

# An allergy-associated polymorphism in a novel regulatory element enhances *IL13* expression

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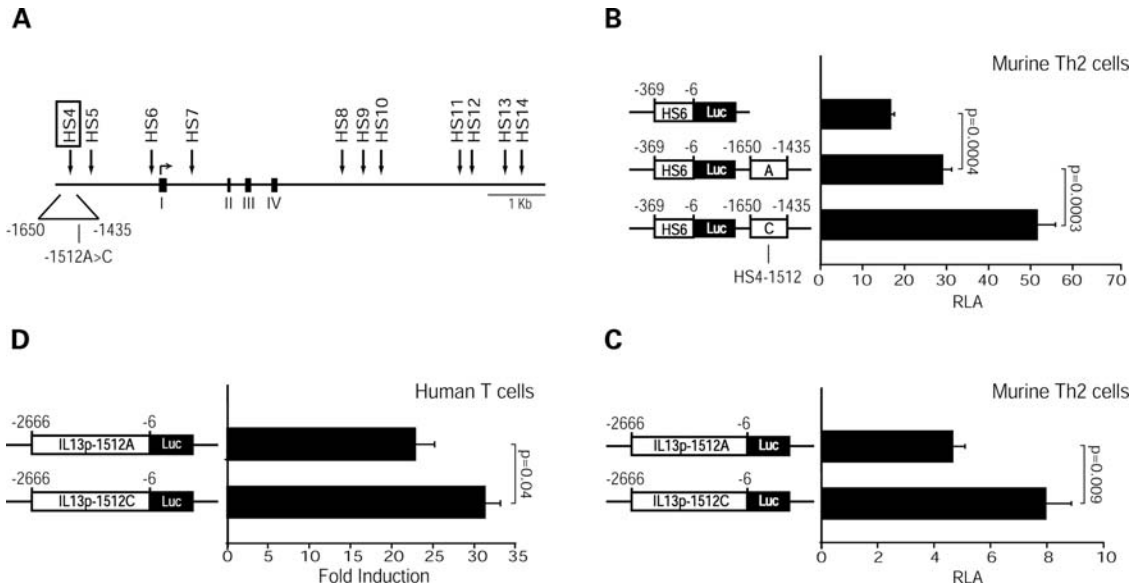
**IL-13 is a central effector of Th2-mediated allergic inflammation and is critical for the induction of IgE synthesis. Common *IL13* variants are associated with allergy phenotypes in populations of distinct ethnic background. We recently demonstrated that *IL13* expression by human CD4<sup>+</sup> T cells is paralleled by extensive *IL13* locus remodeling, which results in the appearance of multiple DNase I hypersensitive sites. Among these, HS4 in the distal promoter is constitutive in both naïve and polarized Th1 and Th2 cells, and spans a common single nucleotide polymorphism, *IL13*-1512A>C (rs1881457), strongly associated with total serum IgE levels. We recently characterized HS4 as a novel *cis*-acting element that upregulates *IL13* transcription in activated human and murine T cells. Here we show that *IL13*-1512A>C is a functional polymorphism that significantly enhances HS4-dependent *IL13* expression by creating a binding site for the transcription factor Oct-1. Of note, endogenous Oct-1 was preferentially recruited to the *IL13*-1512C risk allele in primary CD4<sup>+</sup> T cells from *IL13*-1512A>C heterozygous subjects. Moreover, the *IL13*-1512C allele was overexpressed in transfected Th2 cells from *Oct1*<sup>+/+</sup> mice, but not from *Oct1*<sup>+/-</sup> mice, demonstrating that increased activity was exquisitely dependent on physiologic levels of Oct-1. Our results illustrate how a functional variant in a regulatory element enhances transcription of an allergy-associated gene, thereby modulating disease susceptibility.**

## INTRODUCTION

The cytokine IL-13 is a central effector of Th2 responses and is necessary and sufficient to induce all the cardinal features of allergic lung inflammation and experimental asthma. IL-13 promotes allergen-induced airway hyperresponsiveness, epithelial cell damage, goblet cell metaplasia with mucus hyperproduction and eosinophilia (1,2). Furthermore, IL-13 shares with another cytokine, IL-4, a unique capacity to stimulate human B lymphocytes to synthesize IgE (3), which further amplifies allergen-specific inflammatory responses. Long-term lung inflammation is sustained by the IL-13-dependent activation of resident lung cells, which release chemokines, cytokines (4) and other effector molecules (5) and are involved in airway fibrosis (6,7). Of note, IL-13 is readily detectable in the human placenta (8) and is vigorously secreted by neonatal T cells (9), in line with the notion that early life immunoregulatory events play a critical role in the inception of asthma and allergy (10).

Because of its critical role as an effector of Th2 immunity, *IL13* is a strong candidate gene for allergy and asthma. Indeed, the associations between allergy-related phenotypes (such as IgE levels, asthma and atopy) and individual *IL13* variants (particularly *IL13*-1112C>T, rs1800925 and *IL13*+2044G>A, rs20541) are robust and widely replicated (reviewed in 11). However, dissection of *IL13* genotype/phenotype associations and identification of causative variants require functional studies, because the *IL13* locus harbors numerous common single nucleotide polymorphisms (SNPs) and complex patterns of linkage disequilibrium (LD). We recently showed that *IL13*-1112C>T in the promoter increases *IL13* transcription in transfected murine and human Th2 cells by creating a YY1 binding site and relieving STAT6-mediated repression (12). *IL13*+2044G>A, a non-synonymous SNP in the fourth exon in modest LD with *IL13*-1112C>T, leads to the expression of an IL-13 variant with increased biological activity (13).

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**Figure 1.** *IL13*-1512C enhances HS4-dependent *IL13* expression. (A) Schematic representation of DNase I HS sites (arrows) within the human *IL13* locus. Exons I–IV are marked by black boxes. The location of *IL13*-1512A>C within HS4 is depicted. (B and C) Actively proliferating murine Th2 cells polarized *in vitro* for 9–10 days were nucleofected with HS4/Luc constructs (B) or -2666*IL13p*/Luc constructs (C) carrying an A or a C at position -1512. Cells were harvested 16 h post-transfection. Results are expressed as RLA (mean  $\pm$  SE) measured in 10 (B) or 6 (C) independent experiments. (D) Jurkat T cells were transiently transfected with -2666*IL13p*/Luc constructs carrying an A or a C at position -1512 and harvested after 16 h of culture in the presence or absence of PMA (20 ng/ml) and ionomycin (1  $\mu$ M). Results are expressed as the mean  $\pm$  SE of fold induction values, i.e. the ratio between RLA in stimulated and unstimulated cells measured in six independent experiments. Statistical significance was calculated using the Wilcoxon two-sample test.

Studies in mouse models have shown that expression of *IL13* and the other Th2 cytokine genes, *IL5* and *IL4*, is orchestrated by multiple conserved and developmentally regulated nuclease hypersensitive (HS) sites that are scattered throughout the 160 kb Th2 locus (14) and include gene-specific enhancers as well as a locus control region (15). HS sites typically map to *cis*-regulatory elements and reflect the DNA binding activity of sequence-specific *trans*-acting factors that induce destabilization or displacement of local nucleosomes (16). We extensively analyzed chromatin remodeling at the human *IL13* locus in naïve and differentiating CD4<sup>+</sup> Th cells (17). Among the novel HS sites we identified was HS4, which resides in the distal *IL13* promoter and co-localizes with CpG hypomethylation in naïve and *in vitro* polarized Th1 and Th2 cells. Early and persistent accessibility at HS4 suggests that occupancy by constitutive transcription factors may poise the promoter for rapid *IL13* expression upon T cell activation and/or differentiation.

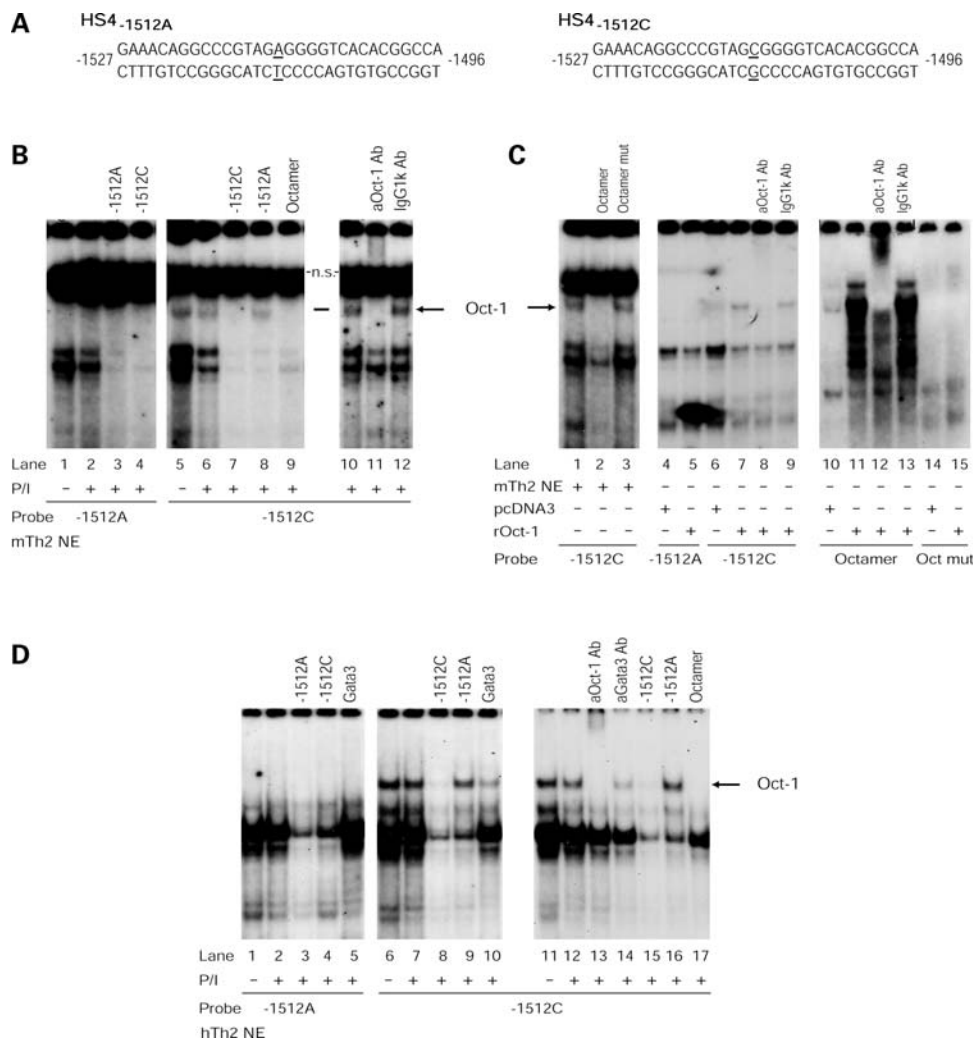
We recently showed that HS4 marks the location of a *cis*-regulatory region which is highly conserved in primates and enhances *IL13* promoter activity in transfected human Jurkat T cells and murine Th2 cells (P. Kiesler *et al.*, submitted). Interestingly, HS4 harbors a common SNP, *IL13*-1512A>C (rs1881457, minor allele frequency >20%), which is strongly associated with total serum IgE levels in unselected populations from the United States and Germany (18). Moreover, an analysis of the association between several *IL13* SNPs and total IgE levels in the British 1958 Birth Cohort showed *IL13*-1512A>C accounted for 85% of the phenotypic variance attributable to *IL13* polymorphisms (19). An association between *IL13*-1512A>C and IgE levels was also found in atopic asthmatic children from Korea (20). These strong

genetic associations, and the localization of *IL13*-1512A>C within a *cis*-acting element, prompted us to investigate whether *IL13*-1512A>C alters the *IL13* regulatory properties of HS4.

## RESULTS

### *IL13*-1512A>C is a functional polymorphism that enhances HS4 activity

We recently provided evidence that the *IL13* promoter region marked by HS4 (-1650 to -1435; numbering is relative to the *IL13* ATG) acts as a position-independent, positive regulator of *IL13* promoter activity in transfected human Jurkat T cells and primary, *in vitro* differentiated murine Th2 cells (P. Kiesler *et al.*, submitted). *IL13*-1512A>C lies in the central portion of HS4 (Fig. 1A). To directly test the impact of *IL13*-1512A>C on the regulatory properties of this *cis*-acting element, we took a reductionist approach that relied on two sets of reporter vectors: one in which HS4 was linked to the *IL13* proximal promoter (HS6/Luc), and one in which HS4 resided at its genomic location within the distal *IL13* promoter. In order to characterize HS4 function in a significant nuclear environment, we compared the ability of the HS4 variants to upregulate the *IL13* proximal promoter in transiently transfected, *in vitro* differentiated primary murine CD4<sup>+</sup> Th2 cells. These cells are programmed for high-rate *IL13* expression *in vivo* and are endowed with a transcriptional machinery ideal for investigating allele-specific differences in human *IL13* expression (12). Figure 1B shows that both HS4<sub>-1512A</sub> and HS4<sub>-1512C</sub> significantly enhanced *IL13* promoter activity. However, the HS4 variant carrying the -1512C



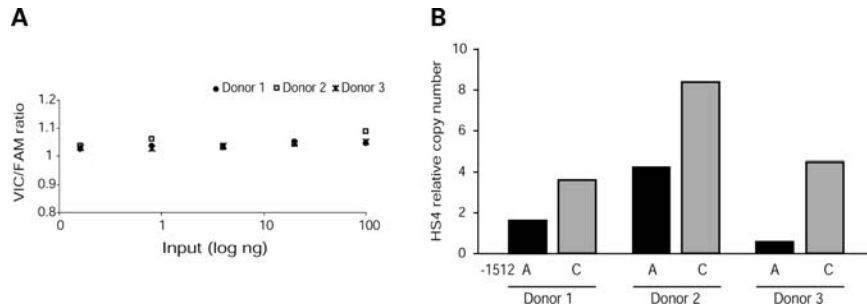
**Figure 2.** HS4<sub>-1512C</sub> selectively binds Oct-1. Radiolabeled HS4 probes carrying an A or a C at position -1512 (A) were incubated with nuclear extracts from *in vitro* polarized murine Th2 cells (mTh2 NE) (B and C) or human Th2 cells (hTh2 NE) (D) cultured for 3 h in the presence or absence of PMA (20 ng/ml) and ionomycin (1  $\mu$ M) (P/I) or *in vitro* translated recombinant Oct-1 (rOct-1) (C). EMSA probes and experimental conditions are noted below the gels. Competitors (100-fold molar excess) and supershifting antibodies added to each reaction are noted above the relevant lanes. A Gata3 consensus oligonucleotide and IgG1 $\kappa$  or an anti-Gata3 antibody were used as unrelated controls. Ns: non-specific.

risk allele was significantly ( $P = 0.0003$ ) more active than the A allele. Moreover, a full length (2.7 kb) *IL13* promoter construct carrying -1512C was significantly more active than the A variant in both murine Th2 cells (Fig. 1C) and human Jurkat T cells (Fig. 1D). Collectively, these results demonstrate that *IL13*-1512A>C is a functional polymorphism that enhances HS4 activity and HS4-driven *IL13* expression.

#### The *IL13*-1512C allele selectively binds Oct-1 *in vitro*

In order to start characterizing the molecular mechanisms underlying the differential activity of the *IL13*-1512 alleles in CD4<sup>+</sup> T cells, we searched for allele-specific patterns of human *IL13* promoter/nucleoprotein interactions. Figure 2B shows that EMSA analysis with -1512 allele-specific probes and competitors (Fig. 2A) and murine Th2 cell nuclear extracts identified a specific complex, which constitutively bound HS4<sub>-1512C</sub> (lanes 5–6) but not HS4<sub>-1512A</sub> (lanes 1–2)

and was cross-competed by -1512C (lane 7) but not -1512A (lane 8) unlabeled oligonucleotides. This complex contained Oct-1, because it was specifically competed by an oligonucleotide carrying a canonical octamer motif (21) (Fig. 2C, lane 2), but not by an oligonucleotide in which the motif had been disrupted (22) (lane 3). *IL13*-1512C allele-specific binding of Oct-1 was further confirmed by supershifting experiments with Oct-1-specific and control antibodies (Fig. 2B, lanes 11–12), as well as by using *in vitro* translated recombinant Oct-1 (Fig. 2C, lane 7). The ability of recombinant Oct-1 to interact with a -1512C and a bona fide octamer probe (lane 11) also suggests that Oct-1 may form a complex directly with DNA. Of note, EMSA analysis with nuclear extracts from primary, *in vitro* differentiated human Th2 cells (Fig. 2D) or human Jurkat T cells (data not shown) also showed that the -1512C (lanes 6–7), but not the -1512A (lanes 1–2), allele bound Oct-1. Collectively, these analyses show that the replacement of an A with a C at



**Figure 3.** Allele-specific recruitment of Oct-1 to HS4. Chromatin from primary human CD4<sup>+</sup> T cells heterozygous at *IL13*-1512A>C and activated with PMA (20 ng/ml) and ionomycin (2  $\mu$ M) for 3 h was crosslinked, sonicated and immunoprecipitated with an anti-Oct-1 antibody or an IgG isotype control antibody. Target enrichment was assessed by allele-specific, real time PCR using a custom TaqMan SNP genotyping assay that amplified a 68 bp region spanning *IL13*-1512A>C (nucleotides -1547 to -1479) and discriminated between the A (VIC) and the C (FAM) alleles with reporter probes. **(A)** VIC:FAM signal ratios obtained from standard curves for each allele generated with serial dilutions of sonicated genomic DNA (input) from each experiment. **(B)** Ratio between number of HS4 targets immunoprecipitated with anti-Oct-1 Ab and control IgG Ab in three independent experiments, each with chromatin from a different donor.

position -1512 of the distal *IL13* promoter creates an Oct-1 binding motif.

#### Oct-1 is preferentially recruited to the *IL13*-1512C allele *in vivo*

To validate the preferential interaction between Oct-1 and the *IL13*-1512C allele, allele-specific ChIP was used to test the ability of Oct-1 to bind the endogenous HS4 variants *in vivo*. Cross-linked chromatin harvested from primary, activated CD4<sup>+</sup> T cells from *IL13*-1512A>C heterozygotes was immunoprecipitated with anti-Oct-1 or IgG isotype control antibodies. Real-time PCR with a custom TaqMan SNP genotyping assay, which was designed to amplify a 68 bp region of the human *IL13* distal promoter spanning *IL13*-1512A>C (nucleotides -1547 to -1479), tested for enrichment of the -1512A or -1512C alleles using two specific reporter probes: VIC recognized the A allele, whereas FAM recognized the C allele. Results were expressed as HS4 relative copy number, i.e. the ratio between HS4 copies immunoprecipitated by anti-Oct-1 and IgG isotype control. Figure 3A shows that this approach resulted in accurate quantification of both HS4 alleles, because the VIC (-1512A)/FAM (-1512C) signal ratio was consistently equal or close to 1 over several orders of magnitude of input DNA (Fig. 3A). Figure 3B shows that endogenous Oct-1 was preferentially recruited to the *IL13*-1512C promoter allele in three independent experiments, each relying on chromatin from a different *IL13*-1512A>C heterozygous donor. These results support a critical role of Oct-1 in the differential activity of the *IL13*-1512 alleles.

#### The increased activity of HS4<sub>-1512C</sub> requires physiologic levels of Oct-1 in the nuclear environment

Because Oct-1 binds selectively the -1512C allele, and this allele exhibits higher activity, we hypothesized that Oct-1 is required for enhanced HS4 activity. To test our hypothesis, we turned to genetically modified mice in which the nuclear environment contains different levels of Oct-1. *Oct1* deletion by gene targeting is embryonically lethal because of defective erythropoiesis, but heterozygous *Oct1*<sup>+/-</sup> mice are fully

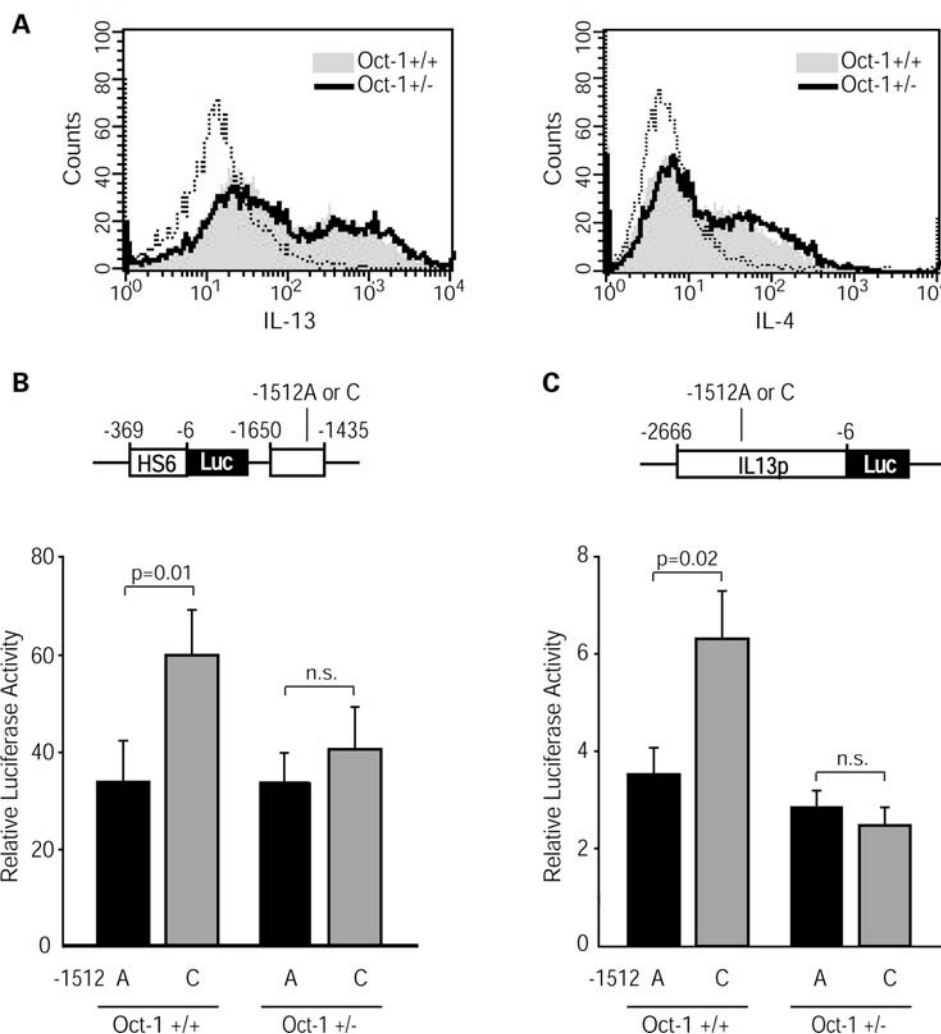
viable (21). We compared the activities of HS4<sub>-1512A</sub> and HS4<sub>-1512C</sub> reporter constructs following transfection into Th2 cells from *Oct1*<sup>+/+</sup> and *Oct1*<sup>+/-</sup> C57BL/6 mice. Figure 4A shows that endogenous Th2 cell differentiation, as revealed by intracellular IL-4 and IL-13 protein expression, was fully preserved in *Oct1*<sup>+/-</sup> cells. Of note, HS4<sub>-1512C</sub> was significantly ( $P = 0.01$ ) more active than HS4<sub>-1512A</sub> in upregulating the human *IL13* promoter in *Oct1*<sup>+/+</sup> Th2 cells. In contrast, the partial decrease in Oct-1 binding activity caused by heterozygosity for the *Oct1* null mutation ( $\approx 60\%$  as assessed by EMSA, data not shown) was sufficient to abrogate the increased activity of the -1512C allele (Fig. 4B). Comparable results were obtained by transfecting *Oct1*<sup>+/+</sup> and *Oct1*<sup>+/-</sup> Th2 cells with -1512 allelic variants of the full-length *IL13* promoter construct (Fig. 4C). These results show that the enhanced activity of HS4<sub>-1512C</sub> was exquisitely dependent on physiologic levels of Oct-1.

## DISCUSSION

Extensive studies in mouse models have shown that the expression of Th2 cytokines, including *IL13*, is controlled by an array of conserved DNase I HS regulatory elements that are positioned throughout the Th2 locus (reviewed in 23). Few of these elements have been functionally characterized in mice (24–30) and virtually none in humans. Our current analysis focused on HS4, an element located in the distal human *IL13* promoter which is detectable in naïve CD4<sup>+</sup> T cells and persists throughout T cell differentiation. Our group has shown that HS4 is a functional regulatory element that strongly upregulates *IL13* transcription in response to T cell activation cues (P. Kiesler *et al.*, submitted).

The *IL13*-1512C allele creates an Oct-1 binding site that is essential for the enhanced activity of the -1512C HS4 variant. Although the site contains a non-canonical octamer motif, its ability to specifically bind Oct-1 was supported by functional studies in murine Th2 cells and by DNA/protein interaction analysis *in vitro* and *in vivo*. To our knowledge, this is the first time that allele-specific binding of a transcription factor to a polymorphic site in the human *IL13* locus is demonstrated *in vivo* and shown to have a significant transcriptional impact.





**Figure 4.** The increased activity of HS4.<sub>-1512C</sub> requires physiologic levels of Oct-1 in the nuclear environment. (A) Th2 cells from C57BL/6 wild-type or *Oct1*<sup>+/-</sup> mice were generated by *in vitro* polarization for 9–10 days, and analyzed by immunofluorescence for intracellular Th2 cytokine expression. The dotted lines correspond to the negative control. Th2 cells were nucleofected with HS4/Luc constructs (B) or -2666*IL13p*/Luc constructs (C) carrying an A or a C at position -1512. Cells were harvested 16 h after transfection. Results are expressed as RLA (mean ± SE) measured in 8 (B) or 11 (C) independent experiments. Statistical significance was calculated using the Wilcoxon two-sample test.

Oct-1 belongs to a family of transcription factors characterized by the POU domain, whose peculiar bipartite structure confers to these proteins great flexibility in their interactions with DNA and other transcriptional regulators (31). Because of this functional versatility, the role played by Oct proteins in the regulation of individual genes varies with the sequence of the octamer motif, the promoter context and the identity of the regulators that engage in interactions with the POU domain. In T lymphocytes, Oct-1, a ubiquitously expressed protein, has been shown to activate tissue-specific gene transcription by cooperatively interacting with lineage-selective factors, particularly NFAT family proteins, within DNase I HS regions (32–34). Interestingly, HS4 contains several putative NFAT binding sites, one of which lies immediately upstream of *IL13*-1512A>C and may provide a platform for productive NFAT/Oct-1 interactions. Further studies are in progress to characterize the molecular events responsible for Oct-1-dependent *IL13* upregulation. It is also noteworthy

that Oct-1 had not been previously linked to *IL13* regulation (21), and our experiments showed endogenous *IL13* expression was fully preserved in Th2 cells from *Oct1*<sup>+/-</sup> mice. The low degree of conservation of the human distal *IL13* promoter, and the absence of *IL13*-1512A>C from the mouse *IL13* promoter, may explain the latter finding. Our results illustrate how creation of a transcription factor binding site within an important *cis*-element may redirect the regulatory program, thereby offering evolutionarily novel opportunities for modulating gene expression and disease susceptibility.

To what extent do the gain-of-function properties of *IL13*-1512A>C explain the previously reported (18–20) associations between this polymorphism and allergy-related phenotypes, particularly elevated IgE levels? The answer to this question depends on both LD patterns and magnitude of the variant's functional effects. In Caucasians, *IL13*-1512A>C is in strong, albeit not perfect, LD with *IL13*-1112C>T (12), which

also resides within the distal *IL13* promoter. Indeed, our LD analysis, which focused on a population of non-Hispanic white children from Tucson, AZ ( $n = 558$ ), and relied on a widely used algorithm (Haploview: ref. 35), yielded an  $r^2$  value of 0.804 for these polymorphisms (D. Stern and M. Halonen, unpublished), and a comparable LD estimate ( $r^2 = 0.71$ ) was recently reported for the ISAAC cohort ( $n = 526$ ). Because of strong LD, the phenotypic effects of *IL13*-1512A>C and *IL13*-1112C>T are not readily separable at the population level, at least in Caucasians. LD estimates for large populations of other ethnicities are currently unavailable. In terms of transcriptional effects, both *IL13*-1512A>C and *IL13*-1112C>T are gain-of-function polymorphisms. However, the increases in *IL13* transcription dependent on the -1512C or the -1112T allele (12) were statistically significant but admittedly modest, a functional outcome which is quite typical of common regulatory variants (36–38). Therefore, taken in isolation, neither *IL13*-1512A>C nor *IL13*-1112C>T are likely to dysregulate *IL13* expression intensely enough to cause an allergic disease phenotype. On the other hand, both *IL13*-1512A>C and *IL13*-1112C>T lie within an LD block that extends  $\approx 87$  kb upstream of the *IL13* promoter, reaching beyond the Th2 locus control region located in *RAD50* (39). Remarkably, several of the SNPs in this block fall within highly conserved HS sites that have been shown to be critical for the coordinated regulation of Th2 cytokine gene expression in mice (15). It is therefore tempting to speculate that, by acting in concert, linked variants of individually modest effects may profoundly alter Th2 cytokine expression, thereby modifying susceptibility to Th2-mediated allergic inflammation.

## MATERIALS AND METHODS

### Mice

*Oct1*<sup>+/-</sup> mice on a C57BL/6 background (21) and C57BL/6 control mice (The Jackson Laboratory) were maintained under specific pathogen-free conditions. All experiments were performed according to institutional and federal guidelines.

### T-cell isolation and Th2 differentiation

To generate murine Th2 cells, CD4<sup>+</sup> T lymphocytes were isolated from splenocyte suspensions using the CD4<sup>+</sup> T cell isolation kit (Miltenyi) as recommended by the manufacturer. Cells ( $\sim 5 \times 10^6$ ) were resuspended in DMEM supplemented with FCS (10%), HEPES (10 mM), 2- $\beta$ -ME (0.1 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and L-glutamine (2 mM), and stimulated for 3 days with plate-bound anti-CD3 mAb (clone 145-2C11, 1  $\mu$ g/ml) and anti-CD28 mAb (clone 37.51, 1  $\mu$ g/ml) in the presence of IL-4 (1000 U/ml) and neutralizing antibodies against IFN- $\gamma$  (clone R4-6A2, 5  $\mu$ g/ml) and IL-12 (clone C17.8, 3  $\mu$ g/ml) (40). After expansion in the presence of IL-4, neutralizing antibodies and IL-2 (20 U/ml) for 4 days, cells were re-stimulated with plate-bound anti-CD3 and anti-CD28 mAb, and nucleofected at day 9–10. Th2 cell polarization was assessed by intracellular cytokine staining (17).

Human primary CD4<sup>+</sup> T cells were isolated from peripheral blood samples using the RosetteSep human T cell enrichment

cocktail (Stemcell technologies) following the manufacturer's directions and resuspended in RPMI 1600 supplemented with FBS (10%), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and L-glutamine (2 mM). *In vitro* polarized human Th2 cells were generated as previously described (12). Briefly, naïve cord blood CD45RO<sup>-</sup>CD4<sup>+</sup> T cells were obtained by negative selection and depletion of memory T cells (Miltenyi). Cells were stimulated for 4 days with plate-bound anti-CD3 mAb (UCHT1, 2  $\mu$ g/ml) and soluble anti-CD28 mAb (1  $\mu$ g/ml) in the presence of IL-2 (5 ng/ml), IL-4 (4 ng/ml) and neutralizing antibodies to IL-12 (2  $\mu$ g/ml) and IFN- $\gamma$  (2  $\mu$ g/ml) and then rested for 3 days in IL-2, IL-4 and anti-IL-12. Subsequently, cells were restimulated for 4 days on anti-CD3-coated plates with IL-4 and anti-IL-12 mAb. Experiments were performed on day 16. Th2 cell polarization was assessed by intracellular cytokine staining (17).

Jurkat T cells (ATCC clone E6-1) were cultured in RPMI 1640 supplemented with fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml) and L-glutamine (2 mM). These cells exhibit DNase I hypersensitivity at HS4 (data not shown) and vigorously up-regulate *IL13* mRNA upon activation (12).

### Reporter constructs

2666*IL13*p/Luc, a luciferase reporter construct driven by the major *IL13*-1512A promoter variant (12), was used as a template to generate -2666*IL13*p<sub>-1512C</sub>/Luc through QuickChange site-directed mutagenesis (Stratagene). HS6/Luc consisted of a 363 bp region encompassing the human *IL13* proximal promoter (-369 to -6) cloned into pGL3 Basic (Promega) (41). PCR amplification of HS4 using -2666*IL13*p/Luc or -2666*IL13*p<sub>-1512C</sub>/Luc as templates and primers HS4<sub>-1650</sub> (5'-ATACTCGTGCACATAAGGGGCGTTGACTCAC) and HS4<sub>-1435</sub> (5'-TTGATGTCGACTCTGACTCCCAGAAGTCTG) and cloning of these amplicons into the *Sa*I restriction site of HS6/Luc in the forward orientation generated HS4<sub>-1512A</sub>/Luc and HS4<sub>-1512C</sub>/Luc, respectively. All constructs were verified by sequencing.

### Transient transfections

Anti-CD3/anti-CD28 mAb-activated murine Th2 cells ( $1 \times 10^6$ ) were nucleofected with equimolar amounts of endotoxin-free reporter constructs (1  $\mu$ g) and pRL-TK (50 ng; Promega) using the Amaxa nucleofector and the mouse T-cell nucleofector kit (Amaxa). Firefly and renilla luciferase activities in cell lysates were assessed 16 h after nucleofection using the Dual Luciferase Assay System (Promega). Total protein concentrations in cell lysates were determined using the BCA assay (Pierce). Results were expressed as RLA (relative luciferase activity) units, i.e. firefly luciferase activity corrected for renilla luciferase (transfection efficiency) and protein content of each sample.

Endotoxin-free plasmids (5  $\mu$ g) were electroporated into Jurkat T cells ( $5 \times 10^6$ ) in log phase of growth using the BTX ECM830 square wave electroporator (1 pulse, 250 V, 50 ms). Cells were co-transfected with pRLTK (25 ng, Promega) to normalize for transfection efficiency. After electroporation, cells were incubated in the presence or absence of

PMA, phorbol 12-myristate 13-acetate, (20 ng/ml, Sigma) and ionomycin (1  $\mu$ M, Sigma). Results were expressed as fold induction values, i.e. the ratio between RLA in stimulated and unstimulated cells.

### EMSA

Murine Th2 cells, human Th2 cells or Jurkat T cells ( $15 \times 10^6$ ) were incubated in the presence or absence of PMA (20 ng/ml) and ionomycin (1  $\mu$ M) for 3 h. After stimulation, cells were collected by centrifugation and washed twice with PBS. Cell pellets were resuspended (1:5 v/v) in buffer A (3 mM MgCl<sub>2</sub>, 10 mM NaCl, 10 mM Tris pH 7.5, 0.1 mM EGTA, 0.5 mM DTT, 1  $\mu$ g/ml aprotinin, 2  $\mu$ M leupeptin, 1 mM PMSF) and incubated on ice for 10 min. NP40 (0.5%) was added and nuclei were pelleted by centrifugation. Nuclear pellets were resuspended (1:2.5 v/v) in buffer C (1.5 mM MgCl<sub>2</sub>, 20 mM HEPES, pH 7.0, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1  $\mu$ g/ml aprotinin, 2  $\mu$ M leupeptin, 0.5 mM PMSF) and incubated on ice for 30 min. After centrifugation, supernatants were collected, snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . A pcDNA3.1-Oct-1 expression vector or empty pcDNA3.1 control (Invitrogen) were *in vitro* translated using the TNT Quick Coupled Transcription/Translation System (Promega) following the manufacturer's directions.

Nuclear extracts (5  $\mu$ g) or *in vitro* translated Oct-1 were incubated with <sup>32</sup>P-labeled HS4<sub>-1512A</sub> or HS4<sub>-1512C</sub> probes in binding buffer (10 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, 0.5 mM MgCl<sub>2</sub>, 100 mM NaCl, 10% glycerol) and poly dI-dC (50 ng/ $\mu$ l) for 30 min at  $4^\circ\text{C}$ . Oligonucleotide competitors (Octamer: 5'-GGATGCAAATATGCAAATATGCAAATGG (21) and Octamer mut: 5'-GGcgGCAAATcgGCAAATcgGCAAATGG (22), 100-fold molar excess) or antibodies (anti-Oct-1, C-21X, anti-Gata3, B-10X, from Santa Cruz Biotechnologies, or IgG1 $\kappa$ , PMP48, from Serotec; 2  $\mu$ g) were pre-incubated with nuclear extracts for 30 min at  $4^\circ\text{C}$  before addition of radiolabeled probe. DNA/protein complexes were separated on 5% (w/v) non-denaturing polyacrylamide gels in TBE (0.5 $\times$ ) for 5 h at  $4^\circ\text{C}$ .

### Allele-specific ChIP

Freshly isolated peripheral blood CD4<sup>+</sup> T cells ( $\sim 7 \times 10^6$ /condition) from *IL13-1512A>C* heterozygotes were stimulated in the presence of PMA (20 ng/ml) and ionomycin (2  $\mu$ M) for 3 h. Chromatin immunoprecipitation experiments were performed using the ChIP assay kit (Millipore) following the manufacturer's directions with few modifications. Specifically, paraformaldehyde fixation was stopped by the addition of 125 mM glycine (RT, 5 min). Crosslinked chromatin was sonicated six times for 10 s each time with a Microson XL200 sonicator (Misonix). An aliquot of chromatin (1/20th) was set aside and used as input DNA. After preclearing, chromatin samples were immunoprecipitated with a rabbit anti-Oct-1 antibody (20  $\mu$ g, sc-232X, Santa Cruz Biotechnology) or a rabbit IgG isotype control antibody (20  $\mu$ g, 12-370, Millipore).

Allele-specific enrichment of the polymorphic HS4 region in immunoprecipitated samples was quantified using a

custom TaqMan SNP genotyping assay (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System. PCR primers (5'-GGCCCTCTACTACAGATTAGGAAAC and 3'-CCTGGAGTGCCGCTACTTG) were designed to amplify a 68 bp region of the human *IL13* distal promoter that spans *IL13-1512A>C* (nucleotides -1547 to -1479). Two TaqMan probes discriminated between the two alleles. The -1512A allele was recognized by the reporter dye VIC (5'-CGTGTGACCCCTCTAC), whereas the -1512C allele was recognized by the reporter dye FAM (5'-CGTGTGACCCCGCTAC). Real-time PCR amplifications were performed in triplicate with the following cycling conditions: 10 min at  $95^\circ\text{C}$  followed by 40 cycles of 15 s at  $92^\circ\text{C}$  and 30 s at  $60^\circ\text{C}$ . Serial dilutions of input DNA were used to generate standard curves for each allele in each experiment and PCR efficiency was determined with the formula:  $E = 10^{(-1/k)} - 1$ , where  $k$  represents the slope of each standard curve. Efficiency values were essentially equal to 1 and closely comparable between reporter dyes. Moreover, as expected for heterozygous genomic DNA, VIC:FAM signal ratios were essentially 1:1 and remained constant across several orders of magnitude of input DNA. Copy number values were calculated with the formula:  $N = (1 + E)^{40 - C_{\text{(sample)}}$ , where  $E$  represents the PCR efficiency. For each allele, results were expressed as relative copy numbers, that is, as the ratio between the numbers of HS4 targets immunoprecipitated with anti-Oct-1 and the numbers of HS4 targets immunoprecipitated with control IgG.

### Human subjects

Cord blood was obtained from anonymous cesarean deliveries. Peripheral blood was drawn from healthy non-allergic subjects of known *IL13-1512A>C* genotype after written informed consent was obtained. All protocols were approved by the Institutional Review Board of the University of Arizona.

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