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IMMUNOPATHOGENESIS OF ASTHMA AND ATOPIC DISEASES – THE SPECIFIC ROLE OF A SELECTED PANEL OF GENES IN HUMAN T HELPER CELL DIFFERENTIATION

by

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Jos et aseta itsellesi päämäärää, pysyt paikallasi tai taannut.
– Paavo Nurmi

ABSTRACT

Maritta Löytömäki

Immunopathogenesis of Asthma and Atopic Diseases – The specific role of a selected panel of genes in human T helper cell differentiation

Turku Centre for Biotechnology, University of Turku and Åbo Akademi University; Department of Medical Genetics, University of Turku; Turku Graduate School for Biomedical Sciences. 2007.

T helper cell (Th) functions are crucial for proper immune defence against various intra- and extracellular pathogens. According to the specific immune responses, Th cells can be classified into subtypes, Th1 and Th2 cells being the most frequently characterized classes. Th1 and Th2 cells interact with other immune cells by regulating their functions with specific cytokine production. IFNγ, IL-2 and TNF-β are the cytokines predominantly produced by Th1 cells whereas Th2 cells produce Th2-type cytokines, such as IL-4, IL-5 and IL-13. Upon TCR activation and in the presence of polarizing cytokines, Th cells differentiate into effector subtypes from a common precursor cell. IFNγ and IL-12 are the predominant Th1 polarizing cytokines whereas IL-4 directs Th2 polarization. The cytokines mediate their effects through specific receptor signalling.

The differentiation process is complex, involving various signalling molecules and routes, as well as functions of the specific transcription factors. The functions of the Th1/Th2 cells are tightly regulated; however, knowledge on human Th cell differentiation is, as yet, fairly poor. The susceptibility for many immune-mediated disorders often originates from disturbed Th cell responses. Thus, research is needed for defining the molecular mechanisms involved in the differentiation and balanced functions of the Th cells. Importantly, the new information obtained will be crucial for a better understanding of the pathogenesis of immune-mediated disorders, such as asthma or autoimmune diseases.

In the first subproject of this thesis, the role of genetic polymorphisms in the human *STAT6*, *GATA3* and *STAT4* genes were investigated for asthma or atopy susceptibility in Finnish asthma families by means of association analysis. These genes code for key transcription factors regulating Th cell differentiation. The study resulted in the identification of a *GATA3* haplotype that associated with asthma and related traits (high serum IgE level). In the second subproject, an optimized method for human primary T cell transfection and enrichment was established. The method can be utilized for functional studies for the selected genes of interest. The method was also utilized in the third subproject, which aimed at the identification of novel, IL-4, IL-12 and TGFβ induced, genes involved in early human Th cell polarization (0-48h) using genome-wide oligonucleotide arrays. As a result, numerous genes and ESTs with known or unknown functions were identified in the study. Using an shRNA knockdown approach, a panel of novel IL-4/STAT6 regulated genes were identified in the functional studies of the genes. Moreover, one of the genes, NDFIP2, with a previously uncharacterized role in the human Th differentiation, was observed to promote IFNγ production of the differentiated Th1 cells.

Taken together, the results obtained have revealed potential new relevant candidate genes serving as a basis for further studies characterizing the detailed networks involved in the human Th cell differentiation as well as in the genetic susceptibility of Th-mediated immune disorders.

Keywords: T helper cell differentiation, Th1/Th2, STAT6, GATA3, STAT4, polymorphism, association analysis, asthma, IgE, haplotype, cytokine, oligonucleotide array

TIIVISTELMÄ

Maritta Löytömäki

Astman ja atooppisten tautien immunopatogeneesi – Tiettyjen ihmisen T-solujen erilaistumiseen vaikuttavien geenien spesifinen merkitys

Turun Biotekniikan keskus, Turun Yliopisto ja Åbo Akademi; Lääketieteellisen genetiikan laitos, Turun Yliopisto; Turun Biolääketieteellinen tutkijakoulu. 2007.

T-auttajasolut (Th) ovat tärkeitä puolustauduttaessa erilaisia solunsisäisiä tai ulkoisia taudinaiheuttajia vastaan. Erikoistuneiden tehtäviensä perusteella Th-solut voidaan jaotella eri alaluokkiin, joista tunnetuimpia ovat Th1- ja Th2-tyypin solut. Th-solut vuorovaikuttavat muiden immuunijärjestelmän solujen kanssa puolustus- ja tulehdusreaktioissa ohjaten näiden toimintaa sytokiinituotantonsa avulla. Th1-solut tuottavat antigeenistimulaation seurauksena tyypillisesti IFNγ-, IL-2- ja TNF-β sytokiineja kun taas IL-4, IL-5 sekä IL-13 ovat pääasiallisia Th2-solujen tuottamia sytokiineja. Th-solut erilaistuvat kantamuodostaan T-solureseptoriaktivaation sekä solunulkoisen sytokiiniympäristön vaikutuksesta. IFNγ ja IL-12 ovat tärkeimpiä Th1-suuntaan ohjaavia sytokiineja, IL-4 puolestaan ohjaa solujen kehitystä Th2-suuntaan. Sytokiinien välittämät vasteet kulkeutuvat soluun sen pinnan spesifisten reseptoreiden kautta.

Th-solujen erilaistumisprosessi on monimutkainen, useiden eri viestinvälittäjämolekyylien, signalointireittien sekä spesifisten transkriptiotekijöiden säätelemä vuorovaikutusverkosto. Th1/Th2-solujen toiminta on immuunivasteessa tarkasti säädeltyä, joskin tieto ihmisen T-auttajasolujen erilaistumismekanismeista on vielä puutteellista. Häiriytyessään Th-soluvasteet voivat johtaa alttiuteen sairastua moniin immuunivälitteisiin tauteihin. Lisätutkimusta tarvitaankin paitsi Th-solujen erilaistumisreittien ja toiminnan tutkimiseksi, myös monien immuunivälitteisten sairauksien, kuten astman ja autoimmuunitautien, syntymekanismien selvittämiseksi.

Tämän väitöskirjan ensimmäisessä osatyössä tutkittiin assosiaatioanalyysin avulla Th-erilaistumisessa olennaisten transkriptiotekijöiden, STAT6, GATA3 ja STAT4, geenipolymorfioiden yhteyttä astma- ja atopia-alttiuteen suomalaisissa astmaperheissä. Tuloksena havaittiin *GATA3*-geenin haplotyypin assosioituvan astmaan tai siihen liittyviin ominaisuuksiin (seerumin korkea IgE-taso). Toisessa osatyössä pystytettiin menetelmä ihmisen primääri-T-solujen transfektoimiseksi ja rikastamiseksi Th-solujen erilaistumisessa vaikuttavien geenien toiminnannallisia tutkimuksia varten. Kolmannen osatyön tavoitteena oli tunnistaa uusia, ihmisen Th1/Th2-solujen erilaistumisen varhaisvaiheissa (0-48h) aktivoituvia IL-4:n, IL-12:n sekä TGFβ:n säätelemiä geenejä. Tutkimuksen tuloksena identifioitiin useita geenejä, joista monien toiminta Th-solujen erilaistumisessa on ennestään tuntematon. shRNA-tutkimusten avulla tutkimuksessa löydettiin myös uusia IL-4/STAT6-signaalitien säätelemiä geenejä. Lisäksi havaittiin NDFIP2-proteiinin, jonka merkitys Th-solujen erilaistumisessa ei ole aiemmin ollut tiedossa, vaikuttavan Th1-solujen IFNγ-tuotantoon. Tämän osoittamiseksi käytettiin toisessa osatyössä kehitettyä menetelmää.

Tutkimustulokset tuovat tietoa uusista mahdollisista Th-solujen erilaistumiseen sekä Th-soluvälitteisiin sairauksiin liittyvistä mekanismeista ja luovat uusia lähtökohtia ja hypoteesejä jatkotutkimuksille.

Avainsanat: T-auttajasolut, Th1/Th2, STAT6, GATA3, STAT4, polymorfia, assosiaatioanalyysi, astma, IgE, haplotyyppi, sytokiini, oligonukleotidisiru

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ABBREVIATIONS

AHR	airway hyperresponsiveness
APC	antigen-presenting cell
bp	base pair
b-ZIP	basic-region leucine zipper
CD	cluster of differentiation
cDNA	complementary DNA
ChIP	Chromatin Immunoprecipitation
CTLA-4	cytotoxic T lymphocyte-associated 4
DC	dendritic cell
DHPLC	denaturing high-performance liquid chromatography
DN	dominant negative, double negative
DNA	deoxyribonucleic acid
(d)dNTP	(di)deoxynucleoside triphosphate
DTH	delayed-type hypersensitivity
EGF(R)	epidermal growth factor (receptor)
EGFP	enhanced green fluorescent protein
ENaC	epithelial sodium channel
ER	endoplasmic reticulum
ERM	ets-related molecule
FACS	fluorescence activated cell sorter
FOXp3	Forkhead Winged-Helix Transcriptional Factor Box p3
GAS	gamma-activated sequence/site
GATA	GATA binding protein
GFP	green fluorescent protein
GO	gene ontology
HDR	syndrome of familial hypoparathyroidism, sensorineural deafness, and renal dysplasia
HLA	human leucocyte antigen
HPM	haplotype pattern mining
HS	DNase I hypersensitive site
ICOS	inducible T cell co-stimulator
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
IL4R	interleukin 4 receptor
IL12R	interleukin 12 receptor
JAK	janus kinase
kb	kilobase
kDa	kilodalton
KO	knockout
LB	Luria-Bertani medium
LD	linkage disequilibrium
LOD	lod score, logarithm of odds

MCS	multiple cloning site
MHC	major histocombatibility complex
mRNA	messenger RNA
MS	multiple sclerosis
MVB	multivesicular bodies
NFAT	nuclear activator of activated T cells
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B cells
NK	natural killer cell
NPL	nonparametric linkage
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 12-acetate
RA	rheumatoid arthritis
RAST	radioallergosorbent test
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase polymerase chain reaction
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
Ser/Thr	serine/threonine
SH2	src homology 2 domain
shRNA	short-hairpin RNA
s-IgE	serum IgE
siRNA	short interfering RNA
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
T1D	Type 1 diabetes
Tc	cytotoxic T cell
TCR	T cell receptor
TGF	transforming growth factor
Th(p)	T helper cell (progenitor)
TMHA	temperature-modulated heteroduplex analysis
TNF	tumour necrosis factor
Treg	T regulatory cell
UTR	untranslated region
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-III).

I Pykäläinen M, Kinos R, Valkonen S, Rydman P, Kilpeläinen M, Laitinen LA, Karjalainen J, Nieminen M, Hurme M, Kere J, Laitinen, T & Lahesmaa R. (2005) Association analysis of common variants of STAT6, GATA3 and STAT4 to asthma and high serum IgE phenotypes. *The Journal of Allergy and Clinical Immunology 115(1):80-87.*

II Tahvanainen J, Pykäläinen M, Kallonen T, Lähteenmäki H, Rasool O, Lahesmaa R. (2006) Enrichment of nucleofected primary human $CD4^+$ T cells: a novel and efficient method for studying gene function and role in human primary T helper cell differentiation. *Journal of Immunol Methods 310(1-2):30-39.*

III Lund R*, Löytömäki M*, Naumanen T, Dixon C, Chen Z, Ahlfors H, Tuomela S, Tahvanainen J, Scheinin J, Henttinen T, Rasool O, Lahesmaa R. (2007) Genome wide identification of novel genes involved in early Th1 and Th2 cell differentiation. *The Journal of Immunology 178(6):3648-60.* *Equal contribution

Some additional unpublished data are presented.

INTRODUCTION

Effective immune responses are crucial for overcoming the various types of intra- or extracellular pathogens that are encountered daily. Therefore, the immune system has evolved to specifically respond to attacks by foreign pathogens. Various specialized white blood cells, such as T and B lymphocytes, dendritic cells, NK cells, mast cells, macrophages, eosinophiles and neutrophiles interact in immune responses. The immune system can be divided into non-specific (innate immunity) and specific acquired (adaptive) immunity, the latter including more specific defence reactions. Along with B cells, T cells form a major class of lymphocytes functioning in adaptive immunity. They function in various manners in the immune defence by regulating cell-mediated or humoral immune reactions that are activated depending on the nature of the pathogen. First born in the bone marrow, T cells migrate into the thymus (T=thymus) to mature and finally wander to peripherial lymph organs, such as the lymph nodes, blood and spleen.

CD4+ T helper cells (Th) are one major class of T lymphocytes. Based on differing biological effector functions, Th cells can be further classified into different subtypes, such as Th1 and Th2 cells as well as T regulatory (Treg) and Th17 cells, the two latter subtypes being more recently described. Th cells function by secreting a set of subtype-specific cytokines which have major immune-regulative effects on other cells in the immune system. Th1 cells are involved in cell-mediated immune reactions against intracellular bacteria and viruses, whilst Th2 cells mediate extracellular defence against pathogens, such as parasites. Th2 cells also promote allergic inflammation and IgE production, thus being also tightly involved in the pathogenesis of asthma and atopic traits. Upon antigen stimulus, Th cells differentiate from common lymphoid progenitor cells into effectors with memory functions for secondary antigen responses. Cytokine microenvironment and cellular signals are key regulators of the Th cell lineage development. Differential cytokine production within Th1 and Th2 cells is controlled at the level of gene transcription. IL-12 and IFNγ are the key cytokines directing Th1 polarization, IL-4 in turn being a major determinant of the Th2 cell fate. These cytokines mediate their effects through specific receptor binding and transcription factor signalling. *STAT6, GATA3, STAT4* and *T-bet* are central transcription factors for Th cell differentiation that activate cytokine-induced gene transcription.

The nature of an immune response is determined by balanced functions of Th1/Th2 cells. Thus, some immune-mediated disorders, such as autoimmune diseases or allergic and asthmatic response, may result from imbalanced and dysregulated Th1/Th2 responses. Thus, Th cell differentiation must be tightly regulated by various genetic and environmental factors that form a complex regulative signalling network. Understanding the detailed machinery controlling the early Th cell differentiation provides valuable information on the molecular and genetic background of these immune disorders. However, the detailed signalling networks and hierarchies leading to Th cell differentiation are so far poorly characterized, particularly in the human system, and many novel players with yet unknown functions may be involved in the process.

Atopic asthma has a multifactorial nature. Besides environmental factors, also genetic factors, too, play a part in disease susceptibility. Association analysis is a tool to investigate the role of genetic variations, such as single nucleotide polymorphisms (SNPs), in genes of asthma predisposition. Association studies are based on comparisons of SNP allele frequencies between family or population-based study samples.

This thesis has three main objectives. Firstly, the significance of SNPs in human *STAT6*, *STAT4* and *GATA3* genes in the development of atopic diseases in the Finnish asthmatic families was examined. The studied genes were chosen based on the candidate gene approach. Secondly, the aim was to establish a method for human primary CD4+ T cell transfection. Once optimized, the novel method is utilized for gene silencing studies for selected genes of interest. Thirdly, a panel of novel genes regulated by IL-4 and IL-12 during the early stages of human Th cell differentiation was identified using the whole-genome transcriptomics analysis. The functions and regulative role of *STAT6* was further investigated for a subset of the genes involved in early human Th2 polarization.

REVIEW OF THE LITERATURE

1 T helper cell differentiation and immune functions

1.1 T helper cells and their functions

CD4+ helper T (Th) cells regulate the immune defence reactions by producing cytokines, molecules mediating inflammatory responses. By their expression of CD surface molecules (Cluster of Differentiation), they are distinguished from CD8+ cytotoxic killer T cells (Tc), which can directly kill the targeted pathogen-infected cells. Th cells recognize the foreign particles specifically via their T cell receptor (TCR) on the surface of antigen-presenting cells (APCs), such as dendritic cells (DC), macrophages or B cells. The different antigens are presented to T cells in the T cell zones in secondary lymph organs as peptides processed by APCs, bound to a class II major histocompatibility complex (MHC) molecule on the APC (Tc cells recognize the antigen in the context of MHC I) (Janeway *et al*. 2001). This, together with accessory molecules on the surface of T and APC cells, results in activation of signalling cascades in T cells and initiates their differentiation into effector or memory cells.

Th lymphocytes can be classified into different effector subpopulations, such as type 1 (Th1) or type 2 (Th2) cells, regulatory T cells with suppressive functions (Tregs), and also very recently reported (Harrington *et al*. 2005, Park *et al*. 2005) pro-inflammatory Th17 cells (Figure 1). Th1 and Th2 type cells with differing immunobiological activities have for a long time been the best characterized Th cell subsets (Mosmann *et al*. 1986, Abbas *et al*. 1996). Distinct CD4+ Th1 and Th2 cell clones, once differentiated from the common naïve lymphoid CD4+ Th progenitor (Thp) into effector cells upon activation caused by the antigen exposure, were at first discovered in mice (Mosmann *et al*. 1986) and later also in humans (Del Prete *et al*. 1991, Romagnani *et al.* 1991, Lahesmaa *et al*. 1992). Th1 and Th2 cells mediate their specific immune functions by the specialized patterns of cytokines they produce after antigen activation (Mosmann & Coffman 1989). Typical cytokines secreted by Th1 cells are interferon gamma (IFNγ), IL-2 and TNF-β (lymphotoxin), whereas Th2 cells, in turn, produce cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann & Coffman 1989, Mosmann & Sad 1996, Romagnani 1996). Th1 cells participate in the reactions of cellular immunity against intracellular microbes, such as *Leishmania major* (Mosmann & Sad 1996, Ho & Glimcher 2002). By producing cytokines, Th1 cells regulate immune defence by activating macrophages and CD8+ cytotoxic T cells to kill the harmful organisms, as well as enhance the immune response by further recruiting other Th1 cells to the site of inflammation (Abbas *et al*. 1996). Th1 cells also mediate delayed-type hypersensitivity (DTH) reactions (Cher & Mosmann 1987, Mosmann & Coffman 1989). Th2 cells, in turn, act as a defence against extracellular parasites, such as helminths. IL-4 and IL-13 production are important mediators of humoral immune responses by promoting the antigen-specific antibody production of B cells (IgE and IgG1) (Sher & Coffman 1992, Romagnani 1994, Abbas *et al*. 1996, O´Garra 1998). By producing IL-5, Th2 cells promote the development of eosinophilic

cells. In addition, IL-5 activates basophils to release histamines and leucotrienes (Coffman *et al*. 1989). Th2 cytokines IL-4, IL-9 and IL-10 also regulate the growth of mast cells and basophils (Romagnani 2000).

The effective functions of T helper cells in changing everyday situations are necessary for the generation of a proper immune response and host defence (Sher & Coffman 1992). Excessive or unbalanced activation of Th1 or Th2 subsets can lead to pathogenesis or progression of certain immune-mediated and inflammatory diseases such as allergic conditions or autoimmunity. Thus, the balance formed between Th1/Th2 type cells is thought to be the key determinant of the outcome of different immune responses (Peltz 1991, Romagnani 1994, Umetsu *et al*. 1997). Therefore, understanding the regulation of the molecular mechanisms of T helper cell differentiation in healthy and diseased subjects is the keystone for the characterization of the errors in the function of the immune system, which may predispose to the certain immune-diseases, such as asthma or autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthiris (RA) and Crohn´s disease. Th1-primed immune reactions such as delayed hypersensitivity have been linked to the progression of autoimmune diseases, and Th1 cytokine production may be important in their pathogenesis (Mosmann & coffman 1989, Mosmann & Sad 1996, Glimcher and Murphy 2000). Pathogenic Th2 cell functions, in turn, play a triggering role in the development of asthma and allergic cascades and overproduction of the allergen-associated antibody, IgE. In addition, Th2-type cytokines have been observed in individuals with asthmatic or allergic symptoms (Romagnani 1994, 2000, Larche *et al*. 2003). With the discovery of the different types of T regulatory cells and the recently decribed Th cell subset, Th17, (see Chapter 1.6) new insights into Th cell biology and immunopathology have been gained. Besides effector Th1 and Th2 cell functions, the immune responses mediated by the cytokine production of different types of T regulatory cells and the Th17 subclass are crucial and tightly involved in the Th-mediated host defence. By suppressing immune functions, Tregs are thought to modulate Th1/Th2 balance and control excessive inflammation and maintain the tolerance and homeostasis (Wan & Flavell 2006, Bopp *et al*. 2007). The Th17 cells, in turn, display pro-inflammatory effects and appear to be potential mediators of autoimmune diseases (Bettelli *et al*. 2007).

1.2 T helper cell activation and polarization

Th cell fate is very much dependent on the nature of the environmental stimuli and modulatory factors, in particular the presence of polarizing cytokines produced by the neighbouring cells, such as APCs or NK cells. Furthermore, the activation of costimulatory molecules, the nature (type, concentration, dosage) of the antigen, duration and strength of TCR signalling, type of APC, the number of postactivation cell divisions as well as the presence and functions of many accessory molecules, chemokines and cell-specific transcription factors have been shown to affect the process (Abbas *et al*. 1996, Constant & Bottomly 1997, O´Garra 1998, Glimcher & Murphy 2000, Murphy & Reiner 2002, Ho & Glimcher 2002, Szabo *et al*. 2003, Mowen & Glimcher 2004, Romagnani 2004). The genetic background of the host also

plays an important role in Th cell differentiation (Hsieh *et al*. 1995, Romagnani 1996). Moreover, information obtained from studies focusing on epigenetic changes during Th cell differentiation will be crucial for understanding the detailed mechanisms of the process (see Chapter 1.5) (Murphy & Reiner 2002, Ansel *et al*. 2003, 2006).

1.2.1 Cytokine microenvironment in Th differentiation

The environmental cytokines are the key determinants of T helper cell differentiation. Released upon antigen stimulation mainly by the neighbouring immune cells, such as macrophages, APC or NK cells, cytokines regulate the initiation of a differentiation programme towards Th1 or Th2 lineage in a naïve Thp cell (Romagnani 1992, Seder & Paul 1994, O´Garra 1998). IL-12 and IFNγ (promoting Th1) and IL-4 (promoting Th2 lineage) are the most important polarizing cytokines of Th1 and Th2 cell commitment, selectively regulating the Th effector functions (Sher & Coffman 1992, Abbas *et al*. 1996, O´Garra 1998) (Figure 1). The effects of IL-4 in inducing Th2 development are dominant over Th1 polarizing cytokines and Th2 cells will differentiate if IL-4 levels reach a certain threshold at the beginning of an immune response (Seder & Paul 1994, O´Garra 1998). By inducing the differentiation of Th subtypes and the Th1/Th2 cytokine production, IL-12, IFNγ and IL-4 also induce their own expression in the cells (Le Gros *et al*. 1990, Swain *et al*. 1990, Szabo *et al*. 2003). The effects induced by these cytokines are mediated through binding to specific receptors which trigger intracellular signal transduction pathways. IL-4 mediates its biological effects in Th2 cells via the IL-4 receptor (IL-4R) through specific transcriptional regulator proteins, STAT6, GATA3 and c-maf, whereas Th1-type cytokine (IL-12 and IFNγ) signalling is mediated by the functions of STAT4, STAT1 and T-bet transcription factors (discussed in more detail in the following chapters) (Abbas *et al*. 1996, Glimcher & Murphy 2000, Rengarajan *et al*. 2000). Interestingly, the differentiating Th subsets, besides having the ability to stimulate cells of the same subsets to enhance their effector functions, also at the same time suppress the differentiation and effector functions of the opposing lineage via cytokine and transcription factors (Swain *et al*. 1990, Seder *et al*. 1992, Seder & Paul 1994, Rengarajan *et al*. 2000, Ansel *et al*. 2003) (Figure 1). Overall, the T helper cell differentiation process into functionally diverse Th subsets involves cytokine-induced effects mediated through different signalling pathways as well as epigenetic mechanisms involving chromating remodelling and transcriptional regulation, to commit and maintain the Th cell fate at multiple developmental stages (Szabo *et al*. 2003, Mowen & Glimcher 2004).

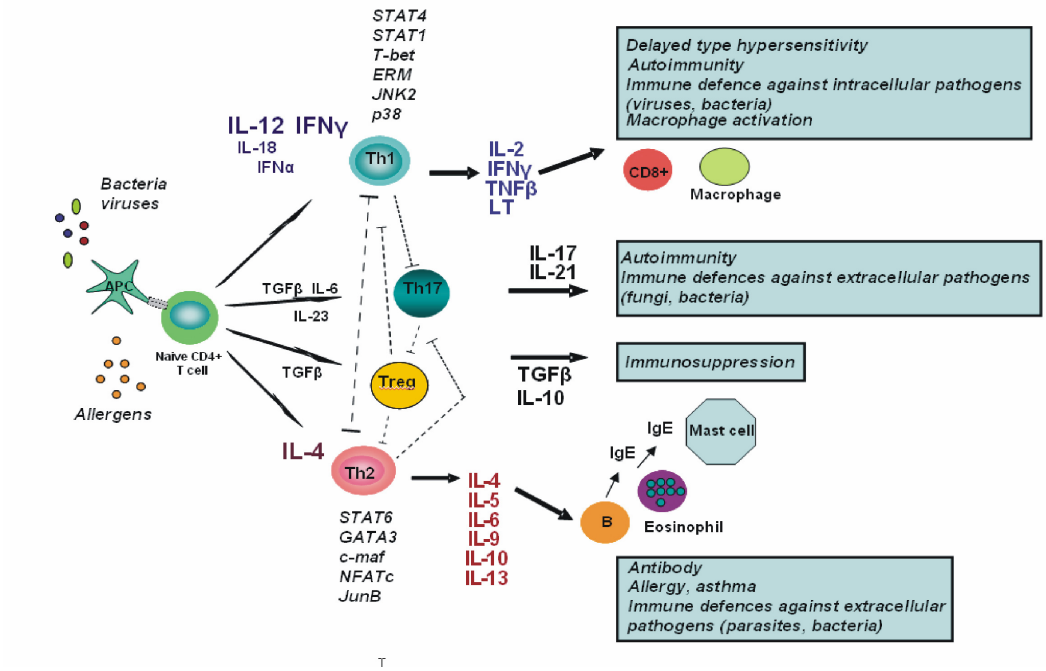


Figure 1. Schematic overview of the differentiation and effector functions of the T helper cells. Upon APC stimulation, a naïve CD4+ T cell starts to differentiate towards Th1 or Th2 type effector functions with the presence of polarizing cytokines and functions of regulating transcription factors. Th1 and Th2 type cells produce different cytokines, which further regulate other types of immune cells. Th1 type cells are important effectors for cell-mediated immunity whilst Th2 cells are crucial for humoral immunity reactions. Pathogenic Th2 responses are linked to an allergic response mediated by IgE. In concert with Th1 and Th2 subsets, T regulatory (Treg) and IL-17 cells are key T cell subtypes with immunosuppressive or pro-inflammatory functions. The antagonizing effects between the cells are shown with dashed lines. (Modified from Tato & O´Shea 2006, Deenick & Tangye 2007, Schmidt-Weber *et al*. 2007)

1.2.2 Costimulatory factors in Th cell activation and differentiation

Upon antigen presentation via TCR-APC, full T cell activation and differentiation requires secondary activation signals provided by various costimulatory molecules. These secondary signals are provided through costimulatory and accessory adhesion molecule interactions, such as CD28/B7-1, CTLA4/B7-1, ICOS/B7-H2 and OX40/OX40L, which are glycoproteins attached to the cell surface of T cells and APCs. The task of these molecules is to strengthen the activation signals from APCs to T cells and fine-tune signals mediated through TCR (Glimcher & Murphy 2000, Chen & Shi 2006). The CD28 molecule is expressed on naïve and activated CD4+ T cells, and it has been shown to be important for Th cell priming (Gonzalo *et al*. 2001). CD28, and its partial homologue CTLA-4, bind to B7-1 (CD80, CD86) ligands on APCs. CTLA-4 (Cytolytic T lymphocyte-associated antigen 4), being present on activated T cells, is an inhibitory cell surface receptor of the CD28 family which down-regulates T cell responses, inhibits further T cell activation and blocks IL-12 signalling (Chen &

Shi 2006). ICOS, sharing about 20% homology with CD28, is up-regulated after T cell activation, being also expressed on many memory T cells. The effects of ICOS are more linked to the regulation of Th2 effector responses than to differentiation (Hutloff 1999, Gonzalo *et al.* 2001, Tesciuba 2001, Dong 2001, Tafuri 2001, Chen & Shi 2006). Interestingly, asthmatic patients have been observed to have elevated concentrations of CD28 or CTLA-4 ligand B7 showing the importance of the accessory molecules in the development of inappropriate functions of Th1/Th2 cells (Hofer *et al.* 1998). Costimulatory molecules also play a role in autoimmunity; for instance, disruption of CD28/B7 interactions was shown to associate with a reduction in the severity of the autoimmune disease (Anderson *et al*. 1999).

1.3 Key cytokines and transcription factors regulating Th2 cell commitment

1.3.1 IL-4-mediated biological processes

A number of factors have been linked to Th2 cell development (Mowen & Glimcher 2004). However, IL-4 has a central role in the regulation of immune response having pleiotrophic effects on a variety of cells in the immune system. IL-4 is the key cytokine, priming Th lymphocytes towards IL-4 producing Th2 cells (Swain *et al*. 1990, Kuhn *et al*. 1991, Seder *et al*. 1992, Kopf *et al*. 1993, Paul & Seder 1994, Seder & Paul 1994, Noben-Trauth *et al*. 1997). IL-4 mediates its effects specifically through its receptor (IL4-R) which is known to be expressed on naïve CD4+ T cells and upregulated by IL-4 (Nelms *et al*. 1999). IL-4 is produced mainly by the activated CD4+ and CD8+ T cells (Th2, Tc2) and also some non-T cells such as basophils, eosinophils, mast cells and NKT cells, which produce small amounts of IL-4. However, it is still not clear which cells provide the early source of IL-4 required for the initiation of Th2 commitment *in vivo* (Brown *et al.* 1987, Moqbel *et al*. 1995, Yoshimoto *et al*. 1995, von der Weid *et al*. 1996, Launois *et al*. 1997, Noben–Trauth *et al*. 2000, Akbari *et al.* 2003, Mowen & Glimcher 2004). Naïve CD4+ T cells produce less IL-4 when compared to differentiated Th2 cells but they may still undergo Th2 differentiation without exogenous IL-4 upon continuous TCR stimulation, thus generating a positive feedback loop (Croft & Swain 1995, Demeure *et al*. 1995, Noben–Trauth *et al*. 2000, 2002).

IL-4 is also a central mediator of allergic inflammation by regulating antibody isotype class-switching of B cells to immunoglobulin IgE and IgG1 producing cells (Swain *et al.* 1990, Nelms *et al*. 1999). IL-4-deficient mice do not tend to develop an allergic inflammatory response after airway challenge (Corry *et al*. 1996, Herrick & Bottomly 2003). IL-4 induces chemokines too, which are essential for maintaining the Th2 response by recruiting other Th2 cells *in situ* (Mackay 2001). Besides being crucial cytokine for Th2 differentiation, IL-4 also powerfully suppresses Th1 differentiation by inhibiting the generation of IFNγ-producing cells (Nelms *et al*. 1999).

The IL-4 receptor is heterodimeric. There are two types of IL-4R, of which the type I receptor is composed of the IL-4 receptor α chain (IL-4Rα) and the γ_c chain, the latter chain also being shared with other cytokine receptors (IL-2, IL-7, IL-9, IL-15, IL-21).

The IL-4Rα is able to bind IL-4 with high affinity (Galizzi *et al*. 1990). The type II receptor is formed of the IL-4Rα chain associating with the IL-13Rα chain (Schindler & Darnell 1995). The type I receptor is expressed on haematopoietic and the type II receptor mainly in nonhaematopoietic cells (Keegan *et al*. 1994, Murata *et al*. 1998, Nelms *et al*. 1999, Mowen & Glimcher 2004). IL-4 mediates its functions via two pathways, acting through two cytoplasmic proteins, Signal transducer and activator of transcription 6 (STAT6) and IL4-induced phosphotyrosine substrate/insulin receptor substrate 2 (4PS/IRS2) acting upon IL-4R stimulation, of which the IL-4/STAT6 pathway is better known for IL-4-mediated responses (Keegan *et al*. 1994, Hou *et al*. 1994, Nelms *et al*. 1999). STAT6 also mediates IL-13-induced signals (Murata *et al*. 1996).

1.3.2 IL-4/STAT6 signalling

STAT6 structure and activation of transcription

Specific activation of the transcription factor STAT6 upon stimulus through IL-4R is crucial for a number of IL-4-mediated effects, including induction of Th2 and IgE responses, the gene expression regulative events, and antigen-induced airway inflammation and hyperresponsiveness (Kotanides & Reich 1993, Kaplan *et al*. 1996, Shimoda *et al*. 1996, Akimoto *et al*. 1998, Kuperman *et al*. 1998, Hebenstreit *et al*. 2006). STAT6 belongs to the STAT family, consisting of seven mammalian members (STAT1, 2, 3, 4, 5a 5b and 6) which are specifically activated by cytokines (Hou *et al*. 1994, Schindler & Darnell 1995, O´Shea *et al*. 2002). The domain structure of STAT proteins is conserved (Figure 2). STAT6 is expressed in both Th1 and Th2 cells, but its full activation occurs predominantly in Th2 cells (Huang *et al*. 1998). In the quiescent stage, STAT6 proteins remain latent in the cell cytoplasm as monomers. Upon IL-4R activation, STAT6 is rapidly (within minutes) recruited to the receptor complex, where it binds to a receptor via its SH2 domains and is phosphorylated on a specific tyrosine residue by intracellular, receptor-associated Janus tyrosine kinases 1 and 3 (JAK 1, 3) (Kotanides & Reich 1993, Hou *et al*. 1994, Schindler *et al*. 1994) (Figure 3). JAKs phosphorylate each other in addition to the phospho-tyrosine residues of the IL-4Rα chain generating a docking site for STAT6 (Schindler & Darnell 1995, O´Shea *et al*. 2002, Hebenstreit *et al*. 2006). Activated STAT6 is released from the receptor, dimerized through its SH2 domain and rapidly translocated into the nucleus in order to activate transcription of IL-4-responsive genes (Seidel *et al*. 1995, Leonard & O´Shea 1998). STAT6 binds specifically to a GAS-like (γ activated sequence) palindromic consensus element (5´-TTC$(N)_4$GAA-3´) (O´Shea *et al*. 2002). STAT6 binding sites have been found in the promoter of several IL-4-responsive genes, such as IL-4R, CD23, class II MHC, P selectin, eotaxins 1 and 3 and GATA3 (Kaplan *et al*. 1996, Kotanides & Reich 1996, Lederer *et al*. 1996, Takeda 1996, Curiel *et al*. 1997, Park *et al*. 1997, Georas *et al*. 1998, Ouyang *et al*. 1998, Khew-Goodall *et al*. 1999, Kurata *et al*. 1999, Matsukura *et al.* 1999, Hoeck *et al*. 2001). STAT6 also activates genes involved with the allergen-specific IgE production, such as the germline epsilon promoter (Ig germline ε) (Messner *et al*. 1997, Linehan *et al.* 1998). Recently, numerous novel STAT6 target genes have been identified in B and T cells using

microarrays (Schroder *et al*. 2002, Chen *et al*. 2003). STAT6 can positively or negatively regulate gene expression, such as expression of many of the allergen-induced chemokine genes (Fulkerson *et al*. 2004).

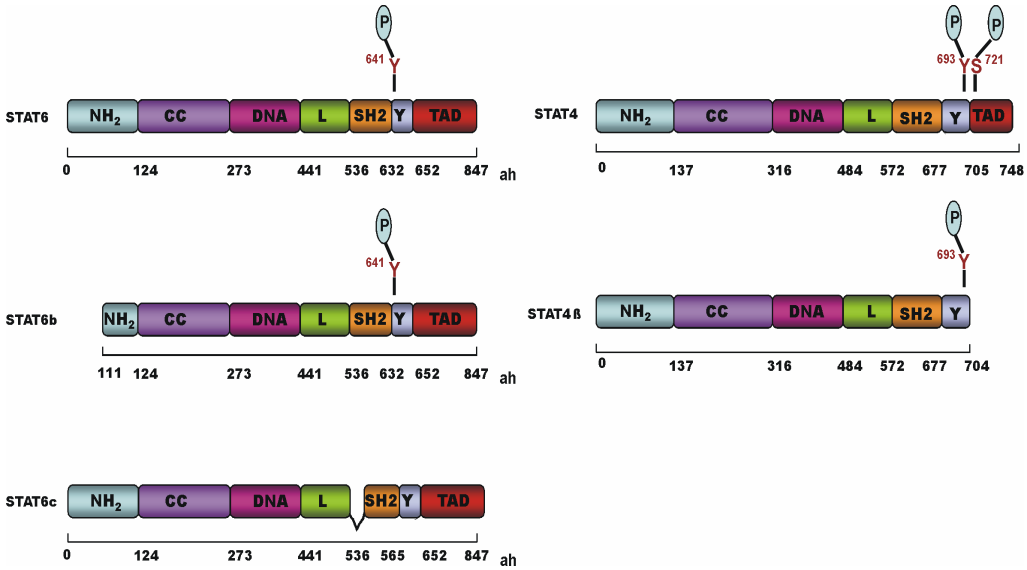


Figure 2. The structure of human STAT6 (110 kDa) and STAT4 (89 kDa) proteins and their functional domains. All STATs (1-6) share the same conserved overall structure including the following domains: N-terminal (NH_2), coiled-coil (cc), DNA-binding (DNA)**,** linker (L)**,** SH2, and C-terminal/Transactivation (TAD) domain. The NH2 domain is shown to be responsible for STAT interactions and dimerization, the coiled-coil for protein interactions and the SH2-domain for dimerization and activation upon phosphorylation. TAD is crucial for STAT-mediated gene transcription, including a conserved single tyrosine residue (Y641 for STAT6; Y693 for STAT4) enabling dimerization and SH2 interactions and specific binding to target genes. Two naturally occurring splice variants of STAT6 (STAT6b and c, 95 and 102 kDa) and one for STAT4 (STAT4β, 83 kDa) exist. STATb has an N-terminal truncation, whereas STAT6c contains a deletion in its SH2 domain, thus failing to be tyrosine phosphorylated upon IL-4 stimulation. STAT4β is shorter than WT STAT4 at the C terminus. All STATs except for STAT2 and STAT6 contain also a conserved phosphor-Ser residue in the TAD domain. (Modified from Kisseleva *et al*. 2002, O´Shea *et al*. 2002, Hebenstreit *et al*. 2006, Lim & Cao 2006)

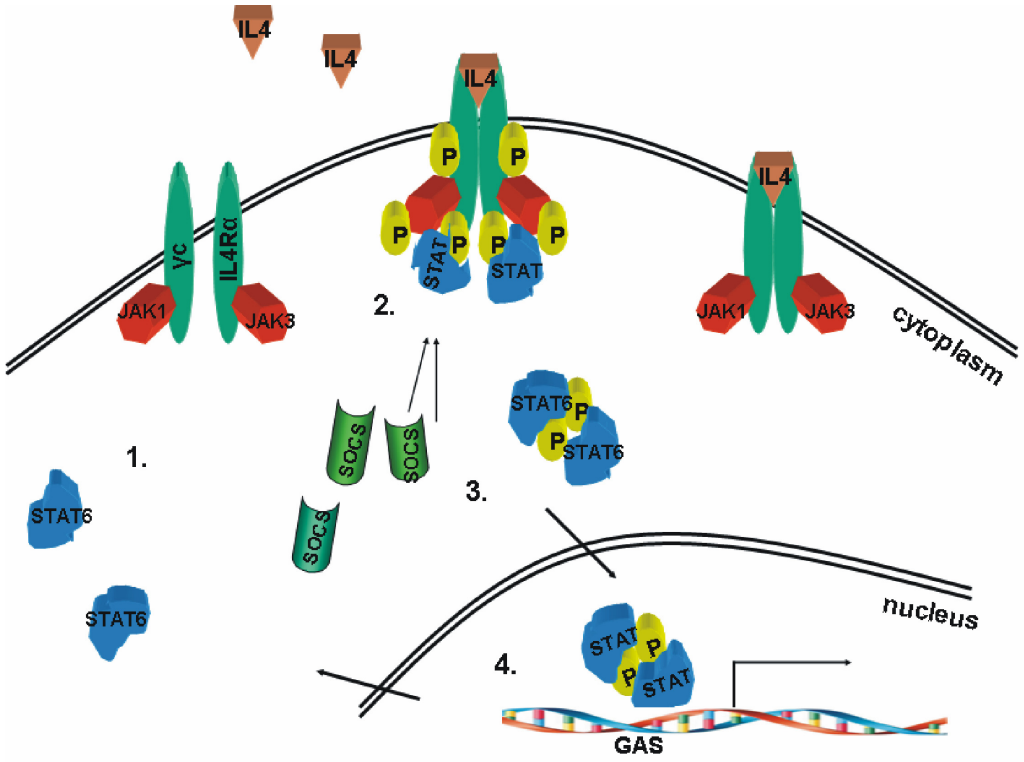


Figure 3. IL-4/STAT6 signalling route. Binding of IL-4 to its specific receptor, composed of IL4Rα and common γ chain (γc), activates the JAK/STAT signalling cascade. When unactivated, STAT6 proteins remain latent in the cytoplasm (1). Activation initiates the functions of JAK1 and JAK3 kinases, which phosphorylate the cytoplasmic domains of IL4R. STAT6 proteins migrate to the IL-4 receptor and become phosphorylated by JAKs (2). Phosphorylated STAT6 is able to dimerize and translocate to the nucleus (3), where it activates its specific target genes by binding to specific promoter sequences (GAS element) (4).

STAT6 in Th2 differentiation and disease pathogenesis

STAT6 also mediates functions of the IL-4-related cytokine IL-13, which activates the IL-4R complex in addition to cytokine IL-4. This is shown by STAT6-deficient mice, which display impaired IL-13 responses (Takeda *et al*. 1996, Chomarat *et al*. 1998, Nelms *et al*. 1999). IL-13 has also been shown to induce the production of IgE, the major mediator molecule of allergic responses, and the activation and functions of STAT6 are closely linked to IL-4 and IL-13-mediated disorders such as allergy (Punnonen *et al*. 1993, Emson *et al*. 1998). IL-13 also mediates Th2 responses independently of IL-4 (Wills-Karp 1998). IL-4-, IL-4R- or STAT6-deficient mice fail to develop proper Th2 responses, thus demonstrating the importance of IL-4R/STAT6 signalling for normal Th2 differentiation and functions (Kuhn *et al*. 1991, Kopf *et al*. 1993, Noben-Trauth *et al*. 1997, Zhu *et al*. 2001). IL-2/STAT5-mediated signals have been shown to have a role in Th2 development by stabilizing IL-4 expression, and STAT5a seems to work in the absence of STAT6 in Th2 differentiation and allergic

airway inflammation (Zhu *et al*. 2003, Cote-Sierra *et al*. 2004, Takatori *et al*. 2005). STAT6$^{-/-}$ mice show impaired Th2 effector cell development and immune responses, such as IL4-mediated IgG1 and IgE class switch recombination by B cells (Kaplan *et al*. 1996, Shimoda *et al.* 1996, Takeda *et al*. 1996). In addition to being an important promoter of Th2 functions, STAT6 also downregulates Th1 functions. Ectopic expression of STAT6 results in production of Th2-specific cytokines in Th1 cells and suppression of IFNγ production (Kurata *et al*. 1999). IFNγ has been shown to inhibit STAT6 signalling (Heller *et al*. 2004). In addition to IL-4 and IL-13, alternative pathways to activate STAT6 also exist (Hebenstreit *et al*. 2006).

STAT6 plays an essential role in the pathogenesis of asthma by regulating airway eosinophilia, allergic airway inflammation and Th2 cytokine and chemokine production (Akimoto *et al*. 1998, Kuperman *et al*. 1998, Mathew *et al*. 2001, Trifilieff *et al*. 2000). STAT6 functions have been associated with human immune-mediated diseases, and differential STAT6 expression in people with and without allergy has been observed (Ghaffar *et al*. 2000, Christodoulopoulos *et al*. 2001, Mullings *et al*. 2001, Skinnider *et al*. 2002). Dysregulation of STAT pathways may cause allergic inflammation (Sampath *et al*. 1999). STAT6 functions have also been implicated in cancer: STAT proteins have been linked to tumorigenesis and cancer progression and the STAT6 pathway has been activated in some tumours. In short hairpin RNA (shRNA) experiments, STAT6 has been suggested to inhibit apoptosis of human colon cancer cells (Zhang *et al*. 2006). IL4/STAT6 signals have also been shown to enhance activation-induced cell death in T cells (Zhang *et al*. 2003). Small changes in the *STAT6* gene can result in hyperactivation and constitutive expression of STAT6 and STAT6-dependent genes, thus causing a possible genetic predisposition for atopic diseases (Daniel *et al*. 2000).

STAT6 interactions

The detailed mechanisms of STAT6-activated regulation of transcription are not fully known. Direct and indirect interactions with other co-operative transcriptional activators are usually needed for sufficient activation of gene transcription, and STAT6 also interacts with transcriptional co-activators with its TAD domain (Goenka *et al*. 2003). CBP and p300 have been shown to interact and to form transcriptional complex with STAT6 (McDonald & Reich 1999, Välineva *et al*. 2005). In addition, other interaction partners of STAT6 are known (Hebenstreit *et al*. 2006). Recently, an unphosphorylated form of STAT6 was also shown to be able to localize in the nucleus and to regulate gene expression (Cui *et al*. 2007).

Negative regulation of STAT proteins

Upon activation, STAT6 is rapidly translocated into the nucleus and after termination of the signal translocated back to the cytoplasm (Kisselewa *et al*. 2002). The kinetics of STAT6 deactivation is also rapid, STAT6 half-life in the nucleus being less than one hour (Andrews *et al*. 2002). Negative feedback mechanisms are needed for STAT6 inactivation. Suppressors of cytokine signalling (SOCS) are phosphatases, playing an important role in the rapid inactivation of STAT-mediated cytokine signals. There are eight family members of SOCSs, including SOCS-1 – SOCS-7 as well as the cytokine-

inducible SH2-containing protein CIS. SOCS proteins inhibit the functions of different STAT members through negative feedback mechanisms, such as suppressing JAK activity or competing with STAT for receptor docking sites (Matsumoto *et al*. 1997, Nelms *et al*. 1999, Alexander *et al*. 2004, O´Sullivan *et al*. 2007). These negative regulators of cytokine signalling also play an important role in Th2-mediated allergic responses by controlling the Th1/Th2 balance. The importance of SOCSs in the control of immune responses has been shown in reports where inadequate SOCS expression has lead to autoimmune diseases (Fujimoto *et al*. 2004). In addition to SOCSs, there are also other pathways leading to inhibition of IL4/STAT6 signalling, such as PIAS and the TNFα/NF-kB pathway (Nelson *et al*. 2002, O´Shea *et al*. 2002, Hebenstreit *et al*. 2006).

However, even though STAT6-mediated signalling is critical for a sufficient number of Th2 effector cells, STAT6 seems not to be absolutely required for Th2 cell development, and STAT6-independent pathways for generation of IL-4 secreting Th2 cells have been shown to exist (Kaplan *et al*. 1999, Jankovic *et al*. 2000). It is known that another crucial transcription factor for Th2 differentiation, GATA-binding protein 3 (GATA3), has a major role in mediating Th2 differentiation signals. IL-4 up-regulates activation of GATA3 via STAT6 (Zheng & Flavell 1997, Lee *et al.* 1998, Ouyang *et al*. 1998). However, autoactivation of GATA3 is one of the major factors of alternative, IL4/STAT6-independent Th2 differentiation responses (Kaplan *et al.* 1999, Ouyang *et al.* 2000).

1.3.3 GATA3 and other important transcription factors

The role of GATA3 in Th2 differentiation

The Th2 cell-specific transcription factor GATA3 was first cloned in 1991 (Ho *et al*. 1991). It belongs to a highly conserved GATA zinc-finger superfamily of DNA-binding proteins, of which only GATA3 is expressed in T cells (Ting *et al*. 1996, Lee *et al*. 2000, Ho & Pai 2007). GATA family proteins contain two highly conserved zinc fingers which can bind DNA containing a consensus sequence (WGATAR) (Merika *et al*. 1993). GATA3 is expressed at low levels in naïve primary CD4+ T cells and is highly up-regulated after TCR activation. GATA3 is predominantly expressed in Th2 cells (Zhang *et al*. 1997, Ray & Cohn 1999, Yagi *et al.* 2002, Hernandez-Hoyos *et al*. 2003). The functions of GATA3 seem to be absolutely required for CD4+ Th2 cell lineage switch and maintenance, and thus it has been reported to be the master controller of Th2 cell differentiation (Zheng & Flavell 1997, Ouyang *et al*. 1998, Ferber *et al*. 1999, Kurata *et al*. 1999, Ho & Pai 2007). GATA3 functions and interactions with DNA and proteins have shown that GATA3 has an essential role in many stages of T cell development (Ho & Pai 2007). It has been demonstrated to be necessary in developmental pathways, for instance in vertebrate embryonic survival, since GATA3 deficiency is lethal, causing abnormalities in the central nervous and haematopoietic system (Pandolfi *et al*. 1995). GATA3 is also absolutely required for the early stage of T cell lineage development, thymopoiesis and Th2 cell development (Pai 2003, 2004, Zhu *et al*. 2004). Using targeted disruption of the GATA3 gene, a complete developmental block occurring early in T cell development was seen in

chimeric mice, and the development of thymocytes was arrested at the double negative (DN) stage (Ting *et al*. 1996). GATA3 is one of the known downstream target genes of STAT6, and is rapidly induced by the IL-4/STAT6 pathway. However, it can also autoactivate its own expression in a STAT6-independent manner. GATA3 has been shown to direct gene expression and Th2 cytokine production and even fully reconstitute Th2 cell development in $STAT6^{-/-}$ mice (Ouyang *et al*. 2000, Farrar 2001, Zhou *et al*. 2001). Thus, GATA3 may be both a necessary and sufficient inducer of Th2 responses (Zheng & Flavell 1997).

GATA3 in asthma and allergic diseases

In atopic patients, GATA3 expression has been shown to be upregulated (Nakamura *et al*. 1999). Human patients with only one functional copy of GATA3 have lower levels of GATA3 protein as well as deficiencies in Th2 responses and IgE levels (Skapenko *et al*. 2004). Studies with the DN form of GATA3 show reduced Th2 cytokine expression, resistence to allergic asthma, inhibited pulmonary allergic responses, decreased levels of Th2 cytokines and impaired Th2 differentiation (Zheng & Flavell 1997, Zhang *et al*. 1999). Experiments with GATA3 antisense oligos, siRNA knockdown or a DN mutant of GATA3 in mice also show diminished Th2 cytokine (IL-4, IL-5, IL-13) production and allergic asthma responses, which suggest the importance of GATA3 in allergic inflammation and hyperresponsiveness (Zhang *et al*. 1998, 1999, Finotto *et al*. 2001). Furthermore, human haploinsufficiencies of *GATA3* has been reported to result in the hypoparathyroidism, deafness and renal dysplasia (HDR) syndrome (Van Esch *et al*. 2000). The syndrome is inherited in an autosomal dominant manner (Bilous *et al*. 1992) and shows a wide phenotypic spectrum. Using a deletion-mapping approach, Van Esch *et al*. (2000) defined a 200 kilobase region containing the *GATA3* gene, which is critical for the disease (Van Esch *et al*. 2000). Different mutations in the gene have been identified as affecting the zinc finger domain functions of GATA3, thus resulting in a loss of DNA binding of the gene or disrupting its interaction with other transcription factors, such as FOG2 (Van Esch & Devriendt 2001, Nesbit *et al*. 2004, Zahier *et al*. 2005).

GATA3-induced epigenetic changes during Th cell differentiation via chromatin modelling

Upon naïve T cell activation, GATA3 translocates rapidly into the nucleus and induces a switch for Th2 cell differentiation through chromatin conformation changes by binding to functional GATA3 binding promoter elements (Takemoto *et al*. 2000, Seki *et al*. 2004, Maneechotesuwan *et al*. 2007). By controlling chromatin remodelling of Th2 cytokine loci, which contain promoters for IL-4, IL-5 and IL-13 genes, GATA3 seems to be a crucial inducer of Th2 differentiation, and a regulator of Th cell phenotype maintenance (Zhang *et al*. 1997, Zheng *et al*. 1997, Lee *et al*. 2000, Ouyang *et al*. 2000, Takemoto *et al*. 2000, Lee *et al*. 2001, Takemoto *et al*. 2002, Lavenu-Bombled *et al*. 2002, Yamashita *et al*. 2004, Ansel *et al*. 2006). Loss of GATA3 expression resulted in decreased Th2 cytokine production and reduction of histone hyperacetylation of the IL-5 locus as well as increased DNA methylation of the IL-4 locus (Yamashita *et al*. 2004). Nuclear translocation of GATA3 from the cytoplasm has been reported to be dependent on its serine phosphorylation by p38 MAPK and direct interaction with the nuclear protein importin-α (Maneechotesuwan *et al*. 2007).

GATA3 regulates cytokine gene transcription by binding directly to the promoter of the IL-5 gene, IL-4 locus regulation probably happening via an indirect effect (Zhang *et al*. 1997, Zhang *et al*. 1998, Lee *et al*. 1998, Takemoto *et al.* 2000, Schwenger *et al*. 2001). IL-4 and IL-13 loci have been reported to have GATA3 enhancer activities (Ranganath *et al*. 1998). In addition to the Th2-promoting role of GATA3, it has also been reported to repress the expression of Th1 cytokines and transcription factors and thus negatively regulate the development of the counter cell lineage Th1. GATA3 regulates Th1/Th2 balance by down-regulating the expression of IFNγ and IL-12 and the Th1-promoting transcription factor STAT4 through IL-12R (Ouyang *et al*. 1998, Ferber *et al*. 1999, Lee *et al*. 2000, Usui *et al*. 2003). In addition, IL-12 has been shown to repress GATA3 (Ouyang *et al*. 1998). In retroviral overexpression studies, GATA3 has been shown to induce Th2 cytokines and to repress the Th1 cytokine expression in polarized Th1 cells (Zheng & Flavell 1997, Ouyang *et al*. 1998, Lee *et al*. 2000).

GATA3 and T-bet interactions

GATA3 and its Th1-specific counterpart T-bet (described in Chapter 1.4.2), regulate their target genes both transcriptionally and epigenetically, thereby promoting the differentiation and effector functions of Th2 and Th1 cells, respectively. In addition to functioning in the positive feedback loops autoactivating and stabilizing their "own" lineage commitment, these transcription factors also inhibit each other´s function (Ansel *et al*. 2003). Thus, interaction of these transcription factors is thought to regulate the balance of Th1/Th2 cell responses (Kiwamoto *et al*. 2006). Recently, T-bet and GATA3 were shown to interact during Th1 differentiation via a Tec kinase Itk that interferes with the DNA binding of GATA3 by phosphorylating T-bet. Itk itself does not affect IFNγ production and is also needed for GATA3 induction and Th2 development (Miller *et al*. 2004, Hwang *et al*. 2005). GATA3 and T-bet may be essential for the initial establishment but not necessarily for the maintenance of cytokine gene activity.

Besides T-bet, GATA3 is known to interact at least with some nuclear proteins expressed in Th cells, such as ROG (repressor of GATA3), FOG-1 (Friend of GATA), Smad3 and PU.1 (Ho & Pai 2007). Both ROG and FOG-1 are shown to repress GATA3 activity (Miaw *et al*. 2000, Zhou *et al*. 2001, Kurata *et al*. 2002). So far, detailed information about the downstream targets of GATA3 in T cells is unknown (Ho & Pai 2007).

c-Maf

Proto-oncogene and Th2-specific transcription factor c-maf belongs to a group of b-ZIP (basic-region leucine zipper) transcription factors which mediate DNA binding and protein interactions. It has been shown to be critical for maximal IL-4 transcription by transactivating the IL-4 promoter; however, it does not have the transactivating effect upon the IL-5 or IL-13 promoters that GATA3 has (Ho *et al*. 1996, Blank *et al*. 1997). Being induced by IL-4, c-maf is expressed at low levels in naïve cells, selectively expressed in differentiating and mature Th2 cells, and is absent from Th1 cells (Ho *et al*. 1996, Lee *et al*. 2000). When overexpressed, c-maf has been observed to skew Th1 cells into the Th2 type, with increased s-IgG1 and s-IgE levels (Ho *et al*. 1998). The

exact role of c-maf in Th2 differentiation is as yet largely unclear, but it seems to have an important role in IL-4 production after Th2 cell commitment (Ho *et al*. 1996, O´Garra 1998). c-Maf has also been demonstrated to interact with the Th2-promoting transcription factor JunB *in vitro,* thereby enhancing the IL-4 promoter activity (Li *et al*. 1999, Hartenstein *et al*. 2002). c-Maf$^{-/-}$ mice have impaired *in vivo* Th2 responses and defects in IL-4 production; however, other Th2 cytokines are produced at normal levels (Kim *et al*. 1999). Thus, c-maf seems to be required for IL-4 expression in addition to GATA3, and it has also been shown to repress the Th1 pathway together with GATA3 (Ho *et al*. 1998, Ouyang 1998).

NFAT proteins

The NFAT (the nuclear factor of activated T cells) family consists of 4 members, NFATc1 (or NFATc/NFAT2), NFATc2 (or NFATp/NFAT1), NFATc3 (or NFAT4/NFATx) and NFATc4 (Szabo *et al*. 2002). Being activated by intracellular calcium influxes, NFAT members translocate into the cell nucleus and regulate the expression of various cytokines, such as IL-2 and IL-4 (Szabo *et al*. 1993, Northrop *et al*. 1994, Rao 1994, Rooney *et al*. 1995). NFAT proteins are not selectively expressed in Th1 and Th2 cells and thus have an important role in Th1 and Th2 cell cytokine production (Yoshida *et al*. 1998, Rengarajan *et al*. 2002). NFAT members also interact with many transcriptional regulators and co-factors, such as AP-1 proteins (e.g. c-Jun, JunB and JunD) and IRF-4 (Interferon regulatory factor 4) (Agnello *et al*. 2003).

Bcl-6

BCL-6, a zinc finger protein, is a transcriptional repressor of Th2 acting through inhibition of STAT6 by competing for its DNA binding sites (Dent *et al*. 1997). Bcl-6$^{-/-}$ mice have enhanced Th2 cytokine responses, and they suffer from Th2-type inflammation. The task of Bcl-6 may be controlling Th cell differentiation in an IL-4/STAT6-independent manner (Dent 1998). Moreover, it has been shown to post-translationally repress GATA-3 protein levels in T cells, independently of STAT6 (Kusam *et al*. 2003).

1.4 Th1 cell commitment – cytokine signalling and key transcription factors

1.4.1 IL-12/STAT4 and IFNγ/STAT1 signalling

Molecular mechanisms regulating Th1 differentiation including different cytokines, costimulatory molecules, transcriptional regulators and different pathways have been thoroughly reviewed by Szabo *et al*. (2003). For Th1 cell commitment, both IFNγ/STAT1 as well as IL-12/STAT4 signalling routes are relevant (Zhang *et al*. 2001). The immunoregulatory cytokine IL-12, mainly produced by the activated APCs, macrophages and DCs (Macatonia *et al.* 1993, Trinchieri 1998), is crucial in promoting Th1 cell fate by being the major inducer of pleiotropic cytokine IFNγ. IL-12 directs Th1 development via the IL-12R/STAT4 pathway. Transcription factor STAT4 also belongs to the STAT family (described in the Chapter 1.3.2) and is an important mediator of IL-12 signalling (Jacobson *et al*. 1995, Wurster *et al*. J. 2000, O´Shea *et al*. 2002). The IL-12/STAT4 route is activated via JAK/STAT pathway, as described in

the IL-4/STAT6 signalling section (Chapter 1.3.2), and JAK kinases Jak2 and Tyk2 mediate activation through IL-12R (Bacon *et al*. 1995, Leonard & O´Shea 1998). STAT4 is also required for expression of IL-12R and IL-18R on Th1 cells (Lawless *et al*. 2000). Naïve T cells lack the IL-12 receptor, which is composed of the IL-12Rβ1 and β2 chains activated by TCR signals (Presky *et al*. 1996). In developing Th2 cells, expression of IL-12R β2 is rapidly lost due to down-regulation by IL-4 (Szabo *et al*. 1997). The requirement of IL-12/STAT4 signalling in the development of Th1 cells has been shown by KO mouse studies where both $IL12^{-/-}$, $IL12R^{-/-}$ or $STAT4^{-/-}$ mice had markedly diminished IL-12-induced Th1 responses, such as IFNγ production, enhanced Th2 differentiation and impaired cell-mediated immunity (Kaplan *et al*. 1996, Magramm *et al*. 1996, Thierfelder *et al*. 1996, Wu *et al*. 1997). $STAT4^{-/-}$ mice also had decreased airway hyperreactivity and peribronchial eosinophils, and they were resistant to certain autoimmune diseases (Chitnis *et al*. 2001, Raman *et al*. 2003). Furthermore, while promoting Th1 type cell development, STAT4 negatively regulates Th2 cell differentiation (Ouyang *et al*. 1998, Murphy & Reiner 2000). STAT4-independent pathways for IFNγ production exist, which has been shown by $STAT4^{-/-}$ $STAT6^{-/-}$ double negative mice (Thierfelder *et al.* 1996, Kaplan *et al*. 1998, Ouyang *et al*. 1999). In human cells, in contrast to mouse cells, cytokine IFNα has also been shown to induce STAT4 and Th1 differentiation (Cho *et al*. 1996, Rogge 1998).

IFNγ induces a variety of immune responses in different cells functioning in the adaptive or innate immune system. It is produced predominantly by Th1, CD8+ and NK cells, and it is crucial for Th1 response (Scott 1991, Scharton & Scott 1993, Lighvani *et al*. 2001, Szabo *et al*. 2003, Laouar *et al*. 2005). IFNγ mediates its effects through IFNγR, being expressed on many lymphoid and nonlymphoid cells, as well as Jak1 and Jak2 kinases and the STAT1 transcription factor (Bach *et al*. 1997, Boehm *et al*. 1997, O´Shea 2002, Szabo *et al.* 2003). When produced, IFNγ directs STAT1-dependent formation of IL-12R and IL-12 signals to the cells. It also functions in a positive feedback loop by inducing its own expression by activating STAT1-mediated T-bet expression, which is in turn negatively regulated by IL4/STAT6 (Nelms *et al*. 1999, Kurata *et al*. 1999, Lighvani *et al*. 2001, Afkarian *et al*. 2002, Szabo *et al*. 2003).

Th1 cytokines IL-12 and IFNγ are able to antagonize the development of Th2 cells by inhibiting the effects of IL-4 and IL-13 and thereby also allergic responses caused by Th2 cell effects (Lack *et al*. 1996, Chung 2001, Leonard & Sur 2003, Wen *et al*. 2003). The absence or low expression of IFNγ or transcription factor T-bet (discussed below) may result in Th2 differentiation (Wang *et al*. 1994, Finotto *et al*. 2002).

1.4.2 T-bet transcription factor

Th1-specific transcription factor T-bet (T-box expressed in T cells), which belongs to a family of T-box transcription factors, is rapidly induced upon TCR activation and during Th1 differentiation by IFNγ/STAT1 signalling, thereby driving Th1 differentiation (Szabo *et al*. 2000, Lighvani 2001, Afkarian 2002). T-bet is also known to be induced independently of IL-12/STAT4 (Mullen *et al*. 2001), and, in addition, both IL-12 and IFNα have been shown to up-regulate T-bet independently of IFNγ in

human CD4+ T cells (Ylikoski *et al*. 2005). Besides Th cell differentiation, T-bet also controls the development and functions of NK, DC and CD8+ cells (Glimcher & Murphy 2000, Sullivan *et al*. 2003, Lugo-Villarino *et al*. 2005, Townsend *et al*. 2004). T-bet is a master controller of Th1 development, regulating IFNγ production by directly binding to the IFNγ promoter and simultaneously repressing Th2 development and Th2 cytokine genes and by interacting with GATA3 (Glimcher & Murphy 2000, Szabo *et al*. 2000, 2002, Tong *et al*. 2005). T-bet not only strongly transactivates IFNγ, but is also involved in the induction of IL12Rß2 chain expression, thus generating more IL-12 signals to developing Th1 cells (Afkarian *et al*. 2002, Ansel *et al*. 2006). T-bet promotes remodelling of the IFNγ locus by interacting with a cofactor, Hlx, which is needed for optimal IFNγ locus activation. In addition to T-bet, Hlx too, is expressed in naïve CD4+ T cells, up-regulated during Th1 cell differentiation and down-regulated in Th2 cells. (Mullen *et al*. 2002, Zheng *et al*. 2004, Mikhalkevich *et al*. 2006). Retroviral expression of T-bet has been shown to promote Th1-type cytokine profiles in Th2 cells, too (Szabo *et al*. 2000). T-bet is critical for the normal development of Th1 effector cells *in vitro*. Its absence impairs Th1 differentiation and results in increased predisposition to the development of airway hyperresponsiveness (AHR), production of Th2 cytokines, and multiple features of asthma (Szabo *et al*. 2002, Finotto *et al*. 2002). Asthmatic patients have reduced numbers of T-bet expressing cells in bronchial biopsies (Finotto *et al*. 2002). T-bet deficiency was also seen to be protective of Th1 cell-mediated diseases such as inflammatory bowel disease or autoimmune encephalitis (Neurath *et al*. 2002, Bettelli *et al*. 2004). A genetic association between variations in the *T-bet* gene and airway responsiveness in asthma has been observed (Raby *et al*. 2006). Moreover, a functional pharmacogenetic variant in the *T-bet* gene for airway responsiveness in asthmatic children has been found by Tantisira *et al*. (2004). They showed that the nonsynonymous *T-bet* gene SNP, coding for the replacement of histidine 33 with a glutamine, enhances the effects of inhaled corticosteroid usage on airway hyperresponsiveness (Tantisira *et al*. 2004). However, in Finnish patients with asthma, T-bet polymorphisms were not associated with high s-IgE levels or asthma (Ylikoski *et al*. 2004). Recently, it was shown by Höhler *et al*. (2005) that human T-bet expression was strongly influenced by genetic factors in a twin study, where differences in T-bet production were observed to be strongly genetically determined. In contrast to T-bet, GATA3, NFAT and NF-κB expression did not show such a strong effect. It was also speculated that subtle changes in T-bet expression and Th1 cytokine regulation directed by T-bet can predispose to Th2-associated diseases (Höhler *et al*. 2005).

1.4.3 Other cytokines and transcription factors regulating Th1 development

In addition to the above-mentioned cytokines and transcription factors, a variety of other molecules have also been shown to be involved in the initiation and maintenance of Th1 cell differentiation. These are, for instance, cytokines IL-18, IL-23 and IL-27 as well as transcription factors ERM, NF-kB, P38MAPK and Jun. Cytokine IL-18, produced by various immune and non-immune cells, belongs to the IL-1 family of cytokines (Nakanishi *et al*. 2001). Its receptor, IL-18R, is absent from naïve CD4 T

cells but is induced on Th1 cells (Yoshimoto *et al*. 1998, Szabo *et al*. 2003). IL-18 has a role, though not essential, in the development of Th1 cells by acting in synergy with IL-12 and increasing IFNγ production of differentiated Th1 cells (Robinson *et al*. 1997). Thus, IL-18 is needed for the full IFNγ production in Th1 cells by augmenting the effects of IL-12 (Robinson *et al*. 1997, Szabo *et al*. 2003). In IL-18 deficient cells, Th1 responses (IFNγ production) have been diminished, however, being more severe in IL-18$^{-/-}$ IL-12$^{-/-}$ cells (Takeda *et al*. 1998). IL-18, together with its family member IL-1, mediates its signals through the IRAK/NF-kb/TRAF/MAPK pathway as well as the JNK pathway (Szabo *et al*. 2003). Interestingly, in addition to enhancing Th1 cell responses, IL-18 has also been shown to have the potential to stimulate Th2 differentiation, thus suggesting its functions in the Th cell polarization are complex (Nakanishi *et al*. 2001a, 2001b). In the absence of IL-12, IL-18 enhances Th2 differentiation by stimulating the production of Th2 cytokines IL-4 and IL-13, thus having a role in the development of atopic and allergic responses, such as IgE production (Hoshino *et al*. 2000, Yoshimoto *et al*. 2000, Nakanishi *et al*. 2001a, 2001b). The other cytokines regulating Th1 development are interleukins IL-23 and IL-27, of which IL-27 has been shown to potentiate IFNγ production by synergizing with IL-12. IL-23, interacting with the IL-12Rβ1 subunit, however, seems to have a role at the later stages of Th1 development (Szabo *et al*. 2003). JNK2 and p38 MAPK kinase pathways have also been reported to be required for the differentiation and effector functions of Th1 cells (Yang *et al.* 1998, Rincon *et al.* 1998). The Ets-related molecule ERM is a Th1-specific transcription factor belonging to the Ets family. ERM is induced by IL-12 in a STAT4-dependent manner. ERM has been shown to augment IFNγ production by interacting with other co-factors (Ouyang *et al*. 1999); however, the exact role of ERM in Th1 development has not been shown.

TGFβ

Transforming growth factor beta (TGFβ) is a potent immunosuppressive cytokine which has been shown to be able to inhibit and modulate Th1 and Th2 cell differentiation, proliferation and functions (Li *et al*. 2007). In addition to predominantly being a negative regulator of T cells, TGFβ also promotes the generation and functions of some T cell subsets, such as T regulatory cells (Schmidt & Weber *et al*. 2007). TGFβ has various functions in the immune systems, and it is secreted by a variety of immune cells, including leukocytes such as activated T cells and T regulatory cells (discussed in Chapter 1.6) (Gorelik & Flavel 2002, Laouar *et al*. 2005). TGFβ has been shown to inhibit Th2 differentiation through inhibiting GATA3 expression and IL-4 locus in developing Th2 cells at the transcriptional level (Gorelik *et al*. 2000, Heath *et al*. 2000). Furthermore, TGFβ also blocks Th1 differentiation by silencing the transcription of *T-bet* (Gorelik *et al*. 2002). It also suppresses Th1 development by regulating IFNγ production of NK cells (Laouar *et al*. 2005). TGFβ promotes the differentiation of T regulatory cells and in combination with IL-6 TGFβ promotes the development of a recently-described Th cell subtype, Th17 cells (discussed in Chapter 1.6) (Bettelli *et al*. 2006, Mangan *et al*. 2006, Veldhoen *et al*. 2006, Li *et al*. 2007). Using oligonucleodite arrays, Lund *et al*. (2003b) reported the effects of TGFβ in the early polarization of human Th1 and Th2 cells. Interestingly,

many potential candidate molecules regulating the balance of Th1 and Th2 cell responses were identified (Lund *et al*. 2003b).

1.5 Postdevelopmental control of Th differentiation by epigenetic mechanisms

Epigenetic mechanisms take place in the initiation, reinforcement and maintenance of Th cell differentiation, thereby generating changes into Th-mediated immune responses (Ansel *et al*. 2006). They may also have an effect on disease susceptibility or unbalanced Th1/Th2 effector functions. Therefore, understanding epigenetic functions is crucial for detailed characterization of molecular mechanisms involved in Th1/Th2 differentiation. Cytokine genes become accessible to the relevant transcription factors via dynamic epigenetic changes which remodel chromatin structure by causing changes in DNA methylation or acetylation status (Ansel *et al*. 2003, Sanders 2006). Via these modifications, heritable and stable patterns of cytokine accessibilities are also established, which enables rapid secondary immune responses (Avni *et al*. 2002, Fields *et al*. 2002). Furthermore, various positive and negative feedback loops and distal control regions, such as silencer and enhancer elements, work in co-ordination and control the Th differentiation machinery (Agarwall & Rao 1998, Ansel *et al*. 2006). The detailed epigenetic mechanisms are still mainly unknown, and changes for Th1 cytokine locus (IFNγ locus) in particular are poorly characterized compared to the Th2 (IL-4) locus (Glimcher & Murphy 2000). In both human and mouse genes, coding for the cytokines IL-4, IL-13 and IL-5 are clustered together into one chromosome (5q23-31 in human; chromosome 11 in mouse) (Le Beau *et al*. 1989). The cytokines sharing some common functions, IL-13 and IL-4, are separated by 10 kb in CNS-1, located in the intergenic region (Chomarat *et al*. 1998, Loots *et al*. 2000, Mohrs *et al*. 2001). The Rad50 element is located upstream of IL-13 and IL-4, encoding a ubiquitously expressed DNA repair protein expressed both in naive Th cells, and Th1/Th2 cells. Its 3´ end contains a potent Th2 Locus Control Region (LCR), which includes a cluster of regulatory elements. The LCR contains DNaseI hypersensitive (HS) sites. During Th2 differentiation, HS sites appear over the IL-4 locus during remodelling and they are acetylated, which facilitates access of regulatory enzymes and transcription factors, such as NFAT and AP-1 factors, into the IL-4 locus (Agarwal & Rao 1998, Glimcher & Murphy 2000). During Th2 differentiation, transcription of certain genes is activated in the developing Th2 cells but is in turn silenced in Th1 cells, and vice versa. IFNγ and IL-4 regulate chromatin remodelling (Rengarajan *et al*. 2000). IL-4 becomes demethylated upon activation in the initiation of Th2 differentiation; however, in developing Th1 cells, the IL-4 locus is silenced. In addition, expression of GATA3 is increased during Th2 development, while the IFNγ locus is silenced. (Agarwal & Rao 1998, Ansel *et al*. 2003, 2006). Recently, it was reported that interchromosomal interactions between regulatory elements also take place in naïve CD4+ T cells between Th1 and Th2 specific cytokine genes, such as Th2 cytokine cluster and IFNγ promoter. However, these interactions are lost upon activation (Spliniakis *et al*. 2005).

Epigenetic processes may be regulated by various environmental factors, such as diet, medication or ageing, being crucial for an individual for adaptation to changing environment (Sanders 2006). Due to epigenetic modifications, gene expression

between identical twins with the same genetic code can be different (Martin *et al*. 2005). Recent techniques, such as the Chromatin Immunoprecipitation (ChIP) assay-based genomic array or the chromosome conformation capture assay (3C) and its applications, are valuable in studying the specific transcription factor binding sites as well as chromating interactions in the human genome (Dekker *et al*. 2002, Weinmann 2004, Dostie *et al*. 2006).

1.6 T regulatory and Th17 cells

Th1 and Th2 cells are the best-characterized subtypes of T helper cells differentiated upon selective activation. Over recent years, reports of other T cell subpopulations, such as diverse types of T regulatory cells (Tregs), IL-17 producing Th cells (Th17), follicular Th cells (T_{FH}), TGFβ-producing T_H3 cells (Weiner 2001), and NK1.1+ T cells (Jian & Lechler 2003, Park *et al*. 2005, Vinuesa *et al*. 2005, Wan & Flavell 2006, Woodfolk 2007) have been published. However, it has been speculated that the number of T cell subsets will still be expanding and that additional subsets, such as Th5, Th9, Th13 and Th31, involved in the specific pathologies may also exist (Schmidt-Weber *et al*. 2007).

1.6.1 T regulatory cells

The existence of suppressor T cells (Treg) was first hypothesized a few decades ago (Gershon & Kondo 1970, Gershon 1975) and finally discovered in the 1990´s (Sakaguchi *et al*. 1995). Autoregulatory Treg cells have been widely studied during recent years. Their task seems to be controlling the allergic response, but little is still known about their functions and development. They are, however, considered to be a distinct subset of CD3+ CD4+ T cells. Tregs are comprised of diverse populations, such as peripherally induced or thymically derived Tregs. Tregs can be either naturally occurring (nTregs) or induced by antigens (iTreg) (Reviewed by Izcue *et al*. 2006) and include various subtypes such as CD4+CD25+ Tregs, CD4+CD25- T cells (TR1), T-helper type 3 (TH3), the TR1 memory phenotype, CD4-CD25+DX5+ natural killer T cells (TRNKT), CD4-CD25+CD8+ cytotoxic T cells (TRCTC) and CD4+CD25+ NO-Tregs, the three first types representing the best characterized subclasses (Shevach 2002, Wan & Flavell 2006, Niedbala *et al*. 2007). The CD4+CD25+ Tregs represent approximately 5-10 % of the peripheral CD4+ T cell population (Wan & Flavell 2006). The Treg cells constitutively express CD25 (high affinity IL2Rα chain), and typical molecular markers for the Treg cells are CD3, CD25, CD62L, CD69, BTLA, GITR, ICOS, Neuroplin-1 (Nrp-1), and PD-1. However, the best marker for Treg cells is Forkhead Winged-Helix Transcriptional Factor Box p3 (Foxp3), specifically expressed in murine and human Treg cells and shown to be crucial for their development (Fontenot *et al*. 2003, Hori *et al*. 2003, Wan & Flavell 2006). Defects in the Foxp3 gene have been shown to cause impaired development and function of nTregs as well as autoimmunity both in humans and mice (Wan & Flavell 2006). Cytokines including IL-2, IL-10 and TGFβ have been shown to be important for Treg development. TGFβ has been shown to promote the generation of Tregs, for example, by increasing Foxp3 expression in the cells, whereas IL-2 and IL-10 promote the development and

maintenance of nTreg and Tr1 cells in the periphery (Maloy & Powrie 2005, Wan & Flavell 2006). T regulatory cells are thought to be modulators of Th1 and Th2 balance by suppressing immune functions and controlling excessive inflammation by secreting IL-10 and TGFβ. Treg cells also participate in downregulation of antigen presentation and interact with various types of cells in the immune system (Thompson & Powrie 2004, Wan & Flavell 2006). CD3+CD4+FOXP3+ Tregs act as natural inhibitors of normal immune responses, whereas CD4+ CD25+ Tregs are important in immune system homeostasis, and their defect is associated with various immune disorders (Shi *et al*. 2005). IL-2/STAT5 signalling has also been shown to be important in Treg development and maintenance as well as upregulating FOXP3 in human CD4+CD25+ Tregs (Setoguchi *et al*. 2005, Cohen *et al*. 2006, Wan & Flavell 2006, Zorn *et al*. 2006). With their immunosuppressive functions, Tregs are crucial in balancing Th cell responses and inhibiting various autoimmune and inflammatory diseases (Thompson & Powrie 2004). Thus, by understanding their functions, it is also possible to manipulate CD4+ CD25+ T regulatory cells for therapeutic purposes targeting these immune disorders (Mottet *et al*. 2003, Maloy *et al*. 2005).

1.6.2 Th17 cells

A recently identified new Th cell lineage, IL-17-expressing T cells (Th17), is a distinct and early lineage of effector T cells, differing from Th1 and Th2 cells by its development, effector functions and cytokine production profiles. Transcriptional regulation of the initiation of these cells also seems to differ from Th1/Th2 cells, being independent of T-bet, STAT4, STAT1, STAT6 and GATA3 (Harrington *et al*. 2005, Park *et al*. 2005, Wynn 2005). The functions (IL-17 production) of Th17 cells are thought to be important in autoimmune pathogenesis: IL-17 has been reported to increase inflammation and have harmful effects in autoimmune and autoinflammatory diseases, such as asthma, RA and SLE. IFNγ and type I interferons, as well as IL-4-mediated Th2 development, have been shown to suppress Th17 cell development (Harrington *et al*. 2005, Park *et al*. 2005, Wynn 2005). TGFβ is known to promote development of Th17 cells and suppress IFNγ producing cells (Mangan *et al*. 2006) Interestingly, recently both Treg and Th17 cells were reported to be linked to a reciprocal developmental pathway for the generation of pathogenic T_H17 T cells inducing autoimmunity and protective Foxp3+ Treg cells inhibiting autoimmune-related tissue injury. The pathway is dependent on the production and presence of IL-6 and TGFβ: in the presence of TGFβ alone, CD4+ T cells differentiate into Foxp3 expressing Tregs, whilst the Th17 phenotype results when both TGFβ and IL-6 are present (Bettelli *et al*. 2006, Veldhoen *et al*. 2006). TGFβ has been shown to inhibit the expression of Foxp3 together with the cytokine IL-6. It has also been reported to induce the development of the Th17 lineage (Mangan *et al*. 2006). This is also supported by experiments with TGFβ transgenic mice in which the mice had increased Th17 cell numbers and more severe autoimmune disease (Bettelli *et al*. 2006). In addition, the cytokines IL-21, IL-22 and IL-23 have been reported to have a role in IL-17 production and Th17 maintenance (Harrington *et al*. 2005, Langrish *et al*. 2005, Park *et al*. 2005, Deenick & Tangye 2007). IFNγ inhibits the expression of IL-23R

(Harrington *et al*. 2005). A recent report also showed that IL-2 actually inhibits the development and differentiation of Th17 cells (Laurence *et al*. 2007). The details of Th17 cell maturation and the role of other factors and cytokines in their development still remain to be clarified (Laurence *et al*. 2007). Both the retinoic acid-related orphan receptor γt (RORγt) as well as STAT3 have been shown to be the key transcription factors for Th17 cell generation (Ivanov *et al*. 2006). Interestingly, Chen *et al*. (2007) recently reported the differential regulation of IL-17 and RORγt between humans and mice. Furthermore, according to the study, the differentiation of Th17 cells differs in human and murine systems since human Th17 cells cannot be generated from the naïve CD4+ T cells in the presence of the cytokines IL-6 and TGFβ, shown to represent optimal conditions for murine Th17 cell differentiation. Finally, the differing effects of the cytokine IL-23 in humans and mice were reported in that study.

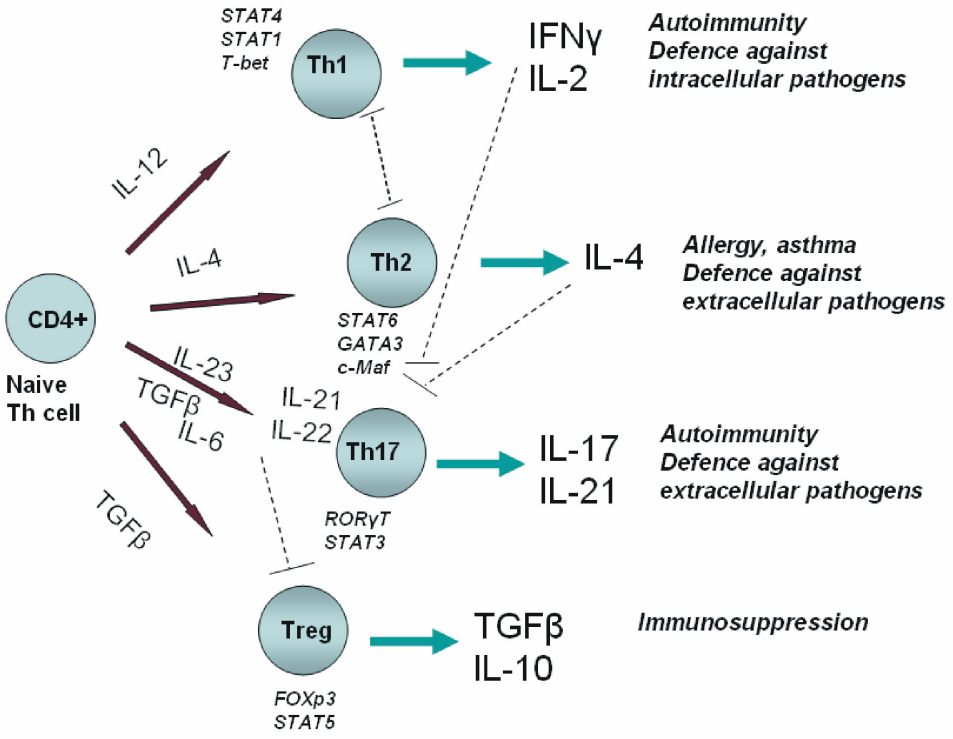


Figure 4. A simplified picture of the differentiation of various types of Th cells and their immune functions. An undifferentiated Th cell can develop towards the Th1, Th2, Th17 or Treg lineages in the presence of polarizing cytokines. IFNγ and IL-4, produced by Th1 and Th2 cells, respectively, can inhibit the development of Th17 cells. Development and maintenance of the different Th cell classes is also dependent on the functions of specific transcription factors. The antagonizing effects between the cells are shown with dashed lines. (Modified from Tato & O´Shea 2006, Deenick & Tangye 2007, Schmidt-Weber *et al*. 2007).

2 Genetics of asthma and atopic diseases

2.1 Overview and prevalence of asthma

Chronic inflammation and hyperreactivity of the airways, mucus hypersecretion and reversible bronchoconstriction in addition to episodes of coughing, wheezing and breathlessness are typical symptoms of the terminal lung disease, asthma. Allergic or immunoglobulin E (IgE)-mediated processes play a crucial role in the immunopathogenesis of asthma, and elevated serum IgE (s-IgE) levels and lymphocyte and eosinophil influx are involved in the disease (Roche *et al*. 1989, Bousquet *et al*. 1990). Total s-IgE has also been suggested to be under genetic control and may predict the development of allergic diseases (Kjellman 1976, Gerrard *et al*. 1978). Based on the disease-triggering factor, asthma can be divided into different types, such as atopic or allergic asthma. Atopy, in turn, is a familial trait characterized by raised s-IgE levels and is strongly linked to asthma being present in the majority of asthma patients (Koh & Irving 2007). In addition to asthma, atopy underlines allergic diseases such as rhinoconjunctivitis and eczema (Yazdanbakhsh *et al*. 2002). Asthma is a highly heterogenous disease with an unknown aetiology and the disease symptoms may vary during one´s lifetime, thus the disease classification is difficult. The immunopathogenesis of asthma involves the interplay of various inflammatory cells and regulatory factors in addition to genetic and non-genetic (environmental) factors tightly involved in the disease susceptibility.

Asthma has become highly prevalent during the last few decades, particularly in Western societies. In Finland, ~6 % of the population are asthmatic (Allergy and Asthma Foundation 2006).

2.2 Th2 cell functions in asthma and allergy

Allergic responses result from an inappropriate immune response caused by harmless environmental pathogens leading to activation and recruitment of various immune cells. Th2 cells are activated and start to secrete specific cytokines, mainly IL-4, IL-5 and IL-13, which activate B cells to produce antibodies (IgE) against the allergen. An allergic reaction is caused when mast cells begin to release histamine and leukotrienes and the immune response is further enhanced by recruiting other immune cells to the site of inflammation. Th2 cytokines, predominantly IL-4, are important in the pathogenesis of allergy and asthma by maintaining and amplifying the allergic response (Robinson *et al*. 1992, Larche *et al*. 2003). Cytokines IL-4 and IL-13 are also produced in large quantities in the asthmatic lung. In addition, the transcriptional activation of certain genes is initiated (Robinson *et al*. 1992, Mosmann & Sad 1996, Drazen *et al*. 1996, Hogan *et al*. 1998, Corry 1999, Ray & Cohn 1999, Webb *et al*. 2000, Wills-Karp 2001).

Both asthma and atopy are biologically associated with unfafourable and pathogenic Th2 cell-driven immune functions potentially caused by unbalanced Th2 differentiation or over-strong effector responses. The Th cell responses of patients

suffering from allergy and atopy were shown to be biased towards Th2, Th1 responses being too low (Singh ym. 1999) (Renauld 2001, Lordan *et al*. 2002). The role of CD4+ Th2 cells in asthma immunopathophysiology has been demonstrated by clinical studies, in which a strong correlation between Th2 cells and asthma severity was observed (Robinson *et al*. 1992, Hogan *et al*. 1998).

Sufficient priming of the immune system to Th1 responses is needed during early life through exposure to environmental pathogens since these may be critical in controlling abnormal Th2 responses, which otherwise possibly cause allergic symptoms in susceptible individuals (Holt 1995). Some factors have been described as inducing either Th1 or Th2 differentiation-favouring cytokines and later responses. Alterations in Th1/Th2 balance can either increase susceptibility to or prevent Th2-mediated immune responses and the tendency to develop allergic diseases (Romagnani 2004). According to the "hygiene hypothesis" theory, early childhoold infections are critical determinants of the outcome of the later Th cell responses (Martinez *et al*. 2001). The theory has been used to explain the explosion of allergy and asthma in urbanized environments. According to the "hygiene hypothesis", the reactions of the immune system are modulated during early life, being dependent on the different environmental and pathogenic stimuli: if the immune stimulus is too weak during the critical developmental period, caused by reduced childhoold infections, this may result in increased risk for allergic diseases (Strachan 1989, von Mutius *et al*. 1994, Romagnani 1994, Yazdanbakhsh *et al*. 2002, Romagnani 2007). However, the "hygiene hypothesis" is still subject to controversy. In addition, the incidence of autoimmune diseases, such as MS, T1D and Crohn´s disease has strongly increased during the past decades, and it has been thought that the disturbed Th1/Th2 dichotomy as such is not a sufficient explanation for the increase of Th1- or Th2-mediated diseases. Recently, the discovery of Treg cell functions has added new insights into the "hygiene hypothesis": the increased prevalance of the diseases could be explained by reduced activity of T regulatory cells and reduced immune suppression rather than by unbalanced Th1/Th2 functions alone. However, further experimental evidence is needed for more conclusive hypotheses and for future therapeutic implications (Yazdanbakhsh *et al*. 2002, Vercelli *et al*. 2004, Romagnani 2004, 2007)

2.3 Asthma genetics

In addition to the environment, susceptibility to asthma or atopy is also strongly dependent on genetic factors: family and twin studies have shown that a genetic effect for asthma and atopy exists and the trait aggregates in families (Hopp *et al*. 1984, Duffy *et al*. 1990). The heritability, also supported by studies using a murine asthma model (Wills-Karp 1997), has been reported to even reach the range of 40-60% and first order relatives also showed a 4-5-fold increase in asthma prevalence (Manian *et al*. 1997, Bossé & Hudson 2007). In a Finnish twin cohort, the asthma heredity was estimated to be ~36 % (Nieminen *et al*. 1991). However, the genetic mechanisms behind the asthma pathogenesis are still largely unknown and thus the pattern for asthma inheritance is difficult to determine. Since multiple genetic and environmental factors are involved in asthma predisposition, the basis of this genetically complex

disease has been complicated to study. Current asthma studies have also included gene-environment interactions in order to reveal detailed mechanisms in the disease pathogenesis (Contopoulos-Ioannidis *et al*. 2007, Martinez 2007).

Numerous genes and factors together contribute to asthma susceptibility and thus no particular "asthma gene" exists. Rather, various different genes contribute to the disease with individually small effects. The genetic component of asthma, as with many other multigenic diseases, has been thought to be caused in part by common DNA variations within genes (Lander 1996, Risch & Merikangas 1996, Chakravarti 1999, Reich & Lander 2001). Several attempts have been made in different ethnic populations to reveal and localize chromosomal regions and genes predisposing to asthma (Bossé & Hudson 2007, Contopoulos-Ioannidis *et al*. 2007). Two traditional methods, genome-wide screens and the candidate gene approach, have been widely used. With the former strategy, the disease-predisposing chromosomal regions are localized by genetic scans using linkage analysis between genomic markers spanning the human genome and asthma-related phenotypes (Bossé & Hudson 2007). The chromosomes and genes identified are positional candidates and are next studied with a denser marker set. In the candidate gene strategy, the genes to be investigated are selected based on their known functions.

2.3.1 Genome-wide screens

Genome-wide screens for asthma and asthma-related traits have been carried out in different study populations resulting in the identification of several (>20) chromosomal areas linked to asthma, atopy or related traits (Daniels *et al.* 1996, Hoffjan & Ober 2002, Hoffjan *et al*. 2003, Wills-Karp & Ewart 2004, Malerba & Pignatti 2005, Morar *et al*. 2006, Ober & Hoffjan 2006, Bossé & Hudson 2007). Some chromosomal areas have been identified in different screens and replicated, thus supporting the contribution of these areas to the genetic susceptibility of asthma. For instance, the chromosomal regions 2q, 5q23-31, 5q31-33, 6p21.3, 7p, 7q, 11q13, 12q14.3-24 and 12q have shown a consistently replicated linkage. Particularly, the loci 5q and 12q have been widely studied since these contain many candidate genes known to be involved in Th cell differentiation and thus have a potential effect on asthma predisposition (Postma *et al*. 1995, Cookson & Moffatt 2000, Laitinen *et al*. 2001, Hoffjan & Ober 2002, Wills-Karp & Ewart 2004, Malerba & Pignatti 2005, Ober & Hoffjan 2006). For instance, locus ch 5q23/5q31 contains a cytokine gene cluster composed of genes coding for *IL-3*, *IL-4*, *IL-5*, *IL-13* and *IL-12β chain* whereas the *IFNγ* and *STAT6* genes are located in 12q (van Leeuwen *et al*. 1989, Marsh *et al*. 1994, Barnes *et al*. 1999). Several meta-analyses have also been performed for a genome-wide screen for asthma, for full genome scans and for the chromosome 5 region. However, no highly significant linkage has yet been found (Contopoulos-Ioannidis *et al*. 2007). In addition, only few individual screens, including the chromosomes 2, 7p, 14q and 20q, have met the criteria of strong linkage (LOD 3.7, $p<2x10^{-5}$), which likely results from the multigenic nature of asthma as well as from the possible lack of adequate statistical power and homogeneous study population (Wills-Karp & Ewart 2004). The disadvantages of the genome-wide screen as an asthmagenetic tool are its

cost and its labour-intensive nature. Results obtained from the screens contain wide chromosomal areas including several genes. Thus, fine mapping with a denser molecular marker map, which is performed using positional cloning, is needed to narrow down the chromosomal area (Wills-Karp & Ewart 2004). A set of genes has been identified from the genome-wide linkage studies and studied further for genetic variation. These are, for instance, the genes located in the 5q, 6p and 12q chromosome regions, such as *IL-4*, *IL-13*, *CD14*, *ADRB2*, *SPINK5*, *LTC4*, *ADRB2*, *TNF*, *TIM1*, *IFNγ*, *ADAM33* as well as genes coding for the human MHC, and the human leucosyte antigen system (HLA) (Ober & Hoffjan 2006).

2.3.2 Asthma candidate gene studies

Candidate gene association analysis has been a common and straightforward way to test whether genetic variation occurs more often in cases than controls and whether it has a role in asthma or atopy predisposition. A certain gene can be selected as a candidate based on either its chromosomal location or function. The advantage of the method is its applicability to both population and family-based samples without prior knowledge of the biological basis of the disease as well as its independence of inheritance models. The cases and controls must be clinically well characterized in a population-based or family-based sample, both of which can be used for the analysis. The advantage of the former is that large population-based sample panels are easier to obtain than family samples. However, in family-based studies, family members share a common genetic background, and families are more homogeneous as a study sample containing less environmental variation (Evangelou *et al*. 2006). Previous studies have shown that significant genetic variants may be missed with too small a sample. Thus, population-based candidate gene analysis may sometimes be more powerful and informative (Hintsanen *et al*. 2006). For understanding the genetic basis of asthma, several candidate gene analyses in different ethnic populations are needed.

Single Nucleotide Polymorphisms

Single Nucleotide Polymorphisms (SNPs) are the most common type of genetic variation (1/400 – 1/1000 bp) in the human genome. They are widely utilized as genetic markers in complex disease candidate gene studies for establishing the role of the studied gene in disease susceptibility (Risch & Merikangas 1996, Collins *et al*. 1997, Wang *et al*. 1998). Since SNPs are publicly available and improved methods for their discovery and genotyping have been developed, they are valuable tools for identification of the genomic variants predisposing to the common disorders and traits, such as asthma and atopy.

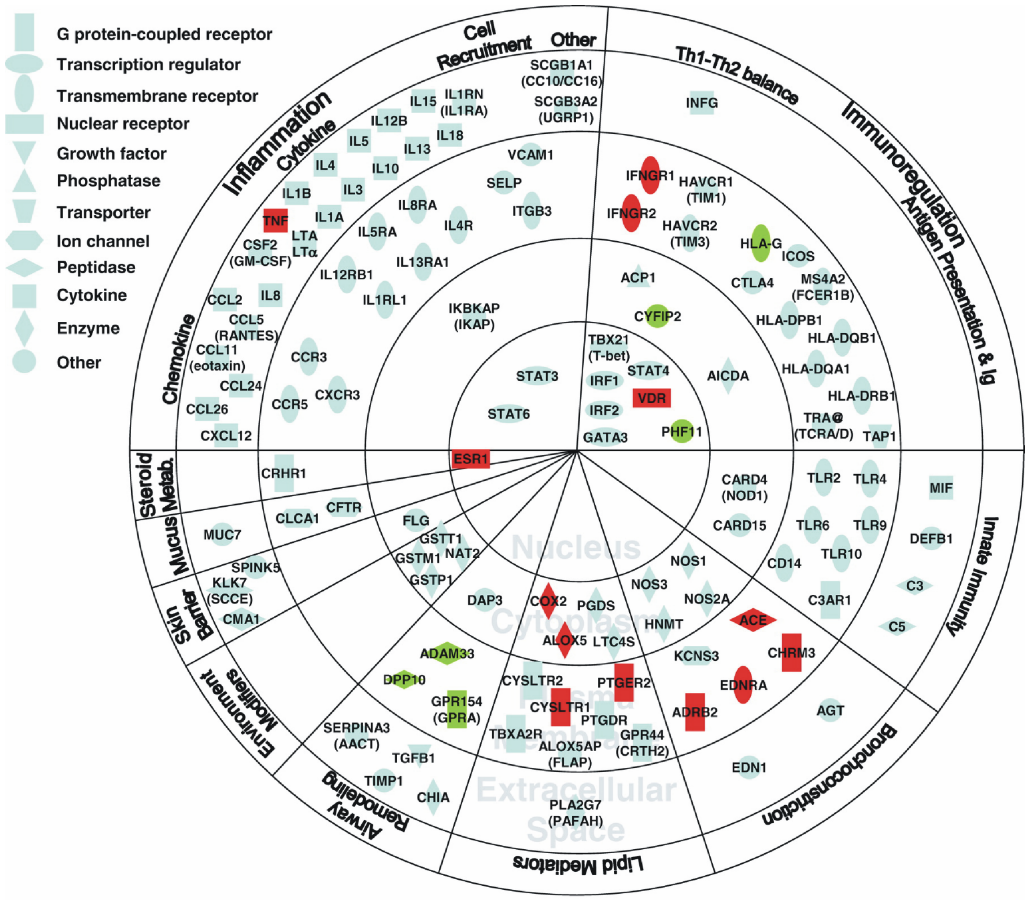
Recent candidate genes of asthma and related traits

According to a recent review, at least 372 gene-disease association studies have been published which include at least 150 different candidate genes for asthma and at least 124 candidate genes for atopy (Contopoulos-Ioannidis *et al*. 2007). The genes associated with asthma and atopy phenotypes in at least one published study, categorized by function and subcellular locations, are shown in Figure 5. For various cytokine genes and their receptors, of which *IL-4* (5q31) and *IL-4Rα* (16p12) are among the most studied, association with asthma or atopy (serum-IgE levels) has been

found (Marsh *et al*. 1994, Deichmann *et al*. 1998, Izuhara & Shirakawa 1999, Ober & Hoffjan 2006, Contopoulos-Ioannidis *et al*. 2007*)*. An allelic variant of *IL-4* receptor was also strongly associated with allergic inflammatory disorders (Hershey *et al*. 1997). Moreover, a functional role of genetic variants for IL-4/STAT6 signalling and IgE synthesis has been found by Kruse *et al*. (1999) and Mitsuyasu *et al*. (1998, 1999). In the studies, polymorphisms in the *IL4R α-chain* gene have been shown to influence to the enhancement of IgE synthesis *(*Mitsuyasu *et al*. 1999) and to reduce the phosphorylation rate of STAT6 (Kruse *et al*. 1999). A polymorphism in the 5´ promoter region of the *IL4R α-chain* has also been reported to associate with decreased soluble IL-4R protein levels (Hackstein *et al*. 2001). In addition, polymorphisms in the promoter regions of the *IL-13* (5q31) and *IL-13R* genes (Xq) have been shown to strongly associate with asthma and atopy susceptibility (Wills-Karp 2000, Wills-Karp & Ewart 2004, Contopoulos-Ioannidis *et al*. 2007). Functional variants of *IL-13* and *IL-13R* have also been detected (Heinzmann *et al*. 2000, Chen *et al*. 2004).

Furthermore, variations in other cytokine genes, such as *IL-10* and *IL-18* have also been associated with atopic phenotypes (Rosenwasser & Borish 1997, Kruse *et al*. 2003, Lyon *et al*. 2004). Also costimulatory molecules *CTLA-4* (2q33) and *CD28* (2q33) have been widely studied as candidate genes of asthma or atopy (Howard *et al*. 2002, Munthe-Kaas *et al*. 2004).

Meta-analyses of variations in asthma have also been performed for the widely studied asthma candidate genes, such as *TNFα*, *IL-4*, *IL-4R*, *IL-13* and *B2AR*, the most evident results, however, were obtained for the *ADAM33* and *TNFα* genes (reviewed in Contopoulos-Ioannidis *et al*. 2007). The *ADAM33* gene codes for a zinc-dependent metalloproteinase (20p13) and has been a widely studied asthma gene during recent years. The gene was first identified by a genome-wide linkage study and positional cloning (Van Eerdeweigh *et al*. 2002). The *ADAM33* gene is highly polymorphic and, to date, there are >50 SNPs identified in the gene and several replicative studies support its association with asthma phenotypes (Wills-Karp & Ewart 2004). However, heterogeneity between studies in different study populations have been obtained (Wills-Karp & Ewart 2004, Blakey *et al*. 2005, Contopoulos-Ioannidis *et al*. 2007). Recently, in Finnish asthma families, Laitinen *et al*. reported a *GPRA* (7p14.3) gene which showed association with high serum IgE levels and asthma in populations from Finland (Kainuu subpopulation, North Karelia) and from Canada (Northeastern Quebec) (Laitinen *et al*. 2004). Moreover, these observations are further supported by the reported positive association results in other ethnic groups (Kormann *et al*. 2005, Feng *et al*. 2006, Malerba *et al*. 2007). However, the molecular mechanisms involved need further elucidation (Laitinen *et al*. 2004, Vendelin *et al*. 2005, Bossé & Hudson 2007). In addition, for *GPRA* negative associations have been found in some populations (Shin *et al*. 2004, Veal *et al*. 2005, Bossé & Hudson 2007).



Bossé Y, Hudson TJ. 2007.
Annu. Rev. Med. 58:171–84

Figure 5. Genes associated with asthma and atopy phenotypes (in at least one published study) categorized by function and subcellular localizations (http://www.ingenuity.com). Green= Genes identified by positional cloning. Red = FDA-approved drug acts against the gene product. (Bossé & Hudson 2007.) Reprinted, with permission, from the Annual Review of Medicine, Volume 58 ©2007 by Annual Reviews www.annualreviews.org).

2.3.3. Future aspects

Besides candidate gene analyses determining the role of genetic variations in atopic traits, gene-gene interactions and SNPs in genes functioning in pathways have also been the focus of considerable interest. For instance, variants in the *IL-4Rα* and *IL-13* genes have been shown to enhance IL-13 responsiveness, the effect being, however, greater when variants in the both genes were present and thus acting synergistically (Chen *et al*. 2004). Howard *et al*. (2002) also reported a significant gene-gene interaction between genetic variations in the genes *IL-4Rα* and *IL-13*, where individuals with risk genotypes for both genes were markedly more susceptible to

asthma (Howard *et al*. 2002). Gene-environment interactions should be also taken into account in future asthma genetic study design, and various models have been described for these types of approaches. However, gene-environment interaction studies may be challenging and require, for instance, large study populations, high quality information on environment and lifestyle, in addition to increased statistical power (Hunter 2005, Martinez 2007). New technologies, such as genome-wide association tools, databases and microarrays, can be utilized in candidate gene studies (Altshuler *et al*. 2005, Syvänen 2005, Evans 2006). Genes shared between immune disorders have also been the subject of interest because these may provide new insights into the rising prevalence of the disorders (Wills-Karp & Ewart 2004).

In contrast to individual SNPs, haplotypes give more information (Johnson *et al*. 2001). A freely available panel of genotype data and haplotype block structure from different worldwide populations, the International HapMap project (www.hapmap.org), has made it possible to find genetic variations across the genome, and thus is helpful for designing association studies (Altshuler *et al*. 2005, Bossé & Hudson 2007). Further, the costs of genotyping will be reduced and the effect of causal variants can be indirectly tested by selecting the informative SNPs (tagging SNPs) which provide information in a specific region (Neale & Sham 2004, de Bakker *et al*. 2005, Bossé & Hudson 2007).

Frequently, association results in different study populations are not consistent, and bias may be caused by various factors, such as the lack of a standard definition of asthma, phenotype misclassification, genotyping errors, a heterogenic or too small sample, or technical problems. Therefore, asthma candidate gene analysis results should be interpreted cautiously. Features of the study population should be planned carefully and genetic heterogeneity between ethnic groups also plays an important role in candidate gene association analyses (Xu *et al*. 2001, Hirschorn *et al*. 2002, Xu *et al*. 2002, Ioannidis *et al*. 2004). Insufficient statistical power has been another major problem in asthma genetics, adding inconsistencies between the results; therefore, standards are needed to minimize false positives. Multiple testing may also elicit problems since numerous markers, genes and phenotypes or gene-gene/gene-environment factors are often tested. (Lander & Kruglyak 1995.)

2.3.4. STAT6, GATA3 and STAT4 as candidate genes

The human *STAT6* gene (19 kb) is located in chromosome 12q13.3-14.1, and contains 23 exons (Patel *et al*. 1998). Chromosomal linkage of asthma and atopy has been obtained for chromosomes 2q and 12q13-24 by several genome-wide screens (Barnes *et al*. 1996, 1999, CSGA 1997, Nickel *et al*. 1997, Wilkinson *et al*. 1998, Wjst *et al*. 1999). Further evidence supporting the importance of the IL-4R signalling pathway in allergic diseases comes from studies indicating an increased frequency of single nucleotide polymorphisms (SNPs) in allergic patients (Deichmann *et al*. 1997, Hershey *et al*. 1997, Mitsuyasu *et al*. 1998). These SNPs were shown to result in enhanced *STAT6* activation and IL-4R signalling. Thus, based both on its genomic location and the biological properties in functioning in the IL-4-mediated responses in Th cell differentiation, *STAT6* is one of the strong candidate genes for asthma and atopy

susceptibility. Over recent years, several variants of *STAT6* have been reported and genotyped in different ethnic groups (Gao *et al*. 2000, Tamura *et al*. 2001, Duetsch *et al*. 2002, Nagarkatti & Ghosh 2002, Shao *et al*. 2004). The GT repeat in the first exon of *STAT6* has been shown to be associated with elevated eosinophil levels among Caucasians (Duetsch *et al*. 2002) as well as to allergic phenotypes (Tamura *et al*. 2001) and childhood asthma (Shao *et al*. 2004) among a Japanese study population. A SNP in intron 18 has previously been shown to associate with total IgE levels (P=0.0070) in one study (Duetsch *et al*. 2002). Gao *et al*. (2000) reported a positive association between a 3´ UTR SNP and atopic asthma in a Japanese study population. However, negative associations for the variant have also been reported (Gao *et al*. 2000, Tamura *et al*. 2001, Duetsch *et al*. 2002, Shao *et al*. 2004). Thus, the biological importance of the described polymorphisms in *STAT6* is not yet clear.

Due to their functional importance in the Th1 and Th2 cell commitment process (Kaplan *et al*. 1996, Ouyang *et al*. 1998, Murphy *et al*. 2000), *STAT4* and *GATA3* genes are also potential candidate genes for asthma predisposition. *GATA3*, being located in chromosome 10p15, consists of six exons and is 19 kb in size (Joulin *et al*. 1991, Zheng & Flavell 1997). *STAT4* spans 120 kb on chromosome 2q32.2-q32.3 across one large intron, and the gene consists of 24 exons (Yamamoto *et al*. 1997). In addition, 2q has been reported as linked to atopy susceptibility or asthma related phenotypes (CSGA, 1997, Wjst *et al*. 1999, Koppelman *et al*. 2002). Recently, Li *et al*. (2007) reported that significant gene-gene interactions among the *STAT6*, *STAT4* and *IFNγ* genes may be possible risk factors for asthma susceptibility in the Chinese population. Furthermore, a *STAT4* intron polymorphism has been observed to associate with asthma in two studies (Park *et al*. 2005, Li *et al*. 2007).

AIMS OF THE PRESENT STUDY

The overall goal of this study was to examine the role of selected genes in human Th1 and Th2 cell differentiation as well as in genetic predisposition to immune-mediated disorders, such as asthma. Furthermore, since human Th cell differentiation involves many unknown factors, the aim was to identify novel IL-12 and IL-4-regulated genes which are induced during the early stages of Th differentiation, thus being potential new players for the Th differentiation process. To study the influence of selected genes of interest in Th1/Th2 cell differentiation, an optimized transfection method for human primary CD4+ T cells was established. The method enables the generation of a pure population of transfected primary CD4+ T cells for functional studies of the genes.

The specific aims of the thesis were:

- **To investigate the role of genetic variations in three candidate genes for predisposition to asthma/high s-IgE levels in the Finnish study populations**
    - To screen single nucleotide polymorphisms (SNP) in the selected genes, *STAT6*, *GATA3* and *STAT4*, involved in central signalling pathways for human T helper cell differentiation.
    - To study by association analysis the significance of the identified SNPs in the development of asthma and related traits in a Finnish founder population.

- **To optimize a novel method for human T cell transfection for studying the role of genes of interest in Th1/Th2 cell differentiation.**

- **To identify novel factors directing the early differentiation of human Th cells.**
    - To perform a genome-wide screening (after 0-48h of Th1/2 cell polarization) using Affymetrix human genome oligonucleotide arrays for identifying novel IL-12 and IL-4-regulated genes potentially involved in the early stages of human Th2 differentiation. The study is a continuation of the previous Affymetrix array studies of our group and particularly focuses on the identification of novel genes and ESTs that are interesting candidates for further studies.
    - To elucidate, using the optimized method for T cell nucleofection and gene silencing with specific siRNA, the influence of a selected set of genes on human Th cell differentiation.
    - To test whether a subset of the novel identified genes are regulated in STAT6-dependent manner.

MATERIALS AND METHODS

1 SNP screening in the human *STAT6*, *GATA3* and *STAT4* genes (I)

1.1 Study subjects

To detect genetic polymorphisms in the *STAT6*, *GATA3* and *STAT4* genes, a number of Finnish unrelated study subjects were screened: 14 for *STAT6*, 22 for *GATA3* and *STAT4*. Seven of them were non-atopic individuals (average IgE 143 kU/l, range from 50 to 310 kU/L) which were studied as controls. They had no history of asthma/atopy, they had normal pulmonary function, showed no bronchial hyperreactivity in a histamine challenge test and had negative RAST and skin prick test results. The others were young atopic adults (average IgE 4,697 kU/L, range from 1,143 to 27,716 kU/L), of which ten had asthmatic symptoms. These patients studied were tested in a similar manner to the non-atopic group. They had a history of asthma and atopy symptoms, a clinically diagnosed significant reversible bronchus obstruction and/or bronchial hyperreactivity during the histamine challenge test and at least one positive RAST and/or skin prick test result. Genomic DNA was extracted from peripheral white blood cells by propanol/ethanol extraction. The serum total IgE level was measured by Diagnostics CAP FEIA (Kabi Pharmacia, Sweden).

1.2 DHPLC analysis of PCR fragments

For discovery of SNPs in genes, the temperature-modulated heteroduplex analysis (TMHA) based DHPLC method was carried out on the automatic Wave^{TM} DNA Fragment Analysis System (Transgenomic). The method is successful for detecting novel genetic variations in DNA fragments (Kuklin *et al*. 1997, Oefner & Underhill 1998) (Figure 6). For the *STAT6*, *GATA3*, and *STAT4* genes, all the exons, 1–2 kb of the 5´ and 3´ flanking sequences of the genes, and intron-exon boundaries were screened for polymorphisms. Screening covered Gene bank sequences NT_029419 for *STAT6*; NT_077569 for *GATA3* and NT_005403 for *STAT4*.

The PCR primers (Table I) were designed by the Primer Express software (Applied Biosystems, Foster City, CA). The amplicons were 300–800 bp in size and, when possible, the amplicons were designed to overlap by 50–200 bp. PCR reactions were carried out in 50–100 µl volumes containing 100–200 ng of template DNA, 0.2 mM of dNTP (MBI Fermentas, Vilnius, Lithuania), 400–800 nM of each PCR primer (Medprobe, Norway), 2–3.5 U of Dynazyme EXT polymerase, and 10 X Dynazyme Tritonfree polymerase buffer (Finnzymes, Espoo, Finland). For a better PCR specificity, 5% DMSO (Finnzymes, Espoo, Finland) was added to the reactions. For DHPLC analysis, the denaturing melting temperatures for each fragment were predicted with WAVEMAKER software (Transgenomic) and Stanford´s DHPLC Melt Program (http://insertion.stanford.edu/melt.html).

Table Ia-c. SNP positions of the human STAT6 (a), GATA3 (b) and STAT4 (c) genes and the primer sequences used for PCR and SnaPshot genotyping.

Table Ia.

STAT6 specific markers named based on their position on NT_029419	Gene Bank SNP ID no.	Location in the Stat6 gene	PCR primers 5´-> 3´	SNaPshot primer
G19647081A	rs167769	intron	GTCCTGGTCAAGCCAATCGA GATGAGGGAGAGGATGGGATG	GCATGAAGGTCTTGGAGCAGAAAC
G19645488A	rs324011	intron	GATGCCCCTGGTTTAAGGTG CTCTTCGGCTCAGCCTCCTA	(T^{15})CCATGAGTGGTGGTGGGGAC
G19633406A	rs324015	3´UTR	TGCTCTGGACACTTGCTCATGC CAGCTATACACGAAGAATCTCAGC	(T^{5})GGGAAGTTCAGGCTCTGAGACAC
A19633134G	rs703817	3´UTR	TGCTCTGGACACTTGCTCATGC CAGCTATACACGAAGAATCTCAGC	CTGGGTGGGAGCATAGGAGG
A19632954G	rs4559	3´UTR	TTAGCCAGGTGGAGGCTTC GCAGCACCTATCTGAGCAGTT	(T^{5})CAACTAAGGTGCCAGCTATA

Table Ib.

GATA3 specific markers named based on their position on NT_077569	GenBank SNP ID no.	Location in the Gata3 gene	PCR primers 5´-> 3´	SNaPshot primer
C2450477T	new	5´flanking	5'- TCTGTCAGTTTCACACACCCCT´-3' 5'- ATCCACACACTCACACACCGTAC -3'	CTCTCCAAAGGGCAGTCC
G2458625A	new	5´flanking	5´- CGAGCAGAGAGTGGATTTGG -3´ 5´- GAGTACGCTCAGTGTAGACGGAC -3´	(T^{13})GTTTCCTTGACTGTGGGAGAAAC
T2458701G	new	5´flanking	5´- CGAGCAGAGAGTGGATTTGG -3´ 5´- GAGTACGCTCAGTGTAGACGGAC -3´	(T^{41})CCCTAGAGTGAAGACCGAGTTCT
A2459095G	rs1269486	5´flanking	5´- TTCTGATCAATTTAACGCGCA -3´ 5´- GCACAAGGAAACTGCAACCC -3´	(T^{65})CTCCCAAACACCCTGCATT
G2460264A	new	5´UTR	5´- ACTCAACTCTCCTCTTTTGGAGGT -3´ 5´- TCGTTGAATGATTTGCTTTCG -3´	(T^{24})GGTCCCGTTTTATTCTGCC
C2460466T	new	5´UTR	5´- TTTGCTCACCTTTGCTTCCC -3´ 5´- TGTTAAAAAGCACATCCACCTCC -3´	(T^{61})GACCGCCCTCCCTCCC
C2463543T	rs2229359	exon, synonymous	5´- CCGCACCTCTTCACCTTCC -3´ 5´- TCAGCAGGCTTTGGGACAC -3´	(T^{54})GTGGCAGCATGACCGC
C2474305T	rs422628	intron	5´- TCTGGTGTACTTTGTGGGTTGAG -3´ 5´- TGACGCTGTTTGCTGGTCA -3´	(T^{30})GTGGGGAGAGAGGAGAGGGT
C2478975T	rs2229360	3´UTR	5´- CCAGCTGAAATGGAAACAGATC -3´ 5´- ATGCCTTACAGCTACCCAGATG -3´	(T^{33})AGGAGCAGTATCATGAAGCCTAAA

Table Ic.

STAT4 specific markers named based on their position on NT_005403	Gene Bank SNP ID no.	Location in the Stat4 gene	PCR primers 5´-> 3´	SNaPshot primer
G42225344A	rs2278940	5´flanking	5´- GTGTTTTGATCCTGGCTACCC -3´ 5´- GGCCACTTACCTAGCGCTCT -3´	TCACCGGGCGTCCTCTTC
C42132257T	rs3024866	intron	5´- TCATAGAAAGTTATTCCTAGATCTATTAAATAGC -3 5´- AATTATAAGGCGTATTAGTAAAGATGTCTG -3´	ACAAAGTACAAATCTTCGGTTG
T42128770C	new	intron	5´- CAGCATGATTTCCTTTTTCGAA -3´ 5´- TTGAAAAACTTCCTCATATTGTCTGC -3´	GAAAATATGTTGCTGTTCTAGA
C 42113438T	new	exon, nonsynonymous	5´- TATGCATGCTCAGAAAGTTCCAT -3´ 5´- GCAGATGTGTTTGTTGGCAATA -3´	(T^{17})ATCACCACAGGCAATGAGCTG
T42110400C	new	exon, synonymous	5´- GAAGCTGCTGAACAGTTTTAGGTG -3´ 5´- GGTAGAATAAGCTGAGCTTTGCAG -3´	(T^{25})CCTCCACCTGCCACATTGAG
C42105770T	rs3024898	intron	5´- CACATGCCCTTAGATCTTACAGAA -3´ 5´- ATGTCACTTCAATGTCAGACCTTC -3´	(T^{30})CTGTGTATCACCGAACAACTGC
C42105023T	rs3024903	intron	5´- AATGGGACATTGCACATTACAA -3´ 5´- TTGTTCGGTGATACACAGAAATG -3´	(T^{37})AAAGGGGCTGAAATACTGCCTG
T42103557C	rs3024908	3´flanking	5´- GCTAATGTCCCATCACGACTT -3´ 5´- ATGACAGGATAAACTCTGACGC -3´	(T^{44})GAGGGAAAAGCCAAATAAGCCA
T42103821C	rs2280236	3´UTR	5´- AACCTCTTTCCCCAGTCTCA -3´ 5´- TAGCAGGCATGGTGATCAC -3´	(T^{54})ACTCCCGTGGCACTCCTCA

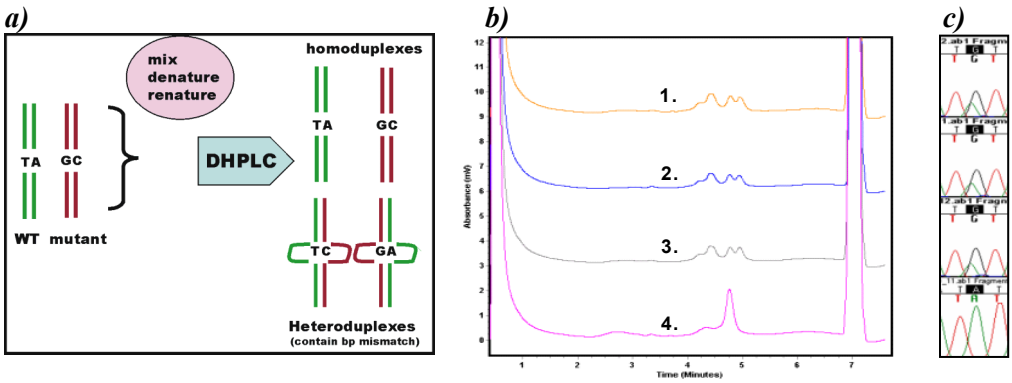


Figure 6a-c. The principle of base pair mutation detection by DHPLC (Wave system) in PCR products. DNA duplexes differing by one or more base pairs are separated based on Temperature Modulated Heteroduplex Analysis (TMHA). After rehybridization (heating to +95°C and slow cooling), both heteroduplex and homoduplex fragments are formed in heterozygotic individuals due to base pair mismatch. Only homoduplexes are formed in wild type (WT) individuals (a). Genetic variations between samples can be identified according their heteroduplex (samples 1-3) or homoduplex (sample 4) peak profiles in chromatograms when heteroduplexes are resolved ahead of homoduplexes in DHPLC in denaturing run temperatures (b) (modified from Kuklin *et al*. 1998). Direct DNA sequencing was carried out to confirm the identified heteroduplexes (c).

1.3. Sequencing

Observed heteroduplexes were confirmed with direct sequencing, both the group of samples giving the heteroduplex as well as those giving homoduplex profile (ABI PRISM 3100 Genetic Analyser using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) (Figure 6c).

2 Association analysis of SNPs in *STAT6*, *GATA3* and *STAT4* genes (I)

2.1 DNA material

For the association study, Kainuu study families were recruited based on asthma (Laitinen 1997, 2000). Phenotypes available for this cohort (average age 43 years: 37 years among individuals with high and 45 among individuals with low serum IgE) were asthma status, serum total IgE level (Diagnostics CAP FEIA, Kabi Pharmacia, Sweden), and blood eosinophil level. For *STAT6*, *STAT4* and *GATA3*, an association analysis was performed with a subset of the cohort including all the trio families (father, mother, offspring) with full phenotypic and genotypic information (a total of 120 nuclear families, 360 study subjects). For *GATA3,* the initial association was replicated in a population-based case-control study including 245 Finnish asthma patients and 405 control subjects (Karjalainen *et al*. 2002). The studied phenotypes for this cohort were the asthma status, serum total IgE level, skin prick test results, and blood eosinophil level. Total serum IgE was measured by the immunoluminometric method (Ciba Corning Diagnostics, Halsted, UK). Genomic DNA of all study

individuals was extracted from peripheral white blood cells using a standard non-enzymatic method.

2.2 Genotyping: SNaPshot minisequencing

After SNP discovery, genotyping was performed by SNaPshot™ (Applied Biosystems, Foster City, CA) (Turner *et al*. 2002) according to the manufacturer´s directions. The system is based on dideoxy single-base primer extension, enabling multiplexing of up to 10 primer/template combinations simultaneously in a single tube. Primer sequences are shown in Table 1. PCR samples were purified with the shrimp alkaline phosphatase (SAP) and Exonuclease I (ExoI) enzymes (UBS, Cleveland, Ohio). The SnaPshot reactions were carried out in multiplex reactions in a total volume of 10 μl containing 5 μl of ready reaction mix (diluted 2:3 with 20 mM of Mg2+ and 800 mM of Tris-HCl, pH 9.0), 0.1–2.8 μM of each genotyping primer and 1:10–1:20 dilutions of the SAP/ExoI purified PCR products. The thermal cycling step consisted of the denaturing step at 96°C for 10 s, the annealing step at 50°C for 5 s and 25 cycles of the extension step at 60°C for 30s. For post-extension treatment, the samples were purified with 1 U SAP. The genotype data were analysed with GeneScan 2.1 software (Applied Biosystems, Foster City, CA). The SNP genotype data was checked for Mendelian errors by the PedCheck program (O´Connell & Weeks 1998).

3 Statistical analyses (I)

3.1 Linkage analysis between chromosome 12q markers, including STAT6 SNPs, and asthma (unpublished)

For the linkage analysis of asthma markers of chromosome 12, including *STAT6* SNPs, the nonparametric multipoint linkage analysis (NPL) was performed using the GENEHUNTER 2.0 computer package (Kruglyak *et al*. 1996). In the study, linkage was analysed between a total of 15 previously reported microsatellites (Laitinen *et al*. 2001), five STAT6 SNP markers (rs167769, rs324011, rs324015, rs703817, rs4559) identified in *Report I* and asthma in the Finnish Kainuu study population. The microsatellite marker information is available at http://www.genome.helsinki.fi/eng/research/asthma/.

3.2 Association analysis

The Haplotype Pattern Mining (HPM) algorithm (Toivonen *et al*. 2000) was used for the association analyses. Haplotypes were constructed using HPM and the analysis was performed for dichotomized traits: asthma (affected and control) and high serum total IgE level (serum IgE level >100 kU/L as high responder, and serum IgE level ≤100 kU/L as low responder), or the combinations of asthma with different serum total IgE levels and associated blood eosinophil counts. Haplotyping was done within each trio and four independent chromosomes were obtained. When there were ambiguities resulting from missing genotype data, identical heterozygotic genotypes in all of the family members, or Mendelian incompatibilities, the alleles were discarded. If the child

was affected, the transmitted chromosomes were considered disease-associated and the non-transmitted chromosomes as controls. If one of the parents was affected, his/her chromosomes were considered disease-associated and the spouse´s chromosomes as controls. When both the parent and the child were affected, only the non-transmitted chromosome of the unaffected parent was considered as the control and the remaining three as disease-associated. The input for HPM consists of these haplotypes (Toivonen *et al*. 2000).

The maximum length of the haplotype patterns and the number of gaps allowed were given as parameters for the algorithm. For the population-based case-control replication data set, the haplotypes were constructed from the data of unrelated individuals using the SNPhap algorithm (http://www-gene.cimr.cam.ac.uk/clayton/software/), and those haplotypes were used in a similar way as input data for HPM. The HPM finds all the phenotype-associated haplotype patterns that exceed a given threshold value by a chi-square test. Each marker is given a marker-wise score based on the number of qualified patterns spanning across the marker. The significance of the observed P value can be tested by permutation where the grouping of chromosomes is done randomly and the scores are re-calculated several times to obtain marker-wise P values that are comparable with each other (Toivonen *et al*. 2000).

Quantitative statistical analysis methods were employed to evaluate associations between specific haplotype carrier status and the elevated total serum IgE levels detected by HPM. For this purpose, total serum IgE measurements were transformed into a normally distributed form by $\log_e$ transformation and adjusted for age and gender. The resulting new variable was analysed using independent samples t-test and linear regression analysis

4 Transfection of T cells (II, III)

4.1 Plasmid constructs

STAT6 shRNA (II, III)
The H-2K^k sequence was amplified by PCR from a pMACSK^k II plasmid (Miltenyi Biotech) containing the sequence coding for a mouse MHC class I H-2K^k protein with a truncated cytoplasmic domain and being used as a cell surface selection marker. The amplified H-2K^k sequence (1185 bp) contains the region between 3563-2378 of the plasmid sequence. The H-2K^k sequence was amplified by using the PfuUltra HF DNA polymerase (Stratagene, La Jolla, CA). For cloning into the pIRES2-EGFP plasmid (BD Biosciences Clontech, Mountain View, CA), the primers (DNA Technology A/S, Aarhus, Denmark) H2K-PCR-F and H2K-PCR-R1 (Report II, Table 1), containing *Bsp1407I* and *BstXI* restriction sites, respectively, were used. For cloning the same H-2K^k sequence into the pSuper-GFP-Neo plasmid (Oligoengine, Seattle, WA), the corresponding primers were H2K-PCR-F and H2K-PCR-R2, the latter of which contains a *BshT1* restriction site.

PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler DNA Engine Tetrad (MJ Research, Inc., Watertown, MA) under the following conditions: an initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. The PCR products were purified from 1% agarose/ethidium bromide gels using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). Aliquotes of purified PCR products and plasmids were either digested with the restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany) *BstXI* and *Bsp1407I* (for cloning into the pIRES2 vector) or with *Bsp1407I* and *BshT1* (for cloning into the pSuper vector). Digestion of the plasmids with these enzymes releases the EGFP fragment, which was discarded. The digested products were ligated, thus creating a pIRES2-H-2K^k construct and a pSuper-H-2K^k construct. TOP10 chemically competent *E. coli* cells (prepared in house) were transformed with the ligation products and plated on a Luria-Bertani (LB) medium containing either 30 μg/ml of kanamycin (for the pIRES2-H-2K^k ligation) or 100 μg/ml of ampicillin (for the pSuper-H-2K^k ligation). Recombinant clones containing an insert of the correct size were selected and confirmed by PCR using the H2K^k-PCR primers (Report II, Table 1) and colonies as a template. Selected clones were grown overnight in an LB liquid medium with an appropriate antibiotic followed by plasmid DNA purification using the Qiaprep Spin Miniprep Kit (Qiagen). The plasmids were sequenced using both the H-2K^k and vector sequence specific primers (Report II, Table 1). To clone the STAT6-shRNA into the pSuper-H-2K^k plasmid, a restriction fragment between *EcoRI* and *XhoI* containing the sequences of the H1 promoter and a STAT6-shRNA was removed from pSuper-STAT6-shRNA construct (prepared and validated in the lab earlier using the first version of the commercially available pSuper vector). The fragment was ligated into the *EcoRI/XhoI* cleaved pSuper-H-2K^k vector. Cleavage of the pSuper-H-2K^k with these enzymes released a fragment (which was discarded) containing the H1 and the sequence of the multiple cloning site (MCS) before the *XhoI* site. The cells were transformed with the ligation products as described above and recombinant clones were detected by PCR and sequenced using M13R and pSuper-1 primers (*Report II, Table 1*). The STAT6-shRNA targets the 5′-GAATCAGTCAACGTGTTGTCAG-3′ sequence inside the STAT6 open reading frame. shRNAs were designed based on Gene Script Corporation software.

Similarly, the pSuper-H2K-scramble2-shRNA (5′-GCGCGCTTTGTAGGATTCG-3′) was designed and used as a shRNA control. Furthermore, another pSuper-H2K-STAT6-shRNA plasmid, targeting 5′-CAGTTCCGCCACTTGCCAAT-3′, was also used in replicative experiments.

NDFIP2 shRNA (III)

The NDFIP2 short hairpin RNA (shRNA) plasmid construct, targeting (5′-GAGGAAGAGTGTCCACCAAGA-3′), was generated by cloning the NDFIP2 shRNA oligonucleotide into the *BglII* and *XhoI* sites of the previously modified pSuper-H-2K^k-pIRES2 plasmid, containing a truncated H2K^k sequence. pSuper-H-2K^k-pIRES2-scramble1-shRNA (5′-AATTCTCCGAACGTGTCACGT-3′) was designed and used as a control. In addition, two synthetic siRNA oligos (one of which

targeting the same sequence as the NDFIP2 shRNA shown above and the other targeting the 5´-CUGGAUAUUUCAAUGGACAUU-3´ NDFIP2 sequence (Sigma-Aldrich)) were used to knock down NDFIP2.

4.2 Isolation and *in vitro* polarization of primary human Th1/Th2 cells (II, III)

Human mononuclear cells were isolated from cord blood (Turku University Central Hospital) or from peripheral blood (buffy coats) from healthy blood donors (Finnish Red Cross) with Ficol Paque Isolation (Amersham Pharmacia Biotech, Uppsala, Sweden). The CD4+ cells were further enriched with Dynabeads (Dynal, Oslo, Norway). Cultures and analysis of lymphocytes have been previously established and optimized in the laboratory (Hämäläinen *et al*. 2000, 2001a, 2001b, Lund *et al*. 2003a, 2003b). The cells were further activated with plate-bound α-CD3 (0.5-1 μg/ml for coating) and 0.5 μg/ml of soluble α-CD28 (Immunotech, Marseille, France). This CD3 and CD28 costimulation was used to mimic T cell activation by APCs (Li *et al*. 1999). The cells were *in vitro* polarized into either Th1 or Th2 cells using IL-12 or IL-4 as polarizing cytokines: Th1 cultures were supplemented with 2.5 ng/ml of IL-12, whereas Th2 cultures were supplemented with 10 ng/ml of IL-4 (R&D Systems, Minneapolis, MN). A subset of the cells was cultured in "neutral conditions" without polarizing cytokines. Some cultures (report III) were also supplemented with 3 ng/ml of TGFβ (R&D Systems). The T cells were cultured in Yssel´s medium (Yssel *et al*. 1984) containing 1 % AB serum (Red Cross, Helsinki, Finland). After 48 hours, 40 U/ml of IL-2 (R&D Systems) was added to the cultures. The cells were harvested at certain time points (0, 2, 6, 48h or 5-7 d) for gene or protein expression analyses and cytokine production assays.

4.3 Nucleofection method and enrichment of transfected cells (II, III)

For T cell transfection, isolated CD4+ human T cells obtained from buffy coats were nucleofected (Martinet *et al*. 2003) using the commercial Nucleofector Kit (Amaxa) and optimized protocol. A total of 5 x 10^6 CD4+ cells were suspended in 100 μl of T cell Nucleofector™ solution (Amaxa) and mixed with 10 μg of plasmid DNA. The cells were then given an electric pulse (program U-14) in a cuvette using a Nucleofector™ device (Amaxa). After the pulse, the cells were resuspended in 1 ml of RPMI-1640 medium (Sigma-Aldrich, St.Louis, MO) and supplemented with 10% FCS, pen/strep and L-glutamine. The cells were left to rest and incubated in 24-well plates o/n (for ~16 hours) in 37°C (2.5 x 10^6 cells/well). After the o/n incubation, the cells were harvested and parallel samples were pooled.

Dead and apoptotic cells were removed from the cultures (Dead Cell Removal Kit) and H-2K^k-positive cells enriched (magnetic separation) using an optimized protocol (MACSSelect K^k.I, Miltenyi Biotech). MACSelect K^k MicroBeads coated with the H-2K^k antibody (Miltenyi Biotech) were incubated with the cells for 15 min to magnetically label the transfected cells. Subsequently, magnetic separation of H-2K^k-positive cells was carried out with a positive LS+/VS+ selection column placed in the magnetic field of a MACS separator.

For the nucleofection experiments, the purified H-2K^k-positive cells and non-nucleofected control cells were polarized into Th1 and Th2 cells as described above. Cells were cultured of a density of 0.3-0.5 x 10^6cells/200 µl in 96-well plates precoated with 0.5 µg/ml of anti-CD3 and in the presence of 0.5 µg/ml of soluble anti-CD28 (Immunotech, Marseille, France). When transfecting the T cells with shRNAs, the enriched H-2K^k-positive cell populations were incubated at 37 °C for 24 hours prior to the polarization.

4.4 RNA isolation and cDNA synthesis (II, III)

For Real-Time quantitative RT-PCR analysis (described below), the cells were harvested at certain timepoints of polarization from biological replicates. The total RNAs were isolated either with the RNA Easy Minikit (Qiagen, Oslo, Norway) or the PicoPureTM RNA Isolation Kit (Arcturus, Mountain View, CA) according to manufacturer's instructions. DnaseI (Qiagen) treatment was included in the RNA isolation procedure to eliminate genomic DNA from the samples. Subsequently, cDNA, prepared using either a Superscript II kit (Gibco BRL, Life Technologies, Paisley, Scotland) or with the Transcription First Strand cDNA Synthesis Kit (Roche, Germany), was used as a template for gene expression analyses.

4.5 Real-Time quantitative PCR (II, III)

After 0-7 days of polarization, the cells were harvested for the RT-PCR analysis of Th1/Th2 marker gene expression (Gibson *et al*. 1996, Heid *et al*. 1996). Gene expression levels for selected genes previously described as markers for either differentiated Th1 or Th2 cells were measured using the TaqMan ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as described before by Hämäläinen *et al*. (2000). Furthermore, gene expression of siRNA silenced samples vs. controls was studied by RT-PCR. To validate the oligonucleotide array results for the selected genes, either probe-based or SYBR Green primer-based quantitative RT-PCR was performed. All measurements were performed in duplicate in two separate runs using samples derived from four different individuals. The housekeeping gene Elongation factor 1 alpha (EF1α) was used as a reference transcript (Hämäläinen *et al*. 2001). Primers and probes (reports II and III, Table I) used for the quantification of gene expression (MedProbe, Oslo, Norway or DNA Technology, Aarhus, Denmark) were designed using the Primer Express software (Applied Biosystems). The quantitative value obtained from TaqMan Real-Time RT-PCR is a threshold cycle (CT). The fold differences between different conditions can be calculated from the normalized CT values ($CT_{gene\ X}$ - $CT_{housekeeping\ gene}$), ΔCT values, with the formula: fold difference= $2^{(|\Delta CT1-\Delta CT2|)}$. Statistical significances between the differences in gene expression were evaluated with t-tests.

4.6 Intracellular cytokine staining and flow cytometry (II, III)

The transfection efficiency and cell viability were analysed before and after H-2K^k enrichment by FACS flow cytometric analysis. After nucleofection and o/n incubation,

the cells were counted and aliquotes of 0.3 x 10^6cells nucleofected with H-2K^k plasmid constructs were collected for determination of transfection efficiency. Similarly, after dead cell removal and the H-2K^k enrichment, equal samples were taken for cell viability and purity (H-2K^k-positivity) analyses of the enriched cells. Non-nucleofected cells were used as negative controls. The collected cell samples were suspended in 100 µl of PBS and stained for 10 min with 10 µl of either H-2K^k-FITC or Control-FITC antibody (Miltenyi Biotech). The cells were washed twice with PBS and the samples were analysed with the FACScan and CellQuest software (BD Biosciences, San Jose, CA).

After 7-8 days of polarization, the intracellular cytokine production (IFNγ and IL-4 cytokines) was measured in transfected and control samples with flow cytometry. To induce the cytokine production, 0.5 x 10^6 cells were incubated for 5 hours with or without (stimulated cells and non-stimulated controls, respectively) 5 ng/ml of PMA and 0.5 µg/ml of ionomysin (Sigma-Aldrich) in the density of 1 x 10^6 cells /ml. After 2 hours of stimulation, 10 µg/ml of Brefeldin A was added and incubation was continued for 3 hours. The cells were washed twice with PBS buffer containing 0.5% BSA and 0.01% azide. The cells were fixed with 4% paraformaldehyde for 15 min, washed with the PBS buffer and permeabilised with 0.5% saponin for 10 min. Cytokine staining was done using anti-IFN-γ-FITC and anti-IL-4-PE (Caltag Laboratories, Burlingame, CA) for 20 min, after which the cells were washed 3 times with PBS buffer. The cytokine production profiles of the cells were measured with the FACScan and CellQuest software (BD Biosciences). Cytokines were measured in the control (non-nucleofected) cells cultured in an identical manner.

4.7 Cytokine secretion assay (III)

For measuring IFNγ and IL-4 cytokine secretion by the control cells (scramble) or cells transfected with NDFIP2-shRNA, the cells were incubated with or without (stimulated cells and non-stimulated controls, respectively) 5 ng/ml of PMA (Calbiochem, San Diego, CA) and 0.5 µg/ml of ionomycin (Sigma-Aldrich). The culture medium from each well was collected after 24 h of re-stimulation with PMA and ionomycin. The culture medium was separated from the cells by centrifugation and stored at -70°C until used for cytokine determination. Secreted cytokines were measured using a Luminex assay and multiplex bead kits from LINCO Research, Inc. (USA) and from Bio-Rad Laboratories, Inc. (Hercules, CA). The assays were conducted in duplicate according to the manufacturer's instructions. Measurements and data analysis were performed with the Bio-Plex system in combination with the Bio-Plex Manager software (Bio-Rad Laboratories).

4.8 Jurkat cell transfection (II)

Ten million Jurkat cells (Tag derivatives expressing SV40 large T antigen, (Northrop *et al*. 1993) were transfected with 10 µg of plasmid DNA in 400 µl of culture medium (RPMI-1640 medium supplemented with 10% FCS, pen/strep and L-glutamine) by electroporation (250 V, 975 µF, Gene Pulser^R II Electroporation system, Bio-Rad

Laboratories, Hercules, CA). The transfected cells were incubated at 37°C for 16h, after which the dead cells were removed and the H-2K^k-positive cells were enriched as described above. The enriched H-2K^k-positive cells were activated with 5 ng/ml of PMA (Calbiochem, San Diego, CA) and 1 μg/ml of PHA (Sigma-Aldrich) in the presence of 10 ng/ml of IL-4 (R&D Systems, Minneapolis, MN). The cells were cultured in 12-well plates at the density of 2.5 x 10^6 cells / 2.5 ml of culture medium. Samples were harvested for Western blotting 48 and 72 hours after transfection.

4.9 Western blotting of Jurkat cells (II)

To study the functionality of Stat6-shRNA by western blotting, Jurkat cells were harvested 48 and 72 hours after transfection and lysed in SDS buffer. The lysates were sonicated and equal amounts of proteins were loaded on the 10% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane (Hybond™-ECL™, Amersham Biosciences, Piscataway, NJ), probed with rabbit anti-STAT6 and anti-β-actin (both from Sigma-Aldrich) and subsequently incubated with HRP-conjugated goat anti-rabbit Ig (BD Biosciences) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnologies, Santa Cruz, CA). The protein bands were visualized with ECL (Amersham Biosciences).

5 Affymetrix oligonucleotide array experiments (III)

5.1 Sample preparation for Affymetrix oligonucleotide arrays

Human genome U133A and B arrays, containing approximately 33 000 probes for different transcripts, were rehybridized with the previously prepared samples (Lund *et al*. 2003b, 2005). Before the hybridizations, the successful polarization of the cells was confirmed with Real-Time RT-PCR. Sample preparation and data analysis was performed according to the instructions and recommendations provided by the manufacturer (Affymetrix, Santa Clara, CA). The total RNA (4 - 5 μg) pooled from different individuals was used as starting material for the Affymetrix sample preparation.

5.2 Data analysis

Two biological repeats for each microarray experiment were performed. After hybridization and scanning, GeneChip Microarray Suite software version 5 (MAS5, Affymetrix), GeneSpring (SiliconGenetics, Redwood, CA) and Microsoft Access for Windows softwares were used to evaluate the quality of the data and for routine data analysis and processing. The normalized numerical data were filtered according to the statistical classifications performed by the MAS5 software based on 16 probes for each gene (Lund *et al*. 2003b, 2005). At the detection level, all the genes that were assigned as “absent” in both samples compared, as well as all the genes that were classified as “no change”, were removed. Genes that presented a consistent change (≥2-fold) in two separate biological repeats were considered to be differentially expressed: the gene expression was considered to be up-regulated if the signal log ratio between the

reference and the target samples was higher than one (2-fold increase) and the target sample was "present". Similarly, a gene was defined as down-regulated if the signal log ratio was < minus one (2-fold decrease) and the reference sample was "present". All the genes fulfilling these criteria in at least one of the comparisons and one of the time points were selected for further analysis where the expression of the genes was explored in parallel in different conditions without a fold change threshold. The gene annotations were obtained from NetAffx-database (Liu *et al*. 2003). The normalized microarray raw data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2770.

5.3 Functional validations of Affymetrix results – measuring gene and protein expression by RT-PCR and Western blotting

For validation of the oligonucleotide array results with Real-Time RT-PCR, additional Th1 and Th2 primary cultures were generated *(Report III)*. The isolated human cord blood CD4+ T cells cells were activated with 0.1 μg/ml of PHA (Murex Diagnostics, Chatillon, France) and irradiated CD32-B7 transfected fibroblasts (Hämäläinen *et al*. 2001b, Lund *et al*. 2003a). Th1 and Th2 cultures were further induced as described above. The cultures were generated from four individuals. Samples were collected at time points 0, 6, 24 and 48h or 7d.

For protein expression studies with Western blotting, human CD4+ T cells were isolated as described and polarized to the Th0, Th1 or Th2 direction. The cells harvested after 2, 6 and 48h of polarization were lyzed in SDS buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT). Equal protein amounts of whole-cell lysates were loaded on the gel. Alternatively, cells were lysed in HEPES buffer (20 mM HEPES, 0.2% Tween-20, 1 mM DTT) containing Complete Protease Inhibitor (Roche, Germany). From the centrifuged lysates, the supernatants were discarded, and a second buffer (20 mM HEPES, 420 mM NaCl, 20% glycerol, 1mM DTT, Roche Complete Protease Inhibitor) was mixed with the cell pellets. After one hour of incubation on ice, the samples were re-centrifuged and supernatants containing nuclear proteins were obtained. The protein concentrations were then quantified from the samples by BioRad protein assay (BioRad, USA). Equal amounts of proteins were loaded into SDS-PAGE and transferred to a Hybond ECL membrane (Amersham Biosciences, Sweden). Uniform protein transfer was verified with Ponceau S staining (Sigma, UK). The membranes were probed for TBX21 (sc-21749, Santa Cruz, USA) and GATA3 (sc-268) to confirm successful induction of Th1/Th2 polarization. Western blotting was then performed for DACT1 (ab5977-100, Abcam, Cambridge, UK), RAB11FIP1 (RCP 11-A, Alpha Diagnostic Int. Inc.), FOSL2/Fra-2 (sc-604), CISH (sc-1529), ZF9 (sc-7158), FLT1 (sc-9029) and RAB27B (sc-22991), all from Santa Cruz.

RESULTS AND DISCUSSION

1 Role of genetic variations in human *STAT6*, *GATA3*, and *STAT4* genes in asthma (I)

1.1 Identification of SNPs in the *STAT6*, *GATA3* and *STAT4* genes

We have used the candidate gene approach for studying the role of genetic variants in three genes, *STAT6* (12q13.3-14.1), *GATA3* (10p15) and *STAT4* (2q32.2-q32.3), for the predisposition of human asthma and atopy. The candidate genes were chosen based on their important functions in the differentiation of T helper 1 and 2 lymphocytes. Furthermore, in particular, *STAT6* as well as *STAT4* loci located in chromosomes 12q and 2q, respectively, are relevant positional candidates according to genome-wide linkage studies (Barnes *et al*. 1996, CSGA 1997, Nickel *et al*. 1997, Wilkinson *et al*. 1998, Barnes *et al*. 1999, Wjst *et al*. 1999, Koppelman *et al*. 2002). At the beginning of the study, in 2000-2001, no SNP screens or asthma association analyses had yet been published for these genes. To identify SNPs in human *STAT6*, *GATA3* and *STAT4* loci, a panel of 14 unrelated Finnish individuals for *STAT6* study and 22 for *GATA3* and *STAT4* study were screened for polymorphisms, thus including 28-44 chromosomes. The material included nonatopics without asthma/atopy history, as well as atopic patients, of whom some also had asthmatic symptoms. To improve the chances of finding functionally important SNPs in IgE mediated disorders, the pool of screened individuals was enriched with atopy patients with exceptionally high serum total IgE values. The screen covered all the exons, the exon-intron boundaries and 1-2 kb of the 5′ and 3′ flanking sequences of the genes. The overlapping PCR fragments covered 9.8 kb (~51.6%) of the *STAT6*, 8 kb (~42.1 %) of the *GATA3*, and 11 kb (~9.1 %) of *STAT4* loci. For the SNP discovery, a DHPLC method based on the temperature-modulated heteroduplex analysis (TMHA), was performed (Kuklin *et al*. 1997). Direct DNA sequencing was used for the verification of all identified heteroduplexes.

As a result, a total of 23 SNPs were identified, five of them located in the *STAT6*, nine in the *GATA3*, and nine in the *STAT4* gene locus *(Report I, Figure 1)*. Eight of the detected polymorphisms were revealed to be novel and were not previously reported in the SNP database (dbSNP/NCBI, http://www.ncbi.nlm.nih.gov/SNP/). Two intronic and three 3′UTR SNPs were identified in the *STAT6* gene. In the *GATA3* locus, the majority of the detected polymorphisms were located in the flanking, or 5′or 3′ UTR, sequences. One synonymous SNP was identified in the second exon of *GATA3*. In the *STAT4* gene, two exon polymorphisms were detected in exons 16 and 17, the first (C42113438T) causing an amino acid substitution and the other being a synonymous SNP. The nonsynonymous SNP in exon 16 was rare and was identified in one of the studied individuals as a heteroduplex in DHPLC screening and verified by sequencing. The remainder of the *STAT4* polymorphisms were located in noncoding sequences. Since no additional coding variants leading to amino acid changes were identified, it suggests the exon structures of the screened genes are highly conserved. This is also consistent with the previously published SNP screening studies for the *STAT6* gene (Gao *et al*. 2000, Heinzmann *et al*. 2000, Tamura *et al*. 2001, Duetsch *et al*. 2002,

Nagarkatti & Ghosh.2002). At the time of submission of the manuscript, no SNP screening studies were published for the *GATA3* or *STAT4* genes. Recently, Park *et al*. (2005) have identified 12 sequence variants of *STAT4*. However, none of the published polymorphisms were located in the coding sequences.

1.2 The association analysis of STAT6, GATA3 and STAT4 SNPs for human asthma and high s-IgE

All the 23 identified SNPs were chosen for the association analysis. Genotyping was performed using the automated SNapShot primer extension method. The DNA material for the association analysis consisted of a total of 120 Finnish asthmatic trio families (360 individuals), representing a subset of the cohort recruited from central eastern Finland (Kainuu province) based on asthma (Laitinen *et al*. 1997, Kauppi *et al*. 1998). Phenotypes available were the asthma status, total s-IgE and blood eosinophils. The Kainuu study population represents a Finnish population isolate and its population history has also been extensively studied (de la Chapelle 1993, Laitinen *et al*. 1997, Kauppi *et al*. 1998). Founder population as a material is thought to be more advantageous compared to outbred populations when studying complex disorders and genetic linkage due to smaller number of expected susceptibility alleles (Kruglyak 1999). All the SNPs detected in the *STAT6* gene were relatively common among the genotyped subjects (28–50%). In GATA3, three out of nine SNPs were rare (<5%), while in *STAT4* all except one SNP were rare (<6%). The genotyping success rate varied from 94.8 to 99.1%. All the markers were in Hardy-Weinberg equilibrium, and Mendel errors were found <0.01%.

Possible association of genotyped SNPs and asthma or high (>100kU/L) serum total IgE level was examined for individual SNPs and SNP haplotypes. The data set revealed 199 affected and 161 control chromosomes for asthma; moreover, there were 273 high serum IgE response and 199 low IgE response-associated chromosomes. The study resulted in no significant allele association for any of the individual SNP loci studied. The most common haplotypes in the *STAT6*, *GATA3* and *STAT4* genes in affected and controls are shown in Tables E1-E3 *(Report I, supplementary data)*. The most common haplotypes formed the majority of haplotype diversity of both affected and control individuals. For *STAT6* and *GATA3*, the three most common haplotypes formed 66% and 63% of all haplotypes, respectively, whereas for *STAT4,* the two most common haplotypes formed 93% of all haplotypes *(Report I, supplementary data)*. The genotyped markers were inherited in linkage disequilibrium (LD). For either *STAT6* or *STAT4*, no haplotype association was found in the study. For the *STAT6* gene, the linkage of chromosome 12q microsatellite markes to asthma was also investigated, in this case by combining our *STAT6* SNP marker data with the previously reported data from the Finnish Genome centre. As a result, no evidence of linkage was observed in the nonparametric linkage (NPL) analysis of a total 15 microsatellite and five SNP markers in the Finnish study population (unpublished).

Interestingly, for the *GATA3* gene, the three potentially associated haplotypes were identified in the family data in HPM haplotype analysis when different asthma-

associated phenotypes were compared *(Report I, Table II)*. Six out of nine alleles were shared between the three partially differing haplotypes. One of the haplotypes, CGTAGCTCC, was observed to associate with high total s-IgE levels. This haplotype was studied further by dividing the study subjects into haplotype carriers and non-carriers. To analyse the association with quantitative methods, the total s-IgE values were transformed into a normally distributed form by $\log_e$ transformation and adjusted for age and gender. As a result, haplotype CGTAGCTCC carriers showed significant association with elevated total s-IgE levels in the t-test *(Report I, Table III)* as well as in linear regression analysis ($p=0.01$). Age, gender, and asthma status were used as covariates. Most of the results remained statistically significant after 10 000 permutations.

Since multiple testing can cause problems, often false positive results, in association analyses of common traits, we decided to evaluate the significance of the results by genotyping the *GATA3* SNP markers in independent population-based study material. The DNA material consisted of a Finnish case-control data set including 245 asthma patients and 405 controls (Karjalainen *et al*. 2002). The studied phenotypes for this cohort were asthma status, total s-IgE level, skin prick test results and blood eosinophil levels. This data set also expresses very similar clinical characteristics (onset of chronic asthma in adulthood and demand for daily medication) as the previously analysed families. For the population-based data set where study individuals are unrelated, the haplotypes were constructed from the data using the SNPhap algorithm, a program that helps to estimate the haplotype frequencies from population samples (http://www-gene.cimr.cam.ac.uk/clayton/software/).

The frequencies of the observed haplotypes in the family data set and predicted haplotypes in the case-control data set were comparable. Possibly due to the nature of the ethnic backgrounds of the two data sets analysed, isolated and outbred populations, there were allelic differences between the related haplotypes. Interestingly, one of the three haplotypes already showing association in the family data was also observed to associate in the replication case-control data set *(Report I, Table II)*, which suggests the potential importance of this haplotype in susceptibility to asthma-related traits.

Conclusions regarding the role of the SNPs identified in the human *STAT6*, *GATA3* or *STAT4* genes are difficult to make. All the SNPs we found in *STAT6* had been previously reported (Gao *et al*. 2000, Duetsch *et al.* 2002) and are relatively common (frequency >22 %) *(Report I, Table I)* (Marth *et al.* 2001). Thus far, several reports have been published in which association of genetic variants in the STAT6 gene and asthma or related traits have been investigated (see page 58, Table II). Duetsch *et al*. (2002) reported the widest SNP screening for the gene, in which association of altogether 16 SNPs were studied for asthma and related traits. In our study, not all the publicly described variants were included since the genotyping was concentrated on the SNPs found among the Finnish individuals (Table II). For instance, we did not genotype the GT repeat in the first exon of *STAT6*, previously reported to associate with elevated eosinophil levels in Caucasian populations (Duetsch *et al*. 2002) as well as associating with allergic phenotypes and childhood asthma in Japanese populations

(Tamura *et al*. 2001, Shao *et al*. 2004). Intron 18 polymorphism of the *STAT6* locus has previously shown association with total s-IgE levels in Caucasian populations (Duetsch *et al*. 2002). However, in our study, the intron 18 SNP was not identified from the screened samples. We found no positive association for the intron 2 SNP (rs324011), reported to be associated with serum IgE levels in the German population (Weidinger *et al*. 2004). According to the study it was hypothesised that it was located in the NF-κB (nuclear factor-kappa B) binding region, where it was suggested that it alters the NF-κB binding properties. Moreover, in our Finnish study material, we did not find a positive association between the 3´UTR SNP G19633406A, previously reported to be associated with mild atopic asthma (Gao *et al*. 2000). However, some negative reports of the role of this variant have also been published (Duetsch *et al.* 2002, Tamura *et al*. 2001, Shao *et al*. 2004, Li *et al*. 2007) supporting our findings and suggesting that this variant does not seem to have an important contribution to the genetics of asthma or atopy, at least in the populations studied. Therefore, even though STAT6 is a crucial mediator of Th cell differentiation, the contribution of polymorphisms in the gene locus is so far unclear and both expression level and transcription factor binding assays are needed to elucidate their role. However, ethnic differences between the studied populations may cause variations to the results and make their interpretation more complicated.

For both the *GATA3* and *STAT4* genes, there were no previously reported asthma association analyses at the time of the publication of our results. For the *STAT4* gene, there are currently two published asthma association studies (Park *et al*. 2005, Li *et al*. 2007). In the previous report, the association of seven common SNPs in the gene was investigated with reference to the risk of asthma in Korea asthmatic patients. No association was found when age, gender and smoking status were analysed as covariates. However, when association with specific IgE production to mite allergens was investigated, a SNP in intron 11 of the *STAT4* locus and two haplotypes showed positive associations, which were, however, lost after Bonferroni corrections (Park *et al*. 2005). The latter study, in which the intron 11 polymorphisms also showed association with asthma in Chinese population, supports the putative role of this variant in the predisposition to asthma. Recently Remmers *et al*. (2007) also reported an association between the haplotype in the third intron of *STAT4* and susceptibility to rheumatoid arthritis in North American and Swedish populations. The inconsistencies between these reports and our work can, however, be explained by ethnic differences or the statistical tests used and, to assure the role of intronic variants in the *STAT4* gene, other study populations should also be investigated.

To our knowledge, in addition to our study there are no other SNP association analyses for *GATA3* and asthma-related traits. Li *et al*. (2006) determined whether sequence variants in murine *GATA3* gene were present between mice with genetic susceptibility to AHR and less responsive C3H/HeJ mice. However, even though sequence variants were detected, no functional polymorphism was detected between the strains. Since we found a positive association with asthma and related traits, it would be interesting to know the biological significance of the *GATA3* polymorphisms. However, since the identified *GATA3* variants were non-coding and were mostly located in the promoter or

intronic sequences and not in the functional domains of the gene *(Report I)*, elucidation of their functional role is not straightforward. Therefore, further studies, for instance at the mRNA level, are needed to elucidate the biological importance of the SNPs.

Taken together, for *STAT6*, *GATA3* and *STAT4* genes, the present study is the first asthma association study among Finnish asthma families. It was observed that SNPs in these genes do not have a significant role in the predisposition to asthma and high serum IgE, at least in the Finnish population. Previously, SNP screening for *GATA3* counter regulator, T-bet, was also carried out in our laboratory (Ylikoski *et al*. 2004). Altogether, 23 SNPs were identified in the gene; however, none of the variants coded for an amino acid change and no positive association between *T-bet* SNPs and asthma-related traits was found, based on a total of 15 SNPs genotyped in this same patient cohort used in our study. In the *GATA3* study, it seems that haplotypes rather than single variants were responsible for association to asthma-related traits as a putative haplotype association to high total s-IgE as well as asthma traits was identified.

Table II (next page). The identified variants in the human STAT6 gene in different asthma and asthma-related trait association studies (Gao *et al.* 2000, Tamura *et al.* 2001, Amoli *et al.* 2002, Duetsch *et al.* 2002, Weidinger *et al.* 2004, Schedel *et al.* 2004, Pykäläinen *et al.* 2005, Negoro *et al.* 2006, Li *et al.* 2007, Yabiku *et al.* 2007). The variants significantly associated with asthma or related traits in different populations are shown in grey.

Variation	Position on NT_029419 /AF067572	Allele	Gao et al. 2000 Japanese (n=300) British (n=400)	Tamura et al. 2001 Japanese (n=60)	Amoli et al. 2002 Caucasian (UK) (n=71)	Duetsch et al. 2002 Caucasian (n=948)	Weidinger et al. 2004 German (n=1407)	Schedel et al. 2004 German (n=1120)	Pykäläinen et al. 2005 Finnish (n=360)	Negoro et al. 2006 Japanese (n=240)	Li et al. 2007 Chinese (n=180)	Yabiku et al. 2007 Japanese (n=333)
5´flanking	G1882A	G A				no association						
5´flanking	C2438T	C T				no association	no association					
exon 1 GT repeat	exon 1			allergic diseases		eosinophilia						s-IgE in patients with Grave´s autoim. disease
intron 2	G19647081A /C5021T	C T				no association			no association			
intron 2	C5814T	C T				no association						
intron 2	G19645488A /C6613T	C T				s-IgE (weak)	total IgE	s-IgE	no association			
intron 8	C9114T	C T				no association						
intron 8	C9234G	C G				no association						
intron 12	C105936T	C T				no association						
intron 16	A763C	A C				no association						
intron 17	A1309G	A G				s-IgE, weak						
intron 18	C1570T	C T				s-IgE	no association					
3´UTR	G19633406A /G4219A	G A	atopic asthma (Japanese) no association (British)	no association	risk of nut allergy	no association			no association	correlated with s-IgE and eosinophil levels	no association	
3´UTR	A19633134G /A4491G	A G							no association			
3´UTR	A4610G	A G				no association	no association					
3´UTR	A19632954G /A4671G	A G				s-IgE (weak)	no association	s-IgE	no association			
3´UTR	A4703G	A G				no association						

2 Establishment of a novel method for human primary CD4+ T cell gene transfection (II)

2.1 Transfection efficiency and purity of nucleofected human primary CD4+ T cells

Human primary T lymphocytes have been difficult to transfect, especially with non-viral approaches. Thus, viral systems, such as lentiviral or retrovirus-based vectors, have been often used for introducing foreign DNA into cells (Rudoll *et al*. 1996, Costello *et al*. 2000, Bai *et al*. 2003, , Marodon *et al*. 2003, Qin *et al*. 2003). However, even though reasonable transfection efficiencies have been often obtained with viral systems, the approach has faced some problems, such as safety issues. In addition, producing viruses is often also time consuming and the viral vectors may have harmful effects upon the cells or cell-signalling pathways (Flaherty *et al*. 2004). Thus, we were interested in further optimizing the non-viral plasmid-based method for gene overexpression or siRNA knockdown studies. Electroporation is a non-viral method which has been successfully used for transfecting human primary T cells (Herndon *et al*. 2002, Zhao *et al*. 2006). We have used the recently-introduced Nucleofector™ technology, based on an improved electroporation method where an electric pulse is given to the cells, for transfection human primary T cells. The method has been used for many hard-to-transfect cell lines (Lai *et al*. 2003, Martinet *et al*. 2003, Trompeter *et al*. 2003, Maasho *et al*. 2004, Schakowski *et al*. 2004). Our previous experiments have shown that, since human primary CD4+ T cells are difficult to transfect, the population of untransfected cells is often >50%. When cultured, these cells also usually proliferate more efficiently compared to transfected cells, thus causing noise in the results obtained when the effects of a transient transfection of a certain gene or shRNA transfection have been measured in the cultured cells (unpublished). Thus, the enrichment of transfected cells is crucial in order to increase the purity of the cell population. Fluorescence-activated cell sorting (FACS) and vectors containing Green Fluorescent Protein (GFP) are ways to sort the cells of interest. However, using the FACS-based method is time-consuming. Further, the GFP-based system, even though widely used, can cause unwanted activating effects on cellular signalling events and thus influence the data (Zhang *et al*. 2003). In our previous experiments, the STAT6 and GATA3 genes were cloned into pIRES-EGFP vectors and overexpressed in human CD4+ peripheral blood cells by transfecting the cells using nucleofection (unpublished). Since we faced some problems with the viability of GFP vector-transfected primary cells after sorting, we were interested in developing alternative ways to more efficiently enrich the number of transfected cells.

Thus, our aim was to design and optimize a cell surface H-2K^k-marker-encoding plasmid as a vector for CD4+ T cell nucleofection studies. In the optimized system, a truncated mouse H-2K^k cell surface molecule is located in the same plasmid vector as the transfected gene/shRNA and is thus efficiently cotransfected with a gene of interest. As the selection marker is a truncated receptor, it does not interfere with the cell functions. During the protocol, nucleofected cells can be magnetically enriched

based on their H-2K^k expression using antibodies conjugated to magnetic particles. The protocol was therefore tested and optimized for H-2K^k-positive cell selection of transfected T cells. With optimizing an H-2K^k selection method for the nucleofection technique, highly pure (>97%) populations of the transfected human CD4+ T lymphocytes are obtained. This is ideal for generating longer-lasting (7-8 days) Th cell cultures under Th1 or Th2 polarizing conditions for further functional studies in which the influence of the selected genes of interest will be studied.

The transfection efficiency of the nucleofected primary human CD4+ T cells with the H-2K^k plasmid constructs varied from 24-51% when the cells were transfected with the pIRES2-H-2K^k overexpression vector. Similarly, the efficiency was between 43-69% when the pSuper-H-2K^k-Stat6-shRNA or pSuper-H-2K^k-scramble vector was used *(Report II)*. The purity of the H-2K^k-positive cells was > 97 % after the enrichment *(Report II, Figure 2b)*.

2.2 The nucleofected and enriched CD4+ T cells are able to differentiate in Th1 and Th2 direction

To study whether the nucleofected and enriched pure H-2K^k-positive T cell cultures were still able to proliferate and *in vitro* differentiate towards Th1 and Th2 cells under the polarizing conditions, both RT-PCR and flow cytometry were used to determine the expression of Th1/Th2 marker genes and the intracellular cytokine production profiles of the cells, respectively. The expression of Th1 and Th2 marker genes in the cells was validated with RT-PCR after 5-8 days of culturing. IL-12Rβ2 and IFNγ, known to be preferentially expressed in Th1 cells compared to Th2 cells, were studied as Th1 cell markers, whereas GATA3, being expressed more in Th2 cells, was studied as a Th2 marker (Lederer 1996, Rogge *et al*. 1997, Zheng and Flavell 1997). According to our results, the nucleofected and enriched H-2K^k-positive cells polarized towards the Th1 direction express more IFNγ and IL-12Rβ2 when compared to the cells polarized towards the Th2 direction. In the cells polarized towards Th2 the levels of GATA3 expression were higher compared to Th1 cells. This was seen both in H-2K^k-positive samples as well as in controls *(Report II, Figure 3)*.

After 7-8 days of polarization, the intracellular cytokine staining was performed for both the enriched human primary CD4+ cells overexpressing the pIRES2-H-2K^k plasmid and for the non-nucleofected control cells, both of which were *in vitro* activated and cultured in Th1 and Th2 polarizing contiditions. IFNγ and IL-4 are the hallmark cytokines produced by Th1 and Th2 cells, respectively, and the determination of the cytokine profiles of these two cell subsets is used to phenotypically distinguish them from each other. Thus, IFNγ and IL-4 production profiles of the cells were analysed in order to determine whether the cells were able to properly differentiate towards Th1 or Th2. As a result, both in control samples and in nucleofected H-2K^k-positive samples, the number of IFNγ producing cells was higher, and the number of IL-4 producing cells was negligible in Th1 cultures *(Report II, Table II)*. In Th2-polarizing conditions, the number of IL-4-producing cells was higher and the cells produced less IFNγ compared to the Th1 cultures. However, the cytokine production

profiles varied between different individuals. The amounts of IL-4 producing cells were quite low in all of the Th2 cultures. However, according to previous reports, IL-4 production is typically less than 10% after the first round of human Th2 cell polarization (Sornasse *et al*. 1996).

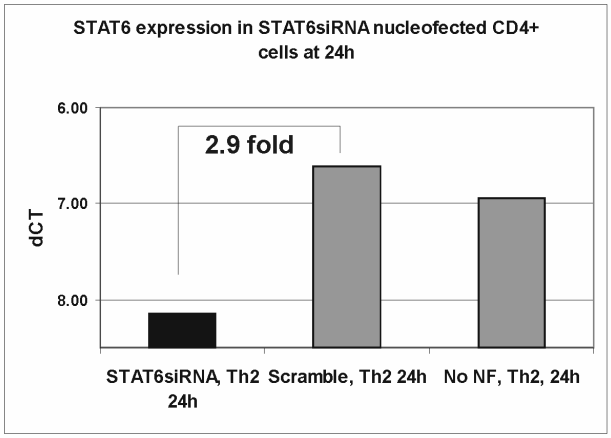
2.3 Nucleofection of pSuper-H-2K^k-Stat6-shRNA into primary human T helper cells impairs Th2 differentiation

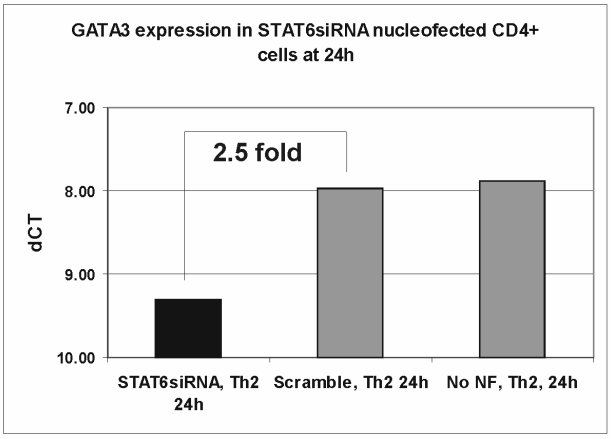
To test whether the optimized nucleofection and enrichment method is also applicable for gene knockdown experiments, a pSuper RNA interference (RNAi) plasmid was modified to create a pSuper-H-2K^k vector *(report II)*. Thereafter, a short hairpin RNA (shRNA) targeting STAT6, a key molecule driving the Th2 cell differentiation, was subcloned into pSuper-H-2K^k vector.

The functionality of the used pSuper-H-2K^k-STAT6-shRNA construct was clearly verified at the protein level in a Jurkat cell line 48 and 72h after transfection *(Report II, Figure 4a)*. To study the influence of STAT6 knockdown during the human Th2 cell differentiation, pSuper-H-2K^k-STAT6-shRNA was introduced in the human primary T helper cells using a nucleofection procedure and the H-2K^k-positive cells were enriched and polarized as previously described. As with the overexpressed cells, both the pSuper-H-2K^k-STAT6-shRNA and the pSuper-H-2K^k-scramble-shRNA (shRNA control) nucleofected and non-nucleofected cells were *in vitro* polarized towards Th1 and Th2. The functionality of Stat6-shRNA in these cells was verified after 24 hours of polarization by measuring the Stat6 mRNA levels with TaqMan RT-PCR. The level of STAT6 mRNA was decreased by 2.9-fold in the STAT6-shRNA sample compared to the control (scramble). The influence of the STAT6 knockdown can also be seen in the expression levels of the STAT6-target gene GATA3 as well as in the levels of IFNγ (see Figure 7, unpublished).

After 7 days of polarization, the intracellular cytokine staining and RT-PCR were performed to characterize how siRNA knockdown influences the cytokine and gene expression profiles in the *in vitro* polarized human Th1/2 cell cultures. The expression of GATA3, IFNγ and IL-12Rβ2 were measured in the samples by RT-PCR. As previously shown, in response to IL-4, STAT6 rapidly induces the expression of GATA3. GATA3, in turn, has been shown to inhibit IL-12Rβ2 expression and to act as a repressor of Th1 development and IFNγ production (Ouyang *et al*. 1998, Kurata *et al*. 1999). As expected, the expression of GATA3 was reduced in Th2 cells expressing the STAT6-shRNA compared to the control cells *(Report II, Figure 4b)*. Furthermore, both IFNγ and IL-12Rβ2 were up-regulated at mRNA level in Th2 cells in response to down-regulation of STAT6 *(Report II, Figure 4b)*.

As expected, since STAT6 is a key molecule promoting the Th2 cell differentiation, in addition to the RT-PCR results, the cytokine and the gene expression profiles of the Th2 cells were also skewed towards Th1 type. When Th2 cultures expressing Stat6-shRNA and scramble-shRNA were compared, STAT6 downregulation resulted in a decrease in IL-4 production and an increase in IFNγ production by the Th2 cells. *(Report II, Figure 4c)*.





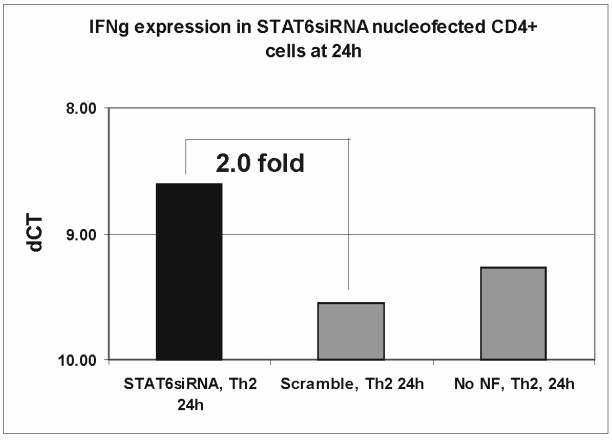


Figure 7. The levels of STAT6, GATA3 and IFNγ mRNAs in a Stat6-shRNA sample compared to the control (scramble-shRNA) after 24 hours of Th2 cell polarization (unpublished data). No NF= no nucleofected.

2.4 Advantages of the optimized method

Using cell transfection, the effects of an introduced transgene can be followed in the different functions of cells in controlled conditions. Over the years, several techniques, including lipofection, calcium phosphate precipitation, electroporation and viral infection, have been utilized. Since the effective transfection of human primary T lymphocytes has been problematic, in particular when using non-viral approaches, our optimized method enables functional studies to be carried out on the given gene of interest in T helper cell differentiation. Manipulation of gene expression in primary T cell cultures is an important step towards understanding the molecular mechanisms and the role of a selected gene of interest in the differentiation of $CD4^+$ T helper cells towards the Th1 and Th2 subtypes.

Taken as a whole, our experimental system enables efficient transfection and enrichment of the human Th cells expressing the selection plasmid. The designed plasmid pIRES2-H-$2K^k$ construct allows both the gene of interest and the H-$2K^k$ gene to be translated from a single bicistronic mRNA and thereby makes an efficient cotransfection possible. Besides gene overexpression, by modifying the pSuper RNAi plasmid to create a pSuper-H-$2K^k$ vector, the H-$2K^k$ system can be also utilized for gene RNAi knockdown studies in isolated human CD4+ T cells. With the optimized H-$2K^k$ enrichment method, highly pure (>97-99%) populations of the transfected human CD4+ T lymphocytes are obtained for further functional studies.

3 Genome-wide identification of novel genes involved in early Th1 and Th2 cell differentiation (III)

3.1 Identification of genes regulated by IL-12 and IL-4 during the early polarization of human Th1/Th2 cells

Affymetrix oligonucleotide arrays were used to obtain a genome-wide view of the early steps of the human Th cell differentiation process. Previously, gene microarray approach has been utilized for studying the differential gene expression between the human or murine CD4+ Th1 and Th2 lymphocytes (Rogge *et al*. 2000, Chtanova *et al*. 2001, Lu *et al*. 2003, Freishtat *et al*. 2005) as well as for identification of the transcriptome expression in CD4+ or CD8+ T cells upon activation (Hess *et al*. 2004, Stentz & Kitabchi 2004, Shu *et al*. 2006). Lund *et al*. (2003b, 2005) have used Affymetrix oligonucleotide arrays for identification of genes differentially regulated by IL-12, IL-4 or TGFβ during the early differentiation of human Th1 and Th2 cells. However, since many of the previous studies have been carried out in mice and unknown factors are still likely to be involved in the human Th cell differentiation process, we have extended the previous study by Lund *et al*. (2003b, 2005) by using the genome-wide arrays, (Affymetrix human genome U133A and U133B) recognizing approximately 33 000 transcripts, for examining whether there are novel, previously unreported factors regulated by IL-12 and IL-4. Furthermore, the study focussed on identification of the factors whose expression is differentially regulated during the

early stages (0-48h) of human Th cell differentiation. Interestingly, among the genes identified there may be novel players directing the differentiation process.

In the study, the previously prepared samples (Lund *et al*. 2003b, 2005), activated and polarized towards Th1 and Th2 type of cells for 2, 6 and 48h, were rehybridized with the genome-wide arrays. Two biological replicates for each array were performed. Furthermore, the genes coregulated by TGFβ and IL-12 or IL-4 during the early polarization were also investigated. Our study resulted in the identification of numerous genes, including many novel players, and ESTs with a known or unknown function in human T helper cell differentiation. Altogether, 171 genes or ESTs were shown to be regulated by IL-12 or IL-4 during the early differentiation of human Th1 and Th2 cells in addition to the previous results by Lund *et al*. (2003b, 2005) (*Report III*).

3.2 Genes regulated by IL-12

The response to IL-12 was observed to be fairly slow, and there were no genes regulated by IL-12 at the 2h time point of Th1 polarization. This was consistent with the previous study (Lund *et al*. 2005). After 6h, a weak induction by IL-12 was observed for the genes encoding T-bet and GTPase GBP5 *(Report III, Table II)*. However, after 48h, the effects of IL-12 became clear and a total of 41 genes were regulated by this cytokine *(Report III, Table II)*. These include the genes *SOCS3*, *CEBPB* and *IL7R* that had a previously described role in Th1 and Th2 cell responses (Li-Weber *et al*. 1997, Chtanova *et al*. 2001, Seki *et al*. 2003). Interestingly, many of the identified genes were novel in this context. The majority of the IL-12-induced genes were also regulated by IL-4 and/or activation *(Report III, Figure 1A)*. Moreover, a subset of genes, including *GIMAP4*, *IL7R*, *PPAP2A*, *MGC4677* and *GBP5*, were regulated in an opposite manner by IL-12 and IL-4.

3.3 Genes regulated by IL-4

The effects of IL-4 were seen earlier compared to the IL-12-induced effects, after only 2h of polarization. According to the results, a total of 153 genes were regulated during the early Th2 polarization (*Report III, Figure 1A-B*). Similarly to the IL-12 regulated genes, the majority of the genes identified did not have a previously described role in the Th cell differentition. The genes *CD47, PRNP, SOCS3, FOSL2, COL6A3, PTPRA, TNFSF10, IL10, CCR7, CEBPB, IL7R, GBP3* and *TBX21* have previously been linked to the process (Li-Weber *et al*. 1997, Rincon *et al*. 1999, Avice *et al*. 2000, Rogge *et al*. 2000, Szabo *et al*. 2000, Chtanova *et al*. 2001, Nagai *et al*. 2001, Chen *et al*. 2003, Seki *et al*. 2003). Interestingly, for a subset of genes, regulation by IL-4 was observed at all of the time points (2, 6 and 48h) (*Report III, Table III*), thus suggesting the importance of these genes for the early Th2 differentiation. These genes include *AI969697*, *NDFIP2*, *CD47*, *AKAP2*, *CISH*, *TMEM49*, *DACT1* and *ELL2*.

3.4 Genes co-regulated by IL-12/IL-4 and TGFβ

The influence of the immunosuppressive factor and known inhibitor of Th cell differentiation, TGFβ, was also studied during the early polarization of Th cells. In

addition to the previously identified TGFβ-regulated genes by Lund *et al*. (2003b, 2005) in human Th differentiation, we identified 110 additional genes, including 63 genes co-regulated by TGFβ and IL-12/IL-4. A total of 25 genes were co-regulated by TGFβ and IL-12 (*Report III, Table IV*), and the effect of TGFβ in Th1 polarizing conditions was either enhancing (5 genes) or antagonizing (19 genes); in Th2 conditions, in turn, the expression of altogether 21 genes was enhanced and that of 25 genes antagonized in the presence of TGFβ (*Report III, Table V*). Co-regulation by TGFβ and both IL-12 and IL-4 (up-regulation) was seen for the genes *ZBED2*, *LMNA, NDFIP2, TncRNA, SYTL3, SLC2A3* and *TRIB3*, of which the expression of *ZBED2, LMNA* and *NDFIP2* was also upregulated by TGFβ. However, for the genes *TncRNA, SYTL3, SLC2A3* and *TRIB3*, up-regulated by IL-12 and IL-4, a repressive effect by TGFβ was observed. As TGFβ is known to be tightly involved in the T regulatory as well as in Th17 cell differentiation, it would be interesting to elucidate the potential role of these identified genes in the functions of these newer subsets of Th cells.

3.5 Validation of the oligonucleotide array results by RT-PCR and Western blot

To validate the Affymetrix gene expression results, the expression of a subset of genes was studied in the Th1 or Th2 polarizing conditions using RT-PCR during various time points up to 7 days. As IL-12 had little effect at the initiation of the differentiation, our special focus was on the genes or ESTs regulated by IL-4. The selected panel for the studies included a total of 14 ESTs or genes which were rapidly regulated by IL-4 according to the Affymetrix results (*AA088177, AA237039, AA489100, AI494573, AW139719, AW152437, AW629527, AL389942, BF056901*, *R98767*, *BE748563, AI674404, ZNF443*, *DACT1)*. Moreover, these genes were also previously poorly characterized. For 12 out of 14 genes/ESTs, the IL-4 mediated regulation was confirmed by RT-PCR (*Report III, Figure 2*). For *BF056901* and *AI674404,* the Affymetrix results could not be confirmed.

In addition, at the protein level, validation of the differences in gene expression under Th1/Th2 conditions was performed for the genes that had commercial antibodies available. However, since most of the genes identified in this study were poorly characterized, only a limited number of antibodies were obtained. The proteins studied included *DACT1, RAB11FIP1, FOSL2, CISH, KLF6, FLT1 and RAB27B*. According to our results, *FOSL2* was differentially expressed at the protein level, with a higher expression in Th2 cells detected at 48 hours (*Report III, Figure 3*). FOSL2 is a member of the Fos family which can dimerize with the Jun family proteins, forming the AP-1 transcription factor complex (Nishina *et al*. 1990, Chen *et al*. 1999). The role of AP-1 factors in the Th1/Th2 differentiation process has previously been discussed (Rincon *et al*. 1997). Working in the AP-1 complex, FOSL2 has also been reported to be important for the regulation of cytokine IL-5 synthesis (Schwenger *et al*. 2002). TBX21 and GATA3 were used as control genes to confirm the polarization of the cells towares the Th1 or Th2 direction. There were no differences in the expression levels of other proteins during early polarization of Th1 or Th2 cells (data not shown). As seen also previously, the transcriptional differences are not always seen at the protein level (Lund *et al*. 2005). However, the lack of properly working antibodies at the time of

study also had an influence upon the results, and, thus, additional functional studies with the identified genes, including FOSL2, at the protein level would be of interest.

3.6 Genes regulated by IL-4/STAT6

Since very little was known about the selected panel of IL-4 induced genes and ESTs (described in the previous chapter), we were interested to study whether these novel factors are regulated through IL-4/STAT6 signalling. Thus, CD4+ cells derived from human peripheral blood were nucleofected with two different pSuper-H2K-STAT6-shRNA plasmids or a control (pSuper-H2K-scramble-shRNA) plasmid, cultured *in vitro* under Th2 polarizing conditions and harvested after 24h or 48h of differentiation. To investigate the effects of STAT6 knockdown on the expression of the selected genes or ESTs regulated by IL-4, mRNA expression was measured from the samples with quantitative, or SYBR Green-based, RT-PCR. According to our results, a subset of the genes/ESTs, including *AW629527*, *AA088177*, *AA489100*, *ZNF443* and *DACT1* was clearly dependent on STAT6 in 3-4 biological replicates. The functionality of the pSuper-H2K-STAT6-shRNA was validated by RT-PCR and *STAT6* and *GATA3* mRNA levels were down-regulated by the shRNAs (*Report III, Figure 4*). Previously, Chen *et al*. (2003) have reported the genes regulated by IL-4/STAT6 dependent manner in murine Th cells and Schroder *et al*. (2002) in murine B lymphocytes. Importantly, our data in the human *in vitro* polarized Th cells provides new information about the STAT6-regulated genes in the human system.

3.7 NDFIP2 promotes IFNγ production in human Th1 cells

NDFIP2 was one of the genes regulated by IL-4 during the early Th2 differentiation (2h, 6h, 48h) (*Report III, Table III*). NDFIP2 was observed to be highly up-regulated in response to activation alone as previously described in T cells (Cristillo *et al*. 2003). Using RT-PCR studies, the differential mRNA expression of NDFIP2 was also seen between the Th1 and Th2 cells polarized for 7 days (*Report III, Figure 5A*). As the role of NDFIP2 was unknown in Th cell differentiation, it was selected for further functional studies. Using the optimized nucleofection and H-2k^k selection method (described in Chapter 2), the influence of NDFIP2 for the differentiation and cytokine production of the *in vitro* polarized human Th1/Th2 cells was investigated with specific shRNA-mediated gene silencing. Peripheral-blood-derived human CD4+ T cells were nucleofected with a pSuper-pIRES2-H2K-NDFIP2-shRNA or pSuper-pIRES2-H2K-scramble-shRNA control vector and the enriched transfected cells were activated and induced to polarize towards the Th1 or Th2 direction with the presence of the polarizing cytokines IL-12 and IL-4 for 7 days. After PMA/ionomycin stimulation, the cytokine production of the non-transfected Th1 and Th2 cells as well as cells expressing scramble-shRNA or NDFIP2-shRNA was measured using the Luminex cytokine assay.

According to the results from three biological replicates, knockdown of NDFIP2 down-regulated IFNγ production by the polarized human Th1 cells, thus suggesting the possible role of NDFIP2 in the differentiation process by promoting the production of

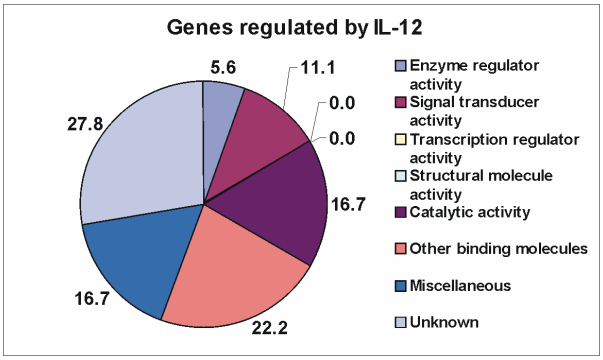
the hallmark Th1 cytokine, IFNγ. However, no clear conclusions can yet be made about the role of this protein without additional functional studies. According to previous reports, NDFIP2 belongs to a conserved group of Nedd4-interacting proteins that are crucial enzymes in ubiquitin-mediated protein sorting, and functions as an ubiquitin-protein ligase (Konstas *et al*. 2002, Cristillo *et al*. 2003, Shearwin-Wyatt *et al*. 2004). NDFIP2 has also been reported to predominantly localize to the Golgi complex, Golgi-derived vesicles and multivesicular bodies (MVB), thus suggesting it has a role in the regulation of intracellular protein trafficking, probably by modulating the Nedd4 ubiquitin activities (Cristillo *et al*. 2003, Shearwin-Wyatt *et al*. 2004). Overexpressed and endogenous NDFIP2 has also been reported to localize to the endoplasmic reticulum (ER) and mitochondria (Cristillo *et al*. 2003). Konstas *et al*. (2002) reported that NDFIP2 regulates the the expression of the epithelial sodium channel (ENaC), the known target of Nedd4, and prevents its sodium feedback inhibition. Furthermore, Shearwin-Wyatt *et al*. (2004) showed that, when ectopically expressed, NDFIP2 inhibits receptor-mediated endocytosis of the epidermal growth factor (EGF) and may affect EGF receptor (EFGR) uptake by regulating receptor recycling and endocytosis within the MVB. The exact mechanism of the enhancing effect of NDFIP2 for IFNγ production needs to be further elucidated. It is possible that NDFIP2 regulates cytokine production through some novel mechanism related to protein trafficking. However, it can be also hypothesized to have similar functions as described by Shearwin-Wyatt *et al*. (2004) in IFNγR-mediated trafficking, but these need futher experimentation. One of the possible mechanisms for NDFIP2-regulated Th cell differentiation and IFNγ production may also involve the NF-κB pathway, as NDFIP2 has been suggested to activate NF-κB signalling (Matsuda *et al*. 2003). As recently reported by Oliver *et al*. (2006), the loss of NDFIP2 family member NDFIP1 in T cells biased the cells towards a Th2 phenotype, supporting our finding that the NDFIP members may be involved in the Th1/Th2 cytokine production. The fact that NDFIP2 is preferentially induced during early Th2 differentiation and also regulates IFNγ production by Th1 cells could be a matter of controversy. However, the Th cell differentiation and functions may involve many mechanisms, such as Th1/Th2 cytokine cross-regulation (Kriegel *et al*. 2006), that remain to be clarified.

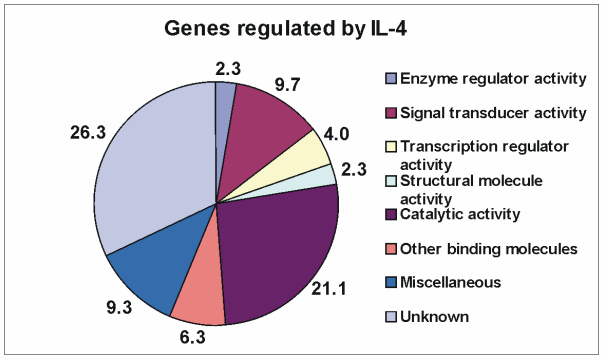
In conclusion, in the present work, a genome-wide oligonucleotide array analysis was performed in order to obtain a more comprehensive view of the genes regulated by IL-12 and IL-4 during the first two days of human Th cell differentiation. The screen resulted in the identification of 171 genes differentially regulated by the human *in vitro* polarized Th1 and Th2 cells during 0-48h of polarization. When added to the previous studies by our group, a total of 288 genes, representing ~1-1.5 % of the genes in the human genome, were observed to be differentially regulated by cytokines involved in early Th1 and Th2 polarization (Lund *et al*. 2003b, 2005) *(Report III)*.

3.8 Functions of the identified genes

To characterize the putative or known functions of the factors involved in early Th1 and Th2 cell differentiation, the newly identified genes were analysed with the GO (Gene Ontology) annotation tool in NetAffx (Liu *et al*. 2003) (Figure 8). Most of the

genes regulated during the early differentiation of Th1 and Th2 cells code for factors involved in the signal transduction from cell surface to the nucleus. Some of the identified genes are involved in well-known intracellular signalling cascades, such as RAS and GPCR. The genes *RAB27B, RAB30* and *RHOQ* were among the IL-4-regulated genes known to be involved in the RAS signalling pathway. The RAS pathway has also been previously shown to promote IL-4R signalling and be essential for Th2 responses *in vivo* (Yamashita *et al*. 1999, 2000, Shibata *et al*. 2002). GPCR signalling is implicated in the regulation of Th1 and Th2 responses playing a role in Th2-mediated diseases such as asthma (Johnson *et al*. 2002). Induction of GPCR signalling may also enhance the activation of the RAS pathway, thus promoting IL-4 signalling. Based on our current and previous studies (Lund *et al*. 2003b, 2005), a panel of factors involved in GPCR signalling are rapidly regulated in response to Th2 polarizing stimuli. Genes including *GNAI1*, *CD47*, *CXCR4, PTGER2, CYSLTR1, EDG1, EBI2, FLJ11856* and *HRH4* become up-regulated within 2 or 6h and *GPRK6* within 48h. In contrast, genes such as *CD97, PTGER4, ADORA2A, CCR7, GPR18, GPRK5* and *P2Y5* are rapidly repressed by IL-4 (Lund *et al*. 2003b, 2005). Another predominant functional group consisted of genes coding for receptors or receptor binding proteins. These included components of GPCR signalling (*CYSLTR1, HRH4, CD47, CCR7* and *GPR18*). Numerous transcriptional regulators, such as *ELL2, ZNF443, PHF20L1, LOC360030, EZH2, ZBED2, FOSL2* and *FRBZ1* also became differentially regulated during the early Th1 and Th2 differentiation. As expected, IL-12 and/or IL-4 regulated several genes involved in the immune response, such as *IL10, CCR7, CD7, CEBPB* and *IL7R*. Other common functional groups consisted of genes involved in apoptosis, cell proliferation, adhesion, metabolism, motility, cell cycle, protein localization, transport, and regulation of protein activity. Importantly, the function of the majority of the genes differentially regulated during early Th1 and Th2 cell differentiation was still unknown (Figure 8). Among these were numerous ESTs that were rapidly up-regulated by IL-4 in the cells induced to polarize towards the Th2 direction. These genes were given particular attention and were selected for further studies, in which their functions for Th cell differentiation will be elucidated. Our hypothesis is that the novel genes are likely to include factors that are critical regulators of the Th1 and Th2 differentiation process, together with the previously identified factors, such as STAT4, T-bet, STAT6 and GATA3. For instance, since the constant maintenance of the IL4-mediated regulation was observed for a subset of the identified genes, these are potential crucial factors in the Th2 cell differentiation process and definitely demand further characterization. This study provides a characterization of the gene regulation during early Th1 and Th2 cell differentiation and numerous new candidates for further studies. Although all of these genes may not be important for the differentiation process, our findings indicate that the regulation of the Th1 and Th2 differentiation process is much more complex than the current models suggest.





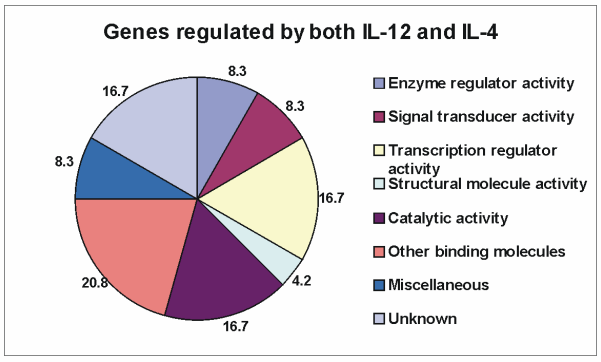


Figure 8. The known/unknown functions (%) of genes involved in the early Th1/Th2 polarization. The genes were analysed with the NetAffx gene ontology annotation tool.

SUMMARY AND CONCLUSIONS

The maturation and selective activation of functionally distinct subsets of Th lymphocytes (Th1, Th2) from human naïve CD4+ T cells are critical for proper immune responses. Pathogenic Th1/Th2 responses have also been linked to several immune-mediated diseases. For elucidating the immunopathogenesis of asthma, atopy and many other immune-mediated and inflammatory disorders, it is important to define the molecular mechanisms that are involved and that direct the differentiation process of human Th lymphocytes. To date, the detailed knowledge of the human Th cell biology is still fairly limited.

The first goal of this thesis was to investigate whether genetic variations in a panel of key factors involved in the Th cell differentiation, STAT6, GATA3 and STAT4, are associated with asthma or related traits in Finnish asthmatic families. Due to their importance in the differentiation process, all of the studied genes were relevant functional candidate genes for human asthma. It can be hypothesized that inherited errors in the key cellular processes may predispose certain individuals for asthma and allergy. Clear evidence of the genetic effect of atopic disoders has been reported to exist, and in order to elucidate the complex background of these disorders, several candidate gene analyses in different ethnic populations are needed. Comparison of polymorphic sites, such as SNPs within a gene, between cases and controls may be useful for establishing the role of the candidate gene in disease susceptibility. The study resulted in an identification of a total of 23 SNPs in the *STAT6*, *GATA3* and *STAT4* genes in the screened 28-44 chromosomes. No significant association for the individual polymorphisms and either asthma or high serum IgE levels was found when a total of 120 Finnish trio families, collected based on asthma, were studied. However, a haplotype analysis of *GATA3* showed an association to asthma and atopy-related phenotypes for three related haplotypes. The haplotype association was further confirmed in an independent Finnish case-control data set consisting of 245 asthma patients and 405 controls. The functional relevance of the identified polymorphisms will be of interest but needs further investigation. Our study is the first asthma association analysis among Finnish individuals for all of the candidate genes studied. Furthermore, for *GATA3*, no other SNP association studies have been yet published.

For studying the influence of a given gene of interest in human Th cell differentiation, an optimized method for human CD+ T cell transfection was established. The novel method can be utilized for further functional studies on how a given exogenously expressed or down-regulated gene influences T helper cell differentiation. With this newly established method, highly enriched (>97%) transiently transfected human primary T cell cultures can be obtained. Our results also show that the nucleofected H-2K^k-positive primary T helper cells are able to differentiate into Th1 and Th2 cells *in vitro*. In addition, when human primary T helper cells were nucleofected with pSuper-H-2K^k-Stat6-shRNA, an impaired Th2 cell differentiation was seen, thus suggesting the applicability of the optimized method for gene knockdown studies, too.

In the light of the present results, many factors not previously recognized may be involved in the Th cell differentiation process. Identification of novel genes differentially regulated during the early human Th cell polarization provides new insights into the process. A more comprehensive picture of the genes involved in the early events of human Th1 and Th2 cell polarization was obtained through a genome-wide oligonucleotide array analysis carried out after 0-48h of polarization. According to the results, a number of IL-12 and/or IL-4 regulated genes were identified, with either previously characterized or unknown functions. Interestingly, a majority of the identified genes were novel in the context of human Th cell differentiation and so of particular interest. A subset of the identified genes was chosen for further functional studies, in which their role in the IL-4/STAT6 signalling pathway or in early Th cell differentiation was studied. Through STAT6-shRNA knockdown studies, we demonstrated that a subset of the ESTs was regulated via IL4/STAT6. By using shRNA-mediated knockdown of human NDFIP2, its promoting effect for IFNγ production of the human polarized Th1 cells was observed. Further functional assays of the newly identified genes are needed to further determine their role in Th cell differentiation process.

To conclude, we have identified a panel of novel candidates and new susceptibility genes and pathways important for human Th cell differentiation and for the development of atopic diseases. Thus, the results provide a basis for functional studies that aim at characterizing the molecular mechanisms and signalling networks involved in human Th cell subset differentiation.

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