Published in final edited form as:

Allergy. 2012 January ; 67(1): 33–40. doi:10.1111/j.1398-9995.2011.02707.x.

Increased expression of IRF4 and ETS1 in CD4⁺ cells from patients with intermittent allergic rhinitis

Sören Bruhn^{1,*}, Fredrik Barrenäs^{1,*}, Reza Mobini, PhD^{1,*}, Bengt A. Andersson², Sreenivas Chavali³, Brian S. Egan⁴, Eivind Hovig^{5,6,7}, Geir Kjetil Sandve⁷, Michael A. Langston^{8,9}, Gary Rogers^{8,9}, Hui Wang¹, and Mikael Benson^{1,10}

¹The Centre for Individualized Medication, Linköping University Hospital, 58185, Linköping, Sweden

²The Department of Clinical Immunology and Transfusion Medicine, Sahlgrenska University Hospital, 41346, Gothenburg, Sweden

³Structural Studies Division, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK

⁴Genepathway, Inc., San Diego, CA 92121, USA

⁵Department of Tumor Biology, Institute of Cancer Research, the Norwegian Radium Hospital, 0310, Oslo, Norway

⁶The Institute for Medical Informatics, Rikshospitalet, Oslo University Hospital, 0514, Oslo, Norway

⁷Department of Informatics, University of Oslo, 0316, Oslo, Norway

⁸Department of Electrical Engineering and Computer Science, University of Tennessee, Knoxville, TN 37996, USA

⁹Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

¹⁰Unit for Pediatric Allergology, Queen Silvia Children's Hospital, Göteborg, 40530 Sweden

Abstract

Background—The transcription factor (TF) IRF4 is involved in the regulation of Th1, Th2, Th9 and Th17 cells, and animal studies have indicated an important role in allergy. However, IRF4 and its target genes have not been examined in human allergy.

Methods—IRF4 and its target genes were examined in allergen-challenged CD4⁺ cells from patients with IAR using combined gene expression microarrays and chromatin immunoprecipitation chips (ChIP-chips), computational target prediction and RNAi knockdowns.

Results—*IRF4* increased in allergen-challenged CD4⁺ cells from patients with IAR and functional studies supported its role in Th2 cell activation. IRF4 ChIP-chip showed that IRF4 regulated a large number of genes relevant for Th cell differentiation. However, neither Th1 nor Th2 cytokines were the direct targets of IRF4. To examine if IRF4 induced Th2 cytokines via one or more down-stream TFs, we combined gene expression microarrays, ChIP-chips and computational target prediction and found a putative intermediary TF, namely ETS1 in allergen-challenged CD4⁺ cells from allergic patients. *ETS1* increased significantly in allergen-challenged

Corresponding author: Mikael Benson, MD, PhD The Centre for Individualized Medication Linköping University Hospital 58185 Linköping Sweden Tel: +46 10-1031337 Fax: +46 31 786-3232 mikael.benson@liu.se.

*These authors contributed equally to the work.

CD4⁺ cells from patients compared to controls. Gene expression microarrays before and after *ETS1* RNAi knockdown showed that *ETS1* induced Th2 cytokines as well as disease-related pathways.

Conclusions—Increased expression of IRF4 in allergen-challenged CD4⁺ cells from patients with intermittent allergic rhinitis, leads to activation of a complex transcriptional program, including Th2 cytokines.

Keywords

Allergy; transcription factor

INTRODUCTION

The transcription factor (TF) Interferon Regulatory Factor 4 (IRF4) has a critical role in Th2 cell regulation, by controlling cytokine release and apoptosis (1-4). IRF4 is also involved in the regulation of Th1, Th9, Th17 and T regulatory cells (5-10). This pleiotropy indicates that IRF4 induces different pathways in different cell types and contexts (4). An important implication is that IRF4 could be used to trace context-dependent pathways in, for example, different diseases. Indeed IRF4 has been implicated both in malignant and inflammatory diseases (11). To our knowledge, IRF4 has not been studied in allergic patients, but its potential relevance is supported by studies of animal models (12). In this study we aimed to analyse IRF4 and IRF4-induced pathways in allergen-challenged CD4⁺ cells from patients with intermittent allergic rhinitis (IAR). For this purpose, we combined chromatin immunoprecipitation chips (ChIP-chips), gene expression microarrays and novel computational methods with short interfering RNA (siRNA) knockdowns. Neither ChIP-chip technology, nor the analytical principles have been previously applied to allergy research. However, the principles are likely to be generally applicable to elucidate disease-relevant pathways in allergy, as well as the TFs that regulate those pathways. Detailed descriptions of the methods are beyond the size restrictions of this journal, and have been given elsewhere (13). Briefly, to determine which genes were regulated by IRF4 we performed IRF4- and RNA polymerase II ChIP-chip analyses of allergen-challenged CD4⁺ cells. The IRF4 ChIP-chip was used to determine IRF4 binding sites and the RNA polymerase II ChIP-chip to determine which of those sites were associated with active transcription. We found that IRF4 was involved in activation or inhibition of some 400 genes, but those genes did not include Th1 and Th2 cytokines. This indicated that IRF4 induced Th2 cytokine release via one or more down-stream TFs. To find such TFs we developed a novel analytical method, which combined gene expression microarrays and ChIP-chip with sequence-based predictions. This resulted in the identification of *ETS1* as an IRF4-induced TF. This TF increased greatly in allergen-challenged CD4⁺ cells. siRNA mediated knockdown of *ETS1* resulted in decreased levels of Th2 cytokines and gene expression microarrays before and after *ETS1* knockdown showed altered expression of several pathways of relevance for allergy. Those pathways included novel candidate genes in allergy, which were validated in an independent gene expression microarray study of allergen-challenged CD4⁺ cells from patients with IAR.

METHODS

Patients

Blood samples were gathered from 35 asymptomatic patients with grass pollen-induced allergic rhinitis outside of the season. CD4⁺ cells from these patients were used for gene expression profiling (n = 23), quantitative real-time PCR (n = 6) and ChIP-chip analyses (n = 6). Patient characteristics, inclusion criteria, *in vitro* stimulation of allergen-challenged

CD4⁺ cells, Q-PCR, flow cytometry, CHIP-chip, sequence-based computational methods, immunoblotting and statistical methods are described in the Supporting Information

Gene expression microarray analysis of CD4⁺ cells

Allergen- and diluent challenged CD4⁺ cells from 20 of the 23 patients, as well as Th2 polarized cells were analyzed with Illumina's Sentrix® Human-6 Expression BeadChips (Illumina Inc, San Diego, CA). CD4⁺ cells from the remaining three patients were analyzed with Affymetrix HuGe U133A (Affymetrix Inc., Santa Clara, CA) (14).

Small interfering RNA (siRNA) mediated gene knockdown of *ETS1*

CD4⁺ Th cells and memory CD4⁺ Th cells were isolated from PBMCs from healthy blood donors using isolation kits from Miltenyi (Bergisch Gladbach, Germany). Isolated cells were nucleofected either with nucleofection buffer, 1 μ M human on target plus SMARTpool siRNA against *ETS1* (Dharmacon, Lafayette, CO) or non-targeting siRNA (Dharmacon, Lafayette, CO) using the AMAXA nucleofection program U-014. Six hours after the nucleofection cells were washed, activated and polarized either towards Th1 or Th2 (see the supporting information for details).

RESULTS

Analysis of IRF4 in allergen-challenged or Th2 polarized CD4⁺ cells

Q-PCR analysis showed a 27.1 ± 5.6 fold increase of *IRF4* in allergen-challenged CD4⁺ cells from patients with IAR, compared to diluent-challenged CD4⁺ cells ($P < .01$). This increase was much higher than in controls, which showed a 5.3 ± 1.7 fold increase compared to the diluent, $P < .05$ (Fig. 1A and 1B). The increase of IRF4 was associated with significantly increased release of IL-5. IL-5 increased from 15.6 ± 0.8 to 54.4 ± 3.9 pg/mL ($P < .001$) (Fig. 1C). In healthy controls, however, no significant change of IL-5 was found. Since glucocorticoids (GCs) are known to have a beneficial effect in allergic inflammation, we examined if GCs would down-regulate *IRF4* and IL-5. Indeed, Q-PCR showed that GC treatment resulted in a significant decrease in *IRF4* expression level in the allergen stimulated CD4⁺ cells ($P < .01$) (Fig. 1B). This was associated with a decrease of IL-5, to 23.4 ± 4.4 pg/mL ($P < .001$) (Fig. 1C).

The protein expression of IRF4 in CD4⁺ cells was studied by flow cytometry under the following conditions: CD4⁺ cells stimulated with diluent, anti-CD3 + anti-CD28, and Th2 polarizing conditions. This showed that IRF4 is induced by the activation of the CD4⁺ cells in both Th2 polarizing and anti-CD3 + anti-CD28 culture conditions (see Fig S1).

Combined IRF4 ChIP-chip and gene expression microarray analysis to find putatively IRF4-regulated genes in allergen-challenged CD4⁺ cells from patients with IAR

Putatively IRF4-regulated genes were identified by ChIP-chip analysis after validation of the anti-IRF4 antibody using immunoprecipitation and Q-PCR of two known IRF4 targets and two negative control targets (Fig. S2). The ChIP-chip analysis resulted in the identification of 2861 binding sites for IRF4. In order to find actively transcribed sites, RNA polymerase II ChIP-chip experiments were performed. This showed that 1595 of the 2861 sites were active binding sites for IRF4. These genes were compared with independent gene expression microarray data from allergen- or diluent challenged CD4⁺ cells from patients with IAR to identify IRF4-induced or -repressed target genes. Genes were considered IRF4-induced if they a) bound IRF4 and RNA polymerase II in the ChIP-chip analyses, b) showed significant increases in expression and were positively correlated with IRF4 in the gene expression microarray data from allergen-challenged CD4⁺ cells ($n = 296$, Table SI). Conversely, genes were considered IRF4-repressed if they a) bound IRF4 but not RNA

polymerase II, b) were differentially expressed and negatively correlated with IRF4 in allergen-challenged CD4⁺ cells (n = 91, Table SII). The IRF4 induced or repressed genes were analyzed for pathway enrichment using the Ingenuity Pathway Analysis tool (Fig. 2). This showed that the most significant pathways were involved in Th cell differentiation (p<0.001) and T cell receptor signaling (p<0.01). IRF4 also regulated a wide range of other cellular functions, including proliferation, metabolism and DNA methylation. Three genes from the T cell pathways, *GATA3*, *IL2RA* and *IL2RG*, were validated with Q-PCR. All three increased in expression following allergen-challenge, and decreased after treatment with GC (Fig. S3). While the Th cell differentiation pathway included the Th2 promoting genes *IL4R* and *GATA3*, none of the IRF4 - regulated genes were Th1 or Th2 cytokines. This suggested that IRF4 interacted with one or more other TFs to induce Th2 cytokine production rather than directly regulating this production. We found that the IRF4 induced genes included 28 TFs, of which only *GATA3* had previously been described as IRF4 induced. Several of those TFs had been reported to induce Th2 cytokines (*ETS1*, *HIF1A*, *MAF*, *MYB*, *RORA*, *STAT5B* (15-22). Another TF, *PRDM1*, was known to inhibit Th1 cytokines (23). IRF4, however, also induced the Th1 promoting TF *STAT4* (this is commented upon in the discussion).

Combined ChIP-chip, gene expression microarrays and computational target prediction to find IRF4 -induced transcription factors that regulate Th2 cytokine release

In order to determine if any of the IRF4 induced TFs directly regulated Th2 cytokine release, we combined the IRF4 ChIP-chip and the gene expression microarrays of allergen-challenged CD4⁺ cells with computational target gene prediction. Briefly, we used sequence-based target prediction to define IRF4-containing *cis*-regulatory modules (CRMs) within a 200 bp window around IRF4 binding sites. This resulted in 57 CRMs, which were used to find modules of putatively co-regulated target gene sets (henceforth called transcriptional modules (TMs)) using gene expression data (Fig. 3). This resulted in 25 TMs, $P < .0001$, compared to randomly generated gene sets (Fig. S4 and Table SIII-SV). The 25 TMs contained 11 TFs, all of which were differentially expressed (Fig. S5). In order to find which of those 11 TFs that were most likely to regulate Th2 cytokine release, the promoter regions of the Th2 cytokines *IL-4*, *IL-5* and *IL-13* as well as the Th1 cytokine *IFN-γ* were analyzed to find predicted TF binding sites (TFBSs) for the 11 TFs. TFs that had predicted TFBS in the promoter regions of the Th2 cytokines, but not in the promoter region of the Th1 cytokine were considered as likely Th2-regulators. The TF that best matched this criterion was *ETS1*. This TF had previously been implicated in Th2 cytokine regulation (19-22) but had not been described in human allergy.

Analysis of the role of *ETS1* in allergen-challenged CD4 cells from patients with intermittent allergic rhinitis

The mRNA expression of *ETS1* in allergen-challenged CD4⁺ cells from patients increased significantly compared to diluent treated cells. *ETS1* also increased in controls although this was less pronounced (Fig. 4). siRNA mediated knockdown of *ETS1* in Th2 polarized cells resulted in significant decreases of *ETS1* expression (Fig 5A and Fig S6). To check for off-target effects we analyzed the Th2 polarized cells with gene expression microarrays at two time points after knockdown and found no significant changes in genes that may be induced by small RNAs (24). In addition, Q-PCR analysis showed no decrease of *IRF4* and *GATA3*, following knockdown (Fig. S6). However, the *ETS1* knockdown did result in significant decreases of *IL-5* and *IL-13* protein levels (Fig. 5B and 5C).

To identify *ETS1* induced pathways, we performed pathway analysis of the gene expression microarray data from Th2 polarized cells at two time points following knockdown of *ETS1*. The most significant pathways were found at the first time point, and included Role of

Cytokines in Mediating Communication Between Immune Cells ($P < 0.0001$), *T cell receptor signalling* ($P < .001$), and *Airway inflammation in asthma* ($P < 0.01$) (Fig. S7). We examined if the genes that were differentially expressed in Th2 polarized cells following *ETS1* knockdown were also differentially expressed in allergen-challenged cells from patients with IAR. We found a significant overlap between the two datasets ($P < 0.01$, Table SVII.).

DISCUSSION

Allergic inflammation is a highly complex process that involves multiple interacting pathways and hundreds of genes (25-27). The comprehensive mapping of those pathways is therefore a formidable challenge. One approach to address this problem may be to use transcription factors (TFs) as tracers to define disease-causing pathways (28). In this study, we applied this principle to allergen-challenged CD4⁺ cells from patients with IAR. We combined ChIP-chip, gene expression microarrays, computational predictions and siRNA knockdowns. ChIP-chip technology has not been previously been used in allergy research, and we also developed novel computational methods to interpret the data. We focused on IRF4, which has an important role in regulating Th2 cells and other T cells but has not been examined in human allergy. We found that IRF4 regulated almost 400 genes. This number is similar to that found in a study of IRF4 induced genes in myeloma cells (29). The genes found in our study were involved in several different pathways, of which the most significant included T helper cell differentiation and T cell receptor signalling. IRF4 also regulated pathways related to cellular proliferation, metabolism and DNA methylation. This indicates that IRF4 has an important role in activating transcriptional programs that integrate immune responses with general cellular activation processes. However, neither Th1 nor Th2 cytokines were the direct targets of IRF4. This suggested that IRF4 interacted with other TFs to regulate these cytokines. Indeed, the IRF4 induced genes included several TFs previously described to regulate Th2 cytokines, namely *ETS1*, *GATA3*, *HIF1A*, *MAF*, *MYB*, *RORA*, and *STAT5B* (15-22). Another TF, *PRDM1* inhibited Th1, while *STAT4* is an important Th1 cell activator. Of these TFs, only *GATA3* has previously been described as IRF4 regulated. The increase of *STAT4* does not agree with the paradigm that Th1 cells have a counter-regulatory role in allergy. This novel finding could, however, explain recent studies reporting concurrent increases of Th1 and Th2 cytokines in allergic inflammation (14, 25, 26, 30, 31).

Limitations of the study include that gene expression microarrays do not detect low-abundance genes and that poorly annotated genes may not be included in the pathway-analysis. ChIP-chip technology detects TF bound to the promoter regions, but such TF do not necessarily regulate transcription. To identify genes that were regulated by IRF4 we combined RNA polymerase II ChIP-chip and gene expression microarrays. The rationale was that an active binding site associated with a differentially expressed gene would indicate that IRF4 regulated that gene. Another problem was to identify which IRF4 induced TFs that directly regulated Th2 cytokines. We therefore developed a novel computational method that integrated the ChIP-chip and gene expression microarray data with sequence-based predictions. This method was based on identifying groups of genes with shared combinations of TF binding sites (TFBS) adjacent to the ChIP-chip defined IRF4 binding sites, whose expression levels were correlated in the allergen-challenged CD4⁺ cells. This resulted in the identification of several groups of genes, which we refer to as transcriptional modules (TMs). Finally, those TMs were searched to find TFs with predicted binding sites in the promoter regions of Th2, but not Th1 cytokines. This resulted in the identification of *ETS1*. This TF has previously been demonstrated to regulate differentiation of Th2 and Th17 cells (19-22), but has not been described in allergy. We found a significant increase of *ETS1* in allergen-challenged CD4⁺ cells from patients compared to controls. In Th2 polarized

cells, siRNA mediated knockdown of *ETS1* resulted in decreased release of Th2 cytokines. This decrease was modest, but statistically significant. A likely explanation for the modest effects was the involvement of several TFs other than ETS1 in the regulation of Th2 cytokines (24). A possible confounding factor could be off-target effects of the siRNA knockdown (24). However, gene expression microarray and Q-PCR analysis did not indicate any such effects. By contrast, pathway analysis of the gene expression microarray data showed that several pathways relevant for allergy were significantly affected by the ETS1 knockdown. Those pathways were involved in Th2 cell differentiation, as well as Th2 cell regulation of effector cells like eosinophils. Moreover, airway inflammation in asthma was one of the most significant pathways. We found a significant overlap between the genes that were differentially expressed after ETS1 knockdown and those found in allergen-challenged CD4⁺ cells.

For future studies, we propose that the analytical principles in this article can be applied to comprehensively map disease-associated pathways in allergy, as well as the TFs that regulate those pathways. The increasing availability of gene expression microarray and ChIP-chip data in the public domain will facilitate such mapping.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jörgen Nedergaard Larsen of ALK-Abello for providing allergen extract. This research has been supported by the European Commission under the Seventh Framework Programme, grant agreement number 223367, MultiMod, the Swedish Medical Research Council and by the US National Institutes of Health under grants P01-DA-015027-01, R01-MH-074460-01, U01-AA-013512 and U01-AA-013641-04, by the US Department of Energy under the EPSCoR Laboratory Partnership Program.

References

1. Lohoff M, Mittrucker HW, Prechtel S, Bischof S, Sommer F, Kock S, et al. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99(18):11808–11812. [PubMed: 12189207]
2. Hu CM, Jang SY, Fanzo JC, Pernis AB. Modulation of T cell cytokine production by interferon regulatory factor-4. *The Journal of biological chemistry*. 2002; 277(51):49238–49246. [PubMed: 12374808]
3. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *The Journal of experimental medicine*. 2002; 195(8):1003–1012. [PubMed: 11956291]
4. Honma K, Kimura D, Tominaga N, Miyakoda M, Matsuyama T, Yui K. Interferon regulatory factor 4 differentially regulates the production of Th2 cytokines in naive vs. effector/memory CD4⁺ T cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(41):15890–15895. [PubMed: 18836070]
5. Lund RJ, Loytomaki M, Naumanen T, Dixon C, Chen Z, Ahlfors H, et al. Genome-wide identification of novel genes involved in early Th1 and Th2 cell differentiation. *Journal of immunology*. 2007; 178(6):3648–3660.
6. Ahyi AN, Chang HC, Dent AL, Nutt SL, Kaplan MH. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *Journal of immunology*. 2009; 183(3):1598–1606.
7. Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*. 2010; 33(2):192–202. [PubMed: 20674401]

8. Biswas PS, Gupta S, Chang E, Song L, Stirzaker RA, Liao JK, et al. Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. *The Journal of clinical investigation*. 2010; 120(9):3280–3295. [PubMed: 20697158]
9. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*. 2009; 458(7236): 351–356. [PubMed: 19182775]
10. Biswas PS, Bhagat G, Pernis AB. IRF4 and its regulators: evolving insights into the pathogenesis of inflammatory arthritis? *Immunological reviews*. 2010; 233(1):79–96. [PubMed: 20192994]
11. Mudter J, Yu J, Zufferey C, Brustle A, Wirtz S, Weigmann B, et al. IRF4 regulates IL-17A promoter activity and controls ROR γ mat-dependent Th17 colitis in vivo. *Inflammatory bowel diseases*. 2011; 17(6):1343–1358. [PubMed: 21305677]
12. Kim BS, Kim IK, Park YJ, Kim YS, Kim YJ, Chang WS, et al. Conversion of Th2 memory cells into Foxp3⁺ regulatory T cells suppressing Th2-mediated allergic asthma. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107(19):8742–8747. [PubMed: 20421479]
13. Park PJ. ChIP-seq: advantages and challenges of a maturing technology. *Nature reviews. Genetics*. 2009; 10(10):669–680.
14. Wang H, Barrenas F, Bruhn S, Mobini R, Benson M. Increased IFN- γ activity in seasonal allergic rhinitis is decreased by corticosteroid treatment. *The Journal of allergy and clinical immunology*. 2009; 124(6):1360–1362. [PubMed: 19910031]
15. Nakata Y, Brignier AC, Jin S, Shen Y, Rudnick SI, Sugita M, et al. c-Myb, Menin, GATA-3, and MLL form a dynamic transcription complex that plays a pivotal role in human T helper type 2 cell development. *Blood*. 2010; 116(8):1280–1290. [PubMed: 20484083]
16. Jaradat M, Stapleton C, Tilley SL, Dixon D, Erikson CJ, McCaskill JG, et al. Modulatory role for retinoid-related orphan receptor alpha in allergen-induced lung inflammation. *American journal of respiratory and critical care medicine*. 2006; 174(12):1299–1309. [PubMed: 16973978]
17. Yamashita M, Katsumata M, Iwashima M, Kimura M, Shimizu C, Kamata T, et al. T cell receptor-induced calcineurin activation regulates T helper type 2 cell development by modifying the interleukin 4 receptor signaling complex. *The Journal of experimental medicine*. 2000; 191(11): 1869–1879. [PubMed: 10839803]
18. Ho IC, Lo D, Glimcher LH. c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. *The Journal of experimental medicine*. 1998; 188(10):1859–1866. [PubMed: 9815263]
19. Blumenthal SG, Aichele G, Wirth T, Czernilofsky AP, Nordheim A, Dittmer J. Regulation of the human interleukin-5 promoter by Ets transcription factors. Ets1 and Ets2, but not Elf-1, cooperate with GATA3 and HTLV-I Tax1. *The Journal of biological chemistry*. 1999; 274(18):12910–12916. [PubMed: 10212281]
20. Moisan J, Grenningloh R, Bettelli E, Oukka M, Ho IC. Ets-1 is a negative regulator of Th17 differentiation. *The Journal of experimental medicine*. 2007; 204(12):2825–2835. [PubMed: 17967903]
21. Grenningloh R, Miaw SC, Moisan J, Graves BJ, Ho IC. Role of Ets-1 phosphorylation in the effector function of Th cells. *European journal of immunology*. 2008; 38(6):1700–1705. [PubMed: 18465773]
22. Stempel JM, Grenningloh R, Ho IC, Vercelli D. Phylogenetic and functional analysis identifies Ets-1 as a novel regulator of the Th2 cytokine gene locus. *Journal of immunology*. 2010; 184(3): 1309–1316.
23. Cimmino L, Martins GA, Liao J, Magnusdottir E, Grunig G, Perez RK, et al. Blimp-1 attenuates Th1 differentiation by repression of ifng, tbx21, and bcl6 gene expression. *Journal of immunology*. 2008; 181(4):2338–2347.
24. Marques JT, Williams BR. Activation of the mammalian immune system by siRNAs. *Nature biotechnology*. 2005; 23(11):1399–1405.
25. Woodfolk JA. T-cell responses to allergens. *The Journal of allergy and clinical immunology*. 2007; 119(2):280–294. quiz 295-286. [PubMed: 17291848]

26. Bosco A, McKenna KL, Firth MJ, Sly PD, Holt PG. A network modeling approach to analysis of the Th2 memory responses underlying human atopic disease. *Journal of immunology*. 2009; 182(10):6011–6021.
27. Sel S, Henke W, Dietrich A, Herz U, Renz H. Treatment of allergic asthma by targeting transcription factors using nucleic-acid based technologies. *Current pharmaceutical design*. 2006; 12(25):3293–3304. [PubMed: 17020535]
28. Mobini R, Andersson BA, Erjefalt J, Hahn-Zoric M, Langston MA, Perkins AD, et al. A module-based analytical strategy to identify novel disease-associated genes shows an inhibitory role for interleukin 7 Receptor in allergic inflammation. *BMC systems biology*. 2009; 3:19. [PubMed: 19216740]
29. Shaffer AL, Emre NC, Lamy L, Ngo VN, Wright G, Xiao W, et al. IRF4 addiction in multiple myeloma. *Nature*. 2008; 454(7201):226–231. [PubMed: 18568025]
30. Cho SH, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *American journal of respiratory and critical care medicine*. 2005; 171(3):224–230. [PubMed: 15502111]
31. Pedicini M, Barrenas F, Clancy T, Castiglione F, Hovig E, Kanduri K, et al. Combining network modeling and gene expression microarray analysis to explore the dynamics of Th1 and Th2 cell regulation. *PLoS computational biology*. 2010; 6(12):e1001032. [PubMed: 21187905]

Figure 1.

(A) Q-PCR analysis of *IRF4* in $CD4^+$ cells from patients with IAR ($n = 12$) and (B) healthy controls ($n = 3$) treated with diluent or allergen for one week. The cells from the patients were also treated with allergen and glucocorticoids (GC). The bar graph illustrates the mean $\pm$ SEM fold changes of gene expression between allergen and diluent -challenged cells. $*P < 0.05$, $**P < 0.005$. (C) The release of IL-5 was measured in supernatants from patients with IAR ($n = 12$) treated for one week with diluent, allergen and allergen in combination with GC (mean $\pm$ SEM, $*P < 0.05$).

Figure 2. Pathway analysis of IRF4 induced or repressed genes using the Ingenuity Pathway Analysis tool. The figure represents significant pathways with a P value $< .05$ (represented by the orange line).

Figure 3. Identification of IRF4 induced transcriptional modules. **(A)** Prediction of IRF4 containing cis-regulatory modules (CRMs) based on sequence in a 200 bp window around IRF4 binding sites. **(B)** Identification of putative target genes for each CRM. **(C)** Selection of modules with correlated putative target genes. **(D)** Clique-based analysis to extract highly correlated transcriptional modules (TMs).

Figure 4.

Q-PCR analysis of *ETS1* in CD4⁺ cells from patients with IAR (n = 6) and healthy controls (n = 6). The bar graph illustrates the mean ± SEM fold changes of gene expression between allergen and diluent -challenged cells. * $P < 0.05$

Figure 5. siRNA mediated knockdown of *ETS1* in polarized Th2 cells affects Th2 cytokine production. Polarized Th2 cells were transfected with either diluent (M) *ETS1*-specific siRNA pool (E) or non-targeting control siRNA pool (C). **A**) The expression of ETS1 protein in polarized Th2 cells 36 hours after transfection with either diluent (M) *ETS1*-specific siRNA (E) or non-targeting control siRNA (C). The positive control (pos Ctrl) shows the expression of the ETS1 in non-transfected polarized Th2 cells (one representative experiment out of five). **B**) and **C**) The levels of the Th2 cytokines IL-5 and IL-13 in cell supernatants after 36 and 60 hours after (n = 5) (mean ± SEM, * $P < .05$, compared to the non-targeting control siRNA and $^{\dagger}P < .05$, compared to the diluent).