Turun yliopisto
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GENE REGULATION IN THE HUMAN IMMUNE SYSTEM

Gene Expression Signatures of Th2 Cell Differentiation, Type 1 Diabetes, and Intrauterine Immune Adaptation

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ABSTRACT

Henna Kallionpää

Pathogenesis of human immune-mediated diseases – Gene expression signatures of Th2 cell differentiation, type 1 diabetes and intrauterine immune adaptation

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The human immune system is constantly interacting with the surrounding stimuli and microorganisms. However, when directed against self or harmless antigens, these vital defense mechanisms can cause great damage. In addition, the understanding the underlying mechanisms of several human diseases caused by aberrant immune cell functions, for instance type 1 diabetes and allergies, remains far from being complete. In this Ph.D. study these questions were addressed using genome-wide transcriptomic analyses.

Asthma and allergies are characterized by a hyperactive response of the T helper 2 (Th2) immune cells. In this study, the target genes of the STAT6 transcription factor in naïve human T cells were identified with RNAi for the first time. STAT6 was shown to act as a central activator of the genes expression upon IL-4 signaling, with both direct and indirect effects on Th2 cell transcriptome. The core transcription factor network induced by IL-4 was identified from a kinetic analysis of the transcriptome.

Type 1 diabetes is an autoimmune disease influenced by both the genetic susceptibility of an individual and the disease-triggering environmental factors. To improve understanding of the autoimmune processes driving pathogenesis in the prediabetic phase in humans, a unique series of prospective whole-blood RNA samples collected from HLA-susceptible children in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study was studied. Changes in different time-windows of the pathogenesis process were identified, and especially the type 1 interferon response was activated early and throughout the preclinical T1D.

The hygiene hypothesis states that allergic diseases, and lately also autoimmune diseases, could be prevented by infections and other microbial contacts acquired in early childhood, or even prenatally. To study the effects of the standard of hygiene on the development of neonatal immune system, cord blood samples from children born in Finland (high standard of living), Estonia (rapid economic growth) and Russian Karelia (low standard of living) were compared. Children born in Russian Karelia deviated from Finnish and Estonian children in many aspects of the neonatal immune system, which was developmentally more mature in Karelia, resembling that of older infants.

The results of this thesis offer significant new information on the regulatory networks associated with immune-mediated diseases in human. The results will facilitate understanding and further research on the role of the identified target genes and mechanisms driving the allergic inflammation and type 1 diabetes, hopefully leading to a new era of drug development.

Keywords: asthma, allergy, Th2, STAT6, interleukin 4, type 1 diabetes, interferon, hygiene hypothesis, cord blood, gene expression

TIIVISTELMÄ

Henna Kallionpää

Ihmisen immuunivälitteisten sairauksien syntymekanismit – Th2 solujen erilaistumiseen, tyypin 1 diabetekseen sekä raskausaikaisen immuunijärjestelmän sopeutumiseen liittyvät geeniekspressiomuutokset

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Immuunijärjestelmämme on jatkuvassa vuorovaikutuksessa sekä kehon sisäisten että sitä ympäröivien ärsykkeiden ja mikro-organismien kanssa. Elintärkeät puolustusmekanismit voivat kuitenkin aiheuttaa myös suurta tuhoa reagoidessaan harmittomia antigeeneja tai omaa kehoa vastaan. Silti monien immuunivälitteisten sairauksien, kuten astman ja tyypin 1 diabeteksen, tarkempi molekylaarinen syntymekanismi on vielä epäselvä. Näiden prosessien ymmärtämiseksi tässä väitöskirjatutkimuksessa selvitettiin genominlaajuisesti ihmisen immuunijärjestelmän geeniekspressio- ja viestintäreittejä.

Aktivoituneet tyypin 2 auttaja T-lymfosyytit (Th2-solut) ovat keskeisessä asemassa allergioita ja astmaa aiheuttavissa reaktioissa. Väitöskirjan ensimmäisessä osatyössä kuvattiin ensi kertaa STAT6 transkriptiotekijän kohdegeenit ihmisellä. Tutkimuksessa osoitettiin STAT6-molekyylin suorat ja epäsuorat kohdegeenit, sekä sen keskeinen tehtävän interleukiini 4:n (IL-4) signalointireitin säätelemien geenien aktivoinnissa. IL-4:n indusoima transkriptiotekijöiden ydinverkko tunnistettiin yksityiskohtaisen aikasarja-analyysin avulla.

Tyypin 1 diabetes on autoimmuunisairaus, jonka puhkeamiseen vaikuttavat sekä yksilön geneettiset alttiustekijät sekä autoimmuunireaktion laukaisevat ympäristötekijät. Väitöskirjatutkimuksen toisessa osatyössä tunnistettiin ihmisen tyypin 1 diabeteksen etenemisestä kertovia geenejä DIPP (Tyypin 1 diabeteksen ennustaminen ja ehkäisy) -tutkimukseen osallistuvilta lapsilta kerätyistä kokoveren RNA-näytteistä. Työssä tunnistettiin eri prekliinisen taudin vaiheille ominaiset muutokset ja eristyisesti interferonivasteen havaittiin aktivoituvan jo varhain ja läpi tutkittujen aikaikkunoiden.

Nk. hygieniahypoteesin mukaan varhaislapsuudessa ja jopa raskauden aikana kohdatut mikrobit ja infektiot ehkäisevät immuunivälitteisten tautien kehittymistä. Kolmannessa osatyössä tutkittiin elintason vaikutusta vastasyntyneiden lasten immuunijärjestelmään vertaamalla Suomessa (korkea hygienia ja elintaso), Virossa (nopeasti kehittyvä yhteiskunta) sekä Venäjän Karjalassa (alhainen elintaso ja hygienia) syntyneiden lasten napaverinäytteitä. Venäjän Karjalassa syntyneiden lasten immuunijärjestelmän havaittiin olevan kehityksellisesti kypsempi kuin Suomessa ja Virossa syntyneistä lapsilla.

Tämän väitöskirjatutkimuksen tulokset tarjoavat merkittävää uutta tietoa ihmisen immuunivälitteisten sairauksien säätelymekanismeista, merkiten uutta aikakautta niin tutkimuksessa kuin lääkekehityksessä; tunnistettuja kohdegeenejä ja mekanismeja muokkaamalla on mahdollista muuttaa mm. allergisen tulehduksen ja tyypin 1 diabeteksen syntyyn vaikuttavia viestintäreittejä.

Avainsanat: astma, allergia, Th2, STAT6, interleukiini 4, tyypin 1 diabetes, interferoni, hygieniahypoteesi, napaveri, geeniekspressio

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ABBREVIATIONS

AIRE	autoimmune regulator
APC	antigen presenting cell
BCR	B cell receptor
CD	coeliac disease
cDC	conventional DC
cDNA	complementary DNA
cGAS	cyclic guanosine monophosphate–adenosine monophosphate synthase
ChIP	chromatin immunoprecipitation
CT	cycle of treshold
CVB	coxsackievirus B
DC	dendritic cell
DNA	deoxiribonucleid acid
EAE	experimental autoimmune encephalomyelitis
EST	expressed sequence tag
eQTL	expressed quantitative trait loci
FCS	fetal calf serum
FDR	false discovery trait
GWAS	genome-wide association study
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFNα	interferon alpha
Ig	immunoglobulin
IL-4	interleukin 4
ILC	innate lymphoid cell
IRF	interferon regulatory factor
ISGF3	interferon stimulated gene factor 3 complex
LD	linkage disequilibrium
LPS	lipopolysaccharide
LTi	lymphoid tissue inducer cell
MAPK	mitogen activated protein kinase
miRNA	micro RNA
mRNA	messenger RNA
MS	multiple sclerosis
mTEC	thymic epithelial cells of medulla
NET	neutrophil extracellular chromatin trap

NLR	Nod-like receptor
NK	natural killer cell
Nt	nucleotide
NOD	non-obese diabetic mouse
OVA	ovalbumin
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pDC	plasmocytoid dendritic cell
RA	rheumatoid arthritis
RNA	ribonucleid acid
RNAi	RNA interference
rRNA	ribosomal RNA
RLR	RIG-I-like receptor
RSV	respiratory syncytial virus
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
RV	rhinovirus
siRNA	small interfering RNA
SLE	systemic lupus erythromatosus
SNP	single nucleotide polymorphism
STAT6	signal transducer and activator of transcription 6
T1D	type 1 diabetes
T2D	type 2 diabetes
TCR	T cell receptor
Tfh	T follicular helper cell
Th	T helper cell
Treg	T regulatory cell
TLR	toll-like receptor
VP1	viral capsid protein 1

LIST OF ORIGINAL PUBLICATIONS

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

I 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. (2010) Genome-wide Profiling of Interleukin-4 and STAT6 Transcription Factor Regulation of Human Th2 Cell Programming. *Immunity*. 32(6):852-862.

II Kallionpää H.*, Elo L.L.*, Laajala E.*, Mykkänen J., Ricaño-Ponce I., Vaarma M., Laajala T.D., Hyöty H., Ilonen J., Veijola R., Simell T., Wijmenga C., Knip M., Lähdesmäki H., Simell O., Lahesmaa R. (2014) Innate Immune Activity Is Detected Prior to Seroconversion in Children with HLA-conferred T1D Susceptibility. *Diabetes*. 63(7):2402-2414.

III Kallionpää H.*, Laajala E.*, Öling V., Härkönen T., Tillmann V., Dorshakova N.V., Ilonen J., Lähdesmäki H., Knip M.#, Lahesmaa R.#, and the DIABIMMUNE study group. (2014) Standard of Hygiene and Immune Adaptation in Newborn Infants. *Clinical Immunology*. 155(1):136-147.

* and # Authors have an equal contribution to this article

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1. INTRODUCTION

T helper (Th) cells orchestrate the responses of both the adaptive and innate immune system by guiding the function and differentiation of other immune cells with secreted signals, such as cytokines, or with cell-to-cell contact through their cell surface receptors. Subsequently, their aberrant activation and behavior can result in immune-mediated pathologies, such as allergic reaction towards harmless antigens or autoimmune attack against self-antigens. Such illnesses constitute a high burden to the patients and society, especially in view of their increasing incidence in the past decades. Although the first T helper cell subsets were discovered 20 years ago, their detailed differentiation programs remain unsolved, especially in human. Due to the high interest for therapeutic interventions on Th cell activity, more information on the differentiation programs of human T cells is needed.

Type 1 diabetes (T1D) is a multifactorial disease, where insulin-producing β cells in the pancreatic islets are destroyed by autoimmune mechanisms. Only 50% of the disease risk can be currently explained by genetic factors, suggesting that the environmental factors, still largely unidentified, play a significant role in the disease pathogenesis. A majority of the studies on the disease mechanisms of T1D have been performed with animal models, mainly with the non-obese diabetic (NOD) mouse. Human studies focusing on established, diagnosed patients are of importance, but only a handful of studies have so far focused on pre-clinical T1D, during which an autoimmune cascasde of years in durations may occur before the appearance of clicnical symptoms. Clearly, characterization of pre-clinical patients before and after the onset of the beta cell autoimmunity is warranted for a more detailed understanding of the disease triggering factors in human.

The increasing incidence of atopic and autoimmune disease during the last few decades has been explained by several theories, such as the hygiene hypothesis and the virus theory. The hygiene hypothesis originally postulated that the lack of infections and proper immune system education early in life would result in immune-mediated diseases later in life. The theory has been debated since its proposal, and clear-cut evidence in support or opposing are still missing. The study of selected cohorts of children born in contrasting standards of living should provide an appropriate means of testing this theory. In addition, evidence on the effect of the *in utero* period in the context of the hygiene hypothesis warrants further exploration.

2. REVIEW OF THE LITERATURE

2.1 Immune system

The immune system protects us from invading pathogens of the outside world, such as viruses, bacteria, fungi and parasites, as well as against internal threats such as cancerous cells. It acts by recognizing the presence of a pathogen and aims at containing and eradicating it. To prevent overreaction and to protect self-tissues, balanced regulation and termination of these inflammatory responses must take place. Ideally, immunological memory is formed, so that the pathogen will be eradicated more quickly on the second encounter in order to prevent re-infection. The immune system is so effective, that it also acts as a barrier to modern-day treatments, such as transplantation and gene therapy. Unbalanced immune-responses can also lead to the development of variety of immune-mediated inflammatory diseases. During evolution, the mammalian immune system has developed to become an intricate system of several elements. In the primary line of defense are the epithelial linings, consisting of both physical skin and other epithelial barriers, as well as antimicrobial peptides secreted at the mucosal surfaces. At the cellular and molecular level the immune system can be divided into humoral immunity, such as antibodies and cytokines secreted by the immune cells, and cell-mediated immunity, governed by the direct interactions of these cells. Traditionally, considerations of the immune system have been divided into innate and adaptive immunity, although the interaction between the two parts is crucial. (Murphy et al. 2012).

All cells of the immune system arise from a multipotent progenitor, hematopoietic stem cell, residing in the bone marrow (**Figure 1**). Upon environmental stimuli, this cell can divide and initiate a differentiation programme either towards an" adaptive" lymphocyte fate, or towards an "innate" myeloid fate. A myeloid progenitor can differentiate into erythrocyte/megakaryocyte progenitor. This gives rise to erythrocytes, which constitute ~99% of all blood cells, as well as megakaryocytes which produce platelets. The myeloid progenitor can also give rise to a granulocyte/monocyte progenitor, from which monocytes and macrophages are derived. Granulocytes include basophils, neutrophils and eosinophils. The lymphocyte progenitor gives rise to T and B lymphocytes of the adaptive immune system, as well as natural killer (NK) cells and other innate lymphoid cells (ILC). Besides the bone marrow, several other tissues participate in the functions of the immune system. Immune cells circulate in the blood and lymph, and in particular the lymphocytes can be found in the lymphoid organs and tissues. Fetal liver, bone marrow and thymus are the primary lymphoid organs of lymphopoiesis, and peripheral lymphoid organs, such as lymph nodes, spleen and the gut-associated lymphoid tissue,

are the location for maturation and initiation of lymphocyte responses. (Murphy et al. 2012).

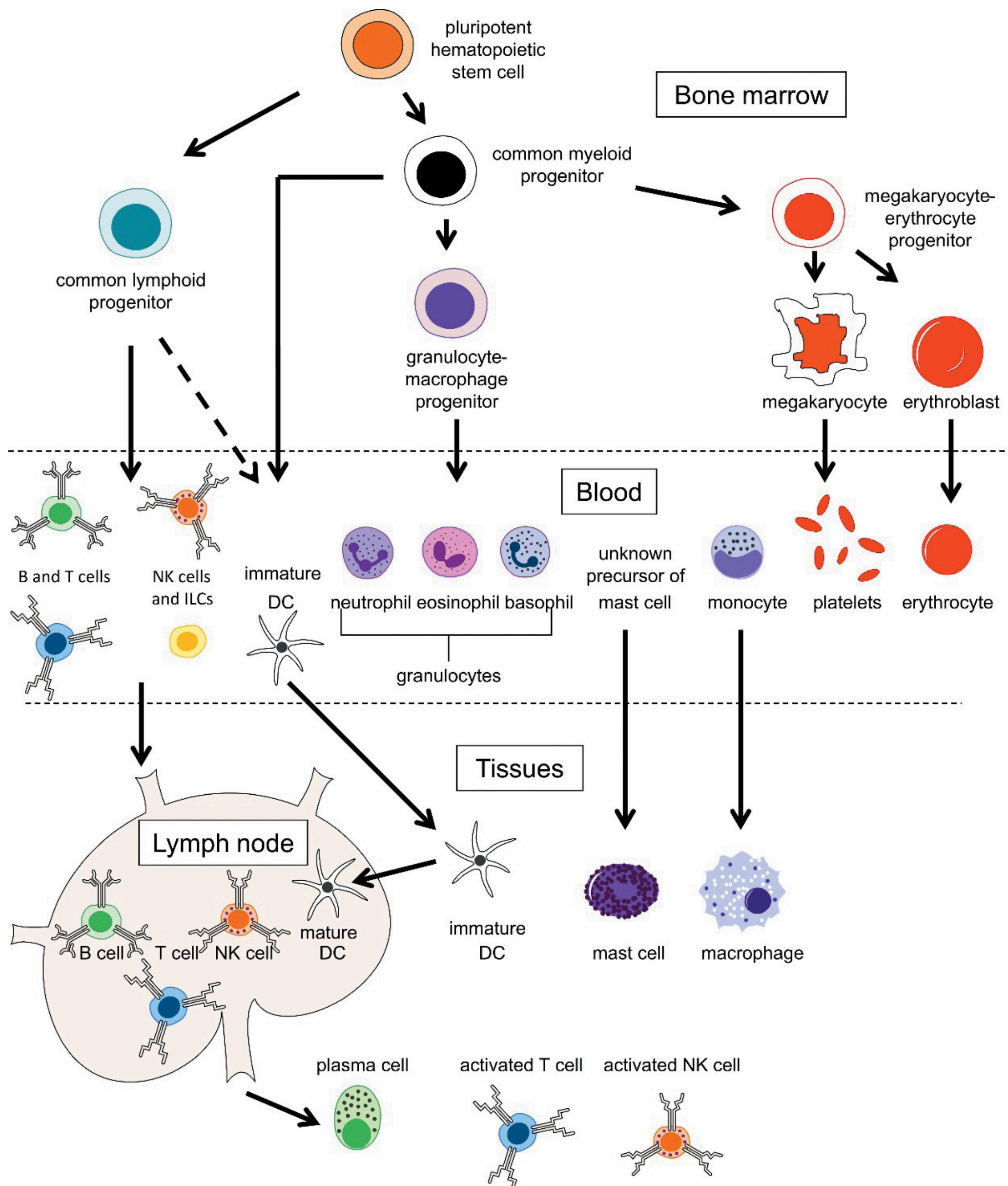

Figure 1. Overview on hematopoiesis – generation of immune cells. The immune cells are formed in the bone marrow, and circulate through the blood and lymph to the tissues. B and T cells are generated in the bone marrow, and then educated in the primary lymphoid organs, bone marrow and thymus, respectively. Lymphocytes are activated in the lymph nodes and other secondary lymphoid organs. Mast cells, dendritic cells (DC) and macrophages mature in the tissues and/or bone marrow in response to cues from their surroundings. Modified from Murphy et al. 2012.

2.1.1 Innate Immunity

Innate immune reactions are the immediate, pattern-recognition capabilities of the body, such as activation of the complement system, production of antimicrobial peptides, or phagocytosis by specialized cells. This is the first line of defense mechanisms activated upon encounter with a pathogen. The cells of the innate immune system are derived from the myeloid lineage and include the granulocytes, monocytes that differentiate into macrophages, platelets, mast cells, and dendritic cells (DCs).

There are three subtypes of granulocytes, i.e**. basophils**, **eosinophils**, and **neutrophils**. These are short-lived cells that circulate in the blood, but migrate to the periphery in response to inflammation. All of these cell types have granules that contain antimicrobial enzymes and toxins. Basophils and eosinophils are thought to contribute to the immune reactions against parasitic worms, whereas neutrophils are specialized in phagocytosis, i.e. internalizing and destroying variety of microbes in their internal vesicles. Microbes are engulfed in phagosomes, which fuse with several types of granules in the neutrophil cytoplasm, such as lysosome and the primary and secondary granules, inducing production of toxic peptides and reactive oxygen species through oxidative burst, eventually killing the engulfed microbe. Additionally, utilization of neutrophil extracellular chromatin traps (NETs), a complex network of nuclear chromatin and antimicrobial proteins, assists in immobilization and degradation of the pathogens. The second type of phagocytic cell with similar phagocytic machinery are the **monocytes** and **macrophages**. Macrophages are differentiated from monocytes in the peripheral tissues, and are long lived and relatively immotile. In the tissues their role is to clear debris from the dying cells, as well as scout for the presence of microbes. (Boltjes and van Wijk 2014). As the antimicrobial components produced by granulocytes and macrophages are highly toxic to the host cells, the phagocyte activation must be tightly controlled. Although the macrophages can continue their phagocytic activities by producing new lysosomes, the neutrophils die after one round of phagocytosis. (Murphy et al. 2012). It is thought, that successful resolution of inflammation requires inhibition of neutrophil influx, rapid clearance of neutrophils, and regeneration of disrupted tissue structure. Monocyte recruitment is preferred, as macrophages engulf the dying neutrophils. Neutrophils are destined to a short half-life due to their constitutive expression of apoptotic molecules, and many disease states are associated with either increased (Influenza A virus) or suppressed (sepsis, RA) neutrophil apoptosis that unbalances the steady state. (El Kebir and Filep 2010).

Platelets are small unnucleated cells produced from megakaryocytes that have been mainly considered as the cell type responsible for blood coagulation upon tissue injury. However, as they are rapidly recruited to sites of tissue trauma where microbial invasion often takes place, their antimicrobial activities are becoming more appreciated (Yeaman

2014). Although anucleated, they have stable mRNAs descending from megakaryocytes and are thus capable of translation and protein production. Platelets express several chemotactic receptors and also release chemokines for recruitment of other immune cell types. Similarly to granulocytes, platelets possess granules that in addition to the modulators of the fibrin clot reaction contain histamine and platelet antimicrobial proteins. They also produce reactive oxygen species, although less than neutrophils. Through their cell surface markers, such as CD40, platelets can interact with monocytes, neutrophils, T, and B cells. For example, platelets and neutrophils act in synergy in killing of bacteria, leading to enhanced activity of both cell types. (Yeaman 2014).

Mast cells are thought to be derived from the myeloid lineage, but the details of their differentiation pathway and especially their relation to basophils are still under debate (Ribatti and Crivellato 2014). Mast cell progenitors migrate into peripheral tissues, where they maturate. They are especially abundant in tissues exposed to the outside environment, such as the skin, lungs, and intestine. Mast cells process and secrete several lipid mediators and a variety of cytokines, and thus are centrally involved in regulating the adaptive immune responses. Perhaps the most widely recognized mediator released by mast cells is histamine, which promotes vasodilation and bronchoconstriction is asthmatic reactions. (Reber and Frossard 2014).

Dendritic cells (**DC**) are also phagocytic cells, and have been broadly divided into myeloid or conventional DCs (cDC) and to plasmacytoid DCs (pDC). cDCs mature in the tissues, express high levels of HLA class II, and compared to monocytes and macrophages are superior in antigen presentation to the adaptive T lymphocytes. cDCs meet the naïve T cells in the lymph node, and direct their stimulation and polarization. In addition, the cDCs activated in a particular tissue environment also imprint the T cell for expressing tissue-specific homing receptors, enabling T cell to emigrates from the lymph node and return to the original site of infection. Recent microarray analyses support the division of further subsets of cDCs, which differ in their expression pattern for innate immune receptors, as well as in cytokine production, that thus determine the type of adaptive response to be initiated by the lymphocytes. In contrast, the pDC subset matures in the bone marrow, lacks HLA class II expression at steady state, and constantly migrates in the blood and lymphoid tissue. pDCs are the central cell type in mediating antiviral state through the secretion of antiviral interferons, and are also able to differentiate into efficient antigen presenting cells upon activation. DCs can also be named as tissue resident or migratory, based on their motility between the tissues and lymph nodes. Specialized tissue DCs include, for example, Langerhans cells of the epidermis. (Boltjes and van Wijk 2014).

In addition to the innate immune cells of the myeloid lineage, there are several lymphoid progenitor cell –derived cell types that cannot be classified as belonging to the adaptive

immunity due to their lack of antigen-specific receptors, and that all depend on transcription factor ID2 for their development. Thus, they are collectively described as innate-like or innate lymphoid cells (**ILCs**), and divided into three groups according to their cytokine signature: Group 1 ILCs secrete IFNγ, group 2 ILCs secrete interleukins 5 and 13, and group 3 produces interleukins IL-17 and/or IL-22, although plasticity between different groups is possible (Spits et al. 2013). Their mirroring of the adaptive T helper cell subset (discussed in chapter 2.1.3.1.) suggests evolutionary relationship of ILCs as primordial T cell precursors (Walker et al. 2013). The best known Group 1 ILC cells are the large and granular natural killer (**NK**) cells. The role of the NK cell is to recognize tumor cells, as well as infected cells especially in the early phase of infection, when adaptive CD8+ cytotoxic cell response has not yet been mounted. NK cells recognize infected cells through their cell membrane receptors, such as the killer lectin-like receptors, and secrete IFNγ. There are both activating and inhibitory receptors: Signaling through inhibitory receptors sequesters the intracellular signaling components, preventing the signal cascades from the activating receptors. Thus the balance between the activating and inhibiting signals determines the killing capacity of the NK cells. Most of the autologous cells of the body express the HLA class I molecules that engage the inhibitory receptors, and thus protect them from NK-cell mediated killing. By a mechanism not clearly understood, infection changes the properties of HLA class I presentation and makes infected cells more susceptible NK cell targets. (Murphy et al. 2012). The group 3 ILC subset includes the lymphoid tissue-inducer cells (**LTi**) that are essential for the formation of lymph nodes and intestinal lymphoid tissue structures during embryogenesis. Apart from the NK and LTi cells, most of the other identified ILC populations have only been characterized recently. A common ILC progenitor, giving rise to ILC1-3 cells, but not NK and LTi cells, and expressing transcription factor Plzf, was recently identified in the mouse system (Constantinides et al. 2014). ILC cells are mainly found in the mucosal tissues, and are proposed to induce tissue repair and combat infections, but also contribute to some of the immune-mediated pathologies, such as allergies and asthma. (Walker et al. 2013).

2.1.1.1 Pattern recognition in the centre of innate immune reactions

The innate immune system relies on recognizing the invading pathogen through germline coded receptors that screen for conserved pathogen-associated molecular patterns (PAMPs) not present in the host cells, such as lipopolysaccharide (LPS) of Gram-negative bacteria and unmethylated CpG of bacteria and DNA viruses. These receptors also respond to the so called danger-associated molecular patterns (DAMPs), such as nucleotides and ATP released from dying cells of the body. The best known sensor for these patterns is the **complement system**, a cascade of serine proteases in the plasma, which aims at either inducing lysis of the invading microbe or targeting it for

phagocytosis by coating it with complement proteins. There are more than 30 complement proteins constantly produced in the liver as inactive pro-enzymes that become active upon proteolysis by the previous protease in the cascade. The sequence starts from the initial pattern recognition receptors detecting the pathogen. The classical pathway is induced by the interaction with C1q complement protein with the pathogen or with an antibody bound to the pathogen. In the alternative pathway the cascade is spontaneously activated. The third pathway is called the lectin pathway, as it relies on mannose-binding lectin and ficolin proteins to recognize and bind the pathogen surface. Finally, as a result from all these pathways, C3 is cleaved by the C4b2a convertase, and as a result of this proteolysis the larger fragment C3b binds the pathogen surface, whereas the smaller fragment C3a recruits phagocytic cells to the site. Phagocytic cells express C3b receptors that aid in the phagocytosis of the pathogen. In addition, the complement pathways result in the formation of the membrane attack complex (MAC), which forms a pore in the cell membrane of the pathogen, resulting in lysis. Intriguingly, the complement system remains active unless inactivated. Therefore, the autologous healthy cells of the body express complement inhibitors that prevent complement activation on their cell surface, including C4-bindig protein (C4BP complex) and CD46 (membrane cofactor protein, MCP), that bind C3b and the upstream product C4b, allowing their degradation by the Factor I protein. (Murphy et al. 2012).

The most recognized family of cellular pattern recognition receptors are the **Toll-like receptors** (**TLRs**) that are mainly expressed by the cells of the immune system and epithelial cells. TLRs are transmembrane proteins, and the majority of the TLRs are expressed on the plasma membrane (TLRs 1-2, 4-6, 10) for detection of extracellular pathogen molecules, whereas the rest reside in the endosomal compartment (TLRs 3, 7-9) for the detection of endocytosed and phagocytosed pathogens. Based on their structural properties, they are thought to act as either homo- or heterodimers. For example, TLR2 can form a complex with TLR1 or TLR6 for detection of fungal zymosan and bacterial lipoproteins. Four adaptor proteins mediate all TLR signaling downstream of the receptors. These include myeloid differentiation factor 88 (MyD88), MyD88 adapter-like protein (MAL), TIR domain containing adapter-inducing IFN-β (TRIF), and TRIF-related adapter molecule (TRAM), and association with these dictates the downstream signaling events. For example, upon recognition of dsRNA by TLR3, the receptor associates solely with TRIF, that eventually leads to activation of the transcription factor Interferon regulatory factor 3 (IRF3), activating the production of interferon β (IFN β) cytokine. All other TLRs are able to signal through the MAL-MyD88 pathway, leading to activation of transcriptional regulators, such as NFκB and AP-1, which drive the transcription of proinflammatory cytokines, such as TNF-α and IL-1β. LPS binding to TLR4 can associate either with MAL-Myd88, when signaling from the plasma membrane, or alternatively TLR4 is internalized to

endosomes, from where it can interact with TRAM-TRIF, leading to production of IFNβ. (Beutler 2009).

In addition to membrane bound TLRs, cytosolic pattern-recognition receptors have been identified. The best characterized is the **RIG-I-like receptor family** of RIG-I (coded by *DDX58*), MDA5 (coded by *IFIH1*) and LGP2, which are widely expressed throughout the body and upregulated upon type I interferon signalling. The RIG-I receptor senses the unmodified 5′-triphosphate end of ssRNA of paramyxoviruses, orthomyxoviruses and flaviviruses, whereas the MDA5 senses dsRNA of picornaviruses, such as enteroviruses. In addition to its well-established role as a viral sensor, the latest data shows that RIG-I also recognizes bacterial mRNA (Abdullah et al. 2012, Hagmann et al. 2013), and the immunomodulatory effect was confirmed to be dependent on the 5′-triphosphate (Schmolke et al. 2014). The second family of cytosolic receptors is the **nucleotide-binding domain and leucine-rich repeat containing receptor family** (NLRs, also known as Nod-like receptors). There are of 14 members of this family and they share the same CARD signaling domain with the RLRs. The most recognized family member, Nod2, binds both bacterial (muramyl dipeptide) (Grimes et al. 2012) and viral (ssRNA) ligands, resulting in alternative downstream signalling pathways (Sabbah et al. 2009). A subset of the NLR family proteins with a pyrin domain are called NRLPs. Many of the NRLPs, such as NRLP3, NLRC4, and NLRC5, participate in the formation of inflammasomes, which are protein complexes of NLRPs, adaptor proteins and caspase-1 required for processing and activation of proprotein forms of pro-inflammatory cytokines IL-1β and IL-18. (Rathinam et al. 2010, Ratsimandresy et al. 2013). In addition, NLRs NLRC5 and CTIIA act as transcriptional activators of HLA class I and class II molecules, respectively (Neerincx et al. 2013).

The **AIM2-like receptor family** (ALMs) consists of 4 members which also contain the pyrin domain and participate in the formation of the inflammasome. AIM2 senses cytosolic dsDNA, whereas another family member IFI16 has been shown to bind ssRNA and dsDNA. Activation of both sensors leads to formation of a caspase-1 activating inflammasome with ASC adaptor protein, and mediates IL-1β production. (Ratsimandresy et al. 2013). Inflammasomes are also known to activate a caspase-1 induced cell death pathway called pyroptosis. As opposed to apoptosis which leads to cell death without inducing inflammation, pyroptosis is a highly inflammatory form of programmed cell death, in which the cytoplasmic contents and proinflammatory cytokines, such as IL-1β, are released upon cell lysis to induce further immune responses. It was recently reported that although cell death of permissive activated HIV infected CD4+ cells occurs silently through caspase-3 –mediated apoptosis, cell death of nonpermissive resting CD4+ cells (95% of CD4+ cells in the lymphoid tissues) of HIV infected individuals occurs through pyroptosis (Doitsh et al. 2014). Upon abortive infection of resting CD4+ cells

IFI16 senses incomplete reverse transcripts of HIV DNA accumulating in the cytosol and induces IFNβ production as well as pyroptosis, indicating IFI16 to be central for both chronic inflammation as well as bystander CD4+ T cell depletion driving AIDS in HIV patients (Monroe et al. 2014). Both AIM2 and IFI16 are members of a recently identified and expanding group of **cytosolic DNA pattern recognition receptors**. For example, cyclic guanosine monophosphate – adenosine monophosphate (cGAMP) synthase (cGAS) was discovered to be an essential for detection of retroviral reverse-transcribed and bacterial DNA in the cytoplasm (Ablasser et al. 2013, Gao et al. 2013, Li et al. 2013, Sun et al. 2013, Wu et al. 2013). Activated cGAS catalyzes production of a second messenger 2′3′cGAMP that binds and activates endoplasmic reticulum and mitochondria -associated adaptor protein STING (stimulator of interferon genes), subsequently leading to activation of IRF3 and transcription of type I interferon genes. In addition, a nuclear protein Rad50, which detects doublestranded DNA breaks, was recently shown to translocate to the cytosol, interact with viral dsDNA, and through adaptor protein CARD9 induce NF-κB signaling, leading to transcription of *Il1b* (pro-IL-1β) (Roth et al. 2014). There is evidence supporting the existence of additional cytoplasmic DNA receptors, such as LRRFIP1, and DAI, but their identity remains controversial until further definition beyond the biochemical studies. A common feature of many of the candidate cytoplasmic DNA sensors is signaling through the adaptor protein STING, activation of IRF3 and induction of IFNβ. (Bhat and Fitzgerald 2014, Unterholzner 2013).

The last of the pattern recognition receptor groups so far identified is the superfamily of **C-type lectin-like receptors** (CLRs) of both membrane bound and secreted receptors with diverse functions in the immune system. This receptor family includes Macrophage-inducible C-type lectin Mincle (CLEC4E) that is expressed in monocytes, macrophages, neutrophils, myeloid DCs and B cells, although not in T cells, pDCs and NK cells. Mincle senses a-mannose in fungal species of *Malassezia* and *Candida*, as well as mycobacterial glycolipid trehalose-6,60-dimycolate (TDM). (Sancho and Reis e Sousa 2013). Mincle also binds to Spliceosome associated ribonucleoprotein SAP-130, which is released by necrotic cells, leading to cytokine (TNF-α, IL-6) and chemokine (MIP-2) mediated recruitment of neutrophils to the inflamed tissue (Yamasaki et al. 2008). Mincle expression by neutrophils was also essential for their migration in to the lung upon *Mycobacterium tuberculosis* infection (Lee et al. 2012).

In summary, efficient recognition of pathogens and activation of the downstream signaling cascades are essential for mounting both innate and subsequently adaptive immune responses. (Murphy et al. 2012). Although we are now beginning to understand the complexity of the pattern recognition signaling pathways, we do not understand the regulatory checkpoints and counterbalancing involved, for example

in differentiating between pathogenic nucleic acids and host DNA, present in the cytosol during cell division. In addition to the receptor families described above, there may be pattern recognition receptors that have yet to be identified, functioning, for example, in the intracellular recognition of LPS (Tan and Kagan 2014). Many of the overlapping pathways probably function in a cell type specific or temporal manner in different stages of immune response. (Bhat and Fitzgerald 2014, Ratsimandresy et al. 2013).

2.1.2 Type I interferon signaling

Altogether there are 10 IRF transcription factors in human, out of which the **IRF3** and **IRF7** can induce the transcription of antiviral **type I interferons** α (IFNα) and β (**Figure 2**). IRF7 is phosphorylated upon signaling from the TLR2, TLR7, and TLR9 receptors, whereas IRF3 can be activated by signaling from the viral recognition receptors TLR3, RIG-I, Nod2, cGAS, and possibly by other nuclear DNA receptors, such as IFI16. Also TLR4 signalling can lead to IRF3 activation and type I interferon production in response to bacterial components, once the TLR4 complex is internalized into endosomes, allowing interaction with the TRIF adaptor and downstream IRF3 activation (Kagan et al. 2008). Although there are several overlapping pathways for the induction of type I interferons, it seems that the activating ligand of different TLRs, adaptor protein preference, as well as the downstream signalling cascades are highly cell type and pathogen specific: DCs and macrophages express TLR2, but viral ligands do not induce type I interferon production in these cells, whereas TLR2 is the main inducer of type I interferons in inflammatory monocytes of the bone-marrow and spleen during *in vivo* infection (Barbalat et al. 2009). The pDC and cDC subsets also differ in their TLR expression and/or signaling. The pDCs lack TLR2 and TLR3, but express TLRs 7-9 and IRF7 constitutively, leading to the notorious type I interferon production by these cells. In cDCs TLR7 activation leads to DC maturation and IL-12 production, and the relatively modest type 1 interferon production is induced by TLR3 and TLR4. (Ng and Gommerman 2013). In addition, IRF3 is ubiquitously expressed allowing IFNβ production by almost any cell type, whereas IRF7 is expressed mainly in the immune system, leading to more limited production of IFNα. pDCs can also engage other IRF factors, such as IRF1 and IRF8 downstream of TLR activation. (Beutler 2009, Lester and Li 2014).

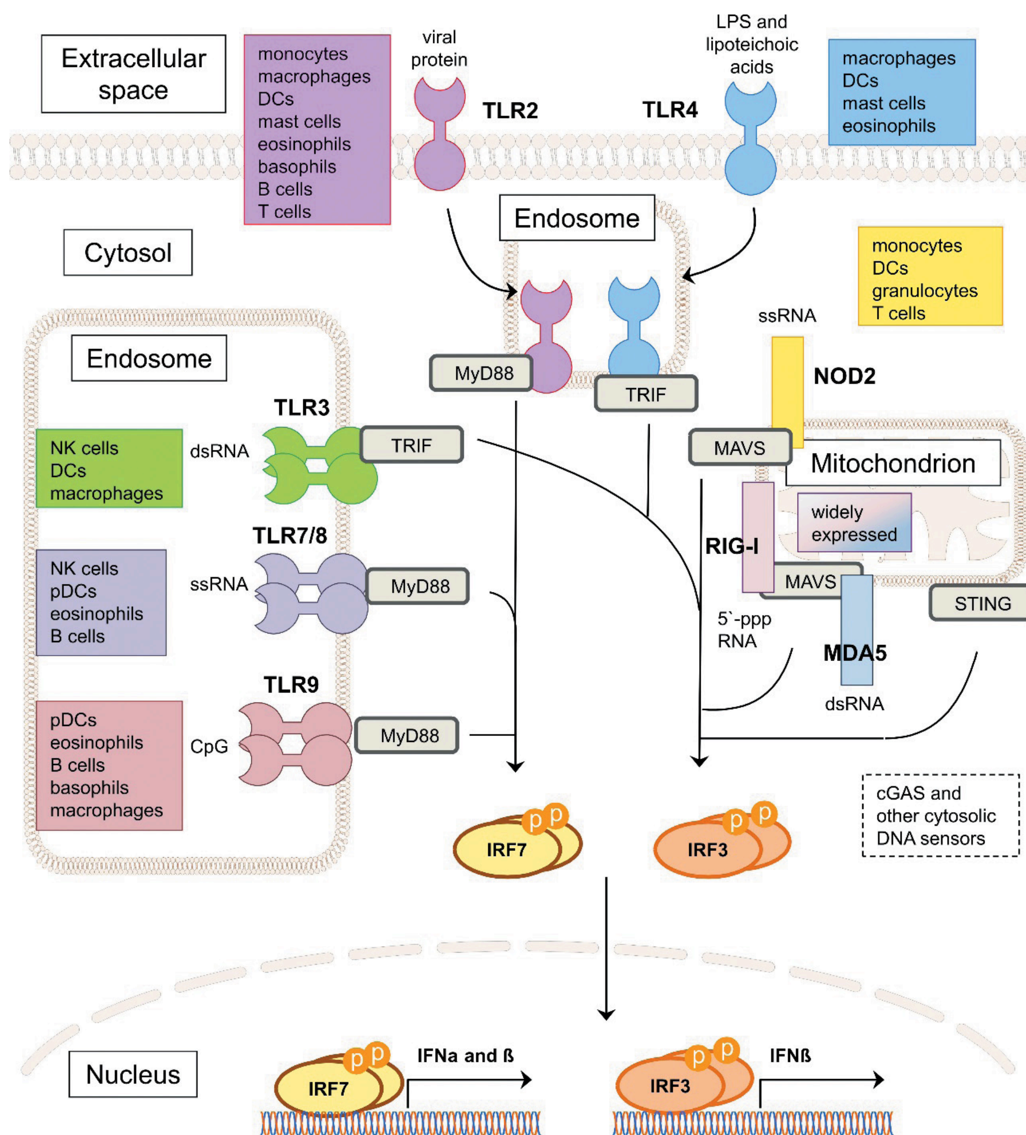

Figure 2. Pattern recognition receptor signalling pathways known to induce the production of type I interferons. Such receptors include several TLRs, RLRs RIG-I and MDA-5, as well as NLR NOD2. The ligand, expression pattern in immune cells, and adaptor molecule associated with each receptor are presented. The endoplasmic reticulum and mitochondria –associated adaptor protein STING (stimulator of interferon genes) is known to be activated by second messenger 2′3′cGAMP, synthesized by cGAS after binding to DNA, and several other candidate receptors for cytosolic DNA have been identified. Eventually the pathways lead to phosphorylation, activation and dimerization of IRF3, which induces IFNβ, or IRF7 that induces both IFNα and β. (Abdullah et al. 2012, Barbalat et al. 2009, Bhat and Fitzgerald 2014, Gutierrez et al. 2002, Hagmann et al. 2013, Kagan et al. 2008, Lester and Li 2014, Murphy et al. 2012, Sabbah et al. 2009, Schmolke et al. 2014, Unterholzner 2013).

Once their production is initiated by an infected cell or a cell recognizing viral or bacterial particles, the type I interferons mediate profound local and systemic effects in the body. IFNα and β signal through widely expressed plasma membrane receptor, a heterodimer composed of IFNAR1 and IFNAR2 proteins, allowing any cell type to respond to the signals. The ligation of the type I interferons with their receptor induces activation of the associated Janus kinases, Jak1 and Tyk2. This leads to phosphorylation of STAT1 (Tyr701) and STAT2 (Tyr689), which together with IRF9 form a transcription factor complex called the Interferon-stimulated gene factor 3 (**ISGF3**). ISGF3 translocates into the nucleus and binds to the **ISRE** (interferon stimulated response element) sequences. IRF9 directs the complex away from the traditional GAS element of STAT binding, and thus is responsible for the binding specificity. However, the DNA binding capacity of STAT1 and STAT2 are needed for optimal stabilization. Over two thousand type I interferon stimulated genes (**ISGs**) have been catalogued (Rusinova et al. 2013), including *DDX58* (RIG-I), *IFIH1* (MDA-5), *IRF7* and *STAT1*, which reinforce the antiviral defense mechanisms of the cells. However, the majority of the ISGs remain poorly characterized. A recent screen for the antiviral role of 389 selected ISGs against six viruses revealed that ISGs contain both broad acting and virus specific modulators. Nucleic acid binding, hydrolase, and helicase activities were the main molecular functions of the ISGs, whereas the most widely used mechanism of antiviral action was translational block (Schoggins et al. 2011). Many ISGs are associated with cell death machinery, as induction of cell death controls infection and pathogen spreading. For example, interferon induced IFIT2 promotes apoptosis by mitochondrial-associated BCL2 family proteins, and AIM2 inflammasome component has been shown to be central for host cell death by pyroptosis upon bacterial infection. However, these mechanisms are also detrimental to the host, leading to immune cell exhaustion, chronic inflammation and collateral tissue damage. Many viruses have evolved to encode proteins that interfere with the cell death pathways to promote the survival of the infected host cell. In contrast, some pathogens preferentially induce cell death as a means of spreading. For example, *Salmonella* induces rapid necroptosis of the phagocytosing macrophages to silence the immune response. (Malireddi and Kanneganti 2013). In addition to ISG transcription and cell death modulation, IFNα and β regulate the immune system at several levels. First of all, they induce proliferation of CD8+ T cells and activity of NK cells (20-100 fold). Additionally, HLA class I expression is induced through direct induction by ISGF3, as well as by upregulation of MHC I transcriptional activator NLRC5 (Neerincx et al. 2013), thus allowing effective recognition of infected cells by the adaptive CD8+ T lymphocytes. Enhanced HLA class I expression also prevents uninfected cells from being killed by the NK cells. (Murphy et al. 2012). As well as priming neutrophils (Martinelli et al. 2004), type I interferons activate DCs and macrophages, and thus induce the development of adaptive immunity, such as Th1 polarization (Cella et al. 2000). Type I

interferons also directly promote the polarization of Th1 cells, even from the established Th2 helper cell subset (Filen et al. 2010, Hegazy et al. 2010, Hibbert et al. 2003, Ray et al. 2014), and have been shown to counteract Th17 and Tfh polarization (Harrington et al. 2005, Ray et al. 2014). TLR ligation also prevents cDC and pDC migration into thymus, preventing induction of T cell mediated central tolerance to pathogens (Klein et al. 2014).

More recently discovered **type III interferons**, IFN-λ1-3 (also known as IL-29, IL-28a and IL-28b), have been shown to regulate antiviral responses through activation of the IFNLR receptor (a complex of IFN- λR1 and IL-10R2 subunits) that, similarly to IFNARs, is able to recruit STAT1 and 2, and induce the formation of the ISFG3 complex leading to induction of the ISGs and HLA class I. Although type III interferons can be expressed by any cell type, their IFNLR receptor is mainly expressed by the epithelial cells in the respiratory and gastrointestinal tract, and might therefore mainly contribute to the defense mechanisms at these sites. A recent study of mouse epithelial fibroblasts infected with influenza virus demonstrated IRF7-mediated induction of both type I and III interferons (Crotta et al. 2013). Using IFNAR1, IFN- λR1, or double knockout cells both type I and III interferons were shown to act redundantly, i.e. regulating identical set of ISGs. The authors suggested that complete redundancy may guarantee induction of antiviral defenses even if the virus blocks one of the pathways through virally encoded antagonists. The restricted expression of type III interferon receptor might allow differential regulation of epithelial vs. systemic immune response, mediated by type I interferons that are able to induce antiviral state throughout the body. (Crotta et al. 2013). Interestingly, high IFNLR expression was reported on human immune cells, with the highest expression observed on B cells, followed by T cells, whilst monocytes and NK cells had the lowest expression levels (Witte et al. 2009). Instead of responding to even high concentrations of type III interferons, immune cells were found to express a short secreted form of the IFNLR (sIFN- λR1, a splice variant lacking the transmembrane and intracellular domains) that was able to bind to type III interferons and inhibit the IFN-λ1 mediated upregulation of HLA class I of a liver cell line in a dose dependent manner *in vitro*. In contrast, a recent study reported high IFNLR expression on human mDCs and pDCs (Dolganiuc et al. 2012). Although alternative splicing was not explored, the use of siRNAs against IFN-λR1 confirmed that in the presence of IFN-λ1, IFN-λ2, or both, monocytes that were differentiated *in vitro* towards DCs acquired an IFNLR-mediated inhibitory DC phenotype. This displayed a diminished capacity to induce T cell proliferation, due to downregulation of IL-2 and IL-12, and an increase in IL-10 and inhibitory receptors PD1 and PD-L1. This environment preferentially promoted proliferation of Tregs, but not their *de novo* generation. Pretreatment of the T cells with type III interferons had no effect on proliferation. (Dolganiuc et al. 2012). Thus it seems that although some immune cells, such as lymphocytes, do not respond to type

III cytokines at least partly due to expression of a soluble inhibitory receptor, they might be indirectly affected by the DCs that do express functional IFNLR, and support Treg survival. Clearly, more studies are warranted for further understanding of the type III interferon signaling in the immune system.

Recently, the canonical type I/III interferon signaling pathway, leading from the interferon receptor to formation of the complex ISGF3 binding on ISRE elements, has been complicated by the accumulating data on heterogeneity of transcription factor complexes mediating the effects of interferons. For example, noncanonical Ser708 phosphorylation of STAT1 was shown to be important for inducing a subset of ISGs with a minimal ISRE element, such as *IFIT2*. Tyr701 (canonical phosphorylation site) was shown to precede Ser708 phosphorylation by IKKε, and the two phosphorylation sites were mutually exclusive, suggesting a temporally different set of target genes for the modified ISGF3. (Fink and Grandvaux 2013). Additionally, ChIP-chip analysis on IFNα-activated STAT1 and STAT2 has revealed sites that were bound by both molecules independently, suggesting that other complexes besides ISGF3 are able to regulate ISGs (Au-Yeung et al. 2013). There are several reports of unconventional ISG inducing complexes in different cell types, which include the unphosphorylated STAT1 dimer complexed with IRF1, STAT2-STAT6-IRF9 complexes, and STAT3/STAT2 as well as STAT6/STAT2 heterodimers, although these remain to be elucidated. (Au-Yeung et al. 2013, Fink and Grandvaux 2013).

2.1.3 Adaptive Immunity – B and T cells

The highly flexible recognition repertoire of the T and B lymphocytes is central to the adaptive immune system. The reactions of these cells are important when innate immunity, the first line of defence, fails or is exhausted and cannot clear the invading pathogens, although can take days to become fully activated. The adaptive immune cells also form the immunological memory, so that on the second encounter with the same pathogen the immune response will be activated more quickly through clonal expansion of memory T and B cells.

Opposed to the fixed, germline encoded selection of receptors expressed by the cells of the innate immune system, the antigen-specific receptors of both T and B lymphocytes are created through genomic recombination. In this process, the heavy and light chain immunoglobulin genes of the **BCR**, as well as α and β chain genes of the **TCR**, each consisting of several interchangeable gene segments, are randomly recombined together in order to form different gene combinations in a αβ T cell. In addition, a minority of the T cells will rearrange TCRγ and TCRδ chains, and become γδ **T cells** with a more restricted TCR profile. Genomic recombination creates the vast repertoire of receptors that are able to recognize any epitope presented to them. The obvious drawback of

creating millions of possible receptor options is that inevitably lymphocytes that are able to react against the body´s own self-antigens or with non-functional receptors will be produced. Therefore, during their maturation, lymphocytes that react with high affinity to self-antigens are removed by a process called negative selection, where autoreactive cells are induced to undergo programmed cell death. It is also possible that the created receptor is non-functional, and thus the proper signaling capacity must be ensured during the lymphocyte education; if a lymphocyte does not receive any signals through its receptor during the maturation phase, it is eliminated. This process is called positive selection, as it enriches only the lymphocytes able to rearrange a functional receptor. (Murphy et al. 2012).

B cell precursors are produced from a lymphoid precursor cell in the bone-marrow and predominantly finish their education process in the same compartment. Firstly, cell-membrane bound IgM immunoglobulin isotypes are transcribed and expressed on the surface of the naïve B cell. If the IgM receptor binds to a self-epitope expressed by the bone-marrow stromal cell, it is eliminated as a result of this negative selection. Otherwise the naïve B cell then migrates to the periphery, where it can be activated upon an encounter with its cognate antigen. In a peripheral lymphoid organ, a B cell can present the antigen captured by its immunoglobulin receptor in the context of the HLA class II molecule, which is then recognized by the Tfh cell. Tfh contact induces proliferation of the B cell, which is associated with an increased somatic hypermutation of the immunoglobulin hypervariable region, aiming towards creating further diversification of high affinity immunoglobulins. B cells with the highest affinity capture the most antigen, which allows them to outcompete lower affinity binding B cells for Tfh help, and are therefore preferentially selected for clonal expansion and somatic hypermutation. (Gitlin et al. 2014). Furthermore, the process of class switching increases immunoglobulin diversity. Here the original constant gene segment C_{μ} (**IgM**) or C_{δ} (**IgD**) of the immunoglobulin heavy chain is replaced by Cα, Cε, or Cγ, leading to expression of **IgA**, **IgE** and **IgG** immunoglobulin isoforms, respectively. Both the hypermutation and class switching processes are active throughout the lifespan of the B cell, and allow a single B cell clone to produce slightly different receptors. IgA, IgE and IgG are secreted molecules and therefore B cells are responsible for a major part of the humoral immunity in the immune system. After diversification, B cells can differentiate into large antibody-producing **plasma cells** or a long-lived memory cell. (Murphy et al. 2012).

T cell precursors formed in the bone marrow migrate in to the thymus, where their maturation takes place. Positive and negative selection of T cells occur in specialized niches of the thymus, i.e. thymic cortex and medulla respectively, ensuring their spatial and temporal segregation. In the thymic cortex the T cell rearranges its TCR β chain, which together with the pre-TCRα chain, expressed before the actual TCRα gene

rearrangement, forms the pre-TCR receptor ready to respond to signals provided by the cortex epithelial cells (cTecs). Passing this β selection checkpoint allows the developing T cell to enter into the next phase of differentiation, the double positive T cell stage, expressing CD4 and CD8 coreceptors. At this stage the T cell rearranges its TCR α chain to produce the functional TCR. Positive selection, e.g. low or medium affinity recognition of self-antigen-HLA complex presented by cortex epithelial cells, allows the T cell to differentiate into single positive, **CD4+** or **CD8+** cells. Single positive cells move to medulla, where the thymic epithelial cells of medulla (mTECs) are specialized in presenting the T cells with all possible self-antigens coded in the genome, due to high expression of transcription factor AIRE (autoimmune regulator) that allows expression of otherwise tissue restricted antigens. Both thymus resident and migratory DCs contribute to the antigen presentation: Resident cDC subsets pick up tissue-antigens produced by the mTECs for cross-presentation, whereas migratory cDCs and pDCs transfer peripheral antigens to be presented in the thymus. These cells mediate the process of negative selection, in which T cells receiving a high affinity signal through their TCR are induced for cell death, and in some cases, towards differentiation to T regulatory cells (nTregs, discussed in the next chapter). (Klein et al. 2014).

As opposed to the B cell receptors, the TCR can also recognize an epitope that is normally hidden or buried, as the antigen is first broken down into peptides, loaded on to the **HLA class I** or **II** molecule and then presented on the cell surface of antigen presenting cells. T cells with the CD4 coreceptor are referred to as T helper cells, which recognize antigens bound to the HLA class II molecules that usually bind peptides derived from intracellular vesicles. In the periphery HLA class II molecules are mainly expressed on phagocytic cells, and thus the CD4+ T cells interact with cells that present antigens picked up from the extracellular environment by phagocytosis or endocytosis, processed into peptides, and loaded onto HLA class II complex. In contrast, The CD8 molecule assists the TCR of the cytotoxic T lymphocytes to recognize the antigen in the context of the HLA class I molecules, which usually bind peptides derived from the cytosol. As HLA class I molecules are expressed by all autologous cells, CD8+ cytotoxic T cells mainly recognize cells infected with intracellular pathogens that present pathogen epitopes on HLA class I. Similarly to the innate NK cell, CD8+ T cells kill their target cell with secreted toxic molecules, such as perforin, released from their intracellular granules. (Murphy et al. 2012).

2.1.3.1 Multiple subsets of CD4+ T lymphocytes

The naïve CD4+ T helper cell precursor (Thp) has the capacity to differentiate into several functional subsets according to the environmental stimuli received through the transmembrane receptors. Primarily these stimuli are innate-derived cytokines secreted

by the antigen presenting cell or present in the lymphoid tissue environment, produced based on the type of pattern recognition receptors activated in the innate immune cells upon pathogen encounter. Both the signal strength of the TCR and the costimulatory environment dictate the net outcome. The seven T helper cell subtypes that have been named so far are classified based on the expression of a transcription factor(s) that modulates their transcriptional network, as well as secretion of a set of cytokines typical for each subset. Thus the nature of the specific pathogen is conveyed to CD4+ T cells through DCs and cytokine environment, subsequently launching pathogen-tailored adaptive immune response. (Kara et al. 2014, Murphy et al. 2012).

The division of CD4+ T helper subsets was initiated when Mossman et al. proposed that mouse T helper cells consisted of two functional subsets, T helper type 1 (**Th1**) and 2 (**Th2**) cells, based on their differing cytokine profiles (Mosmann and Coffman 1989). Th1 cell differentiation was discovered to be induced by IL-12 secreted by macrophages hosting *Listeria monocytogenes* (Hsieh et al. 1993) as well as by IL-12 *in vitro* (Wu et al. 1993). From the IL-12 receptor, signaling is mediated by the Signal transducer and activator of transcription 4 (STAT4) molecules. Activated STAT4 dimers translocate to the nucleus and induce the expression of the central transcription factor TBX21 (also known as T-bet, T-box expressed in T cells) (Thieu et al. 2008), which drives the expression of IFNγ, the key Th1 cytokine (Szabo et al. 2000), and upregulation of IL-12Rb2 (Afkarian et al. 2002). IFNγ through STAT1 signalling furthermore induces T-bet (Afkarian et al. 2002, Zhu et al. 2012), with IL-12 and IFN-γ working redundantly for T-bet expression (Zhu et al. 2012). Type I interferons and IL-27 enhance Th1 polarization (Hibbert et al. 2003, Lucas et al. 2003, Ray et al. 2014). Th1 cells are essential in mounting immune response against cells infected with intracellular bacteria, such as *Mycobacterium tuberculosis*, as well as viruses, through modulating CD8+ T cell and macrophage responses. In the context of the work presented in this Ph.D. thesis Th2 program will be discussed in more detail in the next chapter (2.1.3.2.).

Soon after the proposal of the Th1/Th2 dichotomy, T cells inhibiting antigen-specific T cell responses and autoimmunity were discovered (Chen et al. 1994, Groux et al. 1997, Sakaguchi et al. 1995, Sakaguchi et al. 2011). It is now well established that CD4+ T regulatory cells (**Treg**) are an essential part of the regulatory arm of the immune system in terminating the reactions of other T cell subtypes towards self-molecules, allergens, and commensals. They also play a role in the resolution of the inflammatory reactions. Usually, Treg cells express high amounts of IL-2 receptor (CD25), inhibitory receptor CTLA-4, anti-inflammatory cytokines IL-10 and TGF-β, as well as their signature transcription factor forkhead box P3 (FOXP3). Recently, mouse studies comparing Foxp3 binding and chromatin landscape of resting Tregs and Treg cells activated under *in vivo* inflammatory conditions revealed that in activated Treg cells Foxp3 predominantly

acts as a transcriptional repressor, responsible for establishment of suppressive heterochromatin-associated histone H3 trimethylation at Lys27 (H3K27me3) on several enhancers of effector cell genes, through interaction with methyltransferase Ezh2, which is upregulated in inflammatory conditions (Arvey et al. 2014). CTLA-4 is an important inhibitory receptor that binds CD80 and CD86, and thus inhibit the signaling to CD28 coreceptor of the T cells. Tregs can develop both in thymus (tTreg or natural Treg, nTreg), as discussed in the previous chapter, or in the periphery (pTreg, also called induced Treg, iTreg, or adaptive Treg, aTreg). Although the process is not clearly defined, extrathymic iTregs are generated from other T helper cell subtypes upon appropriate signals, such as suboptimal TCR stimulus, all-trans retinoic acid, TGFβ, and butyrate produced from dietary fiber by microbial fermentation in the colon (Arpaia et al. 2013, Furusawa et al. 2013), and specific commensal bacteria, such as Clostridia species (Atarashi et al. 2011, Atarashi et al. 2013). iTreg cells share characteristics of their precursors, such as the central transcription factors and homing receptors, which is important for efficient suppression of the preceding subtype. (Lehtimäki and Lahesmaa 2013, Povoleri et al. 2013). It is currently unclear whether the nTreg and iTreg subsets are functionally distinct or are defined by a separate TCR repertoire, as clear markers for identification of their site of origin have not been found. However, it has been suggested that nTregs guard central homeostasis and autoimmunity, whereas the iTregs mainly participate in the immune resolution of local inflammation. (Yadav et al. 2013). Amounting data also implies that there are different Treg subpopulations. For example, a Treg cell subset expressing a coinhibitory molecule TIGIT efficiently suppressed Th1 and Th17 cells, but was unable to counter regulate Th2 responses (Joller et al. 2014). This was dependent on TIGIT-ligation induced production of soluble Fgl2, previously shown to skew T helper cells towards the Th2 phenotype. Other types of inhibitory mechanisms utilized by Tregs include disruption of the APC-T cell contact, competition for IL-2, and cytotoxic enzymes, to name a few (Sojka et al. 2008).

The T follicular helper (**Tfh**) cell subset was named for their preferred location in the B cell follicles of the secondary lymphoid organs, where they migrate due to their high expression of chemokine receptor CXCR5 and loss of T-cell zone homing receptor CCR7, and for their support to B cell proliferation and expansion (Breitfeld et al. 2000, Schaerli et al. 2000). In addition to CXCR5, Tfh cells express a variety of signature receptors such as ICOS, CD40L, and PD-1 that are essential for their contact and signaling with the B cells. The signature cytokines of Tfh cells are IL-4, IL-21 and IL-10. Transcription factor Ascl2 was recently shown to initiate the Tfh differentiation process through binding to and inducing *Cxcr5*, and by directly downregulating *Ccr7*, *Il-2r*, as well as a set of Th1 and Th17 lineage genes, such as *Il12rb1*, *Ifng*, *Tbx21*, and *Ahr*. Overexpression of Ascl2 under Th2 conditions also reduced expression of classical Th2 genes, *Gata3*, *Il5*, and *Il13*. (Liu et al. 2014). Additionally, transcription factor Bcl6 is essential for Tfh

differentiation and functions, similarly to Ascl2, as a transcriptional repressor of other T cell lineage transcription factors *Gata3* and *Tbx21* (Johnston et al. 2009, Nurieva et al. 2009, Yu et al. 2009). Stat3 was found to be crucial in promoting Bcl6 expression and downmodulating the type I interferon promoted Th1 differentiation during acute viral infection (Ray et al. 2014). Although the initial differentiation of Tfh cells is driven by the DCs, the following B cell contact in the B cell follicles is thought to reinforce and finalize the Tfh phenotype, for example through upregulating Bcl6 and promoting Tfh survival. Sequential activation of Ascl2 and Bcl6 might provide essential signal checkpoints for amounting effective autoantibody response to infection but preventing autoimmunity (Liu et al. 2014). (Crotty 2011).

Following the discovery of the IL-23 cytokine sharing the same IL-12p40 molecule subunit as IL-12, it was found to be important driver of autoimmunity in a mouse EAE and arthritis models, which had previously been associated to Th1 cells and IL-12 activity (Cua et al. 2003, Murphy et al. 2003). This finding initiated the discovery of the fifth T helper cell subtype, named as **Th17** cells according to their signature cytokines IL-17A and IL-17F (Aggarwal et al. 2003, Harrington et al. 2005, Park et al. 2005). This subset is now established to function in defense against both intra and extracellular pathogens, including fungal pathogens such as *Candida albinos*, especially through recruitment of neutrophils (Kara et al. 2014). Th17 differentiation requires TGF-β, IL-6, and different combinations of additional cytokines (IL-21, IL-23, IL-1β) for efficient polarization. The concentration of TGFβ is important, whereby a low concentration, together with proinflammatory cytokines, was shown to upregulate Il-23R driving Th17 polarization, whereas a high concentration induced Foxp3 promoting Treg differentiation (Zhou et al. 2008). Upon Th17 cytokine stimulation, STAT3 is activated and translocates to the nucleus, and induces lineage-specific transcription factors RORγt (RORC), RORα, and Ahr, leading to upregulation of *Il17*, *Il17f*, *Il22*, and *Il23r* (Yang et al. 2008). Detailed transcriptomics investigation of mouse naïve CD4+ cells revealed Th17 differentiation, induced by TGF-β and IL-6, to be a highly dynamic process (Ciofani et al. 2012). Based on clustering analysis, early (0.5-4h), intermediate (4-20h), and late (20-72h) transcriptional phases were identified. Early phase included *Stat3*, *Irf4*, and *Batf* transcription factors. Batf and Irf4 modify the chromatin landscape accessible for further transcription regulators, whereas Stat3 defines lineage specificity. Together this core initiator set induces Rorc that upregulates the relatively small key Th17 gene set, and downregulates alternative lineage fates by suppressing *Il4ra*, *Il12rb2,* and *Tbx21*. (Ciofani et al. 2012). A kinetic transcriptomic analysis of human Th17 polarized CD4+ cells also considered the early (0.5-4h) and late (6-72h) expression phases, identifying several novel candidates for the regulation of Th17 response (Tuomela et al. 2012). IL-2 signaling through STAT5 inhibits Th17 differentiation (Laurence et al. 2007), and thus STAT3 and Ahr induce expression of *Ikzf3*, coding for transcription factor Aiolos

that binds and suppresses *Il2* promoter (Quintana et al. 2012). *In vivo* mouse models have demonstrated that the microbiome and especially segmented filamentous bacteria (SFB) drive the differentiation of the Th17 subtype, as this subtype is absent in the germ free mice and significantly reduced in SFB negative mice. Recently, SFB were identified to induce solely Th17 responses (Yang et al. 2014). The gut homing Th17 cells are essential for immune defenses and integrity in the mucosal tissues, and at the steady state majority of the Th17 cells are localized in the intestinal lymphoid tissues. The Th17 subtype also drives antigen-specific IgA production by the intestinal B cells upon pathogen challenge, through acquiring a Tfh-cell like phenotype and inducing B cell maturation and induction for somatic hypermutation (Hirota et al. 2013). In contrast to the Th17 cells differentiating in the periphery, existence of a thymic-derived natural Th17 population has been described. This population leaves the thymus fully polarized and secretes IL-17 in response to IL-23 and IL-1β without the need for TCR stimulation (Kim et al. 2011, Marks et al. 2009). The Th17 subtype has been associated with several autoimmune diseases, including RA, MS and CD.

The IL-9 secreting **Th9** subtype was found to be induced by an intricate balance of both IL-4 and TGFβ, where the Stat6-Gata3 pathway antagonizes Foxp3 induction, and TGFβ induced Foxp3 antagonizes Th2 cytokine induction by Gata3 (Dardalhon et al. 2008). IL-9 expression is controlled by the transcription factor Irf4 (Staudt et al. 2010) in co-operation with Batf (Jabeen et al. 2013). IL-9 has been associated with the development of both autoimmunity and atopic diseases. In addition to the IL-9 expressing ILC2 subset, the Th9 subset was shown to be essential for eradication of the parasitic worms *Nippostrongylus brasiliensis*, increasing the numbers of mast cells and basophils, which both express Il9ra (Licona-Limon et al. 2013). Interestingly, the highly transient IL-9 signals in the intestine and lungs preceded the Th2 cytokines, and IL-9 was needed for subsequent IL-5 and IL-13 responses, indicating temporarily distinct but interdependent activities of Th9 and Th2 subtypes in the immunity against helminthes, and possibly in induction of asthma and atopy. Many aspects of this subtype still remain poorly understood. (Schmitt et al. 2014).

Another recently characterized CD4+ subset named **Th22** cells have been described as T helper cells producing IL-22 and expressing skin and epithelial homing receptors CCR4, CCR6 and CCR10 (Duhen et al. 2009, Trifari et al. 2009). Accordingly, Th22 cells are found to be increased in the skin of patients with atopic dermatitis. Polarization is thought to require IL-6 and TNFα, and Ahr has been suggested to drive the Th22 lineage (Ramirez et al. 2010). Recently, IL-21 cytokine was shown to induce STAT3 signaling, leading to induction of *Il22*, *Rorc* and *Ahr*. STAT3 was shown to modulate the *Il22* promoter for binding of Ahr, which showed synergistic effects with both STAT3 and Rorγt. IL-21 stimulation also led to STAT3-dependent upregulation of IL-23R and IL-1R, and thus IL-22 induction

was greatest when IL-21 with IL-23 or IL-1β was used for polarization. T cell –specific deletion of Ahr and IL-21R were associated with more severe colitis, and reduction in intestinal IL-22 *in vivo* (Yeste et al. 2014). Finally, an *in vivo* study demonstrated a role for murine CD4+IL-17A-IL-22+ subtype in protection against *Citrobacter rodentium* infection, especially in the last phase of pathogen clearance (Basu et al. 2012).

Consideration of the number of T cell subtypes identified, with partially overlapping inducing cytokines and transcription factors, has raised questions of their interrelations and **plasticity**. For example, c-Maf is responsible for upregulating IL-21 both in Tfh and Th17 subtypes (Bauquet et al. 2009) and IL-4 in Th2 cells (Ho et al. 1996), and IRF4 that is needed for Th9 differentiation has also been reported to regulate Th2, Th17, Tfh and Treg programs (Ahyi et al. 2009, Huber et al. 2008). The transcriptional model of Th17 cells by Ciofani et al. (2012) suggested Batf and Irf4 to act as TCR-induced initiators of the transcriptional program, and such factors, probably including c-Maf, might be functioning in all Th cell subsets, in co-operation with more restricted transcription factors, such as in the case of Th9 (Jabeen et al. 2013). Additionally, an increasing amount of literature has reported Th cell subtypes with "mixed" identities, especially in inflammatory conditions, and particularly the Th17 subtype seems to display heterogeneity (Sundrud et al. 2003). Th1-like Th17 cells have been implicated to be pathogenic in a variety of autoimmune mouse models, such as in mouse models of arthritis (Nistala et al. 2010) and model of MS (experimentally induced autoimmune encephalomyelitis, EAE) (Hirota et al. 2011). This also extends to humans: CCR6+CXCR3+ T cells are capable of producing IL-17A, IFNγ, or both cytokines simultaneously, and these Th1-like Th17 cells have been found in patients with autoimmune diseases, such as RA and MS (Cosmi et al. 2011, Kebir et al. 2009). Recently, IFNγ production in Th17 cells was shown to require IL-12 mediated co-operative Tbet and Runx1 activity on the *Ifng* promoter (Wang et al. 2014). Cells with Th17 and Th2 cell properties have been characterized in asthma patients and mouse models of asthma (Cosmi et al. 2010, Wang et al. 2010). Additionally, Th17 conversion into Tfh-like cells in the lymphoid tissues of the murine small intestine upon pathogen challenge has been reported (Hirota et al. 2013). Tregs have also been demonstrated to display plasticity. Fate-mapping experiments of Foxp3-expressing cells demonstrated that a substantial part of Treg cells that once expressed Foxp3 become Foxp3 negative under normal conditions, expressing IFNγ or IL-17A. However, the conversion increased in the pancreas of NOD mouse with concomitant decrease in CD25 expression and increase in IFNγ, indicating enhanced Treg instability in the inflammatory environment. (Zhou et al. 2009). In EAE mice, antigen-specific autoreactive Treg cells downregulated Foxp3 expression and secreted IFNγ, and adoptive transfer of these exFoxp3 Th1 cells was able to induce EAE upon immunization to the levels equivalent with conventional T cells (Bailey-Bucktrout et al. 2013). Similarly, in mice with collagen-induced arthritis CD25^{lo}Foxp3+ Treg cells are

prone to differentiate into exFoxp3 Th17 cells, expressing Ccr6 and IL-17. These cells were highly pathogenic, as they accelerated arthritis more efficiently than effector or memory T cells upon adoptive transfer. (Komatsu et al. 2014). In the EAE model, Foxp3 stability was maintained by IL-2 receptor signal, whereas in the arthritis model Treg to Th17 conversion was induced with IL-6 produced by the synovial fibroblasts. IL-17 secreting Tregs, expressing both FOXP3 and RORγt, have also been identified in human peripheral blood and lymphoid tissues (Voo et al. 2009). Therefore, the local cytokine milieu is important not only for initial differentiation signals but also for maintaining established T effector cell populations, and perturbations upon inflammatory conditions might contribute to the plasticity and conversion into alternative lineages or mixed identities. Plasticity could also be an intrinsic preprogrammed property of some cells from the beginning. Sharma et al. (2013) studied transformation of a subset of Foxp3+ Treg cells into T helper cells responsible for activating resting DCs in a vaccination or tumor environment, and noticed that the transformation was accompanied with downregulation of the corepressor Eos. Two populations of "Eos-stable" and "Eos-labile" Tregs could already be identified by specific cell surface markers, both in the thymus and periphery, before inflammatory conditions were induced. Eos-labile Tregs turned to helper Treg cells upon activation by inflammatory DCs secreting IL-6, and IL-6 expression in the thymus during Treg differentiation was shown to be required for the development of the Eos-labile Treg population. Despite of their ability to provide DC help, Eos-labile cells also maintained effector cell suppressor activity. Reprogrammed Tregs provided help very rapidly when a new antigen was presented, and this was suggested to be crucial at the time when conventional T helper cell response had not yet been activated (Sharma et al. 2013). Thus, allowing more plasticity in the T helper cell responses, instead of rigid and fixed states, might increase protection of the host upon pathogen challenge. For example, it was recently demonstrated that differentiated human Th1, Th2, and Th17 subsets respond very differently to IFNα stimulus, inducing a different set of ISGs upon exposure, and subsequently Th1 subset was protected from, Th2 subset compromised, and Th17 subset susceptible for HIV infection (Touzot et al. 2014). Due to this plasticity, it has been suggested that instead of defining rigid boundaries between the terminally "fixed" T helper cell phenotypes, these cells should be considered as a continuum of different plastic fates, highly modulated by the surrounding stimuli (Okoye and Wilson 2011). Although the boundaries are getting blurrier, increasing information will allow a more thorough understanding of immune-mediated diseases and development of novel therapeutic opportunities (Hirahara et al. 2013)

2.1.3.2 T helper cell type 2 development

Early on, Th2 cells and their cytokines were associated to B cell Ig, especially IgE, isotype switching, parasite infections, such as *Leishmania major* and *Toxora canis*, as

well as atopy, and all of these early statements hold true today (Del Prete et al. 1991, Liew et al. 1990, Liew et al. 1990, Liew 2002, Mosmann et al. 1986, Wierenga et al. 1990). It was quickly established, that IL-4 is needed for the development of the Th2 subset (Kopf et al. 1993, Le Gros et al. 1990, Swain et al. 1990). The source for IL-4 has puzzled investigators since the beginning, and DCs, basophils, Tfh cells, γδT cells and NKT cells have been suggested to secrete IL-4 for Th2 polarization. In addition, naïve T cells produce IL-4 upon activation (Noben-Trauth et al. 2000). It was demonstrated recently that initiation of peanut-induced intestinal allergic response relied solely on paracrine IL-4 produced by other intestinal CD4+ T helper cells, and was further reinforced through autocrine loop induced by OX40L expressed by the DCs. In contrast, innate-like lymphocytes, such as NK cells, γδ T cells and ILCs, were totally dispensable for the initiation of Th2 immunity. (Chu et al. 2014).

Naïve T cells express IL-4R, and binding of IL-4 to its receptor leads to activation of Jak1 and Jak3 kinases, and phosphorylation of the signal transducer and activator of transcription protein 6 (STAT6). Stat6 has been shown to be essential for the IL-4-driven Th2 cell phenotype in mouse (Kaplan et al. 1996, Shimoda et al. 1996, Takeda et al. 1996, Zhu et al. 2001). The phosphorylated Stat6 forms a homodimer and translocates into the nucleus, where it binds to DNA sequences with STAT consensus motif 5´-TTC(N)2-4GAA-3´, thereby regulating the transcription of its target genes. Stat6 interacts with transcriptional machinery, such as the CBP and p300 proteins, and p100 adaptor protein was shown to mediate this interaction and enhance transcriptional activity of Stat6. (Hebenstreit et al. 2006). Stat6 induces the expression of the Th2 master transcriptional regulator Gata3, that then positively regulates the transcription of the key cytokines IL-4, IL-5 and IL-13, all situated in the same Th2 cytokine locus on chromosome 5 in humans and chromosome 11 in mice. (Zhang et al. 1997, Zheng and Flavell 1997). Gata3 was shown to be essential for Th2 induction, as deletion of Gata3 led to impairment in IL-4 production and a total block in IL-13 and IL-5 production. Instead, Gata3 deficient cells acquired a Th1 phenotype in the absence of Th1 polarizing cytokines, indicating that Gata3 is essential in downregulation of the alternative Th1 program in Th2 cells. (Zhu et al. 2004). Later discoveries revealed that the suppressive function of Gata3 against Runx3 led to the downregulation of Th1 genes *Stat4*, *Il12rb2*, and *Ifng* in Th2 polarized cells (Yagi et al. 2010).

Genome wide identification of Stat6 target genes using knockout mice revealed that Stat6 mediates upregulation of 16 genes and downregulation of 20 genes in Th2 cells. However, these represented only 14% of the 117 genes identified to be regulated by IL-4 (Chen et al. 2003). Discovery of IL-4 mediated genes in human naïve T cells revealed the response to be rapid, as 65 genes were regulated by IL-4 already at 2h, and 153 genes by 48h. The response was also temporal, as only 5% of genes were discovered to be

differentially regulated throughout the timepoints (2, 6, and 48h) investigated. Silencing STAT6 by shRNA revealed that *GATA3*, *ZNF443*, *DACT1*, and three ESTs are regulated in STAT6-dependent manner in human Th2 cells. (Lund et al. 2007). Wei et al. (2010) investigated Stat6 binding in murine Th2 cells and discovered 4136 Stat6 bound genes, out of which 1200 (29%) were associated with Stat6-induced expression changes. On the genome-wide level Stat6 binding to intergenic sites dominated (59% binding sites), indicating binding to distal enhancers or unknown promoters. Stat6 was additionally shown to regulate the epigenetic landscape of histone modification distribution, when compared to Stat6 deficient mice. In particular, it was shown to antagonize the repressive H3K27me3 histone mark on its target genes, including the major Th2 lineage genes *Il4*, *Gata3*, *Il24* and *Il4ra*, whereas Stat6 binding did not promote the presence of active H3K4me3 mark. (Wei et al. 2010). Further support of the involvement of epigenetic mechanisms in Th2 differentiation came from a study reporting a silencing pathway involving Suv39h1 histone methylase to be activated in polarizing Th2 cells, mediating suppressive trimethylation of histone H3 on lysine 9 (H3K9me3) on the promoter of *Ifng*, thus leading to binding of heterochromatin-associated HP1α and transcriptional silencing. Suv39h1 knockout Th2 cells showed an increase in active histone mark H3K9ac on *Ifng* and *Tbx21* promoters, and were highly susceptible for Th1 reprogramming *in vitro* and *in vivo* (Allan et al. 2012). Hawkins et al. (2013) determined genome-wide histone maps of human Th1 and Th2 polarized cells to find lineage specific enhancers (H3K4me1+ and HK3K4me3-), identifying 2753 Th2-specific enhancers at 72h, out of which 847 were active (H3K27ac+). Th2-specific enhancers were enriched for binding sites of STAT6, PPARG, GATA3, NFIL3, GFI1 and BACH. STAT6 binding on enhancers of six genes, including *RUNX1* and *IL10RA*, were confirmed by STAT6 ChIP-qPCR, revealing appearance of enhancer marks and STAT6 binding already at the 4h timepoint. Enhancers were also overlayed with known immune-mediated disease SNPs. For example, in a Th2 specific enhancer predicted to regulate *IL4R* gene, an asthma linked SNP rs1805012 overlapped with NF-κB binding motif, presumably altering DNA binding capability of NF-κB. (Hawkins et al. 2013).

Kanhere et al. (2012) performed T-bet ChIP-seq in human Th1 cells, as well as GATA3 Chip-seq in human Th1 and Th2 polarized cells. In Th2 cells GATA3 bound to genes with Th2-specific expression and the GATA motif in their locus. Of the Th2-specific GATA3-bound genes only 5.6% were bound by T-bet in Th1 cells. In Th1 cells GATA3 binding was redistributed to overlap with the genes with T-box sequence motif and Th1-specific expression profile. Half of the Th1-specific GATA3 binding sites were also bound by T-bet, perhaps indicating that lineage specificity and/or plasticity in Th1 cells can be established by T-bet sequestering GATA3 away from the Th2-specific sites. Overall, only 12% of GATA3 or T-bet binding sites overlapped with known promoters, and thus 88% were distal regulatory sites. Five or more GATA3 binding sites were found

in 295 genes in Th2 cells, overrepresented in functions of transcriptional regulation and immune system process. Experiments in murine Gata3 deficient cells revealed that loss of Gata3 had no effect on the expression levels of genes bound by Gata3 only in proximal sites, but in contrast the genes bound by Gata3 also in distal sites were five times more likely to depend on Gata3 for their expression. Indeed, extensive Th2-preferred GATA3 binding was discovered in genes such as *IL2*, *IL9*, *IL7*, and *STAT5B*. Th2 marker genes such as *IL4* and *IL13* were bound by both T-bet and GATA3 in both subsets (Kanhere et al. 2012).

Beyond the canonical IL-4 induced STAT6 signalling pathway leading to STAT6 and GATA3 -mediated transcriptional regulation, other IL-4 and STAT6 dependent and independent mechanism have demonstrated to induce Th2 differentiation. Constitutively active Stat5a was enough to induce Th2 polarization in naïve T cells (Zhu et al. 2003), and IL-2 –induced activation of Stat5a was shown to contribute to the Th2 phenotype (Cote-Sierra et al. 2004). Genome-wide ChIP-seq analysis of Stat5a and Stat5b binding sites in Th2 cells demonstrated increasing binding with further rounds of Th2 polarization in Th2 cytokine locus, *Il4ra*, *Maf*, and *Gata3* genes. The authors also demonstrated that IL-7 could induce Stat5 activation in both resting and activated cells, whereas IL-15 could activate Stat5 in activated cells only, as resting cells do not express IL-15R. Therefore, Stat5 activity in Th2 cells might be driven by multiple cytokines, depending on their activation status. (Liao et al. 2008). In addition to Stat5, Stat3 was found to be activated during Th2 differentiation, and cooperate with Stat6 in binding to Th2 cytokine genes (Stritesky et al. 2011). Similarly to Th17 cells, Stat3 bound *Maf*, *Batf*, *Irf4*, *Il21*, and *Gata3* in Th2 cells, but binding to *Il17a* and *Il17f* was greatly reduced. Stat6 bound to *Maf*, *Batf*, *Irf4* and *Gata3* loci in a Stat3-dependent manner, as Stat6 binding to these sites was abolished in the absence of Stat3. Therefore in the presence of both Stat3 and Stat6 signals, the pro-Th17 capacity of Stat3 is diminished, and pro-Th2 capacity of Stat6 is amplified. (Stritesky et al. 2011). Additionally, a low strength of TCR activation is known to favor Th2 responses. For example, components of *Schistoma mansoni* egg antigens, such as omega 1, inhibit DC activation leading to reduced signaling to T helper cells and Th2 differentiation. Also, basophils as antigen presenting cells with lower HLA class II expression are thought to favor Th2 differentiation. Additionally, signals from Notch, Wnt and mTorc2 pathways contribute to the Th2 polarization process. (Paul and Zhu 2010).

As the IL-4 producing Th2 subset was originally described as the initiator of B cell class switching and plasma cell differentiation, identification of the IL-4 producing Tfh subtype as the major CD4+ T cell subset engaging with B cells in the germinal centres has challenged the traditional view. IL-13 can also be produced by Th1 and Th17 cells (Gallo et al. 2012). Therefore it has been suggested, that the IL-5 producing cell might

be the most differentiated state of the Th2 cell. (Hirahara et al. 2013). However, the Th2 phenotype plasticity might offer some explanation for the discrepancies: Th2 cells can acquire Tfh-like phenotype both *in vivo* and *in vitro*, and Mari et al. demonstrated that B cell help provided by Tfh-like Th2 cells, expressing Tfh markers Bcl6, c-Maf and IL-21, was restricted to Th2 polarized cells with phosphorylated Stat3, which is activated by IL-6 and IL-10, that are induced during Th2 polarization (Mari et al. 2013). Additionally, in the literature, Th2 cells have been linked with the Th1 (Hegazy et al. 2010), Th17 (Cosmi et al. 2010, Wang et al. 2010) and Th9 (Veldhoen et al. 2008) phenotypes. Clearly, the latest reports suggest that the Th2 phenotype is more plastic than previously appreciated, and that the Th2 cell differentiation pathway is intricately linked with the other T helper cell lineages, and able to shift according to the pathogen and surrounding stimuli.

2.2 Immune-mediated diseases

The world-wide incidence of immune-mediated diseases, involving several layers of adaptive and immune system, is rising, increasing costs for the society surpassing the costs of cancer treatment. **Autoimmunity** is characterized as a loss of tolerance of the adaptive immune system towards self-antigens or towards commensal bacteria. This could be due to defective negative selection of autoreactive cells in the thymus, or impaired function of the regulatory cells in the periphery. Molecular mimicry between pathogens and autologous antigens could initiate cross reaction towards self-structures. Inflammation is thought to contribute to the activation of autoreactive T cells, as in such an environment many of the normal checkpoint mechanisms against self-recognition become relaxed. Therefore, failure in the clearance of microbes or damaged cells, due to suboptimal activity of the complement or of phagocytic cells, could bring about an environment where proinflammatory cytokines from the innate immune system allow APC activation and efficient T cell activation even when presenting a self-antigen. Also a sustained concentration of an antigen maintains tolerance, and therefore a sudden increase in the self-antigen might alarm especially the receptors of naïve lymphocytes. (Murphy et al. 2012). The American Autoimmune Related Diseases Association (AARDA) catalogues more than 150 autoimmune diseases, stating that over 50 million Americans have at least one autoimmune disease (www.aarda.org). In the context of the work undertaken and presented in this Ph.D. thesis, current knowledge on one of the most common autoimmune diseases, type 1 diabetes (T1D), will be discussed in the next chapter.

Atopy is defined as a predisposition toward developing IgE sensitization towards non-infectious, harmless agents collectively called as allergens, and includes a variety of disease phenotypes such as rhinitis, eczema and asthma. Allergens include a great

variety of environmental substances, such as pollen, nuts, medicines, and metals. Allergic reactions typically involve DCs presenting the allergen, leading to Th2 cell polarization and activation. IL-4 and other cytokines lead to B cell activation and IgE production, and eventually mast cell granulation and release of histamine, leukotrienes and prostaglandins. These mechanisms cause mucus production, swelling, itching, and diarrhea, which are all mechanisms thought to contribute to expelling parasites. Th2-associated cytokines are also involved in tissue repair after parasite destruction, minimizing the number of infiltrating parasites (Allen and Wynn 2011). Therefore, allergy is viewed as a misdirected response of the immune machinery normally responsible for protection against multicellular parasites, such as helminthes and ticks. Opposed to this traditional dogma, Palm and colleagues have recently suggested that allergic immunity is actually intentional and beneficial, as it has evolved to neutralize not only parasitic worms but also small, tissue-damaging chemical compounds, such as toxins and insect venom (Palm et al. 2012). According to their theory, there is no evolutional explanation for the rapidity of the response (within minutes after exposure) or diversity and prevalence of allergens with no unifying chemical structure or properties, if the Th2 responses were merely targeted against slowly replicating parasites and their structural components. This alternative theory for the Th2 evolution remains to be discussed, as opponents have pointed out the lack of definitive evidence of IgE-dependent protection against toxins, as well as the missing proof of poor protection against tissue-damaging toxins in non-allergic individuals (Artis et al. 2012).

Asthma is characterized as a chronic inflammation of the airways in the lung. Symptoms include wheezing, coughing, excess mucus production, and shortness of breath. Asthma is a highly heterogeneous disease and is often categorized in subtypes based on the age of onset (childhood asthma, late-onset asthma), trigger for the symptoms (atopic, allergic, infection, or exercise), the stage of inflammation (acute, chronic), or other parameters (high total IgE, eosinophilia in the airways). Similarly to allergy, Th2 cytokines are thought to initiate the cascade in the lungs. Lately it has been implied that this is not always the case. For example, a study by Woodruff et al. (2009) divided asthma patients into "Th2-high" and "Th2-low" categories based on the high vs. normal ratio of Th2:Th1 cytokines in bronchial biopsies. Patients with "Th2-high" asthma had increased airway hyperresponsiveness, higher serum IgE levels, increased peripheral blood and airway eosinophil counts, and airway remodelling, compared to the patients with "Th2-low" asthma. Both "Th2-high" and "Th2-low" group had increased allergen skin prick test reactivity, when compared to healthy controls. Interestingly, corticosteroid treatment was effective only in the "Th2-high" group. There is also evidence of involvement of other cell types in subtypes of asthma. It has been observed, that the patients not responding to steroids might instead have an activated IL-17 response in the lungs. In particular the role of IL-17F has been implied, as high concentrations of this cytokine are detected in

BAL of asthmatic patients upon allergen challenge, and in the serum of asthma patients. As bronchial epithelial cells, Th17 cells, basophils, mast cells, CD8+ T cells, NK cells, γδ T cells, and LTi cells are all capable of producing the cytokine IL-17F, the cellular source is still unclear. IL-17F has been implicated in various processes characteristic of severe asthma, such as recruitment of neutrophils and macrophages into the lung, goblet cell hyperplasia, mucus production, and airway hyperreactivity. Also, IL-9 is involved in the development of allergic inflammation in the lungs, suggesting involvement of the Th9 cell subset. (Lloyd and Hessel 2010).

Atopy and asthma are multifactorial diseases influenced by both genetic and environmental factors. Depending on the end point studied (eczema, allergic sensitization, types of asthma), different genes have been shown to be associated with these diseases. For example, SNPs in or near *TLR6*, *C11orf30*, *STAT6*, *SLC25A46*, *HLA-DQB1*, *IL1RL1*, *LPP*, *MYC*, *IL2* and *HLA-B* were associated with allergic sensitization in a recent meta-analysis (Bønnelykke et al. 2013). These still explain only 25% of the heritability, which tells us that the majority of the genetic contribution is still unidentified. Novel approaches, such as a recent study identifying asthma genes that interact *in utero* and with childhood tobacco smoke exposure (Scholtens et al. 2014), are needed to identify novel candidates contributing to the heritability. Additionally, epigenetic changes are more easily modified by the environmental factors, and might offer an explanation for "failure" of the genetic studies. A recent preliminary study explored expression and methylation profiles of CD4+ T cells from eight patients with seasonal allergic rhinitis to those of eight healthy controls (Nestor et al. 2014). Data revealed that where mRNA expression profiling of CD4+ T cells was a poor classifier, the overall methylation profiles were able to separate the patients from the healthy controls, both during and outside of the pollen season. A variety of environmental factors, such as diet, levels of vitamin D, an altered balance of the gut microbiome, and viral infections have been associated with allergies and asthma, and will be discussed in more detail later (chapter 2.2.2.). Additionally, yet to be identified cellular processes may contribute to the pathogenesis. Recently a negative association between telomere length of peripheral leukocytes and asthma was reported, suggesting involvement of mechanisms promoting cell senescence (Albrecht et al. 2014).

2.2.1 Type 1 diabetes

Type 1 diabetes is caused by the immunological destruction of the insulin-producing beta cells in the pancreas, resulting in an inability to maintain glucose homeostasis. The onset usually occurs before 15 years of age, and thus T1D is one of the most prevalent chronic childhood diseases in the westernized world. Clinical symptoms include thirst, urination and weight loss, which left unnoticed can lead to acute diabetic ketoacidosis. Treatment of T1D requires daily injections of exogenous insulin to balance the glucose

homeostasis, and well balanced glycemic control reduces the risk for secondary complications, such as retinopathy and nephropathy (Aiello and DCCT/EDIC Research Group 2014, Martin et al. 2014). The incidence of T1D varies between geographical location and countries, and is a record-high in Finland (peak incidence 64.9:100 000 in 2006, Harjutsalo et al. 2013). Its incidence continues to grow with an annual rise of around 4%, with the greatest increase observed in the children under 5 years of age (Patterson et al. 2009). However, a plateau has recently been observed in the countries with highest incidence, Sweden (2005-2007, Berhan et al. 2011) and Finland (2005-2011 Harjutsalo et al. 2013).

T1D is a classical multifactorial disease, influenced by both genetic susceptibility and triggering environmental factors. The concordance rate in monozygotic twins is under 40%, but is also dependent on the age of onset, with an estimated 50% T1D risk for the twins of index cases diagnosed at the age of 9 or younger (Redondo et al. 2001). The HLA class II genes confer around 50% of the genetic risk, and thus the high risk haplotypes encoding DR3-DQ2 *(DRB1*03-DQA1*0501-DQB1*0201)* and DR4-DQ8 *(DRB1*0401-DQA1*0301-DQB1*0302)* are screened to identify individuals at high or increased risk for developing T1D. However, the molecular mechanism linking the high risk haplotypes with the immunopathogenesis is unclear. As HLA class II molecules are involved in antigen presentation to CD4+ T cells, the haplotypes associated with increased risk are thought to more efficiently bind and present T1D triggering peptides, or alternatively shift the balance of Treg vs. effector cell induction. (Howell 2014). In addition to HLA, the candidate gene approach has led to the discovery of polymorphisms in *INS*, *CTLA4*, *PTPN22*, *IL2RA* and *IFIH1* genes. The *INS* polymorphism regulates insulin expression in the thymic epithelial cells, probably contributing to the effectiveness of negative selection, whereas CTLA4 and CD25 are essential in Treg function, contributing to peripheral tolerance. The PTPN22 phosphatase variant downregulates both BCR and TCR signaling, as well as neutrophil activation (Arechiga et al. 2009, Bayley et al. 2014, Vang et al. 2005). *IFIH1* codes for the viral pattern recognition receptor MDA5 that is an essential viral pattern recognition receptor. After the launch of GWA studies, the number of associated loci has risen to 59, many of the associated genes being novel and without known function. It is now appreciated that many of the novel candidates are related to the biology of the β cells, such as GLIS3 modulating β susceptibility to apoptosis (Nogueira et al. 2013). As many of the GWA studies have been performed on SNP arrays designed to capture the variation in populations of European ancestry, newly designed approaches in other populations as well as the next generation sequencing platforms are speeding up the identification of causative gene variants in T1D and the role of the coding and noncoding RNAs in the disease process. (Bakay et al. 2013). The contribution environmental factors has also been vigorously studied. Diet, such as early exposure to foreign complex proteins (Knip et al. 2010) and vitamin

D deficiency, has been associated with development of T1D, and viral infections are believed to trigger autoimmunity (Laitinen et al. 2013, Lönnrot et al. 2000, Oikarinen et al. 2011). Introduction to solid foods early (<4 months) or late (≥6 months) in infancy increases risk for T1D later in life (Frederiksen et al. 2013). The prevalence of T1D is also associated with a westernized lifestyle and the level of hygiene (Patterson et al. 2009). These aspects will be discussed in more detail in the following chapter (2.2.2.).

According to the prevailing dogma, only 20% of the beta cells remain at the time of diagnosis. However, a meta-analysis of histopathological data from 105 T1D patients demonstrated that beta cell reduction correlated with age, the youngest having the greatest degree of beta cell loss: 85% reduction in beta cell mass in infants led to hyperglycemia, whereas only 40% was sufficient for the appearance of clinical symptoms at 20 years of age. This indicated that the threshold for symptoms is dependent on a dynamic balance of insulin production capacity (beta cell mass) and insulin demand (body weight). (Klinke 2008). In addition to the beta cell loss, another histopathological hallmark of T1D is insulitis, i.e. infiltration of inflammatory cells in the islets. Compared to the aggressive and random insulitis observed in the diabetic model NOD mouse, the T1D-associated insulitis in human pancreases was attenuated: insulitis affected less than 10% of the islets in organ donors with multiple T1D-associated autoantibodies (In't Veld et al. 2007). In children recently diagnosed with T1D, the early stage of insulitis was dominated by $CD8^+$ T cell infiltration, followed by the $CD68^+$ macrophages (Willcox et al. 2009). $CD20^+$ B cells were present in the islets where the beta cell loss was more advanced. $CD4^+$ T cell represented the least abundant population, and $FOXP3^+$ Tregs or NK cells were detected only rarely. In the insulin-deficient islets with complete beta cell loss, the number of all immune cells decreases greatly, suggesting that recruitment stimulus for insulitis is originating from the β cells. (Willcox et al. 2009). T1D has been associated with islet HLA class I hyperexpression (Foulis et al. 1987). Pancreatic stainings of HLA class I, CD8+ cell insulitis, and β cell specific CD8+ cells (tetramer stainings) showed, that all of these signs were absent in autoantibody positive subjects, and only found in islets of diagnosed T1D patients (Coppieters et al. 2012). These markers peaked in patients with disease duration under 1 year, and then gradually declined. HLA class I expression, β cell loss, and insulitis showed lobular, variable pattern, and HLA class I positivity did not require presence of insulin or inflammation on the individual islet level. (Coppieters et al. 2012).

β cells are among the most highly specialized cells in the human body, and therefore many proteins are expressed preferentially or exclusively in this cell type. Major T1D-specific **autoantigens** confirmed so far include insulin (also the prefolded preproinsulin), insulinoma-associated antigens 2 (IA-2 and IA-2β), glutamic acid decarboxylase 65 (GAD65), and Zinc transporter 8 (ZnT8). Autoantibodies against these proteins can be

measured years before clinical diagnosis, and seroconversion for two or more confers a greatly increased risk for T1D (70% in the next ten years) (Ziegler et al. 2013). Additional autoantigens include for example islet amyloid polypeptide (IAPP), chromogranin A (ChrA), and PDX1, and more are likely to be discovered. It is thought that upon initiation of the β cell death, further antigens are exposed, leading to an increasing number of targeted autoantigens, e.g. epitope spreading. Interestingly, most T1D autoantigens identified so far are proteins that are at some level associated to the insulin secretory pathway and granules, and are under translational control of glucose due to the response element in the 5`-UTR of their mRNA. Additionally, similarly to GAD65 that produces neurotransmitter GABA, novel β cell autoantigens expressed more widely in the neuroexocrine tissues have been identified, including ICA69 involved in neuroendocrine secretion, and peripherin found throughout the endocrine tissues, suggesting that the pancreatic nervous system tissue might also be targeted along the β cells. (Arvan et al. 2012). The loss of tolerance towards β cell autoantigens is still a mystery, but several mechanisms have been suggested. GAD65 and Znt8A are expressed at a low level in thymic mTECs, which could compromise negative selection (unpublished observations referred to by Klein et al. 2014). As already mentioned for the insulin gene, a VNTR polymorphism on the insulin promoter affects its expression levels specifically in thymus but not in β cells. Additionally, post-translational modifications missing in mTECs but present in β cells, creating neoantigens, might lead to loss of tolerance. Alternatively, misfolded proteins produced due to β cell ER stress could contribute to the production of neoantigens. (Klein et al. 2014). Diabetogenic T cells recognizing modified deaminated chromogranin A peptides are found in the NOD mouse (Delong et al. 2012). Additionally, deamination of number of β cell antigen peptides were shown to improve their binding potency to high-risk associated HLA-DQ8 molecules. T cells from T1D patients reacted more towards deaminated proinsulin peptide (68% vs. 37% in healthy controls) than native peptide (41% vs. 21%), and predominantly with IFNγ whereas controls responded with IL-10. (van Lummel et al. 2014). All of these mechanisms could lead to a vicious cycle where immune cells initiate the β cell loss, leading to a compensatory mechanism for the remaining β cells producing more insulin, leading to ER stress, neoantigen production, epitope spreading, and eventually cell death (Arvan et al. 2012).

Although not the major cell type in insulitis, the strong genetic association of HLA class II haplotypes implies that **CD4+ T cells** have a central role in the T1D pathogenesis. The literature on the role of Th1, Th17 and Treg subtypes in human T1D pathogenesis is controversial. In support of the Th1-driven model of T1D, circulating beta cell antigen -specific CD4+ T cells producing IFNγ can be identified in T1D patients. For example, a recent study reported, that both T1D patients and healthy adults have ZnT8-specific CD4+ cells, but in T1D patients these T cells secrete predominantly Th1 cytokine IFNγ, while Th2 and IL-10-producing cells were prevalent in healthy adults (Chujo et al. 2013). CD4+

cells from patients with established T1D were observed to express more Th1 cytokines IFNγ and TNFα, and demonstrated an increased Th1/Treg ratio (Du et al. 2013). In contrast to the Th1 hypothesis, 50% of recently T1D-diagnosed children had an increase in IL-17 expressing memory CD4+ cells in the peripheral blood (Honkanen et al. 2010). However, a recent study reported comparable levels of blood Th17 cells in patients and controls (Du et al. 2013). Site-specific alterations may provide an explanation for these discordant findings, as a pancreatic draining lymph node -specific increase of the Th17 population, not visible in peripheral blood, was reported in patients with established T1D (Ferraro et al. 2011). Contrasting results might also be explained by the degree of plasticity in the Th17 lineage: islet-antigen specific Th17 caused T1D in the NOD/SCID recipient mice almost as effectively as adoptively transferred Th1 cells. However, this was due to the Th17 cell conversion into Th1-like Th17 cells, that upregulated T-bet and secreted IFNγ, particularly in the pancreatic draining lymph nodes. (Bending et al. 2009). The signatures of both Th1 (*TBX21*, *IFNG*) and Th17 (*RORC*, *IL17A*) cells were found in the purified islets of a recently diagnosed T1D patient, and IL17 was found to exacerbate the apoptotic effect of TNFα, IL-1β and IFNγ in cultured human β cells (Arif et al. 2011). Regarding the regulatory T cells and their central role in maintaining peripheral tolerance, studies from peripheral blood have shown no T1D-associated anomalies in Treg frequencies (Du et al. 2013). On the contrary, pancreatic draining lymph node -specific downregulation of FOXP3 and CD25 in Tregs was observed in T1D patients, manifested by an impaired or lost suppressive activity (Ferraro et al. 2011). Enhanced Treg plasticity might also play a role, as FOXP3$^+$ Treg cells producing IFNγ were elevated in human T1D patients (McClymont et al. 2011). FOXP3$^+$ Treg cells from T1D patients were shown to be antigen experienced and activated, i.e. have the CD45RO$^+$ memory cell phenotype, and express more Th1 cytokines, IFNγ and TNFα (Du et al. 2013). Using fate-mapping, similar Treg conversion into IFNγ producing cells has been seen in the pancreas of NOD mice (Zhou et al. 2009). In conclusion, although T1D is considered to be mainly a Th1-mediated autoimmune disease, Th17 and Treg subsets and their plasticity may play an ancillary pathogenic role. The previous data should therefore be re-evaluated in the light of the novel discoveries (i.e. the possibilities of "double identity"), especially as target organ -specific changes have not been fully explored in human T1D. Additionally, depending on the patient population (children vs. adults), differences in age at onset and/or duration of clinical T1D might yield different results concerning the ratios between different T cell subtypes or circulating and tissue-residing lymphocytes. In comparison to the T helper cells and Tregs, the relevance of the other CD4+ subtypes is underexplored. Both an increase and reduction of the percentage of γδ T cells has been reported in peripheral blood measurements in prediabetes and clinical T1D have been reported (Kretowski et al. 1999, Lang et al. 1991), and evidence in the NOD mouse supports both protective and pathogenic roles (Han et al. 2010,

Markle et al. 2013, Zhang et al. 2010b). One recent study reported increased Tfh cell levels in the peripheral blood of T1D patients (Xu et al. 2013).

In addition to CD4+ T cells, the cytotoxic **CD8$^+$ T cell** population might play a substantial role in the pathogenesis of T1D. As described above, these cells present the most prevalent cell type in the human insulitis, and hyperexpression of HLA class I in the T1D pancreas is thought to enhance their autoreactivity. The frequency of CD8+ cells has been shown to be increased in the pancreatic draining lymph nodes of T1D patients (Ferraro et al. 2011). In fact, a recent study comparing T1D patients to patients with type 2 diabetes (T2D) reported that although CD4$^+$ T cell reactivity to islet antigens was shared between T1D and T2D, CD8$^+$ T cell reactivity was unique to T1D (Sarikonda et al. 2014). Preproinsulin-specific CD8+ cells were observed to be increased in peripheral blood of T1D patients, and capable of killing beta cells *in vitro* (Skowera et al. 2008), mainly recognizing peptides with low binding affinity for HLA (Abreu et al. 2012). The killing mechanism was shown to require release of cytotoxic granule components, but at a suboptimal level when compared to virus-specific T cells (Knight et al. 2013). CD8+ cells prone for IL-17 production were reported in T1D patients (Marwaha et al. 2010). Coppieters et al. demonstrated the presence of autoreactive CD8+ T cells in the islets of cadaveric T1D donors by tetramer staining. Their results indicated that autoreactive CD8+ cells were not detected in the pancreas during the autoantibody-positive period before T1D diagnosis, whereas CD8+ T cells peaked in patients with disease duration under one year, and then gradually declined (Coppieters et al. 2012). Single CD8+ islet-autoreactive specificity could be detected in a single islet when studying recent onset patients, whereas multiple CD8+ T cell reactivities were present in patients with a long-standing disease, indicating epitope spreading. However, CD8+ T cells need DC activation and CD4+ T help to be "licensed" for β cell killing, and therefore the CD8+ response is dependent on other cell types, especially effector Th cells and Tregs (Wong et al. 2008).

B cell depletion with a CD20 antibody was shown to exert a therapeutic effect in both NOD mice (Hu et al. 2007) and humans (Herold et al. 2011, Pescovitz et al. 2009), but only transiently as tolerance is not induced. Generally it is thought that the autoantibody production by B cells is nonpathological, as T1D was reported in a patient with hereditary B lymphocyte deficiency (Martin et al. 2001). In addition, T1D cannot be transferred through plasma, or from a T1D mother to the child during pregnancy. Instead, B cells are thought to act as potent antigen presenting cells. T1D progression is delayed in NOD mice without B cells but enhanced in NOD mice able to produce only transmembrane Ig-receptors (Wong et al. 2004). Such antigen presenting B cells promote CD8+ responses in the late stage of the disease progression by inhibiting CD8+ cell apoptosis in the islets and promoting cytolytic differentiation in the pancreatic draining lymph node.

(Brodie et al. 2008). Similarly, in an autoimmune prone mouse model, B cells were discovered to cross-present autologous β cell antigen to CD8+ cells in the pancreatic lymph node, driving CD8+ differentiation to effector cells (Marino et al. 2012). Some have challenged the idea of B cells acting merely as APCs, as in the mouse models the presence of autoantibodies and their binding to FcγR (expressed by an unidentified cell type) led to expansion of autoreactive CD4+ cells and their enhanced survival, suggestive of autoantigen-autoantibody complex uptake by **DCs** leading to increased autoantigen presenting capacity (Silva et al. 2011). Allen et al. showed a significant increase in pDC counts at the time of T1D diagnosis, and demonstrated that pDCs, but not mDCs or monocytes, showed enhanced antigen presentation in the presence of autoantibody positive patient serum, similarly suggesting immunocomplex capture (Allen et al. 2009). Additionally, Diana et al. suggested that defective clearance of debris from dying β cells in the NOD mouse leads to formation of self-antigen-autoantibody complexes, triggering neutrophils and induction of type I interferon production from the DCs (Diana et al. 2013). However, decreased numbers of both mDCs and pDCs have also been reported both in both newly diagnosed and established disease (Hinkmann et al. 2008, Nieminen et al. 2012, Vuckovic et al. 2007). Increased IL-1β and decreased IL-6 upon TLR ligation of monocytes and mDCs of autoantibody positive subjects was reported, and this effect was most prevalent in seropositive children under 11 years of age, suggesting an age-related dependency of the response (Alkanani et al. 2012). TNFα and IL-1β producing macrophages and DCs are present in the insulitis of newly diagnosed T1D patients (Uno et al. 2007). Moreover, monocytes isolated from T1D patients have increased levels of IL-6 and IL-1β, which could potentially increase Th17 response (Bradshaw et al. 2009). (Wong and Wen 2012).

Upon deletion of Tregs in the NOD mouse, the earliest infiltrating cells in the islets are **NK cells** (Feuerer et al. 2009). Pancreatic NK cells unleashed from Treg IL-2 depriving suppression proliferated and increased their production of IFNγ and granzyme B, indicating a vicious cycle of infiltrating autoreactive CD4+ cells providing NK cells with IL-2, and NK cells contributing to the autoreactive capacity of CD4+ cells by producing IFNγ (Sitrin et al. 2013). This might represent an extreme situation, as in human insulitis NK cells are detected only rarely (Willcox et al. 2009). For a reason not yet understood, starting from the progenitor cell stage β cells stably express an unknown ligand for NK cell receptor NKP46, making them highly susceptible for NK cell killing. However, the mouse studies indicate that NK cells are absent from a healthy pancreas and NK cells only infiltrate concomitantly with T cells. In addition to the contributions from β cells, NK cells in T1D patients seem to be perturbed. For example, NK cells from T1D patients were reduced in numbers, were impaired in cytolytic activation, IFNγ production, and in NK2GD-mediated signaling conferring activating signals to NK cells (Qin et al. 2011). However, the molecular biology of NK cells is not clearly defined, as many activating

ligands for its receptors remain unknown, and the net result is determined by the balance of different activating and inhibiting signals, many of which might be tissue dependent (Enk and Mandelboim 2014). However, it has been observed that frequencies of certain combinations of NK receptor and their HLA class ligand haplotypes are increased in T1D patients (Mehers et al. 2011, Rodacki et al. 2007).

Other innate immune cell types and processes have received less attention in the context of T1D. For example, according to a recent report, both preclinical and established T1D patients have a reduced number of circulating **neutrophils**, when compared to healthy controls or patients with T2D (Valle et al. 2013). In addition, increased **complement** activation, e.g. elevated C4b deposition, was observed in the pancreases of T1D patients (Rowe et al. 2013).

The presence of functional β cells long after clinical T1D diagnosis raises hope for preventive immune modulation. Several clinical intervention trials aiming at immunomodulation or insulin tolerance have been completed, but so far without clear long-term success. Although autoantibodies are widely used to screen preclinical patients (Ziegler et al. 2013), the hope for finding additional and even earlier **biomarkers** for progressing autoimmunity is great. Genetic screens can identify the susceptible population, but the limitation for both the genetic and autoantibody markers is their usefulness for assessing therapeutic outcomes, as they do not change during disease progression. (Jin and She 2012). In the search for other types of early biomarkers, transcriptomics on PBMCs or whole blood samples, as well as serum metabolomic and lipidomic analyses results have been reported. So far, most of the transcriptomic studies have published microarray analysis on cross-sectional samples of whole blood or PBMCs of established T1D patients. A study on autoantibody positive individuals revealed that the type I interferon response pathway is activated in approximately 30% of preclinical T1D cases (Reynier et al. 2010). A more recent study showed that the higher expression levels of a five gene set was able to identify the autoantibody positive cases with greater risk for T1D progression (Jin et al. 2014). Earliest biomarkers identified so far are serum metabolites and lipids. Metabolic analysis revealed that specific signatures could be detected throughout the disease process (Oresic et al. 2008). Increased risk for T1D were conferred by reduced levels of succinic acids and choline phospholipids at birth, increased levels of proinflammatory lysophosphatidylcholines before seroconversion, as well as reduced levels of triglycerides and antioxidant ether phospholipids at all timepoints. Ketoleucines were decreased and glutamic acids were elevated before seroconversion. In addition, a specific signature of seven lipids in the cord blood predicted highly increased risk for T1D progression (Oresic et al. 2013). Metabolite analysis in another cohort of children showed that phosphatidylcholines and phosphatidylethanolamines were decreased in the cord blood samples of children diagnosed with T1D before 4 years

of age (La Torre et al. 2013). These profiling studies suggest that autoimmunity might be a relatively late response to the early metabolic disturbances, partly induced *in utero*, and that specific processes such as increased glutamate load might increase GAD65 enzyme activity in β cells, triggering autoimmunity (Oresic et al. 2008).

2.2.2 Hygiene, macro- and microdiversity, and viral infections - culprits for the rising incidence of immune-mediated diseases

The rising incidence of atopic disease along with the rising standard of living and hygiene was already discussed in the 1970´s (Preston 1970). The concept of the **hygiene hypothesis** started to formulate in 1989 when David P. Strachan first published his epidemiological analysis on a 23-year follow-up cohort of 17 414 British children: an inverse correlation existed between the appearance of hay fever and the number of children in the family. For a possible explanation Strachan concluded that infections in the early childhood, transmitted by older siblings, or prenatally from the mother, could prevent allergy. At a later age, further protection could be contributed by reinfection and other infections through the younger siblings. Together the declining family size and the increasing standard of living and hygiene could thus contribute to the increase in the atopic diseases observed in the past decades. (Strachan 1989). Later on the hypothesis gained further support from the Th1 and Th2 cell paradigm. Infections preferably induce a Th1 type of response, subsequently suppressing the allergy inducing Th2 responses that drive IgE switching. However, research in the area has revealed it to involve a much more complicated scenario. Heterogeneity of the allergic diseases makes it challenging to define the clinical phenotype, there is a great variety of possible contributing environmental factors, and response variation is influenced both by the multifactorial genetic susceptibility and the age of the individual at exposure (von Mutius 2007).

The geographical distribution of the frequency of many immune-mediated diseases is a mirror image of that of infectious diseases common in developing world, such as tuberculosis, helminth infections, and childhood diarrheal diseases (Bach and Chatenoud 2012, Haahtela et al. 2013). The incidence of immune-mediated diseases has increased upon westernization of the lifestyles. For example, the incidence of atopic diseases rose quickly in East Germany after reunification with West Germany (von Mutius et al. 1998), and in rural Poland, where farm-related exposures were dramatically reduced after accession to the European Union, followed by rapid changes in farming practices (Sozanska et al. 2014). In addition, immigration to an allergy-prevalent country increases the risk for atopy and asthma above the prevalence observed in the country of origin (Rottem et al. 2005). Similarly, the risk for developing type 1 diabetes is high for the children born in United Kingdom to immigrant Asian parents (Bodansky et al. 1992) and for children born in Sweden to immigrants originating from the countries with low incidence (Söderström et al. 2012). However, the components of the westernized lifestyle

conferring high susceptibility to immune-mediated diseases are currently unknown, and could include such variables as increased exposure to chemicals, modern healthcare (antibiotics, vaccines), and diet. Modern life style changes also include reduced exposure to nature. A farm environment rich in microbial, plant, and animal antigens protects from asthma and atopy (Ege et al. 2011, Roduit et al. 2011, von Mutius and Radon 2008). For example, consumption of farm milk was associated with a reduced risk for asthma and hay fever (Illi et al. 2012). Additionally, the balance of macro- and microlevel biodiversity might regulate the immune system: Atopic individuals were shown to have lower environmental biodiversity in the surroundings of their homes and significantly lower generic diversity of gammaproteobacteria on their skin (Haahtela et al. 2013).

In addition, a positive latitude gradient exists for both type 1 diabetes as well as for development of asthma and allergies, the incidence being greatest in higher latitudes (Mohr et al. 2008). This is thought to at least partly stem from reduced sun exposure during winter months, as well as from progressively indoor lifestyle in the modern environments, which would affect especially the **vitamin D** levels produced by the skin upon **UV light** exposure. Vitamin D uptake is also low in westernized diets. This has led to prevention trials with vitamin D supplementation, but the results have varied. For example, out of eight observational studies on vitamin D intake during early childhood years, five observed reduced risk for T1D whereas three found no effects (Dong et al. 2013). Studies in mouse have implicated that life-long supplementation with very high doses is necessary for T1D protection (Takiishi et al. 2014). Due to inconsistencies in these results, other sun light -mediated health benefits, and especially of UVB wavelengths, have also recently been highlighted. UVB exposure has been shown to mediate immunosuppression: Treg numbers and function are enhanced, whereas IFNγ and Th17 responses are suppressed. Surprisingly, these affects are mediated through TLR4 (Lewis et al. 2011). Notably, the immunosuppression could be transferred to a recipient mouse through bone marrow transplantation, suggesting long-term imprinting of hematopoietic stem cell precursors giving rise to poorly functional DCs (Ng et al. 2013), possibly through UV-induced prostaglandin E_2 –mediated mechanism (Scott et al. 2014). (Hart et al. 2011, Hart and Gorman 2013).

It is increasingly appreciated that both the **commensal microbiome** and the pathogens of the gastrointestinal tract have important immunoregulatory functions in the immune system. Healthy microbiota are thought to educate the immune system by shaping the balance between pro- and anti-inflammatory mechanisms, as well as to protect from pathogens by competing for resources and creating an antagonistic environment (Licciardi et al. 2013). Conversely, some gastrointestinal infections seem to confer protection. For example, *H. pylori* and *T. gondii* are prevalent in Karelian area of Russia, whereas they are very low across the border in Finland, where there is a higher

incidence of atopic diseases (Seiskari et al. 2007). Allergic infants show an altered composition of gut microbiome (Ouwehand et al. 2001, Stsepetova et al. 2007). For example, a more diverse gut microbiome during the first two months of life conferred protection from the allergy (Sjogren et al. 2009). Intriguingly, *Bifidobacterium* diversity was positively correlated with family size and endotoxin levels in the house dust. Subsequently, modulating the microbiome composition with pre- or probiotics could be used for preventing allergies. *Lactobacillus rhamnosus* GG supplementation during the first 6 months of life reduced development of eczema, but increased development of allergic rhinitis and asthma during the first seven years of life (Kalliomäki et al. 2007). A recent meta-analysis concluded, that at present there is no sufficient evidence to recommend perinatal probiotics in prevention of wheeze and asthma (Azad et al. 2013). In addition, the microbial colonization in the respiratory tract might play a significant role. Metagenomics studies have demonstrated that the sputum samples from asthmatic patients have a greater proportion of proteobacteria and overall bacterial diversity when compared to nonasthmatic controls (Hilty et al. 2010, Huang et al. 2011, Marri et al. 2013). The colonization at an early age seems to play a major role. Colonization of the hypopharyngeal region with *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, or in combination, as early as 1-month of age confers increased risk for recurrent wheeze and asthma by the age of 5 years (Bisgaard et al. 2007). Additionally, for infants born with cesarean section, the subsequent risk for asthma is estimated to be increased by 20% (Thavagnanam et al. 2008), and could entail both reduced rates of microbial colonization and the extensive use of antibiotics related to the alternative delivery mode. A recent study reported additive effects of IL-13 polymorphism and birth by caesarean section combined with antibiotic usage to increased development of atopic dermatitis (Lee et al. 2014). Thus, the underlying genetic susceptibility together with the use of antibiotics, westernized high hygiene diet and other possible modulatory factors in the modern societies may play a role in triggering the immune cascade. On the other hand, specific antibiotic treatments and carefully selected probiotics might serve as a therapeutic tool to modify and maintain the healthy status of the epithelial linings.

Both preclinical and diagnosed T1D patients have an altered gut permeability, associated with structural changes as well as altered expression levels of tight junction proteins, that is thought to affect the absorption of antigens as well as lead to enhanced inflammation in the gut tissue (Vaarala et al. 2008). For example, a study of small intestine biopsies of coeliac disease (CD) and CD+T1D patients discovered that expression of tight junction protein 1 (TJP1) was lowest and FOXP3 expression highest in children with CD+T1D, compared to children with CD or healthy controls (Vorobjova et al. 2011). Duodenal biopsies of T1D patients indicated a reduction of the intestinal Treg cells when compared to healthy controls or patients with coeliac disease, due to defective priming by the lamina propria DCs of T1D patients. (Badami et al. 2011). This was speculated to bias the effector

T cell / Treg balance in the intestine. Mounting evidence suggests instability of the T1D-associated gut microbiome. A study on four autoantibody positive children close to T1D diagnosis from the Finnish DIPP high risk cohort found reducing bacterial diversity and increasing *Bacteroides* vs. *Firmicutes* abundance over time in the autoantibody negative period preceding seroconversion (Giongo et al. 2011). The follow-up study of 18 autoantibody positive Finnish children and their controls confirmed the trend of reduced diversity as well as the *Bacteroides* vs. *Firmicutes* ratio (de Goffau et al. 2013). The low abundance of *Bifidobacterium adolescentis* and *B. pseudocatenulatum* was associated with autoimmunity. Further evidence of the associationof an increased *Bacteroides* vs. *Firmicutes* ratio with T1D was provided by mesurements form 16 Spanish children with established T1D (Murri et al. 2013). In eight Mexican children newly diagnosed with T1D *Bacteroides* were increased and *Prevotella*, *Megamonas* and *Acidaminococcus* decreased, indicating unique T1D-associated phyla with a non-western diet (Mejia-Leon et al. 2014). However, how these changes affect the health of the intestine and its immune system remains to be investigated, especially as the latest study on 22 German autoantibody positive children of a high genetic risk cohort did not confirm any of these findings, as there was no individual phylum or genera that showed significantly different abundances compared to autoantibody negative healthy control children (Endesfelder et al. 2014). All of these microbiome studies have been performed with 16S rRNA gene amplification combined with sequencing, not allowing confident species-level resolution of taxa, or functional analysis of other genes in the bacterial genome. Therefore, shotgun sequencing combined with metatranscriptomic and metabolomic analyses are awaited for more detailed information (Morgan and Huttenhower 2014).

Viral infections have been implicated to play a role in the pathogenesis of the immune mediated diseases. However, whether these infections play a protective or causative role has been debated since the early days of the hygiene theory. In particular, respiratory tract infections by human rhinovirus at an early age have been associated with wheezing and asthma later in childhood (Jackson et al. 2008, Kotaniemi-Syrjänen et al. 2003, Kusel et al. 2007, Lemanske et al. 2005). For example, 90% of children who wheezed with rhinovirus infection at three years of age had asthma at the age of six years (Jackson et al. 2008). Additionally, repeated infections by respiratory syncytial virus (RSV) early in life are seen as inducers of wheezing episodes and asthma (Henderson et al. 2005, Sigurs et al. 2005, Sigurs et al. 2010, Stein et al. 1999). For example, lower respiratory infection by RSV requiring hospitalization conferred a greatly enhanced risk for developing asthma compared to children never hospitalized due to RSV (Sigurs et al. 2005). Also, protective effects of viral infections have been postulated. Recently, preliminary results reported by Lukkarinen et al. (2014) demonstrated that wheezing children with coinfection of rhinovirus and bocavirus, or bocavirus alone, develop reoccurring wheezing later and less often than children with rhinovirus infection. In addition, the serum IL-4/IFNγ ratio

was higher and IL-10/IL-12 ratio lower in children with rhinovirus infection, when compared to coinfected and bocavirus-infected children, suggesting that bocavirus is able to downmodulate the rhinovirus-mediated Th2 response. However, as genetic predisposition to asthma can be linked to TLRs and other pattern recognition pathways, it is currently unclear if the individuals suffering from virus-mediated wheezing are predisposed towards infections due to underlying abnormal lung physiology and/or immune response, eventually leading to development of asthma (Jackson and Linsley 2010, Thomas et al. 2014). It was recently demonstrated that allergic sensitization towards aeroallergen precedes and predisposes to rhinovirus associated wheezing, and not the other way around, suggesting that the preventive measures should be primarily focused towards preventing allergic sensitization (Jackson et al. 2012). In support of this, impairment of type I and III interferon production has been reported in the bronchial epithelial cells and alveolar macrophages of asthmatic patients (Contoli et al. 2006, Edwards et al. 2013, Wark et al. 2005), pDCs of allergic subjects (Tversky et al. 2008), as well as bronchoalveolar lavage cells from asthmatic patients (Sykes et al. 2012). The mechanism is currently unknown, but the observed increased expression of serum IgE as well as its receptor FcεR on the DCs of atopic and asthmatic individuals could play a role. IgE crosslinking was shown to reduce influenza-induced IFN-α production by pDCs of asthmatics (Gill et al. 2010), as well as RSV-induced IFN-α and IFN-λ1 secretion by PBMCs of allergic asthmatic children (Durrani et al. 2012).

Concerning T1D, seasonal variation in the T1D incidence was the first indication of the viral connection. Since then, the autumn/winter seasonality in autoantibody seroconversion (Kimpimäki et al. 2001) and genetic association of essential signaling molecules in the pattern recognition signaling, such as *TLR2* (Bjørnvold et al. 2009), *IFIH1* (Smyth et al. 2006), and IRF7-mediated transcriptional network (Heinig et al. 2010), have further emphasized the possible link with viruses. The most convincing evidence so far has been collected on the enteroviruses, such as the Coxsackieviruses. Pancreatic β cells express the human coxsackievirus and adenovirus receptor (HCAR) and are therefore highly susceptible to enteroviral infection, the cytolytic activity being strain dependent. Seven out of twelve newborn babies who died of fulminant coxsackievirus infection were positive for enterovirus RNA in the pancreatic islets and duct cells, out of which six showed signs of insulitis. (Ylipaasto et al. 2004). Immunohistochemical staining of pancreases demonstrated that enteroviral capsid protein VP1 is present in insulin-producing beta cells in 60% of recently-diagnosed T1D patients (Richardson et al. 2009). Most recent studies have demonstrated that VP1 is also present in the beta cells of patients with established disease, indicating persistent infection (Richardson et al. 2013). VP1+ cells coexpressed high levels of pathogen recognition receptor PKR (protein kinase R), which is induced by enteroviral infection and activates antiviral cascades. Additionally, beta cells positive for enteroviral capsid protein had downregulated levels

of anti-apoptotic Mcl-1 protein (myeloid cell leukemia sequence 1), which is rapidly degraded upon translational arrest in infected cells, indicating enhanced sensitivity to apoptosis. (Richardson et al. 2013). In the pancreas of three extreme patients with fulminant diabetes, e.g. abrupt onset of ketoacidosis due to rapid β cell loss caused by enterovirus infection with almost 100% islets associated with insulitis, viral receptor MDA5 was expressed by both α and β cells, RIG-I was expressed in β cells, and VP1 was expressed by all types of cells. Infiltrating mononuclear cells expressed TLR3 and TLR4. All islet cells expressed IFNα, IFNβ, IL-18, and increased levels of HLA class I. Most residual β cells expressed both IFNγ and IL18, and subsequently serum IFNγ levels were high (3-fold compared to controls). α and β cells expressed IRF7, and Fas was expressed mostly by β cells. DCs and macrophages were observed to clear the β cell debris, as some of the immune cells were positive for insulin. NK cells or Tregs were not detected. (Aida et al. 2011).

Concerning enterovirus markers detected outside of the pancreas, there are both positive and negative findings in the development of T1D. A meta-analysis concluded that the presence of enteroviral RNA or viral capsid protein in blood, stool, or tissues of prediabetic and established patients is linked to an increased risk both for T1D-related autoimmunity as well as clinical T1D (Yeung et al. 2011). The strain specific differences might account for some of the discrepancies between the studies, as the previous assays have mainly searched for a presence of a virus family/genus without further serotyping. Subsequently, in a nested case-control study of a T1D-susceptible birth cohort (The Finnish DIPP study), Coxsackievirus B strain 1 (CBV1) infections, as measured by the presence of neutralizing antibodies in the serum, were observed to increase the risk for developing T1D-specific autoantibodies, whereas genetically closely related strains CBV3 and CBV6 conferred protection, especially if occurring before CBV1 infections (Laitinen et al. 2013). In addition, the presence of maternal neutralizing antibodies against CBV1, detected from the cord blood, demonstrated a trend towards protection from CBV1 and development of autoantibodies. This implies that the infectious history of an individual modulates the susceptibility to viral infections and that different serotypes can modulate susceptibility for subsequent infections by cross-protection by enterovirus-specific T memory cells. Additionally, the data implies that under 5% of CBV1 infected children develop type 1 diabetes, which fits to the low attack rate of poliovirus with less than 1% infected persons developing motor neuron damage and paralysis (Laitinen et al. 2013). Additionally, antibodies against CBV1 were associated with T1D development in an international cohort of Finnish, Swedish, English, French and Greek children (Oikarinen et al. 2014). Recently, differences between a highly and weakly immunogenic CBV1 strains were investigated for their downstream effects on human primary PBMCs, and only the highly immunogenic strain was able to induce IFNα production and subsequent transcriptional response (Hämäläinen et al. 2014). As discussed by the authors, both

scenarios could drive autoimmunity: the highly immunogenic strain could easily infect the target tissue and cause efficient innate and adaptive immune system activation leading to tissue destruction, whereas the weakly immunogenic virus could more easily escape the viral surveillance and be able to mount a persistent infection (Hämäläinen et al. 2014).

As originally postulated by Strachan (Strachan 1989), **age at infection** might be an important modulator of the disease susceptibility. In relation to T1D, it has been suggested that the decreasing incidence of enteroviral infections, similarly to the poliovirus in the end of the 18th century, would lead to lack of protective antibodies from uninfected mothers, or infections later in childhood beyond the maternal protection, thus causing more severe infections in the infants. The poliovirus used to be endemic, leading to infection of the neonate under the protection of maternal antibodies, until the rising standard of living led to a delay in the age of infection and concomitant increase in paralytic complications, as encountered in "the polio epidemic", of the 1920´s. (Viskari et al. 2000). Indeed, the maternal enterovirus antibody titers have been connected to protection of neonates, and are thought to be primarily transferred through breast feeding (Sadeharju et al. 2007). The protective effect by maternal antibodies was also suggested in the case of T1D-associated CBV1 (Laitinen et al. 2013). Conversely, any respiratory infection resulting in hospital admission during the first year of life increased risk for asthma diagnosis after five years of age (Montgomery et al. 2013). A birth cohort study of 95310 children born in USA demonstrated that infant birth approximately four months before the winter virus peak was associated with the highest risk of developing asthma by the age of five years, with 29% increased risk compared with birth 12 months before the seasonal peak (Wu et al. 2008). Colonization of the hypopharynx by *S. pneumonia*, *H. influenzae*, and *M. catarrhalis* at one month of age was associated with an increased risk for reoccurring wheeze and asthma by the age of five years, whereas colonization at the age of one year did not confer increased risk, although the overall colonization increased from 21% of children being colonized at one month of age to 71% children being colonized at the age of one year (Bisgaard et al. 2007). Animal studies provide further proof of the significance of the age at first exposure, but also suggest multiplicity of possible mechanisms. For example, mice who had been challenged with RSV as neonates (1 or 7 days old) developed more severe pulmonary disease and an IL-4 dominated CD4+ cell response upon RSV reinfection at adulthood (12 weeks of age), opposed to the first challenge at 4 or 8 weeks followed by IFNγ dominated CD4+ response upon reinfection at adulthood (Culley et al. 2002). Additionally, it was demonstrated that early life RSV infections and the following Th2-prone inflammatory conditions in the lung might compromise Treg-mediated tolerance to allergen acquired earlier in life, as repeated RSV infections in the young mice tolerized to OVA antigen through mother´s milk as neonates impaired the Treg suppressor function towards OVA-specific T cells,

due to skewing of the Treg cells towards Th2-like program, expressing GATA3 and IL-13 (Krishnamoorthy et al. 2012). Recent discoveries of persistent RSV infection of bone marrow cells and transplacental transmission of RSV from pregnant mice to pups also suggest longer lasting and more pervasive RSV infection, as well as earlier onset for asthma susceptibility, than previously thought (Piedimonte and Perez 2014). Inoculation of pregnant rats with red fluorescent tagged RSV led to presence of the same virus in *in utero* exposed fetuses (30%), as well as lungs of the neonate (40% of pups) and adult offspring (25%), leading to enhanced airway reactivity upon reinfection (Piedimonte et al. 2013).

The unifying concept for bringing together the different aspects of the hygiene theory, protective and causal infections, as well as the other modulating parameters such as the age and previous infectious history, is still missing. Our understanding of the balance between macro- and microbiodiversity is still forming. For example, the *Bifidobacterium* commensals, generally considered as beneficial and essential for a healthy intestinal microbiome, are completely missing in the gut microbiome of a human hunter-gatherer group in Tanzania (Schnorr et al. 2014). It has even been claimed that the modern-day crowd infections, such as virus infections in childhood, were non-existing during the hunter-gatherer period of human evolution, as they either killed the host or induced permanent immunity, and thus were not able to persist in small communities. Instead, harmless infections by "old friends", such as fungi or helminthes persisted, had to be tolerated and hence mediated their beneficial immunomodulatory effects. (Rook et al. 2013). Clearly, more research in this area is needed to fill the gaps in between the different "frontiers of associations" and determine the time-window for effective immune system modulation.

2.2.3 Prenatal and neonatal immune regulation - relation to the hygiene theory

As the onset of allergic diseases and pediatric autoimmune diseases occurs at a very early age, the associated immune system perturbations are thought to partly stem from the *in utero* period. As a well-known example, maternal (Neuman et al. 2012) and grandmaternal (Li et al. 2005) smoking during pregnancy are known to increase atopy and asthma risk in the offspring. In general, a high level of IgE in cord blood increases the risk for allergy and asthma, and association of these levels with certain genetic markers, such as SNPs in the *IL-13* gene, suggests *in utero* gene-environmental interaction (Chang et al. 2012, Hong et al. 2010, Sadeghnejad et al. 2007, Yang et al. 2010). For example, Curtin et al. (2013) reported that the suppressive, methylated status of a CpG island on *IL-2* gene promoter in cord blood cells is associated with asthma exacerbations and more frequent hospital admissions due to wheezing and asthma during the childhood years. In relation to T1D, the associated high risk HLA-DR4-DQ8 haplotypes have been linked to poor *in vitro* induction of type 2 immune responses of cord blood lymphocytes, compared

to the cells of newborns without these haplotypes (Luopajärvi et al. 2007). Maternal enterovirus infection during pregnancy is associated with a slightly increased risk for T1D in the offspring (Elfving et al. 2008, Viskari et al. 2012). In addition, a distinct cord blood lipidomic profile associated with high risk for T1D progression has been identified (La Torre et al. 2013, Oresic et al. 2013). Clearly, the intrauterine environment as well as prenatal immune system maturation should be taken into consideration when investigating immune-mediated diseases of childhood.

From the third trimester onwards the skin of the fetus is covered in a waxy coating called vernix carneosa. This layer, unique to humans, is a mixture of water, proteins and lipids. In addition to functioning as a barrier to water and temperature loss, it contains a variety of antimicrobial proteins, peptides, and free amino acids, such as lysozyme, lactoferrin and psoriasin, and thus is an important barrier against a wide variety of bacterial and fungal infections (Tollin et al. 2006). In addition, the fetal skin is highly equipped in TLR signaling. Expression of TLRs 1-5 are significantly higher when compared to adult skin, with >100 fold differences in mRNA levels observed. Fetal and neonatal keratinocytes are superior in producing IL-8, IP-10 and TNFα upon TLR3 ligand stimulation, when compared to adult skin. Upon TLR3 stimulation, fetal cells also secrete higher levels of CCL3-5 and IFN inducible chemokines CXCL9-11 than neonatal and adult keratinocytes. (Iram et al. 2012). Fetal keratinocytes were also shown to produce large amounts of antimicrobial peptides, such as β-defensins and S100 proteins, when compared to neonatal and adult skin (Gschwandtner et al. 2014).

Defects in neutrophil function in neonates have been studied for a century, as the limited phagocytic activities of neonatal granulocytes were discovered in 1910, and neutropenia in children was associated with bacterial sepsis in 1928. Later studies have revealed further insufficiencies in neonatal neutrophils. In adults, bacterial infection leads to neutrophil release from the bone marrow storage pool, followed by an increase in overall granulopoiesis from the progenitor cells. However, the neonatal storage pool is smaller, and additionally the neonatal bone marrow is unable to increase the neutrophil production due to the lower number and reduced proliferation rate of progenitor cells. Furthermore, reduced chemotaxis, rolling adhesion, transmigration, and formation of neutrophil extracellular traps have been reported in neonates. (Melvan et al. 2010). Modulations of neutrophil activity are observed already in the fetus. Umbilical vein sampling *in utero* revealed that fetal monocytes and neutrophils showed impairment in phagocytosis of opsonized bacteria, compared to the cells of neonates and adults, but that the oxidative burst activity of the granulocytes was enhanced, even at unstimulated level. An inverse correlation was observed between oxidative burst product generation and gestational age, indicating that the NADPH oxidase system matures and functions early to compensate for the immaturity of the phagocytic capacity. IL-6 and TNFα

production were reduced in fetal monocytes upon LPS stimulation, when compared to neonates and adults, but the production of IL-8 was greatly enhanced both in fetuses and neonates, when compared to adult monocytes. (Strunk et al. 2004). The impairment in phagocytic responses is transient, as these are restored to adult levels by the third day of life (Filias et al. 2011).

The presentation of antigens by the neonatal APCs is reduced due to downregulation of HLA class II as well as costimulatory molecules, such as CD40, CD80 and CD86, leading to poor induction of antigen-specific T cell responses. The suboptimal antigen presentation and costimulation are thought to favor Treg development. Compared to adult DCs, a higher amount of mDCs and lower amount of pDCs are detected in cord blood, and the neonatal DCs responded poorly to a variety of bacterial stimuli, such as GpC, LPS, or poly(I:C). However, neonatal pDC responses to viral stimuli are as potent as those of adult cells, as measured by high induction of IFNα, TNFα and chemokines (Zhang et al. 2013). Most notably, the neonatal DCs are prone to drive Th12 and Th17 responses upon bacterial stimulation, as their production of the key Th1 polarizing cytokine IL-12 is heavily restricted due to suppressive nucleosome remodeling of the *IL-12(p35)* promoter (Goriely et al. 2004). However, as the transcription of IL-12(p40) subunit is unaffected, it can dimerize with IL-23(p19) subunit, inducing differentiation of IL-17 producing CD8+ T cells (Vanden Eijnden et al. 2006). The neonatal Th1 subtype is also self-limiting as they upregulate IL-13Rα1 in low concentrations of IL-12. This receptor can form a heterodimer with IL-4Rα, responding to IL-4 and leading to apoptosis of Th1 cells. (Lee et al. 2008). Further restrictions are elicited by T cell intrinsic hypermethylation of the IFNγ promoter in the neonatal CD4+ T cells (White et al. 2002). In addition, naïve CD4+ T cells of preterm and term newborn infants were shown to constitutively express the essential signaling components driving the Th17 differentiation (*IL-23R*, *STAT3*, *RORC*, *TGFβ*) and downregulate the Th1 transcription factor *TBX21*, leading to efficient Th17 polarization in the appropriate cytokine environment (Black et al. 2012). Furthermore, components of the neonatal plasma are further finetuning the cytokine environment in the favor of Th2 or regulatory responses (Belderbos et al. 2012, Belderbos et al. 2013, Pettengill et al. 2013). The various anti-Th1 cell mechanisms in the neonatal immune system are thought to protect from alloimmune reactions between the mother and the fetus, as this would be detrimental to the course of pregnancy. (Willems et al. 2009).

Despite the shortcomings of antigen presentation, a high proportion of fetal CD4+ and CD8+ T cells are preactivated, as 18% of both subsets were found to express early T cell activation marker CD69 in the mesenteric lymph nodes. The CD69+ CD4+ and CD8+ T cells were able to spontaneously proliferate and secrete IFNγ, but were actively suppressed by the fetal Treg cells (Michaëlsson et al. 2006). In fact, fetal T cells are highly prone to proliferate and differentiate into Tregs upon allogeneic stimulation

(Mold et al. 2008, Mold et al. 2010), which is seen as increased percentage of Treg cells (15-20%) in the total peripheral CD4+ population, when compared to healthy neonates (7.5%) and adults (7%) (Takahata et al. 2004). In addition, fetal and adult CD4+ T cells showed distinctive gene expression profiles, perhaps indicating a distinct tolerance-prone progenitor cell of origin (Mold et al. 2010). Fetal T cells were unresponsive to maternal allogeneic APCs but not towards unrelated APCs, and thus the extensive Treg population is considered to induce tolerance towards maternal alloantigens, such as the maternal micro chimeric cells detected in the cord blood and lymph nodes of human fetuses (Mold et al. 2008). Whether the fetal Tregs driving immune tolerance to maternal micro chimeric cells also induce tolerance to self-antigens, autoantigens or infectious antigens encountered *in utero* remains to be investigated. It could be speculated that due their high activation potential the earliest fetal T cells migrating to tissues respond to peripheral autoantigens, and by their default program differentiate into Tregs, thus creating a peripheral Treg repertoire with vast specificity on top of the natural Treg population maturating in the thymus (Mold and McCune 2011).

Intriguingly, enhanced microchimerism of both fetal origin in the mother and maternal origin in the child have been associated with autoimmunity. Increased level of maternal microchimerism descending from the gestational period has been observed in the peripheral blood and pancreases of T1D patients (Nelson et al. 2007). Microscopy analysis revealed that maternal cells contribute to both endocrine and exocrine tissues of all healthy pancreases (0.39%), but that the maternal microchimerism was significantly enhanced in T1D patients (0.68%). In addition, an enrichment of maternal cells among the β cell fraction was observed in T1D pancreases, and was not detected due to the infiltrating immune cells or islet cell replication. Whether the microchimeric β cells contribute to breaking of tolerance after reaching a certain threshold, or simply divide and enrich in an attempt to regenerate the damaged tissue, is unclear at the present. (Vanzyl et al. 2010, Ye et al. 2014). It is also possible, that microchimeric maternal T cells or other immune cell types disturb the fetal Treg development, leading to breaking of tolerance and autoimmunity later in life (Leveque and Khosrotehrani 2011).

In addition to the Treg-mediated regulation, the neonatal immune system seems to employ several, recently discovered mechanisms to actively ensure immunosuppression. The cord blood was reported to be enriched in neutrophilic/granulocytic myeloid-derived suppressor cells (Gr-MDSC), which decreased to adult levels soon after birth. Neonatal Gr-MDSC cells effectively inhibited both CD4+ and CD8+ T cell proliferation and cytokine production, as well as NK cell activity. (Rieber et al. 2013). Recently, a study compared the responses to *Listeria monocytogenes* infection in mice at 6 days and at 8 weeks of age (mimicking human neonates and adults, respectively), showing

impaired responses, reduced survival and 1000 fold bacterial loads in the neonatal mice. Surprisingly, transfer of adult immune cells did not rescue the neonatal mice, instead, the adult cells were suppressed in their ability to produce TNF-α. Conversely, TNFα production of neonatal cells was restored upon transfer to *Listeria* -challenged adult mice. Co-cultures of neonatal and adult cells eventually revealed that the suppression was mediated through arginine depletion. Both neonatal mouse splenocytes and human cord blood population were found to be enriched in cells expressing arginase-2 enzyme, CD71 and erythroid lineage marker (CD235A+ in humans), when compared to adult splenocytes (mouse) or PBMCs (human). Depletion of this cell population in neonatal mice restored defenses against *L. monocytogenes* and *E. coli* infections. The CD71+ erythroid population retracted with age, following reduction in susceptibility to infection. Subsequently, the CD71+ population was shown to be important for facilitating the non-inflammatory colonization of the neonatal intestine by commensal microbes, as the cytokine responses of intestinal immune cells were greatly enhanced in CD71+ depleted animals, and abolished if the microbiota was removed by antibiotics. (Elahi et al. 2013). It seems that as a trade-off for the colonization of the intestine, this population also suppresses protective immune responses to infection. (Bordon 2014).

In the context of the hygiene theory, it is intriguing to hypothesize that the maturing immune system would be able to adapt in the presence of early microbial stimuli. Perhaps the most compelling evidence of the *in utero* effects of microbial exposure so far is that maternal farming activity, associated with high exposure to animal and/or plant material and microbes, protects the offspring from atopic diseases. In a multinational study, prenatal exposure to stables, hay, and farm animals increased the expression of bacterial TLRs 2 and 4 in the peripheral blood cells, as well as reduced the risk for atopic sensitization in the school children (Ege et al. 2006). Maternal farming was also associated with increased cord blood Treg counts and their increased suppressive activity (Schaub et al. 2009). Only a handful of studies have so far studied interpopulation differences in the early immune system development, especially with a focus on the effects modulated by high vs. low standard of hygiene. For example, in comparison to neonates born in North America, Tregs cells are increased by 30-40% in the cord blood of newborn infants born in Kenya, where exposure to malaria and other parasites is high. Kenyan cord blood Tregs were also more likely to express activation marker HLA-DR (Mackroth et al. 2011). Contradictingly, the frequency of Tregs was found to be higher in the cord blood of neonates born in high income Austria vs. low income Gabon (Köhler et al. 2008), as well as in high income Australia vs. low income Papua New Guinea (Lisciandro et al. 2012a). Markers of mature or activated B cells (downregulation of CD5, upregulation of CD40) were detected both in Gabon and Papua New Guinea. The pDC population was also decreased in both low income settings, which could be linked to reduction in Tregs through the production of IL-10 by pDCs (Lisciandro et al. 2012a).

CD4+ cells of neonates born in Gabon also showed an activated or mature phenotype. In addition, although Papua New Guinean neonates had reduced frequencies of B cells, monocytes and pDCs, these cells showed signs of activation: monocytes expressed more markers of activation (HLA-DR and CD86) as well as inhibition (ILT3 and ILT4), and DCs expressed more HLA-DR. Lisciandro et al. also performed functional studies and demonstrated that the proliferative response of Papua New Guinean CD4+ T cells to PHA on autologous APCs was reduced and delayed. As the proliferation of T cells was greatly enhanced in a coculture with allogeneic adult APCs, and as the APCs of Papua New Guinean neonates suppressed proliferation of adult CD4+ cells more potently when compared to Australian APCs, the Papua New Guinean APCs were concluded to actively limit T cell proliferation. In further functional tests, Papua New Guinean monocytes processed less antigen and were incapable to further induce activation markers upon PHA stimulation. An increased percentage of monocytes expressed IFNγ and IL-6 at the steady state, but the cells were unable to further increase their cytokine production upon stimulation. Conclusively the data indicated that although the Papua New Guinean neonatal APCs had a more activated and mature baseline status than the APCs of Australian neonates, the Papua New Guinean APCs were also more quiescent and resistant to further LPS stimulation. This was suggested to reflect an evolutionary mechanism of immune regulation learned *in utero* to prepare newborn infants for the intensity and frequency of the immunological challenges in the postnatal environment and to protect them from infections. (Lisciandro et al. 2012a). Thus, the high microbial burden in low income countries has an effect in tolerazing the developing immune system, the mechanisms of which remain to be explored. Although the fetus itself is not infected, it is able to prime antigen-specific T and B cell responses, as well as cytotoxic CD8+ and NK cell responses upon maternal infection by various pathogens, suggesting that microbial antigens cross the placenta (Dauby et al. 2012). It is possible that the detected differences between the populations could also be partly influenced by the presence of functional TLR mutations due to genetic drift and differences in infectious pressure, but still the most striking difference in developed and developing countries is the microbial burden (van den Biggelaar and Holt 2010). In summary, our limited knowledge of neonatal immune function is predominantly derived from populations living in western societies, although there is much to be learned on how a more traditional lifestyle influences *in utero* immune development away from chronic inflammatory disorders (Lisciandro and van den Biggelaar 2010).

Interestingly, the immune system of humans and other large mammals develops early during gestation, as opposed to mouse who produces the adaptive immune cells around the time of birth (Mold and McCune 2011). The need for the early maturation of the defense mechanisms might be driven by the long gestational period, increasing risk for pathogen encounter *in utero* (Ivarsson et al. 2013). Still, intrauterine environment is

often referred to as a protected and sterile space, lacking of any microbial contacts in healthy pregnancy. As pointed out by Rautava et al. (2012b), evolutionarily the dogma is bizarre as mammals have evolved in a world already inhabited by an enormous variety of microbes, and maybe the *in utero* environment is able to offer only limited and/or selected exposure during the vulnerable periods of early human development. Indeed, several lines of evidence point to the *in utero* origin of bacterial colonization in term, healthy pregnancies without signs of infection in the neonate. Bacterial DNA is present in the cord blood after cesarean section (Jiménez et al. 2005), in the amniotic fluid sampled at caesarean section (Bearfield et al. 2002), placental and fetal membranes (Rautava et al. 2012a, Satokari et al. 2009, Steel et al. 2005), placenta (Aagaard et al. 2014) and in the meconium (first intestinal discharge) of healthy neonates (Ardissone et al. 2014, Gosalbes et al. 2013, Jiménez et al. 2008). The composition of the meconium microbiome has been associated with preterm birth (Ardissone et al. 2014), atopic eczema of the mother, sociodemographic factors such as maternal education level, organic food consumption and smoking (Gosalbes et al. 2013), as well as maternal diabetes (Hu et al. 2013). In addition, the microbial composition of the meconium was very unique, as it differed significantly from the feces collected after one week of life (Moles et al. 2013). The origin and transfer route of the intrauterine bacteria remains unknown, but the fact that intestinal commensals *Bifidobacterium* and *Lactobacillus rhamnosus* were found in almost all of the placentas studied after caesarean delivery suggests a transplacental vertical delivery from the mother, possibly through a similar mechanism to entero-mammary pathway, where immune cells transfer intestinal bacteria to the mammary glands during late pregnancy and lactation (Satokari et al. 2009). Oral administration of stained bacteria to pregnant mice has been shown to lead to the presence of these bacteria in the amniotic fluid and in the meconium of the offspring (Jiménez et al. 2005, Jiménez et al. 2008). Additionally, probiotic supplementation of mothers in the last weeks before caesarian section led to expression changes of TLRs both in the placenta and in the exfoliated intestinal cells in the fetal meconium (Rautava et al. 2012a). Recent whole-genome shotgun sequencing of placental microbiome pointed out similarity to communities rich in oral sites, such as tongue and tonsils (Aagaard et al. 2014). Conclusively the evidence encourage a major paradigm shift away from the sterile womb theory (Funkhouser and Bordenstein 2013). Similarly nonpathogenic viruses or archaea may be transferred from mother to child alongside their bacterial counterparts. As the microbes contribute 99% of all unique genetic information present in the human body, microbial transfer between mother and the fetus has been suggested to present an additional and important mechanism of genetic and functional change in human evolution, having wide implications on human health and disease in the modern era. (Funkhouser and Bordenstein 2013).

3. AIMS OF THE STUDY

The overall goal of this PhD study was to investigate the cells of the immune system to better understand the immune responses and their regulation in human immune-mediated diseases. All of the subprojects utilized modern high-throughput measurement technologies, such as microarrays and massively-parallel sequencing, on either *in vitro* differentiated human primary T cells or clinical blood samples collected from neonates and children.

The detailed aims of this thesis were:

1. To identify the kinetics and extent of IL-4 and STAT6 -mediated transcriptional response in human naïve CD4+ T cells *in vitro* polarized towards Th2 subtype (I)
2. To identify the mRNA transcripts differentially expressed in HLA-susceptible children during the autoimmune cascade towards clinical type 1 diabetes (II)
3. To elucidate the *in utero* induced gene expression differences in the umbilical cord blood of children born in environments with deviating standard of hygiene and infections (III)

4. MATERIALS AND METHODS

4.1 Sampling, cohorts and ethical considerations (I, II, III)

Umbilical cord blood for naïve CD4+ T cell isolation and differentiation (I) was collected from healthy neonates born in Turku University Hospital, Hospital District of Southwest Finland. Sampling from anonymous donors was performed under the permission of the Ethics Committee of Hospital district of South-West Finland.

All the children in the study II were participants in the Finnish Type 1 Diabetes Prediction and Prevention Study (DIPP) (Kupila et al. 2001), where venous blood was drawn into PAXgene Blood RNA tubes (PreAnalytix) 1 – 4 times a year at the study clinic in Turku, Finland. Collection was approved by the Ethics Committee of Hospital district of South-West Finland. After sampling, the PAXgene Blood RNA tubes (PreAnalytix) were incubated for 2 hours at room temperature and then stored at −70 °C until analyzed. Islet cell autoantibodies (ICA), insulin autoantibodies (IAA) and autoantibodies to GAD, IA-2 and ZnT8 were measured from all individuals. Of the 28 subjects in this study, nine were sampled starting before or at the time of the appearance of autoantibodies (seroconversion) (S1-5 and S7-10); cases S3, S6, S7, and S10 progressed to type 1 diabetes and the other six did not. The remaining 18 children (D1-18) were all sampled starting after seroconversion and all progressed to diabetes (D1-18). A persistently autoantibody negative control child was matched with each case, based on the date and place of birth, gender and *HLA-DQB1* genotype. In all, 356 blood samples (191 from seroconverters and TID children, and 165 from healthy controls) were processed for genome-wide transcriptional analysis.

In study III, the samples were collected as part of the international DIABIMMUNE consortium, aimed at testing the hygiene hypothesis in the development of autoimmune and allergic diseases. Umbilical cord blood was drawn into Tempus Blood RNA tubes (Applied Biosystems) from children born between January and May 2010 at the maternity unit of Jorvi hospital (Espoo, Finland; n=48), maternity units of Tartu and Põlva (Estonia; n=25), or two maternity departments in Petrozavodsk (capital of the Republic of Karelia, Russian Federation; n=40). Collection of cord blood samples was approved by the ethical committees of the participating hospitals and the parents gave their written informed consent. Samples were stored at −70 °C until analyzed. All newborn infants were full-term (>36 gestational weeks) and born vaginally. The HLA-DR-DQ genotypes related to type 1 diabetes risk were determined with a lanthanide labelled oligonucleotide hybridization method as described previously (Hermann et al. 2003). The subjects were divided into four groups based on their HLA-associated risk for T1D (Peet et al. 2012).

4.2 Cord blood CD4+ cell isolation and culturing (I)

First, mononuclear cells were separated with Ficoll-Paque gradient centrifugation (Amersham Biosciences), after which CD4+ T cells were purified with magnetic beads (Dynal CD4 Positive Isolation Kit, Invitrogen) in accordance with the manufacturer`s recommendations. Naïve CD4+ cells were activated with plate-bound αCD3 (500 ng/24-well culture plate well, Immunotech) and soluble αCD28 (500 ng/ml, Immunotech) at a density of 2−4 x 10^6 cells/ml of Yssel´s medium (Iscove modified Dulbecco medium, Invitrogen, 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. For Th2 cell polarization, IL-4 (10 ng/ml, R&D Systems) with or without αIL-12 (10 µg/ml, R&D Systems) was added to the culture. At 48 h, IL-2 was added (17 ng/ml, R&D Systems) to all cultures. 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.

4.3 STAT6 knockdown with siRNAs and analysis of target genes with Illumina microarrays (I)

siRNAs (1.5 µg/4 x 10^6 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 (#R5886, Sigma) supplemented with 10% heat inactivated FBS (#C-37360, PromoCell), 2 mM L-Glutamine (#G7513, Sigma) and 50 U/ml Penicillin and 50 µg/ml Streptomycin (#P0781, Sigma) at 37°C in 5% CO_2. Thereafter, cell activation and culturing were performed as described above (4.2). For STAT6 target gene identification, cells were harvested at 12, 24, 48 or 72 h time point.

Total RNA was extracted (RNeasy Mini Kit, #74106, Qiagen) and treated in-column with DNase (RNase-Free Dnase Set, #79254, Qiagen) for 15 minutes. The sample preparation for the microarray hybridizations was performed at the Finnish Microarray and Sequencing Centre, Turku, Finland. Amplification for expression analysis was started from 100 ng total RNA with Illumina RNA TotalPrep Amplification kit (#AMIL1791, Ambion). RNA and cRNA concentrations were measured with a Nanodrop ND-1000 and quality controlled with an Experion electrophoresis station (BioRad). 1.5 µg of amplified and labeled samples was hybridized to Sentrix HumanWG-6 Expression BeadChips,

version 2 (#BD-25-112, Illumina) at 55ºC overnight according to Illumina Whole-Genome Gene Expression Direct Hybridization protocol, revision A. Hybridization was detected with 1 µg/ml Cyanine3-streptavidine (#146065, Amersham Biosciences). Chips were scanned with Illumina BeadArray Reader and numerical results extracted with GenomeStudio v1.0 without any normalization.

4.4 Flow cytometry (I)

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

4.5 Western blotting (I)

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 protease (#4693159001, Roche) and phosphates inhibitors (#8906738001, 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), equal amounts of samples were loaded on 10−12% SDS-PAGE gels, transferred to nitrocellulose 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 (LI-COR), using Goat-anti-Rabbit IRDye800CW (#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 STAT6 ChIP-seq with massively parallel sequencing (I)

CD4+ T cells were cultured in Th0 or Th2 polarizing condition 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 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, Inc.) coupled to the magnetic beads (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 on Illumina Genome Analyzer GAII producing from 4 to 5.2 million reads per sample.

4.7 RT-PCR analysis of STAT6 ChIP-seq target genes (I)

Ten Th2-specific STAT6 Chip-seq sites were selected for kinetic analysis of STAT6 binding. ChIP followed by qPCR was 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 of AbsoluteTM QPCR ROX Mix (Thermo Scientific). Amplification was monitored with an Applied Biosystems 7900HT Fast Real-Time PCR System (15 min enzyme activation and 40 cycles of 15 s 95ºC, 1 min 60ºC). The percent of Input values were calculated with the following equation: 100*2^(Input - Ct (ChIP)), in which Input value was adjusted to 100%. GATA3 and IFNg regions were used as negative controls. The table of primers and probes is presented in article (I), Supplemental experimental procedures.

4.8 Kinetic analysis of IL-4 target genes with Affymetrix microarrays (I)

For transcriptional profiling of IL-4 targets, the samples were cultured as described above (Section 4.1) and cells 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. Three replicate cultures were performed. Total RNA was extracted (RNeasy Mini Kit, #74106, Qiagen) with an in-column DNase (RNase-Free Dnase Set, #79254, Qiagen) treatment step for 15 minutes. The subsequent sample treatments were performed at the Finnish Microarray and Sequencing Centre, Turku, Finland. 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 GeneChip Expression Analysis Technical Manual. 15 µg of biotinylated and fragmented cRNA was hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays overnight (16−18 hours) at 45ºC. GeneChips were washed and stained in the Affymetrix Fluidics Station. GeneChips were scanned using the GeneChip Scanner 3000 with an AutoLoader. Of the 54 hybridizations, two were excluded from further data analysis based on the compromised quality of the samples (Th0 4 h and Th2 6 h).

4.9 RNA isolation and microarray analysis of whole-blood samples (II, III)

4.9.1 RNA isolation from PAXgene RNA tubes (II)

Total whole-blood RNA was extracted from the samples using PAXgene Blood RNA kit (Qiagen), and RNA quality and quantity was determined using a NanoDrop ND-2000 (Thermo Scientific) and Experion Automated Electrophoresis System (Bio-Rad Laboratories). Each sample was hybridized in duplicate both on Affymetrix and Illumina arrays according to the manufacturer´s instructions.

4.9.2 RNA isolation from Tempus RNA tubes (III)

Total whole-blood RNA was extracted from the samples using a Tempus Spin RNA isolation kit (Applied Biosystems) according to the manufacturer’s instructions. RNA quality and quantity was determined using a NanoDrop ND-2000 (Thermo Scientific) and Experion Automated Electrophoresis System (Bio-Rad Laboratories).

4.9.3 Microarray analysis of whole-blood samples (II, III)

For Illumina arrays (II), RNA was processed with RiboAmp OA 1 round RNA amplification kit (case-control pairs S1-S6, Applied Biosystems/Arcturus) or Ovation RNA amplification system v2 including the Ovation whole blood reagent (case-control pairs S7-S10 and all type 1 diabetes case-control pairs, NuGEN Technologies) and hybridized to Illumina Sentrix human WG6 v2 expression bead chips (seroconverted case-control pairs) or Illumina Human HT-12 Expression BeadChips, version 3 (type 1 diabetes case -control pairs). For Affymetrix arrays (II, III), 50 ng of total RNA was processed to cDNA with Ovation RNA amplification system v2, including the Ovation whole blood reagent (NuGEN Technologies). The amplified cDNA was subsequently biotin-labelled and fragmented with Encore biotin module (NuGEN Technologies). Samples were hybridized to GeneChip Human Genome U219 array plate (Affymetrix) in accordance with the manufacturer´s protocols for using the GeneTitan Hybridization, Wash and Stain Kit for 3’ IVT Array Plates (Affymetrix). A GeneTitan MC Instrument (Affymetrix) was used to hybridize, wash, stain, and scan the arrays. The probe cell intensity data was summarized with Affymetrix GeneChip Command Consol 3.1.

4.10 DNA isolation and Immunochip hybridization (II)

Genomic DNA was extracted from whole blood using the salting-out method (Miller et al. 1988). Thereafter, samples were processed at the Department of Genetics, University Medical Centre Groningen (The Netherlands). The DNA quality and concentration

was measured by fluorescence in the Synergy HT Multi-Mode Microplate Reader (Biotek) using the PicoGreen® dsDNA quantitation assay (Invitrogene) according to the manufacturer's instructions. Samples were diluted appropriately to a final concentration of 50 ng/µl and measured with a NanoDrop 2000c (Thermo Scientific). 200 ng of genomic DNA was amplified, enzymatically fragmented and hybridized onto the Human Immuno BeadChips (Illumina) at 48°C overnight (20 h) according to the Illumina's Infinium HD Ultra Assay protocol. The staining of the arrays was performed using the Freedom EVO 100 robot (Tecan) and scanned with the iScan Reader (Illumina). Bead intensity data was analysed using the Genotyping Module version 1.9.4 of Genome Studio version 2011.1 (Illumina).

4.11 Data analysis and mining (I, II, III)

4.11.1 Analysis of STAT6 target genes (I)

The microarray data were quantile-normalized and log2-transformed in each experiment using the Bioconductor affy package. IL-4-regulated genes were first identified by determining significant expression changes between the matched Th2- and Th0-measurements (same time point and culture) over the three replicate control-siRNA experiments. Linear modeling with moderated F- and t-statistics (Bioconductor limma package) was applied. Genes with a FDR <0.05 in the overall F-test and at least in one of the t-tests for the individual time points were defined as changed (Benjamini and Hochberg 1995). The effect of STAT6 knockdown on the IL-4-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 IL-4- and STAT6 regulation across the three biological repeats was identified using the moderated F- and t-statistics at a FDR <0.05, similarly as above.

4.11.2 STAT6 ChIP-seq data analysis (I)

The reads were aligned to the human reference genome (NCBI v36) using the SOAP software (Li et al. 2008b). Only uniquely mapped reads were retained (~3 million reads per sample). Potential binding regions were identified based on their enrichment for reads at FDR <0.05 using the FindPeaks software (version 3.1.9.2) following the recommendations in the FindPeaks manual (Fejes et al. 2008), which showed, on average, the best peak detection performance in this particular dataset (Laajala et al. 2009). To further remove potential false positives due to non-uniform background, a minimum of 10-fold enrichment of reads in the Th2 sample relative to the corresponding location in the Thp sample was required. Moreover, Th2-specific peaks that additionally

showed at least a 2-fold read enrichment in the Th2 sample relative to the corresponding Th0 sample were considered as the final set of IL-4-mediated STAT6 binding sites.

4.11.3 IL-4 target gene analysis (I)

The microarray data were quantile-normalized and log2-transformed using the Bioconductor affy package. IL-4-regulated genes were identified by determining significant expression changes between the matched Th2- and Th0-measurements (same time point and culture) over the three replicate cultures. 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. Probesets with $p < 0.05$ in the overall F-test and further at least in one of the t-tests for the individual time points were defined as changed.

4.11.4 Identification of genes and pathways differentially regulated during the development of type 1 diabetes (II)

The gene expression microarray data were preprocessed using the RMA procedure for the Affymetrix data and the quantile normalization procedure for the Illumina data using R/Bioconductor (Gentleman et al. 2004). Differentially expressed genes were identified by comparing subjects with their matched healthy controls. Since the follow-up series are not fully synchronized in time between the different individuals, a similar approach as in Elo et al. 2010 and Huang et al. 2001 that avoids the need of a direct alignment was applied. More specifically, for each individual, the expression intensity value of a particular probe/probeset x at each time point was given a z-score, defined as $z=(x-m)s^{-1}$, where m is the mean and s is the standard deviation calculated using all the time points in the matched control time series. Such z-scores penalize those probes/probesets that have high variability in the control series. To identify significant up- or down-regulation across the individuals, the rank product algorithm was applied to case-wise maximum/minimum z-scores. Genes with false discovery rate (FDR) below 0.05, estimated as the percentage of false positive predictions (pfp), were considered as differentially regulated. To focus on type 1 diabetes- or seroconversion-specific changes the same procedure was applied to the data after swapping the case and control pairs. Only those probes were retained that did not pass the criterion FDR <0.05 in the swapped analysis. The aim of the swapping procedure was to exclude from further analyses such non-diabetes-related changes that were similarly detected also in the controls.

Finally, the findings from Affymetrix and Illumina arrays were compared, and genes showing concordant changes were regarded as final, validated findings (II, Supplementary

Tables 2 and 3). Additionally, the genes were required to be determined as present in more than 50% of all individuals (at least in one sample per individual) using a two-component Gaussian Mixture model for the Affymetrix data and detection p-values for the Illumina data. Affymetrix and Illumina data were combined using the gene symbols provided by IPA. To combine the data from the Illumina WG-6 v2 and HT-12 arrays, the probes between the array types were matched based on their sequence similarity. Seventy percent of the probes in Human WG-6 v2 array had remained completely identical to the corresponding probes in the Human HT-12 arrays. In addition, 6% of the probes were mapped by requiring a minimum sequence overlap of 25/50 consecutive bases (one mismatch allowed). The probes also had to target the same gene according to annotation. The remaining 24% of the probes were excluded from the analyses, for which the WG-6 v2 and HT-12 data needed to be combined.

In order to identify transcripts active in different phases of the autoimmune process, the rank product method was applied to find differentially expressed genes between the cases and their controls in five time-windows (II, Figure 1). The division was based on the maximal sample representation in each time-window. Inside each time-window, the fold change between the case and the matched control was calculated using linear inter/extrapolation. More specifically, the control sample series were matched to the time points of the case sample series. For the time points which were inside the range of real control time points this was performed by linear interpolation. For the time points needed outside this range, the expression values were approximated by constant extrapolation (set equal to the closest real measurement). Genes with false discovery rate (FDR) below 0.05 were considered differentially regulated. Additionally, the genes were required to be determined as present, as described above. The findings validated with all array types (as described above) were regarded as changed.

In addition, a comparison was made between those seroconverted children (cases S3, S7 and S10) who had later progressed to type 1 diabetes, and those who have not been diagnosed with type 1 diabetes to-date. An unpaired two group rank product analysis was performed for the case-control ratios to compare the affected and the unaffected cases. The analysis was performed for three time-windows: before seroconversion, at seroconversion, and 6−18 months after seroconversion.

4.11.5 DAVID pathway analysis (II)

The functional annotation tool DAVID (Database for Annotation, Visualization and Integrated Discovery) was used to identify enriched biological pathways (BBID, BioCarta, KEGG, Panther, Reactome) among the regulated genes (Huang da et al. 2009a, Huang da et al. 2009b). Pathways with FDR below 20% were reported (modified Fisher's exact test).

4.11.6 Transcription binding motif analysis (II)

Overrepresented transcription factor binding sites in the promoter regions (± 2 kilobases around the transcription start site) of the regulated genes were identified using the transcription factor target sets in the Molecular Signatures Database (MSigDB) (Subramanian et al. 2005). Binding sites with p-value below 0.05 were reported (hypergeometric test).

4.11.7 Transcription module analysis (II)

A module based method modified from Chaussabel et al. (2008) was used to survey coordinately expressed sets of genes (modules) functionally annotated with literature search. The gene symbols belonging to each module were downloaded from www.biir.net/modules, and differentially expressed genes not belonging to any of the modules presented in Chaussabel et al. (2008) were excluded from the analysis. The significance of overlap of each of the 28 modules with each list of differentially expressed genes was calculated using Fisher's exact test. Modules up- or downregulated with the Benjamini-Hochberg corrected p-value below 0.05 were visualized in the module maps with red or blue (up- and downregulation, respectively), the intensity of the color corresponding to the percentage of the regulated probesets that belong to the module.

4.11.8 Immunochip data analysis (II)

NCBI build 36 (hg18) mapping was used (Illumina manifest file Immuno_BeadChip_11419691_B.bpm) for SNP mapping. A cluster set based on 196,524 autosomal and X-chromosome variants was applied to all samples. Quality control of the data and linkage disequilibrium (LD) pruning was performed using PLINK v1.07 (Purcell et al. 2007). First, non-polymorphic markers (N-17216) and markers with duplicated rs identifiers (N-864) were removed. Next, samples with a call rate below 95%, as well as SNPs with a call rate below 98% were excluded. Markers were excluded when they deviated from Hardy- Weinberg equilibrium in controls ($p < 0.0001$). Due to the sample size the focus was set on common markers and thus SNPs with minor allele frequency below 10% were removed (N=80,259). Finally the dataset was pruned based on LD between markers ($r^2>0.8$). The final dataset comprised 30,463 SNPs. The effects of SNPs on gene expression were surveyed within 250 kb to both directions from each differentially expressed gene (case-wise max/min), using Affymetrix gene coordinates. A linear model was fit for genotype ~ gene expression and a p-value was calculated for the null hypothesis that genotype has no effect on gene expression (slope = 0). A Benjamini-Hochberg correction was applied for the p-values and effects with FDR < 0.05 were considered significant (II, Supplementary Table 7). The SNPs and their proxies $r^2 > 0.8$ that had an eQTL effect on the differentially expressed genes were searched for associations with autoimmune diseases in GWA studies, as listed in (Ricaño-Ponce and

Wijmenga 2013) and/or T1Dbase (Burren et al. 2011). The proxies for these SNPs were found based on HapMap3 (release 2) and 1000 Genomes in the CEU population panel by using SNP annotation and proxy search (http://www.broadinstitute.org/mpg/snap/ldsearch.php).

4.11.9 Identification of genes differentially regulated in cord blood of children born in Finland, Estonia, and Russia (III)

The intensity data was pre-processed using robust multi-array averaging (RMA) (Irizarry et al. 2003). The absent call filtering for Affymetrix U219 arrays was performed by determining the threshold value empirically for each sample, as described by (Lee et al. 2010a). A two-component Gaussian Mixture model was fitted for the non-control probe sets of each chip with an Expectation Maximization algorithm, which was implemented using the mixtools-package in R (Benaglia et al. 2009). The assumption is that each component of the distribution would correspond to a different source of signal (background and true expression). A probe set was filtered out if it was absent in at least 50 % of the samples from Espoo, Tartu and Petrozavodsk.

As the Espoo and Tartu RNA samples were hybridized to array plates in December 2011 - January 2012 and the Petrozavodsk samples in March 2013, the differences between Petrozavodsk and the other areas was confounded by a batch effect. In order to correct for this, 16 samples (eight from Petrozavodsk, four from Espoo and four from Tartu) were re-hybridized on the same Affymetrix U219 platform. The batch correction was made by applying the ComBat analysis method (Johnson et al. 2007), which implements an empirical Bayes framework to adjust for the batch effect.

Differential expression was detected by using the R Bioconductor (Gentleman et al. 2004) package Limma (Smyth 2004) to fit a linear model and compute a moderated t-statistic for each present probe set for all three comparisons: Espoo vs. Petrozavodsk, Tartu vs. Petrozavodsk and Espoo vs. Tartu. Gender, pregnancy week, month of birth and HLA risk class were included as confounding factors in the model. A probe set was deemed differentially expressed if its Benjamini-Hochberg-corrected p-value, estimated by the false discovery rate (FDR), was lower than 0.01.

4.11.10 Ingenuity Pathway Analysis (I, II, III)

Ingenuity pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com) was used in annotation of the microarray expression data, as well as for categorization of the genes according to their functional class and cellular location (I, Figure 1D-E and Tables S1, S4-5, S7-8; II, Figure 2B and Supplementary tables 2-4 and 6-7; III, Table S2). In article I, hypergeometric test was used to analyze the statistical significance of the enrichments.

In article III, molecular pathway enrichment was tested using the Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) core analysis tool.

4.11.11 Differentially expressed genes with functions related to the innate immunity responses (II, III)

The human genes with literature-annotated function in the innate immunity were downloaded from www.innatedb.org (Breuer et al. 2013). The enrichment of these genes among the differentially expressed genes was calculated using Fisher's exact test and the p-values were corrected with the Benjamini-Hochberg method.

4.11.12 Developmental regulation of gene expression (III)

Two recent studies report transcriptomic changes in 1-year-old infants and newborn infants. Martino et al. (2012) studied LPS induction in mononuclear cells at birth and at 1 year of age. They reported 549 genes that were differentially expressed between these age groups. The enrichment of these genes among the differentially expressed genes identified in the study (III) was calculated using Fisher's exact test and the Benjamini-Hochberg-corrected p-values are reported in Table S3 in the and Figure 4.

Wynn et al. (2011) performed whole blood transcriptomics upon bacterial sepsis infection in different age groups of children relative to healthy control children. Preprocessed and normalized microarray data sets were downloaded from Gene Expression Omnibus (GSE26440 and GSE26378) and samples that were from neonates (age < 1 month) or from infants (0.5 years < age < 1.5 years) were picked for father analysis. In both age groups, approximately one third of these samples were from healthy controls and two thirds were from patients with bacterial sepsis. Up- and downregulated genes between neonates and infants were identified by fitting a linear model and computing a moderated t-statistic for each probe set (Smyth 2004), taking health status (sepsis/healthy) and survival as confounding factors in the analysis. There were 2205 genes that were identified as upregulated and 1432 genes downregulated in the older infants compared to the neonates. The enrichment of these genes among the differentially expressed genes identified in the study (III) was calculated using Fisher's exact test. The Benjamini-Hochberg-corrected p-values are reported in Table S3 and Figure 4.

4.12 Data accession (I, II, III)

The gene expression and ChIP-seq data discussed in this Ph.D. thesis are accessible through GEO SuperSeries accession numbers GSE18017 (I), GSE30211 (II), and GSE53473 (III).

5. RESULTS

5.1 Identification of IL-4 and STAT6-mediated transcriptional programme of Th2 cell polarization (I)

To identify the kinetics and extent of IL-4 and STAT6 -mediated transcriptional response in human naïve CD4+ T cells *in vitro* polarized towards the Th2 subtype, siRNAs and STAT6 chromatin immunoprecipitation coupled with microarray and sequencing measurements were employed.

All three of the STAT6 targeting siRNAs severely attenuated Th2 polarization, as was indicated by the reduction of GATA3 and G protein-coupled receptor 44, a human specific Th2 cell marker (better known as chemoattractant receptor homologous molecule expressed on T helper type 2 cells, CRTH2), even after 1 week of culture in Th2 polarizing conditions (I, Figure 1A-B). At the genome-wide level, 453 individual IL-4 regulated genes were affected by decreased STAT6 expression (I, Table S1), and the proportional effect varied along the polarization process, being on average 40% of the IL-4 regulated genes at 12h, and more than 80% at 48h (I, Figure 1C). The effect of the STAT6 knockdown was significantly larger among the IL-4-upregulated than among the IL-4-downregulated genes (I, Figure 2B), suggesting that STAT6 primarily drives the activation of transcription, whereas downregulation would mainly remain a downstream effect after IL-4 stimulation. The broad functional distribution of the identified target genes and their localization throughout all cellular compartments reflected the fundamental role of STAT6 in the global regulation of the Th2 cell phenotype (I, Figure 1D-E). A literature search for the identified STAT6-regulated genes indicated that only 6% had been recognized as STAT6 target genes in earlier studies, either in human or mouse T or B cells (at the time of the publication; I, Table S1) (Ahn et al. 2009, Arpa et al. 2009, Büttner et al. 2004, Chen et al. 2003, Filen et al. 2008, Gabay et al. 1999, Hebenstreit et al. 2003, Kim et al. 2006, Kurata et al. 1999, Lund et al. 2007, McGaha et al. 2003, Ohmori et al. 1996, Schaefer et al. 2001, Schroder et al. 2002, Yang et al. 2005, Zhang et al. 2000, Zhu et al. 2002).

To identify the genes regulated by STAT6 directly (through DNA binding) and indirectly (through downstream effectors), chromatin immunoprecipitation with a STAT6 antibody was performed at 1h and 4h of Th2 polarization, after which the STAT6 bound DNA sequences were identified with massively parallel sequencing (ChIP-seq). In response to IL-4 a total of 508 genes were bound by STAT6 within 10 kb of the transcription start or end site (I, Table S2). The RNAi data indicated that STAT6 preferentially bound to

the IL-4 and STAT6 upregulated genes. Across the timepoints measured, 20–28% of the IL-4 upregulated STAT6 target genes were also bound by STAT6, whereas only 4-9% of the IL-4 downregulated genes were discovered as direct STAT6 targets (I, Figure 2C and Figure S2C). One of the four STAT6 consensus motifs (Hebenstreit et al. 2006) was present in 85% of the identified peaks (I, Figure 2D), and STAT6 bound sites were enriched at the transcription start site (I, Figure 2E). Sixtysix percent of the binding sites were intragenic, the first two introns being the most common (I, Table S3). Still, almost 10% of the identified STAT6 binding sites were more than 100 kb away from any known gene.

In order to study the detailed kinetics of IL-4 mediated transcriptional changes, undisturbed naive cells were polarized towards the Th2 phenotype and samples for genome-wide transcriptomics profiling were collected at dense intervals between 0.5h and 72h (I, Figure 3A). In total, 640 genes were upregulated and 460 genes were downregulated by IL-4 at one or more time points (I, Table S4). Clustering of the data demonstrated that IL-4 first induces transient changes in gene expression, followed by the stable Th2 cell signature profile (I, Figure 3B and Table S5). Summary of the IL-4 mediated transcriptional effects (I, Figure 3A) revealed that Th2 polarization is first dominated by gene upregulation (0.5–4h), followed by a period of gene downregulation (6–72h). The upregulated genes were significantly enriched with direct STAT6 targets (ChIP-seq) already at 0.5 hr, whereas the enrichment among the downregulated genes was detected only at later time points and at weaker significance levels.

To explore the regulation of indirect STAT6 target genes (affected by RNAi but not directly bound), a transcription factor binding motif search was performed for the sequences 10 kb upstream from the transcription start site of these genes. Notably, the STAT6 binding motif could not be found enriched among the indirectly regulated genes, further indicating these genes to be secondary or atypical targets of STAT6. Instead, STAT5A homotetramer motif was among the most significantly enriched motifs (I, Table S6). Comparison of the indirect STAT6 genes to the STAT5A ChIP-seq hits from mouse Th2 polarized cells, sampled at 8 and 13h (Liao et al. 2008), revealed a statistically significant overlap at both timepoints, suggesting that STAT5A might be a downstream regulator of the STAT6 target genes.

Overall the transcription factor kinetics followed the trends of the IL-4 stimulation, as the IL-4-mediated upregulation of this functional category occurred between 0.5 and 4h of polarization, whereas the downregulation happened later at 6h and onwards (I, Figure 5A and Figure S4). To build a comprehensive transcription factor network controlling the Th2 polarization under IL-4 and/or STAT6, the top IL-4 induced transcription factors were divided into four groups: (1) STAT6-independent, (2) putative STAT6 targets, (3) STAT6-dependent primary targets, and (4) STAT6-dependent secondary targets (I, Figure 5B). The STAT6-independent arm included *XBP1* and *NCOA3*, although in mouse B

cells these have been demonstrated to be regulated by STAT6 (Schroder et al. 2002). *LRRFIP1*, one of the earliest transcription factors induced by IL-4, was identified as a putative STAT6 target bound by STAT6 in the ChIP-seq experiment, but not detected as STAT6-regulated in the RNAi experiments. Three transcription factors, *BATF*, *EPAS1* and *RUNX1*, were directly regulated by STAT6. The STAT6-dependent but indirect arm included upregulated *GATA3*, *GFI1*, and *NFIL3*, as well as downregulated *BHLHE40*, *ID3*, *IRF8*, and *STAT1*. This core set of transcription factors was then expanded using a pathway analysis tool to include known transcription factor interactions (I, Figure 5C). The identified interaction neighbourhood included several factors determining the different CD4+ T cell fates, e.g. all the STAT family members.

5.2 Identification of gene expression changes associated with the pathogenesis of type 1 diabetes (II)

To identify the mRNA transcripts differentially expressed in HLA-susceptible children during the autoimmune cascade towards clinical type 1 diabetes, genome-wide microarray data describing longitudinal whole blood samples from 10 children sampled at the time of developing T1D-associated autoantibodies (seroconversion, S1-10), and 18 children sampled before the onset of clinical T1D (D1-18), were compared to the data obtained from time and place of birth, gender, and HLA risk genotype matched healthy control children.

When the transcriptional profiles of seroconverted children (S1-10; II, Table 1) were compared to their matched healthy controls, 109 unique genes were differentially expressed (false discovery rate, FDR<0.05; II, Supplementary Table 2), with 66% of genes being upregulated and 34% downregulated. Genes residing in the genomic type 1 diabetes susceptibility loci (T1Dbase, Burren et al. 2011) included the upregulated oncostatin M (*OSM*) and the downregulated killer cell lectin-like receptor subfamily B member 1 (*KLRB1*) (II, Supplementary Figure 1). The upregulated genes were enriched in the retinoic acid inducible gene 1 (RIG-I)-like receptor signaling pathway involved in recognizing viral dsRNA (II, Supplementary Table 2).

Altogether, 12 unique transcription factor genes, as annotated by the Ingenuity Pathway Analysis (IPA) knowledge base, were differentially regulated in the seroconverted children (II, Supplementary Table 2). Analysis of known gene and protein interactions among these transcription factors and other genes in the dataset using the IPA network neighborhood tool showed that interconnected interferon regulatory factors 5 and 7 (*IRF5* and *IRF7*) had several co-regulated network partners (II, Figure 2B and Supplementary Figure 1). Additionally, IRF7 binding sites were enriched among the promoters of the upregulated genes (P=0.000309; II, Figure 2A and Supplementary Table 2). Both *IRF5*

and *IRF7* are highly expressed in lymphoid tissues and drive the activation of a wide range of antiviral genes. Target genes in the identified network (Figure 2B) included the DEAD box protein 58 (*DDX58*, better known as Retinoic acid inducible gene 1, *RIG-I*), which codes a helicase involved in viral double stranded RNA recognition, as well as type 1 diabetes associated 2'-5'-oligoadenylate synthetase (*OAS1*), which codes for an enzyme involved in activating RNAse L, resulting in viral RNA degradation.

A comparison of the samples obtained from the 18 T1D progresses (D1-18; II, Table 1) to their matched controls revealed 472 differentially expressed genes (FDR<0.05; II, Supplementary Table 3). Notably, gene expression was suppressed, as 73% of the differentially regulated genes in the progressors were downregulated. The upregulated genes residing in the genomic type 1 diabetes susceptibility loci (T1Dbase, Burren et al. 2011) included the TRAF-type zinc finger domain containing 1 (*TRAFD1*) and the signal transducer and activator of transcription 2 (*STAT2*). In contrast, the diabetes-associated grancalcin (*GCA*) and the von Hippel-Lindau binding protein 1 (*VBP1*) were downregulated. (II, Supplementary Figure 1). The top downregulated pathways were related to immune system signaling, blood coagulation and complement (II, Supplementary Table 3).

To identify transcripts that are active in different phases of the diabetic disease, the data was divided and analyzed separately within five time-windows (II, Figure 1). The number of samples available in each time-window is summarized in Figure 3 (I). In the time-windows before, at, or 6−18 months after seroconversion, 124, 33, and 551 genes were identified as differentially expressed (FDR<0.05), respectively, and only 14 genes were common to all three time windows (II, Figure 3A and Supplementary Table 4). During the intervals of 1−2 years before clinical diagnosis and at clinical diagnosis, 388 and 211 genes were differentially expressed between the progressors and controls, with 125 common genes (II, Figure 3B and Supplementary Table 4). The two latest time-windows overlapped with 47 genes that were differentially expressed before seroconversion (II, Figure 3B). Different time windows showed enrichment of genes bearing different transcription factor binding sites, for example IRF and/or STAT binding sites were enriched in genes upregulated before seroconversion, and in the interval between seroconversion and diagnosis (II, Supplementary Table 4). The overall suppression of the ribosomal proteins was represented by downregulated pathways named ribosome, metabolism of proteins, and 3`-UTR mediated translational regulation (II, Table 2 and Supplementary Table 4). In addition, by utilizing a whole-blood sample data analysis tool (Chaussabel et al. 2008) that searches for the presence of predefined groups of coordinately expressed transcripts (functionally related, literature-annotated modules), the changes in transcriptional signatures were observed to be not stable but dynamic relative to different stages of type 1 diabetes pathogenesis (II,

Figure 4 and Supplementary Table 5). For example the cytotoxic cell module (M2.1) was first downregulated at the earliest time-window, but then induced at the time of clinical diagnosis. This module included killer cell lectin like receptors subfamily D and F member 1 (*KLRD1*, *KLRF1*) that are expressed on natural killer (NK) cells, as well as granulysin (*GNLY*) that is expressed by both cytotoxic T cells and NK cells. However, a module consisting of interferon-induced transcripts (M3.1), including the antiviral *OAS1* and *DDX58* (described previously), constantly showed changes at almost every stage of the development of type 1 diabetes.

Finally, a separate time-window analysis was performed for the seroconverted children who later progressed to clinical type 1 diabetes (cases S3, S6, S7 and S10; II, Table 1) compared to seroconverted children who have remained non-diabetic for at least 73 months. For cases S3, S7, and S10, data before, at, and 6−18 months after seroconversion were available. Before seroconversion, nine genes were identified as differentially regulated (FDR<0.05; II, Supplementary Table 6). At seroconversion and 6−18 months after seroconversion the difference had increased to 13 and 54 genes, respectively. One of the genes showing constant and high upregulation in the progressors was Ribonuclease, RNase A family, 2 (*RNASE2*, also known as eosinophil-derived neurotoxin, *EDN*; II, Figure 5). It encodes a secreted protein with several immunomodulatory functions. The ribonucleolytic activity of RNASE2 plays an important role in eosinophil-mediated antiviral activity against single-stranded RNA viruses. RNASE2 is an endogenous TLR2 ligand (Yang et al. 2008) that could play a role in the induction of interferons. Most interestingly, *RNASE2* expression has been reported to be upregulated in the PBMCs of patients with autoimmune diseases, such as rheumatoid arthritis (RA) (Bovin et al. 2004) and systemic lupus erythematosus (SLE) (Bennett et al. 2003).

In order to study the effect of possible single nucleotide polymorphisms (SNP) as the cause for the detected expression differences, DNA samples from the subjects were genotyped using the Immunochip SNP array (Illumina) (Cortes and Brown 2011). Using representative (LD pruned) SNP markers residing inside a window of +-250 kb around the gene coordinates, 118 differentially expressed genes had a *cis* eQTL (*cis* acting expressed quantitative trait loci) effect with 1-54 SNPs per gene (FDR<0.05; II, Supplementary Table 7). Comparison to eQTLs recently identified in whole-blood from 5311 individuals (Westra et al. 2013) validated 27% of the detected *cis* effects (II, Supplementary Table 7). Fourteen of the identified eQTL genes (the identified SNPs or their proxies $r^2>0.8$), such as Histone cluster 1 H2bd (*HIST1H2BD*), have also been associated with autoimmune diseases in GWAS, as listed in (Ricaño-Ponce and Wijmenga 2013) and/or the T1Dbase (Burren et al. 2011) (II, Supplementary Table 7). Interestingly, eQTL effects were found with SNPs adjacent to *IRF5*, *OAS1*, *OAS2*, *DDX58*, Transporter 2 ATP-binding cassette sub-family B (MDR/TAP) (*TAP2*), Indoleamine 2,3-dioxygenase 1 (*IDO1*) and Leukocyte immunoglobulin-

like receptor subfamily A (with TM domain) member 5 (*LILRA5*), which were identified as the core of the central interferon stimulatory network in the seroconverted individuals (II, Figure 2B). Previously, SNPs overlapping the *TAP2* gene residing in the human MHC locus have been associated with type 1 diabetes in several studies (Caillat-Zucman et al. 1992, Penfornis et al. 2002) and the *IRF5* polymorphisms have been connected to SLE, RA, and inflammatory bowel disease (IBD) (Dideberg et al. 2007, Sigurdsson et al. 2005, Sigurdsson et al. 2007).

5.3 Gene expression changes in the cord blood of newborns affected by the standard of living and hygiene (III)

To elucidate the *in utero* induced gene expression differences in the umbilical cord blood of children born in environments with deviating standards of hygiene and infections, umbilical cord blood RNA samples were collected from newborn infants born in Espoo, Finland (n=48), Tartu, Estonia (n=25), and Petrozavodsk, Republic of Karelia, Russian Federation (n=40), hereafter called Espoo, Tartu, and Petrozavodsk, and processed for genome-wide microarray analysis.

A majority of the differences in the cord blood transcriptomes were observed between Espoo and Petrozavodsk (3442 probesets, FDR below 0.01; III, Figure 2). The umbilical cord blood transcriptome of infants born in Tartu was more similar to that of infants born in Espoo (only 130 differentially regulated probesets), than to that of infants born in Petrozavodsk (1655 probesets). At the pathway level (III, Table 2), genes associated with B cell receptor signaling, such as *CD79B* coding for Ig-beta protein of BCR and *PAX5* coding for essential transcription factor for B cell lineage commitment, were suppressed in Petrozavodsk. Conversely, pathways associated with FLT3 activity (FLT3 signaling in hematopoietic stem cells and Acute myeloid leukemia signaling) were suppressed in Espoo and Tartu when compared to Petrozavodsk.

Altogether there were 899 similarly regulated genes when Espoo and Tartu were compared to Petrozavodsk (III, Figure 2). These included *ZFP36L1* and *PTCRA*, both highly upregulated in Espoo and Tartu vs. Petrozavodsk and associated with functions of immature immune cells (III, Figure 3A-B). *ZFP36L1* codes for a RNA-binding protein involved in various steps of mRNA processing. It is mainly expressed in lymphoid and myeloid hematopoietic lineages, and has been shown to inhibit both erythroid and plasma cell differentiation by targeting Stat5b and BLIMP1, respectively (Nasir et al. 2012, Vignudelli et al. 2010). Interestingly, polymorphisms of *ZFP36L1* have been associated with coeliac disease (CD), Crohn´s disease, and type 1 diabetes (T1D) (Ricaño-Ponce and Wijmenga 2013). *PTCRA* codes for pre-TCRα, which together with the TCRβ forms a pre-TCR complex expressed on immature T cells, before the rearrangement of TCRα locus.

As the newborn immune system is still immature, neonates rely on their innate immune system to fight the encountered pathogens. Therefore, the overlap of the differentially expressed genes with the innate immunity genes listed by the InnateDB (Breuer et al. 2013) was determined. From this comparison there was an enrichment with the upregulated genes in Petrozavodsk, when compared to Espoo and/or Tartu, and in the downregulated genes in Petrozavodsk, when compared to Espoo (II, Table S2, Innate immunity columns, and Table S3). Several pattern-recognition receptors were upregulated in Petrozavodsk vs. the other sites, including TLR2 receptor for Gram-negative bacteria and AIM2 receptor for dsDNA of bacteria and viruses. In addition, Nod-like receptors (*NLRC4*, *NLRC5*) were upregulated in Petrozavodsk when compared to Espoo. Genes involved in inhibition of the complement cascade werealso upregulated in Petrozavodsk. *C4BPA* codes for protein isoform alpha, that together with the beta isoform C4BPB forms the C4BP (C4 binding protein) complex, the major inhibitor of the classical and lectin complement pathways. The complex can also bind the CD40 receptor and thus induce B cell proliferation and IgE production (Brodeur et al. 2003). Additionally, the complex of the alpha isoforms was recently shown to induce a tolerogenic, anti-inflammatory state in DCs (Olivar et al. 2013). Another complement inhibitor, *CD46* (also known as membrane cofactor protein, MCP), is ubiquitously expressed on the cell surface to protect autologous cells from complement activation by promoting promoting degradation of the C3b and C4b complement proteins. CD46 also has a wide immunomodulatory role in which it is crucial for type 1 interferon production in macrophages (Hirano et al. 2002) and for efficient Th1 cell responses (Le Friec et al. 2012), and it was shown to promote the development of IL-10 secreting Tregs (Kemper et al. 2003) even from the Th1 subtype (Cardone et al. 2010).

Importantly, it was crucial to test whether the detected expression differences indicated changes in developmental and maturation status of the neonatal immune system between Petrozavodsk and other study sites. For comparison, we selected two recent gene expression studies reporting a) LPS-induced changes on monocytes at birth vs. 1 year of age (Martino et al. 2012) and b) the influence of age on septic shock transcriptional profile in whole blood (Wynn et al. 2011), both reporting dramatic differences in newborn response when compared to the other age group(s). These analyses revealed that upregulated genes in Petrozavodsk (downregulated in Espoo and/or Tartu) overlapped with the genes upregulated both by LPS and in children with sepsis at 1 year of age, when compared to the newborn response (III, Figure 4 and Table S3). For example, *ATG5*, which was associated with sepsis response at 1 year of age and upregulated in Petrozavodsk (III, Figure 3D), is also associated with recognition of single-stranded RNA viruses and type 1 interferon production in DCs (Lee et al. 2007), DC antigen presentation and subsequent T cell priming (Lee et al. 2010b), as well as with immune-mediated disease, such as MS (Alirezaei et al. 2009) and asthma (Martin et al. 2012).

6. DISCUSSION

6.1 STAT6 at the core of IL-4 induced Th2 polarization of human naïve CD4+ cells

The data and analyses obtained in the context of the studies (I) clearly demonstrated that STAT6 is an essential and central node in the IL-4 initiated Th2 polarization process of the human CD4+ T cells. The downstream regulatory network of STAT6 included target genes from all cellular compartments and functional gene categories, highlighting the extent of cellular machinery involved in finetuning the differentiation process. Most evidently, STAT6 was shown to activate the transcription upon the IL-4 signalling. Subsequently, the knockdown of STAT6 mainly affected the IL-4 upregulated Th2-specific genes, and these were also more commonly under direct regulation of STAT6. This suggests that the secondary regulators activated by STAT6 are responsible for IL-4 -mediated gene downregulation, perhaps through transcriptional repression and/or modelling the chromatin landscape, subsequently shutting down the other potential differentiation fates, such as Th1 and Th17, of the naive CD4+ cell.

Compared to the published studies on IL-4 mediated transcriptomic changes (Hämäläinen et al. 2001, Lund et al. 2007, Nagai et al. 2001, Rogge et al. 2000), the detailed kinetic analysis of IL-4 -mediated transcriptional changes revealed that the initial steps of the Th2 polarization process are highly dynamic. Firstly, IL-4 first induced a wave of transcriptional induction, followed by a timeframe of transcriptional repression. Secondly, only a subset of the IL-4 target genes differentially regulated within the first hours remained differentially expressed throughout the analysed time frame. This indicated that there are both switch kind of genes needed at specific time point as well as factors important both for transitioning to the Th2 developmental pathway and for maintaining the already acquired phenotype. The overlap of the STAT6 target genes identified in article I with the data generated from the STAT6 knockout mouse was surprisingly low, as only 6% of the STAT6 target genes had been previously identified. Therefore, a majority of the identified STAT6 target genes were novel target genes with potential to regulate the differentiation process downstream of STAT6 through previously unrealized pathways and cellular processes. For example, *ST6GAL1* and *RNF125* link STAT6 to the determination of Th2 cell-specific surface glycoprotein structures and ubiquitin ligase activity, respectively (Toscano et al. 2007, Zhao et al. 2005). SPINT2, one of the earliest induced direct STAT6 target genes,

was shown to be secreted from the Th2 cells (Äijö et al. 2012), where it potentially regulates extracellular or cell surface serine proteases.

Only a small fraction (approximately 30%) of the STAT6 bound genes were associated with gene expression changes in the kinetic IL-4 transcriptomics data, and 10% of the identified STAT6 binding sites were more than 100 kb away from any known gene. Various explanations for such binding exist. Such sites could be "poised" for STAT6 regulation at a later timepoint (e.g. restimulation) when necessary coregulators become available. Alternatively, STAT6 could regulate its target genes via chromosomal looping between the promoters and more distal regulatory elements. Previously STAT6 has been shown to regulate the intrachromosomal interactions of the Th2 cell cytokine locus, which is suggested to coordinate cytokine expression in the effector cells (Spilianakis and Flavell 2004). Non-regulatory binding sites of STAT6 could also act as a reservoir of STAT6 proteins, as a low level of STAT6 binding was readily detected in the nucleus even without any IL-4 stimulus (naive precursors and activated control cells). In addition, such sites could be important for regulation of the epigenetic landscape in Th2 cells, as Wei et al. (2010) demonstrated the central role of STAT6 in directing these processes in murine Th2 cells.

Interestingly *GATA3*, the hallmark transcription factor in Th2 cells, was not identified as a direct STAT6 target gene in our ChIP-seq studies, although both the mRNA and protein expression of GATA3 were severely reduced by STAT6 siRNA. In support of this, 7.5% of STAT6 deficient mouse CD4+ T cells were shown to produce IL-4 (around 50% of the amount in wild type control cells) at day 7, even when IL-4 was neutralized in the culture, due to high expression of GATA3 (Ouyang et al. 2000). This indicates that GATA3 can be induced and function independently of both IL-4 and STAT6. However, as the experiments of study I investigated STAT6 binding at the very early phase of IL-4 stimulation (1h and 4h, one culture without biological replicates), a later direct effect cannot be ruled out. One possibility is that STAT6 regulates the expression of *GATA3* via distant regulatory sites that could not be connected to *GATA3* in this study. Supporting the role of STAT6-controlled indirect regulation, BATF, one of the direct STAT6-dependent transcription factors, has been identified to regulate *Gata3* transcription (Betz et al. 2010) (**Figure 3**). STAT6 also indirectly upregulated the expression of *GFI1* and *NFIL3*, which are linked to the regulation of GATA3 protein stability (Shinnakasu et al. 2008), perhaps allowing further GATA3 induction through autoactivation loop (Ouyang et al. 2000).

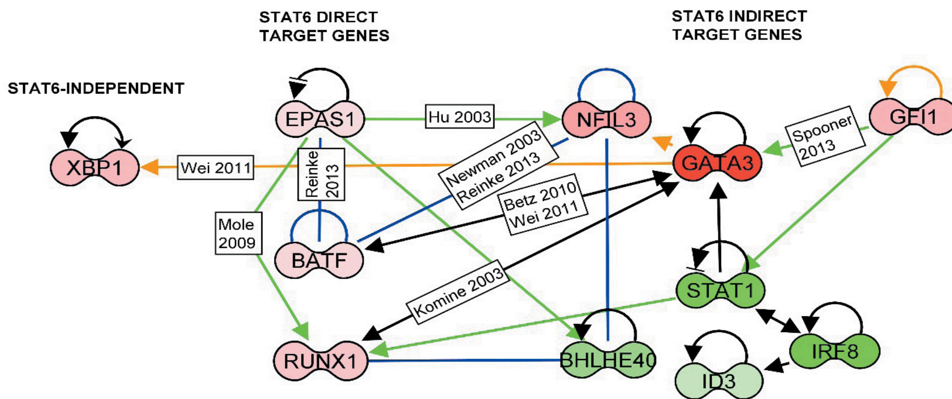

Figure 3. The 2014 update of the interconnected transcriptional network of STAT6 direct target genes, *EPAS1*, *BATF* and *RUNX1*, with the STAT6 indirect target genes identified in article I (Figure 5B). The nodes are coloured based on the average Th2/Th0 signal log ratio at 24h of the IL-4 kinetic culture. Blue edges correspond to protein-protein interaction, green edges to transcriptional regulation, orange edges to promoter binding, and black edges to mixture of these interactions. The network was generated through the use of Ingenuity Pathway Analysis software.

All in all, *BATF*, *EPAS1* and *RUNX1*, the core STAT6-dependent primary transcription factor targets (I, Figure 5B), were identified as the putative key initiators of the IL-4-induced transcriptional program. As mentioned, they show tight molecular association with indirect target genes of STAT6, especially *GATA3* and *NFIL3* (**Figure 3**). RUNX1 has been previously linked to inhibition of Th2 cell polarization via downregulation of GATA3 expression (Komine et al. 2003) and through binding to the IL-4 silencer (Naoe et al. 2007). Interestingly, EPAS1 binds to the promoter of *RUNX1* (Mole et al. 2009) and may amplify the STAT6 effect. EPAS1 has also been shown to interact with BATF at the protein level (Reinke et al. 2013), and to induce *NFIL3* in HEK293 cells upon hypoxia (Hu et al. 2003). BATF, the third transcription factor found to be directly regulated by STAT6, has been shown to be needed for both Th17 (Schraml et al. 2009) and Th2 (Betz et al. 2010) differentiation. Additionally, it has been shown to be induced by Gata3 in mouse Th2 cells (Wei et al. 2011) and to interact with NFIL3 (Newman and Keating 2003, Reinke et al. 2013). The indirect STAT6 target gene Gfi1 is known to increase proliferation of Th2 cells (Zhu et al. 2002) and to induce *Gata3* in type 2 innate lymphocytes (Spooner et al. 2013). Nfil3 has been shown to positively regulate IL-4, although it supresses IL-5 and IL-13 at an early state of Th2 polarization, and negatively affects IL-4 at a later stage of polarization (Kashiwada et al. 2011). In the identified core transcriptional network of Th2 cells, *BHLHE40*, *ID3*, *IRF8*, and *STAT1* were indirectly downregulated by STAT6, suggesting that the expression of these factors is disadvantageous for Th2 cell differentiation. In addition, GATA3 has been observed

to bind the promoter of STAT6-independent IL-4 target gene *XBP1* (Wei et al. 2011), reinforcing the role of GATA3 in regulating the STAT6-independent arm of the Th2 polarization. Interestingly, comparison to recent genome-wide analysis of GATA3 binding in Th1 and Th2 subsets, as well as T-bet in the Th1 subset (Kanhere et al. 2012), indicates that majority of the core regulatory network genes are strongly bound by both T-bet and GATA3 in the human Th1 cells polarized for 10 days, whereas the GATA3 binding in the respective Th2 culture is rather low (**Table 1**). This further indicates their central role in dictating the Th1-Th2 dichotomy. In addition, the absence of GATA3 binding in Th2 subset could indicate either temporal or indirect regulation. The latest genome-wide data on mouse Th2 cells by Wei et al. (2010) supports these findings. Here the core regulatory network genes showed similar IL-4 mediated patterns of up –and downregulation, with the exception of *Gfi1*, which is not very Th2-specific in the mouse data, and *Stat1*, which seems to be more Th2-specific. With the exception of *Gfi1*, the Stat6 induced up- and downregulated pattern is similar, and Stat6 induces also *Xbp1* that was STAT6 independent in the data of article I. Most strikingly, Stat6 directly binds to all of the regulatory network genes, except for *Xbp1*, indicating that the STAT6-independent arm of the core regulatory network might be bound by STAT6 at a later timepoint in more polarized cells.

Table 1. STAT6 core network genes (**Figure 3**) and the number of T-bet and GATA3 binding sites identified with ChIP-seq from human Th1 and Th2 cells polarized for 10 days (Kanhere et al. 2012, Table S1).

Gene	# T-bet sites (Th1)	# GATA3 sites (Th1)	# GATA3 sites (Th2)
XBP1	9	4	0
BATF	5	2	0
RUNX1	15	12	1
NFIL3	5	4	0
BHLHE40	13	14	2
STAT1	7	10	2

The linking of STAT5A to the regulation of the human Th2 transcriptome (I, Table S6) was an intriguing observation, as previous mouse data has shown its crucial involvement in the process: *Stat6*$^{-/-}$*Stat5a*$^{-/-}$ mice have markedly reduced airway inflammation compared to *Stat6*$^{-/-}$ (Takatori et al. 2005) and Stat5 is known to play an important role in IL-2 enhanced Th2 cell polarization (Zhu et al. 2006). STAT5A can be connected to the core transcriptional network through GFI1, which is involved in increasing Stat5a phosphorylation upon IL-2 signalling (Zhu et al. 2002). Additionally, STAT5A is known to bind the *EPAS1* promoter in hematopoietic stem cells (Fatrai et al. 2011) and to interact with EPAS1 at the protein level (Wang et al. 2011). In addition to STAT5A, the core transcription factor genes could be linked to other central factors regulating

alternative Th cell phenotypes (I, Figure 5C), namely STAT1 and STAT4 regulating Th1 cell differentiation, as well as STAT3 acting on the Th17 cell subtype. Interestingly, Stat3 was shown to promote Th2 differentiation in the presence of Stat6, as Stat3 was needed for optimal expression of *Gata3*, *Batf*, and Th2 cytokines, and was shown to control the locus accessibility for effective Stat6 binding (Stritesky et al. 2011). In addition, Stat3 signalling was needed for acquiring a Tfh-like phenotype in Th2 cells needed for optimal B cell help (Mari et al. 2013). Clearly, co-operational role of the STAT family proteins plays a major role in determining the T helper cell fate, and the overlapping regulatory networks should be further studied in the human system.

6.2 Identification of gene expression changes associated with pathogenesis of type 1 diabetes

The results of the article (II) demonstrated that T1D-specific changes are evident in the whole-blood transcriptome, affecting hundreds of genes. Along with the study on German children at high genetic risk for T1D (Ferreira et al. 2014), this was the first time samples before and at the time of seroconversion were studied with genome-wide expression arrays, providing novel information on the immunopathogenesis of type 1 diabetes (**Figure 4**).

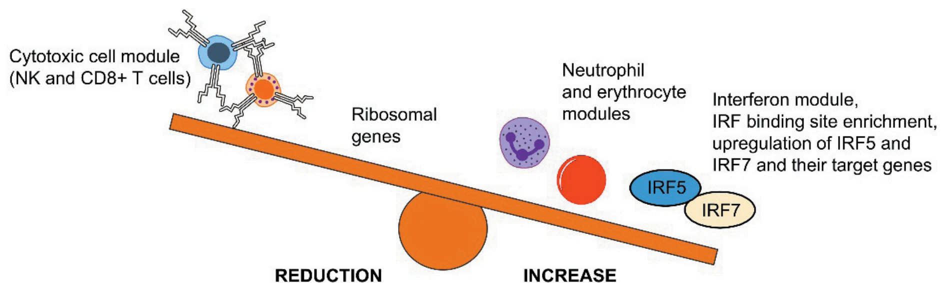

Figure 4. Summary of the transcriptional signatures detected in the whole blood of HLA-susceptible children before the first detection of T1D-associated autoantibodies (II), indicating the components of the imbalanced immune system with a potential contribution to the pathogenesis of T1D.

A module-based data analysis tool, which had been developed for whole-blood gene expression samples, was used to infer the cell types and biological processes important in different phases of the pathogenesis of type 1 diabetes (II, Figure 4 and Supplementary Table 5). For example the neutrophil module, including granule proteins such as lipocalin 2 (*LCN2*) and matrix metalloprotease 9 (*MMP9*), was first activated, after which the neutrophil-associated transcripts were suppressed before diagnosis. This is consistent with a recent report on reduced numbers (7-27%) of circulating neutrophils

during the preclinical autoantibody positive phase until years after diagnosis of T1D, when compared to healthy controls or patients with T2D (Valle et al. 2013). Neutrophils are tightly associated with autoimmune reactions, as they constitute a majority of the immune cell infiltration of the joint in RA, and several abnormalities in neutrophil functions have been demonstrated in both RA and SLE (reviewed in Kaplan 2013), as well as in EAE mouse model for MS (Steinbach et al. 2013). Interestingly, the T1D and autoimmune-associated *PTPN22* gene R620W variant was recently shown to enhance neutrophil functions, such as ROS production and Ca^{2+} release (Bayley et al. 2014).

The platelet module, including coagulation factor XIII A1 polypeptide (*F13A1*), was activated after seroconversion until clinical disease, which is in line with the reports on platelet hyperreactivity in type 1 diabetes (Sobol and Watala 2000). This should be also interpreted in the light of increasingly appreciated role of platelets in various immune-cell contacts and cytokine secretion (Yeaman 2014). Interestingly, the cytotoxic cell module was suppressed before seroconversion, but highly activated at the time of diagnosis, indicating the differential cytotoxic cell activity in the blood during the early vs. late progression of the disease process. However, the cytotoxic cell module consists of both adaptive (CD8+ T cells) and innate (NK cells) cytotoxic cell genes, such as granulysin (*GNLY*) expressed by both cell types, and thus the detailed role of these cannot be further separated. Also the erythrocyte related module was activated in the early time-windows and before diagnosis. Patients with type 1 and type 2 diabetes patients exhibit reduced red blood cell plasticity (also called erythrocyte deformability) and glycated haemoglobin due to elevated glucose levels, and thus the induced activity of the erythrocyte-associated transcripts could be a general indicator of the metabolic stress or a compensatory mechanism common to both T1D and T2D. However, as the erythrocyte module was increased already before seroconversion, the effect of the elevated glucose level in this early time-window is more unlikely, and merits further investigation. Mature erythrocytes are non-nucleated and thus do not synthesize mRNA. Therefore, one possible explanation is an increase in the number of more immature erythrocytes due to increased haemopoiesis.

The interferon module was affected throughout the time-windows analysed. In addition, genes involved in the RIG-I-like receptor signalling pathway were upregulated in the seroconverted individuals, and also the binding sites for interferon regulatory factors (IRFs) as well as interferon response element (ISRE) were enriched in the promoters of the regulated genes. In the type 1 diabetes progressors, IRF7 and IRF8 binding sites were enriched on the upregulated genes. Taken together, the results indicate that type 1 interferon mediated innate immune system is activated even prior to seroconversion and throughout the development of type 1 diabetes. The induction is temporal, as expected for this systemic response, but some individuals show several signature peaks

during the follow-up period (II, Supplementary Figure 1). Importantly, the findings are consistent with the results of by Ferreira et al. (2014), conducted in an independent birth cohort of children genetically susceptible for type 1 diabetes, also reporting a type 1 interferon signature to be temporally increased in susceptible children prior to the development of autoantibodies. In agreement with these results, Reynier et al. (2010) recently reported that 12 interferon response genes are upregulated in whole-blood of 30% of autoantibody-positive prediabetic children, but not in healthy controls or recently diagnosed patients. The data of the article II indicates that the interferon response can be detected before the appearance of autoantibodies (II, Figure 4), and that it consists of a much larger number of genes than previously reported. In fact, comparison of the differentially regulated genes to the genes listed to be involved in functions related to human innate immune responses in the InnateDB (Breuer et al. 2013) gave an overlap of 17.4% for the seroconverted children (19 genes, $P<0.001$) and 9.5% for the progressors (45 genes, $P<0.001$), respectively (II, Supplementary Tables 2 and 3, InnateDB columns). Interferons α and β were not differentially regulated in the data, indicating that the blood cells are showing an indirect response to cytokines produced elsewhere, supporting the viral theory of T1D pathogenesis. Already in 1987 Foulis et al. reported that hyperexpression of HLA class I by the beta cells was strongly associated with the upregulation of interferon alpha in the pancreatic stainings of T1D organ donors (Foulis et al. 1987). More recent studies have detected enteroviral infections in the beta cells of newly diagnosed patients (Dotta et al. 2007) and signs of infections have been observed a few months prior to seroconversion to autoantibody positivity (Hiltunen et al. 1997) and during the 6-month period preceding seroconversion (Laitinen et al. 2013, Oikarinen et al. 2011). In addition, respiratory infections during the first 6 months of life were shown to increase the risk for autoantibody seroconversion (Beyerlein et al. 2013). Moreover, Ferreira et al. (2014) demonstrated an association between upper respiratory tract infections and upregulation of interferon responsive genes in children developing autoantibodies or progressing towards clinical T1D.

In addition, bacterial DNA, LPS, and flagellin are efficient triggers of TLR signalling and interferon response, providing an intriguing link between the gut immune system and autoimmunity. Both preclinical and established type 1 diabetes patients exhibit increased gut permeability (Bosi et al. 2006, Kuitunen et al. 2002), leading to enhanced immune responses in the intestinal tissues (Westerholm-Ormio et al. 2003). Also, a preliminary study has shown that gut microbiome diversity is reduced in children progressing towards type 1 diabetes compared to healthy controls (Giongo et al. 2011). Moreover, the interferon response can be activated without infection by bacteria or viruses; for example, in SLE, IFNα production is driven by TLR7-mediated signaling and is induced by autoantibody-protein-RNA complexes derived from apoptotic cells. Also, endogenous TLR ligands such as RNASE2, that was identified to be upregulated in the seroconverters who later presented

with type 1 diabetes, could play a role as it has been shown that spontaneous interferon production drives autoimmune diabetes in NOD mice (Li et al. 2008a). In SLE, antinuclear autoantibodies activate neutrophils to die by forming neutrophil extracellular traps (NETs) that consist of DNA and antimicrobial proteins. This leads to the release of self-DNA immunocomplexes, as well as stimulation of pDCs, that robustly secrete type I interferons, both markers of SLE. IFNα treated healthy neutrophils were also susceptible to NETosis upon autoantibody exposure. (Garcia-Romo et al. 2011).

In this study, the matching of the cases and controls was based on the *HLA-DQB1* genotype. To take into account further susceptibility alleles and alterations affecting the transcriptional changes, Immunochip SNP detection was performed for combinatorial analysis of the genome and transcriptome. This revealed that approximately 10% of the genes differentially expressed between the cases and controls were affected in *cis* by the genetic variation. The affected variants and genes have previously been linked to several autoimmune diseases, highlighting the shared genetic basis for these disorders. For example in the case of SLE, where strong type 1 interferon signature is also observed, the associated variations in *IRF5* have been shown to control cytokine responses upon TLR ligation (Hedl and Abraham 2012). This pathway could be also genetically linked to type 1 diabetes, since only a small part of the genetic factors conferring susceptibility to this disease have been identified. In support of the findings of article II, Heinig et al. (2010) performed an expression quantitative trait loci (eQTL) analysis across seven rat tissues combined with transcription factor binding site enrichment analysis, resulting in the identification of Irf7 and 23 Irf7 target genes mapping to a single eQTL. They subsequently identified a human IRF7-driven network using genome-wide expression and SNP data collected from monocytes. Eventually, SNPs close to genes in the human and rat IRF7 network were found to be associated with T1D. Most importantly, of the 12 direct IRF7 target genes (identified by the IPA tool) detected as differentially expressed (II, Figure 2B), eight (66%) are present in the human IRF7-regulated network identified by Heinig et al. through a completely different complementary approach further highlighting the role of this pathway in T1D pathogenesis.

6.3 Intrauterine immune adaptation to the standard of hygiene

The study (III) presented the first transcriptional investigation of the changes occurring in the expression levels of immunologically active molecules of umbilical cord blood as an adaptation to the surrounding *in utero* environment. The results were clear, with the modern and developing societies (Espoo and Tartu) deviating from the more traditional environment (Petrozavodsk) in many aspects of the neonatal immune system (**Figure 5**), providing further support for the hygiene hypothesis and the effect of the *in utero* period in the maturation of the neonatal immune system.

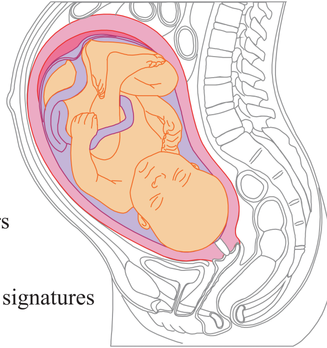

Figure 5. Differences observed in the cord blood transcriptome of newborn infants born in traditional environment (Petrozavodsk, Russian Karelia) as opposed to modern (Espoo, Finland) and developing (Tartu, Estonia) societies. Reprinted from Clinical Immunology, Volume 155, Issue 1, Kallionpää et al., Standard of hygiene and immune adaptation in newborn infants, 136-137, 2014, graphical abstract, with permission from Elsevier.

Upregulation of many central genes of the innate immune response, such as *AIM2*, *ATG5* and *IRF7*, and *TLR2* was evident in the cord blood samples from Petrozavodsk. IRF7 plays a central role in viral defense and the induction of type 1 interferon response (Honda et al. 2005), and the IRF7 regulatory network shows genetic association to T1D (Heinig et al. 2010). *TLR2* expression has been reported to be upregulated in neonate mononuclear cells upon infection with Gram-positive bacteria (Zhang et al. 2010a), and conversely be downregulated in cord blood $CD34^+$ progenitor cells of newborns at high atopic risk due to maternal allergy (Reece et al. 2011). The activation of the innate immunity has been shown to protect from allergy. A multinational study demonstrated an association between maternal contact with farm animals during pregnancy, higher expression of Toll-liker receptors 1-9, and a protective effect on developing atopic dermatitis in the first 2 years of life (Roduit et al. 2011). Therefore, the detected activation of innate immune genes in Petrozavodsk, usually associated with subsequent Th1 cell immune responses, might protect these infants from aberrant Th2 cell immunity, associated with atopy and asthma, later in life.

There was also a clear overlap with the upregulated genes in Petrozavodsk and the genes previously identified to be induced by LPS or bacterial sepsis at 1 year of age, but not in newborns (III, Figure 4 and Table S3). This indicates, that newborn infants born in Petrozavodsk have been exposed to TLR ligands *in utero*, and that the subsequent immune response results in more matured phenotype of the immune system, resembling that of 1 year old infants. In support of this, it was recently reported that Papua New Guinean (traditional environment) neonatal antigen presenting cells (APC) had a more activated and mature baseline status than the APCs of Australian (modern environment) neonates,

as determined by increased expression of activation markers HLA-DR and CD86, but that the APCs were also more quiescent and resistant to further LPS stimulation (Lisciandro et al. 2012b). This would indicate that the surrounding bacterial environment is preprogramming the immune responses *in utero*, and that this environment would differ in Petrozavodsk versus the other study sites. In fact, data on house dust sample analysis in Finnish and Russian Karelian homes revealed striking differences, as both the amount of bacterial content and the diversity were higher in Russian Karelia than in Finland (Pakarinen et al. 2008). Lisciandro et al. (2012b) suggested that the differences in the state of activation of antigen presenting cells from neonates between modern and traditional environments reflect an evolutionary mechanism of immune regulation learned *in utero* to prepare newborn infants for the intensity and frequency of the immunological challenges in the postnatal environment and to protect them from infections.

Markers of more immature immune cell phenotypes, such as *ZFP36L1* inhibiting plasma cell and erythroid differentiation, or pre-T cell receptor alpha (*PTCRA*) expressed in immature T cells, were upregulated in newborn infants from Espoo and Tartu (III, Figs 3A-B). *Zfp36l1* downregulation, similar to that seen in samples from Petrozavodsk, has been observed in murine B cells after LPS stimulation and during the shift from mature B cell to plasma cell (Nasir et al. 2012). This suggests that not only innate immune cells, but also adaptive T and B cells in newborn infants from Espoo and Tartu have a more immature phenotype and have not been activated and/or differentiated to the extent observed in Petrozavodsk infants. On the other hand, it also seemed that inhibitory or homeostasis mechanisms were activated in Petrozavodsk, such as CD46 with a role of suppressing Th1 responses in infection (Nasir et al. 2012). CD46-induced Tregs have been hypothesized to ensure unresponsiveness to commensal bacteria while maintaining defence against invading pathogens by allowing DC maturation (Barchet et al. 2006). Interestingly, CD46 responsiveness is defective in T cells from patients with multiple sclerosis (Astier et al. 2006), asthma (Xu et al. 2010) and juvenile and rheumatoid arthritis (Cardone et al. 2010). In this respect, observations with the multifunctional C4BPA, highly upregulated in Petrozavodsk, was also an interesting finding. This protein has immunomodulatory downstream effects on DCs, as treatment with LPS together with C4BPA failed to induce activation markers and Th1 programming, and instead promoted Treg differentiation (Olivar et al. 2013). It is intriguing to speculate that such a tolerant state of the APCs and lymphocytes induced *in utero* would lead to different immune responses upon colonization with the commensal flora or infections after birth, and that a similar protective shift in the prenatal host-microbe interaction might be achieved by probiotics in the modern environments (Rautava et al. 2012b).

6.4 Limitations of the studies and theoretical implications for further exploration

In the timeframe of the study I (until 72h), STAT6 was shown to be an essential transcriptional activator of human Th2 cell polarization. However, the kinetic qPCR validation of the STAT6 bound ChIP-seq peaks illustrated that the binding sites of STAT6 measured early at 1h and 4h might not be occupied by STAT6 at the later timepoints (I, Figure 4). It is clear that the data presented by the article (I) covers only the initial hours and days of Th2 polarization, and thus cannot be directly extrapolated to more distant timeframes and fully polarized Th2 effector cells. If indeed STAT6 mainly directly activates the Th2 specific genes early on, and the indirect signalling shuts off the alternative differentiation paths, the question arises whether STAT6 action is required anymore in the reinforcement state of the Th2 cells, once the upregulation of GATA3 and other downstream transcriptional regulators has already been successfully established. Therefore, STAT6 binding and target genes should also be explored at a later stage of polarization to further delineate the timeframe and dynamics of STAT6 regulation. In addition, the role of the core transcriptional network under STAT6 (*BATF*, *EPAS1*, and *RUNX1*) requires further validation and exploration, possibly as a combinatorial modulation. Similarly, the role of other STAT family members in human Th2 cell polarization remains to be explored.

Differences between the existing data on *Stat6* knockout mice and the results from human cells in study I were clear. However, the experimental setup, culture conditions, polarization efficiency, sampling timepoints, as well as the analysis platforms in the mouse and human studies differed, which makes comparisons somewhat challenging. In addition, in-bred mouse strains (Watanabe et al. 2004) are certainly not comparable to the extent of genetic variation present in human population and primary cell assays. Several key aspects of human and mouse cell biology are different (Seok et al. 2013), warranting studies on human cells. On the other hand, the experimental systems available for human primary cells are somewhat limited. For example, with the current experimental model of primary CD4+ cells, obtaining a complete and long-lasting knockdown is challenging, and therefore some of the weaker effects of the STAT6 downregulation might have been missed.

Further limitations of study I were introduced during the data analysis, such as determining the cut-off thresholds and genomic windows for signifigance. Assigning the ChIP-seq peaks simply to a nearest gene is problematic especially in the case of the intergenic binding sites. Therefore, the global chromatin landscape of the cells should be taken into account (Hawkins et al. 2013). Such information would allow more finetuned analysis of a promoter binding, gene activity status, as well as information of the transcriptional blocks determined by the CTCF protein insulator, which all could bring

crucial validation for the parallel transcriptomic and ChIP-seq analyses. Furthermore, although the chromatin binding of STAT6 was dissected using the massively parallel sequencing, the detection of IL-4 and STAT6 -induced transcripts relied on the use of microarrays, complicating the inter-experimental annotations. Recent reports of STAT6 as a chromatin remodeller also warrant investigations of the chromosomal 3D structure, which could further clarify the role of the intergenic STAT6 binding.

The relatively low number of cases (n=18) could have limited the power and false discovery rates in the study II, especially when comparing the progressors and non-progressors before seroconversion. However, technical replicates were used, as each sample was hybridized on two different array formats. Due to low power, the extent of information and analyses gained with Immunochip genotyping was limited to the differentially expressed genes only. Furthermore, the Immunochip measures a preselected panel of polymorphisms and copy number variations, and is thus unable to provide genome-wide coverage of the genetic variation. However, the initial findings of the study (II) encourage further exploration combining genomic, transcriptomic and epigenetic data. A vast majority of differentially regulated genes were not affected in *cis* by genetic variation, suggesting additional levels of regulation, such as *trans* effects, epigenetic or miRNA mediated regulation. Interestingly, *cis* eQTLs were found in the genes of the core IRF5 and IRF7 –mediated transcriptional network, further supporting the strong influence of genetic variants, such as in the case of *IFIH1* (Nejentsev et al. 2009) and the IRF7 network (Heinig et al. 2010), in the regulation of the type I interferon response. Additionally, other levels of regulation may play a role, as demonstrated in genome wide CpG island methylation analysis of T cells, B cells, and monocytes from patients with SLE. This study revealed hypomethylation near the genes involved with type I interferon signalling, both in active and quiescent phases of the disease (Absher et al. 2013). Based on this data the authors suggested that the SLE-specific methylation defects lead to hyper-sensitization towards interferons, and such a poised state quickly leads to overexpression of the interferon response genes upon increasing levels of interferon α and β. The DNA methylation status of T1D patients has only been explored in a handful of studies, and certainly not in pre-clinical patients. In addition, the inducer and source of the type I interferons remains to be validated. The correlation of gene expression profiles to markers of virus infections in susceptible children should be explored more carefully. Almost all cell types in the body are capable for producing type I interferons, but pDCs are particularly specialized for this function. Interestingly, several studies have reported a reduction in pDC counts in the peripheral blood of T1D patients when compared to healthy controls (Chen et al. 2008, Hinkmann et al. 2008, Nieminen et al. 2012, Vuckovic et al. 2007), and pDCs are known to accumulate in the pancreas of NOD mice at the early phases of disease process (Li et al. 2008a). The use of type I interferon signature as a disease biomarker might be limited, as in the case of

SLE, where interferon signature is detected during flares, the active state of the disease, and complicates the identification of active disease vs. infections. In addition, the T1D-associated type I interferon signature was not as frequent as seen in SLE patients (Ferreira et al. 2014), perhaps suggesting it to be more easily detectable in systemic disease than in organ-specific autoimmunity.

A limitation of the studies II and III relates to the use of whole blood samples. First, the presence of relatively abundant transcripts, such as globins, might mask changes in the levels of more low-abundant mRNAs. In practice, the globin is usually removed from whole blood samples prior to the transcriptomic analyses. However, the samples in studies II and III were processed with a kit that "tolerates" globin, supplies the polymerase reaction with extra reagents usually consumed by the globin amplification, and produces cDNA instead of cRNA thus allowing more stringent hybridization and washes. In addition, the fetal globin present in newborn infants and very young children would not be removed by commercial kits, and the introduction of such extra handling steps in the sample preparation protocol could create undesired biases and batch effects. Secondly, the detected genes and potential biomarkers are presently limited to the use of whole blood, as pinpointing the cell population/s responsible for the detected changes is subject to biological interpretation and the use of data mining tools. As the detailed recording of the proportions of different cell populations at the time of the blood sample draw was not available, the conclusions based on heterogeneous whole blood samples should be further explored in future studies. In addition, the signatures detected in the peripheral blood do not necessarily mirror the events taking place in the target organ(s) and associated lymphoid tissue, and might simply reflect changes in the tissue homing capacity of the immune cells. That said, the whole blood analysis is simultaneously an advantage for the practical implications of the study, as whole blood is an easily accessible material for both novel discoveries and diagnostic testing for disease susceptibility. If PBMCs or sorted cells had been used, the differences in the erythrocyte-associated transcripts would have been missed. In addition, correlation of additional levels of data from serum or plasma, such as serum metabolomics and proteomics measurements, is more straightforward with the use of unsorted whole blood transcriptomics data.

The samples of the cord blood transcriptomics study (III) were collected at three different study sites, although possible batch effects were kept at minimum by stabilizing the RNA immediately after the blood draw into the preservative buffer present in the collection tubes, as well as storing the samples at −70 °C until processing and analysis in the same laboratory. Any batch effects related to hybridization were eliminated during the data analysis as well as possible. It would certainly be of interest to repeat the study in another cohort of neonates born in contrasting standards of living as a validation for this study, and take into account also prenatal variables, such as maternal diet, microbiome,

infections and the course of pregnancy and child birth. For example, consumption and intake of proteins from sour milk products, and intake of fat from fresh milk products during pregnancy were associated with an increased risk of pre-clinical T1D in the offspring (Niinistö et al. 2014). Additionally, a recent study demonstrated that maternal blood biomarker status of substrates and cofactors required for methyl-donor pathways at the time of conception was associated with DNA methylation patterns in the neonates (Dominguez-Salas et al. 2014). Such variables will be further delineated in the Australian ENDIA study that samples mothers from the early pregnancy in the search for early environmental triggers for T1D (Penno et al. 2013). Additionally, neonatal immune system modulation through the use of probiotics in the modern societies should be investigated further, but with care, as maternal LGG supplementation in the final weeks of gestation was observed to reduce vaccine-specific immunity in infants (Licciardi et al. 2013).

7. CONCLUSIONS

In these studies transcriptional responses mediated by IL-4 and STAT6 were analyzed in detail for the first time in human. STAT6 was shown to act as a central activator of the early gene expression upon IL-4 signalling, with both direct and indirect effects on the dynamic Th2 cell transcriptome. The data presented in this study provides a solid basis for subsequent research on the role of STAT6 in the committed and effector Th2 cells driving the allergic inflammation. For example, immediate STAT6 target genes might be eligible for drug development.

The study on longitudinal whole-blood samples from T1D-susceptible children revealed the dynamic regulation of the transcriptome along the disease process, highlighting the importance of studies performed throughout the preclinical phase of T1D. Early changes were found that even preceded the appearance of the first autoantibodies, the best biomarkers of the activated disease process at the moment. This might facilitate the development of better diagnostic tools for monitoring autoimmune activity and progression, allowing earlier identification of the high-risk children for future intervention trials.

The findings from the cord blood analyses bring further support to the hygiene theory and extend the immunomodulatory window to the prenatal period. The environmental factors that play a role in the susceptibility/protection towards immune-mediated diseases begin to shape the neonatal immunity already *in utero* and direct the maturation of both the adaptive and the innate immune responses in accordance with the surrounding microbial milieu. This could then have far reaching effects on immune functions later in childhood, and dictate the outcome of the balance between efficient immune responses against pathogens and development of immune mediated diseases. Based on this observation, it could be speculated that the prevalency of these diseases keep increasing together with growing industrialization and modernization, warranting further confirmatory studies and exploration of immunomodulatory possibilities during gestation.

The studies concerning primary human cells and clinical samples are of great potential value, providing a resource for new avenues of research in tackling harmful immune reactions. The data presented reveals gene expression signatures related to health and disease of the immune system, and clearly justify the need for high-throughput experimental tools in the discovery phase studies on the human immune responses. Together, they demonstrate the vast complicity of the pathways and functions related to disease processes, and encourage the development of better and more detailed experimental and analysis tools for full utilization of the valuable human samples and

data. The analyses performed also highlighted the importance of inter-experimental and inter-species comparisons for novel insights and their validation. To initiate further exploration, the data produced in this Ph.D. thesis can be openly accessed in gene expression data repository.

In conclusion, our remarkable immune system has been shaped by the co-evolution with the surrounding microbes, pathogens and commensal flora for millions of years. The efficient defense mechanisms developed for this microbe-rich environment, together with the hygiene and health care practices of the modern societies, have ensured us the lifespan and the quality of life we enjoy today. Unfortunately, the selective pressure for genes coding for effective immune responses against pathogens, together with several bottle necks in the history of mankind, have simultaneously allowed enrichment of alleles conferring risk for immune-mediated diseases. Reduction of microbial contacts in the modern era has further tipped the balance in the favor of improper immune reactions contributing to disease. We are only beginning to understand the intricate pathways and functional mechanisms of the human immune system, which is crucial for diagnosis, interpretation, and amelioration of the disease processes.

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