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NEW MECHANISMS REGULATING HUMAN TH1 AND TH2 CELL DIFFERENTIATION

by

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To my family

ABSTRACT

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New mechanisms regulating human Th1 and Th2 cell differentiation

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Selective development of human T helper (Th) cells into functionally distinct Th1 and Th2 subtypes plays an essential role in the host immune response towards pathogens. However, abnormal function or differentiation of these cells can lead to development of various autoimmune diseases as well as asthma and allergy. Therefore, identification of key factors and the molecular mechanisms mediating Th1 and Th2 cell differentiation is important for understanding the molecular mechanisms of these diseases. The goal of this study was to identify novel factors involved in the regulation of Th1 and Th2 differentiation processes. A new method was optimized for enrichment of transiently transfected resting human primary T lymphocytes, that allowed the study of the influence of genes of interest in human Th1/Th2 cell differentiation and other primary Th cell functions. Functional characterization of PRELI, a novel activation-induced protein in human Th cells, identified it as a mitochondrial protein involved in the regulation of Th cell differentiation and apoptosis. By influencing the intracellular redox state, PRELI induces mitochondrial apoptosis pathway and downregulates STAT6 and Th2 differentiation. The data suggested that Calpain, an oxidative stress induced cysteine protease, is involved as a mediator in PRELI-induced downregulation of STAT6. PIM serine/threonine-specific kinases were identified as new regulators of human Th1 cell differentiation. PIM1 and PIM2 kinases were shown to be preferentially expressed in Th1 cells as compared to Th2 cells. RNA interference studies showed that PIM kinases enhance the production of IFNγ, the hallmark cytokine produced by Th1 cells. They also induce the expression of the key Th1-driving factor T-bet and the IL-12 signaling pathway during early phases of Th1 cell differentiation. Taken together, new regulators of human T helper cell differentiation were identified in this study, which provides new insights into the signaling mechanisms controlling the selective activation of human Th cell subsets.

Keywords: T helper cell differentiation, cytokine, STAT6, mitochondrial apoptosis pathway, PIM kinase, siRNA

TIIVISTELMÄ

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Th1- ja Th2-tyypin valkosolujen erilaistumista säätelevät uudet soluviestintäreitit

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Toiminnaltaan erilaiset T-auttajasolutyypit (Th1 ja Th2) ovat keskeisiä tekijöitä immuunivasteen muodostumisessa erilaisia taudinaiheuttajia vastaan. Häiriöt normaalissa Th-solujen vasteessa tai kehittymisessä voivat johtaa autoimmuunisairauksien tai astman ja allergisten tulehdussairauksien syntyyn. Uusien, Th-solujen kehittymistä säätelevien proteiinien identifiointi sekä säätelymekanismien selvittäminen auttavat ymmärtämään näiden sairauksien syntymekanismeja. Tämän väitöskirjatutkimuksen tavoitteena oli identifioida uusia Th1- ja Th2-solujen erilaistumiseen vaikuttavia tekijöitä. Väitöskirjatyössä kehitimme uuden tutkimusmenetelmän, joka perustuu ihmisen naivien Th-solujen transfektoimiseen sekä transfektoitujen solujen rikastamiseen. Menetelmän avulla voidaan tutkia uusien kandidaattigeenien vaikutusta ihmisen primaari-Th-solujen erilaistumiseen ja toimintaan. Väitöskirjatutkimuksessa identifioitiin uusi, Th-solujen erilaistumiseen ja solukuolemaan vaikuttava proteiini, PRELI, joka sijaitsee solun mitokondrioissa. Säätelemällä reaktiivisten happiradikaalien tuottoa mitokondrioissa PRELI lisää solukuolemaa sekä estää Th2-solujen erilaistumista. Tutkimuksissa selvisi myös, että PRELI:n vaikutus Th2-erilaistumiseen mahdollisesti välittyy Calpain-proteaasin ja transkriptiotekijä STAT6:n kautta. PIM-kinaasiperheen tutkimus osoitti, että PIM-kinaasit ilmenevät enemmän Th1- kuin Th2-soluissa sekä edistävät Th1-solujen erilaistumista. RNA-interferenssikokeet osoittivat, että PIM-kinaasit lisäävät Th1-soluille tyypillisen välittäjäaineen, interferoni gamman tuottoa Th1-soluissa. Ne lisäävät myös Th1-soluille erittäin tärkeän transkriptiotekijän, T-bet:in, määrää sekä interleukiini-12 -soluviestintäreitin aktiivisuutta solujen varhaisessa erilaistumisvaiheessa. Tässä väitöskirjatutkimuksessa identifioitiin uusia T-auttajasolujen erilaistumiseen vaikuttavia tekijöitä. Tulokset auttavat ymmärtämään ihmisen T-auttajasolujen kehittymiseen johtavia mekanismeja.

Avainsanat: T-auttajasolujen erilaistuminen, välittäjäaine, STAT6, mitokondriaalinen apoptoositie, PIM kinaasi, siRNA

CONTENTS

ABBREVIATIONS

AP1	activator protein 1
APAF1	apoptotic protease activating factor 1
APC	antigen presenting cell
ATF	activating transcription factor
BCL	B-cell lymphoma
BCR	B cell receptor
BRG1	brahma-related gene 1
BSA	bovine serum albumin
CBP	CREB binding protein
C/EBP	CCAAT/enhancer binding protein
cFLIP	cellular Fas-associated death domain-like interleukin-1-beta converting enzyme inhibitory protein
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
$\Delta\psi_m$	mitochondrial membrane potential
DC	dendritic cell
DHR	dihydrorhodamine
DIC	differential interference contrast
DMEM	Dulbecco modified eagle medium
ECL	enhanced chemiluminesence
EF1α	elongation factor 1 alpha
(E)GFP	(enhanced) green fluorescent protein
EGR1	early growth response protein 1
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell-sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FOG	friend of GATA
FOXP3	forkhead box P3
GADD45	growth arrest and DNA-damage inducible 45
GATA3	GATA binding protein 3
GFI1	growth factor independent 1
GTPase	guanosine triphosphatase
HeLa	Cervical cancer cell line taken from Henrietta Lacks
HEK293	Human embryonic kidney 293 cell line
HLX	H2.0-like homeobox protein
HP1	heterochromatin protein 1
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
ICOS	inducible T cell co-stimulator
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin

IL-12Rβ2	interleukin-12 receptor beta 2 subunit
IL-18RAP	interleukin-18 receptor accessory protein
IRAK	interleukin-1 receptor-associated kinase
IRES	internal ribosome entry site
IRF	interferon regulatory factor
ITK	IL-2-inducible T cell kinase
iTreg	induced regulatory T cell
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Jurkat	Human T lymphocyte cell line derived from acute leukemia
kDa	kilodalton
LEA	late embryogenesis abundant
LEF1	lymphoid enhancer factor 1
LFA1	lymphocyte function-associated antigen 1
MACS	magnetic-activated cell-sorting
MAPK	mitogen activated protein kinase
MCID	microcomputer imaging device
MCS	multiple cloning site
MHC	major histocompatibility complex
NAC	N-acetyl-cysteine
NCoA	nuclear receptor co-activator
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa light chain enhancer in B cells
NK cell	natural killer cell
NKT cell	natural killer T cell
nTreg	naturally occurring regulatory T cell
O-GlcNAc	O-linked N-acetylglucosamine
OPA1	optic atrophy 1
OX40L	OX40 ligand
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA	phytohemagglutinin
PI	propidium iodide
PIM	proviral integration site for Moloney murine leukemia virus
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PP2A	protein phosphatase 2A
PRELI	protein of relevant evolutionary and lymphoid interest
PTP1B	protein tyrosine phosphatase 1 B
RAC2	Ras-related C3 botulinum toxin substrate 2
RIBP	Rlk/ITK binding protein
RNAi	RNA interference
ROG	repressor of GATA
RORγt	retinoid-related orphan receptor gamma t

ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
RUNX	runt-related transcription factor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of mean
SHP1	SH2-containing phosphatase 1
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tc2	Type 2 cytotoxic T cell
TCCR	T cell cytokine receptor
TCF1	T cell factor 1
TCR	T cell receptor
Tfh	follicular T helper cell
Th	T helper cell
Thp	T helper precursor cell
TIM	T cell immunoglobulin- and mucin-domain containing
TNF	tumor necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
YY1	Yin-Yang 1

LIST OF ORIGINAL PUBLICATIONS

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

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

II Tahvanainen J., Kallonen T., Lähteenmäki H., Heiskanen K.M., Westermarck J., Rao K.V.S and Lahesmaa R. PRELI is a mitochondrial regulator of human primary T-helper cell apoptosis, STAT6 and Th2-cell differentiation. *Blood* 2009; 113:1268-1277.

III Tahvanainen J., Gupta B., Lähteenmäki H., Kallonen T., Rasool O., Koskinen P.J., Rao K.V.S. and Lahesmaa R. PIM kinases promote human T helper 1 cell differentiation. Manuscript.

1 INTRODUCTION

The immune system has developed to protect the host against various types of pathogens. Several types of cells have specialized functions and co-operate either non-specifically (innate immunity) or specifically (adaptive immunity) to eliminate different pathogens such as bacteria, viruses and parasites. T helper (Th) cells are central part of the adaptive immune response. They are activated in the lymph nodes by antigen presenting cells. In response to antigen encounter and the surrounding cytokine milieu, naive Th cells differentiate into different subsets including Th1, Th2, Th17 and regulatory T (Treg) cells. Th1 and Th2 subtypes were discovered more than 20 years ago and they form the classical paradigm of Th cell differentiation. Th1 cells mainly protect the host from intracellular pathogens promoting cell-mediated immune responses, whereas Th2 cells are important mediators of humoral immune response protecting host against extracellular pathogens. The most potent stimuli influencing the differentiation process are the cytokines. In the presence of interleukin 12 (IL-12), naive T cells develop into Th1 cells. Conversely, IL-4 triggers the development of Th2 type cells. Signal transducers and activators of transcription (STAT) proteins are class of transcription factors responsible for mediating the cytokine-induced responses. STAT4 and STAT6 are important in transducing the intracellular signaling induced by IL-12 and IL-4, respectively. Several other transcription factors, such as T-box expressed in T cells (T-bet) and GATA binding protein 3 (GATA3) have been shown to be critical factors driving Th1 and Th2 cell polarization, respectively.

In addition to cytokines, the strength of the T cell receptor (TCR) stimulus has been shown to influence the direction of the Th1/Th2 polarization. A strong TCR activation tends to enhance Th1 differentiation, whereas weak TCR signals favor Th2 polarization. It is also well established that Th1 and Th2 cells differ in terms of their susceptibility to apoptosis, with Th2 cells being more resistant to apoptotic cell death than are Th1 cells. IL-4 has been shown to protect the cells from apoptosis by regulating proteins mediating the mitochondrial apoptosis pathway and by maintaining the mitochondrial membrane potential. The signaling networks and exact mechanisms regulating Th1/Th2 cell production are not fully defined. Identification of key factors mediating the differentiation of naive $CD4^+$ T helper cells into Th1 and Th2 subsets is important for understanding the molecular mechanisms of the development of autoimmune diseases as well as asthma and allergy.

Most of the previous studies on Th1/Th2 differentiation have been performed using gene-targeted mouse models or murine cells. It is also important to be able to study these questions in human cells. However, the functional importance of selected genes in the initiation of human Th cell differentiation has been hard to study due to the difficulty in transfecting primary resting human T lymphocytes. One objective of this thesis was to develop a method for studying the influence of gene overexpression or knockdown

on Th1/Th2 cell differentiation process and other primary human T cell functions. The second aim was to characterize the function of PRELI, a novel TCR activation-induced protein in human CD4+ cells, and investigate its role in Th1/Th2 differentiation process. Thirdly, the role of serine/threonine-specific PIM kinases in regulation of human T helper cell differentiation was investigated.

2 REVIEW OF THE LITERATURE

2.1 T HELPER CELLS IN THE IMMUNE SYSTEM

CD4+ Th cells play a central role in the development of immune responses against pathogens. Proper function and development of different Th cell subsets play an essential role in a balanced immune response. However, dysregulated or abnormal function or differentiation of these cells often leads to development of inflammatory and autoimmune diseases. Several subsets of CD4+ Th cells have been characterized to date. The different lineages originate from a common naive precursor cell and they are classified based on their cytokine production profiles (Figure 1). These different Th cell populations are either specialized to promote or suppress immune responses.

Th1 type cells are defined as producers of pro-inflammatory cytokines including interferon gamma (IFN-γ), lymphotoxin, IL-2, tumor necrosis factor alpha (TNF-α) and TNF-β (Figure 1). Th1 cells promote cell-mediated immune responses protecting host from intracellular pathogens as well as provide help in tumour rejection (Micallef et al., 1997). Aberrant Th1 response can cause tissue damage or contribute to several inflammatory and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes, inflammatory bowel disease and graft-versus-host disease. Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 (Figure 1). They are important mediators of humoral immune response inducing immunoglobulin E (IgE) production as well as proliferation of eosinophils. Th2 cells protect host against extracellular pathogens and they are important in mucosal immune responses in the lung. Extensive Th2 response plays a crucial role in the development of asthma and allergic diseases. (Reviewed in Glimcher and Murphy, 2000; Mosmann and Sad, 1996; Romagnani, 1996; Wan and Flavell, 2009)

Characterization of a more recently identified lineage of Th cells, Th17 cells, has been both a very active and controversial area of investigation. Th17 cells produce IL-17, IL-21, IL-22 and TNF-α (Figure 1). The differentiation process of Th17 cells appears to be different in the human and mouse system. In mouse, the developmental programs of Th17 and Treg cells are reciprocally interconnected. In humans, Th17 cells are suggested to be developmentally related to Th1 cells. They have also been shown to exhibit plasticity by being able to shift to Th1 type cells. Transcription factor retinoid-related orphan receptor gamma t (RORγt) is considered to be the master regulator driving Th17 polarization. Th17 cells are required for the protection against specific extracellular pathogens and fungal species. Like Th1 cells, Th17 cells have been associated with several autoimmune diseases, but the respective roles of these two subsets behind these diseases remain unclear. Furthermore, Th17 cells have been suggested to be involved in allergic responses. (Reviewed in Annunziato et al., 2007; Annunziato and Romagnani, 2009; Korn et al., 2009; Romagnani, 2008; Wan and Flavell, 2009)

Follicular Th cells (Tfh) and a newly described subset, Th9 cells (Figure 1), are also involved in promoting immune responses, yet relatively little is known about their contribution to pathogen clearance and inflammatory diseases. Tfh cells are enriched in B cell follicles and germinal centers. They secrete IL-21, a cytokine promoting B cell differentiation, and have been shown to be effective inducers of B-cell humoral responses. Differentiation of Tfh cells from naive CD4+ cells is induced by cytokines IL-6 and IL-21 and driven by transcription factor B-cell lymphoma 6 (BCL-6) (Bryant et al., 2007; Linterman and Vinuesa, 2010; Nurieva et al., 2008; Suto et al., 2008; Vinuesa et al., 2005; Yu et al., 2009). Th9 cells produce large amounts of IL-9 and IL-10 and have been shown to be involved in Th2-type responses. These cells can be derived from Th2 cells in the presence of TGF-β, or they can be produced directly from naive T helper precursor (Thp) cells in the presence of IL-4 and TGF-β. Whether there is a Th9 cell-specific transcription factor driving the polarization of this subset, remains to be determined. (Dardalhon et al., 2008; Soroosh and Doherty, 2009; Veldhoen et al., 2008)

Treg cells, including naturally occurring Treg cells (nTreg) and induced Treg cells (iTreg), are specialized to suppress immune functions (reviewed in Curotto de Lafaille and Lafaille, 2009; Josefowicz and Rudensky, 2009; Roncarolo et al., 2001; Stassen et al., 2004). iTreg cells, including Tr1 and Th3-type cells, mediate immunosuppressive functions through secretion of large amounts IL-10 and TGF-β. iTreg cells differentiate from naive Thp cells by TGF-β and IL-2, and their function is dependent on the transcription factor forkhead box P3 (FOXP3) (Figure 1). Unlike the other Tregs, nTregs are developed in thymus and do not actively secrete large amounts of cytokines. The common task of different Treg populations is to maintain self-tolerance and immune homeostasis, although their individual contributions have not yet been fully determined. Interestingly, Tregs have also been implicated in promoting immune response by recruiting natural killer (NK) cells, dendritic cells and T cells to the site of inflammation (Lund et al., 2008).

Thus, a proper regulation of the differentiation and function of these different subsets of Th cells is essential for a balanced immune response against pathogens. The known mechanisms and molecular networks leading to the differentiation of Th1 and Th2 subsets, the two lineages studied in this thesis, are reviewed.

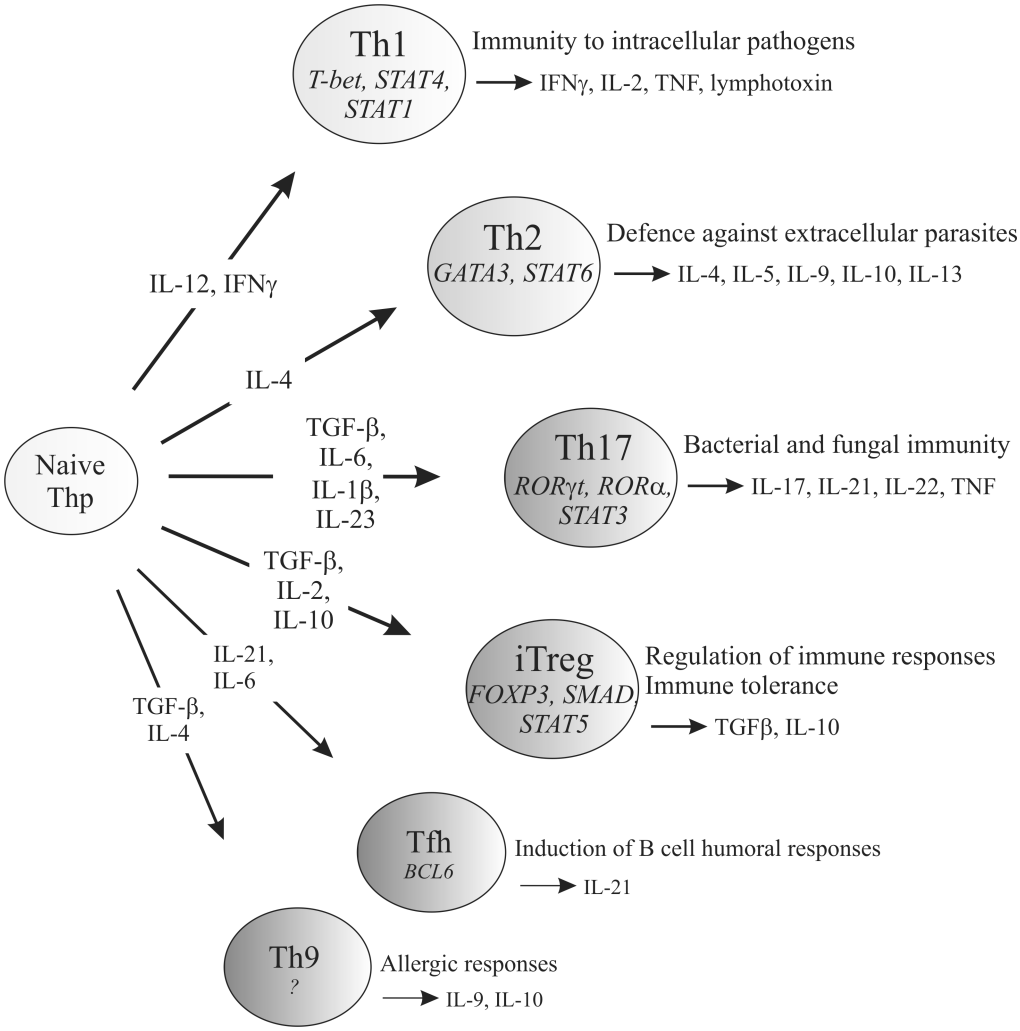

Figure 1. Differentiation of T helper cell subsets. In response to antigen encounter, naive CD4+ cells can differentiate into Th1, Th2, Th17, iTreg, Tfh or Th9 cells. The surrounding cytokine milieu strongly influences the direction of this differentiation process. IL-12 and IFNγ produced by antigen-presenting cells promote Th1 cell differentiation. IL-4 induces Th2 cell polarization. TGF-β together with IL-6, IL-1β and IL-23 promotes Th17 cell polarization, whereas TGF-β, IL-2 and IL-10 induce iTreg development. Tfh and Th9 cells are differentiated from naive Th precursor (Thp) cells in response to IL-21 and IL-6 or TGF-β and IL-4, respectively. Shown are the main transcription factors driving the polarization of these different cell subsets and the main effector cytokines and functions for these cells.

2.2 REGULATION OF TH1 AND TH2 CELL DIFFERENTIATION

2.2.1 T cell receptor stimulus and co-stimulatory signals

2.2.1.1 Strength of the TCR stimulus and calcium signaling

Differentiation of naive CD4+ T cells from a common precursor cell into distinctive Th1 and Th2 cell subsets is mediated by a complex interplay between the cytokine environment and receptor-ligand interactions between a naive T cell and an antigen presenting cell (APC). The interaction between a TCR and major histocompatibility complex II (MHC II) on the surface of an APC is necessary for activation of CD4+ T cells. In addition, the strength of this TCR stimulus, defined as an affinity between TCR and peptide-bound MHC II, influences the direction of Th cell polarization. A strong TCR stimulus usually promotes Th1 differentiation, whereas weak signals favor Th2 polarization (Constant et al., 1995; Leitenberg and Bottomly, 1999). The intensity of TCR-induced calcium signaling influences the direction of Th cell polarization. Stimulation of calcium signaling or inhibition of protein kinase C (PKC) favors Th1 differentiation, whereas inhibition of Calcineurin or stimulation of PKC results in Th2 polarization (Noble et al., 2000). CD4+ cells have been shown to lose the calcium signaling pathway while polarizing towards Th2 direction (Sloan-Lancaster et al., 1997).

Calcium signaling is an important regulator of nuclear factor of activated T cells (NFAT) family of transcription factors. Three of the NFAT proteins that are expressed in T cells are regulated by calcium: NFAT1 (also known as NFATp, NFATc2), NFAT2 (NFATc, NFATc1) and NFAT4 (NFATx, NFATc3). Increased intracellular Ca^{2+} levels activate a protein phosphatase, Calcineurin, leading to dephosphorylation of NFAT, allowing it to enter the nucleus and activate transcription. NFAT proteins are implicated in the regulation of Th1 and Th2 differentiation. NFAT1 has been considered to induce Th1 differentiation and act as negative regulator of Th2 differentiation. It has been shown that, NFAT1 deficient mice show a bias towards Th2 differentiation and decreased IFNγ production (Hodge et al., 1996; Kiani et al., 2001). Mice lacking both NFAT1 and NFAT4 showed extensive overproduction of Th2 cytokines (Ranger et al., 1998b). In contrast, NFAT2 deficient mice show defect in IL-4 production, indicating that NFAT2 acts as an inducer of Th2 polarization (Ranger et al., 1998a; Yoshida et al., 1998). Furthermore, it has been shown that NFAT1 is able to induce transcription of both IL-4 and IFNγ. It could bind to IL-4 promoter only in polarized Th2 cells and IFNγ promoter in Th1 cells (Agarwal et al., 2000). Thus the role of NFATs in the regulation of Th1 vs. Th2 differentiation is complex and the role of specific NFAT on Th1/Th2 polarization depends on the cell-type as well as other regulatory factors present.

NFAT regulates transcription synergistically or in direct interaction with other transcription factors such as activator protein 1 (AP1), c-Maf, interferon regulatory factor 4 (IRF4), early growth response protein 1 (EGR1) and GATA3. NFAT co-operates

with STAT proteins at IFNγ and IL-4 cytokine regulatory regions to induce changes in DNase I hypersensitivity and histone modification in these sites. In addition to regulating the cytokine locus, NFAT proteins may be required to induce Th1 or Th2-specific transcription factors. It has been suggested that, together with STAT molecules, NFATs induce the expression of Th1 and Th2 specific transcription factors T-bet and GATA3. (Agarwal and Rao, 1998; Ansel et al., 2003; Hogan et al., 2003; Savignac et al., 2007; Scheinman and Avni, 2009)

NFAT proteins also mediate the effect of the strength of the TCR stimulus on Th cell differentiation. A weak TCR signal has been shown to induce nuclear localization and DNA binding of NFAT2, a known inducer of IL-4 expression and thereby promote Th2 cell differentiation. Instead, a strong stimulus increased the level of NFAT1, which is known to positively regulate IFNγ production and inhibit Th2 polarization (Brogdon et al., 2002).

2.2.1.2 Tec kinases

Tec family of non-receptor tyrosine kinases is another family of proteins induced by TCR stimulation that have been shown to influence Th1/Th2 differentiation processes. They are activated by Src kinases after TCR ligation and are required for a sustained calcium flux in cells (Readinger et al., 2009). IL-2-inducible T-cell kinase (ITK), a member of this family, is induced during Th2 cell differentiation and in peripheral blood T cells of patients with atopic dermatitis (Matsumoto et al., 2002; Miller et al., 2004). It is required for nuclear localization of NFATc and development of IL-4 producing Th2 cells (Fowell et al., 1999) Specifically, ITK has been shown to be required for Th2 cytokine production upon restimulation, and not for regulation of the Th2 differentiation process itself (Au-Yeung et al., 2006). However, in another study, ITK was proposed to induce Th2 differentiation after weak TCR stimulation by suppressing the expression of T-bet (Miller et al., 2004). TXK (Rlk in mouse), another member of Tec kinase family, is a Th1 cell-specific transcription factor. It has been shown to induce transcription of IFNγ (Takeba et al., 2002). Sequences similar to the TXK-responsive element in the IFNγ enhancer region, are present in several other Th1-associated genes, supporting the significance of TXK for Th1 cell differentiation (Takeba et al., 2002). Despite these findings, the exact mechanism by which ITK and TXK regulate Th1 and Th2 differentiation remains disputed. It has been shown that TXK can partially compensate for the deficiency of ITK, suggesting that instead of the specific functions of these kinases, their expression patterns determine their effect on Th cell differentiation (Readinger et al., 2009; Sahu et al., 2008). In addition, a Rlk/ITK-binding protein, RIBP, has been shown to influence the IFNγ production in activated T cells (Rajagopal et al., 1999).

2.2.1.3 MAPK pathways

TCR stimulation leads to the activation of components of the mitogen activated protein kinase (MAPK) signaling cascade. The key factors of this pathway, extracellular signal-

regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, are constitutively expressed in T cells. They become activated upon phosphorylation by upstream kinases and have been reported to be involved in Th1 and Th2 differentiation (Figure 2 and 3; Table 1). JNK1 deficient mice show a bias towards Th2 type response, possibly through increased levels of nuclear NFAT2, without effect on IFNγ production (Chow et al., 2000; Dong et al., 1998). Instead, JNK2 is selectively activated in Th1 cells following TCR activation and is involved in the induction of IFNγ and interleukin-12 receptor beta 2 subunit (IL-12Rβ2) during early stages of Th1 differentiation (Yang et al., 1998).

In mouse, p38 MAP kinase has been shown to regulate IFNγ production. Inhibition of p38 by specific inhibitors or with a dominant negative form of p38 resulted in reduced IFNγ production in Th1 cells without affecting IL-4 secretion by Th2 cells. Activation of the p38 pathway by a constitutively active form of MKK6, a MAP kinase upstream of p38, increased IFNγ levels during Th1 differentiation (Rincon et al., 1998). In human, p38 has also been shown to promote the expression of Th2 cytokines (Dodeller et al., 2005; Mori et al., 1999) and stimulate IL-4-induced, STAT6-mediated transcription (Pesu et al., 2002). Also the ERK pathway has been shown to positively regulate STAT6 activity and IL-4 expression, as well as play a role in the stabilization of GATA3 protein (Shinnakasu et al., 2008; So et al., 2007; Yamashita et al., 2005).

The growth arrest and DNA-damage inducible 45 (GADD45) family of proteins are mediators of stress-induced signaling events. GADD45γ is preferentially expressed in Th1 cells. It induces activation of JNK and p38 MAPK pathways upon TCR stimulation thereby leading to increased IFNγ production by effector Th1 cells (Lu et al., 2001). In addition, GADD45β, which is induced by Th1-driving cytokines IL-12 and IL-18, promotes cytokine-induced IFNγ production (Yang et al., 2001). Furthermore, Ras-related C3 botulinum toxin substrate 2 (Rac2), an activator of nuclear factor kappa light chain enhancer in B cells (NF-κB), JNK and p38 pathways, has been shown to be selectively expressed in Th1 cells and promote IFNγ production, providing an additional mechanism by which these MAPK pathways are selectively activated in Th1 cells (Li et al., 2000).

Two of the main signaling pathways induced upon T cell activation, the calcium/ Calcineurin pathway and the MAPK pathway are interconnected by the cooperation between NFAT proteins and AP1. AP1 is formed by Fos-Jun heterodimers or Jun-Jun homodimers, which are proteins induced by the MAPK pathway. The nature of AP1 is important for the induction of IFNγ and IL-4 expression (Cippitelli et al., 1995; Penix et al., 1996; Rooney et al., 1995). JunB, which is selectively induced in Th2 cells, induces IL-4 production in synergy with c-Maf (Li et al., 1999).

2.2.1.4 Co-stimulatory signals

In addition to TCR signal, effective T cell activation requires a second signal, mediated by an array of cell-surface co-stimulatory molecules on APCs engaging their corresponding receptors on T cells. Both positive and negative co-stimulatory signals determine and fine-

tune the outcome of the TCR-engagement. Some of the co-stimulatory signals are coupled to calcium signaling and NFAT activation, whereas others are linked to activation of AP1 and NF-κB, or influence both pathways to varying extents (Macian, 2005). Different co-stimulatory signals have been implicated in the regulation of Th1 vs. Th2 differentiation (Figure 2 and 3; Table 1). Signaling through the best characterized and most important co-stimulatory pathway in T cells, CD28/B7, has been shown to be more essential for Th2 than Th1 cell differentiation and critical for Th2-mediated inflammation (Corry et al., 1994; Kallinich et al., 2005; Lenschow et al., 1996). Inducible T cell co-stimulator (ICOS), which is structurally and functionally closely related to CD28 receptor, has also been shown to promote Th2 differentiation and IL-4 production (McAdam et al., 2000; Nurieva et al., 2003). CD28 and ICOS have been shown to have complementary, non-overlapping roles in the development of Th2-type response in vivo (Shilling et al., 2009). The strength of TCR stimulus influences the co-stimulation needed for Th1 and Th2 cell polarization. It has been shown that CD28 ligation induces IL-4 production only after a weak TCR stimulus (Tao et al., 1997).

The OX40 co-stimulatory molecule is mainly expressed on activated T cells. The OX40 / OX40 ligand (OX40L) pathway is important in triggering inflammatory Th2 response induced by thymic stromal lymphopoietin (TSLP)-activated dendritic cells or *Leishmania major* infection. Furthermore, it is important for the maintenance of the Th2 memory cell pool (Akiba et al., 2000; Ito et al., 2005; Wang and Liu, 2007). In addition, CD30/CD30L-mediated signaling promotes the polarization of Th2-type cells (Del Prete et al., 1995).

Lymphocyte function-associated antigen 1 (LFA1) / intercellular adhesion molecule (ICAM) interaction has been shown to suppress Th2 cell differentiation (Salomon and Bluestone, 1998). In addition, it favors Th1 cell differentiation under low cytokine conditions accompanied by a dose-dependent decrease in GATA-3 expression and an increase in T-bet expression (Smits et al., 2002). Also the CD27/CD70 co-stimulatory pathway promotes Th1 cell polarization by enhancing T-bet expression and subsequent induction of IL-12Rβ2. It also promotes survival of activated effector cells (van Oosterwijk et al., 2007).

The T cell immunoglobulin- and mucin-domain containing (TIM) family of cell-surface receptors regulate T cell activation, apoptosis and tolerance. They are involved in both Th1 and Th2-mediated immune responses and have been shown to regulate autoimmune and allergic diseases. TIM3 is specifically expressed in Th1 cells and it downregulates Th1 responses, whereas TIM1 is preferentially expressed in Th2 cells and is associated with Th2 responses (Khademi et al., 2004; Meyers et al., 2005; Rodriguez-Manzanet et al., 2009). TIM1 costimulates T cell activation and it has been suggested to act on Th2 responses by activating ITK, the Th2-promoting Tec kinase (Binne et al., 2007).

Notch signaling is an important regulator of Th cell differentiation, but its specific role has been controversial. Delta-like ligand on APC induces Th1 differentiation, possibly by

inducing Notch to promote the expression of T-bet. Alternatively, Notch may induce Th1-specific genes through interaction with the NF-κB-family proteins p50 and p65. Delta-like ligands also inhibit Th2 polarization by suppressing IL-4 signaling. Expression of Jagged ligands by APCs induces Notch to directly transactivate GATA-3 and IL-4 expression and thereby induce Th2 differentiation. (Reviewed in Amsen et al., 2009)

2.2.2 Th1-inducing cytokines and transcription factors

In addition to several TCR-induced signaling pathways and co-stimulatory signals, Th cell differentiation is regulated by specific cytokines (Table 1). The most potent cytokines driving the Th1 polarization are IFNγ and IL-12. In addition, IL-18 and IL-27 promote Th1 cell development. By binding to their receptors, these cytokines activate and induce expression of Th1-driving transcription factors such as STAT1, STAT4, and T-bet, leading to expression of Th1-specific genes.

2.2.2.1 IFNγ and STAT1

IFNγ plays an essential role in regulating both innate and adaptive immune responses. Major sources for IFNγ are Th1, NK and CD8+ T cells. Naive T and B cells as well as macrophages and dendritic cells are also able to produce IFNγ. One important function of IFNγ is to activate macrophages to promote the elimination of intracellular pathogens (reviewed in Szabo et al., 2003).

IFNγ is the hallmark cytokine produced by Th1 cells. Many factors control the expression of IFNγ in differentiating Th1 cells (Figure 2). It is upregulated by the main signaling pathways induced upon T cell activation; the calcium/Calcineurin pathway and the MAPK and NF-κB pathways. Specifically, p38 MAP kinase as well as the transcription factors NFAT and NF-κB have been shown to induce transcription of IFNγ (Aronica et al., 1999; Corn et al., 2003; Kiani et al., 2001; Peng et al., 2001; Porter and Clipstone, 2002; Rincon et al., 1998; Sica et al., 1997). In addition, AP1, Jun- activating transcription factor 2 (ATF2) complex and ATF3 have been shown to play enhancing role in the IFNγ promoter and positively regulate IFNγ expression (Barbulescu et al., 1997; Filen et al., 2010; Glimcher and Murphy, 2000; Samten et al., 2008; Zhang et al., 1998). Whereas, nuclear factor Yin-Yang 1 (YY1), a ubiquitous DNA-binding protein has been shown to both activate and inhibit the activity of the IFNγ promoter (Soutto et al., 2002; Sweetser et al., 1998; Ye et al., 1996). The IRF family of transcription factors have also shown to regulate Th cell differentiation. IRF1 is induced by IL-12/STAT4 signaling pathway (Coccia et al., 1999) and it has been shown to promote IFNγ production and Th1 cell polarization. However, IRF1 doesn't seem to directly regulate the IFNγ promoter (Lohoff et al., 1997). Instead, IRF1 and IRF2 have been shown to act as direct repressors of the IL-4 promoter (Elser et al., 2002).

After being induced by factors related to TCR-stimulus, other factors, specifically T-bet and STAT4, further induce and maintain the expression of IFNγ during Th1 differentiation.

Table 1. Signaling pathways and molecules regulating Th1 and/or Th2 polarization

Signaling pathway/ molecule	Effect on Th1 or Th2 response	References
Co-stimulatory signals		
CD28/B7	Th2↑	Corry et al., 1994; Lenschow et al., 1996
ICOS/B7h	Th2↑	McAdam et al., 2000; Nurieva et al., 2003
OX40/OX40L	Th2↑	Akiba et al., 2000; So et al., 2006; Wang and Liu, 2007
CD30/CD30L	Th2↑	Del Prete et al., 1995
LFA1/ICAM	Th1↑, Th2↓	Salomon and Bluestone, 1998; Smits et al., 2002
CD27/CD70	Th1↑	van Oosterwijk et al., 2007
Notch/Delta-like	Th1↑, Th2↓	Amsen et al., 2009
Notch/Jagged	Th2↑	Amsen et al., 2009
TIM1/TIM4	Th2↑	Binne et al., 2007; Meyers et al., 2005
TIM3	Th1↓	Meyers et al., 2005
TCR-activated kinases		
ITK	Th2↑	Au-Yeung et al., 2006; Fowell et al., 1999; Miller et al., 2004
TXK	Th1↑	Takeba et al., 2002
ERK	Th2↑	So et al., 2007; Yamashita et al., 2005
JNK1	Th2↓	Dong et al., 1998
JNK2	Th1↑	Yang et al., 1998
p38	Th1↑, Th2↑	Dodeller et al., 2005; Mori et al., 1999; Rincon et al., 1998
Transcription factors		
NFAT1	Th1↑, Th2↑/Th2↓	Agarwal et al., 2000; Hodge et al., 1996; Kiani et al., 2001
NFAT2	Th2↑	Ranger et al., 1998a; Yoshida et al., 1998
NFAT4	Th2↓	Ranger et al., 1998b
NF-κB	Th1↑	Aronica et al., 1999; Corn et al., 2003
JunB	Th2↑	Li et al., 1999
c-Maf	Th2↑	Ho et al., 1996; Kim et al., 1999
Jun-ATF2	Th1↑	Samten et al., 2008; Zhang et al., 1998
ATF3	Th1↑	Filen et al., 2010
IRF1	Th1↑	Lohoff et al., 1997
IRF4	Th2↑	Lohoff et al., 2002; Rengarajan et al., 2002
T-bet	Th1↑, Th2↓	Finotto et al., 2002; Szabo et al., 2000; Szabo et al., 2002
GATA3	Th1↓, Th2↑	Ferber et al., 1999; Ouyang et al., 1998; Zhang et al., 1999; Zheng and Flavell, 1997
EGR-1	Th2↑	Lohoff et al., 2010
RUNX1	Th2↓	Komine et al., 2003
RUNX3	Th1↑	Djuretic et al., 2007
HLX	Th1↑	Mullen et al., 2002
BCL-6	Th2↓	Dent et al., 1997; Ye et al., 1997
TCF1	Th1↓, Th2↑	Yu et al., 2009
LEF-1	Th2↓	Hossain et al., 2008
Cytokines/cognate transcription factors		
IFNγ/STAT1	Th1↑	Afkarian et al., 2002; Lighvani et al., 2001; Szabo et al., 1997
IL-12/STAT4	Th1↑, Th2↓	Kaplan et al., 1996b; Ouyang et al., 1998; Thierfelder et al., 1996
IL-18/NF-κB	Th1↑	Micallef et al., 1996; Robinson et al., 1997; Takeda et al., 1998
IL-27/STAT1	Th1↑	Owaki et al., 2006; Pflanz et al., 2002; Takeda et al., 2003
IL-4/STAT6	Th1↓, Th2↑	Kaplan et al., 1996a; Kaplan et al., 1998; Kuperman et al., 1998; Tamachi et al., 2009
IL-2/STAT5	Th2↑	Cote-Sierra et al., 2004; Liao et al., 2008
TSLP	Th2↑	Al-Shami et al., 2005; Ito et al., 2005; Liu, 2006; Soumelis et al., 2002)
Others		
GADD45γ	Th1↑	Lu et al., 2001
GADD45β	Th1↑	Yang et al., 2001
RAC2	Th1↑	Li et al., 2000
RIBP	Th1↑	Rajagopal et al., 1999
SOCS-1	Th2↓	Losman et al., 1999; Yu et al., 2004
GFI1	Th2↑	Shinnakasu et al., 2008; Zhu et al., 2002
FOG	Th2↓	Kurata et al., 2002
ROG	Th2↓	Hirahara et al., 2008
Mina	Th2↓	Hemmers and Mowen, 2009; Okamoto et al., 2009

GATA3, in turn has been shown to inhibit IFNγ expression. Although GATA3 has the capacity to bind the IFNγ promoter (Penix et al., 1993), it has been suggested that it suppresses IFNγ through downregulation of STAT4 signaling (Kaminuma et al., 2004; Ouyang et al., 1998; Usui et al., 2003).

IFNγ promotes the differentiation of Th1 cells. After being upregulated by TCR activation, secreted IFNγ forms a positive autoregulatory loop to enhance Th1 polarization. It binds to its receptor on the cell surface leading to receptor dimerization and association with the janus tyrosine kinases (JAK1 and JAK2). This leads to recruitment and phosphorylation of STAT1 (Figure 2). Upon activation STAT1 homodimerizes and translocates to the nucleus and activates the transcription of its target genes. STAT1 is an important inducer of T-bet, the key transcription factor driving Th1 polarization (Afkarian et al., 2002; Lighvani et al., 2001). In addition, STAT1 is known to co-operate with Ets-1 to optimize the transcription of ICAM-1, a Th1-promoting cell surface co-stimulatory molecule (Yockell-Lelievre et al., 2009).

2.2.2.2 T-bet, RUNX and HLX

T-bet, also known as Tbx21, is regarded as the master regulator of Th1 cell differentiation. T-bet was originally cloned in a study aiming at identification of transcription factors responsible for the Th-subset specific expression of cytokines (Szabo et al., 2000). T-bet is upregulated in Th1 cells in response to TCR activation and Th1-driving signals. NFAT binding is suggested to play a role in the initiation of chromatin remodeling at the T-bet locus (Placek et al., 2009). Also Notch and NF-κB signaling have been proposed to induce the expression of T-bet (Corn et al., 2003; McCracken et al., 2007; Minter et al., 2005). Moreover, an ubiquitous transcription factor, Sp1, has been shown to activate T-bet expression (Yu et al., 2007). IFNγ/STAT1 signaling plays the most important role to control the Th1 cell-specific expression of T-bet (Figure 2). Also the IL-12/STAT4 pathway has been reported to induce T-bet at the transcriptional level (Placek et al., 2009; Szabo et al., 2000; Usui et al., 2006; Ylikoski et al., 2005), although this mechanism has been shown to take place in the late phase of Th1 cell differentiation (Schulz et al., 2009).

T-bet is an important inducer of IFNγ. Retrovirus-mediated expression of T-bet in Th2 cells induced IFNγ production and shut down the expression of Th2 cytokines (Szabo et al., 2000). In addition, T-bet knockout mice had defects in IFNγ production and Th1 cell development and displayed increased Th2 responses and spontaneously develop asthma-like diseases (Finotto et al., 2002; Szabo et al., 2002). T-bet induces IFNγ directly by binding to its regulatory elements and also by inducing chromatin remodeling of the IFNγ locus (Mullen et al., 2001; Szabo et al., 2000). In addition, another T-box family member, Eomesodermin, is upregulated in Th1 cells and has been proposed to be involved in regulation of IFNγ expression. IL-21 was shown to inhibit IFNγ production during Th1 differentiation through repression of Eomesodermin and this was not due to effects on IFNγ, STAT4, STAT1 or T-bet (Suto et al., 2006).

The role of T-bet on the expression of IL-12Rβ2 has been disputed. T-bet has been reported to promote the function of the important Th1-driving IL-12/STAT4 signaling pathway by enhancing the expression of IL-12Rβ2. Retrovirus-mediated expression of T-bet induced the expression of IL-12Rβ2 in Th2 conditions independently of IFNγ/STAT1 or IL-12/STAT4 pathways (Afkarian et al., 2002; Mullen et al., 2001). Whereas other studies have suggested that T-bet is not a significant inducer of IL-12Rβ2 (Letimier et al., 2007; Usui et al., 2006). Letimier *et al.* (Letimier et al., 2007) reported that retrovirus-mediated expression of T-bet in human Th2 cells only slightly induced expression of IL-12Rβ2 and the enhancer and promoter of the human IL-12Rβ2 gene do not contain consensus binding sites for T-bet. Furthermore, neither genetic deletion nor the retroviral expression of T-bet influenced the expression of IL-12Rβ2 in polarized Th1 cells (Usui et al., 2006).

T-bet has been shown to regulate Th1 differentiation and IFNγ production in collaboration with two other transcription factors, runt-related transcription factor 3 (RUNX3) and H2.0-like homeobox protein (HLX). These proteins belong to RUNX and homeodomain protein families, respectively, which often interact with other transcription factors to regulate transcription in a context-dependent manner. RUNX3 and HLX are expressed specifically in Th1 cells. T-bet upregulates the expression of both of these proteins and interacts and cooperates with them to induce maximal IFNγ production (Djuretic et al., 2007; Mullen et al., 2002) (Figure 2). T-bet also acts together with RUNX3 to silence IL-4 promoter in Th1 cells (Djuretic et al., 2007).

Negative regulation by factors driving the development of an opposite Th cell subset is an important mechanism influencing the outcome of the Th1/Th2 differentiation process. The ability of T-bet to suppress Th2 cell development has been proposed to be mediated by inhibiting both the function and the expression of GATA3 (Usui et al., 2006), a major regulator of Th2 cell polarization, suggesting that T-bet mediated induction of Th1 differentiation and repression of Th2 polarization are functionally separated events. Tyrosine phosphorylation of T-bet by the Tec family kinase, ITK, did not influence induction of IFNγ expression, whereas it induced T-bet to interact with GATA3 thereby inhibiting its binding to Th2 cytokine locus (Hwang et al., 2005). Furthermore, Tamachi *et al.* have shown that while Th2 cell differentiation is enhanced in T-bet$^{-/-}$ mice, it is diminished in STAT6$^{-/-}$T-bet$^{-/-}$ mice to the level of STAT6$^{-/-}$ mice, suggesting that suppression of Th2 differentiation by T-bet is mediated through regulation of STAT6 signaling (Tamachi et al., 2009).

2.2.2.3 IL-12 and STAT4

IL-12 is mainly produced by dendritic cells, monocytes and macrophages in response to infection. It enhances both innate and adaptive immunity by inducing proliferation of NK and T cells, IFNγ production by APCs, NK and B cells and by promoting Th1 type responses. (Chan et al., 1991; Frucht et al., 2001; Gately et al., 1998; Kobayashi et al., 1989; Valiante et al., 1992; Yoshimoto et al., 1997)

IL-12 has been considered to be the main cytokine driving the Th1 cell differentiation. IL-12Rβ2, the signaling component of the IL-12 receptor, is not expressed in naive T cells. TCR stimulus induces recruitment of brahma-related gene 1 (BRG1), the ATPase subunit of the BAF chromatin remodeling complex, to the enhancer and promoter of the *IL-12Rβ2* gene leading to increased histone acetylation and low-level transcription at the *IL-12Rβ2* locus (Letimier et al., 2007). In addition, expression of IL-12Rβ2 has been suggested to be induced by T-bet (Afkarian et al., 2002; Mullen et al., 2001) and maintained by IFNγ (Chang et al., 1999; Szabo et al., 1997). Once the expression of IL-12Rβ2 is upregulated, IL-12, produced by APCs, is able to bind its receptor and subsequently activate STAT4 (Figure 2). STAT4 is considered to be more important for amplifying rather than initiating Th1 responses. The mechanism by which STAT4 mediates its effects is uncertain. In addition to its direct effects on IFNγ expression, it may interact with general transcriptional co-activators, such as CREB binding protein (CBP) (Barbulescu et al., 1998; Mullen et al., 2001). STAT4 also enhances the expression of cytokine receptors and signaling molecules required for the induction of IFNγ, such as IL-12Rβ2 and IL-18R (Lawless et al., 2000; Letimier et al., 2007; Nishikomori et al., 2002). STAT4 also induces the expression of HLX, the transcription factor that promotes Th1 polarization in co-operation with T-bet (Thieu et al., 2008). In addition to T-bet, STAT4 also represses Th2 differentiation. IL-12 and STAT4 signaling were shown to be required for full inhibition of GATA3 in developing Th1 cells (Ouyang et al., 1998).

Thus, Th1 cell differentiation is regulated by an interrelated signaling network, with multiple factors positively regulating each other. However, the order of events that take place after the polarization is initiated and the relative roles of STAT4 and T-bet in Th1 lineage commitment have been under debate. The main function of T-bet in the regulation of Th1 cell differentiation is still subject to discussion. Some studies have shown the importance of T-bet in promoting the expression of IL-12Rβ2 and IFNγ, while others have demonstrated that the main function of T-bet is to repress GATA3 thus preventing its downregulation of Th1 differentiation, rather than to induce chromatin remodeling of the IFNγ locus and transcription of IFNγ (Usui et al., 2006). Two distinct linear models have been proposed for the development of Th1 cells. In one model, IL-12 and STAT4 initially induce IFNγ leading to activation of STAT1, T-bet expression and further upregulation of IFNγ (Usui et al., 2003). In the other IFNγ and TCR signaling promote T-bet expression leading to IL-12Rβ2 expression and subsequent activation of STAT4 (Mullen et al., 2001). However, STAT4 has been shown to promote Th1 differentiation even in the absence of T-bet, in opposition of the latter model (Usui et al., 2006). In fact, several findings oppose the idea of these simple linear models behind the function of STAT4 and T-bet.

A more recent study by Thieu *et al.* proposes a parallel model, in which both IFNγ and IL-12 pathways are required for the complete activation of Th1 phenotype (Thieu et al., 2008). By studying a panel of Th1-associated genes in $CD4^+$ T cells from wild-type, STAT4-deficient or T-bet-deficient mice, they identified three subsets of genes that required either STAT4 or T-bet, or both factors for their expression. Exogenous IFNγ did not rescue STAT4-dependent gene expression. In addition, ectopic T-bet

expression could rescue expression of Th1-specific genes in T-bet$^{-/-}$ T cells, but not in T-bet/STAT4 double-deficient T cells. Both of these factors induced unique changes in chromatin modifications on common target genes, but they also had specific effects on their specific target genes. These results supported a model, in which both STAT4 and T-bet are required for complete Th1 polarization.

2.2.2.4 IL-18 and IL-27

IL-18 is a pro-inflammatory cytokine produced by activated macrophages, monocytes and dendritic cells (Okamura et al., 1995; Stoll et al., 1998). It plays an important role both in innate immunity and enhancing specific Th1 responses. IL-18Rα, a receptor for IL-18, is induced by IL-12/STAT4 signaling and IFNγ at later stages of Th1 differentiation (Sareneva et al., 2000; Yoshimoto et al., 1998). IL-18 has been shown to be a selective activator of IFNγ in Th1, but not in Th2, cells. However, it is not necessary for Th1 differentiation, but facilitates IL-12-induced Th1 differentiation by optimizing IFNγ production (Micallef et al., 1996; Robinson et al., 1997). IL-18 deficient mice show impaired IFNγ production in response to *Propionibacterium acnes*, and mice lacking both IL-18 and IL-12 had a more severe defect in IFNγ production than either strain alone (Takeda et al., 1998). IL-18-induced signaling is mediated by interleukin-1 receptor-associated kinase (IRAK), leading to activation of NF-κB (Figure 2). IL-18 has also been shown to activate AP1 via the JNK pathway (Barbulescu et al., 1998; Matsumoto et al., 1997; Robinson et al., 1997).

IL-27 shares several characteristics with IL-12 and IL-18. It is produced by monocytes, activated macrophages and dendritic cells. It has been reported to regulate the activities of several cell types including CD8+ T cells, NK cells, B cells, monocytes and activated macrophages. IL-27 induces Th1 differentiation from naive CD4+ T cells by synergizing with IL-12 to dynamically induce IFNγ production (Pflanz et al., 2002). It mediates its effects via the T cell cytokine receptor (TCCR)$^{/WSX-1}$. IL-27/TCCR$^{/WSX-1}$ stimulation induces activation of STAT1 (Figure 2) leading to increased expression of T-bet and subsequent induction of IL-12Rβ2 and IFNγ expression (Hibbert et al., 2003; Takeda et al., 2003). In addition, IL-27 promotes IFNγ production through STAT1-dependent activation of the ICAM1/LFA1/ERK1/2 pathway (Owaki et al., 2006). TCCR$^{/WSX-1}$ deficient mice have impaired Th1 responses and increased Th2 cytokine production (Artis et al., 2004; Chen et al., 2000; Yoshida et al., 2001). Defective IFNγ production was shown to be limited only to the early phases of infection and it has been suggested that IL-27 signaling may play an important role in early Th1 differentiation, before the expression and function of the IL-12R pathway (Owaki et al., 2005; Yoshida et al., 2001). IL-27 is a unique cytokine in that it also has anti-inflammatory properties. Its immunosuppressive effects are mediated through IL-2 suppression, inhibition of Th17 cell development and induction of IL-10 production (Yoshida and Miyazaki, 2008; Yoshida et al., 2009).

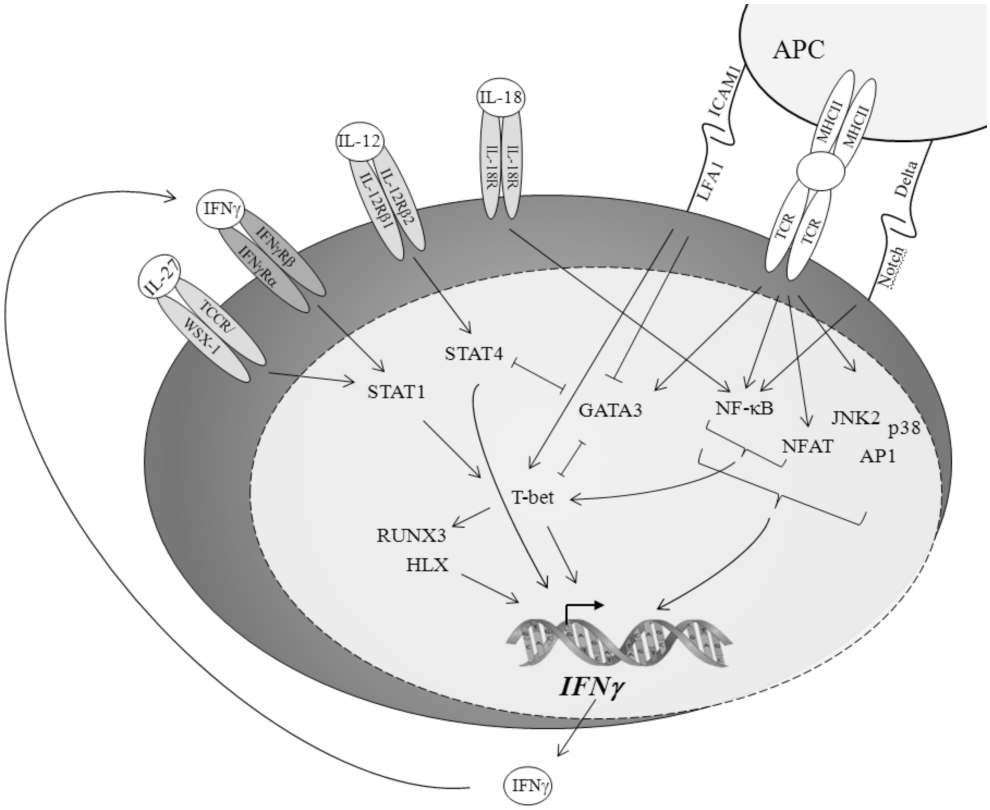

Figure 2. Pathways and signaling molecules regulating Th1 differentiation. Expression of the Th1 signature cytokine, IFNγ, is regulated by several factors. Th1-promoting STATs, STAT1 and STAT4, are activated by IL-12, IL-27 and IFNγ. STAT4 and STAT1 induce expression of IFNγ and T-bet, the master regulator of Th1-polarization. T-bet and IFNγ are also induced by certain co-stimulatory signals as well as TCR-induced signaling pathways, such as NFAT, NF-κB and MAPK (JNK2, p38) pathways. GATA3 inhibits Th1 polarization and it is downregulated by several Th1-driving factors.

2.2.3 Cytokines and transcription factors important for Th2 cell polarization

2.2.3.1 IL-4 and STAT6

IL-4 plays an important role in the immune system where it affects many cell types. It is produced mainly by Th2 and type 2 cytotoxic T cells (Tc2),, eosinophils, basophils, mast cells and natural killer T (NKT) cells (Nelms et al., 1999; Sad et al., 1995). It has a central role in the regulation of Th2 and Tc2 cell differentiation and has several effects in B cells including the regulation of immunoglobulin class switching to IgE and IgG4. IL-4 also promotes expression of endothelial adhesion molecules and production of chemokines by epithelial cells, which is essential for maintenance of Th2 responses. (Reviewed in Li-Weber and Krammer, 2003; Nelms et al., 1999)

IL-4 has a variety of effects in CD4+ cells including effects on cell growth, differentiation and resistance to apoptosis (reviewed in Nelms et al., 1999). In addition

to suppressing the development of IFNγ-producing Th1 cells, IL-4 is the main cytokine driving the Th2 cell polarization. However, it is still unclear what cells are the first source of IL-4 required for the initiation of Th2 cell differentiation in the lymph node. NKT cells, producing large amounts of IL-4, have been suggested to direct the development of Th2 type immune responses (Singh et al., 1999; Yoshimoto et al., 1995). However, several studies have shown that Th2 response can develop in the absence of NKT cells suggesting that NKT cells are not required for the initiation of Th2 cell differentiation (Akbari et al., 2003; Brown et al., 1996; Smiley et al., 1997; von der Weid et al., 1996). Activated naive CD4+ cells themselves have been considered as a initial source of IL-4 sufficient for Th2-polarization, but this is still subject to discussion (Mohrs et al., 2001; Xin et al., 2007). Also the epithelial cell-derived cytokine TSLP and the OX40 pathway have been suggested to provide the initial signal for naive CD4+ cells to polarize into Th2 cells (Ito et al., 2005; So et al., 2006) (described in chapter 2.2.3.4).

The wide range of effects of IL-4 are mediated through its receptor complex consisting of the IL-4 receptor α subunit (IL-4Rα) and the common gamma chain. Binding of IL-4 to its receptor results in series of phosphorylation events mediated by receptor-associated JAK kinases. These kinases phosphorylate tyrosine residues on the cytoplasmic tails of the receptor generating docking sites for several signaling molecules (Nelms et al., 1999). Of the IL-4Rα-mediated signaling pathways, STAT6 pathway has been described to be critical for Th2 cell differentiation (Kaplan et al., 1996a) (Figure 3). Stat6-deficient mice are resistant to antigen induced airway inflammation and hyperresponsiveness, thus illustrating the importance of this pathway in the development of allergic inflammation (Akimoto et al., 1998; Kuperman et al., 1998).

Once phosphorylated on tyrosine residue 641, STAT6 disengages from the IL-4 receptor and forms homodimers. Dimerized STAT6 translocates to the nucleus where it binds to specific DNA motifs in the promoters of its target genes (reviewed in Nelms et al., 1999). STAT6 can act as a transcriptional activator or repressor, depending on the specific target gene and the cellular context. Known targets of STAT6 include genes encoding CD23, MHC II and IL-4Rα chain (Nelms et al., 1999). STAT6 also binds directly to IL-4 promoter and enhancer regions and is required for the histone hyperacetylation in the IL-4 locus. Furthermore, STAT6 is needed for the NFAT binding to the same regions (Avni et al., 2002). As the key Th2-driving factor, the essential function of STAT6 is to upregulate the expression of GATA3, the master regulator of Th2 cell development and c-Maf, a Th2-specific inducer of IL-4 expression (Ho et al., 1996; Kurata et al., 1999) (Figure 3). In vivo, it has been observed that Th2 responses can be obtained also independently of STAT6 or IL-4 (Finkelman et al., 2000; Huang et al., 1997; Jankovic et al., 2000). Based on these studies, STAT6 was suggested to be more important in stabilizing the Th2 populations and development of Th2 memory cells rather than in the development of the primary response.

STAT6 also plays a role in the inhibition of Th1 polarization. Th1 cell differentiation was partly recovered in mouse CD4+ cells deficient in both STAT6 and T-bet as compared

to cells deficient with T-bet only. In addition, IL-12-induced phosphorylation of STAT4 was increased in these cells relative to T-bet$^{-/-}$ cells, suggesting that STAT6 suppresses T-bet independent Th1 differentiation by inhibiting IL-12/STAT4 signaling (Tamachi et al., 2009). In addition, another study suggests that STAT6 can also repress a STAT4-independent pathway driving Th1 polarization (Kaplan et al., 1998).

2.2.3.2 Regulation of STAT6-mediated transcription and activity of STAT6

In addition to IL-4, various other stimuli can also lead to the activation of STAT6, including B cell receptor (BCR), TCR, CD40 and CD28 engagement. In addition, IFN-α, IL-3 and IL-15 as well as ligands of c-Kit and platelet-derived growth factor (PDGF) receptor tyrosine kinases have been shown to induce STAT6 phosphorylation (reviewed in Hebenstreit et al., 2006). Also unphosphorylated STAT6 has been shown to form homodimers through its amino-terminal part (Ota et al., 2004). STAT6 can also activate transcription in its unphosphorylated form (Cui et al., 2007).

STAT6 interacts and collaborates with several transcriptional co-factors to activate transcription. The presence of a set of co-regulatory proteins is required for the interaction of STAT6 with the basal transcription machinery. Ubiquitously expressed nuclear proteins CBP and p300 bridge STAT6 with the basal transcription machinery, thereby promoting the expression of STAT6 target genes (Gingras et al., 1999; McDonald and Reich, 1999). Interaction of STAT6 with CBP/p300 is dependent on another co-activator protein, p100 (Välineva et al., 2005). In addition, two members of the p160/nuclear receptor co-activator (NCoA) family, NCoA-1 and NCoA-3 have been shown to interact and/or positively regulate the transcriptional activity of STAT6 (Arimura et al., 2004; Litterst and Pfitzner, 2001; Litterst and Pfitzner, 2002).

Several other transcription factors are involved in the regulation of STAT6 target genes. Depending on the cell type and the target gene, these factors can either positively or negatively influence STAT6-mediated transcription by binding close to the STAT6 motif. Members of the CCAAT/enhancer binding proteins (C/EBP) family of transcription factors influence STAT6-mediated transcriptional activation, although their specific roles are not yet clearly defined (reviewed in Hebenstreit et al., 2006). NF-κB and STAT6 can act synergistically as well as inhibit each others function. The well known STAT6 target, germline epsilon promoter, is cooperatively activated by NF-κB and STAT6 (Messner et al., 1997). In contrast, NF-κB and STAT6 counter-regulate E-selectin gene, by competing for an overlapping binding site (Bennett et al., 1997). Two members of the Ets family of transcription factors, Ets-1 and PU.1 are implicated in STAT6 signaling. Whereas Ets-1 was able to interact with STAT6 and repress the expression of the STAT6 target gene suppressor of cytokine signaling 1 (SOCS-1), PU.1 synergized with STAT6 to induce the expression of several genes (Pauleau et al., 2004; Pesu et al., 2003; Stutz and Woisetschlager, 1999; Travagli et al., 2004). BCL-6 transcriptional repressor is an important negative regulator of STAT6-mediated transcription. Mice deficient with BCL-6 develop extensive Th2-type

inflammatory responses (Dent et al., 1997; Ye et al., 1997). BCL-6 has been shown to inhibit STAT6-mediated activation of germline epsilon promoter and proposed to act as an STAT6 antagonist, because of the similarities between their binding motifs (Dent et al., 1997; Harris et al., 1999). BCL-6 can also repress the transcriptional activity of STAT6 through inhibition of transcription factor IRF4, which has been shown to directly interact with STAT6 and promote the activation of STAT6 target genes (Gupta et al., 1999).

The activity of STAT6 is tightly regulated through several mechanisms, of which the IL-4R/JAK-mediated phosphorylation of STAT6 on tyrosine 641 is the most thoroughly described (Nelms et al., 1999). Serine phosphorylation also regulates the transcriptional activity of STAT6. IL-4 induces phosphorylation of the serine 756 in the transactivation domain of STAT6 (Wang et al., 2004). Moreover, TCR-mediated calcium signaling influences STAT6 activity through regulation of serine phosphorylation events (Schmidt-Weber et al., 2000). However, the effect of the serine phosphorylation of STAT6 is currently unclear, since it has been shown to influence STAT6 transcriptional activity both positively and negatively (Maiti et al., 2005; Pesu et al., 2000; Schmidt-Weber et al., 2000; Woetmann et al., 2003).

Methylation of STAT6 at Arg^{27} is necessary for optimal STAT6 tyrosine phosphorylation, nuclear translocation and DNA-binding activity (Chen et al., 2004a). Moreover, STAT6 has been shown to be modified by acetylation as well as O-linked N-acetylglucosamine (O-GlcNAc) modification (Gewinner et al., 2004; Shankaranarayanan et al., 2001). In addition to bridging STAT6 with the basal transcription machinery, the histone acetyltransferase CBP/p300 is able to acetylate STAT6, which has been shown to be required for the transcriptional activation of the 15-lipoxygenase-1 gene (Shankaranarayanan et al., 2001). IL-4-induced O-GlcNAc modification was observed on STAT6, but the functional significance of this modification is not known. On STAT5, O-GlcNAc modification is required for the interaction of STAT5 with the co-activator protein CBP (Gewinner et al., 2004).

Inhibition of STAT6 signaling is controlled by specific phosphatases (Hanson et al., 2003) as well as indirectly via SOCS proteins (Yu et al., 2004). The tyrosine phosphatase SHP-1 (SH2-containing phosphatase-1) has been shown to negatively regulate STAT6 activity as well as STAT6-mediated transcription, but its specific target in the IL-4R/JAK/STAT6 pathway remains unclear (Hanson et al., 2003; Haque et al., 1998). Another tyrosine phosphatase, protein tyrosine phosphatase 1B (PTP1B), directly interacts with and dephosphorylates STAT6 in an IL-4-inducible manner leading to decreased STAT6-mediated transcriptional activation (Lu et al., 2008). In contrast, the serine/threonine protein phosphatase 2A (PP2A) enhances the transcriptional activity of STAT6, as serine phosphorylation can inhibit the activity of STAT6 (Woetmann et al., 2003).

SOCS-1 is an important inhibitor of the IL-4/STAT6 signaling pathway. It suppresses IL-4-induced activation of STAT6 by inhibiting the activation of IL-4R-associated JAK1, subsequently leading to downregulation of the expression of STAT6-responsive

genes. IL-4 and activated STAT6 has been shown to induce the expression of SOCS-1, indicating that STAT6 is involved in a negative feedback regulatory mechanism by inducing the expression of its own inhibitor. (Dickensheets et al., 2007; Hebenstreit et al., 2005; Losman et al., 1999; Yu et al., 2004)

Several studies indicate that STAT6 is negatively regulated by proteolytic degradation, both by proteasome and directly by proteases. Proteasome inhibitor MG132 decreased the decay rate of the tyrosine-phosphorylated STAT6 but it did not influence the levels of total STAT6, indicating that the proteasome is not acting on STAT6 itself, but rather on other proteins involved in the dephosphorylation phase (Hanson et al., 2003). The serine proteases that regulate STAT activity show both STAT and cell-type specificity. In mouse, but not in human mast cells, STAT6 is proteolytically cleaved by a serine protease belonging to the elastase family. This cleavage generates a truncated product of 65 kDa which lacks the C-terminal transactivation domain and functions as a dominant-negative molecule to STAT6 (Suzuki et al., 2002; Suzuki et al., 2003). Furthermore, STAT6 has been shown to be completely degraded by a mechanism sensitive to the serine protease inhibitor 4-(2-aminoetyl)-benzenesulfonyl fluoride, suggesting the existence of different STAT6 serine proteases (Perez-G et al., 2008). STAT6 is also cleaved in mouse mast cells by a cysteine protease Calpain, generating a protein of 70 kDa (Suzuki et al., 2003). In contrast, in mouse T cell lines activated STAT6 is completely degraded by Calpain (Zamorano et al., 2005). The physiological importance of Calpain-mediated cleavage of STAT6 is unknown. Calpains are implicated in cell proliferation, apoptosis and differentiation (Perrin and Huttenlocher, 2002). They are activated by a strong TCR stimulus and enhanced calcium signaling in T lymphocytes (Rock et al., 1997; Selliah et al., 1996), which all, in turn, have been shown to favor Th1 over Th2 differentiation (Constant et al., 1995; Imam et al., 2007; Noble et al., 2000). It has been suggested that strong TCR signals may promote Th1 cell polarization through promoting strong calcium signaling, Calpain activation and a subsequent degradation of STAT6 (Imam et al., 2007; Suzuki et al., 2003; Zamorano et al., 2005).

2.2.3.3 GATA3 and other factors

GATA3 is the master regulator of Th2 cell differentiation. Downregulation of GATA3 or expression of a dominant negative mutant of GATA3 greatly diminish Th2 cytokine production and allergic responses, whereas elevated GATA3 induces Th2 cytokine expression in polarized Th1 cells (Zhang et al., 1999; Zheng and Flavell, 1997). Conditional deletion of GATA3 in CD4+ cells further confirmed its importance in the development and maintenance of Th2 cells (Pai et al., 2004; Zhu et al., 2004). GATA3 regulates Th2 cytokine production by binding to several sites and controlling the acetylation and accessibility of the IL-4 locus containing the IL-4, IL-5 and IL-13 genes (Avni et al., 2002; Fields et al., 2002; Tykocinski et al., 2005; Yamashita et al., 2002). GATA3 was also shown to play important role in the maintenance of the Th2 phenotype and continuous chromatin remodeling at the Th2 cytokine locus (Yamashita et al., 2004).

Although preferentially expressed in Th2 cells, GATA3 is also expressed in human Th1 cells (Figure 2). GATA3 represses Th1 polarization distinct from its positive effects on Th2 cytokine production (Ouyang et al., 1998). It has been reported to inhibit the expression of IL-12Rβ2 (Ouyang et al., 1998) and Th1 differentiation (Ferber et al., 1999; Ouyang et al., 1998) by downregulating STAT4 (Usui et al., 2003). Furthermore, GATA3 has been shown to occupy several of the same genes as T-bet, including ones differentially expressed between Th1 and Th2 cells, such as IFNγ, IL-18 receptor accessory protein (IL-18RAP), IL-4 and ITK. This suggests that T helper lineage decision is regulated by the opposing actions of GATA3 and T-bet at a shared set of target genes (Jenner et al., 2009). The ability of GATA3 to induce the Th2 phenotype or to downregulate IFNγ expression is weakened as Th cells differentiate into Th1 type cells.

Although GATA3 is induced by IL-4/STAT6 signaling pathway, it is also able to induce Th2 cytokine expression independently of STAT6. Notch has been shown to regulate IL-4 production by inducing GATA3 expression from the distal promoter (Amsen et al., 2007; Fang et al., 2007). In addition, T cell factor 1 (TCF1) and its cofactor β-catenin mediate STAT6 and Notch-independent, TCR-induced early GATA3 expression from the proximal promoter leading to increased IL-4 production. TCF1 was also able to inhibit Th1 polarization by negatively regulating IFNγ expression (Yu et al., 2009). NFAT1 has also been shown to bind both GATA3 promoters and induce its expression (Scheinman and Avni, 2009). Furthermore, GATA3 is able to activate its own expression creating a positive feedback loop stabilizing Th2 commitment and providing an additional mechanism for IL-4 and STAT6 independent Th2 development (Ouyang et al., 2000). Thus, expression of GATA3 is induced by different combinations of transcription factors downstream of TCR and IL-4R as well as by autoregulatory mechanism for optimal Th2 differentiation (Figure 3). Expression of GATA3 is negatively regulated by RUNX1. This was suggested to take place only in naive CD4+ cells and during the very early phases of Th cell differentiation because expression of RUNX1 is downregulated after TCR stimulation (Komine et al., 2003).

Expression and activity of GATA3 are also regulated by post-transcriptional mechanisms. MAPK signaling cascade stabilizes GATA3 protein by inhibiting ubiquitin-mediated GATA3 degradation (Yamashita et al., 2005). This has been suggested to be mediated by growth factor independent 1 (GFI1), a TCR and STAT6-induced transcriptional repressor. GFI1 is a downstream target of the ERK MAPK cascade and it has been shown to control IL-5 and IFNγ production in Th2 cells by regulating GATA3 protein stability likely by repressing the ubiquitination of GATA3 (Shinnakasu et al., 2008; Zhu et al., 2002) (Figure 3).

Tyrosine phosphorylated T-bet has been shown to inhibit the binding of GATA3 to the Th2 cytokine locus by interacting with GATA3 (Hwang et al., 2005). Friend of GATA (FOG) is expressed in naive Th cells where it interacts with GATA3 repressing GATA3-mediated Th2 cytokine production (Kurata et al., 2002), whereas repressor of GATA (ROG) is induced upon TCR-stimulation and interacts with and represses the GATA3-

mediated transcription and cytokine production (Miaw et al., 2000). ROG has also been shown to inhibit Th2-type allergic inflammation (Hirahara et al., 2008). In addition, lymphoid enhancer factor 1 (LEF-1) interacts with GATA3 and inhibits its DNA-binding activity leading to suppression of Th2 cytokine production (Hossain et al., 2008).

Other transcriptional regulators important for Th2 cell development include c-Maf and IRF4 (Figure 3). c-Maf is a member of the AP1 family of transcription factors and it is expressed selectively in Th2 cells. It is required for the production of IL-4, but not other Th2 cytokines (Ho et al., 1996; Kim et al., 1999). IRF4 has also shown to be required for Th2 differentiation. It has been shown to synergize with NFAT1 and c-Maf to enhance the expression of IL-4 (Rengarajan et al., 2002). It was also shown to directly interact with STAT6 and promote the activation of STAT6 target genes (Gupta et al., 1999) as well as suggested to mediate the IL-4-induced upregulation of GATA3 (Lohoff et al., 2002). Mina, a member of the jumonji C protein family, has been recently identified as an important regulator of Th2 cell bias. Overexpression of Mina inhibited IL-4 expression, whereas its knockdown led to upregulation of IL-4 in primary CD4+ cells. Mina was shown to bind to IL-4 promoter in co-operation with NFAT leading to inhibition of IL-4 expression (Okamoto et al., 2009). Mina was identified as a candidate gene for regulating the susceptibility to *Leishmania major* infection and the regulatory mechanisms controlling Mina expression and activity were suggested to determine at least in part the capacity for initial IL-4 production and susceptibility to Th2-driven pathologies (Hemmers and Mowen, 2009).

2.2.3.4 IL-2/STAT5 pathway and TSLP

IL-2 is mainly produced by activated CD4+ T cells in response to activation. It has multiple roles in regulating T cell growth, survival as well as activation-induced cell death (Lenardo et al., 1999). IL-2 is also necessary for the development of Treg cells (Malek, 2003). IL-2 mediates its effects through activation of STAT5 and STAT3 (Akaishi et al., 1998; Nakajima et al., 1997). IL-2/STAT5 signaling pathway plays an important role in Th2 differentiation. This pathway has been shown to regulate the chromatin accessibility at the *IL-4* locus (Cote-Sierra et al., 2004; Liao et al., 2008; Zhu et al., 2003) (Figure 3). It has also been demonstrated that STAT5 binds to *Il4ra* locus (Liao et al., 2008). STAT5 was shown to mediate IL-2-dependent early induction as well as maintenance of IL-4Rα, thereby increasing T cell responsiveness to IL-4. Furthermore, STAT5 was observed to bind the Th2 cytokine locus at several sites and *Maf* and *Gata3* loci after two rounds of Th2 polarization indicating the importance of STAT5 both at early and later stages of Th2 differentiation (Liao et al., 2008).

TSLP is an epithelial cell-derived cytokine that is highly expressed in human lung and skin (Soumelis et al., 2002). It has been shown to play a key role in allergen-driven Th2 responses. TSLP receptor knockout mice fail to develop an inflammatory lung response to inhaled antigen (Al-Shami et al., 2005). In addition, its expression has been shown to be increased in asthmatic airways and correlate with the severity of the disease (Ying et

al., 2005). TSLP influences B cell development and regulates the activities of dendritic cells (DCs) and monocytes (Al-Shami et al., 2005; Reche et al., 2001; Sims et al., 2000). Importantly, it promotes DCs to induce Th2-type responses. TSLP-activated DCs have been shown to secrete Th2 cell-attracting chemokines as well as induce CD4+ cells to produce Th2-type cytokines (Ito et al., 2005; Liu, 2006; Soumelis et al., 2002). This has been shown to be mediated by OX40L-OX40 interaction between a DC and a CD4+ cell in the absence of IL-12 (Ito et al., 2005) (Figure 3). TSLP-induced OX40L has been suggested to represent the initial signal from DCs for naive CD4+ cells to polarize into Th2 cells, because TSLP-activated DCs do not produce IL-4 (Ito et al., 2005).

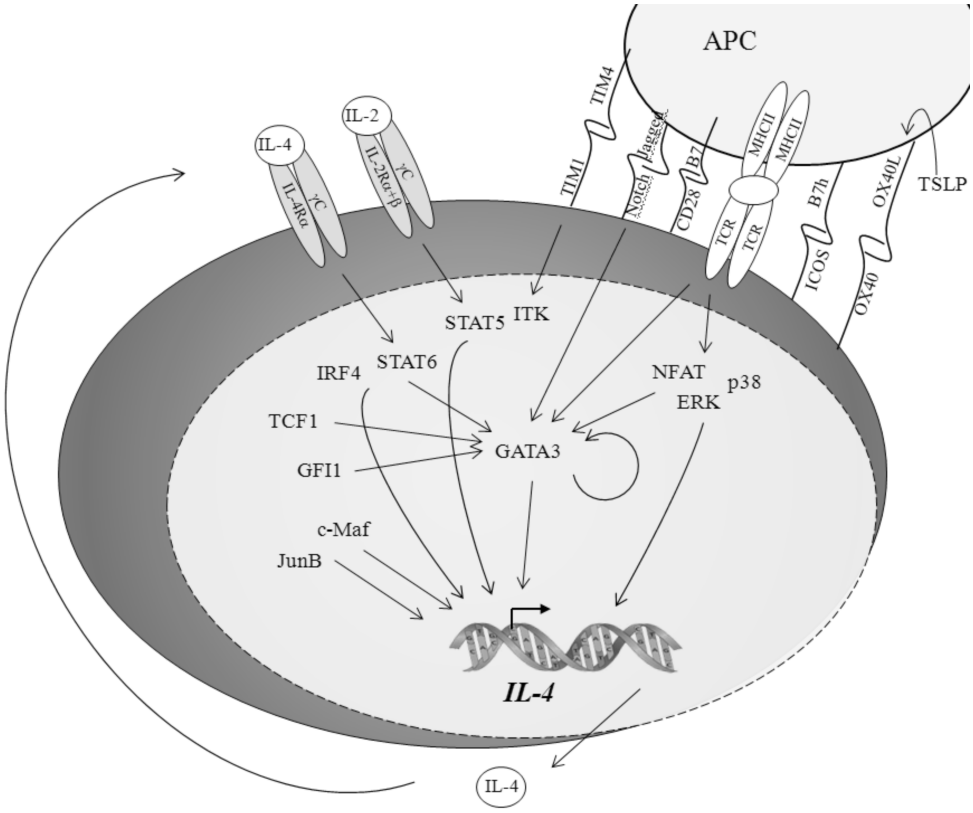

Figure 3. Signals and factors promoting Th2 polarization. The master regulator of Th2 differentiation, GATA3, is upregulated by TCR signaling, certain co-stimulatory signals, STAT6 signaling pathway as well as by other transcription factors such as TCF1. GATA3 together with STAT6, c-Maf and NFAT are important inducers of expression of the Th2 cell hallmark cytokine IL-4.

2.2.3.5 IL-4 and regulation of apoptosis

The two main routes leading to apoptosis are the death receptor mediated (extrinsic) pathway and the mitochondrial (intrinsic) pathway. Both involve activation of caspases leading to cleavage of multiple intracellular substrates. In addition to their role in the regulation of apoptosis, caspases have been associated in cell differentiation, proliferation and NF-κB activation (Lamkanfi et al., 2007). In developing Th cells, death receptor

signaling and caspase activity have been shown to promote Th1 differentiation and prevent Th2 development. FAS receptor stimulation of CD4+ T cells led to increased IFNγ production, whereas inhibition of caspases enhanced IL-4 production (Maksimow et al., 2003; Maksimow et al., 2006; Sehra et al., 2005). Moreover, Caspase-8 has been shown to inhibit Th2 responses and is required for protective T cell-mediated immunity against an intracellular parasite *Trypanosoma cruzi* (Silva et al., 2005).

Various death or stress signals induce the loss of mitochondrial membrane potential resulting in mitochondrial membrane permeabilization, generation of reactive oxygen species (ROS) and the release of pro-apoptotic members of the BCL-2 family from the mitochondrial inter-membrane space into the cytosol. These pro-apoptotic factors further promote the membrane permeabilization and release of cytochrome *c* from mitochondria. Cytochrome *c* associates with apoptotic protease activating factor 1 (APAF1) to form apoptosome, which activates pro-Caspase-9 to trigger the final cascade of the apoptotic process (reviewed in Brenner and Mak, 2009; Chen et al., 2003). The exact mechanism behind the membrane permeabilization remains obscure. The involvement and significance of channels such as the mitochondrial permeability transition pore or the voltage-dependent anion channel have been under debate (Brenner and Mak, 2009). Pro-apoptotic BCL-2 family members have also shown to regulate the mitochondrial dynamics during apoptosis. This may act as a positive feedback loop that amplifies membrane permeabilization and release of mitochondrial pro-apoptotic proteins (Suen et al., 2008).

IL-4 has been shown to prevent apoptosis in several cell types, but the molecular mechanisms involved are largely unknown. In T cells, STAT6 does not seem to be required for the anti-apoptotic effect of IL-4 (Wurster et al., 2002b), indicating that the IL-4 induced Th2 differentiation and protection from apoptosis are in this sense distinct signaling cascades emanating from the same receptor. Instead, IL-4 has been shown to inhibit Caspase-3 activity during the early phases of human Th2 differentiation (Rautajoki et al., 2007). IL-4 also decreased FAS receptor expression and upregulated the expression of cFLIPs, a Caspase-8 inhibitor, as well as the mitochondrial anti-apoptotic BCL-2 family members BCL-2 and BCL-XL (Rautajoki et al., 2007). It has also been shown that STAT6 promotes cell survival through regulation of BCL-XL expression (Ritz et al., 2008). Other studies have also demonstrated that the IL-4 signaling pathway regulates apoptosis through regulation of mitochondrial proteins (Wurster et al., 2002a), and by maintaining the mitochondrial membrane potential (Lemaire et al., 1999).

2.3 PRELI

PRELI (protein of relevant evolutionary and lymphoid interest) is an evolutionarily conserved 25 kDa protein, which has been reported to be highly expressed in adult lymph nodes and peripheral blood leukocytes. In addition, based on its high expression level in fetal liver and germinal center B lymphocytes, PRELI has been proposed to be important for B cell development (Guzman-Rojas et al., 2000).

In mice, PRELI is co-ordinately regulated with *Rab24* from a strong bi-directional promoter (Fox et al., 2004). Rab24 is a member of the Rab GTPase family (Fox et al., 2004), that is thought to be involved in autophagy-related processes by directing proteins to degradative pathways (Munafo and Colombo, 2002). PRELI contains tandem repeats of a late embryogenesis abundant (LEA)-motif, characteristic of a group of stress-controlling proteins. It also contains a coiled-coil domain, which are often associated with protein-protein interactions. PRELI also has a PRELI/MSF1 motif, the function of which is unknown but has been suggested to have a role in intra-mitochondrial protein sorting and may also have a function associated with cellular membranes (Anantharaman and Aravind, 2002). Consistent with this, the N-terminal domain of the PRELI protein contains a mitochondrial targeting sequence and PRELI has been reported to localize in mitochondria in HeLa cells (Fox et al., 2004). (Figure 4)

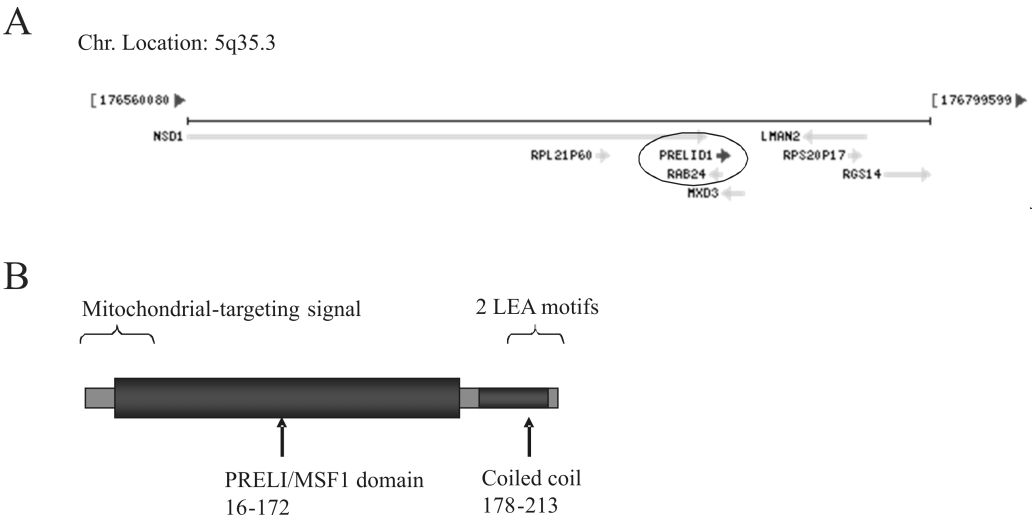

Figure 4. Genomic location of *preli* and schematic picture of PRELI protein structure. (A) *Preli* is located on human chromosome 5 and it is co-expressed with *Rab24,* a member of the Rab GTPase family. Figure is taken from www.ncbi.nlm.nih.gov; Entrez Gene; GeneID:27166. (B) PRELI protein motifs. Mitochondrial targeting signal is located in the N-terminus of PRELI, whereas the coiled-coil structure and 2 stress-related LEA-motifs are located in the C-terminus of PRELI. PRELI/MSF1 domain covers amino acids of positions 16-172.

In *Saccahromyces cerevisiae* the homologue of human PRELI, Ups1p (31% identity), regulates mitochondrial shape and the processing of the GTPase Mgm1p via an unknown mechanism (Sesaki et al., 2006). Because human PRELI was able to replace Ups1p in Mgm1p processing in yeast cells it was considered to be likely that in human cells PRELI acts on OPA1 (optic atrophy 1), the human homologue of Mgm1p. OPA1 has been associated in the regulation of mitochondrial dynamics as well as cytochrome *c* release and apoptosis.

Mammalian *opa1* gene has eight transcript variants resulting from alternative splicing. These variants are differentially processed to yield several different OPA1 isoforms in

mitochondria. The distinct isoforms have different effects on mitochondrial function and apoptosis and their abundance greatly varies between organs (Olichon et al., 2007), therefore the differential processing of these isoforms plays an important role in the regulation of mitochondrial function and susceptibility to apoptosis. Processing of OPA1 is activated by the loss of the mitochondrial membrane potential and is associated with progression of apoptosis (Ishihara et al., 2006). Interestingly, the variant that induces cytochrome *c* and caspase-mediated apoptosis is found predominantly in liver, kidney and thymus (Olichon et al., 2007). This resembles the expression of PRELI, which has also been suggested to be important for the development of vital and immunocompetent organs (Guzman-Rojas et al., 2000). The potential role for PRELI in the regulation of OPA1 has not been studied. Furthermore, neither the expression nor the cellular role for PRELI in primary human T helper cells have not been characterized.

2.4 PIM KINASES

The proto-oncogene *pim1* was identified as a proviral insertion site of the Moloney murine leukemia virus in experiments aiming at identification of novel genes involved in tumorigenesis. *Pim1* codes for a serine/threonine-specific kinase. The oncogenic potential of Pim1 was shown using *pim1* transgenic mice. Pim1 predisposed mice to T cell lymphomas in co-operation with Myc (van Lohuizen et al., 1989). Two other PIM1-like kinases have been identified since, termed as PIM2 and PIM3. The members of this kinase family are evolutionarily highly conserved and have largely overlapping functions, but differ in their tissue distribution (Eichmann et al., 2000; Mikkers et al., 2004). PIM kinases are predominantly expressed in hematopoietic tissues, such as thymus, spleen, bone marrow and fetal liver. Non-hematopoietic expression exists mainly in testis, brain, oral epithelia and kidney (Eichmann et al., 2000; Feldman et al., 1998). PIM kinases have been implicated in early transformation and tumor progression in hematopoietic malignancies and prostate carcinomas (Shah et al., 2008).

Expression of PIM kinases is induced upon TCR activation (Wingett et al., 1996) as well as by wide variety of cytokines, growth factors and mitogens (reviewed in Bachmann and Moroy, 2005; Mikkers et al., 2004). PIM kinases have diverse biological roles in cell survival, proliferation and differentiation. They have been implicated in signal transduction, transcriptional regulation as well as cytokine-dependent proliferation and survival in hematopoietic cells (Bachmann and Moroy, 2005; Fox et al., 2005; Wang et al., 2001; White, 2003).

Enzymatic activity of PIM kinases is regulated at the level of transcription, translation and protein stability/degradation (Wang et al., 2001). An association between protein level and overall kinase activity has been reported for PIM1 and PIM2 (Fox et al., 2003; Zhu et al., 2002), indicating that once their expression is induced, they act as constitutively active kinases. In addition to phosphorylating various exogenous substrates, PIM kinases autophosphorylate. Phosphorylation of PIM1 is not required for its kinase activity but contributes to its stability (Qian et al., 2005).

Mechanisms underlying the effects of PIM kinases are not well understood, although they have been reported to interact with several adapter proteins and substrates. PIM1 protein is predominantly located in the cytoplasm, although nuclear localization patterns have also been reported (Ionov et al., 2003; Saris et al., 1991; Zippo et al., 2007). PIM1 has been shown to phosphorylate and co-operate with p100 to enhance the activity of c-Myb transcription factor, a known regulator of cell proliferation, differentiation and apoptosis (Leverson et al., 1998). It has also been shown to phosphorylate and enhance the transcriptional activity of NFAT-c1, a mediator of TCR signaling and a regulator of Th cell polarization (Rainio et al., 2002). PIM kinases have also been implicated in the regulation of transcription through influencing chromatin modifications. PIM1 phosphorylates the heterochromatin protein 1 (HP1) influencing its transcriptional repression activity (Koike et al., 2000). PIM1 has also been shown to phosphorylate histone H3 on the Myc-binding sites contributing to Myc-dependent transcriptional activation and cellular transformation (Zippo et al., 2007). PIM kinases have also shown to enhance the transcriptional activity of c-Myc through stabilizing c-Myc protein by serine phosphorylation (Zhang et al., 2008).

PIM kinases promote cell survival via their effects on the anti-apoptotic protein BCL-2. They enhance the expression of BCL-2 in co-operation with c-Myc (Shirogane et al., 1999) as well as inhibit the pro-apoptotic protein BAD, a negative regulator of BCL-2 (Aho et al., 2004; Yan et al., 2003). PIM has also been shown to positively regulate cell-cycle by enhancing the activity of several cell cycle promoters such as Cdc25a and Cdc25c as well as inactivating cell-cycle inhibitors such as C-TAK1 and $p21^{Cip1/WAF1}$ (Bachmann et al., 2004; Mochizuki et al., 1999; Wang et al., 2002; Zhang et al., 2009).

PIM kinases have also been implicated in the inhibition of STAT signaling through regulation of the SOCS proteins. PIM1 has been shown to inhibit phosphorylation and transcriptional activity of STAT5 through phosphorylation-mediated stabilization of SOCS1 and SOCS3 (Peltola et al., 2004). Similarily, PIM kinases inhibited STAT6-mediated transcription by maintaining the levels of SOCS1 protein (Chen et al., 2002). PIM kinases have also been implicated in the regulation of RUNX proteins, α-subunits of heterodimeric transcription factors important for hematopoietic cell proliferation and differentiation. PIM1 was shown to phosphorylate and increase the transcriptional activity of RUNX1 and RUNX3. RUNX1 and RUNX3 have been shown to inhibit Th2 differentiation by repressing GATA3 expression and promoting Th1 polarization in co-operation with T-bet, respectively (Djuretic et al., 2007; Komine et al., 2003).

Some of the data for PIM kinases suggest a role for these proteins in the regulation of Th cell differentiation. In addition, *PIM* family genes have been reported to be upregulated by Th1-polarizing cytokines (Aho et al., 2005). PIM1 was preferentially expressed by Th1 cells at both mRNA and protein level. Also PIM2 mRNA was shown to be upregulated in human Th1 cells. Specific role for PIM kinases in the regulation of human Th1 and/or Th2 cell differentiation has not been studied in detail.

3 AIMS OF THE STUDY

The overall goal of this PhD thesis was to elucidate signaling mechanisms behind human Th1 and Th2 cell differentiation processes and to identify novel regulators of these pathways. Furthermore, since resting human primary T cells are known to be difficult to transfect, our aim was to develop and optimize a novel method for transfection and enrichment of these cells, facilitating the investigation of the function of a candidate gene of interest.

The aims of the subprojects included in this thesis were:

I To develop a fast and efficient method for transfection of human primary CD4+ cells for studying the function of genes of interest in T helper cell differentiation.

II To characterize the function of PRELI in human CD4+ cells and investigate its role in Th1/Th2 differentiation process.

III To investigate the role of serine/threonine-specific PIM kinases in regulation of human T helper cell differentiation process, particularly in Th1 polarization.

4 MATERIALS AND METHODS

4.1 CELL ISOLATION AND CULTURING

4.1.1 Isolation and culturing of human CD4+ cells (I-III)

Human mononuclear cells were isolated from cord blood of healthy neonates or peripheral blood (buffy coats) of healthy blood donors (Finnish Red Cross) using Ficoll-Paque isolation (Amersham Pharmacia Biotech, Piscataway, NJ). CD4+ cells were further purified using DYNAL magnetic beads (Invitrogen, Carlsbad, CA). Cells from several individuals were pooled after the isolation.

CD4+ cells were cultured on 24-well plates at a density of $2x10^6$ cells/ml in Yssel medium (Iscove modified Dulbecco medium [IMDM, Invitrogen] supplemented with Yssel medium concentrate, pen/strep and 1% AB-serum). Cells were activated with plate-bound α-CD3 (0.5 μg/well) and soluble α-CD28 (0.5 μg/ml; both from Immunotech, Marseille, France) and cultured in neutral conditions (Th0) or polarized towards Th1 cells with 2.5 ng/ml of IL-12 or Th2 cells with 10 ng/ml of IL-4 (both from R&D Systems, Minneapolis, MN). IL-2 (40 U/ml, R&D Systems) was added into the cultures after 48 h of priming.

When indicated (II), the cells were cultured in the presence of 0.2 to 1 mM H_2O_2 (Merck, Darmstadt, Germany), 1.25 mM N-acetyl-cysteine (NAC) or 5 μM Calpastatin peptide (both from Calbiochem, San Diego, CA).

4.1.2 Cell lines (I, II)

Human-derived renal epithelial HEK293 (human embryonic kidney 293) cells (ATCC, Manassas, VA) were cultured in Dulbecco modified eagle medium (DMEM; Sigma-Aldrich, St.Louis, MO) supplemented with pen/strep, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal calf serum (FCS). HeLa cells (ATCC) - human epithelial cells from cervical carcinoma - were cultured in modified eagle medium (MEM) with Earle salts (both Invitrogen, Carlsbad, CA), supplemented with pen/strep, 2 mM L-glutamine and 10% FCS. Jurkat cell line, human T lymphocyte cells derived from acute leukemia (Tag derivatives expressing SV40 large T-antigen; Northrop et al., 1993), were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with pen/strep, 2 mM L-glutamine and 10% FCS.

4.2 PLASMID CONSTRUCTS AND SIRNA OLIGONUCLEOTIDES (I-III)

To create plasmid constructs allowing enrichment of transfected cells, a gene coding for a cell surface selection marker, mouse MHC class I H-$2K^k$, was cloned and ligated into

modified pIRES2- and pSUPER vectors suitable for gene overexpression and knockdown studies, respectively. H-2K^k sequence was amplified by PCR from pMACSK^k II plasmid (Miltenyi Biotec). H2K-PCR-F and H2K-PCR-R1 primers (I, Table 1; DNA Technology A/S, Aarhus, Denmark) were used in PCR for cloning the sequence into the pIRES2-EGFP plasmid (BD Biosciences Clontech, Mountain View, CA). For cloning the same sequence into the pSuper-GFP-Neo plasmid (Oligoengine, Seattle, WA), PCR primers were H2K-PCR-F and H2K-PCR-R2. PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler DNA Engine Tetrad (MJ Research, Inc., Watertown, MA) under the following conditions: an initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 2 min followed by a final elongation step at 72°C for 10 min. The vectors (pIRES2-EGFP and pSuper-GFP-Neo) were digested with *BstXI* and *Bsp14071* or *Bsp14071* and *BshT1*, respectively. This released the EGFP fragments, which were discarded. PCR products were digested with the same enzymes and ligated with the vectors, creating pIRES2-H2K^k and pSUPER-H2K^k constructs.

To clone the STAT6-shRNA (short hairpin RNA; targeting the sequence 5´-GAATCAGTCAACGTGTTGTCAG-´3) into the pSuper-H2K^k plasmid, a fragment containing the STAT6-shRNA was removed from pSuper-STAT6-shRNA construct (prepared and validated in our lab earlier using the first version of the commercially available pSuper vector) and ligated into pSuper-H2K^k vector. PRELI cDNA was amplified from human Th2 cDNA with PRELI-forward and -reverse primers (II, Table 1) and cloned into the pFLAG-CMV-2 vector (Kodak, New Haven, CT), creating a pFlag-PRELI construct. pIRES2-H2K^k-PRELI was made by amplifying PRELI with PRELI-forward and -reverse2 primers (II, Table 1), using pFlag-PRELI as a template and subsequently ligating the PCR product to the pIRES2-H2K^k vector.

Small interfering RNA (siRNA) oligonucleotides and siRNA pools (II, III: Table 1) were purchased from Sigma-Aldrich and Dharmacon (Lafayette, CO), respectively.

4.3 CELL TRANSFECTIONS

4.3.1 Nucleofection of human CD4+ cells (I-III)

Freshly isolated CD4+ cells were suspended in T cell Nucleofector solution™ (Amaxa, Cologne, Germany; subproject I) or in Optimem I (Invitrogen; II, III) in a density of 5x10^6 cells/100 µl for transfection with plasmid DNA (I, II) or 4x10^6 cells/100 µl for transfection with siRNA oligonucleotides (II, III). Subsequently, the cells were mixed with 10 µg of plasmid DNA or 1.5 µg of siRNA. Cells were transfected using the nucleofection technique (Amaxa, Cologne, Germany) according to instructions provided by the manufacturer. After being nucleofected with plasmid DNA, cells were incubated in RPMI 1640 medium supplemented with pen/strep, 2 mM L-glutamine and 10% FCS at 37°C for 16 h (2.5x10^6 cells/ml), after which dead cells were removed and the transfected cells were enriched as described below. In siRNA experiments, nucleofected

cells were let to rest in 37°C for 24 h ($2x10^6$ cells/ml) and subsequently activated and polarized as described in section 4.1.1.

4.3.2 Removal of dead cells and enrichment of transfected cells (I, II)

Dead cells, apoptotic cells and debris were depleted from nucleofected cells 16 h after the nucleofection by using magnetic-activated cell-sorting (MACS) Dead Cell Removal Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Enrichment of the H-$2K^k$-positive cells was then done for the remaining cells according to the manufacturer's instructions (Miltenyi Biotec). Briefly, after the dead cell removal, the cells were incubated with MACSelect K^k MicroBeads coated with $H2K^k$-antibody (Miltenyi Biotec) for 15 min to magnetically label the transfected cells. The magnetic separation of H-$2K^k$-positive cells was done with a positive selection column placed in the magnetic field of a MACS separator. After the enrichment, cell viability and purity ($H2K^k$-positivity) were determined by flow cytometry and the cells were cultured as described in section 4.1.1.

4.3.3 Transfection of cell lines (I, II)

HEK293 and HeLa cells were plated on cover slips (precoated with poly-L-lysine) in 6-well plates at a density of $0.7x10^6$ cells/2 ml of DMEM (HEK293) or $0.4x10^6$ cells/2 ml of MEM (HeLa) without antibiotics. After 24 h, the cells were transfected with 1.5 µg (HEK293) or 4 µg (HeLa) of empty pFlag vector or pFlag-PRELI using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Jurkat cells were transfected with 10 µg of pSuper-$H2K^k$-scramble or pSuper-$H2K^k$-STAT6-shRNA in culture medium ($10x10^6$ cells/400µl) by electroporation (250 V, 975 µF, GenePulser R II Electroporation system, Bio-Rad Laboratories, Hercules, CA). The transfected cells were incubated at 37°C for 16 h after which the dead cells were removed and the H-$2K^k$ positive cells were enriched as described in section 4.3.2. The enriched $H2K^k$-positive cells were activated with 5 ng/ml of phorbol 12-myristate 13-acetate (PMA; Calbiochem, San Diego, CA) and 1 µg/ml of phytohemagglutinin (PHA) (Sigma-Aldrich) in the presence of 10 ng/ml of IL-4 (R&D Systems, Minneapolis, MN). The cells were cultured in 12-well plates in the density of $2.5x10^6$ cells/ 2.5 ml of culture medium.

4.4 FLOW CYTOMETRY

4.4.1 Transfection efficiency and purity of sorted cells (I, II)

To measure the transfection efficiency, 300 000 cells nucleofected with H-$2K^k$ -plasmid constructs were collected 16 h after the nucleofection. Subsequently, dead cells were removed and $H2K^k$-positive cells were enriched after which an equal number of cells was

collected to determine the purity of sorted cell population. Non-nucleofected cells were used as negative controls. Cells collected before or after the enrichment were suspended in 100 µl of phosphate buffered saline (PBS) and stained for 10 min with 10 µl of either $H2K^k$-fluorescein isothiocyanate (FITC) or Control-FITC antibody (Miltenyi Biotech), respectively. The cells were washed twice with PBS and the samples were measured either with FACScan and analyzed with CellQuest software (I) or with FACSCalibur™ system and CellQuest Pro software (II; BD Biosciences, San Jose, CA).

4.4.2 Intracellular cytokine staining (I, II)

IFNγ and IL-4 production by the polarized cells was measured after 7 to 8 days of culture by restimulation and intracellular cytokine staining. To induce cytokine production, $0.5x10^6$ cells were incubated for 5 h with or without 5 ng/ml of PMA and 500 ng/ml of ionomycin (Sigma-Aldrich) at a density of $1x10^6$ cells/ml. Non-stimulated cells were used as negative controls. After 2 h of incubation, 10 µg/ml of Brefeldin A (Alexis Biochemicals, Farmingdale, NY) was added and incubation was continued for 3 h. The cells were then washed twice with PBS buffer containing 0.5% bovine serum albumin (BSA) and 0.01% azide, fixed with 4% paraformaldehyde for 15 min, washed with PBS buffer and permeabilised with 0.5% saponin for 10 min. Cytokine stainings were done by incubating cells with α-IFNγ-FITC (Caltag Laboratories, Burlingame, CA; 3 µl) and α-IL4-phycoerythrin (PE; Caltag Laboratories or BD Pharmingen; 3 µl or 1 µl, respectively) in 100 µl of permeabilization buffer for 20 min, after which cells were washed 3 times with PBS buffer. The cytokine production profiles of the cells were analyzed as described in section 4.4.1.

4.4.3 AnnexinV and Caspase-3 analysis (II)

To stain the cells with FITC AnnexinV (BD Pharmingen, San Jose, CA), 300 000 to 500 000 cells were washed with FACS buffer (2% FCS and 0.01% atzide in PBS), resuspended in 100 µl of 1X binding buffer (10X buffer: 0.1 M HEPES (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$, diluted with FACS buffer) and mixed with 5 µl of FITC Annexin V. The cells were incubated for 20 min in dark at +4°C and subsequently washed twice with 1X binding buffer. Cells were suspended in 300 µl of binding buffer and measured with the FACSCalibur™ system and analyzed with CellQuest Pro (BD Biosciences). 5 µl of propidium iodide (PI; Clontech, Mountain View, CA) was mixed with the cells 20 sec before measurement. Staining with α-active Caspase3-PE antibody (#550914; BD Pharmingen) was performed according to the manufacturer's instructions.

4.4.4 Surface staining for CRTH2 and IL-12Rβ2 (II, III)

Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), also known as prostaglandin D2 receptor DP-2 or G protein-coupled receptor-44, has quite recently emerged as a marker for Th2 cell phenotype and function (Cosmi et al., 2000;

De Fanis et al., 2007; Iwasaki et al., 2002; Nagata et al., 1999). CRTH2 is selectively expressed in Th2 but not in Th1 cells. To measure the expression of CRTH2 on polarized Th cells, 0.3 to 0.5 x 10^6 cells were first washed with PBS, then with MACS buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.2) and subsequently incubated with 5 µl of CRTH2-PE antibody for 10 min at 4°C. Next, the cells were washed twice with MACS buffer and resuspended in 1% formalin/PBS.

To stain the IL-12Rβ2 on Th1-polarized cells, 300 000 to 500 000 cells were washed with FACS buffer and incubated with 6 µl of Rat α-human IL-12Rβ2 (CD212) antibody or of Rat IgG2a (isotype control; both from BD Pharmingen, San Jose, CA) in 100 µl of FACS buffer at +4°C for 15 min. Subsequently, the cells were washed twice with FACS buffer and incubated with 2 µl of biotin-conjugated α-rat-antibody (BD Pharmingen) for 15 min at 4°C. After two more washes, the cells were incubated with 1 µl of Streptavidin-peridinin chlorophyll protein (PerCP; BD Pharmingen) for 15 min at 4°C. The cells were then washed twice and resuspended in FACS buffer. Samples were analyzed as described in section 4.4.3.

4.5 MICROSCOPY

4.5.1 Localization of PRELI and mitochondrial membrane potential (II)

To study the localization of PRELI in HEK293 or HeLa cells, the cells were washed with the culture medium 24 h after transfection and incubated with 20 to 200 nM MitotrackerRed CMXRos (Molecular Probes, Eugene, OR) for 30 min at 37°C. The cells were washed with cold PBS, fixed with methanol for 5 min at -20°C, washed twice with PBS, and blocked with goat serum (Gibco) for 15 min. The cells were stained for Flag-PRELI with 25 µg/ml mouse α-Flag-M2 antibody (Sigma) for 1 h. The cells were then washed with PBS (3 x 5 min), incubated with 5 µg/ml goat α-mouse-FITC antibody (Caltag Laboratories, Burlingame, CA) for 45 min, and washed with PBS. The cells were subsequently analyzed with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany) using Plan-Apochromat 63x/1.4 Oil differential interference contrast (DIC) objective. FITC was excited with a 488 nm laser line and emission was collected with a 500 to 530 nm bandpass filter. MitotrackerRed was excited with a 543 nm laser line and emission was collected with a 560 nm long pass filter. Images were analyzed with LSM5 software (Carl Zeiss).

To examine PRELI's effect on mitochondrial membrane potential ($\Delta\psi_m$) in primary Th cells, the nucleofected cells were harvested after 24 h of activation and incubated with 50 nM Mitoprobe $DilC_1(5)$ (Molecular Probes) in PBS (~0.3x10^6 cells/500 µl) for 30 min at 37°C, and subsequently washed and resuspended in 300 µl of PBS. Cells were measured with the FACSCalibur™ system and analyzed with CellQuest Pro (BD Biosciences).

4.5.2 Reactive oxygen species (II)

To study the effect of PRELI on ROS production in HeLa cells, the cells were treated as described in section 4.5.1 except that, instead of using Mitotracker, the cells were incubated with 20 μM dihydrorhodamine 123 (DHR123; Molecular probes) for 30 min at 37°C. Alexa Fluor 568-conjugated goat α-mouse antibody (5 μg/ml) (Molecular Probes) was used as a secondary antibody to detect overexpressed PRELI. Cells were analyzed with a Zeiss LSM 510 META confocal microscope (Carl Zeiss). DHR123 was excited with a 488 nm laser line and emissions were collected with a 500 to 530 nm bandpass filter. Alexa568 was excited with a 543 nm laser line and emission was collected with a 560 nm long pass filter.

To examine PRELI's effect on ROS production in primary Th cells, the nucleofected cells were harvested after 24 h of activation and incubated with 20 μM DHR123 in Yssel medium (~$0.3x10^6$ cells/500 μl) for 30 min at 37°C, and subsequently washed and resuspended in 300 μl of PBS. Cells were measured with the FACSCalibur™ system and analyzed with CellQuest Pro (BD Biosciences).

4.6 REAL-TIME RT-PCR

4.6.1 RNA isolation and cDNA synthesis (I-III)

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. DnaseI (Qiagen) treatment was included in the RNA isolation procedure to eliminate genomic DNA from the samples. Subsequently, cDNA, prepared using a Superscript II kit (Gibco BRL, Life Technologies, Paisley, Scotland), was used as a template for gene expression analyses.

4.6.2 Gene expression analysis (I-III)

Gene expression levels were measured using TaqMan ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) (Hämäläinen et al., 2001). All measurements were performed in duplicate in two separate runs. Housekeeping gene Elongation factor 1 alpha (EF1α) was used as a reference transcript (Hämäläinen et al., 2001). The primers and probes (I-III: Table 1; MedProbe, Oslo, Norway) were designed using Primer Express software (Applied Biosystems). Universal Probe Library probes (Roche, Mannheim, Germany) were designed using ProbeFinder™ software.

4.7 WESTERN BLOTTING (I-III)

The cells were lysed in sodium dodecyl sulfate (SDS) buffer, boiled for 5min, sonicated and subsequently equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis. Proteins were detected using the following antibody dilutions:

1:1000 sheep α-PRELI-antiserum (kind gift from Dr. Elisabeth Fox, Leeds, UK), 1:500 mouse α-STAT6 (BD Biosciences, San Jose, CA), 1:1000 rabbit α-phospho-STAT6 (Tyr641; Cell Signaling Technology, Beverly, MA), 1:200 rabbit α-STAT1 p84/p91 (E-23; Santa Cruz Biotechnology, Santa Cruz, CA), 1:200 rabbit α-STAT4 (C-20; Santa Cruz Biotechnology), 1:500 rabbit α-Calpain 1 large subunit (μ-type; Cell Signaling Technology), 1:500 rabbit α-PIM2 (ATLAS antibodies, Stockholm, Sweden), 1:200 mouse α-PIM1 (12H8; Santa Cruz Biotechnology, Santa Cruz, CA), 1:200 mouse α-T-bet (4B10; Santa Cruz Biotechnology), 1:200 mouse α-GATA3 (HG3-31; Santa Cruz Biotechnology) or 1:20000 mouse α-β-actin (Sigma-Aldrich). Horseradish peroxidase-conjugated goat α-mouse IgG (1:10000; Santa Cruz Biotechnology), α-rabbit Ig (1:10000; BD Biosciences) or donkey α-sheep (1:20000; Jackson ImmunoResearch Laboratories, Baltimore Pike, PA) were used as secondary antibodies. The protein bands were visualized with enhanced chemiluminesence (GE Healthcare, Piscataway, NJ), quantified with a microcomputer imaging device (MCID) and normalized against β-actin.

4.8 BIO-PLEX CYTOKINE ASSAY (III)

To measure IFNγ produced by Th1-polarized cells, cell culture supernatants were diluted 1:10. Duplicate samples were stained on 96-well plates according to instructions by the manufacturer (Bio-Plex Cytokine assay; Bio-Rad, Hercules, CA) and measured using Luminex® 100™ system (Luminex, Austin, TX).

5 RESULTS AND DISCUSSION

5.1 DEVELOPMENT AND OPTIMIZATION OF A NOVEL METHOD FOR STUDYING GENE FUNCTION IN HUMAN PRIMARY CD4+ CELLS (I)

5.1.1 Efficient transfection and enrichment of human primary CD4+ cells

Manipulation of gene expression in primary T cell cultures is an important step towards understanding the molecular mechanisms and the role of a selected gene in the differentiation of $CD4^+$ T helper cells to Th1 and Th2 subtypes. However, resting primary T lymphocytes are known to be difficult to transfect. Lentiviral-based vectors have been successfully used for introducing DNA into these cells (Bai et al., 2003; Marodon et al., 2003; Qin et al., 2003). However, viral-based techniques have several disadvantages, including laboratory safety precautions and time demanded. In addition, the vectors introduced may affect several signalling pathways (Flaherty et al., 2004) or may introduce viral elements into the host cell. Electroporation has been the most promising nonviral method for introducing DNA into primary T lymphocytes (Herndon et al., 2002). Nucleofector™ technology (Amaxa, Cologne, Germany), based on an improved electroporation method, offered an advanced method for transfecting primary cells and hard-to-transfect cell lines.

The success of experiments based on transfection of cells depends on the transfection efficiency. Usually transfected cell cultures contain a high background of more than 50 % of untransfected cells. We have observed that the untransfected cells are usually in a better condition and proliferate more efficiently compared to the transfected cells in the culture (unpublished data). Therefore, 7 to 8 days after the nucleofection most of the viable cells in the culture originate from non-transfected cells. One way to avoid this problem is to enrich the transfected cells. Fluorescence activated cell sorting (FACS) is one, however a very expensive and time-consuming way to enrich the cells of interest. In many studies, vectors containing GFP have been cotransfected or used as a fusion protein for tracking the cells containing the gene of interest. However, one can not ignore the possibility that GFP may influence the data by affecting cellular signalling events. Another way to enrich the transfected cells is to co-transfect the cells with plasmid encoding a cell surface marker followed by sorting using antibody-coated magnetic beads (Schneider and Rusconi, 1996). However, in this method, one has to optimize the conditions for the simultaneous co-transfection of several plasmids because this may influence the amount of cells expressing both the selection marker and the DNA of interest.

To study the effect of genes of interest in human primary T helper cell differentation, we developed and optimized an assay for enrichment of transiently transfected primary human T cells. We combined the benefits of two commercial expression vectors introducing a single plasmid construct containing a multiple cloning site (MCS) for the gene of interest, an internal ribosome entry site (IRES) and a gene coding for a truncated mouse MHC class l H-2K^k molecule which is a cell surface marker. This plasmid construct (pIRES2-H-2K^k; I, Figure 1A) allowed both the gene of interest and the H-2K^k gene to be translated from a single bicistronic mRNA and the possibility that the cells might take up different amounts of different vectors was excluded. In addition, as the selection marker was a truncated receptor and lacked its cytoplasmic tail, it did not interfere with the cell functions. To be able to use this novel approach also in gene knockdown experiments, we modified a pSuper RNA interference (RNAi) plasmid to create a pSuper-H-2K^k vector (I, Figure 1B). Transfection efficiencies of the cells nucleofected with the H-2K^k -plasmid constructs were measured 16 hours after nucleofection. The transfection efficiency for the pIRES2-H-2K^k plasmid varied from 24% to 51% between different individuals in 6 independent experiments (I, Figure 2A). When the cells were transfected with the pSuper-H-2K^k-STAT6-shRNA or pSuper-H-2K^k-scramble-shRNA, the efficiency varied between 43% and 69%. Importantly, cells transfected with these H-2K^k containing plasmids could be enriched gently and fast using H-2K^k antibody based MACS separation method. The purity of the enriched, H-2K^k-positive, cells was always > 97% after the enrichment (I, Figure 2B).

5.1.2 Nucleofected and sorted CD4+ cells are able to polarize into Th1 and Th2 cells

In order to be sure that we could use this assay for investigating the role of the genes of interest in T helper cell differentiation, we needed to establish whether the enriched H-2K^k-positive T cells could differentiate into Th1 and Th2 subtypes. Enriched primary T helper cells overexpressing pIRES2-H-2K^k plasmid and non-nucleofected control cells were cultured under Th1 or Th2 polarizing conditions for 5 to 7 days. After that, we measured the expression of selected Th1 and Th2 marker genes, IL-12Rβ2, IFN-γ and GATA3, in these cells with TaqMan RT-PCR. Our results showed that, like control cells, nucleofected and enriched H-2K^k-positive cells polarized to Th1 direction expressed more IFN-γ and IL-12Rβ2 compared to Th2-polarized cells (I, Figure 3). Furthermore, GATA3 was preferentially expressed in Th2-polarized cells compared to Th1 cells in H-2K^k-positive samples as well as in controls (I, Figure 3).

IFN-γ and IL-4 are the signature cytokines produced by Th1 and Th2 cells, respectively, and the determination of the cytokine profiles of these two cell subsets is used to phenotypically distinguish them from each other. To study if the enriched H-2K^k-positive cells are able to differentiate into Th1 and Th2 cells based on their cytokine production profiles, non-nucleofected control cells and the enriched CD4+

cells overexpressing pIRES2-H-2K^k plasmid were cultured in Th1 and Th2-polarizing contiditions for 7 to 8 days and subsequently subjected to intracellular cytokine staining. The cytokine production profiles varied between different individuals in different experiments. However, in Th1 culturing conditions, the amount of IFN-γ producing cells was high and the number of IL-4 producing cells was negligible, both in controls and in nucleofected H-2K^k-positive samples (I, Table 2). In Th2 polarizing conditions, the amount of IL-4 producing cells was higher compared to the Th1 cultures and there were less IFN-γ producing cells than in the Th1 cultures. This was seen both in control samples as well as in nucleofected H-2K^k-positive samples (I, Table 2). Taken together, our results showed that the nucleofected H-2K^k-positive primary T helper cells were able to differentiate into Th1 and Th2 cells.

5.1.3 Introduction of STAT6 shRNA in CD4+ cells by the optimized nucleofection method downregulates Th2 cell differentiation

To demonstrate that this assay is applicable in studying the influence of a given gene on T helper cell differentiation, we nucleofected human primary T helper cells with pSuper-H-2K^k vector containing a shRNA targeting STAT6 (pSuper-H-2K^k-STAT6-shRNA), a key transcription factor driving Th2-polarization, and showed that this resulted in impaired Th2 cell differentiation. The functionality of the shRNA was first validated in a Jurkat cell line (I, Figure 4A). Nucleofected CD4+ cells were enriched and polarized to Th2 cells for 7 days, after which the expression of GATA3, IFN-γ and IL-12Rβ2 was measured. STAT6 is known to rapidly induce the expression of GATA3 in cells in response to IL-4 stimulation. GATA3, in turn, has been shown to inhibit the expression of IL-12Rβ2 and to act as a repressor of Th1 development and IFN-γ production (Kurata et al., 1999; Ouyang et al., 1998). As expected, the expression of GATA3 was reduced in cells expressing the STAT6-shRNA compared to the control cells (I, Figure 4B). Furthermore, both IFN-γ and IL-12Rβ2 were upregulated at mRNA level in response to downregulation of STAT6 (I, Figure 4B). The level of STAT6 mRNA was decreased by 2.9-fold in STAT6-shRNA sample compared to the control cells (transfected with pSuper-H-2K^k-scramble; data not shown).

To further demonstrate that the introduction of pSuper-H-2K^k-STAT6-shRNA in human primary T helper cells by our method has an expected effect on Th2 differentiation, we showed that this treatment influenced the Th2-type cytokine production profile. Intracellular cytokine staining was performed for the enriched cells after 7 days of polarization. As predicted, the amount of IL-4 producing Th2 cells was diminished in response to downregulation of STAT6. Whereas, the amount of IFN-γ producing cells was much higher in Th2 cultures expressing STAT6-shRNA compared to the control cells (I, Figure 4C). Taken together, these results verified the usefulness of our system when studying the influence of a gene of interest on T helper cell differentiation.

5.1.4 Comparison with other methods

The method described above provides advantages compared to previous methods used with human primary CD4+ cells. Nucleofector™ electroporation technology itself was a great advancement, because the transfection efficiencies of nucleofected cells were significantly higher compared to cells transfected with other methods such as lipofection or traditional electroporation. However, even though the transfection efficiency was improved, it was still important to be able to enrich the transfected cell population and study only the cells that contained the gene or shRNA of interest, because the untransfected cells in the sample proliferated faster than the transfected cells. Enrichment of transfected cells based on the magnetic selection of $H2K^k$-positive cells proved to be very efficient and gentle way to perform the sorting. This was a clear improvement compared to other sorting methods used previously, such as enrichment of GFP-positive cells using flow cytometry, which seemed to be too stressful method for human primary CD4+ cells, at least according to our experiences.

Since the development of the method described in this thesis, nucleofection of cells with siRNA oligonucleotides has proven to be very good and feasible method to knock down genes of interest in human primary Th cells. Time-consuming cloning steps are avoided when using siRNAs and by using pooled siRNAs, the knockdown efficiency is usually improved. In addition, the cells nucleofected with siRNA oligos are in better condition compared to cells transfected with plasmid DNA and therefore this method has replaced vector based shRNA nucleofection in our gene knockdown studies. The combination of siRNA oligonucleotide-mediated gene inactivation and gene overexpression using the method described in this thesis, provides an effective strategy for studying the effect of specific genes on primary Th cell function and differentiation.

5.2 IDENTIFICATION AND CHARACTERIZATION OF PRELI AS A NEW REGULATOR OF HUMAN T HELPER CELL APOPTOSIS, STAT6 AND TH2 CELL DIFFERENTIATION (II)

5.2.1 PRELI is highly induced in mitochondria in response to T cell activation

PRELI was identified as an activation-induced gene in primary human Th cells in our study aiming at identifying novel genes involved in the regulation of early human primary Th cell differentiation and activation using Affymetrix oligonucleotide arrays. Expression of PRELI mRNA was highly increased when naive CD4+ cells (Thp) were subjected to TCR activation, with a maximum expression at 24 h. PRELI was not differentially regulated by the Th1/Th2 polarizing cytokines IL-12 or IL-4 (II, Figure 1A). Consistent data was obtained also at protein level (II, Figure 1B).

The cellular role of PRELI was previously unknown. It is an evolutionarily conserved protein whose expression is co-ordinately regulated in mice with *Rab24*

(Fox et al., 2004), a gene associated with autophagy-related processes (Munafo and Colombo, 2002). *PRELI* and *RAB24* are located at human chromosome 5q34-q35, a region that has been implicated in several hematopoietic malignancies (Fox et al., 2004). PRELI has been suggested to be important for development of B lymphocytes based on its expression in germinal center B cells and fetal liver (Guzman-Rojas et al., 2000). To clarify the cellular function of PRELI, we studied its localization. Consistent with the description of mitochondrial localization of PRELI in HeLa cells and a mitochondrial targeting sequence in the N-terminal domain of the PRELI protein (Fox et al., 2004), exogenously expressed PRELI co-localized with Mitotracker in the mitochondria of HeLa, HEK293 and primary CD4+ cells (II, Figure 2A and data not shown).

5.2.2 PRELI regulates membrane potential and production of reactive oxygen species in mitochondria and induces apoptosis in CD4+ cells

Interestingly, the localization study also revealed that in mitochondria, PRELI reduced the mitochondrial membrane potential, $\Delta\Psi_m$ (II, Figure 2A). The fluorescence intensity of the membrane potential sensitive Mitotracker was lower in HeLa cells overexpressing pFlag-PRELI when compared to cells where PRELI was not overexpressed, indicating that the $\Delta\Psi_m$ was reduced by PRELI (II, Figure 2A). To further study this in human primary CD4+ cells, we used two approaches; PRELI overexpression and knockdown by specific siRNAs. Primary human CD4+ cells were nucleofected either with PRELI overexpression vector or PRELI-specific siRNA oligonucleotides, activated and cultured for 24 hours and subsequently stained with Mitoprobe and analyzed with flow cytometry. Overexpression of PRELI increased the proportion of cells with reduced $\Delta\Psi_m$, whereas its silencing significantly decreased the number of these cells, confirming that PRELI reduced the $\Delta\Psi_m$ in human primary Th cells (II, Figure 2A).

The loss of $\Delta\Psi_m$, induced by various stress signals, leads to mitochondrial membrane permeabilization and generation of ROS by the mitochondria. After this, cytochrome *c* is released from mitochondria and Caspase3 is activated resulting in activation of apoptotic cell death. PRELI's potential to reduce the $\Delta\Psi_m$ suggested that it could also influence ROS production by mitochondria. Indeed, our studies with HeLa cells indicate that the production of ROS was slightly induced by PRELI (II, Figure 2B). This was further confirmed in human CD4+ cells both by PRELI overexpression and knockdown experiments. (II, Figure 2B)

These findings suggested that PRELI could also influence primary Th cell apoptosis. Moreover, since IL-4 has been shown to protect cells from apoptosis by regulating proteins mediating the mitochondrial apoptosis pathway (Rautajoki et al., 2007; Wurster et al., 2002a) and by maintaining the mitochondrial membrane potential (Lemaire et al., 1999), we also examined the combined effects of IL-4 and PRELI on $\Delta\Psi_m$ and apoptosis.

AnnexinV intensity, as well as the number of cells with active Caspase3 and reduced $\Delta\Psi_m$, were higher in the samples overexpressing PRELI compared to the control cells after 24h of culture (II, Figure 3A), indicating that PRELI induced the mitochondrial apoptosis pathway in human Th cells. PRELI also reduced cell viability. IL-4 partly inhibited the effects of PRELI, indicating that while PRELI and IL-4 have opposing effects on the mitochondrial apoptosis pathway, IL-4 cannot completely reverse the effect of PRELI. Consistent results were obtained in PRELI knockdown experiments (II, Figure 3B). Taken together, these results suggested that PRELI induces the mitochondrial apoptosis pathway in human primary Th cells and the addition of IL-4 partly reverses the effect of PRELI.

The mechanism by which PRELI affected the $\Delta\Psi_m$ and ROS production is presently unknown. In a previous study using yeast cells it was proposed that in human cells PRELI is involved in processing of OPA1, an important regulator of mitochondrial membrane dynamics and apoptosis (Frezza et al., 2006; Olichon et al., 2003). Distinct isoforms and cleavage products of OPA1 have different effects on mitochondrial function and apoptosis and their quantity varies greatly between different organs (Olichon et al., 2007). Differential processing of these isoforms is important for the regulation of mitochondrial function and susceptibility to apoptosis. Our preliminary results (Figure 5) suggested that PRELI did not affect the expression levels of OPA1 isoforms in human CD4+ cells. However, whether PRELI influenced the function of OPA1, was not determined. It is important to remember that there are differences in the processing of OPA1 between human and yeast and therefore, the mechanisms involved in yeast do not necessarily apply to human (Griparic et al., 2007). Furthermore, the yeast homologue of PRELI, Ups1p, was also suggested to have additional targets in the mitochondria (Sesaki et al., 2006), suggesting that PRELI may also engage other substrates than OPA1.

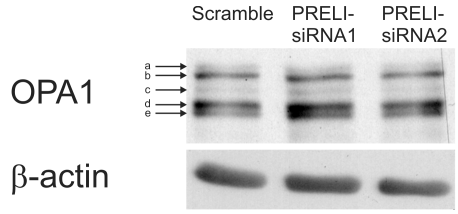

Figure 5. PRELI does not influence the expression of different OPA1 isoforms (a-e) in human CD4+ cells. Cord blood CD4+ cells were nucleofected with scrambled or PRELI siRNAs, activated and analyzed by Western blotting at 24 h after activation.

5.2.3 PRELI downregulates Th2 cell differentiation and STAT6 - likely via regulation of oxidative stress and Calpain-activation

After finding out that PRELI and IL-4 counter-regulate the mitochondrial apoptosis pathway in primary Th cells, the role of PRELI on Th cell differentiation was examined. CD4+ cells were nucleofected with a control or PRELI overexpression vector and

differentiated into Th1 and Th2 cells for 7 days. After this, the production of IFNγ and IL-4, the signature cytokines produced by Th1 and Th2 cells, were measured. PRELI decreased the number of IL-4-producing Th2 cells as compared to control cells (II, Figure 4A). Consistent data was obtained in PRELI knockdown experiments. Since the number of IL-4-producing cells after the first round of human Th2 cell polarization is typically less than 10%, as previously reported (Sornasse et al., 1996), we analyzed PRELI´s effect on the expression of another Th2 cell marker; the cell surface molecule CRTH2. CRTH2 is expressed on Th2, but not in Th1 cells and its expression correlates strongly with the production of IL-4 and IL-13 in human Th2-polarized cultures (Cosmi et al., 2000; De Fanis et al., 2007). As expected, downregulation of PRELI increased the amount of CRTH2-positive Th2 cells compared to the control cells (II, Figure 4B). No reproducible effect on IFNγ production by Th1 cells could be detected in experiments involving PRELI overexpression (n=3, p-value 0.28) or knockdown (n=9, p-value 0.42) (data not shown).

To further investigate the mechanism behind PRELI's effect on Th2 differentiation, we examined whether PRELI has any effect on STAT6, the key transcription factor behind Th2-polarization. Knocking down of PRELI in cord blood or peripheral blood CD4+ cells led to increase in STAT6 protein levels, whereas STAT6 mRNA levels were not notably affected (II, Figure 5A), indicating that PRELI regulated STAT6 at the post-transcriptional level. In contrast, downregulation of PRELI had no reproducible effect on STAT1 or STAT4 proteins (II, Figure 5A and data not shown). In addition, PRELI's effect on IL-4 induced tyrosine (Y641) phosphorylation of STAT6 was investigated in CD4+ cells. Our results indicated that PRELI downregulated STAT6 protein levels with no additional effect on its IL-4-dependent phosphorylation (II, Figure 5B).

Since PRELI was shown to regulate oxidative stress in cells, we investigated whether STAT6 was regulated by the cellular redox state to elucidate the mechanisms by which PRELI downregulated STAT6. Exposure of cells to H_2O_2 was used to model oxidative stress. The amount of STAT6 protein was observed to decrease in a dose-dependent manner in response to increasing cellular ROS concentration, whereas the expression of STAT6 mRNA was not similarly affected (II, Figure 5C), indicating that STAT6 is downregulated by oxidative stress at the post-transcriptional level in human primary Th cells. In contrast, levels of STAT1 and STAT4 were not similarly affected by H_2O_2 as the level of STAT6 was (II, Figure 5C and data not shown), excluding the possibility of non-specific, general protein degradation induced by the H_2O_2. Consistent data was obtained in experiments utilizing an antioxidant NAC to neutralize cellular ROS production (II, Figure 5D).

Based on earlier observations by others, Calpain, a calcium-dependent cysteine protease, seemed as a possible mediator of PRELI-induced downregulation of STAT6 in human primary Th cells. Calpains are oxidative stress-activated proteases implicated in cell proliferation, apoptosis and differentiation (Ishihara et al., 2000; Perrin and Huttenlocher, 2002) (II, Figure 5E). STAT6 has been reported to be negatively regulated

by Calpain in mouse T cell lines and mast cells (Suzuki et al., 2003; Zamorano et al., 2005). In our study, by using Calpastatin, an endogenous Calpain inhibitor (Melloni et al., 1996), STAT6 was shown to be downregulated by Calpain in human primary Th cells (II, Figure 5F). Importantly, PRELI was shown to induce the cleavage/activation of Calpain in HEK293 cells (II, Figure 5G). Taken together, our results suggested that downregulation of STAT6 by PRELI could be mediated by oxidative stress and Calpain activation.

5.2.4 The strength of the TCR stimulus influences the expression of PRELI and Th2 differentiation

Strength of the TCR signal influences the direction of Th1/Th2 polarization. A strong TCR stimulus enhances Th1 differentiation, whereas weaker stimuli favor Th2 polarization (Brandt et al., 2002; Constant et al., 1995). Interestingly, the intracellular levels of PRELI correlated with the strength of the TCR activation. The amount of PRELI, at both the protein and mRNA levels, was clearly in proportion to the concentration of α-CD3 utilized for stimulation (II, Figure 6A). In another experiment, CD4+ cells, nucleofected with scramble or PRELI siRNA oligonucleotides, were activated with different concentrations of α-CD3 and cultured under Th1 or Th2 polarizing conditions for 7 days. Subsequently, the expression of CRTH2, the Th2 cell marker, was analyzed by flow cytometry. Th2 differentiation was observed to be inhibited when primary Th cells were activated with a strong TCR stimulus, whereas it was enhanced when PRELI was downregulated (II, Figure 6B). These results thus indicated that the strength of the TCR activation, as well as the amount of PRELI in cells, influenced the ability of a naive CD4+ cell to differentiate into Th2 cell (II, Figures 4 and 6B). Importantly, our results suggested that PRELI is involved, at least in part, in the mechanism by which a strong TCR stimulus influences Th2 differentiation. Figure 6 summarizes the data obtained in this study and illustrates the function of PRELI in human primary CD4+ cells.

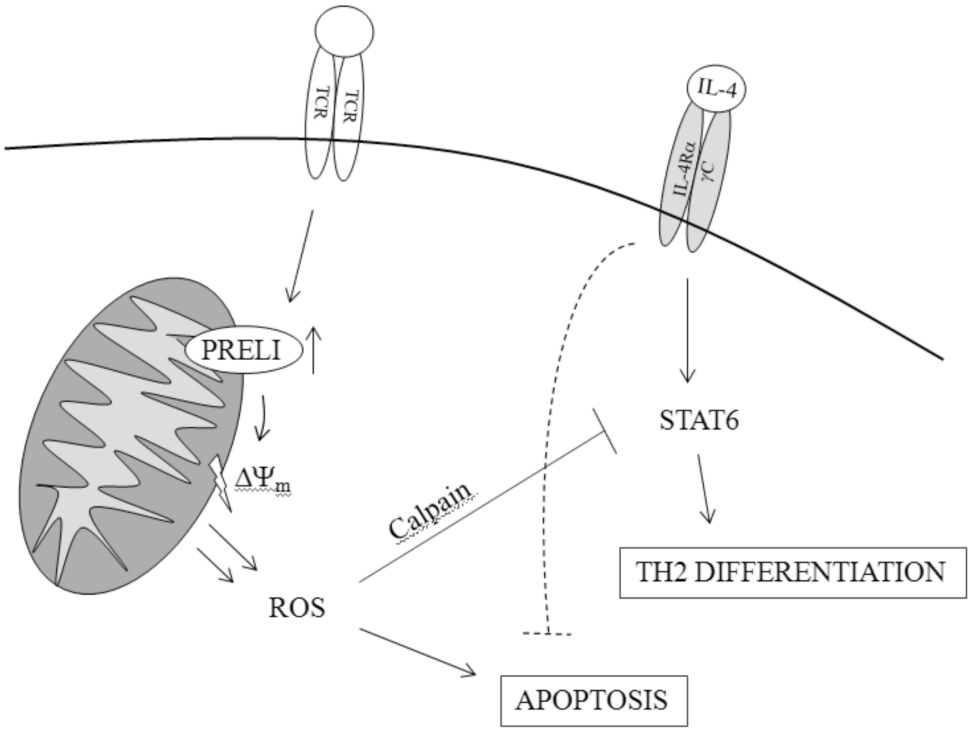

Figure 6. Summary of the effects induced by PRELI in human CD4+ cells. PRELI is highly upregulated in response to T cell activation. It is localized in mitochondria, it alters the $\Delta\Psi_m$ and induces mitochondrial ROS production. We also observed that PRELI influences T helper cell apoptosis and differentiation. PRELI induced apoptosis and this was partly inhibited by IL-4. PRELI downregulated STAT6 at protein level and repressed Th2 cell polarization. Our results suggest that these new functions for PRELI are mediated by an overlapping mechanism, by the regulation of the cellular redox state. Data obtained suggests that oxidative stress and Calpain are involved in the PRELI-induced downregulation of STAT6.

5.3 IDENTIFICATION OF PIM KINASES AS ENHANCERS OF HUMAN TH1 CELL DIFFERENTIATION (III, UNPUBLISHED)

5.3.1 Expression of PIM kinases is upregulated in Th1 cells

PIM family of serine/threonine-specific kinases are predominantly expressed in hematopoietic cells and they are known to regulate cytokine-dependent proliferation and survival in lymphocytes, with largely overlapping functions (Bachmann and Moroy, 2005; Eichmann et al., 2000; Mikkers et al., 2004). Expression levels of *PIM* family genes have been previously shown to be upregulated by Th1-inducing cytokines, suggesting a role for PIM kinases in the regulation of Th1/Th2 differentiation process (Aho et al., 2005). Consistently, in our study, expression levels of PIM kinases were induced in Th1 cells. Naive human CD4+ cells were activated and cultured either in neutral conditions (Th0) or under Th1 or Th2-polarizing cytokines, IL-12 or IL-4,

respectively. Levels of PIM1 and PIM2 proteins were measured at different time-points by Western blotting. PIM1 and PIM2 were induced in response to TCR activation and further upregulated by the Th1-polarizing cytokine, IL-12. PIM2 was found to be upregulated more rapidly in Th1 cells as compared to PIM1 (III, Figure 1A and data not shown).

Furthermore, PIM kinases were shown to be downregulated during early Th2 differentiation by a STAT6-dependent mechanism. STAT6 was specifically knocked down in naive CD4+ cells and the levels of PIM kinases were determined by Western blotting at different time-points during the Th0/Th2 culture (III, Figure 1B).

5.3.2 Silencing of PIM2 downregulates expression of IFNγ and T-bet

After establishing that PIM kinases are preferentially expressed in Th1 cells as compared to Th2 cells, we wanted to determine their effect on Th1 differentiation. We analyzed the expression of IFNγ and T-bet, the hallmark cytokine expressed by Th1 cells and the key transcription factor driving Th1 polarization. To address this question, we used an siRNA approach to specifically knock down expression of individual PIM kinases in naive human CD4+ cells. After nucleofecting the cells with specific siRNAs, cells were polarized to Th1 direction. The knockdown efficiencies were controlled by measuring the PIM mRNA and protein levels at different time-points during the culture (III, Figure 2A and data not shown). Knockdown of PIM2 was very efficient. PIM2 siRNA1 almost completely inhibited the TCR and IL-12-induced upregulation of PIM2. The siRNA-mediated downregulation of PIM2 lasted up to 3 to 4 days after which the effect of the siRNA was decreased, most probably because of the proliferation of the cells. Therefore we focused on studying the early phases of Th1 cell differentiation.

Analysis of IFNγ produced to cell culture supernatants indicated that PIM2 siRNA inhibited IFNγ production in Th1-polarized cells. Reduction was most remarkable at 24 hour time-point, after which the effect was slowly diminished (III, Figure 2B). Level of IFNγ mRNA was significantly downregulated by PIM2 siRNA already after 6 hours of culture, indicating that PIM2 regulates IFNγ production at the transcriptional level (III, Figure 2C). Depletion of PIM2 also inhibited the upregulation of T-bet during Th1 cell polarization, both at protein and mRNA levels (III, Figure 2D-E). T-bet was significantly downregulated at the protein level by PIM2 siRNA after 12 and 24 hours of polarization. Consistently, at mRNA level the induction of T-bet was delayed by PIM2 knockdown, with effects observed already 6 hours after the polarization. Thus, taken together, our data suggests that upregulation of PIM2 under Th1 polarizing conditions is likely to be important for the proper induction of both IFNγ and T-bet during early phases of Th1 cell differentiation.

The effects of the PIM2 knockdown were generally transient. This is likely due to the fact that the siRNA oligonucleotides are diluted after the cells start to proliferate. In addition, since PIM1 and PIM2 have overlapping functions, it is possible that the

increasing expression of PIM1 at 48 hour time-point and onwards (III, Figure 1A and data not shown) reduced the effects of PIM2 knockdown at those later time-points. Furthermore, T-bet and IFN-γ themselves were very highly upregulated during the early phases of Th1 polarization, which also could reduce the effects of the PIM2 knockdown at the later time-points.

5.3.3 PIM2 regulates IL-12Rβ2/STAT4 signaling pathway

After PIM2 was shown to regulate the expression of T-bet and IFNγ during early Th1 cell differentiation, we decided to examine whether PIM2 has any effect on IL-12Rβ2 or STAT4, which constitute another critical Th1-driving pathway. IL-12Rβ2 is not expressed in naive T cells but is induced by TCR activation and T-bet and maintained by IFNγ (Afkarian et al., 2002; Chang et al., 1999; Letimier et al., 2007; Mullen et al., 2001; Szabo et al., 1997). Once expression of IL-12Rβ2 is upregulated, IL-12, a key cytokine driving Th1 differentiation, is able to activate STAT4, an important inducer of IFNγ and IL-12Rβ2 expression (Barbulescu et al., 1998; Lawless et al., 2000; Letimier et al., 2007; Mullen et al., 2001; Nishikomori et al., 2002). IL-12Rβ2 was shown to be significantly downregulated by PIM2 knockdown both at mRNA and protein levels (III, Figure 3A-B). This was seen later as compared to the effects on T-bet and IFNγ. STAT4, but not STAT1, was transiently downregulated by PIM2 siRNA at 24h time-point (III, Figure 3C). Because T-bet has been shown to induce the expression of IL-12Rβ2 as well as affect STAT4 levels in Th1 cells (Afkarian et al., 2002; Mullen et al., 2001; Usui et al., 2006), it is possible that effect of PIM2 on IL-12Rβ2 and/or STAT4 is mediated at least partly via T-bet. Expression of IL-12Rβ2 was still inhibited by PIM2 siRNA at days 4 to 6 (III, Figure 3B). Since STAT4 and IFNγ are known to induce and maintain the expression of IL-12Rβ2 (Chang et al., 1999; Lawless et al., 2000; Letimier et al., 2007; Szabo et al., 1997), it is possible that the long-term influence of PIM2 on IL-12Rβ2 is mediated through its effects on IFNγ and STAT4.

5.3.4 PIM2 transiently regulates GATA3 in Th1 cells, but not STAT6 or Th2 differentiation

GATA3, a major regulator of Th2 cell polarization, is an important inhibitor of Th1 differentiation. GATA3 has been shown to inhibit the expression of IL-12Rβ2 (Ouyang et al., 1998) and Th1 differentiation (Ferber et al., 1999; Ouyang et al., 1998) by downregulating STAT4 (Usui et al., 2003). The ability of GATA3 to induce the Th2 phenotype or to downregulate IFNγ expression is weakened as Th cells differentiate into Th1 type cells. T-bet has been shown to inhibit both the expression and the function of GATA3, thereby suppressing Th2 cell development (Hwang et al., 2005; Usui et al., 2006). Also STAT4 has been reported to negatively regulate the expression of GATA3 (Ouyang et al., 1998).

We examined the influence of PIM kinases on the expression of GATA3 and STAT6 in Th1 polarized cells. Knocking down of PIM2 transiently upregulated GATA3 protein at 48h time-point whereas mRNA levels of GATA3 were not significantly changed (III, Figure 4 and data not shown). Since T-bet has been shown to regulate GATA3 mainly at protein rather than mRNA level (Usui et al., 2006), it is possible that the effect of PIM2 on GATA3 was mediated at least partly via T-bet. Also the transient regulation of STAT4 at 24h time-point could have had an effect on GATA3. Unlike GATA3, STAT6 was not significantly regulated by PIM2 knockdown at any of the time-points studied (III, Figure 4).

We also investigated whether PIM2 influences Th2 cell development, as defined by expression of a Th2 cell surface marker CRTH2 on Th2-polarized cells. CD4+ cells, nucleofected with non-targeting or PIM2-specific siRNAs, were polarized into Th1 and Th2 cells for seven days. After this, the cells were stained with CRTH2 antibody and analyzed by flow cytometry. PIM2 knockdown didn't have any influence on CRTH2 expression on Th2-polarized cells. Taken together, although PIM2 had a transient effect on the expression of GATA3 in Th1 polarized cells, it didn't influence Th2 cell differentiation, at least based on the expression of CRTH2 after 7 days of Th2 cell polarization. Thus, consistent with their preferential expression in Th1 cells, also the functional role of PIM kinases seemed to be more evident in human Th1 than in Th2 cell differentiation.

5.3.5 PIM1 promotes Th1 polarization

Considering that both PIM1 and PIM2 are preferentially expressed in Th1 cells and they often have overlapping functions, also the role of PIM1 in Th1 differentiation was studied. The reason for mainly focusing on PIM2 in our experiments was that the PIM2-specific siRNA was much more efficient than any of the PIM1 siRNAs used (III, Table 1). In spite of this, their effects were rather similar. Both IFNγ and IL-12Rβ2 mRNAs were downregulated at the 24h time-point by PIM1 siRNA1 in three independent experiments. In addition, depletion of PIM1 with several PIM1-specific siRNA's (PIM1 siRNA2 or PIM1 siRNA pool; III, Table 1) led to downregulation of IFNγ mRNA, T-bet protein and cell surface IL-12Rβ2 (III, Figure 5 and data not shown). Altogether, our results indicated that, similar to PIM2, also PIM1 regulates important Th1-promoting factors during Th1 cell differentiation.

5.3.6 Possible mechanism behind the effects of PIM kinases

Our results suggested that PIM2 induces the transcription of T-bet and IFNγ already during the early hours after the activation and Th1 cell polarization. The effect on IL-12Rβ2 was seen later, at the 24h time-point and onwards. The expression of STAT4 was transiently regulated at 24h, whereas GATA3 was inhibited at 48h after Th1 priming (summarized in III, Figure 6). Although the regulation of T-bet could at least partly

explain the effects seen on IL-12Rβ2, STAT4 and/or GATA3, the exact order and the mechanisms behind these events are still unclear.

The mechanism through which PIM kinases regulate T-bet and IFN-γ transcription during the early hours of Th1 polarization is presently unknown. The expression of T-bet is regulated by TCR activation and the IFNγ/STAT1 signaling pathway (Afkarian et al., 2002; Lighvani et al., 2001). Also IL-12 and STAT4 have been shown to induce T-bet at the transcriptional level (Szabo et al., 2000; Usui et al., 2006; Ylikoski et al., 2005), although this mechanism was shown to take place in the late phase of Th1 cell differentiation (Schulz et al., 2009). The expression of IFNγ is also regulated by TCR activation and STAT4 (Barbulescu et al., 1998; Mullen et al., 2001). Whether PIM kinases can regulate the activity of STAT1 or STAT4 is not known. Our preliminary data suggest that PIM2 does not influence the serine phosphorylation of STAT1 at Ser^{727} or STAT4 at Ser^{721}, sites important for their transcriptional activities (Chen et al., 2004b). In addition, there are no conserved substrate recognition sequences for PIM kinases (Palaty et al., 1997; Peng et al., 2007) in STAT1 or STAT4. Therefore, it is not very likely that PIM1 or PIM2 would directly regulate the activities of STAT1 or STAT4 by serine phosphorylation.

NFAT and NF-κB, important inducers of several cytokine genes, have been implicated in the Th1/Th2 differentiation process. They have been shown to be involved in the early induction of expression of T-bet and IFNγ (Kiani et al., 2001; McCracken et al., 2007; Sica et al., 1997). Interestingly, both NFAT and NF-κB have been reported to be targets of PIM kinases. PIM1 was shown to phosphorylate NFATc1 on serine residues and thereby enhance its transcriptional activity (Rainio et al., 2002). PIM2, in turn, was shown to stimulate NF-κB-dependent transcription through phosphorylation of the oncogenic serine/threonine kinase Cot that activates NF-κB (Hammerman et al., 2004). Thus, it is possible that the induction of T-bet and IFN-γ expression by PIM kinases is mediated at least partly via NFAT and /or NF-κB activation.

A possibility based on earlier observations is that PIM kinases influence Th1 cell polarization by regulating the RUNX family of transcription factors. PIM1 has been shown to phosphorylate RUNX proteins and enhance their transcriptional activity (Aho et al., 2006). RUNX proteins, in turn, regulate Th1/Th2 differentiation. RUNX1 has been shown to repress GATA3 expression and to inhibit Th2 differentiation (Komine et al., 2003). RUNX3, in turn, promotes Th1 polarization in co-operation with T-bet. T-bet induces the expression of RUNX3 and they both are required for the maximal production of IFNγ in Th1 cells (Djuretic et al., 2007).

Collectively, it is possible that PIM kinases promote Th1 polarization by several mechanisms. They increase the activities of RUNX proteins as well as promote the early induction of important Th1-driving factors T-bet, IFNγ and the IL-12/STAT4 signaling pathway (shown in Figure 2). Figure 7 shows hypothetical mechanism of the effects of

PIM kinases during early Th1 polarization. The figure is based on the data obtained in this thesis and other published literature.

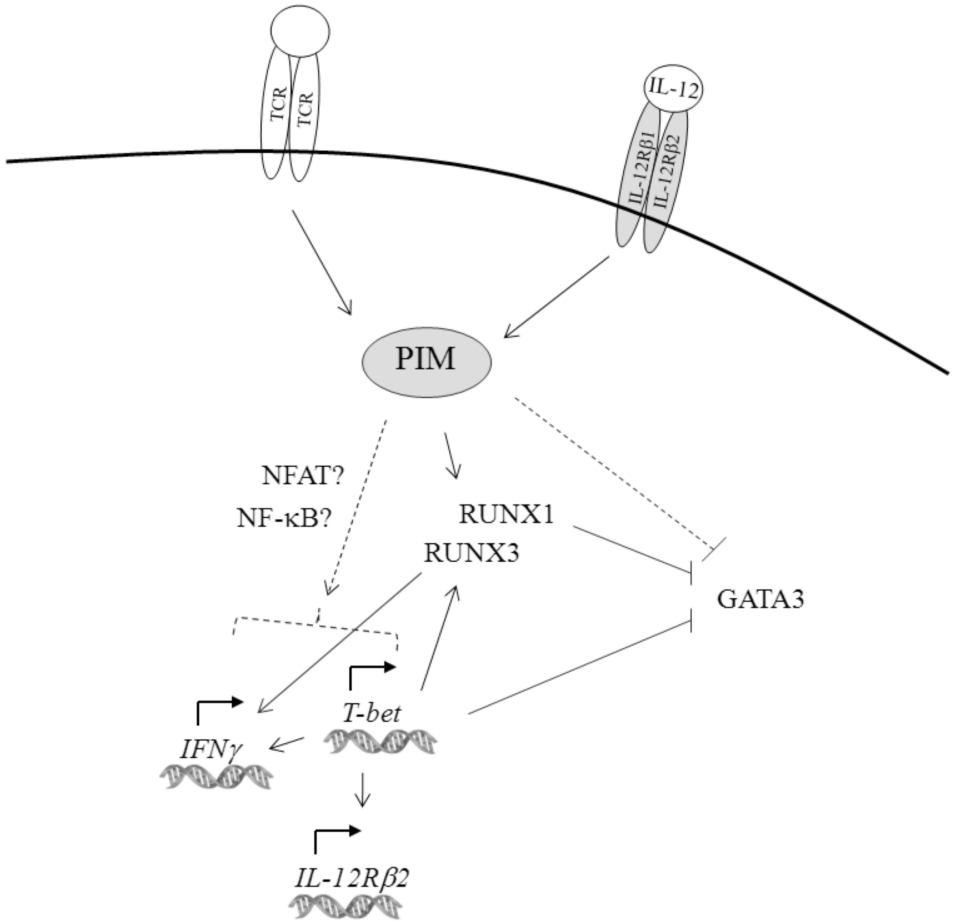

Figure 7. Summary of effects of PIM kinases during human Th1 cell polarization. In this study, PIM kinases were shown to induce expression of T-bet, IFNγ and IL-12Rβ2 as well as transiently inhibit GATA expression. Previously, PIM kinases have been shown to activate RUNX proteins, which influence Th1/Th2 polarization by downregulating GATA3 (RUNX1) or inducing IFNγ expression (RUNX3). In addition, PIM kinases have been shown to positively regulate the activities of both NFAT and NF-κB, known inducers of early T-bet and IFNγ expression. Thus, it is possible that PIM kinases promote Th1 differentiation both by stimulating NFAT or NF-kB pathways as well as via RUNX proteins. Figure represents data obtained in this thesis (dashed line) and data obtained previously by others (solid lines).

6 CONCLUSIONS

The selective development of Th1 and Th2 cell subsets plays an essential role in the immune system identifying different pathogens and protecting against diseases. The relative proportion of Th1 and Th2 cells defines the host defence mechanism but has also been implicated in the development of pathogenic responses and many autoimmune and inflammatory diseases. Identification of factors regulating the early phases of Th1 and Th2 cell differentiation processes is essential for understanding the signaling networks and mechanisms behind the development of these diseases as well as for identification of new potential targets for the development of novel interventions and therapeutics.

The aim of this thesis was to identify novel factors regulating Th1 and Th2 differentiation processes. In order to achieve this, one aim was to set up a feasible assay for transfecting resting human primary T lymphocytes – an essential tool for being able to study the effects of genes of interest in Th cell differentiation. By combining the benefits of two commercial expression vectors and Nucleofector™ technology, we optimized an assay for enrichment of transiently transfected unstimulated primary human Th cells expressing the gene or shRNA of interest. Nucleofected and enriched cells were shown to differentiate into Th1 and Th2 cells as well as the non-transfected control cells. To show the functionality of the assay, we demonstrated that introduction of an shRNA targeting STAT6, a key molecule driving the Th2 cell development, resulted in impaired Th2 cell differentiation, as expected.

PRELI was identified as a novel, activation-induced mitochondrial protein in human Th cells, influencing Th cell apoptosis and differentiation. Our results suggested that PRELI, by regulating the intracellular redox state, influences Th2 cell development at two levels. It induces mitochondrial apoptosis pathway in these cells, counteracting the anti-apoptotic effect of IL-4. PRELI also downregulates STAT6 and Th2 differentiation. The data suggested that Calpain, an oxidative stress induced cysteine protease, is involved as a mediator in PRELI-induced downregulation of STAT6. In addition, the strength of the TCR stimulus was observed to determine the intracellular level of PRELI in cells as well as influence Th2 development. This suggested that PRELI is involved – at least in part – in the mechanism through which the strength of the TCR stimulus influences the polarization of Th2 cells.

PIM serine/threonine-specific kinases were identified as new regulators of human Th1 cell differentiation. PIM1 and PIM2 kinases were established to be preferentially expressed in Th1 cells as compared to Th2 cells. During Th2 cell polarization, the expression of PIM1 and PIM2 were found to be downregulated by STAT6. Focusing on the early initiation stage of Th1 cell polarization, we demonstrated that PIM kinases positively influence production of IFNγ, the hallmark cytokine produced by Th1 cells. They were also found to induce the expression of the key Th1-driving factor T-bet and

the IL-12Rβ2/STAT4 signaling pathway during early phases of Th1 cell differentiation. Furthermore, downregulation of PIM kinases during the initiation of Th1 differentiation was found to transiently upregulate GATA3, a transcription factor important for Th2 cell differentiation and an inhibitor of Th1 polarization. Our results together with earlier observations suggest that PIM kinases could influence IL-12/STAT4 signaling pathway and GATA3 at least partly through the early regulation of T-bet.

In conclusion, this thesis describes the development and application of an assay that enables preparation of highly pure transfected primary Th cell cultures for studying the effect of overexpression or knockdown of selected genes on T helper cell differentiation and other primary human T cell functions. PRELI and PIM kinases were identified as new factors involved in the regulation of human primary Th cell differentiation and function, thus providing new information on the mechanisms leading to the selective development Th1 and Th2 cell subsets.

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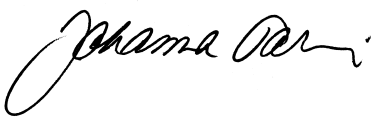

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