Immunity
Resource

Genome-wide Profiling of Interleukin-4 and STAT6 Transcription Factor Regulation of Human Th2 Cell Programming

Laura L. Elo,[1,2,13] Henna Järvenpää,[2,3,13] Soile Tuomela,[2,3,13] Sunil Raghav,[2,13] Helena Ahlfors,[2,4] Kirsti Laurila,[5] Bhawna Gupta,[2] Riikka J. Lund,[2,6] Johanna Tahvanainen,[2,7] R. David Hawkins,[8] Matej Orešič,[9] Harri Lähdesmäki,[5,10] Omid Rasool,[2] Kanury V. Rao,[11,14] Tero Aittokallio,[1,2,14] and Riitta Lahesmaa[2,12,*]

[1]Biomathematics Research Group, Department of Mathematics, University of Turku, FI-20014 Turku, Finland
[2]Turku Centre for Biotechnology, University of Turku and Åbo Akademi, P.O. Box 123, FI-20521 Turku, Finland
[3]Turku Graduate School of Biomedical Sciences, Kiinamyllynkatu 13, FI-20520 Turku, Finland
[4]The National Graduate School in Informational and Structural Biology, Åbo Akademi University, FI-20520 Turku, Finland
[5]Department of Signal Processing, Tampere University of Technology, P.O. Box 553, FI-33101 Tampere, Finland
[6]Department of Biological Science, University of Sheffield, S10 2TN, Sheffield, UK
[7]Drug Discovery Graduate School, University of Turku, FI-20014 Turku, Finland
[8]Ludwig Institute for Cancer Research, University of California, San Diego, CA 92037, USA
[9]VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 Espoo, Finland
[10]Department of Information and Computer Science, Helsinki University of Technology, P.O. Box 5400, FI-02015 TKK, Finland
[11]International Centre for Genetic Engineering and Biotechnology, P.O. Box 10504, 110067 New Delhi, India
[12]Immune Disease Institute, Harvard Medical School, Boston, MA 02115, USA
[13]These authors contributed equally to this work
[14]These authors contributed equally to this work
*Correspondence: riitta.lahesmaa@btk.fi
DOI 10.1016/j.immuni.2010.06.011

SUMMARY

Dissecting the molecular mechanisms by which T helper (Th) cells differentiate to effector Th2 cells is important for understanding the pathogenesis of immune-mediated diseases, such as asthma and allergy. Because the STAT6 transcription factor is an upstream mediator required for interleukin-4 (IL-4)-induced Th2 cell differentiation, its targets include genes important for this process. Using primary human $CD4^+$ T cells, and by blocking STAT6 with RNAi, we identified a number of direct and indirect targets of STAT6 with ChIP sequencing. The integration of these data sets with detailed kinetics of IL-4-driven transcriptional changes showed that STAT6 was predominantly needed for the activation of transcription leading to the Th2 cell phenotype. This integrated genome-wide data on IL-4- and STAT6-mediated transcription provide a unique resource for studies on Th cell differentiation and, in particular, for designing interventions of human Th2 cell responses.

INTRODUCTION

T helper (Th) cells are a subgroup of lymphocytes that are crucial in the immune system defense against intracellular and extracellular pathogens. The naive Th precursor (Thp) cells are functionally immature until activated. After activation, they can differentiate into different subtypes, among which the most studied are Th1 and Th2 cells. More recently described lineages are Th17 and regulatory T (Treg) cells (Weaver et al., 2006). Because the different subtypes have distinct functional roles in the immune system, disturbances in their balance have been linked to various immune-mediated diseases. In particular, enhanced Th1 cell-type responses along with Th17 cell activity are implicated in several autoimmune diseases, such as type 1 diabetes, whereas inappropriate Th2 cell-type responses might lead to the development of asthma and atopic disorders (Bettelli et al., 2007).

Th2 cell differentiation is induced by interleukin 4 (IL-4). Binding of IL-4 to its receptor leads to activation of janus kinase 1 and 3, and phosphorylation of the signal transducer and activator of transcription protein 6 (STAT6). The phosphorylated STAT6 forms a homodimer and translocates into the nucleus, where it binds to specific DNA sequences, thereby regulating the transcription of its target genes. It is shown that STAT6 is important for IL-4-driven Th2 cell phenotype in mouse (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996; Zhu et al., 2001).

Because STAT6 is required as an upstream mediator of IL-4-receptor-induced Th2 cell differentiation, identification of its downstream targets is of particular interest. These factors are likely to include candidates important for eliciting Th2 cell response. The present study systematically utilized the state-of-the-art genome-scale measurement technologies together with efficient computational methods to investigate IL-4-receptor signaling as a STAT6-centered network that initiates and dynamically regulates Th2 cell differentiation. STAT6-regulated genes were identified using RNA interference (RNAi) technology and the primary target genes of STAT6 were

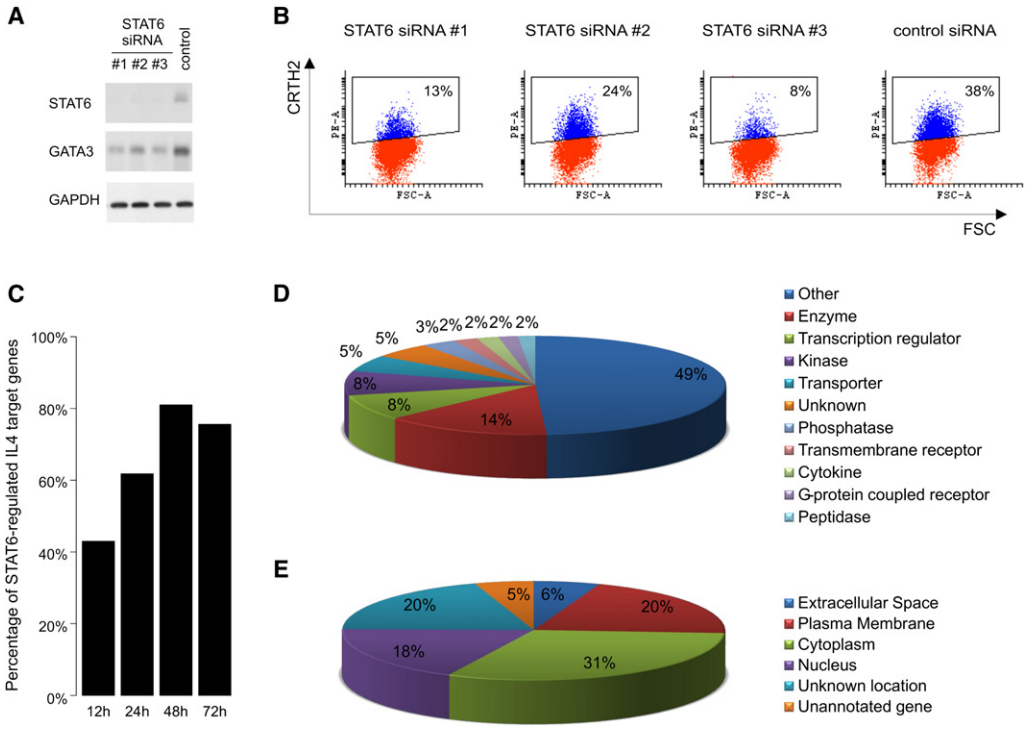


Figure 1. The Overall Effect of STAT6 Knockdown on IL-4-Mediated Regulation during the Human Th2 Cell Differentiation Process

(A) Immunoblotting data showing the expression of STAT6 and GATA3 after introduction of three different STAT6-siRNAs into naive $CD4^+$ T cells and culturing for 24 hr in Th2 cell polarizing condition. Nontargeting control-siRNA was used as RNAi control and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The representative result from two independent experiments is shown. See also Figure S1A.

(B) The percentage of cells treated with STAT6-siRNA or control-siRNA expressing CRTH2 after 1 week of polarization. The results correspond to the experiment in Figure 1A. Living cells were gated for the analysis based on forward (FSC) and side scattering. The mean CRTH2 fluorescent intensities of the living cells were as follows: siRNA #1 456, siRNA #2 742, and siRNA #3 412, and control-siRNA 968.

(C) The percentage of IL-4-regulated genes whose expression was affected by STAT6 knockdown after 12–72 hr of polarization. STAT6 target genes were identified by determining IL-4 target genes (control-siRNA Th2/Th0, FDR < 0.05) and, subsequently, the genes regulated by STAT6-RNAi (STAT6-siRNA Th2/Th0 compared to control-siRNA Th2/Th0, FDR < 0.05) among the IL-4 targets. Both the IL-4- and the STAT6-regulated genes were identified across three independent biological replicates of the time series. See also Experimental Procedures and Figure S1B.

(D and E) Molecular function (D) and cellular localization (E) of the identified STAT6 target genes, according to Ingenuity Pathway Analysis software. See also Supplemental Experimental Procedures for further explanation of the classifications.

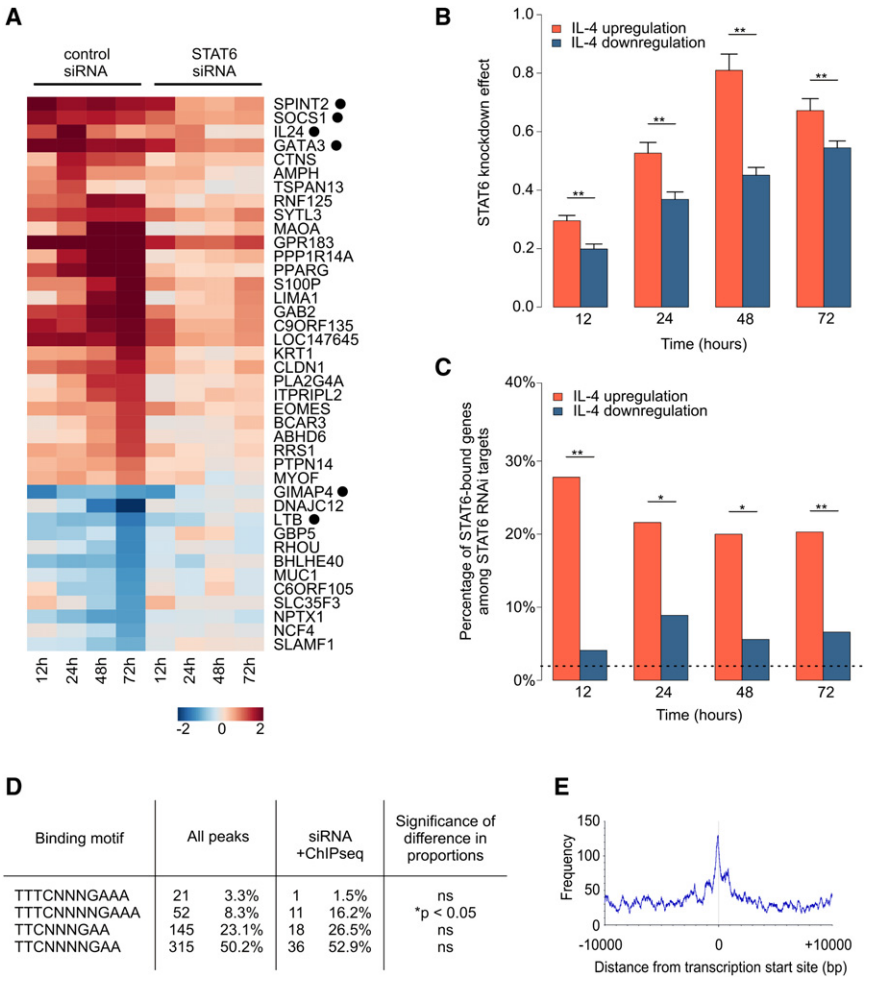
dissected from the downstream mediators of the signaling cascade by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq). Integrating these data on a comprehensive map of detailed transcriptional kinetics of undisturbed differentiation of naive human $CD4^+$ T cells enabled us to draw an experimentally validated pathway of molecular events that mediates IL-4-STAT6 signaling toward Th2 cell phenotype. All the experimental data have been gathered from human primary cells, further highlighting the value of the findings and providing powerful resource of knowledge, for example, for rational design of therapy for pathogenic human immune responses.

RESULTS

STAT6 in the Center of IL-4-Mediated Transcription

In order to assess the role of STAT6 in the human Th2 cell differentiation process, we suppressed its expression with RNA interference and investigated the genome-wide effects of this perturbation on IL-4-mediated transcriptional regulation. Samples were collected at 0, 12, 24, 48, and 72 hr time points. The robustness against technical biases was enhanced by repeating the experiment separately with three different small interfering RNAs (siRNA). The decreased protein expression of STAT6 and its known target GATA binding protein 3 (GATA3) (Ouyang et al., 1998) verified the efficacy of the RNAi-mediated knockdown (Figure 1A and Figure S1A available online). In addition, STAT6 knockdown decreased the number of cells expressing the human-specific Th2 cell differentiation marker G protein-coupled receptor 44 (also known as chemoattractant receptor homologous molecule expressed on T helper type 2 cells, CRTH2) (Cosmi et al., 2000), indicating that the cells could not acquire the normal Th2 cell phenotype when the expression of STAT6 was suppressed (Figure 1B).

The effect of STAT6 on the differentiation process was investigated by identifying those IL-4-regulated genes whose IL-4 effect—either up- or downregulation—changed when STAT6 was knocked down (Table S1). The proportion of IL-4-regulated genes affected by STAT6-RNAi increased with time, being over 80% after 48 hr of polarization (Figure 1C and Figure S1B). Altogether, the expression of 492 probes representing 453 known



Binding motif	All peaks		siRNA +ChIPseq		Significance of difference in proportions
TTTCNNNGAAA	21	3.3%	1	1.5%	ns
TTTCNNNNGAAA	52	8.3%	11	16.2%	*p < 0.05
TTCNNNGAA	145	23.1%	18	26.5%	ns
TTCNNNNGAA	315	50.2%	36	52.9%	ns

Figure 2. STAT6 Mediates IL-4-Induced Transcription Directly and Indirectly

(A) Heat map presentation of the strongest STAT6 target genes (signal log-ratio between Th2 and Th0 > 1, and between STAT6-siRNA and control-siRNA > 1). Genes previously identified as STAT6 targets in genome-wide *Stat6*$^{-/-}$ mice studies (Chen et al., 2003; Schröder et al., 2002), RNAi or EMSA experiments are marked with black circles.

(B) The effect of STAT6 knockdown among the IL-4-driven target gene expression changes was calculated using the statistic Th2/Th0 − sTh2/sTh0, where Th2/Th0 and sTh2/sTh0 denote the signal log ratios between the matched Th2 and Th0 measurements in the control and knockdown samples, respectively. The histograms present the average STAT6 knockdown effect for the up- and downregulated genes in RNAi experiments, and the error bars represent the standard error of the mean. The difference in the average STAT6 knockdown effects among the IL-4-upregulated STAT6 target genes (red bars) and the IL-4-downregulated target genes (blue bars) is shown (**Welch two sample t test, $p < 0.01$).

(C) The proportion of STAT6-regulated genes identified from the RNAi data (statistically significant targets across three independent biological replicates) that were bound by STAT6 in the ChIP-seq experiment was determined separately for the IL-4-up- and IL-4-downregulated genes (difference between proportions **$p < 0.01$, *$p < 0.05$). The dashed line illustrates the random expectation as defined by the expected value of the hypergeometric distribution. See also Figures S2A and S2B for STAT6 ChIP controls, Tables S1 and S2 for the STAT6-regulated and STAT6-bound genes, respectively, and Figure S2C for the numbers of detections in each category.

(D) The STAT6 ChIP-seq target genes and the genes which were in addition regulated by STAT6-siRNA were analyzed for the presence of STAT6 binding motifs (ns, not significant).

(E) Graphical representation of the STAT6 binding sites related to the position of the transcriptional start sites (TSSs). All the ChIP-seq detections were analyzed using the Genomatix RegionMiner tool (http://www.genomatix.de, Genomatix Software GmbH). TSS is defined to be at position zero in the graph.

genes were affected by decreased STAT6 expression. The broad functional distribution of the identified target genes (Figure 1D) and their localization throughout all cellular compartments (Figure 1E) reflected the fundamental role of STAT6 in the global regulation of the Th2 cell phenotype.

Immediate Target Genes of STAT6

To our knowledge, only 6% of the target genes in our RNAi data are reported to be regulated by STAT6, by using genome-wide screens performed either on T or B cells of *Stat6*$^{-/-}$ mice or STAT6-RNAi experiments, or bound by STAT6 in electrophoretic mobility shift assay (EMSA) (Table S1) (Ahn et al., 2009; Arpa et al., 2009; Büttner et al., 2004; Chen et al., 2003; Filen et al., 2009; Gabay et al., 1999; Hebenstreit et al., 2003; Kim et al., 2006; Kurata et al., 1999; Lund et al., 2007; McGaha et al., 2003; Ohmori et al., 1996; Schaefer et al., 2001; Schröder et al., 2002; Yang et al., 2005; Zhang et al., 2000; Zhu et al., 2002). In addition, few of the genes, such as *MAOA*, are suggested to be regulated by STAT6 by using indirect methods such as in silico predictions (Chaitidis et al., 2004). Of the target genes having the largest, over 2-fold, STAT6 knockdown effects in our study (Figure 2A), only six, namely *GATA3*, *GIMAP4*, *IL24*, *LTB*, *SOCS1*, and *SPINT2*, were among the genes identified in the previous studies. The previously not-reported target genes suggested additional functions and processes mediated by STAT6 signaling (Table S1). For example, the downregulation of *ST6GAL1* and *RNF125* by STAT6-siRNA linked STAT6 to the determination of Th2 cell-specific surface glycoprotein structures and ubiquitin ligase activity, respectively (Toscano et al., 2007; Zhao et al., 2005). STAT6-siRNA upregulated the expression of *NCF4*, which is linked to Crohn's disease (Rioux et al., 2007), rheumatoid arthritis (Olsson et al., 2007), and chronic granulomatous disease (Matute et al., 2009). *MUC1*, also upregulated by STAT6-siRNA, is reported to be induced by IL-12 in human T cells (Agrawal and Longenecker, 2005), as well as to interact with STAT1 in a cooperative manner (Khodarev et al., 2010). The data presented showed that genome-wide expression analysis combined with RNAi can efficiently provide new candidates with potential to influence the differentiation process and to be further studied and used for building gene regulatory networks.

The RNAi experiments demonstrated that the effect of the STAT6 knockdown was, in general, significantly larger among the IL-4-upregulated than among the IL-4-downregulated genes (Figure 2B). This suggested that STAT6 primarily drives the activation of transcription, whereas downregulation would mainly remain a downstream effect after IL-4 stimulation. To distinguish the direct STAT6 targets from the secondary effects of STAT6, we exploited ChIP-sequencing (ChIP-seq) (Johnson et al., 2007; Robertson et al., 2007). Because the level of phosphorylated STAT6 (Tyr641) reached its maximal level readily after IL-4 stimulation in our experimental setup (Figure S2A) and because STAT6 is shown to bind to its targets quickly after the IL-4 stimulus in previously published data (Andrews et al., 2002), the binding was measured within 4 hr after initiation of polarization. The success of the STAT6 ChIP experiment used for sequencing was validated with detection of STAT6 binding to the promoter of its known target gene *SOCS1* (Figure S2B) (Hebenstreit et al., 2006).

In total, 508 genes were bound by STAT6 IL-4-dependently within 10 kb of the transcription start or end site (Table S2). Twenty percent to nearly 30% of the upregulated target genes observed with STAT6-RNAi across the time points analyzed were confirmed to be direct targets of STAT6 on the basis of the ChIP-seq results (Figure 2C and Figure S2C). Among the genes downregulated by STAT6, the proportion of direct STAT6 targets was significantly lower, less than 10%, than among the upregulated genes. These observations suggested that most of the downregulated target genes were not direct targets of STAT6, but the regulation of their expression depends on secondary regulatory factors.

Seventy-nine percent of the identified STAT6 binding sites contained STAT family sequence motif (Table S3). The closer investigation of the known STAT6 consensus motifs ($TTTCN_3GAAA$, $TTTCN_4GAAA$, $TTCN_3GAA$, $TTCN_4GAA$) (Hebenstreit et al., 2006) (Figure 2D) revealed that $TTCN_3GAA$ and $TTCN_4GAA$ motifs were the ones that were most commonly found, 23% and 50% respectively, within the ChIP-seq peaks. Interestingly, when comparing the frequency of the motifs between the STAT6-regulated genes and all the STAT6-bound genes, we found a statistically significant increase in the occurrence of the one-base extended N_4 motif among the STAT6-regulated genes ($p < 0.05$).

Of the detected STAT6 binding sites, 66% resided in the intragenic regions and 34% were located either upstream of the transcription start site or downstream of the transcription end site, as assessed using the Genomatix RegionMiner tool (http://www.genomatix.de, Genomatix Software GmbH) (Table S3). The great majority of the identified intragenic STAT6 binding sites were located in the introns of the genes, the first two introns being the most common. Further investigation of the detected STAT6 binding sites along the target genes revealed that there is a clear enrichment of the peaks in close proximity of transcription start site (Figure 2E) as noticed with other STAT factors (Kwon et al., 2009; Robertson et al., 2007). Almost 10% of the identified STAT6 binding sites were more than 100 kb away from any known gene based on the CisGenome annotation software (Table S3) (Ji et al., 2008), which most probably reflects the overall complexity of the regulation of human gene expression. These hits may indicate that there are still novel transcripts to be found close by these binding sites or that these sites are used as reservoir of STAT6 molecules. An intriguing option is also that these sites are needed for regulation of the genes located far away when measured directly by the distance along the DNA strand but which are brought together by the regulation of looping of the DNA.

Transcriptional Regulation by IL-4

Because our ultimate goal was to understand in detail how IL-4 drives the Th2 cell differentiation, we constructed a more comprehensive kinetic profile of the genome-wide transcriptional response of human cord blood Thp cells to IL-4 stimulation combined with T cell receptor (TCR) activation. The early differentiation process at nine time points between 0.5 and 72 hr after polarization was studied (Figure 3A). In total, 640 genes were upregulated and 460 genes were downregulated by IL-4 at one or more time points (Table S4). Clustering of the data demonstrated that IL-4-specific signaling was initiated with transient changes in gene expression followed by the stable Th2 cell signature profile (Figure 3B and Table S5). Compared to previous studies (Hämäläinen et al., 2001; Lund et al., 2007; Nagai et al., 2001; Rogge et al., 2000), our results revealed that IL-4-induced regulation of transcription in human cells was highly dynamic.

The overall expression kinetics revealed that the early IL-4-mediated signaling can be roughly split into two phases: the rapid wave of upregulation from 0.5 to 4 hr is followed by downregulation starting at 6 hr after polarization (Figure 3A). The upregulated genes were significantly enriched with direct STAT6 targets already at 0.5 hr, whereas the enrichment among the downregulated genes was detected only at later time points and at weaker significance levels. Overlay of the STAT6 ChIP-seq hits to the IL-4 target genes within the first 4 hr of polarization displayed the group of genes directly downstream of STAT6 likely to be responsible for mediating the effects of IL-4. The genes selected based on signal log-ratio (Th2/Th0 > 1) are presented in Figure 3C.

The fact that STAT6 also had direct target genes which are regulated only at the later time points, speaks for the importance of cofactors and putatively also epigenetic changes determining the role of STAT6. For example, of the known STAT6 coactivators (Goenka and Boothby, 2006; Hebenstreit et al., 2006), *NCOA3* was upregulated peaking at 4 hr, whereas *PARP14* was downregulated from 24 hr onward (Table S4). Moreover, the presence of competing transcription factors that can recognize similar binding sites as STAT6, such as BCL6 (Hebenstreit et al., 2006), can influence the availability of DNA binding sites. In our data, the expression of *BCL6* and its corepressor *BCOR* were downregulated by IL-4, supporting the importance of turning down of this transcription factor, possibly competing with STAT6 DNA binding during the Th2 cell differentiation. Further investigation of the kinetics of STAT6 binding to its primary target genes identified in this study (Figure S3) suggested that STAT6 occupancy at its target sites may change during the differentiation process (Figure 4). This again reinforced the notion that human Th2 cell differentiation is a strictly regulated step-wise process.

To detect potential secondary factors needed for enhancement of Th2 cell polarization, we identified STAT6-regulated genes that were not among our ChIP-seq hits, i.e., indirect

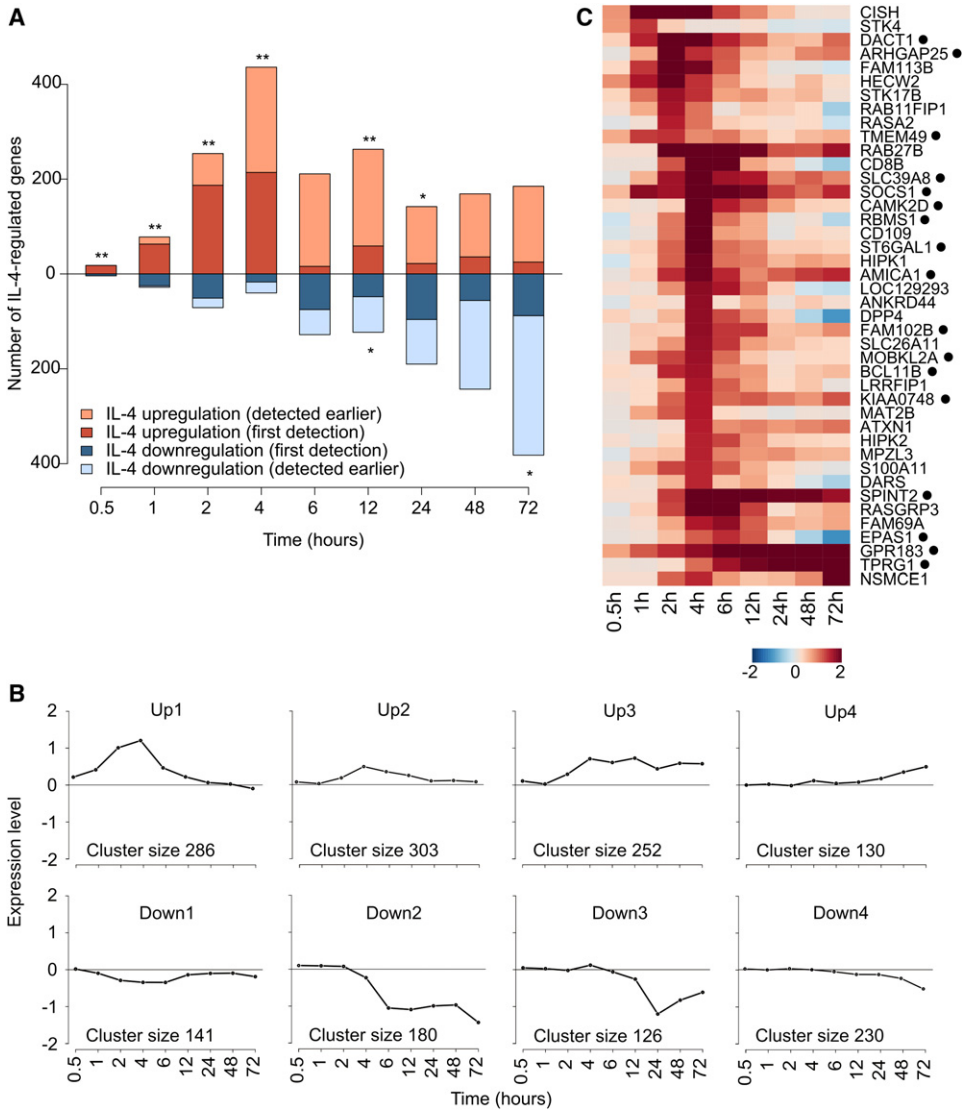


Figure 3. Dynamics of IL-4-Mediated Gene Expression

(A) The number of IL-4-regulated genes identified from the transcriptomics experiments is shown at each time point (see Table S4). The height of a bar shows the total number of detections at a particular time point, while the height of the darker inner part represents those genes that have not been detected at earlier time points. Enriched STAT6 binding, on the basis of the ChIP-seq experiment, is indicated with stars above the bars (hypergeometric test $^{**}p < 0.01$ or $^{*}p < 0.05$), referring to the genes detected to be differentially expressed for the first time at a specific time point. The ChIP-seq peaks were linked to the genes with maximum distance of 10 kb up- or downstream from the transcribed regions.

(B) Transcription profiles of the differentially expressed genes between the Th2 and Th0 conditions. The genes were clustered using the hopach algorithm (Bioconductor hopach package) with cosine angle as a dissimilarity measure. See also Table S5.

(C) Heatmap of selected IL-4-regulated genes (signal log-ratio between Th2 and Th0 > 1) within 4 hr of Th2 polarization found to be bound by STAT6. Genes regulated by STAT6 in RNAi experiments are marked with black circles. STAT6 ChIP-seq targets were identified based on one experiment. IL-4-mediated transcriptomics and RNAi data represent statistically significant changes over three independent experiments.

STAT6 target genes. We investigated these genes for the presence of enriched transcription factor binding motifs based on the PreMod database (Table S6), focusing on high-scoring sites in modules located within 10 kb upstream of the transcription start sites (Blanchette et al., 2006). Notably, STAT6 binding motif could not be found enriched among the indirectly regulated genes, further indicating these genes to be secondary or atypical targets of STAT6. Instead, we found STAT5A homotetramer motif to be among the most significantly enriched motifs (Table S6). STAT5 is known to play an important role in Th2 cell polarization (Zhu et al., 2006). As the binding of this factor is studied with ChIP-seq in mouse $CD4^{+}$ T cells at 8 and 13 hr after IL-4 stimulation (Liao et al., 2008), we compared the identified STAT5A target genes to the STAT6 secondary target genes identified in the present study. The statistically significant overlap found supports the idea that STAT5A may be a downstream regulator of STAT6 target genes. Furthermore, by comparing the STAT6-regulated ChIP-seq target genes to the reported

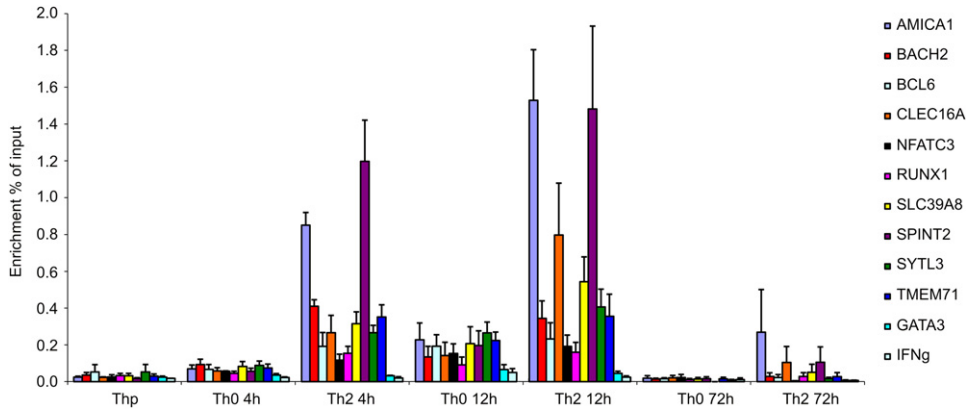


Figure 4. Kinetics of STAT6 Occupancy on Its Target Genes

qPCR results of the selected STAT6 ChIP-seq sites measured at 0, 4, 12, and 72 hr after cell stimulation. *GATA3* and *IFNg* were used as negative controls. Percent of input value is an average of two to three cultures showing enrichment, with the bars representing the corresponding standard error. The primers and probes used are listed in Supplemental Experimental Procedures. See also Figure S3.

STAT5A target genes (Liao et al., 2008), we found a significant overlap between these data sets, further proposing that STAT6 and STAT5A might regulate even the same genes (Table S6).

Core Transcription Factor Interactions

Categorization of the IL-4-controlled and STAT6-regulated genes into functional groups along the time points (Table S7) supported the hypothesis that Th2 cell polarization is initiated by fast regulation of transcription factors promoting phenotypic differentiation (Figure 5A and Figure S4). The activation of transcription factors followed the same kinetics as the overall IL-4-dependent signaling (Figure 3A), emphasizing its role in determining the timing of the differentiation process. Based on the results from complementary approaches used in this study, we categorized the mediators of the IL-4-specific transcriptional program into four groups of transcription factors: (1) STAT6-independent, (2) putative STAT6 targets, (3) STAT6-dependent primary targets, and (4) STAT6-dependent secondary targets. The selected members of each category are presented in Figure 5B.

In the mouse system, the expression of *Xbp1* and *Ncoa3* is regulated via STAT6 in B cells (Schröder et al., 2002), but in our data, both of these genes were STAT6 independent. *NCOA3*, and especially *XBP1*, were among the earliest transcription factors induced by IL-4 within the first hours after stimulation (Table S4). XBP1 regulates the unfolded protein response (UPR) needed for increased expression of secreted and membrane proteins, and it is required for maturation of plasma cells (Brunsing et al., 2008). UPR is active in immature $CD4^+CD8^+$ double positive thymocytes, but inactive in spleen $CD4^+$ T cells (Brunsing et al., 2008). This suggested that XBP1 can have a role independent of UPR in polarization of Th cells or that IL-4 stimulation reactivates this pathway. *LRRFIP1*, in turn, which was also among the earliest transcription factors induced by IL-4, was identified as a putative STAT6 target bound by STAT6 in the ChIP-seq experiment, but not detected as STAT6-regulated in the RNAi experiments. This gene is a repressor of tumor necrosis factor expression (Suriano et al., 2005) and an enhancer of β-catenin mediated transcription (Lee and Stallcup, 2006).

We identified three transcription factors, *BATF*, *EPAS1*, and *RUNX1*, to be directly regulated by STAT6. RUNX1 is previously linked to inhibition of Th2 cell polarization via downregulation of GATA3 expression (Komine et al., 2003) and through binding to the IL-4 silencer (Naoe et al., 2007). RUNX1 can also form a complex with FOXP3 and RORC and is indispensable for Treg and Th17 cell function, respectively (Kitoh et al., 2009; Zhang et al., 2008). Due to the general importance of RUNX proteins in different Th cell subtypes, they are suggested to function as core modifiers of effector $CD4^+$ T cell functions (Kitoh et al., 2009). Interestingly, EPAS1 binds to the promoter of *RUNX1* (Mole et al., 2009) and may amplify STAT6 effect. BATF, the third transcription factor found to be directly regulated by STAT6, is shown to be needed for Th17 cell differentiation (Schraml et al., 2009), and more recently, for Th2 cell development as well (Betz et al., 2010). These STAT6-dependent primary targets are putative key initiators of IL-4-induced transcriptional program.

Intriguingly, *GATA3* could not be recognized as a primary STAT6 target gene in our ChIP-seq analysis, although *GATA3* transcription was regulated by IL-4 shortly after initiation of polarization. This can indicate that there is still, in addition to STAT6, room for other regulators of early *GATA3* expression or that STAT6 regulates the expression of *GATA3* via distant regulatory sites which could not be connected to *GATA3* in this study. Interestingly, BATF, one of the direct STAT6-dependent transcription factors, is identified to regulate *Gata3* transcription (Betz et al., 2010). STAT6 also indirectly upregulated the expression of *GFI1* and *NFIL3*, which are linked to regulation of GATA3 protein stability (Shinnakasu et al., 2008) and natural killer cell development (Gascoyne et al., 2009; Kamizono et al., 2009), respectively. *BHLHE40*, *ID3*, *IRF8*, and *STAT1* were indirectly downregulated by STAT6, suggesting that the expression of these factors is disadvantageous for Th2 cell differentiation.

In addition to their putative individual role in the differentiation process, the identified IL-4- and STAT6-regulated transcription factors formed a compact core interaction network (Figure 5C). This suggested that Th cell commitment is defined by combinatorial signaling pathways, acting together to determine the functional outcome. The core network also illustratively revealed that

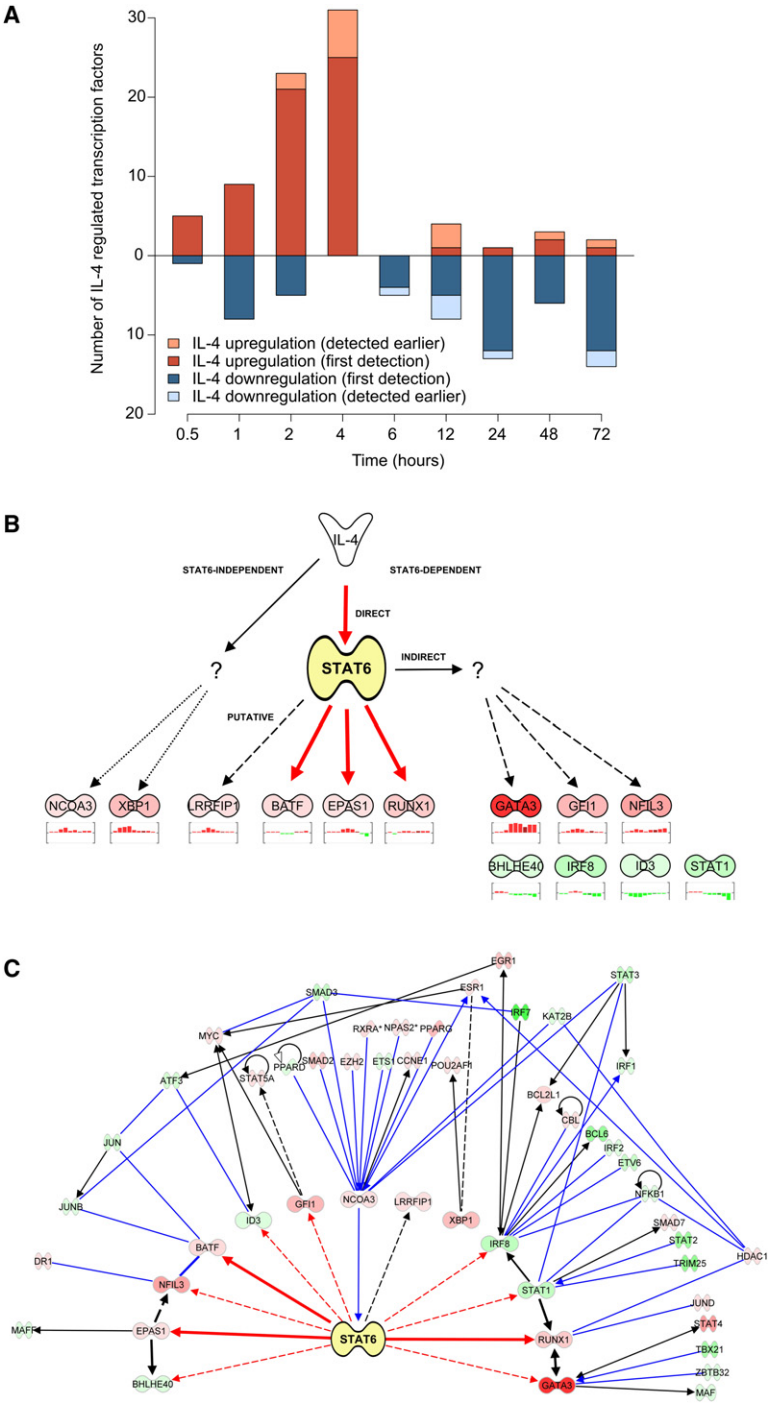


Figure 5. IL-4-Mediated Dynamic Network of Transcription Factors

(A) IL-4 triggers the Th2 cell-specific transcriptional program via regulation of transcription factors. The height of a bar shows the total number of detections at a particular time point, while the height of the darker inner part represents those transcription factors that have not been detected at any earlier time point. The expression profiles of these transcriptional regulators are listed in Table S8. See also Figure S4.

(B) IL-4 signal transmission through STAT6-dependent and independent transcription circuitries. STAT6-dependent genes were selected based on their IL-4-dependent regulation and induction, when compared to Thp sample (signal log ratio Th2/Thp > 1). Although the STAT6-independent genes were not regulated by STAT6-siRNA, they were induced by IL-4 in the control-siRNA experiments (FDR < 0.05). STAT6-independent genes were also required to have at least 2-fold expression difference between Th2 and Th0 sample. Putative STAT6 target gene LRRFIP1 was induced by IL-4 in our kinetic data (signal log-ratio Th2/Thp and Th2/Th0 > 1) and bound by STAT6 in ChIP-seq experiment but was not regulated by STAT6 in the RNAi experiments. The average signal log-ratios between the Th2 and Th0 conditions from 0.5 to 72 hr are visualized as bar charts next to the nodes.

(C) IL-4- and STAT6-regulated transcription factors formed a complex core network of interacting nodes. The core transcription factor network represented in Figure 5B (inner circle) was further expanded with known transcription factor interactions (outer circle). STAT6-mediated regulation detected in this study is marked with red edges (solid edge for direct regulation, dashed edge for indirect regulation). Known direct interactions between the putative downstream transcriptional regulators in human were added to the figure. Blue edges correspond to protein-protein interactions, and black ones correspond to other type of interaction or regulation. The networks were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). The data presented shows the statistically significant changes over three independent experiments.

there was a close relationship between genes engaged in determining the different Th cell fates, as all known STAT family members could be connected to each other via a relatively limited number of molecules.

DISCUSSION

Despite the fact that STAT6 plays a fundamental role in the processes associated with the Th2 cell branch of the immune system, its target genes in human are not known. To get a more systematic knowledge about the mechanisms involved, we identified STAT6 target genes during the early stages of human Th2 cell differentiation process. We showed that STAT6 resided in the core of IL-4-driven transcriptional events in human CD4$^+$ T cells. Importantly, a marked association was observed between the STAT6-upregulated genes and its direct targets, indicating that after IL-4 stimulation, STAT6 was primarily needed for the activation of pathways leading to the Th2 cell phenotype. In our data, only a subset of the genes differentially regulated within the first hours remained differentially expressed throughout the analyzed time frame. This indicated that there are both switch kind of genes needed at specific time point and factors that are important both for the transition to the new developmental pathway and for maintaining the already acquired phenotype. Detailed dissection of the functional role of these downstream factors may require analysis within a very specified timeline, as well as modulation of combinations of factors.

As reported in other previous genome-wide ChIP experiments with other transcription factors, only a relatively small fraction (~30%) of the STAT6-bound genes were evidently associated with gene expression changes (Massie and Mills, 2008). One plausible cause for the DNA binding, which is not connected to gene regulation, is that the loci might not permit the recruitment of cofactors that are required to modulate the transcriptional activity. In contrast, STAT6 may also regulate its target genes via long-distance interactions, for example, via looping of the chromatin, and these interactions cannot be identified computationally. Previously STAT6 has been shown to regulate the intrachromosomal interactions of Th2 cell cytokine locus, which is suggested to coordinate cytokine expression in the effector cells (Spilianakis and Flavell, 2004). In addition to the transcription regulation mediated directly via DNA binding, STAT6 may regulate its target genes via sequestration of transcriptional cofactors (Ohmori and Hamilton, 2000) or by modulating epigenetic marks, such as posttranslational modifications of histones or DNA methylation. Furthermore, a possibility that is completely unexplored on a genome-wide scale is that the role of STAT6 may change during the commitment and effector phase of Th2 cell lineage. Our kinetic ChIP-PCR data, although with a limited target gene set, already indicated that STAT6 occupancy on its target genes varies during time.

The present study provides a unique source of IL-4-STAT6-mediated regulation of gene expression during the early human Th2 cell differentiation process. Interpreting the initial steps of this process is also crucial for understanding the Th cell polarization in general because our results underlined the close connection between alternative Th cell phenotypes. STAT1 and STAT4 regulating Th1 cell differentiation, STAT3 acting on Th17 cell subtype, and STAT5 and STAT6 needed for Treg and Th2 cell development (Adamson et al., 2009), respectively, could all be connected to each other. This highlighted the importance of gathering detailed information of the role of all STAT family members, in order to thoroughly understand the complexity of determination of Th cell phenotype. In mouse, the STAT5A is shown to be responsible for STAT6 independent Th2 cell commitment, and *Stat6*$^{-/-}$*Stat5a*$^{-/-}$ mice have markedly reduced airway inflammation compared to *Stat6*$^{-/-}$ (Takatori et al., 2005). The overlap of our STAT6 results to previously published STAT5A data (Liao et al., 2008) suggests that the co-operational role of these factors should be further studied in human system.

Our data set illustrated the diverse effects of STAT6, from the level of DNA binding to the control of transcription during the first steps of Th2 cell polarization. The data presented in this study provides a solid basis for subsequent research on the role of STAT6 in the committed and effector Th2 cells. In addition, the data presented allow more detailed modeling of the role of STAT6 in the initiation of Th2 cell differentiation process. While genome-wide systems are beyond the reach of detailed predictive models, selected subnetworks could be used as starting points, for instance, for differential equation models of specific subsystems. Undoubtedly, our data of STAT6-mediated transcriptional control alone and combined to the genome-wide datasets acquired with other STAT molecules (Liao et al., 2008; Robertson et al., 2007) provide important insight to the steps needed for developing and regulating Th cell responses. Importantly, the findings on primary human cells, such as those presented in this study, are of particular value providing resource for new avenues for tackling the harmful immune reactions.

EXPERIMENTAL PROCEDURES

Human CD4$^+$ T Cell Purification and Culturing

Umbilical cord blood was collected from healthy neonates born in Turku University Hospital, Hospital District of Southwest Finland. Mononuclear cells were isolated (Ficoll-Paque, Amersham Biosciences), after which CD4$^+$ T cells were collected (Dynal CD4 Positive Isolation Kit, Invitrogen). Cells were activated with plate-bound αCD3 (500 ng/24-well culture plate well, Immunotech, France) and soluble αCD28 (500 ng/ml, Immunotech) in density of 2 to 4 × 10^6 cells/ml of Yssel's medium (Yssel et al., 1984) containing 1% human AB serum (PAA). Th2 cell polarization was initiated with IL-4 (10 ng/ml, with or without neutralizing αIL-12 10 μg/ml, both R&D Systems). Cells activated without differentiating cytokines were also cultured (Th0). At 48 hr, IL-2 was added to the cultures (17 ng/ml, R&D Systems).

RNAi-Mediated STAT6 Knockdown

STAT6-siRNAs #1 (5′-AAGCAGGAAGAACTGAAGTTT-3′), #2 (5′- GAATCAGT CAACGTGTTGTCA-3′), or #3 (5′-CAGTTCCGCCACTTGCCAAT-3′), or nontargeting control-siRNA (5′-GCGCGCTTTGTAGGATTCG-3′) were introduced to the cells (Sigma,1.5 μg/4 × 10^6 cells) with Nucleofector (Amaxa Biosystems), after which cells were rested 24 hr before culturing. For STAT6 target gene identification, cells were harvested at 12, 24, 48, or 72 hr time point. The culture was repeated three times, each time with different siRNA targeting *STAT6*. Total RNA (RNeasy Mini Kit, QIAGEN) was processed and hybridized on Illumina BeadChip Human-6 v2 arrays (Illumina Inc., San Diego, USA). All the microarray samples included in this study have been prepared at the Finnish Microarray and Sequencing Centre, Turku, Finland. CRTH2-PE staining (no. 130-091-238, Miltenyi Biotech) was performed after 1 week of polarization and analyzed with LSR II flow cytometer (BD Biosciences) and Cyflogic software (CyFlo Ltd, Finland). Western detections were probed with the following antibodies: STAT6 (no. 611291, BD Biosciences), GATA3 (no. 558686, BD PharMingen), and GAPDH (no. 5G4, MAb 6C5, HyTest).

Identification of IL-4-STAT6 Targets

The microarray data were quantile-normalized (Bioconductor affy package) and log2-transformed in each experiment. IL-4-regulated genes were identified between the matched Th2- and Th0-measurements (same time point and culture) in the control-siRNA data using linear modeling with moderated F- and t-statistics (Bioconductor limma package). Genes with false discovery rate (FDR) < 0.05 in the overall F-test and further at least in one of the t tests for the individual time points were defined as changed (Benjamini and Hochberg, 1995). The effect of STAT6 knockdown on the IL-4-regulated genes was then assessed using the statistic Th2/Th0 - sTh2/sTh0, where Th2/Th0 and sTh2/sTh0 denote the signal log-ratios between the matched Th2- and Th0-measurements in the control and knockdown data, respectively. Consistent IL-4-STAT6 regulation across the biological repeats was identified using the moderated F- and t-statistics at FDR < 0.05, similarly as above.

STAT6 ChIP-seq Studies

CD4$^+$ T cells were cultured in Th0 or Th2 cell polarizing condition for 1 and 4 hr, and naive cells were used as a control. ChIP was performed as described previously (Li et al., 2003). The cells were sonicated using Bioruptor sonicator (Diagenode) to obtain chromatin fragments of 100–500 bp. 500 μg of sonicated chromatin was incubated with 10 μg of STAT6 antibody (M-20, Santa Cruz Biotechnology, Inc.) coupled to the magnetic beads (no. 112.04 Dynal Biotech). The crosslinks were reversed (65°C for 12 hr), and DNA was treated sequentially with Proteinase K and RNase A and purified (QIAquick PCR purification kit, QIAGEN). The library preparation was performed according to the Illumina recommendations (Fasteris Life Sciences, Switzerland). Sequencing was performed on Illumina Genome Analyzer GAII producing from 4 to 5.2 million reads per sample. The reads were aligned to the human reference genome (NCBI v36) using SOAP software (Li et al., 2008). Only uniquely mapped reads were retained (~3 million reads per sample). Potential binding

regions were identified based on their enrichment for reads at FDR < 0.05 using the FindPeaks software (version 3.1.9.2) following the recommendations in the FindPeaks manual (Fejes et al., 2008), which showed, on average, the best peak detection performance in this particular data set (Laajala et al., 2009). To further remove potential false positives because of nonuniform background, a minimum of 10-fold enrichment of reads in the Th2 sample relative to the corresponding location in the Thp sample was required. Moreover, we focused only on the Th2 cell-specific peaks that additionally showed at least a 2-fold read enrichment in the Th2 sample relative to the corresponding Th0 sample.

Kinetic qPCR Analysis of Selected ChIP-seq Targets

We selected 10 ChIP-seq STAT6 binding regions for kinetic analysis of STAT6 binding at 0, 4, 12, and 72 hr. ChIP followed by qPCR was performed using either FAM and TAMRA labeled probes (negative controls *GATA3* and *IFNg*) or Universal ProbeLibrary probes (Roche Applied Science) with custom ordered oligos (Supplemental Experimental Procedures; STAT6 ChIP-seq section) designed with Universal ProbeLibrary Assay Design Centre (Roche), in Absolute QPCR ROX Mix (Thermo Scientific). The qPCR runs were analyzed with 7900HT Fast Real-Time PCR System (Applied Biosystems). Percent of input values were calculated with the following equation: $100 \times 2^{([\text{Input}] - [\text{ChIP}])}$, where input was adjusted to 100%.

Transcriptional Profiling of IL-4 Targets

Samples were collected at 0, 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 hr time points. RNA (RNeasy Mini Kit, QIAGEN) from three cultures was processed and hybridized on Affymetrix GeneChip HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, USA). Of the 54 hybridizations, two were excluded from further data analysis based on the compromised quality of the samples (Th0 4 hr and Th2 6 hr). The microarray data were quantile-normalized (Bioconductor affy package) and log2-transformed. IL-4-regulated genes were identified between the matched Th2 and Th0 measurements (same time point and culture) using the probe-level expression change averaging procedure PECA (Elo et al., 2005) together with linear modeling (Bioconductor limma package). The probe-level estimates of the moderated F and t statistics were summarized into probeset-level values using the Tukey biweight average, and the significance of an expression change was determined based on the analytical p value of the estimated probeset-level statistic. Probesets with $p < 0.05$ in the overall F-test and further at least in one of the t tests for the individual time points were defined as changed.

Ethical Aspects

The usage of blood of unknown donors was approved by the Ethics Committee of the Hospital District of Southwest Finland.

ACCESSION NUMBERS

The data discussed in this publication are accessible through GEO SuperSeries accession number GSE18017 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18017).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, eight tables, Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2010.06.011.

ACKNOWLEDGMENTS

We thank the technical personnel for assistance; the staff of the Finnish Microarray and Sequencing Centre, Turku, Finland for microarray hybridizations; all voluntary blood donors; the personnel of Turku University Hospital Department of Obstetrics and Gynaecology, Maternity Ward (Hospital District of Southwest Finland) for the sample collection; and A. Rao for the critical reading of the manuscript. The work was supported by the Academy of Finland (grants 77773, 203725, 207490, 116639, 115939, 123864, 126063, 127575 [L.L.E.], and 120569 [T.A.]), the European Commission Seventh Framework grants (EC-FP7-SYBILLA-201106, EC-FP7-NANOMMUNE-214281 and EC-FP7-DIABIMMUNE-202063), CIMO/Sitra grant (S.R. and R.L.), The Sigrid Jusélius Foundation (R.L. and K.V.R.), The Department of Biotechnology, Government of India (K.V.R), and Turku University Hospital Grant.

Received: December 11, 2009
Revised: April 20, 2010
Accepted: May 26, 2010
Published: June 24, 2010

REFERENCES

Adamson, A.S., Collins, K., Laurence, A., and O'Shea, J.J. (2009). The Current STATus of lymphocyte signaling: new roles for old players. Curr. Opin. Immunol. *21*, 161–166.

Agrawal, B., and Longenecker, B.M. (2005). MUC1 mucin-mediated regulation of human T cells. Int. Immunol. *17*, 391–399.

Ahn, H.J., Kim, J.Y., Ryu, K.J., and Nam, H.W. (2009). STAT6 activation by Toxoplasma gondii infection induces the expression of Th2 C-C chemokine ligands and B clade serine protease inhibitors in macrophage. Parasitol. Res. *105*, 1445–1453.

Andrews, R.P., Ericksen, M.B., Cunningham, C.M., Daines, M.O., and Hershey, G.K. (2002). Analysis of the life cycle of stat6. Continuous cycling of STAT6 is required for IL-4 signaling. J. Biol. Chem. *277*, 36563–36569.

Arpa, L., Valledor, A.F., Lloberas, J., and Celada, A. (2009). IL-4 blocks M-CSF-dependent macrophage proliferation by inducing p21Waf1 in a STAT6-dependent way. Eur. J. Immunol. *39*, 514–526.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Series B Stat Methodol. *57*, 289–290, 300.

Bettelli, E., Korn, T., and Kuchroo, V.K. (2007). Th17: the third member of the effector T cell trilogy. Curr. Opin. Immunol. *19*, 652–657.

Betz, B.C., Jordan-Williams, K.L., Wang, C., Kang, S.G., Liao, J., Logan, M.R., Kim, C.H., and Taparowsky, E.J. (2010). Batf coordinates multiple aspects of B and T cell function required for normal antibody responses. J. Exp. Med. *207*, 933–942.

Blanchette, M., Bataille, A.R., Chen, X., Poitras, C., Laganière, J., Lefèbvre, C., Deblois, G., Giguère, V., Ferretti, V., Bergeron, D., et al. (2006). Genome-wide computational prediction of transcriptional regulatory modules reveals new insights into human gene expression. Genome Res. *16*, 656–668.

Brunsing, R., Omori, S.A., Weber, F., Bicknell, A., Friend, L., Rickert, R., and Niwa, M. (2008). B- and T-cell development both involve activity of the unfolded protein response pathway. J. Biol. Chem. *283*, 17954–17961.

Büttner, C., Skupin, A., and Rieber, E.P. (2004). Transcriptional activation of the type I collagen genes COL1A1 and COL1A2 in fibroblasts by interleukin-4: analysis of the functional collagen promoter sequences. J. Cell. Physiol. *198*, 248–258.

Chaitidis, P., Billett, E.E., O'Donnell, V.B., Fajardo, A.B., Fitzgerald, J., Kuban, R.J., Ungethuem, U., and Kühn, H. (2004). Th2 response of human peripheral monocytes involves isoform-specific induction of monoamine oxidase-A. J. Immunol. *173*, 4821–4827.

Chen, Z., Lund, R., Aittokallio, T., Kosonen, M., Nevalainen, O., and Lahesmaa, R. (2003). Identification of novel IL-4/Stat6-regulated genes in T lymphocytes. J. Immunol. *171*, 3627–3635.

Cosmi, L., Annunziato, F., Nagata, K., Romagnani, S., Nagata, K., and Romagnani, S.; Galli MIG; Maggi RME. (2000). CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. Eur. J. Immunol. *30*, 2972–2979.

Elo, L.L., Lahti, L., Skottman, H., Kyläniemi, M., Lahesmaa, R., and Aittokallio, T. (2005). Integrating probe-level expression changes across generations of Affymetrix arrays. Nucleic Acids Res. *33*, e193.

Fejes, A.P., Robertson, G., Bilenky, M., Varhol, R., Bainbridge, M., and Jones, S.J. (2008). FindPeaks 3.1: a tool for identifying areas of enrichment from massively parallel short-read sequencing technology. Bioinformatics *24*, 1729–1730.

Filen, J.J., Filen, S., Moulder, R., Tuomela, S., Ahlfors, H., West, A., Kouvonen, P., Kantola, S., Björkman, M., Katajamaa, M., et al. (2009). Quantitative proteomics reveals GIMAP family proteins 1 and 4 to be differentially regulated during human T helper cell differentiation. Mol. Cell. Proteomics *8*, 32–44. Published online August 12, 2008. 10.1074/mcp.M800139-MCP200.

Gabay, C., Porter, B., Guenette, D., Billir, B., and Arend, W.P. (1999). Interleukin-4 (IL-4) and IL-13 enhance the effect of IL-1beta on production of IL-1 receptor antagonist by human primary hepatocytes and hepatoma HepG2 cells: differential effect on C-reactive protein production. Blood *93*, 1299–1307.

Gascoyne, D.M., Long, E., Veiga-Fernandes, H., de Boer, J., Williams, O., Seddon, B., Coles, M., Kioussis, D., and Brady, H.J. (2009). The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat. Immunol. *10*, 1118–1124.

Goenka, S., and Boothby, M. (2006). Selective potentiation of Stat-dependent gene expression by collaborator of Stat6 (CoaSt6), a transcriptional cofactor. Proc. Natl. Acad. Sci. USA *103*, 4210–4215.

Hämäläinen, H., Zhou, H., Chou, W., Hashizume, H., Heller, R., and Lahesmaa, R. (2001). Distinct gene expression profiles of human type 1 and type 2 T helper cells. Genome Biol. *2*, RESEARCH0022.

Hebenstreit, D., Luft, P., Schmiedlechner, A., Regl, G., Frischauf, A.M., Aberger, F., Duschl, A., and Horejs-Hoeck, J. (2003). IL-4 and IL-13 induce SOCS-1 gene expression in A549 cells by three functional STAT6-binding motifs located upstream of the transcription initiation site. J. Immunol. *171*, 5901–5907.

Hebenstreit, D., Wirnsberger, G., Horejs-Hoeck, J., and Duschl, A. (2006). Signaling mechanisms, interaction partners, and target genes of STAT6. Cytokine Growth Factor Rev. *17*, 173–188.

Ji, H., Jiang, H., Ma, W., Johnson, D.S., Myers, R.M., and Wong, W.H. (2008). An integrated software system for analyzing ChIP-chip and ChIP-seq data. Nat. Biotechnol. *26*, 1293–1300.

Johnson, D.S., Mortazavi, A., Myers, R.M., and Wold, B. (2007). Genome-wide mapping of in vivo protein-DNA interactions. Science *316*, 1497–1502.

Kamizono, S., Duncan, G.S., Seidel, M.G., Morimoto, A., Hamada, K., Grosveld, G., Akashi, K., Lind, E.F., Haight, J.P., Ohashi, P.S., Look, A.T., and Mak, T.W. (2009). Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. J. Exp. Med. *206*, 2977–2986.

Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity *4*, 313–319.

Khodarev, N., Ahmad, R., Rajabi, H., Pitroda, S., Kufe, T., McClary, C., Joshi, M.D., MacDermed, D., Weichselbaum, R., and Kufe, D. (2010). Cooperativity of the MUC1 oncoprotein and STAT1 pathway in poor prognosis human breast cancer. Oncogene *29*, 920–929.

Kim, S.H., Gunst, K.V., and Sarvetnick, N. (2006). STAT4/6-dependent differential regulation of chemokine receptors. Clin. Immunol. *118*, 250–257.

Kitoh, A., Ono, M., Naoe, Y., Ohkura, N., Yamaguchi, T., Yaguchi, H., Kitabayashi, I., Tsukada, T., Nomura, T., Miyachi, Y., et al. (2009). Indispensable Role of the Runx1-Cbfbeta Transcription Complex for in vivo-suppressive function of FoxP3+ regulatory T cells. Immunity *31*, 609–620.

Komine, O., Hayashi, K., Natsume, W., Watanabe, T., Seki, Y., Seki, N., Yagi, R., Sukzuki, W., Tamauchi, H., Hozumi, K., et al. (2003). The Runx1 transcription factor inhibits the differentiation of naive CD4+ T cells into the Th2 lineage by repressing GATA3 expression. J. Exp. Med. *198*, 51–61.

Kurata, H., Lee, H.J., O'Garra, A., and Arai, N. (1999). Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. Immunity *11*, 677–688.

Kwon, H., Thierry-Mieg, D., Thierry-Mieg, J., Kim, H.P., Oh, J., Tunyaplin, C., Carotta, S., Donovan, C.E., Goldman, M.L., Tailor, P., et al. (2009). Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. Immunity *31*, 941–952.

Laajala, T.D., Raghav, S., Tuomela, S., Lahesmaa, R., Aittokallio, T., and Elo, L.L. (2009). A practical comparison of methods for detecting transcription factor binding sites in ChIP-seq experiments. BMC Genomics *10*, 618.

Lee, Y.H., and Stallcup, M.R. (2006). Interplay of Fli-I and FLAP1 for regulation of beta-catenin dependent transcription. Nucleic Acids Res. *34*, 5052–5059.

Li, Z., Van Calcar, S., Qu, C., Cavenee, W.K., Zhang, M.Q., and Ren, B. (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc. Natl. Acad. Sci. USA *100*, 8164–8169.

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: short oligonucleotide alignment program. Bioinformatics *24*, 713–714.

Liao, W., Schones, D.E., Oh, J., Cui, Y., Cui, K., Roh, T.Y., Zhao, K., and Leonard, W.J. (2008). Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. Nat. Immunol. *9*, 1288–1296.

Lund, R.J., Löytömäki, M., Naumanen, T., Dixon, C., Chen, Z., Ahlfors, H., Tuomela, S., Tahvanainen, J., Scheinin, J., Henttinen, T., et al. (2007). Genome-wide identification of novel genes involved in early Th1 and Th2 cell differentiation. J. Immunol. *178*, 3648–3660.

Massie, C.E., and Mills, I.G. (2008). ChIPping away at gene regulation. EMBO Rep. *9*, 337–343.

Matute, J.D., Arias, A.A., Wright, N.A., Wrobel, I., Waterhouse, C.C., Li, X.J., Marchal, C.C., Stull, N.D., Lewis, D.B., Steele, M., et al. (2009). A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity. Blood *114*, 3309–3315.

McGaha, T.L., Le, M., Kodera, T., Stoica, C., Zhu, J., Paul, W.E., and Bona, C.A. (2003). Molecular mechanisms of interleukin-4-induced up-regulation of type I collagen gene expression in murine fibroblasts. Arthritis Rheum. *48*, 2275–2284.

Mole, D.R., Blancher, C., Copley, R.R., Pollard, P.J., Gleadle, J.M., Ragoussis, J., and Ratcliffe, P.J. (2009). Genome-wide association of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha DNA binding with expression profiling of hypoxia-inducible transcripts. J. Biol. Chem. *284*, 16767–16775.

Nagai, S., Hashimoto, S., Yamashita, T., Toyoda, N., Satoh, T., Suzuki, T., and Matsushima, K. (2001). Comprehensive gene expression profile of human activated T(h)1- and T(h)2-polarized cells. Int. Immunol. *13*, 367–376.

Naoe, Y., Setoguchi, R., Akiyama, K., Muroi, S., Kuroda, M., Hatam, F., Littman, D.R., and Taniuchi, I. (2007). Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. J. Exp. Med. *204*, 1749–1755.

Ohmori, Y., and Hamilton, T.A. (2000). Interleukin-4/STAT6 represses STAT1 and NF-kappa B-dependent transcription through distinct mechanisms. J. Biol. Chem. *275*, 38095–38103.

Ohmori, Y., Smith, M.F., Jr., and Hamilton, T.A. (1996). IL-4-induced expression of the IL-1 receptor antagonist gene is mediated by STAT6. J. Immunol. *157*, 2058–2065.

Olsson, L.M., Lindqvist, A.K., Källberg, H., Padyukov, L., Burkhardt, H., Alfredsson, L., Klareskog, L., and Holmdahl, R. (2007). A case-control study of rheumatoid arthritis identifies an associated single nucleotide polymorphism in the NCF4 gene, supporting a role for the NADPH-oxidase complex in autoimmunity. Arthritis Res. Ther. *9*, R98.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., and Murphy, K.M. (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity *9*, 745–755.

Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W., et al. (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat. Genet. *39*, 596–604.

Robertson, G., Hirst, M., Bainbridge, M., Bilenky, M., Zhao, Y., Zeng, T., Euskirchen, G., Bernier, B., Varhol, R., Delaney, A., et al. (2007). Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat. Methods *4*, 651–657.

Rogge, L., Bianchi, E., Biffi, M., Bono, E., Chang, S.Y., Alexander, H., Santini, C., Ferrari, G., Sinigaglia, L., Seiler, M., et al. (2000). Transcript imaging of the development of human T helper cells using oligonucleotide arrays. Nat. Genet. *25*, 96–101.

Schaefer, G., Venkataraman, C., and Schindler, U. (2001). Cutting edge: FISP (IL-4-induced secreted protein), a novel cytokine-like molecule secreted by Th2 cells. J. Immunol. *166*, 5859–5863.

Schraml, B.U., Hildner, K., Ise, W., Lee, W.L., Smith, W.A., Solomon, B., Sahota, G., Sim, J., Mukasa, R., Cemerski, S., et al. (2009). The AP-1 transcription factor Batf controls T(H)17 differentiation. Nature *460*, 405–409.

Schröder, A.J., Pavlidis, P., Arimura, A., Capece, D., and Rothman, P.B. (2002). Cutting edge: STAT6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. J. Immunol. *168*, 996–1000.

Shimoda, K., van Deursen, J., Sangster, M.Y., Sarawar, S.R., Carson, R.T., Tripp, R.A., Chu, C., Quelle, F.W., Nosaka, T., Vignali, D.A., et al. (1996). Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature *380*, 630–633.

Shinnakasu, R., Yamashita, M., Kuwahara, M., Hosokawa, H., Hasegawa, A., Motohashi, S., and Nakayama, T. (2008). Gfi1-mediated stabilization of GATA3 protein is required for Th2 cell differentiation. J. Biol. Chem. *283*, 28216–28225.

Spilianakis, C.G., and Flavell, R.A. (2004). Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. Nat. Immunol. *5*, 1017–1027.

Suriano, A.R., Sanford, A.N., Kim, N., Oh, M., Kennedy, S., Henderson, M.J., Dietzmann, K., and Sullivan, K.E. (2005). GCF2/LRRFIP1 represses tumor necrosis factor alpha expression. Mol. Cell. Biol. *25*, 9073–9081.

Takatori, H., Nakajima, H., Hirose, K., Kagami, S., Tamachi, T., Suto, A., Suzuki, K., Saito, Y., and Iwamoto, I. (2005). Indispensable role of Stat5a in Stat6-independent Th2 cell differentiation and allergic airway inflammation. J. Immunol. *174*, 3734–3740.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996). Essential role of Stat6 in IL-4 signalling. Nature *380*, 627–630.

Toscano, M.A., Bianco, G.A., Ilarregui, J.M., Croci, D.O., Correale, J., Hernandez, J.D., Zwirner, N.W., Poirier, F., Riley, E.M., Baum, L.G., and Rabinovich, G.A. (2007). Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. Nat. Immunol. *8*, 825–834.

Weaver, C.T., Harrington, L.E., Mangan, P.R., Gavrieli, M., and Murphy, K.M. (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity *24*, 677–688.

Yang, Y., Ochando, J., Yopp, A., Bromberg, J.S., and Ding, Y. (2005). IL-6 plays a unique role in initiating c-Maf expression during early stage of CD4 T cell activation. J. Immunol. *174*, 2720–2729.

Yssel, H., De Vries, J.E., Koken, M., Van Blitterswijk, W., and Spits, H. (1984). Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J. Immunol. Methods *72*, 219–227.

Zhang, S., Lukacs, N.W., Lawless, V.A., Kunkel, S.L., and Kaplan, M.H. (2000). Cutting edge: differential expression of chemokines in Th1 and Th2 cells is dependent on Stat6 but not Stat4. J. Immunol. *165*, 10–14.

Zhang, F., Meng, G., and Strober, W. (2008). Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. Nat. Immunol. *9*, 1297–1306.

Zhao, H., Li, C.C., Pardo, J., Chu, P.C., Liao, C.X., Huang, J., Dong, J.G., Zhou, X., Huang, Q., Huang, B., et al. (2005). A novel E3 ubiquitin ligase TRAC-1 positively regulates T cell activation. J. Immunol. *174*, 5288–5297.

Zhu, J., Guo, L., Watson, C.J., Hu-Li, J., and Paul, W.E. (2001). Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. J. Immunol. *166*, 7276–7281.

Zhu, J., Guo, L., Min, B., Watson, C.J., Hu-Li, J., Young, H.A., Tsichlis, P.N., and Paul, W.E. (2002). Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. Immunity *16*, 733–744.

Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L., and Paul, W.E. (2006). GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. *16*, 3–10.