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SYSTEM-WIDE APPROACHES TO UNCOVER TH2 CELL LINEAGE COMMITMENT

by

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System-wide approaches to uncover Th2 cell lineage commitment
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ABSTRACT

T helper (Th) cells are vital regulators of the adaptive immune system. When activated by presentation of cognate antigen, Th cells demonstrate capacity to differentiate into functionally distinct effector cell subsets. The Th2 subset is required for protection against extracellular parasites, such as helminths, but is also closely linked to pathogenesis of asthma and allergies. The intracellular molecular signal transduction pathways regulating T helper cell subset differentiation are still incompletely known. Moreover, great majority of studies regarding Th2 differentiation have been conducted with mice models, while studies with human cells have been fewer in comparison. The goal of this thesis was to characterize molecular mechanisms promoting the development of Th2 phenotype, focusing specifically on human umbilical cord blood T cells as an experimental model. These primary cells, activated and differentiated to Th2 cells in vitro, were investigated by complementary system-wide approaches, targeting levels of mRNA, proteins, and lipid molecules. Specifically, the results indicated IL4-regulated recruitment of nuclear protein, and described novel components of the Th2-promoting STAT6 enhanceosome complex. Furthermore, the development of the activated effector cell phenotype was found to correlate with remodeling of the cellular lipidome. These findings will hopefully advance the understanding of human Th2 cell lineage commitment and development of Th2-associated disease states.

Keywords: T helper cell, Th2, allergy, interleukin 4, transcriptomics, proteomics, lipidomics, systems biology

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Th2-tyypin valkosolujen erilaistumisen tutkimus systeeminlaajuisin menetelmin

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TIIVISTELMÄ

T-auttajasolut (Th) ovat elintärkeitä adaptiivisen immuunijärjestelmän säätelijöitä. Aktivoituessaan kohdattuaan tunnistamansa antigeenin, Th-solut erilaistuvat toiminnallisesti eroaviksi effektorisoluiksi. Th2-tyypin solut puolustavat elimistöä solujen ulkopuolisia loismatoja vastaan, mutta ovat toisaalta yhteydessä myös astman ja allergian kehittymiseen. T-auttajasolujen erilaistumista säätelevät solunsisäiset molekulaariset viestintätiet tunnetaan yhä puutteellisesti. Lisäksi suurin osa Th2-solujen erilaistumisen tutkimuksesta on suoritettu hiirimallien avulla, ja ihmisen soluja on tutkittu verrattain vähän. Tämän väitöskirjatutkimuksen tavoitteena oli selvittää ihmisen Th2-solujen kehitystä edistäviä molekyylitason mekanismeja käyttäen tutkimusaineistona napanuoran verestä eristettyjä T-soluja. Näiden primaarisolujen in vitro -stimuloitua erilaistumista Th2-tyypin soluiksi tutkittiin toisiaan täydentävin systeeminlaajuisin menetelmin, jotka kohdistuivat lähetti-RNA-, proteiini-, ja lipidimolekyyleihin. Tuloksena havaittiin IL4:n säätelemiä muutoksia tumaproteiinien määrissä, sekä tunnistettiin uusia Th2-solujen erilaistumista edistävän STAT6-enhanseosomikompleksin osia. Lisäksi aktivoidun effektorisolun fenotyypin kehitykseen havaittiin liittyvän solun lipidikoostumuksen selektiivisiä muutoksia. Nämä tulokset toivottavasti edistävät ymmärrystä ihmisen Th2-solujen erilaistumisen ja Th2-soluvälitteisten sairauksien mekanismeista.

Avainsanat: T-auttajasolu, Th2, allergia, interleukiini 4, transkriptomiikka, proteomiikka, lipidomiikka, systeemibiologia

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ABBREVIATIONS

2DE, two-dimensional electrophoresis
ACACA, acetyl-OcA carboxylase alpha
ACN, acetonitrile
AHR, aryl hydrocarbon receptor
AP-1, activator protein 1
APC, antigen-presenting cell
ATF, activating transcription factor
BATF, basic leucine zipper transcription factor, ATF-like
BCL10, B-cell CLL/lymphoma 10
CARD11, caspase recruitment domain family, member 11, synonym CARMA1
CD, cluster of differentiation
CDP, CCAAT displacement protein
CIA, collagen-induced arthritis
CN, calcineurin
CREB, cyclic adenosine monophosphate responsive element binding protein
CREBBP, CREB binding protein, synonym CBP
DG, diacylglycerol
EAE, experimental autoimmune enchaphalomyelitis
ELOVL, fatty acid elongase
ERK, Extracellular signal-regulated kinase
ESI, electrospray ionization
FAS, Fas (TNF receptor superfamily, member 6)
FCM, flow cytometry
FOXP3, forkhead box P3
GATA3, GATA binding protein 3
GFI1, growth factor independent 1 transcription repressor
GTP, Guanosine triphosphate
HNRNPK, heterogeneous nuclear ribonucleoprotein K
HS2, hypersensitive site 2
IBD, inflammatory bowel disease
ICOS, inducible T-cell co-stimulator
IFI16, interferon, gamma-inducible protein 16
IFN, interferon
i.d., inner diameter
Ig, immunoglobulin
IKZF1, IKAROS family zinc finger 1, synonym Ikaros)
IL, interleukin
ILR, IL receptor
IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

IRF4, interferon regulatory factor 4
ITAM, immunoreceptor tyrosine-based activation motif
ITK, IL2-inducible T-cell kinase
iTRAQ, Isobaric tags for relative and absolute quantitation
iTreg, inducible regulatory T cell
JAK, Janus kinase
JNK, JUN N-terminal kinase
LAT, linker for activation of T cells
LC, liquid chromatography
LCK, lymphocyte-specific protein tyrosine kinase
LPCAT, lysophosphatidylcholine acyltransferase
LTQ, hybrid linear ion trap-quadrupole
MAF, v-maf musculoaponeurotic fibrosarcoma oncogene homolog, synonym c-MAF
MAPK, mitogen-activated protein kinase
MHC, major histocompatibility complex
MMTS, methylmethanethiosulfonate
MS, mass spectrometry
mTOR, mammalian target of rapamycin
NCL, nucleolin
NFAT, nuclear factor of activated T-cells
NFKB, nuclear factor of kappa light polypeptide gene enhancer in B-cells
NICD, notch intracellular domain
NKX3, NK3 homeobox
nTreg, natural regulatory T cell
OX40, tumor necrosis factor receptor superfamily, member 4
PAGE, polyacrylamide gel electrophoresis
PC, phosphatidylcholine
PCR, polymerase chain reaction
PCYT2, phosphate cytidylyltransferase 2, ethanolamine
PE, phosphatidylethanolamine
PI3K, phosphoinositide-3-kinase
PKC, protein kinase C
PU.1, spleen focus forming virus (SFFV) proviral integration oncogene spi1
QTOF, hybrid quadrupole-time of flight
RA, rheumatoid arthritis
RAS, Rat sarcoma
RBPJ, recombination signal binding protein for immunoglobulin kappa J region
RHA, RNA helicase A
RORC, RAR-related orphan receptor C
RT, reverse transcriptase
SAP, sphingolipid activator protein-1
SATB1, SATB homeobox 1
SCD, stearoyl-CoA desaturase

SCX, strong cation exchange
SDS, sodium dodecyl sulphate
SH2, Src homology 2 domain
SHC, SHC (Src homology 2 domain containing) transforming protein
SLAM, signaling lymphocytic activation molecule
SM, sphingomyelin
SND1, staphylococcal nuclease and tudor domain containing 1
SRC, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT, signal transducer and activator of transcription
TBX21, T-box 21, synonym T-bet
TCF7, transcription factor 7 (T-cell specific, HMG-box)
TCR, T cell receptor
TEC, tec protein tyrosine kinase
Tfh, follicular T helper cell
TG, triacylglycerol
TGFβ, transforming growth factor beta
Th, T helper cell
TPM, tropomyosin
Treg, regulatory T cell
TRIM22, tripartite motif containing 22
TSLP, thymic stromal lymphopoietin
YB1, Y box binding protein 1
ZAP70, zeta-chain (TCR) associated protein kinase 70kDa

LIST OF ORIGINAL PUBLICATIONS

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

I Moulder, R.*, Lönnberg, T.*, Elo, L.L., Filén, J.J., Rainio, E., Corthals, G., Orešič, M., Nyman, T.A., Aittokallio, T., Lahesmaa, R. (2010) Quantitative Proteomics Analysis of the Nuclear Fraction of Human $CD4^+$ Cells in the Early Phases of IL-4-induced Th2 Differentiation. *Mol Cell Proteomics* 9:1937-1953 (*Equal contribution)

II Lönnberg, T., Yetukuri, L., Seppänen-Laakso, T., Lahesmaa, R.*, Orešič, M.* (2013) T-cell activation induces selective changes of cellular lipidome. *Front Biosci* E5, 558-573 (*Equal contribution)

III Lönnberg, T, Filén, J.J., Moulder, R., Goo, Y.A., Kantola, S., Jalonen, J., Rasool, O., Goodlett, D.R., Lahesmaa, R. (2012) Proteomic analysis identifies HNRNPK as a member of STAT6 enhanceosome in human $CD4^+$ T-cells. Manuscript

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1. INTRODUCTION

The immune system is formed by a complex hierarchy of cell types with specialized roles. Efficient functioning of such system requires tight regulation and communication between the constituent components. T helper (Th) cells, characterized by the expression of CD4 surface marker, form the topmost regulatory layer of the adaptive immune system. They activate and recruit downstream immune cells, such as macrophages, cytolytic (CD8+) T cells, and in particular "help" B cells to produce antibodies.

T cells arise from the hematopoietic stem cells of the bone marrow, and mature in the thymus, hence the name T cells. Their most distinctive feature is the expression of the cell surface T cell receptor, responsible for antigen recognition. During their maturation, genes coding for T cell receptor undergo somatic recombination. As a result, the receptors expressed on T cells differ between T cell clones, and the spectrum of antigens specifically recognized by the T cell repertoire is practically unlimited. While there are approximately $2.5*10^8$ T cell clones in an individual, estimated theoretical diversity of T cell receptor structures is in the order of 10^{16} (Robins et al., 2009). Dysfunctional and self-reactive T cell clones are cleared by consequent processes of positive and negative thymic selection, which in fact results in apoptotic elimination of vast majority of newly generated T cells (Scollay et al., 1980). Mature T cells leave thymus and circulate in the periphery, until encountering antigen-presenting cells in the lymphoid tissues. Presentation of cognate antigen activates the T cell, inducing clonal expansion and development to functionally active phenotype. The activated cells develop into alternative effector subsets, as instructed by signals from cells of the innate immunity. By subset differentiation, the T helper cell compartment can selectively launch different arms of the immune system that are best suited for overcoming the particular type of pathogen in question.

The regulatory functions performed by T helper cells are vital. In untreated HI virus infection, depletion of the CD4+ T cell compartment leads to fatal debilitation of the adaptive immune system, while insufficiently regulated T helper cell activity is associated with various autoimmune diseases. In particular, strong evidence exists for involvement of cells of Th2 subset in atopy and allergies. Hence, elucidation of molecular mechanisms regulating the development of Th2 cells would improve our understanding of the pathogenesis of these disorders. In the present work, system-wide approaches were applied to investigate transcriptomics, proteomics, and lipidomics aspects of Th2 cell differentiation. These studies focused specifically on primary human T cells to achieve results representative of normal human physiology.

2. REVIEW OF THE LITERATURE

2.1. Characterization of functionally distinct T helper cell subsets

By the mid 1970s, it had been demonstrated that antibody-mediated and delayed-type hypersensitivity responses were distinct and potentially regulated by different types of T cells (Liew and Parish, 1974). Subsequently, existence of T helper cell populations with different functional properties was reported in the late 1970s and early 1980s by several groups (Liew, 2002; Coffman, 2006).

Terms Th1 and Th2 were first used by Tada et al. to divide T helper cells based on the capacity to trigger B cells only by cognate interaction (Th1) or in a polyclonal manner (Th2) (Tada et al., 1978). In 1986, by application of newly established molecular immunology methods, Mosmann et al. provided the first, authoritative characterization of two functionally distinct murine T helper cell clones, and adopted the nomenclature proposed by Tada et al. A key difference between the clones was characteristic cytokine secretion profiles, which supported the hypothesis that the interferon-γ (IFNγ)- and Interleukin-2 (IL2)-producing Th1 cells activate cell-mediated immune responses and the IL4-producing Th2 cells activate humoral responses (Mosmann et al., 1986). Further experiments with T cell clones verified that Th1 and Th2 cells arose from a common precursor cell type, and that the differentiation was not predetermined by antigen specificity (Hsieh et al., 1992; Röcken et al., 1992b; Sad and Mosmann, 1994). The existence of corresponding human Th1 and Th2 lineages was confirmed relatively shortly after the original findings with mice (Wierenga et al., 1990; Del Prete et al., 1991; Parronchi et al., 1991; Romagnani, 1991; Lahesmaa et al., 1992).

The dichotomy of T helper cells to Th1 and Th2 populations remained as the universally accepted paradigm for nearly twenty years, although data from studies with some Th1-specific disease models, such as experimental autoimmune enchaphalomyelitis (EAE) and collagen-induced arthritis (CIA), were increasingly in conflict with the predictions from the Th1/Th2 hypothesis (Harrington et al., 2006; Steinman, 2007). These discrepancies were reconciled by discovery of IL23 (Oppmann et al., 2000) and subsequent characterization of a separate subset of IL-17 producing T helper cells (Cua et al., 2003; Murphy et al., 2003; Langrish et al., 2005; Park et al., 2005; Chen et al., 2006). For cells of this novel subset, term Th17 was coined (Harrington et al., 2005; McKenzie et al., 2006). Furthermore, naïve T cells were found to be capable of developing into inducible regulatory T cells (iTreg) (Apostolou and Boehmer, 2004; Cobbold et al., 2004; Curotto de Lafaille et al., 2004). The functional properties of the iTreg cells resembled those of the so called natural regulatory cells (nTreg), but instead of thymus, their differentiation took place in the periphery as part of

activation of the adaptive immune response (Curotto de Lafaille and Lafaille, 2009).

In the past few years, the repertoire of distinct T helper cell subsets has further expanded with the discovery of subsets of IL9-producing Th9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008) and IL22-producing Th22 cells (Duhen et al., 2009; Trifari et al., 2009), and description of follicular T helper cells (Tfh) as a lineage distinct from the Th1, Th2, and Th17 populations (Nurieva et al., 2008).

With the description of the new CD4+ T cell populations, and concurrent improvement in understanding of underlying molecular mechanisms that regulate T cell diversity, the boundaries between the subsets have in many cases become less obvious. Subsets such as Th2 and Th9 have been shown to be related both in terms of immunological function and regulatory intracellular pathways utilized (Soroosh and Doherty, 2009; Staudt et al., 2010; Goswami and Kaplan, 2011; Goswami et al., 2012). In addition, while originally T helper cell differentiation was seen as strictly irreversible process (Murphy et al., 1996), this view has recently been challenged (Bluestone et al., 2009; Zhou et al., 2009; Murphy and Stockinger, 2010). In particular, the subset identities of Th17 and Th1 cells in vivo have turned out to be remarkably interchangeable (Hirota et al., 2011). All things considered, despite more than 20 years of investigation, the full extent of human T helper cell subset diversity, as well as the regulatory signaling pathways, remain incompletely known.

2.2. Immunological roles of T helper subsets

Responding to signals from the immune immunity, the differentiated T helper cell subsets perform highly specialized functions in the immune system, acting primarily through secretion of cytokines characteristic to each subset. Th1 cells activate cell-mediated immune responses against intracellular viruses and bacteria (Buchmeier and Schreiber, 1985; Kobayashi et al., 1997; Sareneva et al., 1998; Fujioka et al., 1999; Mastroeni et al., 1999; Tanaka-Kataoka et al., 1999), protozoal parasites (Sadick et al., 1987), and tumors (Micallef et al., 1997), while Th17 activity targets extracellular bacteria and fungi (Chung et al., 2003; Huang et al., 2004; Happel et al., 2005; LeibundGut-Landmann et al., 2007). Th2 cells respond primarily against extracellular parasites, such as helminths (Sher and Coffman, 1992). The recently discovered Th9 subset has also been accredited with this function (Richard et al., 2000; Khan et al., 2003; Forbes et al., 2008; Annunziato and Romagnani, 2009). Th22 cells express skin-homing chemokine receptors CCR4 and CCR10 and their function might involve immunosurveillance of the skin (Duhen et al., 2009; Trifari et al., 2009). Tfh cells are found in germinal centers of secondary lymphoid tissues, associated with B cells and regulating their proliferation and immunoglobulin class switching (Vinuesa et al., 2005; Fazilleau et al., 2009; King and Mohrs, 2009).

iTreg cells, together with the thymus-generated nTreg cells, regulate homeostasis of lymphocyte populations, promoting immune tolerance and preventing autoimmunity (Wohlfert and Belkaid, 2008).

Deviations in polarization of T helper cell response are correlated with various disease states. Such associations have been highlighted by numerous studies with mice strains deficient for key factors regulating T helper cell development. Depending on the specific effect of the perturbations on the balance of T helper cell subsets, such phenotypes may either be predisposing or protective to a particular disease. Th1 immunity plays a role in type-1 diabetes (Wang et al., 1997; Pakala et al., 1999), rheumatoid arthritis (RA) (Leung et al., 2000; Yamada et al., 2008), inflammatory bowel disease (IBD) (Davidson et al., 1996; Parronchi et al., 1997), and graft-versus-host disease (Hu et al., 1999), whereas defects in Th1 signaling pathways lead to susceptibility to *Salmonella typhirium* and mycobacterial infection, including *Mycobacterium tuberculosis* (Filipe-Santos et al., 2006). In line with its relation to Th1 phenotype, the Th17 subset is also involved in RA and IBD (Bush et al., 2002; Nakae et al., 2003; Yen et al., 2006; Nistala et al., 2010; van Hamburg et al., 2011). Even more significant is the role of Th17 cells in the pathogenesis of multiple sclerosis, and the corresponding mouse model EAE (Hofstetter et al., 2005; Langrish et al., 2005; Komiyama et al., 2006). Tfh cells have also been implicated in autoimmunity, in particular systemic lupus erythematosus (Simpson et al., 2010) and autoimmune thyroid disease (Zhu et al., 2012). Th2 responses, when uncontrolled and persistent, can lead to chronic inflammatory airway diseases, namely allergy and atopic asthma (Durham et al., 1992; Robinson et al., 1992; Yssel et al., 1992; Ebner et al., 1993). In contrast, mice deficient for Th2 cells are protected from airway hyperreactivity but prone to disorders involving Th1 and Th17 subsets (Akimoto et al., 1998; Kuperman et al., 1998; Chitnis et al., 2001). In addition to Th2, Th9 cells have been shown to contribute to atopy and allergic inflammation (Shimbara et al., 2000; Erpenbeck et al., 2003; Soroosh and Doherty, 2009; Bullens et al., 2011; Kearley et al., 2011; Yao et al., 2011). Elevated levels of IL22 and Th22 cells have been detected in patients with psoriasis, ankylosing spondylitis and RA (Lo et al., 2010; Zhang et al., 2012). Development of naïve T cells to Treg fate is induced in cancer tissues as means of inhibiting activity of tumor-targeting effector response (Liu et al., 2007). On the other hand, lack of regulatory T cell function is observed in patients with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (Wildin et al., 2001). Detailed understanding of how CD4+ T cell responses are regulated could thus offer numerous therapeutic and diagnostic possibilities.

2.3. Common features in T helper cell lineage commitment

The current paradigm views T helper cell lineages as alternative developmental programs, any of which the uncommitted precursor cell can undergo in response

to specific external stimuli. Although all T helper cell lineages represent separate directions of effector cell differentiation with considerably complex individual characteristics, there are several common unifying themes, which can at least provide a useful conceptual simplification. These features are presented in table 1 and discussed below in detail. Essentially, T helper cell lineages are populations with characteristic cytokine production, distinctive gene expression profile regulated by a lineage-specific transcription factor, and to a lesser extent, express lineage-specific cytokine and chemokine receptor phenotype (Bluestone et al., 2009; Guo et al., 2009; O'Shea and Paul, 2010). Whether all of the newly discovered subsets (Th9, Th22, Tfh) truly fulfill these criteria remains to be confirmed (Zhu and Paul, 2010).

2.3.1. Cytokines

The defining property of T helper cells subsets is the unique profile of secreted cytokines. Th1 cells produce mainly IFNγ, while the hallmark Th2 cytokines are IL4, IL5, and IL13, and Th17 cells secrete IL17a and IL17f (Zhu and Paul, 2008; Annunziato and Romagnani, 2009; Zhu et al., 2010), and Th9 cells produce high levels of IL9 (Dardalhon et al., 2008; Veldhoen et al., 2008). The main cytokines generated by Tfh and Th22 cells are IL21 and IL22, respectively, although both are produced also by Th17 cells (Nurieva et al., 2008; Duhen et al., 2009; Trifari et al., 2009; Zhu et al., 2010). iTreg produce TGFβ and in common with the Th9 cells, also IL10 (Dardalhon et al., 2008; Veldhoen et al., 2008; Zhu and Paul, 2008; Annunziato and Romagnani, 2009). In addition to these subset-specific signature molecules, cytokines such as IL2 are produced in varying amounts by multiple subsets.

Local cytokine milieu is the key regulatory component of T helper cell differentiation. Early on it was observed that the cytokines produced by the Th cells themselves promote the proliferation of the secreting subset in an autocrine and paracrine manner, while specifically restricting the proliferation of the opposing subset (Horowitz et al., 1986; Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988; Fiorentino et al., 1989; 1991). Thus, the effector cytokines play dual roles, serving also as key regulators driving T helper cell lineage commitment. This concept of reciprocal regulation by opposing cytokines has in its part strengthened the notion of T helper cell subsets as mutually exclusive separate cell lineages.

Naïve Th cells differentiate to Th1 cells in response to IL12 and IFNγ whereas IL4 induces Th2 differentiation (Le Gros et al., 1990; Swain et al., 1990; Kühn et al., 1991; Hsieh et al., 1993; Kopf et al., 1993; Seder et al., 1993; Magram et al., 1996; Noben-Trauth et al., 1997; Wu et al., 1997; Lighvani et al., 2001). Both IFNγ and IL12 contribute to downregulation of Th2 subset (Manetti et al., 1993; Mountford et al., 1999). Differentiation of both Th1 and Th2 cells is inhibited by

TGFβ (Gorelik et al., 2000; 2002; Park et al., 2007). IL4 suppresses differentiation of Th1, Th17, and iTreg cells (Zhu et al., 2006; 2009), but in combination with TGFβ promotes Th9 differentiation (Dardalhon et al., 2008; Veldhoen et al., 2008). Th17 differentiation can be induced in mouse with TGFβ and either IL6 or IL21 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007), and in human with TGFβ, IL6, and IL1β (Manel et al., 2008; Tuomela et al., 2012). Th17 differentiation is suppressed by IL2 (Laurence et al., 2007). Human Th22 cells can be generated using IL6 and TNF (Duhen et al., 2009). Differentiation of iTreg cells requires TGFβ and IL2 (Chen et al., 2003a; Burchill et al., 2007; Davidson et al., 2007; Yao et al., 2007; Burchill et al., 2008), and Tfh cells IL21 (Nurieva et al., 2007; Vogelzang et al., 2008).

The mechanistic role of cytokines in lymphocyte differentiation was for long debated. Whether cytokine signals truly instructed the development of naïve cells to alternative fates or merely acted as selective signals, specifically permitting proliferation of pre-committed cells, was unclear (Farrar et al., 2002; Murphy and Reiner, 2002). However, recent reports have unambiguously shown that cytokines can indeed control developmental decisions in individual cells in an instructive manner (Rieger et al., 2009; Sarrazin et al., 2009).

2.3.2. STAT pathways

Ligation of cytokine receptors activate transcriptional factors of the Signal transducer and activator of transcription (STAT) family, composed of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Stark and Darnell, 2012). Each IL activates specific STAT molecule, and to a certain extent these pathways are characteristic for the T helper cell subsets. In Th1 cells, IL12 and IFNγ activate STAT4 and STAT1, respectively (Greenlund et al., 1994; Thierfelder et al., 1996; Kaplan et al., 1996b; Ramana et al., 2002). In Th2 cells, IL4 signaling is mediated by STAT6. STAT6 is required also for Th9 differentiation(Goswami et al., 2012). STAT3 is the major regulator in Th17 and Tfh cells and STAT5 in iTreg cells, although both of these STATs are also involved in differentiation of Th2 cells (Holland et al., 2007; Minegishi et al., 2007; Ma et al., 2008; Milner et al., 2008; Stritesky and Kaplan, 2011). In the case of the recently discovered Th22 subset, the relative contributions of the intracellular signaling pathways are still under investigation, but STAT1 and STAT3 are likely to play major roles (Heinrich et al., 1998; Annunziato and Romagnani, 2009; Stark and Darnell, 2012).

2.3.3. Lineage-specific transcription factors

For each of the “classical” T helper cell subsets, i.e. Th1, Th2, and Th17 cells, but also for most of the more recently discovered subsets, lineage-specific

transcription factors have been identified, sometimes also referred to as master regulators. These factors are induced in response to the signals mediated by the STAT pathways, and in turn upregulate the production of the subset-specific cytokines. Thus, positive feedback loops are formed, resulting in reinforcement of subset identity. In Th1 differentiation, the transcription factor T-box 21 (T-bet) is highly expressed in response to IFNγ (Szabo et al., 2002). In Th2 cells, the counterpart regulator is GATA3 (Zhang et al., 1997; Zheng and Flavell, 1997). In Th17, Th9, and iTreg cells, the corresponding factors are RORC (Ivanov et al., 2006), PU.1 (Chang et al., 2010), and FOXP3 (Sakaguchi et al., 2006), respectively. For Tfh cells, the critical transcription factor appears to be BCL6 (Nurieva et al., 2008; Vogelzang et al., 2008; Fazilleau et al., 2009). A master regulator for the Th22 program has not been characterized, although both RORC and AHR appear to be involved (Trifari et al., 2009). Importantly, the expression of these transcription factors appears to be mutually exclusive. This has been widely documented especially in the case of T-bet and GATA3 (Zheng and Flavell, 1997; Ouyang et al., 2000).

2.3.4. Epigenetic changes

In fully differentiated cell populations, the instructive cytokine signals are no longer required to maintain the subset identity. Instead, cells exhibit subset-specific patterns of cytokine expression in response to re-stimulation by cognate antigen alone. According to current knowledge, this subset memory is largely based on epigenetic regulation of key cytokine loci, including both permissive and silencing modifications (Grogan et al., 2001) (Ansel et al., 2006; Lee et al., 2006; Janson et al., 2009; Wilson et al., 2009).

Table 1. Functional and regulatory characteristics of lineages derived from naïve CD4+ T cells.

Feature	Th1	Th2	Th9	Th17	Th22	Tfh	iTreg
Hallmark cytokine secretion	IFNγ	IL4, IL5, IL13	IL9	IL17A, IL17F, IL21, IL22	IL22, TNFα	IL10, IL21	TGFβ
Primary instructing cytokines	IL12, IFNγ	IL4	IL4, TGFβ	IL6, TGFβ, IL21	TNF, IL6	IL21	TGFβ, IL2
Main STAT mediator(s)	STAT1, STAT4	STAT6	STAT6	STAT3	unknown	STAT3	STAT5
Master transcription factor	T-bet	GATA3	PU.1	RORC	AHR?	BCL6	FOXP3

2.4. Cytokine-induced signaling pathways promoting the development of Th2 phenotype

The focus of this thesis was elucidation of intracellular molecular signal transduction mechanisms that lead to the development of Th2 phenotype. Since the original characterization of Th2 cells, a multitude of factors affecting Th2 subset polarization has been described. These factors are organized into several, partly crosstalking pathways, the most important of which are discussed below and summarized in figure 1. This signaling network processes environmental information that is delivered primarily in the form of cytokines by the cells of the innate immunity, although the exact in vivo conditions that regulate Th2 responses are still incompletely understood (Fowell, 2009).

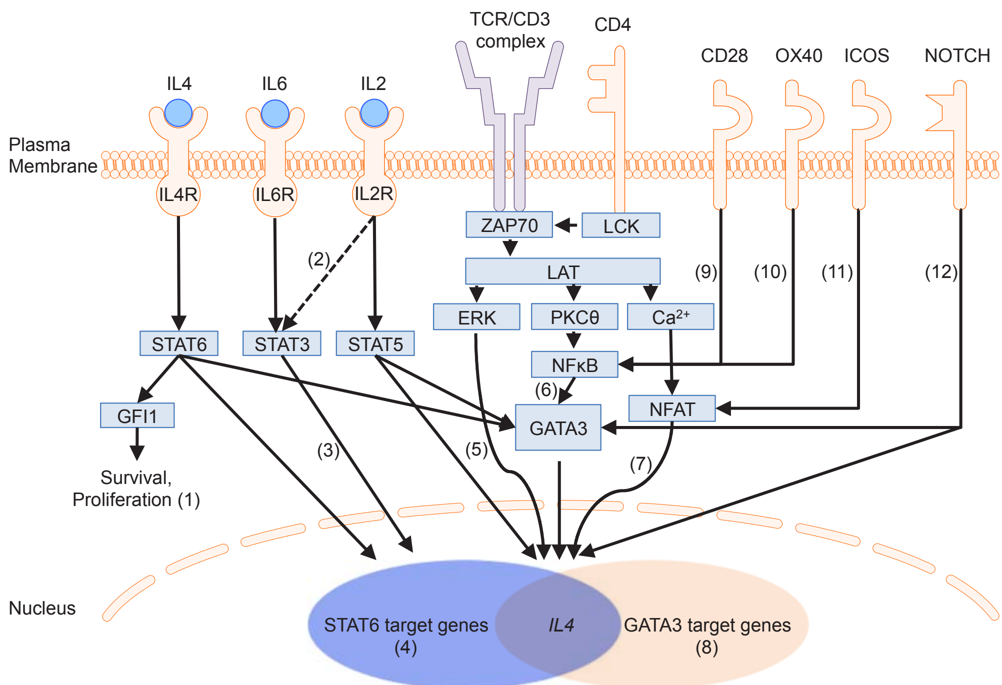

Figure 1. A simplified model of signaling pathways promoting the development of Th2 cells. References: (1) (Zhu et al., 2002); (2) (Malek, 2008); (3) (Stritesky et al., 2011); (4) (Elo et al., 2010); (5) (Tripathi et al., 2011); (6) (Corn et al., 2005); (7) (Hutloff et al., 1999); (8) (Jenner et al., 2009; Horiuchi et al., 2011; Wei et al., 2011); (9) (Verweij et al., 1991); (10) (Watts, 2005); (11) (Nurieva et al., 2003); (12) (Amsen et al., 2004; 2007; Fang et al., 2007).

2.4.1. IL4-STAT6 pathway

IL4 is considered as the hallmark Th2 cytokine. Naïve CD4+ T cells primed in presence of IL4 differentiate to cells of Th2 phenotype (Betz and Fox, 1990;

Le Gros et al., 1990; Swain et al., 1990; Hsieh et al., 1992; Seder et al., 1992). In IL4 knockout mice, production of Th2 cytokines IL-5, IL-9, and IL-10, as well as IgE, is diminished (Kühn et al., 1991; Kopf et al., 1993). IL4 signals through heterodimeric type I or type II receptors. The receptors found on naïve T cells and Th2 cells are of type I (Newcomb et al., 2011), and consist of IL4R-α and common γ chain subunits (Nelms et al., 1999). The IL4R-α subunit is common with the IL13 receptor (Lin et al., 1995; Zurawski et al., 1995; Hilton et al., 1996), while the γ chain is shared with the receptors for IL2 (Takeshita et al., 1992), IL7 (Noguchi et al., 1993; Kondo et al., 1994), IL9 (Russell et al., 1994), IL15 (Giri et al., 1994), and IL21 (Asao et al., 2001). The more recently discovered type II IL4 receptor is composed of IL4R-α and IL13R-α1 proteins (Murata et al., 1998), and its expression is confined mostly to non-hematopoietic cells. While the IL4 receptor is expressed in naïve T cells, the expression of the IL4R-α subunit is rapidly upregulated in response to IL4 (Ohara and Paul, 1988; Dokter et al., 1992).

IL4 signaling leading to Th2 polarization is mediated primarily by STAT6 (Boothby et al., 1988; Kotanides and Reich, 1993; Hou et al., 1994). In STAT6 deficient mice, Th2 development is impaired even more completely than in IL4 knockout mice (Shimoda et al., 1996; Takeda et al., 1996; Kaplan et al., 1996a). In human Th2 cells, more than 80% of IL4 target genes are regulated by STAT6 (Elo et al., 2010). Inactive, unphosphorylated STAT6 resides preferentially in the cytosol. Contrary to early notion of latent STAT6 existing only in monomeric form, it has since been shown, like other STAT proteins, to form dimeric or multimeric complexes referred to as statosomes (Haan et al., 2000; Ota et al., 2004; Sehgal, 2008). Activated STAT6 dissociates from the IL4 receptor and translocates to the nucleus. Recent study has shown that also inactive STAT6 is continuously shuttled to the nucleus, but the activated form accumulates in the nucleus due to DNA binding and thus longer time of nuclear retention (Chen and Reich, 2010).

STAT6 is activated as a consequence of sequential phosphorylation events initiated by binding of IL4 to its receptor (Hebenstreit et al., 2006). IL4 interacts first with the IL4R-α, which then dimerizes with the γ chain (Mueller et al., 2002). Upon dimerization, conserved tyrosine residues Y575, Y603, and Y631 on cytoplasmic tail of the IL4R-α chain become phosphorylated by Janus Kinases (Jak) associated with the receptor (Pernis et al., 1995; Ryan et al., 1996; Wang et al., 1996). Mediated by an SH2 domain, inactive STAT6 docks with the phosphotyrosine residues on the receptor and subsequently becomes itself phosphorylated on tyrosine 641 by Jak3 or Jak1 (Witthuhn et al., 1994). Once phosphorylated, the Y641 residue interacts with the SH2 domain in a neighboring molecule, resulting in the formation of an activated STAT6 homodimer. Although phosphorylation of tyrosine residue 641 is the key determinant of STAT6 activity, a number of

other post-translational modifications have been characterized. Independent of Y641, also S756 is phosphorylated in IL4-responsive manner, however, this this phosphorylation is required neither to nuclear translocation nor DNA binding activity of STAT6 (Wang et al., 2004b). Recently, phosphorylation of S707 by JNK has been to shown to negatively regulate STAT6 function by inhibiting its binding to DNA (Shirakawa et al., 2011). Instead of IL4, this phosphorylation is induced by stress response or IL1β. In addition to phosphorylation, STAT6 is subject to methylation on R27 (Chen et al., 2004). Both IL4 induced phosphorylation and nuclear translocation of STAT6 seem to be dependent on this modification, although contradictory evidence exists (Chen and Reich, 2010). Moreover, inducible lysine acetylation of STAT6 might, at least in some cases, be required for its transcriptional regulatory activity (McDonald and Reich, 1999; Shankaranarayanan et al., 2001; Wieczorek et al., 2012).

In the nucleus, STAT6 regulates expression of a pattern of genes by binding directly to their regulatory elements, promoting local chromatin modification, or by suppressing activity of transcription factors of opposing function (Ohmori and Hamilton, 2000). The target genes of STAT6 in Th2 cells have been identified with DNA microarrays (Chen et al., 2003b; Lund et al., 2007), and recently by more direct approaches based on chromatin immunoprecipitation and sequencing (ChIP-Seq) (Elo et al., 2010; Wei et al., 2010). The canonical STAT6 consensus binding sequence is 5′-TTCN$_{(3-4)}$GAA-3′ (Schindler et al., 1995). In the human STAT6 target genes, the consensus sequence was found in 73% of the cases (Elo et al., 2010). While this implies that STAT6 can directly bind many of its target genes, efficient transcriptional induction by STAT6 requires recruitment of coactivator proteins resulting in formation of STAT6 enhanceosome complex (Goenka and Kaplan, 2011). Best-characterized components of the complex include SND1 (Yang et al., 2002), CREBBP (Gingras et al., 1999; McDonald and Reich, 1999), and RHA (Välineva et al., 2006). These coactivators bridge STAT6 with the basal transcriptional machinery, and recruit histone deacetylase activity to the target loci. Importantly, co-operation with inducible or cell-type specific factors can provide context-dependent regulation and fine-tuning to IL4-STAT6 signaling. A recent comparison of STAT6 targets identified by ChIP-Seq in B cells, Th2 cells, and epithelial cells has demonstrated that these targets often are indeed cell-type specific (Kanai et al., 2011).

In the context of T helper cell lineage commitment, arguably the most important of the STAT6 target genes is GATA3, the Th2 master transcription factor (Zhang et al., 1997; Zheng and Flavell, 1997; Ouyang et al., 1998). The induction of GATA3 expression is achieved by STAT6 mediated displacement of repressive polycomb protein complex (Onodera et al., 2010). Transcription factor IRF4 contributes to GATA3 induction, and is necessary for Th2 differentiation (Lohoff et al., 2002; Rengarajan et al., 2002). GATA3 itself is required for T cell maturation, and its

deletion causes to embryonic lethality (Ting et al., 1996). Consequently, direct genetic evidence for its significance for Th2 differentiation has been difficult to obtain. However, evidence from studies with conditional knockout models demonstrates that disruption of GATA3 inhibits development of Th2 responses, measured as diminished production of Th2 cytokines IL4, IL5, IL10, and IL13 (Pai et al., 2004; Zhu et al., 2004). Accordingly, in patients with haploinsufficiency of GATA3, Th2 differentiation is reduced (Skapenko et al., 2004).

GATA3 has been shown to bind directly to promoter regions of both *IL5* and *IL13*, but, somewhat surprisingly, not to *IL4* (Siegel et al., 1995; Zhang et al., 1998; Kishikawa et al., 2001; Lavenu-Bombled et al., 2002; Yamashita et al., 2002; Klein-Hessling et al., 2003). While regulation of the *IL4* locus involves GATA3 (Tanaka et al., 2011), also contributions by other proteins, including STAT6 and the Th2-specific transcription factor c-MAF, seem to be required (Kim et al., 1999; Lee and Rao, 2004). The collaboration of GATA3 and STAT6 has been further confirmed by systematic study of GATA3 targets with constitutively active or absent STAT6 (Horiuchi et al., 2011). Genome-wide analyses of GATA3 targets have illustrated that while approximately 100 genes are bound by GATA3 in cells of all Th subsets, the number of subset-specific targets is more than 10-fold higher in the case of Th2 cells, highlighting the importance of accessory factors and epigenetic mechanisms (Horiuchi et al., 2011; Wei et al., 2011).

In addition to regulating the expression of Th2-specific genes, GATA3 actively suppresses the differentiation of Th1 cells, and in absence of GATA3, T cells can develop into Th1 phenotype even in absence of IL12 or IFNγ (Zhu et al., 2004). Interestingly, some of the GATA3 target genes are in common with T-bet (Jenner et al., 2009). This suggests that these factors have opposing influence on a common set of genes that ultimately determine the direction of effector subset differentiation.

The STAT6-mediated upregulation of GATA3 and the regulatory effects of these factors on their downstream targets represent a straightforward instructive pathway promoting differentiation to Th2 phenotype. In addition, The IL4-STAT6 signaling pathway positively influences cellular viability and proliferation (Ben-Sasson et al., 2000; Zhu et al., 2001). These effects are mediated by growth factor independent-1 (GFI1), expression of which is up-regulated in response to IL4 in activated T-cells (Zhu et al., 2002). GFI1 prevents apoptosis and promotes proliferation of cells that express high levels of GATA3. Altogether, the IL4-STAT6 pathway can be seen as serving complementary dual roles by providing instructive signals to trigger the Th2 transcriptional program, as well as selectively promoting the proliferation of cells already committed to the Th2 lineage.

2.4.2. STAT5 and STAT3 pathways

Since the discovery of IL4 as the hallmark cytokine of the Th2 subset, the source of early IL4 as the initial promoter of Th2 differentiation has remained controversial. Moreover, development of Th2 cells was reported to take place also in absence of both IL4 and STAT6, albeit in most studies at a decreased frequency (Noben-Trauth et al., 1997; Finkelman et al., 2000; Jankovic et al., 2000; van Panhuys et al., 2008). Thus, from early on it was likely that complementary or compensatory pathways independent of IL4 existed.

In vitro differentiation of mouse Th2 cells, even in presence of IL4, requires IL2 (Le Gros et al., 1990). The receptor for IL2 (composed of IL2Rα, IL2Rβ, and γc subunits) is not present on naïve T cells, but its expression is induced by TCR and costimulatory signaling pathways, namely NFκB, NFAT, AP-1, and CREB/ATF (Kim et al., 2006; Malek, 2008). IL2 signals through STAT5 with mechanism analogous to that employed in IL4-STAT6 signaling. Ligation of IL2 with its receptor leads to JAK1/JAK3-mediated phosphorylation of cytoplasmic receptor tails and STAT5, which subsequently translocates to nucleus to exert its regulatory activity. Simultaneously, MAPK and PI3K pathways are activated by recruitment of adaptor protein SHC (Nelson and Willerford, 1998; Gaffen, 2001).

While IL2 is a general growth factor for T cells, its functions in Th2 differentiation extend beyond promotion of proliferation and survival. The proliferative effects of IL2 are mediated by the MAPK and PI3K pathways (Nelson and Willerford, 1998; Gaffen, 2001), but the Th2-specific regulatory effect is promoted specifically by STAT5, as constitutively active STAT5 can induce Th2 differentiation in absence of IL2 or without induction of GATA3 expression (Cote-Sierra et al., 2004). However, disruption of GATA3 abrogates this effect, suggesting that both transcription factors are needed for Th2 development (Zhu et al., 2004). The mechanisms by which STAT5 promotes differentiation of Th2 cells include maintenance of GATA3 expression (Guo et al., 2009), up-regulation of IL4Rα expression (Liao et al., 2008), and in particular, direct binding to the HS2 enhancer region of the *IL4* gene, facilitating its expression (Zhu et al., 2003; Cote-Sierra et al., 2004). Notably, in addition to IL2, STAT5 can be activated by IL7 and thymic stromal lymphopoietin (TSLP). Of these, TLSP is readily produced e.g. during allergic reactions by cells of the innate immunity, making this cytokine a potentially significant in vivo regulator of Th2 responses (Paul and Zhu, 2010).

Ligation of IL2 to its receptor can also activate STAT3, although to a lesser extent than STAT5 (Malek, 2008). While its activity is typically associated with the Th17 and Tfh subsets, STAT3 is phosphorylated also during Th2 differentiation with a mechanism involving combination of signals from IL2, IL6, and IL21 (Stritesky et al., 2011).

Conditional deletion of STAT3 decreased the expression of GATA3, MAF, and BATF, reduced the production of Th2 cytokines, and inhibited the development of allergic inflammation (Stritesky et al., 2011). Regulation of MAF and BATF expression has been demonstrated to result from direct binding of STAT3 to the corresponding gene loci (Yang et al., 2005; Durant et al., 2010). Using ChIP assay, STAT3 was shown to bind multiple Th2-specific loci and through permissive chromatin remodeling facilitate subsequent binding by STAT6, thus promoting STAT6-mediated Th2 differentiation (Stritesky et al., 2011). Furthermore, IL6 signaling through STAT3 induces expression of SOCS1 and NFATc1, factors associated with inhibition of Th1 responses and induction of IL4 production (Diehl et al., 2000; Diehl and Rincón, 2002).

2.5. Th2-promoting pathways engaged by T cell activation

T cells are activated in response to recognition of cognate antigen peptide-MHC complex and co-stimulatory ligands on the surface of an antigen-presenting cell. The activation through T cell receptor (TCR) is prerequisite for effector cell development and differentiation to Th2 subset (Swain et al., 1988; Röcken et al., 1992a). The nature of antigen itself does not restrict the direction of T helper cell differentiation, as cells from T cell clones have been shown to be capable of committing to either Th1 or Th2 lineage (Hsieh et al., 1992; Röcken et al., 1992b; Sad and Mosmann, 1994). Instead, the influence of nature of antigen on T cell subset differentiation is typically mediated by the antigen-presenting cell (Kaiko et al., 2008). In contrast, the direct effect of TCR stimulation strength on T helper cell lineage commitment has been recognized for long (Constant et al., 1995; Hosken et al., 1995; Tao et al., 1997). As a generalization, strong TCR signals, associated with high affinity and dose of antigen, lead to generation of Th1 response, while weaker signals favor Th2 differentiation (Constant and Bottomly, 1997; Leitenberg and Bottomly, 1999). However, the specific evidence from various experimental models based on different antigen peptides has proven difficult to reconcile, and in part has seemed all but contradictory. These observed discrepancies have been proposed to be related to different absolute ranges of stimulatory signals used, in vivo differentiation follow a bi-phasic distribution where both low and very high antigen dosages promote Th2 differentiation and intermediate dosage Th1 differentiation (Nakayama and Yamashita, 2010). The preferential in vivo Th2 differentiation in very high antigen concentrations might be explained by repeated stimulation of naïve T cells by APCs, leading to enhanced production of IL4 correlating with accelerated differentiation and proliferation of developing Th2 cells. Such positive feedback effect would not be seen on Th1 cells, as early Th1 differentiation is dependent of exogenous source of IL12 (Magram et al., 1996). A wealth of experimental evidence describes mechanisms by which specific nodes of T cell activation and

co-stimulation networks can contribute to the determination of T helper cell fate.

2.5.1. T cell receptor signaling

Proper T cell function requires infallible discrimination between non-self and self peptides, reacting strongly to rare pathogenic antigens present among highly abundant innocuous peptides. Due to this requirement of exceptional specificity and sensitivity, strength of TCR signaling cannot rely on traditional mass action dynamics alone, where rate of reaction is in linear correlation with concentration of substrate molecules. Instead, intricate mechanisms that control the triggering of T cell activation have evolved (Davis et al., 2007; Morris and Allen, 2012). A key discriminatory feature is the difference in duration of occupancy of the receptor by the peptide, referred to as the dwell time. For self peptides, this time is typically only 0.1-2 seconds, while antigenic peptides contact the TCR for 2-10 seconds (Davis et al., 1998; Baker and Wiley, 2001; Gascoigne et al., 2001). A related regulatory mechanism is employed on the level of lateral organization of TCRs. The TCRs are distributed as both monovalent receptors as well as multivalent nanocluster complexes consisting from two to over 20 individual TCRs (Schamel et al., 2005). Importantly, mono- and multivalent TCRs have been shown respond differently to varying doses of antigen, full activation requiring binding to multiple juxtaposed receptors (Minguet et al., 2007; Kumar et al., 2011; Blanco and Alarcón, 2012). The organization of TCRs parallels with distinct plasma membrane organization in the immediate surroundings of the TCRs, referred to as lipid microdomains or lipid rafts (Harder and Engelhardt, 2004; Choudhuri and Dustin, 2010). The nature and biological importance of lipid rafts has been for long disputed. However, recent direct analyses have corroborated the existence of distinct lipid environment surrounding the TCR (Brügger et al., 2006; Zech et al., 2009; Saito et al., 2010).

The human T cell receptor complex consists of variable α and β chains associated with three pairs of CD3 molecules (γ-ε, δ-ε, and ζ-ζ), required for signal transduction to the cytoplasm (Moss et al., 1992). Ligation of cognate antigen-MHC complex to the α/β subunits of the TCR initiates a cascade of phosphorylation events, commonly referred to as the proximal TCR signaling network, or signalosome. The signals from this module further diversify into several distinct pathways, broadly categorized as calcium-mediated and GTPase-Ras-mitogen-activated protein kinase (MAPK) signaling pathways (Acuto et al., 2008; Smith-Garvin et al., 2009; Morris and Allen, 2012). However, the exact mechanisms by which the conformational change induced by antigen binding is translated as the biochemical change on the cytoplasmic side of the receptor are still debated (Smith-Garvin et al., 2009; Gascoigne et al., 2011; van der Merwe and Dushek, 2011).

In addition to the TCR/CD3 complex itself, the essential components of the proximal TCR signalosome are LCK, ZAP70, and LAT. LCK is a SRC-family protein tyrosine kinase, which in response to antigen binding, phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the CD3 molecules. Another protein tyrosine kinase, ZAP70, then binds to the phosphorylated ITAM tyrosine residues by dual SH2 motifs. This interaction alters the conformation of ZAP70, leading to its phosphorylation by LCK and ZAP70 itself. Once activated by phosphorylation, ZAP70 phosphorylates LAT, a scaffold protein required for activation of downstream calcium- and MAPK-mediated pathways (Smith-Garvin et al., 2009).

Each of the components of the proximal TCR signaling network, when experimentally perturbed, can influence the outcome of Th differentiation. Mice expressing dominant negative LCK have impaired Th2 but normal Th1 responses (Yamashita et al., 1998). In contrast, inhibition of ZAP70 activity favors Th2 development(Tanaka et al., 2003). In mice defective for LAT function, abnormally dominant Th2 responses develop. However, the maturation of T cells is significantly impaired in these mice (Aguado et al., 2002; Sommers et al., 2002). Thus, whether the observed effect on Th2 development is attributed to the strength of TCR signaling or to altered T cell homeostasis due to lymphopenic environment, has been questioned (Nakayama and Yamashita, 2010).

Downstream of LAT, the activity of the calcium mediated signaling is directly involved in development of Th2 effector cells. Induction of calcium release from the endoplasmic reticulum is dependent on the recruitment of the Tec family kinase ITK to the LAT complex. Deletion of ITK, in certain experimental models, leads to impairment of Th2 responses (Fowell et al., 1999). Release of calcium ions from intracellular stores triggers activation of numerous factors including the phosphatase calcineurin (CN) (Savignac et al., 2007). Inhibition of CN activity impairs Th2 responses more profoundly than Th1 (Yamashita et al., 2000). Consistently, CN-activated transcription factors of the NFAT family regulate directly both GATA3 and IL4(Agarwal et al., 2000; Rengarajan et al., 2002; Scheinman and Avni, 2009).

Of the MAPK pathways activated in response to TCR stimulation, ERK is required for Th2 differentiation, promoting stability of GATA3 through inhibition of proteosomal degradation (Yamashita et al., 1999; 2005). ERK has also been shown to regulate expression of IL4 by direct binding to the *IL4* promoter (Tripathi et al., 2011). On the other hand, high ERK activity resulting from strong TCR stimulation was reported to lead to reduced Th2 differentiation and IL4 production. In this case, the effect on IL4 expression was based on selective modification of AP1 complex, and was negated by ERK inhibitors (Jorritsma et al., 2003). These findings might be explained by differential requirements for ERK in different phases of Th2 differentiation (Zhu et al., 2010). In addition to ERK, the p38 MAPK

pathway plays a role in Th2 signaling, phosphorylating GATA3 and promoting its localization to the nucleus (Maneechotesuwan et al., 2007).

In parallel with the MAPK pathways, PKCθ is activated, triggering the pleiotropic NFκB pathway (Barnes and Karin, 1997; Schulze-Luehrmann and Ghosh, 2006). Disruption of NFκB signaling has been demonstrated to inhibit the development of Th2-associated eosinophilia (Yang et al., 1998). NFκB promotes production of IL2 and regulates GATA3 (Verweij et al., 1991; Corn et al., 2005). Activation and nuclear translocation of NFκB is achieved by degradation of the inhibitory IκB factor. Multiple factors contribute to IκB degradation. Of these, SLAM, SAP, Bcl10, CARD11, and PKCθ itself have been shown to be required for proper Th2 differentiation (Czar et al., 2001; Wu et al., 2001; Cannons et al., 2004; Marsland et al., 2004; Wang et al., 2004a; Medoff et al., 2006).

2.5.2. CD4 signaling

The CD4 receptor is required for recognition of class II MHC molecule on the APC, and thus plays a crucial role in T cell activation. Interestingly, while the expression of CD4 in Th2 cells is only half of that in Th1 (Itoh et al., 2005), CD4 is required for development of Th2 but not Th1 cells (Fowell et al., 1997). The dependence of Th2 differentiation on CD4 is explained by the association of LCK with the cytoplasmic part of the molecule, as Th2 differentiation is impaired by mutation of the LCK-interacting cytoplasmic tail even in presence of intact CD4 ectodomain (Brown et al., 1997).

2.5.3. CD28 signaling

Stimulation through TCR/CD3 alone leads to anergy. For proper activation, simultaneous signaling through co-stimulatory receptors is required, referred to as the two-signal model (Mueller et al., 1989; Schwartz, 1990; Shahinian et al., 1993). Like the MHCII complex, the ligands for the co-stimulatory receptors are expressed on the antigen-presenting cell surface. However, unlike antigen recognition by the TCR, the ligation of the co-stimulatory receptors is not clonotypic. Of the known co-stimulatory molecules, the ones important for Th2 cell development include CD28, CD4, OX40, ICOS, and Notch (Mowen and Glimcher, 2004; Amsen et al., 2009). CD28 signaling has been shown to be indispensable for Th2 differentiation, enhancing IL4-STAT6 signaling, activating NFκB and promoting expression of GATA3 (Verweij et al., 1991; Shahinian et al., 1993; Rulifson et al., 1997; Kubo et al., 1999; Rodríguez-Palmero et al., 1999). The CD28 receptor is activated by ligands CD80 (B7-1) and CD86 (B7-2), inducibly expressed on the APCs (Hathcock et al., 1994). Rather than being a single linear signaling cascade, CD28 regulates a number of pathways controlling survival, metabolism, and transcription, significantly overlapping with targets of TCR

signaling (Riley et al., 2002; Acuto and Michel, 2003; Riha and Rudd, 2010). Notably, CD28 upregulates both glycolysis and glucose uptake required for intense proliferation undergone by the activated cells (Frauwirth et al., 2002).

2.5.4. OX40 (CD134) signaling

The co-stimulatory OX40 is not present in naïve T cells, but transiently expressed upon activation (Gramaglia et al., 1998). Ligation of OX40 with OX40L (CD252) has effects complementary to CD28, promoting cell survival, proliferation, and cytokine secretion (Redmond et al., 2009). OX40 has been shown to selectively augment the IL4-induced differentiation of Th2 cells(Flynn et al., 1998; Ohshima et al., 1998). However, OX40L-/- dendritic cells fail to induce neither proper Th2 nor Th1 responses (Chen et al., 1999). Thus, while OX40 seems to be required for Th2 development, its exact function is still unclear. Rather than being a straightforward polarizing signal OX40, among other co-stimulatory pathways, has been proposed to mediate the effect of antigen dose and affinity, regulating overall magnitude of Th response (Rogers and Croft, 2000; Jenkins et al., 2007; Redmond et al., 2009).

2.5.5. ICOS (CD278) signaling

Expression of the ICOS receptor is induced by CD28 stimulation, with expression levels higher in Th2 than Th1 cells (Hutloff et al., 1999; Coyle et al., 2000). The ICOS ligand B7RP-1, is constitutively expressed by APCs. ICOS regulates expression of Th2-promoting transcription factor c-Maf, and increases early IL4 production, through NFAT (Nurieva et al., 2003). However, ICOS has functions also in Th1 subset and augments secretion of cytokines of both Th1 and Th2 subsets (Hutloff et al., 1999; Wilson et al., 2006). Instead of instructive regulation of Th differentiation, the primary role of ICOS might be related to enhancing the size of lymphocyte populations in the lymph node and promoting survival of the effector cell populations. The need for such positive signals would be more required in cases of weak TCR stimuli typically associated with Th2-type responses (Loke et al., 2005; Burmeister et al., 2008; Tesciuba et al., 2008).

2.5.6. Notch signaling

Evolutionarily conserved Notch receptors regulate cell fate decisions during embryonic and hematopoietic development (Maillard et al., 2003; Stanley and Guidos, 2009). Accumulating evidence implicates them also in T helper cell subset differentiation. Of the four mammalian Notch receptors, naïve T cells express Notch1 and Notch2, and expression of Notch3 is induced by activation (Adler et al., 2003; Amsen et al., 2004). The Notch ligands are encoded by two gene families, Jagged and Delta. The ligation of Jagged or Delta to the Notch receptor induces

proteolytic cleavage and nuclear translocation of the Notch intracellular domain (NICD). In the nucleus, NICD interacts with the repressory DNA-bound RBPJ complex leading to recruitment of coactivator Mastermind/Lag-3 and MED8 complex and induction of gene expression (Kopan and Ilagan, 2009).

Although according to current knowledge Delta and Jagged ligands activate similar intracellular signaling pathways, when expressed on surface of APCs they seem to promote development of opposing T helper cell subsets, Delta instructing differentiation to Th1 and Jagged instructing differentiation to Th2 subset (Amsen et al., 2004). Notch1, Notch2, and RBPJ has all been shown to be required for proper in vivo Th2 development (Amsen et al., 2007). The notch-mediated Th2 differentiation involves direct regulation of both *IL4* and *GATA3* loci (Amsen et al., 2004; 2007; Fang et al., 2007). Interestingly, the binding pattern to *GATA3* resembles that of TCR-induced NFAT1 (Scheinman and Avni, 2009). These regulatory effects are dependent on RBPJ pathway but independent of STAT6, as demonstrated by gene deletion experiments (Amsen et al., 2004). Thus, acting upstream of both IL4 and STAT6, the Notch-mediated up-regulation of GATA3 has been proposed as an alternative initiation mechanism for Th2 lineage commitment (Amsen et al., 2009). However, the full importance of this pathway for in vivo Th2 differentiation is still under debate (Zhu and Paul, 2008).

2.6. T helper cell differentiation from a systems biology point of view

Large majority of the original fundamental studies of T helper cell differentiation were conducted using models such as knock-out mice, in which genes of interest were investigated in isolation in a dualistic setting. The findings from these studies have provided the groundwork for the current model of T helper cell lineage commitment. Nevertheless, with recent reports suggesting newfound diversity and plasticity among the T helper cell populations, increasing appreciation of the importance of quantitative effects on multiple levels in regulation of T helper cell lineage commitment has emerged.

The original model of Th cell differentiation where one cytokine induces development of particular subset, activating a master transcription factor through a specific JAK-STAT pathway, is proving inadequate. This view has been challenged with the discovery of the novel Th subsets together with improved understanding of the molecular underpinnings of pathways regulating Th1 and Th2 differentiation. Nowadays it is evident that many of the cytokines are involved in development of multiple Th cell fates, and for each T helper subset more than one cytokine seem to be required (Zhu and Paul, 2010). In general, the same observation applies for JAK-STAT pathways. For example, while Th2 differentiation has been for long held as a process directed uniquely by STAT6, it is now turning out that in fact, STAT6, STAT5, and STAT3 are all involved.

Similarly, both STAT1 and STAT4 are required for Th1 differentiation (Stritesky and Kaplan, 2011). Thus, to accurately describe transcriptional regulation in Th cells, the combinatorial effects of distinct STAT pathways should be considered.

In particular, the understanding of function of lineage-specific master regulator transcription factors is becoming more refined. The original monolithic view has gradually shifted towards one emphasizing network-like regulation, where even the master regulators are, to a certain extent, dispensable (O'Shea and Paul, 2010; O'Garra et al., 2011). For example, constitutionally active STAT5 can compensate for low level of GATA3 (Zhu et al., 2004), and IFNγ production requires presence of only either T-bet or STAT4 (Lighvani et al., 2001; Thieu et al., 2008). To truly understand how Th subsets arise in vivo, transcription factors should, instead of digital inputs, be considered as a regulatory network in which both quantitative and temporal information play important roles (O'Shea and Paul, 2010).

The importance of quantitative changes is especially pronounced in the context of T helper cell subset plasticity. In contrast with the original notion of irreversible T helper cell lineage commitment, Th subsets have been found, in some cases, to have the capacity interconvert (Sundrud et al., 2003; Hirota et al., 2011). Consequently, the question has been raised to which extent the original Th clone phenotypes represent in vivo Th responses. It seems possible that Th differentiation proceeds through phases during which plasticity to change subset fate is gradually lost, and original Th clones correspond to terminally differentiated cells normally associated only with chronic inflammation (Bluestone et al., 2009; Murphy and Stockinger, 2010; O'Shea and Paul, 2010).

Altogether, while reductionistic models where individual factors are studied in isolation have provided us with valuable mechanistic insight on function of most of the molecules associated with the T helper cell signaling network, they might no longer always represent the optimal strategy for addressing questions about the in vivo human T cell function. Hence, it has been proposed that quantitative system-level measurement of immune responses might be required for further advancing our understanding of the immune system(O'Shea and Paul, 2010). A major challenge will be describing how the environmental cues are integrated on the level of crosstalking and partly redundant signaling pathways, and translated as cell fate decisions leading to the establishment of alternative cellular phenotypes that can be qualitatively categorized as distinct T helper cell subsets.

2.7. System-wide studies on human Th2 differentiation

The development of high throughput analytical methods, such as microarrays (Hoheisel, 2006), deep sequencing (Wold and Myers, 2008; Metzker, 2010), and mass spectrometry (Aebersold and Mann, 2003), has for the first time allowed

to study the structure and function of whole biological systems, including genomes, cells, and even entire organs (Noble, 2002). In parallel, biology as a scientific discipline has evolved, increasingly assuming characteristics of informational science. The specific concept of systems biology has been coined to describe the attempt to characterize flow of information in biological networks, defined by Leroy Hood as "the study of all the elements in a biological system (all genes, mRNAs, proteins, and so on) and their relationships to one another in response to perturbations" (Hood, 2002). The promise of systems biology is in characterization of emergent properties of a system that cannot be discovered by study of behavior of individual components in isolation (Schlitt and Brazma, 2005). Arguably, the outcome of T helper cell lineage commitment can be viewed as such property.

Systems biology approaches typically involve computational models which are based on integration of data acquired with system-wide experimental platforms targeting different levels of cellular organization (Germain et al., 2011). Such methods are often conceptually divided on basis of targeted analytes to categories including, but not limited to, genomics, transcriptomics, proteomics, and metabolomics (Figure 2).

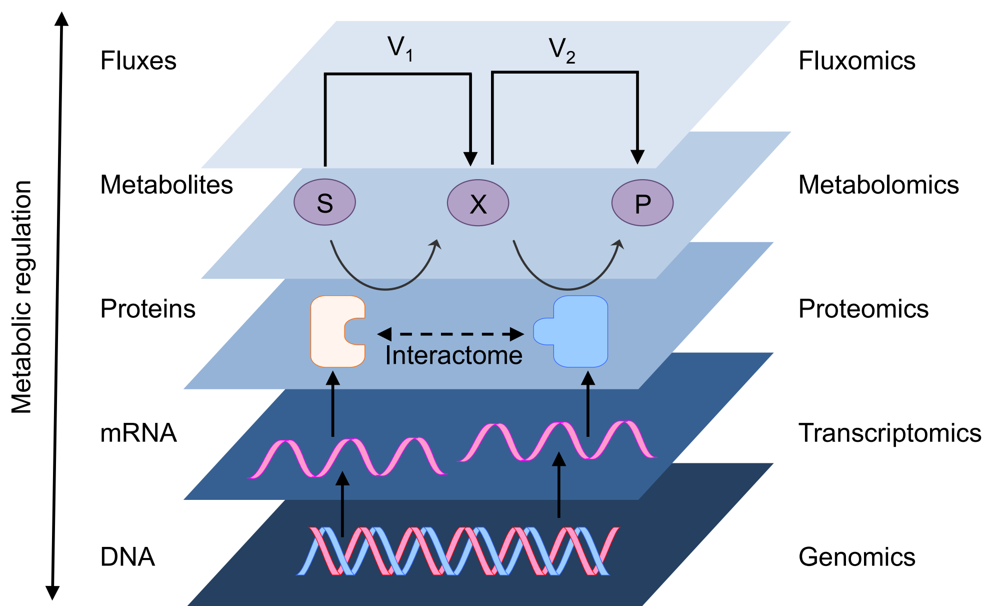

Figure 2. Levels of biological organization targeted by system-wide experiments. Adapted from Nielsen & Oliver (Nielsen and Oliver, 2005).

Some of the "-omics" approaches have been applied to study of T helper cell differentiation rather extensively, while others have received less attention, for technological limitations and otherwise. Studies in humans have been more

limited in number than those in mice. Some of the most important system-wide studies of development of human Th2 cells are listed in table 2 and discussed herein.

Table 2. Selected system-wide studies relevant to human Th2 development. Abbreviations: 2DE, two-dimensional gel electrophoresis; LC, liquid chromatography; MS, mass spectrometry (peptide mass fingerprinting); MS/MS, tandem mass spectrometry.

Reference	Material	Cytokine stimuli	Analytes	Platform(s)
(Rogge et al., 2000)	Cord blood	IL4, IL12	mRNA	Oligonucleotide array
(Hämäläinen et al., 2001)	Cord blood	IL4, IL12	mRNA	Oligonucleotide array
(Nyman et al., 2001)	Peripheral blood	-	Total protein	2DE, MS
(Lund et al., 2003a)	Cord blood	IL4, IL12, TGFβ	mRNA	Oligonucleotide array
(Rautajoki et al., 2004)	Cord blood	IL4, IL12	Total protein, mRNA	2DE, MS, oligonucleotide array
(Stentz and Kitabchi, 2004)	Peripheral blood	-	Total protein, mRNA	Oligonucleotide array, MS
(Kronfeld et al., 2005)	Peripheral blood	-	Total protein, phosphoprotein	2DE, MS/MS
(Nikula et al., 2005)	Cord blood	IL4, IL12, TGFβ	mRNA	cDNA array
(Lund et al., 2007)	Cord blood	IL4, IL12, TGFβ	mRNA	Oligonucleotide array
(Rautajoki et al., 2007)	Cord blood	IL4	Total protein	2DE, MS/MS
(Wang et al., 2008a)	Peripheral blood	-	mRNA	Oligonucleotide array
(Wang et al., 2008b)	Peripheral blood	-	mRNA	Oligonucleotide array
(Carrascal et al., 2008)	Peripheral blood	-	Phosphoprotein	MS/MS
(Filén et al., 2009)	Cord blood	IL4, IL12	Microsomal protein	MS/MS
(Haudek et al., 2009)	Peripheral blood	-	Cytoplasmic protein	2DE, MS/MS
(Elo et al., 2010)	Cord blood	IL4	mRNA	Oligonucleotide array
(Lichtenfels et al., 2012)	Cord blood	-	Total protein	2DE, MS
(Orr et al., 2012)	Peripheral blood	-	Chromatin-bound protein	MS/MS
(Ruperez et al., 2012)	Peripheral blood	-	Phosphoprotein	MS/MS

2.7.1. Transcriptomics

Of all system-wide techniques, the ones targeting nucleic acids have matured most quickly (Lander, 1999). Together with the widely accepted view of T helper cell lineages fundamentally representing alternative transcriptional programs, the interest for applying array-based transcript profiling methods to investigate T helper cell differentiation has been high. In 2000, Rogge et al. reported the first systematic comparison of transcription kinetics in human Th1 and Th2 cells, resulting in identification of more than 200 differentially expressed genes (Rogge et al., 2000). However, this as well as the other earliest studies, were conducted using arrays with limited scope of probe sequences (Hämäläinen et al., 2001; Lund et al., 2003a; Nikula et al., 2005). With subsequent advances in microarray technology, genome-wide studies of both activation (Stentz and Kitabchi, 2004; Wang et al., 2008a; 2008b) and Th2 differentiation (Lund et al., 2007; Elo et al., 2010) of human primary cells have been published. Although the mRNA kinetics of human Th2 cells have been described in detail, additional insight on transcriptional regulation will be achieved through genome-wide studies of transcription factor binding (Jenner et al., 2009; Elo et al., 2010) and epigenetic regulatory mechanisms (Roh et al., 2006).

2.7.2. Proteomics

Proteins represent the end products of gene expression and carry out innumerable enzymatic, regulatory, and structural functions. However, the abundance of proteins and corresponding mRNAs exhibit only modest correlation, and the former cannot be universally predicted from the latter (Gygi et al., 1999; Lundberg et al., 2010). With analogy to genomics and transcriptomics, proteomics is defined as the large scale investigation of proteins using biochemical methods (Wilkins et al., 1996; Pandey and Mann, 2000). In comparison to the study of DNA and RNA, several factors make proteomics inherently more challenging. Unlike the genome, the proteome is highly transient in nature. Average protein turn-over rate is approximately only 20 hours, varying from minutes to over 100 hours (Ohsumi, 2006; Boisvert et al., 2012). In addition to temporal variation, proteomes are also highly specific to tissue and cell types (Geiger et al., 2012). Complexity of proteomes is exponentially increased by numerous possible post-translational modifications (PTMs), including phosphorylation, methylation, acetylation and proteolytic cleavage. On average, each protein is subject to at least 2.5 modifications, which can also be highly transient and context-dependent. The total extent and importance of PTMs might still be underestimated (Papin et al., 2005). Finally, whereas nucleic acids can be amplified by polymerase chain reaction, no such methods are available for proteins.

Early proteomics studies relied on application of two-dimensional gel electrophoretic separation (2DE) and visualization of proteins to identify

relative differences in protein expression. The first such study in human T helper cells afforded identification of 91 proteins from activated cells by peptide mass fingerprinting (Nyman et al., 2001). Subsequent comparison of whole cell lysates from polarized Th1 and Th2 cultures resulted in detection of more than 70 differences, 14 of which were reproducible over the time points under examination (7 and 14 days). The observed differences were in partial agreement with the results of a transcriptomic analysis (Rautajoki et al., 2004). A related study targeting an earlier stage (24h) of IL4-induced Th2 differentiation provided a panel of 35 IL4-regulated proteins, as identified by a shotgun proteomics approach (Rautajoki et al., 2007). Not accounting for the role of IL4, proteomic changes in activated primary T cells induced by TCR(Stentz and Kitabchi, 2004) and CD28 signaling have been characterized (Kronfeld et al., 2005; Lichtenfels et al., 2012).

Variation in the levels of cellular protein abundance has been measured to reach seven orders of magnitude (Beck et al., 2011; Geiger et al., 2012). Mass spectrometry-based proteomics has only recently begun to even approach this dynamic range (Picotti et al., 2009). Moreover, the complete proteome has remained prohibitively complex for a single-step analysis. Hence, methods for targeting of spatially limited sub-proteomes, e.g. organelles, have been developed and utilized. In addition to the advantage of reduced sample complexity, such approaches can provide valuable information of protein localization, which cannot be extrapolated from results of transcriptomic analyses (Cox and Mann, 2011). With human T cells, IL4 and IL12 associated changes were identified using the microsomal protein fraction (Filén et al., 2009). Outside the context of cytokine signaling, the cytoplasmic fraction of primary T cells (Haudek et al., 2009), as well as protein bound to chromatin or nuclear matrix, have been characterized (Orr et al., 2012). In addition, with the use of human Jurkat T cell leukemia model, studies of the nuclear (Hwang et al., 2006), mitochondrial (Rezaul et al., 2005), and lipid raft (Bini et al., 2003; Haller et al., 2003; Kobayashi et al., 2007; de Wet et al., 2011) protein fractions have been reported.

Of the post-translational protein modifications, reversible phosphorylation of serine, threonine, and tyrosine residues is particularly significant as a mechanism of signal transduction and modifier of biological activity. Consequently, effective methods for enrichment and analysis of phosphorylated proteins and peptides have been developed (Bodenmiller et al., 2007). In context of T cell biology, the cascade of phosphorylation events triggered by stimulation of T cell receptor and CD28 has received most attention. Majority of the studies have relied on the use of the Jurkat cell line (Brill et al., 2004; Cao et al., 2006; Kim and White, 2006; Matsumoto et al., 2009; Mayya et al., 2009; Nguyen et al., 2009; Brockmeyer et al., 2011), whereas primary cells have been analyzed in only few cases (Kronfeld et al., 2005; Carrascal et al., 2008; Ruperez et al., 2012). Notably, the effects of IL4

signaling have not been investigated with high-throughput phosphoproteomics. Together with mass spectrometry-based tools, recent advent of multiplexed phospho-specific flow cytometry (Krutzik and Nolan, 2003; Perfetto et al., 2004; Irish et al., 2006; Krutzik and Nolan, 2006) and mass cytometry offer promising tools for such experiments (Bandura et al., 2009; Bendall et al., 2011).

2.7.3. Metabolomics

Metabolomics is a diverse field covering investigation of biomolecules not directly encoded by the genome. Like current proteomics, metabolomics relies heavily on the use LC-MS instrumentation (Lee et al., 2010a; Theodoridis et al., 2012). Being an emerging field, the number of applications in study of human T cells is still very limited. In relation to T helper cell activation and differentiation, most significant are experiments targeting the membrane fraction associated with the T cell receptor (Zech et al., 2009). As T cell activation is known to cause alterations in energy metabolism (Vander Heiden et al., 2009), and recent reports indicate crosstalk between pathways regulating metabolism and subset differentiation (Lee et al., 2010b), further metabolomics investigation of T helper cell differentiation would seem justified.

3. AIMS OF THE STUDY

The overall goal of this study was to uncover novel molecular mechanisms underlying the IL4-induced development of human T helper cell subset 2 phenotype using unbiased system-wide techniques. These methods, based on untargeted parallel detection and quantification of biomolecules such as RNA, protein, and lipids, allow hypothesis-independent measurement of systemic responses to experimental perturbations, e.g. in vitro stimulation of Th2 differentiation. The tools used in this study included liquid chromatography-coupled mass spectrometry as well as DNA microarrays, with the following specific aims:

1. Quantification of IL4-induced changes in the T cell nuclear proteome during early Th2 differentiation (*I*)

2. Isolation and characterization of proteins interacting with STAT6 in human Th2 cells (*III*)

3. Determination of lipid composition of human CD4+ T cells and quantification of changes in lipid concentrations and metabolic pathways associated with the development of Th2 effector phenotype (*II*)

4. Dissection of mRNA-level regulation of lipid metabolism in activated T cells (*II*)

4. MATERIALS AND METHODS

4.1. Isolation of CD4+ cells from umbilical cord blood

To obtain naïve non-stimulated T helper cells, umbilical cord blood was used as sample material. The samples were obtained from healthy neonate donors born in Turku University Hospital. Mononuclear cells were isolated using Ficoll gradient centrifugation (Amersham). CD4+ cells were further purified using positive selection with anti-CD4-coated magnetic Dynal beads (Invitrogen). The typical purity of such samples has been determined to be in the order of 98-99% (Rautajoki et al., 2007).

4.2. Culture and stimulation of primary T cells

CD4+ cells were cultured in Yssel's medium (Iscove's modified Dulbecco's medium [IMDM; Invitrogen] supplemented with Yssel's medium concentrate, penicillin, streptomycin, and 1% AB-serum) at a density of 2-4*10^6 cells/ml on 24-well plates. Cells were activated using plate-bound anti-CD3 (0.5µg/well) and soluble anti-CD28 (0.5µl/ml), (both antibodies from Beckman Coulter). Th2 polarization was induced with the addition of IL4 (10 ng/ml, R&D Systems). At 48h after activation, IL2 was added (17 ng/ml, R&D Systems) in all cases. Cells were cultured for up to 72 hours, as described in table 3.

Table 3. Cell stimulation times used in the subprojects. The roman numerals refer to time points studied in the corresponding original publications.

	0.5h	1h	2h	4h	6h	12h	24h	48h	72h
Th0 (no cytokines)	II	II	II	II	I, II	II	I, II	II	II
Th2 (IL4)	II	II	II	II	I, II	II	I, II, III	II	II

4.3. siRNAs and transfection

Transfection of primary CD4+ T cells was performed with Amaxa Nucleofector equipped with a 96-well shuttle (Lonza), in each well mixing 5*10^5 cells with 40 pmol of RNA and using program EO-115. (Tahvanainen et al., 2006). After transfection, cells were rested in RPMI (supplemented with penicillin, strepomycin, 2mM L-glutamine and 10% FCS) at 37°C for 40 hours. Subsequently, prior to activation, cells were resuspended in Yssel's medium at density of 2-3*10^6 cells per ml. The sequences of siRNA molecules used are listed in table 4.

Table 4. siRNA sequences used in nucleofection of primary CD4+ T cells.

Target	Sequence
HNRNPK (Dharmacon Smartpool M-011692-00-0005, Thermo Scientific)	5′-GAGCGCAUAUUGAGUAUCA-3′
	5′-GAUCUUGGUGGACCUAUUA-3′
	5′-GGUCAGCGGAUUAAACAAA-3′
	5′-GUCGGGAGCUUCGAUCAAA-3′
NCL	5′-GGAAGGUCAGCAGUCUUCCAUGAGA-3′
STAT6	5′-AAGCAGGAAGAACUCAAGUUU-3′

4.4. Isolation of nuclear protein

Nuclear fractions were prepared according to Andrews and Faller, with some modifications (Andrews and Faller, 1991). Cells were lysed to hypotonic lysis buffer (0.2% Nonidet P-40, 10mM HEPES pH 7.9, 0.5mM DTT, 1.5mM $MgCl_2$ 10mM KCl, and protease and phosphatase inhibitors from Roche) by incubating on ice for 10-20 minutes. Lysates were centrifuged and supernatants (cytoplasmic fractions) were recovered. Pelleted nuclei were washed once more with lysis buffer and suspended to and incubated 10-60 min in high-salt nuclear extraction buffer (20mM HEPES pH 7.9, 420mM NaCl, 0.5mM DTT, 1.5mM $MgCl_2$, 0.2mM EDTA, and protease and phosphatase inhibitors from Roche) on ice. Samples were centrifuged 15 min at full speed in a tabletop centrifuge at +4°C. Supernatants (nuclear fractions) were recovered and pelleted debris were discarded. Enrichment of nuclear protein was confirmed with immunodetection of PARP1.

4.5. DNA affinity purification

Biotinylated and antisense bait oligonucleotides were supplied by Oligomer. Sequences for STAT6-specific and negative control baits were 5′-Biotin-GGATC CGAGAGGTTTCCGGTGAATGTTAGA-3′, and 5′-Biotin-GGATCCGAGA GGTTATCGGTCTATGTTAGA-3′, respectively. Antisense strands were of complementary sequence, and were not biotin labelled. Oligonucleotides were annealed by heating to 95°C and letting cool down slowly over night. Annealing efficiency was verified by acrylamide gel electrophoresis. Beads (Ultralink immobilized neutravidin protein, Pierce) were washed with buffer A (10mM HEPES pH 7.9, 60mM KCl, 2mM EDTA, 1mM EGTA, 0.1% Triton X-100, 1mM DTT, and protease and phosphatase inhibitors from Roche). Bait oligonucleotides were conjugated to beads by incubating 250 pmol of annealed oligos with 10μl beads in 200μl buffer A for 1h in a 360° rotator at +4°C. Oligo-coated beads were washed four times with buffer A. Nuclear fractions were diluted to 60mM KCl using buffer B (10mM HEPES pH 7.9, 2mM EDTA, 1mM EGTA, 0.1% Triton X-100, 1mM DTT, and protease and phosphatase inhibitors

from Roche) and pre-cleared by incubating with uncoated beads for 1h in a 360° rotator at +4°C. Precleared supernatants were combined with beads coated with oligonucleotides and further incubated for 1h in a 360° rotator at +4°C. Beads were washed four times with buffer A, and precipitated proteins were eluted by incubating twice for 5 minutes at 95°C in 2xSDS buffer (125mM Tris-HCl pH 6.8, 4% w/v SDS, 20% glycerol, 100mM DTT).

4.6. SDS-PAGE and Western blot

Protein concentrations were measured with Bio-Rad detergent compatible protein assay according to manufacturer's instructions. 10-40 µg of each sample was resolved with either precast Bio-Rad Criterion 10% Bis-Tris gels, or with 10% SDS-PAGE minigels. Following electrophoresis, the proteins were either visualized with silver-staining, or transferred to Amersham Hybond ECL membrane, Millipore Immobilon-FL membrane, or Millipore Immobilon-P membrane. Primary antibodies used in the immunodetections are listed in table 5. Proteins were visualized using chemiluminsecence, or fluorescence-based detection with the Odyssey system (Li-cor). Silver-staining was performed using a MS-compatible protocol as follows: gels were fixed over night with 30% ethanol and 10% acetic acid, followed by 20 min washes with 20% ethanol and deionized water. Proteins were sensitized 2 min with 1mM sodium thiosulphate, and stained 2 h with 12 mM silver nitrate. Gels were rinsed twice with deionized water, and developed in 37% formaldehyde, 200 mM potassium carbonate, and 0.06 mM sodium thiosulphate. Development was stopped with 2.5% acetic acid in 400 mM Tris.

Table 5. Primary antibodies used in immunodetections.

Antibody	Supplier
GAPDH	Hytest
GATA3	BD Biosciences
HNRNPK	Santa Cruz Biotechnology
IKZF	Santa Cruz Biotechnology
MAPK (Erk1/2)	Cell Signaling Technologies
PARP1	Cell Signaling Technologies
Phospho-MAPK (Thr-202/Tyr-204)	Cell Signaling Technologies
Phospho-STAT6 (Tyr-641)	Cell Signaling Technologies
SATB1	Santa Cruz Biotechnology
STAT1	Santa Cruz Biotechnology
STAT6	BD Biosciences
TBX21 (T-bet)	Santa Cruz Biotechnology
TCF7	Upstate
YB1	Abcam

4.7. Flow cytometry

For staining of surface epitopes, cells were washed with FCM buffer (PBS, 2% FCS, 0.01% NaN_3), incubated with antibodies at +4°C protected from light, washed with FCM buffer and PBS, and resuspended in 1% formalin in PBS. For intracellular staining, cells were fixed by adding an equal volume of Phosflow fix buffer I (BD Biosciences), and incubating 10 minutes at +37°C. After fixation, cells were washed with PBS, permeabilized with -20°C Phosflow Perm Buffer III (BD Biosciences), and stored at -80°C. Staining of permeabilized cells was then performed similarly as surface epitopes. Antibodies used in staining of surface and intracellular epitopes are listed in table 6. Analyses were performed with Facscalibur and LSR2 instruments (BD Biosciences). Data were analyzed with Cellquest (BD Biosciences) or Flowing Software (www.flowingsoftware.com).

Table 6. Primary antibodies used in flow cytometry studies.

Antibody	Supplier
CD4-FITC/CD8-PE	BD Biosciences
CD69-FITC	BD Biosciences
Phospho-STAT6 (Tyr-641)	Cell Signaling Technologies

4.8. Real time quantitative RT-PCR

Real time quantitative RT-PCR was performed as previously described (Hämäläinen et al., 2000; Lund et al., 2003b). Briefly, total RNA was extracted using RNeasy Mini kit (Qiagen), genomic DNA was degraded with DNase I (Gibco), and cDNA was prepared using Transcriptor First Strand cDNA synthesis kit (Roche). Reactions were analyzed using a TaqMan ABI Prism 7900 HT Instrument (Applied Biosystems). The primer sequences used are listed in table 7.

Table 7. Primer and probe sequences used in RT-PCR assays.

Gene	Primer 1 Primer 2 **Probe**
EF1α	5′-CTGAACCATCCAGGCCAAAT-3′ 5′-GCCGTGTGGCAATCCAAT-3′ **5′-(FAM)-AGCGCCGGCTATGCCCCTG-(TAMRA)-3′**
GATA3	5′-GGACGCGGCGCAGTAC-3′ 5′-TGCCTTGACCGTCGATGTTA-3′ **5′-(FAM)-TGCCGGAGGAGGTGGATGTGCT-(TAMRA)-3′**
IKZF1	5′-CCTTCCGGGCACACTGTA-3′ 5′-TCTCTCTGATCCTATCTTGCACA-3′ **Roche Universal ProbeLibrary #29**
SATB1	5′-ACCAGTGGGTACGCGATGA-3′ 5′-TGTTAAAAGCCACACGTGCAA-3′ **5′-(FAM)-AACGAGCAGGAATCTCCCAGGCG-(TAMRA)-3′**
STAT1	5′-GGATTGAAAGCATCCTAGAACTCA-3′ 5′-GATGAAGCCCATGATGCAC-3′ **Roche Universal ProbeLibrary #32**
STAT6	5′-TGGGCCGTGGCTTCAC-3′ 5′-CCGGAGACAGCGTTTGGT-3′ **5′-(FAM)-CAGGACACCATCAAACCACTGCCAAA-(TAMRA)-3′**
TBX21 (T-bet)	5′-ACAGCTATGAGGCTGAGTTTCGA-3′ 5′-GGCCTCGGTAGTAGGACATGGT-3′ **5′-(FAM)-TCAGCATGAAGCCTGCATTCTTGCC-(TAMRA)-3′**
TCF7	5′-CTGCAGACCCCTGACCTCTCT-3′ 5′-ACACCAGAACCTAGCATCAAGGAT-3′ **5′-(FAM)-CTCCCTGACCTCAGGCAGCATGG-(TAMRA)-3′**
YB1	5′-GGAGGGTGCTGACAACCA-3′ 5′-GCTGTCTTTGGCGAGGAG-3′ **Roche Universal ProbeLibrary #2**

4.9. Microarray analysis

Total RNA was extracted using RNeasy Mini kit (Qiagen). cRNA was hybridized on GeneChip HG-U133 Plus 2.0 arrays (Affymetrix). The raw microarray data were processed using robust multi-array average normalization and log2-transformed in R (version 2.12.0) using the Bioconductor affy package (version 1.28.0).

4.10. Alkylation and in-gel tryptic digestion of silver-stained proteins

SDS-PAGE Gel lanes were excised to pieces which were further sliced to approximately 1mm^3 cubes, and washed twice for 15 minutes with 40mM ammonium bicarbonate/50% acetonitrile at +37°C. Gel pieces were dehydrated with acetonitrile and dried using vacuum centrifuge. Dried gel

pieces were rehydrated and reduced using 20mM DTT for 30min at 56°C and again dehydrated using acetonitrile. Gel pieces were alkylated with 55mM iodoacetamide for 15 min at room temperature, protected from light, followed by two rounds of washing with 40mM ammonium bicarbonate, and dehydration with acetonitrile. For each sample, 0.4µg Sequencing grade modified trypsin (Promega) was added, incubated for 10 min on ice, and overlaid completely with 40mM ammonium bicarbonate/10% acetonitrile. Digestion was performed at +37°C for 16 to 18 hours. After digestion, peptides were extracted by adding an equal volume of acetonitrile and incubating 15 min at +37°C. Extraction was repeated twice more using 50% acetonitrile/5% formic acid. Peptides were dried with vacuum centrifuge, and prior to MS analysis, desalted using Empore C_{18} (3M) packed in GELoader pipet tips (Eppendorf).

4.11. iTRAQ labeling and peptide fractionation

Relative quantification based on labeling with iTRAQ reagents (Applied Biosystems) was performed to compare nuclear proteomes of activated T helper cells cultured for 6 and 24 hours in presence or absence of IL4. Equal amounts of protein (50 –100 µg) from the four compared states were used for the labeling. Proteins were precipitated by mixing with 6 volumes of acetone and incubating for 4 h at -20 °C and re-dissolved in 40 µl of triethylammonium bicarbonate buffer containing 0.1% SDS. Labeling with iTRAQ reagents was performed according to manufacturer's protocol. Briefly, this included reduction with tris(2-carboxyethyl)phosphine and derivatization of free cysteines with methyl methanethiosulfonate (MMTS), followed by overnight digestion with sequencing grade modified trypsin (Promega). The resulting peptides were labeled with the iTRAQ reagents for 1 h at room temperature. In all three biological replicates, the 114 and 116 reagents were used to label the peptides from the cells activated for 6 and 24 h, respectively, and likewise the 115 and 117 reagents were used to label the activated and IL4-treated cells for 6 and 24 h, respectively. The labeled peptides were combined and acidified (pH 2.9–3.1). The resulting peptide mixtures were fractionated with a 200 × 4.6-mm-inner diameter polysulfoethyl A (Poly LC) strong cation exchange (SCX) column using a BioCAD chromatograph (PerSeptive Biosystems). The peptides were eluted at 0.7 ml/min from the cation exchange column during a two-step gradient from 0 to 30% B in 14 min and then to 100% B in 10 min (maintained for 15 min). The A and B phases consisted of 5 mM KH_2PO_4 and 25% acetonitrile, pH 3 with the B phase containing 0.6 M KCl. The eluted peptides were subsequently collected in 20 sequential fractions. The SCX fractions were dried in a HetoVac vacuum centrifuge (Heto-Holten) and desalted using Empore C_{18} material (3M).

4.12. Proteomics analysis by LC-MS/MS

Proteomics analyses were performed by tandem mass spectrometry (LC-MS/MS) using a shotgun proteomics approach (Yates, 2004). In these studies, four different instrument set-ups were used, as specified in table 8.

Identification of in-gel digested proteins was performed using set-ups 2, 3, and 4. The data acquired by MS/MS were searched against a human Uniprot database supplemented with common contaminant peptide sequences using Mascot software (Matrix Science). Iodoacetamide derivative of cysteine was specified as a fixed modification, and deamidation of asparagine and glutamine and oxidation of methionine as variable modifications. Scaffold (Proteome Software) was used to validate the MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003).

Analysis of iTRAQ-labeled peptides was performed with instrument set-up 1 (as described in table 8). The MS/MS data were searched against a Swiss-Prot database composed of human proteins plus known contaminants (trypsin fragments and BSA; 11 entries), with fixed modifications iTRAQ labeling at the N terminus and lysine, and methylmethanethiosulfonate (MMTS) modification of cysteine. Variable modifications included iTRAQ labeling of tyrosine, deamidation of asparagine and glutamine, and methionine oxidation. One missed tryptic cleavage was permitted. The data analysis was done with two methods, using the Analyst script ProQuant (Applied Biosystems) in combination with the ProGroup algorithm (Applied Biosystems), as well as a complementary analysis by Mascot (Matrix Science). Quantification of iTRAQ reporter ions was achieved by comparison of weighted average values of the corresponding reporter ion peak area ratios as described by Gan *et al.* (Gan et al., 2007).

Table 8. LC-MS/MS set-ups used for identification of in-gel digested proteins. Abbreviations: ACN, acetonitrile; ESI, electrospray ionization; i.d., inner diameter; LC, liquid chromatography; LTQ, hybrid linear ion trap-quadrupole; MS, mass spectrometer; QTOF, hybrid quadrupole-time of flight.

<table>
<tr><th></th><th>Set-up 1</th><th>Set-up 2</th><th>Set-up 3</th><th>Set-up 4</th></tr>
<tr><td>MS system</td><td>QSTAR Pulsar (Applied Biosystems)</td><td>QSTAR Pulsar (Applied Biosystems)</td><td>QSTAR Elite (Applied Biosystems)</td><td>LTQ-Orbitrap (Thermo Scientific)</td></tr>
<tr><td>Ionization and MS type</td><td colspan="3">ESI-QTOF</td><td>ESI-ion trap-Orbitrap</td></tr>
<tr><td>LC system</td><td>Famos/Switchos-II/Ultimate (LC Packings)</td><td>Cap-LC (Waters)</td><td>Ultimate 3000 capillary LC (Dionex)</td><td>NanoAcquity (Waters)</td></tr>
<tr><td>Loading column</td><td>5 × 0.3-mm PepMap C_{18} µ-precolumn (LC Packings)</td><td>Atlantis C18 NanoEase Trap Column 5um (Waters)</td><td>5 × 0.3-mm PepMap C_{18} µ-precolumn (LC Packings)</td><td>18 mm × 100 µm i.d. precolumn packed with 200 Å (5 µm) Magic C_{18} particles (C_{18}AQ; Michrom BioResources)</td></tr>
<tr><td>Analytical column</td><td colspan="4">15 cm × 75 µm i.d. fused silica capillary column packed with 5 µm Magic C_{18}AQ 100Å particles (Michrom BioResources)</td></tr>
<tr><td>Phase A</td><td>MilliQ water with 5% ACN, 0.1% HCOOH</td><td colspan="2">MilliQ water with 5% ACN, 0.2% HCOOH</td><td>MilliQ water, 0.1% HCOOH</td></tr>
<tr><td>Phase B</td><td>95% ACN, 0.1% HCOOH, MilliQ water</td><td colspan="2">95% ACN, 0.2% HCOOH, MilliQ water</td><td>ACN, 0.1% HCOOH</td></tr>
<tr><td>Flow rate</td><td>200 nl/min</td><td colspan="2">200 nl/min</td><td>250 nl/min</td></tr>
</table>

4.13. Lipid extraction and analysis

Cell harvesting and metabolic quenching was performed with the protocol of de Koning et al. (de Koning and van Dam, 1992), with some modifications. In brief, culture medium was removed; cells were re-suspended to -20°C 50% methanol, and pelleted by centrifugation (770g 20min) at -20°C. The supernatant was discarded and cells were stored in liquid nitrogen. Aliquots of cultured T cells containing ca. 2 million cells were spiked with a standard mixture consisting of 10 lipid compounds (0.2 µg/sample) and mixed with 100 µl of chloroform/methanol (2:1) by vortexing for 2 min. After 1 h standing the tubes were centrifuged at 10 000 rpm for 3 min and the lower organic phase was separated into a vial insert and mixed with a standard mixture containing 3 labelled lipid compounds (0.1 µg/sample).

For the lipidomics analysis, the lipid extracts were run on a Q-Tof Premier mass spectrometer (Waters) combined with an Acquity Ultra Performance Liquid Chromatography (Waters) by using a solvent system phase A of water with 1%

1M NH_4Ac and 0.1% HCOOH and phase B of acetonitrile/isopropanol (5:2) with 1% 1M NH_4Ac, 0.1% HCOOH. An Acquity UPLC BEH C_{18} 1 × 50 mm column with 1.7 µm particles was used at 50°C at a flow rate of 200 µl/min. The lipid profiling was carried out using ESI+ mode and the data was collected at mass range of m/z 300-1200. Data was processed using MZmine 2 software (Pluskal et al., 2010). Lipid identification was performed using in-house spectral library as described previously (Laaksonen et al., 2006). The lipidomics data were normalized using the lipid standard mixture: all monoacyl lipids except cholesterol esters, such as monoacylglycerols and monoacylglycerophospholipids were normalized with PC(17:0/0:0), all diacyl lipids except ethanolamine phospholipids were normalized with PC(17:0/17:0), ceramides with Cer(d18:1/17:0), the diacyl ethanolamine phospholipids were normalized with PE(17:0/17:0), and the TG and cholesterol esters with TG(17:0/17:0/17:0).

5. RESULTS AND DISCUSSION

5.1. Identification and relative quantification of nuclear proteins in activated and IL4-stimulated cells

To characterize the nuclear proteome of activated human T helper cells, and to detect protein-level responses induced by IL4, a quantitative proteomics approach was used. Nuclear protein was extracted from activated CD4+ T cells cultured for 6 and 24 hours in presence or absence of IL4. The analysis was performed with triplicate biological sample material, each replicate representing a pool of cord blood CD4+ T cells from multiple individuals. With LC-MS/MS, 843 proteins were reproducibly identified, each identification being supported by at least two distinct peptides. These results represented so far most extensive coverage of the primary human T cell nuclear proteome. However, the nuclear proteome of the Jurkat cell line has been extensively studied (Hwang et al., 2006; Wu et al., 2007). Of the proteins identified from primary T cells, approximately three quarters were reported also in the Jurkat studies. While some of the discrepancies between the datasets might result from differences in sample preparation, others might signify biologically relevant differences between these experimental models. IL4-induced differences arising from regulation of expression and nuclear translocation were studied by comparing activated cells harvested after 6 and 24-hour culture. Relative quantification of 815 proteins was achieved by use of peptide labeling with iTRAQ reagents (Applied biosystems). IL4 has been shown to regulate expression of numerous genes (Lund et al., 2007; Elo et al., 2010), while the differences observed at the level of nuclear proteome were limited in comparison. According to related iTRAQ-based studies, significant differences were filtered by p-value of less than 0.05 combined with at least 20% quantitative difference (Seshi, 2006; Unwin et al., 2006; Duthie et al., 2007). With these criteria, 30 proteins were considered to be regulated by IL4, excluding keratins and ribosomal proteins (Table 9). As expected, the list of IL4-induced proteins included STAT6, being preferentially recruited to nucleus upon IL4-dependent phosphorylation. Correspondingly, levels of Th1-associated proteins TRIM22, STAT1, IFI16, TBX21 (T-bet), and TCF7 were decreased by IL4.

Table 9. IL4-regulated proteins identified from nuclear extracts of human CD4+ T cells. Adapted from I.

Gene symbol	Uniprot accession	Th2/Th0 at 6h	Th2/Th0 at 24h
S10A9	P06702	1.27	1.82
RPL5	P46777	1.36	1.66
YB1	P67809	1.23	1.65
GRAP2	O75791	1	1.27
BUD31	P41223	0.97	1.24
AKAP8	O43823	1.1	1.24
RFC1	P35251	1.01	1.24
SATB1	Q01826	1.12	1.24
IKZF1	Q13422	1.04	1.21
CD3E	P07766	1.25	1.16
GNAS	P63092	0.72	1.14
HS105	Q92598	1.24	1.09
GLU2B	P14314	1.23	1.07
STAT6	P42226	1.32	1.07
TXLNA	P40222	1.38	1.01
MP2K3	P46734	1.82	0.92
TAF10	Q12962	1.59	0.9
RPB1	P24928	1.45	0.86
PSB8	P28062	1.03	0.83
APT	P07741	0.99	0.83
TRIM22	Q8IYM9	0.82	0.81
STAT1	P42224	0.95	0.81
IFI16	Q16666	0.99	0.81
CD45	P08575	0.99	0.8
CD44	P16070	0.91	0.77
TCF7	P36402	0.95	0.75
ATP5J	P18859	1.23	0.74
CD5	P06127	0.9	0.73
PSME3	P61289	0.93	0.73
TBX21	Q9UL17	0.98	0.64

5.2. Validation of proteomic changes and correlation with mRNA kinetics

The measured proteomic changes were successfully validated by quantitative western blotting in the cases of STAT6, SATB1, YB1, IKZF1, STAT1, TCF7, and TBX21 (Figure 3). Of these proteins, STAT6, SATB1, and IKZF1 have documented positive influence on Th2 differentiation, whereas TBX21, STAT1, and TCF7 are involved in pathways promoting Th1 development (Quirion et al., 2009; Ahlfors et al., 2010; Thomas et al., 2010). YB1 is a multifunctional protein with currently no reported implications in either Th1 or Th2 cells. To further elaborate the mechanisms regulating these proteins, quantitative RT-PCR assays were

performed. For *SATB1* and *TBX21*, significant mRNA-level differences were measured between the Th2 and Th0 states, while other mRNAs, including *YB1*, were induced in response to CD3/CD28-activation regardless of IL4, highlighting the importance of regulation at post-transcriptional and spatial levels.

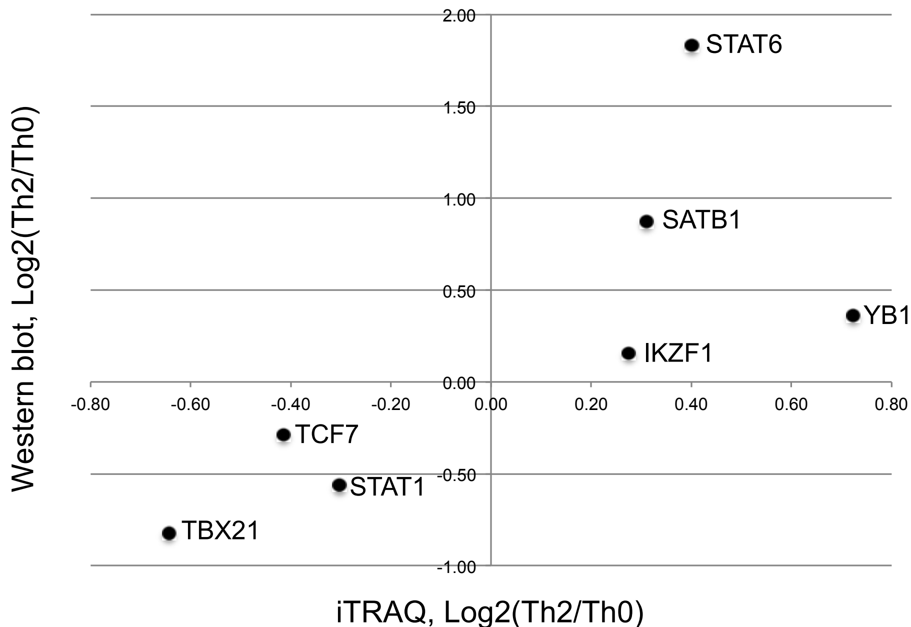

Figure 3. Relative quantification of selected nuclear proteins by iTRAQ and western blot. The data points represent the mean values of 3 replicate experiments in case of iTRAQ data, and at least 5 independent experiments performed by western blotting.

5.3. Enrichment and analysis of endogenous STAT6 protein complex

Study of protein-protein interactions can provide valuable information about protein function. To identify proteins associated with the IL4-induced STAT6 enhanceosome complex in human Th2 cells, a DNA affinity purification-based workflow was optimized. The results indicated that STAT6 protein could be specifically precipitated using bait oligonucleotide sequences based on the consensus STAT6 binding motifs. Importantly, comparison to samples produced with mutated decoy sequences provided efficient negative control for estimating specificity of precipitation. The enriched proteins were analyzed by LC-MS/MS, resulting in identification of altogether more than 200 proteins from three replicate experiments. Spectral count-based relative quantification of proteins identified in STAT6-containing and decoy samples afforded identification of specific putative STAT6 interacting proteins, including known cofactor SND1. Importantly, two novel proteins, HNRNPK and NCL, were identified with equal confidence of binding specificity.

HNRNPK is a multifunctional protein, localized in nuclear ribonucleoprotein particles, but also carrying out regulatory functions (Bomsztyk et al., 2004). Interestingly, HNRNPK has been categorized as a network hub molecule with more than 100 identified interacting proteins including NCL and the IL4-induced protein YB1 (Shnyreva et al., 2000; Mikula et al., 2006). NCL, in turn, has roles in processes such as PolII transcription and chromatin remodeling (Mongelard and Bouvet, 2007). To investigate the involvement of HNRPNK and NCL in signaling pathways regulating Th2 differentiation, siRNA-based gene silencing was used, and expression levels of GATA3, the master Th2-specific transcription factor, were measured. Notably, disruption of HNRNPK lead to notable reduction of GATA3. For NCL, the results were negative, suggesting that its enrichment might result from secondary binding to HNRPNK. While literature suggests that association of HNRNPK with STAT6 enhanceosome is potentially mediated by SND1, the exact mechanism as well as full implications for Th2 differentiation will require more conclusive functional evidence.

5.4. Lipidomic profiles of CD4+ T cells

Lipid molecules carry out important biological functions as structural components, energy storages, barriers, and signaling intermediates. Correspondingly, cellular lipid composition is subject to active regulation. To investigate lipidomic composition and lipid homeostasis in human CD4+ T cells, an LC-MS approach was used. While the coverage achieved by such approach is still limited, data could be acquired from most of the major lipid classes, including phosphatidylcholines (PC), phosphatidylethanolamines (PE), sphingomyelin (SM), triacylglycerols (TG), and diacylglycerols (DG). From resting CD4+ cells, 41 distinct molecular species representing these classes were identified. Importantly, as lipid species were identified on the level of sum formulas (numbers of side chain carbon molecules and double bonds), true chemical variation among the analytes is considerably greater, accounting for differential combinations of side chains as well as differential spatial organization of double bonds. Nevertheless, the data represented so far most extensive catalogue of human primary T cell lipidome.

5.4.1. Relative lipid kinetics in activated and IL4-stimulated cells

Importantly, the LC-MS approach allowed relative quantification of lipids by the use of spiked lipid standards (Figure 4). Lipid kinetics was measured during a time course of 72 hours following activation, with time points correlating with the transcript-level data. The activation was performed in both presence and absence of IL4. However, no statistically significant effects were measured associated specifically with IL4. In contrast, activation through CD3 and CD28

lead to notable remodeling of the cellular PC and PE lipids at the 48 and 72-hour time points, correlating with activation-induced proliferation. Interestingly, the upregulated species were typically shorter (36 or less side chain carbons) and more saturated (3 or less double bonds) than the downregulated ones. The time scale of the observed alterations of lipid composition correlated with the time of intense activation-induced cellular proliferation, possibly resulting from increased de novo lipid synthesis, as mammalian fatty acid biosynthesis produces predominantly relatively saturated species with only 14-18 carbons (Smith, 1994). T cell activation has been previously shown to lead to increased synthesis of molecules localizing to lipid rafts (Martin et al., 2001; Tuosto et al., 2001; Tani-ichi, 2005; Bensinger et al., 2008). However, only some of the observed lipidomic changes corresponded to known lipid raft components. Whether the changes correlate with expansion or reduction of some other subcellular compartment could not be concluded from the current global data alone.

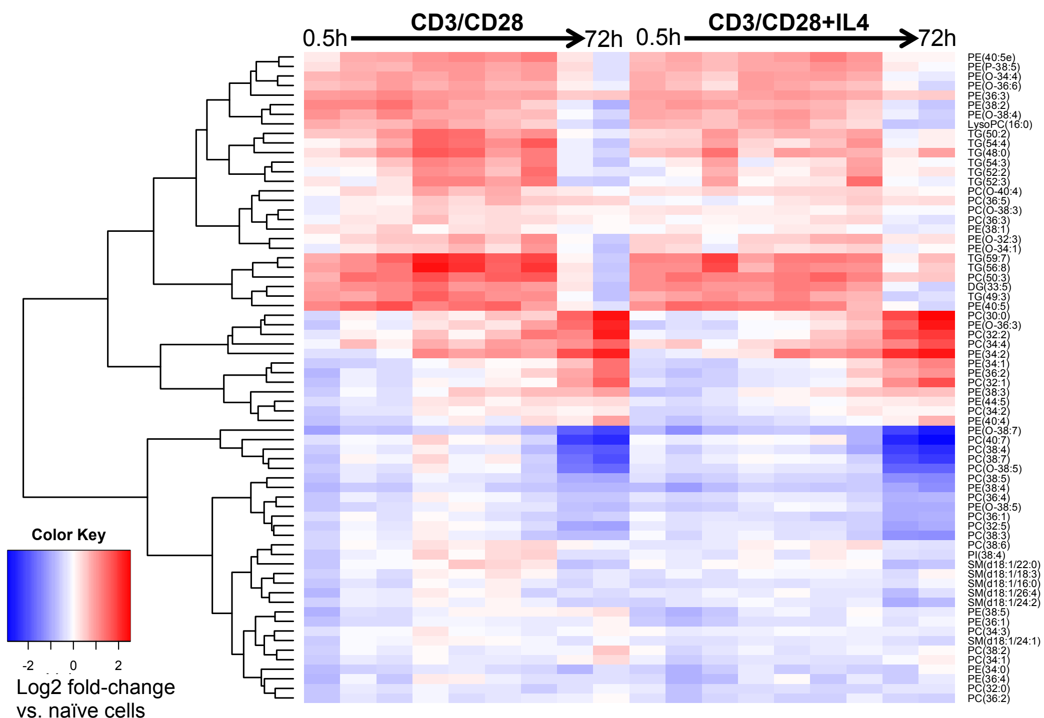

Figure 4. Relative concentrations of lipid species in activated CD4+ T cells cultured in presence or absence of IL4. Samples were collected at 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 hours post activation, and concentrations were normalized against concentration of corresponding species in unstimulated cells. Abbreviations: DG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; TG, triacylglycerol. Adapted from II.

5.4.2. Transcriptional regulation of lipid metabolism

The transcriptomic dataset was mined in order to find possible explanatory mechanisms of regulation. To this end, mRNA expression levels in activated cells were compared to the unstimulated state, focusing on genes with known roles in lipid metabolism, as annotated in the KEGG knowledgebase (Kanehisa and Goto, 2000). The most significant activation-induced change was the upregulation of stearoyl CoA-desaturase (*SCD*). *SCD* is required for generation of monounsaturated fatty acids, and functions as a regulatory hub of lipid metabolism (Paton and Ntambi, 2009). Deletion of *SCD* leads to deficiency in TG, cholesterol esters, wax esters, and alkyldiacylglycerols (Miyazaki et al., 2001; Ntambi et al., 2002). The upregulation of *SCD* expression has been validated on also protein level by mass spectrometry. In addition to *SCD*, upregulation of acetyl-OcA carboxylase α (*ACACA*) and *PCYT2* suggested induction of lipid synthesis. In parallel, the expression of several enzymes specific for saturated or monounsaturated substrates were upregulated, including elongases *ELOVL1* and *ELOVL6*, and lysophospholipid acyltransferases *LPCAT1* and *LPCAT4*. Altogether, the mRNA data suggested active remodeling of cellular lipidome on multiple levels.

5.5. Immunological significance

Whereas the hallmark functions of Th2 cells are related to eradication of extracellular parasites such as helminths, Th2 responses can, in addition, be promoted by non-microbial stimuli including allergens, venoms, and vaccine adjuvants (Coffman et al., 2010; Palm et al., 2012; Pulendran and Artis, 2012). The motivation of the experiments discussed herein was to describe molecular mechanisms that underlie the development of human Th2 cells, and thus provide opportunities for development of diagnostic markers or therapeutic modulators of Th2 cell function.

Important information about dissecting molecular underpinnings of cell fate determination can be obtained by identification of molecules that are expressed in a lineage-specific manner, and by determination of their relative kinetics of expression. When successful, this strategy can provide a list of components regulating the lineage commitment process, as well as possible causal relationships based on the temporal order of expression changes. The transcriptomic signatures of human Th cells have been investigated relatively extensively before the presented studies, effectively illustrating the principal kinetics of Th2 differentiation (Rogge, 2002; Lund et al., 2003a; 2007; Elo et al., 2010).

Building on the results of the transcriptomic analyses, a more targeted approach to regulation of Th2 differentiation was taken with the analysis of the nuclear

proteome in response to IL4 stimulation. As nuclear proteins in many cases function as signaling molecules, transcription factors or epigenetic modifiers, preferential expression or nuclear recruitment in response to IL4 may be indicative of regulatory role in Th2 differentiation. In addition to known Th2-promoting factors such as STAT6, the resulting panel of IL4 targets included several proteins with no previous implications in Th2 development. One of these proteins was YB1, a known transcriptional repressor of the apoptosis-related *FAS* receptor (Lasham et al., 2000). Th2 cells have been reported to express decreased levels of multiple components of the Fas pathway, including the FAS receptor, leading to relative inhibition of cell death (Rautajoki et al., 2007). Thus it seems possible that YB1 is involved in this differential regulation of apoptotic pathways in Th cells. However, this hypothesis could not be experimentally confirmed by use of siRNA targeting YB1. Of note, in parallel with the herein reported identification as a Th2-associated nuclear protein, IKZF1 was demonstrated to bind directly to regulatory regions within the *IL4* locus, and to be required for proper Th2 differentiation in mice (Quirion et al., 2009), and to silence expression of T-bet and IFNG (Thomas et al., 2010). Further characterization of such proteins might ultimately lead to identification of potential targets for regulation of Th2 responses.

STAT6, the key mediator of IL4-signaling, was studied in more detail, namely in context of protein interactions. Due to its central role in orchestration of Th2 responses, artificial modulation of STAT6 activity has been proposed as a therapeutic possibility for allergic disorders (Darcan-Nicolaisen et al., 2009; Tian et al., 2011). These approaches might benefit from more detailed characterization in molecular interactions of STAT6 in cell-type specific physiological context. Using IL4-stimulated primary T cells, HNRNPK and nucleolin were identified as potential novel cofactors for STAT6. As HNRNPK was found to regulate expression of GATA3, it seems a potential inducer of Th2 differentiation deserving more detailed investigation. Unfortunately, as HNRNPK plays a central role in mRNA processing, deletion of HNRNPK is likely to cause embryonic lethality, hindering its studies with mouse models (Bomsztyk et al., 2004).

In the final part of the thesis, the lipid metabolism was investigated in activated and IL4-stimulated T cells using untargeted LC-MS approach. Consistent trends in kinetics of lipid concentrations were detected, which appeared to be at least partially regulated on the level of the transcriptome. Deeper insight into the immunological significance of these changes could potentially be obtained by studying T cell development in mice deficient for selected regulatory enzymes, such as SCD.

In general, the overall importance of metabolic regulation for T helper cell differentiation is poorly understood. However, some lines of evidence, in particular regarding the mTOR pathway, highlight the interrelatedness of

metabolism and cellular differentiation (Wullschleger et al., 2006; Powell and Delgoffe, 2010; Lee et al., 2010b). Although in the present experiments no specific IL4-associated differences were observed, the possibility should not be entirely excluded by these data alone, as coverage of the method still has room for improvement. In the future, application of lipidomics and metabolomics methodology to T cell populations isolated from peripheral blood of healthy individuals as well as patients with T cell associated disorders might provide important clues to overall role of metabolic regulation in T cell biology.

6. SUMMARY

This study focused on system-wide measurements of mRNA, protein, and lipid compositions of human CD4+ T cells, and importantly, resulted in description of specific changes in these compositions occurring during the development of Th2 subset phenotype. By using exclusively umbilical cord blood T cells, these studies aimed at results representative of normal human immunology.

The nuclear proteins of IL4-stimulated cells were targeted by expression and interaction proteomics. The former resulted in identification of more than 800 proteins, representing the most extensive catalogue from human primary cells. Such datasets can provide useful "parts lists" for modeling-based computational systems biology (Schlitt and Brazma, 2005). In comparison to mRNA level, the IL4-induced proteomic differences were limited. Nevertheless, both known regulators of Th2 differentiation as well as proteins novel in this context were consistently quantified from triplicate samples.

By application of affinity purification and mass spectrometry, the Th2-promoting STAT6 enhanceosome complex was enriched and characterized. The use of semi quantitative proteomics approach allowed for use of low stringency purification conditions and isolation of the endogenous complex from primary human cells. Importantly, two previously undocumented proteins HNRNPK and NCL were found specifically associated with STAT6.

On the level of cellular metabolites, the lipid profiles of both resting and activated T cells were characterized. Importantly, following activation by T cell receptor and CD28, phosphatidylcholine and phosphatidylethanolamine lipids exhibited consistent gravitation towards more saturated and shorter molecular species. Integration with genome-wide transcriptomics data suggested that the observed lipidomic remodeling is regulated by acceleration of lipid synthesis as well as selective activation of metabolic pathways favoring generation of saturated lipid species.

In its part, this thesis provided a view of Th2 differentiation as a complex transition, where biological information initially delivered through transmembrane receptor molecules is mediated by diverse pathways and ultimately translates to responses observed on both protein and metabolite levels. In these studies the developmental process of Th2 lineage commitment was investigated using diverse complementary techniques. However, rapid methodological progress presents researchers with ever increasing selection of tools, many of which might be particularly informative in case of T cell differentiation. Integration and interpretation of these system-wide datasets will present an important challenge.

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