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REGULATORY MECHANISMS INVOLVED IN TH2 CELL DIFFERENTIATION

A PROTEOMICS APPROACH

by

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ABSTRACT

Asthma and allergies are prevalent and severe diseases that affect millions of individuals worldwide. *In vivo* animal studies indicate that interleukin-4 (IL-4) is important for the development and perpetuation of allergic asthma and allergy. It is needed for the proper development of Th2 type effector cells that have a key role in the onset of these diseases. IL-4 is also produced by Th2 cells and it promotes the subsequent stages of these diseases. The signal transducer and activator of transcription 6 (STAT6), which is activated by IL-4 stimulation, is especially needed to acquire and maintain Th2 type responses in antigen induced asthmatic airway inflammation.

The general aim of this doctoral thesis was to identify novel proteins important for Th2 cell differentiation using two-dimensional electrophoresis (2-DE) based proteomics and mass spectrometry. Undifferentiated T helper cells were isolated from human umbilical cord blood or mouse spleen. Cells were activated through T cell and co-stimulatory receptors and differentiated in the Th1 or Th2 direction with the polarizing cytokines IL-12 and IL-4, respectively. In the first study, the proteomes of *in vitro* differentiated human Th1 and Th2 cells were compared to identify differences in the protein expression levels or in the protein modifications. The subsequent studies focused on IL-4 induced protein regulation within 24 hours after T cell activation. In the first of these, IL-4 induced differences were identified from activated human T helper cells. IL-4 was found to regulate several proteins in caspase mediated pathways and to enhance T cell survival and activation. In the other, the proteomes of STAT6 deficient mouse lymphocytes were compared to the wild-type control cells after T cell activation and IL-4 stimulation. In these studies overall, we have characterized several new IL-4 and STAT6 target proteins and revealed new regulatory networks, which has provided new hypothesis on the mechanisms of Th2 cell differentiation.

Key words: T helper cell differentiation, interleukin-4, cytokine, STAT6, two-dimensional electrophoresis, mass spectrometry, proteomics, asthma, allergy

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Th2-solujen erilaistumista ohjaavat säätelyverkostot ja niiden tutkiminen proteomiikan avulla

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TIIVISTELMÄ

Astma ja allergiat ovat laajalle levinneitä ja vakavia sairauksia, joista kärsivät miljoonat ihmiset ympäri maailmaa. Koe-eläimillä tehdyt tutkimukset osoittavat, että interleukiini-4 (IL-4) on tärkeä allergisen astman ja allergioiden kehittymiselle ja kroonistumiselle. Se ohjaa T-auttajasolujen (Th-solujen) kehittymistä Th2-tyypin soluiksi, joilla on merkittävä rooli näiden tautien puhkeamisessa. Th2-solut tuottavat myös itse IL-4:ä, joka edesauttaa taudin seuraavien vaiheiden kehittymistä. Erityisesti STAT6-proteiini, joka aktivoituu IL-4-stimulaation seurauksena, on tarpeen Th2-vasteen syntymiselle ja kroonistumiselle antigeenin aiheuttamassa keuhkoputkien astmaattisessa tulehduksessa.

Väitöskirjatyöni tarkoituksena oli käyttää kaksidimensionaaliseen elektroforeesiin (2-DE) perustuvaa proteomiikkaa ja massaspektrometriaa uusien Th2-solujen erilaistumista säätelevien proteiinien tunnistamiseksi. Erilaistumattomat Th-solut eristettiin vastasyntyneen napaverestä tai hiiren pernasta. Solut aktivoitiin T-solureseptorin ja ns. ko-stimulatoristen reseptorien kautta ja erilaistettiin joko Th1- tai Th2-suuntaan vastaavasti erilaistavien IL-12- ja IL-4-sytokiinien avulla. Ensimmäisessä tutkimuksessa *in vitro* -erilaistettujen Th1- ja Th2-solujen proteomeja verrattiin keskenään proteiinien ilmenemisessä tai proteiinimodifikaatioissa olevien erojen tunnistamiseksi. Kaksi muuta päätutkimusta keskittyivät IL-4:n aiheuttamaan proteiinitason säätelyyn ensimmäisen vuorokauden aikana T-soluaktivaation jälkeen. Näistä ensimmäisessä IL-4:n aiheuttamia eroja tunnistettiin aktivoiduista ihmisen Th-soluista. IL-4:n todettiin säätelevän useita proteiineja kaspaasien välittämissä signalointiteissä sekä lisäävän T-solujen elävyyttä ja aktivoitumista. Toisessa tutkimuksessa STAT6-poistogeenisten hiirien lymfosyyttien proteomia verrattiin villityypin kontrollisoluihin T-soluaktivaation ja IL-4-stimulaation jälkeen. Näissä tutkimuksissa karakterisoitiin useita uusia IL-4:n ja STAT6:n kohdeproteiineja ja löydettiin uusia säätelyverkostoja. Tutkimustulokset ovat johtaneet uusiin Th2-erilaistumismekanismeja koskeviin hypoteeseihin.

Avainsanat: T-auttajasolujen erilaistuminen, interleukiini-4, sytokiini, STAT6, kaksidimensionaalinen elektroforeesi, massaspektrometria, proteomiikka, astma, allergia

TABLE OF CONTENTS

ABBREVIATIONS

2-DE	two-dimensional electrophoresis
Act	activated
ADAMa	disintegrin and metalloproteinase
Akt	thymoma viral proto-oncogene 1
AML	acute myeloid leukemia
αNAC	nascent-polypeptide-associated complex alpha subunit
APC	antigen presenting cell
Bad	BCL2-antagonist of cell death
Bax	BCL2-associated X protein
Bcl	B-cell leukemia/lymphoma
Bid	BH3 interacting domain death agonist
BSA	bovine serum albumin
cAMP	cyclic AMP
CBF	core binding factor
CBF1	recombination signal binding protein for immunoglobulin kappa J region
CCR	chemokine (C-C motif) receptor
cDNA	complementary DNA
c-FLICE	cellular FADD-like IL-1β-converting enzyme
c-FLIP	c-FLICE inhibitory protein
ChIP	chromatin immunoprecipitation
CNBP	cellular nucleic acid protein
COX-2	prostaglandin-endoperoxide synthase 2
CRTH2	chemoattractant receptor-homologous molecule expressed on TH2 cells
CSTF	cleavage stimulation factor
CSL	CBF1-suppressor of hairless-Lag1
CXCR	chemokine (C-X-C motif) receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DLL1	Delta-like 1
DNMAML1	dominant negative form of MAML1 protein
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
ELISA	enzyme-linked immunosorbent assay
ERK	elk-related tyrosine kinase
ESI	electrospray ionization
FasL	Fas ligand
FCS	fetal calf serum
FELASA	Federation of European Laboratory Animal Science Associations
FOG	Friend of GATA
FSC	forward scatter

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	GATA binding protein 3
γc	gamma common chain
Gfi	growth factor independent
H3K4	Histone 4 lysine 3 methylation
hNRNP	heterogeneous nuclear ribonucleoprotein
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HS	hypersensitive site
ICAT	isotope-coded affinity tag
ICOS	inducible costimulator
IEF	isoelectric focusing
IFN	interferon
Ig	Immunoglobulin
IκB	Inhibitor of NF-κB kinase
IκBα(ΔN)	dominant negative form of IκB-α
IL	interleukin
IL-4R	interleukin-4 receptor
IP	immunoprecipitation
IRS	insulin receptor substrate
Itk	IL2-inducible T-cell kinase
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
LAT	linker for activated T cells
LPS	lipopolysaccharide
Ly-GDI	Rho GDP dissociation inhibitor (GDI) β
Mac-1	integrin alpha M
MALDI	matrix-assisted laser desorption/ionization
MAML1	mastermind-like 1
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MNC	mononuclear cell
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
Ndfip	Nedd4 family interacting protein
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor of kappa light chain gene enhancer in B-cells
NIP	NFAT interacting protein
NK cell	natural killer cell
NKT cell	natural killer T cell
o/n	over night
OVA	Ovalbumin
OX40L	OX40 ligand
p27Kip1	cyclin-dependent kinase inhibitor 1B
p56lck	lymphocyte-specific protein tyrosine kinase

pb	plate bound
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffer solution
PGE2	Prostaglandin E2
pI	isoelectric point
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PMF	peptide mass fingerprinting
RBP-J	recombination-signal-binding-protein-J
Rel	reticuloendotheliosis oncogene
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
Runx	Runt related transcription factor
SAP	SH2 domain protein 1A
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SILAC	stable isotope labeling with amino acids in cell culture
siRNA	small interfering RNA
SLAT	SWAP-70-like adapter of T cells
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
SSC	side scatter
STAT	signal transducer and activator of transcription
Stat6-/-	signal transducer and activator of transcription 6 deficient
T1/ST2	interleukin 1 receptor-like 1
TACE	TNF-alpha converting enzyme
T-bet	T-box 21
Tc2 cell	T cytotoxic type 2 cell
TCR	T cell receptor
TGF	Transforming growth factor
Th cell	T helper cell
TIM-1	T cell, Ig domain, and mucin domain-1
TNF	tumor necrosis factor
TOF	time-of-flight
T_{reg}	regulatory $CD4^{+}CD25^{high}$ cells
TSLP	thymic stromal lymphopoietin
TSS	transcription start site
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
UbcH5B	ubiquitin-conjugating enzyme E2D 2
ZAP-70	zeta-chain (TCR) associated protein kinase 70kDa

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. Rautajoki, K.*, Nyman, T.A.*, Lahesmaa, R. (2004) Proteome characterization of human T helper 1 and 2 cells. *Proteomics* 4(1):84-92 (*Equal contribution)

II. Rautajoki, K.J., Marttila, E.M., Nyman, T.A., Lahesmaa, R. (2007) Interleukin-4 inhibits caspase-3 by regulating several proteins in the Fas pathway during initial stages of human T helper 2 cell differentiation. *Mol Cell Proteomics* 6(2):238-51

III. Tuomela, S.*, Rautajoki, K.*, Nyman, T.A., Lahesmaa, R. Identification of novel STAT6 regulated proteins in IL-4 treated mouse lymphocytes with 2-DE based proteomics. Manuscript (*Equal contribution)

This thesis also includes unpublished data presented only in the thesis (IV). The original publications have been reprinted with permissions of the copyright holders.

1. INTRODUCTION

T helper (Th) cells mature in the thymus and are characterized by CD4 expression on their cell surface. When undifferentiated T helper cell recognizes an antigen on an antigen presenting cell (APC), this leads to the T cell activation through T cell receptor and co-stimulatory receptors. T cell activation can also be induced *in vitro* by stimulating the cells through CD3 and CD28 receptors. Cytokines surrounding activated T helper cells have a potential to induce the differentiation (i.e. polarization) of activated T helper cells into distinct T helper cell subsets, such as Th1, Th2, or Th17 cells. For example, interleukin-12 (IL-12) promotes Th1 development and IL-4 Th2 development. Also additional co-stimulatory signals and signals originating from the T cell receptor can regulate T helper cell differentiation. The inappropriate T helper cell activation and polarization has been linked to different diseases. Differentiated Th1 and/or Th17 cells are mainly responsible for the onset and perpetuation autoimmune diseases, such as type-1 diabetes and multiple sclerosis, whereas harmful Th2 cell activity leads to allergies and allergic asthma.

Despite the very active research on T helper cell differentiation, most of the studies have been conducted in mice, and there is still only a limited amount of information on the differentiation of human T helper cells. Since several differences between human and mouse systems have been reported, e.g. in the expression of CRTH2 protein (1-3), and the induction of Th17 development (4-7), studies on the molecular mechanisms of human T helper cell differentiation are important for a better understanding of T cell mediated human diseases. In this thesis, the majority of experiments have been done by using primary human T helper cells. This broadens our knowledge on human T helper cell differentiation.

The proteome refers to all the proteins that are expressed in a certain cell type or population within a certain time frame and/or condition. The aim of proteomics is analysis of the proteome, although technical limitations do not allow the whole proteome to be in the scope of all proteomics studies. Proteomics covers several different approaches. For example, proteomic methodologies can be utilized for the identification of proteins expressed in a certain cell type. Differential display proteomics is in turn based on the relative quantitative measurements of protein expression. Proteomic approaches are also used to study isolated protein complexes, subcellular protein fractions, and protein modifications. In this thesis, gel-based proteomics has been applied to identify novel relevant factors that are regulated during T helper cell differentiation.

2. REVIEW OF THE LITERATURE

2.1. The role of T helper cells in the immune system

The progenitors of the haematopoietic cell lineage arise in the bone marrow, from where T cell progenitors enter the thymus for their maturation. In the thymus, primarily double $CD4^-CD8^-$ negative cells develop into single positive $CD4^+$ or $CD8^+$ T cells through a double positive ($CD4^+CD8^+$) stage. (8, 9) T cells that are nonfunctional or reactive against self proteins are eliminated in the thymus during the processes of positive and negative selection (10, 11). In the periphery, T cells function as a part of an adaptive immune system that works through the specific recognition of foreign antigens. Antigens are recognized by a T cell receptor (TCR), when the specific antigen is presented together with a major histocompatibility complex (MHC) molecule on the surface of presenting cell. T helper cells are a subpopulation of T cells that are defined by CD4 expression on their cell surface. CD4 binds to MHC class II molecule, which is constitutively expressed on thymic epithelial cells as well as on so called professional APCs, such as dendritic cells (DCs), B lymphocytes, and cells from monocyte-macrophage lineage (12). In addition, MHC II can be induced in certain cell types, such as fibroblasts, astrocytes, endothelial cells, and epithelial cells, especially in response to IFNγ (12). CD4 interaction with MHC II requires the antigen to be presented on the MHC II molecule on the surface of the APC.

Antigen presentation to the naive T cell in the lymph node is mainly conducted by DCs. This is partly due to the fact that the interactions of naive T lymphocytes with B cells or macrophages are extremely rare in the lymph node. In addition, naive T cells reside in the blood and secondary lymphoid organs, such as lymph nodes, spleen and mucosal lymphoid organs, so they are not usually present in the tissues, the common site for infection and macrophage interaction. (13) During invasion with a pathogen, such as virus or parasite, the cells of the innate immune system become activated first (14). DCs are stimulated by proinflammatory cytokines and/or pathogenic structures. They then start to maturate and migrate to the lymph node, where they present the antigen on the MHC II to the specific T helper cell (13). When a naive T cell encounters the specific antigen on an APC, it forms a very strong contact that lasts for several hours and leads to the full activation of the Th cell (15). During the interaction period, the APC provides additional signals to Th cell through other cell-cell interactions (through a so-called co-stimulatory receptors) and by secreting stimulating cytokines to the intercellular space. CD4–MCH II and TCR–Antigen interactions, as well as other receptor mediated interactions, are spatially organized in the cell-cell contact area called the immunological synapse (16, 17). According to the current model, an activated Th cell has a potential to differentiate into several different cell types. Th1, Th2, or Th17 cells are associated with a proinflammatory phenotype, whereas Th3 cells, Tr1 and regulatory $CD4^+CD25^{high}$ (T_{reg}) cells are involved in inhibiting proinflammatory cell types and in sustaining immune tolerance (18, 19). The

development of $CD4^{+}CD25^{high}$ T_{reg} cells is mainly taking place in thymus, but also in the periphery (18, 20-25). All the other cell types are mainly generated from mature activated naive T helper cells after thymic development (18-20). The TCR mediated and co-stimulatory signals, as well as cytokine environment, determine the differentiation pathway that the activated Th cell will follow. In the lymph node, activated T helper cells also provide costimulation to B cells, as they transiently migrate to the B zone a couple of days after activation (26). Differentiated T helper cells migrate to the site of inflammation via blood stream (26, 27). At the inflammation site, they become reactivated by the APCs, which leads to the secretion of pro-inflammatory cytokines and upregulation of stimulatory receptors. Signals provided by Th cells activate other inflammatory cells to enhance their response against pathogens (28). Proinflammatory Th cell subtypes are characterized by distinct cytokine profile that they produce during the reactivation phase, so the differentiation of T helper cells enables them to boost the immune response suitable for a given situation. The role of T helper cells is to guide the stimulation of other cells to act in an appropriate manner against the pathogens in question.

Proinflammatory T helper cells have a profound role in a variety of immune responses. Through their cytokine production, effector Th1 cells activate macrophages, natural killer cells and cytotoxic $CD8^{+}$ T cells, and stimulate B cells to produce IgG antibodies that are involved in opsonization and phagocytosis. Th1 cells are important for the eradication of intracellular pathogens, including bacteria, parasites, yeast and viruses. Newly discovered Th17 cells are in turn functioning during the infection against extracellular bacteria. Both Th1 and Th17 cells are also associated with autoimmune diseases, such as multiple sclerosis. (28-30) Effector Th2 cells activate mast cells and eosinophils. Their cytokines induce B lymphocytes to switch to IgE producing cells. Th2 cells are important in defense against certain helminths and other extracellular parasites. Th2 response is also linked to atopic diseases and allergies. (28, 29, 31)

2.2. The differentiation of proinflammatory T helper cells

The differentiation of effector T helper cells is controlled by TCR activation, co-stimulatory molecules on the surface of APC, and polarizing cytokines in the surrounding of T cells. Th1, Th2, or Th17 cells have a proinflammatory role in the immune system, although they also tend to inhibit the immune responses of the alternative T helper cell type. (18, 32, 33) Certain cytokines have a strong effect on T helper differentiation, and they are most commonly used to induce the differentiation *in vitro*. The main cytokines driving Th1 and Th2 differentiation are IL-12 and IL-4, respectively (Figure 1). (32) The development of Th17 cells is induced by IL-6 and Transforming growth factor β (TGF-β) in mice, whereas IL-1β and IL-6 synergistically drive Th17 development in humans (5-7, 34). As there are several mechanisms and factors that simultaneously determine the direction of differentiation in a certain direction and inhibit the alternative differentiation pathways, these developmental fates are more or less mutually exclusive. (18, 32, 33)

It has been shown that the differentiation process of Th cells is irreversible after long-term stimulation (35). It takes several days *in vitro* and requires a certain amount of cell divisions to be completed (36-39). T helper cell differentiation is accompanied by epigenetic changes especially in the cytokine loci and most likely also elsewhere in the genome (40-43).

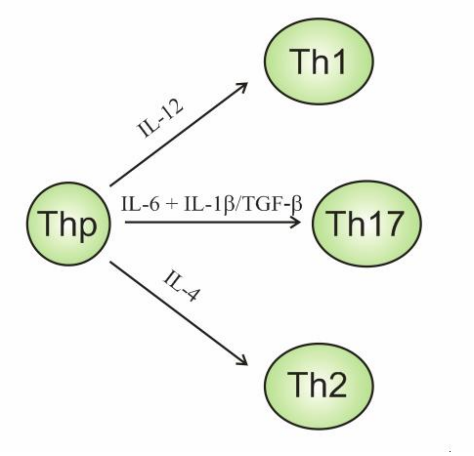


Figure 1. Cytokines are able to induce the development of different proinflammatory T helper cell types. TCR activation and certain costimulatory signals are also needed for proper T helper cell differentiation. The main cytokines driving Th1 or Th2 differentiation are IL-12 and IL-4, respectively. The development of Th17 cells is induced by IL-6 and TGF-β in mice, whereas IL-6 acts together with IL-1β to drive Th17 development in humans.

2.3. Definition and characteristics of Th2 cells

Effector T helper cells are traditionally characterized by a typical set of cytokines that they secrete upon the restimulation in the infected tissue. Th1 cells produce mainly interferon-γ (IFNγ), but also IL-2, tumor necrosis factor-α (TNF-α), and lymphotoxin, whereas Th2 cells produce IL-4, IL-13, IL-9, and IL-5 (29, 31, 32). In reality, cells are not that clearly categorized to distinct T helper cell subtypes, as their cytokine pattern might not be identical: only a few cells express all the cytokines typical for the subtype and they might also secrete cytokines of the opposing subtype (44). Researchers have generally designated T helper cells producing both Th1 and Th2 type cytokines, such as both IFN-γ and IL-4, as Th0 cells, which are thought to represent partly unpolarized cells (28, 44). In *in vitro* cultures, Th0 cells producing both IFNγ and Th2 cytokines are rare (45). *In vivo*, heterogeneous cytokine patterns are more common at early phases of diseases, and the cytokine profile becomes more typical for a certain polarized Th subtype with the chronicity of the immune response (28).

Cytokines that are produced by Th2 cells are functionally important for regulating the immune response. As IL-4, and to lesser extent IL-9 and IL-13, induce B-cells to produce IgE, they are thus needed for proper IgE dependent, mast cell mediated reactions. IL-5 in turn activates eosinophils. Both IgE production and recruitment of

eosinophils are characteristic for Th2-dominated immune reponses. In addition, Th2 cytokines, such as IL-4, IL-9, and IL-13, are able to induce mucus and chemokine production in epithelial cells. (31)

In addition to their characteristic cytokine secretion pattern, Th2 cells can also be identified according to their cell surface marker expression. Chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) is designated as a Th2 marker gene in humans but not in mice (1-3). GATA3 (GATA binding protein 3), which is the major transcription factor driving Th2 differentiation, can upregulate the expression of chemokine receptor CRTH2 (46). In humans, stimulation through CRTH2 leads to an enhanced Th2 response *in vitro* (47). The expression of human CRTH2 protein correlates strongly with the production of IL-4 and IL-13 as well as increased mRNA and protein levels of GATA3. Furthermore, the expression of Th1 markers T-box 21 (T-bet) and IFNγ are decreased in CRTH2 positive cells. (48)

In addition to CRTH2, Th1 and Th2 cells also show differential expression of several other chemokine receptors leading to their differential migration to the sites of inflammation (49). In general, chemokine (C-X-C motif) receptor 3 (CXCR3), CXCR6, and chemokine (C-C motif) receptor 5 (CCR5) are expressed more in Th1 cells, whereas CCR4 and CCR8 are expressed more in Th2 cells (49-56). Higher CCR4 expression in Th2 cells correlates with significantly increased migration and responsiveness to CCR4 binding chemokines TARC and MDC (50, 52, 54, 57). The expression of CCR8 and the responsiveness to its ligand I-309 are increased by TCR restimulation in Th2 cells (51, 57, 57). CCR3 expression is also reported to be higher in Th2 than Th1 cells, but its expression is generally very low, at least in naive, memory, and Th1/Th2/Th0 effector cells in the blood as well as in polarized cells within the first 2 weeks of polarization *in vitro* (50, 56, 58, 59). CCR3 is not expressed by T cells, but instead by eosinophils, in the asthmatic lungs after allergen exposure (60). Low to moderate expression of CCR3 is more characteristic of Th2 cell clones, but it is more of a marker gene for a subset of Th2 cells than for the whole Th2 cell population (53, 58).

With the exception of CRTH2, the expression of any of the above chemokine receptors is not totally specific or characteristic for Th1 or Th2 subset (1, 2, 54-56, 61). In other words, there are cells with IFN-γ/IL-4 production pattern typical for Th1 or Th2 cell subtype expressing chemokine receptors for the opposite subtype, and all Th1 or Th2 type cells do not necessarily express the chemokine receptors typical for them. Clearly, the $CCR4^{+}CXCR3^{-}$ phenotype is a more specific marker for Th2 type cells than mere CCR4 expression alone (56).

The expression of T1/ST2, an IL-1 receptor like cytokine receptor, is restricted to Th2 cell clones and Th2 type *in vitro* polarized cells, both in human and mouse (62). Its surface expression is increased by TCR activation in human Th2 cell clones, but remains very low, making it a less useful marker protein for Th2 cells, at least for scientific purposes (63). Its expression is also rather low in activated human Th2 cell lines polarized for several weeks *in vitro* (64). In mouse, its proper surface expression

requires several rounds of restimulation, so it is a rather late marker for Th2 cells (65). T1/ST2 is neither a global marker for IL-4 producing effector cells, since a subpopulation of them are T1/ST2 negative (64, 66). T1/ST2 expression correlates better, but not absolutely, with IL-5 production in these cells (66).

2.4. Extra-cellularly induced signaling pathways in Th2 cell differentiation

When naive T helper cells are activated by APCs in the lymph node, several of the signals that they receive will lead them to one of the several differentiation pathways, to an undifferentiated stage, or to cell death. The strength of TCR activation, as well as stimulation through certain cytokine and costimulatory receptors, has an effect on Th2 differentiation. APCs have a profound role in providing these signals, although some signals, such as certain cytokines, might originate from the activated T helper cells themselves or some other cells in the surroundings. Mature DCs, the main APCs for naive T cells, can be divided into different subsets according to their potential to induce certain effector responses on target Th cells (67, 68). The maturation of DCs into subtypes that favor either Th1 or Th2 differentiation is in turn regulated by the signals they receive. Different microbial compounds, stimulation through certain Toll-like receptors or cytokines such as thymic stromal lymphopoietin (TSLP) are known to control DC maturation and the signals that DCs provide to T helper cells during the antigen presentation phase (69-72). The focus of the following chapter will be on selected known stimuli that have the potential to regulate Th2 differentiation.

2.4.1. IL-4

Signaling through the IL-4 receptor is needed for adequate differentiation in the Th2 direction and proper Th2 responses (73-76). Especially signal transducer and activator of transcription 6 (STAT6) mediates the signals that induce Th2 development in response to IL-4 (77-80).

IL-4 can be produced by many cell types, such as mast cells, basophils, eosinophils, natural killer T (NKT) cells and differentiated T cytotoxic type 2 (Tc2) and Th2 cells (76, 81). Yet the source of IL-4 in the lymph node during the initial activation of naive T helper cell is presently unclear. Proper Th2 development can be acquired in the absence of NKT cells, suggesting that they are not required for Th2 differentiation *in vivo* (82-85). $CD4^+$ T helper cells produce IL-4 in a STAT6-independent manner in the lymph nodes during the initial activation, and they are the main producers of IL-4 in the lymph node at that stage (86, 87). IL-4 produced by activated naive T helper cells might be necessary for driving Th2 development, since Th2 responses can be mounted in mice in which only $CD4^+$ cells are capable of producing IL-4 (88). Consistently, Th2 development of human and mouse T cells has been shown to occur in the absence of any exogenous IL-4 at least *in vitro* (89-91). Nonetheless, whether the amount of IL-4 produced by activated $CD4^+$ T cells in the lymph node is sufficient to generate potent

Th2 responses remains a matter of debate (86). Instead, it has been argued that IL-4 is not as essential at the very beginning of the polarization as it is at the later stages by stabilizing and enhancing Th2 development (68, 92). On the other hand, delay in IL-4 stimulation after the primary TCR activation shows gradual inhibition of Th2 development, and proportion of IL-4 producing cells is markedly decreased, if IL-4 induction is delayed for 3-4 days after activation (93, 94). This suggests that IL-4 is needed already during the first days of polarization at least *in vitro*.

Despite the fact that Th2 differentiation has been shown to be IL-4 dependent in several studies, IL-4 is not always necessary for Th2 development or Th2 mediated immune responses (95). IL-4 independent Th2 responses have been reported especially in mice infected with live pathogens, but also in certain allergic disease models (96-99). These IL-4 independent immune responses tend to be mediated by IL-13 (98-102). In addition, IL-4/STAT6 signalling pathway is not needed for IL-4 production by activated naive T helper cells or differentiated Th2 cells. Instead, IL-4 secretion is dependent on TCR activation induced calcium mobilization at these stages of cell development. (39, 103, 104)

2.4.1.1. STAT6 mediated signaling pathway

In haematopoietic cells types, the IL-4 receptor is formed by the dimerization of interleukin-4 receptor α (IL-4Rα) to the gamma common chain (γc). The binding of IL-4 to its receptor will cause the binding of Janus kinase 1 (Jak-1) and Jak-3 to the receptor and their consequential activation through phosphorylation. This leads to the phosphorylation of IL-4Rα chain, which serves as docking site for many signalling molecules such as STAT6. (76) As IL-4 binds to its receptor, STAT6 becomes phosphorylated on Tyr-641, which leads to its homodimerization through a Src homology 2 (SH2) domain - Tyr-641 interaction followed by nuclear localization, where it regulates the transcription of its target genes (Figure 2) (105). IL-13 is also capable of activating STAT6 mediated transcription under certain circumstances (100, 101, 105), but there are few reports of other stimuli that can result in STAT6 activation. The importance of STAT6 for Th2 differentiation has been documented in several studies with STAT6 deficient mouse strains (77-80) and also by knocking down STAT6 in human primary T helper cells (106). STAT6 deficient effector cells show decreased expression of cytokines IL-4, IL-5, IL-10, and IL-13, and IgE responses are abolished in these mice, implicating a severely impaired Th2 development (77-80, 107). Consistently, the conditionally active form of STAT6 can induce Th2 cell type cytokine expression, upregulating IL-4, -5, -6, -10 and -13 and downregulating IL-2 and IFN-g expression in developing Th1 cells (108).

STAT6 deficient mice are resistant to experimental asthma and susceptible to several parasite infections (80). In humans, certain STAT6 single nucleotide polymorphism (SNP) variants have been linked to asthma and high IgE, although this linkage has not been detected in all studies (109). However, STAT6 is not absolutely necessary of Th2 development (95), and STAT6 dependent immune responses are partly explained by the role of STAT6 in IL-13 signaling (98, 99, 102). Although IL-4/STAT6 signaling

pathway is highly important for Th2 development or Th2 responses in certain immune responses, its role cannot be generalized to all Th2 type situations, which highlights the actual diversity and flexibility of the immune system.

It is generally thought that many effects of STAT6 are mediated by GATA3, which is upregulated in a STAT6 dependent manner during Th2 development in human and mouse (108, 110-112). This image of Th2 differentiation might be oversimplified, as enforced expression of GATA3 does not fully rescue IL-4 production or chromatin accessibility in the IL-4 locus in STAT6 deficient cells (113, 114). STAT6 directly binds to the Th2 cytokine locus, e.g. to the RAD50 gene region as well as to hypersensitive site 1 (HS1) in the IL-4 locus and IL-4 enhancer V_A, in mouse. STAT6 also regulates the accessibility of RAD50-C HS, HS1, and IL-4 enhancer V_A sites in the Th2 cytokine locus region. (115, 116)

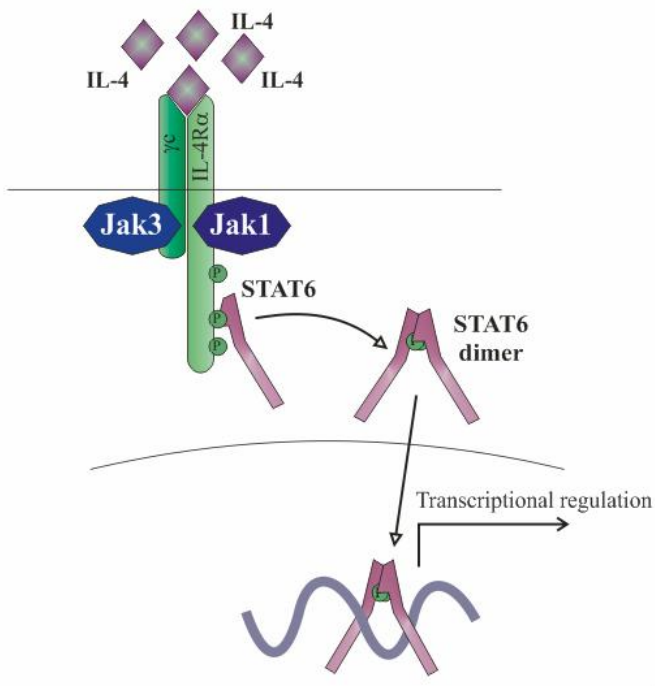


Figure 2. Overview of STAT6 mediated IL-4 induced signaling pathway. IL-4 receptor consists of IL-4Rα and gamma common chain (γc). The binding of IL-4 to its receptor will cause the binding of Jak-1 and Jak-3 to the receptor and their subsequent activation through phosphorylation. This will lead to the phosphorylation of IL-4Rα chain, which serves as docking site for many signalling molecules such as STAT6. When STAT6 binds to the receptor, it is phosphorylated and forms a homodimer through its SH2 domain - Tyr-641 interaction, after which it is translocated into the nucleus. In the nucleus, STAT6 regulates a number of IL-4 target genes by binding to specific target sites, especially to $TTCN_4GAA$ sequences.

2.4.2. Other cytokines promoting Th2 responses

The significant role of thymic stromal lymphopoietin (TSLP) in Th2 mediated disease states, such as asthma and allergies, has been discovered in recent years (72, 117). TSLP is highly expressed by epithelial cells, especially by keratinocytes, in skin

lesions of patients with atopic dermatitis (118). TSLP expression is also increased in the bronchial epithelium and submucosa of asthmatics compared to healthy controls and its expression is correlated with disease severity (119). TSLP could not be detected from lesions of patients with nickel-induced contact allergic dermatitis or cutaneous lupus erythematosus, indicating that its expression is restricted to Th2 mediated diseases (118). TSLP binds weakly to TSLPR, but has higher affinity to the dimer of TSLPR and IL-7Rα, and only the latter receptor is capable of properly activating target cells (120-122). Transgenic mice expressing TSLP in either the lungs or the skin spontaneously develop asthma or atopic dermatitis, respectively (123, 124). Human and mouse TSLP regulate T helper cell differentiation mainly by triggering DCs to induce Th2 development, which is mediated at least partly by OX40L-OX40 interaction between DCs and $CD4^+$ T cells (72, 118, 125, 126). Additionally, it has been reported that TSLP can directly, although quite weakly, induce Th2 development in mouse (127). TSLPR is not expressed on the surface of human T helper cells, so direct regulation of T helper cell polarization is not possible in humans (72). Restimulated Th2 cells that have been polarized with TSLP triggered DCs produce high levels of TNF-α in addition to traditional Th2 cytokines IL-4, IL-5 and IL-13, whereas IL-10 and IFN-γ expression are decreased (118). Therefore they slightly differ from traditional Th2 cells, which are thought to express IL-10 but less TNF-α (29, 128). When naive T helper cells were polarized with TSLP triggered DCs, neutralization of IL-4 from the culture medium lead to decreased Th2 development, when measured by production of IL-4, IL-5 and IL-13 cytokines, but it had no effect on the amount of TNF-α or IL-10 producing cells (126). Inhibition of OX40L-OX40 interaction had a counter-effect on all the measured cytokines, implicating that it regulates IL-10 and TNF-α expression in addition to traditional Th2 cytokines. IL-4 and OX-40L have a synergistic effect on these cells, as neutralization of both synergistically inhibited Th2 development as well as TNF-α production. As TSLP triggered DCs do not produce IL-4, TCR induced IL-4 might be responsible for inducing Th2 development in these experiments. (126)

In addition to IL-4 and TSLP, there are also other cytokines involved in Th2 differentiation or Th2 effector functions. IL-2 and STAT5a, which is activated in response to IL-2 stimulation, are needed for proper Th2 differentiation and Th2 mediated responses in mouse (129-134). This is partly due to a direct regulation of the Th2 cytokine locus by STAT5a, which binds to and opens up the chromatin from HSII and HSIII sites in the locus during Th2 development (134, 135). IL-6 has been suggested to play a role in Th2 differentiation by enhancing IL-4 production in an NFAT1 dependent manner during the initial TCR activation (136, 137). However, IL-6 is not needed for Th2 differentiation, since Th2 development is not impaired in IL-6 deficient mice (138). Similarily in humans, IL-6 increases the production of IL-4 and IL-5 in activated naive T helper cells, but it does not increase Th2 development or Th2 cytokine production at the effector phase (139). IL-6 is also reported to drive Th17 development in the presence of TGF-β and IL-1β in mice and humans, respectively (7, 34). Both IL-33, an interleukin-1-like cytokine that signals through T1/ST2, and T1/ST2 mediated signaling in general, enhance Th2 responses in mouse, but they do

not regulate the Th2 differentiation itself (62, 140). T1/ST2 expression is induced more slowly than Th2 cytokine secretion during differentiation, so it is unlikely to be involved in the early regulation of T helper cell differentiation (65, 141).

2.4.3. The strength of TCR activation and calcium influx mediated signaling

The strength of TCR stimulation influences T cell differentiation. Usually strong TCR activation promotes Th1 and weak activation promotes Th2 differentiation (142-145). There are several proteins in the TCR signaling pathway that are reported to control Th2 differentiation. Full activation of zeta-chain (TCR) associated protein kinase 70kDa (ZAP-70) seems to prefer Th1 development in mouse, since its slight inhibition by Piceatannol leads to increased Th2 development whereas Th1 differentiation is decreased (146). ZAP-70 activity is also biologically inhibited by SLAT protein (SWAP-70-like adapter of T cells), which is selectively upregulated during Th2 development in mouse (146). Although general T cell maturation is impaired transgenic mice carrying Y136F mutation in LAT (linker for activated T cells) protein, which is one of the targets for ZAP-70, they develop exaggerated and spontaneous Th2 response (147). Y136F mutation in LAT leads to decreased Phospholipase C γ 1 (PLC-γ1) phosphorylation and calcium mobilization, implicating that decreased calcium stimuli drives T helper cell differentiation in the Th2 direction (148). In support of this notion, Th2 differentiation is enhanced by the cyclosporine A induced inhibition of calcineurin, an important component of the calcium pathway, whereas induction of calcium mobilization with ionomycin prefers Th1 differentiation (149, 150). NFAT (nuclear factor of activated T-cells) transcription factors, which become activated by calcineurin after calcium influx, also have a regulatory role in T helper cell differentiation (151). Targeted deletion of NFAT1 increases Th2 development, which is further augmented by combined loss of NFAT1 and NFAT4 genes, suggesting a negative role in Th2 differentiation (152, 153). On the other hand, NFAT2 is needed for proper Th2 responses, such as IL-4 secretion by the effector cells or IgE production (154). FOXP3, which is a T_{reg} specific transcription factor, in turn inhibits NFAT activity, thereby downregulating NFAT dependent cytokine gene transcription (155, 156). To conclude, different stages of the calcium mediated signaling pathway seem to regulate the differentiation fate of the activated naive T helper cell.

2.4.4. Costimulatory signals

2.4.4.1. CD28

CD28 is the most effective known costimulatory molecule for T cells, and it is expressed by both naive and already primed T helper cells (157). CD28 binds to the CD80 (B7-1) and CD86 (B7-2) receptors on the surface of APC. Stimulation through CD28 activates Vav-JNK (Vav and c-Jun N-terminal kinase), phosphatidylinositol 3-kinase (PI3K), NFAT and nuclear factor of kappa light chain gene enhancer in B-cells (NF-κB) mediated signaling pathways (158, 159). TCR binding in the absence of

CD28 leads to apoptosis or anergy, and CD28 ligation has been reported to lower the threshold for T cell activation (157). In mice, costimulation through CD28 leads to enhanced Th2, but not Th1, differentiation (160). Consistently, IL-4 production and the amount of IL-4 producing reactivated cells are decreased in CD28 deficient mice (161). Cell proliferation and IL-2 production are also reduced in these mice (161, 162). More specifically, B7-2 interaction with CD28 is linked to Th2 differentiation (163-166). CD28 stimulation is also required for full TCR induced upregulation of GATA3 transcription and activity (167, 167).

2.4.4.2. OX40

OX40, also known as CD134 or TNFRSF4, is a member of TNF-receptor family. Its expression is induced by T cell activation in human and mouse T cells (168-170). OX40 delivers survival signals on activated Th cells by upregulating anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl2) and Bcl-xL proteins through a thymoma viral proto-oncogene 1 (Akt) mediated pathway (171, 172). OX40 costimulation enhances Th2 development *in vitro* in both human and mouse (170, 173). *In vivo*, inhibition of OX40-OX40L interaction, or the removal of either receptor, leads to impaired Th2 differentiation and Th2 mediated responses as well as decreased production of Th2 cytokines upon primary TCR activation (174-179). OX40 has also been linked to Th1 differentiation in mouse *in vivo* studies, but its Th1 promoting property is dependent on adjuvants, whereas the capacity to drive Th2 development is adjuvant independent (174, 180-182). The significance of TSLP in allergic diseases makes OX40 an important costimulatory molecule for Th2 differentiation, as the Th2 driving effect of TSLP primed DCs is mediated by OX40-OX40L interaction (72, 126). The role of TSLP in Th2 type responses is described in more detail on Chapter 2.4.2.

2.4.4.3. Notch

The Notch family includes four mammalian Notch receptors, designated as Notch1-4, which can bind to five different canonical Notch ligands, called Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3, and DLL4. A few non-canonical Notch ligands have been described, but they have been studied mainly with other cell systems. Binding of a Notch ligand to its receptor leads to sequential cleavage of Notch receptor. First Notch receptor is cleaved by ADAM-like (a disintegrin and metalloproteinase) protease TNF-alpha converting enzyme (TACE), then it is mono-ubiqutinylated, and finally proteolytically cleaved by γ-secretase, which leads to its release from the membrane and allows it to transmigrate to the nucleus. In the nucleus, it associates with transcriptional repressor CBF1-suppressor of hairless-Lag1 (CSL, mouse homolog is called recombination-signal-binding-protein-J, RBP-J), which induces the transcription of CSL repressed genes in a CSL dependent manner. (183, 184) Expression of DLL1 is lower than that of Jagged1 or Jagged2 in splenic DCs, but higher than expression of Jagged proteins in splenic macrophages (185). Jagged1 and Jagged2 are upregulated in DCs that are maturated with signals promoting Th2 response, and DLL4 upregulation in DCs is linked to Th1 inducing potential. Expression of DLL1 and DLL3 remain very low or undetectable, at least when Prostaglandin E2 (PGE2), cholera toxin or

lipopolysaccharide (LPS) are used to maturate DCs (186). TCR activation with anti-CD3 and anti-CD28 antibodies is able to upregulate Notch receptor expression as well as activate Notch signaling through a mechanism that is still unknown (187, 188).

In addition to regulating proliferation (187-189), Notch signaling is important for proper Th2 development and Th2 responses (184). RBP-J deficiency leads to increased Th1 differentiation and decreased Th2 differentiation, shifting the balance into Th1 side (189). Similarly, expression of a dominant negative form of mastermind-like 1 (MAML1) protein (i.e. DNMAML1), which should prevent the transcriptional activation of all four Notch members (although Notch4 less efficiently) (190, 191), impairs Th2 development (192). DNMAML1 mice fail to develop a protective Th2 mediated response against *Trichuris muris*, while having a protective Th1 mediated response during *Leishmania major* infection (192). At least one Notch ligand has been reported to prime Th2 responses: Jagged1 is able to promote Th2 development in a manner that is mediated by Notch signaling (186). Jagged1 costimulation also induces IL-4 and GATA3 expression in activated T helper cells independently of STAT6 (186). GATA3 expression after primary activation has also been reported to be dependent on RBP-J and MAML in other studies (189, 193, 194). However, the significance of IL-4 induction is somewhat controversial, as IL-4 secretion upon primary activation is independent of γ-secretase and RBP-J, two important components of Notch signaling (189, 195). Although no defects in T helper cell differentiation were discovered in Notch1 deficient cells, it has been shown to efficiently induce GATA3 expression and Th2 differentiation in two recent studies (188, 193, 194, 196, 197). The phenotype of Notch1 deficient cells might be partly explained by the capability of other Notch proteins, such as Notch2, to compensate the loss of Notch1 (193).

The role of Notch signaling in Th1 differentiation is less consistent. DLL1-Notch3 interaction or DLL1 stimulation has been shown to favor Th1 differentiation over Th2 differentiation (186, 196). Notch3 is not expressed in naive T helper cells but it is upregulated after TCR stimulation and is expressed in $CD4^+CD25^+$ cells (186, 187, 198). Pharmacological γ-secretase inhibitors decrease IFN-γ expression in activated naive T helper cells leading to impaired T-bet upregulation (195). Inhibitor treated mice were also more resistant to experimental autoimmune encephalomyelitis (EAE) (195). In the presence of another γ-secretase inhibitor IL-CHO, IFN-γ production was similarly reduced in activated splenocytes, but not in restimulated $CD4^+$ cells (188). This suggests that Notch signaling is not needed for Th1 differentiation although it might regulate activation induced IFNγ expression at beginning of differentiation. Furthermore, both activation induced IFN-γ production and Th1 differentiation are actually increased in RBP-J deficient cells, and impaired Notch signaling in DNMAML1 cells leads to unaffected or increased Th1 differentiation, depending on the experimental setup used (186, 189, 192). Overall, the evidence suggests that Notch signaling is more important for Th2 than for Th1 development, although additional studies are needed to ascertain the role of Notch proteins in Th1 differentiation.

2.4.4.4. ICOS

The Inducible Costimulator (ICOS) protein is reported to promote Th2 responses, although it is also linked to Th1 mediated diseases and enhanced IFNγ production by human Th1 cells (199-202) ICOS expression shows species specific differences, being higher in mouse Th2 and human Th1 cells, when compared to mouse Th1 and human Th2 cells, respectively (200, 203, 204). In both species, ICOS expression is induced by TCR activation (200, 201, 203, 204). The production of IL-4 and IL-13 by T helper effector cells is selectively reduced in ICOS knockout mice (205, 206). Furthermore, activation of wt $CD4^+$ T cells with ICOS ligand B7h deficient APCs leads to impaired expression of IL-4 and IL-13 upon restimulation of Th cells (207). In humans, inhibition of ICOS signaling with ICOS-Ig fusion protein leads to decrease of Th2 cytokine secretion, both during the primary activation and at the effector phase (201). Consistently, allergic sensitization and the production of Th2 cytokines IL-4, IL-13, IL-5 are enhanced in individuals with alternative SNP variants in the promoter region of ICOS gene leading to increased ICOS expression (208). ICOS has also been shown to induce IL-10 production in several studies, although IL-10 secretion was not ICOS dependent in ICOS deficient mice (200, 201, 204, 206, 207, 209). However, studies using ICOS-Ig have shown that ICOS is not needed for Th2 development but rather for efficient cytokine production during restimulation both in humans and mice (201, 209). ICOS mediated signaling also selectively enhances the clonal expansion of human Th2 cells (201). The effect of ICOS on IL-4 production by effector cells might be mediated by c-Maf. TCR induced expression of c-Maf, but not that of JunB, T-bet, or GATA3, is reduced in ICOS deficient T helper cells, and c-Maf overexpression restores the normal IL-4 levels in differentiated ICOS knockout cells (210). If ICOS deficient activated T helper cells are differentiated in the presence of IL-4, both the induction of c-Maf and the IL-4 production by effector cells are restored (206, 210).

2.5. Specific factors involved in Th2 cell differentiation

2.5.1. GATA3

GATA3, a member of GATA family of transcription factors, has a significant regulatory role during the embryogenesis, especially in the generation of nervous system, as well as in several stages of T cell development (211-216). Following TCR activation, GATA3 mRNA and protein levels are induced by IL-4 in both humans and mice, and this induction is dependent on STAT6 (108, 110-112, 217). However, STAT6 does not appear to be obligatory either for GATA3 induction or Th2 development, since low frequencies of Th2 type cells can be generated in the absence of either STAT6 or IL-4R (95). Both STAT6 and IL4R deficient murine cells show increased expression of GATA3 during Th2 type development, suggesting the presence of other upstream regulators for GATA3 (113). Among the potential candidates are the NF-κB and Notch signaling pathways, which have been reported to regulate GATA3 expression (186, 189, 193, 194, 218, 219). Notch signaling selectively induces a certain GATA3 splice variant, whose transcription start site is nearly 10 kb upstream from the

most common start site (193, 194). The expression of this splice variant is selectively induced during Th2 differentiation, approximately 3 days after initial activation (220).

GATA3 binds to IL-13 and IL-5 core promoters and directly regulates their expression (221-223). In addition, GATA3 also binds to several sites in Th2 cytokine locus and controls locus DNA conformation and accessibility (224-228). GATA3 overexpression in humans induces or enhances the production of IL-4, IL-5, and IL-13 in developing Th2 cells and in memory cells (46). Additionally, GATA3 downregulates the expression of Th1 type chemokine receptor CXCR3 and upregulates that of Th2 type chemokine receptor CCR4 (46). One GATA3 haplotype has been associated with asthma related traits, especially with high serum IgE (229). Individuals with defective GATA3 expression have a lower proportion of IL-4 secreting memory Th2 cells (230). Their serum IgE and IgG4 levels are decreased and IgG1 levels increased, implicating a defective Th2 response as well (230). Consistently, the knockdown of GATA3 with small interfering RNA (siRNA) methodology in primary human memory cells, the Th2 type cell line HUT-78 and malignant Jurkat cells decreases the production of Th2 cytokines (223, 230-232).

GATA3 is required for the optimal IL-4 production in Th2 cells, but IL-4 production is not totally dependent on it: Deletion of GATA3 in differentiated Th2 cells has only a slight effect on the proportion of IL-4 producing cells. However, the amount of IL-4 produced by these cells is significantly decreased. The effect of GATA3 deletion on IL-13 and IL-5 secretion is more severe. (233-235)

As well as inducing Th2 development, GATA3 also inhibits T cell differentiation in the Th1 direction independently of STAT6 or IL-4 (110, 113, 214, 236). GATA3 is reported to mediate its negative effect on IFNγ production by downregulating STAT4 both in mouse and in the Jurkat cell line (232, 237). Both in human and mouse, the competence of GATA3 to induce Th2 phenotype or to shut down IFNγ expression weakens progressively, as T helper cells differentiate into Th1 type effector cells (46, 114).

Despite GATA3 expression being markedly higher in mouse *in vitro* differentiated Th2 cells than in Th1 cells at the 7 day timepoint, it is more similarly expressed between polarized human Th1 and Th2 cells (48, 230, 238). However, the minor differences in GATA3 levels appear to correlate with the lower IL-4 production in human compared to mouse cells (48). GATA3 is able to upregulate the expression of chemokine receptor CRTH2 (46). CRTH2 is differentially expressed between human Th1 and Th2 cells, but shows similar expression in mouse Th1 and Th2 cells (1-3). The expression of human CRTH2 correlates strongly with the production of IL-4 and IL-13 as well as the decreased expression of Th1 markers T-bet and IFNγ (48). Although the mRNA and protein levels of GATA3 are also increased in CRTH2 positive cells, they are still lower in human $CRTH2^{+}$ Th2 cells than mouse Th2 cells (48). This might indicate that Th2 differentiation in the *in vitro* system is not as efficient in humans as it is in mouse. Of course it should be taken into a consideration that mouse strains, which are used to

study Th2 differentiation, are generally Th2 prone, whereas the genetic background is more diverse in humans.

In addition to the IL-4 induced increase in GATA3 transcription, there are also post-translational mechanisms involved in the regulation of GATA3 expression and activity. Thus, it has been reported that the Ras and elk-related tyrosine kinase (ERK) mediated mitogen-activated protein kinase (MAPK) signaling cascade, as well as *Polycomb* Group gene bmi-1, stabilizes the GATA3 protein by decreasing ubiquitin mediated GATA3 degradation (239, 240). Friend of GATA (FOG) as well as tyrosine phosphorylated T-bet has been shown to suppress both GATA3 activity and GATA3 mediated Th2 cytokine production by binding to GATA3 (241-243). Interestingly, acetylation of GATA3 appears to be necessary for its proper function both in humans and mice (244, 245). Furthermore, the intracellular localization of GATA3 is regulated by serine phosphorylation, the unphosphorylated form localizing to the cytoplasm and the phosphorylated form to the nucleus (231). Serine phosphorylation of GATA3 is dependent on P38 MAPK activity that is induced by stimulating either HUT-78 cells or peripheral blood mononuclear cells (PBMCs) through CD3 and CD28 or by the elevation of cyclic AMP (cAMP) levels in mouse cells (231, 246).

2.5.2. c-Maf

c-Maf was originally found to be differentially expressed between mouse Th1 and Th2 cell clones and to induce IL-4 production synergistically with NFAT1 (247). In humans, c-Maf is upregulated at protein level in bronchial biopsies and in induced sputum of asthmatic patients (248, 249). c-Maf mRNA expression is also increased in the bronchoalveolar cells of asthmatics after allergen exposure, and the expression correlates strongly with the amount of IL-4 producing $CD4^+$ T cells in the alveoli (250). Similarly, IL-4 increases c-Maf mRNA expression in differentiating human Th2 cells (217, 251). In mice, c-Maf is needed for proper IL-4 production, but it does not directly regulate the expression of IL-13 or IL-5 genes (247, 252, 253). c-Maf has been shown to bind to several sequences within the Th2 cytokine locus upon restimulation of mouse Th2 effector cells (254). The role of c-Maf in human T helper cell differentiation has not been studied in detail. c-Maf interacts with several transcription factors, e.g. with JunB, which is selectively upregulated during Th2 differentiation and promotes Th2 development, and they synergistically induce IL-4 in mouse (255, 256). Mouse c-Maf is also capable of inducing IL-4 transcription in a Th1 clone AE7 and in M12 B lymphoma line that are not spontaneously producing IL-4, and this induction is synergistically increased by NFAT1 (247). IL-4 production is similarly induced by c-Maf and NFAT1 in a human Jurkat T cell line not producing IL-4, and IL-4 production is further increased by the NFAT interacting protein 45kDa (NIP45) protein (257).

2.5.3. Caspases

Caspase activity is generally associated with apoptotic cell death, but it appears that certain caspases are also needed for proper T cell activation and development (258-

260). During the initial activation of primary human T cells, Caspase-3, -6, -7, and -8 become activated in a manner that is linked to selective substrate cleavage and is not related to apoptosis of these cells (261, 262). Similar observations have also been made in mouse effector cells, and, in this case, Th2 cells show both higher caspase activity and better survival than Th1 cells (263). Caspase inhibition has been shown to increase IL-4 production in restimulated cells, suggesting that caspase activity is inhibitory to Th2 differentiation, (264). Consistently, stimulation of $CD4^+$ T cells through Fas receptor during the primary TCR activation leads to increased Th1 differentiation as measured by IFNγ production 6 days after the primary activation (265). Also TCR activation induced IFNγ production is increased in Fas stimulated cells (266). Cell survival and increased IFNγ production in Fas stimulated cells is dependent on NF-κB activity (265). Furthermore, Caspase-8 activity has been shown to prevent Th2 responses, and active Caspase-8 is required for effective T cell mediated immunity against the intracellular parasite *Trypanosoma cruzi* (267). However, transgenic mice expressing the long form of c-FLIP show elevated Caspase-8 activity and enhanced Th2 responses: Th2 differentiation is augmented, allergic inflammation is enhanced and mice are protected from Th1 type experimental autoimmune encephalomyelitis (EAE) (268-270). Interestingly, NB-κB activity was decreased in c-FLIP long transgenic cells after the initial TCR activation (269). Caspase-8 and c-FLIP long are also needed for proper T cell activation and TCR induced proliferation (271-274). Caspase-3 activity has been in turn associated with the regulation of monocyte differentiation, osteogenic differentiation of bone marrow stromal cells, and skeletal muscle differentiation (275-277), but its role in T helper cell differentiation has not been studied in detail.

2.5.4. NF-κB pathway

In mammalian cells, there are five NF-κB family members: reticuloendotheliosis oncogene (c-Rel), RelA (p65), RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100), which form homo- or heterodimers with each other in their active form. RelB is unique in a sense that it cannot homodimerize or form dimers with RelA or c-Rel. In an inactive form, NF-κB proteins retain in the cytoplasm, and RelA, c-Rel, and RelB are bound to one of the Inhibitor of NF-κB kinase (IκB) proteins. Upon NF-κB activation, IκB becomes phosphorylated leading to its proteasomal degradation, which releases active NF-κB subunits and enables them to translocate to the nucleus. NF-κB1 and NF-κB2 do not bind to IκB proteins, but their precursors p105 and p100 need to be proteolytically cleaved to p50 and p52 subunits, respectively, to activate these proteins. However, if p105 or p100 bind to active NF-κB subunits, they can retain these subunits in the cytoplasm, thereby inhibiting their activity. The p50 and p52 subunits can also be located to the cytoplasm, if they are bound by Rel-IκB complexes. RelA, RelB, and c-Rel have a transactivation domain, so dimers containing any of these factors are able to mediate transactivation directly. However, p50 and p52 lack the transactivation domain, and their homodimers act as transcriptional repressors. (278)

Transgenic mice expressing the dominant negative form of IκB-α in T cells (IκBα(ΔN) mice) show decreased DNA binding activity and nuclear localization of c-Rel and RelA containing NF-κB complexes (279). Th1 differentiation is defective in these mice, whereas Th2 response is unaltered or even slightly increased, suggesting that IκB-α regulated signaling is needed for Th1 development but dispensable or even inhibitory for Th2 development (280). However, both IFNγ and IL-4 production are decreased after the primary TCR activation in T cells expressing dominant negative form of IκB-α (281, 282). In addition, susceptibility to apoptosis is increased and TCR activation induced proliferation decreased in these cells (279, 282-284). In accordance with the minor significance of IκB-α regulated factors for Th2 development, TCR induced nuclear localization of both p50 and RelA are reduced in a mouse Th2 cell clone compared to Th1 clones (285). However, activation induced IL-4 production has also been reported to be IκB-α dependent on mouse Th2 cell line EL4 (282).

NF-κB requires signals originating both from the TCR and costimulatory receptors to become fully activated (286). Studies using mice deficient in certain NF-κB family members have elucidated their role in T helper cell differentiation. p50 deficient mice show defective Th2 development and GATA3 expression, and they are resistant to Ovalbumin (OVA) induced allergic airway inflammation (218, 219). Interestingly, when OVA sensitized p50 deficient $CD4^+$ T cells were isolated and restimulated with OVA *in vitro*, no defects in IL-4 production were detected (218). However, when they were polarized *in vitro* using anti-CD3 and anti-CD28 antibodies, IL-4 production was markedly decreased in these cells (219). IL-5 production of the effector cells was clearly p50 dependent in both experimental settings, so it can partly explain resistance to inflammation in p50 deficient mice (218, 219). Additionally, primary T cell proliferation has been reported to be markedly defective in p50 deficient mice both *in vivo* and *in vitro*, which can influence T helper cell differentiation, although this defect could not be detected in OVA stimulated T cells (218, 287). Th1 differentiation is also impaired in p50 deficient mice (287, 288). The SAP/Fyn/PKC-θ mediated signaling pathway has been shown to regulate the nuclear localization of NF-κB1 (p50) upon primary activation, and CD3/CD28 activation induced IL-4 production in the beginning of differentiation, as well as the Th2 differentiation itself, is impaired in cells lacking either SAP or Fyn (289). However, SAP or Fyn deficient T helper cells show normal Th2 differentiation in the presence of exogenous IL-4, suggesting that SAP/Fyn dependent IL-4 production in CD3/CD28 activated cells is responsible for Th2 polarization (289). p52 appears to have a different role in the immune system, as p52 null mice show normal Th1 and Th2 development but are susceptible to Leishmania major infection due to impaired IL-12 production by macrophages (290).

Although the role of RelA in Th2 differentiation has not been studied in a detail, it has been shown to inhibit NFAT or c-Maf dependent IL-4 induction in humans (291, 292). RelA also competes with NFAT for binding to human IL-4 promoter P sequence (291). Since the mouse P sequence only contains a low affinity binding site for RelA, a similar competitive inhibitory effect cannot be observed in mouse (291). NF-κB complexes including c-Rel are less diverse than other NF-κB complexes, as c-Rel

forms only homodimers or heterodimers with p50 or RelA (278). c-Rel is needed for proper Th1, but not Th2, differentiation if the cells are activated using APCs, but it does not regulate Th1 differentiation in CD3/CD28 activated T helper cells (293, 294). RelB is essential for Th1, but not Th2, differentiation in both conditions, and primary IFNγ production is also impaired in RelB deficient T cells (294, 295).

2.5.5. Other factors

The Runt related transcription factor 1 (Runx1), also known as AML-1, CBFα2 or PEBP2αB, is involved in the regulation of several stages of hematopoiesis (296). Runx1 has several splice variants (297-301), and one of its isoforms has been shown to inhibit Th2 development by inhibiting the expression of GATA3 (302). It has also been suggested that Runx1 is involved in the repression of IL-4 gene in Th1 cells (303). Another Runx protein, Runx3, is able to cooperate with T-bet, and is required for the maximal IFNγ production as well as proper repression of IL-4, thereby functioning in a similar manner as Runx1 in differentiating T helper cells (303, 304).

Supressors of cytokine signaling, so called SOCS proteins, are responsible for inhibiting or shutting down the signaling that emerges from the cytokine receptor (305, 306). Some of the SOCS proteins are differentially expressed between Th1 and Th2 cells, SOCS1, SOCS2, and SOCS5 being more abundant in Th1 cells and SOCS3 in Th2 cells (307, 308). Consistently, SOCS1 and SOCS5 inhibit IL-4 induced STAT6 activation, whereas SOCS3 promotes Th2 development and Th2 responses (308-310).

Bcl-6 is reported to have a negative role in Th2 differentiation. This has been shown mainly with Bcl-6 deficient mice that develop a spontaneous Th2 type disease even in the absence of IL-4 or STAT6 (311-313). The expression of GATA3 protein (but not mRNA) is also upregulated in Bcl-6 deficient T helper cells leading to enhanced Th2 development, although only in the presence of exogenous IL-6 (314). Surprisingly, when mouse T helper cells are polarized *in vitro* in the presence of IL-4, Th2 differentiation is not regulated by Bcl-6 (313). Further studies are required to determine, whether the enhanced Th2 response in Bcl-6 deficient mice is caused by altered function of other cell types or whether it is specifically initiated by dysregulated Th2 differentiation.

In mouse, the AP-1 transcription factors JunB and JunD have a positive and negative effect on Th2 polarization, respectively (256, 315). JunB protein expression is also upregulated in developing mouse Th2 cells (255). JNK signaling pathway and Nedd4 family interacting protein 1 (Ndfip1) protein regulate JunB protein levels in mice by enhancing E3 ubiquitin ligase Itch dependent degradation of JunB, the significance of which is indicated by a profound Th2 response, when the inhibitory function of either of these components has been disrupted (316-318). JNK1 deficient cells show preferential Th2 development even under Th1 polarizing conditions, suggesting also that JNK1 inhibits Th2 development (159).

The expression of IL2-inducible T-cell kinase (Itk) is selectively increased in developing mouse Th2 cells *in vitro,* and in peripheral blood T cells from patients with atopic dermatitis (319, 320). Itk is needed for proper TCR activation, but its deficiency also leads to diminished Th2 responses (321). The latter is mainly due to the fact that Itk deficient Th2 cells are incapable of mounting the proper cytokine response upon restimulation (322, 323). Human T cell, Ig domain, and mucin domain-1 (TIM-1) is reported to act in the same pathway upstream of Itk, suggesting than it might have a similar role in effector T cell functions (324).

Lymphocyte-specific protein tyrosine kinase p56lck and IκB family member Bcl-3 are reported to be necessary for proper Th2, but not for Th1, development in mouse (294, 325).

2.6. Effects of IL-4 on viability and proliferation of polarizing T helper cells

IL-4 has potential to regulate viability of activated or non-activated T helper cells. In murine $CD4^+$ cells, IL-4 enhances the survival of naive T cells at least partly by maintaining Bcl-2 and Bcl-xL protein levels (326-328). IL-4 also enhances long-term survival of activated wt or CD28 deficient $CD4^+$ lymphocytes (329), induces survival of *in vivo* activated $CD4^+$ cells by inhibiting the decay of Bcl-2 and Bcl-xL (330), and rescues T cell clones from cell death induced by CD4 triggering before TCR activation (331). In all these studies, cell death was measured by propidium iodide (PI) staining. However, PI staining is not specific to caspase dependent apoptosis, as it also detects cells that have died by other means, such as through caspase independent apoptosis, necrosis or autophagy (332). When apoptosis is measured by staining of $AnnexinV^+$ PI^- cells, IL-4 has been shown to induce apoptosis in restimulated mouse T helper cells via an IL-2 dependent mechanism (333). When apoptosis is detected with a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, IL-4 also increases TNF-mediated apoptosis of human peripheral blood lymphocytes after stimulation with *Mycobacterium tuberculosis* antigens (334). Further studies are needed to ascertain, through which mechanisms IL-4 increases cell viability and which type of cell death is regulated by IL-4.

In general, IL-4 enhances cell proliferation and expansion of activated T helper cells (335, 336). This IL-4 induced proliferation is dependent on both IRS-2 and STAT6 signaling pathways (336-338). STAT6 mediates its function at least partly by decreasing cyclin-dependent kinase inhibitor 1B (p27Kip1) protein levels and increasing growth factor independent 1 (Gfi-1) expression (337, 339). Consistently, when mouse T helper cells are cultured under Th2 conditions, in the presence of IL-4, they show increased proliferation compared to that in cells cultured under Th1 conditions (39).

2.7. Two-dimensional electrophoresis based proteomics to investigate T helper cell differentiation

Proteomics refers to the term proteome, which covers all the proteins expressed in certain cell type or population within a certain time frame and/or certain condition (340). The research object of proteomics is generally the proteome, although technical limitations do not allow the whole proteome to be in the scope of all proteomics studies. Proteomics can also be utilized to study isolated protein complexes, subcellular protein fractions, and post-translational modifications. Proteomics can be divided into two main branches according to the method used for sample separation, namely into two-dimensional electrophoresis (2-DE) based and non-gel based proteomics. In 2-DE based proteomics, soluble proteins are first separated based on their isoelectric point during isoelectric focusing (IEF) and then according to their molecular weight (MW) with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Once separated, the proteins are visualized with protein dyes or by autoradiography of metabolically labeled proteins (Figure 3) (341, 342). For differential display proteomics, differentially expressed proteins are selected for identification with mass spectrometry (MS) (343). The most common identification methods available for 2-DE separated proteins are peptide mass fingerprinting (PMF) and generation of sequence information by tandem mass spectrometry (MS/MS) (343-345). Both methods require proteolytic cleavage of the proteins, usually with trypsin that cleaves the protein after arginine and lysine residues. For PMF, peptide masses, which are generated by trypsination of the protein, are measured with mass spectrometry and this experimental peptide mass fingerprint is compared to theoretically generated fingerprints in the database to identify the protein (or proteins) in question. For identification using MS/MS, selected peptides are fragmented to acquire sequence information in addition to peptide mass. Similarly, this combined information is compared against theoretical fragmentation pattern of known proteins in the database for protein identification. Because the generation of sequence information increases the specificity of mass information, fewer peptides are needed for reliable identification with MS/MS.

In non-gel approaches, separation is done at the peptide level using different forms of chromatography. When proteins or peptides are labeled for the relative quantification prior to non-gel proteomics, isotope-coded affinity tag (ICAT) (346), iTRAQ (347), and stable isotope labeling with amino acids in cell culture (SILAC) technologies (348, 349) can be used for labeling. For ICAT studies, cysteine containing peptides are labeled with isotope tags, purified with affinity chromatography and quantified as well as identified using mass spectrometry (346). iTRAQ method is in turn based on the labeling of all amine containing peptides with isobaric tags prior to MS (347). There are also additional, so-called label-free non-gel approaches, which do not require labeling of the sample material (350, 351).

There are several reports about gene expression profiles in differentiating Th1 and Th2 cells both in human and mouse. Many studies, including our own, have identified a large number of genes whose mRNA levels are regulated by TCR signalling or by differentiating cytokines either during differentiation (111, 112, 217, 251, 352-354) or

after the cells have been polarized (355-357). Transcriptomics data have been complemented with proteomics studies depicting the regulated proteins in differentiating T helper cells (358-362) or proteome differences between polarized Th1 and Th2 cells (363, 364).

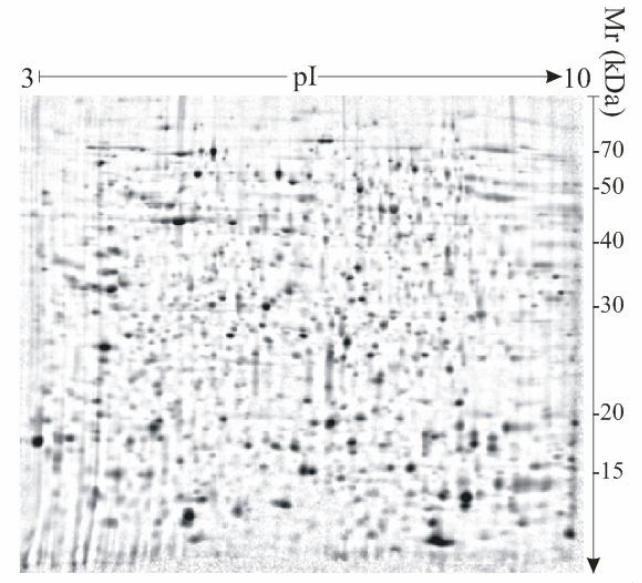


Figure 3. Representative image of a 2-DE gel. During 2-DE, proteins have been separated first according to their pI properties with isoelectric focusing (IEF) and then according to their molecular weight with SDS-PAGE. Proteins have been detected with autoradiography, and the background has been subtracted from the image.

The use of proteomics in the studies on T helper cell differentiation has an advantage of providing a direct insight into the regulation of proteins that are the main active agents in the cell. This is highlighted by the fact that post-translational modifications, which are an important part of cellular regulation, cannot be directly studied using transcriptomic approaches. Furthermore, the total mRNA and protein levels of several genes do not often correlate with each other (365-368). In addition, the proteins that can be studied and identified with 2-DE and MS from total cell lysates are largely different from many differentially expressed genes found from transrciptomics studies, so proteomics provides an important complementation for transcriptomics studies (369, 370).

Although the general view on RNA pool is becoming increasingly complex with the discovery of microsomal and other noncoding RNAs and an increasing number of splicing variants (371-374), the complexity of protein pool is still outstanding (375). This, together with a large dynamic range in protein amounts and protein-to-protein variation in the chemical properties, imposes several challenges to proteomics studies (369, 370, 376). For example, most of the membrane proteins and low abundant proteins cannot be studied with standard 2-DE separation methods because of the limitations of the protein solubility and the sensitivity of detection methods, respectively (369, 377). Many large proteins are not represented in 2-DE gels, and some proteins migrate out of the gel either because of extreme pI values or because of

the low MW of the protein (370). In addition, secreted proteins are lost in the analysis, when cell lysates are used for proteomics studies. The use of non-gel proteomics can circumvent some of the limitations of 2-DE, although they do not provide any information about pI or MW properties of the specific protein form in question. For example large proteins are better detected with an ICAT method (378). However, not all the limitations of 2-DE based proteomics are necessarily bypassed with non-gel approaches. For example the ICAT method has been reported to be strongly biased to detect acidic proteins (with pI <7.0), under-represent small proteins and be unable to show clear superiority over 2-DE in monitoring hydrophobic proteins from cell lysates (378). Sample fractionation can be used to decrease sample complexity in proteomic studies. This can enable the detection of less abundant proteins, but also requires a large amount of sample material and workload (379). The use of ICAT technology is another option to decrease sample complexity, as only cysteine containing peptides are included in the analysis. In this case, decreased complexity also entails the loss of information, as all the other peptides are excluded, which especially reduces the information acquired on protein modifications. The use of iTRAQ, SILAC, or label-free approaches allows in principle all the peptides to be analyzed, but restores the problem of complexity.

3. AIMS OF THE STUDY

IL-4 has a role in driving Th2 differentiation, which is one of the key mechanisms for the onset and perpetuation of allergies and allergic asthma. The overall goal of my thesis was to characterize IL-4 induced differences in primary lymphocytes and especially in T helper cells during the early steps of Th2 differentiation. Differential display proteomics, with 2-DE and protein identification with mass spectrometry, was employed for the identification of regulated proteins. An additional goal was to identify novel mechanisms of Th2 cell differentiation by studying the regulation and function of IL-4 regulated genes and proteins.

The specified aims of my study were

1. to identify the differences in the proteomes of differentiated, reactivated Th1 and Th2 cells (I).
2. to study the effect of IL-4 stimulation on the protein expression pattern of activated naive $CD4^+$ lymphocytes (II).
3. to find out how STAT6 deficiency affects the proteomes of murine lymphocytes (III).
4. to study the functional role of the proteins found from previous proteomics studies (II, III).
5. to characterize the IL-4/STAT6 mediated regulation of GATA3 expression mainly by identifying STAT6 *in vivo* binding sites in GATA3 locus in recently activated IL-4 treated T helper cells (IV).

4. MATERIALS AND METHODS

4.1. Isolation of primary cells

4.1.1. Isolation of human CD4$^+$ lymphocytes from umbilical cord blood

Human neonatal umbilical cord blood was obtained from Turku University Central hospital. $CD4^+$ lymphocytes were isolated from cord blood mainly by using Ficoll Isolation paque (Amersham Pharmacia Biotech Uppsala, Sweden) and $CD4^+$ Isolation kit (Dynabeads M-450 Human, Dynal, Oslo, Norway). RosetteSepTM $CD4^+$ T Cell Enrichment kit (StemCell Technologies, USA) was used for negative $CD4^+$ T cell isolation.

4.1.2. Isolation of mouse MNCs and CD4$^+$ lymphocytes from spleen

STAT6-deficient mice and control wild-type Balb/cJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were bred according to Federation of European Laboratory Animal Science Associations (FELASA) recommendations in the central animal laboratory of Turku. Mononuclear cells were isolated from homogenized spleens with Mouse Lympholyte (Dedarlane, Hornby, Canada). Before culturing the cells for 2-DE separation, the remaining erythrocytes were lysed hypotonically with 0.83 % NH_4Cl. $CD4^+$ T cells were isolated with MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany).

4.2. Activation and culturing of human T cells

4.2.1. PHA and feeder cells

$CD4^+$T cells were plated to final concentration of 1×10^6 cells/ml. Cells were stimulated with irradiated (6400 rad) CD32 and CD80 transfected L-fibroblasts (final concentration 0.5×10^6 cells/ml) obtained from Dr. Hans Yssel (INSERM, Montpellier, France) and with PHA (0.1 μg/ml, Difco, Detroit, MI, USA). Cells were cultured in Yssel's medium (Irvine Scientific, Santa Ana, CA, USA) containing 1% human AB serum (Finnish Red Cross Blood Service, Helsinki) on 24-well flat-bottom plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA). IL-12 (2.5 ng/ml, R & D Systems, Minneapolis, MN, USA) or IL-4 (10 ng/ml, R & D Systems) and anti-IL-12 (10 μg/ml, R & D Systems) were added to differentiate the cells in the Th1 or Th2 direction, respectively.

4.2.2. Anti-CD3 and anti-CD28

When crosslinking of the activating antibodies was performed with anti-mouse F(ab')2 fragment, cells were suspended in Yssel's medium (380) or in RPMI (Sigma, Saint Louis, MO, USA) containing 1 % of human AB serum (the Finnish Red Cross Blood Service) to the concentration $10x10^6$ cells/ml, incubated for 15 minutes at +4 °C with mouse anti-CD3 (10 µg/ml, Immunotech, Marseille, France) and mouse anti-CD28 (10 µg/ml, Immunotech), and then washed with Dulbecco's phosphate buffer solution (D-PBS). Cells were cultured in RPMI (Sigma) medium containing cross-linking dialyzed goat anti-mouse F(ab')2 (final concentration 10 µg/ml, Biosource, Camarillo, CA, USA) and 1 % human AB serum (the Finnish Red Cross Blood Service). Recombinant human IL-4 (10 ng/ml, R & D Systems, Minneapolis, MN, USA) was added to the culture media of IL-4 treated samples.

For plate-bound anti-CD3 activation, cells were activated with plate-bound anti-CD3 (pb anti-CD3) and soluble anti-CD28 so that 200 µl anti-CD3 (0.5 or 2.5 µg/ml in D-PBS, Immunotech) was incubated on 24-well plate wells for 2h at 37 °C and washed twice with 0,5 ml D-PBS before the cells were added to the wells. Cells were cultured at 37°C in 5 % CO_2 in Yssel's (380) or RPMI medium (Sigma) containing 1 % human AB serum and 500 ng/ml mouse anti-CD28 (Immunotech).

4.2.3. Long-term T helper cell differentiation culture

T helper cells were activated in one of the above methods. To induce the differentiation in the Th1 direction, cells were cultured in the presence of IL-12 (2.5 ng/ml, R & D Systems). To induce Th2 differentiation, cells were cultured in the presence of IL-4 (10 ng/ml, R & D Systems) with or without anti-IL-12 (10 µg/ml, R & D Systems). IL-2 (17 ng/ml, R & D Systems) was added into the cultures 48h after the initiation of polarization. Thereafter the cells were fed every other day and the cell concentration was kept in the range of 0.5-2 x 10^6 cells/ml. Cells were polarized for either 7 or 14 days. Only cells activated with PHA feeder cells were cultured for 14 days. Cells were restimulated on day 7 by using the same method as at the beginning of the culture except that the final concentration of $CD4^+$ lymphocytes was 0.5 x 10^6 cells/ml. After restimulation on day 7, cells were cultured as during the first round of polarization.

4.2.4. Inhibiting Fas pathway

Cells were activated with plate-bound anti-CD3 and soluble anti-CD28 as described above. Cells were cultured for 24h and collected. Mouse anti-Fas antibody (5 µg/ml, ALX-805-010-C100, Alexis Biochemicals, Switzerland) or mouse anti-FasL antibody (10 µg/ml, #556371, BD Biosciences Pharmingen, San Jose, CA, USA) was included to the culture medium to inhibit Fas-Fas ligand (FasL) interaction.

4.2.5. Detection of mRNA stability

Cells were isolated and either treated directly with actinomycin D as indicated below, or activated with plate-bound anti-CD3 (2.5 μg/ml) and soluble anti-CD28 as described above. Cells were cultured for either 4 or 20 h in the presence or absence of polarizing cytokines IL-12 and IL-4. Actinomycin D (10 μg/ml, Sigma) was added to the culture medium to inhibit RNA synthesis and cells were collected 0, 30, 60, 90, and 120 min after addition of actinomycin D. RNA was isolated with RNAeasy kit (Qiagen) and cDNA synthesized with Superscript kit (Invitrogen, Foster City, CA, USA). RNA levels were quantitated with Taqman real-time reverse transcription polymerase chain reaction (RT-PCR) as previously described (238) with slight modifications. Absolute QPCR ROX Mix was from ABgene (Surrey, UK). The initial heating step during PCR was 15 min at 95 ^{o}C followed by 40 cycles of 15 sec at 95 ^{o}C and 1 min at 60 ^{o}C. The levels of elongation factor 1 α (EF1α) and Ubiquitin-conjugating enzyme E2D 2 (UbcH5B) mRNA were used for the normalization.

4.3. Activation and culturing of mouse MNCs and CD4^{+} T cells

Mononuclear cells were activated with plate bound anti-mouse CD3 (clone 500A2, Pharmingen, San Diego, CA). Anti-CD3 (325 ng/250 μl/well) was incubated for 2h at 37 ^{o}C and washed three times with 0,5 ml D-PBS before the cells were added to the wells. Cells were cultured in RPMI (Sigma, St. Louis, MO, USA) containing 5 % heat inactivated fetal calf serum (FCS), 1 mM Na-pyruvate (Sigma), 2 mM L-glutamine (Sigma), 50 μM β-mercaptoethanol (Invitrogen, Paisley, Scotland, UK), non-essential amino acids (Invitrogen), and penicillin+streptomycin (Sigma). Cells were stimulated with recombinant murine IL-4 (10ng/ml, Pharmingen, San Diego, CA). The culture density was 5 x 10^{6} cells / 500 μl on 24-well plates. Cells were cultured for 24 hours at 37°C, in a 5 % CO_2 atmosphere.

Isolated CD4^{+} T cells were activated as above with plate-bound anti-CD3 and cultured in the same culture medium in the presence of anti-CD28 (500 ng/ml, Pharmingen, San Diego, CA, USA). For neutralization, 10 μg/ml anti-IL-12, 10 μg/ml anti-IFNγ, and 10 μg/ml anti-IL-4 (all from Pharmingen, San Diego, CA, USA) were added to the culture medium.

4.4. Metabolic labeling of the cells

For metabolic labeling, human and mouse cells were cultured for 24h in methionine free RPMI (Sigma) medium including ^{35}S-methionine and ^{35}S-cysteine (50μCi/ml, Redievue Pro-Mix L-[35S] *in vitro* cell labelling, Amersham Pharmacia Biotech) as well as the supplements mentioned in Chapters 4.2.2. and 4.3, respectively.

4.5. Cytokine production of differentiated Th cells

To measure cytokine concentration in reactivated human T helper cell cultures, cells were cultured for 7 or 14 days, harvested, and reactivated to induce cytokine production.

For enzyme-linked immunosorbent assay (ELISA) measurements, polarized cells were cultured for 16-20 h with or without 5 ng/ml PMA (Phorbol-12-myristate-13-acetate, Calbiochem) and 5 μg/ml Concanavalin A (Pharmacia). The culture medium was separated from the cells by centrifugation and stored at –20°C until used for the measurement of cytokine concentration. Production of IL-4 and IFN-γ was measured with sandwich ELISA using commercially available antibody pairs (Pharmingen, San Diego, CA, USA).

For Multiplex cytokine measurements, polarized cells were cultured for 24h with or without 5 ng/ml PMA (Calbiochem) and 500 ng/ml ionomycin (Sigma-Aldrich). The culture medium was separated from the cells by centrifugation and stored at –70°C until used for the measurement of cytokine concentration. Secreted cytokines were measured using Luminex assay and multiplex bead kits (Bio-Rad). Measurements and data analysis were performed with the Bio-Plex system in combination with the Bio-Plex Manager software (Bio-Rad).

Polarization of the cells that were used for Affymetrix studies was monitored by intracellular cytokine staining with anti-IFNγ and anti-IL-4 antibodies (Caltag Laboratories, Burlingame, CA, USA) as previously described (251).

4.6. Two-dimensional electrophoresis and gel comparison

Cells were lysed and proteins were separated with two-dimensional electrophoresis (2-DE) as previously described (381). Briefly, soluble proteins were absorbed into the 18 cm pH 3-10 non-linear IPG-strips (Amersham Pharmacia Biotech) for 24h at room temperature (RT). Isoelectric focusing to a total of 40 kVh was carried out at 20°C, and the focused strips were equilibrated for 25 min at RT. The second dimension was vertical 12% SDS-PAGE with gel thickness of 1 mm. Proteins were detected with silver staining (382) and metabolically labeled proteins by autoradiography (24h) (383). Autoradiography images were normalized and compared with the PDQuest program for the comparison of protein expression levels in 2-DE gels (BioRad, Hercules, CA, USA).

4.7. Protein identification with mass spectrometry

Proteins that were selected for identification, were in-gel digested with trypsin, and the resulting peptides were analyzed either by peptide mass fingerprinting or with nano-LC-MS/MS as previously described (358, 360, 384, 385). For both methdods, the excised gel spot was first cut into pieces, washed twice, and dehydrated with

acetonitrile. Proteins were reduced with 20 mM dithiothreitol (Sigma), alkylated with 55 mM iodoacetamide (Sigma), and in-gel digested with trypsin (Sequencing Grade Modified Trypsin, Promega Corporation, Madison, WI, USA) at +37 ^{o}C over night (o/n).

For the peptide mass fingerprinting, the peptides were purified and concentrated using reverse phase columns (Poros Oligo R3, PerSeptive Biosystems, Framingham, MA, USA). Peptides were eluted directly onto the sample plate with saturated α-cyano-4-hydroxycinnamic acid (HCCA, Aldrich Chemical Company Ltd, Dorset, UK) in 0.1% trifluoroacetic acid, 60% acetonitrile. Peptide masses were measured with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS, Voyager–DE^{TM} PRO, PerSeptive Biosystems, Houston, Texas, USA) in positive ion reflector mode. Spectra were internally calibrated using autoproteolytic trypsin fragments, or with standard peptides (Cal Mix 2, Sequazyme peptide mass standard kit, PE Biosystems, Framingham, MA, USA). The masses of the acquired peptides were generated with Data explorer V4 program (Applied Biosystems). Database searches were pefromed using either MS-Fit (http://prospector.ucsf.edu/) or Mascot (http://www.matrixscience.com) software against NCBI and SWISS-PROT databases.

For nano-LC-MS/MS identification, peptides were extracted with 5% formic acid, 50 % acetonitrile and dried with a vacuum centrifuge. Peptides were dissolved to 2 % HCOOH prior to MS analysis. Analyses were made with a QStar Pulsar^{TM} electrospray ionization (ESI) - hybrid quadrupole - TOF instrument (Applied Biosystems/MDS Sciex, Toronto, Canada) coupled on-line with nano-scale high performance liquid chromatography (nanoHPLC) (Famos, SwitchosII and Ultimate, LC Packings, Amsterdam, Netherlands) as previously described (360). Peak lists from the MS/MS spectra were created with the Analyst QS program (Version 1.1, Applied Biosystems) using the mascot.dll script. Peak lists were analysed using Mascot software (www.matrixscience.com) and searched against a Swiss-Prot or TrEMBL databases.

4.8. Affymetrix studies

For transcriptomics studies, CD4^{+} lymphocytes were polarized for 7 days as described above and reactivated by plate-bound anti-CD3 (500 ng/well for coating) and 0.5 µg/ml soluble anti-CD28 (Immunotech, Marseille, France) for 6 hours.

Two replicates were hybridized on Affymetrix oligonucleotide microarrays. The total RNA of the samples was isolated using the Trizol method (Invitrogen Co., Carlsbad, CA) and was further purified with Qiagen's RNAeasy minikit (Qiagen, Valencia, CA). 4-5 µg of total RNA was used as starting material for the Affymetrix sample preparation. The sample preparation was performed according to the instructions and recommendations provided by the manufacturer (Affymetrix, Santa Clara, CA, USA, http://www.affymetrix.com/). The samples were hybridized to HG-U133A arrays containing ~20 000 probe sets. The data was analyzed on three consecutive levels. At the detection level, each probe was assigned a call of present, absent or marginal. The

comparison level of analysis includes the determination of the signal log ratio between activated Th1 and Th2 cell samples. At the third level of data analysis, the log ratio was changed to the fold difference of the expression itself. The fold difference of a specified gene was measured as a mean of two individual fold difference results. In each case where the expression was either upregulated or downregulated at least by 2-fold, this difference was seen in both experiments.

4.9. Western blotting

Cells were lysed in 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10 % glycerol, 50 mM dithiothreitol (DTT), 0.1 % w/v bromophenyl blue. SDS-PAGE and protein transfer to nitrocellulose membranes were carried out according to standard protocols (386). If the proteins, that were separated with 2-DE, were transferred to a nitrocellulose membrane, 2-DE gels were cut into convenient parts that were transferred independently.

The following antibodies were used for Western blots: rat anti-GRP94 (RT-102-P1ABX, NeoMarkers, Fremont, CA, USA), goat anti-hnRNPK (sc-16554, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-hnRNPE1 (sc-16504, Santa Cruz Biotechnology), rabbit anti-Ly-GDI (#556511, BD Pharmingen), mouse anti-D4-GDI (anti-Ly-GDI, cleavage product specific, ALX-804-264, Alexis Biochemicals), mouse anti-c-FLIP (ALX-804-428, Alexis Biochemicals), mouse anti-CBFb 1:250 (BD Transduction Laboratories #610514, recognizes a peptide sequence specific for isoform 2), rabbit CNBP antiserum 1:500, mouse anti-GATA3 (sc-268, Santa Cruz Biotechnology) and mouse anti-β-actin (#A5441, Sigma). Members of Bcl-2 family were detected with Bcl-2 family sampler kit (#612742, BD Biosciences). Rabbit anti-αNAC was a generous gift from Dr. René St-Arnaud (Genetics Unit, Shriners Hospital for Children, Montréal, Québec, Canada). Secondary antibodies used in these studies: horse radish peroxidase conjugated (HRP) goat anti-mouse (sc-2005, Santa Cruz Biotechnology), HRP goat anti-rabbit (#554021, BD Pharmingen), HRP donkey anti-goat (sc-2020, Santa Cruz Biotechnology), HRP goat anti-mouse IgG1 (Cat. 1070-05, Southern Biotech, Birmingham, AL, USA), and HRP goat anti-rat (#81-9520, Zymed laboratories, San Francisco, CA, USA).

4.10. Flow cytometry

In the order of 0.1–0.5 $x10^6$ cells were used for each flow cytometry sample. Staining was performed at +4 ^oC and the cells were incubated in the dark. Samples were measured with FACScan or FACSCalibur™ System (BD Biosciences) and analyzed with CellQuest or CellQuest Pro (BD Biosciences), respectively. At least 10 000 cell counts were measured per sample.

For cell surface stainings, cells were washed once with FACS buffer (2% FCS, 0,01% atzide in D-PBS) and incubated for 15 min with specific antibodies or their isotype

controls, washed once with FACS buffer, once with FACS buffer w/o FCS, and finally resuspended to 1% formalin in D-PBS.

The amount of intracellular cleaved Rho GDP dissociation inhibitor (GDI) β (Ly-GDI) was measured with flow cytometry in the following way: Cells were washed twice with staining buffer (0,5% BSA, 0,01% atzide in D-PBS) and fixed with 4% paraformaldehyde in D-PBS for 15 min. The cells were washed once with staining buffer, permeabilized for 10 min with a permeabilization buffer (0,5% saponin, 0,5% BSA, 0,01% azide in D-PBS) and incubated for 20 min with 5 µl mouse anti-D4-GDI (cleavage product specific, Alexis Biochemicals). Cells were then washed 4 times with permeabilisation buffer and incubated with goat F(ab')2 anti-mouse-FITC (M35001, Caltag) for 20 min. Finally, the cells were washed 4 times with permeabilisation buffer, once with staining buffer, and then resuspended in the staining buffer.

Active Caspase-3 was measured according to the protocol provided by the manufacturer (#550914, BD Biosciences Pharmingen).

4.11. Chromatin immunoprecipitation

Cells were fixed with 1% formaldehyde for 3-10 min at room temperature followed by incubation in 125 mM glycine in D-PBS for 5 min. The cells were then harvested, washed once with ice-cold PBS, pelleted and stored at -70 ^{o}C. DNA was sonicated, until the maximum length of the majority of DNA fragments was below 500 bps. Precipitation was mainly performed according to Upstate's Chromatin immunoprecipitation assay kit protocol, with slight modifications. The third washing buffer was 10% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.1), 20 mM Tris (pH 8.1). Protease inhibitors (Complete, Roche) and phosphatase inhibitors (1mM NaF and 1 mM Na_3VO_4) were added to all the lysis and precipitation buffers. Precipitated samples were incubated with RNAse A at 65 ^{o}C o/n, treated with Proteinase K for 2 h at 42 ^{o}C, after which Protein G sepharose beads were discarded and DNA purified by pheonol extraction followed by ethanol precipitation. DNA was further purified with magnetic beads (genopure ds kit, Bruker, Leibzig, Germany). Isolated DNA from input and precipitated samples was quantified with Taqman real-time RT-PCR as previously described (238) with slight modifications. Absolute QPCR ROX Mix was from ABgene (Surrey, UK), primers and probes from DNA Technology (Aarhus C, Denmark) or Oligomer (Helsinki, Finland). The initial heating step during PCR was 15 min at 95 ^{o}C, followed by 40 cycles of 15 sec 95 ^{o}C and 1 min 60 ^{o}C.

The following antibodies were used for immunoprecipitation: rabbit anti-STAT6 (sc-1698 and sc-621, both from Santa Cruz Biotechnology), rabbit anti-mono/di/trimethyl-Histone H3 (Lys 4) (#05-791, Upstate, Lake Placid, NY, USA), rabbit anti-dimethyl-Histone H3 (Lys 9) (#07-441, Upstate), rabbit anti-dimethyl-Histone H3 (Lys 27) (#07-452, Upstate), and rabbit serum (Sigma).

A genomic region from human CSTF2 locus was used as a negative control for enriched DNA, as there are no putative STAT6 binding sites in the proximity of that specified region. Ct values were normalized both against the immunoprecipitation sample with rabbit serum and against signals acquired CSTF2 locus with following equation (IP, immunoprecipitation):

$$[Ct(\text{CSTF2 region after antibody IP}) - Ct(\text{CSTF2 region after serum IP})] - [Ct(\text{STAT6 site after antibody IP}) - Ct(\text{STAT6 site after serum IP})]$$

4.12. Immunohistochemistry and confocal microscopy

In the order of 0.1-0.5 x 10^6 cells / sample were fixed with 3.65 % formaldehyde for 15 min at RT and permebilized with 0.2 % saponin. Unspesific binding was blocked with 10 % BSA. Primary antibody incubations were done in 1.0 % BSA, 0.2 % saponin buffer o/n at 4 ºC and secondary antibody incubations in the same buffer for 45min at RT. The antibodies used were: CBFb (BD #610514, 1:25), Runx1 (Active motif, #39000, 1:100), FITC conjugated goat F(ab')2 anti-mouse IgG (H+L), (Caltag #M35001 1:200) and goat anti-rabbit Alexa 568nm (Molecular Probes 1:400). The cell nuclei were stained separately with 4',6-diamidino-2-phenylindole (DAPI) or by using DAPI containing mounting medium (Molecular probes SlowFade #S36938). Zeiss LSM510 META laser scanning confocal microscope was used for image acquisition.

5. RESULTS AND DISCUSSION

5.1. The proportion of naive cells in freshly isolated $CD4^+$ T cells

Cell surface marker expression of human and mouse $CD4^+$ T cells was measured with flow cytometry. In human cord blood samples, 90.8 ± 4.0 % of the freshly isolated $CD4^+$ cells were $CD45RA^+$ and 20.8 ± 7.1 % $CD45RO^+$ (consisting mainly of $CD45RO^{low}$ cells), so approximately 90 % of the cells were naive in these samples. These figures are typical for human umbilical cord blood samples (387).

In mouse $CD4^+$ T cells, approximately 77.9 ± 1.0 % of the cells were $CD62L^+$ and 21.6 ± 2.1 % $CD44^{high}$. The proportion of $CD45RB^{high}$ cells was 68.4 ± 2.5 %. In mouse $CD8^+$ T cells, approximately 91.0 ± 0.3 % of the cells were $CD62L^+$ and 9.6 ± 1.0 % $CD44^{high}$. The proportion of $CD45RB^+$ cells was 90.5 ± 2.6 %. These results indicated that the proportion of naive cells was nearly 80 % in mouse $CD4^+$ cells and approximately 90 % in $CD8^+$ cells. There were no significant differences between wt and STAT6 deficient (Stat6-/-) cells (Table 1 and data not shown).

Table 1. Cell surface expression of freshly isolated mouse splenic $CD4^+$ T cells. No significant differences were detected between wt and STAT6-/- cells.

	Balb/cJ %	**Stat6 -/- %**
$CD62L^+$	78.7	77.1
$CD45RB^{high}$	70.3	66.6
$CD44^{high}$	20.5	22.7

5.2. The proportions of different cell types in mouse mononuclear cell populations

As a heterogenous mononuclear cell population was used in the proteomics studies to identify STAT6 target proteins (III), we aimed to confirm that the proportions of different cell types were similar between activated IL-4 stimulated wt and Stat6-/- cells. There were on average 41.0 ± 2.7 % $CD4^+$ cells, 20.5 ± 3.0 % $CD8^+$ cells, 43.3 ± 9.9 % $CD19^+$ cells, 1.5 ± 1.0 integrin alpha M positive ($Mac\text{-}1^+$) cells and 7.9 ± 3.2 natural killer (NK) cells in the mononuclear cell populations (Table 2). The proportion of cells expressing early activation marker CD69 in $CD4^+$ and $CD4^-$ cell populations is also indicated in the table. No significant reproducible differences were detected between wt and STAT6-/- cells.

Table 2. The proportions of different cell types in wt and STAT6-/- splenic mononuclear cell populations. Cells were isolated from the spleens of wt and STAT6-/- mice, stimulated through CD3 (Act) and cultured for 24h in the presence or absence of IL-4. The cell surface marker expression of wt and STAT6-/- mononuclear cells was studied with flow cytometry. No reproducible differences were detected between wt and STAT6-/- cells. The proportion of Mac-1^+ cells was slightly decreased in cultured samples. The mean values from 2 independent experiments are shown in the table.

		wt	Stat6-/-	wt		Stat6-/-	
		0h	0h	Act	Act + IL-4	Act	Act + IL-4
		%	%	%	%	%	%
CD4		42.9	43.4	39.1	42.8	39.2	38.6
CD8		20.3	17.4	22.9	22.5	18.8	21.2
CD19		39.0	37.0	42.9	44.0	53.8	43.1
NK		7.2	9.0	6.6	6.9	7.6	10.1
Mac-1		2.8	2.8	0.9	0.7	1.0	1.0
CD69	**CD4+**	14.2	12.2	33.2	39.1	33.3	37.9
	CD4-	5.5	5.2	38.6	33.0	38.8	39.5

5.3. Differences in the proteomes of activated Th1 and Th2 cells

The differences in the proteomes of differentiated human Th1 and Th2 cells were studied after 7 and 14 days of polarization (I). Differentiated cells were reactivated for 24h in the presence of radioactivate ^{35}S-Met/Cys, harvested, and lysed, followed by 2-DE analysis and peptide mass fingerprinting to identify differentially expressed proteins. There were altogether 14 protein spots, whose expression differed between polarized Th1 and Th2 cells, and 12 unique proteins were identified from these spots (Table 3 and I, Figure 1 and Table 1). The mRNA expression of these genes was also studied with Affymetrix 6h after restimulation (Table 3 and I, Table 2). Nearly all the proteins, whose expression was higher in Th1 than in Th2 cells in both 2-DE and Affymetrix samples (Table 3), were known to be regulated by interferons (358, 388-392). Proteins in spots that were upregulated in Th2 cells were not differentially expressed between Th1 and Th2 cells at RNA level (Table 3). The secretion of IFNγ is markedly induced by reactivation in differentiated Th1, but not in Th2, cells. Therefore, the higher expression of interferon target genes in Th1 than Th2 cells is consistent with enhanced IFNγ signaling in reactivated Th1 cells.

Proper differentiation of Th1 and Th2 cells was confirmed for proteomics studies with ELISA and for transcriptomics studies with intracellular staining of cytokines (data not shown). The production of IFNγ and IL-4 were used as indicators of Th1 and Th2 polarization, respectively. More profound differences between Th1 and Th2 cells were detected in IFNγ production, although IL-4 was produced more in Th2 than Th1

samples. Smaller differences in IL-4 production may originate from the fact that generally 5-20 % of the Th2 polarized cells produce IL-4 after 7 and 14 days of culturing (393, 394). This suggests that the generation of human Th2 cells is not very efficient with this kind of *in vitro* polarization approach, although Th1 differentiation is clearly inhibited by IL-4 during the first two weeks of polarization (393). On the other hand, the proportion of $CRTH2^+$ cells is larger than that of IL-4 producing cells at least after 7 days of culturing, so IL-4 production might be a too strict criterion for the detection of Th2 cells (48). Still, only a proportion of cells show Th2 type of cytokine and CRTH2 expression after 7-14 days of culturing. The enrichment of IL-4 producing or $CRTH2^+$ cells prior to the comparison between Th1 and Th2 cells is likely to facilitate the detection of genes with a Th2 specific expression profile.

Table 3. Differentially expressed proteins in polarized Th1 and Th2 cells and their expressional difference at protein level in 2-DE gels and at RNA level in Affymetrix experiments. Spot numbers in the first column correspond to the ones in I, Figure 1. Accession numbers of the identified proteins in Swiss-Prot database are also indicated in the table. γ-actin (accession number P63261) was identified with the same peptide mass fingerprint as β-actin from protein spots number 10 and 11. Because the RNA expression of β- and γ-actin was similar, only the expression of β-actin is indicated in the table. LI: could not be detected because of the low intensity. *In 7 and 14b, two proteins were identified from one spot, and the fold difference refers to the corresponding spot. This table is reproduced from tables 1 and 2 in the original article I.

Spot no.	Identified proteins	Accession no.	Fold change	
			Protein	RNA
	Th1 > Th2			
1.	Tryptophanol-tRNA synthetase	P23381	2.0	6.4
2.	Vacuolar ATP synthase subunit E	P36543	1.7	1.2
3.	Proteasome subunit alpha type 4	P25789	2.9	1.5
4. U	Interferon-induced 35 kDa protein (IFP 35)	P80217	12.4	3.9
L	Interferon-induced 35 kDa protein (IFP 35)	P80217	7.8	3.9
5.	Proteosome activator subunit 1	Q06323	2.8	2.4
6.	Histone H2A	P20670	7.1	LI
7.	UCRP	P05161	4.1*	3.3
	Histone H2A	P20670		LI
	Th2 > Th1			
9.	Prohibitin	P35232	2.4	0.9
10.	Actin, cytoplasmic 1 (β-actin)	P60709	4.7	1.1
11.	Actin, cytoplasmic 1 (β-actin)	P60709	3.4	1.1
	Modificational difference			
13. a	Actin-regulatory protein CAP-G	P40121	1.1	0.8
b	Actin-regulatory protein CAP-G	P40121	0.3	0.8
14. a	Cyclophilin A	P62937	1.7	0.9
b	Cyclophilin A	P62937	1.7*	0.9
	UbcH8	O14933		1.9
c	Cyclophilin A	P62937	0.6	0.9
d	Cyclophilin A	P62937	0.7	0.9

5.4. IL-4/STAT6 regulated proteins in proteomics studies

In publications II and III, 2-DE based proteomics was used to identify novel IL-4 target proteins in activated human $CD4^+$ T cells as well as STAT6 target proteins in anti-CD3 activated IL-4 stimulated mouse mononuclear cells, respectively. In both studies, the cells were stimulated for 24h in the presence of radioactive ^{35}S prior to 2-DE analysis. In human $CD4^+$ T cells, the expression of 20 proteins were reproducibly regulated by IL-4, 7 of which were upregulated and 13 downregulated in IL-4 treated cells (II, Figure 1). Altogether 35 unique proteins were identified from these protein spots using tandem mass spectrometry (Table 4 and II, Table 1). In mouse mononuclear cells, 21 proteins were differentially expressed between wt and STAT6-/- cells, 16 of which being more highly expressed in wt and 5 in STAT6-/- cells (III, Figure 1). Altogether 49 unique proteins were identified from these protein spots (Table 5 and III, Tables 1 and 2). No specific protein identification could be assigned from 3 regulated protein spots in these studies, which was likely due to the low amount of protein in 2-DE gels. The number of regulated proteins is rather low in both human and mouse. However, comparable numbers of proteins have also previously been found to be regulated by cytokines in activated $CD4^+$ T cells, when 2-DE based proteomics has been used to detect differential expression (359, 360). In addition, the number of IL-4 or STAT6 regulated genes (approximately 40-150) is not very high in $CD4^+$ T or B cells according to transcriptomics studies published to date (111, 112, 217, 251, 395). Furthermore, most of the regulated genes reported in transcriptomics studies are beyond the scope of 2-DE based proteomics, because they are expressed as trans-membrane, low abundant or secreted proteins.

There were altogether four proteins identified from protein spots that were regulated both by IL-4 in activated human $CD4^+$ T cells and by STAT6 in mouse mononuclear cells: Actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Histone H2A, and Lamin B1 (Tables 4 and 5). These are all abundant proteins, and their different forms are easily identified from several positions in 2-DE gels. Additional proteins were generally also identified from these same protein spots, and they might be responsible for the differences in protein expression in 2-DE gels. In addition, the identified Actin, GAPDH, Histone H2A, and Lamin B1 proteins migrated to different positions in the gel in human and mouse samples. As all these proteins are highly conserved between human and mouse, differential migration of these proteins suggest that different protein forms are represented in identified human and mouse samples. There might be several reasons for the identification of different target proteins in these studies. First of all, the mouse and human cell populations are different: $CD4^+$ cells represent only approximately 40 % of the mouse mononuclear cell population, whereas purified $CD4^+$ T cells were used for human studies. Human and mouse cells were also isolated from different organs, and the cells were activated in different ways. It is notable that all the STAT6 regulated proteins need not be targets of IL-4, as although the cellular localization and transcriptional activity of STAT6 is regulated by IL-4, it might have additional, IL-4 independent regulatory functions at protein level, for example in the

cytosolic protein compartment. In addition, unphosphorylated STAT6 has been reported to bind to prostaglandin-endoperoxide synthase 2 (COX-2) promoter and upregulate its expression (396). IL-4 also induces signaling pathways other than the STAT6 mediated one (76), which might have STAT6 independent targets in the cell. Interestingly, transcriptomics studies conducted in our group have revealed very few genes that are regulated both in a IL-4/STAT6 dependent manner in mice and by IL-4 in humans (111, 217, 251).

In the experiments included in the thesis, autoradiography has been used to detect the IL-4/STAT6 regulated proteins. In addition to providing good sensitivity and linear range (383, 397), this method also enables the detection of newly expressed proteins, i.e. those containing the radiolabeled aminoacids. However, the differential expression of proteins in autoradiography images does not implicate that the proteins in question are transcriptionally regulated, as protein expression can be regulated at multiple levels. Additionally, since we have used the 24h timepoint in our proteomics studies, this increases the likelihood that the proteins identified are not direct targets of IL-4 induced signaling pathway or STAT6 mediated transcription. Actually, none of the proteins identified in these studies are reported to be IL-4/STAT6 regulated at RNA level (111, 112, 217, 251, 395). Although this could be due to the experimental differences in proteomics and transcriptomics studies, many of the differences detected in 2-DE gels are likely to result from post-translational regulation of these proteins. For example, many proteins identified in publications II and III, such as hnRNP K (II), αNAC (II), and CNBP (III), are differentially expressed in 2-DE gels but their total protein expression is similar in all samples studied. Also all of the proteins, whose expression was studied with Western blotting after 2-DE separation, migrated to several different positions in the gel, indicating the presence of different forms of these proteins in the cell. As post-translational regulation is an important aspect of cellular responses, the identification of post-translationally regulated IL-4 or STAT6 target proteins can provide important information on the cellular events during Th2 type responses.

Table 4. IL-4 regulated proteins in activated human T helper cells. Differentially expressed proteins were identified with tandem mass spectrometry and database searches. Average fold change, the reproducibility of at least 2-fold difference, and accession numbers in Swiss-Prot database are indicated in the columns. Spot numbers in the first column correspond to the ones in II, Figure 1. a, a 2-fold difference was seen once in the opposite direction. b, P58876, Q99880, Q99877, Q99879, Q93079, O60814, and P57053 were also identified from the same sample with same peptide identification. c, P04908, Q93077, P20671, P28001, Q96KK5, Q99878, P0C0S8, Q6FI13, Q16777, Q7L7L0, and P16104 were also identified from the same sample with same peptide identification. *, Paired t-test value <0.05 for the effect of IL-4. **, Paired t-test value <0.01 for the effect of IL-4. This table is a modified version of table I in the original article II.

Upregulated by IL-4:

Spot No.	Reproducibility of ≥2-fold differences	Fold difference	Accession Number	Identified Proteins
1	3/5**	2.1	P61978	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)
2	3/6*	1.8	Q15365	Poly(rC)-binding protein 1 (Alpha-CP1, hnRNP E1)
			P09972	Fructose-bisphosphate aldolase C (EC 4.1.2.13)
3	4/5**	2.7	Q13765	Nascent polypeptide-associated complex alpha subunit (αNAC)
			P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 (Transducin beta chain 1)
4	3/5	1.7	P07195	L-lactate dehydrogenase B chain (LDH-B)
			P05388	60S acidic ribosomal protein P0 (L10E)
5	3/6	2.2	-	Not identified
6	4/6	1.8	P25398	40S ribosomal protein S12
			P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
			P62807[b]	Histone H2B
			P00338	L-lactate dehydrogenase A chain (LDH-A)
			P49773	Histidine triad nucleotide-binding protein 1 (Adenosine 5'-monophosphoramidase)
			P23528	Cofilin-1 (Cofilin, non-muscle isoform, 18 kDa phosphoprotein)
			P10606	Cytochrome c oxidase polypeptide Vb, mitochondrial precursor (EC 1.9.3.1)
7	3/6[a]	1.6	Q96QV6[c]	Histone H2A

Downregulated by IL-4:

8	2/4*	1.9	P14625	Endoplasmin precursor (94 kDa glucose-regulated protein, GRP94)
9	4/5	2.7	P13796	L-plastin (Plastin-2, Lymphocyte cytosolic protein 1)
			P20700	Lamin-B1
			P02768	Serum albumin precursor
10	3/5	3.0	P13796	L-plastin (Plastin-2, Lymphocyte cytosolic protein 1)
			P20700	Lamin-B1
			P02768	Serum albumin precursor
11	3/5	1.4	P35527	Keratin, type I cytoskeletal 9 (Cytokeratin-9, CK-9)
			P60709, P63261	Actin, cytoplasmic 1/2 (β/γ-actin)
12	3/5	3.1	P07437	Tubulin beta-2 chain
13	3/4*	2.8	P61158	Actin-like protein 3 (Actin-related protein 3)
14	4/6	2.4	Q9UL46	Proteasome activator complex subunit 2 (Proteasome activator 28-beta subunit)
15	4/6*	2.3	P52566	Ly-GDI (Rho GDP-dissociation inhibitor 2, Rho GDI 2, Rho-GDI beta)
			P37802	Transgelin-2 (SM22-alpha homolog)
			O94833	Bullous pemphigoid antigen 1, isoforms 6/9/10 (Trabeculin-beta)
16	3/5	2.9	P08758	Annexin A5 (Annexin V, Lipocortin V, Endonexin II)
17	2/3	2.6	P08758	Annexin A5 (Annexin V, Lipocortin V, Endonexin II)
			P63165	Small ubiquitin-related modifier 1 precursor (SUMO-1)
18	3/6[a]	1.1	P62937	Peptidyl-prolyl cis-trans isomerase A (PPIase A, Rotamase A, Cyclophilin A)
			P30044	Peroxiredoxin-5, mitochondrial precursor (EC 1.11.1.15, Prx-V)
			Q16695, P68431, P84243	Histone H3
19	2/3	1.5	P61088	Ubiquitin-conjugating enzyme E2 N (EC 6.3.2.19, Ubiquitin-protein ligase N)
20	3/4	4.4	P02042	Hemoglobin delta subunit (Hemoglobin delta chain)
			P60709, P63261	Actin, cytoplasmic 1/2 (β/γ-actin)
			Q96QV6[c]	Histone H2A
			P69905	Hemoglobin alpha subunit (Hemoglobin alpha chain)

Table 5. Stat6 target proteins in TCR activated and IL-4 stimulated mouse mononuclear cells at 24h. Differentially expressed proteins were identified with tandem mass spectrometry and database searches. Average fold change, the reproducibility of differential expression, and Swiss-Prot/TrEMBL entry name for the proteins are indicated in the columns. Spot numbers in the first column correspond to the ones in III, Figure 1. a, Different peptides are identified from entries Q3UYR3_MOUSE and PEBB_MOUSE. Entry Q3UYR3_MOUSE contains isotype 2 specific peptide. b, ACTC_MOUSE, ACTG_MOUSE and/or ACTS_MOUSE were also identified from the same sample with same peptide identification. c, H2A1F_MOUSE, H2A1H_MOUSE, H2A1K_MOUSE, H2A2A_MOUSE, H2A2C_MOUSE, H2A3_MOUSE, H2AV_MOUSE, H2AX_MOUSE, and/or H2AZ_MOUSE were also identified from the same sample with same peptide identification. This table is reproduced from table 1 in the original article III.

Proteins that are more expressed in wt than Stat6-/- cells:

Spot no.	Fold change wt / Stat6-/-	Reproducibility	Entry name	Identified proteins
1	2.2	3/3	COR1A_MOUSE	Coronin-1A (Clipin-A)
			FKBP4_MOUSE	FK506-binding protein 4
			CH60_MOUSE	60 kDa heat shock protein, mitochondrial precursor (Hsp60)
2	2.2	3/3	ACTB_MOUSE[b]	Actin
			HNRPF_MOUSE	Heterogeneous nuclear ribonucleoprotein F (hnRNP F)
3	2.0	2/3	PSD13_MOUSE	26S proteasome non-ATPase regulatory subunit 13
			Q9CQT1_MOUSE	RIKEN library, clone:3010001P22 product:hypothetical Initiation factor 2B containing protein
			SHLB1_MOUSE	SH3 domain GRB2-like protein B1 (Endophilin-B1)
4	2.4	3/3	IF33_MOUSE	Eukaryotic translation initiation factor 3 subunit 3 (eIF-3 gamma)
			TWF2_MOUSE	Twinfilin-2 (mA6RP)
			COR1A_MOUSE	Coronin-1A (Clipin-A)
			CTBP1_MOUSE	C-terminal-binding protein 1 (EC 1.1.1.-, CtBP1)
5	2.3	2/2	G3P_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPDH)
			MDHM_MOUSE	Malate dehydrogenase
6	3.6	3/3	GBLP_MOUSE	Guanine nucleotide-binding protein subunit beta 2-like 1
			CH1B2_MOUSE CH1B1_MOUSE	Charged multivesicular body protein 1b (Chromatin-modifying protein 1b, CHMP1b)
			RSU1_MOUSE	Ras suppressor protein 1 (Rsu-1, RSP-1)
			PSA4_MOUSE	Proteasome subunit alpha type
7	2.4	3/3	PSB1_MOUSE	Proteasome subunit beta type
			GSTP1_MOUSE	Glutathione S-transferase P 1
8	2.0	3/3	Q3UYR3_MOUSE[a]	Core-binding factor subunit beta (CBF-beta), isoform 2
			PEBB_MOUSE	
9	2.4	3/3	Q3UIQ7_MOUSE Q71LX8_MOUSE	Heat shock protein HSP 90-beta (HSP 84)
			LMNB1_MOUSE	Lamin-B1
			PGK1_MOUSE	Phosphoglycerate kinase 1
			ACTB_MOUSE[b]	Actin
10	2.5	2/3	LMNB1_MOUSE	Lamin-B1
			GDIR_MOUSE	Rho GDP-dissociation inhibitor 1 (Rho GDI 1, Rho-GDI alpha, GDI-1)
			ACTB_MOUSE[b]	Actin
			HS90A_MOUSE HS90B_MOUSE	Heat shock protein HSP 90
			LASP1_MOUSE	LIM and SH3 domain protein 1 (LASP-1, MLN 50)
11	2.8	2/2	Q9QY93_MOUSE	RS21-C6 (RIKEN cDNA 2410015N17 gene)
			PSB9_MOUSE	Proteasome subunit beta type 9
			VIME_MOUSE	Vimentin
			Q6PFA2_MOUSE Q3THU7_MOUSE	Clathrin, light polypeptide
			CBX3_MOUSE	Chromobox protein homolog 3
			DBLOH_MOUSE	Diablo homolog, mitochondrial precursor (Smac protein)
			ACTB_MOUSE[b]	Actin
12	3.5	2/2	Not identified	
13	2.4	3/3	MTND_MOUSE	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase (Aci-reductone dioxygenase, ARD)
			PEBP1_MOUSE	Phosphatidylethanolamine-binding protein 1 (PEBP-1, HCNPpp)
14	10.6	2/2	Q3U935_MOUSE Q3ULK8_MOUSE	Cellular nucleic acid binding protein
			H2A1_MOUSE[c]	Histone H2A
15	2.4	3/3	Not identified	
16	4.8	2/2	UB2D3_MOUSE	Ubiquitin-conjugating enzyme E2 D3
			H2A1_MOUSE[c]	Histone H2A

Table 5. (continued)

Proteins that are more expressed in Stat6-/- than wt cells:

Spot no	Fold change Stat6-/- / wt	Reproducibility	Entry name	Identified proteins
17	**2.8**	**2/2**	DOCK2_MOUSE	Dedicator of cytokinesis protein 2
			TPIS_MOUSE	Triosephosphate isomerase
			THY1_MOUSE	Thy-1 membrane glycoprotein precursor
			PSB3_MOUSE	Proteasome subunit beta type 3
18	**2.4**	**3/3**	RB11A_MOUSE RB11B_MOUSE	Ras-related protein Rab-11
			RHOA_MOUSE	Transforming protein RhoA
19	**2.1**	**3/3**	GRP78_MOUSE	78 kDa glucose-regulated protein precursor (GRP 78, Heat shock 70 kDa protein 5)
20	**2.5**	**3/3**	H2A1_MOUSE[c]	Histone H2A
			ACTC_MOUSE[b]	Actin
			COR1A_MOUSE	Coronin-1A
			UBE2A_MOUSE	Ubiquitin-conjugating enzyme E2 A
21	**2.2**	**3/3**	NUCL_MOUSE	Nucleolin (Protein C23)
			PSA1_MOUSE	Proteasome subunit alpha type 1

5.5. CNBP and CBFb2, two STAT6 regulated proteins in mouse lymphocytes

In our search for novel STAT6 target proteins in mononuclear cells after T cell activation and IL-4 stimulation, we identified two interesting proteins, namely CNBP and core binding factor b, isoform 2 (CBFb2), both of which were more expressed in wt mononuclear cells (III, Table 1, Figure 1). We could also confirm their differential expression observed in 2-DE gels with Western blotting (III, Figures 2c and 3b). Interestingly, the total expression of neither CNBP nor CBFb2 was regulated by STAT6 in the mononuclear cell population (III, Figures 2b and 3a). However, STAT6 was found to downregulate the total expression of CBFb2 in $CD4^+$ T cells (III, Figure 3a). CBFb dimerizes with Runx proteins, which allows them to bind to DNA and regulate target gene transcription (398). Runx proteins are important regulators of cell development ((399-403), and especially Runx1 is reported to inhibit Th2 differentiation, GATA3 expression and Th2 cytokine production (302, 303). Our discovery of STAT6 dependent downregulation of CBFb2 protein suggested that STAT6 could have a negative effect on Runx DNA binding activity. However, when we measured Runx1 activity *in vitro*, it was observed to be independent of STAT6 expression (III, Figure 4a). This might be partly explained by the differential cellular localization of Runx1 protein and the STAT6 regulated form of CBFb2 (III, Figures 4b and 5).

5.6. Effect of IL-4 on caspase signaling pathways

When we used 2-DE proteomics to identify novel IL-4 target proteins in activated human $CD4^+$ T cells, the levels of fragmented Ly-GDI protein were detected to be downregulated by IL-4 (II, Figures 1-2 and Table 1, and data not shown). Ly-GDI is a known target of Caspase-3 (404-406), and Caspase-3 activity, when measured by flow cytometry (II, Figure 3a) or Western blotting (data not shown), was consistently decreased in IL-4 treated cells. IL-4 was also seen to induce changes in the expression of the following upstream regulators of Caspase-3: it decreased the expression of Fas receptor (II, Figure 5) and increased the expression of Bcl-2, Bcl-xL, and noncleaved form of BH3 interacting domain death agonist (Bid) (II, Figure 6). Interestingly, the protein expression of c-FLIP short (c-FLIPs) was also upregulated by IL-4 (II, Figure 7). Three different isoforms of c-FLIP are reported to be expressed in human cells: c-FLIPs, c-FLIPl (long), and c-FLIPr (Raji). Of those, c-FLIP long can both stimulate and inhibit Caspase-8 and -10, but c-FLIPs and c-FLIPr have only an inhibitory effect on caspase activity (407-409). All the above IL-4 induced differences are consistent with decreased caspase activity. Caspase activity has been reported to favor Th1 differentiation in developing Th cells, since caspase inhibition or stimulation leads to increased IL-4 or IFNγ production, respectively (264-266). In addition, Caspase-8 activity has been shown to prevent Th2 responses, and active Caspase-8 is required for effective T cell mediated immunity against the intracellular parasite *Trypanosoma cruzi* (267). It is tempting to speculate that IL-4 induced regulation of caspase-mediated signaling pathway could have a role in Th2 differentiation in addition to its effect on cell viability.

IL-4 also increased the levels of IκB-α in early stages of Th2 development (II, Figure 7). IκB-α is degraded after Caspase-8 induction leading to the activation of NF-κB (407, 410). Increased IκB-α levels thereby suggested decreased caspase activity in IL-4 treated cells, although the effect of IL-4 on IκB-α expression might also be caused by other factors regulating the expression and turn-over of this protein. Studies using dominant negative form of IκB-α in T cells have shown that Th1 differentiation is defective but Th2 differentiation unaltered or even slightly increased, if IκB-α degradation is blocked, implicating that IκB-α regulated signaling is needed for Th1 development but dispensable or even inhibitory for Th2 development (280). The IL-4 induced upregulation of IκB-α observed in our experiments is interesting in this context, and suggests that IL-4 induced modulation of IκB-α regulated NF-κB activity might be one mechanism, through which IL-4 is enhancing Th2 differentiation.

IL-4 induced differences in the expression of the described proteins in the caspase mediated signaling pathways are summarized in Figure 4.

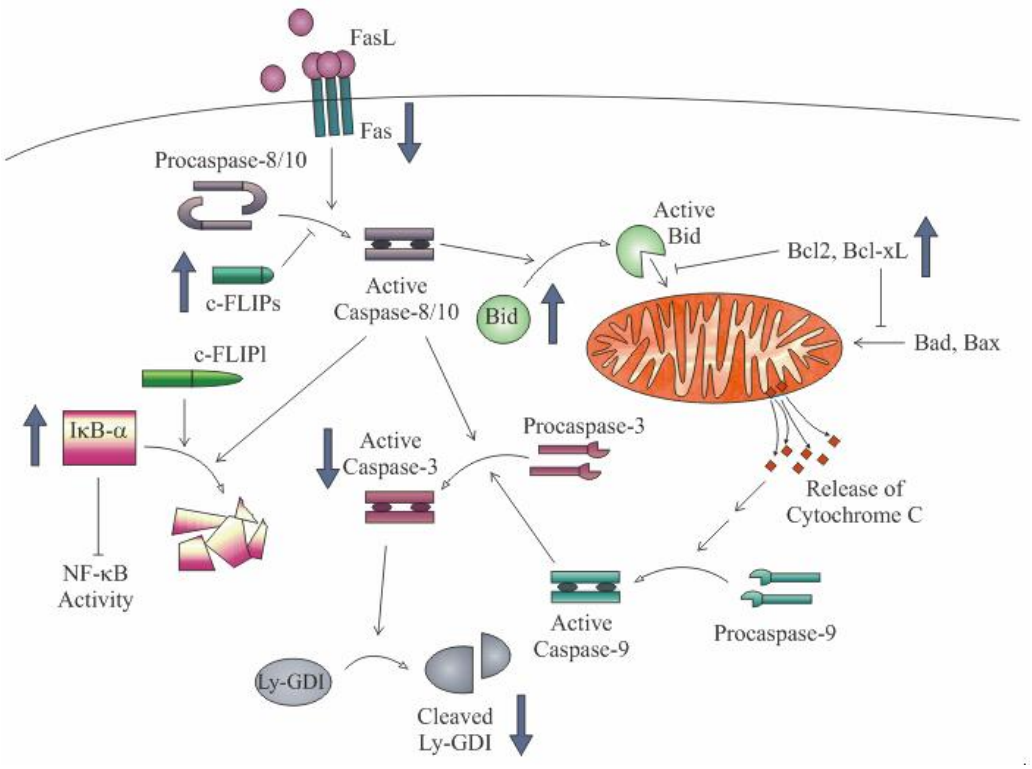


Figure 4. The overview of the effects of IL-4 on the expression of key factors in the caspase signaling pathways. Upon Fas receptor mediated stimulation with FasL, Fas receptor oligomerizes, which leads to the activation of initiator caspases, i.e. Caspase-8 or -10. These initiator caspases can directly activate Caspase-3 through a proteolytic cleavage, and they can also initiate the depolarization of mitochondrial membrane potential by cleaving Bid protein. Bid, BCL2-antagonist of cell death (Bad), and BCL2-associated X (Bax) proteins have the potential to form permeability transition pores in the outer mitochondrial membrane, which leads to the release of proapoptotic substances, such as cytochrome c, to the cytosol. Bcl2 and Bcl-xL can inhibit the function of Bid, Bad, and Bax and thereby stabilize the mitochondrial membrane potential. Release of cytochrome c induces the activation of Caspase-9, which is in turn capable of activating Caspase-3. Ly-GDI is one target protein of Caspase-3. c-FLIP short (c-FLIPs) inhibits the activity of initiator caspases, whereas c-FLIP long (c-FLIPl) can together with Caspase-8 induce IκB-α degradation leading to NF-kB activation. IL-4 induced differences in protein expression are marked with bold arrows in the figure. This figure is a modified version of Figure 8 in original article II.

5.7. TCR activation dependent effects of IL-4 in human $CD4^+$ T cells

TCR activation using soluble anti-CD3 antibody, anti-CD28 antibody and goat anti-mouse F(ab')2 fragment is a mild activation method. The function of F(ab')2 fragment is to crosslink receptor-bound antibodies thereby enhancing the activation signal (411), but it does not result in full activation of the cells according to CD69 expression (see chapter 5.8. for further information), whereas activation of cells with high amounts of plate bound anti-CD3 and soluble anti-CD28 leads to markedly higher CD69 expression. Stronger TCR activation was also evident, when morphology and colony formation under microscopy was observed.

To study the combined effect of TCR activation and IL-4, Caspase-3 activity was measured either from unactivated cells or from activated cells after weaker or stronger TCR stimulation. IL-4 was also able to decrease Caspase-3 activity in unactivated naive $CD4^+$ T cells, but its effect was slightly weaker than after weak TCR stimulation (Figure 5a). However, when strong plate-bound anti-CD3 activation was used to activate the cells, IL-4 could decrease the proportion of active Caspase-3 positive cells only by 18% (Figure 5a). The use of a lower plate-bound anti-CD3 concentration (0.5 μg/ml) led to similar results to the activation method using F(ab')2 fragment (data not shown). Similarly, IL-4 induced downregulation of Fas receptor (II, Figure 5) was dependent on the strength of TCR activation, as IL-4 was unable to downregulate Fas expression after strong plate-bound anti-CD3 (2.5 μg/ml) activation (data not shown).

Engagement of CD4 before TCR triggering has been shown to induce apoptosis in T cell clones, and IL-4 was able to revert this induction (331). To clarify the role of T cell isolation in Caspase-3 activation, we isolated the cells either positively with anti-CD4 beads or negatively with RosetteSepTM $CD4^+$ T Cell Enrichment kit and cultured them for 24h with or without TCR activation. Positive $CD4^+$ cell isolation increased the amount of active Caspase-3 positive cells in the absence of TCR triggering and slightly also after TCR activation (Figure 5b). However, stimulation through CD3 and CD28 led to increased Caspase-3 activity in negatively isolated $CD4^+$ T cells. The effect of IL-4 was similar both after positive and negative isolation, so it is not dependent on the method used for T cell isolation. To summarize, both the positive cell isolation method and TCR activation induce Caspase-3 activity in primary human $CD4^+$ cells.

The percentage of active Caspase-3 positive cells is rather low irrespective of cell isolation or strength of TCR activation. In all stainings, Caspase-3 positive cells were clearly separated from negative cells (II, Figures 3a and 4a). Although Caspase-3 activity has also been detected during the initial activation of primary human T cells without apoptosis (261, 262), the distinctive caspase 3 staining on positive cells in our experiments indicates that these cells most likely represent apoptotic cells. We also studied the effect of IL-4 on cell viability by using forward/side scattering (FSC/SSC)

of the fixed cells to detect dying cells (412). According to this analysis, IL-4 was able to significantly increase cell viability in mildly activated cells (Figure 6), which is in agreement with the observed change in Caspase-3 positive cells. Interestingly, the effect of IL-4 was lower and not statistically significant, when the cells were either left unactivated or when they were activated strongly with plate-bound anti-CD3 (Figure 6). Caspase-3 is able to inhibit proliferation of B cells (413), so controlled Caspase-3 activity might also regulate other cellular responses than cell death. Low Caspase-3 activity and its regulation by IL-4 have not been studied, and potential alternative roles of Caspase-3 in proliferation or T helper cell differentiation need to be investigated with additional experiments.

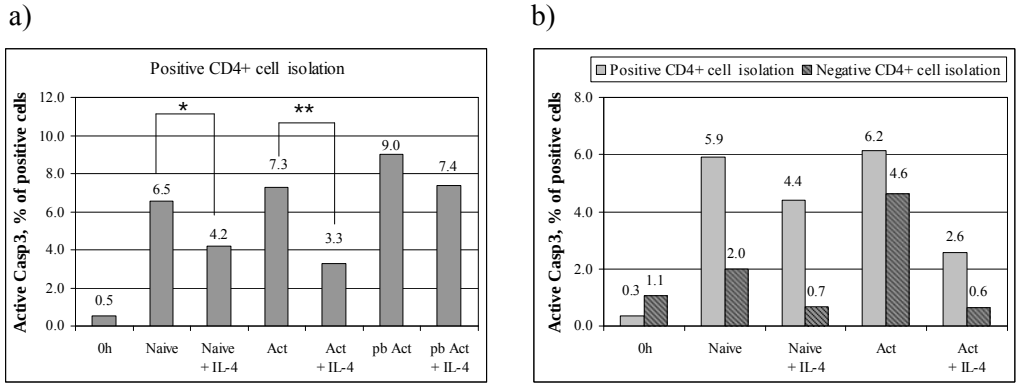


Figure 5. Both T cell activation and positive CD4$^+$ cell isolation induce Caspase-3 activation. The effect of IL-4 on Caspase-3 activity is dependent on the strength of TCR activation: the potential of IL-4 to inhibit Caspase-3 activity is strongest after weak TCR stimulation. (a) CD4$^+$ cells were isolated positively with anti-CD4 beads. Isolated cells were either left unactivated (Naive), activated mildly with anti-CD3, anti-CD28 and anti-mouse F(ab')2 fragment (Act) or activated strongly with high concentration of plate bound anti-CD3 (2.5 μg/ml) together with soluble anti-CD28 (pb Act). Cells were cultured for 24h in either the presence or absence of IL-4, harvested and their Caspase-3 activity was measured with flow cytometry. The proportion of active Caspase-3 positive cells was slightly induced by T cell activation in positively isolated CD4$^+$ cells (paired t-test values 0.068 and 0.084 for the effect of weaker and stronger activation, respectively). The inhibitory effect of IL-4 was statistically significant in unactivated (Naive) and in mildly activated (Act) samples but was reduced in strongly activated cells. The Naive and Act results are the mean values from 6 independent experiments, pb Act results from 3 independent experiments. *, paired t-test <0.05; **, paired t-test <0.01 **(b)** CD4$^+$ T cells were isolated either positively with anti-CD4 beads or negatively with RosetteSep kit and either left unactivated (Naive) or activated mildly with anti-CD3, anti-CD28 and anti-mouse F(ab')2 fragment (Act). TCR activation clearly increases Caspase-3 activity after negative CD4$^+$ cell isolation. Negative CD4$^+$ cell isolation decreases Caspase-3 activity, implicating that positive CD4$^+$ isolation activates Caspase-3 in the cultured cells. The results are the mean values from two independent experiments with similar results.

Overall, the effects of IL-4 on Caspase-3 activity, cell viability, and downregulation of Fas receptor (Figures 5-6; II, Figure 7, and data not shown) were dependent on the strength of TCR stimulation in activated naive $CD4^+$ T cells. We therefore studied the role of TCR stimulation in the Th2 differentiation in the *in vitro* cultures. $CD4^+$ T cells were isolated from umbilical cord blood, activated with two different concentrations of plate-bound anti-CD3 (either 0.5 or 2.5 μg/ml) and polarized in the Th2 direction. Cells were reactivated as described in materials and methods, and their cytokine production measured with Multiplex assay. Control cells were just replated at 7 day timepoint and cultured for 24h without any restimulation. IL-4 production was increased, when weaker CD3 stimulation (0.5 μg/ml) was used for the primary activation (Table 6), implicating an enhanced Th2 polarization. Johanna Tahvanainen et al. (manuscript submitted) in our group have detected a similar phenomenon, i.e. Th2 polarization is increased, when anti-CD3 concentration is decreased from 2.5 μg/ml to 0.6 μg/ml. The effects of IL-4 on Caspase-3 activity, cell viability, and downregulation of Fas are evident if 0.5 μg/ml anti-CD3 is used for TCR activation. These results are consistent with earlier reports showing that weak TCR activation favors Th2 cell differentiation (142-145).

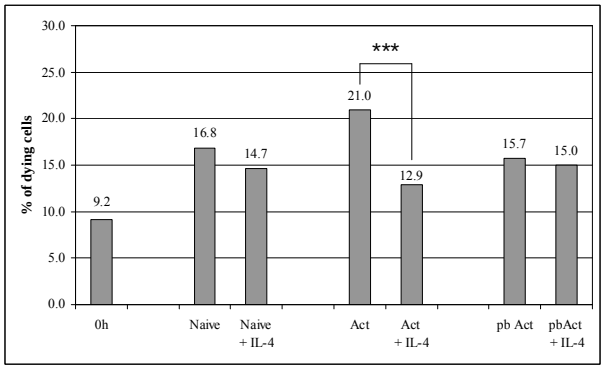


Figure 6. IL-4 decreases cell death in weakly activated T helper cells. Isolated $CD4^+$ cells were either left unactivated (Naive), activated mildly with anti-CD3, anti-CD28 and anti-mouse F(ab')2 fragment (Act) or activated strongly with high concentration (2.5 μg/ml) of plate bound anti-CD3 together with soluble anti-CD28 (pb Act). Cells were cultured for 24h in the presence or absence of IL-4, harvested, fixed and their FSC/SSC scattering was measured with flow cytometry. IL-4 significantly reduced the proportion of dying cells in weakly activated samples. The effect of IL-4 was lower and not significant in naive or strongly activated (pb Act) cells. The Naive and Act results are the mean values from at least six independent cultures, pb Act results from four independent cultures. ***, paired t-test < 0.0001

Table 6. IL-4 production is increased in restimulated Th2 polarized cells, when lower CD3 concentration is used for the primary activation. Isolated $CD4^+$ T cells were activated with two different concentrations of plate-bound anti-CD3 (0.5 or 2.5 μg/ml) and polarized in the Th2 direction. Cells were reactivated as described in Materials and Methods, and their cytokine concentration in the supernatants measured with Multiplex assay. Without reactivation, cells did not produce any IL-4, and IFNγ production was extremely low (measured concentration 1.46 pg/ml). The mean values from 2 independent cultures with similar results are shown in the table.

	Anti-CD3 μg/ml	**IL-4** pg/ml	**IFNγ** pg/ml	**IL-4/IFNγ**
Th2	0.5 μg/ml	47.2	938	0.053
Th2	2.5 μg/ml	11.2	877	0.013

5.8. The effects of IL-4 on T cell activation

Since IL-4 was shown to decrease T cell activation induced Caspase-3 activity (Figure 5), the effect of IL-4 on Caspase-3 activity could result from lower T cell activation in IL-4 treated cells. We therefore studied how IL-4 influences T cell activation. For this, the isolated $CD4^+$ cells were TCR activated with either weaker or stronger activation stimulus, cultured for 24h in the absence or presence of IL-4, and the expression of early activation marker CD69 measured with flow cytometry. IL-4 increased the amount of $CD69^+$ cells as well as the mean fluorescence intensity of CD69 expression in $CD69^+$ cells (Figure 7), thereby acting more as a stimulating than an inhibitory factor for T cell activation. The expression of activation marker CD25 was similarly upregulated by IL-4 (data not shown). Our data is consistent with earlier results, where IL-4 has been shown to act as an activating cytokine for TCR activated $CD4^+$ T cells (337).

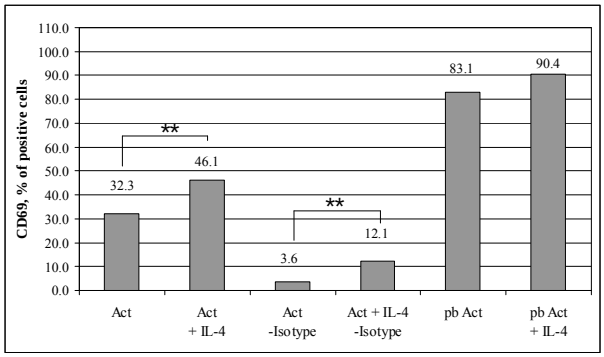


Figure 7. IL-4 enhances the expression of T cell activation marker CD69. Isolated $CD4^+$ cells were activated mildly with anti-CD3, anti-CD28 and anti-mouse F(ab')2 fragment (Act) or strongly with a high concentration (2.5 µg/ml) of plate bound anti-CD3 together with soluble anti-CD28 (pb Act). Cells were cultured for 24h and the surface expression of the early activation marker CD69 was measured with flow cytometry. IL-4 increases CD69 expression irrespective of the strength of TCR activation. IL-4 also increases the mean intensity of CD69 from 72.0 ± 25.0 to 91.6 ± 30.7 in mildly activated cells (Act), and from 430 ± 138 to 652 ± 200 in strongly activated cells (pb Act), respectively. Since cell surface bound anti-mouse F(ab')2 fragment was also binding to mouse antibodies used for staining, the signal in isotype control samples was rather high (on average 28 %). "–Isotype" bars represent the mean values after isotype signal has been deducted from the samples. Since activation without F(ab')2 fragment led to CD69 expression in about 20 % of cells (data not shown) and treatment with F(ab')2 fragment should enhance TCR activation, unspecific signal caused by cell surface bound F(ab')2 fragment is partly masking the specific signal. The effect of IL-4 on CD69 expression is independent of the binding of F(ab')2 fragment to staining antibodies, as it was also detected, when the isotype signal was deducted from the samples (-Isotype bars). "Act" results are the mean values from 10 independent experiments and "pb Act" results from 5 independent experiments, respectively. **, paired t-test < 0.01, the paired t-test for the effect of IL-4 on the proportion of $CD69^+$ cells was 0.085 after plate-bound CD3 activation.

5.9. The effects of IL-4 on GATA3 expression

5.9.1. STAT6-dependent regulation of GATA3 transcription

GATA3 is one of the few genes that has been shown to be upregulated in an IL-4/STAT6 dependent manner both in humans and mice during the early stages of Th2 differentiation (108, 110-112, 217). IL-4 increases the levels of GATA3 mRNA as early as 2h after activation and this induction is also STAT6 dependent in mouse $CD4^+$ T cells already at that point (111, 112, 217). We therefore wanted to study the mechanism of STAT6 mediated upregulation of GATA3 transcription. Our collaborate Eija Nordlund (University of Turku) used computational methods to search for putative STAT6 binding sites from the GATA3 locus in human and mouse. The canonical binding site for STAT6 consists of two halves of palindromic sequence separated by four nucleotides ($TTCN_4GAA$) (N4-site). STAT6 is unique in a sense that it prefers N4-sites (105, 414), although it is also able to bind to a so-called N3-site sites where the palindromic sequence is separated by three nucleotides. STAT6 may also recognize the sites with mismatches in the palindromic sequence (415, 416). Altogether 11 putative STAT6 human binding sites fulfilled the selected criteria: binding sites should be in the genomic regions that are conserved between human and mouse, and there should be an aligned STAT6 binding site in the same region in mouse. Also some atypical STAT6 binding sites, such as N3 sites and nonpalindromic sites were included in the analysis. The binding of STAT6 to these sites *in vivo* was studied with the chromatin immunoprecipitation (ChIP) method. Altogether eight STAT6 binding site regions (marked as a-h in Figure 8) including all putative STAT6 binding sites were included in the analysis. The binding of STAT6 to these regions was studied in human $CD4^+$ T cells prior to, or 2h after TCR activation in both the presence and absence of IL-4. We did not detect DNA bound STAT6 on any of the selected binding sites (Figure 9). This might be due to selected timepoint or because the amount of sample material was too small for ChIP detection. Additional studies will be conducted with more sample material and different culture periods to rule out these possibilities. Proper fixing rate as well DNA fragmentation was confirmed from all samples, so they should be appropriate for ChIP experiments. Interestingly, there are no reports on the mechanism of STAT6 induced upregulation of GATA3. This might be due to the fact that others have also been unsuccessful in identifying *in vivo* STAT6 binding sites in GATA3 locus, which in turn implicates that STAT6 dependent regulation of GATA3 is either indirect or the functional regulatory regions are located outside the scope of earlier studies.

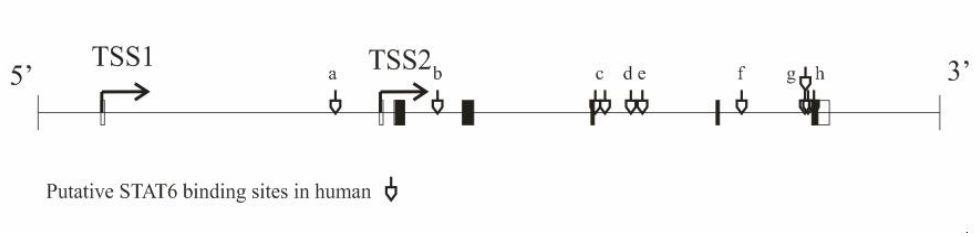


Figure 8. Schematic representation of human GATA3 gene locus. GATA3 is located in chromosome 10. Two alternative transcription start sites (TSS1 and TSS2) and eight putative STAT6 binding site regions (a-h) are indicated in the figure. GATA3 has 6 exons (marked with squares), 5 of which are coding ones. The DNA region 15 kb upstream of TSS2 and 5 kb downstream of the 3' end of the transcript is shown in the figure. STAT6a-h refer to the regions that are quantified with Taqman real-time RT-PCR after chromatin immunoprecipitation. The transcribed regions are marked with squares, white boxes representing UTR regions and black boxes coding regions.

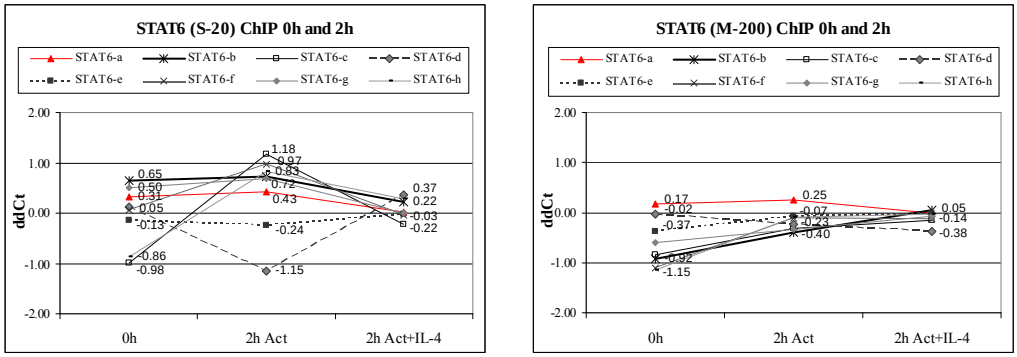


Figure 9. The *in vivo* binding of STAT6 to its putative target sequences in GATA3 gene region was not detected in IL-4 stimulated cells. Freshly isolated human $CD4^+$ T cells were activated (Act) for 2h either in the presence or absence of IL-4. Protein-DNA interactions were fixed with formaldehyde and STAT6 bound DNA enriched with chromatin immunoprecipitation (ChIP) using two different anti-STAT6 antibodies (S-20 and M-200). Rabbit serum was used as a negative control for immunoprecipitation. Isolated DNA was quantified with Taqman real-time RT-PCR. The signals measured from putative STAT6 binding sites were normalized against both the serum sample and signals from a DNA region not containing any putative STAT6 binding sites. No significant IL-4 induced binding of STAT6 was detected. Scale is logarithmic (base 2), so the difference of 1 ddCt means 2-fold difference in the amount of enriched DNA. The higher the ddCt value, the larger the amount of enriched DNA. The mean values from 3 independent cultures are shown in the figure.

As it is possible that the binding of STAT6 could be prevented by a closed, inaccessible chromatin structure, we used ChIP method to study the chromatin conformation in the STAT6 binding site regions. Histone 4 lysine 3 methylation (H3K4) is generally localized in the areas of open, active chromatin, whereas H3K9 and H3K27 methylations are characteristic for closed, inactive chromatin regions (417). H3K9 modification is also associated with elongation in transcribed DNA regions (418). When ChIP approach with histone methylation specific antibodies was used to study DNA conformation, H3K4, H3K9, and H3K27 associated regions were enriched with ChIP either from freshly isolated cells or 2h and 48h after TCR activation both in the presence and absence of IL-4. No reproducible differences in H3K9 and H3K27 methylation were detected between putative STAT6 binding site regions (Figure 10). According to H4K3 ChIP results, genomic regions in the proximity of GATA3 main transcription start site TSS2 (regions STAT6a-b) showed more open chromatin structure at 0h and 2h timepoints compared to the other putative STAT6 binding sites, but their chromatin structure had changed into a less permissive form 48h after activation (Figure 10). As GATA3 transcription is initiated from an alternative transcription start site TSS1 approximately at this stage of differentiation (220), this might lead to partial inactivation of TSS2 site, as our results suggest.

5.9.2. IL-4 and the stability of human GATA3 mRNA

GATA3 is already expressed at the mRNA level in freshly isolated human cord blood cells and its expression is downregulated by TCR activation and upregulated by IL-4 (Figure 11a and (238, 419)). Transcription machinery is thus likely to be active in GATA3 locus already before activation or IL-4 stimulation. Therefore we hypothesized that one way by which IL-4 might upregulate the GATA3 expression could be the stabilization of GATA3 mRNA. The stability of GATA3 transcripts were measured either from freshly isolated $CD4^+$ T cells or at 4h and 20h after TCR activation. No significant activation induced differences in the mRNA stability were detected. Surprisingly, IL-4 decreased the mRNA stability 20h after TCR activation (Figure 11b-c). IL-12 appeared to have a similar although weaker effect on GATA3 mRNA stability at that timepoint, but the effect of IL-12 was not statistically significant. However, IL-4 did not have an effect on the mRNA degradation at 4h after TCR activation, although IL-4 was already then capable of upregulating the mRNA levels of GATA3 (Figure 11). Since IL-4 is destabilizing GATA3 mRNA 20h after initial TCR activation, even more GATA3 transcripts needs to be produced to upregulate GATA3 mRNA levels in IL-4 treated cells at that point of differentiation. Decreased stability of GATA3 mRNA might enable the cells to respond more rapidly to changes in upstream signals regulating GATA3 expression.

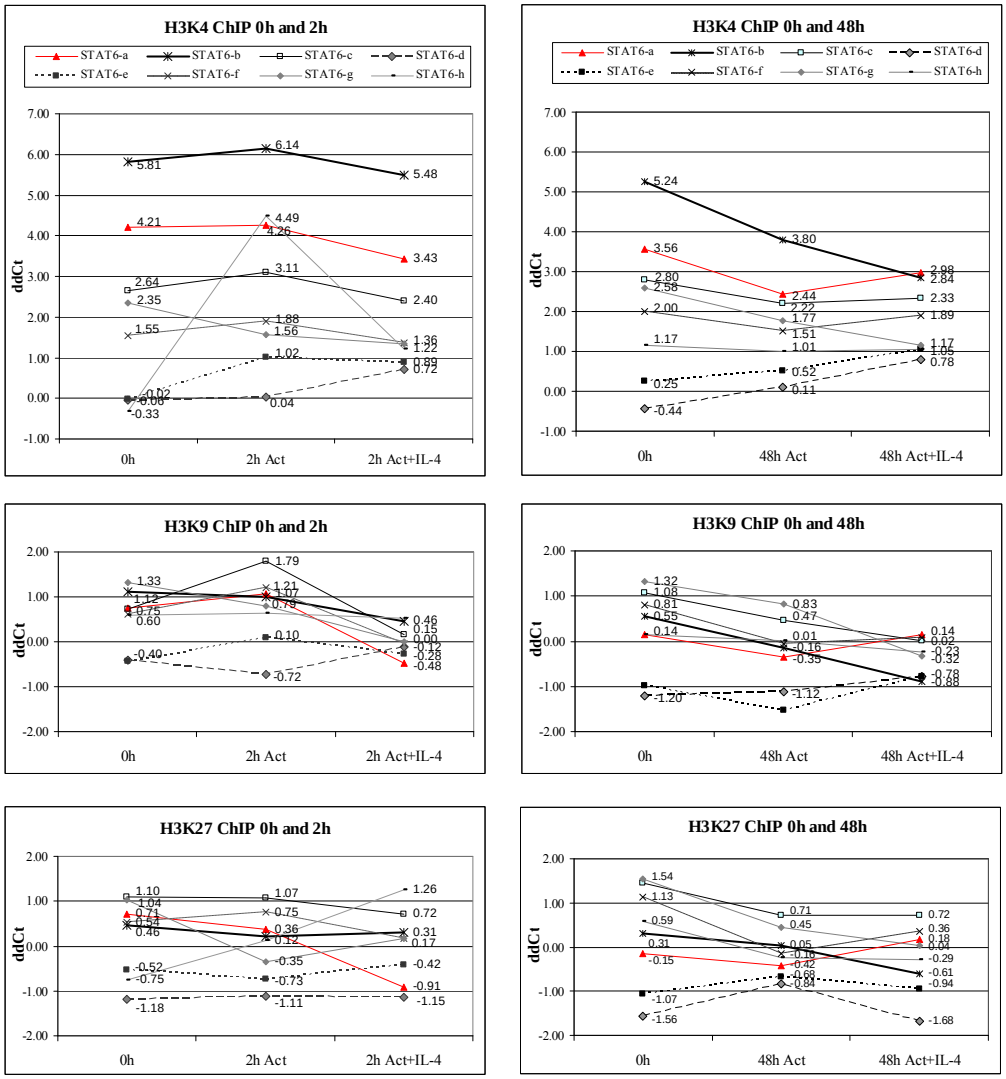
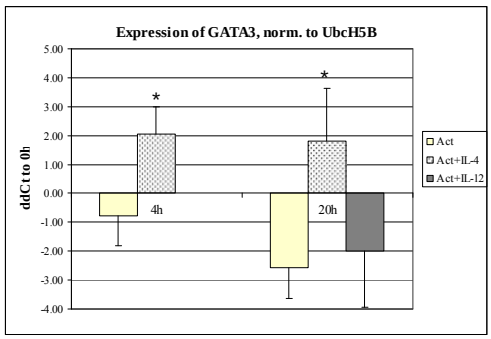
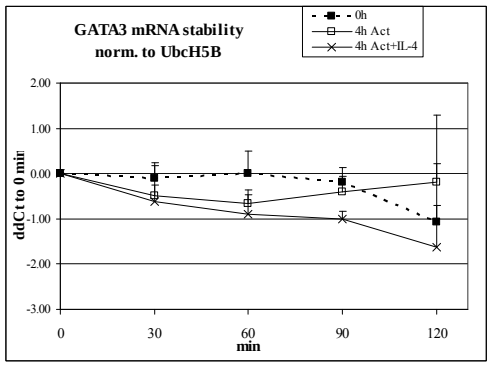


Figure 10. DNA conformation in the STAT6 binding site regions located in the proximity of the main transcription start site TSS2 is more permissive than in other regions studied, but this difference decreases by prolonged TCR activation. Freshly isolated human CD4$^+$ T cells were activated (Act) for 2h or 48h in the presence or absence of IL-4. The DNA conformation in putative STAT6 binding sites was studied with the ChIP method. Isolated DNA was quantitated with Taqman real-time RT-PCR. The signals measured from putative STAT6 binding sites were normalized against both the rabbit serum sample (a negative control for immunoprecipitation) and signals of a DNA region not containing any putative STAT6 binding sites. According to H4K3 ChIP, genomic regions in the proximity of GATA3 main transcription start site (regions STAT6a-b) showed more open chromatin structure at 0h and 2h compared to other putative STAT6 binding sites, but their chromatin structure had changed into a less permissive form at 48h after activation. No major differences in H3K9 and H3K27 methylation were detected between putative STAT6 binding site regions. The scale is logarithmic (base 2), so the difference of 1 ddCt means 2-fold difference in the amount of enriched DNA. The mean values from either 2 (2h) or 3 (48h) independent experiments are shown in the figure.

a)



(b)



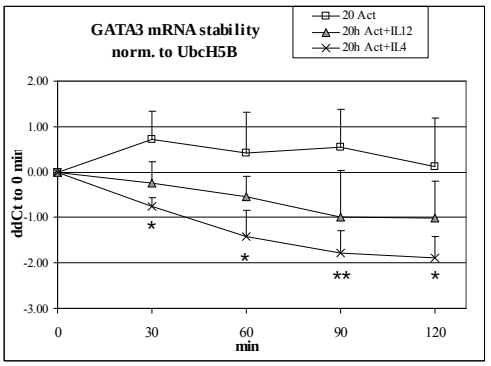


Figure 11. IL-4 slightly decreased the stability of GATA3 mRNA 20h after TCR activation. (a) GATA3 expression is decreased by TCR activation and upregulated by IL-4 in $CD4^+$ T cells isolated from human umbilical cord blood. GATA3 expression was measured with Taqman real-time RT-PCR either prior to or 4h and 20h after TCR activation. Ct signal values were normalized to the signals acquired from housekeeping gene UbcH5B (420). Signal differences (ddCt) to 0h sample are indicated in the figure. The mean values from 3 independent experiments are shown in the figure. *, paired t-test < 0.05 for the effect of IL-4 **(b)** IL-12 and especially IL-4 decrease the half life of GATA3 mRNA 20h after activation. The effect of IL-4 at 20h timepoint was statistically significant (t-test <0.05) in all actinomycin D treatments (30-120 min) tested. The stability of GATA3 mRNA was measured in freshly isolated $CD4^+$ T cells as well as at 4h (on the left side) and 20h (on the right side) after TCR activation. mRNA synthesis was blocked with actinomycin D and the levels of degrading mRNA were measured with Taqman real-time RT-PCR. Ct signal values were normalized to the signals acquired from UbcH5B (b) or EF1α (data not shown). Similar results were acquired irrespective of the normalization. When the ddCt values are below 0, GATA3 is degrading faster than house-keeping gene UbcH5B used for normalization. The scale is logarithmic (base 2), so the difference of 1 ddCt means 2-fold difference in the amount of mRNA. The mean values from 3 independent experiments are shown in the figure. *, paired t-test < 0.05 for the effect of IL-4; **, paired t-test <0.01 for the effect of IL-4

5.9.3. Different forms of GATA3 protein in human IL-4 stimulated CD4$^+$ T cells

In addition to IL-4 induced upregulation of GATA3 mRNA expression, GATA3 is also known to be regulated at the protein level. For example, Ras-ERK MAPK signaling cascade, as well as *Polycomb* Group gene bmi-1, stabilizes GATA3 protein by decreasing ubiquitin mediated GATA3 degradation (239, 240). Furthermore, acetylation of GATA3 appears to be necessary for its proper function both in humans and mice (244, 245), and intracellular localization of GATA3 has been reported to be dependent on serine phosphorylation (231). Since protein regulation is generally mediated by the post-translational modification, we aimed to study, how many different forms of GATA3 could be detected after 2-DE separation and consecutive Western blotting. GATA3 migrated to several positions in the 2-DE gels, but the different forms of GATA3 were localized very close to each other (Figure 12). Small differences in pI values between different spots suggest that the modifications in question are not very basic or acidic. Acetylation for example does not cause dramatic changes to protein pI (421). The molecular weight of GATA3 did not differ very much between the different forms, either. Further studies are needed to identify the modifications that are responsible for the changes in the migration of different GATA3 forms.

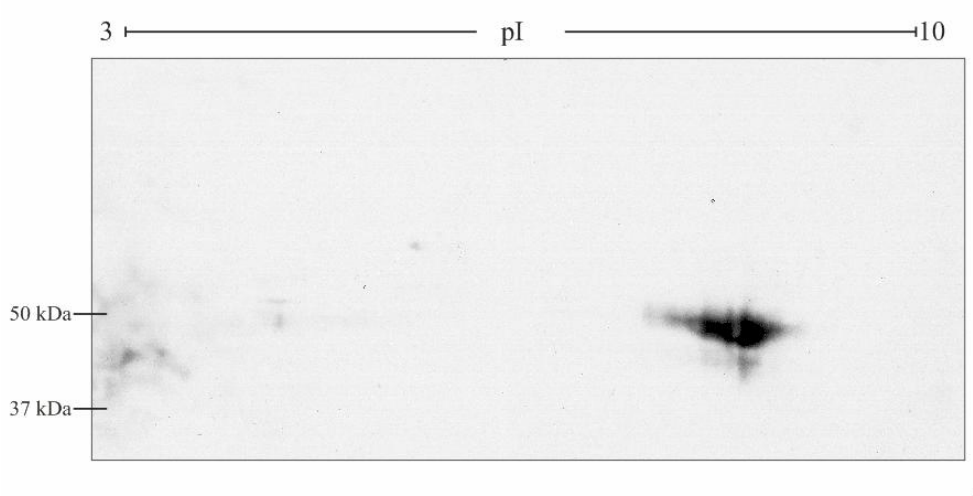


Figure 12. The detection of GATA3 with Western blotting after 2-DE separation of proteins. Human CD4$^+$ cells were activated through CD3 and CD28 and cultured for 24h in the presence of IL-4. GATA3 migrated to the basic pI range. GATA3 was detected from several spots with a similar molecular weight and pI. No signal was detected from TCR activated samples not treated with IL-4. Nonlinear pI range in the gel is from 3 to 10. The results were similar in 3 independent experiments.

6. SUMMARY

The differentiation of Th2 cells can be regulated by several different extracellular stimuli, and a variety of intracellular factors participate in Th2 cell development both *in vivo* and *in vitro*. The significant role of IL-4 for Th2 differentiation has been implicated especially in allergies and allergic asthma. In the studies included in this thesis, undifferentiated T helper cells were isolated from human umbilical cord blood or mouse spleen, activated through T cell and co-stimulatory receptors and differentiated in the Th1 or Th2 direction with polarizing cytokines IL-12 and IL-4, respectively. If no polarizing cytokines were added to the culture medium, Th cells were activated but they were unable to properly develop to either of the above T helper cell subtypes.

One of the aims of my thesis was to reveal the mechanism for IL-4/STAT6 induced upregulation of GATA3, the major transcription factor driving Th2 differentiation. GATA3 expression is upregulated in a STAT6 dependent manner after IL-4 stimulation both at the level of mRNA and protein. We first studied the *in vivo* binding of STAT6 to its putative target sequences in GATA3 promoter at 2 hours after TCR activation and IL-4 stimulation, but we could not detect DNA bound STAT6 in the promoter regions studied. We also investigated, whether IL-4 could stabilize GATA3 mRNA leading to its upregulation. Surprisingly, IL-4 was observed to destabilize GATA3 mRNA after TCR activation, so IL-4 induced upregulation of GATA3 mRNA is dependent on increased *de novo* transcription of the gene.

In the proteomics studies included in this thesis, proteins regulated by IL-4/STAT6 during the early stages of Th2 differentiation were identified using 2-DE and mass spectrometry. We also compared the proteomes of *in vitro* differentiated and reactivated Th1 and Th2 cells to find out differences in the protein expression levels and protein modifications. The regulated proteins in our proteomics studies were largely different from genes that have been detected to be regulated during Th2 differentiation or between differentiated Th1 and Th2 cells in transcriptomics studies. Proteomics thus has an important complementary role in identifying novel genes and proteins that are regulated during T helper cell differentiation.

When 2-DE based proteomics was used to identify novel STAT6 target proteins, STAT6 was shown to regulate the expression of CBFb2 protein. CBFb dimerizes with Runx proteins, thus enabling them to bind to DNA and regulate target gene transcription. As Runx1 protein is reported to inhibit Th2 differentiation and Th2 cytokine production, STAT6 dependent downregulation of CBFb2 protein suggested that STAT6 could have a negative effect on Runx DNA binding activity. However, Runx1 activity was not regulated by STAT6, which might be partly due to the differential localization of Runx1 protein and STAT6 regulated form of CBFb2.

With the use of proteomics methodology to identify novel IL-4 target proteins in human $CD4^+$ T cells during the first 24 hours of polarization, IL-4 was found to regulate several proteins in caspase mediated pathways and to enhance T cell survival and activation. Based on this study, we have proposed new hypothesis on the mechanisms of IL-4 induced caspase and NF-κB mediated regulation of T helper cell differentiation, the validity of which will be studied further.

In summary, this thesis covers the use of multiple approaches, where proteomics type screening methods are used in combination with functional studies of selected proteins. It provides new information on proteins that are regulated during T helper cell differentiation and other IL-4 induced cellular responses.

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