TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1085

MEDICA - ODONTOLOGICA

REGULATION OF LYMPHOCYTE RESPONSE *IN VITRO* AND *IN VIVO*

by

Minna Kyläniemi

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2013

From the Institute of Biomedicine, Department of Medical Biochemistry and Genetics,
University of Turku
Turku Centre for Biotechnology
University of Turku and Åbo Akademi University
The National Doctoral Programme in Informational and Structural Biology

Supervised by

Professor Riitta Lahesmaa
Turku Centre for Biotechnology
University of Turku and Åbo Akademi University
Turku, Finland

Reviewed by

Docent Marko Pesu, MD, Ph.D.
Institute of Biomedical Technology
University of Tampere
Tampere, Finland

And

Docent Jukka Partanen, Ph.D.
Finnish Red Cross Blood Service
Helsinki, Finland

Opponent

Professor Hilde Cheroutre, Ph.D.
Division of Developmental Immunology
La Jolla Institute for Allergy and Immunology
La Jolla, California, USA

The originality of this dissertation has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service

ISBN 978-951-29-5510-7 (PRINT)
ISBN 978-951-29-5511-4 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2013

To my family

"We make our choices.
Then our choices make us."
-Anne Frank

ABSTRACT

Minna Kyläniemi

Regulation of lymphocyte response *in vitro* and *in vivo*

Faculty of Biomedicine, Department of Medical Biochemistry and Genetics, University of Turku
Turku Centre for Biotechnology, University of Turku and Åbo Akademi University
The National Doctoral Programme in Informational and Structrural Biology

CD4+ T helper (Th) cells have an important role in the defence against diverse pathogens. Th cells can differentiate into several functionally distinct subtypes including Th1 and Th2 cells. Th1 cells are important for eradicating intracellular pathogens, whereas Th2 cells protect our body against extracellular parasites. However if uncontrolled, Th cells can mediate immunopathology such as asthma or allergies, but inappropriate Th response can also lead to autoimmune diseases such as multiple sclerosis or type 1 diabetes. Deeper knowledge of the regulation of the lymphocyte response both *in vitro* and *in vivo* is important for understanding the pathogenesis of immune-mediated diseases and microbe-host interactions.

In the work presented in this thesis, the first goal was to elucidate the role of novel factors, PIM kinases and c-FLIP in the regulation of human Th cell differentiation. The oncogenic serine-threonine kinases of the PIM family were shown to be preferentially expressed in Th1 cells and in addition, by using RNA interference, they were also shown to be positive regulators of Th1 differentiation. The PIM depletion experiments suggest that PIM kinases promote the expression of the hallmark cytokine of Th1 cells, IFNγ, and influence the IL12/STAT4 pathway during the early Th1 cell differentiation.

In addition to cytokine and T cell receptor (TCR) induced pathways, caspase activity has been shown to regulate Th cell proliferation. In the work presented in this thesis, the two isoforms of the caspase regulator protein, c-FLIP, were shown to be differentially expressed in Th1 and Th2 cells. Both of the isoforms were up-regulated in response to TCR activation, but the expression of the short isoform was selectively induced by IL4, the Th2 inducing cytokine. Furthermore, the c-FLIP isoforms had distinct and opposite roles during the early differentiation of Th1 and Th2 cells. The knockdown of the long isoform of c-FLIP led to the induction of Th1 marker genes, such as IFNγ and *TBET,* whereas the depletion of c-FLIP short down-regulated Th2 marker genes IL-4 and *GATA3.*

The third goal was to elucidate the gene expression profiles characterizing the T- and B-lymphocyte responses *in vivo* during experimental infection by intracellular bacterium *Chlamydia pneumoniae*. Previously, it has been shown that CD8+ and CD4+ T cells are important for the protection against *Chlamydia pneumoniae*. In this study, the analysis revealed up-regulation of interferon induced genes during recurrent infection underlining the importance of IFNγ secreted by Th1 and CD8+ T cells in the protection against this pathogen. Taken together, in this study novel regulators of Th cell differentiation were discovered and in addition the gene expression profiles of lymphocytes induced by *Chlamydia pneumoniae* infection were characterized.

Keywords: T helper cell, lymphocyte, differentiation, kinase, *Chlamydia pneumoniae*

TIIVISTELMÄ

Minna Kyläniemi

Lymfosyyttivasteen säätely *in vitro* ja *in vivo*

Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Turun yliopisto
Turun Biotekniikan keskus, Turun yliopisto ja Åbo Akademi
ISB tutkijaohjelma

CD4+ T-auttajasoluilla (Th-solut) on tärkeä rooli puolustuksessa erilaisia patogeenejä vastaan. Th-solut voivat erilaistua moniin funktionaalisiin alaluokkiin, joihin kuuluvat muun muassa Th1- ja Th2-solut. Th1-solut suojaavat kehoamme solunsisäisiltä patogeeneiltä, kun taas Th2-solut suojelevat meitä solun ulkoisia parasiitteja vastaan. Kontrolloimaton Th-soluvaste voi kuitenkin johtaa immuunipatologiaan, kuten astmaan ja allergisiin oireisiin. Häiriöt Th-soluvasteessa voivat myös johtaa autoimmuunisairauksiin, kuten multippeli skleroosiin (pesäkekovettumatauti) tai tyypin 1 diabetekseen. Lymfosyyttivasteen säätelyn mekanismien tunteminen *in vitro* ja *in vivo* on tärkeää selvitettäessä immuunivälitteisten sairauksien patogeneesiä ja mikrobien ja isäntäsolujen välistä vuorovaikutusta.

Tässä työssä ensimmäinen tavoite oli selvittää PIM-kinaasiperheen ja c-FLIP proteiinin roolia ihmisen Th-solujen erilaistumisessa. Onkogeenisen PIM-kinaasiperheen todettiin ilmenevän enemmän Th1- kuin Th2-soluissa. Lisäksi RNA-interferenssikokeiden avulla PIM-kinaasien todettiin vaikuttavan positiivisesti Th1-solujen erilaistumiseen. Niiden hiljentäminen vaikutti Th1-solujen tuottaman sytokiinin, IFNγ:n, tuottoon sekä myös Th1-soluille tärkeään signaalinvälitysreittiin, joka kulkee IL12:n ja STAT4 transkriptiotekijän kautta.

Sytokiinien ja T-solureseptorin aktivoimien signaalinvälitysreittien lisäksi kaspaasi-aktiivisuuden on todettu säätelevän T-solujen proliferaatiota. Tässä työssä tutkittiin kaspaasi-aktiivisuuden säätelijän, c-FLIPin, kahden eri muodon vaikutusta Th-solujen erilaistumiseen. c-FLIPin kahden muodon todettiin ilmentyvän eri tavalla Th2-solujen erilaistumiselle tärkeän IL4:n vaikutuksesta. c-FLIP short-muodon ilmenemisen todettiin indusoituvan IL4:n vaikutuksesta. Lisäksi RNA-interferenssikokeissa todettiin, että c-FLIP long-muodon hiljentäminen johtaa voimakkaampaan Th1-solujen erilaistumiseen, kun taas Th2-solujen erilaistuminen on heikentynyt c-FLIP short-muodon hiljentämisen seurauksena.

Väitöskirjatyön kolmantena tavoitteena oli selvittää T- ja B-lymfosyyttien geenieksprессio-profiileja keuhkoklamydia (*Chlamydia pneumoniae)* infektion aikana. Aiemmin on näytetty, että CD8+ ja CD4+ T-soluilla on tärkeä rooli immuunipuolustusreaktioissa keuhkoklamydiainfektion aikana. Tässä työssä selvitettiin, että toistuvan keuhkoklamydian aikana hiiren lymfosyyteissä ilmenee enemmän erityisesti interferonin indusoimia geenejä. Tämä korostaa Th1- ja CD8+ T-solujen tuottaman IFNγ:n tärkeää merkitystä immuunireaktiossa keuhkoklamydian aiheuttamaa infektiota vastaan. Yhteenvetona voidaan sanoa, että tässä työssä löydettiin uusia Th-solujen erilaistumiseen vaikuttavia tekijöitä sekä selvitettiin hiirimallissa keuhkoklamydian aiheuttamia muutoksia lymfosyyttien geenien ilmenemisessä.

Avainsanat: T-auttajasolu, lymfosyytti, erilaistuminen, kinaasi, *Chlamydia pneumoniae*, keuhkoklamydia

TABLE OF CONTENTS

ABBREVIATIONS

AICD	activation induced cell death
ALN	axillary lymph node
AP1	activator protein 1
APC	antigen presenting cell
ATF	activating transcription factor
BCL6	B-cell lymphoma 6
BCR	B cell receptor
BRG1	brahma related gene 1
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
CFLAR	CASP8 and FADD apoptosis regulator (c-FLIP)
ChIP	chromatin immunopresipitation
c-FLIP	cellular FLICE inhibitory protein
c-MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
DE	differentially expressed
DED	death effector domain
DISC	death inducing signalling complex
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EB	elementary body
ERK	extracellular signal-regulated kinase
FADD	Fas-associated protein via death domain
FC	fold change
FDR	false discovery rate
FAM	carboxyfluorescein
FASLG	FAS ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLICE	FADD-like ICE
FOXP3	forkhead box P3
GATA3	GATA binding protein 3
GFI1	growth factor independent-1
GZMA	granzyme A
HLX	H2.0-like homeobox protein
HSP	heat shock protein
IFN	interferon
IFNGR	interferon gamma receptor
IFU	inclusion forming unit
Ig	immunoglobulin
IL	interleukin
IL2Rα	interleukin-2 receptor α subunit
IL4Rα	interleukin-4 receptor α subunit
IL12Rβ2	interleukin-12 receptor β2 subunit
IL18R	interleukin-18 receptor
IRF	interferon regulatory factor

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAT	linker of activated T cells
LFC	log fold change
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MLN	mediastinal lymph node
mRNA	messenger RNA
NCoA	nuclear receptor co-activator
NFAT	nuclear factor of activated T cell
Nf-κB	nuclear factor kappa enhancer binding protein
NT	non-targeting
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
PIM	proviral integration site for Moloney murine leukemia virus
PKC-θ	protein kinase-θ
PLC-γ	phospholipase C-γ
PMA	phorbol 13-myristate 12-acetate
PP2A	protein phosphatase 2A
RB	reticulate body
RNA	ribonucleic acid
RORC	RAR-related orphan receptor C
RT-PCR	reverse transcriptase PCR
RUNX	runt related transcription factor
SDS	sodium dodecyl sulfate
siRNA	short interfering RNA
SLPI	serine leukocyte protease inhibitor
SMAD2	SMAD family member 2
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
SOX4	SRY (sex-determining region Y) box 4
STAT	signal transducer and activator of transcription
TAMRA	tetramethylrhodamine
TBET	T-box expressed in T cells
TBX21	T-box 21, synonym T-BET
Tc	cytotoxic T cell
TCF1	T cell factor 1
TCR	T cell receptor
Tfh	follicular T helper cell
TGFβ	transforming growth factor β
TGIF1	TGFβ-induced factor homeobox 1
Th	T helper cell
TNF	tumour necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
TYK	tyrosine kinase
ZAP-70	ζ-chain associated protein 70

LIST OF ORIGINAL PUBLICATIOS

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

I Tahvanainen, J.T.*, Kyläniemi, M.K.*, Kanduri, K., Gupta, B., Lähteenmäki, H., Kallonen, T., Rajavuori, A., Rasool, O., Koskinen, P.J., Rao, K.V.S., Lähdesmäki H., Lahesmaa, R. (2013) Proviral Integration Site for Moloney Murine Leukemia Virus (PIM) kinases promote human T helper 1 cell differentiation. *J Biol. Chemistry,* 288: 3048-3058 **equal contribution*

II Kyläniemi, M.K., Kaukonen, R., Myllyviita, J., Lahesmaa, R. The regulation and role of c-FLIP in human T helper cell differentiation. *manuscript*

III Kyläniemi, M.K., Haveri, A., Vuola, J.M., Puolakkainen, M., Lahesmaa, R. (2009) Gene expression signatures characterizing the development of lymphocyte response during experimental *Chlamydia pneumoniae* infection. *Microb. Pathog*., 46:235-242.

1 INTRODUCTION

From birth, our bodies are constantly bombarded by diverse pathogens ranging from viruses and bacteria to multicellular parasites. The immune system must be able to detect the variety of pathogens as well as to distinguish them from the body's own tissues. In humans, there are two branches of the defence against pathogens, the innate and the adaptive immune system. The functional immune system needs appropriate functionality of both branches as well as their mutual co-operation and tight regulation of response to avoid harmful self-destructive actions. The innate immune system cells, such as dendritic cells, neutrophils, eosinophils, basophils and macrophages, provide the first line of defence when we encounter a new pathogen. However, many pathogens can escape from the innate immune cells and an adaptive immune response is activated when the initial innate immune response fails to clear the infection. After encountering an antigen, the antigen presenting cells (APC) are activated and migrate into lymph nodes to activate the cells of the adaptive immune system, i.e. lymphocytes. The activation and proliferation of lymphocytes will then lead to B cell-mediated humoral responses as well as to cell-mediated responses by T cells.

The lymphocyte response is based on the clonal expansion of lymphocytes recognising a specific antigen with their unique antigen receptor. Lymphocytes express a vast variety of antigen receptors arising from somatic gene recombinations occurring during their maturation. The lymphocyte population in the body will collectively have a huge repertoire of antigen receptors enabling the body to recognize and respond to virtually any antigen it is exposed to. To avoid the self-reactivity of lymphocytes they will be selected positively and negatively to eradicate the non-functional or self-reactive antigen receptor bearing lymphocytes during the maturation. The antigen receptor expressed by naive B cells is called the B cell receptor (BCR), which is able to recognize intact micro-organisms or proteins such as bacterial toxins. On the other hand, the T cell receptor (TCR) will only recognize processed peptide antigens bound by major histocompatibility (MHC) class II or class I molecules on the cell surface of APC or any cells, respectively. The CD8+ cytotoxic T (Tc) cells will recognize antigens bound by MHC I whereas the CD4+ helper T (Th) cells recognize antigens bound by MHC II. When naive lymphocytes encounter the antigen that is specifically recognized by their TCR or BCR, they will proliferate and differentiate into effector cells that can eliminate the pathogen. Part of the lymphocyte population will proliferate to memory cells, which then reside in the body and will be activated if the same pathogen is encountered again providing a faster response and proliferation

of effector cells. This unique phenomenon of the adaptive immune system is called the immunological memory.

The effector B cells (plasma cells) secrete antibodies, which are the soluble form of their BCR having the identical specificity. They provide defence against pathogens in the extracellular spaces of the body. CD8+ T cells are able to directly kill their target cells, such as virus infected cells, whereas CD4+ T cells can provide help to other cells of the adaptive and innate immune system through the production of cytokines and expression of cell surface molecules. CD4+ T cells can activate macrophages, attract neutrophils to the site of infection, promote allergic responses by activating e.g. mast cells and inducing IgE production, activate CD8+ T cells and provide help to B cells for antibody production and immunologlobulin class switching. Therefore, CD4+ T cells are important for the cell-mediated and humoral immune response of the adaptive immunity as well as for activating the innate immune cells. The differentiation of CD4+ T cells into functionally distinct effector subtypes is crucial for the proper function of the immune system.

A deeper understanding of regulation of the lymphocyte response both *in vitro* and *in vivo* could help us to understand the pathogenesis of immune-mediated diseases and microbe-host interactions. In this study, the focus has been on the different aspects of the lymphocyte response. The differentiation of CD4+ T cells into T helper 1 (Th1) and Th2 cells is a finely balanced and tightly regulated process. Th cells are important in the regulation of immune responses against a variety of pathogens, but if uncontrolled these cells may mediate immunopathology such as type 1 diabetes or asthma. Several factors have been identified as regulators of Th cell differentiation, but the picture is far from complete. In this work, the role of the PIM family of serine threonine kinases in the human Th1 cell differentiation was studied. In addition, the role of apoptosis related protein, c-FLIP, was elucidated in the early differentiation of human Th1 and Th2 cells. Furthermore, the nature of lymphocyte response evolving *in vivo* during experimental infection by intracellular bacterium *Chlamydia pneumoniae* was studied.

2 REVIEW OF THE LITERATURE

2.1 Lymphocytes

The formation of the initial lymphocyte response is slow in comparison with the innate immune response (Chaplin, 2010). It will take from 4-6 days from the first encounter of a new antigen to the development of fully active plasma cell and effector T cell populations (Murphy et al., 2012). However, when the memory cells encounter a previously met antigen, the response will be fast and efficient (Delves and Roitt, 2000a). This unique feature of the adaptive immune system is called immunological memory or acquired immunity (Murphy et al., 2012).

Lymphocytes differentiate from a common hematopoietic precursor cell in the bone marrow. The B cell progenitors continue their maturation in the bone marrow, whereas T cell progenitors migrate into thymus (Delves and Roitt, 2000a). During their maturation, B and T lymphocytes go through several steps of selection where the formation of an antigen receptor is confirmed to be effective and the self-tolerance of the developing lymphocyte is tested (Carpenter and Bosselut, 2010; Welner et al., 2008). In the thymus, T cell progenitors differentiate to form CD4+ and CD8+ T cells (Carpenter and Bosselut, 2010). A fully matured lymphocyte that has not encountered its cognate antigen is called naive B or T cell, and it will leave the bone marrow or thymus, respectively, and migrate into lymph nodes and lymphoid organs. For T cell differentiation, encounter with an APC bearing a cognate antigen to their TCR is crucial for the initiation of the differentiation, and similarly naive B cells start their proliferation into plasma cells when they encounter their cognate antigen (Chaplin, 2010; Delves and Roitt, 2000a).

2.1.1 B cells

B cells form the humoral defence mechanism of the adaptive immune system. The B cell response is based on the production of soluble forms of their BCR, which are called antibodies. B cells produce antibodies in response to bacteria, bacterial toxins and viruses that are present in extracellular spaces. Antibodies mediate their effects by neutralizing toxins and other harmful particles, or by binding to the cell surface of bacteria or viruses thus activating phagocytosis by macrophages (called opsonisation) or leading to the activation of the complement system (Figure 1) (Delves and Roitt,

2000b; Murphy et al., 2012). A variety of antibodies will be generated during B cell maturation by somatic DNA recombination of Immunoglobulin (Ig) genes, and millions of naive B cells bearing different antibody molecules are formed (Li et al., 2004). The naive B cell expresses the BCR on the cell surface and will be activated if it encounters an antigen that it can recognize. In addition to the BCR-antigen stimulus, a B cell will need additional stimulation, for example, from Th cells, which express co-stimulatory molecules, such as CD40L (CD40 ligand), on their cell surface and produce B cell-activating soluble mediators, i.e. cytokines (Crotty, 2011). The activated B cell will then go through clonal expansion to form a population of antibody secreting plasma cells and a smaller population of long lasting memory cells, which will allow a faster response if the same pathogen is encountered again.

The effector B cells produce different classes of antibodies characterized by their constant regions called IgM, IgD, IgG, IgE and IgA (Kenter, 2005; Li et al., 2004). The variable regions of antibodies are specific to a certain antigen, but the constant region will define the secondary actions that will occur. The first class of antibodies that are expressed on the surface of a naive B cell are the IgM immunoglobulins (Delves and Roitt, 2000b). After activation, B cells can also secrete soluble IgM antibodies, which activate the complement pathway (Murphy et al., 2012). In the early phases after activation, B cells also express IgD on their cell surface and can secrete the soluble form as well, but the function of IgD antibodies is still unclear (Delves and Roitt, 2000b; Murphy et al., 2012). The gene recombination process that allows B cells to change the constant region of their specific immunoglobulin during the immune response is called class switching (Delves and Roitt, 2000a). Interleukin 4 (IL4), produced by Th2 or follicular Th (Tfh) cells, induces the antibody class switching to IgG1 or IgE (Crotty, 2011; Kühn et al., 1991), whereas interferon-γ (IFNγ), produced by Th1 cells, induces the class switching to IgG3 and IgG2 (Mond et al., 1995). IgE antibodies are mainly involved in allergic responses and activate innate immune cells in defence against multicellular organisms (Gould and Sutton, 2008), whereas IgG antibodies activate the complement system and opsonisation by phagocytes (Mond et al., 1995). IgA antibodies are secreted to mucosal surfaces and to mother's milk, providing protection against pathogenic bacteria (Cerutti et al., 2011). All the plasma cells derived from one naive B cell have the same specificity for the antigen, but the B cell can be instructed by Th cells to produce different antibody classes to achieve the most effective clearance of the encountered pathogen.

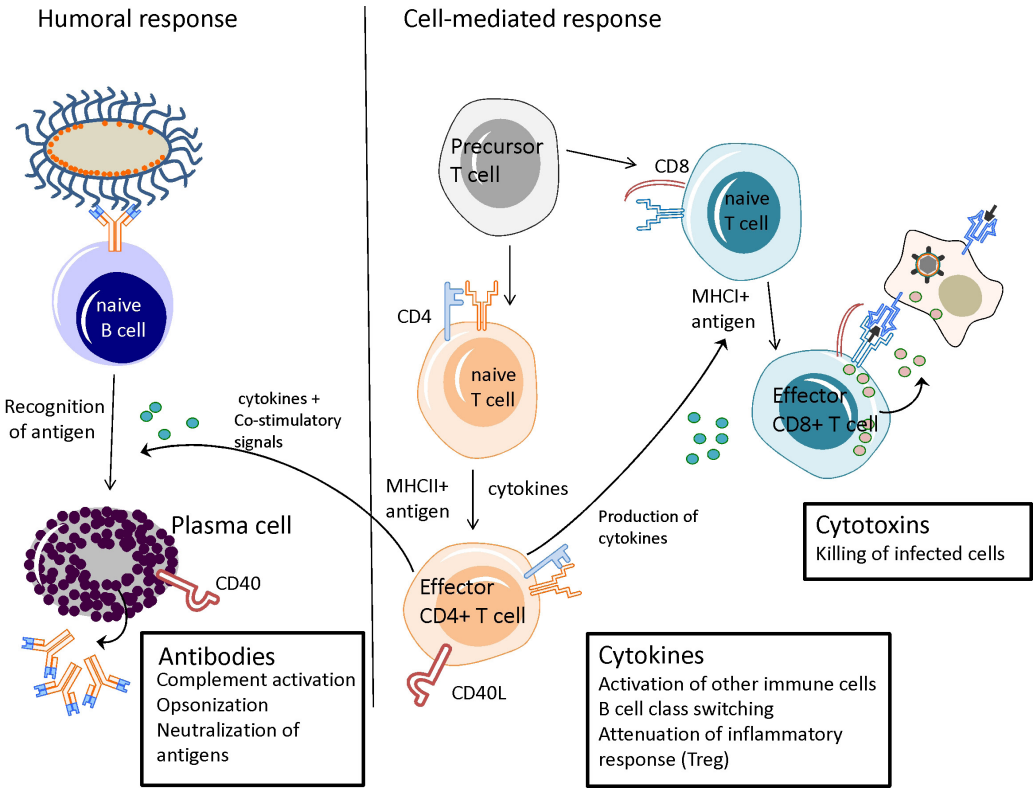

Figure 1. Lymphocytes and their roles in the immune system. The CD4+ and CD8+ T cells differentiate from a common precursor cell in the thymus and are the mediators of the cell-mediated adaptive immune response. B cells mature in the bone marrow and mediate humoral immune responses through the production of antibodies. Mature lymphocytes migrate to lymph nodes and lymphoid organs as naive cells and, if activated, will differentiate into effector cells. Effector CD4+ T cells regulate the development of plasma cells and effector CD8+ T cells by the production of cytokines and expression of co-stimulatory molecules such as CD40L (CD40 ligand). Effector CD8+ T cells secrete cytotoxins, whereas plasma cells mediate the humoral responses by secreting antibodies.

2.1.2 T cells

T cells differentiate from common precursor cells, called thymocytes, in the thymus. Thymocytes begin their development from a double negative (DN) stage, at which they do not express either CD4 or CD8 and the TCR gene rearrangements will start (Murphy et al., 2012). The TCR is a dimer that is formed by either α and β or γ and δ chains (Carpenter and Bosselut, 2010). The formation of αβ TCR predominates and the majority of T cells have the αβ receptor and they also express either CD8 or CD4 (Carpenter and Bosselut, 2010; Murphy et al., 2012). Only a small population of thymocytes will develop into γδ T cells, which differ from the αβ T cells by having both innate- and adaptive-like properties and are found for example, in the gut mucosa (Vantourout

and Hayday, 2013). The αβ T cells will begin their maturation from the DN stage by rearrangement of the β gene (Delves and Roitt, 2000a). Formation of a functional β chain is confirmed by β-selection and the T cells bearing unfunctional β chains will die. The DN thymocytes will continue with the α chain gene rearrangement, and at the same time the cells will up-regulate both CD4 and CD8 expression (Carpenter and Bosselut, 2010). The formation of a functional αβ TCR complex is then tested in a process called positive selection, where only those cells capable of recognizing MHC I or MHC II-molecules complexed with a peptide will survive (Carpenter and Bosselut, 2010; Delves and Roitt, 2000a). Most of the thymocytes will die by apoptosis during maturation and only a small proportion will develop into single positive CD4+ or CD8+ T cells. The final stage in the thymus will lead to the development of single positive CD4+/CD8+ T cells, which will be selected by negative selection for functional TCR that do not show autoreactivity (Carpenter and Bosselut, 2010).

2.1.2.1 CD8+ T cells

CD8+ T cells can only recognize processed peptide antigens presented by MHC I molecules on the surface of an APC (initial activation) or other cells (Delves and Roitt, 2000a). The CD8 (cluster of differentiation 8) molecule on a surface of CD8+ T cell serves as a necessary co-receptor for the recognition of the MHC I in the MHC I-antigen complex (Chaplin, 2010). CD8+ T cells leave the thymus as naive T cells, expressing their unique TCR on the cell surface. In lymph nodes or lymphoid organs, CD8+ T cells become activated if they encounter an APC bearing an antigen-MHC I complex that their TCR can recognize. Activated effector CD8+ T cells are called cytotoxic T lymphocytes (Tc). Similarly, as in B cell differentiation, a part of the Tc cell population will differentiate to form a population of memory CD8+ T cells (Murphy et al., 2012). The Tc cell response is critical against viruses and some intracellular pathogens because Tc cells are able to recognize and kill the infected host cells. The bacteria or virus-infected cells present processed peptides originating from pathogenic proteins bound to MHC I-molecules on their cell surface, and activated Tc cells can recognize these complexes. Tc cells also have antitumor activity since they are able to recognize tumor-associated antigens presented by the MHC I on the surface of cancer cells (Chaplin, 2010; Murphy et al., 2012). Tc cells kill their target cells by secreting cytotoxins such as granzymes and perforin, and they can also induce target cell MHCI expression by secreting IFNγ, thus making infected cells more susceptible to cytotoxic killing (Russell and Ley, 2002). IFNγ also has an anti-viral function, making the adjacent cells more resistant to infection (Mosmann et al., 1997). Cytotoxins are released in the immunological synapse that is formed between Tc cell and its target cell. Cytotoxins

are able to penetrate the lipid bilayer of the cell surface, and once inside the cell they will trigger the apoptosis of the target cell (Russell and Ley, 2002). Because the Tc cell response is very harmful when it is inappropriately activated, the naive CD8+ T cell needs strong co-stimulatory signals from the APC, such as B7-CD28 ligation, to become an active effector cell. In addition, the IFNγ secreted by Th1 cells is important for the activation of Tc cell responses (Whitmire et al., 2005).

2.1.2.2 CD4+ T cells

CD4+ T cells can differentiate into several different effector subsets. The mouse effector CD4+ T cells were originally divided into two functionally distinct effector subtypes; Th1 and Th2 cells, based on their cytokine profiles (Mosmann et al., 1986). Similar subtypes were also found in humans, and they were shown to be derived from a common precursor cell (Del Prete et al., 1991; Parronchi et al., 1992; Rocken et al., 1992; Sad and Mosmann, 1994; Zhu et al., 2010). The original Th1 and Th2 subtypes still remain the most extensively studied, although in recent years a tremendous amount of new knowledge has been gained about the new subtypes, Th17 (Bettelli et al., 2006; Harrington et al., 2005), Th9 (Dardalhon et al., 2008; Veldhoen et al., 2008), Tfh (Crotty, 2011; Nurieva et al., 2008) and inducible regulatory T cells (iTreg) (Josefowicz and Rudensky, 2009; Sakaguchi et al., 2008).

The different effector CD4+ T cell subtypes play distinct roles in the regulation of the immune responses. In contrast to the Tc cells, the targets for the CD4+ T cells are other immune cells, which they regulate by the secretion of cytokines and expression of co-stimulatory molecules. Th1 cells activate the cell-mediated responses against intracellular bacteria and viruses and against some protozoan parasites like *Leishmania major* (*L. major*) as well as tumours (Szabo et al., 2003; Wan and Flavell, 2009). Th2 cells activate the humoral responses and B cell class-switching to IgE (Paul and Zhu, 2010). In addition, Th2 cells activate eosinophils, basophils and mast cells. Th2 responses are crucial against extracellular parasites such as helminths and nematodes (Paul and Zhu, 2010). Th17 cells protect the host from extracellular bacteria and fungi (Annunziato et al., 2007), whereas Th9 cells are involved in the host response to intestinal helminths (Kaplan, 2013). Tfh cells reside in the B cell follicles in germinal centres and seem to be important for B cell help (Crotty, 2011), whereas the regulatory T cells are important for self-tolerance and immune homeostasis by suppressing immune functions through the production of IL-10 and TGFβ (Josefowicz and Rudensky, 2009; Sakaguchi et al., 2008). The appropriate differentiation and balance between the CD4+ T cell subtypes are important, since aberrant Th responses may mediate immunopathology. Th1 and Th17 cells have been associated with different autoimmune diseases, such as multiple

sclerosis, rheumatoid arthritis and type 1 diabetes, although their respective roles behind these diseases remain to be solved (Annunziato et al., 2007; Wan and Flavell, 2009). Aberrant Th2 type responses are crucial mediators of asthma and allergies, while Th17 and Th9 cells have also been suggested in playing a role in allergic responses (Kaplan, 2013; Paul and Zhu, 2010; Wan and Flavell, 2009).

The effector CD4+ T cells are classified on the basis of their cytokine profiles, the expression of different transcription factors and cell surface molecules and the differentiation of each subtype is driven by a combination of cytokines (Table 1). The inducing cytokines upregulate the expression of cell-type specific master regulator transcription factors, whereas the expression levels of signal transducer and activator of transcription (STAT) proteins do not generally change, but they are activated by cytokine signaling. Naive CD4+ T cells differentiate into Th1 cells in the presence of IL12 and IFNγ and the transcription factors T-box expressed in T cells (TBET), STAT1 and STAT4 are important for the differentiation (Szabo et al., 2003). IL4 induces the differentiation of Th2 cells, and the most important transcription factors for Th2 cells are GATA binding protein 3 (GATA3), STAT5 and STAT6 (Paul and Zhu, 2010). Th1 cells produce mainly IFNγ, but also TNFα and TNFβ, lymphotoxin and IL2, whereas Th2 cells produce IL4, IL5, IL13 and IL9 (Paul and Zhu, 2010). Transforming growth factor β (TGFβ) suppresses the differentiation of Th1 and Th2 cells, but together with IL6 and IL23 drives Th17 cell differentiation, and in the presence of IL4 drives the differentiation of Th9 cells (Annunziato et al., 2007; Veldhoen et al., 2008). Th17 cells are characterized by the production of IL17A, IL17F and IL21, while the important transcription factors are RAR-related orphan receptor C (RORC) and STAT3 (Ivanov et al., 2006; Stockinger and Veldhoen, 2007). Th9 cells produce IL9 and the main transcription factors important for their differentiation are PU.1 and STAT6 (Chang et al., 2010; Goswami et al., 2012). Furthermore, iTreg cell differentiation is driven by TGFβ and IL2 and they are also producers of TGFβ (Curotto de Lafaille and Lafaille, 2009; Josefowicz and Rudensky, 2009). The differentiation of iTreg cells is driven by forkhead box P3 (FOXP3) and STAT5 transcription factors (Sakaguchi et al., 2008). Tfh cells produce IL21 and IL10, whereas their differentiation is dependent on IL21 and B-cell lymphoma 6 (BCL6) transcription factor (Crotty, 2011). In addition to inducing one or more subtypes of Th cells, cytokines also suppress the differentiation of other subtypes. Thus the differentiation is dependent on the complex cytokine milieu present in the lymph nodes or lymphoid organs during the naive CD4+ T cell activation. Interestingly each Th cell subset produces at least one cytokine that is important for the differentiation of that subset, thus providing a possible positive feedback loop for the differentiation. Although the Th cell subtypes were originally considered as irreversible

states of cell differentiation (Murphy et al., 1996), in recent years the plasticity of Th subtypes has become more obvious (Murphy and Stockinger, 2010; Nakayamada et al., 2012; Sundrud et al., 2003; Takimoto et al., 2010). Th17 cells can be induced to produce IFNγ or Tfh cells to produce the cytokines of other subtypes (Nakayamada et al., 2012) In addition, the simultaneous expression of two different master regulator transcription factors has also been demonstrated (Hegazy et al., 2010; Koch et al., 2009). Thus there are still a number of open questions after over two decades of intensive research. Nevertheless, the focus of this thesis has been on the first-characterized Th subtypes; Th1 and Th2 cells, and the main factors regulating the Th1 and Th2 cell differentiation will be reviewed in more detail in the following chapter.

Table 1. Classification of CD4+ T cell effector cell lineages

	Th1	**Th2**	**Th17**	**Th9**	**iTreg**	**Tfh**
Inducing cytokines	IL12, IFNγ	IL4	IL6, IL23, TGFβ	IL4, TGFβ	TGFβ, IL2	IL21
Activated STATs	STAT4, STAT1	STAT6, STAT5	STAT3	STAT6	STAT5	STAT3
Master regulator TF	TBET	GATA3	RORC	PU.1	FOXP3	BCL6
Signature cytokines	IFNγ TNFα	IL4, IL5, IL9, IL13	IL17 IL21	IL9	TGFβ IL10	IL21 IL10
Target pathogens	Intracellular bacteria, viruses	Extracellular parasites	Extracellular bacteria, fungi	Helminths	Regulation of immune responses	-
Target immune cells	B cells, CD8+ T cells	B cells, Eosinophils, basophils	Neutrophils	B cells	Attenuation of inflammation	B cells in GC

TF; Transcription factor, GC; Germinal centres

2.2 Factors regulating Th1 and Th2 cell differentiation

Although Th cell differentiation requires TCR activation and co-stimulatory signals, the cytokine milieu surrounding the cell at the time of the activation is the main factor defining the fate of the differentiating cell. Recently it has been shown that gene expression is also regulated by the epigenetic changes in the chromatin. These changes have been shown to have an especially important role in fully polarized cells, for

example, in the regulation of the Th2 and Th1 hallmark cytokine genes, *Il4* and *Ifng*, respectively (Ansel et al., 2006; Lee et al., 2006; Wilson et al., 2009). The chromatin status will affect the ability of a cell to express certain genes and the permissive or repressive marks enable cell-type specific gene expression by ubiquitously expressed transcription factors (Lee et al., 2006; Wilson et al., 2009). In this way the epigenetic regulation of genes, together with cell-type specific and ubiquitously expressed transcription factors define the gene expression pattern of each cell type (Lee et al., 2006; Wilson et al., 2009). The focus in this thesis has been on the TCR and cytokine induced pathways, which form an interrelated signalling network together with the co-stimulatory pathways. These signalling pathways will be discussed in more detail in the following chapters.

2.2.1 T cell receptor and co-stimulation induced signalling pathways

The initiation of Th cell differentiation requires the stimulation of TCR as well as co-stimulatory signals provided by APC. The strength of the TCR stimulus has been shown to influence the differentiation of naive Th cells: strong signals favouring Th1 and weak signals favouring Th2 differentiation (Constant et al., 1995; Leitenberg and Bottomly, 1999). The main signalling cascades activated by the TCR stimulus include the nuclear factor of activated T cells (NFAT), the nuclear factor kappa light chain enhancer in B cells (NF-κB) and the mitogen activated protein kinase (MAPK) pathways (Figure 2 and Figure 3).

The binding of TCR and CD4 to the MHC II-antigen complex activates a signalling cascade, which also needs a co-stimulatory signal by CD28-B7 ligation. The CD28 molecule on the T cell surface will bind to the inducible expressed B7 ligands (also called CD80 and CD86) on the surface of APC. The co-stimulatory signal provided by the CD28-B7 complex is crucial for activation, since T cells activated only through the TCR will either die or go to anergy. TCR ligation leads to the activation of a signalling cascade mediated by Lck kinase, tyrosine kinase ZAP-70 (ζ-chain associated protein), transmembrane scaffold protein LAT (linker of activated T cells) and phospholipase C-γ (PLC-γ). The activation of PLC-γ also needs a co-stimulatory signal by CD28-B7 ligation, mediated by the activation of Tec kinase Itk, which in turn activates PLC-γ. (Murphy et al., 2012; Smith-Garvin et al., 2009)

Activation of the TCR together with CD28 co-stimulation will signal through three different pathways to the activation of NFAT, NF-κB and AP1 transcription factors. First, the activation of PLC-γ leads to a change in the intracellular calcium levels and thus to the activation of calmodulin-calcineurin pathway (Gallo et al.,

2005). Calcineurin is a protein phosphatase and its target proteins include the NFAT proteins. NFAT proteins are retained in the cytosol in their phosphorylated form, but dephosphorylation by calcineurin enables their translocation into the nucleus to regulate the transcription of their target genes (Gallo et al., 2005; Macian, 2005). From the five members of the NFAT family, NFAT1 (also known as NFATc2, NFATp), NFAT2 (NFATc1, NFATc) and NFAT4 (NFATc3, NFATx) have been reported to have a role in the Th cell differentiation. Mice deficient of NFAT1 have a mild bias towards Th2 whilst the Th1 differentiation is reduced (Hodge et al., 1996; Kiani et al., 2001). However, the effect of NFAT1 on Th1/Th2 differentiation seems to be dependent on other factors present in the cell or the chromatin state, since NFAT1 can bind to the *Ifng* promoter and collaborate with TBET in activated Th1 cells, whereas it can bind to the *Il4* promoter in activated Th2 cells (Agarwal et al., 2000; Lee et al., 2004). Mice deficient of both NFAT1 and NFAT4 have exacerbated Th2 responses (Ranger et al., 1998b), whereas NFAT2 deficient mice show diminished Th2 cytokine production, indicating that NFAT2 is a positive regulator of Th2 differentiation (Ranger et al., 1998a; Yoshida et al., 1998). On the basis of these observations NFAT appears to play a complex role in Th cell differentiation that is related to other cellular factors and chromatin accessibility in the cell.

PLC-γ activates the family of NF-κB transcription factors through protein kinase C θ (PKC-θ) (Sun et al., 2000). Similarly to NFAT proteins, NF-κB seems to promote and inhibit Th1 and Th2 differentiation depending on other factors in the cell. The different NF-κB family members have been shown to positively influence Th1 differentiation by inducing the expression of IFNγ, TBET and STAT4 (Aronica et al., 1999; Corn et al., 2003; McCracken et al., 2007), whereas other studies have suggested that they have also a role in Th2 differentiation in promoting the IL2 production and regulating the GATA3 expression (Corn et al., 2005; Verweij et al., 1991).

The third pathway activated by the TCR/CD28 signalling is the MAPK pathway. The MAPK pathway consists of a number of kinases that activate each other by phosphorylation and finally activate Fos or Jun proteins that form a heterodimeric transcription factor activator protein 1 (AP1) (Rauscher et al., 1988). One of the TCR activated MAPK kinases is the extracellular signal-regulated kinase (ERK). ERK activity is required for Th2 differentiation, as has been shown by its role in regulating the IL4 expression and promoting the stability of GATA3 through the inhibition of proteasomal degradation (Tripathi et al., 2012; Yamashita et al., 2004; Yamashita et al., 2005). In addition, the p38 MAPK can phosphorylate GATA3 to promote its nuclear localization (Maneechotesuwan et al., 2007). p38 MAPK and another MAPK kinase, c-Jun N-terminal kinase (JNK),

have also been shown to promote IFNγ production (Dong et al., 2000; Rincón et al., 1998). In addition, AP1 activated by MAPK signalling as well as NFAT activated by calcium signalling and NF-κB, co-operate with each other and furthermore with other factors such as TBET, GATA3 or c-MAF (Macian, 2005; Zhu et al., 2010). Thus the TCR stimulated pathways regulate the differentiation of Th cells in collaboration with other signalling pathways activated in the cell.

In addition to the TCR stimulus, co-stimulatory signals provided by the APC determine the outcome of the activation and differentiation process. Important co-stimulatory signals for the Th cell development include CD28/B7, OX40-OX40L, LFA-1/ICAM and Notch signalling. The most important and best characterized is CD28-B7 signalling, which has been shown to be more important for Th2 than Th1 differentiation by enhancing the production of Th2 cytokines (Corry et al., 1994; Lenschow et al., 1996; Rulifson et al., 1997; Verweij et al., 1991). In addition, OX40-OX40L pathway seems to drive Th2 differentiation (Ito et al., 2005), whereas LFA-1/ICAM has been shown to inhibit Th2 responses (Salomon and Bluestone, 1998) and to induce Th1 responses (Smits et al., 2002). Furthermore, signalling through the Notch receptor interaction with different ligands on the cell surface of the APC has been shown to affect Th1/Th2 polarization. The binding of the Notch receptor to Delta-like ligand favours Th1 differentiation, whereas its binding to Jagged ligands has opposite action by activating GATA3 and IL4 expression and thus inducing the Th2 response (Amsen et al., 2009).

2.2.2 Factors regulating Th1 cell differentiation

In addition to the TCR signalling, cytokines and cytokine induced signalling pathways are crucial for the differentiation of the Th cell subtypes. Cytokines, IFNγ, IL12, IL18 and IL27 as well as transcription factors, TBET, STAT1, STAT4 and RUNX3, have been shown to be important for the development of the optimal Th1 cell response (Figure 2). IL12 signalling through STAT4 as well as IFNγ signalling mediated by STAT1 drive the differentiation of Th1 cells. IFNγ is also the hallmark cytokine produced by Th1 cells, thus forming a positive autoregulative loop for the differentiation. The expression of the master regulator transcription factor of Th1 cells, TBET, is induced by IFNγ/STAT1 signalling. IL18 co-operates with IL12 in inducing Th1 cell differentiation, whereas IL27 can activate STAT1.

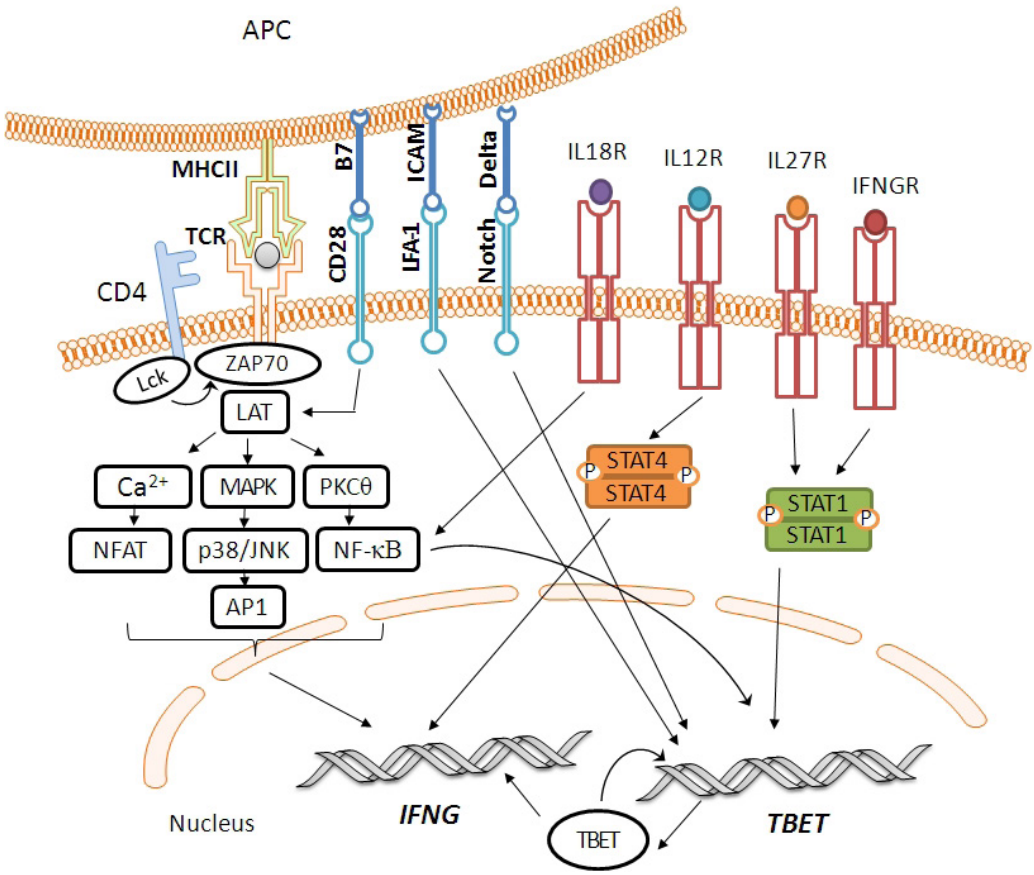

Figure 2. An overview of the signalling pathways promoting Th1 cell differentiation. The expression of IFNγ is induced by several different pathways. TCR activation and co-stimulation lead to activation of NFAT, NF-κB and AP1, which can all induce IFNγ expression. IFNγ signalling through STAT1, IL18 signalling through NF-κB, as well as stimulation of the cell through LFA-1 or Notch induces the expression of TBET. TBET in turn is a strong positive regulator of IFNγ expression forming a positive feedback loop. TBET is also able to activate its own expression. STAT1 can also be activated by signalling from IL27 receptor (IL27R). IL12/STAT4 signalling further induces the expression of IFNγ and enables the sustained IFNγ secretion.

2.2.2.1 IL12 and STAT4

IL12 was the first cytokine shown to be important for Th1 differentiation (Hsieh et al., 1993). IL12 is produced mainly by macrophages and dendritic cells, but also by monocytes, neutrophils and B cells in response to different pathogens (Watford et al., 2004). However, IL12 signalling is impaired in naive Th cells due to the lack of IL12Rβ2 expression, which forms the IL12 receptor together with IL12Rβ1 (Szabo et al., 1997). The initial activation of TCR leads to the recruitment of Brahma related gene 1 (BRG1), which is a component of the BAF chromatin remodelling complex, to the enhancer and promoter region of *Il12Rb2,* followed by histone hyperacetylation and a low level of IL12Rβ2

expression (Letimier et al., 2007). In addition, IFNγ/STAT1 signalling through TBET has been suggested to induce IL12Rβ2 expression (Afkarian et al., 2002; Chang et al., 1999; Mullen et al., 2001; Szabo et al., 1997). When the expression of IL12Rβ2 is established, IL12 is able to bind to its receptor and activate the downstream signalling by IL12R bound Janus kinase 2 (JAK2) and Tyrosine kinase 2 (TYK2), which phosphorylate STAT4 (Watford et al., 2004). Furthermore, IL12 and STAT4 form a positive autoregulative loop and active phosphorylated STAT4 is able to directly induce the expression of IL12Rβ2 (Lawless et al., 2000; Letimier et al., 2007; Nishikomori et al., 2002), thus maintaining the IL12 responsiveness of developing Th1 cells. In addition to IL12Rβ2, STAT4 induces the expression of other Th1 lineage genes, such as IL18R1 (Lawless et al., 2000; Wei et al., 2010), H2.0-like homeobox protein HLX (Thieu et al., 2008) as well as IFNγ (Barbulescu et al., 1998; Wei et al., 2010). In line with this, mouse deficient of STAT4 exhibit a diminished Th1 response, IFN-γ production and an increased susceptibility to infection by intracellular pathogen *Listeria monocytogenes* (Kaplan et al., 1996b), and similarly patients with deficient STAT4 expression have reduced Th1 responses (Chang et al., 2009).

Furthermore, IL18 can co-operate with IL12 to promote the induction of IFNγ expression, although its role seems to be complex. IL18 is not necessary for Th1 differentiation but it enhances the effect of IL12 in inducing IFNγ production in both human and murine Th1 cells (Micallef et al., 1996; Okamura et al., 1995). IL18 not only activates NF-κB signalling in Th1 cells, but also AP1 and JNK pathways (Barbulescu et al., 1998; Matsumoto et al., 1997; Robinson et al., 1997). However, it is also able to promote Th2 cytokine production in the absence of IL12 (Yoshimoto et al., 2000) and thus its role in promoting Th1 differentiation seems to be dependent on IL12.

2.2.2.2 IFNγ/STAT1 and IL27

IFNγ is considered as the hallmark cytokine produced by Th1 cells and it is also an important cytokine for the differentiation of Th1 cell lineage. NK, CD8+ and CD4+ Th1 cells are the major sources of this cytokine. In addition, naive T cells, B cells, macrophages as well as dendritic cells are able to produce IFNγ. It regulates both innate and adaptive immune responses to promote the elimination of intracellular as well as other pathogens. (Szabo et al., 2003)

The expression of IFNγ during Th1 differentiation is regulated by several mechanisms. Naive T cells can produce IFNγ in response to TCR activation. This is mediated by NFAT and NF-κB transcription factors (Aronica et al., 1999; Corn et al., 2003; Kiani et al., 2001; Peng et al., 2001; Sica et al., 1997). In addition, TCR activation induces the MAPK pathway and especially the p38 MAP kinase has been shown to induce IFNγ expression (Rincón et al., 1998). Furthermore, transcription factors activating transcription factor 2

(ATF2) and ATF3 in a complex with JUN and AP1 have also been shown to induce IFNγ expression by positively regulating the *IFNG* promoter (Barbulescu et al., 1997; Filen et al., 2010; Jones and Chen, 2006). In addition, the IL12/STAT4 induced transcription factor Interferon regulatory factor 1 (IRF1) promotes the expression of IFNγ, although it seems not to be able to directly regulate the *IFNG* promoter (Lohoff et al., 1997).

IFNγ induces the differentiation of Th1 cells through STAT1 signalling (Szabo et al., 2003). The binding of IFNγ to its receptor (IFNGR) leads to the activation of JAK1 and JAK2, and to the subsequent phosphorylation and activation of STAT1 (Greenlund et al., 1994; Ramana et al., 2002). Phosphorylated STAT1 is then able to homodimerize and relocate into the nucleus to induce the expression of its target genes, such as TBET (Afkarian et al., 2002; Lighvani et al., 2001). This signalling cascade forms an autoregulative loop for IFNγ production because TBET is an important inducer of IFNγ production (Szabo et al., 2000). Furthermore, IL12 induced STAT4 together with TBET maintains IFNγ expression during Th1 cell differentiation (Barbulescu et al., 1998; Lawless et al., 2000; Letimier et al., 2007; Nishikomori et al., 2002), whereas GATA3 is shown to act as a repressor of IFNγ production. GATA3 seems to mediate the negative effect on IFNγ production by down-regulating STAT4 both in human and mouse (Kaminuma et al., 2004; Usui et al., 2003).

Another cytokine, IL27, is also able to induce STAT1 phosphorylation (Owaki et al., 2005; Pflanz et al., 2002; Takeda et al., 2003). IL27 is related to IL12 and IL18 and the receptor for IL27, IL27R (composed of T cell cytokine receptor (TCCR)/WSX-1 and gp130 chains), is expressed early on in the development of the T cell (Chen et al., 2000; Pflanz et al., 2004; Yoshida et al., 2001). IL27 signalling through STAT1 has been shown to induce the expression of IFNγ and IL12 receptor β2 (IL12Rβ2) (Hibbert et al., 2003; Takeda et al., 2003) and IL27 synergizes with IL12 to induce the Th1 cell differentiation (Pflanz et al., 2002). However, IL27R is down-regulated after TCR activation (Owaki et al., 2005), which implicates that it might be important for the initiation of Th1 differentiation but not required for further development. In line with this, WSX-1 deficient mice show an impaired IFNγ response and an insufficient early responses to *L. major* infection, but this impairment in the Th1 response is transient and mice will eventually clear the infection as well as wild-type mice (Yoshida et al., 2001). In addition, IL27 seems also to have an anti-inflammatory role since IL27 has been shown to induce the production of the anti-inflammatory cytokine IL10, and to suppress the differentiation of Th17 cells (Diveu et al., 2009; Murugaiyan et al., 2009; Yoshida and Yoshiyuki, 2008). In fact, IL27 induced IL10 production by Th1 cells has been shown to be crucial for protection against immunopathology during *Malaria* infection in mice (Findlay et al., 2010; Freitas do Rosário et al., 2012). Thus, the role of IL27 seems not only to be driving Th1 differentiation but also to suppress excessive immune responses.

2.2.2.3 TBET, HLX and RUNX3

The IFNγ/STAT1 pathway induces and maintains a high level of TBET expression (also called TBX21), the well-characterized master regulator transcription factor of Th1 cells and important factor for the IFNγ production (Lighvani et al., 2001; Mullen et al., 2001; Szabo et al., 2000; Szabo et al., 2002). The ectopic expression of TBET in both murine and human Th2 cells was shown to induce IFNγ production while down-regulating the expression of Th2 cytokines (Lametschwandtner et al., 2004; Sundrud et al., 2003; Szabo et al., 2000). The importance of TBET on Th1 cell differentiation was further characterized by using knockout animals. The $TBX21^{-/-}$ mice show decreased IFNγ secretion, impaired Th1 response and clearance of *L. major* infection as well as increased Th2 response and asthmatic like condition (Finotto et al., 2002; Szabo et al., 2002). TBET has been shown to directly bind to conserved sequences at the *Ifng* locus and induce active chromatin marks (Mullen et al., 2002; Mullen et al., 2001; Shnyreva et al., 2004; Szabo et al., 2000; Zhu et al., 2012). Furthermore, TBET induces the expression of Th1 specific chemokine receptor CXCR3 (Thieu et al., 2008) and up-regulates the IL12Rβ2 expression, thus enabling IL12/STAT4 signalling (Afkarian et al., 2002; Mullen et al., 2001). Some contradictory data also exist showing only slightly increased TBET-mediated IL12Rβ2 expression (Letimier et al., 2007; Usui et al., 2006) or no change in the IL12Rβ2 expression by the deletion of TBET or by its retroviral expression in mature Th1 cells (Usui et al., 2006). If IL4 is neutralized, IFNγ production can be obtained from TBET deficient CD4+ T cells, and thus it has been proposed that the most important role of TBET in Th cell differentiation is the inhibition of GATA3 activity and not the activation of IFNγ expression (Usui et al., 2006). However, recently Zhu *et al.* (Zhu et al., 2012) showed with the ChIP-Seq (Chromatin immunopresipitation followed by sequencing) method that TBET binding is required for active chromatin marks for several Th1 specific genes, including *Ifng, Il12Rb2* and *Cxcr3,* and in turn TBET suppressed other lineage specific genes, such as Th17-specific chemokine receptor *Ccr6* and Th2-specific genes *Gata3, Il4, Il5* and *Ccr4*.

The expression of TBET has been shown to be induced by other factors such as TCR activation, Notch signalling, NFAT signalling, the NF-κB pathway, as well as the IL12/STAT4 pathway. NF-κB induces TBET expression by binding to the *TBX21* promoter (Corn et al., 2003; McCracken et al., 2007), whereas TCR signalling through NFAT activates chromatin remodelling at the *TBX21* locus (Placek et al., 2009). In addition, the Notch signalling pathway and a ubiquitously expressed transcription factor Sp1 have been shown to positively influence TBET expression (Minter et al., 2005; Yu et al., 2007). Ectopic expression of TBET is also able to induce the endogenous TBET expression in a STAT4-independent and STAT1-dependent manner (Afkarian et al., 2002; Mullen et al., 2001;

Szabo et al., 2000). Recently it was shown that TBET is also able to directly autoregulate its own expression by binding to the *TBX21* promoter (Kanhere et al., 2012).

TBET mediates the regulation of transcription by interacting with other transcription factors such as Runt related transcription factor RUNX3 and H2.0-like homeobox protein HLX. The expression of both of these factors is also positively regulated by TBET (Djuretic et al., 2007; Martins et al., 2005; Mullen et al., 2002). RUNX3 co-operates with TBET to enhance IFNγ expression in Th1 cells, although it is not compulsory for the production of IFNγ (Djuretic et al., 2007). However, for the repression of IL4 expression, RUNX3 protein-protein interaction with TBET is required (Djuretic et al., 2007; Naoe et al., 2007). TBET can also co-operate with the HLX transcription factor to promote optimal IFNγ production (Mullen et al., 2002). HLX is selectively expressed in Th1 cells, and similarly to TBET, is positively regulated by STAT4 (Kaneko et al., 2007; Thieu et al., 2008).

The suppression of the Th2 lineage is another major role for TBET. Tyrosine phosphorylated TBET can interact with GATA3 to suppress the Th2 lineage gene expression by preventing the binding of GATA3 to DNA (Hwang et al., 2005; Usui et al., 2006), as well as directly suppressing the *Gata3* expression by binding to its gene locus (Jenner et al., 2009; Zhu et al., 2012). In addition, TBET and GATA3 have been shown to bind the same genomic regions especially in Th1 cells. TBET seems to mediate GATA3 binding for most of these sites since GATA3 binding sites are not present (Kanhere et al., 2012). Thus it seems that the role of TBET in this GATA3 binding context is to sequester GATA3 away from its own Th2 specific binding sites. GATA3 also modifies the *Tbx21* locus during Th2 differentiation (Wei et al., 2011), thus directly suppressing TBET expression. Together these data highlight the importance of TBET in orchestrating the Th1 cell differentiation and active transcription of lineage specific genes as well as suppressing the other lineages.

The differentiation of Th1 cells is regulated by a network of signalling pathways that co-operate or antagonize each other to achieve the desired phenotype. IL12 and STAT4 can induce TBET expression, whereas TBET is able to induce IL12Rβ2 expression. In addition, STAT4 and TBET seem to co-operate and be both critical for the optimal IFNγ production (Thieu et al., 2008; Zhu et al., 2012). TBET and STAT4 also collaborate in the up-regulation of IL18R1, HLX and IL12Rβ2 (Thieu et al., 2008). Similarly to TBET, STAT4 is able to suppress the Th2 lineage, and in line with this, $STAT4^{-/-}$ mice show an enhanced Th2 response (Kaplan et al., 1996b). In the light of recent studies it seems that although STAT4 has been shown to promote the Th1 differentiation in the absence of TBET (Usui et al., 2006), the co-operation of both of these factors is required for the optimal Th1 differentiation and suppression of Th2 lineage genes in developing Th1 cells both *in vitro* and *in vivo* (Thieu et al., 2008; Zhu et al., 2012).

2.2.3 Factors regulating Th2 cell differentiation

The differentiation of naive T cells into Th2 cells is induced by several factors in addition to the initial TCR activation. Cytokines IL4, TSLP and IL2 as well as transcription factors STAT6, GATA3 and STAT5 all play a role in this process. IL4 signalling through STAT6 and IL2 that activates STAT5 are required for the optimal polarization of Th2 cells. IL4/STAT6 signalling induces the expression of the Th2 master regulator transcription factor, GATA3, which in turn is important in the expression of the Th2 cytokines, IL4, IL5 and IL13. Thymic stromal lymphopoietin (TSLP) is also able to activate STAT5 (Figure 3).

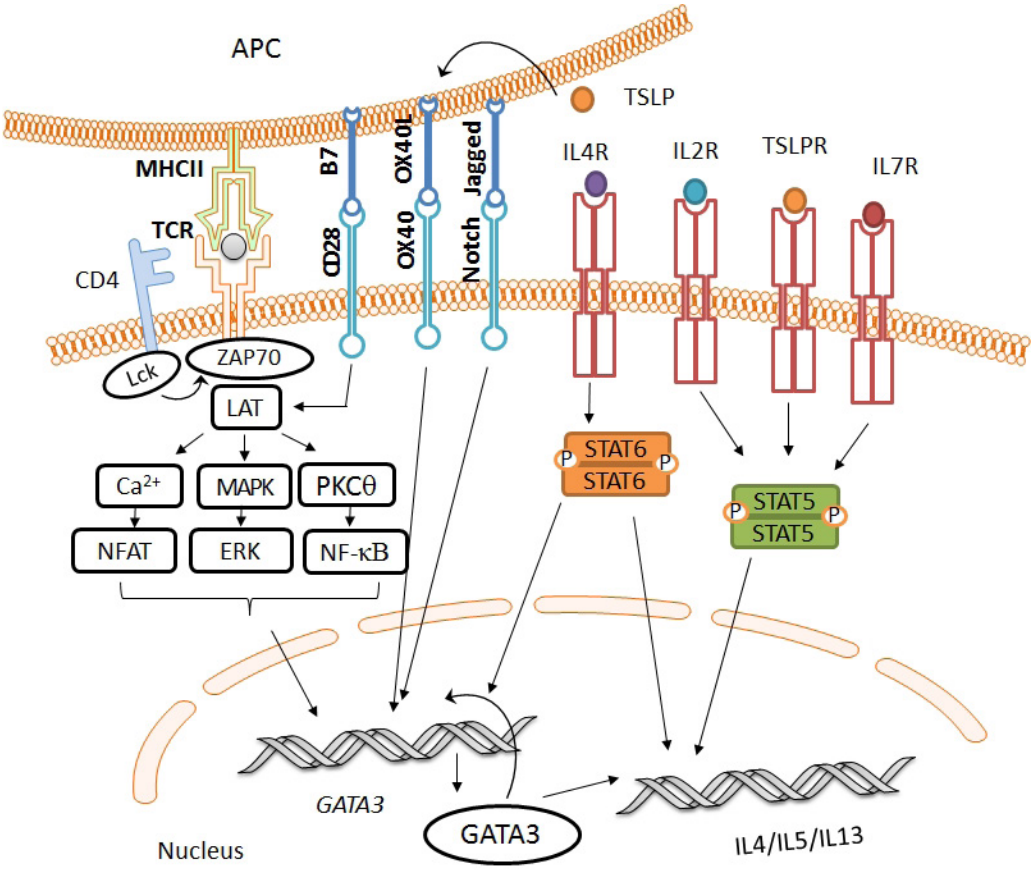

Figure 3. An overview of the signalling pathways promoting the Th2 cell differentiation. The Th2 cytokine locus consisting of *Il4, Il5* and *Il13* genes is regulated by several pathways. TCR activation and co-stimulation induced pathways through NFAT, NF-κB and MAPK induce the expression of Th2 cytokines. IL4 signalling through STAT6 induces the expression of GATA3, which in turn can induce the Th2 cytokine production but also its own expression. Furthermore, STAT5, which can be activated by IL2, TSLP and IL7, is also a positive regulator of Th2 cytokine production. TSLP can also act on the APC to induce the expression of OX40L, which in turn enhances the Th2 differentiation by OX40L-OX40 ligation.

2.2.3.1 IL4 and STAT6

IL4 is the hallmark cytokine produced by the Th2 cells (Nelms et al., 1999), and in addition, naive Th cells can be differentiated into Th2 cells in the presence of IL4 (Le Gros et al., 1990; Swain et al., 1990). The *Il4*$^{-/-}$ mice show reduced levels of IgE as well as diminished production of Th2 cytokines, highlighting the role of IL4 in the Th2 differentiation (Kopf et al., 1993; Kühn et al., 1991). Naive Th cells express some IL4R complex, but its expression is rapidly up-regulated by IL4 (Dokter et al., 1992; Ohara and Paul, 1988), thus making the cells more responsive to IL4. IL4 signalling through STAT6 is crucial for the Th2 response and mice deficient of STAT6 have an even more impaired Th2 development than IL4 knockout mice (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996). STAT6 knockout mice are also resistant to allergen induced airway inflammation (Kuperman et al., 1998). Furthermore, the ectopic expression of STAT6 in polarized Th1 cells is sufficient to induce the production of Th2 cytokines and up-regulate the transcription of GATA3, the master regulator of Th2 cells (Kurata et al., 1999; Zhu et al., 2001). The importance of STAT6 as a mediator of IL4 signalling was also shown in human Th2 cells where 80% of the IL4 induced genes were STAT6-dependent (Elo et al., 2010). Although STAT6 is mainly described as a transcriptional activator, it mediates the transcriptional repression of several genes and can induce epigenetic modifications in the chromatin (Elo et al., 2010; Wei et al., 2010). In addition, STAT6 binding sites were shown to co-localize with mainly accessible chromatin marks and on some of the sites modifications were dependent on the presence of STAT6 (Wei et al., 2010). Therefore, it is clear that STAT6 regulates the gene expression in several ways.

The STAT6 level in Th cells remains relatively constant, but it is activated by phosphorylation in response to the binding of IL4 to the IL4R complex consisting of IL4Rα chain and the common γ chain (Mueller et al., 2002). The binding of IL4 to IL4R complex activates the IL4R-bound JAK1 and JAK3, which in turn will phosphorylate STAT6 molecules (Nelms et al., 1999). Phosphorylated STAT6 molecules dimerize and are transported to the nucleus to regulate the gene expression of their target genes (Chen and Reich, 2010; Nelms et al., 1999). In addition to tyrosine phosphorylation by JAK1 and JAK3, STAT6 is also subject to other post-translational modifications such as serine phosphorylation, methylation and acetylation. The serine phosphorylations of the STAT6 transactivation domain may influence the DNA binding of STAT6 (Pesu et al., 2000; Shirakawa et al., 2011; Wang et al., 2004), whereas the acetylation seems to control the transcriptional regulator activity of STAT6 (Mcdonald and Reich, 1999; Shankaranarayanan et al., 2001). The methylation of STAT6 seems to regulate both the IL4 induced tyrosine phosphorylation and nuclear transportation of STAT6 (Chen et al., 2004) although contradictory data also exists (Chen and Reich, 2010).

STAT6 target genes have been identified by microarrays (Chen et al., 2003; Chen et al., 2003; Lund et al., 2007), and more recently also by ChIP-Seq methods (Elo et al., 2010; Wei et al., 2010). The most important of the STAT6 target genes in the context of Th2 cell differentiation, is the master regulator of the Th2 cell lineage, GATA3. STAT6 is both sufficient and necessary to induce high levels of GATA3, thus mediating the Th2 polarization (Chen et al., 2003; Elo et al., 2010; Kurata et al., 1999; Lund et al., 2007; Ouyang et al., 1998; Zhu et al., 2001). In addition to GATA3, STAT6 regulates a number of genes required for the initiation and maintenance of the Th2 lineage. These genes include IL4Rα (Nelms et al., 1999) and the AP1 family transcription factor, c-MAF, which is selectively up-regulated in Th2 cells, enhancing the expression of IL4 but not other Th2 cytokines (Ho et al., 1996; Kim et al., 1999). c-MAF has also been shown to regulate the expression of IL2Rα (CD25) in developing Th2 cells making the cells more responsive to IL2 (Hwang et al., 2002). STAT6 can also collaborate with transcriptional co-activators including members of the basal transcription machinery, such as CBP, p100 and p300 (Gingras et al., 1999; Mcdonald and Reich, 1999; Yang et al., 2002), as well as with two members of the p160/nuclear receptor co-activator (NCoA) family, NCoA-1 and NcoA-3 (Arimura et al., 2004; Litterst and Pfitzner, 2001; Litterst and Pfitzner, 2002), to promote the expression of STAT6 target genes.

2.2.3.2 GATA3

The transcription factor GATA3 is a well-defined master regulator of the Th2 cell lineage (Pai et al., 2004; Zheng and Flavell, 1997; Zhu et al., 2004). It has been shown to be up-regulated specifically in Th2 cells, while its expression is not induced in developing Th1 cells (Zhang et al., 1997; Zheng and Flavell, 1997). High levels of GATA3 expression are induced by IL4/STAT6-dependent pathways, but some GATA3 expression can be achieved independent of IL4/STAT6 (Ho et al., 2009; Zheng and Flavell, 1997). The ectopic expression of GATA3 in the polarized Th1 cells is able to promote the Th2 cytokine expression (Ho et al., 2009; Kurata et al., 1999; Zheng and Flavell, 1997). Whereas some Th2 development can be achieved in the absence of STAT6, IL4 or IL2/STAT5 pathway, GATA3 seems to be indispensable for the Th2 development both in human and mouse (Ouyang et al., 2000; Pai et al., 2004; Zhu et al., 2004). Apart from Th2 differentiation, GATA3 has other roles in embryonic development, e.g. as demonstrated by the embryonic lethality of GATA3 knockout mice (Pandolfi et al., 1995). However, by using chimeric mice defective of GATA3 as well as a T cell specific GATA3 conditional knockout animals, it has been shown that GATA3 is required for the CD4+/CD8+ T cell lineage commitment, CD4+ T cell development as well as for the Th2 cell differentiation both *in vitro* and *in vivo* (Hendriks et al., 1999; Pai et al., 2004; Ting et al., 1996; Zhu

et al., 2004). CD4+ T cells deficient of GATA3 differentiate into Th1 cells even in the absence of polarizing cytokines, thus emphasizing the importance of GATA3 for Th2 differentiation (Yamashita et al., 2004; Zhu et al., 2004). Accordingly, in humans GATA3 haploinsufficiency leads to a reduced Th2 response (Skapenko et al., 2004).

In addition to STAT6, the expression of GATA3 is regulated by other factors such as the Notch signalling, TCR activation and Interferon regulatory factor 4 (IRF4) transcription factor. The Notch signalling pathway has been shown to up-regulate GATA3 expression in a STAT6-independent manner (Amsen et al., 2007; Fang et al., 2007). A weak TCR activation leads to IL4-independent up-regulation of GATA3 and initiation of Th2 polarization. A TCR activation-induced transcription factor, NFAT1, has been shown to bind to the GATA3 promoter and to induce the GATA3 expression (Scheinman and Avni, 2009). Furthermore, T cell factor 1 (TCF1) mediates the TCR-induced early GATA3 expression in a STAT6- and Notch-independent manner and down-regulates the expression of IFNγ (Yu et al., 2009a). A transcriptional repressor, Growth factor independent 1 (GFI-1), is up-regulated by TCR activation and further induced by IL4 signalling (Zhu et al., 2002). GFI-1 is important for increasing the proliferation of Th2 cells expressing high levels of GATA3 in response to IL2 and it is also involved in preventing proteasomal degradation of GATA3 protein, thus stabilizing GATA3 (Shinnakasu et al., 2008; Zhu et al., 2002; Zhu et al., 2006). GFI-1 also suppresses the differentiation of Th1, Th17 and Treg lineages (Zhu et al., 2002; Zhu et al., 2009). IRF4 is another transcription factor that has been shown to be important for Th2 differentiation (Lohoff et al., 2002; Rengarajan et al., 2002). IRF4 deficient Th cells have impaired IL4 production, but this can be rescued by GATA3 overexpression (Lohoff et al., 2002) suggesting that IRF4 might be a positive regulator of GATA3 expression. The expression of IRF4 was also shown to be elevated in allergic asthma patients and the levels of IRF4 positively correlated with the elevated levels of GATA3 (Xia et al., 2012).

GATA3 has been shown to bind to the Th2 cytokine locus consisting of *Il4, Il5* and *Il13* genes controlling the acetylation and accessibility of the locus (Yamashita et al., 2004). Whereas the deletion of GATA3 during Th2 polarization impairs the production of all the Th2 cytokines, in committed Th2 cells only IL5 and IL13 expression is abolished by the GATA3 deletion, although the expression of IL4 is mildly diminished (Zhu et al., 2004). Although GATA3 directly binds to the *Il5* and *Il13* promoters, no GATA3 binding site has been found in the *Il4* promoter (Jenner et al., 2009; Kishikawa et al., 2001; Lee et al., 2001; Spilianakis and Flavell, 2004; Zhang et al., 1998). However, GATA3 is an important regulator of IL4 expression, and the binding of GATA3 to a HS2 enhancer region in the *Il4* intronic region regulates the chromatin modifications of

the *Il4* locus (Tanaka et al., 2010). The binding of GATA3 to this region was required for the expression of IL4 but not for IL13 or IL5 expression. In addition, a genome-wide analysis of GATA3 binding sites has shown that it binds to several genes in the different Th lineages, and in a cell-type specific manner can either activate or repress the expression of its target genes (Wei et al., 2011). GATA3 was also shown to induce both repressive and active chromatin marks in a cell-type specific manner (Wei et al., 2011). While GATA3 was shown to bind 100 genes in both Th1 and Th2 cells, the number of GATA3 bound genes specific to Th2 cells was 10 times higher (Horiuchi et al., 2011), indicating the importance of GATA3 in Th2 lineage specific gene expression. Furthermore, the up-regulation of many Th2 induced genes was shown to require both GATA3 and STAT6 binding, implying that co-operation between these two factors is important for the efficient Th2 differentiation (Horiuchi et al., 2011).

GATA3 is not only important for the initiation of Th2 differentiation, but it also actively represses the expression of Th1 lineage genes. GATA3 represses the expression of IL12Rβ2 and STAT4 by directly binding to these gene loci (Ouyang et al., 1998; Usui et al., 2003). In addition, the expression of IFNγ is repressed by several mechanisms under the Th2 polarizing conditions. First, GATA3 can bind to the distal regions in *Ifng* gene, although it does not bind to the *Ifng* promoter (Wei et al., 2011). Second, GATA3 can impair the expression of IFNγ by protein-protein interaction with RUNX3 or TBET (Yagi et al., 2010). RUNX3 mediates the production of IFNγ in GATA3 deficient cells even under Th2 polarizing conditions in an IL12/STAT4- and IFNγ/TBET-independent manner (Yagi et al., 2010). The latter indicates that GATA3 actively represses the RUNX3-mediated IFNγ production under the Th2 polarizing conditions.

2.2.3.3 IL2/STAT5 and TSLP

Another signalling pathway important for the Th2 cell differentiation is IL2 signalling through STAT5. Mouse CD4+ cells fail to differentiate into Th2 cells *in vitro* in the absence of IL2 even if IL4 is present (Le Gros et al., 1990). Although IL2 is known to regulate Th cell proliferation, survival and activation-induced cell death (Le Gros et al., 1990), it has also been shown to be required for the differentiation of Th2 cells through the activation of STAT5 (Liao et al., 2011a). STAT5 has been shown to regulate the IL4Rα expression during the initiation of Th2 differentiation, thus making the cells more responsive to IL4 (Liao et al., 2008). This regulation of IL4Rα is IL2 dependent and an impaired Th2 differentiation in *Il2*$^{-/-}$ mice could be restored by the forced expression of IL4Rα (Liao et al., 2008). Furthermore, STAT5 has been shown to augment IL4 production in an IL2 dependent manner by directly binding to the *Il4* locus making it more accessible (Cote-Sierra et al., 2004; Takatori et al., 2005; Zhu et al., 2003). In

addition to the priming of the Th2 differentiation, at later stages of the Th2 polarization, STAT5 binds to the *Maf* and *Gata3* promoters to enhance their expression (Liao et al., 2008). The retroviral expression of STAT5 is able to drive Th2 differentiation without enhancing the expression of GATA3 (Cote-Sierra et al., 2004; Zhu et al., 2003). However, a basal expression level of GATA3 is required for the differentiation of Th2 cells since the ectopic expression of constitutively active STAT5 is not sufficient to drive Th2 differentiation in the GATA3 deficient cells (Zhu et al., 2004). This indicates that co-operation between STAT5 and GATA3 is required for a proper Th2 response. However, although the activity of STAT5 has been shown to be important for Th2 differentiation, under the Th1 polarizing conditions IL2/STAT5 can also induce *Il12Rbβ2* and *Tbx21* loci and inhibit Th17 differentiation (Liao et al., 2011b).

Thymic stromal lymphopoietin (TSLP) and IL7 can also activate STAT5 signalling. TSLP is produced by epithelial cells and innate immune cells (Paul and Zhu, 2010), and it is shown to induce the allergic airway inflammation (Soumelis et al., 2002; Zhou et al., 2005). In line with this, the expression of TSLP is increased in asthmatic airways correlating with the severity of disease (Ying et al., 2005). This indicates that it might be an important regulator of the Th2 cell responses *in vivo.* The effect of TSLP on Th2 cells is at least partly mediated by its effect on triggering the dendritic cells to induce the Th2 development by OX40-OX40L interaction (Liu et al., 2007), but not by IL4 since TSLP activated dendritic cells do not produce IL4 (Ito et al., 2005). TSLP activated dendritic cells induce the production of IL4 by Th2 cells as well as produce the Th2-attracting chemokines thus attracting Th2 cells to the site of allergic inflammation (Ito et al., 2005; Soumelis et al., 2002). TSLP has also been shown to directly regulate Th2 cell differentiation through binding to its receptor and thus activating STAT5 (Kitajima et al., 2011). Direct binding of TSLP to its receptor was shown to enhance the proliferation of Th2, but not Th1 or Th17 cells (Kitajima et al., 2011).

2.3 Caspase activity and T helper cell differentiation

2.3.1 Role of caspase activity in T helper cell differentiation

In addition to the TCR stimulus and the cytokine milieu, T cell development is also regulated by caspase pathways, which usually regulate programmed cell death, i.e. apoptosis. Apoptosis pathways can be activated by death receptors such as CD95 (also known as FAS), which is expressed by thymocytes and activated T cells, as well as in other cell types. Activation of CD95 leads to the recruitment of caspase proteases and adaptor proteins, such as FAS-associated via death domain (FADD), to the receptor

and to formation of Death inducing signalling complex (DISC) (Riedl and Shi, 2004). Caspase activity is tightly regulated, because excessive, non-regulated cell death would be harmful to any individual or animal. However, increasing data have shown that the caspase activity is not only important for the regulation of apoptosis, but also required for the regulation of cell proliferation and growth. First, *CASP8* knockout mice were found to die at embryonic stage (Varfolomeev et al., 1998) and an inherited genetic deficiency in *CASPASE-8* in humans showed defects in T cell, B cell and NK T cell activation leading to increased susceptibility to viral infections (Chun et al., 2002). Humans have a close homolog of CASPASE-8, CASPASE-10, which is absent from mouse and this might compensate for the CASPASE-8 deficiency in humans (Kischkel et al., 2001). The conditional knockout mice of *Casp8* have defects in the NF-κB activity (Lemmers et al., 2007; Su et al., 2005) as well as in the T cell activation and proliferation and are unable to clear viral infection (Salmena et al., 2003). An important regulator of CASPASE-8 activity in its apoptotic and non-apoptotic functions is cellular FLICE inhibitory protein (c-FLIP; gene name *CFLAR*), which is discussed in more detail below.

2.3.2 Cellular FLICE inhibitory protein

c-FLIP (gene name *CASP8 and FADD apoptosis regulato*r; *CFLAR*) was originally cloned as a cellular homologue of viral FLIPs expressed by herpesviruses and molluscipoxviruses (Goltsev et al., 1997; Han et al., 1997; Hu et al., 1997; Inohara et al., 1997; Irmler et al., 1997; Rasper et al., 1998; Shu et al., 1997; Srinivasula et al., 1997; Thome et al., 1997). The v-FLIPs have the ability to inhibit CASPASE-8 activity and thus promote the survival of the virus infected cell (Thome et al., 1997). The location of *CFLAR* gene close to its homologues *CASPASE-8* and *CASPASE-10* in human chromosome 2q33-34 (Hadano et al., 2001; Rasper et al., 1998) indicates that these genes may have arisen by gene duplication.

A wide variety of cells from hematopoietic cells, kidney and heart to skeletal muscle cells express c-FLIP constitutively (Irmler et al., 1997; Rasper et al., 1998) and the expression of c-FLIP is regulated by several signalling pathways or transcription factors including ERK (Yeh et al., 1998a), NF-κB (Kreuz et al., 2001; Micheau et al., 2001), NFAT1 and NFAT2 (Ueffing et al., 2008) and STAT3 (Kovalovich et al., 2001). Although c-FLIP was originally cloned as an inhibitor of CD95 induced CASPASE-8 activity, it has also been shown to regulate many signalling cascades such as NF-κB and ERK in response to TCR activation (Kataoka et al., 2000). In fact, studies with c-FLIP transgenic mice have shown that c-FLIP is required for T cell survival and proliferation (Dohrman et al., 2005; Lens et al., 2002).

The *CFLAR* gene has been characterized in multiple different mRNA splice variants, but in humans only three of them have been reported to be translated at the protein level, the long isoform (c-FLIP$_L$, 55kDa) and two short isoforms, c-FLIP short (c-FLIP$_S$; 26 kDa) and c-FLIP raji (c-FLIP$_R$; 24 kDa) (Golks et al., 2005; Irmler et al., 1997; Shu et al., 1997) (Figure 4). All three isoforms have a common N-terminal region of 202 amino acids and a unique C-terminal tail. c-FLIP$_L$ is a CASPASE-8/10 homolog having two death effector domains (DED) and an inactive caspase domain in the C-terminal end (Irmler et al., 1997). Two shorter isoforms, c-FLIP$_S$ and c-FLIP$_R$ have two DED-domains, but their C-terminal tails are only 19 and 11 amino acids, respectively, and do not contain the inactive caspase domain (Golks et al., 2005; Irmler et al., 1997). The DED-domains mediate dimerization with CASPASE-8 and the formation of DISC (Irmler et al., 1997). The c-FLIP isoforms arise from the same transcript by alternative splicing and the expression of two short isoforms is regulated by a single nucleotide polymorphism (SNP) located in the 3' splice site of intron 6 of the human *CFLAR* gene (Ueffing et al., 2009). Ueffing *et al.* analysed the evolutional conservation of the two short isoforms and showed that the c-FLIP$_R$ isoform is the more conserved and the only short c-FLIP isoform expressed in for example mice, rats and dogs (Ueffing et al., 2009). However, in humans both the transcription and translation of c-FLIP$_R$ is less efficient than that observed for the c-FLIP$_S$ and thus its expression level is normally lower (Golks et al., 2005; Ueffing et al., 2009). An exception would be some of the B cell lymphoma cell lines like Raji, which was the original source of identification and naming of c-FLIP$_R$ isoform (Golks et al., 2005).

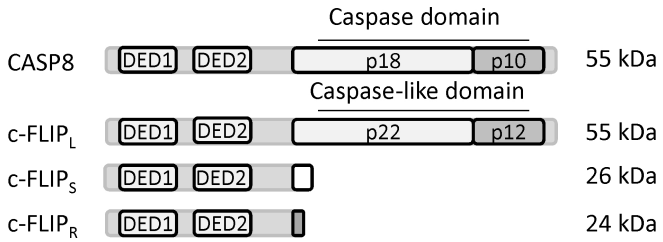

Figure 4. A simplified diagram of c-FLIP splice variants. c-FLIP$_L$ is a homologue of CASPASE-8, but instead of a caspase domain, it has an inactive caspase-like domain. The two short isoforms, c-FLIP$_S$ and c-FLIP$_R$, lack the caspase-like domains and have unique C-terminal tails of only 19 and 11 amino acids, respectively. All c-FLIP isoforms contain two DED domains through which they are able to interact with CASPASE-8.

In T cells, TCR activation rapidly up-regulates the expression of c-FLIP$_S$, which protects cells from activation induced cell death (AICD) (Kirchhoff et al., 2000a; Kirchhoff et al., 2000b). In addition, several cytokines have been shown to regulate the expression of c-FLIP. In CD8+ T cells, IL6 was shown to induce c-FLIP$_S$ (von Rossum et al., 2011)

and STAT3, which is activated by IL6, was shown to up-regulate c-FLIP expression in hepatocytes (Kovalovich et al., 2001). Interestingly, Th17 cells, which are differentiated in response to IL6 and TGFβ, were shown to express a higher level of c-FLIP$_L$ than Th1 cells (Yu et al., 2009b). In addition, Th17 and Th2 cells have been shown to be more resistant to apoptosis than Th1 cells (Fang et al., 2010) and the Th2 driving cytokine, IL4, was also shown to up-regulate c-FLIP$_S$ in human Th cells (Rautajoki et al., 2007) as well as in cancer cells (Conticello et al., 2004). Contradictory data also exists showing the down-regulation of c-FLIP by IL4 in mouse cells (Zhang et al., 2003). Furthermore, the IL12 and IL10 cytokines have also been shown to up-regulate the c-FLIP expression, whereas IL2 was shown to down-regulate the expression of c-FLIP$_L$ (Eslick et al., 2004; Lee et al., 2003; Refaeli et al., 1998). Therefore, several cytokines may regulate the expression of c-FLIP in Th cells, but its role in human Th cell differentiation is not clear.

CFLAR knockout mice are similar to *CASP8* and *FADD* knockout mice dying at embryonic day 10.5 mainly because of failure in the heart development (Varfolomeev et al., 1998; Yeh et al., 1998b; Yeh et al., 2000). Because of the embryonic lethality, conditional knockout mice of *CLAR* have been developed to gain more information on the function of c-FLIP in T cells (Chau et al., 2005; Zhang and He, 2005). These studies have shown that c-FLIP is essential for T cell survival and proliferation in response to the TCR activation (Chau et al., 2005; Zhang and He, 2005). ERK and NF-κB signalling pathways seemed to function normally in c-FLIP conditional knockout T cells, indicating that although c-FLIP has been shown to regulate these pathways, it is not essential for their regulation.

Transgenic mice overexpressing c-FLIP$_L$ show enhanced Th2 cell differentiation and are protected from the Th1 and Th17 cell driven autoimmune disease, EAE (experimental autoimmune encephalomyelitis) (Tseveleki et al., 2004) and sensitized to allergic airway inflammation (Wu et al., 2004). However, the overall numbers of peripheral T cells and thymocytes are normal (Lens et al., 2002). The c-FLIP$_L$ transgenic mice T cells proliferate more efficiently than wild-type T cells but are also more sensitive to apoptosis because of augmented CASPASE-8 activity (Dohrman et al., 2005; Lens et al., 2002) thus leading to normal cell numbers. Activation of resting T cells has been shown to lead to the migration of CASPASE-8 and c-FLIP$_L$ into lipid rafts together with the signalling molecules of NF-κB pathway (Misra et al., 2007). This migration to a restricted cellular compartment might be important for the regulation of TCR activation induced NF-κB activation by c-FLIP$_L$ and CASPASE-8 and to avoid any activation of the apoptosis pathway. TCR activation induces CASPASE-8 activation, which also leads to the cleavage and activation of c-FLIP$_L$ to p43-c-FLIP by CASPASE-8 in a CD95-independent manner (Misra et al.,

2007). In contrast to the transgenic expression of c-FLIP$_L$, the overexpression of c-FLIP$_S$ leads to the inhibition of NF-κB and CASPASE-8 activation in T cells and increased cell death both spontaneously and after T cell activation (Hinshaw-Makepeace et al., 2008). No effect on ERK activation was observed (Hinshaw-Makepeace et al., 2008). Thus, although there is data indicating that c-FLIP has a role in T cell proliferation and might also have a role in the differentiation, many aspects are still unknown.

2.4 PIM kinases

The serine/threonine kinase family of Proviral integration site for Moloney murine leukemia virus (PIM) kinases is evolutionarily highly conserved and the members, PIM1, PIM2 and PIM3, have largely overlapping functions, but differ in their tissue distribution (Eichmann et al., 2000; Mikkers et al., 2004). PIM kinases have been widely studied for their oncogenic properties and their expression is increased in many cancer cells (Brault et al., 2010). The kinase activity of PIM proteins is not dependent on post-translational modification but they are mainly regulated at the transcriptional level (Fox et al., 2003; Qian et al., 2005). In fact, PIM1 and PIM2 protein levels and overall kinase activity have been shown to be associated (Fox et al., 2003; Zhu et al., 2002). PIM kinase activity is also regulated by protein stability through ubiquitylation and protein degradation. The binding of PIM1 and PIM3 to the protein phosphatase 2A (PP2A) results in their dephosphorylation and targeting for protein degradation (Losman et al., 2003). PIM1 binding to heat shock protein 90β (HSP90) was found to stabilize PIM1 by preventing its proteasomal degradation, whereas the binding of HSP70 to PIM1 promotes the proteasomal degradation of PIM1 (Mizuno et al., 2001; Shay et al., 2005). PIM1 has also been shown to autophosphorylate itself although this phosphorylation seems not to regulate its activity (Qian et al., 2005).

The expression of PIM kinases has been demonstrated in a variety of cell types and tissues. PIM1 and PIM2 are expressed mainly in the hematopoietic tissues including thymus, spleen and bone marrow (Bachmann and Moroy, 2005; Mikkers et al., 2004). PIM3 has been reported to be mainly expressed in brain, kidney, liver and epithelia (Eichmann et al., 2000; Feldman et al., 1998), but very recently also in mouse and human CD4+ lymphocytes (Jackson et al., 2012). The expression of PIM kinases has previously been shown to be regulated by several cytokines and growth factors (Bachmann and Moroy, 2005) and by TCR activation and B cell activation through the CD40 co-receptor (Wingett et al., 1996; Zhu et al., 2002). In addition, JAK/STAT pathways as well as NF-κB signalling are implicated in the up-regulation of PIM kinase expression (Li et al.,

2001; Matikainen et al., 1999; Miura et al., 1994; Shirogane et al., 1999; Wang et al., 2001; Zhu et al., 2002).

Two of the PIM family members, PIM1 and PIM2, have been characterized as regulators of the cytokine-dependent lymphocyte proliferation and survival in hematopoietic cells (Fox et al., 2005; Wang et al., 2001; White, 2003). So far several substrates of PIM kinases have been identified that link PIM kinase activity to cell survival, proliferation and migration. The substrates and binding partners identified for PIM kinases include Bcl2 associated death promoter (BAD) protein, a pro-apoptotic protein; p100, an activator of c-Myb transcription factor; NFAT2, a mediator of TCR signalling; chemokine receptor CXCR4 as well as several factors involved in cell cycle regulation (Bachmann and Moroy, 2005; Brault et al., 2010). PIM1 has also been shown to activate c-Myc target genes by chromatin phosphorylation (Zippo et al., 2007). In addition, PIM kinases inhibit STAT5 and STAT6 signalling through the regulation of Suppressor of cytokine signalling proteins SOCS1 and SOCS3 (Chen et al., 2002; Peltola et al., 2004).

In human Th cells, PIM1 and PIM2 are up-regulated by Th1 polarizing cytokines both at mRNA and protein levels (Aho et al., 2005), whereas this was not observed in mouse (Jackson et al., 2012). In addition, PIM1 has been shown to phosphorylate RUNX1 and RUNX3 proteins and enhance their transcriptional activity (Aho et al., 2006). RUNX3 and RUNX1 in turn negatively regulate the Th2 cell differentiation (Djuretic et al., 2007; Komine et al., 2003). Collectively, these data suggests that PIM kinases might have a role in the regulation of the Th cell differentiation.

2.5 Lymphocyte response to *Chlamydia pneumoniae*

2.5.1 *Chlamydia pneumoniae*

In developed countries the intracellular bacterium *Chlamydia pneumonia*e is a common respiratory tract pathogen infecting most people at some time of their life. *C. pneumoniae* typically causes mild respiratory tract infections, but also pneumonia (Kuo et al., 1995). Although most people are infected by *C. pneumoniae* at least once during their life the infection results only in partial immunity and recurrent infections are common (Ekman et al., 1993; Kuo et al., 1995). Although acute *C. pneumoniae* infections are usually mild, the infection may become persistent. Chronic *C. pneumoniae* infections have been associated with severe consequences such as coronary heart disease, asthma and atherosclerosis (Belland et al., 2004; Campbell and Kuo, 2004; Hansbro et al., 2004). *C. pneumoniae* exists in two forms, as the infective, but metabolically inactive elementary

bodies (EB) and the replicative, non-infective metabolically active reticulate bodies (RB) (Kalayoglu et al., 2002). These form the two stages of the life cycle of this obligate intracellular bacterium. *C. pneumoniae* EBs attach to the cell membrane receptor of the respiratory epithelial cells of the host and are internalized into the cell by endocytosis (Hammerschlag, 2002). Inside the cell, EB differentiates to form RB and the bacteria will start to replicate (Hammerschlag, 2002). After a few days, RBs condense to form new EBs, which will be released after the lysis of the host cell thus starting a new infectious cycle (Hammerschlag, 2002). During the infection, if the environment is not favouring the replication of the bacteria, *C. pneumoniae* can adopt a persistent form which will stay inactive until the conditions again favour replication (Beatty et al., 1994; Hogan et al., 2004). Persistence can be induced in *in vitro* conditions by, for example, IFNγ, certain antibiotics and deprivation of nutrients. In addition, IFNγ has been shown to be an important factor for the development of persistent, chronic infection *in vivo* as well (Hogan et al., 2004). The more severe consequences of the persistent infection, such as coronary heart disease and atherosclerosis, have made it important to gain deeper knowledge of the factors favouring the persistent infection as well as development of better diagnostics for the low bacterial numbers found in chronic infections.

2.5.2 Host response

In experimental models for *C. pneumoniae* infection, the intranasal inoculation of the bacteria results in mild, self-restricted acute respiratory infection, partial immunity and susceptibility to reinfection (Kaukoranta-Tolvanen et al., 1993; Penttilä et al., 1998; Yang et al., 1993), thus resembling infection in humans. Although intracellular bacteria are commonly thought to induce Th1 type response and CD8+ T cell responses are more common against virus infections, the studies of *C. pneumoniae* infections have shown that both CD4+ and CD8+ T cells are important for protection against this pathogen. Athymic nude mice lacking T cells cannot clear the infection, although they are able to restrict the infection to a state where 10^4-10^5 inclusion forming units (IFU) are recovered from lungs constantly (Penttilä et al., 1999). Specifically, the CD8+ T cells have been shown to be important both in protection against primary infection and reinfection (Penttilä et al., 1999; Rottenberg et al., 2000), whereas CD4+ T cells have been shown to have both early deleterious and later protective roles (Rottenberg et al., 1999). Studies exploiting the IFNGR knock-out mice and *in vivo* neutralization of IFNγ, have demonstrated IFNγ-mediated protection to be important for the control of infection (Rottenberg et al., 1999; Rottenberg et al., 2000; Vuola et al., 2000), although it is also an important factor for the development of persistent infection both *in vitro* and *in vivo* (Hogan et al., 2004). However, CD8+ T cells have shown to control the *Chlamydia*

infection by IFNγ secretion (Rottenberg et al., 2002), but CD4+ T cells and macrophages can also participate as a source of IFNγ (Rothfuchs et al., 2004). In addition, IFNGR knockout mice, as well as IFNγ knockout mice, are highly susceptible to *C. pneumoniae* infection (Rottenberg et al., 2002) and the mediator of IFNγ signalling, STAT1, has also been shown to be important for the protection against *C. pneumoniae* (Rothfuchs et al., 2006). Thus the level of IFNγ might be important in the outcome of the immune response; suboptimal levels of IFNγ lead to persistence, whereas higher levels of IFNγ augment the clearance of infection.

3 AIMS OF THE STUDY

The goal of this study was to study the lymphocyte response *in vitro* and *in vivo*. The first part of this project focused on the roles of new players in the early differentiation of Th cells, namely the PIM family of kinases and the apoptosis related gene c-FLIP. The other part of this project aimed at characterizing the lymphocyte response during *Chlamydial* infection in a mouse model.

The specific aims of subprojects included in this PhD thesis were:

I To study the role and regulation of PIM family of kinases during the early Th1 differentiation

II To study the regulation and role of c-FLIP isoforms in Th cell differentiation

III To characterize the lymphocyte response during experimental *Chlamydia pneumoniae* infection in mouse.

4 MATERIALS AND METHODS

4.1 Cell culture and transfections

4.1.1 Isolation and culturing of human CD4+ cells (I, II)

As a source of human naive CD4+ T cells the umbilical cord blood of healthy neonates born in Turku University Hospital was used. Mononuclear cells were isolated using Ficoll gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) and CD4+ cells were further purified using DYNAL magnetic beads (Invitrogen, Carlsbad, CA). Cells from several individuals were pooled after the isolation. Cells were cultured in Yssel's medium (IMDM [Invitrogen] supplemented with Yssel medium concentrate, penicillin/streptomysin and 1% AB-serum). Cells were activated with plate-bound α-CD3 (0.125µg/well or 0.500µg/well) and soluble α-CD28 (0.5µg/ml; both from Immunotech, Marseille, France) and at the same time polarized towards Th1 cells with 2.5ng/ml of IL12 or Th2 cells with 10ng/ml of IL4 (both from R&D Systems, Minneapolis, MN) or cultured without addition of cytokines (Th0 cells). IL2 (40U/ml, R&D Systems) was added into all of the cultures after 48 hours of priming.

Research involving the use of blood from anonymous donors was permitted by the Finnish Ethics Committee.

4.1.2 siRNAs and transfections (I, II)

Freshly isolated CD4+ cells from cord blood were transfected with siRNA oligonucleotides (Sigma-Aldrich, St Louis, MO) using the nucleofection technique (Lonza). $4x10^6$ cells were transfected with 1.5 µg – 4.5 µg of siRNA oligo. The following siRNA amounts were used: c-FLIP and STAT6 siRNAs 1.5 µg, PIM1, PIM2 and PIM3 siRNAs simultaneuously 1.5 µg each, STAT4 siRNA1+siRNA2 (1:1) 1.5µg. Non-targeting (NT) siRNA was used accordingly to gene specific siRNAs. The siRNA sequences used are listed in Table 2. The nucleofected cells were allowed to rest for 20h-24h in RPMI 1640 medium (Sigma-Aldrich) supplemented with penicillin/streptomysin, 2 mM L-glutamine and 10% FCS at 37ºC, 5% CO_2 ($2x10^6$ cells/ml). The cells were subsequently activated and cultured in Yssel's medium as described in earlier in this section.

Table 2. siRNA sequences.

siRNA sequence	
Non targeting (NT)	5' -GCGCGCUUUGUAGGAUUCG- 3'
c-FLIP_Long siRNA	5'-GAGCUUCUUCGAGACACCUUC-3'
c-FLIP_Short siRNA	5'-CACCCUAUGCCCAUUGUCCU-3'
PIM1 siRNA1	5' -GAAGGUGAGCUCGGGUUUC- 3'
PIM2 siRNA1	5' -GUGGAGUUGUCCAUCGUGACA- 3'
PIM3 siRNA1	5' -GGCGTGCTTCTCTACGATA- 3'
STAT4 siRNA1	5' -GGUACAACGUGUCAACCAA- 3'
STAT4 siRNA2	5' -GGCAACGAUUCUUCUUCAA- 3'
STAT6	5' -AAGCAGGAAGAACUCAAGUUU- 3'

4.1.3 Sorting and analysis of human memory Th1 cells (I)

CD4+ lymphocytes from buffy coats of healthy donors (Red Cross Finland Blood Service, Helsinki, Finland) were isolated as described earlier in this section. After isolation, CD4+ cells were stained with α-CD183(CXCR3)-APC and α-CD4-PE (both from BD Pharmingen, San Jose, CA), and the CXCR3+CD4+ cell population was isolated using FACSaria Ilu Cell Sorter (BD BioSciences, Franklin Lakes, NJ). A fraction of the CXCR3-CD4+ cell population was collected as control. α-mouse-IgG1,κ-APC and α-mouse-IgG1,κ-PE (both from BD Pharmingen) were used as isotype controls. Sorted cell populations were activated with platebound α-CD3 and soluble α-CD28, and cultured in Yssel's medium (as described earlier in this section). After 24h and 48h of culturing, samples were collected for western blotting. The intracellular IFNγ production of the CXCR3 positive and negative populations was analyzed after 40h of activation by intracellular cytokine staining, as described later in this section.

4.2 Mouse lymphocytes (III)

4.2.1 Mice (III)

Female inbred BALB/c mice were obtained from Bomholtgård Breeding and Research Centre Ltd. (Ry, Denmark) for characterization of the *C.pneumoniea* infection and from Harlan Netherland (Horst, The Netherlands) for the microarray study. The mice were given food and water ad libitum and they were kept in ventilated containers (Scantainer). This study was approved by the institutional animal care and use committee.

4.2.2 Isolation of CD4+, CD8+ and CD19+ lymphocytes (III)

A single cell suspension was prepared by mechanical homogenization through a 70 μm filter from lungs, mediastinal lymph nodes (MLN) and axillary lymph nodes (ALN). Erythrocytes were lysed with hemolytic Gey's solution and mononuclear cells were counted. Pulmonary cells were further purified using Ficoll-Paque isolation (Amersham Pharmacia Biotech).

CD4+ and CD8+ cell populations were positively selected from homogenized samples using a magnetic cell separation system (MACS; Miltenyi Biotec) according to the manufacturer's instructions. CD19+ cell population was either positively or negatively selected using the MACS beads. The purity of the isolated cell population was analyzed by flow cytometry. Lymphocyte populations used in the microarray study were 67% to 97.9% pure. Cell purities of ALN derived lymphocytes were: CD4+ 95.3% and 97.3%; CD8+ 90.1% and 94%; CD19+ 77.1% and 67%, for experiment 1 and 2, respectively. The purity of cell populations from primary infected mice MLNs were: CD4+ 96.5% and 95.9%; CD8+ 83.5% and 97.6%; CD19+ 90.5% and 86.4%, for experiment 1 and 2, respectively. The purity of cell populations from reinfected mice MLNs were: CD4+ 91.4% and 97.9%; CD8+ 71.5% and 92.1%; CD19+ 90.2% and 95.0%, for experiment 1 and 2, respectively.

4.3 *Chlamydia pneumoniae* preparation and experimental infections (III)

Chlamydia pneumoniae isolate Kajaani 6 (Ekman et al., 1993) stocks were propagated and purified as previously described (Penttilä et al., 1998). The mice were challenged intranasally with 10^6 IFU of *C. pneumoniae* in 40 µl of sucrose-phosphate-glutamate (SPG) under Metofane® (Sigma) anesthesia. In rechallenge, the same dose was given via the same route, 42 to 49 days after the primary challenge. Samples were taken at defined time points after primary infection and reinfection (3-7 mice/time-point). The level of infection was measured by culturing *C. pneumoniae* from lungs, as described earlier (Penttilä et al., 1998). For *in vitro* assays, 5 x 10^5 UV-inactivated *C. pneumoniae* EB/ml, corresponding to approximately 0.5 µg protein/ml, were used to stimulate cells. For microarray analysis, samples were collected at 10 days after primary infection (12 mice) and 3 days after reinfection (12 mice).

4.4 Lymphoproliferation assay (III)

Triplicate samples of freshly isolated mononuclear cells (2 x 10^5 cells per well) were stimulated with UV-inactivated *C. pneumoniae* elementary bodies (EB) for 4 days at +37°C, 5 % CO_2 atmosphere. Proliferation activity of the cells was measured *in vitro* as previously described (Tammiruusu et al., 2005).

4.5 Real-time quantitative RT-PCR (I-III)

Real-time quantitative RT-PCR was performed as previously described (Hämäläinen et al., 2000; Lund et al., 2003). Briefly, the total RNA from human Th cells was isolated using RNeasy minikit (Qiagen, Valencia, CA) applying the in-column DNAse treatment (Qiagen). The total RNA of the mouse samples was isolated using the Trizol method (Invitrogen) and further purified with RNeasy minikit. Total RNA was treated with DNAse I (Amplification

grade, Invitrogen) to remove any genomic DNA. The cDNA was prepared using Superscript II kit (Gibco BRL, Life Technologies, Paisley, Scotland) or with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Gmbh). RT-PCR reactions were analysed using ABI PRISM 7900HT or ABI Prism 7700 instrument (Applied Biosystems). Ef1a was used as a housekeeping gene (Hämäläinen et al., 2001). The primer sequences used are shown in Table 3.

Table 3. Primers and probes used in Real-time RT-PCR

Taqman RT-PCR		Sequence 5'-3'
HUMAN	c-FLIP_L-Probe	Universal probelibrary #14 (Roche)
	c-FLIP_L-F	GCTCACCATCCCTGTACCTG
	c-FLIP_L-R	CAGGAGTGGGCGTTTTCTT
	c-FLIP_R-Probe	6(FAM)-CCAGACTCACCCTGAAGTTATTTGAAGGATCCT-(TAMRA)
	c-FLIP_R-F	CAAGCAGCAATCCAAAAGAGTCT
	c-FLIP_R-R	TCATGCTGGGATTCCATATGTTT
	c-FLIP_s-Probe	6(FAM)-TTCAGGATGATAACACCCTATGCCCATTGTC-(TAMRA)
	c-FLIP_s-F	TCTCCAAGCAGCAATCCAA
	c-FLIP_s-R	TCACATGGAACAATTTCCAAGAATTTT
	EF1a-Probe	6(FAM)-AGCGCCGGCTATGCCCCTG-(TAMRA)
	EF1a-F	CTGAACCATCCAGGCCAAAT
	EF1a-R	GCCGTGTGGCAATCCAAT
	Gata3-Probe	6(FAM)-TGCCGGAGGAGGTGGATGTGCT-(TAMRA)
	Gata3-F	GGACGCGGCGCAGTAC
	Gata3-R	TGCCTTGACCGTCGATGTTA
	IFNg-Probe	6(FAM)-TGCTGGCGACAGTTCAGCCATCAC -(TAMRA)
	IFNg-F	TGTCCAACGCAAAGCAATACA
	IFNg-R	CTCGAAACAGCATCTGACTCCTT
	IL-12Rb2-Probe	6(FAM)-TGCATTGCTATCATCATGGTGGGCAT-(TAMRA)
	IL-12Rb2-F	CGTTTGTGGCACCAAGCA
	IL-12Rb2-R	GCTGGAAGTAATGCGTTGAGAA
	T-bet-probe	6(FAM)-TCAGCATGAAGCCTGCATTCTTGCC-(TAMRA)
	T-bet-F	ACAGCTATGAGGCTGAGTTTCGA
	T-bet-R	GGCCTCGGTAGTAGGACATGGT
	STAT4-Probe	6(FAM)-AGTCTCGCAGGATGTCAGCGAATGG-(TAMRA)
	STAT4-F	GCTGAGAGCTGTAGTGTTTACCGA
	STAT4-R	AATAAAGGCCGGTTGTCTGCT
MOUSE	EF1a1-probe	Universal probelibrary #38 (Roche)
	EF1a1-F	AGCAAAAACGACCCACCA
	EF1a1-R	GCCTGGATGGTTCAGGATAA
	Gbp2-probe	Universal probelibrary #6 (Roche)
	Gbp2-F	GGGGTCACTGTCTGACCACT
	Gbp2-R	ATGCATTCTGCACACAGAGG
	Gzma-probe	Universal probelibrary #75 (Roche)
	Gzma-F	GGCCATCTCTTGCTACTCTCC
	Gzma-R	CGTGTCTCCTCCAATGATTCT
	Ifit1-probe	Universal probelibrary #2 (Roche)
	Ifit1-F	TGAAATGCCAAGTAGCAAGGT
	Ifit1-R	GCCTGCTAGACAGGATCAGAA
	Ifit3-probe	Universal probelibrary #4 (Roche)
	Ifit3-F	GAGTGCTGCTTATGGGGAGA
	Ifit3-R	AGAGCAGTTTGTCAGCAATCC
	Irf7-probe	Universal probelibrary #25 (Roche)
	Irf7-F	CTTCAGCACTTTCTTCCGAGA
	Irf7-R	TGTAGTGTGGTGACCCTTGC
	Irs2-probe	Universal probelibrary #53 (Roche)
	Irs2-F	TTCCCCTTCCTCCTTACAGC
	Irs2-R	AGGCAGGCGGTATAGGTCTC
	Slpi-probe	Universal probelibrary #12 (Roche)
	Slpi-F	ATCCTGGCACCCTGGACT
	Slpi-R	GCAGGCTCCGATTTTGATAG
	Usp18-probe	Universal probelibrary #88 (Roche)
	Usp18-F	GACGCAAAGCCTCTGAAAAC
	Usp18-R	ACTCTTTGGGCTGGACGAA

4.6 Flow Cytometry

4.6.1 Intracellular cytokine staining (I,II)

The cultured cells were harvested and washed with PBS. Half of the cells were stimulated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA; Calbiochem, San Diego, CA) and 0.5 pg/ml ionomycin (Sigma-Aldrich) in Yssel's medium ($1x10^6$ cells/ml), and the other half was incubated in Yssel's medium and used as an unstimulated control. After 2h of incubation, 10 μg/ml brefeldin A (Alexis Biochemicals, Lausanne, Switzerland) was added and the incubation was continued for 3h. Cells were washed with 0.5%BSA/PBS (w/v), 0.01% NaN_3, fixed with 4% paraformaldehyde/PBS and permeabilized with 0.5% saponin/PBS. For stainings α-human-IFNγ-FITC (Invitrogen) and α-human-IL-4-PE (BD Pharmingen, San Jose, CA) were used. α-mouse-IgG1-FITC (Invitrogen) and α-rat-IgG1-PE (BD Pharmingen) were used as isotype controls. Cells were analysed with the FACSCalibur or the LSR II Flow cytometer (both from BD Biosciences).

4.6.2 Surface staining of IL12Rβ2 (I)

To measure the expression of IL12Rβ2 on the surface of Th1-polarized cells, cells were first incubated with Rat α-human IL12Rβ2 (CD212) antibody or Rat IgG2a (isotype control; both from BD Pharmingen) and then with biotin-conjugated α-rat-antibody (BD Pharmingen) and finally labeled with Streptavidin-PerCP (BD Pharmingen). Cells were analysed with the FACSCalibur Flow cytometer (BD Biosciences).

4.6.3 Analysis of mouse CD4+, CD8+ and CD19+ populations (III)

Freshly isolated mononuclear cells, positively or negatively isolated lymphocyte populations from Balb/c mice were stained with FITC- or PE-conjugated antibodies to CD4, CD8α or CD19 or matching isotype control antibodies (all antibodies Caltag or Pharmingen) and were analyzed by FACScan (Becton Dickinson).

4.7 Western blotting (I, II)

Cells were lysed in Triton-X-100 lysis buffer (TXLB; 50mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton-X-100, 5% glycerol, 1% SDS, 1 mM Na_3VO_4, 10 mM NaF) or SDS lysis buffer (62.5 mM Tris-Hcl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.1% (w/v) bromphenol blue), denatured in 95ºC (5 min) and sonicated. Equal amounts of proteins were subsequently separated by SDS-PAGE electrophoresis and transferred

to nitrocellulose membranes. The proteins studied were detected using the following primary antibodies: α-PIM1 (12H8; Santa Cruz Biotechnology, Santa Cruz, CA), α-PIM2 (ATLAS antibodies, Stockholm, Sweden or D1D2; Cell Signaling Technology, Beverly, MA), α-PIM3 (D17C9; Cell Signaling Technology), α-T-BET (4B10; Santa Cruz Biotechnology), α-STAT1 p84/p91 (E-23; Santa Cruz Biotechnology), α-STAT4 (C-20; Santa Cruz Biotechnology), α-STAT6 (BD Biosciences), α-cFLIP (NF6: Alexis Biochemicals, Lausanne, Switzerland), α-GAPDH (#5G4, 6C5, HyTest, Turku, Finland) or α-β-ACTIN (Sigma-Aldrich). Suitable HRP-conjugated secondary antibodies were selected. The proteins were visualized with enhanced chemiluminescence (GE Healthcare), quantified with a microcomputer imaging device (MCID; M5+, Imaging Research Inc., St. Catharines, Canada) and normalized against β-ACTIN or GAPDH. For quantification a non-overexposed film was used for each protein studied.

4.8 Microarray (I, III)

4.8.1 Human CD4+ lymphocytes (I)

Total RNA was isolated using the RNeasy minikit (Qiagen) and sample preparation for GeneChip oligonucleotide array hybridizations was performed according to Affymetrix's intructions with GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA). Samples from three biological replicates were hybridized to HG-U219 arrays using the GeneTitan® Instrument (Affymetrix). The data were normalized using robust multi-array average algorithm (Irizarry et al., 2003) and duplicate and un-annotated probe sets were removed using the *genefilter* package in R. Present and absent calls for probe sets were generated by fitting the chip-wide expression data to a two-component Gaussian distribution function using the standard EM algorithm implemented in *mixtools* package in R (Benaglia et al., 2009). A probe set was defined as present if it had an expression value higher than the threshold where the two components of the Gaussian distribution densities meet (Lee et al., 2010). Combat with non-parametric empirical priors was used to correct for the batch effect (Johnson et al., 2007). Differential expression analysis was done using the moderated paired t-test as implemented in *limma* (Smyth, 2004). Genes were considered differentially expressed when the Benjamini-Hochberg adjusted false discovery rate (FDR) was < 0.1 and the log fold change (LFC) was <-0.5 or >0.5. Over-representation analysis of transcription factor binding sites was done using Genetrail (Backes et al., 2007).

4.8.2 Mouse lymphocytes (III)

The total RNA was isolated using the Trizol method (Invitrogen) and further purified with RNAeasy minikit (Qiagen). The sample preparation was performed according to the protocol for the Affymetrix two-cycle GeneChip Eukaryotic small sample target labeling assay version II (Affymetrix). The samples were hybridized to a MG-U74Av2 array and the biological replicate samples to a MOE430 2.0 array (Both from Affymetrix). The Affymetrix statistical data analysis softwares: Microarray Suite (MAS) and GeneChip operating software (GCOS), were used to normalize and process the results according to Affymetrix's recommendations. Because the samples were hybridized to two different arrays, results of these different sample series were compared using Best Match probe set-tables provided by Affymetrix.

4.9 Bio-plex cytokine assay (I, II)

To measure IFNγ production from Th1 polarized cells, duplicate samples were stained on 96-well plates (Milliplex Map Kit; Millipore, Billerica, MA) according to the manufacturer's instructions and measured using the Luminex® 100™ system (Luminex, Austin, TX). The cytokine concentrations of cell culture supernatants were normalized against relative cell counts obtained by flow cytometry.

5 RESULTS AND DISCUSSION

5.1 Identification of PIM kinases as regulators of early human Th1 cell differentiation (I)

5.1.1 PIM kinases are predominantly expressed in Th1 cells

The PIM family of serine/threonine kinases is known to regulate cytokine-dependent cell proliferation and survival in lymphocytes (Bachmann and Moroy, 2005; Eichmann et al., 2000; Mikkers et al., 2004). The three kinases of this family, PIM1, PIM2 and PIM3, have largely overlapping functions. PIM1 and PIM2 have been reported as being predominantly expressed in hematopoietic cells (Bachmann and Moroy, 2005; Mikkers et al., 2004), whereas PIM3 is expressed mainly in the brain, kidney, liver and epithelia (Eichmann et al., 2000; Feldman et al., 1998), and has very recently also been reported to be expressed in mouse and human CD4+ T cells (Jackson et al., 2012). PIM family genes have previously been shown to be up-regulated by Th1 polarizing cytokines at the mRNA level (Aho et al., 2005). Consistently, we found all three PIM kinases to be expressed more in Th1 than Th2 cells. Human naïve Th cells were activated and cultured in neutral conditions (Th0) or polarized with IL12 (Th1) or IL4 (Th2). PIM kinase expression was measured at different time-points by western blotting. PIM3 was found to be more rapidly up-regulated by TCR activation than PIM1 or PIM2 (I, Figure 1A), and all three were more expressed under Th1 polarizing conditions. Furthermore, *in vivo* developed CXCR3+ Th1 cells expressed more PIM1 and PIM2 than the CXCR3- cells (I, Figure 1B). It has been shown that a high level of CXCR3 chemokine receptor expression distinguishes Th1 cells from Th2 and Th17 cells (Groom and Luster, 2011; Yamamoto et al., 2000). CXCR3 has also been shown to be essential for effector and memory Th1 cell migration and its expression is regulated by T-BET (Groom and Luster, 2011). Although our results did not show any difference in PIM3 expression between these two cell populations, these results indicate that at least PIM1 and PIM2 might have a potential role in *in vivo* developed Th1 cells.

Both STAT4 and STAT6 were found to regulate PIM expression. In Th2 polarizing conditions, STAT6 appears to down-regulate PIM1 and PIM2 expression, whereas in Th1 conditions the depletion of STAT4 leads to down-regulation of all three PIM kinases (I, Figure 1C-D). Our results suggest that in addition to up-regulation in Th1 conditions, the expression of PIM kinases is also negatively regulated in Th2 cells. Very recently it was reported that all PIM kinases are up-regulated in response to activation in murine

and human CD4+ T cells (Jackson et al., 2012). Similarly, as in our results, Jackson *et al.* observed a more pronounced and faster up-regulation of PIM3, in comparison to PIM1 and PIM2, in activated murine CD4+ T cells (Jackson et al., 2012). However, in contrast to our data, no difference in PIM1 expression was found between Th1, Th2 and Th17 cells. Notably, however, they have used murine CD4+ cells where the expression of PIM kinases may differ from that observed in human cells.

5.1.2 The knockdown of PIM kinases down-regulates early Th1 differentiation pathways

Having observed that PIM kinases were preferentially expressed in Th1 cells, when compared with Th2 cells, we examined whether they have any role in the regulation of the Th1 differentiation process. Thus, the potential influence of the PIM knockdown on the key Th1 differentiation pathways, i.e. IFNγ/TBET and IL12/STAT4, were analysed. To address these questions, an siRNA approach was used to knockdown the expression of all three PIM family members in human naive CD4+ cells. Cells were nucleofected with specific siRNAs and polarized in the Th1 direction. Efficient knockdown was confirmed by measuring the PIM protein levels (I, Figure 2A). The siRNA-mediated knockdown of three PIM kinases lasted up to 48 to 72 hours, after which its effect decreased, most likely because of the proliferation of the cells. The knockdown data obtained by these siRNAs was also confirmed with two other siRNA sequences for each PIM kinase (data not shown).

The measurements of IFNγ at the protein and mRNA level indicated that PIM knockdown decreased IFNγ expression at both the transcriptional and translational level (I, Figure 2B-D), supporting the hypothesis that the PIM kinases affect the induction of *IFN*γ expression. Furthermore, although the knockdown effect of siRNA is transient, the intracellular IFNγ staining of nucleofected cells revealed that there was still a small but statistically significant reduction in intracellular IFNγ after 6 days of culture and restimulation (I, Figure 2D), indicating that the initiation of Th1 polarization is impaired by PIM knockdown. In line with the IFNγ results, the early induction of *TBET* mRNA was delayed by the PIM knockdown (I, Figure 3A). Consistently, the western blotting analysis of TBET protein levels demonstrated down-regulation in response to the PIM depletion (I, Figure 3B). The PIM knockdown reduced TBET protein levels to half of the control cells and strong effects were seen after both 12 and 24 hours of culture, although *TBET* mRNA was not affected at the 24h time-point. However, the level of T-BET was highly up-regulated under Th1 polarizing conditions during the first 24 hours of culture, which may have reduced the effects of PIM siRNAs after the 24 hour time point. Taken

together, our data indicate that the depletion of PIM kinases slows down the induction of IFNγ and T-BET both at the mRNA and protein levels.

IL12/STAT4 signalling is a critical pathway for Th1 cell differentiation. Since PIM kinases were observed to regulate the expression of T-BET and IFNγ, it was of interest to study if they have any influence on the expression of IL12Rβ2 and/or STAT4, which have been shown to be direct or indirect targets of T-BET and IFNγ signalling (Afkarian et al., 2002; Mullen et al., 2001; Szabo et al., 1997; Usui et al., 2006). Indeed, the depletion of PIM kinases down-regulated both IL12Rβ2 and STAT4 at mRNA and protein levels (I, Figure 4A-D). The level of surface IL12Rβ2 in PIM knockdown Th1 cells was less than half of the level of control cells at least until 72h after priming (I, Figure 4B). The expression of STAT4 was transiently down-regulated by the triple knockdown of PIM kinases at the 24 hour time-point, whereas the expression of STAT1, the mediator of IFNγ signalling, was not influenced by the PIM siRNAs (I, Figure 4C). Although the effects seen in TBET and STAT4 were reversed relatively quickly, the effect on IL12Rβ2 surface expression was still strong three days after priming. This might be explained by the fact that STAT4 and IFNγ are known to induce and maintain the expression of IL12Rβ2 (Chang et al., 1999; Lawless et al., 2000; Letimier et al., 2007; Szabo et al., 1997) and thus the influence of PIM kinases on STAT4 and IFNγ may mediate the effect seen on IL12Rβ2. Taken together, our results show that the depletion of PIM kinases from human Th1-polarized cells down-regulates both the IFNγ/TBET and IL12/STAT4 signalling and this was not due to a defect in proliferation since the cell proliferation and viability seen with PIM knockdown was similar to control cells.

5.1.3 The knockdown of PIM kinases alters the transcriptional profiles of Th1 and Th2 cells

The observation that the knockdown of PIM kinases impairs the important pathways crucial for Th1 differentiation, i.e. IFNγ/T-BET and IL12/STAT4 pathways, led to the question which transcription factors might be the mediators of these observed changes. To address this question we performed a genome-wide analysis of transcriptional changes caused by the PIM triple knockdown 6h after the induction of Th1 differentiation or Th2 differentiation. This early time-point was selected to avoid the secondary effects of STAT4 and TBET down-regulation induced by PIM knockdown, and a parallel analysis was also performed in Th2 cells in order to see if PIM knockdown induces similar transcriptional changes in Th2 cells than Th1 cells. For these measurements, naive CD4+ T cells were nucleofected with the specific siRNAs and polarized with IL12 (Th1) or IL4 (Th2) and the microarray analysis was performed form three independent cultures. This analysis showed

that altogether 241 genes were differentially expressed in Th1 cells by PIM knockdown (I, Figure 5 and Table S1) and that the majority of the differentially expressed genes found in Th1 cells were not affected by the PIM knockdown in Th2 cells (I, Figure S1). Among the genes found differentially expressed in Th1 cells, there were TGFβ signalling related transcription factors *Smad2, TGIF1* and *Sox4*, which were all up-regulated (I, Figure 5C). While important for Th17 and Treg cell differentiation, TGFβ represses the differentiation of other Th subtypes (Bettelli et al., 2006; Li and Flavell, 2008; Lund et al., 2003; Veldhoen et al., 2006). *Smad2* knockout mice studies have also shown that SMAD2 inhibits IFNγ production by Th1 cells (Takimoto et al., 2010). The up-regulation of these genes under Th1 polarizing conditions is in line with the impaired Th1 response seen when the expression of PIM kinases has been down-regulated.

Interestingly, NFAT2, NF-κB, STAT5 and ATF2 target genes were enriched among the differentially expressed genes (I, Figure 5D-E). In addition, 8 of the 9 target genes of these transcription factors were down-regulated, and only FAS ligand (FASLG) was up-regulated. Two of these transcription factors, NFAT and NF-κB, are induced upon TCR activation and contribute to the early induction of *T-BET* and *IFN*γ expression (Kiani et al., 2001; McCracken et al., 2007; Sica et al., 1997). Interestingly, both NFAT and NF-κB have been reported being targets of PIM kinases. PIM1 phosphorylates NFAT2 on several serine residues and thereby enhances its transcriptional activity (Rainio et al., 2002). PIM2 stimulates NF-κB-dependent transcription through the phosphorylation of oncogenic serine/threonine kinase Cot that activates NF-κB (Hammerman et al., 2004). Interestingly, Cot has been linked to Th1 cell differentiation. Cot deficient mouse T cells show diminished IFNγ, TBET and STAT4 expression in response to IL12 and TCR activation induced signalling (Watford et al., 2008), and Cot expression was also up-regulated in human Th1 cells (Aijo et al., 2012). ATF2 has both serine and threonine phosphorylation sites that have been reported being phosphorylated by several different kinases such as p38 and c-Jun terminal kinase (JNK) (Gozdecka and Breitwieser, 2012), and ATF-cJUN has been shown to bind to *IFNG* promoter (Filen et al., 2010; Jones and Chen, 2006). In addition to the data obtained in this study, earlier observations suggest that PIM1 can phosphorylate RUNX proteins to enhance their transcriptional activity (Aho et al., 2006). Furthermore, RUNX proteins have been implicated in the regulation of Th1 and Th2 differentiation. RUNX3 has been shown to promote IFNγ production in co-operation with TBET (Djuretic et al., 2007), whereas RUNX1 has been shown to inhibit GATA3 expression and Th2 differentiation (Komine et al., 2003). Thus, taken together, these results support the idea that the effects of PIM kinases on the expression of T-BET and IFNγ, are mediated at least partly through the enhanced activation of NFAT and /or NF-κB but they can also be mediated by other transcription factors such as RUNX proteins.

5.2 The regulation and role of c-FLIP during human Th differentiation (II)

5.2.1 c-FLIP short is selectively upregulated by IL4 during early Th cell differentiation

Previous studies have shown that in addition to apoptosis, CASPASE-8 and its regulator, c-FLIP, have a role in T cell activation and proliferation (Chau et al., 2005; Chun et al., 2002; Salmena et al., 2003; Salmena and Hakem, 2005) and the inhibition of Caspase-8 leads to an enhanced Th2 response in mice (Sehra et al., 2005). Although studied in mice, the role of c-FLIP in human Th cell differentiation has not been previously studied (Tseveleki et al., 2004; Wu et al., 2004). In our study, the kinetics of c-FLIP isoform expression during the early polarization of human Th1 and Th2 cells were characterized in detail. For this purpose naive CD4+ T cells were activated and cultured with either IL12 (Th1), IL4 (Th2) or without cytokines (Th0). The expression of c-FLIP isoforms c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$ was studied by real-time RT-PCR (II, Figure 1A-C). This revealed that although all three c-FLIP isoforms are up-regulated by TCR activation, only c-FLIP$_S$ was induced by the Th2 polarizing cytokine, IL4 (II, Figure 1A). In addition, the level of c-FLIP$_S$ was found to be up to 7 times higher than the level of c-FLIP$_R$ in the cells polarized in the Th2 direction (II, Figure 1D).

The expression of c-FLIP isoforms was also studied at the protein level. In western blot analysis of Th0, Th1 and Th2 samples from multiple time-points, only c-FLIP$_S$ and c-FLIP$_L$ were detected at the protein level (II, Figure 2A). This observation was in line with the mRNA result showing much higher expression levels of c-FLIP$_S$ than c-FLIP$_R$ in the pooled human Th samples (II, Figure 1D). It has also been shown that all the c-FLIP isoforms arise from the same mRNA transcript by alternative splicing and the expression of c-FLIP$_S$ and c-FLIP$_R$ isoforms is determined by a single nucleotide polymorphism that is not present in all individuals (Ueffing et al., 2009). In addition, although the c-FLIP$_R$ isoform is the evolutionarily more conserved of the two short isoforms and the only short isoform present in for example mice, rats and dogs (Ueffing et al., 2009), the levels of c-FLIP$_S$ are usually higher than the levels of c-FLIP$_R$ in human T cells (Golks et al., 2005; Ueffing et al., 2009). Thus, it might be that c-FLIP$_R$ is expressed at a very low level as a protein, or that in the pooled naive Th cells that were used, the majority of the individuals did not express c-FLIP$_R$ at all.

At the protein level, the expression of c-FLIP$_L$ and c-FLIP$_S$ was induced by TCR activation, but only c-FLIP$_S$ was further induced by IL4 (II, Figure 2A-2B). In addition, STAT6, an important mediator of the IL4 signalling pathway, seems at least partly to be involved in the IL4 induced c-FLIP$_S$ up-regulation, on the basis of the STAT6 knockdown experiments

(II, Figure 2C). STAT6 was down-regulated by siRNA and the naive cells were cultured under Th0 or Th2 inducing conditions. The knockdown of STAT6 led to decreased levels of c-FLIP$_S$, when compared with control cells, thus indicating that STAT6 might be at least partly mediating the effect of IL4 in c-FLIP$_S$ expression. In fact STAT3, another STAT family member, has been shown to regulate c-FLIP$_L$ in hepatocytes (Kovalovich et al., 2001). Either NFAT2 or NFAT1 could be another possible candidate for IL4 induced c-FLIP$_S$ expression, since NFAT2 is a positive regulator of Th2 differentiation (Yoshida et al., 1998) and NFAT1 can bind to *IL4* promoter in activated Th2 cells (Agarwal et al., 2000). Both NFAT1 and NFAT2 have been shown to bind the *CFLAR* promoter and to selectively up-regulate c-FLIPs expression (Ueffing et al., 2008). Although NFAT proteins have not been shown to be induced by IL4 signalling, NFAT1 an NFAT2 co-operate with IL4 induced transcription factors, GATA3, IRF4 and c-MAF, to promote gene expression of their target genes (Avni et al., 2002; Avni et al., 2002; Ho et al., 1996; Rengarajan et al., 2002). Thus it is possible that NFAT proteins could act in co-operation with other IL4 induced factors to selectively up-regulate the c-FLIP$_S$ expression.

5.2.2 Down-regulation of c-FLIP$_S$ and c-FLIP$_L$ has distinct and opposite effects on early Th1 and Th2 cell differentiation

Mouse studies of c-FLIP$_L$ and c-FLIP$_S$ have indicated that these proteins might be involved in the regulation of Th differentiation (Tseveleki et al., 2004; Wu et al., 2004). To further elucidate the possible roles of c-FLIP$_S$ and c-FLIP$_L$ in the early differentiation of human Th1 and Th2 cells, we used isoform specific siRNAs. Human naive Th cells were nucleofected with isoform specific siRNAs and then polarized in Th1 or Th2 direction. The knockdown efficiency was confirmed by measuring the c-FLIP protein levels by Western blot (II, Figure 3A). The ability of the siRNA treated cells to proliferate was confirmed by CFSE staining and the c-FLIP knockdown cells proliferated at the same rate as control cells (data not shown). To study the effects of c-FLIP$_S$ and c-FLIP$_L$ knockdown on Th1/Th2 differentiation, the expression of *TBET, IL12RB2, IFNG* and *GATA3* were measured at the mRNA level using RT-PCR (II, Figure 3B-E). *TBET, IL12RB2* and *IFNG* are important factors for Th1 differentiation, whereas *GATA3* is a crucial transcription factor for Th2 polarization. The mRNA expression of *TBET* was up-regulated by the knockdown of c-FLIP$_S$ and c-FLIP$_L$, whereas *IL12RB2* and *IFNG* were expressed at a higher level in c-FLIP$_L$ knockdown Th1 cells, when compared with control cells. The c-FLIP$_S$ knockdown Th2 cells had a lower level of *GATA3* expression when compared with control cells.

The higher expression of IFNγ in c-FLIP$_L$ knockdown Th1 cells was also confirmed at the protein level. The secreted IFNγ was measured from the cell culture supernatants of

Th1 polarized cells from days 1, 2 and 4 (II, Figure 4). The amount of secreted IFNγ was clearly increased by c-FLIP$_L$ knockdown Th1 cells compared with control (II, Figure 4). Furthermore, the intracellular cytokine levels of Th1 and Th2 polarized c-FLIP knockdown cells were studied at day 7 after priming. Although the effect of siRNA oligos is only transient and the knockdown efficiency of siRNAs is already diminished after 7 days of culture, the effect of c-FLIP knockdown on cytokine expression of Th1 and Th2 polarized cells was sustained for at least 7 days (II, Figure 5A-B). In line with the mRNA and luminex results, the number of IFNγ positive Th1 c-FLIP$_L$ knockdown cells was increased (II; Figure 5A), whereas the knockdown of c-FLIP$_S$ in Th2 cells led to decreased IL4 production by these cells (II, Figure 5B).

These data thus demonstrate that by using isoform specific siRNAs for c-FLIP$_S$ and c-FLIP$_L$ in human Th cells, the knockdown of c-FLIP$_L$ leads to the induction of Th1 marker genes such as *IFNG* and *IL12Rβ2*, whereas the knockdown of c-FLIP$_S$ leads to the down-regulation of Th2 related genes *IL4* and *GATA3*. In line with our results the transgenic mice expressing c-FLIP$_L$ in the T cell compartment secrete decreased levels of IFNγ and have a diminished expression of TBET (Wu et al., 2004). However, contradictory to our result, c-FLIP$_L$ transgenic mice produced elevated levels of GATA3 and Th2 cytokines (Tseveleki et al., 2004; Wu et al., 2004), whereas our c-FLIP$_L$ knockdown Th2 cells produced more IL-4 than control cells. This contradiction could be due to different systems (human/mouse) or due to a different approach. Nonetheless, our observation that knockdown of c-FLIP$_L$ in human Th cells leads to induced Th1 differentiation is in line with the mouse studies showing elevated Th2 differentiation in c-FLIP$_L$ transgenic mice (Tseveleki et al., 2004; Wu et al., 2004). In addition, c-FLIP$_S$ is a known inhibitor of CASPASE-8 activity, and CASPASE-8 inhibition in mouse Th cells leads to an elevated expression of GATA3 and IL4 (Sehra et al., 2005), which is in line with the decreased IL4 and GATA3 expression observed in c-FLIP$_S$ knockdown Th2 cells in this study.

The c-FLIP proteins have been shown to regulate the ERK and NF-κB signalling pathways in response to activation in Jurkat T cells overexpressing c-FLIP$_S$ and c-FLIP$_L$, respectively (Kataoka et al., 2000). In addition, ERK activation has been shown to be involved in Th2 cell differentiation by up-regulating IL4 expression by direct binding to the *Il4* promoter (Tripathi et al., 2012), and by promoting GATA3 stability (Yamashita et al., 2005). NF-κB activation is involved in the induction of Th1 response by directly regulating the expression of IFNγ (Sica et al., 1997) and reduced NF-κB activity leads to diminished levels of TBET (Corn et al., 2003; McCracken et al., 2007). Thus, it is possible that modulation of ERK or NF-κB activation by c-FLIP$_S$ and c-FLIP$_L$ might

result in decreased Th2 polarization in c-FLIP$_S$ deficient Th2 cells and induced Th1 polarization in c-FLIP$_L$ deficient cells.

In summary, we have demonstrated that the c-FLIP isoforms, c-FLIP$_S$ and c-FLIP$_L$, are differentially expressed during the early polarization of human Th1 and Th2 cells. In addition, using an siRNA approach we were able to show that the knockdown of c-FLIP$_L$ and c-FLIP$_S$ had distinct and opposite effects on Th1/Th2 polarization. c-FLIP$_L$ knockdown led to enhanced Th1 differentiation, while the knockdown of c-FLIP$_S$ reduced the expression of genes important for Th2 polarization. Interestingly, although the c-FLIP$_L$ knockdown Th2 cells produced elevated levels of IL4, the cells did not show elevated *GATA3* mRNA expression. Thus, the c-FLIP$_L$ knockdown seems to affect both Th1 and Th2 cell differentiation, whereas the effect of c-FLIP$_S$ knockdown was seen only in Th2 cell polarization. This gives an interesting basis for further studies. This study provides new insight into the roles of c-FLIP proteins in Th cell differentiation. Therefore it seems that c-FLIP isoforms are both differentially expressed and have distinct roles during the early differentiation of human naive Th cells.

5.3 Characterization of lymphocyte response during experimental *Chlamydia pneumoniae* infection (III)

5.3.1 *C. pneumoniae* infection leads to accumulation of lymphocytes into mediastinal lymph nodes

Chlamydia pneumoniae causes usually mild respiratory tract infections although pneumonia can also develop (Kuo et al., 1995). Host response to *C. pneumonia* involves both innate and adaptive immunity while T lymphocytes are crucial for the clearance of the infection (Penttilä et al., 1999; Rottenberg et al., 1999). Although acute infections are typically mild, infection may become persistent with more severe consequences, such as coronary heart disease, asthma and atherosclerosis (Belland et al., 2004; Campbell and Kuo, 2004; Hansbro et al., 2004). In experimental models for *C. pneumoniae* infection, intranasal inoculation of bacteria results in mild acute respiratory infection, partial immunity and susceptibility to reinfection (Penttilä et al., 1998), thus resembling the infection in humans. In this study, the infection was first characterized by three independent infection experiments. Mice were infected intranasally with 10^6 IFU of *C.pneumoniae* and in rechallenge, the same dose was given by the same route 42-49 days after the primary challenge. The kinetics of the primary and the reinfection were similar to those reported previously (Penttilä et al., 1998). The number of bacteria recovered from the lungs peaked between days 6 and 10 after primary infection (III, Figure 1A).

The primary infection led to partial immunity detected as a faster clearance of bacteria after rechallenge. In addition, the lymphocyte composition as well as lymphocyte numbers in lungs, axillary lymph nodes (ALN) and mediastinal lymph nodes (MLN) of infected mice were studied. We detected a dramatic lymphocyte influx to MLN and lungs during primary infection and at the early phase of reinfection. Interestingly, a large proportion of the lymphocyte influx to MLN consisted of CD19+ B cells (III, Figure 1C). The number of CD4+ and CD8+ T cells also increased although their proportion decreased (III, Figure 1C). Whilst the MLNs in uninfected mice were hardly visible, the MLNs of infected mice were clearly increased in size. In contrast, ALNs enlarged only modestly and the lymphocyte proportions in ALNs did not change during the infection.

The increase in B cell numbers was contradictory to the previous findings (Penttilä et al., 1998). However, a different B cell marker (B220) was used in the earlier study, which might explain the contrary results. Although previous studies have suggested that specific circulating antibodies have little or no role in protection against *C. pneumoniae* (Penttilä et al., 1999; Rottenberg et al., 2000), contradictory data also exist suggesting that B cells may have other functions in addition to antibody production against intracellular bacteria (Elkins et al., 1999; Yang and Brunham, 1998). Possible B cell-mediated mechanisms could be antigen presentation to T cells, the production of cytokines, interaction with macrophages or natural killer cells, and the regulation of cell trafficking.

5.3.2 Experimental *C. pneumoniae* infection induces transcriptional changes in IFN response genes in lymphocytes

To further study the differencies in lymphocyte response during *C. pneumoniae* primary and reinfection, we studied the gene expression profiles of MLN derived B cells and CD4+ and CD8+ T cells. MLN lymphocyte populations of infected mice from two independent experiments were studied by microarrays, with ALN lymphocyte populations of uninfected mice used as controls. The ALN lymphocytes were selected as controls because the mediastinal lymph nodes of uninfected mice were hardly visible and could not be used as a source of control cells. Two different microarrays were used, MG-U74Av.2 and MOE430 2.0-arrays. The comparability of results obtained by these arrays has been demonstrated (Elo et al., 2005) and the results can be compared by using the best match probe-set tables provided by Affymetrix (www.affymetrix.com). The majority (four fifths) of the differentially regulated genes in CD4+ and CD8+ T cells were up-regulated during primary and reinfection, whereas in B cells almost half of the altered genes were down-regulated during primary infection and two thirds of the genes during reinfection. The number of differentially expressed genes in B cells was higher

than in CD4+ or CD8+ T cells. In all lymphocytes the number of differentially regulated genes was higher when primary or reinfection was compared with control than when reinfection was compared with primary infection. However, the gene expression profiles of lymphocytes during primary and reinfection were different. There were altogether 148 differentially regulated genes in CD19+ cells when reinfection was compared with primary infection (III, Table 1A and 1B). In addition, there were 7 (III, Table 2) and 12 (III, Table 3) differentially regulated genes in CD4+ and CD8+ T cells, respectively. Selected genes were also confirmed by RT-PCR from the lymphocytes of infected mice from three independent experiments (III, Figure 2).

Four of the genes that were confirmed by RT-PCR; Gbp2, Usp18, Gzma and Slpi, have not previously been reported to be differentially regulated in host cells during *C. pneumoniae* infection. Some of the other genes that were verified have also been reported to be differentially regulated in mouse lungs during *C. pneumoniae* infection (Ifit1, Ifit3, Irf) (Rodriguez et al., 2007), as a part of a "common host response"-cluster of genes that were induced in different cell types in response to various pathogens (Irf7) (Jenner and Young, 2005) and up-regulated in macrophages during *C. pneumoniae* infection (Irs2) (Lim and Chow, 2006). In summary, our data is in line with previous studies showing the importance of IFNγ in the control of *C. pneumoniae* infection (Rottenberg et al., 2002; Rottenberg et al., 1999; Rottenberg et al., 2000; Vuola et al., 2000) and we also observed up-regulation of *Ifng* in the CD4+ T lymphocytes during both primary and reinfection (data not shown). Furthermore, granzyme A (Gzma) and serine leukocyte protease inhibitor (Slpi) were found to be down-regulated in reinfection compared with primary infection. CD8+ T cells secrete granzymes and perforin to kill their target cells (Russell and Ley, 2002), but the role of CD8+ T cell mediated killing in the host response to *C. pneumoniae* is not clear (Wizel et al., 2008), which is in line with the observed down-regulation of Gzma during reinfection. In addition, Slpi has a role in attenuating the excessive inflammatory innate immune responses (Nakamura et al., 2003). Similarly as in our results, Slpi expression was elevated in murine lungs during *Streptococcus pneumoniae* primary infection (Abe et al., 1997) and it might have a role in attenuating the inflammatory response during primary infection of *C. pneumoniae.* However, the transcription level of Slpi returns to the control level during reinfection.

In summary, we demonstrated an accumulation of lymphocytes into MLN in response to *C. peumoniae* infection and characterized the gene expression of MLN derived lymphocytes during infection. These novel gene expression signatures provide new insights to the nature of protective immunity established during experimental *C. pneumoniae* infection.

6 CONCLUSIONS

The lymphocyte cells of the adaptive immune system are important for host defence to various different pathogens. T helper cells orchestrate both cell-mediated and humoral immune responses against a variety of pathogens, but if uncontrolled they can also mediate immunopathology. Th2 cells activate basophils and eosinophils and induce B cell antibody class-switching to IgE whereas Th1 cells produce pro-inflammatory cytokines IFNγ and TNFα and activate CD8+ T cell mediated antiviral responses as well as NK T cells. IFNγ and CD8+ T cells together with CD4+ T cells have also been shown to be important for protection against intracellular pathogen *C. pneumoniae,* which causes respiratory tract infections resulting only in partial immunity. The differentiation of Th1 and Th2 cells is a finely balanced and tightly regulated process and although several regulators have been identified, the picture is far from complete. In the work presented in this thesis the role of novel factors, PIM kinases and c-FLIP, were studied in the regulation of human Th1 and Th2 cell differentiation. In addition, the gene expression signatures characterizing the lymphocyte response during *C. pneumoniae* infection were studied. The family of PIM serine/threonine kinases as well as anti-apoptotic c-FLIP protein have been shown to affect the cell proliferation and survival. In addition, c-FLIP is involved in the regulation of T cell activation and activation induced cell death.

The family of PIM kinases were identified as new regulators of human Th1 cell differentiation. We were able to show that all three members of this family are preferentially expressed in *in vitro* polarized Th1 cells, and in addition, that PIM1 and PIM2 are also expressed in memory Th1 cell population isolated from peripheral blood. Using an siRNA approach, we demonstrated that PIM kinases act as positive regulators of IFNγ/TBET and IL12/STAT4 pathways. In addition, by genome-wide analysis of transcriptional changes caused by PIM knockdown, we were able to show that NFAT2, NF-κB, STAT5 and ATF2 target genes were enriched among the differentially regulated genes in Th1 cells. Interestingly, NFAT and NF-κB have been reported to be PIM targets, thus providing an interesting basis for further studies on the role of PIM kinases in the regulation of Th1 cell differentiation. In summary, we have identified a novel role for PIM kinases in regulating the Th1 cell differentiation, thus providing new insights into the mechanisms controlling the early development of human Th1 cell subset.

The apoptosis regulator protein, c-FLIP has three isoforms. We have shown that one of these isoforms, c-FLIP$_S$, is selectively up-regulated by IL4, whereas all three isoforms were induced by TCR activation at the mRNA level. In addition, we demonstrated by

STAT6 knockdown that this up-regulation might be at least partly mediated by STAT6. To further characterize the roles of two of the isoforms that were detected at protein level, c-FLIP$_S$ and c-FLIP$_L$, we used isoform specific siRNAs. The knockdown of c-FLIP$_L$ led to enhanced IFNγ production and IL12Rβ2 expression by Th1 polarized cells, whereas knockdown of c-FLIP$_S$ diminished GATA3 expression as well as IL-4 production by Th2 cells. In summary, these measurements indicated a role for both c-FLIP$_L$ and c-FLIP$_S$ in Th1/Th2 polarization, which interestingly influence differentiation in a distinct and opposite fashion. This gives an interesting basis for future studies to further characterize the isoform specific roles of c-FLIP in Th cell differentiation as well as to study in more detail the mechanisms behind the selective up-regulation of c-FLIP$_S$ in response to IL4 stimulation.

The characterization of the gene expression profiles of MLN derived lymphocytes during experimental *C. pneumoniae* infection provided new insights to the nature of protective immunity established during infection. A panel of IFNγ induced genes were found to be upregulated in the lymphocytes isolated from mice during reinfection (in comparison with primary infection), thus indicating the importance of Th1 type response in the protection against this pathogen. These genes could be used as markers for detecting protective immune response. In addition, further studies with, for example, knockout mice could provide more information of the roles of these genes in the immunity against *C. pneumoanie* infection.

In conclusion, these studies presented in this thesis have provided new information into the mechanisms of human Th cell differentiation *in vitro* and to the nature of lymphocyte response evolving *in vivo* during experimental *C. pneumoniae* infection. PIM kinases and c-FLIP were identified as new factors involved in the regulation of human Th cell differentiation, and a panel of genes characterising the protective lymphocyte response to *C. pneumoniae* infection was identified.

ACKNOWLEDGEMENTS

This Ph.D. thesis was carried out at the Turku Centre for Biotechnology, University of Turku and Åbo Akademi University and in the department of Medical Biochemistry and Genetics, University of Turku. I would like to warmly acknowledge a number of people who have made this achievement possible.

I wish to express my sincere gratitude to my supervisor, Professor Riitta Lahesmaa for giving me the opportunity to do research in her group. I also want to thank her for all her support and guidance, as well as for having trust in me and for providing me excellent opportunities to grow as a scientist.

The National Doctoral Programme in Informational and Structural Biology and its Director Mark Johnson are acknowledged for financial support and providing me a possiblitiy to interact with other PhD students in ISB meetings. Docent Marko Pesu and Docent Jukka Partanen are warmly acknowledged for a careful review of my thesis and providing valuable comments. Dr. Robert Moulder is acknowledged for the language revision of this thesis. I also wish to thank the members of my supervisory committee, Dr Kanury Rao, Professor Jukka Pelkonen and Dr Gobardhan Das, for their valuable comments and advice during these years.

This work would not have been possible to do without a number of great co-authors and collaborators. Anu Haveri, Jenni Vuola, Mirja Puolakkainen, Johanna Tahvanainen, Kartiek Kanduri, Bhawna Gupta, Teemu Kallonen, Anna Rajavuori, Hanna Lähteenmäki, Johanna Myllyviita, Riina Kaukonen, Omid Rasool, Harri Lähdesmäki, Kanury Rao and Päivi Koskinen are all warmly acknowledged for their invaluable contribution in these projects.

I also wish to thank the skillful personnel and technical staff of Turku Centre for Biotechnology and its core facilities, especially all the people in the office, it-support, technical and intrument maintenance, and Finnish Microarray and Sequencing Centre for their work. I especially want to thank Marjo Hakkarainen, Sarita Heinonen, Elina Pietilä and Marjo Linja for their excellent technical assistance.

Importantly, all the past and current members of the ATLAS group are acknowledged for making the working atmosphere so nice. It has been a great pleasure to share the scientific as well as other activities with you. I have learned a lot from you during these years and I feel priviledged that I have had the chance to get to know you all.

I also want to thank my dear friends for their friendship and support. We have shared the ups and downs of life together and with you I have been able to forget all the science and concentrate on the life itself. It is great to have you all in my life! I also wish to thank Tommi's whole family for their support and being there for us.

I owe my deepest gratitude to my dear family for your unconditional support and love throughout my life. Tommi, I could not have completed this without you! Thank you for your love, support and being there for me. Kiitos Ronja ja Arttu, kun muistutatte äidille mikä todella on tärkeää elämässä ja saatte minut nauramaan!

This work was financially supported by The National Doctoral Programme in Informational and Structural Biology, the Turku University Foundation, Väinö and Laina Kivi Foundation, Hospital District of Southwest Finland, Waldemar von Frenckell Foundation, Turku Postgraduate School of Health Sciences, The Finnish Society for Immunology, The Academy of Finland, The Systems Biology Research Program, The Finnish Concordia Fund, Konkordialiitto, Sigrid Jusélius foundation.

Turku, September 2013

Minna Kyläniemi

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