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Th2 Cell-Selective Enhancement of Human *IL13* Transcription by *IL13*-1112C>T, a Polymorphism Associated with Allergic Inflammation

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Abstract

IL-13 is a central mediator of allergic inflammation. The single nucleotide polymorphism *IL13*-1112C>T (rs1800925) is associated with allergic phenotypes in ethnically distinct populations, but the underlying mechanism(s) remain unknown. Using in vivo, in vitro, and in silico analysis, we show that the *IL13*-1112T allele enhanced *IL13* promoter activity in primary human and murine CD4$^+$ Th2 lymphocytes. Increased expression of *IL13*-1112T in Th2 cells was associated with the creation of a Yin-Yang 1 binding site that overlapped a STAT motif involved in negative regulation of *IL13* expression and attenuated STAT6-mediated transcriptional repression. Because IL-13 secretion was increased in *IL13*-1112TT homozygotes, we propose that increased expression of *IL13*-1112T in vivo may underlie its association with susceptibility to allergic inflammation. Interestingly, *IL13*-1112T had opposite transcriptional effects in nonpolarized CD4$^+$ T cells, paralleled by distinct patterns of DNA-protein interactions at the *IL13* promoter. Our findings suggest the nuclear milieu dictates the functional outcome of genetic variation.

Allergic diseases have a strong genetic component. Genome-wide linkage studies have identified several loci associated with increased susceptibility to allergy and/or asthma, including one on chromosome 5q.31 that contains the Th2 cytokines *IL5, IL13*, and *IL4* (1). *IL13* is a strong candidate for allergic disease. Elegant studies in animal models have

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shown that IL-13 is sufficient to mediate all of the cardinal features of Th2 inflammation in the lung, such as airway hyperresponsiveness, inflammatory cell infiltration, mucus hypersecretion, and airway fibrosis (2, 3). In humans, IL-13 and its receptors are highly expressed in the respiratory tract of patients with asthma and rhinitis (4, 5). The primary sources of IL-13 are Th2 cells, but eosinophils, mast cells, and basophils also produce this cytokine (6). IL-13 stimulates human B cells to synthesize IgE (7), a key effector in Th2-mediated disease, while suppressing the expression of CD14 (8), a molecule involved in protection from atopy (9). *IL13* is expressed in the placenta (10) and is actively secreted by T cells in the neonatal period (11), a time critical for susceptibility to allergic disease.

Analysis of genetic variation across the *IL13* locus in European ancestry subjects identified a block of common single nucleotide polymorphisms (SNPs)[6] in complete linkage disequilibrium (LD) extending from *IL13*-1923C>T in the third intron to *IL13*-2749C>T in the 3′ untranslated region (12). This block includes *IL13*-2044G>A, a coding SNP resulting in the expression of a gain-of-function variant (IL-13 *R130Q*; Ref. 13) strongly associated with allergic inflammation in several ethnically distinct populations (14). This polymorphism is also in high, albeit not complete, LD with two promoter polymorphisms, *IL13*-1512A>C and *IL13*-1112C>T (rs1800925, also referred to as −1055 and −1111) (12). The *IL13*-1112TT genotype was more prevalent in individuals with asthma and atopic dermatitis and has been associated with increased risk of sensitization to food and outdoor allergens in several studies (15-18). Associations between the *IL13*-1112T allele and allergic phenotypes, such as high IgE serum levels, bronchial hyperresponsiveness and positive skin tests, have also been demonstrated (12, 15, 18).

Although these results strongly suggest that genetic dysregulation of *IL13* expression and/or function may be a critical determinant of susceptibility to allergy and asthma, the extensive LD at the *IL13* locus prevents the tools of genetic epidemiology from deciphering the contribution of individual polymorphisms to increased disease risk. Stratified analysis of *IL13* haplotypes in a large Caucasian population did suggest an effect of *IL13*-1112C>T on IgE levels independent of *IL13*-2044G>A (19), but functional studies are required to characterize the impact of *IL13*-1112C>T on the regulation of *IL13* expression.

To this purpose, we used a combination of in vivo, in vitro, and in silico approaches. We show herein that *IL13*-1112C>T is a functional polymorphism that results in increased *IL13* transcription in primary Th2 cells through Yin-Yang 1 (YY1)-dependent attenuation of STAT6-mediated promoter repression. Analyses in a large population confirmed that IL-13 secretion was increased in *IL13*-1112TT homozygotes, suggesting that increased expression of the *IL13*-1112T allele may underlie its association with increased susceptibility to allergic inflammation.

[6]Abbreviations used in this paper: SNP, single nucleotide polymorphism; ChIP, chromatin immunoprecipitation; LD, linkage disequilibrium; RLA, relative luciferasc activity; YY1, Yin Yang 1; DT, divergence threshold; rh, recombinant human.

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Materials and Methods

Phylogenetic shadowing

The *IL13* promoter was sequenced in genomic DNA samples (Coriell Cell Repository) from 12 primate species consisting of hominoids. Old World and New World monkeys. PCR primers for amplifying the primate promoters were designed based on the human *IL13* sequence (GenBank accession No. AC004041 and L42080). Phylogenetic shadowing (20) was conducted using a multiple sequence alignment of human and primate sequences by Clustal W, and conservation plots were generated with *eShadow* (www.eshadow.dcode.org) (21). Settings were a 25-bp sliding window, 800-bp layer, default Hidden Markov Model Islands (0.85/0.77/0.1), and a divergence threshold (DT) of 15% variation within a minimum length of 70 bp (DT 15/70).

T cell isolation and culture

Human naive cord blood $CD45RO^{-}CD4^{+}$ T cells were obtained by negative selection and depletion of memory T cells using Ab against CD45RO (Miltenyi Biotec). Purity was typically 95%. All cytokines and mAbs were from R&D Systems, unless otherwise specified. Cells were primed for 4 days with plate-bound anti-CD3 mAb (UCHT1, 2 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) under Th2 polarizing conditions (recombinant human (rh) IL-2, 5 ng/ml; rhIL-4, 4 ng/ml; neutralizing Abs to IL-12, 2 μg/ml; and IFN-γ, 2 μg/ml). Cells were rested for 3 days in rhIL-2 (5 ng/ml), rhIL-4 (4 ng/ml), and anti-IL-12 mAb (1 μg/ml). For subsequent weeks, cells were restimulated for 4 days on anti-CD3-coated plates with IL-4 (20 ng/ml) and anti-IL-12 mAb. Experiments were performed on day 16 or 21. Th2 cell polarization was assessed by intracellular cytokine staining.

Jurkat T cells (clone E6-1) and murine D10.G4.1 cells were obtained from American Type Culture Collection. The D10.G4.1 Th2 clone was cultured in RPMI 1640 (Cellgro) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and murine rIL-2 (5 U/ml). Cells were restimulated with 100 μg/ml conalbumin (Sigma-Aldrich) and irradiated syngeneic AKR/J splenocytes every 3 wk. Experiments were conducted 6–12 days after restimulation.

Vector construction and transient transfections

A 2666-bp IL13 promoter construct (−1112/Luc; numbering relative to the *IL13* ATG) was generated using genomic DNA obtained from an *IL13*-1112C homozygote as PCR template and cloned into the pGL3 Basic luciferase reporter vector (Promega). QuickChange site-directed mutagenesis (Stratagene) was performed to create a construct with a T at position −1112 (−1112T/Luc), to represent the minor allele, as well as the −1112C STATmut/Luc and −1112T STATmut/Luc constructs that contained a 3-bp mutation (TTC/gca, −1123/−1121) abolishing the STAT motif. Fidelity of PCR reactions was confirmed by sequencing.

Th2 cells (2×10^{4}/μl) were transiently nucleofected with the −1112/Luc reporter constructs (1 μg) and pRL-TK (Promega: 50 ng) using program T23 (Amaxa). Efficiencies 45% were achieved, as determined by flow cytometric analysis of cells transfected with pCMV-

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GFP (eGFP_C1; BD Clontech). Freshly isolated CD4+ T cells (1.25×10^4 cells/μl) were transiently nucleofected with the −1112/Luc reporter constructs (5 μg) and pRL-TK (5 ng) using program U14 (Amaxa) and achieving 75% transfection efficiency. Jurkat T cells (5 $\times 10^6$) were transiently cotransfected with the −1112/Luc reporter construct (10 μg), STAT6 (TPU-388, 1–10 μg) or STAT1 (a gift from Dr. P. Rothman, Columbia University, New York, NY; 10 μg) expression vectors and pRL-TK (10 ng) by square wave electroporation using one pulse of 50 ms, 240 V (BTX, ECM 830, Genetronics). These conditions resulted in transfection efficiency 40%. In all experiments, cells were stimulated with PMA (20 ng/ml) and ionomycin (1 μM) immediately after transfection. IL-4 (10 ng/ml) was added to the STAT6/STAT1 cotransfections. Luciferase activity was assessed after 16–18 h incubation using the Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce). Results were normalized for transfection efficiency (as determined by *Renilla* luciferase activity) and protein concentrations, and expressed as relative luciferase activity (RLA; luciferase counts per microgram of protein).

Murine D10.G4.1 cells (2×10^6 cells/sample) were nucleofected with *IL13*-1112/Luc reporter constructs or pGL3 Basic (2 μg) and pRL-TK (50 ng) using program T01 (Amaxa). When indicated, a neutralizing anti-murine IL-4 mAb or an IgG1 isotype control (10 μg; BD Pharmingen) were added during the culture. Luciferase activity was assessed 16–18 h after nucleofection. Results were expressed as fold increase in the RLA detected in unstimulated cells transfected with *IL13* reporter constructs relative to cells transfected with pGL3 Basic.

EMSA

To prepare nuclear extracts, cells (10×10^6) were pelleted, washed in cold 1× PBS and resuspended in cold buffer A (10 mM HEPES, 1.2 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF, 5 mM β-glycerophosphate, 1 mM benzamidine, 1 mM orthosodium vanadate, 1 mM sodium fluoride, 10 μg/ml antipain, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin; 120 μl). Cell lysis was monitored by trypan blue, and once swollen, cells were centrifuged at 14,000 rpm for 30 s at 4°C. Nuclear pellets were resuspended in an appropriate volume of ice-cold buffer C (420 mM NaCl, 20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 10 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 5 mM β-glycerophosphate, 1 mM benzamidine, 1 mM orthosodium vanadate, 1 mM sodium fluoride, 10 μg/ml antipain, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin), incubated on ice for 20 min, and centrifuged at 14,000 rpm for 25 min at 4°C. Supernatants containing nuclear proteins were recovered, and aliquots were frozen immediately and stored at −80°C.

Gel shift assays were performed using a 31-bp oligonucleotide probe (5′-GGACTTCTAGGAAAACGAGGGAAGAGCAGGA, position −1127/−1097 of the *IL13* 5′-flanking region) with either a C or a T at position −1112 (underlined). Double-stranded oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP. The binding reaction included nuclear extract (5 μg), 100 mM NaCl, 10% glycerol, 50 ng/μl poly(deoxyinosinate-deoxycytidylate) (Pierce Chemical Co.), 1× binding buffer (10 mM Tris, 0.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT), and 1 μl of probe (0.5–1 ng), and was incubated for 30 min on ice. Competition or supershift were performed by preincubating nuclear proteins with

unlabeled oligonucleotides (50- to 100-fold molar excess) or transcription factor-specific Abs (4 μg) for 30 min before addition of the probe. The following oligonucleotides were used as competitors: STAT3N, AGAGTTTCCCAGAAGGATG (−889/−910 of the *IL13* promoter); STATmut, GGACGCATAGGAAAACGAGGGAAGAGCAGGA; Oct-1, TGTCGAATGCAAATCACTAGAA; NFAT IL2, CGGAGGAAAAACTGTTTCATACAGAAGGCGTG. Abs against STAT1 (E-23 or M-22), STAT6 (S-20), YY1 (C-20), STAT2 (C-20), STAT3 (H-190), STAT4 (H-119), STAT5a (L-20), and STAT5b (N-20) were obtained from Santa Cruz Biotechnology. Reactions were loaded onto 5% nondenaturing polyacrylamide gels and run for 5–6 h at 18–19 mA with 0.5× Tris-buffered EDTA at 4°C.

Chromatin immunoprecipitation (ChIP)

Human naive peripheral blood CD4+ T cells, differentiated for 2 wk under Th2 conditions, were cultured in the presence or absence of PMA (20 ng/ml) and ionomycin (1 μM) for 3 h. Cells (5×10^7 per sample) were fixed for 10 min at 37°C with 1% formaldehyde. After incubation, glycine was added to a final concentration of 125 mM. Cells were washed twice with ice raid PBS and resuspended at 5×10^7/ml in ChIP lysis buffer (Upstate Biotechnology) supplemented with 1× EDTA-free protease inhibitor mixture (Roche) and 1 mM PMSF. Chromatin was sheared by sonication to an average length of 600 bp (six pulses for 10 s each at 30% maximum output; Microson XL200) and diluted 2-fold in ChIP dilution buffer (Upstate Biotechnology) supplemented with protease inhibitors as above. One-twentieth of the total sample was removed to be used as input DNA. Before immunoprecipitation, samples were precleared with salmon sperm DNA/protein A agarose slurry (Upstate Biotechnology) for 30 min. Soluble chromatin was immunoprecipitated overnight at 4°C with 10 μg of anti-STAT6 (sc-621) or anti-YY1 (sc-281) (both from Santa Cruz Biotechnology) or control mouse IgG1 (M5284; Sigma-Aldrich). Chromatin-Ab complexes were collected with salmon sperm DNA-protein A agarose beads and washed sequentially with low salt, high salt, LiCl, and Tris-EDTA (ChIP Assay Kit; Upstate Biotechnology). Complexes were then eluted (1% SDS and 0.1 M $NaHCO_3$), incubated at 65°C overnight to reverse cross-links, and deproteinated with proteinase K. DNA samples were purified by phenol-chloroform extraction followed by ethanol precipitation in the presence of glycogen (20 μg) and resuspended in 20 μl of nuclease-free water. PCR amplification of a 202-bp amplicon encompassing position −1112 of *IL13* was performed with primers 1112ChiPF (5′-GGGTAGGGGAGAAATCTTGACATC) and 1112ChIPR (5′-ATCAACCCCTGCCGTCTGG). Amplification of CNS-1 was performed using primers CNS1ChIPF (5′-CACAGCGTCGTTCAGAAACAC) and CNS1ChIPR (5′-CAGCCCCCGCACAGTTG) to yield a 152-bp amplicon. PCR was performed under the following conditions: 95°C for 15 min followed by 30 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 45 s, ending with a final extension at 72°C for 5 min.

Analysis of IL-13 secretion by **IL13-*1112 genotype***

Peripheral blood samples were obtained from women at the time of their enrollment in the Infant Immune Study (22), at about the eighth month of pregnancy. PBMC (2×10^6 cells/ml) were incubated in the presence or absence of Con A (10 μg/ml) and PMA (10 ng/ml: Sigma-Aldrich). After 18–24 h, supernatants were harvested and stored at – 70°C.

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IL-13 levels were assayed using a commercially available ELISA kit (Diaclone Research). Genotyping for *IL13*-1112C>T and *IL13*2044G>A was performed as previously described (12).

Statistical analysis

The relationship between *IL13*-1112 genotypes and IL-13 production was assessed by one-way ANOVA, whereas the differences in IL-13 levels between individual genotypes were assessed with a Bonferroni multiple comparison test. Statistical significance of differences in fold induction between transfected vectors was determined using the Wilcoxon two-sample test.

Human subjects

Cord blood was obtained from anonymous cesarean deliveries. Peripheral blood was drawn from healthy nonallergic subjects after written informed consent was obtained. All protocols were approved by the Institutional Review Board of the University of Arizona.

Results

IL13-*1112T is the ancestral allele and resides within a putative primate-specific* cis-*regulatory element*

To investigate the functional relevance of *IL13*-1112C>T to *IL13* regulation, we initially relied on comparative genomic analysis. Genomic segments strongly conserved during evolution frequently exhibit regulatory properties (20), implying that SNPs located in such regions are more likely to be functional. A human/mouse sequence alignment revealed poor conservation of the region containing *IL13*-1112C>T (Fig. 1). However, comparisons among distant species, although effective for the identification of highly constrained elements, invariably miss more recent changes in DNA sequence, such as those accounting for biological traits unique to primates (23). Therefore, we turned to phylogenetic shadowing, an approach that was recently developed to analyze sequence conservation profiles among closely related species (20) and accurately predicted primate-specific exons and regulatory elements arisen after divergence from the mouse lineage (21).

A 2-kb region of the *IL13* promoter was sequenced in 12 primate species representative of the main clades of hominoids. Old World and New World monkeys. Analysis of multiple sequence alignments using *eShadow* (21) showed that *IL13*-1112C>T falls within a peak of high intraprimate conservation that spans ~80 bp and predicts the existence of a primate-specific *cis*-regulatory element (Fig. 1, *top*). This element maps to the vicinity of a region that exhibits constitutive DNA hypomethylation and hypersensitivity to DNase I digestion in human naive, Th1 and Th2 $CD4^+$ T cells (R. B. Webster, Y. Rodriguez, W. Klimecki, and D. Vercelli, in press), suggesting this region may be endowed with regulatory properties. These findings provide indirect but suggestive evidence for a potential role of *IL13*-1112C>T in the regulation of *IL13* expression. All 12 nonhuman primate species had a T at position −1112 (Fig. 1, *bottom*). Therefore, the disease-associated *IL13*-1112T allele is the ancestral allele.

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Transcription of the IL13-*1112T allele is enhanced in primary CD4⁺ Th2 cells, but not in nonpolarized CD4⁺ T cells*

Because *IL13*-1112C>T disrupts a CpG site (Fig. 1), we explored the possibility that loss of the C at −1112 may alter DNA methylation patterns throughout the region, rendering the minor promoter variant more accessible to the transcriptional machinery. Bisulfite sequencing analysis revealed virtually identical CpG methylation profiles in $CD4^+$ T cells of distinct *IL13*-1112 genotypes (data not shown), ruling out this possibility.

To examine more directly whether *IL13*-1112C>T affects *IL13* transcription, we generated luciferase reporter constructs driven by a 2.7-kb *IL13* promoter fragment carrying either the major (C) or minor (T) allele at position −1112. Initially, the −1112/Luc reporter constructs were transfected into the Jurkat T cell line, a well-established model to study transcriptional regulation of cytokine genes. Fig. 2 (*left*) shows that −1112C/Luc activity was upregulated 22-fold in response to T cell activation. In contrast, the −1112T/Luc construct was induced only 15-fold ($n = 26$; $p = 0.006$). Thus, *IL13*-1112T was significantly less active than the major allele in Jurkat T cells.

Although these data suggested that *IL13*-1112CT is a functional SNP, the lower activity of the T allele could not be readily reconciled with its reported association with allergic phenotypes and increased asthma susceptibility (1). To reassess these findings in primary T cells, we studied transcription of the *IL13*-1112 alleles after nucleofection into $CD4^+$ T cells freshly isolated from normal peripheral blood. Activation of $CD4^+$ T cells up-regulated transcription of both allelic variants to a comparable extent (4.4- vs 3.8-fold; $n = 9$, $p > 0.05$: Fig. 2, *left*), a result that was again inconsistent with the association between *IL13*-1112T and susceptibility to Th2-dependent inflammation. However, the true transcriptional impact of the −1112 polymorphism might only become apparent within a cytokine/nuclear environment leading to high level *IL13* expression. Jurkat and $CD4^+$ T cells up-regulate *IL13* mRNA levels in response to activation (80-fold for Jurkat cells and   140-fold for primary T cells, as assessed by real time RT-PCR), but only ~3% of these cells expressed detectable levels of intracellular IL-13 protein (data not shown). Thus, the majority of luciferase activity in nonpolarized $CD4^+$ T cells was likely generated from a nuclear environment inadequate to promote optimal *IL13* expression.

Because *IL13* is typically expressed by polarized $CD4^+$ Th2 cells and these cells play a critical effector role in human and experimental allergic inflammation, the transcriptional effect of *IL13*-1112C>T was examined in two independent, primary Th2 cell models. In one set of experiments, human neonatal naive $CD45RO^-CD4^+$ T cells were differentiated in vitro under Th2-polarizing conditions. After 2–3 wk of culture, cells acquired a highly polarized Th2 phenotype, as demonstrated by elevated levels of intracellular IL-13 and virtually absent IFN-γ (data not shown). Nucleofection of primary human Th2 cells with *IL13*-1112C and T reporter constructs led to significantly higher transcriptional induction of the −1112T allele in response to T cell activation (Fig. 2, *left*). Likewise, the −1112T variant was significantly more active in the murine D10.G4.1 Th2 clone, a well-established model of Th2 cell polarization (24) (Fig. 2, *right*). Our results demonstrate that the nuclear environment can dictate the transcriptional outcome of genetic variation. In the context of a Th2 milieu that drives high *IL13* expression, but not within nonpolarized $CD4^+$ T cells, the

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−1112T allele conferred higher activity to the *IL13* promoter, consistent with the reported association between this allele and increased susceptibility to allergic inflammation (1).

IL13-*1112 allele- and cell type-specific patterns of transcription factor binding parallel profiles of transcriptional activity*

To identify the mechanisms underlying higher transcription of *IL13*-1112T in Th2 cells, we initially used EMSA analysis to compare and contrast patterns of DNA-protein interactions occurring at the *IL13* −1112 promoter variants in distinct T cell nuclear environments. We reasoned that such comparisons could provide an indirect but powerful tool to tease out the interactions involved in increased transcription of the −1112T allele.

Using oligonucleotides corresponding to the C or T allelic variants of the −1127/−1097 *IL13* promoter region (Fig. 3A, *top*), we demonstrated that nuclear extracts from activated primary Th2 cells (in which the −1112T allele was transcriptionally more active) contained two specific, slowly migrating complexes that bound both the C and the T allele (Fig. 3A, *lanes 2* and *8*) and were cross-competed by cold C and T 1127/−1097 *IL13* oligonucleotides (Fig. 3A, *lanes 3, 4, 9,* and *10*). The upper complex contained STAT6, because formation of the complex was abrogated by a STAT3N (*lanes 6* and *12*), but not an NFAT competitor (*lanes 5* and *11*). Furthermore, the band was supershifted by an anti-STAT6 (*lanes 14* and *18*), but not an anti-STAT1 (Fig. 3A, *lanes 15* and *19*) or an anti-C/EBPγ (Fig. 3A, *lane 16*) Ab. Conversely, the lower complex was formed by STAT1, because it was fully competed by a STAT3N oligonucleotide (Fig. 3A, *lanes 6* and *12*) and selectively supershifted by an Ab against this STAT family member (Fig. 3A, *lanes 15* and *19*). No other STAT proteins were found to participate in complex formation, as assessed by supershifting with Abs specific for individual family members (data not shown). The −1112T but not the C probe bound an additional fast-migrating complex that was competed by a cold −1112T, but not −1112C, oligonucleotide (Fig. 3A, *lanes 9* and *10*). The complex contained YY1 because it was supershifted by an anti-YY1 Ab (Fig. 3A. *lane 20*), but not by Abs to STAT6 or STAT1 (Fig. 3A, *lanes 18* and *19*). Comparison of nuclear extracts from resting and activated Th2 cells revealed that the YY1 complex bound constitutively to the −1112T allele (Fig. 3A, *lane 23*), whereas formation of the STAT6- and STAT1-containing complexes was dependent on T cell activation (Fig. 3A, *lanes 22* and *24*). Increased activity of *IL13*-1112T in Th2 cells was therefore paralleled by allele-specific binding of the transcription factor YY1.

When we analyzed nuclear factor binding to the −1127/−1097 *IL13* promoter region using nuclear extracts from nonpolarized primary CD4$^+$ T cells (in which the T allele was transcribed less actively), Ab supershift analysis again demonstrated the presence of activation-induced complexes containing STAT6 (Fig. 3B, *lanes 4 and 10*) and STAT1 (Fig. 3B, *lanes 5 and 11*), that interacted equivalently with the −1112C and T allele (Fig. 3B, *lanes 2 and 8*). Interestingly, in CD4$^+$ T cell extracts the T allele selectively bound not only constitutively expressed YY1 (Fig. 3B, *lanes 7 and 8*), but also an additional inducible complex. The latter contained NFAT2, because its formation was inhibited by an anti-NFAT2 Ab (Fig. 3B, *lane 9*), but not by Abs to STAT6 (Fig. 3B *lane 10*), STAT1 (Fig. 3B, *lane 11*) or C/EBPγ (Fig. 3B, *lane 12*). Moreover, pretreatment of PMA-ionomycin-stimulated CD4$^+$ T cells with cyclosporine A (50 μg/ml) selectively prevented complex

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formation (data not shown), further supporting the identification of NFAT as a main complex constituent.

Finally, EMSA analysis of nuclear extracts from Jurkat T cells (which, like fresh CD4$^+$ T cells, supported weaker transcription of the −1112T allele) showed strong specific binding of STAT1 to both alleles (Fig. 3C, *lanes 3, 4, 8*, and *9*) and selective binding of YY1 to −1112T (Fig. 3C, *lanes 11* and *13*). However, no STAT6-containing complex was detected, consistent with deficient STAT6 activity in Jurkat T cells (25). A slowly migrating complex supershifted by an anti-Oct-1 Ab (Fig. 3C, *lane 12*), but not by Abs to YY1 (Fig. 3C, *lane 13*), C/EBPγ (Fig. 3C, *lane 14*), or Oct-2 (data not shown), was also detected in Jurkat T cell extracts using a −1112T allele probe. The same complex was selectively competed by a bona fide Oct consensus oligonucleotide (data not shown), and thus contained Oct-1.

These results show that the higher activity of the *IL13*-1112T allele in Th2 cells correlated with a unique pattern of DNA-protein interactions marked by the combination of STAT6/STAT1 and YY1, and provide a molecular rationale for the differential transcription of the −1112 alleles in distinct T cell nuclear environments.

IL13-*1112T attenuates STAT6-mediated repression of IL13 transcription*

YY1 is a ubiquitously expressed nuclear protein that can either activate or repress transcription (26). Because the sequences flanking the YY1 core motif (TCAT) can be quite variable, many promoters contain YY1 sites that overlap those for other transcriptional regulators (26). This topology fosters interactions between YY1 and other factors, the outcome of which depends on the function of the proteins involved. In silico analysis of the *IL13* promoter sequence and mutational EMSA analysis by nucleotide transversions (data not shown) demonstrated that the YY1 motif created by −1112T overlaps the 3′ end of a STAT palindrome that is present on both alleles and binds STAT6 or STAT1 in Th2 cells (Fig. 3A, *top*). In view of the dual role of YY1 in transcriptional regulation, two models may explain the role of YY1 in the increased activity of the *IL13*-1112T allele in Th2 cells. Both YY1 and STAT proteins may act cooperatively as transcriptional activators, as reported for YY1 and STAT5b binding to the serine protease inhibitor 2.1 gene (27). Alternatively, STAT binding to the *IL13*-1112 region may repress transcription, as reported for the human *IL4* promoter (25), and YY1 may relieve STAT-mediated repression by displacing STATs or recruiting STAT corepressors.

We reasoned that understanding the role played by YY1 in the increased activity of the *IL13*-1112T allele required the functional characterization of the −1123/−1115 STAT motif overlapping the YY1 site. Therefore, the *IL13*-1112C and T reporter vectors were mutated in the 5′ half of the STAT motif. EMSA analysis confirmed that the mutation disrupted binding of STAT1/STAT6, but not YY1 (data not shown). Fig. 4 (*left*) shows that the −1112C STAT mutant construct was markedly more active than either allelic variant when nucleofected into primary human Th2 cells. In contrast, the activity of the minor −1112T allele was only marginally altered by the STAT site mutation. These results demonstrate that the STAT motif upstream of the polymorphism plays a strong negative regulatory role in the context of the −1112C *IL13* promoter and indicate that YY1 binding to its newly created site is virtually sufficient to relieve STAT-dependent promoter repression.

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Strong independent support for both these conclusions was provided by the experiments shown in Fig. 4 (*center*). Inhibition of STAT6 activation by neutralization of endogenous IL-4 significantly up-regulated transcription of the common −1112C promoter variant in D10.G4.1 Th2 cells, raising the activity of the −1112C allele to the levels achieved by the −1112T allele. In contrast, IL-4 blockade did not affect the activity of the −1112T promoter.

STAT6, not STAT1, was involved in transcriptional attenuation through the −1123/−1115 site adjacent to *IL13*-1112C>T, because overexpression of STAT6, but not STAT1, significantly decreased −1112C/Luc activity in Jurkat T cells (Fig. 4, *right*). Interestingly, STAT6 overexpression did not affect transcription from −1112T/Luc, consistent with blockade of the STAT6 site by YY1, which is highly expressed in Jurkat T cells (Fig. 3C). We conclude that binding of YY1 to a motif created by *IL13*-1112T relieves STAT6-mediated repression, leading to increased activity of the −1112T allele in Th2 cells.

STAT6 and YY1 bind at the IL13-*1112 region in vivo*

To confirm the role of STAT6 and YY1 as critical determinants of *IL13* transcription in vivo, the ability of these factors to bind the endogenous *IL13*-1112 promoter region in primary human Th2 cells was assessed by ChIP. Fig. 5 (*top*) shows that the region of interest was readily immunoprecipitated by anti-STAT6 and anti-YY1 Abs but not control IgG, demonstrating STAT6 and YY1 interact with the *IL13* promoter in chromatin. STAT6-anti-STAT6 interactions were specific, because the anti-STAT6 Ab failed to immunoprecipitate CNS-1 (Fig. 5, *bottom*), a regulatory element located in the *IL13/IL4* intergenic region, that does not encompass bona fide STAT6 motifs. A weak YY1 signal was detected in CNS-1, in line with the presence of several predicted YY1 sites (J. M. Strempel and D. Vercelli, unpublished observations). Detection of DNA-bound STAT6 in resting Th2 cells, although at variance with our EMSA findings, may reflect the continuous presence of IL-4 in Th2 cell cultures and the sensitivity of ChIP to modifications in chromatin architecture.

Given that the DNA fragments generated by sonication were on average 600 bp long but exhibited some expected heterogeneity, and at least 6 YY1 and 9 STAT putative binding motifs are located within 1 kb of *IL13*-1112C>T (data not shown), ChIP analysis could not formally prove that the docking sites for the immunoprecipitated STAT6- and/or YY1-containing complexes were those immediately adjacent to, or overlapping, *IL13*-1112C>T. However, our results point to an involvement of both STAT6 and YY1 in the regulation of *IL13* promoter activity in the endogenous nuclear environment and as such lend further support to the view that the interplay between these factors is critical for the functional outcome of *IL13*-1112C>T.

Increased IL-13 secretion in IL13-*1112TT homozygotes*

The *IL13*-1112T allele resulted in increased *IL13* transcription, (Figs. 2 and 4) and was associated with allergic phenotypes in multiple studies (14), but the existence of a direct correlation between *IL13*-1112 genotypes and levels of IL-13 production was never tested in a large population. We assessed IL-13 secretion in subjects enrolled in the Tucson Infant Immune Study, a large prospective study of the development of immunological markers of asthma risk (22). We focused on 174 women at the third trimester of pregnancy, unselected

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for atopy and asthma. Fig. 6 shows that mitogen-activated PBMC from *IL13*-1112TT homozygotes secreted significantly higher levels of IL-13 compared with −1112CC and CT individuals (ANOVA, $p = 0.015$). The effect was strengthened after adjusting for ethnicity and *IL13*-2044 genotype in a multivariate linear regression (−1112TT vs CC, $p = 0.007$: −1112TT vs CT, $p = 0.003$). The latter adjustment was required because *IL13*-2044G>A, a common coding SNP in strong LD with *IL13*-1112C>T in the Tucson population (12), results in the expression of an IL-13 variant (IL-13 *R130Q*) with increased biological activity and altered antigenic properties that decrease its ELISA-based detection by a factor of 25.7% (13). Therefore, the data shown in Fig. 6, if anything, would represent under detection of IL-13 in carriers of *IL13*-2044A. Although the complex patterns of LD in the *IL13* locus warrant caution in the interpretation of these data, our findings strongly support the possibility that *IL13*-1112C>T may contribute to increased *IL13* expression in vivo. The low IL-13 levels detected in −1112CT heterozygotes may result from monoallelic *IL13* expression, a pattern well documented in mouse strains (28), and preferential expression of the C allele.

Discussion

The locus encoding IL-13, a central mediator of allergic inflammation, harbors numerous common polymorphisms in strong LD, and distinct *IL13* haplotypes are associated with allergic phenotypes in ethnically distinct populations (1). Because of this genetic complexity, functional studies are necessary to dissect the mechanisms underlying the contribution of natural genetic variation to *IL13* dysregulation and susceptibility to allergy. Our study demonstrates *IL13*-1112C>T is a functional polymorphism that enhances *IL13* promoter activity in primary human Th2 lymphocytes, cells programmed for high *IL13* expression, but has opposite effects in nonpolarized $CD4^+$ T cells. The nuclear milieu may therefore dictate the functional outcome of genetic variation.

Gene-environment interactions in the nucleus are a phenomenon we previously observed for *CD14*-159C>T, a SNP which results in different patterns of *CD14* promoter activity in monocytes and hepatocytes depending on the Sp1:Sp3 ratio (29). However, our current data are more remarkable in that differential *IL13* expression was observed not in distinct cell types but in distinct $CD4^+$ Th cell phenotypes and that they correlated with distinct patterns of transcription factor binding to the *IL13* promoter. That the gain-of-function associated with the *IL13*-1112T allele emerged only in differentiated Th2 cells eloquently shows how subtle the functional impact of genetic variation can be and how essential it is to choose experimental models able to capture it. Furthermore, these results suggest that *IL13*-1112C>T is likely to influence risk of allergy and/or asthma in the context of an established Th2 response. Thus, this polymorphism may contribute to the maintenance and/or exacerbation of allergic inflammation more than to its inception.

Gene-environment interactions in the nucleus may also offer a rationale for the common but disquieting finding that many published associations could not be replicated (30-32). If the functional outcome of genetic variation contributing to disease risk is determined not only by the genetic but also by the biological context, as our data indicate, the conditions under which biological samples are collected for phenotyping may become

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critically important, and failing to account for gene-environment interactions in the nucleus may hamper detection of susceptibility loci. Interestingly, there are now many examples of established associations with different functional variants within the same gene or with opposite alleles at the same SNP in different populations (1). For example, IgE levels are associated with *IL13*-1112C>T in some populations (33, 34) and with *IL13*-2044G>A (12, 33, 35) or *IL13*-1512A>C (36) in others. Protection from allergy and/or asthma is associated with both the T (9) and the C (37) allele of *CD14*-159. It is tempting to speculate that these seemingly contradictory results may often represent an outcome of gene-environment interactions in the nucleus.

Increased transcription of *IL13*-1112T was associated with the creation of a binding site for YY1 that overlapped a STAT motif and attenuated STAT6-mediated repression. Although necessary for Th2 cell differentiation and expression of Th2 cytokine genes, including *IL13* (38), STAT6 can also act as a negative transcriptional regulator. STAT6 overexpression was shown to suppress the activity of an *IL4* reporter construct in both Jurkat (25) and polarized murine Th2 (39) cells. Furthermore, comparative mRNA profiling of IL-4-stimulated B cells from wild-type and *STAT6*$^{-/-}$ mice demonstrated that more than one-half of STAT6-controlled genes were down-regulated by this transcription factor in vivo (40). Last but not least, STAT6 inhibited *IL4* promoter activity in differentiated D10.G4.1 murine Th2 cells (39). Our work further extended these findings by showing that STAT6 negatively affects *IL13* expression in Th2 cells, and furthermore identified the STAT site adjacent to −1112C>T as being critical for STAT6-dependent suppression of *IL13* transcription. Indeed, the replacement of −1112C with a T, and the concomitant creation of a YY1 site overlapping the STAT motif, was sufficient to fully counteract STAT6-dependent promoter inhibition. STAT6 may be part of a complex feedback circuit, acting initially as a permissive factor essential for Th2 cytokine locus remodeling (24) leading to *IL4* and *IL13* expression in Th2 cells. Partnering with a different set of factors, STAT6 may then become an inhibitor that fine tunes or even limits *IL13* expression in fully differentiated Th2 effector cells. Our data suggest that the dynamic transcription factor context of differentiating Th2 cells plays a major role in modulating the regulatory effects of *IL13*-1112C>T. Whereas STAT6 and YY1 docking on the −1112T allele was detected in both nonpolarized CD4^{+} T cells (in which the T allele was less active) and Th2 cells (in which the T allele was more active), other factors (NFAT, Oct-1) were available for binding only in nonpolarized cells. In view of the topology of the relevant binding sites in the polymorphic *IL13* promoter region, the latter factors may impair the ability of YY1 to interfere with STAT6-mediated promoter repression (Fig. 7). The molecular interactions underlying these effects remain to be determined.

The relatively modest impact of *IL13*-1112C>T on transcription reflects the nature of single nucleotide variations, subtle differences that alter fine tuning or sensitivity thresholds of promoters and regulatory elements rather than impose the drastic effects of loss- or gain-of-function mutations seen in Mendelian disorders. Indeed, the magnitude of the effect was similar to other regulatory polymorphisms such as the SNP in *SLC22A* associated with rheumatoid arthritis and loss of transcriptional activity (41) and the variant *CD14* and *TGF-β* promoters (29, 42). Because the functional effects of individual polymorphisms may be small, risk for complex diseases is substantially increased by synergism between multiple SNPs arrayed along a single regulatory pathway. Indeed, asthma risk was 5-fold

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higher in carriers of *IL13*-1112T and *IL4RA 478Ser* (43), compared with individuals with both nonrisk genotypes, and the effects of the *IL13*-1112TT genotype on risk for food sensitization were modified by *IL4RA S478P* and *IL4RA Q155R* (17). Risk for high IgE and asthma was increased even further by the combination of *IL13*-1112C>T, *IL4*-589C>T, *IL4RA*-148A>G and *STAT6 IL4RA*-2892C>T (44).

Comparative analysis of the *IL13* promoter showed the *IL13*-1112T allele that increases risk for allergic disease is the ancestral allele, whereas the derived −1112C allele is protective. Furthermore, this analysis revealed the topology of STAT6 and YY1 motifs resulting in increased *IL13* promoter activity has been fully conserved through at least 30 million years of evolutionary history, and all the replacements found in the STAT motif in Old World and New World monkeys occurred within the 3N spacer, not in the TTC/GAA palindrome critical for DNA-protein interactions (Fig. 1, *bottom*). These findings and their relevance to common diseases are best interpreted in the framework of the ancestral susceptibility model (45), according to which ancestral alleles reflect ancient adaptations to the lifestyle of ancient human populations. In that context, derived alleles were deleterious. With the shift in environment and lifestyle that has occurred in modern populations, ancestral alleles can increase the risk of common diseases, as exemplified by variants involved in energy metabolism and sodium homeostasis (45). An equivalent role of *IL13*-1112C>T among immunity genes is suggested by its current associations with allergy and asthma susceptibility in Western environments (1) in the face of strong associations between *IL13*-1112T and protection from *Schistosoma hematobium* in Africa (46) and severe malaria in Thailand (47). Although it is unclear why *IL13*-1112C rose abruptly in frequency to become the common allele in most human populations, the *IL13* locus shows signatures of a recent selective sweep in the Caucasian and Chinese populations (48). We speculate that a genetically determined propensity for high *IL13* expression may have become detrimental through deleterious effects on reproduction. Indeed, endometriosis, which increases the risk of infertility, has been associated with elevated *IL13* mRNA and protein expression within the ectopic endometrium (49). *IL13* may therefore be the first immunity gene that conforms to the ancestral susceptibility model.

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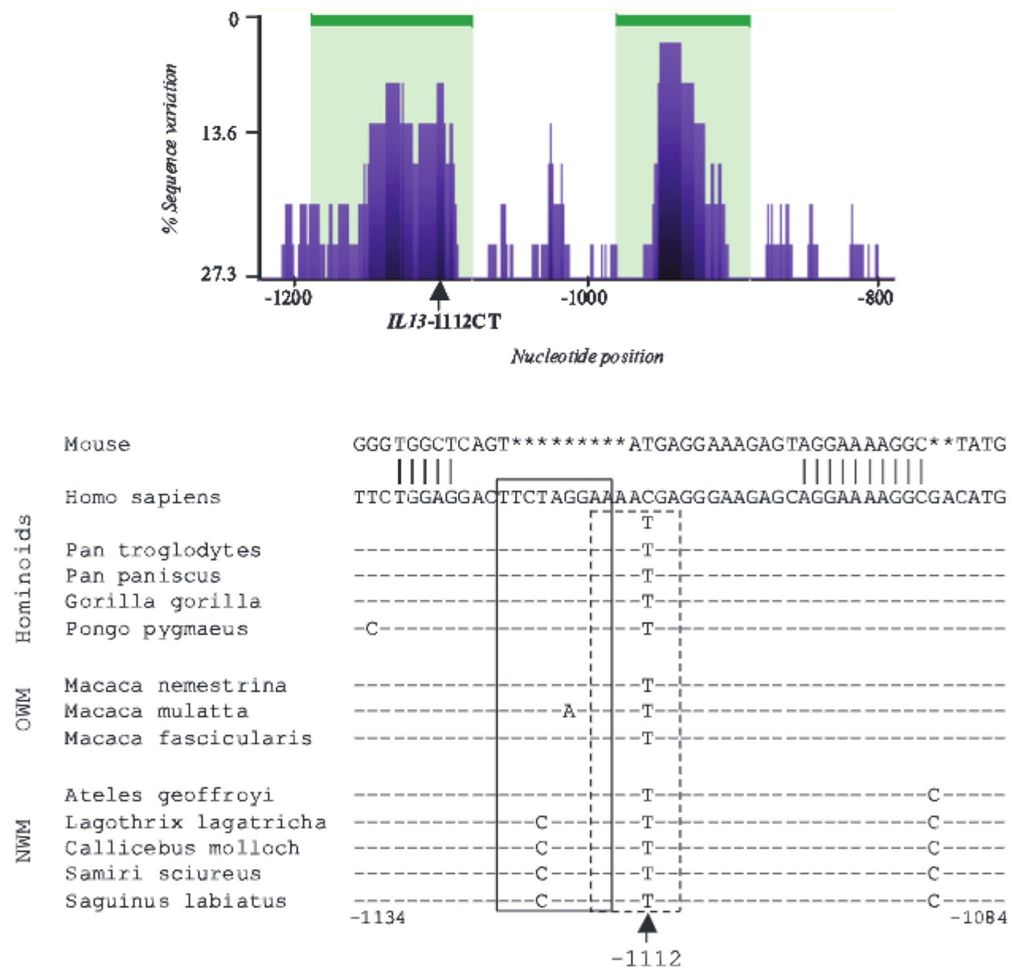

FIGURE 1.

IL13-1112T is the ancestral allele and resides within a putative primate-specific *cis*-regulatory element. *Top*, *eShadow* conservation plot (www.eshadow.dcode.org) for the region surrounding IL13-1112C>T (arrow) in humans and 12 primate species representative of distinct clades. Peaks and valleys correspond to regions of low and high variation, respectively, with 0% variation = 100% sequence identity. Areas shaded in green represent regions below the DT (15/70). *Bottom*, Sequence alignment for the region surrounding *IL13*-1112C>T (arrow) in mice, humans, and primates. Numbering is relative to the human *IL13* ATG. The pairwise alignment of human (GcnBank accession No. NC_000005:132016718-132031715) and mouse (GenBank accession No. NC_000077:53392544-5337166) promoter sequences for the IL13 –1112 region was performed with AlignX, a feature of the Vector NTI suite, which is based on the Clustal W algorithm. The dashes and asterisks mark conserved positions and gaps in the sequence, respectively. The STAT6 and YY1 binding motifs revealed by the DNA-protein interaction analysis discussed later in this paper are boxed by continuous or dashed lines, respectively. OWM, Old World monkeys; NWM, New World monkeys.

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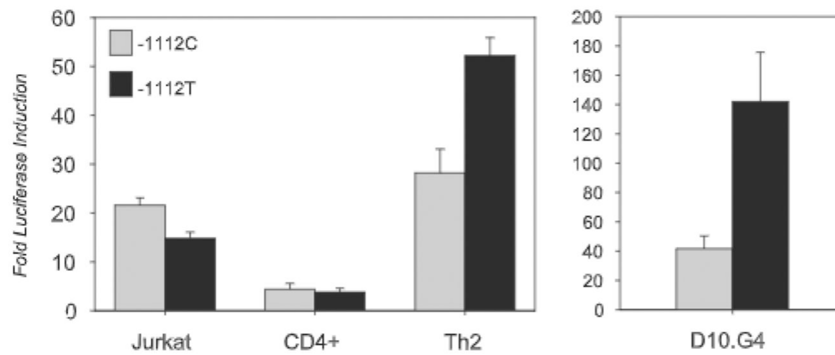

FIGURE 2.

IL13-1112T enhances IL-13 promoter activity in polarized $CD4^+$ Th2 cells but not in nonpolarized $CD4^+$ T cells. *Left*, Jurkat T cells ($n = 26$), freshly isolated $CD4^+$ T cells ($n = 9$), or in vitro polarized human Th2 cells ($n = 5$) were transiently transfected with 2.7-kb *IL13* promoter reporter constructs carrying the major (C) or minor (T) −1112 allele. Cells were left in medium or stimulated with PMA (20 ng/ml) and ionomycin (1 μM) and harvested after 16–18 h. Results are expressed as fold-increase in RLA (mean ± SE) after stimulation. *Right*, Murine D10.G4.1 Th2 cells were nucleofected with pGL3 Basic or −1112 allelic variants of an *IL13* reporter construct ($n = 8$). Cells were left unstimulatcd for 16 h after transfection. Results are expressed as fold increase in the activity of the *IL13* reporter constructs relative to pGL3 Basic. Statistical significance of all results was determined using the Wilcoxon two-sample test.

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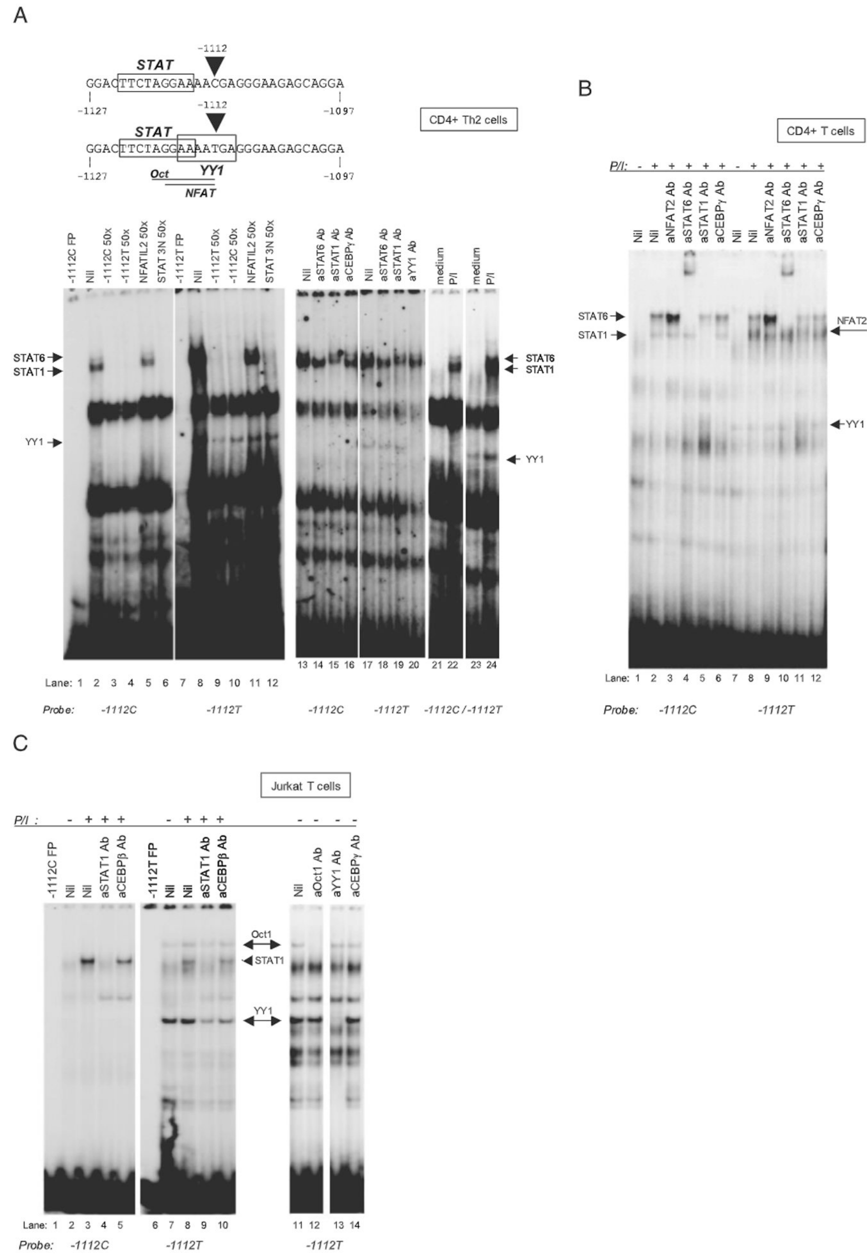

FIGURE 3.

IL13-1112 allele-specific patterns of transcription factor binding in distinct T cell nuclear environments. EMSA analysis with *IL13*-1112C and T oligonucleotide probes (*top*) and nuclear extracts from primary polarized human Th2 cells (*A*), freshly isolated CD4+ T cells (*B*), and Jurkat T cells (*C*) cultured for 3 h in medium or PMA (P; 20 ng/ml) and ionomycin (I; 1 μM). The nuclear extracts used in lanes *1–20* of *A* were prepared from PMA-ionomycin-activated cells. STAT and YY1 binding sites in the probe sequence are boxed; NFAT and Oct motifs are underlined. The competitors (fold molar excess) and supershifting Abs used for each experiment are noted above the corresponding lanes in the gels. Probes are noted below the gels. FP, free probe; a (as in aSTAT), anti.

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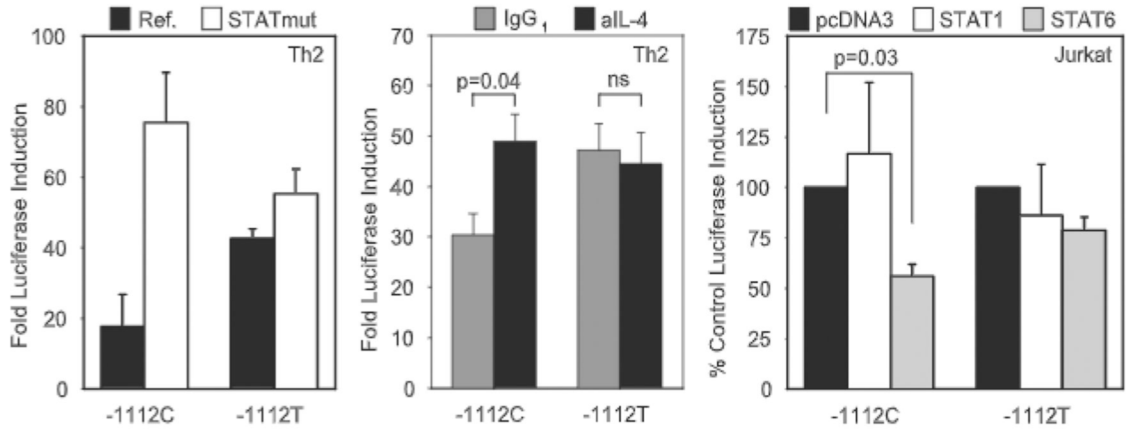

FIGURE 4.

IL13-1112T altcnuatcs STAT6-mediated repression of IL-13 transcriplion. *Left*, Primary polarized human Th2 cells were transienlly transfected with −1112 allelic variants of the reference 2.7-kh *IL13* promoter reporter construct (−1112C/Luc or −1112T/Luc) or equivalent constructs in which the STAT site adjacent to the SNP had been disrupted (−1112C STATmut/Luc and −1112T STATmut/Luc) ($n = 2$). Cells were left in medium or stimulated with PMA (20 ng/ml) and ionomycin (1 μM) and harvested after 16–18 h. Results are expressed as fold increase in RLA (mean ± SE) after stimulation. *Center*, Murine D10.G4.1 cells were harvested 6–12 days after antigenic stimulation and nucleofected with pGL3 Basic or *IL13*-1112C or T promoter reporter variants. Cells were incubated with neutralizing anti (a)-murine IL-4 or control IgG1 Ab for 16 h before harvesting ($n = 6$). Results are expressed as fold increase in the activity of the *IL13* reporter constructs relative to pGL3 Basic. *Right*, Jurkat T cells were cotransfected with the −1112/Luc *IL13* promoter constructs and pcDNA3, STAT1 ($n = 4$), or a STAT6 ($n = 11$) expression vectors. Cells were incubated with medium or PMA (20 ng/ml) and ionomycin (1 μM). IL-4 (10 ng/ml) was added to the STAT6 cotransfections. Cells were harvested after 16–18 h. Results arc expressed as percentage of fold luciferase induction in response to stimulation for STAT-transfected cells relative to cells transfected with pcDNA3 (mean ± SE). For all panels, statistical significance was determined using the Wilcoxon two-sample test.

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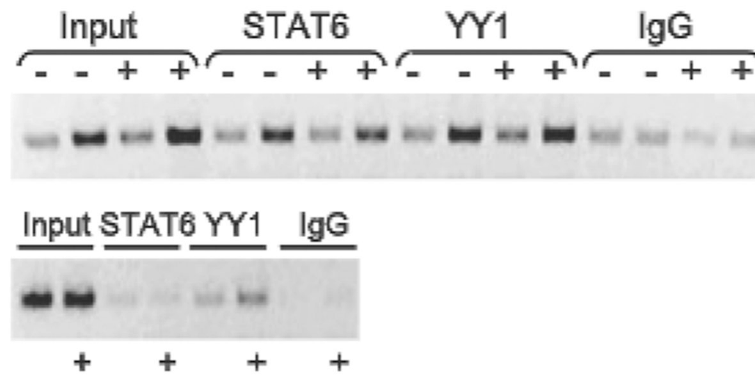

FIGURE 5.

STAT6 and YY1 bind at the *IL13–1112* region in vivo. Naive peripheral blood $CD4^+$ T cells from a healthy *IL13*–1112T heterozygote were differentiated in vitro for 2 wk under Th2 conditions. ChIP assays with an anti-STAT6, anti-YY1, or control Ab (mouse IgG1) were performed on cells (5×10^7 per immunoprecipitation) cultured in the presence (+) or absence (–) of PMA and ionomycin (P/I) for 3 h. *Top*, Input DNA (5 or 10 ng) or immunoprecipitated DNA (one-twentieth and one-tenth of total) was used as template for PCR amplification of a 202-bp amplicon encompassing *IL13*-1112. *Bottom*, Input DNA (10 ng) or immunoprecipitated DNA (one-tenth of total) was used as template for PCR amplification of a 152-bp amplicon corresponding to CNS-1. Results are from one representative experiment of three independent immunoprecipitations.

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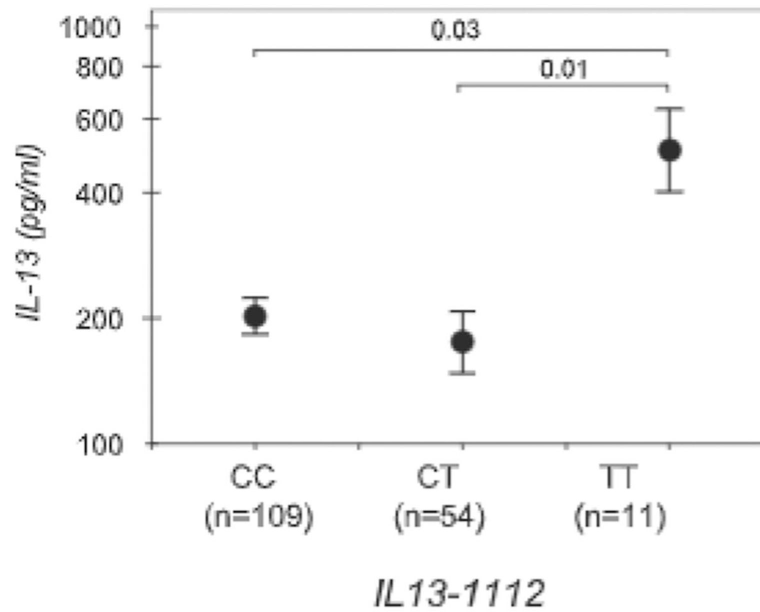

FIGURE 6.

Increased IL-13 secretion in *IL13*-1112T homozygotes. PBMC from pregnant women enrolled in the Infant Immune Study study and genotyped for *IL13*-1112C>T and *IL13*-2044G>A were incubated with Con A (10 μg/ml) and PMA (10 ng/ml) for 18–24 h. IL-13 concentrations in culture supernatants were assessed by ELISA. Results are the mean ± SE of IL-13 concentrations measured in each *IL13*-1112 genotype group. Statistical significance was assessed by one-way ANOVA for the relation between *IL13*-1112 genotypes and IL-13 production, and a Bonferroni multiple comparison test for differences in IL-13 levels between individual genotypes.

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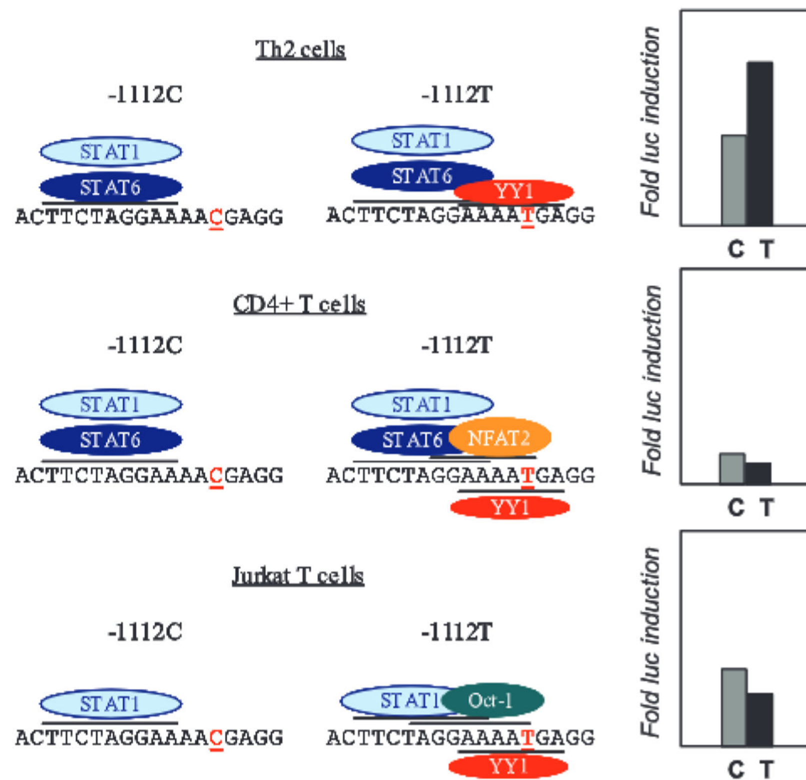

FIGURE 7.

Model of gene-environment interactions in the nucleus at *IL13*-1112C>T. The results of the DNA/protein interaction analysis are summarized on the left. The corresponding *IL13*-1112C>T transcription data are presented on the right.