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SYSTEM-LEVEL CHARACTERIZATION OF TH2 CELL DEVELOPMENT AND IMMUNE CELL RESPONSES TO ZnO AND TiO_2 NANOPARTICLES

by

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ABSTRACT

Asthma and allergy are common diseases and their prevalence is increasing. One of the hypotheses that explains this trend is exposure to inhalable chemicals such as traffic-related air pollution. Epidemiological research supports this theory, as a correlation between environmental chemicals and allergic respiratory diseases has been found. In addition to ambient airborne particles, one may be exposed to engineered nanosized materials that are actively produced due to their favorable physico-chemical properties compared to their bulk size counterparts. On the cellular level, improper activity of T helper (Th) cells has been connected to allergic reactions. Th cells can differentiate into functionally different effector subsets, which are identified according to their characteristic cytokine profiles resulting in specific ability to communicate with other cells. Th2 cells activate humoral immunity and stimulate eradication of extracellular pathogens. However, persistent predominance of Th2 cells is involved in a development of number of allergic diseases. The cytokine environment at the time of antigen recognition is the major factor determining the polarization of a naïve Th cell. Th2 cell differentiation is initiated by IL4, which signals via transcription factor STAT6. Although the importance of this pathway has been evaluated in the mouse studies, the signaling components involved have been largely unknown. The aim of this thesis was to identify molecules, which are under the control of IL4 and STAT6 in Th cells. This was done by using system-level analysis of STAT6 target genes at genome, mRNA and protein level resulting in identification of various genes previously not connected to Th2 cell phenotype acquisition. In the study, STAT6-mediated primary and secondary target genes were dissection from each other and a detailed transcriptional kinetics of Th2 cell polarization of naïve human CD4+ T cells was collected. Integration of these data revealed the hierarchy of molecular events that mediates the differentiation towards Th2 cell phenotype. In addition, the results highlighted the importance of exploiting proteomics tools to complement the studies on STAT6 target genes identified through transcriptional profiling. In the last subproject, the effects of the exposure with ZnO and TiO_2 nanoparticles was analyzed in Jurkat T cell line and in primary human monocyte-derived macrophages and dendritic cells to evaluate their toxicity and potential to cause inflammation. Identification of ZnO-derived gene expression showed that the same nanoparticles may elicit markedly distinctive responses in different cell types, thus underscoring the need for unbiased profiling of target genes and pathways affected. The results gave additional proof that the cellular response to nanosized ZnO is due to leached Zn^{2+} ions. The approach used in ZnO and TiO_2 nanoparticle study demonstrated the value of assessing nanoparticle responses through a toxicogenomics approach. The increased knowledge of Th2 cell signaling will hopefully reveal new therapeutic nodes and eventually improve our possibilities to prevent and tackle allergic inflammatory diseases.

Keywords: T helper cell, IL4, STAT6, ZnO, TiO_2, nanoparticle, system-level analysis

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Th2-solujen kehityksen sekä ZnO- ja TiO_2-nanopartikkelien aiheuttaman vasteen tutkiminen immuunisoluissa systeemitason menetelmin
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TIIVISTELMÄ

Astma ja allergia ovat yleisiä tauteja, joiden esiintyvyys lisääntyy. Yhtenä selittävänä tekijänä on pidetty hengitysteiden kautta tapahtuvaa altistumista kemikaaleille kuten pakokaasuille. Epidemiologiset tutkimukset ympäristökemikaalien ja hengitystiesairauksien välisestä yhteydestä tukevat tätä teoriaa. Passiivisesti syntyvien partikkelien lisäksi hengitysilmassa voi olla myös nanopartikkeleita, joita erityisesti tuotetaan hyödyllisten ominaisuuksiensa takia. Solutasolla T-auttajasolujen (Th-solu) häiriintynyt aktiivisuus on liitetty allergisiin reaktioihin. Th-solut voivat erilaistua toiminnaltaan erikoistuneiksi alatyypeiksi, joita määrittelee niille ominainen sytokiinien eritys ja tästä seuraava tyypillinen kyky kommunikoida muiden solujen kanssa. Th2-solut aktivoivat vasta-ainevälitteisen immuunivasteen ja käynnistävät solun ulkoisten taudinaiheuttajien hävittämisen. Th2-solujen pitkäaikainen ylivalta immuunijärjestelmässä liittyy kuitenkin myös lukuisten allergisten sairauksien kehittymiseen. Th-solujen erilaistumisen suunnan määrittävät suurelta osin sytokiinit, jotka ympäröivät solua sen tunnistaessa oman antigeeninsä. IL4, jonka signalointi välittyy STAT6-transkriptiotekijän kautta, käynnistää Th2-solujen erilaistumisen. Vaikka tämän signalointireitin merkitys on tiedetty hiirikokeiden perustella, kaikkia siihen osallistuvia komponentteja ei tunneta. Tämän väitöskirjatyö tarkoituksena oli tunnistaa molekyylejä, joita säädellään IL4- ja STAT6-välitteisesti Th-soluissa. Tutkimuksessa käytettiin systeemitason menetelmiä, joilla STAT6-transkriptiotekijän kohdegeenejä tunnistettiin genomi-, lähetti-RNA- ja proteiinitasoilla. Työssä havaittiin lukuisia geenejä, joita ei ole aiemmin liitetty Th2-solujen ilmiasun määräytymiseen. Tutkimuksessa STAT6-kohdegeenit jaoteltiin välittömiin ja epäsuoriin, sekä kerättiin ajallisesti yksityiskohtainen tieto transkriptomin muutoksista naivien CD4-solujen erilaistumisesta Th2-alatyypiksi. Näiden tietojen yhdistäminen paljasti Th2-solujen erilaistumiseen osallistuvien molekyylitason muutosten keskinäisen suhteen. Tämän lisäksi tutkimuksessa saadut tulokset korostivat proteiinitason analyysien merkitystä transkriptomiikan täydentäjänä. Viimeisessä osatyössä tutkittiin ZnO- ja TiO_2-nanopartikkelien aikaansaamaa vastetta Jurkat T-solulinjassa ja ihmisen primääreistä monosyyteistä erilaistetuissa makrofageissa ja dendriittisoluissa. Työssä selvitettiin näiden nanopartikkelien toksisuutta ja kykyä aiheuttaa tulehdusreaktio. ZnO-nanopartikkelien aiheuttaman geenien ilmenemisvasteen huomattiin olevan solutyypistä riippuvainen. Tämä löydös painottaa, että nanopartikkelien vaikutuksia on tutkittava genominlaajuisesti. Tulokset tukivat havaintoja, joiden mukaan ZnO-nanopartikkelien toksisuus määräytyy partikkeleista irtoavien sinkki-ionien perusteella. Kokonaisuutena työ osoitti, että toksikogenominen lähestymistapa on hyödyllinen nanopartikkelien aikaansaamien vasteiden analysoinnissa. Th2-solusignaloinnista kertyvän tiedon toivotaan johtavan uusien terapeuttisten kandidaattigeenien tunnistamiseen ja lopulta mahdollistavan haitallisten allergisten tulehdusreaktioiden estämisen tai muokkaamisen.

Avainsanat: T-auttajasolu, IL4, STAT6, ZnO, TiO_2, nanopartikkeli, systeemitason analyysi

TABLE OF CONTENTS

ABBREVIATIONS

2DE, two-dimensional electrophoresis
ACN, acetonitrile
AHR, aryl hydrocarbon receptor
APC, antigen-presenting cell
AV, annexin V
β-ME, β-mercaptoethanol
BAL, bronchoalveolar lavage
BATF, basic leucine zipper transcription factor, ATF-like
BCL6, B-cell CLL/lymphoma 6
BSA, bovine serum albumin
bp, base pair
BTLA, B and T lymphocyte associated
CD, Crohn's disease
cDNA, complementary DNA
CBF, core binding factor
CCL, C-C chemokine ligand
CCR, C-C chemokine receptor
ChIP, chromatin immunoprecipitation
CIA, collagen-induced arthritis
CNBP, cellular nucleic acid binding protein
cRNA, complementary RNA
CRTH2, chemoattractant receptor homologous molecule expressed on Th2 cells
Ct, cycle threshold
CXCL, C-X-C chemokine ligand
CXCR, C-X-C chemokine receptor
DAPI, 4',6-diamidino-2-phenylindole
DBD, DNA binding domain
DEP, diesel exhaust particles
DIC, differential interference contrast
DLS, dynamic light scattering
DMSO, dimethyl sulfoxide
DNA, deoxyribonucleic acid
DTT, ditiotreitol
EAE, experimental autoimmune encephalomyelitis
EDTA, ethylenediaminetetraacetic acid
eGFP, enhanced green fluorescent protein
EN, engineered nanoparticle
EMSA, eletromobility shift assay
F-AAS, flame atomic absorption spectroscopy

FAM, carboxyfluorescein
Fc, constant region of an antibody
FC, fold change
FCS, fetal calf serum
FDR, false discovery rate
FELASA, Federation of European Laboratory Animal Science Associations
FSC forward scatter
FT-IR, Fourier transform infrared spectroscopy
EN, engineered nanoparticle
EPAS1, endothelial PAS domain protein 1
ESI, electrospray ionization
FOXP3, forkhead box P3
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GATA3, GATA binding protein 3
GFI1, growth factor independent 1 transcription repressor
GO, gene ontology
H1N1, swine influenza A virus
H, histone
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIES, hyper-immunoglobulin E syndrome, Job's syndrome
HMDM, human monocyte-derived macrophage
HPLC, high performance liquid chromatography
HRP, horseradish peroxidase
HUVEC, human umbilical vein endothelial cell
ICOS, inducible T-cell co-stimulator
ID4, inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
IEF, isoelectric focusing
IFN, interferon
Ig, immunoglobulin
IL, interleukin
ILR, IL receptor
iTreg, inducible regulatory T cell
JAK, Janus kinase
JNK, c-Jun N-terminal kinase
LC, liquid chromatography
LCMV, lyphocytic chorimeningitis virus
lincRNA, long intergenic non-coding RNAs
LPS, lipopolysaccharide
MAF, v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MDDC, monocyte-derived dendritic cell
me, methylation
MHC, major histocompatibility complex
miRNA, microRNA

MNC, mononuclear cell
mRNA, messenger RNA
MS, multiple sclerosis
MS/MS, tandem mass spectrometry
MT, metallothionein
MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW, molecular weight
NC, nitrocellulose
ncRNA, non-coding RNA
NK cell, natural killer cell
NR3C1, glucocorticoid receptor
NP, nanoparticle
nTreg, natural regulatory T cell
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PD-1, programmed cell death 1
PDI, polydispersity index
PE, phycoerythrin
PKA, cAMP-dependent protein kinase
pI, isoelectric point
PI, propidium iodide
PU.1, spleen focus forming virus (SFFV) proviral integration oncogene spi1
qPCR, quantitative PCR
RA, rheumatoid arthritis
RMA, robust multi-array average
RNA, ribonucleic acid
RNAi, RNA interference
RORC, RAR-related orphan receptor C
ROS, reactive oxygen species
RPMI, Roswell Park Memorial Institute medium
RT, room temperature
RT-PCR, reverse transcriptase polymerase chain reaction
RUNX, runt-related transcription factor
SAP, SH2 domain containing 1A
SDS, sodium dodecyl sulphate
Seq, massively parallel sequencing
SH2, Src homology 2 domain
shRNA, short hairpin RNA
siRNA, small interfering RNA
SLAM, signaling lymphocytic activation molecule
SLC, solute carrier family
ss, single strand

SSC, side scatter
STAT, signal transducer and activator of transcription
STING, transmembrane protein 173
T1D, type 1 diabetes
TAD, transactivation domain
TAMRA, tetramethylrhodamine
TBK1, TANK-binding kinase 1
TBX21, T-box 21, synonym T-bet
Tc, cytoxic T cell
TCR, T cell receptor
TEM, transmission electron microscopy
Tfh, follicular T helper cell
TGA, thermogravimetric analysis
TGFβ, transforming growth factor beta
Th, T helper cell
TLR, Toll-like receptor
TOF, time-of-light
tRNA, transfer RNA
TSLP, thymic stromal lymphopoietin
TSS, transcriptional start site
UPR, unfolded protein response
VST, variance stabilizing transform
WT, wild type
XRD, X-ray diffraction

LIST OF ORIGINAL PUBLICATIONS

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

I. Tuomela, S.*, Rautajoki, K.J.*, Moulder, R., Nyman, T.A., Lahesmaa, R. Identification of novel Stat6 regulated proteins in IL4-treated mouse lymphocytes. Proteomics. 2009;9(4):1087-98. (*Equal contribution)

II. Elo, L.L.*, Järvenpää, H.*, Tuomela, S.*, Raghav, S.*, Ahlfors, H., Laurila, K., Gupta, B., Lund, R.J., Tahvanainen, J., Hawkins, R.D., Oresic, M., Lähdesmäki, H., Rasool, O., Rao, K.V.[#], Aittokallio, T.[#], Lahesmaa, R. Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming. Immunity. 2010;32(6):852-62. (*Equal contribution, [#]Equal contribution)

III. Tuomela, S., Autio, R., Buerki-Thurnherr, T., Arslan, O., Kunzmann, A., Andersson-Willman, B., Wick, P., Mathur, S., Scheynius, A., Krug, F. H., Fadeel, B., Lahesmaa, R. Gene Expression Profiling of Immune-Competent Human Cells Exposed to Engineered Zinc Oxide or Titanium Dioxide Nanoparticles. (Submitted manuscript)

1. INTRODUCTION

The immune system protects higher organisms from foreign intrusions, such as bacteria, and eliminates malfunctioning host cells. It is hierarchically composed of different cell types having their own specialized function, but also working in synergy to provide targeted and tunable response. Distinction between "self" and "non-self" forms the basis of the immune system. Cells from innate immunity are specialized in recognizing foreign structures of pathogens and transformed host cells. They can destroy the intruder via phagocytosis or complement cascade, but also importantly activate the cells of adaptive immune system. Antigen-presenting cells (APC) digest the pathogens to peptides and activate the T helper (Th) cells bearing the cognate T cell receptor (TCR). This leads to clonal expansion of antigen-specific Th cells and ultimately to the development of a long term immunological memory against the pathogen. Th cells are characterized by co-expression of TCR and CD4 on their cell membrane, the latter mediating the specific interaction of major histocompatibility complex (MHC) II bearing antigen-presenting cells. Th cells orchestrate both the cell and the antibody mediated cytotoxic and humoral immune responses, respectively. This makes them indispensable for proper function of adaptive immune system.

The nature of pathogen instructs the cells of the innate immune system to produce different cytokines and express activating co-receptors. The local cytokine milieu, the strength of TCR engagement and the instructive signals provided by co-receptors determine the differentiation path, which naïve Th cell will take. This specific differentiation of Th cells is essential for targeted elimination of pathogens because each Th cell subset has distinct functional capabilities that are derived from their specific cytokine profile. The balance between the activities of different Th cell subsets is pivotal for efficient and controlled immune response. The proper function of different Th cell subtypes is needed for a specific and efficient eradication of various pathogens. Whereas, persistent predominance of a certain Th cell subtype can lead to immunological disease, such as allergic inflammation in the case of Th2 cells. Interleukin 4 (IL4) promotes the differentiation of Th2 cells via activation of signal transducer and activator of transcription protein 6 (STAT6). The importance of STAT6 signaling in the development and function of Th2 cells was identified in studies with *Stat6*-deficient mice. These mice are unable to produce functional Th2 cells and furthermore are resistant to antigen-induced airway inflammation and hyperresponsiveness. In human, Th2 cells have been shown to be elevated in the lungs of allergic patients (Meyer et al., 2008). In addition, the genetic locus coding STAT6 has been associated with asthma or atopy phenotypes in several populations (Ober and Hoffjan, 2006). Collectively, these results provide convincing evidence on the importance of IL4/STAT6 pathway in the development of allergic inflammation.

There are several hypotheses that have been used to explain the high prevalence of allergic reactions. The hygiene hypothesis was formulated on the basis of an observed inverted

correlation between level of microbes in our environment and allergic symptoms. It follows the assumption that infections during childhood educate the immune system to react properly, and without exposures the immune system becomes incorrectly activated in response to harmless substances such as pollen grains. At the same time, inhaled particles from air pollution have been shown to modulate immune cell activity. In addition to particulate matter coming as a byproduct of human activity, nanoscale particles are also actively produced to be utilized for example in biomedical applications. As the size of engineered nanoparticles (EN) is comparable to biological structures, they can be introduced in biological systems to carry drugs and other active compounds. However, the large reactive surface area of ENs is thought to pose a more severe risk of adverse effects on organisms than microscale materials. Metal oxide nanoparticles, such as zinc (ZnO) or titanium dioxide (TiO_2), are produced and used in large quantities in consumer products such as sunscreens. Although exposure to these materials happens mainly via the skin, the unprotected manufacture workers may inhale TiO_2 or ZnO during chemical production or when using galvanized metal in welding. Inhalation of ZnO can lead to acute metal fume fever and work-related asthma. Correspondingly, pulmonary exposure to TiO_2 has been shown to modulate airway reactivity.

The aim of the work presented in this thesis was to characterize the IL4/STAT6 signaling pathway in Th2 cell differentiation and identify new factors putatively participating in the downstream signal transduction. In addition, the effects of ZnO and TiO_2 EN exposure to the transcriptional profiles of selected immune cells were investigated.

2. REVIEW OF THE LITERATURE

2.1 Overview of the immune system

Human body is an ecosystem of "self" cells and microbes forming so called normal microbiota. The coexistence of these different cells is harmless and can be even symbiotic. However, there is also a multitude of microorganisms which can be pathogenic. Colonization by these microbes is inhibited by the complex cooperation of different organs, tissues and cells, which together participate in processes needed to protect the host. Collectively this entity is called the immune system.

Innate immunity

Unspecific protection mechanisms are responsible for the first line of defense. Tissues such as skin form protective barriers, which physically inhibit foreign intrusions. However, after microbial invasion inside the body, the cells of the innate immune system get immediately engaged to protect the host. Dendritic cells, neutrophils, monocytes and macrophages nonspecifically recognize foreign structures. Unmethylated CpG motifs, fungal chitins, bacterial lipopolysaccharides and formylated methionine among other foreign structures act as a pathogen-associated immunostimulants. In addition, soluble serum proteins of the complement system can become activated by microorganisms. NK cells are activated by the detected lack of MHC I, which is expressed on every nucleated host cell type. Basophils, mast cells and eosinophils degranulate releasing their antimicrobial compounds after being crosslinked by antibodies or complement proteins. The cells participating in the innate immune reactions protect the host via cytolysis, phagocytosis, and by releasing antimicrobial peptides and inflammation mediators. Importantly, macrophages and dendritic cells work as main professional antigen-presenting cells, which activate the next level of protection called adaptive immune system.

Adaptive immunity

T and B cells have antigen receptors, which have high specificity to a certain processed or native antigen, respectively. In addition, T cells can recognize their cognate antigen only in the context of MHC molecule. T cells are further classified into cytotoxic CD8+ cells (Tc) and CD4+ T helper cells (Th). Tc cells interact with MHC I molecules, and are needed for elimination of cells damaged or infected by viruses. Activated Tc cells release proteins that disrupt the cell membrane and lead to the targeted elimination of cells presenting altered antigens. Th cell are instead activated by antigen-presenting cells expressing MHC II molecules. Th cells can differentiate into various functional subtypes distinctively orchestrating immune reactions by regulating the activity and recruitment of other immune cells. The course of activation and functional differentiation of naïve adaptive immune cells takes several days. However, once fully recruited B and T cell populations expand clonally and produce massively pathogen-specific antibodies or cytokines, respectively.

Characteristically a part of the cells of the adaptive immune system become long-lived memory cells. Upon subsequent infection with the same pathogen, these memory cells can be recruited faster and their response is stronger than when the antigen was encountered for the first time. γδ T cells and NK T cells work in the interface of innate and adaptive immunity and share phenotypical and functional properties with cells from both of the systems. Innate and adaptive immune reactions are tightly interconnected and essential for successful elimination of pathogens. (Alberts et al., 2002; Janeway et al., 2001)

2.2 T helper cell subsets in the immune system

Presentation of intra- or extracellular pathogens has been thought to lead to either a Th1 or Th2 cell response, respectively (Coffman, 2006; Liew, 2002). This simple dichotomy was broken by the observations of immune responses that could not be explained by this model (Gor et al., 2003), and ultimately by the identification of new Th cell subsets. First, naïve Th cells were shown to be capable of acquiring extrathymically immune regulatory iTreg cell phenotype (inducible T regulatory, adaptive T regulatory) (Apostolou and von Boehmer, 2004; Chen et al., 2003; Cobbold et al., 2004; Curotto de Lafaille et al., 2004; Kretschmer et al., 2005). Subsequently, Th17 cells were accepted to represent a new Th cell fate (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005; Wilson et al., 2007). Later, Th9, Th22 and follicular helper T cells (Tfh) joined the family of CD4+ effector cells (Breitfeld et al., 2000; Dardalhon et al., 2008; Duhen et al., 2009; Eyerich et al., 2009; Schaerli et al., 2000; Staudt et al., 2010; Trifari et al., 2009; Veldhoen et al., 2008). Different Th cell subsets are characterized by their phenotypic cytokine and trafficking receptor expression determining the function and the migration of the activated cells, respectively. However, all Th cell subsets express more than one cytokine and chemokine receptor and none of the signature molecules is exclusively expressed only in one subset of Th cells. Thus only the combinatorial expression can be used to determine the cell identity and function (**Table 1**). The cytokines produced by each Th cell subset are needed to prime or enhance the differentiation of naïve Th cells towards the same direction and inhibit the alternative fates. Thus the specific cytokine secretion not only determines the functional outcome but provides a principle of the subtype intrinsic amplification of the activated Th cell population. Similarly, the preferential expression of chemokine receptors on each subtype guides the cell to the inflammatory site predominated by the presence of the members of the same subtype, which have stimulated the expression of the attracting chemokine (Bonecchi et al., 1998; Wilson et al., 2007; Zhang et al., 2000). This forms the second loop of the subtype-specific amplification. Equivalently as the immune system needs to be properly activated in response to pathogen encounter, the response has to be dampened after eradication of the foreign intruder. The unresolved activity of Th cells is detrimental to the integrity of the host, and sustained predominance of different Th cell subtypes is known to be correlated with several autoimmune and inflammatory diseases. Although different Th cells have been connected to various diseases, it should be remembered that

all cells work in the context of the whole immune system in which the overall balance of the cells, reactions and signaling determines the final outcome.

Th2, Th9 and Th22 cells

Th2 cells produce IL4, IL5 and IL13 (**Table 1**) (Cherwinski et al., 1987; Lewis et al., 1988; Mosmann et al., 1986; Wierenga et al., 1990; Wierenga et al., 1991) and are important for eradication of extracellular parasites such as helminthes (Katona et al., 1988; Mohrs et al., 2001; Vignali et al., 1989). All Th2 cell cytokines have pleiotropic functions. IL4 is the most important cytokine priming and amplifying Th2 cell differentiation and it controls antibody production of B cells by inducing immunoglobulin (Ig) class switching to IgE (Nelms et al., 1999). Crosslinking of IgE in turn efficiently activates degranulation of mast cells and other cell types bearing Fcε receptors leading to release of inflammation mediators needed for elimination of parasites (Gould et al., 2003). IL5 stimulates growth, differentiation, activation, survival and mobilization of eosinophils, which regulate mast cell function and release cytotoxic compounds (Rothenberg and Hogan, 2006). IL13 regulates mucus secretion and integrity of extracellular matrix, but has also a partly redundant function with IL4 (Wynn, 2003). Th2 cells express C-C chemokine receptor (CCR) 4 on their surface (Bonecchi et al., 1998; Sallusto et al., 1998) along with G protein-coupled chemoattractant receptor CRTH2, which is a marker for human Th2 cells (Cosmi et al., 2000; De Fanis et al., 2007; Nagata et al., 1999). CCR4 ligands CCL17 and CCL22 (chemokine (C-C motif) ligand) (Imai et al., 1997; Imai et al., 1998) are highly induced upon allergen challenge in the airway epithelial cells of asthmatic patients and in the skin biopsies of atopic dermatitis patients (Galli et al., 2000; Panina-Bordignon et al., 2001). CRTH2 binds prostaglandin D2 (Hirai et al., 2001), which is produced in high amounts by mast cells activated by IgE during allergic inflammation (Gyles et al., 2006; Lewis et al., 1982; Xue et al., 2009). Prostaglandin D2 engagement of CRTH2 both stimulates the Th2 cell cytokine production (Xue et al., 2005), but also prevents Th2 cell apoptosis (Xue et al., 2009), thus amplifying the Th2 cell mediated inflammation.

The Th9 cell subset is named according to their signature cytokine (**Table 1**). Along with Th2 cells, Th9 cells may participate in elimination of extracellular parasites, as has been supported with the observation that the mice with defective TGFβ signaling, which is needed for Th9 cell development, show compromised immunity against *Trichuris muris* infection (Veldhoen et al., 2008). Concomitantly, Th2 and Th9 cell cytokine predominance has been linked to the same allergic inflammatory disorders such as asthma and atopy (Robinson et al., 1993; Robinson et al., 1992; Stassen et al., 2012; Staudt et al., 2010; Temann et al., 1998; Wenzel, 2012; Wierenga et al., 1991). However, *in vivo* IL9 production of IL9 fate-reporter mouse model was mainly assigned to innate lymphoid cells during papain-induced lung inflammation with only a minor contribution of CD4+ cells (Wilhelm et al., 2011). So, although Th9 cells can be generated *in vitro*, direct evidence for the existence of this subset *in vivo* is still lacking (Wilhelm et al., 2012). This could be due to the extremely transient nature of IL9 expression, and on the other hand the usage of experimental setups biased for non-Th9 cell associated inflammation. (Wilhelm et al., 2011; Wilhelm et al., 2012)

Th22 cells express CCR10 and CCR4 (**Table 1**) (Duhen et al., 2009; Eyerich et al., 2009), and are thus skin-homing (Soler et al., 2003). It has been suggested that they participate in epithelial innate immune reactions and regeneration, but also contribute to inflammatory skin diseases like psoriasis, atopic eczema and allergic contact dermatitis. (Boniface et al., 2007; Duhen et al., 2009; Eyerich et al., 2009; Fujita et al., 2009; Nograles et al., 2009; Zhang et al., 2011). Recently, Th22 cell activity was also shown to be important for mucosal immunity against enteropathogenic *Citrobacter rodentium* bacteria (Basu et al., 2012).

Th1 and Th17 cells

The main cytokine produced by Th1 cells is IFNγ (**Table 1**) (Mosmann et al., 1986; Rotteveel et al., 1988), which activates macrophages to destroy the cells infected by intracellular viruses or bacteria (Suzuki et al., 1988). Defective Th1 cell responses are detected among the patients susceptible to mycobacterial and *Salmonella* infections (Altare et al., 1998; de Jong et al., 1998; Filipe-Santos et al., 2006). The chemokine receptor most characteristic to Th1 cells is C-X-C chemokine receptor CXCR3 (Langenkamp et al., 2003; Sallusto et al., 1998; Yamamoto et al., 2000). It recognizes three interferon inducible C-X-C chemokine ligands, namely CXCL9, CXCL10 and CXCL11, recruiting Th1 cells to the site of inflammation (Groom and Luster, 2011) and guiding the localization of a developing Th1 cells in the lymph node (Groom et al., 2012). Th17 cells, named according to their archetypical expression of IL17 and IL17F (**Table 1**), are needed for eradication of extracellular bacterial and fungal infections (Korn et al., 2009). Deficiency in Th17 cell development found in the patients suffering from hyper-immunoglobulin E syndrome (HIES, Job's syndrome), leads to the increased susceptibility to *Staphylococcus aureus* and *Candida albicans* infections (Berger et al., 1980; Ma et al., 2008; Milner et al., 2008; Szczawinska-Poplonyk et al., 2011). Th17 cells express CCR6 (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007). CCL20 is the ligand for CCR6 (Baba et al., 1997) and it directs the homing of Th17 cells to the specific parts of mucosal lymphoid tissue in intestine such as Peyer's patches (Wang et al., 2009). Besides the cells of mucosal surfaces, several other cell types can secrete CCL20, thus being potential recruiters of CCR6 expressing Th17 cells (Schutyser et al., 2003). Chemokine receptor expression on Th17 cells is heterogeneous and the combinatorial expression may ultimately determine the tissue distribution of Th17 cells in different inflammatory conditions (Lim et al., 2008). In addition, human Th17 cells express cluster of differentiation 161 (CD161) lectin receptor (Cosmi et al., 2008; Maggi et al., 2010), the role of which in Th17 cells is not known.

Uncontrolled Th1 and Th17 cell responses are connected to several autoimmune diseases. The distinction of the contribution of Th1 and Th17 cells, and the cells sharing properties of both of these subtypes in several autoimmune diseases, especially in human, is still to be elucidated in detail (Brand, 2009; O'Connor et al., 2010; Yamada, 2010). The difficulty of specifying the pathogenic subtype originates from two main reasons. Th1 cell differentiation inducing IL12 and Th17 cell phenotype promoting IL23 cytokines share the p40 subunit, and consequently in the studies in which p40 expression is modified the differentiation

and function of both of these subtypes is disturbed. Furthermore, the IL12 and IL23 receptors include IL12Rβ1 subunit. (Watford and O'Shea, 2003) In addition, both IL12 and IL23 induce IFNγ expression from T cells. (Oppmann et al., 2000) Also, Th17 cells have been shown to be phenotypically plastic and the cytokine environment surrounding Th17 cells can modify their function from protective to pathogenic (Marwaha et al., 2012). Improper Th1 cell dominance has been associated with several autoimmune diseases such as rheumatoid arthritis (RA) (Li et al., 2012; Yamada et al., 2008), type I diabetes (T1D) (Katz et al., 1995; Martin-Orozco et al., 2009) acute graft-versus-host disease (Yi et al., 2008) and Crohn's disease (CD) (Fuss et al., 1996; Parronchi et al., 1997). In all these diseases, IFNγ producing CXCR3 positive Th cells have been reported to be the dominating CD4+ cells and the expression of CXCR3 ligands is increased. (Groom and Luster, 2011) Thus CXCR3 antagonists are being developed for therapeutic intervention of these diseases (Jenh et al., 2012; Wijtmans et al., 2008). Human Th17 cells were identified using samples from CD patients and it was shown that the percentage of IL17 producing CD4+ cells was increased in the inflamed gut. However, a significant proportion of IL17 producing cells coexpressed IFNγ. (Annunziato et al., 2007) In support of the pathogenic potential of Th17 cells, the number of CD161+ cells producing IL17 but not IFNγ was higher in the blood of CD patients than in the blood of the healthy donors (Kleinschek et al., 2009). Convincing evidence of the pathogenicity of Th17 cells has been gathered in mouse EAE (experimental autoimmune encephalomyelitis) model of multiple sclerosis (MS). IL12 has been shown to be dispensable (Becher et al., 2002) and IL23 essential for the development of EAE (Cua et al., 2003). Concomitantly, the percentage of Th17 cells is increased in the subjects having active MS compared to subjects with inactive state of MS, patients with other non-inflammatory neurological diseases, or healthy controls (Brucklacher-Waldert et al., 2009; Durelli et al., 2009). However, in the clinical trial, intravenous administration of IFNγ exacerbated MS (Panitch et al., 1987), underlining the importance of non-classical Th17 cells or Th1 cells in the disease pathology. In children with T1D, the Th17 cell mediated immunity in peripheral blood is increased (Honkanen et al., 2010). In patients with RA the occurrence of Th17 cells and IL17 has been reported to correlate with the disease activity (Leipe et al., 2010). In addition, in the collagen-induced arthritis mouse model of RA, the *Il23*-deficient mice were protected from the disease and the lack of IL12 aggravates autoimmune joint inflammation (Murphy et al., 2003). However, it has been shown that the Th17 phenotype is unstable and gives rise to Th1 effector cells in the inflamed joint (Nistala et al., 2010). A recent report showed that Th17 cell derived Th1 cells and the classical Th1 cells can be distinguished from each other by measuring the expression of *Rorc*, *Il17re*, *Il4i1*, CCR6 and CD161, which were almost exclusively expressed in the Th17 cell derived Th1 cells. This gene expression signature may provide a novel tool for better dissection of the role of Th17 versus Th1 cells in the human autoimmune diseases. (Maggi et al., 2012)

Tfh cells

Tfh cells are needed for B cell development in germinal centers to achieve high affinity antibody production and immunological memory. They secrete IL21 and express

CXCR5 (**Table 1**), which facilitates Tfh cell migration out of the T-cell zone to the B cell area of the lymph node. (Breitfeld et al., 2000; Chtanova et al., 2004; Nurieva et al., 2008; Schaerli et al., 2000) In addition, germinal center Tfh cells express high levels of ICOS, SAP, BTLA and PD-1, which are important for Tfh cell interaction with B cells and modulation of Tfh cell function (Crotty, 2011). Deregulation of Tfh function has been implicated both in autoimmune diseases and immunedeficiencies due to increased pathogenic autoantibody production or inadequate humoral response, respectively (King et al., 2008; Morita et al., 2011; Simpson et al., 2010). Increased frequency of Tfh cell has been found in patients suffering from autoimmune thyroid disease (Zhu et al., 2012) and systemic lupus erythematosus (Simpson et al., 2010; Vinuesa et al., 2005).

iTreg cells

Treg cells limit the effector T cell responses and endow immunological tolerance (Brunkow et al., 2001; Chatila et al., 2000; Wildin et al., 2001). They express immunosuppressive cytokines IL10 and TGFβ (**Table 1**) (Goodman et al., 2012). The specific roles of iTreg and natural Treg (nTreg) cells in the regulation of immunological homeostasis are beginning to be established (Bilate and Lafaille, 2012). By using a colitis mouse model and adaptive transfer of T cells, it was shown that synergistic function of nTreg and iTreg cells was needed to achieve the full tolerance on otherwise lethal *Fox3*-deficient background. This finding supports a separate role for nTreg and iTreg cells. The authors suggest that iTreg cells provided an increased TCR repertoire needed to protect the host. (Haribhai et al., 2011) Generation of iTreg cells has been observed in several inflammation mouse model systems. For example in the asthma model, iTreg cells downregulated IgE production and the level of chronic inflammation although they were not able to prevent the disease (Curotto de Lafaille et al., 2008).

Table 1. Hallmark characteristics of T helper cell subsets.

	Th1	Th2	Th9	Th17	Th22	Tfh	iTreg
Cytokine secretion	IFNγ	IL4, IL5, IL13	IL9	IL17, IL17F	IL22	IL21	TGFβ, IL10
Priming cytokines	IL12, IFNγ	IL4	IL4, TGFβ	IL6, TGFβ	TNFα, IL6	IL6, IL21	IL2, TGFβ
Important transcription factors	STAT1, STAT4, TBX21	STAT6 STAT5 GATA3	STAT6, PU.1	STAT3, RORC	AHR	STAT3, BCL6	STAT5 FOXP3
Chemokine receptor pattern	CXCR3+ CCR4- CCR6-	CCR4+ CXCR3- CCR6-		CCR6+ CCR4+ CXCR3-	CCR10+ CCR4+ CCR6+	CXCR5+	
Characteristic surface molecule		CRTH2*		CD161*		PD-1**, ICOS**, SAP**, BTLA**	

*In human

**Germinal center Tfh cells

2.2.1 Molecular mechanisms of cytokine-induced T helper cell differentiation

A naïve CD4+ T cell has to recognize its cognate antigen, presented as peptide-MHC complex on APC, and engage co-stimulatory molecules for the formation of an immunological synapse in order to become responsive to polarizing cytokine signals. Cytokine stimulation alone does not drive differentiation (Geginat et al., 2001), and TCR activation without co-stimulation leads to anergy (Chappert and Schwartz, 2010; Schwartz, 2003). In addition, the kinetics of TCR activation and cytokine stimulation determines whether the priming is successful; these two parameters cannot be completely temporally separated from each other (Iezzi et al., 1999; Jelley-Gibbs et al., 2000; Mohrs et al., 2003; Nakamura et al., 1997; Richter et al., 1999; Seki et al., 2004). The characteristics of APCs, concentration of the antigen and chemokines, as well as signaling through co-stimulatory molecules modulate Th cell differentiation process (Burr et al., 2001; Constant and Bottomly, 1997; Filippi et al., 2003; Gu et al., 2000; Liu et al., 2001; Long et al., 2011; McAdam et al., 2000; Paulos et al., 2010; Purvis et al., 2010; Rothoeft et al., 2003). Selective outgrowth of the cells of a certain Th subset also plays a role in the augmentation of biased immune response (Liu et al., 2010; Vieira et al., 2004; Zhu et al., 2002). However, the acquisition of functionally different Th cell phenotypes is predominantly regulated by the cytokines surrounding the naïve Th cell at the time of activation (Constant and Bottomly, 1997). Th cell polarization is regulated via various cytokine inducible signaling pathways, employing multiple regulatory mechanisms at each level of gene expression (**Figure 1**).

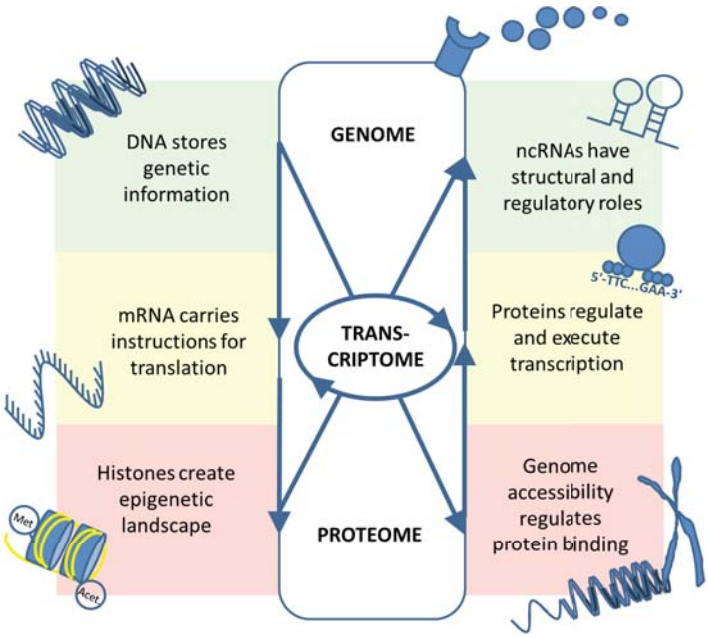

Figure 1. Molecular mechanisms of Th cell differentiation. External signals are drivers of the differentiation process, which is regulated at all levels of gene expression. The regulatory mechanisms are tightly interconnected and cross-regulate each other to create a complex network of circuitries and feedback loops. The figure gives an overview on the mechanisms discussed further in detail in this thesis. (ncRNA, non-coding RNA).

Transcriptional regulation

Binding of a cytokine to its receptor leads to phosphorylation-dependent intracellular spreading of the stimulus, which along with TCR-mediated signaling causes subsequent activation of transcription factors. Recognition of the specific instructive cytokine or combination of cytokines, and quintessential transcription factors driving the downstream signaling has been used as one of the criteria for definition of Th cell lineages. Identification of the key transcription factors for the newest Th cell subtypes is partly still ongoing or under debate. However, all Th cell subsets can be separated from each other based on their distinct transcriptional profile (Chen et al., 2003; Chtanova et al., 2001; Chtanova et al., 2004; Ciofani et al., 2012; Elo et al., 2010; Eyerich et al., 2009; Haribhai et al., 2009; Langrish et al., 2005; Lu et al., 2004; Lund et al., 2005; Lund et al., 2007; Park et al., 2005; Rogge et al., 2000; Tuomela et al., 2012). Th1 cell differentiation is regulated by IL12 stimulating the phosphorylation of STAT4, and IFNγ activating STAT1-mediated induction of TBX21 (Afkarian et al., 2002; Hsieh et al., 1993; Kaplan et al., 1996; Lighvani et al., 2001; Schulz et al., 2009; Szabo et al., 2000; Thierfelder et al., 1996; Ylikoski et al., 2005). IL4 is needed for STAT6-dependent differentiation of GATA3 expressing Th2 cells (Kaplan et al., 1996; Le Gros et al., 1990; Ouyang et al., 1998; Shimoda et al., 1996; Swain et al., 1990; Takeda et al., 1996; Zheng and Flavell, 1997). In the presence of TGFβ, IL4 drives the expression of PU.1 and IRF4, which along with STAT6 promote the Th9 phenotype (Chang et al., 2010; Goswami et al., 2012; Staudt et al., 2010). Combination of proinflammatory cytokines IL6, IL1β, IL21 or IL23 with TGFβ leads to phosphorylation of STAT3 and induction of RORC assigning the path towards IL17 secreting Th17 cells (de Jong et al., 2010; Ivanov et al., 2006; Ma et al., 2008; Manel et al., 2008; Milner et al., 2008). IL6 and IL21-derived activation of STAT3 and BCL6 is shown to be important for Tfh cell development, although there are still many open questions regarding Tfh cell priming and development (Crotty, 2011; Eto et al., 2011; Johnston et al., 2009; Kroenke et al., 2012; Nurieva et al., 2008; Nurieva et al., 2009; Yu et al., 2009). iTreg development is dependent on TGFβ and IL2, which activate transcription factors FOXP3 and STAT5, respectively (Chen et al., 2011; Chen et al., 2003; Davidson et al., 2007; Josefowicz et al., 2012). The acquisition of Th22 cell specific characteristics is mediated by TNFα and IL6 via AHR (Baba et al., 2012; Duhen et al., 2009; Ramirez et al., 2010; Trifari et al., 2009). The upstream transcription factors promote the Th cell subtype deviation via inducing the expression of the other phenotypic transcription factors and by suppressing the transcription factors important for alternative phenotypes. For example cells primed towards the Th1 direction fail to repress *Gata3* expression in the absence of STAT1 and STAT4 (Ouyang et al., 1998). On the other hand GATA3 inhibits the expression of STAT4 (Usui et al., 2003; Yagi et al., 2010). The expression of key transcription factors is regulated at several levels as can be illustrated for example with BCL6, which inhibits Th2 cell development by competing with STAT6 of the genomic binding sites (Dent et al., 1997; Harris et al., 1999; Harris et al., 2005) and by inhibiting GATA3 expression at a posttranscriptional level (Kusam et al., 2003). Lineage-specific transcription factors also regulate each other's function by

protein-protein interactions. DNA binding of GATA3 is diminished in a protein complex with TBX21 (Hwang et al., 2005). TBX21 and GATA3 have also been shown to bind to the same genomic regions and thus putatively regulate the same genes (Jenner et al., 2009). However, in a follow-up study, it was shown that although GATA3 binding sites are shared between the different Th cell subsets the expression of these genes is largely regulated in a cell type specific manner (Wei et al., 2011).

In addition to protein coding RNA, the genome is transcribed into non-coding RNA (ncRNA) molecules of various sizes and functions (Amaral et al., 2008). MicroRNAs (miRNA) are small RNA molecules that downregulate gene expression posttranscriptionaly by inhibiting translation or by tagging mRNA for degradation. Importantly, as miRNA need only be partially complementary with the target mRNA, one miRNA can regulate several target transcripts. DICER and DROSHA are RNase III-like enzymes, which cleave immature miRNAs into their active form. (Bartel, 2004) miRNAs have been shown to be especially important for the development and function of Treg cells as the *Dicer* or *Drosha*-deficient Treg cells lose their suppressive capacity during inflammation (Chong et al., 2008; Cobb et al., 2006; Liston et al., 2008; Zhou et al., 2008). Analogous to their distinct mRNA profiles, naïve CD4+ cells, Th1, Th2, Th17, Tfh and Treg cell subsets have their characteristic miRNome (Cobb et al., 2006; Kuchen et al., 2010; Rossi et al., 2011). In a comparison between the human and mouse miRNome, it has been found that the concordance between the signatures is relatively poor (Rossi et al., 2011). It remains to be studied to what extent the low correlation was derived from the different experimental set ups of the studies, as the human Th cell subsets were *ex vivo* isolated from peripheral blood (Rossi et al., 2011) and in the mouse study the cells were differentiated *in vitro* (Kuchen et al., 2010). There are some studies in which the Th cell differentiation process has been shown to be modulated by the specific miRNA. *miR-125b* upregulated in naïve CD4+ cells was shown to inhibit IFNγ and IL13 expression of activated CD4+ cells (Rossi et al., 2011). Selective blockade of *miR-126* expression impaired Th2 cytokine expression and most importantly development of airway inflammation (Mattes et al., 2009). *miR-155*-deficient CD4+ cells showed potentiation of Th2 cytokine expression and an increase in airway remodeling (Rodriguez et al., 2007). Importantly, blood and lung T cells of asthmatic patients have been shown to have disease-associated miRNA expression patterns (Seumois et al., 2012). In addition to miRNAs, a wealth of conserved long intergenic noncoding RNAs (lincRNAs) is transcribed throughout genome (Cabili et al., 2011; Guttman et al., 2009). Recently, it was reported that *Tmevpg1* lincRNA is selectively expressed in mouse and human Th1 cells contributing to the IFNγ expression (Collier et al., 2012).

Epigenetic regulation

Epigenetic modifications of DNA, histones and nucleosome positioning along with high-order chromatin structure define the activity of genomic loci and contribute to coordinated intra- and interchromosomal gene expression. These mechanisms are needed for maintaining the acquired phenotype in the absence of external differentiation cues.

Analysis of epigenetic regulation of Th cell differentiation started with studies of the IFNγ and Th2 cytokine loci (Ansel et al., 2003), but has since been revolutionized with new methods enabling genome-wide analysis (Kanno et al., 2012). Histone methylation and acetylation, nucleosome location and DNase hypersensitivity site analysis have been performed for CD4+ T cells before or after activation (Barski et al., 2007; Boyle et al., 2008; Schones et al., 2008; Wang et al., 2008). In addition to these studies, Th cell subtype-specific epigenetic modifications have been analyzed. A Treg lineage-specific DNA methylation pattern has been reported and shown to correlate with the subtype-specific enhancer activity (Schmidl et al., 2009). Chromatin immunoprecipitation (ChIP) based histone modification analysis has been used to recognize distinguishing sites of the active promoters (H3K4me3) and the repressed sites (H3K27me3) in various Th cell subtypes, and that STAT4, STAT6 and GATA3 regulate the specificity of various histone modifications (Wei et al., 2009; Wei et al., 2011; Wei et al., 2010). High-order chromatin structures regulate coordinated expression within the Th2 cytokine locus, and its interchromosomal interaction with the IFNγ gene, which is lost upon activation of the subtype-specific signature gene expression (Cai et al., 2006; Spilianakis and Flavell, 2004; Spilianakis et al., 2005). Of the transcription factors essential for Th cell differentiation, STAT6, GATA3 and FOXP3 have been suggested to play a role in the regulation of gene expression via DNA looping (Bandukwala et al., 2011; Chen et al., 2012; Spilianakis and Flavell, 2004). Importantly, the linkage between epigenetic regulation and diseases originating from uncontrolled Th cell activity are also beginning to be recognized (Lovinsky-Desir and Miller, 2012; Ngalamika et al., 2012).

Regulation of transcription of coding and noncoding RNAs and epigenetic modifications is highly interconnected. The epigenetic landscape regulates the expression of mRNA and noncoding RNA molecules during Th cell differentiation (Kuchen et al., 2010; Wei et al., 2009). Mutually, transcription factors regulate the epigenetic printing of their target loci (Durant et al., 2010; Wei et al., 2011; Wei et al., 2010). The detailed mechanisms that are utilized in this regulation are becoming to be revealed in Th cells. DNA binding domain of TBX21 interacts directly with the histone modifying proteins enabling sequence and Th1 cell specific chromatin remodeling (Lewis et al., 2007; Miller et al., 2008). The STAT6 transcription complex also contains proteins with histone acetyl- and deacetylasetransferase activity (Dong et al., 2011; Välineva et al., 2005). Although yet to be proven in a wider perspective, the expression level of the target mRNA may also affect the expression of its regulatory miRNA (Kuchen et al., 2010). Of the key transcription factors for the Th cell subsets, BCL6, which contributes to the Tfh phenotype, has been shown to repress many miRNAs (Yu et al., 2009). Similarly, STAT4 and TBX21 are needed for the expression of *Tmevpg1* lincRNA, which in turn, in collaboration with TBX21, induces Th1 cell specific IFNγ expression (Collier et al., 2012). Although knowledge of the importance of lincRNAs in the immune system is still in its infancy, further studies will most likely increase our understanding on their role in the regulation of chromatin modifications and higher order structures as previously reported in the other cell types (Tsai et al., 2010; Wang et al., 2011). The interaction of different levels

of gene expression regulation is no doubt going to be an area of intensive research in the coming years. These studies will uncover the wealth of regulation circuitry defining the functional outcome of Th cells.

2.2.2 Plasticity of T helper cell phenotypes

One of the key characteristics of adaptive immunity is the formation of memory cells, which can immediately respond to a subsequent intrusion of the previously recognized pathogen. Although development of memory cells is quintessential for immunological homeostasis, the pathways guiding towards memory cell phenotypes have not been thoroughly elucidated. According to the current knowledge a small percentage of the differentiated effector Th cells become long-lived effector memory T cells (T_{EM}) and Tfh cells may develop into central memory T cells (T_{CM}) (Pepper and Jenkins, 2011). The original idea of Th effector cell differentiation was that it is a step-wise process leading to a fixed phenotype, which could not be altered by removing the inducing cytokine environment or even when the cytokine instructing the polarization towards the alternative fate was introduced to the repeatedly stimulated cells (Del Prete et al., 1991; Mosmann et al., 1986; Murphy et al., 1996; Swain et al., 1990). This would be rational for preserving the optimized reactivity against recurrent infections. However, although Th1 and Th2 cells have been seen as fully differentiated lineages, there have never been generally accepted criteria which unequivocally define Th1 and Th2 phenotypes. One reason for this is that both Th cell clones derived from *ex vivo* isolated cells or *in vitro* differentiated cells have heterogeneous cytokine expression pattern (Firestein et al., 1989; Openshaw et al., 1995; Paliard et al., 1988; Picker et al., 1995; Sornasse et al., 1996; Street et al., 1990; Umetsu et al., 1988). In addition to heterogeneity, cells co-expressing IL4 and IFNγ were observed (Openshaw et al., 1995; Paliard et al., 1988; Picker et al., 1995). The identification of new Th cell subtypes has partly explained the noticed heterogeneity, but beyond this, reports indicating that Th cells might retain some functional plasticity started to gather already some time ago. For example, human Th2 cell clones derived from the skin biopsies of atopic dermatitis patients were shown to be able to express IFNγ when stimulated via SLAM co-stimulatory receptor without a need for external cytokines (Aversa et al., 1997; Carballido et al., 1997). In theory, functional plasticity could be a mechanism to enhance immunological response against new pathogens at an older age (Bluestone et al., 2009; Zhu and Paul, 2010) when the pool of naïve cells is shrunken (Fulop et al., 2007; Haynes and Lefebvre, 2011) leading to an increased importance of cross-reactivity of the previously formed memory T cells (Mason, 1998; Wooldridge et al., 2012). Accordingly, memory T cells have been shown to have adjustable cytokine expression pattern (Adeeku et al., 2008; Ahmadzadeh and Farber, 2002; Krawczyk et al., 2007; Messi et al., 2003). In addition, a growing evidence of plasticity in gene expression and function of effector Th cells has been gathered. Studies *in vitro*, and *in vivo* fate-reporter mice and adoptive transfer experiments have shown that Th17 cells can transform into Th1 cells (Annunziato et al., 2007; Bending et al., 2009; Hirota et al., 2011; Lee et al., 2009; Martin-Orozco et al., 2009; Shi et al.,

2008). An opposite phenotypic destabilization from Th1 cells to IL17 and IL17/IFNγ co-producers has also been reported to occur in mesenteric lymph nodes (Kurschus et al., 2010). Effector memory Th17 cells can also acquire characteristics of Th2 cells and contribute to chronic asthma (Cosmi et al., 2010; Wang et al., 2010). iTreg cells can convert into Th17 cells in the presence of TGFβ and IL6, the process being enhanced by IL23 and inhibited by retinoid acid, and leading to the expression of Th17 cell specific genes and lack of suppressive function (Yang et al., 2008). IFNγ secreting Th1 effector cells have also been shown to be able to acquire Treg cell functions and concomitantly express TBX21, IFNγ and FOXP3 (Chowdary Venigalla et al., 2012). Importantly, although mouse Th2 cells are resistant to *in vitro* re-differentiation to Th1 cells they can acquire stable mixed Th2/Th1 phenotype *in vivo* during an infection with lyphocytic chorimeningitis virus (LCMV) (Hegazy et al., 2010). In addition, some human Th2 cell clones have been shown to switch on IFNγ expression in response to the appropriate costimulation (Shanafelt et al., 1995). The stability of Th2 cells is also tilted by TGFβ, which leads to reduced GATA3 expression and induction of IL9 in reprogramming to Th9 cell phenotype (Veldhoen et al., 2008).

The mechanisms controlling Th cell stability or flexibility are the same ones that control their differentiation, i.e. environmental signals, transcriptional regulation and epigenetic imprinting. *In vivo*, Th cells are exposed to a more variable environment than *in vitro*. They can be surrounded with various cytokines and interact with APCs providing a range of co-stimulatory signals at different stages of activation and differentiation. The sum of instructing and restricting signals is translated into intracellular signaling cascades leading to specification or final determination of a phenotype. Exposure to an instructing cytokine in a permissive environment can lead to an activation of positive feedback loops, which may become self-sufficient from external stimulation. Signaling circuitry can also shut down expression of molecules important for alternative development. In the case of Th2 cells, GATA3 can autoactivate its own expression (Ouyang et al., 2000) and the loss of expression of IL12Rβ2 makes the differentiating cell unresponsive to IL12, the key Th1 cell priming cytokine (Szabo et al., 1995; Szabo et al., 1997). However, ectopic expression of IL12Rβ2 in Th2 cells is not sufficient for conversion to a Th1 cell phenotype in the presence of IL12 (Heath et al., 2000; Nishikomori et al., 2000). Introduction of GATA3 to the cells differentiated to Th1 direction causes an upregulation of Th2 cell marker genes without a need for external cytokines (Lee et al., 2000; Sundrud et al., 2003). However, ectopic expression of GATA3 can only partly restore the global IL4/STAT6-dependent transcription and epigenetic marks (Vahedi et al., 2012). This highlights the importance of using genome-wide unbiased analysis methods in the analysis of different phenotypes. Analysis of epigenetic modifications has led to the hypothesis that epigenetic imprinting determines the stability of the acquired phenotype. The analysis of the key loci such as Th2 cytokine locus support this concept as it is marked with activating and repressing marks in Th2 and Th1 cells, respectively. (Wilson et al., 2009) Interestingly, the situation is more complex beyond the cytokine genes and when the other Th cell subsets are analyzed. Genome-wide

analysis of H3K4me3 and H3K27me3 histone marks revealed that *Gata3* and *Tbx21* genes were marked with the permissive modification in the cells of their specific subtype and with the repressive marks in the opposite subtype. However, bivalent H3K4me3 and H3K27me3 modification of these genes was found in the other Th subsets analyzed. This argues for retained potential to upregulate the expression of these genes upon appropriate stimulation. (Wei et al., 2009) Similarly, it has been interpreted that permissive H3K4me3 epigenetic mark of *Bcl6* in Th1, Th2 and Th17 cells, and *Tbx21*, *Gata3*, and *Rorc* loci in Tfh cells allow reciprocal plasticity between these phenotypes (Lu et al., 2011). External cytokine cues have been shown to affect the epigenetic landscape of *Il17a/Il17F* and *Ifng* loci correlating with the known plasticity of the Th17 cell phenotype (Mukasa et al., 2010). Importantly, the causal effect of the epigenetic modifications on the stability of Th2 cell phenotype has been shown by analyzing *Suv39h1* knockout mice. SUV39H1 is a histone methyltransferase that trimethylates histone 3 on lysine 9 (H3K9me3) enabling heterochromatin formation. The loss of SUV39H1 did not have an impact on Th2 cell polarization, but caused a substantial increase in plasticity allowing the expression of IFNγ and TBX21 during re-culturing in the Th1 polarizing conditions. (Allan et al., 2012) DNA methylation has also been implicated in the regulation of Th cell plasticity. *Dnmt3a* DNA-methylase deficient Th2, Th17 and iTreg cells have an increased capability to express IFNγ upon re-culture in an IL12 containing medium. The reactivation of IFNγ expression was accompanied by increase of the active H3K4me3 and decrease of the repressive H3K27me3 histone marks. (Thomas et al., 2012) Evidence of miRNA-dependent regulation of Th cell plasticity has also been mounted. Exogenous expression of *miR-10a* inhibits the re-differentiation of the *in vitro* generated iTreg cells into Tfh cells in Peyer's patches (Takahashi et al., 2012). When DICER, an enzyme central for miRNA synthesis, is absent in CD4+ cells, differentiation into Th1 or Th2 cells is not affected, as judged based on their signature cytokine expression. However, *Dicer*-deficient Th2 cells express reduced level of *Gata3* and produce more IFNγ than the wild type Th2 control cells indicating a conversion towards a Th1 phenotype. (Muljo et al., 2005)

The concept of plasticity of Th cells is not completely astonishing, as for example it has been shown that fibroblast can be reprogrammed into pluripotent stem cell (Takahashi and Yamanaka, 2006). The technological improvements allowing the analysis of a wider range of readouts, integration of the results, and fate mapping of single cells will facilitate the analysis of functional plasticity and heterogeneity of Th cells along with elucidation of causality of the regulatory mechanisms. It has already been proposed that Th cell differentiation should be seen as a varying gradient of probabilities rather than a direct path to a fixed endpoint (O'Shea and Paul, 2010). Plasticity and heterogeneity among Th cells can also be seen as risk management of the host, as a fixed phenotype might pose an increased risk in changing conditions (**Figure 2**). Although the occurrence and the importance of plasticity *in vivo* remain to be studied, reprogramming of Th cells could putatively be utilized in therapeutic interventions to redirect an already established pathogenic Th cell response.

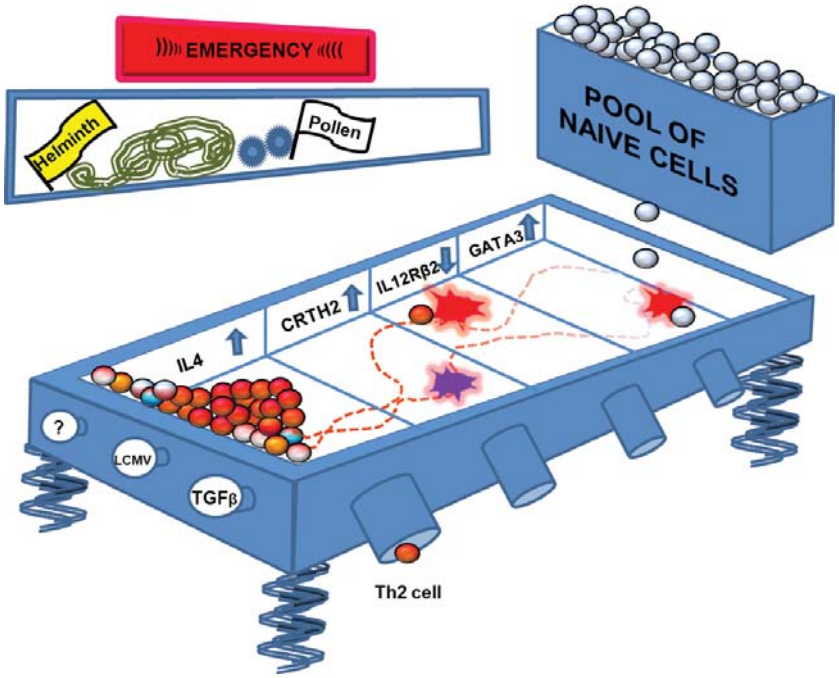

Figure 2. A Pinball analogy of Th2 cell differentiation. The genetic background forms the pillars or the framework of the immune system, which protective function is optimized according to detected environmental triggers. Helminth infection initiates polarization of naïve cells towards Th2 cell phenotype. However, harmless substances such as pollen may also activate the process. The cytokine environment created by activated innate immune cells, and the interaction of naïve T cells with antigen-presenting cells determine the direction of the differentiation. Modulation of the characteristics of developing cells, such as induction of GATA3, CRTH2 and IL4, and suppression of IL12Rβ2 expression function as molecular thresholds during the differentiation process increasing the likelihood of a prototypical polarization. Nevertheless, there are several routes, which can lead to the same functional outcome, and differences in the interaction with the environment may create a spectrum of nuances in effector cell phenotype and its stability. The stability of Th2 cells is known to be modulated for example with TGFβ and LCMV-infection, which can trigger Th9 and Th2/Th1 cell development, respectively (Dardalhon et al., 2008; Hegazy et al., 2010; Veldhoen et al., 2008).

2.3. STAT6 in Th2 cell differentiation

STAT6 belongs to the family of signal transducer and activator of transcription proteins with seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. The characterization of the STAT-mediated signaling started from the observation of a fast IFN-inducible DNA binding complex, formation of which was independent on protein synthesis. A few years after, STAT1 and STAT2 were cloned in 1992, followed by the identification of all the STATs within three years. (Stark and Darnell, 2012) Full length STAT6 has N-terminal, coiled-coil, DNA-binding (DBD), linker, Src homology 2 (SH2), and C-terminal transactivation (TAD) domains, a structure that is conserved in the other STATs (O'Shea et al., 2002). The coiled-coil domain of STAT6 contains its nuclear localization signal (Chen and Reich, 2010). The SH2 domain is important

for STAT6 binding to the activated IL4R and dimerization (Mikita et al., 1998). STAT6 molecules preferentially form homodimers (Nelms et al., 1999; O'Shea et al., 2002), but in response to IFNα STAT6:STAT2 heterodimers have been observed in hepatocytes, and T and B cells (Eriksen et al., 2004; Gupta et al., 1999; Wan et al., 2008). In addition, STAT6 dimers have been suggested to form tetramers, although their existence has not yet been validated (Harris et al., 2005).

The STAT6 gene consists of 23 exons spanning in a 16 kb region in the human chromosome 12. Various splice variants are produced from the gene from which three different proteins (STAT6/STAT6a, STAT6b and STAT6c) are known to be translated in human (Hebenstreit et al., 2006; Patel et al., 1998). The function of the different isoforms is largely unexplored (Hebenstreit et al., 2006). STAT6b and STAT6c, are an amino-terminal truncation and a partial SH2 domain deletion forms of STAT6, respectively. STAT6c has been shown to be a dominant negative variant of STAT6, inhibiting the dimer formation of the tyrosine phosphorylated STAT6. Instead, STAT6b is tyrosine phosphorylated upon IL4 stimulation, but its function is attenuated compared to the full length STAT6. (Patel et al., 1998) In addition to alternative splicing, different protein forms of STAT6 are formed from proteolytic processing. In mast cells, a 65 kDa form of STAT6 lacking TAD is cleaved in the nucleus and works as a dominant negative inhibitor of STAT6 signaling (Sherman et al., 1999; Sherman et al., 2002; Suzuki et al., 2002).

The expression of *STAT6* mRNA is regulated via CCAAT and ATF elements at a minimal STAT6 promoter region located at -148 to +34 of human *STAT6* gene. In addition, DNA methylation regulates the expression of STAT6. (Kim et al., 2010) Calpains and proteasome are known to downregulate STAT6 expression via proteolysis (Hanson et al., 2003; Suzuki et al., 2003; Tahvanainen et al., 2009; Zamorano et al., 2005). The main cascade for STAT6 activation is initiated by extracellular cytokines bound to their receptor on the cell membrane leading to the activation of JAK-STAT6 pathway (Leonard and O'Shea, 1998), which is discussed more in detail in the following section. However, there are also a few studies reporting an alternative activation of the STAT6-mediated signaling. A recent study identified a mechanism of viral infection induced STAT6 activation, which was found in a variety of cell types. This activation was independent of JAKs, but requited TBK1 kinase and took part in endoplasmic reticulum after STAT6 recruitment by STING protein. The induction of STAT6 by the viral infection lead to the expression of genes related to immune cell homing such as *CCL2*, *CCL20*, and *CCL26*. (Chen et al., 2011) Beyond the novel mechanism of the virally activated STAT6, unphosphorylated STAT6 has been shown to be active regulator of COX2 expression in human non-small cell lung cancer (Cui et al., 2007). Interestingly, in *Drosophila melanogaster* unphosphorylated STAT6 ortholog STAT (STAT92E) controls heterochromatin stability and STAT phosphorylation allows transcriptional machinery to access chromatin (Shi et al., 2008). STAT6 is known to be phosphorylated at various sites (Hebenstreit et al., 2006), however the function of only few of them is known (Wang et al., 2004). Phosphorylation of Tyr641 is needed for human STAT6

activation and dimerization. Phosphorylation of several serine residues within the TAD have been reported to inhibit STAT6 DNA binding in IL4-treated cells (Maiti et al., 2005). Ser707 has been reported to be phosphorylated by JNK in response to stress or IL1β stimulation, and inhibit the IL4/STAT6 signaling (Shirakawa et al., 2011). STAT6 has also been reported to be modified by acetylation, which positively regulated the transcriptional activity of STAT6 (Shankaranarayanan et al., 2001).

2.3.1 IL4 and STAT6-dependent Th2 cell differentiation

IL4, which initiates Th2 cell differentiation (Le Gros et al., 1990; Swain et al., 1990), is produced by basophils, mast cells, NKT cells, γδ T cells and eosinophils (Nelms et al., 1999), but also by naïve CD4+ T cells (Noben-Trauth et al., 2000; Noben-Trauth et al., 2002) and Th2 cells (Mosmann et al., 1986). IL4 receptor (IL4R) consists of IL4Rα and common γc chains (Kondo et al., 1993; Russell et al., 1993). Binding of IL4 to IL4Rα leads to dimerization of the receptor subunits, which activates the receptor-associated Janus kinases JAK1 and JAK3 (Leonard and O'Shea, 1998). These kinases subsequently phosphorylate the five tyrosine residues in the IL4Rα subunit, three of which are essential for STAT6 activation and downstream gene expression. (Nelms et al., 1999) Phosphorylated tyrosines form the docking sites for the SH2 domain of the STAT6 molecule (Hou et al., 1994; Kotanides and Reich, 1993; Quelle et al., 1995; Schindler et al., 1994), which becomes immediately tyrosine phosphorylated (Quelle et al., 1995) by the receptor associated Jak kinases. Activated STAT6 molecules disengage from the receptor, assemble into homodimers and translocate from the cytoplasm into the nucleus (Nelms et al., 1999). The signal transduction from the external environment into the nucleus is fast as phosphorylated STAT6 can be found in nucleus shortly following IL4 stimulation (Andrews et al., 2002). STAT6 dimers recognize the specific DNA sequence 5'-TTCN$_{(3-4)}$GAA-3' (Ehret et al., 2001; Schindler et al., 1995), which leads to accumulation of activated STAT6 into the nucleus (Chen and Reich, 2010). Transcriptional regulation by STAT6 requires interaction with the basal transcriptional machinery and is modulated by several co-factors (Hebenstreit et al., 2006). The presence or absence of cofactors may explain cell type and developmental stage specific gene expression (Kanai et al., 2011; Tozawa et al., 2011) and contribute to the fact that only a fraction of the STAT6 binding sites seem to be associated with changes in gene expression (Elo et al., 2010; Kanai et al., 2011; Tozawa et al., 2011; Wei et al., 2010).

The essential role of the IL4/STAT6 signaling in Th2 cell development and function is supported by several observations. Deficiency of IL4 or IL4Rα leads to a dramatic defect in Th2 cell differentiation potential (Barner et al., 1998; Kopf et al., 1993). Furthermore, mice with disrupted *Stat6* gene are almost completely defective in their ability to polarize CD4+ cells into Th2 cell direction both *in vitro* and *in vivo* (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). In addition, Th cells expressing constitutively active STAT6 differentiate into a Th2 cell phenotype without a need for external cytokines (Bruns et al., 2003; Zhu et al., 2001). The genes regulated by IL4/STAT6 signaling in Th cells have been mainly characterized using a transcriptomics

approach. It has been shown that IL4 regulates a specific subset of genes belonging to diverse functional categories both in human and in mouse (Chtanova et al., 2001; Lund et al., 2003; Lund et al., 2005; Lund et al., 2007; Rogge et al., 2000; Zhu et al., 2002). Although based on the analysis of *Stat6*-deficient mice it was recognized that both differentiation and proliferation of T cells was affected (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996), only very few STAT6 target genes were known especially in T cells at the time. Electromobility shift assay (EMSA) provided evidence that STAT6 can directly bind to the IL4Rα promoter and regulate its expression when overexpressed together with the reporter construct in HeLa cells (Kotanides and Reich, 1996). The labor intensive candidate gene search revealed that STAT6-regulated T cell proliferation by downregulating $p27^{Kip1}$ protein expression (Kaplan et al., 1998). Transcription factor GATA3 was known to be stimulated by IL4 and both induce Th2 cell cytokine expression and repress IL12Rβ2 expression (Kurata et al., 1999; Zhang et al., 1997; Zheng and Flavell, 1997). Shortly afterwards, GATA3 was reported to be regulated by STAT6 (Kurata et al., 1999; Ouyang et al., 2000). Improvements of microarray technology have since revolutionized target gene identification. The specific role of STAT6 in the IL4-mediated transcriptome has been analyzed by using the mice with *Stat6* deletion (Chen et al., 2003; Zhu et al., 2002). The search for growth-related genes affected by STAT6, resulted in identification of failed IL4-derived induction of *Il4rα*, *Cis*, *Cd44*, *Plagl2*, *Gfi-1* and *EST(AA261222)* genes in *Stat6*$^{-/-}$ mice. It was especially pinpointed that GFI-1 enhances Th2 cell proliferation and inhibits apoptosis. (Zhu et al., 2002) The role of STAT6 in the Th2 cell differentiation was then assessed (Chen et al., 2003). It was shown that although STAT6 signaling was prerequisite for Th2 cell differentiation, it regulated less than 20% of the IL4 target genes during Th2 cell polarization. Altogether 36 genes were identified to be regulated by STAT6 in this study. By using an updated microarray version, the number of STAT6-regulated genes detected in mouse was thereafter increased substantially (Wei et al., 2010). In this study it was highlighted that STAT6 has a dual role in Th2 cell differentiation by both upregulating the genes associated to Th2 cell polarization and by repressing the genes inhibiting the process. Furthermore, the direct and indirect target genes of STAT6 were distinguished from each other by using the ChIP-seq (chromatin immunoprecipitation followed by deep sequencing) technique. Most importantly, it was shown for the first time that STAT6 regulates the global epigenetic histone modification pattern, which controls the Th2 cell-specific gene expression. For example, *Gata3* gene was shown to be bound and regulated by STAT6, which furthermore regulated the permissive epigenetic modification of the loci. In general, STAT6 was shown to have the strongest impact on elimination of the repressive H3K27me3 marks, although the underlying mechanism needed for this function remained to be clarified.

In human CD4+ cells, STAT6 target genes have been dissected from the selected IL4-regulated genes by utilizing gene downregulation with shRNA (short hairpin RNA) (Lund et al., 2007). It was noted that most of the genes regulated by IL4 during the early polarization of human Th2 cells were poorly characterized. Out of these, *AW629527*,

AA088177, *AA489100*, *ZNF443*, and *DACT1* transcripts were induced by IL4-activated STAT6. In addition, it has been shown that introduction of *STAT6*-siRNA (small interfering RNA) into human CD4+ cells during Th2 polarization upregulates the expression of GTPase IMAP family member 4 (GIMAP4) (Filen et al., 2008). Proteomics has also been used to analyze IL4-induced differences in human cord blood cells at different stages of Th2 cell polarization. Twelve proteins were identified to be differentially expressed between Th1 and Th2 polarized cells at 7 and 14 days after initiation of the culture. Importantly, most of the observed protein level changes were not detected at RNA level highlighting the complementarity of the approach. (Rautajoki et al., 2004) In addition, 35 proteins were identified from 20 protein spots representing the IL4-specific proteome of activated cord blood cells at 24 h timepoint. In this study, identification of reduced fragmentation of GDP dissociation inhibitor expressed in lymphocytes (Ly-GDI), led to the validation of IL4-dependent inhibition of caspase activity during the priming of Th2 cells. (Rautajoki et al., 2007)

2.3.2 Alternative pathways promoting Th2 cell differentiation

Although there is a compelling amount of data showing that IL4 and STAT6 are needed for Th2 cell differentiation, there are also reports indicating that alternative pathways exist. Especially *in vivo* differentiation appears to be more complex than could be interpreted based on the *in vitro* data alone. For example, although STAT6 is needed for the Th2 cell cytokine secretion *in vitro*, Th2 cell cytokine response can be seen in *Bcl6*$^{-/-}$*Stat6*$^{-/-}$ double knockout mice *in vivo* (Dent et al., 1998). Equivalently, by analyzing e*GFP*/*IL4* knock-in reporter mice crossed with *Stat6*-deficient mice in several *in vivo* Th2 cell differentiation model systems (van Panhuys et al., 2008), it was revealed that the lack of IL4 or STAT6 did not affect to the kinetics or degree of Th2 cell differentiation (Mohrs et al., 2001; van Panhuys et al., 2008). These results indicate that the microenvironment *in vivo*, which drives Th2 cell polarization at least in certain infections and putatively accompanied with suitable genetic predisposition (Bancroft et al., 2000), is yet to be defined.

The defect in Th2 cell differentiation is more pronounced in *Stat6*$^{-/-}$ than in *Il4*$^{-/-}$ mice (Kaplan et al., 1996). Consecutively, it was found that IL13 can also signal through IL4Rα and guide cells towards the Th2 phenotype *in vivo* (Barner et al., 1998). Indeed, *Il13*$^{-/-}$ mice have impaired IL4 and IL5 production (McKenzie et al., 1998). However, IL13 cannot initiate Th2 cell differentiation of naïve human CD4+ cells (Sornasse et al., 1996). It is generally thought that T cells lack type II IL4R complex consisting of IL4Rα and IL13Rα chains, and are thus non-responsive to IL13 (Wills-Karp and Finkelman, 2008). Nevertheless, conflicting results also exist, e.g. peripheral blood T cells have been reported to express *IL13Rα* mRNA, which is downregulated upon activation (Gauchat et al., 1997), and IL13 stimulation of peripheral blood derived T cell blasts has been shown to lead to the formation of STAT6 DNA binding complex (Curiel et al., 1997). In addition, viral infection induces IL13Rα expression on transformed T cells (Waldele et al., 2004). IL4Rα (Noben-Trauth et al., 1997) and *Stat6*-deficient (Jankovic et al., 2000;

Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996) mice have substantially diminished Th2 cell responses, but there is still some residual production of Th2 cell cytokines. There are several factors that may be involved in this IL4/STAT6-independent differentiation. IL2 has been shown to promote IL4 production following CD3 crosslinking. Importantly, the effect was suggested to be instructive, rather than based on selective outgrowth of proliferating cells or prevention of cell death (Ben-Sasson et al., 1990), and work in synergy with IL4 to induce IL4 production (Le Gros et al., 1990). IL2 activates STAT5 (Heltemes-Harris and Farrar, 2012), which plays a hallmark role in differentiation of Treg cells (Josefowicz et al., 2012). However, STAT5 also participates in Th2 cell polarization, as the data acquired with *Stat5a*-deficient mice have revealed. Deletion of *Stat5a*, both *in vitro* and *in vivo*, causes reduced IL4 production without disabling IL4/STAT6 signaling (Kagami et al., 2001). Importantly, by comparing IL4 production of the cells from *Stat5a*$^{-/-}$, *Stat6*$^{-/-}$ and *Stat5a*$^{-/-}$*Stat6*$^{-/-}$ double knockout mice, it was concluded that STAT5A was responsible for the residual IL4 production seen in *Stat6*$^{-/-}$ mice (Takatori et al., 2005). Multiplicity of cytokine signaling in Th2 cell development is increased by the observation that also IL6 induces IL4 expression in CD4+ cells (Rincon et al., 1997). It phosphorylates STAT3 upon TCR activation and induces the expression of MAF, which in turn transactivates the IL4 gene (Yang et al., 2005). Alternatively, IL6 has been shown to induce IL4 expression via NFATc2 (Diehl et al., 2002). Overexpression of constitutively active STAT6 cannot upregulate IL4, IL5 and IL13 expression without STAT3. Furthermore, it was shown that STAT3 binding to the specific target genes, such as *Gata3* and *Maf*, was needed to prime the loci for STAT6. (Stritesky et al., 2011) BCL6 has been identified to be the repressor of the IL6-induced Th2 cell differentiation (Kusam et al., 2003).

Thymic stromal lymphopoietin (TSLP), IL25 and IL33 cytokines have mainly been reported to play an indirect role in Th2 cell differentiation *in vivo*, for example via priming DCs to initiate Th2 cell differentiation program, and by amplifying already established Th2 cell responses (Oliphant et al., 2011; Paul and Zhu, 2010; Saenz et al., 2008; Williams et al., 2012). However, there are also reports showing that these cytokines may be directly recognized by naïve Th cells. IL17BR, the IL25 receptor subunit, is expressed on naïve CD4+ T cells and repressed or induced upon Th1 or Th2 cell differentiation, respectively. In addition, IL25 has been shown to induce IL4, IL5 and IL13 during Th cell priming. (Angkasekwinai et al., 2007) TSLP increases the proliferation of CD4+ cells during *in vitro* activation (Al-Shami et al., 2004). However, it can also induce early IL4 production from naïve CD4+ T cells, the effect being independent of cell division (Omori and Ziegler, 2007). In the studies showing that IL25 and TSLP can directly prime Th2 cell differentiation, the effect has nevertheless been dependent on IL4/STAT6 signaling (Angkasekwinai et al., 2007; Omori and Ziegler, 2007). The role of TSLP in the initiation of Th2 cell polarization has been challenged based on its expression primarily on mucosal surfaces and skin epithelia, and not in the lymph node at the time when DCs present the antigens to naïve cells (Paul, 2010). However, basophils have been reported to produce TSLP along with IL4, enter the lymph node after immunization with

papain, and prime Th2 cell differentiation (Sokol et al., 2008). IL33 is mainly produced by the cells forming the barrier tissues, such as epithelial cells and fibroblasts, although its expression can be detected in various tissues. IL33 signals through heterodimeric receptor consisting of ST2 and IL1 receptor accessory protein (IL1RAcP). (Liew et al., 2010; Palmer and Gabay, 2011) ST2 expression is specifically induced during Th2 cell differentiation (Lecart et al., 2002; Lohning et al., 1998), but it is indispensable for the process (Hoshino et al., 1999; Kropf et al., 2003; Xu et al., 1998). IL33R is not expressed on naïve T cells (Lohning et al., 1998; Meisel et al., 2001). However, CD3 activated and IL33 stimulated naïve human and mouse cells have been reported to express increased amounts of IL5 and IL13 in an IL4/STAT6-independent manner (Kurowska-Stolarska et al., 2008).

Apart from cytokine signaling, TCR-mediated cascades also participate in Th2 cell differentiation. The variables affecting to cumulative TCR stimulation are potency (affinity and half-life), density (dose and concentration), and duration (stability of interaction and antigen persistence) of TCR-peptide/MHC interaction (Corse et al., 2011). Different *in vitro* and *in vivo* approaches have given variable results on the impact of the TCR signal strength to Th2 cell polarization. In addition, extrapolation of the findings acquired with monoclonal T cells to a real life *in vivo* situation in which there are large variety of TCR specificities, and thus diverse outcomes, is challenging (Corse et al., 2011). However, two models for TCR activation dependent Th2 cell differentiation have been proposed. According to the first one, low TCR signal strength leads to Th2 cell priming (Yamane and Paul, 2012). The regulation of STAT5 activity and the expression of GATA3 are the central nodes in this model. Strong TCR engagement leads to enhanced ERK activation, which inhibits TCR-driven GATA3 expression and STAT5 phosphorylation both of which have been shown to be important for Th2 cell differentiation (Cote-Sierra et al., 2004; Jorritsma et al., 2003; Kagami et al., 2001; Takatori et al., 2005; Yamane et al., 2005; Zhu et al., 2003). During a helminth infection leading to a high antigen load, Th2 cell differentiation could be acquired by inhibition of the antigen presentation potency of APCs by parasitic extracts, as reported with Omega-I glycoprotein secreted by *Schistosoma mansoni* (Everts et al., 2009; Everts et al., 2012; Steinfelder et al., 2009). The second model suggests that the effect of TCR is bimodal, and both low and high activation favors Th2 cell differentiation. The preferential Th2 cell differentiation during high antigen load is explained in this model by the ability of Th cells to produce IL4 themselves in contrast to IL12, which is supplied by other cell types in response to activation. Thus the repeated stimulation of naïve and developing Th2 cells by APCs would lead to auto- and paracrine amplification of Th2 cell polarization and proliferation. (Nakayama and Yamashita, 2010) The putative importance of self-reinforcement of Th2 cell polarization is supported by the observations of preferential Th2 cell phenotype acquisition in the experimental setting where both IL12 and IL4 are present (Perez et al., 1995) and by the absence of IL12R on naïve Th cells (Igarashi et al., 1998; Szabo et al., 1997).

2.4 Crosstalk of nanoparticles and the immune system

Nanomaterials are structures with at least one dimension less than 100 nm (**Figure 3**). Nanoscale particles can be naturally occurring like volcanic ash, originate as a result from everyday life such as traffic, be byproducts from industrial processes or be engineered to meet specific needs. Due to their size, nanomaterials have unique properties compared to their bulk size counterparts. Increased relative surface area and number of atoms at material's surface change the physical characteristics such as melting temperature and conductivity of the nanosized materials. Nowadays, engineered nanomaterials are produced for several applications of which the usage in food industry, cosmetics and paints are probably the best known to the general public. Altogether, there are more than 1300 nanotechnology-based consumer products available worldwide on the market (Woodrow Wilson International Center for Scholars, The Project on Emerging Nanotechnologies). Nanotechnology is also applied in medicine. Nanoparticles (NP) are already tested and used as drug delivery vehicles, and in diagnosis and imaging applications. Future expectations for nanomaterials include a wide range of medical uses such as in nano-welding to stop bleeding during surgery or in neuro-electronics to give external impulses for treatment of paraplegia. (Chang, 2010)

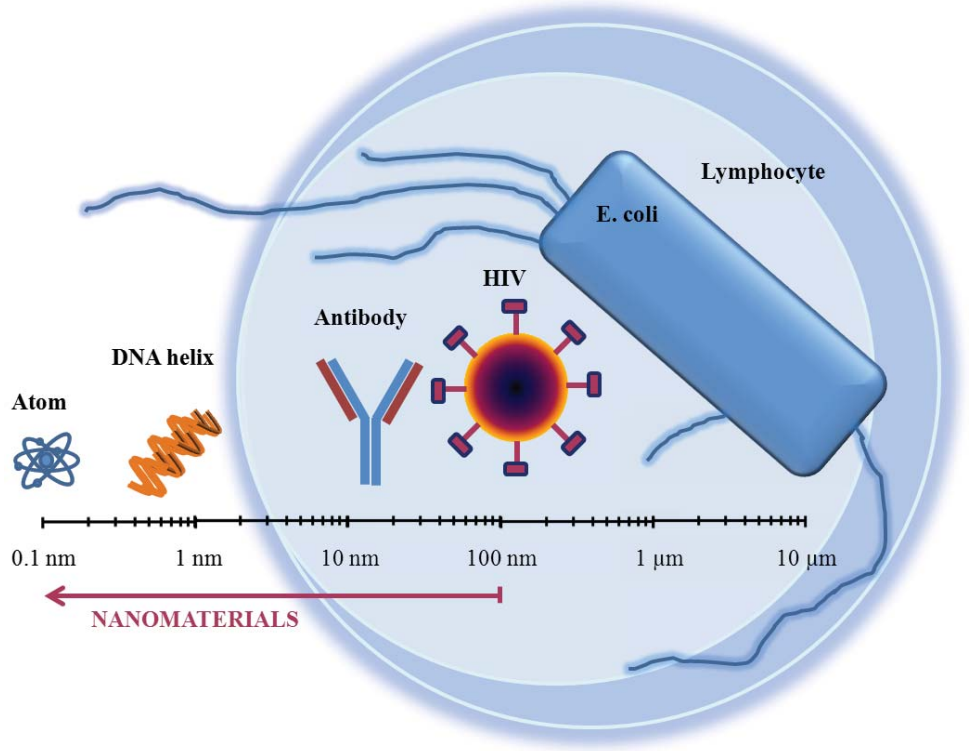

Figure 3. Scale of nanomaterials. Diameter of atom (0.1 nm), DNA helix (2 nm), antibody (15 nm), human immunodeficiency virus, HIV (145 nm), E. coli (1 µm) and lymphocyte (8 µm) drawn next to the logarithmic scale (Alberts et al., 2002; Briggs et al., 2003). Nanomaterials have at least one dimension less than 100 nm. The sizes of the different components in the illustration are not strictly in scale.

Concerns about potential health effects of NPs are rooted in the known harmful effects of micrometric airborne particulate matter. Epidemiological studies have revealed a clear connection between airborne particles and morbidity and mortality. (Borchers et al., 2006) Similarly, the causal effect of asbestos to lung cancer is well known. Due to their size, which is comparable to naturally occurring biological molecules, nanomaterials can be internalized into biological systems and interact with cell surface or internal molecules potentially interfering with cellular signaling (Shvedova et al., 2010). The toxic potential of NPs is thought to be inversely correlated to their size (Nel et al., 2006). New disciplines, such as nanotoxicology and toxicogenomics, are emerging fields in the study and assessment of the health and environmental risks of nanomaterials. Many diseases have already been associated with some forms of NPs including autoimmune, neurological and inflammatory diseases. However, a conclusive link between engineered nanoparticle (EN) exposure and disease outcome has not yet been reported in human (Li and Nel, 2011). Diesel exhaust particles (DEP), a heterogeneous group of ambient airborne particles, have been used as a model in studies on immune effects of inhaled particles. More than 80% of DEP is estimated to have an aerodynamic diameter of 1 μm or less (Kerminen et al., 1997), thus containing fine and ultrafine particles, i.e. nanoparticles. DEPs are known to modulate the Th cell balance leading to Th2 cell biased immunity via direct modulation of gene expression or indirectly by altering the phenotype of DCs. (Bezemer et al., 2011; Ohtani et al., 2005) The composition of DEPs included a range of polyaromatic hydrocarbons and metals (Laks et al., 2008; Wichmann, 2007). Transition metals in the ambient particulate matter samples, especially zinc, have been shown to cause toxicity by mediating pulmonary cell reactivity and inflammation (Adamson et al., 2000; Costa and Dreher, 1997). Thus, epidemiological and experimental studies have found a correlation between exposure to nanosized particles, transition metals, Th2 cell function and allergic lung inflammation.

2.4.1 Immune recognition of nanoparticles

Inhaled NPs escape from clearance by airway ciliary mucosa, and can penetrate into the lower respiratory tract (Chang, 2010). In addition, NPs can enter our body via ingestion or epithelial exposure. NPs can activate the immune system similarly as any other foreign substances. As the symptoms from particle exposure can be both acute and chronic, the mechanisms of their action may also vary from local irritation to improper priming of the immune system. (Borchers et al., 2006). There are several properties of NPs that influence their immunological recognition. For example, size, charge and shape of a particle determine the pathway of cellular uptake. (Kunzmann et al., 2011; Scherbart et al., 2011) Phagocytes, macrophages, neutrophils, DCs and mast cells, can recognize and ingest NPs. However, phagocytosis of NPs smaller than 200 nm is less efficient than bigger particles and cellular uptake appears to happen via passive mechanisms. (Geiser et al., 2005; Peters et al., 2006) NPs also form agglomerates and complex with host proteins. Depending on the route of exposure the proteins from serum, lung or bile fluids can cover the particles. This so called “protein corona” has an effect on cellular

uptake and immunological recognition of NPs. (Lesniak et al., 2010; Yang et al., 2010) For example, the human plasma proteome is a mixture of almost two thousand proteins with varying concentrations (Farrah et al., 2011), and thus the composition of NP protein corona could be complex. However, the studies in which the proteins attached to NP have been identified have revealed that the protein corona is composed of less than 200 proteins (Lundqvist et al., 2008; Martel et al., 2011; Tenzer et al., 2011; Zhang et al., 2011). The quantitative analysis of protein corona formed on silica NPs revealed significant enrichment of proteins involved in the regulation of complement activation and blood coagulation, and belonging to the family of lipoproteins. In contrary, the relative abundance of immunoglobulins and some acute phase proteins was lower than in human plasma in general. (Tenzer et al., 2011)

2.4.2 Mechanisms of nanoparticle-mediated immune modulation

Induction of oxidative stress, disruption of Th cell balance, and adjuvant effect are believed to be the main mechanisms by which NPs modulate homeostasis of the immune system (Chang, 2010). The hierarchical oxidative stress model has been proposed to explain the defense responses activated in order to maintain cellular and eventually organism-wise homeostasis in an altered redox milieu (Xiao et al., 2003). According to the model, oxidative stress response can be broken down to three partly overlapping phases: 1) NRF2-mediated antioxidative defense reactions, 2) inflammation leading to expression of cytokines and chemokines via MAP kinase and NF-kβ pathways, and 3) cytotoxicity leading to apoptosis. NP-derived oxidative stress may be induced by reactive oxygen species (ROS) generated on the surface of NPs, metal NPs via generation of hydroxyl radicals, leakage of damaged mitochondria or release of ROS from phagocytes. (Buzea et al., 2007) In the healthy lung antioxidant reactions keep the redox state primarily reducing, in spite of the direct exposure to inhaled oxygen and the active oxidizing defense reactions needed to control the airborne microorganisms. On the contrary, enhanced oxidative reactions and decreased expression of antioxidant enzymes are reported in asthmatic patients correlating with the disease severity. (Comhair and Erzurum, 2010)

Adjuvants enhance, prolong or accelerate the immune response to the target antigen without being immunogenic themselves. They are used to boost the recipient's immune response to drugs and vaccines while the amount of foreign material can be kept in a minimum. Environmental and engineered nanosized materials have both been shown to have adjuvant activity. Ambient ultrafine particles and DEPs have been shown to prime allergic response to allergens (Diaz-Sanchez et al., 1997; Kleinman et al., 2007; Li et al., 2009), and potentiate the recall immune response (Li et al., 2010). Adjuvant effect has been associated with the oxidizing potential of inhalable material, although the antioxidant pretreatment cannot completely diminish the allergic sensitization (Li et al., 2009). NPs are being intensively studied for their use as vehicles in the development of novel vaccines. For example, poly(D,L-lacticco-glycolic acid) (PLGA) nanoparticles loaded with TLR4 and TLR7/TLR8 ligands and administered together along with the

antigen have been shown to provide efficient protection against H1N1 influenza in rhesus macaques (Kasturi et al., 2011). Similarly, NPs containing siRNA against *ID4* are promising candidates to treat ovarian cancer (Ren et al., 2012). Although NP adjuvants are being successfully developed and used in experimental setups, there isn't yet any clear-cut information regarding the connection of the physico-chemical properties and immunological potential of the particles, and the experimental parameters. However, a number of mechanisms have been connected to adjuvant activity of NPs: nanoparticles may enhance the uptake of antigen by APCs, serve as a depot for sustained release of antigen, direct the types of immune responses by changing the size of an antigen or protect antigen from degradation. (Oyewumi et al., 2010)

Exposure with DEPs and ambient particles has been shown to result in a Th2 cell bias leading to allergic reactions (Bezemer et al., 2011; Finkelman et al., 2004; Ohtani et al., 2005), but predominant Th1 cell activity and association to the regulation of autoimmunity has been also reported (Chang, 2010). The connection between ENs and priming of Th2 cell mediated immunity is less clear, and it has been criticized that the results acquired with environmental particle exposures cannot be applied to engineered nanoparticles (Fadeel et al., 2012). In addition, the earlier observations of the allergy promoting properties of ENs such as ultrafine elemental carbon particles and carbon nanotubes (Alessandrini et al., 2006; Inoue et al., 2009) have been explained by the organic or metallic impurities in the nanomaterial preparations (Hardy et al., 2012). However, despite the differences between ENs and ambient NPs, certain ENs also share characteristics with ambient particles, such as induction of inflammation via oxidative stress. This means that the knowledge gathered with ambient NPs can, to some extent, be utilized in EN research. (Li and Nel, 2011) Interestingly, glycine coated polystyrene ENs, have recently been shown to suppress allergic airway inflammation via inhibiting DC expansion after allergen challenge. Importantly, the results were not strain-specific (Hardy et al., 2012), an aspect that has been criticized in other mouse studies (Fadeel et al., 2012). In summary, the composition, and the capacity to adsorb chemicals and to produce oxidative stress were suggested to determine the inflammatory potential of an EN (Hardy et al., 2012).

The aforementioned mechanisms by which ambient and engineered nanoparticles may activate the host immune system are tightly interconnected. For example, NP-derived ROS production can modify the activity of APCs thus altering the polarization of Th cells (Chan et al., 2006; Porter et al., 2007; Sharma et al., 2008). Interestingly in the context of Th2 cells, IL4R signaling is endogenously regulated by reactive oxygen as the receptor engagement leads to generation of ROS. Inhibition of this ROS production inhibits STAT6-mediated gene expression. As activation of other cytokine receptors, such as IL3R, also leads to ROS production and as ROS are diffusible molecules, there may be several signals by which ROS-dependently modify STAT6 activity. (Sharma et al., 2008) In astrocytes, ROS have been shown to increase STAT6 target gene expression by inducing STAT6 phosphorylation (Park et al., 2012). However, in human cord blood

CD4+ Th cells oxidative stress has been reported to posttranscriptionally downregulate STAT6 expression (Tahvanainen et al., 2009). Collectively these results suggest that nanoparticles may interfere with the intrinsic cellular signaling at several stages and disrupt the balance leading to altered gene expression.

2.4.3 ZnO and TiO_2 nanoparticles

Metal oxide ENs, such as ZnO or TiO_2, are produced and used in large amounts in consumer products. In the inventory of customer products containing nanomaterials, there are 32 products containing nanosized TiO_2 produced to be used as sunscreens, UV-light protecting clothing, cosmetics, computer hardware, self-cleaning coating or cleaning products. Similarly, there are 24 customer products, mainly sunscreens, containing nanosized ZnO. (Woodrow Wilson International Center for Scholars, The Project on Emerging Nanotechnologies) In addition, TiO_2 and ZnO ENs can be found in food and food packages, respectively (Weir et al., 2012). For example TiO_2 (E171) is used in several candies and chewing gums, and more than 30% of this is nanosized (Weir et al., 2012). As, TiO_2 and ZnO ENs are already used in several consumer products, the knowledge of their impact on human health is urgently needed.

There are reports showing that ZnO ENs need to be physically in touch with the exposed cells to alter their gene expression underlining the importance of physico-chemical properties of the surface of ZnO ENs in determining their cellular response (Moos et al., 2010). However, although there is an ongoing debate on the details of ZnO EN dissolution, i.e. whether it takes place extra or intracellularly or both, it is known that ZnO ENs release Zn^{2+} ions which are cytotoxic (Buerki-Thurnherr et al., 2012; Franklin et al., 2007; George et al., 2010; Gilbert et al., 2012; Xia et al., 2008; Xia et al., 2011). The relative importance of particle and ionic effects might depend of the composition of ZnO ENs and the experimental conditions (Cho et al., 2012; Sohaebuddin et al., 2010).

Titanium dioxide has three main mineral forms in nature: rutile, anatase and brookite. TiO_2 cannot pass undamaged skin, and even when inhaled or ingested TiO_2 is not thought to have serious effects on humans. Bulk size TiO_2 is considered as physiologically inert based on the epidemiological studies. (Iavicoli et al., 2011) However, in rats high doses of inhaled nanosized TiO_2 is reported to cause cancer (Heinrich et al., 1995), due to which the compound has been classified as possibly carcinogenic to humans (International Agency for Research on Cancer, 2010) and putative occupational carcinogen (National Institute for Occupational Safety and Health, 2011). Nevertheless epidemiological studies have not found conclusive evidence for increased risk for cancer among the workers exposed to TiO_2 dust (Fryzek et al., 2003; Ramanakumar et al., 2008). However, additional systematic risk assessment of nanosized TiO_2 has been requested for example to evaluate the risks of occupational TiO_2 exposure (Iavicoli et al., 2011; National Institute for Occupational Safety and Health, 2011). Mechanistically the malignant potential of TiO_2 has been explained to be derived from lung overload after an exposure with a higher dose of TiO_2 than the lung clearance processes can manage leading to

destructive inflammation being the causal reason for genotoxicity (ILSI Risk Science Institute Workshop Participants, 2000).

2.4.3.1 ZnO in regulation of pulmonary inflammation

Elevated zinc concentration in ambient air has been epidemiologically linked to the increased morbidity of asthmatic children (Hirshon et al., 2008). Inhalation of ZnO-containing fume during the welding of galvanized metal can cause metal fume fever a syndrome characterized by muscle pain, weakness, shaking chills, fever and irritation of nose and throat. The symptoms disappear in a few days. Repeated exposures to zinc fumes cause a short term tolerance to this disease, but the symptoms return upon new exposure. (Swiller and Swiller, 1957) A high zinc level in blood is associated with deficiencies of copper and calcium, and metal fume fever among the exposed galvanization workers (El Safty et al., 2008). Cases of work-related asthma, originating from the exposure to zinc when working with galvanized metal, has also been reported (Malo and Cartier, 1987; Malo et al., 1993; Weir et al., 1989). In rats, intratracheal instillation of ZnO ENs has been shown to cause reversible neutrophilic lung inflammation (Ho et al., 2011; Sayes et al., 2007). Other studies have confirmed the acute neutrophilic inflammation in response to *in vivo* ZnO EN exposure, but in addition reported eosinophilia associated with increased expression IL13 and EOTAXIN (Cho et al., 2010; Cho et al., 2011). Eosinophilia was reported to be caused by the exposure to well-dispersed ZnO ENs, as the agglomerated ZnO particles recruited significantly less eosinophils (Cho et al., 2011). Lung histological findings after ZnO EN instillation show fibrosis, massive epithelial cell proliferation and goblet cell hyperplasia. Besides, serum IgE levels were increased in response to a single high dose exposure with the ZnO ENs. (Cho et al., 2011) At the mechanistic level, zinc ions released from ZnO ENs cause oxidative stress via radical formation, which activates macrophages and neutrophils in the lung. Subsequently these phagocytes generate ROS that further enhance the oxidative stress. (Fukui et al., 2012) Intriguingly, although zinc exposure causes acute lung inflammation, systemic zinc supplementation reduced airway reactivity and serum IgE levels in a murine model of experimental allergic airway inflammation (Morgan et al., 2011). In human, there is also evidence suggestive of the protective role of zinc for asthma (Nurmatov et al., 2011). However, it needs to be highlighted that zinc is an essential trace element, the concentration of which is strictly controlled, and both excess and deficiency can cause negative effects (Chasapis et al., 2012; Feske et al., 2012).

2.4.3.2 TiO_2 in regulation of pulmonary inflammation

Workers in the TiO_2 industry haven been reported to have reduced lung function (Daum et al., 1977; Garabrant et al., 1987). In addition, one case report describes a metal fume fever after an accidental inhalation of TiO_2 (Otani et al., 2008). Human H441 Clara-like cell line has been reported to have a defect in adhering to fibronectin in extracellular matrix after an hour of exposure to 100 μg/ml anatase TiO_2 ENs indicating that even a short exposure may lead to structural alterations in bronchial epithelia (Zarogiannis et al., 2012). Chen et al. reported that the human bronchial epithelial cell line ChaGo-K1

secretes mucin in response to exposure to a mixture of anatase and rutile nanosized TiO_2 (Chen et al., 2011). In contrast to the relatively scarce human studies, there are several reports on experimental animals showing that nanosized TiO_2 exposure can lead to the development of asthma and other symptoms of airway inflammation. In the analysis of chronic lung toxicity, mice were exposed to TiO_2 ENs for 90 consecutive days via intratracheal instillation. The exposure generated dose-dependent oxidative stress caused by compromised antioxidant capacity, and increased the expression of various cytokines such as IL4. (Sun et al., 2012) In the diisocyanate-induced mice asthma model, anatase TiO_2 ENs have been reported to increase airway reactivity, macrophage and neutrophil count in bronchoalveolar lavage (BAL) and macrophage infiltration into the lung. In this study the EN exposure was made to sensitized mice a day before the challenge. Importantly, the authors state that the dose of TiO_2 used, was not unrealistically high compared to the putative occupational exposure of workers in TiO_2 industry. (Hussain et al., 2011) In a different experimental setup, administration of TiO_2 along with OVA allergen led to a higher specific IgE levels than the exposure with $Al(OH)_3$ indicating the high Th2 cell dominant adjuvant potential of TiO_2 ENs (Larsen et al., 2010). Interestingly, pulmonary exposure of pregnant mice to respirable-sized TiO_2 has been shown to cause neutrophilic lung inflammation. In addition, a risk of asthma susceptibility among the offspring of the exposed mothers was increased. (Fedulov et al., 2008)

However, there are also opposing studies showing that nanosized TiO_2 exposure inhibits features of allergic asthma (Rossi et al., 2010; Scarino et al., 2012) First, it was reported that continuous exposure with nanosized TiO_2 particles reduce several parameters of allergic inflammation, such as leucocytes count in BAL, number of mucus secreting goblet cells, and the expression Th2 cytokines in the lungs of asthmatic mice. TiO_2 ENs caused not only a reduction of inflammation in lung, but also a systemic immune suppression as the level of OVA-specific IgE and cytokine expression by OVA-stimulated spleen cells was also reduced. Interestingly, at the same time asthmatic mice exposed with TiO_2 ENs were reported to have reduced *foxp3* and *IL10* expression in the lung, indicating that the effects of TiO_2 ENs were not due to an increased activity of suppressive Treg cells. (Rossi et al., 2010) Importantly, the same research group had earlier reported that when healthy mice inhaled the same silica coated TiO_2 ENs pulmonary neutrophilia was induced (Rossi et al., 2010). However, the authors underline that it should be noted that the TiO_2 ENs used were endotoxin-free, which is rarely the case in real life exposure, and the results cannot be generalized to non-allergic asthma as the study concentrated on Th2 cell associated parameters (Rossi et al., 2010). The protective role of TiO_2 nanoparticles on pulmonary inflammation was also reported in an independent study with rats. The study showed a decreased number of leucocytes and eosinophils in BAL accompanied with reduced levels of IL4, IL6 and IFNγ in plasma of the OVA-challenged asthmatic rats when exposed to nanosized TiO_2. (Scarino et al., 2012)

The data gathered with TiO_2 exposures of asthmatic vs. healthy (Rossi et al., 2010) and pregnant vs. non-pregnant mice (Fedulov et al., 2008) suggest that the outcome

of exposure to TiO_2 is dependent on the immunological status at the time of exposure. The opposite functions of TiO_2 in modulation of lung inflammation has also been explained by the different timing of exposure; an exposure before challenge promotes inflammation (Hussain et al., 2011; Larsen et al., 2010) and an exposure after a challenge suppresses inflammation (Rossi et al., 2010; Scarino et al., 2012). It has also been proposed that TiO_2 can form bigger agglomerates when administered via aerosol inhalation than when ENs are introduced into the lungs via instillation. This may have an effect on phagocytosis efficiency of macrophages, and also on local TiO_2 concentration in lung. (Scarino et al., 2012) The shape of TiO_2 ENs has also been reported to affect the clearance of particles from the lung. Rat alveolar macrophages were compromised in clearing the TiO_2 nanobelts compared to spherical TiO_2 ENs from the lung, which caused pulmonary inflammation. (Porter et al., 2012) However, in the study in which the TiO_2 ENs were much more comparable in size and shape, neither the surface area, primary or aggregate size nor the capacity to form radicals explained their inflammatory potential. Instead, the surface coating, in this case silica, led to lung inflammation characterized by accumulation of neutrophils. In the same study, it was also reported that toxicity of the TiO_2 ENs correlated with their location in the alveolar macrophages; the inert nanoparticles were entrapped in the phagosomes and inflammatory silica-coated ENs were free in the cytosol. (Rossi et al., 2010) Interestingly, a recent study gives evidence for a variable TiO_2 EN toxicity related to subject's age; young mice were shown to be more susceptible for the oral toxicity compared to the adult rats (Wang et al., 2012). Similarly, variable sensitivity towards oral toxicity was reported between male and female mice, female being more sensitive (Wang et al., 2007). These studies add yet more aspects to the list of parameters which may play a role in determining the outcome of TiO_2 exposure. In addition to *in vivo* experiments on TiO_2 toxicity, several *in vitro* evaluations have been performed. However, it is difficult to directly extrapolate the observed effects acquired with cell cultures containing only one cell type to an exposure via inhalation in a dose and a duration-wise realistic setting relevant, for example, to occupational safety. (Iavicoli et al., 2011)

3. AIMS OF THE STUDY

The overall goal of this PhD thesis was to identify molecules and signaling pathways involved in the regulation of allergic inflammation in the cells of the immune system. The main emphasis was put on the characterization of IL4 and STAT6-regulated molecules. In addition, the transcriptional response to ZnO and TiO_2 EN exposure was investigated.

The specific goals of the subprojects included into the PhD thesis were to:

1. Identify STAT6-regulated proteins in mouse lymphocytes by using proteomics approach. (I)

2. Characterize the role of STAT6 in human IL4-initiated Th2 cell polarization by identifying its primary and secondary target genes. (II)

3. Profile the gene expression changes derived from ZnO or TiO_2 nanoparticle exposure in immunological *in vitro* model systems. (III)

4. MATERIALS AND METHODS

4.1 Mouse cell isolation and culturing (I)

Stat6-deficient and control wild type Balb/cJ mice were from the Jackson Laboratory. Mice were bred according to the Federation of European Laboratory Animal Science Associations (FELASA) recommendations in the Central Animal Laboratory of the University of Turku. The permission for conduction the experiments was given by the Lab-Animal Care and Use Committee of Turku University. Mononuclear splenocytes were collected with Mouse Lympholyte (Cedarlane). The remaining erythrocytes were lysed with 0.83% NH_4Cl before the cells were cultured for analysis with two-dimensional electrophoresis (2-DE). CD4+ T cells were isolated with positive selection with MACS beads (Miltenyi Biotech).

Cells were activated with plate-bound CD3 antibody (clone 500A2) coated on 24-well culture plates (325 ng/well). Unattached antibody was washed away before use. Cells were cultured in RPMI (Sigma) supplemented with 5% heat inactivated FCS, 1 mM Na-pyruvate (Sigma), 2 mM L-glutamine (Sigma), 50 mM β-mercaptoethanol (β-ME) (Invitrogen), 1X non-essential amino acids (Invitrogen), and penicillin/streptomycin (Sigma). The cells were stimulated with 10 ng/ml of IL4. Metabolic labeling was achieved by adding Redievue Pro-Mix L ^{35}S-methionine and ^{35}S-cysteine (50 mCi/ml, Amersham Pharmacia Biotech) to the supplemented methionine-free RPMI (Sigma). For activation of CD4+ cells, CD28 antibody was included in the culturing medium (500 ng/ml). IL4-induced polarization was enhanced with neutralizing antibodies: 10 mg/ml of anti-IL12 and anti-IFNγ. In addition, 10 mg/ml of anti-IL4 was added to samples not treated with IL4. All stimulating and neutralizing antibodies were from Pharmingen. The cells were cultured in a density of $5x10^6$ cells/500 µl on 24-well plates for 24 h at 37 °C with constant 5% CO_2 concentration.

4.2 Human cell isolation and culturing (II, III)

Umbilical cord blood was from healthy neonates born in Turku University Hospital, Hospital District of Southwest Finland. The usage of blood of unknown donors was approved by the Finnish Ethics Committee. CD4+ T cells were isolated with magnetic beads (Dynal CD4 Positive Isolation Kit, Invitrogen) from Ficoll-Paque (Amersham Biosciences) enriched mononuclear cells. Cells were activated with plate-bound CD3 (500 ng/24-well culture plate well, Immunotech) and soluble CD28 (500 ng/ml, Immunotech) antibodies, and cultured at a density of $2\text{-}4x10^6$ cells/ml in Yssel´s medium (Iscove's Modified Dulbecco's Medium supplemented with Yssel medium concentrate (Yssel et al., 1984), 1% human AB serum (PAA) and 100 U/ml Penicillin and 100 µg/ml Streptomycin (Sigma) at 37°C in 5% CO_2. Cells were stimulated with IL4 (10 ng/ml)

with or without neutralizing IL12 (10 μg/ml). After two days of culture, IL2 was added (17 ng/ml) to all the cells. All cytokines were from R&D Systems. Thereafter cells were fed and divided every other day maintaining the polarizing conditions throughout the culture. All the cell cultures consisted of pooled cells from several neonates.

Jurkat A3 (ATCC, CRL-2570) cells were cultured in RPMI 1640 culture medium (Sigma) supplemented with 10% FCS, 2 mM L-glutamine (Gibco) and 1% penicillin-streptomycin-neomycin (Gibco). The detailed protocol for generation of the human monocyte-derived macrophages (HMDM) and monocyte-derived dendritic cells (MDDC) and the controlling of the phenotypes have been previously described (Kunzmann et al., 2011). Briefly, peripheral blood mononuclear cells were prepared from buffy coats obtained from healthy blood donors (Karolinska University Hospital, Stockholm, Sweden) by density gradient centrifugation using Lymphoprep (Axis-Shield) or Ficoll-Pacque PLUS (GE Healthcare Bio-Science AB) for HMDM or MDDC, respectively, and positively selected for CD14 expression (CD14 MicroBeads, Miltenyi Biotec). To obtain HMDM, CD14+ monocytes were cultured in supplemented RPMI 1640 medium (Sigma) with 50 ng/ml recombinant M-CSF (Novakemi) for three days. To obtain immature MDDC, CD14+ monocytes were cultured at a density of 4×10^5 cells/ml at 37 °C in a humidified atmosphere containing 6% CO_2 in supplemented RPMI 1640 medium (Sigma Aldrich) with IL4 (800 IU/ml) and GM-CSF (550 IU/ml) (Biosource International). After three days, half of the culture medium was exchanged with fresh medium containing the cytokines. After six days, the cells were collected for nanoparticle exposure.

4.3 Two-dimensional electrophoresis and protein detection (I)

Cells were lysed and proteins separated with 2-DE as previously described (Nyman et al., 2001). Briefly, soluble proteins were absorbed into the 18 cm 3–10 NL IPG-strips (Amersham Pharmacia Biotech) for 24 h at room temperature (RT). Isoelectric focusing was carried out at 20 °C, and the focused strips were equilibrated for 25 min at RT. Subsequently, the proteins were separated with vertical 12% SDS-PAGE gel with thickness of 1 mm. Proteins were detected with silver staining (O'Connell and Stults, 1997) and autoradiography (24 h) (Johnston et al., 1990). The gels were dried with GelAir Drying System (Bio-Rad). Gel image normalization and comparison was made with the PDQuest program (version 6.2, BioRad).

4.4 Protein identification (I)

The proteins differentially expressed between IL4-treated *Stat6*$^{-/-}$ and wild type cells, were in-gel digested, and the resulting peptides analyzed by nanoLC-MS/MS as previously described (Nyman et al., 2000; Rosenfeld et al., 1992; Rosengren et al., 2005; Shevchenko et al., 1996). Briefly, the excised gel spot was cut into pieces, washed, and dehydrated with acetonitrile (ACN). Proteins were reduced with 20 mM dithioreitol

(DTT) (Sigma), alkylated with 55 mM iodoacetamide (Sigma), and in-gel digested with trypsin (Sequencing Grade Modified Trypsin, Promega) at 37 °C overnight. Peptides were extracted with 5% formic acid, 50% ACN, and dried with a vacuum centrifuge. Peptides were dissolved to 2% HCOOH prior to MS analysis. Analyses were made with QStar Pulsar ESI-hybrid quadrupole-TOF instrument (Applied Biosystems/MDS Sciex) coupled on-line with nanoHPLC (Famos, SwitchosII and Ultimate, LC Packings) as previously described (Rosengren et al., 2005). Peak lists from the MS/MS spectra were created with Analyst QS program (version 1.1, Applied Biosystems) using the MASCOT. dll script. These spectra were analyzed using MASCOT software (www.matrixscience. com, version 02.01.02) via MASCOT Daemon 2.1.6 software (Matrix Science) and searched against Swiss-Prot (version 51.5) and TrEMBL databases (version 34.5).

4.5 Western blotting (I, II)

Mouse samples (whole cells and insoluble protein pellet fractions) were boiled with 62.5 mM Tris-HCl (pH 6.8), 2% w/v sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue. Cytosolic and nuclear protein fractions were boiled with 5xSDS sample buffer. The following antibody dilutions were used for Western blots: mouse anti-CBFβ 1:250 (#610514, BD Transduction Laboratories), rabbit CNBP antiserum 1:500, and anti-β-actin 1:10 000 (#A-5441, Sigma). CNBP antiserum was a generous gift from Dr. Paola Pierandrei-Amaldi. Secondary antibodies used in this study were 1:10 000 HRP goat anti-mouse (#sc-2005, Santa Cruz Biotechnology), 1:5000 HRP goat anti-rabbit (#554021, BD Pharmingen).

Human cell samples were first boiled in Triton-X sample buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% Triton-X-100; 5% glycerol; 1% SDS), containing proteinase and phosphatase inhibitors (Roche). After adding 6x loading dye (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β-ME; 170 μM bromophenol blue; 30% glycerol), samples were loaded on 10-12% SDS-PAGE gels, transferred to NC membranes, and probed with antibodies: STAT6 (#611291, BD Biosciences), pSTAT6(Tyr641) (#9361, Cell Signaling Technology), GATA3 (#558686, BD Pharmingen), and GAPDH (#5G4, 6C5, HyTest). For detection and quantitation with infrared imaging system (Odyssey, LI-COR Biotechnology), the blocking and antibody hybridizations were performed in Odyssey blocking buffer (#927-40000, LI-COR), using Goat-anti-Rabbit IRDye 800CW (#926-32211, LI-COR) and Goat-anti-Mouse IRDye680 (#926-32220, LI-COR) as secondary antibodies. The blots were scanned and quantified using the Odyssey Application Software (LI-COR, version 1.2.15).

4.6 Nuclear fractionation and RUNX1 DNA binding activity (I)

Cells were lysed with a hypotonic lysis buffer (0.2% Tween, 1 mM NaF,1 mM Na_3VO_3, 1 mM DTT and complete protease inhibitor cocktail (Roche Diagnostics) in 22 mM Hepes, pH 7.9) for 2 min on ice, the nuclear fraction was spun down, and cytosolic

fraction collected. Nuclei were incubated in the extraction buffer (1.0% glycerol, 430 mM NaCl, 1 mM NaF, 1 mM Na_3VO_3, 1 mM DTT and complete protease inhibitor cocktail in 22 mM HEPES, pH 7.9) for 1 h on ice. Extracted nuclear proteins were collected after the cell debris was spun down. Alternatively, after removal of the cytosolic fraction and incubation in the nuclear extraction buffer, the nuclear protein pool was sonicated with the cell debris, samples centrifuged at 4 °C and supernatants collected. Runx1 DNA binding activity was measured with TransAM AML-1/Runx1 kit (#47396, Active Motif).

4.7 Confocal microscopy (I)

0.1-0.5x10^6 cells/sample were fixed with 3.65% formaldehyde for 15 min at RT and permeabilized with 0.2% saponin or 0.1% Triton X-100. Unspecific binding was blocked with 10% bovine serum albumin (BSA). Primary antibody incubations were made in 1.0% BSA/permeabilization buffer overnight at 4 °C and secondary antibody incubations in the same buffer 45 min at RT. The antibodies used were: CBFβ (#610514, 1:25, BD) and Runx1 (#39000, 1:100, Active Motif), goatF(ab')2 anti-mouse IgG (H + L), FITC conjugate (#M35001, 1:200, Caltag), and goat anti-rabbit Alexa 568 nm (1:400, Molecular Probes). Nuclei were stained separately with 4',6-diamidino-2-phenylindole (dapi) or by using dapi-containing mounting medium (Slow-Fade #S36938, Molecular Probes). A Zeiss LSM510 META laser scanning confocal microscope was used for image acquisition.

4.8 RNAi-mediated STAT6 knockdown (II)

siRNAs (1.5 µg/4x10^6 cells, Sigma) were introduced into the freshly isolated CD4+ T cells with Nucleofector (program U-14, Amaxa Biosystems). Three replicate cultures were produced, using a different *STAT6*-siRNA in each: #1 (5´-AAGCAGGAAGAACTGAAGTTT-3`), #2 (5`- GAATCAGTCAACGTGTTGTCA-3`) or #3 (5'-CAGTTCCGCCACTTGCCAAT-3'). The same non-targeting control-siRNA (5´-GCGCGCTTTGTAGGATTCG-3´) was used in every culture. After nucleofection the cells were rested for 24 h in RPMI (Sigma) supplemented with 10% heat inactivated FBS (PromoCell), 2 mM L-Glutamine (Sigma) and 50 U/ml Penicillin and 50 µg/ml Streptomycin (Sigma) at 37 °C in 5% CO_2. Thereafter, cell activation and culturing were performed as described above.

4.9 Microarray sample preparation for identification IL4 and STAT6-regulated genes (II)

For transcriptional profiling of IL4 targets, the samples were harvested at 0.5, 1, 2, 4, 6, 12, 24, 48 and 72 h. Cells without any treatments were collected as 0 h controls. For STAT6 target gene identification, cells were harvested at 12, 24, 48 or 72 h time point. Total RNA was extracted (RNeasy Mini Kit, Qiagen) and treated in-column with DNase (RNase-Free Dnase Set, Qiagen) for 15 min.

For the identification of IL4 target genes, the amplification was started from 100 ng of total RNA using the Affymetrix Two-Cycle cDNA Synthesis Kit (P/N 900432), and cDNA/cRNA synthesis reactions and sample cleanup steps were performed according to Affymetrix´s GeneChip Expression Analysis Technical Manual. Biotinylated and fragmented cRNA (15 μg) was hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays overnight (16-18 h) at 45 ºC. GeneChips were washed and stained in the Affymetrix Fluidics Station. GeneChips were scanned using the GeneChip Scanner 3000 with AutoLoader.

Amplification of RNAi study samples was started from 100 ng total RNA using an Illumina RNA TotalPrep Amplification kit (#AMIL1791, Ambion). 1.5 μg of amplified and labeled samples was hybridized to Sentrix HumanWG-6 Expression BeadChips, version 2 (#BD-25-112, Illumina) at 55 ºC overnight according to Illumina Whole-Genome Gene Expression Direct Hybridization protocol, revision A. Hybridization was detected with 1 μg/ml Cyanine3-streptavidine (Amersham Biosciences). Chips were scanned with Illumina BeadArray Reader and numerical results extracted with GenomeStudio v1.0 without any normalization.

4.10 Identification of IL4 and STAT6-regulated genes (II)

The IL4 and STAT6-regulated genes were identified from the quantile-normalized Illumina microarray data. IL4-regulated genes were first identified by determining significant expression changes between the matched Th2- and Th0-measurements. The effect of STAT6 knockdown on the IL4-regulated genes was then assessed using the statistic Th2/Th0 - sTh2/sTh0, where Th2/Th0 and sTh2/sTh0 denote the signal log-ratios between the matched Th2- and Th0-measurements in the control and knockdown data, respectively. Consistent regulation was identified using the moderated F- and t-statistics.

The kinetics of IL4-regulated genes in non-disturbed CD4+ cells was identified with quantile-normalized Affymetrix microarray data. The probe-level expression change averaging procedure PECA (Elo et al., 2005) was applied together with linear modeling (Bioconductor limma package). The probe-level estimates of the moderated F- and t-statistics were summarized into probeset-level values using the Tukey biweight average and the significance of an expression change was determined based on the analytical p-value of the estimated probeset-level statistic.

4.11 Flow cytometry (II)

CRTH2-PE staining (#130-091-238, Miltenyi Biotech) was performed after one week of polarization and analyzed with a LSR II flow cytometer (BD Biosciences) and CyflogicTM software (CyFlo Ltd). CRTH2 expressing cells were analyzed from the viable cell population, determined based on the forward (FSC) and side scattering (SSC).

4.12 STAT6 ChIP-seq (II)

CD4+ T cells were cultured in Th0 or Th2 polarizing conditions for 1 and 4 h and freshly isolated Thp cells were used as a control. ChIP was performed as described previously (Li et al., 2003). The cells were sonicated using a Bioruptor sonicator (Diagenode) to obtain chromatin fragments of 100-500 bp. 500 µg of sonicated chromatin was incubated with 10 µg of STAT6 antibody (#M-20, Santa Cruz Biotechnology) coupled to the magnetic beads (#112.04, Dynal Biotech). The cross-links were reversed (65 °C for 12 h) and DNA was treated sequentially with Proteinase K and RNase A, and purified (QIAquick PCR purification kit, Qiagen).

The library preparation was performed according to the Illumina recommendations by Fasteris Life Sciences, Switzerland. Sequencing was performed with an Illumina Genome Analyzer GAII producing from 4 to 5.2 million reads per sample. The reads were aligned to the human reference genome (NCBI v36) using the SOAP software (Li et al., 2008). The binding regions were identified with FindPeaks software (version 3.1.9.2) (Fejes et al., 2008). Only the Th2 cell-specific peaks were considered.

4.13 Quantitative PCR analysis (II, III)

Quantitative PCR (qPCR) reactions were performed using Universal ProbeLibrary probes (Roche Applied Science) and custom ordered oligos designed with Universal ProbeLibrary Assay Design Centre (Roche) or with FAM (reporter), TAMRA (quencher) double labeled probes in 10 µl reaction volume. The reaction mix used was Absolute QPCR ROX Mix (Thermo Scientific) and amplification was monitored with Applied Biosystems 7900HT Fast Real-Time PCR System (15 min enzyme activation and 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC).

In ChIP-qPCR the percent of input values were calculated with the equation: 100*2^(Input - Ct (ChIP)), in which Input value was adjusted to 100%. GATA3 and IFNγ regions were used as negative controls. The primers and probes are listed in **Table 2**.

For qRT-PCR validation of the ZnO-1 responsive genes, RNA was treated with DNase I Amplification Grade (Invitrogen). cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche). The primers and probes are listed in the **Table 3.** The fold changes of the transcripts were calculated using the equations: $\Delta Ct=(CtGene-CtEF1\alpha)$, $\Delta(\Delta Ct)=(\Delta Ct(EN)-\Delta Ct(Ctr))$ and fold change=$2^{|\Delta(\Delta Ct)|}$. In the equations, the Ct is a cycle threshold value at which the RT-PCR signal exceeds the detection threshold. The Ct-values of the transcripts studied were normalized against the signals acquired with *EF1*α (Hämäläinen et al., 2001). If the expression of a gene was undetectable in the untreated control samples, the Ct-value 35 was given to it before fold change calculations.

Table 2. Primers and probes for qPCR for STAT6 ChIP-seq hits (II).

Target gene	Primers, 5'-sequence-3'	Roche Universal ProbeLibrary probe
AMICA	CCCTCTGTTCCCCATACAGAC ACAAACACGTCAACAGAAAACC	66
BACH2	CCTGTGCTCATCTCTACATCAAA TGGAAATACAGGCAGCAATTT	6
BCL6	CGATGGTATGGCCTGCTC GGCCGAGCTTTGCTACAG	63
CLEC16A	CATTTGAGCTACATTAGAAACTACACG CCCGAATATGCCTTCTCTCA	34
NFATC3	CCTTTTTCCAGGCTCCATT GGGGAGATGTGGGAAATACA	52
RUNX1	ATTTTTCGACAAACAGGATGC CTTTCAAAGAGCCTGGGATG	90
SLC39A8	GCCGCAAACTTAGAAGTGAGA CCAGGACTCCGGAAGAGC	17
SPINT2	GCCTTCGCTCTAGGACTGG AACCACAGGCTTCCAAGAAA	52
SYTL3	TGAGTGCGTGTGAATGTGAC CTTGTGGGCTTTGAGAACG	9
TMEM71	ACACTTCCTGTTGGGCTCAC GCCCAGGAAACTCTCTGAAAT	59
Control gene	**Primers, 5'-sequence-3'**	**6(FAM)-probe-(TAMRA)-3**
GATA3	GGACGCGGCGCAGTAC TGCCTTGACCGTCGATGTTA	TGCCGGAGGAGGTGGATGTGCT
IFNγ	TGTCCAACGCAAAGCAATACA CTCGAAACAGCATCTGACTCCTT	TGCTGGCGACAGTTCAGCCATCAC

Table 3. qRT-PCR reagents for validation of ZnO-1 target genes (III).

Target gene	Primers, 5'-sequence-3'	Roche Universal ProbeLibrary probe
MT1P3	ACCTCCTGCAAGAAGAGCTG GCCCCTTTGCAGACACAG	18
MT1E	AAAGGGGCATCGGAGAAGT AAATCCAGGTTGTGCAGGTT	7
MT1F	GACTGATGCCAGGACAACC TCCAGGTTTGTACATGTCTCTCTG	14
MT1G	AAAGGGGCATCGGAGAAGT AATCCTAGATTTTACGGGTCACTC	32
MT1H	TGGGAACTCCAGTCTCACCT CATTTGCACTTTTTGCACTTG	68
MT1X	CTTCTCCTTGCCTCGAAATG ACAGGCACAGGAGCCAAC	15
MT1A	TGGGATCTCCAACCTCACC ATTTGCAGGAGCCAGTGC	68
Control gene	**Primers, 5'-sequence-3'**	**6(FAM)-probe-(TAMRA)-3**
EF1α	CTGAACCATCCAGGCCAAAT GCCGTGTGGCAATCCAAT	AGCGCCGGCTATGCCCCTG

4.14 Data annotation and pathway analysis (II, III)

Ingenuity pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com) was used in annotation of the microarray expression data. Annotations for the IL4 and STAT6 target gene study were exported in June 2009 and for ZnO/TiO_2 study in February 2012). Ingenuity's Knowledge Base annotation was also used in categorization of the genes into functional classes and specification of cellular location. Hypergeometric testing was used to analyze the statistical significance of the enrichments.

4.15 Nanoparticles (III)

ZnO-1 (IBU-tec advanced materials AG) was compared to ZnO-2 (mandelic acid modified), ZnO-3 (mercaptopropyl-trimethoxysilane modified), ZnO-4 (methoxyl modified), ZnO-5 (diethylene glycol modified), ZnO-6 (mandelic acid modified), ZnO-7 (gluconic acid modified), ZnO-8 (citric acid modified) and ZnO-9 (folic acid modified). Commercial TiO_2 was from Evonik Degussa (Aeroxide® TiO2 p25). The synthesis of the ENs is either described in the manuscript (III, Supplementary data and methods) or published earlier (Buerki-Thurnherr et al., 2012).

4.16 Nanoparticle characterization (III)

Transmission electron microscopy (TEM) investigations were carried out on a LEO 912 Omega (Zeiss). Zeta potentials and hydrodynamic light scattering (DLS) of the nanoparticle dispersions were measured using a Zetasizer Nano ZS (Malvern Instruments). For the measurements, the NP suspensions (1 mg/ml) were prepared in deionised water or RPMI-medium and were sonicated for 3 min using an ultrasonic probe (70W, 20 kHz, Ultrasonic Homogenizer Bandelin Sonopuls UV 70). For DLS experiments, stock suspensions were further diluted to 100 ng/ml of ZnO in H_2O or RPMI-medium and immediately measured at 25 °C. X-ray diffraction (XRD) patterns of the ENs were measured with a STOE-STADI MP vertical system in transmission mode using Cu Kα (α=0.15406 nm) radiation. Fourier transform infrared (FT-IR) analyses were carried out with a PerkinElmer Spectrum 400 with Universal ATR Sampling Accessory in the 400-4000 cm-1 range. Thermogravimetric analysis (TGA) of the obtained particles was performed at a temperature range from 30 °C to 800 °C with a heating rate of 10 °C/min under nitrogen atmosphere (flow rate 25 ml/min) using a Mettler Toledo TGA/DSC 1 Stare system.

4.17 Analysis of ZnO dissolution (III)

The ENs were introduced into the cell culturing medium at a concentration of 10 µg/ml and ultrasonicated for 5 min (70 W, 20 kHz, Ultrasonic Homogenizer Bandelin Sonopuls UV 70). The mixtures were kept in an oil bath at 37 °C in capped centrifuge tubes with

constant stirring for 30 min, 6 h or 24 h. For the removal of the solid particles, the tubes were centrifuged altogether three times at 11000 rpm for 10 min with Eppendorf-Centrifuge 5804. After the separation, the supernatant was carefully transferred into another tube for two consecutive centrifugations to ascertain the purity of the soluble fraction. An iCE 3500 Atomic Absorption Spectrometer (Thermo Fisher Scientific) was used to determine the amount of dissoluted zinc.

4.18 Exposure of HMDM, MDDC and Jurkat cells to nanoparticles (III)

HMDM and Jurkat cells were seeded in 24-well plates at a density of $1x10^6$ cells/well and $1.5x\ 10^5$ cells/well, respectively, in a final volume of 1 ml. Immature MDDC were seeded in 6-well plates at a density of $4x10^5$ cells/ml in a final volume of 3 ml. Nanoparticle exposures were conducted in the culturing mediums without any cytokines. Nanoparticles were dispersed by sonication in ultra-pure water to a stock solution of 1 mg/ml and added to the cells. The nanoparticles were controlled for lipopolysaccharide (LPS) contamination (Vallhov et al., 2006) by using the chromogenic LAL test method (Limulus Amebocyte Lysate Endochrome, Charles River Endosafe) according to the manufacturer's instructions.

4.19 Analysis of cell viability (III)

HMDM cell viability was determined using 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich). Monocytes were seeded into a 96-well plate and differentiated to HMDM as described previously (Kunzmann et al., 2011). After exposure, the supernatant was removed, cells washed and 100 µl of MTT solution (0.5 mg/ml) added. After 3 h, 50 µl of dimethyl sulfoxide (DMSO) (Sigma Aldrich) was added to dissolve the formazan crystals. MTT conversion was quantified by measuring the absorbance at 570 nm using a spectrophotometer (Infinite F200, Tecan). Cell viability of MDDC and Jurkat cells was analyzed with PI/Annexin V staining as previously described (Andersson-Willman et al., 2012; Buerki-Thurnherr et al., 2012).

4.20 Sample preparation of ZnO or TiO_2 treated cells (III)

Cells were collected in RNAlater buffer (Ambion) and total RNA isolated with RNAqueous Small Scale Phenol-Free Total RNA Isolation Kit (Ambion).

For Illumina microarray analysis 300 ng of RNA was amplified with Illumina RNA TotalPrep Amplification kit (Ambion). *In vitro* transcription, during which cRNA was biotinylated, was carried out for 16 h. Labeled cRNA (0.75 µg) was hybridized to Illumina's Sentrix HumanHT-12 Expression BeadChips, version 3 (#BD-103-0603) at 58 °C for 18 h according to Illumina Whole-Genome Gene Expression Direct Hybridization protocol, revision A. Hybridization was detected with 1 µg/ml Cy3-Streptavidin

(GE Healthcare). The arrays were scanned with Illumina BeadArray Reader, BeadScan software version 3.5 and the numerical results extracted with GenomeStudio 2009.2 without any normalization.

For Affymetrix analysis 250 ng of total RNA was processed with GeneChip 3' IVT Express Kit (#901229) and hybridized to GeneChip Human Genome U219 array plate with specific protocols for using the GeneTitan Hybridization, Wash and Stain Kit for 3' IVT Array Plates (#901530). GeneTitan Instrument was used to hybridize, wash, stain and scan the arrays. Affymetrix GeneChip Command Console 3.1 was used to control the process and to summarize probe cell intensity data. Hybridization quality was checked with Affymetrix GeneChip Command Console and Expression ConsoleTM 1.1s.

4.21 Identification of genes regulated in response to ZnO or TiO_2 exposure (III)

The data from the samples measured with Illumina's Sentrix HumanHT-12 Expression BeadChips were analyzed using R and the Bioconductor Lumi package (Du et al., 2008). The raw data values were pre-processed with the Variance Stabilizing Transform (VST) of Illumina data (Lin et al., 2008). Further, the values were normalized between-chip using a quantile normalization method (Bolstad et al., 2003). Samples analyzed with the Affymetrix platform were preprocessed using the Robust Multi-array Average (RMA) algorithm using the R package affy (Gautier et al., 2004). The probe values were linked directly into the ENSEMBL genes with Brainarray CDF-files Version 14 (Dai et al., 2005). To ensure the independent analyses of EN-specific gene expression changes in MDDC, HMDM, and Jurkat cells, the different cell types were preprocessed separately. In contrast, to compare the untreated 6 h samples, all the samples from each cell type were preprocessed together from the raw values. Differentially expressed genes were detected with the limma package (Smyth et al., 2005) of R using empirical Bayes moderated t-test and the p-values were adjusted with the FDR method (Smyth, 2004). The limma analyses were performed as paired analysis.

5. RESULTS AND DISCUSSION

5.1. Identification of STAT6-regulated proteins in mouse lymphocytes (I)

5.1.1 STAT6-dependent protein expression

A proteomics approach was selected to complement the studies on the effects of *Stat6* knock-out done at transcriptional level (Chen et al., 2003). Mononuclear cells from spleens of *Stat6*$^{-/-}$ and wild type control Balb/cJ mice were isolated with gradient centrifugation. Cells were activated with plate-bound anti-CD3 antibody and stimulated with IL4 for 24 h, during which they were metabolically labeled with radioactive ^{23}S. 2-DE gels were used to illustrate how STAT6 influences the proteomes of the cells. Altogether, 21 protein spots were found to be reproducibly differentially expressed between the mouse strains (I, Figure 1); 16 with a higher expression in the wild type cells and 5 preferentially expressed in the *Stat6*$^{-/-}$ cells. Mass spectrometric analysis of the protein spots identified 49 different proteins as candidates to be differentially expressed (I, Table 1). Comparison of the results to the findings obtained at transcriptomics level (Chen et al., 2003) revealed that the results acquired with these two methods are very different emphasizing the importance of exploiting complementary approaches and methodologies. At the time of the publication, a relatively low number of genes was known to be regulated by STAT6. For example, microarray analysis of the transcriptomes of *Stat6*$^{-/-}$ and wild type mouse CD4+ splenocytes, had revealed only 36 genes to be regulated by this transcription factor (Chen et al., 2003). A majority of these genes were out of the reach of the technology used in this study as they are low abundant proteins such as transcription factors, secreted or membrane-bound. However, strict comparison of the results between these two studies is not appropriate due to the differences in the sample material, timepoint of analysis, and the usage of metabolic labeling in protein detection.

5.1.2 Regulation of CNBP and CBFβ2

Two of the differentially expressed proteins that were identified, namely CNBP (cellular nucleic acid binding protein) and CBFβ2 (core binding factor β, isoform 2), were selected for further studies according to their expression pattern and previously reported function. These transcription factors were identified to be more expressed in the *in vivo* labeled wild type mononuclear cells compared to *Stat6*$^{-/-}$ cells (I, Table 1). Both differences could also be confirmed with 2-DE western blotting (I, Figure 2b, Figure 3a). The difference seen in CNBP expression is probably due to a posttranslational modification or alternative splicing because the same protein was identified from several protein spots very close to each other in 2-DE gels (I, Figure 2a), and because the total CNBP amount didn't differ between the mouse strains (I, Figure 2c). However, the attempts to identify posttranslational modification didn't succeed, most likely because the expression level

of the protein was so low. The expression of CBFβ2 in 2-DE western blotting was diverse suggesting that in addition to alternative splicing CBFβ gene products are also regulated posttranslationally (I, Figure 3a). Both proteins chosen for further studies had been previously shown to be involved in the regulation of transcription, but they had not been reported to be regulated by STAT6.

Interestingly, the expression pattern of both CNBP and CBFβ2 was different when analyzed with 1-DE compared with 2-DE western blotting, again indicating that the original differences found were due to some modifications (I, Figure 2b and c, Figure 3a and b). Most notably, the total expression of CBFβ2 was induced in *Stat6*$^{-/-}$ CD4+ cells although in MNCs the expression level was constant between the mouse strains (I, Figure 3b). RUNX1 is known to be activated by its interaction with CBFβ2 (Cohen, 2009) and it had been reported to inhibit Th2 cell differentiation by repressing GATA3 expression (Komine et al., 2003). This suggested that one mechanism, by which STAT6 could enhance Th2 cell development, might be downregulation of the RUNX1-mediated inhibitory pathway (**Figure 4**).

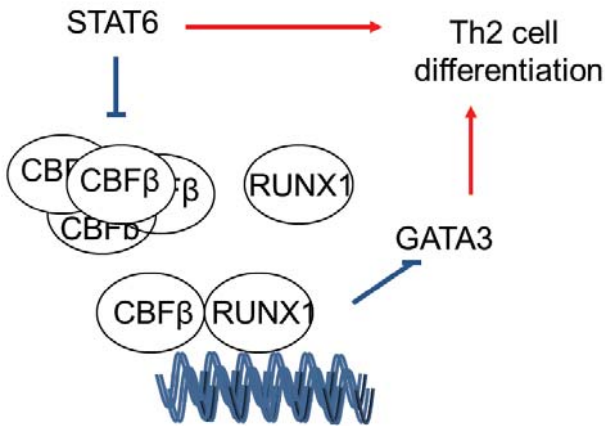

Figure 4. The tested hypothesis of the mechanism, which STAT6 could utilize in the regulation of Th2 cell differentiation. In the *Stat6*$^{-/-}$ CD4+ cells, the expression of CBFβ2 was increased compared to wild type cells. Because the interaction with CBFβ enhances RUNX1 DNA binding activity, in *Stat6*$^{-/-}$ cells RUNX1-mediated effects in gene expression could be enhanced. GATA3 is known to be negatively regulated by RUNX1 (Komine et al., 2003). Hence, it was studied whether STAT6 could enhance Th2 cell development by inhibiting RUNX1 activity.

This hypothesis was tested by analyzing RUNX1 DNA binding activity. However, no significant differences in RUNX1 DNA binding activity were detected between wild type and *Stat6*$^{-/-}$ cells before or after activation or IL4 stimulation (I, Figure 4a). Localization studies showed that the differentially expressed form of CBFβ2 and RUNX1 are mainly separated in different cellular compartments (I, Figure 4b, Figure 5a and b). This suggests that these proteins cannot freely interact, providing a possible explanation why altered CBFβ2 protein expression didn't directly lead to changes in RUNX1 DNA binding activity.

5.2 Characterization of IL4 and STAT6-mediated signaling in human Th2 cell priming (II)

Undisturbed *in vitro* Th2 cell polarization and *STAT6*-siRNA perturbed transcription was analyzed and compared by using oligonucleotide microarrays and bioinformatics,

respectively. The direct role of STAT6 in the regulation of IL4-specific transcriptome was further dissected by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq). Integration of the data resulted in detailed characterization of the IL4-dependent alterations of mRNA abundances during human Th2 cell priming and revealed the essential role of STAT6 in this process.

5.2.1 Identification of genes regulated by STAT6

The importance of STAT6 to the human Th2 cell priming was analyzed by comparing the effect of IL4 stimulation between the cells into which *STAT6*-specific siRNA or non-targeting control-siRNA was introduced with electroporation. The transcriptional responses were analyzed within three days of polarization, as the aim was to identify the genes important in initiating the differentiation process. The most prominent and reliable identification of the STAT6-regulated genes was ensured by using different siRNAs (II, Figure 1a and b) and statistical filtering of the data. Altogether around 450 genes were identified to be regulated in a STAT6-dependent manner (I, Figure S1, Table S1). siRNA-mediated downregulation of STAT6 expression was very efficient, as the protein level effect remained clearly visible throughout the first week of Th2 cell polarization (data not shown). Compared to the studies made earlier in mouse for STAT6 target gene identification (Chen et al., 2003), a much higher proportion of the IL4-regulated genes was under the control of STAT6 in human. At the 48 h timepoint over 80% of the IL4-controlled genes were significantly affected by perturbation with *STAT6*-siRNA (I, Figure 1c). The broad importance of STAT6 was also evident from the wide functional distribution and cellular localization of its recognized target genes (II, Figure 1d and e). In a later study, STAT6 has been shown to regulate a comparable percentage (79%) of the IL4-regulated genes in human umbilical vein endothelial cells (HUVEC) (Tozawa et al., 2011). At the time of publication (II), less than 10% of the genes identified to be under the control of STAT6 had been previously shown to be STAT6 targets in any cell type (II, Figure 2a and Table S1).

The identified STAT6 target genes contained approximately the same number of up- and downregulated genes indicating that STAT6 regulates transcription by inducing and repressing target gene expression (II, Table S1). When the magnitude of the *STAT6*-siRNA treatment was further analyzed separately among the up- and the downregulated target genes, it was noticed that the effect of STAT6 knockdown was, in general, significantly stronger among the IL4-upregulated than among the IL4-downregulated genes (II, Figure 2b). This suggested that STAT6 primarily drives the activation of transcription, and downregulation would be a secondary effect. At the same time as the present study was published, Wei et al. described the role of STAT6 in the regulation of the epigenetic marks in mouse Th2 cells and correlated the data to the changes in the transcriptome (Wei et al., 2010). Direct comparison of the annotated gene level data of the identified STAT6 targets genes between human (II, Table S1) and mouse (the genes having at least two-fold difference between *Stat6*$^{-/-}$ and wild type mice), reveals that more than 70% of the genes regulated in human are not regulated in mouse. However, the timepoint of analysis was different between these experiments; the human study

focused on the initiation of the polarization and the mouse study analyzed polarized cells after restimulation. Thus, there is a possibility that the relatively low concordance of the datasets is due to the different polarization status of the cells. This aspect remains to be analyzed. Beyond this, it is of interest that there is a group of genes which are coordinated by STAT6 both in human and in mouse irrespective of the timepoint of analysis. In general, the transcriptional response mediated by IL4/STAT6 signaling is now known to be largely cell type specific (Kanai et al., 2011; Tozawa et al., 2011).

5.2.2 Definition of genomic landscape of STAT6 binding

The above mentioned interpretation that different mechanisms are involved in STAT6-dependent up- and downregulation of gene expression was further supported by the observed higher overlap between the STAT6-upregulated than downregulated genes and the STAT6-bound genes (II, Figure 2c, Figure S2c). Altogether around 30% of the genes to which STAT6 binding was associated (II, Table S2 and S3) were found out to be regulated in a STAT6-dependent manner (II, Table S1). It has been known for a relatively long time that STAT6 binding to DNA does not necessarily associate with gene regulation (Kotanides and Reich, 1996). The nonfunctional DNA binding may be explained by the limiting expression of vital cofactors. Another alternative is that the binding could not be associated to the regulated genes. This could happen if the STAT6-modulated alteration in the gene expression would happen via long-distance DNA interactions, as previously reported to take place in Th2 cytokine locus (Spilianakis and Flavell, 2004). This kind of a role of STAT6 as a genomic organizer may also be one explanation for the observed binding sites which were more than 100 kb away from any known gene (II, Table S3). The study of STAT6-dependent regulation of epigenetic marks (Wei et al., 2010), confirmed afterwards the presented hypothesis of STAT6-mediated regulation of gene expression by epigenetic mechanisms. Recently, STAT6 has also been shown to be the major regulator of Th2 cell-specific enhancer activity (Vahedi et al., 2012). In addition, STAT6 has been suggested to regulate its target genes via sequestration of transcriptional cofactors (Ohmori and Hamilton, 2000). The putative target genes controlled via this mechanism would have also stayed unidentified when DNA binding and gene expression data were overlaid. In general, STAT factors recognize similar gamma-activated sequence (GAS) motif (Decker et al., 1997). The specificity of STAT signaling is largely regulated via external stimuli leading to activation of a specific member of the STAT family. However, based on the STAT6 ChIP-seq data, DNA sequence in its natural context may also regulate STAT6 binding. 79% of the identified STAT6 binding sites had the previously determined STAT family recognition sequence, indicating that one fifth of the binding occurred via atypical DNA sequence (II, Table S3). Interestingly, it was observed that the extended N_4-GAS motif, $TTTCN_4GAAA$, was preferentially found within the genes which were directly regulated by STAT6 (II, Figure 2d). STAT6 binding sites were enriched around the transcription start sites of its target genes (II, Figure 2e). The same phenomenon had previously been observed with STAT3 and STAT1 (Kwon et al., 2009; Robertson et al., 2007).

5.2.3 Kinetics of IL4 and STAT6-dependent transcription

To dissect the role of STAT6 in IL4-regulated gene expression, a detailed dataset of the transcriptional dynamics during the early Th2 cell polarization was collected. In total, 640 and 460 genes were detected to be upregulated or downregulated by IL4, respectively, within the nine selected timepoints between 0.5 and 72 h (II, Figure 3a, Table S4). The data illustrated the highly dynamic pattern of the gene expression involved in the priming of Th2 cells (II, Figure 3b, Table S5). The data uncovered that the early IL4-mediated signaling is biphasic: rapid gene upregulation until 4 h is followed by more preferential repression of target gene expression (II, Figure 3a). The comparison of IL4-induced gene expression and the STAT6 ChIP-seq data revealed that STAT6 was most distinguishingly bound to the genes which were transcriptionally modulated within 4 h of initiation of Th2 cell polarization (II, Figure 3a and c). However, there were also genes which were bound by STAT6 within 4 h, but regulated only at the later stages of differentiation. This might be explained by restricting expression of cofactors or lack of permissive chromatin structure at the time of the observed DNA binding. Interestingly, it was observed that STAT6 binding to the part of its newly identified primary target genes varied between the analyzed 4, 12 and 72 h timepoints (II, Figure 4 and Figure S3). The data suggested that this could be a general phenomenon. More recently, it has been shown in the genome-wide fashion that in HUVECs the binding of IL4-activated STAT6 to its target genes can be either transient and sustained (Tozawa et al., 2011). In this study the STAT6 binding was analyzed at 1, 4 and 16 h after IL4 stimulation. The majority of the STAT6 binding sites were detected at 1 h after IL4 stimulation, and altogether only 3% of the STAT6 binding sites observed at 4 or 16 h were not occupied by STAT6 at 1 h. In addition, approximately half of the 10611 binding sites detected at 1 h were transient and not in use at the later timepoints. The number of STAT6 binding sites decreased during the sustained IL4 stimulation being 2668 at 16 h of culture. By analyzing the consensus motifs among the STAT6 ChIP-seq peaks, the study provided evidence that both the nature of STAT6 binding site and the presence of other transcription factors participate in determining the sustainability of STAT6 binding. In this study, the correlation between IL4-dependent gene induction and STAT6 binding within the proximity of the target gene was greatest at 16 h timepoint. (Tozawa et al., 2011) Interestingly, another recent report has shown that the outcome of the DNA binding might also differ depending on the time of transcription factor occupancy on its target DNA. It was reported that the overall occupancy and the binding dynamics had a low correlation, and the long lasting residence on the target loci was a better indicator of functional binding than the total occupancy (Lickwar et al., 2012). In the future, it would be interesting to analyze whether STAT6 target genes vary during the human Th2 cells differentiation process, and what is the mechanism regulating selectivity.

5.2.4 Transcriptional network programming Th2 cell priming

Functional categorization of the IL4-induced and STAT6-regulated genes indicated that Th2 cell polarization is triggered by fast modulation of transcription factor expression (II,

Figure 5a and Figure S4, Table S7). Based on the gene expression profiling, RNAi and ChIP-seq data, the transcription factors were classified as (1) STAT6-independent, (2) putative STAT6 targets, (3) STAT6-dependent primary targets, and (4) STAT6-dependent secondary targets (II, Figure 5b). *BATF*, *RUNX1* and *EPAS1* were identified to form the core of the early STAT6-mediated transcription as these factors were both bound by STAT6 and regulated in an IL4 and STAT6-dependent manner. In mouse, RUNX1 has previously been linked to inhibition of Th2 cell polarization via downregulation of GATA3 expression (Komine et al., 2003). Thus, it remains to be studied why STAT6 upregulates *RUNX1* during human Th2 cell programming. EPAS1 has been reported to bind the promoter of RUNX1 (Mole et al., 2009) and may thus form a transcriptional circuitry amplifying STAT6 effect. BATF, the third of the directly via STAT6-regulated transcription factors identified, has been reported to induce *Il4* and *Gata3* transcription during the skewing towards Th2 cell phenotype (Betz et al., 2010).

STAT6-regulated genes that were not among ChIP-seq hits, i.e., indirect STAT6 target genes were further analyzed for the presence of enriched transcription factor binding sites (II, Table S6). The examination of the results revealed that STAT5A homotetramer motif was among the top hits (II, Table S6). The significant overlap between the reported STAT5A bound genes (Liao et al., 2008) and the secondary STAT6 target genes suggested that STAT5A may regulate these genes. Moreover, it was also observed that the genes directly regulated by STAT6 had a significant overlap with the reported STAT5A target genes (Liao et al., 2008). Based on these observations, STAT6 and STAT5A may synergistically regulate some target genes (II, Table S6). In summary, the data collected supported the idea that positive and negative interplay of signaling pathways underlines the Th2 cell polarization (II, Figure 5c).

5.3 Toxicogenomic characterization of engineered ZnO and TiO_2 nanoparticles (III)

A comprehensive cell viability measurements and genome-wide microarray profiling was combined with bioinformatics analysis to reveal the signaling pathways affected by ZnO or TiO_2 EN exposure of human primary monocyte-derived macrophages (HMDM), monocyte-derived dendritic cells (MDDC), and Jurkat T cells. In addition, the toxicity, dissolution and surface functionalization of modified ZnO-ENs was compared.

5.3.1 Nanomaterial characterization

Altogether nine ZnO and one TiO_2 ENs were used to analyze their cellular effects (III, Table 1). The ENs included in the study had varying characteristics; morphology, surface chemistry and size (III, Table 1, Figure S1-3). ZnO ENs analyzed were either commercial (ZnO-1), or lab made or modified (ZnO-2 to ZnO-9). The surface chemistry of the modified particles was altered in reaction either with mandelic acid (ZnO-2 and ZnO-6), mercaptopropyl-trimethoxysilane (ZnO-3), methoxyl (ZnO-4), diethylene

glycol (ZnO-5), gluconic acid (ZnO-7), citric acid (ZnO-8) or folic acid (ZnO-9). To verify the ligand and the coverage of the ENs, Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analysis (TGA) were performed, respectively (III, Table S1). The greatest weight loss was observed with mandelic acid modified ZnO-2 (Buerki-Thurnherr et al., 2012) and ZnO-6. The analyses revealed that also the commercial ZnO-1 was covered with some unknown organic compound (Buerki-Thurnherr et al., 2012). ZnO-9 had a rod shape and ZnO-3 a core-shell structure while all other ENs were spherical (III, Figure S2). X-ray diffraction (XRD) analysis confirmed that all the ZnO particles form hexagonal wuertzite crystals (III, Figure S3, Table S1) (Buerki-Thurnherr et al., 2012). The average size of the spherical ENs varied between 5 to 35 nm (III, Table 1, Figure S1). All ENs formed strong agglomerates of uniform size in water and in the cell culture medium (III, Table 1) with stable characteristic dispersion patterns as indicated by invariable Zeta potential, polydispersity indexes (PDI) or dynamic light scattering (DLS) results upon storage (III, Table S2).

5.3.2 Toxicity and dissolution of ZnO nanoparticles

Previous mechanistic analysis with zinc chelators and $ZnCl_2$ controls had revealed that in exposure with ZnO-1, ZnO-2, ZnO-3 or ZnO-4 the degree of extracellular dissolution largely determines the resulting toxicity (Buerki-Thurnherr et al., 2012). To further analyze this interdependency, immortalized Jurkat T-cells were exposured to varying doses of ZnO-5, ZnO-6, ZnO-7, ZnO-8 and ZnO-9 and the consequential effects on cell viability analyzed (III, Figure 1). In contrast to the previous study (Buerki-Thurnherr et al., 2012), the cell death and the amount of released Zn^{2+} did not directly correlate. However, the data could be explained when the surface ligand and the EN synthesis method were taken into account in the analysis (III, Table 2).

5.3.3 Effects of TiO_2 and ZnO-1 nanoparticles on cell viability and transcriptome

Microarray profiling of ZnO-1 and TiO_2 exposed MDDC, HMDM and Jurkat cells (Figure S4, Table S3) showed that the commercial TiO_2-ENs did not disturb gene expression at the level of transcription (III, Figure S6). Instead, commercial ZnO-1 caused dose-dependent cell death (III, Figure S5) (Andersson-Willman et al., 2012; Buerki-Thurnherr et al., 2012) and altered transcription in all cell types studied (III, Figure S6). The transcriptional response to ZnO-1 exposure varied both between the analyzed cell types (III, Figure 2a, Figure 4a and b, Table 5 and 6) and the cells from different individuals (III, Figure 2b, Figure S10 and S11). In response to exposure to 10 µg/ml of ZnO-1, 2703, 980 and 12 genes were found to be differentially expressed in HMDM, Jurkat and MDDC samples, respectively, as measured with Illumina microarrays (III, Figure 2a, Table S4). Metallothioneins were the only genes constantly affected in all cell types (III, Figure 3, Figure S7). The validation of the results with the Affymetrix platform largely confirmed the observed gene expression patterns (III, Table S4, Table S8). Cell viability of MDDC, HMDM and Jurkat cells after 24 h of exposure with 10 µg/ml of ZnO-1 was around 70%, 55% and 70%, respectively (III, Figure S5) (Andersson-Willman et al., 2012; Buerki-Thurnherr et al., 2012). Although

the cell viability of MDDC and Jurkat cells was at the same level, the transcriptional response varied substantially, showing that these outcomes do not directly correlate in all cell types. The reason for varying sensitivity to ZnO exposure was investigated by analyzing the expression of zinc transporter genes. Significant differential expression between the analyzed cell types was found in the expression of *SLC30A1*, *SLC30A3*, *SLC30A7*, *SLC39A11*, *SLC39A3*, *SLC39A8* and *SCL39A9* transcripts (III, Figure 5, Table S5). It remains to be studied whether the differential expression of these genes plays a role in the regulation of the observed transcriptomes. The low transcriptional responsiveness of MDDCs was in line with the reported unaltered phenotype of these cells in response to ZnO-1 exposure with the same dose used in the present study (Andersson-Willman et al., 2012). The genes regulated in HMDM and Jurkat cells in response to ZnO-1 exposure are largely known to regulate cell death and growth, but some play also a role in the development of the immune system (III, Table 4). Ingenuity Pathway Analysis software was used to identify the transcription factors which could control the observed ZnO-1-derived transcriptomes. The best candidate identified was glucocorticoid receptor, NR3C1. Altogether, it had 106 and 45 target genes in HMDM and Jurkat cells, respectively (III, Table S4). Interestingly from the perspective of Th cell differentiation, NR3C1 has been shown to interact both with STAT6 and TXB21 resulting in decreased transcriptional activity of these factors (Biola et al., 2000; Liberman et al., 2007).

5.3.4 Analysis of transcriptional response to modified ZnO nanoparticles

Jurkat cells were exposed to ZnO-2, ZnO-3, ZnO-4, ZnO-5 and ZnO-9 (III, Table S3) with different physic-chemical properties, dissolution and toxicity patterns (III, Table 1 and 2, Figure 1, Figure S2, Table S2) and analyzed along with ZnO-1 exposed cells with Affymetrix microarrays. In addition, ZnO-4 dose-response at four sublethal concentrations (Buerki-Thurnherr et al., 2012) was used to extrapolate the effects of different concentrations of released zinc. Of the modified ENs analyzed, diethylene glycol modified ZnO-5 (10 µg/ml) disrupted transcription most strongly (III, Figure 6). ZnO-5 along with ZnO-1 (10 µg/ml) and ZnO-4 (100 µg/ml) exposures differed from the rest of the samples based on the highest number of EN-responsive genes (III, Figure 6, Table S8). Thus, the global gene expression data reflected the cell viability results after the exposure with these nanoparticles (III, Figure 1, Table 2). The genes regulated by each ZnO-EN were largely the same with varying magnitude (III, Figure 6). In addition, comparison of the obtained results to the previously reported zinc responsive gene expression signature in THP-1 cell line (Cousins et al., 2003) further indicated that Zn^{2+} ions released from ZnO ENs impel the cells to alter their transcription (III, Figure S13, Table S9). However, it cannot be completely excluded that there are still some nanoparticle-specific effects, as previously reported in other experimental setups (Gilbert et al., 2012; Moos et al., 2010). In conclusion, ZnO EN toxicity can be modified by altering particle surface chemistry affecting dissolution properties. Similarly, doping into the ZnO nanoparticles has been reported to enable modification of their toxicity (George et al., 2010; Li et al., 2011; Xia et al., 2011).

5.4 Possible future improvements and applicability of the results

STAT6-regulated proteins (I) were identified from the mononuclear cell population, which complicates the interpretation of the results. An obvious improvement to the experimental setup used, would be the purification of CD4+ cells for the analysis. This could be done either before *in vitro* culture or alternatively after the culture. One interesting option would be also to infect the mice with a parasite such as, *Schistosoma mansoni*, to induced Th2 cell polarized immune reaction *in vivo*. The separation of the proteins could be improved by fractionating cellular compartments and by using narrower pH range isoelectric focusing. Fractionation of the samples would mean that the amount of cells needed for the analysis should be higher than in the published study. Ultimately this might limit the study to some specific cell fraction due to the practical reasons. However, reduction of the coverage of analyzed proteins, would most likely give results with higher information content as such. Protein separation with 2-DE could also be replaced with a quantitative LC-MS/MS analysis of either label-free or stable isotope labeled peptides. This approach has successfully been used in characterization of STAT6-dependent proteome of mouse liver cells concluding that STAT6 regulates lipid homeostasis (Iff et al., 2009). A recent study reevaluated the correlation between the mRNA and protein abundances and reported that unexpectedly even around 40% of the variations at the protein level could be associated with the changes at the mRNA concentrations. However, the translation rate was a better predictor of protein abundance than transcription. (Schwanhausser et al., 2011) This clearly underlines the importance of the efforts aiming at characterizing the protein compartment regardless of the known technical difficulties (Bell et al., 2009; Cox and Mann, 2011; Domon and Aebersold, 2010). It would be valuable to follow up the STAT6 proteomics study presented in this thesis using the latest technology enabling further in depth analysis of STAT6-dependent protein regulation. This is still clearly an underexplored area of research.

The identification of IL4 and STAT6-regulated genes was achieved by using the Affymetrix and Illumina microarrays (II). The comparison of the results would have been more straightforward, if only one platform had been used. In addition, there is a possibility that the different probe designs used on the arrays masks a part of the correlation in the current set up. In general, combination of different arrays may expand the candidate gene selection in cases when as high as possible identification coverage is needed. As the main interest was to identify the STAT6 target genes initiating the polarization, the samples for ChIP-seq analysis were collected within 4 h after stimulation of Th2 cell polarization. qChIP-PCR of the selected target genes revealed that STAT6 binding to DNA might be regulated in a kinetic fashion during continuous IL4 stimulation (II, Figure 4). Thus, it would have been good to have also an overlapping timepoint in the RNAi and ChIP-seq experiments. STAT6 is an important upstream mediator of IL4-induced Th2 cell differentiation and thus its downstream targets include candidates important for eliciting Th2 cell responses. The high prevalence of asthma and other allergic inflammatory diseases (Anandan et al., 2010; Masoli et al., 2004)

mediated by Th2 cells reason the characterization of this pathway. However, therapeutic targeting of Th cells is recognized to be challenging (Lloyd and Hessel, 2010). It should also be remembered that allergic inflammation is not solely regulated or driven by Th2 cells. In addition, for example asthma is a very heterogeneous disease with varying clinical outcomes, and the mechanisms resulting in the disease manifestation are not universal. (Bhakta and Woodruff, 2011; Lloyd and Hessel, 2010) In mouse experimental asthma models, the absence of STAT6-mediated signaling has been shown to lead to alternative gene expression and leucocyte infiltration pattern, possibly leading to another kind of lung pathology (Zimmermann et al., 2004). However, use of local STAT6 inhibitors has been shown to be effective in inhibiting experimental asthma (Darcan-Nicolaisen et al., 2009; McCusker et al., 2007). As all the data presented in the study was gathered from human primary cells, the work provides valuable information needed for identification of druggable nodes in the Th2 cell signaling. In addition, detailed analysis of IL4/STAT6 signaling can be used in evaluation of the potency and the downstream effects of the novel candidate modifiers designed. Although mRNA molecules are not directly the active compounds in the cells, microarrays provide the only genome-wide analysis method that is available for wide research community at the moment. Thus the mRNA expression patterns can be exploited as biomarkers in further experiments. As a general conclusion, the study highlighted that a kinetic genome-wide analysis of signaling pathways is feasible with human primary T cells. Usage of surrogate cell types should be carefully considered as, similar to many other transcription factors, the role of STAT6 varies depending on the cell type (Kanai et al., 2011). In addition, there are several known differences for example between human and mouse immune system and physiology, which emphasize the particular importance of the analysis of human cells in order to understand immune-mediated diseases (Germain and Schwartzberg, 2011; Karp, 2012; Khanna and Burrows, 2011; Mestas and Hughes, 2004).

The last study included in this thesis aimed at identifying the ZnO and TiO_2 EN-derived transcriptional signatures in three different cell types (III). In respect to applicability of the results, it would have been interesting to analyze the same cell types as primary cells and as transformed cell line. This could have better revealed the extent at which the cell lines may be utilized in the preliminary EN-characterization screens. In addition, it would have been interesting to analyze how inflammation affects ZnO or TiO_2 EN-induced transcriptional program. Overall, the study provided an extensive resource of transcriptional markers for mediating ZnO EN-induced toxicity for further mechanistic studies. The cells of the immune system, such as the ones used in the present study, are the ones recognizing and getting activated by foreign intrusions thus forming a crucial bio-nanomaterial interface for research.

6. SUMMARY

The selective differentiation of T helper cells is needed for efficient eradication of pathogens. Th2 cells are important in elimination of extracellular parasites such as helminthes. However, these same cells can also drive development of allergic inflammatory diseases if their differentiation and activity is not properly controlled. The crucial role of IL4 and STAT6 in the development and function of Th2 cells is well reported. Nevertheless, the molecules participating in this signal transduction remained largely unidentified before establishment of moderate and high-throughput analysis methods enabled by technological improvements and increased knowledge of genomes. The present study characterized IL4 and STAT6 driven Th2 cell polarization by using systems level measurements at the genome, mRNA and protein level. In addition, the effects of TiO_2 and ZnO nanoparticle exposure were evaluated by using a toxicogenomics approach.

Identification of STAT6 target proteins was achieved using proteomics tools. Mononuclear cells isolated from the spleens of Balb/cJ control and *Stat6*$^{-/-}$ mice were cultured in the Th2 cell polarizing conditions, whereupon the soluble proteins of the cells were separated with two-dimensional electrophoresis. The proteins that were expressed differentially between the control and the knock-out mice were identified with mass spectrometry. The study resulted in observation of 21 protein spots out of which 49 proteins were identified to be either up- or downregulated by STAT6. Specific isoforms of CNBP and CBFβ2 were found to be preferentially expressed in wild type mononuclear cells compared to *Stat6*-deficient cells. Interestingly, the expression CBFβ2 was increased in *Stat6*-deficient compared to wild type cells when CD4+ cells were analyzed. The differential expression of CBFβ2 was not reflected in the DNA binding activity of RUNX1, which uses CBFβ2 as its cofactor. Thus the functional consequence of the observed difference remains to be resolved. The results highlighted the importance of exploiting proteomics in characterization of STAT6 signaling.

The role of STAT6 in the regulation of IL4-dependent transcriptome was investigated by downregulating its expression with siRNAs and analyzing the resulting changes in gene expression. The hierarchy of STAT6-regulated genes was resolved by identification of STAT6 primary target genes with chromatin immunoprecipitation. The study indicated that STAT6 mainly drives the transcriptional upregulation needed for Th2 cell programming and its role in repressing alternative signaling is predominantly a secondary effect. A detailed analysis of IL4-regulated transcripts showed that Th2 cell priming is highly dynamic in respect of time, and that the great majority of the IL4-responsive genes were regulated by STAT6. The overlap of the STAT6-mediated primary and secondary target genes revealed that *BATF*, *RUNX1* and *EPAS1* form the core of the early STAT6-mediated transcriptional regulation. The data produced provides a unique resource of IL4 and STAT6-regulated transcriptional markers characterizing primary human CD4+ cell polarization towards a Th2 cell phenotype.

Nanosized particulate matter has been proposed to alter Th cell polarization and predispose to allergic inflammation. In the last study, the cellular toxicity and transcriptional changes of primary monocyte-derived macrophages, monocyte-derived dendritic and Jurkat T cells exposed ZnO and TiO_2 nanoparticles were assessed. TiO_2 nanoparticles were inert, but the commercial ZnO caused dose-dependent cell death in all cell types studied. Comparison of the differentially modified ZnO nanoparticles gave additional proof that the cellular response to nanosized ZnO is due to leached Zn^{2+} ions. In addition, when the data was interpreted in the context of the particle surface chemistry, a correlation between the toxicity of ZnO nanoparticles and their dissolution was found. The analysis of ZnO EN-regulated genes showed that the nanoparticles elicit cell type specific transcriptional response. The upregulation of metallothionein genes was the only shared signature of the ZnO exposure in all cell types tested.

The use of the complementary approaches illustrated the diversity of the mechanisms participating in the initiation of Th2 cell responses. In addition, the analysis of ZnO EN-derived transcriptional changes underlined the importance of unbiased testing of the effects of nanomaterials. All the data analyzed in the projects included in the thesis was gathered from human cells or primary cells increasing applicability of the results. Hopefully, in the future new innovative methods and integration of datasets will provide us tools for even deeper system-level understanding of Th2 cell differentiation and function.

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