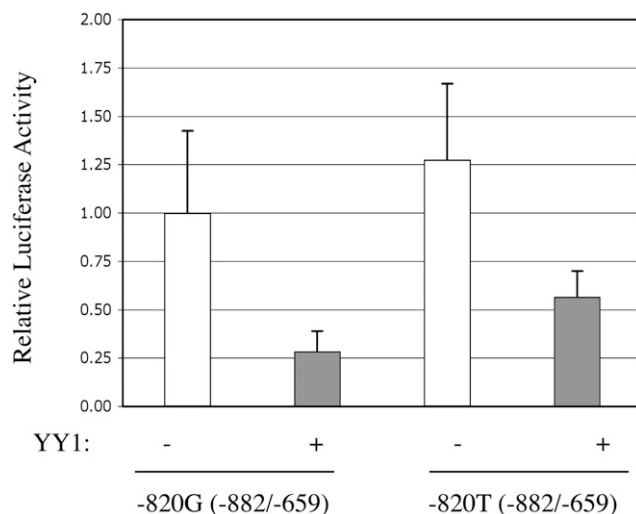


FIGURE 7. Overexpression of YY1 represses the *SOCS-1* promoter. The –882/+659 constructs with either a G or T at the –820 position were used in luciferase experiments in RAW cells. Ten nanograms of expression vector encoding YY1 or empty expression vector were transfected along with 500 ng luciferase and 100 ng *Renilla* construct per transfection. Luciferase activities were first normalized to the *Renilla* transfection control and then subsequently graphed relative to the value of the –882/–659 region. Values are given as the mean of three independent experiments $\pm$ SD.

morphisms in the promoter region of the *SOCS-1* gene; although they did not find association between the SNPs and total serum IgE level, they showed significant association between an SNP at position –1478 and adult asthma susceptibility in a Japanese population as well as increased *SOCS-1* transcription in vitro (49).

The results obtained from mouse studies prompted us to assess whether alterations in the *SOCS-1* locus might be associated with altered IgE levels in children. We identified 21 SNPs in the ~3.5-kb genomic region of human chromosome 16 that contains *SOCS-1*. No association with either the prevalence or severity of asthma was found with the 21 SNPs; however, a significant association was found between lower mean IgE levels and the *SOCS1*+1125G > C SNP. Moreover, this SNP was found to be in high LD with the *SOCS1*–269C > T (LD = 0.8) and in very high LD with the *SOCS1*–820G > T SNP.

The *SOCS1*–820T is located in the promoter region of *SOCS-1* and has been identified as a “protective” variant. Characterization of this polymorphism identified YY1 as a transcriptional regulator of *SOCS-1*. YY1 is a transcription factor that is capable of regulating a plethora of cellular and viral genes as either a transcriptional activator or repressor. YY1 is highly conserved among humans, mice, and *Xenopus* (50); the polycomb group protein pleiohomeotic, the *Drosophila* homolog of YY1, is necessary for the maintenance of homeotic gene repression (51, 52). YY1 deletion in mice is embryonically lethal due to a peri-implantation defect, consistent with the presumed role of YY1 in the regulation of developmental genes (53). Published evidence has demonstrated that the regulation by YY1 depends on the promoter context that in turn influences the interaction of coactivators and corepressors with other transcription factors or constituents of the basal transcription machinery. The –820 position in the YY1 binding site of the *SOCS-1* promoter corresponds with an important base in the sequence of the adeno-associated virus P5 initiator sequence that makes contacts with the DNA binding domain of YY1 in the cocrystal structure (46). Based on this homology, the *SOCS1*–820T SNP is therefore unlikely to be tolerated and is expected to prevent the interaction of the *SOCS-1* promoter with YY1. This prediction was confirmed by a failure of the *SOCS1*–820T allelic variant to interact with YY1 in vitro. Further, the *SOCS1*–820T alteration within the YY1 response element suggested a decrease in the ability of YY1 to inhibit the *SOCS-1* promoter as indicated by the relative increase in transcriptional activity shown by luciferase experiments. YY1 can also interact with a promoter variant (IL13–1112T) of the IL-13 gene (54). This SNP was shown to be associated with high serum IgE levels and bronchial hyperresponsiveness (55). The T at position –1112 creates a YY1 binding site and results in increased promoter activity in primary human and murine CD4⁺ Th2 lymphocytes due to an overlap with a STAT motif, thus attenuating STAT6-mediated repression of the IL-13 gene (54). Together with our findings, these results point to the importance of YY1 as an important regulator of IgE levels.

The increased transcriptional activity observed with the *SOCS1*–820T SNP is consistent with the murine data that demonstrated that *SOCS-1* has an important in vivo role in limiting IgE levels. Hence, the observed increase in *SOCS-1* transcriptional activity suggests a role for the *SOCS1*–820T variant in the lower levels of IgE observed with patients with the promoter variant. Which pathway is affected by the increased *SOCS-1* transcriptional activity, however, remains unclear. *SOCS-1* negatively regulates the action of both IL-4 and IFN- γ , thus the lower IgE level observed with the *SOCS1*–820T variant might suggest that *SOCS-1* has a more pronounced effect on the IL-4 pathway. To determine if IL-4 regulation was the critical factor, the CD19⁺

samples described in Fig. 5B were also cultured in the presence of IL-4 stimulation, after which mRNA levels of *SOCS-1* were measured and compared with the genotype at *SOCS1*-820. We may have predicted that the samples with the -820T variant would have impaired upregulation of *SOCS-1* expression after IL-4 stimulation. This would be consistent with their increased baseline *SOCS-1* expression (shown in Fig. 5B) serving to block signaling by the IL-4 receptor and hence subsequent upregulation of IL-4-dependent pathways such as SOCS-1. However, our findings were inconclusive as samples showed no significant difference in expression of *SOCS-1* after IL-4 stimulation, regardless of genotype at *SOCS1*-820. There are a number of possible explanations for this finding. First, it may be that the importance of the *SOCS1*-820T variant is in cells at their resting, not stimulated, state. Second, although the decreased levels of IgE found in patients suggests a possible role for B cells in this phenotype, it may be the result of regulation of *SOCS-1* expression in other cell types. For example, the increased *SOCS-1* associated with the TT genotype is likely also to be found in CD4⁺ T cells. These CD4⁺ cells may then be less capable of autocrine responses to IL-4, resulting indirectly in decreased IgE production by B cells. IL-13 is another important cytokine that has functions that are separate from as well as overlapping with those of IL-4. Receptors for IL-13 are expressed on B cells, and thus it may be the B cell response to IL-13 alone or in combination with IL-4 or other cytokines that finally regulates *SOCS-1* expression. In addition, the finding that mutations in STAT3 are found in patients with hyper-IgE syndrome (56) suggests that other cytokines are also essential in controlling IgE levels in humans. Thus, SOCS-1 may perform a role in regulating all, or some, of these pathways and thereby affect IgE levels and perhaps atopy.

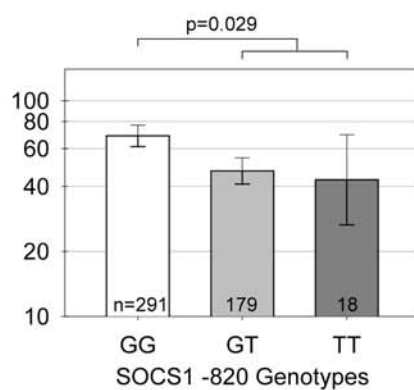
Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure. *Decreased levels of IgE in non-selected children with the SOCS1-820T allele.*

Total serum IgE levels were measured at 11 years of age in the Tucson Children's Respiratory Study. IgE levels for the three SOCS-1 820 genotypes are shown along with the number of subjects with each genotype (n).

Supplemental Figure