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THE EFFECTS OF ENVIRONMENTAL AND DIETARY FACTORS ON THE DEVELOPMENT OF THE IMMUNE SYSTEM IN CHILDREN AT GENETIC RISK OF TYPE 1 DIABETES

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ABSTRACT

Epidemiological studies have shown an association between the increasing incidence of immune-mediated diseases and improved standard of living and hygiene. Studies claim that decreased microbial burden and diversity in our environment may affect the maturation of immune regulation, contributing to the development of both allergies and type 1 diabetes (T1D). Genetic factors play a role in autoimmune diseases. As genetic factors can hardly alone explain the rapid increase in either disease, environmental factors are considered to affect the induction of autoimmune diseases. Our environment, and our way of life, have changed over the last decades, such as changes in the infection rates and the types of infections. Dietary habits have as well changed. In autoimmune diseases, the pathogenesis is considered to result from a breakdown of immunologic tolerance. Immunologic tolerance is maintained by two major phenomena; central and peripheral tolerance. The balance between effector T cells and so-called regulatory T cells (Treg) is delicate, dictating the outcome.

This doctoral thesis set out to explore the effect of environmental factors on the development of the immune system by studying how gut microbiota and viral infections affect the Treg cells in infants at genetic risk of T1D. In the DIABIMMUNE study, we observed infants from Estonia and Finland, two neighboring countries, with a quite similar genetic risk of T1D but differences in the standard of living and the incidence of T1D. We followed the infants for three years from birth, and biological samples were collected at specified time points during the follow-up. Treg cells from whole blood samples were analyzed by flow cytometry and by enrichment of Treg cells to analyze the Treg cell-associated transcription factors. Also, the gut microbiome was studied by microbial DNA extraction and pyrosequencing and the virome by viral RNA sequencing.

This thesis also studied the effect of early diet on the appearance of T1D-linked autoantibodies and progression to T1D in the FINDIA trial. In that study, the infants were randomized to three different infant formula groups (Cow's milk formula (CMF), whey-based hydrolyzed formula (WHF), whey-based FINDIA formula, virtually free of bovine insulin), and the test formulas were used during the first six months of life. Infants who were only breastfed and did not consume any

formula formed the fourth group. The follow-up was up to at least three years of age. Blood samples were collected for antibody and the following T1D associated autoantibody analyses: insulin autoantibodies, antibodies to Glutamic acid decarboxylase autoantibody (GADA), and antibodies to islet antigen 2 with specific radiobinding assays. In individuals positive for at least one of the three initial autoantibodies, additional analyses of zinc transporter 8 autoantibodies and islet cell antibodies were performed. We wanted to study our hypothesis that immunization to bovine insulin during early infancy when the maturation of the gut is incomplete could explain the findings implicating cow milk as a risk factor for T1D.

The study demonstrated a two-step maturation process in the circulating Treg cell population in the first two years of life. This maturation process coincides with the development of the microbiota from a *Bifidobacteria* dominated to an adult-like microbiota dominated by butyrate-producing species, including the *Faecalibacterium* genus, Roseburia-like-, Coprococcus-like-, and various *Eubacteria* genus. We showed that the abundance of *Bifidobacteria*, and the relative abundance of *B. longum* alone, at three months of age, showed an inverse association with atopic sensitization and development of allergy. This association suggests that the richness of *B. longum* among Bifidobacteria is an important factor protecting from atopic sensitization. The switch in the microbiota occurred earlier in Estonian children than in Finnish children, associated with an earlier maturation of the circulating Treg population and a lower risk of allergic diseases.

The study also demonstrated that enterovirus infected infants had a decreased FOXP3 expression in Treg cells, particularly in highly activated $Treg^{FOXP3High}$ cells, with a cytokine profile with increased activation of Th1 and Th17 response and reduced activation of CCL22, a chemokine associated with Th2 response, and this effect lasted up to 60 days after the infection. These findings provide evidence of the potential of immunopathology induced by enterovirus infections. This immunopathology might be a sign of an early event in the breaking of tolerance.

The study showed that weaning to the FINDIA formula reduced the incidence of autoantibody positivity when compared with CMF. This result might reflect a dysfunctional immune system, where bovine insulin peptides from the infant

formula may activate autoreactivity against the primary autoantigen, i.e., human insulin, resulting in non-tolerogenic T cells.

The study shows that environmental factors, such as the development, the timing of the development, and the diversity of the gut microbiota or the viruses encountered can influence the development of regulatory T cells and immunotolerance. Also, the nutrition, i.e., breast milk or type of infant formula in early infancy, can influence the development of the immune system or autoantibodies to T1D in infants genetically at risk. The new-born infant encounters all of these environmental factors simultaneously when the immune system is maturing. Most likely, the interplay of all of these factors, or some of them, might be needed for the induction of autoimmunity in T1D or atopic sensitization.

Keywords: type 1 diabetes, allergy, regulatory T cells, autoantibodies, viruses, diet

TIIVISTELMÄ

Epidemiologisissa tutkimuksissa on osoitettu yhteys immuunivälitteisten sairauksien lisääntyneen esiintymisen ja parantuneen elintason ja hygienian välillä. Tutkimukset väittävät myös, että vähentynyt mikrobikuormitus ja monimuotoisuus ympäristössämme voi vaikuttaa immuunisäätelyn kehittymiseen edistämällä sekä allergioiden että tyypin 1 diabeteksen (T1D) kehittymistä. Vaikka geneettisillä tekijöillä on merkitys autoimmuunisairauksissa ne tuskin yksinään voivat selittää kummankin taudin nopeaa lisääntymistä. Ympäristötekijöiden, kuten sairastuvuuden infektiosairauksiin, sekä muuttuneiden ravitsemustottumusten, uskotaan vaikuttavan autoimmuunisairauksien syntyyn. Autoimmuunisairauksien patogeneesin katsotaan johtuvan immunologisen toleranssin murtumisesta.

Tässä väitöskirjatyössä tutkittiin ympäristötekijöiden vaikutusta immuunijärjestelmän kehitykseen tutkimalla, kuinka suoliston mikrobiota ja virusinfektiot vaikuttavat Treg-soluihin pikkulapsilla, joilla on geneettinen riski tyypin 1 diabetekseen. DIABIMMUNE-tutkimuksessa seurasimme lapsia Virosta ja Suomesta, kahdesta naapurimaasta, joilla on melko samanlainen geneettinen T1D-riski, mutta erot elintason ja T1D:n esiintyvyyden välillä. Seurasimme lapsia kolmen vuoden ajan syntymästään saakka, ja biologiset näytteet kerättiin tiettyinä ajankohtina seurannan aikana. Kokoverinäytteiden Treg-solut analysoitiin virtaussytometrialla ja rikastamalla Treg-soluja niihin liittyvien transkriptiotekijöiden analysoimiseksi. Suoliston mikrobiomia tutkittiin bakteerien DNA-uutolla ja pyrosekvensoinnilla ja viromia virus-RNA-sekvensoinnilla.

Tässä väitöskirjassa tutkittiin myös varhaisen ruokavalion vaikutusta diabetekseen-kytkettyjen auto-vasta-aineiden esiintymiseen ja etenemiseen diabetekseksi FINDIA-tutkimuksessa. Kyseisessä tutkimuksessa imeväiset satunnaistettiin kolmeen erilaiseen äidinmaidonkorvikeryhmään (lehmänmaitokorvike (CMF), herapohjainen hydrolysoitu korvike (WHF), herapohjainen FINDIA-korvike, joka oli käytännössä vapaa naudan insuliinista), ja korvikkeita käytettiin ensimmäisen kuuden kuukauden aikana. Neljännen ryhmän muodostivat täysimetetyt lapset, jotka eivät käyttäneet mitään äidinmaidonkorviketta. Lapsia seurattiin vähintään kolmen vuoden ikään asti, ja verinäytteet kerättiin vasta-aine ja diabetes-autovasta-aineanalyysejä varten.

Analysoidut autovasta-aineet olivat: insuliiniautovasta-aine, Glutamaattidekarboksylaasiproteiini (GADA) -vasta-aine ja saarekesolu 2 vasta-aine jotka detektoitiin spesifisillä radiositoutumismäärityksillä (RIA). Näytteistä, jotka olivat positiivisia ainakin yhdelle kolmesta alkuperäisestä vasta-aineesta, analysoitiin lisäksi zinc transporter 8 autovasta-aineet (ZnT8A) ja saarekesolu vasta-aineet.

Tutkimus osoitti kaksivaiheisen kypsymisprosessin perifeerisessä Treg-solupopulaatiossa kahden ensimmäisen elinvuoden aikana. Tämä kypsymisprosessi on yhtäaikainen suoliston bakteerikannan kehityksen kanssa. Bakteerikanta kehittyy *Bifidobakteeri*-valtaisesta bakteerikannasta aikuisen kaltaiseksi bakteeripopulaatioksi, jota hallitsevat voihapon suoloja ja estereitä (butyraattia) tuottavat lajit, kuten suvut *Faecalibacterium*, *Roseburia*, *Coprococcus*, ja useita *Eubacteria* sukuja. *Bifidobakteerien* runsaus yleensä ja *B. longumin* suhteellinen runsaus yksistään, kolmen kuukauden näytteissä, korreloi käänteisesti atooppiseen herkistymiseen ja allergian kehittymiseen. Tämä bakteerikannan kehitys oli varhaisempi virolaisilla lapsilla verrattuna suomalaisiin lapsiin. Virolaisilla lapsilla oli myös alhaisempi allergisten sairauksien riski verrattuna suomalaisiin lapsiin, mikä viittaa Treg populaation aikaisempaan kypsymiseen.

Tutkimuksessa havaittiin myös, että enterovirus-tartunnan saaneilla imeväisillä oli vähentynyt FOXP3-ilmentyminen Treg-soluissa, etenkin voimakkaasti aktivoiduissa $Treg^{FOXP3high}$-soluissa. Tähän Treg solujen expressioon liittyi sytokiiniprofiili, jossa oli nähtävissä lisääntynyt Th1- ja Th17-vasteen (Th =T auttajasolu) aktivaatio ja vähentynyt CCL22:n aktivoituminen, ja tämä vaikutus kesti jopa 60 päivää infektion jälkeen. Nämä havainnot saattavat kuvastaa enterovirusinfektioiden aiheuttamaa immunopatologiaa, joka voi olla merkki varhaisesta tapahtumasta toleranssin murtumisessa.

Tutkimus osoitti lisäksi, että FINDIA-korvikkeen käyttö vähensi autovasta-aineiden esiintyvyyttä verrattuna lehmänmaito-korvikkeeseen. Tämä tulos voi heijastaa immuunijärjestelmän häiriötä, jossa äidinmaitokorvikkeesta peräisin olevat naudan insuliinipeptidit saattavat aktivoida autoreaktiivisuuden ensisijaista autoantigeeniä, ts. Ihmisen insuliinia vastaan ja johtaa ei-tolerogeenisiin T-soluihin.

Tutkimus osoitti, että ympäristötekijät, kuten suolen mikrobiotan kehitys, sen monimuotoisuus ja kehittymisen ajoitus, sekä sairastetut virusinfektiot voivat vaikuttaa säätelevien T-solujen ja immunotoleranssin kehittymiseen varhaislapsuudessa. Myös varhaislapsuuden ravitsemus, eli rintamaito tai äidinmaidonkorviketyyppi, voivat vaikuttaa diabeteksen autovasta-aineiden kehittymiseen näillä tyypin 1 diabeteksen geneettisen riskin omaavilla lapsilla. Vastasyntynyt vauva kohtaa kaikki nämä ympäristötekijät samanaikaisesti aikana jolloin immuunijärjestelmä on vasta kypsymässä. Todennäköisesti yhteisvaikutusta joidenkin tai kaikkien näiden tekijöiden välillä voidaan tarvita tyypin 1 diabeteksen tai allergioiden kehittymiseen.

Avainsanat: tyypin 1 diabetes, allergia, säätelevät T-solut, autovasta-aineet, virukset, imeväisruokinta

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LIST OF ORIGINAL PUBLICATIONS

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II Ruohtula T, Kondrashova A, Lehtonen J, Oikarinen S, Hämäläinen A-M, Peet A, Tillmann V, Nieminen JK, Ilonen J, Knip M, Vaarala O, Hyöty H, and the DIABIMMUNE Study Group. Immunomodulatory Effects of Rhinovirus and Enterovirus Infections During the First Year of Life. Front Immunol. 2021 Feb 11;11:567046. doi: 10.3389/fimmu.2020.567046. PMID: 33643278; PMCID: PMC7905218.

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The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

Ag	antigen
APC	antigen-presenting cell
AU	arbitrary units
BACH2	transcription regulator protein
BCL11B	B-cell lymphoma/leukemia 11B protein
BSA	bovine serum albumin
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD25	IL-2 receptor α subunit, cluster of differentiation 25
CI	confidence interval
CMF	cow's milk formula
CTLA-4	cytotoxic T-lymphocyte activation antigen 4
DC	dendritic cell
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme-linked solid-phase immunoassay
EST	Estonian child
FACS	fluorecenscence activated cell sorter
FIN	Finnish child
FOXP3	transcription regulator fork-head box P3
GADA	antibodies to glutamic acid decarboxylase
GATA3	transcription factor
GITR	glucocorticoid-induced TNF receptor
HLA	human leucocyte antigen
HAS	human serum albumin
IA-2A	islet antigen 2 autoantibodies
IAA	insulin autoantibodies
IBD	inflammatory bowel disease
ICA	islet cell antibodies
ICOS	inducible co-stimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IRF4	interferon regulatory factor 4

mDC	myeloid dendritic cell
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MS	multiple sclerosis
NK	natural killer cell
NS	non-significant
NOD	non-obese diabetic
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
pTreg	adaptive/peripheral regulatory T cell
PU.1	ETS-family transcription factor (SPI1)
RA	rheumatoid arthritis
RegIII	regenerating islet-derived protein 3
RNA	ribonucleic acid
RLT	lysis buffer for lysing cells prior to RNA isolation
ROR	RAR-related orphan receptor
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RU	relative unit
SLO	secondary lymphoid organs
STAT	signal transducer and activator of transcription
T1D	type 1 diabetes
T-bet	T-box transcription factor TBX21
TCF-1	T-cell factor 1, HMG-box transcription factor 7 (T-cell specific)
TCR	T-cell receptor
Teff	effector T cell
TGF-β	transforming growth factor β
Th	T helper cell
TLR	toll like receptor
TNF-α	tumor necrosis factor α
Tr1	type 1 regulatory T cell
TREC	T-cell receptor excision circle DNA
tRA	*all-trans* retinoic acid
Treg	regulatory T-cell
tTreg	naturally occurring/thymic regulatory T cell
WHF	whey-based hydrolyzed formula
ZnT8A	zinc transporter 8 antibodies

1 INTRODUCTION

Epidemiological studies have shown an association between the increasing incidence of immune-mediated diseases and improved standard of living and hygiene. The hygiene hypothesis, originally postulating that a decreased exposure to infections during childhood increases the susceptibility to immune-mediated diseases, has been expanded to claim, that impaired maturation of the immune regulation, contributing to the development of such diseases, is caused by a reduced microbial burden or diversity. Both in allergies and T1D, a combination of genetic predisposition, environmental factors, and stochastic events are needed for the disease pathology. Genetic factors can hardly alone explain the rapid increase in either disease. Our environment, and our way of life, has changed over the last decades, such as changes in the infection rates and the types of infections. Also, dietary habits have changed. In autoimmune diseases, the pathogenesis is considered to result from a breakdown of immunologic tolerance. Immunologic tolerance is maintained by two major phenomena; central and peripheral tolerance. The balance between effector T cells and so-called regulatory T cells (Treg) is delicate, and it dictates the outcome.

In this study, the effect of environmental factors was explored on the development of regulatory T cells (Treg) in the context of immune-mediated diseases, such as allergies and T1D. The maturation of Tregs was assessed in a prospective study (the DIABIMMUNE study) in the context of both the gut microbiome development during the first three years and the effects of enteral virus infections in early infancy. Also, the effect of the infant diet on the appearance of autoantibodies was analyzed in the FINDIA study. The infants were randomized to three different infant formula groups (Cow's milk formula (CMF), whey-based hydrolyzed formula (WHF), whey-based FINDIA formula virtually free of bovine insulin), and the test formulas were given during the first six months of life, in this intervention trial. The infants were followed for a minimum of three years. The development of T1D specific autoantibodies or progression to T1D was compared between the three formula groups. Antibodies to bovine insulin in children who later developed T1D and T1D specific autoantibodies were also analyzed. Our goal was to understand the maturation of the regulatory T cell population better, and the risk factors and mechanisms for the development of T1D and autoimmune diseases in general.

2 REVIEW OF THE LITERATURE

2.1 The Immune system: innate and adaptive immunity

The immune system consists of two complementary parts leading to very different types of responses to invading microbes. Innate (natural) responses occur every time the infectious agent is encountered irrespective of how many times it happens, whereas acquired (adaptive) responses improve on repeated exposure to a given antigen. The innate immunological responses are rapid and, therefore, the first response. Immunological memory had always been considered a part only of adaptive immunity. However, there is a growing pool of evidence regarding immunological memory in the innate part of the immune system, also called the innate immune memory or trained immunity. There can be certain training of immune cells for a second encounter with the pathogen. The training of innate immunity is mediated mostly by epigenetic modifications and is usually unspecific because there is no production of specific antibodies/receptors. The immunological memory lasts maximally for several months in contrast with the classical immunological memory [1]. Trained immunity has been shown mainly at natural killer cells (NK) cells and monocytes/macrophages, less at γδ T cells and innate lymphoid cells [2].

The innate immune system consists of cells that release inflammatory mediators (basophils, mast cells, and eosinophils), NK cells, and phagocytic cells (neutrophils, monocytes, and macrophages). Macrophages (derived from blood-borne monocytes) can discriminate between “foreign” and “self” molecules by their receptors for carbohydrates that are not normally exposed on the cells of vertebrates [3], such as mannose. Also, both macrophages and neutrophils have receptors for antibodies and complement, so that the coating of microorganisms with antibodies, complement, or both enhances phagocytosis [4]. Macrophages not only phagocyte antigens but also remove the body's own dead or dying cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines such as the interferons. Innate lymphoid cells (ILC cells), a new group of immune cells, have been identified in the territory between the cells of innate and acquired immunity [5]. The central role of ILC cells is to sense danger signals in the body and modify immune responses based on this information. NK

cells are considered to be a subgroup of these cells [6, 7]. A classification of ILCs into five subsets: NK cells, ILC1s, ILC2s, ILC3s, and LTi cells, reflecting their distinct developmental pathways, was recently proposed [7].

The body has different anatomical barriers that are impermeable to most infectious agents, acting as the first line of defense against invading organisms. These barriers include physical, chemical, and biological barriers. The epithelial surfaces form a physical barrier in the respiratory and gastrointestinal tract, movement due to cilia or peristalsis, respectively, helps remove infectious agents. Also, mucus on the epithelial surfaces acts as a trap for infectious agents. The tears and saliva help prevent infection of the eyes and mouth by flushing away the infectious agents. The gut flora can prevent the colonization of pathogenic bacteria by competing with these bacteria for nutrients or attachment to cell surfaces or by secreting toxic substances.

2.2 The adaptive immune system

Adaptive immune responses involve antigen-specific B and T cells that proliferate when their surface receptors bind to an antigen. Specialized cells, called antigen-presenting cells (APC), display the antigen to lymphocytes and collaborate with them in their response to the antigen. B cells mature within the bone marrow, but T cells must travel to the thymus to complete their maturation. The secondary lymphoid tissues (lymph nodes, spleen, and mucosa-associated lymphoid tissue) generate the adaptive immune responses that defend mucosal surfaces. The mucosa-associated lymphoid tissue consists of tonsils, adenoids, and Peyer's patches as well as diffuse collections of lymphoid cells that are present throughout the lung and the lamina propria of the intestinal wall.

2.3 Immune tolerance

Central to the immune system’s ability to mobilize a response to an invading pathogen, toxin or allergen is its ability to distinguish self from non-self. This ability of the immune system to avoid attacking its own tissues and the elimination of potentially self-reactive T and B cells are referred to as immunological tolerance [8, 9].

Immunological self-tolerance is critical for the prevention of autoimmunity and the maintenance of immunological homeostasis. Two mechanisms; central and peripheral tolerance, maintain the immunological homeostasis.

Central tolerance refers to the deletion of T cells with an excessively high T-cell receptor (TCR) affinity for self-peptides, presented on the major histocompatibility complex (MHC) class I and class II molecules in the thymus (reviewed in [10]). Also, T cells with TCR that do not show any affinity for expressed peptide-MHC complexes are eliminated. However, thymocytes that express TCR with a low affinity for self-peptides survive and migrate from the thymus to secondary lymphoid organs.

Gene expression during T-cell development is regulated by the transcription factor known as autoimmune regulator (AIRE). The transcription factor regulates gene expression of tissue-specific antigens in the medullary epithelial cells in the thymus. The expression of the AIRE gene critically regulates the deletion of self-reactive thymocytes [11, 12]. Mutations in the AIRE gene result in a failure of T-cell tolerance [13], as shown in the autoimmune disease autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, also known as autoimmune polyendocrine syndrome type 1). APECED is a severe autoimmune disease characterized by the loss of self-tolerance in multiple endocrine organs and affects central tolerance in the thymus. Immunologically, APECED is characterized by the destruction of the target organs by a cellular- and/or antibody-mediated attack.

It has been suggested that central tolerance is aberrant in patients suffering from an autoimmune disease, and that autoreactive T cells escape from the thymus and prime the immunological systems towards autoimmunity [14]. However, also healthy individuals release autoreactive T cells from their thymuses, but in healthy individuals, these potentially pathogenic autoreactive T cells are controlled in the periphery. This phenomenon is known as peripheral tolerance [13].

T cells develop in the thymus from bone marrow-derived stem cells, which differentiate into multipotent progenitors (MPP) cells, retaining the potential to become both myeloid and lymphoid cells. The MMP cells differentiate into common lymphoid progenitor (CLP) cells [11] and migrate to the thymus, a specialized organ in which the maturation process of the T cells takes place [10]. The cells at each stage have differential potentials to commit to non-T lineages and other cellular properties. Interestingly, these progenitors, termed

multilymphoid progenitors (MLPs), give rise to T cells, B cells, NK cells, dendritic cells (DCs), monocytes, and macrophages, but not granulocytes [12]. The differentiation from multipotent lymphoid progenitor cells to unipotent early T cells is a multiple-stage process. Four stages of development T cells were identified at the early phase of this process; double-negative CD4-CD8-, double-positive CD4+CD8+, single positive CD4+, or CD8+ cells (CD = cluster of differentiation). The progression through these stages is controlled by a significant number of transcription factors, including four core factors, TCF-1, PU.1, GATA3, and BCL11B. Stepwise changes in the levels of these transcription factors in the four developmental stages of T cells were detected [15, 16].

Evidence suggests that, despite the thymus partially degenerating at puberty, T cells continue to develop in the thymus throughout life [11]. In the thymus, T cells recognize their specific antigens bound to self-MHC molecules via their TCR. The early maturation process of T cells consists of several sequential checkpoint events [17]. In the later stages of development, thymocytes are double-positive regarding CD4+ and CD8+ surface antigens [18]. These developing CD4+ CD8+ thymocytes undergo two critical selection processes within the thymus, once their TCR chains are expressed, namely positive and negative selection.

During positive selection, the affinity of TCR is tested on the Ag-MHC complex, and only those thymocytes that possess a high enough, but not excessively high affinity towards the body's peptides, are allowed to live. At this stage, developing thymocytes are differentiated to express only either CD4+ or CD8+ on their surface [10]. Even though the CD4+ lineage or the CD8+ lineage is generally considered to be fixed mature CD4+/CD8+ double positive T cells have been described in the blood and peripheral lymphoid tissues of numerous species, as well as in numerous disease settings, including cancer (reviewed by Overgaard et al. [19]).

In negative selection, the selection is determined by the affinity of TCR to the Ag−MHC complex, and thymocytes with too low or high affinity are directed to the apoptotic pathway. After the selection, fully matured, naïve T cells migrate to the secondary lymphoid organs where they meet their specific antigen and are activated. Peripheral tolerance supplements the central tolerance in regulating the expansion of low-affinity autoreactive T cells or T cells escaping negative selection. Anergy, deletion, and immune suppression are the main mechanisms of peripheral tolerance. Anergy follows the unresponsiveness of T cells

recognizing the self-antigen–MHC complex on APC in the absence of co-stimulatory molecules. Peripheral deletion is based on programmed apoptotic cell death. Also, distinct subsets of T cells with regulatory functions maintain peripheral tolerance [20].

Thymus-derived natural regulatory T cells (tTregs), expressing the signature transcription factor Forkhead box 3 (FOXP3), are considered to be major regulators of the immune system. They function as key moderators in maintaining immunological homeostasis, extinguishing inflammation processes initiated by autoreactive T cells. The role of Tregs in shutting off unwanted, harmful immune reactions has been described in many diseases [reviewed in [21]]. The most significant evidence for the role of Tregs in immunological homeostasis is the fact that both humans and mice deficient in Tregs due to mutations in the *Foxp3* gene suffer from widespread autoimmune diseases, Immunodysregulation polyendocrinopathy enteropathy X-linked (or IPEX) syndrome in humans, and Scurfy in mice [22-24] discussed later.

Tregs are known to develop from moderately high TCR affinity thymocytes. Why some thymocytes with high affinity to the body's peptides escape and differentiate into Treg cells is not known. Neither is the antigen specificity of the cells entering the Treg development pathway known [25]. Treg cells are assumed to be polyclonal populations and that they can recognize an extensive repertoire of both self-derived and exogenous antigens [25].

2.3.1 T lymphocytes

The human adaptive immune system consists of T and B lymphocytes. T cells are divided into two main categories, CD4+ T helper cells, and CD8+ cytotoxic T cells. The adaptive immune cells can form a memory of the encountered pathogen. This capacity gives the immune system a dramatically improved potential to resist pathogens.

CD4+ T cells act as helper T cells (Th) and recognize antigens presented on the surface of MHC class II molecules. Only professional antigen-presenting cells such as DCs, monocytes, macrophages, and B-cells express MHC II molecules.

The cytotoxic CD8+ T cells destroy virus-, or other intracellular-microbe infected cells, and cells that are otherwise damaged, such as cancer cells. These cells are also important in the regulation of activation and differentiation of CD4+ T

cells. CD8+ T cells bind to an invariant part of the MHC class I molecule presenting the antigen for recognition. Practically all nucleated cells express MHC class I molecules. Therefore, the nucleated cells can, if they become infected, present antigens to CD8+ cells. CD8+ cells may kill target cells after the TCR-Ag-MHC I complex is formed, which initiates the death-mediating processes by one of at least three distinct pathways. Either cytotoxicity is exerted directly through the Fas-FasL interaction, or perforin and/or granzyme killing. CD8+ cells can also kill indirectly by the release of soluble cytokines such as IFN-γ and TNF-α, which, for example, activate innate immune responses [13].

2.3.2 T helper cells

CD4+T helper cells (Th) cells were initially divided into two functionally different subsets based on their cytokine profile, Th1, and Th2 cells, first in mice [26] and later in humans [27]. Th1 and Th2 cells have different potential to enhance B-cell responses in vitro [28]. Macrophages are activated in a Th1- type reaction by the cytokine IFN-γ to eliminate intracellular pathogens. IgE production and responses to helminth infections are considered to be a Th-2 type reaction, with the activating eosinophils and basophils by the cytokines IL-4, 5, and 13. The attack on the target tissue in organ-specific autoimmune diseases by autoreactive T cells is perceived as a Th1-polarised- and allergic diseases as a Th2-polarised process characterized by a strong humoral immune response, including IgE antibodies. Observations are indicating that allergic diseases are rarer in patients with an organ-specific autoimmune disease than in unaffected subjects [29].

At the moment, further CD4+ T-cell subsets have been defined. In addition to Th1 and Th2, effector CD4+ T-cell subsets called Th9 [30, 31], Th17 [32], Th22 [33], or f-Th [34] cells, and regulatory T cells a CD4+ T-cell class mediating immune regulation functions, have been characterized.

T helper cell precursors originate from the bone marrow and mature in the thymus from a common precursor cell (Th0). Differentiation of Th0 cells into either Th1, Th2, Th9, Th17, Th22, f-Th effector or the Treg phenotype is mainly controlled by the cytokine stimuli of their environment and the strength and quality of the MHC II-Ag-TCR interaction, but also the nature of the antigen, the type of antigen-presenting (i.e., dendritic) cell, route of antigen exposure, ligation of co-stimulatory molecules and stage of the immune response [35]. Dendritic cells have been

implicated to be the primary type of APCs presenting peptides to naïve CD4+ T cells in an MHC II-restricted fashion [36].

The mature T cells migrate from the thymus into the peripheral blood circulation. Activation of Th cells takes place in the lymph nodes after the interaction of their antigen-specific T-cell receptor with the antigen-MHC class II complex presented by the APCs. APCs are the most important regulators of the cytokine environment in the tissue.

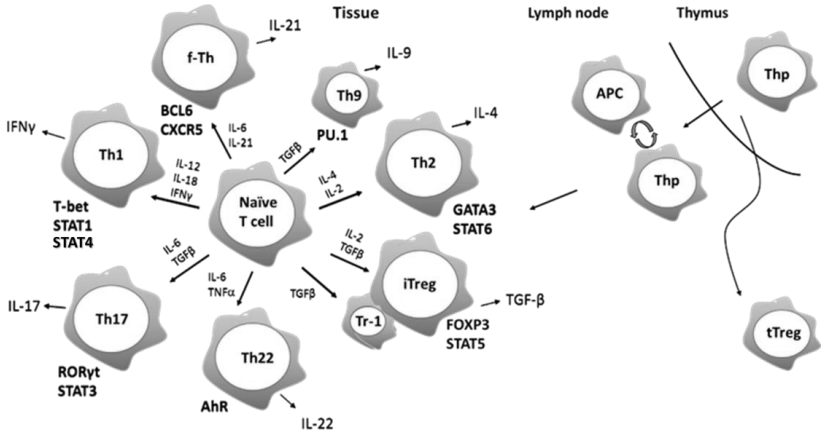

FIGURE 1. Differentiation of human CD4+ T helper cell subsets. T helper cell precursors (Thp) are generated in the bone marrow. After migrating to the thymus, they undergo positive and negative selection. Naïve T cells then migrate to the peripheral blood circulation. In the lymph nodes, the Th cells are activated when they encounter APCs that present peptides bound onto their HLA class II molecules. After the activation of Th cells, T helper type 1 (Th1), type 2 (Th2), type 17 (Th17), type 9 (Th9) and type 22 (Th22), Follicular T helper cells (Tfh), and induced peripheral regulatory T cells (iTreg) as well as type 1 regulatory T cells (Tr1) are induced based on multiple cytokines being produced mainly by dendritic cells and macrophages among other immune subsets. tTregs are already committed to the regulatory phenotype in the thymus. Adapted from Carbo et al. [37].

2.3.3 Th17 cells

A subset of interleukin IL-17-producing T cells (Th17) distinct from Th1 or Th2 cells has been described and shown to have an essential role in host defense against specific extracellular bacteria and fungi such as Candida albicans and Borrelia burgdorferi [38]. The production of selected cytokines, including IL-17A, IL-17F, IL-21, IL-22 (mice), and IL-26 (humans), characterize Th17 cells, thereby

inducing a massive tissue reaction owing to the broad distribution of the IL-17 and IL-22 receptors. Th17 cells recruit neutrophils to the site of inflammation indirectly by the induction of cytokines such as GM-CSF but have also been described as playing a crucial role in the induction of autoimmune tissue injury [39], such as in multiple sclerosis, inflammatory bowel disease, T1D, and rheumatoid arthritis.

As the master regulator for Th17, transcription factor RAR-related orphan receptor (RORγt) has been indicated, and its up-regulation is dependent on transcription factor signal transducer and activator of transcription 3, STAT3, signaling [40, 41].

2.3.4 Th9 cells

A specialized, dedicated subset of T cells (Th9 cells) is suggested to be producing IL-9, a cytokine that may be involved in immune-mediated diseases ranging from autoimmunity to asthma. The precise function of Th9 cells in the inflammatory milieu likely depends upon the tissue microenvironment and other T helper cell cytokines that are present. IL-9 production was first associated with the Th2 phenotype; however, it appears that other Th subsets also have a potential for IL-9 production — the combination of TGF-β and IL-4 prime naive CD4+ T cells to produce high levels of IL-9. TGF-β has been shown to induce IL-9 production in human Th17 cells, and repeated stimulation under Th17 conditions resulted in the co-expression of IL-17A and IL-9. In contrast, IL-23 has inhibitory effects on IL-9 production. IL-23 is a cytokine required for the maintenance of the Th17 phenotype. The transcription factors that regulate Th9 development include TGF-β-induced Sfpi1 and IL-4-induced STAT6, which induces IRF4 as it represses FOXP3 and T-bet [42, 43].

2.3.5 T regulatory cells

CD4+CD25+ regulatory T cells (Treg) are a subset of T cells with suppressive activity. Treg repertoire is biased to recognize “self” agonist ligands. The capacity of Tregs to suppress other T cells is not restricted to inhibition of their early proliferation but also involves the suppression of effector function of activated T cells in inflamed tissues [44, 45].

Tregs can be separated into naturally occurring regulatory T cells, which originate from the thymus (tTreg) after recognition of high-affinity self-antigens, and adaptive regulatory T cells (pTreg), which are induced from naïve CD4+ T cells following antigenic stimulation in the periphery [46-49]. Autoreactive tTreg are selected in the thymus in a highly specific manner through TCR interaction of high affinity on endogenous self-antigens. Presentation defects of self-antigen, either due to MHC polymorphism or impaired expression of this antigen or can create holes in the Treg repertoire. In tTreg selection, distinct molecular mechanisms control the expression and presentation of different (auto)antigens in the thymus. This (auto)antigen presentation involves different sets of transcription factors and antigen-presenting cells [50].

The consistently lower levels of the α chain of the IL-7 receptor (CD127) expression than the majority of other CD4+ T cells characterize pTregs expressing CD25+ [51]. tTregs comprise ~5-10% of CD4+ T cells in the peripheral blood [51]. tTregs characteristically express a high-affinity IL-2 receptor α subunit (CD25) [52]. Unlike effector T cells that upregulate CD25 upon activation through TCR, tTregs constitutively express CD25 at very high levels.

The thymus induced tTregs characteristically express cytotoxic lymphocyte activation antigen (CTLA-4), inducible co-stimulator (ICOS), the glucocorticoid-induced TNF receptor family-related protein TNFRSF18 (GITR), and transcription regulator fork-head box P3 (FOXP3) [49].

The observation that forced FOXP3 transcription factor expression in T cells leads to the acquisition of the regulatory potential of these cells, and mutations in the FOXP3 gene causing IPEX /Scurfy underlines the importance of FOXP3 for the Treg functioning [44, 53]. FOXP3 is the master transcription factor and lineage-specific marker for appropriate Treg cell development [53-56]. FOXP3 activity is crucial to keep the cells on the right developmental pathway towards a regulatory phenotype, and for the suppressive function of Tregs [44, 48, 57]. In addition, it has been shown that FOXP3 stabilizes the Treg cell lineage during an expansion [58, 59]. In contrast to conventional Teff-cells, FOXP3+ Tregs scarcely produce IL-2 and other inflammatory cytokines, including IFN-γ, IL-4, and IL-17 [53, 60].

TCR signals strictly control the differentiation and homeostasis of Treg cells; however, molecular mechanisms that govern these processes are not fully understood. Sidwell et al. showed that Bach2 is an important regulator of Treg cell differentiation and homeostasis, downstream of TCR signaling. This transcription regulator is required for the development of peripherally induced Treg (pTreg) cells,

it prevents premature differentiation of fully suppressive effector Treg (eTreg) cells, and limits IL-10 production in the gastrointestinal tract [61].

The role of the FOXP3 gene was first discovered in the “Scurfy” mouse. These mice suffer from a broad spectrum of autoimmune diseases, which are caused by a mutation in the FOXP3 gene coding region, leading to premature termination of the transcription process, resulting in a truncated and biologically inactive protein lacking an NH2 terminus [23]. The same inheritable mutations in the FOXP3 gene in humans leads to a rare immunological disorder IPEX syndrome, which results in severe, aggressive, and lethal autoimmunity [24].

In the absence of infection or inflammation, transforming growth factor-β (TGF-β1) produced in the immune system induces Foxp3+ regulatory T cells, such as either RORγt+Foxp3+ Treg/Th17 cells or Foxp3− Tr1 cells, and thereby maintain self-tolerance. However, if pro-inflammatory cytokines including either IL-6 or IL-1β and IL-6, produced by the activated innate immune system, are present, the generation of TGF-β-induced Treg cells will be suppressed, and a pro-inflammatory T-cell response is induced by the differentiation of pathogenic Th17 cells from naïve T cells that are being activated through their TCR [62, 63]. In contrast to Tregs, IL-17-secreting Tregs are suppressive *in vitro* but lose this capacity upon stimulation with IL-1β and IL-6, IL-17-secreting Th17 cells, in contrast to Tregs, are suppressive in vitro but lose this capacity upon stimulation with IL-1β and IL-6 [64]. The lifetime of Treg cells is short, and a population of Treg cells is induced de novo from antigen-stimulated memory T cells [65]. Recently, a subset of $FOXP3^+$ T cells with low CD25 expression $CD25^{low}FOXP3^+$ T cells was found to be increased in peripheral blood in patients affected by an autoimmune disease of varying severity, from systemic lupus erythematosus (SLE) and T1D to combined immunodeficiency with active autoimmunity. These T cells share phenotypic features resembling conventional Tregs, including demethylation of the Treg-specific epigenetic control region in *FOXP3*, HELIOS expression, and lack of IL-2 production. Ferreira et al. suggest that these T cells represent a subset of Tregs that are derived from $CD25^{high}FOXP3^+$ T cells, and are a peripheral marker of recent Treg expansion in response to an autoimmune reaction in tissues [66].

Not only the balance between fully committed effector T cells and regulatory T cells but also the plasticity between these phenotypes seems to play a role in autoimmunity and tolerance. Tregs are also more heterogeneous and plastic than were initially thought. The pattern of cytokine secretion may switch from that of one

lineage toward another, i.e., Tregs to Th17 cells, or vice versa, under certain circumstances [67, 68].

2.4 The gut immune system

The gut-associated lymphoid tissue (GALT) is the largest immune system compartment in the body. GALT and the draining lymph nodes are the principal locations for priming adaptive immune cell responses in the intestine. The GALT comprises subepithelial lymphoid aggregates that lie in the mucosa and submucosa and are essential in the ingestion of nutritionally important molecules.

Adaptive immune reactions to luminal perturbations are orchestrated in the gut-draining lymph nodes [69, 70], which are immunologically specific to the functional gut segment that they drain [71]. However, it is unclear how they simultaneously support inflammatory and tolerogenic reactions. Peyer's patches, being located on the antimesenteric side of the small intestine, are important for the development of immune responses in the gut. Peyer's patches consist of numerous B-cell lymphoid follicles (up to ten in mice and several hundred in humans [72]), which are flanked by smaller T-cell areas.

In contrast to lymph nodes, Peyer's patches are not encapsulated and always contain germinal centers, which is indicative of continual immune stimulation, presumably in response to luminal antigens. Also, lymphocytes are found throughout the epithelium and lamina propria of the mucosa. Innate lymphoid cells (ILC) are a subset of these lymphocytes, and they play a protective role against pathogens, including extracellular bacteria, viruses, and helminths. ILC lack an antigen-specific receptor but can respond to environmental stress signals contributing to the rapid orchestration of an early immune response [73]. A single layer of epithelial cells separates the gut microflora from the gut immune system. Antigens must cross the layer of mucus and then the intestinal epithelial cell barrier to induce a mucosal immune response.

Access of antigen from the lumen across the epithelium to underlying immune cells requires specialized transport mechanisms that vary depending on the region of the intestine. In the small intestine, bacteria, viruses, and inert particles are taken up by columnar epithelial cells, and microfold cells (M cells) in the follicle-associated epithelium of Peyer's patches and isolated lymphoid follicles (ILFs) for transportation to dendritic cells in the adjacent subepithelial dome region [74]. Also, DC sample the luminal content by extending their processes through

the epithelium [75]. Fischer et al. demonstrated by using autofluorescence 2-photon microscopy on living intestinal mucosa that lymphocytes associated with M cells were remarkably motile, with average speed rates of 8.2 μm/min. The new M cell-associated lymphocyte clusters were formed within less than 15 min., deforming the M cells' cytoplasm and laterally moving from one lymphocyte cluster to the next [76]. Similar mechanisms may occur in ILFs in the colon and in the caecal patches, which were suggested to be the primary source of IgA-producing plasma cells in the colon [77].

In peripheral organs, such as the gut, the balance between Treg/effector cells is typically achieved by in situ induction of these cells from naïve T cells, recruitment of differentiated Treg/effector cells into the tissue, and reprogramming of already differentiated Treg/effector cells towards other lineages in peripheral tissues [78-80]. T-helper 17 (Th17) and T-regulatory (Treg) cells, such as CD4+CD25+FOXP3 expressing pTregs, CD25+Foxp3+ tTregs, CD25+Foxp3- Type 1 regulatory T (Tr1) cells, CD25+Foxp3- T helper 3 (Th3) TGF-β dependent Tregs, and CD8+ Tregs, are frequently found at barrier surfaces, particularly within the intestinal mucosa [81] [40], where they function to protect the host from pathogenic microorganisms and to restrain excessive effector T-cell responses, respectively. Tr1 cells and Th3 cells are converted from CD4+CD25- T cells into CD25+Foxp3- by naïve B cells. CD25+Foxp3- cells secrete IL-10 and TGF-β. Intriguingly, B-T cell-cell contact but not IL-10 is essential for CD25+Foxp3- cell induction [82]. Even though RORγ+ and Helios+ Treg cells in the colon are phenotypically and functionally distinct, single-cell RNA-seq revealed the sharing of TCR clonotypes between these Treg cell populations. This sharing could indicate a common progenitor in monocolonized and normal mice. Interaction with gut microbiota could drive naïve conventional CD4+ (Tconv) cells, but not pre-existing tTregs, to differentiate into RORγ+ pTregs. Helios+ pTreg cells dominated when the Tconv cells originated from preweaning mice, but generally, only a smaller proportion of Tconv cells converted into Helios+ pTreg cells [83].

2.5 Maturation of the immune system

At birth, the immune system undergoes a comprehensive transition, when adapting from the sheltered intrauterine entity into a new environment followed by age-dependent maturation. The fetal and neonatal immune system has to protect against infection, including bacterial and viral pathogens at the maternal-fetal

interface [84]. Other physiological demands on the immune system are avoidance of potentially harmful pro-inflammatory/Th1-polarising responses that could induce alloimmune reactions between the mother and fetus [85], and the transition of the infant from the ordinarily sterile intrauterine environment to the foreign antigen-rich environment of the outside world, including colonization of the skin and intestinal tract by microorganisms [86].

2.5.1 Prenatal maturation

For a successful pregnancy, it is crucial that the maternal immune system does not reject a genetically different fetus. How this immunological unresponsiveness is achieved is not fully understood. Initially, during fetal life, a skewing of the immune response towards a Th2-biased environment at the maternal-fetal interface has been the central paradigm to explain the generation of fetal tolerance [87]. However, during normal pregnancy, a decreased CD4+/CD8+ ratio compared to the peripheral blood and reduced numbers of CCR6+ Th1, Th2, and Th17 cells in the decidua are seen, while the CD4+CD25^{high}FoxP3^{high} Tregs and CCR6− Th1 cells are increased [88, 89]. In early gestation, ILC1, ILC3, and LTi-like cells are more abundant in the decidua, whereas ILC2s increase at the end of pregnancy [90]. Interestingly, proportions of functional decidual ILC2s and ILC3s increased in women who underwent spontaneous preterm labor [91]. Fetal ILC cells are generated from the CD34+ hematopoietic stem cells in the fetal liver [92], and mature ILC can be found in the liver, secondary lymphoid organs (SLO), intestine, and lung of the fetus, and in the amniotic cavity [90]. These findings suggest that maternal ILCs play central roles in both the initiation and maintenance of pregnancy, and fetal ILCs participate in the development of immunity.

This picture is much more complicated, explaining the immune processes as a balance between Th1, Th2, Th17, and regulatory responses involving both innate and adaptive immune cells [93, 94].

It is well established that there is a placental transfer of maternally produced IgG of various isotypes, with a preferential transfer of IgG (1) > IgG (3) > IgG (4) > IgG (2), to the fetus [95, 96]. Small amounts cross early in gestation, with a rapid increase in transfer after 20 weeks. The mechanisms of IgG transport are not fully understood [96]. The transport pathway across the syncytiotrophoblasts of the

chorionic villi (first cellular layer) is dependent on neonatal Fc receptors. Immune complexes are absorbed in the stroma of the villi.

Immunoglobulin M (IgM) is produced for circulation near the end of the first trimester, around the time of appearance of the thymus, which functions to promote maturation of lymphocytes, especially in the first few years of life (reviewed by Klimovich [97]). IgM and immunoglobulin D (IgD) are the first antibody isotypes expressed during B-cell ontogeny. IgD is co-expressed with IgM on the membranes of most B cells, and membrane IgD has the same antigen-binding specificity as IgM. However, its biological function is not known (reviewed by Chen and Cerutti [98]). Production of immunoglobulin E (IgE) begins first in the fetal liver, followed by the spleen and lung, appearing towards the end of the first trimester [99]. Immunoglobulin A (IgA) is produced for circulation by the end of the first trimester in the fetal liver, and the restrictions of the primary antibody repertoire (IgM) persists in the IgA repertoire. Preterm birth does not measurably accelerate the maturation of the IgA repertoire. After birth, contact with environmental antigens induces the production of IgA, which represents a first line of defense for the neonate [100].

T cells in humans can be detected already at the gestational week 10 in the primary lymphoid organs, thymus, and bone marrow. T cells mature in the thymus and B cells mainly in the liver and bone marrow. Mature lymphocytes migrate to secondary lymphoid tissues (spleen, lymph nodes, tonsils, Peyer's patches, and lamina propria), where they respond to antigens. Interestingly, in mice, the adaptive immune system only starts to develop around birth [13, 101, 102]. T-cell receptors begin to be expressed towards the end of the first trimester, and an intense expansion of T-cell populations occurs in the second trimester; exposures induce such cells during this period [103]. Increasing evidence suggests that antigen-specific priming of the fetal immune system may occur in response to maternal infections, allergens encountered, and maternal vaccination, with studies showing evidence of adaptive antigen-specific cellular immune responses in utero and at birth [104], in contrast to the former view that a neonate is immunologically naïve, and the development of antigen-specific immune responses is restricted to the period after birth. However, this remains a topic of some controversy. Newborn infants are exposed to environmental antigens in utero. This is documented in both cord blood (CB) and amniotic fluid. Several studies have reported that newborn infants already have antigen-specific T-cell reactivity to exogenous antigens such as dietary and inhalant allergens and microbial antigens at birth (reviewed by

Wilcox and Jones [104]). Prescott et al. have shown in lymphocyte stimulation studies that CB mononuclear cells can produce cytokines in response to specific allergens [87]. These immune responses indicate intrauterine sensitization and priming of the fetal immune system. In addition, antigen priming has been implicated in occurring in the fetal gut [105].

Fetal regulatory T cells are now considered as key cells in mediating immune tolerance in the fetus. Fetal Tregs are, however, capable of suppressing both proliferation and cytokine production, suggesting that their function is similar to that of adult Treg cells. Mold et al. showed that fetal T cells are a unique cell population distinct from those found in adults and that this in part reflects differences in the hematopoietic stem and progenitor cell populations which give rise to the T- cell compartment across different stages of development, and that fetal CD4+ T cells are biased towards immune tolerance [106]. In the fetus, CD4+CD25+ thymocytes already have the potential to suppress the proliferation of CD25− cells. After leaving the thymus as naïve cells, CD4+CD25+FOXP3+ Tregs enter the fetal lymph nodes and spleen where they encounter their cognate antigen and acquire a primed/memory phenotype and play an immunoregulatory role in intrauterine life [107].

2.5.2 Postnatal maturation

After birth, there is an age-dependent maturation of the immune response. This maturation is related to deficient activation of the adaptive immune system, characterized by heightened sensitivity to infectious agents, i.e., viruses and bacteria. The susceptibility of newborn infants to infectious diseases might be partly due to the lack of pre-existing immunological memory [108, 109]. During this maturation period of the infant's immune system, maternal antibodies protect neonates and infants. In humans, maternal antibodies are transferred preferentially before birth transplacentally from mother to child. The vast majority of maternal antibodies are of the IgG isotype, and they wane within 6–12 months [110].

The bias towards a Th2-cell response and limited Th1-cell function in neonates is perhaps one of the best characterized immunological differences to adults. After birth, APC are critical for balancing the postnatal Th2 response and Th1 immune responses. The co-stimulatory signals for the induction of Th1 cells are defective, as APCs secrete little IL-12. Hypermethylation at CpG and non-CpG

(CpA and CpT) sites, within and adjacent to the IFN-gamma promoter, results in a reduced capacity to transcribe IFN-γ-specific mRNA leading to selective IFN-γ gene expression in the CD4+/CD45RO- T cell subset in early postnatal life. In contrast, similar hypermethylation of the IFN-γ promoter region has not been detected in adult cells [111].

Tregs are implicated to be less mature in early life than in adulthood. One primary distinction is that the major population (90%) of CB CD25+ T cells has the naive phenotype of CD45RA+, and differs in their expression markers, including CD62L [112]. Freshly isolated CB CD25+ T cells can suppress T-cell proliferation [113]. Mayer et al. depicted that after antigen-specific stimulation, CB Treg cells gained potent suppressive properties [114]. Demethylation of FOXP3 in cord blood was specific for isolated CD4+CD25^{high} Tregs and subsequentially very low in CD4+CD25- T cells. Thus, from early life, FOXP3 methylation seems to be a stable parameter for Treg assessment [115]. In addition, Th17 cells in cord blood are shown to be involved in early immune regulation [116].

The innate responses are important early in the maturation of the immune system because of the natural unresponsiveness of the adaptive response. Neonates have a lower proportion of DCs and specific CD14+ monocyte subsets than adults. In addition, they exhibit a more immature phenotype and have a decreased capacity to release TNF-α and IFN-α than adult cells. On the other hand, neonates are efficient inducers of CD4+ T cells with a Treg phenotype [117, 118]. The first year of life represents a critical period during which an increase to adult-like levels of plasmacytoid dendritic cell (pDC) and myeloid dendritic cell (mDC) responses to TLR4 agonists (bacterial lipopolysaccharide) and TLR9 agonists (CpG oligonucleotides) are reached for most but not all cytokine responses [119].

2.5.3 Postnatal development of the gut immune system

Both endogenous and exogenous factors drive the development and maturation of the intestinal immune system. The formation of secondary lymphoid structures, such as mesenteric lymph nodes and Peyer's patches, occurs before birth. Microbial flora rapidly colonizes the neonatal skin and gut following birth. The principal stimuli of postnatal maturation of the immune function in infants are signals from the microbial environment, particularly the commensal microbiota of

the gastrointestinal tract [120]. The microbiota comprises mainly of bacteria; however, viruses, fungi, and protozoans are also present.

The most significant number of bacterial cells is found in the large intestine (1011 per gram of intestinal content), and the richness of microbiota species characterizes the gastrointestinal microbiome in healthy individuals [121]. Human gut-associated microbiota is dominated by four main phyla of the more than 50 phyla detected on Earth: Gram-positive *Firmicutes* and *Actinobacteria*, and the Gram-negative *Bacteroidetes* and *Proteobacteria* [122, 123]. The *Firmicutes* is the most abundant bacterial phylum, comprising more than 200 genera, including Lactobacillus and Clostridium species. *Bacteroidetes* and *Firmicutes*, constitute over 90% of the known phylogenetic categories and dominate the distal gut microbiota. However, differences in the proportions of these bacterial phyla exist between individuals, as well as over time in an individual [121]. The functions of the microbiota include metabolic functions such as the fermentation of non-digestible dietary substances and vitamin synthesis, the barrier effect, which protects against pathogens, and the control of the immune homeostasis of the gut.

The gut harbors several subsets of lymphocytes. Consequently, the different bacterial strains may be differentially stimulating the lymphocytes that exist in the gut lymphoid tissues [124]. Both the oral and nasal administration of antigens is shown to favor the induction of immunological tolerance towards specific antigens. The actual protective function of the gut requires microbial stimulation by initial bacterial colonization, even though the intestinal mucosal immune system is fully developed after a full-term birth.

The maternal microflora can be a source of bacteria colonizing the intestine in the newborn infant [125, 126]. It has been shown that the way of birth has an impact on the strains that colonize the infant's gut. Infants born through cesarean section had lower numbers of *Bifidobacteria* and *Bacteroides* compared with vaginally born infants. [127, 128]. They also had a significantly more varied microbial composition, including *Citrobacter freundii*, *Clostridium* species, *Enterobacter cloacae*, *Enterococcus faecalis*, *Klebsiella oxytoca, Klebsiella pneumoniae*, and *Staphylococcus aureus*, whereas colonization by *Escherichia coli* was associated with natural birth [129]. Also, the feeding patterns can influence the microbiota composition as exclusively formula-fed infants were more often colonized with *E coli, C difficile*, *Bacteroides*, and *Lactobacilli*, compared with breastfed infants who had more *Bifidobacteria* [130, 131]. Bacteria may use

microvillus membrane glycoconjugates as target cell receptors for adherence to the gastrointestinal surface [132].

2.6 The hygiene hypothesis

Epidemiological studies have shown an association between the increasing incidence of immune-mediated diseases and improved standard of living and hygiene [109, 133], and the hygiene hypothesis originally postulating that a decreased exposure to infections during childhood increases the susceptibility to immune-mediated diseases [134, 135], has been expanded to claim that decreased microbial burden or diversity causes impaired maturation of immune regulation contributing to the development of such diseases [136-138].

2.7 Type 1 diabetes

2.7.1 Epidemiology of type 1 diabetes

The incidence of T1D worldwide has been rapidly increasing since the 1950s, with an increase of 3% per year from the year 1960 to 1996 [139]. In 2019 Patterson and co-workers showed in an analysis of 26 population-based registers in 22 European countries, significant increases in incidence rates in all but two registers over the 25 years from 1989 to 2013 [140]. Their analysis suggests that, despite few high-risk countries showing some slowing in the time period 2004–2008, the overall pattern of an approximately 3% per year increase remains. As it turns out, in general, the actual increase has been higher than predicted by their exponential model. For example, the incidence in Finland in 2010 was predicted to be 50.2 based on the linear model and 57.9 according to the exponential model. However, the actual incidence had risen to 64.2 in Finland by 2006 and 62.5 from 2006 to 2011 [141-144]. There is a close to a six-fold gradient in the incidence of T1D between Russian Karelia and Finland, although the frequency of predisposing HLA DQ genotypes are relatively equal in the two populations. This difference in incidence suggests that environmental factors contribute to this steep difference in the incidence rate between these adjacent regions [145].

A significant number of patients, particularly adults, presenting to their physician with what appears to be non-insulin-requiring diabetes, have an early stage of latent autoimmune diabetes mellitus (LADA) [146]. There have been only a few studies that have examined the incidence or prevalence of LADA, mainly because of the difficulty of distinguishing LADA from insulin-requiring type 2 diabetes in older individuals. The diagnosis of such adult patients is now possible because of the availability of autoantibody assays with high sensitivity and specificity [146, 147].

2.7.2 Classification of diabetes in children

The World Health Organization (WHO) has defined diabetes mellitus as "a group of metabolic diseases characterized by chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both."

The current classification of diabetes, recommended by the WHO and the American Diabetes Association, includes four main categories: T1D, type 2 diabetes, gestational diabetes mellitus and other specific types such as genetic defects of beta-cell function, diseases of the exocrine pancreas, and diabetes induced by drugs, chemicals, or infections [148, 149].

T1D can be further divided into two subclasses; type 1A and a rare type 1B diabetes [150]. T1D requires life-long treatment with exogenous insulin as the body loses its ability to utilize carbohydrates as an energy source without the body's insulin production [151, 152].

Type 1A diabetes results from the autoimmune destruction of β-cells in the pancreas by T cells of the immune system. In children with active β-cell loss, autoantibodies against β-cell structures appear in the peripheral circulation. Type 1A diabetes also has a strong HLA association. The etiology of the β-cell destruction in Type 1B diabetes, called idiopathic diabetes, is not known. Patients with type 1B diabetes have no immunological evidence of β-cell autoimmunity and, no HLA association exists, but they have insulin deficiency and are prone to ketoacidosis. T1DB has been reported in individuals from Africa and Asia. In Japan, it comprises 10% of all T1D cases, but is a very infrequent condition in Caucasian populations.

Immune-mediated type 1A diabetes (T1D hereafter) is the most common form of diabetes among children, comprising about 80% of cases worldwide [153].

In addition to T1D, there are some rare types of diabetes. A familial form of mild, non-ketotic diabetes presenting during adolescence or early adulthood, under the age of 25 years [154, 155], formerly termed maturity-onset diabetes of the young (MODY), is now recognized as a group of disorders which result from dominantly acting heterozygous mutations in genes important for the development or function of β cells [154, 156]. There is considerable overlap in the presentation of type 1, type 2, and monogenic diabetes. Within the diagnostic groups of monogenic diabetes, there is great variation in the degree of hyperglycemia, need for insulin, and risk for future complications.

Transient or persistent neonatal diabetes (TNDM and PNDM, respectively) is insulin-requiring diabetes that is usually diagnosed in infants before the age of six months [157].

2.7.3 Diagnostic criteria of type 1 diabetes

The clinical signs of diabetes include hyperglycemia and osmotic diuresis with the telltale symptoms of polyuria, polydipsia, and weight loss. A blood sugar level higher than 11.1 mmol/l is considered diabetic, but symptoms may not start to become noticeable until even higher values such as 15–20 mmol/l. If the glucose concentration is detected in fasting, a plasma sample of 7.0 mmol/l or over is diabetic [150, 158].

Untreated insulin deficiency leads to the use of fats as an energy source and elevated levels of ketoacids in the body. The elevated levels of ketoacids result in nausea and hyperventilation as clinical symptoms. Unexplained weight loss also often accompanies the symptoms mentioned above. These patients with T1D are rarely obese, and most have circulating autoantibodies against β-cell antigens, typically found in more than 90% of patients. Their plasma C-peptide levels are low, referring to very low insulin secretion. About 50% of the patients who have T1D are usually diagnosed at a young age (<14 years) with acute onset and are at risk for ketoacidosis at diagnosis [159].

T1D and T2D can be distinguished from each other by detecting these specific autoantibodies against the islet cell structures, and by measuring their level of c-peptides, at the time of diagnosis.

2.7.4 Autoimmunity in diabetes

The first suggestions of T1D being an autoimmune disease were in 1965 when Gepts reported seeing peri- and intra-insular inflammatory infiltrates in pancreata from patients with juvenile diabetes [160]. Insulitis is an inflammatory lesion consisting of immune cell infiltrates around and within the pancreatic islets. The primary infiltrating lymphocytes are CD8 positive cytotoxic T cells, but other T-cell subpopulations including CD4 positive helper T cells (Th cells) macrophages and monocytes, natural killer cells, and B-cells are also infiltrating the pancreatic islets [161-164]. In 1974 both autoantibodies and autoreactive T-cells against β-cells were shown to exist in the circulation of patients with T1D, making the autoimmune nature of T1D more evident [165-167].

T1D is considered a T-cell mediated disease. A proportion of the T cells infiltrating the pancreatic islets are activated, as shown by increased expression of HLA class II molecules and interleukin (IL)-2 receptors on their surface [161]. Similarly, the islet cells themselves express increased amounts of HLA class I molecules [161, 163, 164, 168]. The disease can be transferred by autoreactive cells from a diabetic individual to an unaffected individual, as shown by Lampeter et al. in a case report on the transfer of T1D between siblings by bone marrow transplantation [169]. Our knowledge of human antigenic targets for islet-infiltrating T cells in humans is limited due to minimal access to pancreas samples.

Michels et al. studied inflamed pancreatic islets of three young organ donors with type 1 diabetes. The donors had a short disease duration and high-risk HLA genes. The group identified hundreds of T cells infiltrating the islets by using a direct T-cell receptor (TCR) sequencing approach without long-term cell culture. They selected 85 CD4 TCRs for testing of reactivity to preproinsulin peptides presented by diabetes-susceptible HLA-DQ and HLA-DR molecules. They found that one T cell recognized C-peptide amino acids 19-35, and two clones from separate donors responded to insulin B-chain amino acids 9-23 (B:9-23). B:9-23 and C:19-35 are known to be a critical self-antigens driving disease progression in animal models of autoimmune diabetes. These B:9-23-specific T cells from islets responded to whole proinsulin and islets, whereas previously identified B:9-23 responsive clones from peripheral blood did not, highlighting the importance of proinsulin-specific T cells in the islet microenvironment [170].

Immunological therapies such as CTLA4-Ig (Abatacept) that block costimulation between CD80/86 on the APCs and CD28 on the T lymphocytes

[171], or CD20 monoclonal antibody (Rituximab) that reduce B-cell contribution to autoimmunity [172] as well as vaccination with autoantigens [173] have been shown to alter antigen-specific immunity, and initial studies reported some preservation of β-cell function. Short treatment (1–2 wk.) with monoclonal antibodies to CD3, a molecule expressed in almost all T cells, in recently diagnosed patients also maintains or improves the patient's own insulin production for at least one year after the diagnosis in both academic phase II trials [174-177]. In a recently reported phase II study, a single course of Teplizumab, a monoclonal antibody to CD3, delayed the appearance of clinical T1D with around two years in risk individuals with multiple islet autoantibodies and glucose intolerance initially [178]. This is the first observation showing that it is possible to modify the disease process by immunomodulation. Most likely, the effect of this treatment is due to the production of regulatory cells that are capable of suppressing autoreactive T cells (reviewed by Harlan and von Herrath [179]).

Autoantibodies to pancreatic islet cells and individual T1D-associated autoantigens are widely used for the prediction of the disease even though B-cells are not necessarily required for the development of T1D [180, 181]. Patients with recent-onset T1D may show humoral immune responses to as many as 14 different β-cell molecules [182]. T-cell responses have been demonstrated to eight autoantigens including insulin [183], glutamate decarboxylase (GAD) 65 [184], insulinoma-associated protein 2 (IA-2A) [185], heat-shock proteins 60 and 70 [186, 187], islet cell autoantigen of 69 kDa (ICA69) [188], transcription factor jun-B [189] and, cation efflux transporter (ZnT8) [190].

For the prediction of T1D, only four assays are routinely used: insulin autoantibodies (IAAs) [191], GAD autoantibodies (GADAs) [192, 193] and, IA-2 autoantibodies (IA-2As) [194] are measured by radiobinding assays using radiolabeled antigens. Islet cell antibodies (ICAs) are measured basically in the same way as reported in 1974 by using immunofluorescence labeling of frozen pancreatic sections [165]. Zinc transporter 8 antibodies (ZnT8A) are not routinely measured but are used in scientific studies.

When predicting T1D, repeated positivity for multiple (≥2) autoantibodies is associated with an increased risk of T1D, being about 70% over the next ten years [195] and if a subject tests positive for three or four autoantibodies carries the highest HLA risk genotype the risk of T1D is close to 80% [195]. Wenzlau et al. showed that the combined measurement of ZnT8A, GADA, IA2A, and IAA raised

autoimmunity detection rates to 98% at the diagnosis of T1D, a level that is similar to that needed to detect prediabetes in a general pediatric population [190].

2.8 Risk factors of type 1 diabetes

2.8.1 Genetic risk

More than 50 gene loci are associated with susceptibility to T1D [196]. Approximately 50% of the genetic risk is associated with the genomic region containing genes for HLA molecules (Human Leucocyte Antigen, human analog for MHC) [197]. HLA molecules are expressed on the cell surface. HLA molecules are divided into two classes that differ from each other by the structure and the expression signature. HLA class I molecules (HLA-A, B, and C) are expressed by most somatic cells. They present peptides derived from intracellular proteins bound into their peptide-binding cleft and display them on the cell surface for recognition by the cytotoxic CD8+ T cells. In contrast, the expression of HLA class II molecules (HLA-DP, DQ, and DR) is restricted to the APCs, i.e., DC, B lymphocytes, macrophages and thymic epithelial cells, and class II molecules present peptides derived from exogenous proteins to CD4+ cells [198, 199]. The most important locus defining the risk of T1D is located within the HLA class II gene region.

HLA-DQ is a heterodimeric molecule encoded by DQA1 and DQB1 genes. According to the current view, the major factors for the genetic risk of T1D are HLA-DQA1 and HLA-DQB1. HLA-DQB1 and HL-DQA1 are highly polymorphic genes, and the variation especially affects the conformation of the peptide-binding groove of these molecules. These two loci are in linkage disequilibrium, and in most cases, the characterization of HLA-DQB1 genes is sufficient for the estimation of the disease risk [200]. However, the genetic risk associated with the HLA class II gene region is more complicated, and in addition to HLA-DQ, the HLA-DR alleles, among others, have a modifying effect on the risk. HLA class II effect is limited to the early phase of the disease process characterized by seroconversion to islet autoantibody positivity [201].

The genetic component of T1D is polygenic as previously mentioned, and several loci have so far been recognized to contribute to the overall risk of T1D. Among the other genes three loci, whose effects are relatively modest on T1D susceptibility, were well characterized: the insulin gene [202], cytotoxic T-

lymphocyte antigen-4 (CTLA-4) gene [203] and PTPN22 gene [204]. The highest risk genotypes of T1D are DQA1*0301-DQB1*0302 (DQ8) and DQA1*0501-DQB1*0201 (DQ2) [200, 205]. The highest risk is associated with the DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 heterozygote. The actual risk is, however, more complicated to determine, since other loci, such as three alleles in the HLA-DQ gene region protect against T1D. These are DQB1*0602, DQB1*0301, and DQB1*0603, out of which the DQB1*0602 allele has the strongest protective effect. The highest risk alleles, DQB1*0302, and/or DQB1*0201, are found in 90-95% of patients with T1D [200, 206]. These risk alleles are also common in the general population as approximately 40-45% of Finns carry one of these alleles, and half of them have no protective alleles [200, 206]. However, the risk of a Finnish newborn of developing T1D is less than 1% (in 2019, the risk was 0.8%, Ilonen, personal communication), suggesting that environmental factors play a significant role in the pathogenesis of T1D.

2.8.2 Viral infections

Viruses inhabit all surfaces of the human body, and most of them are bacteriophages [207]. Many viral genotypes colonize body surfaces including skin, oral and gastrointestinal tracts, airways, and the bloodstream, that was previously considered a sterile environment. The composition of gut virome is closely related to dietary habits and the surrounding environment. Viruses also have a considerable effect on the evolution of the human genome, as it is estimated that approximately 42 percent of the human genome is composed of viral sequences. The viruses in and around humans not only drove evolution via selective pressure but also contributed novel genetic material during human evolution [208]. The virome also acts as a significant agent in horizontal (i.e., non-reproductive) gene transfer between biomes [209-212].

Viruses may play a causative role in chronic autoimmunity [213] and [214]. However, there is also compelling epidemiologic evidence that infection in humans predisposes to autoimmune disease [213].

The potential mechanisms by which viruses may initiate the autoimmune process include: direct viral infection of β-cells, causing lytic destruction; molecular mimicry, virus-encoded antigen mimics of self-antigens activate cross-reactive T cells; direct bystander activation, where viral infection causes a release of immune

mediators such as cytokines, and the release of self-antigens could lead to activation of autoreactive T cells; epitope spreading, de novo activation of autoreactive T cells by sequestered antigens released secondary to virus-mediated tissue destruction; superantigens, encoded by viruses that stimulate autoreactive T cells with particular Vb receptors; cryptic antigens, self-antigens normally encrypted in tissue are released from tissue during virus-directed tissue damage (reviewed by [213], and [215]).

Three potential mechanisms of β-cell destruction in T1D are molecular mimicry, bystander activation, and low-grade infection of the islets of Langerhans. Molecular mimicry may be involved in the association between both coxsackievirus B4 and rotavirus and T1D. P2-C protein of coxsackievirus B4 shares an amino acid sequence similar to that of GAD [216], and the VP7 protein of rotavirus has similarities with both IA-2 and GAD [217]. However, there is no proven case of molecular mimicry. Bystander activation by viruses in the proximity of β-cells could cause a release of immune mediators such as proinflammatory cytokines and nitric oxide which are harmful to β-cells, and the release of self-antigens from damaged β-cells could lead to activation of autoreactive T-cells (reviewed by Filippi and von Herrath [218]). In the DiViD-study, Krogvold et al. showed that a low-grade enteroviral infection was sustained within the islets of Langerhans in fresh pancreatic tissues collected 3-9 weeks after the time of diagnosis of T1D in adult patients. However, the results do not prove causality between enterovirus infection and T1D [219].

Several viruses have been connected with the risk of T1D, but their role in pathogenesis has been challenging to prove. Congenital rubella infection leads to diabetes in about 20% of affected individuals, and the virus can infect the fetal pancreas [220, 221]. The mumps virus, which can infect human β-cells in vitro, may also be associated with T1D [222, 223]. However, these two viruses cannot be considered as a risk factor for T1D today, since rubella and mumps infections are almost non-existent due to vaccination programs in developed countries. Other viruses occasionally connected with T1D include cytomegalovirus [224, 225], Epstein-Barr virus [226] and retroviruses [227], but the evidence for their role in the pathogenesis of T1D is scarce.

2.8.2.1 Enteroviruses

Virus infections, especially enterovirus and rotavirus infections, have, for a long time, been implicated as triggering factors of the autoimmune process in T1D [228]. Viskari et al. [229] suggested that variation in the incidence rates of T1D might be connected to the geographic spread of enteroviruses. The seasonality of the incidence of β-cell autoimmunity was reported in epidemiological studies, which could reflect the seasonality of viral infections [230].

There are more than 300 serotypes in the enterovirus family after the inclusion of rhinoviruses into the family [231]. Coxsackie B viruses (six serotypes) have most often been implicated as diabetogenic strains. Enteroviral infections are common during childhood and are transmitted through the fecal-oral route. As the enteroviruses are resistant to acidic pH and bile, they pass through the stomach to the intestine. Viremic spread to other organs, such as the pancreas, can occur. In vitro studies showed that some enteroviruses are also able to infect β-cells [232, 233].

Enterovirus infections appear to be the most probable viral trigger of β-cell autoimmunity. Gamble et al. reported, as early as 1969 that enterovirus antibody titers against the coxsackievirus B4 strain were higher in a patient with recent-onset T1D than in unaffected control subjects [234]. Coxsackie B4 viruses, isolated from the pancreas of a child who died in ketoacidosis, induced diabetes when inoculated into mice, causing hyperglycemia, infiltration of immune cells into the pancreatic tissue and β-cell necrosis [232]. Recently, enteroviruses were detected in pancreatic islets of living patients with recent-onset T1D [219]. In a study screening 41 EV serotypes for their T1D association using neutralizing antibodies, only the group B coxsackieviruses, was associated with the appearance of islet autoantibodies [235].

Antibodies against enteroviruses were more often detected from the serum of subjects who tested positive for one or more T1D related autoantibodies, indicating that enteroviral infections may have a causal role in the pathogenesis of the disease [236, 237]. Several studies have reported increased levels of enterovirus antibodies and enterovirus RNA in the peripheral circulation in T1D and prediabetic children (reviewed by Tauriainen et al. [238]).

In follow-up studies of children with increased risk of T1D, findings of the association of coxsackieviruses with T1D are conflicting. In a Finnish study Hyöty et al. reported that the occurrence of coxsackievirus infections was more frequent,

both in pregnant mothers whose children later developed T1D and in the siblings of affected children who were diagnosed with T1D in a follow-up study [239]. Also, the increased enterovirus antibody levels were clustered in the sample collected in intervals in which β-cell-specific antibodies first appeared [240]. However, the hypothesis that T1D is due to maternal enterovirus infection was not supported by a large Finnish study on the association of enterovirus infection during pregnancy and T1D in offspring [241].

Hyöty et al. have introduced the polio hypothesis to explain the inverse association between the frequency of EV infections and the incidence of T1D on the population level [229, 242], and to the marked variation in T1D incidence between countries between the incidence of EV infections and T1D [242]. The hypothesis is named after the enterovirus polio since a similar phenomenon occurred previously in polio. When the circulation of polioviruses decreased in developed countries, an epidemic increase in polio paralysis coincided. Children started to get poliovirus infections at an older age when maternal poliovirus antibodies no longer protected them, and as a consequence, the frequency of polio paralysis being a complication of more severe polio infections increased [243].

Two prospective studies (BABYDIAB in Australia and Germany and the Diabetes Autoimmunity Study in the Young [DAISY] in Denver) have failed to demonstrate any association between enterovirus infections and β-cell autoimmunity [244-246]. A recent report of the TEDDY study showed an association between Coxsackie B infection and beta-cell autoimmunity but not with the development of T1D. The study included the largest cohort of infants at genetic risk of T1D analyzed so far [247]. A new potential approach to prevention of T1D is the development of a Coxsackie B (CVB1) virus vaccine. Formalin inactivation of CVB1 produced a vaccine that induced a strong, virus-neutralizing antibody response in vaccinated mice, which protected against challenge with CVB1 virus [248].

2.8.2.2 Other viruses

The Australian BabyDiab study found a connection between the appearance or increase of T1D-associated autoantibodies and seroconversion of another virus, rotavirus [246] instead. Coulson et al. from the same research group also showed

that rotavirus is able to infect animal pancreatic islets in vitro [249]; a result no other group has been able to confirm.

In a recent study, Huo et al. studied the association between T1D and influenza A virus (IAV) in a mouse model [250]. Clinical observations have shown that T1D patients are more easily infected by IAV and suffer more severe symptoms than non-T1D patients. The research group reported that the T1D-infected mice showed more clinical severe symptoms and lower survival rates than the non-T1D infected mice. The study results revealed an increase in serious pathological damage to the lung and pancreas in T1D-infected mice. Also, higher IAV loads and more extensive distribution of positive signals in the lungs and pancreas of T1D-infected mice than in those of non-T1D infected mice was shown.

Furthermore, according to the real-time quantitative polymerase chain reaction (PCR) results, viral replication appeared to occur more easily in the lungs of T1D-infected mice. Thus, T1D-infected mice exhibited higher susceptibility to IAV than did normal mice [250].

The question of whether virus infections are the cause of β-cell destruction and progression to clinical T1D, or whether T1D or the susceptibility to T1D is predisposing the individual to virus infections, is yet to be answered.

2.8.3 Dietary factors

Dietary factors may contribute to the risk of T1D due to intestinal immune activation. A short period of breastfeeding, the use of cow's milk-based formula and dietary gluten are among the possible environmental triggers of β-cell autoimmunity as discussed in the next paragraphs.

2.8.3.1 Breastfeeding and cow's milk formula

Several groups have studied the possible link between the duration of breastfeeding and protection against T1D, but the results have been controversial. There have been studies showing a protective effect but also reports of no association or even a predisposing link (reviewed by Giwa et al. [251]). Some studies have shown that a short breastfeeding period (<3 mo.) in infancy may be associated with the appearance of β-cell autoimmunity [251]. However, other studies suggest that the duration of breastfeeding does not contribute to the

individual's risk of developing β-cell autoimmunity [251] and T1D. In the nationwide "Childhood Diabetes in Finland" (DiMe) case-control study, the risk of diabetes was related to the age at introduction of supplementary formula feeding, independently of duration of breastfeeding, suggesting that early exposure to cow's milk proteins, and not the protective effect of breastfeeding, is the pivotal factor [252]. Also, other groups have proposed early exposure to cow's milk proteins as one of the possible factors priming β-cell autoimmunity [253, 254] and T1D [255, 256] in genetically predisposed individuals.

Interestingly, exposure to bovine insulin in cow's milk formula induces an immune response to bovine insulin, which cross-reacts with human insulin, and this primary immunization to insulin, when it takes place during the early months of life, could explain the link between cow's milk and T1D. Vaarala et al. proposed that early exposure to bovine insulin associated with impaired insulin tolerance [257, 258].

The first study that showed children with T1D had increased circulating antibody levels to CM proteins was conducted already in 1988 when Savilahti et al. observed that diabetic children had significantly higher levels of serum IgA antibodies to CM and beta-lactoglobulin and IgG antibodies to beta-lactoglobulin than age-matched control subjects [259]. The cellular immune response to the cow's milk proteins β-lactoglobulin and β-casein is also increased in patients with newly diagnosed T1D [260, 261]. However, some studies have shown no association between the early introduction of CM and the risk of β-cell autoimmunity [262, 263].

Visser et al. reported that casein hydrolyzed formula was associated with a decrease in autoimmune diabetes in the disease-prone BB rat, in association with improved integrity of the intestinal barrier and higher production of regulatory cytokine IL-10 in the ileum, as well as beneficial changes in the gut microflora (increased Lactobacilli and reduced Bacteroides levels). This suggested that the presence of specific peptides has a beneficial effect in the prevention of autoimmune diabetes [264]. In a NOD mouse study, casein hydrolyzed formula affected the microbial colonization and reduced proinflammatory T cell activation suggesting a link between dietary factors, microbial colonization and mucosal immune activation [265]. The mechanisms by which hydrolyzed formula reduces and foreign proteins (like cow's milk proteins) increase the risk of diabetes-predictive autoantibodies are not known.

An international, double-blind, randomized clinical trial of 2159 infants from 15 countries, the “Trial to Reduce IDDM in the Genetically at Risk” (TRIGR), studying children with increased risk for T1D (children having a first-degree relative with T1D and a risk HLA genotype) addressed the question of exposure to cow’s milk-proteins by comparing infants weaned to a hydrolyzed casein formula or a conventional cow’s milk-based formula. The study concluded that weaning to a hydrolyzed casein formula did not reduce the cumulative incidence of T1D after a median follow-up of children for 11.5 years [266]. This outcome is consistent with the report of that trial that showed no difference between the study groups in the appearance of islet autoantibodies [267] but is not consistent with data from the pilot study [268], which showed a decreased cumulative incidence of diabetes-associated autoantibodies in children receiving the hydrolyzed formula during the first months of life. This result might be due to the pilot study being conducted in a more homogenous study population, including 230 Finnish children, in comparison with the multicenter TRIGR study.

These discrepancies between the different studies may be due to the variation between countries in the consumption of complementary foods, e.g., types of CM formula, or cereals that infants are first exposed to, or altered immunological response to cow’s milk proteins, gut microbiome, or increased permeability of the intestine to dietary proteins in children who develop T1D.

2.8.3.2 Wheat proteins

Gluten or other cereal-derived proteins have been considered as one of the nutritional risk factors for T1D. Animal models have shown that dietary wheat gluten modulates the intestinal immune system, and may, therefore, also participate in the pathogenesis of T1D [269, 270]. In humans, an association between T1D and celiac disease has been reported [269-271], which is at least partly due to the fact that specific HLA alleles predispose both to celiac disease and T1D. In one study, low cellular reactivity to gluten in patients with newly diagnosed T1D without celiac disease was shown [272]. Wang et al. stimulated jejunal biopsies in vitro from T1D patients and those with celiac disease. The up-regulation of T-cell activation markers CD25 and ICAM-1 was associated with T1D but not with active celiac disease [271]. This indicates that gliadin may be the specific trigger for subclinical inflammation in T1D patients.

In a pilot intervention trial, assessing whether gluten elimination modifies the natural course of β-cell autoimmunity, the gluten-free diet did not affect on the signs of β-cell autoimmunity in first-degree relatives of affected patients. It did not prevent progression to T1D [272].

However, other studies have shown that the risk of development of islet autoimmunity is increased in children who were exposed to cereal proteins [273, 274] during the first 3 months of life compared with children who received gluten between 3 and 6 months of age. In a recent report from the DIPP study based on 5545 children who were followed for six years, Hakola et al. showed that a high intake of gluten, gluten-containing cereals, oats, and dietary fiber was associated with an increased risk of beta-cell autoimmunity [275].

Of interest is that the prevalence of autoimmune disorders, including T1D in patients with celiac disease, was related to the duration of gluten exposure, i.e., the longer the exposure, the higher the prevalence of other diseases [276].

3 AIMS OF THE STUDY

This thesis includes three original studies.

I The first study set out to describe the development of the gut microbiota in early infancy, in healthy infants in a prospective setting, and to characterize the possible effects of the microbiota on the early development of peripheral regulatory T cells.

II The aim of the second study was to determine whether the Treg cell response is aberrant in virus-infected otherwise healthy infants with genetic risk for T1D when comparing to infants without infections. The study included as cases infants with common enterally detectable infections, such as enterovirus, rotavirus, norovirus, parechovirus, and rhinovirus, out of which enterovirus and rhinovirus have previously been associated with T1D.

III In the third study, the aim was to characterize whether bovine insulin in infant formula is a trigger of autoimmunity, by studying the appearance of autoantibodies after exposure to infant formulas containing bovine insulin, whey-based hydrolyzed formula, or a virtually bovine insulin-free whey-based hydrolyzed FINDIA formula during the first six months of life, whenever breast milk was not available.

4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Publications I and II

Children were recruited during the years 2009-2010 in Estonia and Finland in the DIABIMMUNE (Pathogenesis of type 1 diabetes: testing the hygiene hypothesis) study. All children carried T1D associated HLA risk genotypes a combination of (DR3)-DQA1*05-DQB1*02 (DR3-DQ2) and DRB1* 04:01/02/04/05/08- DQA1*03-DQB1*03:02/4 (DR4-DQ8) haplotypes indicating a strongly increased risk or alternatively DR4-DQ8/X (X=DR4-DQ8 or a neutral haplotype) or DR3-DQ2/Y (Y=DR3-DQ2 or a neutral haplotype) indicating moderately increased risk. The children were recruited from the following hospitals: Jorvi Hospital, Espoo, Finland, and Tartu University Hospital, Tartu, Estonia. Local ethics committees of both hospitals approved the study protocols, and the study was carried out in accordance with the Declaration of Helsinki. The parents of the infants gave their informed written consent to the study. In studies, I and II samples from these children were analyzed.

The study cohort in the study I included 161 children between the ages three months and 36 months, and the publication II cohort altogether 136 of the same children who were prospectively followed from birth until the age of 12 months in that study.

Four hundred thirty-two heparinized whole blood samples were used in both studies (study I and II) for flow cytometry analyzes. Blood samples were collected from children at the age of 3 months (111 children; 56 EST), 6 months (45 children; 18 EST), 12 months (100 children; 43 EST), 24 months (84 children; 40 EST), and 36 months of age (92 children; 42 EST).

In study I, we also analyzed samples from 219 children by RT-qPCR to study Treg cell-associated transcription factors. We analyzed a separate set of 6-month (33 children; 19 EST), and 18-month-old children (96 children; 48 EST), and also a part of the same 36-month samples as in flow cytometry (80 children; 31 EST). We also analyzed serum samples from 375 children (178 EST) from the flow

cytometry cohorts for their IgE levels at 6 (142 children; 70 EST), 18 months (111 children; 49 EST), and 36 months of age (120 children; 58 EST).

Serum samples for cytokine analysis (study II) were collected from 136 children (75 EST) at the same time points as Treg samples at 3 (103 children; 56 EST), 6 months (100 children; 51 EST), and 12 months of age (101 children; 45 EST).

Stool samples for microbiota and virus analyses were collected every month, starting at the age of one month. For microbiota analysis (study I), 243 feces samples from 47 children (18 EST) were analyzed from samples from the same infants who were studied in the flow cytometry analyzes, at the same time points as, or as close as possible (SD = 0.7 months). A few additional (n = 18; 7 EST) fecal samples were analyzed as well, if available, in selected cases in whom the gut microbiota was found to be seemingly aberrant at the age of 3 or 6 months. These extra samples were, in most cases, obtained at the age of 2, 4, 5, or 7 months. For virus analysis (study II), 1063 samples from 116 children (54 EST, on average 8 samples/child), were analyzed from the series of stool samples taken every month from birth up to 12 months.

4.1.2 Publication III

Children were recruited in three pediatric hospitals in Finland from May 15, 2002, to November 22, 2005, in the FINDIA (Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes) study which was a randomized, double-blind pilot trial. All 1104 children carried HLA-conferred susceptibility to T1D (the high-risk *HLA-DQB1*02/DQB1*0302* genotype (13.4%), the moderate-risk *DQB1*0302/x* genotype (*x_DQB1*0301* or**0602*; 54.2%), and the *HLA-DQA1*05-DQB1*02 (DR3)/y* genotype (*y_DQB1*0301*, **0602*, or **0603*; 32.5%).

The children were recruited in Jorvi Hospital, Espoo; Department of Pediatrics, Kuopio University Hospital; and Central Finland Central Hospital, Jyväskylä, Finland. The ethics committees of the participating hospitals approved the study protocol, and the study was carried out in accordance with the Declaration of Helsinki. The parents of the infants gave their informed written consent to the study.

The infants were randomly assigned to receive one of three study infant formulas; the cow's milk formula (CMF) (n = 389), whey-based hydrolyzed formula

(WHF) (n = 350), or whey-based FINDIA formula virtually free of bovine insulin (n = 365) during the first 6 months of life whenever breast milk was not available. The bovine insulin was removed from the FINDIA formula according to the method described in patent EP1124436 [277]. Study formulas were prepared and coded by Valio Ltd, which maintained the codes. Breastfeeding was encouraged; thus, a group of infants not exposed to any study formula became available during the study. Newborn infants requiring supplemental feeding in the delivery hospital received frozen collected and pooled breast milk or WHF. At the follow-up visits, blood was drawn at 3, 6, and 12 months of age and annually after that, up to the age of 6 years. Nine hundred eight (908) children provided at least one follow-up blood sample during the study period.

The codes were opened after all children in the follow-up groups had reached the age of three years. At that time, the statistical analysis of the emergence of diabetes-predictive autoantibodies was performed.

4.2 Methods

4.2.1 Detection of Treg cells by Flow cytometry

In studies I and II, fresh heparinized blood samples were stained for flow cytometry. The Foxp3 staining was adapted from a paper by Luopajärvi et al. [278]. We stained the blood samples for surface antigens with the following antibodies: perCP-conjugated anti-CD4 (clone SK3; BD Biosciences, Franklin Lakes, NJ, USA), APC-conjugated anti-CD25 (M-A251; BD Biosciences, Franklin Lakes, NJ, USA), and PE-conjugated anti-CD127 [HIL-7R-M21; BD Biosciences, Franklin Lakes, NJ, USA). After the cell surface staining was completed, we fixed, permeabilized, and stained the cells with anti-human Alexa488-conjugated anti-FOXP3 (clone 206D; BioLegend, San Diego, CA, USA), and used Alexa488-Mouse-IgG1-isotype as a control-isotype antibody (BioLegend, San Diego, CA, USA). We acquired at least 1×10^6 events from each sample on a FACSCalibur™ and analyzed the data with the FlowJo™ software (Tree Star, Ashland, OR, USA). The samples were compensated post-acquisition with FlowJo™ software.

We assessed the number of circulating CD4+CD25^{high}FOXP3+ T cells in the samples by first gating the CD4+ cells, and then the CD25+CD127-/lo population. We analyzed the expression of FOXP3 protein in these cell populations by

quantifying the median fluorescence intensity (MFI) in arbitrary units (AU) of CD4+CD25^{high}FOXP3+ after subtraction of the negative-control antibody intensity. Intensity values over the 97.5 percentile of the negative control antibody were regarded as positive. We calibrated the intensities to a set of particles containing known amounts of fluorescein isothiocyanate (SPHEROTM Easy Calibration Fluorescent Particles, FITC; SPHEROTM Easy Calibration Fluorescent Particles, PE; SPHEROTM Easy Calibration Fluorescent Particles, APC; all from Spherotech, Libertyville, IL, USA).

4.2.2 Analysis of bovine and human insulin antibodies

In study III, we used plasma samples derived from Ficoll-Paque density gradient centrifugation. We analyzed bovine- and human insulin–binding IgG antibodies using an enzyme-linked solid-phase immunoassay method (EIA) described by Vaarala et al. [258]. In short, we coated polystyrene plates (Combiplate Enhanced Binding; Labsystems, Helsinki, Finland) with bovine or human insulin (1 µg/well; Sigma, St. Louis, MO). We used 1% human serum albumin (HSA) in phosphate-buffered saline (PBS) for residual coating and Tween 20-PBS (0.05%) as a washing buffer. The samples were diluted 1:20 for IgG antibodies and 1:10 for IgG1 and IgG2 antibodies in 0.2% HSA/0.05% Tween - PBS. We used alkaline phosphatase-conjugated rabbit anti-human IgG or biotinylated rabbit anti-human IgG1 or IgG2 antibodies (Jackson ImmunoResearch, West Grove, PA, USA) as the secondary antibody. After adding the substrate, we measured the absorbance, and the results were expressed as optical density units. Two known positive and two known negative reference samples were run on each plate as controls.

4.2.3 Analyses of IAA, GADA, IA-2A, ZNT8A, and ICA

In study III, autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated protein-2 (IA-2A) and zinc transporter-8 (ZnT8A) were analyzed on serum samples by a radiobinding assay described by Salonen et al. [279, 280]. Islet cell antibodies (ICA) were measured by a classical immunofluorescence method applied to sections of the human pancreas, blood group O [165]. The cutoff limits for positivity for the different autoantibody tests

were determined as the levels corresponding to the 99th percentile of antibody levels in 354 nondiabetic Finnish children. The cutoff limits used for positivity were 2.80 relative units (RU) for IAA, 5.36 RU for GADA, 0.78 RU for IA-2A, and 0.61 RU for ZnT8A. The cutoff levels for ICA were 2.5 Juvenile Diabetes Foundation units. The disease sensitivity of the IAA assay was 44%, and the specificity was 100%, for GADA assay (82% and 96%), and IA-2A assay (72% and 100%, respectively) in the 2005 Diabetes Autoantibody Standardization Program Workshop [281]. Samples with IAA, GADA, or IA-2A levels between the 97th and 99.5th percentiles were reanalyzed for confirmation. Transplacentally transferred maternal antibodies were disregarded in the analysis. If the amounts of autoantibodies continuously declined to or near zero, starting from the cord blood sample up to samples taken at 12 or 24 months, the antibodies were considered maternal. Islet cell autoantibodies (ICA) and autoantibodies to zinc transporter 8 (ZnT8A) were analyzed in individuals positive for at least one of the three initial autoantibodies.

4.2.4 Analysis of IgE-class antibodies to different allergens

In study I, total IgE and allergen-specific IgE concentrations were analyzed from serum samples by using the ImmunoCAP fluoroenzyme immunoassay (Phadia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. The test is a sandwich immunoassay design. In short, the allergen, allergen component, or a balanced mixture of relevant inhalant allergens are covalently coupled to the solid phase and react with the specific IgE in the serum sample. Enzyme-labeled detection antibodies against IgE are added to form a sandwich complex, and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured. The intensity of the fluorescence from the capture antibody is proportional to the concentration of the specific IgE in the sample. IgE to the following food and air-borne allergens were analyzed: egg, cow's milk, house dust mite, cat, timothy-grass, birch, peanut, and dog.

The children were classified into five groups based on their clinical allergy diagnosis and allergen-specific IgE values: (A) No signs of allergy (no clinical diagnosis, no specific IgE responses); (B) IgE-sensitization during the study period, up to 36 months of age (no clinical diagnosis, at least one specific IgE response);

(C) sensitized, up to the time point analyzed (no clinical diagnosis, at least one specific IgE response); (D) clinical non-IgE allergy (clinical diagnosis, no specific IgE response [282]; and (E) clinical IgE allergy (clinical diagnosis and a specific IgE response to the same allergen).

4.2.5 Enrichment of Treg cells

In study I, CD4+CD25+CD49d- cells were purified from Ficoll-Paque density gradient isolated PBMCs using MACS CD25+CD49d- magnetic beads (Miltenyi Biotec, Auburn, CA, USA). CD4+ cells were negatively selected by incubating PBMCs with magnetic particles coated with antibodies targeting CD8+CD49d+ cells and collecting the flow through fraction. Negatively-selected CD4+ cells were washed with 0.5% BSA in PBS with EDTA. Cells were briefly spun down and resuspended in 97.5 μl of buffer per 10^7 total cells, and 2.5 μl of magnetic microbeads coated with antibodies targeting the CD25 were added. The number of magnetic microbeads was titrated for improved cell purity to 2.5 μl from the 10 μl of CD25 MicroBeads II per 10^7 total cells in the manufacturer's instructions. CD4+CD25- cells were first washed out from the column attached to a magnetic stand. Then the column was removed from the stand and placed in a 15 ml Falcon tube, and the magnetically labeled CD4+CD25+ cells were eluted from the column with the elution buffer according to the manufacturer's instructions. The second enrichment elution with the MS column was omitted as it resulted in too high yield loss. The purity of the eluted CD4+CD25+ cells was confirmed by flow cytometry, resulting in a mean 87.7% purity of the enriched Treg cells. The pellets were frozen at −70°C in RLT-lysis buffer for subsequent RT-qPCR analysis.

4.2.6 Quantitative Real-time PCR

In study I, total RNA was extracted and transcribed from the samples with commercial kits (Qiagen RNeasy Plus Micro kit, Qiagen), and the (High Capacity cDNA Reverse Transcription kit, Applied Biosystems) was used, both according to the manufacturer's instructions. The quantitative PCR assay (TaqMan Gene Expression assay, Applied Biosystems) was used to amplify FOXP3, TGF-beta1, Helios, GATA-3, CTLA-4, IL-10, and IFN-γ. The ABI-Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used in

triplicate wells. Ribosomal 18S RNA was used as an endogenous control. An in-house control sample (calibrator sample) was used to control inter-assay variation. The calibrator sample was prepared from the phytohaemagglutinin-stimulated human PBMC derived RNA. The quantities of the markers were analyzed with a comparative threshold cycle (CT) method and were presented as relative amounts (2-ΔΔCt): ΔCT is calculated by subtracting the CT value of the 18S gene from the CT value of the marker gene, whereas ΔΔCT is the difference between the ΔCT of the analyzed sample and the ΔCT of the calibrator. Calculation of 2-ΔΔCt then gives a relative amount of the analyzed sample compared with the calibrator.

4.2.7 Analysis of fecal samples

In studies I and II, the fecal samples from children were collected using stool collection vials and immediately stored in home freezers (-20°C). Families delivered the frozen samples to the study center as soon as possible, and the samples were stored at −80°C until processing.

4.2.7.1 Microbial DNA extraction and pyrosequencing

In study I, total DNA was extracted from a 0.25 g fecal sample using the repeated bead beating method described in detail by Yu and Morrison [283], with a number of modifications. In brief, four 3 mm instead of 0.5mm glass beads were added during the homogenization step. Bead beating was performed using a Precellys 24 tissue homogenizer/grinder (Bertin Technologies, Montigny le Bretonneux, France). Protein precipitation with 260 µl of ammonium acetate was carried out twice. Elution of DNA from the purification columns was as well performed twice. Columns from the QiaAmp Stool Kit were replaced by those from the QIAamp DNA Stool Mini Kit (Qiagen).

From each sample, the 16S rRNA genes were amplified using a primer set using modified 341F and 806R primers with a 6 nucleotide barcode on the 806R primer. These PCR primers target the V3, and V4 hypervariable regions of the 16 rRNA. Pyrosequencing was carried out using a Roche FLX Genome Sequencer at DNAvision (Liège, Belgium) according to their standard protocol [284]. Sequences were assigned to samples according to sample-specific barcodes. Sequencing Quality Control was carried out by using the Galaxy Tools website [285]. SFF files

from the 454 Genome Sequencer FLX were converted into FASTA files and FASTA quality files. FASTA - formatted files contained an average (± SD) of 12 830 ± 4888 reads per sample. The RDP pyrosequencing pipeline [286] (RDP 10 database, Update 17) was subsequently used to check the FASTA sequence files for the same criteria as described by De Filippo et al. [284], and that the average experimental quality score was at least 20. After this quality check, the FASTA files contained an average (± SD) of 8024 ± 3136 high quality reads.

Taxonomy (phylum, family, and genus level) was assigned using RDP classifier v.2.01 [287]. Richness and diversity analyses were carried out as described by De Filippo et al. [284]. Identification down to the species level was performed using ARB software [288]. For this, an SSU reference database (SSURef_106_ SILVA_19_03_11) was downloaded from the SILVA website [289]. From this database, only sequences of cultured and identified isolates were used. From these sequences, a “PT server” database was built, which was subsequently used to find the closest match for each of our high-quality sequences imported from the FASTA files. For this, the “search next relatives of listed species in PT server” function was applied with the following settings: Oligo length: 12; mismatches: 0; match score: relative; Min score: 10. The average (± SD) match score was 75.2 ± 18.5. Sequences that were identified as being from different strains but belonging to the same species were grouped together. Species that represented more than 0.005% of all sequences were included in statistical analysis, together representing 99.2% ± 0.35 of all high quality FASTA reads per sample.

4.2.7.2 Virus RNA sequencing

In study II, viral RNA for enterovirus, rotavirus, norovirus, parechovirus, and rhinovirus was extracted from the fecal samples using comparative real-time RT-PCR as described in earlier publications [219, 290-293]. In short, a 10% stool suspension was prepared from the original stool sample in RPMI medium, and viral RNA was extracted using the MagNaPure extraction robot (Roche, Applied Science, Mannheim Germany) and Total Nucleic Acid extraction kit (Roche, Applied Science) and performed according to the manufacturers’ instructions.

4.2.8 Genetic analyses

HLA genotyping was performed according to the screening protocols in the DIABIMMUNE and FINDIA studies. The initial HLA-DQB1 typing for risk-associated (DQB1*02, DQB1*0302) and protective (DQB1*0301, DQB1*0602, DQB1*0603) alleles was complemented with DQA1 typing for DQA1*0201 and DQA1*05 alleles in those with DQB1*02 without protective alleles or the major risk allele DQB1*0302. This two-step screening technique is based on the hybridization of PCR products with lanthanide-labeled probes detected by time-resolved fluorometry as described in earlier publications [294, 295].

4.2.9 Statistical analyses

In study I, all tests were performed with PASW Statistics 18. In studies II-III, we performed statistical analysis using IBM® SPSS® Statistics 20.0. For Windows (SPSS Inc., Chicago, IL, USA). For the analysis of Flow cytometry data, in studies I and II, we used Graph Pad Prism 5 software.

All tests were two-tailed, and P values less than 0.05 were considered significant. The data were most often not normally distributed, and therefore we used non-parametric statistical methods. The statistical significance of differences between the groups was tested using non-parametric tests: the Mann-Whitney U-test for comparisons between two unrelated groups and the Kruskal-Wallis test for comparisons among three unrelated groups. The Wilcoxon Signed-Ranks Test was used for the comparison of two related parameters, and Spearman's rank correlation test (rs) for correlation analysis of variables. Variances were analyzed with the Chi-square test ($\chi 2$), ANOVA (R-version 2.15.0), and Fisher LSD test. In study III, cumulative regression analyses were performed using Binary logistic regression analysis, and the results are given as odds ratios (ORs) with 95% CIs. Survivals were analyzed by Cox regression analysis and Kaplan-Meier curves. The proportionality of the hazards was checked using the cumulative hazard plot and the log-minus-log plot, and the results are given as hazard ratios with 95% CIs.

In study I, also, principal component analysis (PCA) was performed to find clusters of similar groups of samples or species. PCA is an ordination method based on multivariate statistical analysis that maps the samples into a reduced number of relevant dimensions of variability.

5 RESULTS AND DISCUSSION

5.1 The characteristics of circulating Treg- and TregFOXP3^{high} cells during the first two years of life (I)

Following birth and during the first year of life, the infant's immune system must rapidly develop to succeed in coping with the infectious milieu of the extrauterine environment, as well as to establish tolerance to nonpathogenic antigens it encounters. Several groups have described the proportions of basic lymphocyte subsets at birth [296, 297], over the first year of life [298, 299], and during childhood and adolescence [300], but there are limited data regarding the longitudinal natural development of these subsets during the first year of life.

In our study, we had a unique opportunity to compare the development of Treg cells between children from Estonia and Finland, two neighboring countries significantly differing in the incidence of allergic diseases, asthma, and T1D [133, 143, 301]. The children took part in the DIABIMMUNE study, a prospective study investigating otherwise healthy infants, who carried HLA conferred susceptibility to T1D. We studied the characteristics of blood Treg cells at the ages of 3, 6, 12, 24, and 36 months in the Estonian and Finnish children who participated in the study (Studies I, and II). The infants from both countries had high numbers of circulating Tregs (CD4+CD127-/loCD25high) at 3 months and 6 months of age. With a drop in Treg frequencies occurring at 12 months of age (from 6 to 36 months $P = 0.0026$, and $P = 0.008$ in Estonia and Finland, respectively; 12 to 36 months $P = 0.0003$, and $P < 0.0001$ in Estonia and Finland, Figure 2C, D). Also, the FOXP3 protein expression showed a significant drop after 12 months of age ($P < 0.0001$ from 6 to 24 months in Finnish infants; $P < 0.0001$ from 12 to 24 and 12 to 36 months, Figure 2E, F) in infants from both countries. This infant Treg phenotype characterized by the high frequency of circulating Treg cells with high FOXP3 expression is in agreement with the few earlier studies reporting increased numbers of circulating Treg with high FOPX3 intensity in infants compared to adults [302, 303].

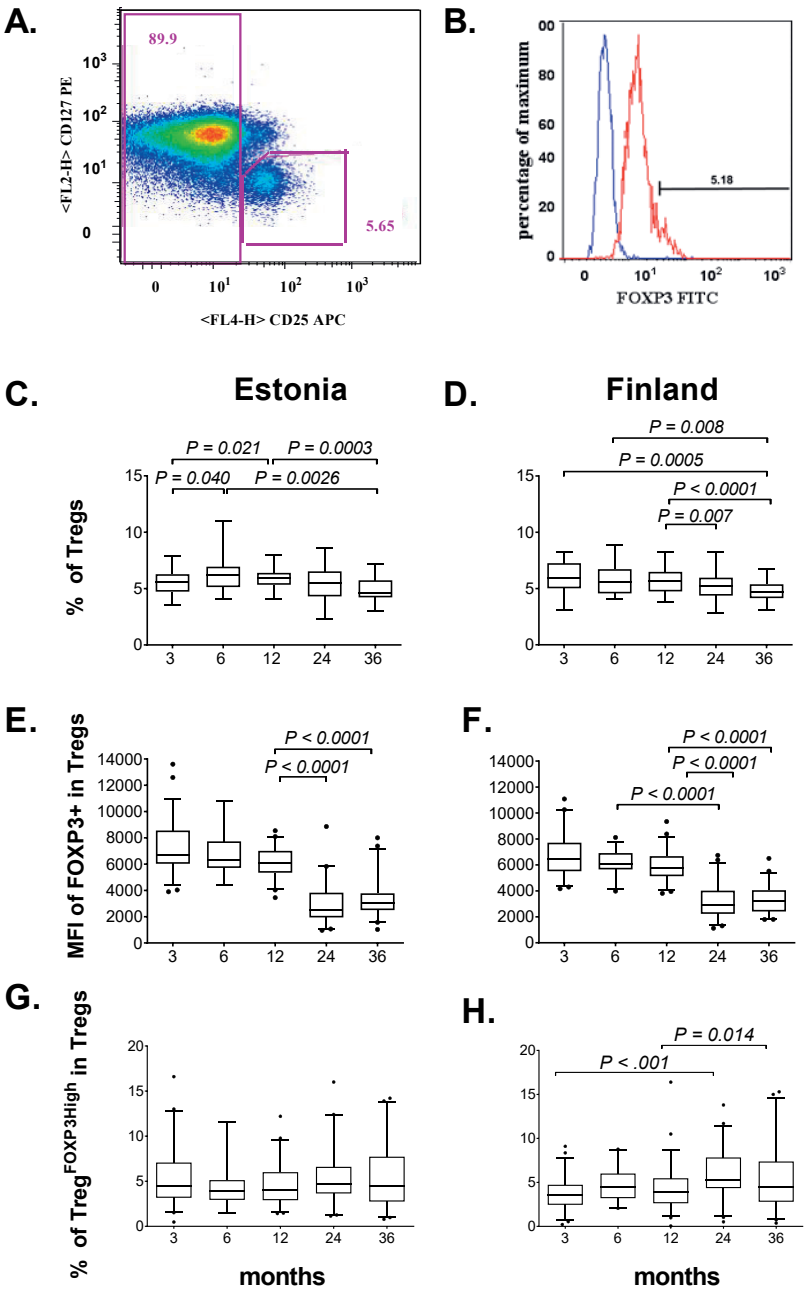
A.
89.9
5.65
<FL2-H> CD127 PE
<FL4-H> CD25 APC
B.
percentage of maximum
5.18
FOXP3 FITC
C.
Estonia
% of Tregs
P = 0.021
P = 0.0003
P = 0.040
P = 0.0026
D.
Finland
P = 0.008
P = 0.0005
P < 0.0001
P = 0.007
E.
MFI of FOXP3+ in Tregs
P < 0.0001
P < 0.0001
F.
P < 0.0001
P < 0.0001
P < 0.0001
G.
% of Treg FOXP3High in Tregs
months
H.
P = 0.014
P < .001
months

FIGURE 2. **A**. Regulatory T cells are gated by gating the CD25+ and CD127- cells from the CD4+ cell population. The gate on the lower right. **C** and **D**. The frequency (%) of Treg cells in the CD4+ cell population shows a significant decrease over time in both countries. **E** and **F.** FOXP3 expression as the median fluorescence intensities (MFI) in Treg cells shows a marked decline from 12 months to 24 and 36 months of age in both countries. No differences between countries could be seen. **B.** A histogram of FOXP3 fluorescence intensity demonstrates a population of Treg cells with a high expression of FOXP3 ($Treg^{FOXP3high}$). The gate is shown as a horizontal line in the graph and the negative isotype control as a histogram on the left. The changes in the proportion (%) of TregFOXP3high cells within the Treg cell population is shown for Estonian **G**. and Finnish children **H**. An increase in the $Treg^{FOXP3high}$ population was seen only in Finland. Medians are shown as horizontal lines in the boxes, and the whiskers show the 5-95 percentiles. Wilcoxon test (two-sided) was used for comparisons.

To address the activation status of Treg cells, we analyzed the proportion of a subpopulation of Treg cells with the highest expression of FOXP3 (Figure 2F). Several groups have shown that Treg cells with a high FOXP3 expression possess an enhanced suppressive activity [303-305]. The proportion of these highly activated (CD4+CD127-/loCD25highFOXP3high) $Treg^{FOXP3high}$ cells increased from 12 to 36 months of age in both Finnish and Estonian cohorts (Figure 2G, H), but the increase in Treg ^{FOXP3High} cells was significant only in the Finnish children. At the same time, a decrease occurred in the relative numbers of circulating Treg cells and their FOXP3 expression.

The expression of FOXP3 and TGF-beta1 transcripts in the blood-derived Treg cell population followed the observed changes in the FOXP3 protein expression showing first a decrease from 6 to 18 months followed by an increase from 18 to 36 months of age (P <0.001 for both decrease and increase; original publication I, Figure 1E) Also, the mRNA expression of Helios, a marker for highly activated and possibly thymus-derived Treg cells [304, 305], and GATA-3, which is considered to stabilize FOXP3 expression in Treg cells in type 2 cytokine environment [306], showed similar age-dependent expression pattern; high expression at 6 months, a decreased expression at 18 months, and an increase again from 18 to 36 months of age (P < 0.001 for both, original publication I, Figure 1E). The expression of CTLA-4 transcripts decreased from 6 to 18 months (P = 0.02, original publication I, Figure 1E). IL-10 and IFN-γ mRNAs were not detectable in the Treg cell population. We suggest that the increase in the highly activated Treg cells reflects the rise in of thymus-derived Treg cells, based on the earlier study by Tulic et al. who showed an age-dependent increase in Treg cell frequency and their proliferation and FOPX3 expression in thymus of children who underwent thymectomy [307]. However, the idea of Helios as a marker of thymus-derived Treg cells has been challenged [308]. HLA genotype or gender did not affect any of the Treg parameters at any of the time points analyzed.

Our results revealed a two-step maturation process in the circulating Treg cells population: a high proportion of Treg cells is seen during the first year of life, and then during the second year of life, a decline in the percentage of circulating Treg cells, and an increase in the highly activated Treg cell subpopulation, characterized by high FOXP3, TGF-beta1, and Helios expression. Results from both countries were combined, as no significant differences between the two countries were seen.

5.2 The associations between Treg- and TregFOXP3^{high} cell maturation and the development of the gut microbiota composition, and later risk of atopic sensitization and clinical allergy (I)

Earlier studies indicate that the gut microbiome plays a pivotal role in the regulation of allergic immune response [109, 133-136, 138]. The gut microbiota composition is determined by our microbial environment, and several studies in animal models show that the gut microbiome modulates the permeability of the intestinal epithelium and gut immune responses [309-313]. Probiotic treatment of rodents reduces or prevents the onset of autoimmune diabetes [314-316]. Antibiotics, which modulate gut microbiota, can also prevent autoimmune diabetes [317, 318]. The incidence of diabetes in NOD mice has been reported to increase in a germ-free environment [319]. But this is challenged by a study showing, that the incidence of diabetes in female NOD mice remained unchanged under germ-free conditions. But a spontaneous monoculture with a gram-positive aerobic bacteria delayed the onset and reduced the incidence of diabetes [320]. Alam et al. reported that lack of intestinal microbiota promotes an imbalance between Th1, Th17, and Treg differentiation in the intestine. But does not affect the recruitment of FOXP3+ Tregs into islets. This imbalance is associated with accelerated insulitis. These results suggest either a microbial dependence of local induction of Treg in the gut and draining lymph nodes, or a potentially compensatory function of naturally occurring Tregs in the islets, which may help control diabetogenic T cells [321]. In a BB-rat model, Roesch et al. showed that the diabetes-prone (BB-DP) rats had a higher number of *Bacteroides*, *Eubacterium*, and *Ruminococcus* before the onset of diabetes. In contrast, the diabetes resistant (DR) rats had higher populations of *Lactobacillus* and *Bifidobacterium* in their intestine [322].

The gut microbiome also modulates differentiation and function of effector and regulatory T cells (Treg) [112, 323-328], which could further influence the development of immune-mediated diseases, including T1D and allergic diseases. Altered gut microbiota composition has been associated with allergic responsiveness in children. It predicts allergic diseases, but the mechanisms linking the gut microbiota and allergic immune deviation have not been revealed in these studies [329].

This study aimed to study the hygiene hypothesis by comparing the colonization of the gut and the changes in the Treg cell population during the first three years of life in both Estonia and Finland. In fecal microbiota analyses, Bifidobacteria comprised the most dominant microbial group at 3 and 6 months of age, with a steady decrease in abundance with age (Figure 3 and Figure 2A in original publication I). A principal component analysis of the 3 and 6-month samples, on the species level, revealed additional details in regards to the importance of the Bifidobacterial composition. Principal component 1 (PC1, a variance of 51%) is the main descriptor of variation at 3 months of age, describing the difference between children who have a normal Bifidobacterium dominated microbiota and those who have an aberrant microbiota dominated by a combination of *Escherichia coli*, *Bacteroides*, *Ruminococcus gnavus* and *Clostridia.* PC1 did not correlate with Treg parameters, however. The linear combination of Bifidobacterial species described by the abundance of *Bifidobacterium breve* minus *Bifidobacterium longum* at 3 months, accurately described by PC2 (16%), showed a positive correlation with the relative numbers of Treg cells at 3 months of age (r_s = 0.40; *P* = 0.006). This particular linear combination of both Bifidobacterial species also showed a positive correlation with the numbers of Treg cells (*P* = 0.002; r_s = 0.44). The abundance of *B. breve* minus *B. longum* at 3 months of age was also reflected in the relative numbers of Treg cells later at 6 and 12 months (r_s = 0.33; *P* = 0.015; and r_s = 0.35; *P* = 0.018, respectively). Thus, the effects of the initial Bifidobacterial composition at 3 months of age on Treg numbers last at least for one year (see Figure. 2C in original publication I). Interestingly, a strong association was seen between the amounts of highly activated $Treg^{FOXP3high}$ cells at 3 months of age and principal component 4 (*P* = 0.003). PC4 is almost totally defined by the abundance of *B. longum* minus the amount of *B. pseudocatenulatum* (rs = 0.80; *P* < 0.001). It follows that *B. longum* minus B. *pseudocatenulatum* was also associated with the proportion of $Treg^{FOXP3high}$ cells (r_s = 0.40; *P* = 0.007). A shift from a Bifidobacterial dominated

microbiota towards one dominated by butyrate producers, and normal maturation of the gut microbiota was seen over the first year of life.

Butyrate is the primary energy source for colonic epithelial cells [323] and thus thought to be beneficial. Butyrate-producing bacteria, despite their heterogeneity, represent a functional group within the microbial community of the human large intestine. Butyrate plays a key role in maintaining human gut health, not only as the major source of energy to the colonic mucosa, but also as an important regulator of gene expression, inflammation, differentiation, and apoptosis in host cells [330]. Furthermore, butyrate has been shown to regulate the assembly of tight junctions and gut permeability [331]. Butyrate affects the intestinal epithelium integrity and modulates Treg and Th17 cells directly [325, 332] or via effects on dendritic cells [333]. The effects of butyrate seem to be local in the intestine, and thus it potentiates extrathymic Treg differentiation [325]. On the other hand, it is also possible that the changes in the intestinal microbiota affect the thymus microenvironment and maturation of thymus-derived Treg cells, as it has been shown that antibiotic treatment-induced changes in the gut microbiota resulted in a concomitant shift in the thymic Treg T cell receptor repertoire [334]. This shift from Bifidobacterial dominated microbiota towards one dominated by butyrate-producing bacteria was inversely correlated with FOXP3 intensity (see Figure 2B in original publication I). Together these results suggest that during early infancy, Bifidobacterial species play an essential role in Treg cell activation, as indicated by earlier studies [335-338]. Our findings especially provide support for the role of *B. longum* abundance in the early microbiota as a promoter of highly activated $Treg^{FOXP3high}$ cells in the maturation of the Treg cell population. The clinical significance of this finding will need further studies.

At the age of 3 months, Estonian children had significantly more highly activated $Treg^{FOXP3high}$ cells than the Finnish children (P = 0.002; Figure 3A in original publication I) in their T-cell population, whereas an increase in these highly activated Treg cells occurred in Finnish children later (data not shown).

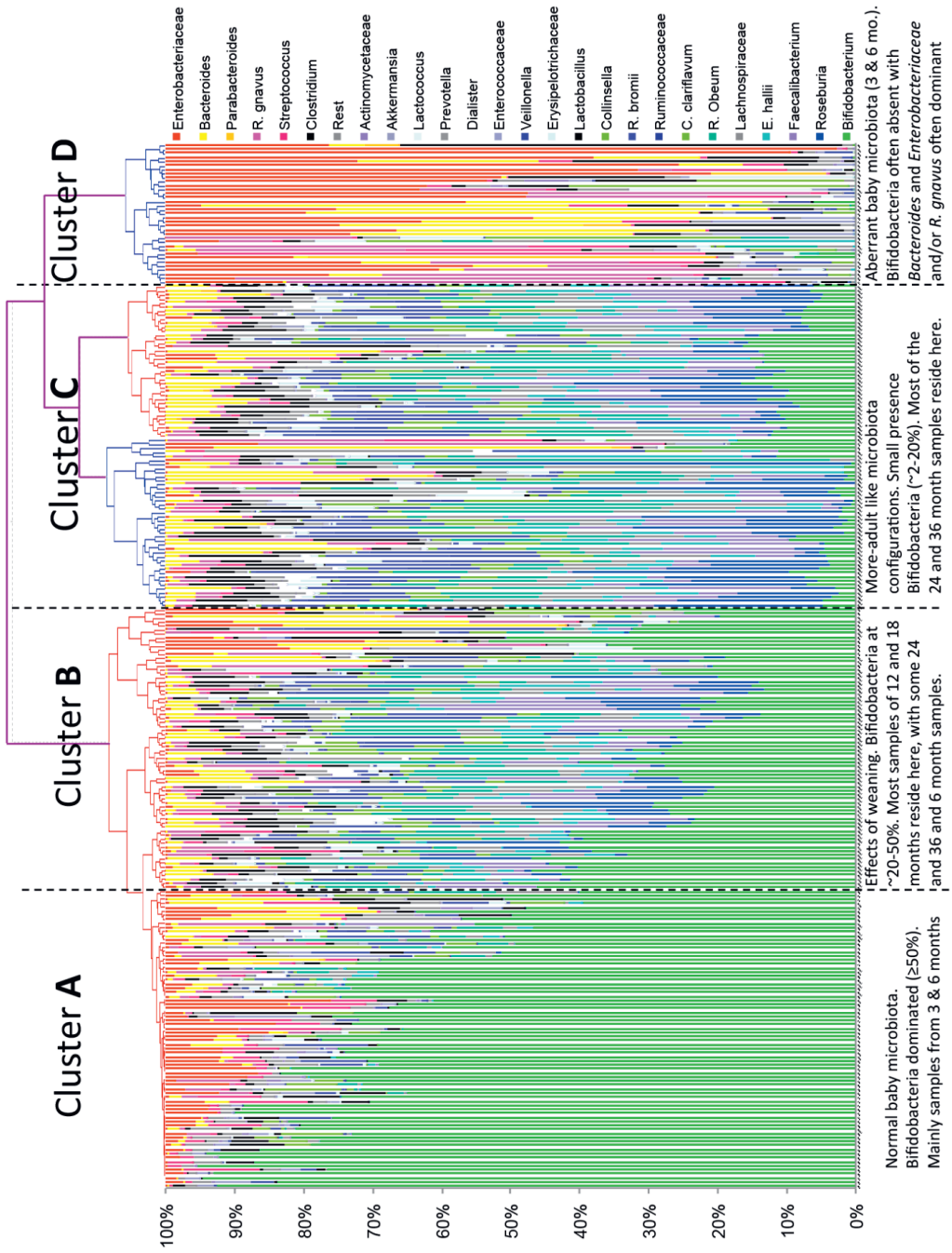
Cluster A
Cluster B
Cluster C
Cluster D
Enterobacteriaceae
Bacteroides
Parabacteroides
R. gnavus
Streptococcus
Clostridium
Rest
Actinomycetaceae
Akkermansia
Lactococcus
Prevotella
Dialister
Enterococcaceae
Veillonella
Erysipelotrichaceae
Lactobacillus
Collinsella
R. bromii
Ruminococcaceae
C. clariflavum
R. Obeum
Lachnospiraceae
E. hallii
Faecalibacterium
Roseburia
Bifidobacterium
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Normal baby microbiota. Bifidobacteria dominated (≥50%). Mainly samples from 3 & 6 months
Effects of weaning. Bifidobacteria at ~20-50%. Most samples of 12 and 18 months reside here, with some 24 and 36 and 6 month samples.
More-adult like microbiota configurations. Small presence Bifidobacteria (~2-20%). Most of the 24 and 36 month samples reside here.
Aberrant baby microbiota (3 & 6 mo.). Bifidobacteria often absent with *Bacteroides* and *Enterobacteriaceae* and/or *R. gnavus* often dominant

FIGURE 3. **A**. Complete linkage clustering, in combination with a microbial profile at the genus level, in children's fecal samples from 3 months to 36 months of age. Cluster A. *Bifidobacteria* dominate (≥50%). Cluster B. *Bifidobacteria* ~20-50%. Cluster C. *Bifidobacteria* ~2-20%, an adult-like configuration. Cluster D. Aberrant microbiota consists mostly of samples taken at 3 and 6 months: *Bifidobacteria* are mostly absent, and a combination of *Bacteroides*, *Escherichia coli*, and/or *Ruminococcus gnavus* is dominant. Reproduced with permission from Frontiers in Immunology [339].

A positive correlation between FOXP3 and CTLA-4 transcripts in Treg cells was observed in Estonian children at the age of 6 months (P = 0.008; r_s = 0.59). FOXP3 and CTLA-4 transcripts did not show any positive correlation in Treg cells from Finnish children at those ages (NS), while a positive correlation was seen in both countries at the age of 3 years (P < 0.001 for both r_s = 0.58 and r_s = 0.70 for Estonian and Finnish children, respectively). These results suggest earlier maturation of the Treg population with cells having an enhanced suppression capacity [340] in Estonian children with a lower risk of allergic diseases. Differences in the development of microbiota between Estonian and Finnish children were also seen. The ratio of *B. longum* vs. *B. breve* at 3 months, which was associated with the numbers of highly activated Treg cells, was higher in Estonian children (P < 0.001). An earlier switch from Bifidobacteria dominated microbiota to a microbiota with increasing numbers of butyrate producers was as well observed in Estonian children. Already at the age of 18 months, a lower Bifidobacterial abundance (13% *vs.* 26%, P = 0.02), and a higher abundance of butyrate-producing bacteria (40% *vs.* 29%, P = 0.01) was observed in the Estonian samples (see Figure 3B in original publication I). The abundance of Bifidobacteria remained stable in Estonian children between 18 and 24 months (13% and 14%), while it was still dropping in Finnish children (26% and 12%), and associated with the simultaneous increase in the abundance of butyrate producers (from 29% to 42%). PC1 at 36 months of age, representing over 50 % of the variation at this time point, primarily reflected the abundance of butyrate producers, and now the abundance of butyrate producers had again become higher in Estonian than in Finnish children (45% *vs.* 36%, P = 0.02, Figure 3C in original publication I). In addition, Finnish children showed a higher abundance of *Bacteroides* than Estonian children at 12 and 18 months of age (9.7% *vs.* 2.4%, P = 0.003 and 6.6% *vs.* 1.6%, P = 0.008, respectively). The bacterial species producing butyrate were detected, but the butyrate levels were not measured, as the focus of the study was in describing the development of the microbial diversity on the species level.

The effect of infant feeding, i.e., breastfeeding vs. CMF formula feeding on Tregs, is not well understood even though it is well-accepted that breastfeeding is

a potent modifier of gut microbiota. In our study, the duration of total breastfeeding was similar in Estonia and Finland, but the duration of exclusive breastfeeding was longer in Finland than in Estonia (median 100 days, range (0-180 days) *vs.* one day, range (0-186 days), $P = 0.029$). The definition of exclusive breastfeeding was strictly based on the full exclusion of any other food substance than breastmilk. The total duration of breastfeeding was, in general, positively correlated with the abundance of Bifidobacteria, *B. longum* in particular, but also with *B. breve*. An association between the lower frequency of Treg cells and an earlier start of infant formula was associated with a lower frequency of Treg cells at 3, 6, and 12 months of age ($P \leq 0.05$), and thus earlier maturation of the Treg population.

In addition to feeding patterns, the mode of delivery affects the colonization of the gut in the infant. We did not find significant associations with Tregs when we compared the children born with vaginal delivery or cesarean section. Among the infants with microbiota analyzes, only one infant was born with a cesarean section preventing any meaningful comparisons.

In the Diabimmune study (study I), atopic sensitization later in life is associated inversely with the abundance of *Bifidobacteria* ($P = 0.014$) at 3 months of age and with PC2 at the genus level ($P = 0.010$, Figure 4A in the original publication I) in Estonian and Finnish children. Furthermore, the relative abundance of *B. longum* alone at 3 months of age shows an inverse association with atopic sensitization, number of allergen-specific IgEs and development of allergy ($P = 0.023$, $P = 0.026$, $P = 0.022$) suggesting, that richness of *B.longum* among *Bifidobacteria* is an important factor protecting from atopic sensitization. As mentioned earlier, Estonian children had a higher *B. longum* vs. *B. breve* ratio than Finnish children. The abundance of *Bacteroides* ($P = 0.009$) and *E. coli* (*Enterobacteriacea*) ($P = 0.015$) is associated with an increased risk of atopic sensitization. PC2, nearly perfectly described by *Bifidobacteria* minus the abundance of *Bacteroides* and *E. coli* ($r_s = 0.96$, $P < 0.001$), represents a clear vector associated with atopic sensitization ($r_s = 0.37$, $P = 0.006$, Figure 4B in original publication I), the number of allergen-specific IgEs ($P = 0.012$), the age of the first allergen-specific IgE ($P = 0.039$) and the clinical diagnosis of allergies ($P = 0.012$). In conclusion, a high abundance of *Bacteroides* and *E. coli* at the expense of a reduced abundance of Bifidobacteria in early infancy increased the risk of atopic sensitization and clinical allergy later in childhood.

Later at 36 months of age, we found several associations between the intestinal microbiome and the occurrence of atopic sensitization. Still, these associations should be cautiously interpreted because the microbiota composition may at least partly reflect the dietary differences between allergic and non-allergic children. Atopic sensitization is nonetheless strongly correlated (r_s = 0.57; *P* < 0.001) with PC1 on the genus level. PC1 accounts for 66% of the variation at this time point and describes the abundance of a trophic network of butyrate producers. PC1 is inversely correlated with the number of allergen-specific IgEs (r_s = -0.50; *P* < 0.001), (Figure 5A in original publication I), with total IgE at 36 months (r_s = -0.48; *P* = 0.007), with the age at the appearance of the first allergen-specific IgE (r_s = 0.58; *P < 0.001*), atopy (r_s = -0.57; *P* < 0.001), food allergies (r_s = -0.58; *P* < 0.001) and inhalation allergies (r_s =-0.39; *P* = 0.031). In children with a food allergy, *Faecalibacterium* (*F. prausnitzii*) (Figure 5B in original publication I), which is directly correlated with PC1 and is one of the most important butyrate-producing groups, was decreased whereas the abundance of *Mogibacteriaceae* (Figure 5C in original publication I) being inversely correlated with PC1, was increased.

Because increased total IgE levels are considered as a predictive marker for the development of later IgE-sensitization and allergies [341] we analyzed the associations between total IgE levels, gut microbiota composition, and characteristics of Tregs. Despite having less IgE mediated allergies, Estonian children had higher total IgE levels than Finnish children at 6 months (median 8.4, range 2-311 vs. 5.2 range 2-3112-93.5 kU/L; *P = 0.02*) and 36 months (62.4; range 2.41-2956 vs. 25.9; range 1.26-456 kU/L; *P = 0.006*, respectively), indicating an earlier maturation of IgE production, which takes place during the first 5 years of life when the IgE levels reach adult levels [342]. In Estonian children, total IgE levels at 6 months did not correlate with later IgE sensitization or allergies as they did in Finnish children (*P* < 0.001, r_s = 0.40). There was an association between the Treg cell phenotype at 6 months of age and later maturation of IgE production. In Finnish children, the markers of Treg activation at 6 months of age, such as FOXP3, Helios (r_s = -0.75; *P* = 0.002; r_s = -0.77 and *P* = 0.001) and GATA-3 correlated inversely with the later total IgE levels suggesting an inhibitory effect of Treg activation on IgE production. The expression of FOXP3, Helios, and GATA-3 transcripts in Treg cells at 6 months of age correlated inversely with total IgE levels at 18 months of age (r_s = -0.59; *P* = 0.032; r_s = -0.63; *P* = 0.021; r_s = -0.55 and *P* = 0.052). FOXP3 intensity in Treg cells at 12 months of age correlated inversely with total IgE levels at 18 and 36 months of age in Finnish children (r_s = -0.31, *P* = 0.028;

r_s = -0.38 , *P* = 0.007, respectively). Similarly, there was an inverse correlation between Helios expression in Treg cells at 6 months and the total IgE production in Finnish children at 6 and 18 months of age (r_s = -0.75, *P* = 0.002 and r_s = -0.63, *P* = 0.021). In Estonian children, GATA-3 expression in Treg cells at 6 months correlated positively with total IgE levels at 18 months (r_s = 0.56, *P* = 0.047) while in Finnish children GATA-3 expression level in Treg cells at 6 months showed an inverse correlation with total IgE levels at 6 (r_s = -0.77, *P* = 0.001) and later at 18 months (r_s = -0.55, *P* = 0.052).

Accordingly, the maturation of IgE production in Finnish children is differentially associated with Treg cell activation in comparison to Estonian children. The expression of FOXP3, GATA-3, and CTLA-4 transcripts at 18 months (Figure 4C in the original publication I; the missing X-axis titles are FOXP3 for the first cluster of boxes, GATA-3, CTLA4, and TGFβ1, respectively), as well as the FOXP3 intensity at the age of 24 months, was increased in Treg cells from children with IgE sensitization compared to children who did not develop IgE sensitization or clinical allergies. Both in the children with IgE allergy [282] and those with non-IgE-allergy the activation markers of Treg cells were decreased at the age of 36 months in comparison to non-allergic children, the expression of FOXP3 and Helios mRNA in the children with non-IgE allergy and that of CTLA-4 in children with IgE-allergy. Accordingly, these findings showing an association of high numbers of Treg cells and later risk of allergy might be related to the delayed maturation of the Treg population in the individuals who develop allergies [343].

The composition of microbiota at 3 months showed associations with later allergic sensitization and disease. Firstly, the relative abundance of *B.longum* at 3 months of age showed an inverse association with later development of IgE sensitization and allergy in both populations. Also, the high abundance of *Bacteroides* and *Escherichia coli* at the expense of Bifidobacteria implied later risk of allergic sensitization and disease in our cohort. A recent study in a US cohort of young children with follow-up for four years also identified a group of infants with a specific neonatal gut microbiota composition at the age of 3 months showing a high risk of atopy and asthma. A lower relative abundance of *Bifidobacterium, Akkermansia* and *Faecalibacterium* and a higher relative abundance of particular fungi, was associated with asthma risk, and fecal water extract from these high-risk infants compromised Treg suppressive activity *in vitro* [344]. Our findings, together with earlier reports [345], emphasize the importance of the neonatal gut

microbiota for later allergy development and the maintenance of Bifidobacteria as the dominant gut microbiota group during the first months of life.

5.3 Virus infections detected in feces during the first year of life and the immunomodulatory effects of viral infections in children (II)

One of the possible risk factors in the etiology of T1D are viral infections. Viral infections are frequent during the first year of life, and their role in the risk of autoimmune diseases, such as T1D, and allergies, and asthma has been studied in recent years [346-349]. Seasonal incidence of T1D and observed case reports have contributed to the generation of the virus hypothesis, and various viruses, including cytomegalovirus, parvovirus, encephalomyocarditis virus, retroviruses, rotavirus, congenital rubella, and mumps infection have been connected to T1D. The most studied association between a virus and T1D has been with enteroviruses, a viral single-stranded RNA (ssRNA) genus belonging to the picornaviruses. The seasonal occurrence of T1D is reviewed by Moltchanova et al. [230], showing the incidence of T1D peaking through fall, winter, and early summer months. Results from 52 countries show the incidence of T1D peaking through fall, winter, and early summer months. The seasonality of T1D occurs worldwide, but this phenomenon is stronger in the northern hemisphere, especially in areas with colder winter [230]. The seasonal pattern of autoantibody seroconversion time has also been reported. In Finland, children turn autoantibody-positive usually during late summer and fall [254]. The seasonal pattern of autoantibody seroconversion was sharper than reported in the development of T1D. Altogether, the seasonal pattern in the incidence of T1D and autoantibody seroconversion resembles that of enterovirus infections. The outcome of these studies is that enterovirus is currently considered as one of the most likely triggers of T1D, but this association still needs further confirmation.

In our study, both enteral and respiratory viruses were tested from stool samples collected during the first 12 months of life. In the DIABIMMUNE study, we tested for enterovirus, norovirus G1 and G2, parechovirus, rhinovirus, and rotavirus. Of the altogether 1063 samples, 433 (40.7 %) were positive for at least one of the tested viruses. Of the children, 108 (93.1 %) were virus-positive at least once, whereas eight (6.9 %) were virus negative in all samples. The most

frequently detected virus was rhinovirus in 280 of the samples (26.3 %) followed by norovirus G1 and G2 (71 samples/6.7 %), rotavirus (42/4.0 %), enterovirus (59/5.6 %), and parechovirus (46/4.3 %; Figure 1 in original publication II). Viruses showed the following seasonality: enterovirus, parechovirus, and rotavirus were most frequently detected during autumn months, while norovirus was most frequent from February to March. Rhinovirus was most prevalent in May-June and September-October (Figure 2 in original publication II). Rhinoviruses were most frequently detected in the children during the first 6 months of life, while positivity for enteroviruses and parechoviruses was highest at the age of 6-12 months (Table 1 in original publication II). Norovirus G2 was detected in all age groups. Rotavirus was most frequent at the age of 2 and 3 months in Finland, while there was only one rotavirus positive sample in Estonia. The rotavirus frequency is likely due to the introduction of live attenuated rotavirus vaccine in Finland at the age of 2, 3, and 5 months. In contrast, rotavirus vaccination was not used in Estonia during the study.

To assess the effects of virus infections on the activation of Treg cells, we analyzed the temporal association of virus infection with the expression of FOXP3 in Tregs. The children who had had at least one virus infection with any virus during the preceding 60 or 30 days showed increased expression of FOXP3 in Tregs in comparison to the children without virus infections during the same period (P = 0.005 and P = 0.124; Figure 3A in original publication II). When we analyzed the various virus infections separately, the children with rhinovirus infection had higher FOXP3 expression in Treg cells (P = 0.001 for 60 days, and P = 0.036 for 30 days period) than children without rhinovirus infection; Rhinovirus infected showed a trend to an increased expression of FOXP3 in $Treg^{FOXP3high}$ cells (P = 0.026 and P = 0.173 for 60 and 30 days, respectively) (Figure 3B and E in original publication II). These results are in line with earlier studies showing activation of Treg cells in virus infections [350, 351]. Rhinoviruses and infection associated wheezing in infancy have been linked to the development of respiratory allergies and asthma later in life [352-354]. On the other hand, a recent study has shown that early exposure to rhinoviruses is inversely associated with later development of IgE sensitization particularly in boys suggesting that rhinovirus infections may protect against IgE-mediated sensitization [355]. The role of the Treg cells in this protection is not known.

In this study, enterovirus (EV) positivity 30 days before Treg analyses showed an inverse trend, with a decrease in the FOXP3 intensity in the Treg

population (P = 0.088: Figure 3C in original publication II) when compared with children without enterovirus infections. Also, in the analyses of the highly activated $Treg^{FOXP3high}$ cells, the infants who had enterovirus infection during the preceding 60 or 30 days period showed lower FOXP3 expression ($P < 0.001$ and $P = 0.008$) in $Treg^{FOXP3high}$ cells than children without infections (Figure 3F in original publication II). In previous reports, a low frequency of Treg cells was associated with the severity of EV71 associated pulmonary edema [356-358]. Valproic acid treatment alleviated myocarditis in a mouse model by upregulating IL-10 in serum and heart tissues and promoting both the differentiation and suppressive function of Treg cells [359]. Valproic acid is a histone deacetylase inhibitor that has anti-inflammatory effects.

On the other hand, reports of EV infection in mucosal tissue show elevated densities of Treg cells, which correlated with the frequency of EV in the mucosal tissue from celiac disease (CD) patients, when compared to biopsies from healthy individuals [360]. One could speculate that a lower expression of FOXP3 in Treg cells after EV infections seen in this study, could lead to increased tissue inflammation and impaired suppression of anti-viral/pathogen responses. This impaired suppression could contribute to the induction of autoimmunity and tissue destruction. The mechanism behind the decrease of Tregs induced by enterovirus infections remains to be elucidated.

Next, we analyzed the levels of circulating cytokines, which could reflect the immunomodulatory effects of viral infections in children from both countries. We detected lower levels for several cytokines in children with preceding rhinovirus infection in comparison to the children without preceding viral infections. The levels of Th2 cytokines, IL-5 and IL-13 (P = 0.006, and P = 0.002 for a 60 day period, respectively), as well as Th1 cytokine IFN-γ (P = 0.005) and Th17 cytokine IL-17 and GM-CSF (P = 0.004, and P = 0.001, respectively) were decreased after a rhinovirus infection. Also, IL-1beta and soluble IL-1R (P = 0.004, and P = 0.005, respectively) (Figure 5 in original publication II), which are related to inflammasome activation, were decreased after rhinovirus infection.

In contrast, enterovirus infected infants had lower FOXP3 expression in Tregs, particularly in highly activated $Treg^{FOXP3high}$ cells. Their cytokine profile after infection was also altered showing increased activation of a Th1 response; CXCL10 (IP-10) (P = 0.002), which is induced by IFN-γ, and elevated Th17 response; IL-17 (P = 0.009) after infection (60 days range prior infection), while CCL4 (MIP-1beta) (P = 0.0001, and P = 0.006 for 60 day and 30 day period,

respectively) and CCL22 (MDC) (P = 0.004, for 60 day period) remained decreased after infection (Figure 6 in original publication II). CCL4 is a chemoattractant for NK cells, monocytes and a variety of other immune cells. B cells and professional APCs recruit regulatory T cells via CCL4. CCL22 is a DC-derived chemokine associated with Th2 deviation.

IL-10 was increased after parechovirus infection (P = 0.009). Norovirus infection was associated with a decrease in CXCL1 (GRO) (P = 0.006), a chemoattractant for neutrophil infiltration. Rotavirus positivity was associated with an increased level of TNFα (P = 0.001).

These findings are interesting, as earlier reports have shown that certain enterovirus infections are associated with low risk of IgE mediated sensitization, namely echo-, or coxsackie-B-viruses [361]. However, the mechanism(s) by which these viruses promote or enhance these immune-mediated conditions is not clear.

5.4 The association of different infant formula feeding and the induction of autoantibody positivity and progression to T1D (III)

Dietary habits, and infant nutrition, in particular, may affect the immune system conspicuously. Several dietary factors, such as a short period of breastfeeding, or the use of cow's milk-based formula, are among the potential environmental triggers of β-cell autoimmunity and the subsequent risk of T1D. Earlier studies have proposed early exposure to cow's milk proteins as one of the possible factors priming β-cell autoimmunity and T1D [252-256] in genetically predisposed individuals. Vaarala et al. proposed that early exposure to bovine insulin may sensitize intestinal T lymphocytes early in life for later participation in the autoimmune destruction of the insulin-secreting β-cells when associated with impaired insulin tolerance [257, 258]. Lymphocytes may also circulate between the gut and pancreas since the Langerhans islets display MAdCAM-1, a ligand to gut-homing receptor α4β7-integrin [362-364]. In an animal model studying disease-prone BB rats feeding of casein hydrolyzed formula was associated with a decrease in autoimmune diabetes and improved integrity of the intestinal barrier, as well as beneficial changes in the gut microflora (increased Lactobacilli and reduced Bacteroides levels) [264]. On the other hand, studies in NOD mice, feeding on the same casein hydrolyzed formula, show an increase in Bacteroides levels [265]. The observation made by Visser et al. suggested that the presence of

specific peptides has a beneficial effect in the prevention of autoimmune diabetes [264], which has not been confirmed by other groups. The mechanisms by which hydrolyzed formula reduces and foreign proteins increase the risk of diabetes-predictive autoantibodies are not known.

The Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA) pilot study aimed to test whether weaning to bovine insulin-free cow's milk formula (CMF) reduces T1D–associated autoantibodies in children at genetic risk. The study was a randomized, double-blind pilot trial. Altogether 1104 children were recruited in three pediatric hospitals in Finland. The infants received one of three study infant formulas; the cow's milk formula (CMF) (n = 389), whey-based hydrolyzed formula (WHF) (n = 350), or whey-based FINDIA formula virtually free of bovine insulin (n = 365) during the first 6 months of life whenever breast milk was not available. A group of infants not exposed to any study formula became available during the study (n=256). The follow-up time was initially for three years, but it was prolonged until the time when the last recruited children reached three years of age. By this time, the first of recruited children had reached six years of age.

We screened for autoantibody positivity for the following auto-antibodies: IAA, GADA, and the tyrosine phosphatase–related IA-2 molecule (IA-2A) with specific radiobinding assays. Islet cell autoantibodies (ICA) and autoantibodies to zinc transporter 8 (ZnT8A) were analyzed in individuals positive for at least one of the three initial autoantibodies.

A total of 908 children provided at least one blood sample during the follow-up from age 3 months for analysis of diabetes-associated autoantibodies (Figure 1 in original publication III). We found that by the age of 3 years, 42 children (4.6%) had seroconverted to positivity for at least one autoantibody. Of these children, 21 children had received CMF (6.3%) in the control group, eight had received FINDIA-formula (2.6%), and 13 WHF (4.9%). The ORs for autoantibody positivity by the age of 3 years are reported in Table 2 (see original publication III).

Weaning to FINDIA-formula, a whey-based formula virtually free of bovine insulin, was associated with a reduced risk of at least one autoantibody (IAA, GADA, or IA-2A) and for repeated autoantibody positivity or positivity for multiple autoantibodies (≥2 of IAA, GADA, IA-2A, ICA, and ZnT8A) compared with the use of CMF (see eTable 1 in original publication III for the appearance of autoantibodies). The reduced OR for autoantibody positivity was more pronounced

when only children who received study formulas were included in the per-protocol analysis.

We next wanted to assess the timing for the first appearance of autoantibody positivity according to the treatment-received analysis. We analyzed the infants without positive autoantibodies by drawing the Kaplan-Meier curves for survival up to age 6 years as shown in Figure 2 (original publication III). Curves represent survival in infants who received cow's milk formula (CMF) (n = 236), whey-based hydrolyzed formula (WHF) (n = 187), or FINDIA-formula (n = 229) and in the group not exposed to any study formula (no exposure) (n = 256). The CMF group was included as a reference group. The number of children studied for beta-cell autoimmunity after the age of 4 years remains low.

The appearance of autoantibody positivity, and especially the appearance of multiple autoantibodies during the first three years of life, is highly predictive for T1D among individuals carrying increased HLA-conferred disease susceptibility [280]. The number of progressors is low in our study, as the development of clinical T1D is a rare phenomenon before the age of 3 years, and because of the short follow-up time. The child's HLA genotype and the exposure time to study formula had no effect on the outcome of the intervention.

Altogether ten children had developed T1D in the whole study group by December 2010. When analyzed according to the treatment-received principle, four children progressed to T1D in the WHF group, and two in the FINDIA group, and none in the CMF group.

In the follow-up group, four children were randomized to the WHF group and three children to the FINDIA group. Three affected children were withdrawn from the study soon after birth without any follow-up data, and one child in the FINDIA group did not receive study formula during the intervention period. During the six-year-follow-up period, 13 children (7.0%) developed either T1D or beta-cell autoimmunity in the WHF group, as well as seven children (3.1%) in the FINDIA group, and 18 (7.6%) in the CMF group. Twelve children (4.7%) who did not receive any study formulas developed either T1D or beta-cell autoimmunity. Because of the small number of children developing T1D during this short follow up time, the results are not statistically significant.

5.5 The induction of bovine insulin–binding antibodies in the FINDIA study (III)

Normally children exposed to dietary antigens, such as bovine insulin, develop oral tolerance to these antigens. Therefore, the dietary antigens, such as bovine insulin, cannot be considered diabetogenic as such.

Dysfunction of oral tolerance against cow's milk proteins in general, and towards bovine insulin in particular, in children who later develop beta-cell autoimmunity or T1D has been reported [258, 365]. In human T1D, the dysregulation of the gut immune system might result in altered immune responses to dietary insulin.

To investigate the immune reactions towards bovine insulin, we measured the antibody levels towards bovine insulin with an enzyme-linked solid-phase immunoassay method (EIA). The antibody levels binding to bovine insulin were higher in the CMF group than in the other groups at both three months, and six months of age. When we compared the antibody levels between other formula groups at three months with the CMF (n = 108), the ratio in the WHF (n = 70) was 0.62 (95% CI, 0.48-0.81; $P < 0.001$), in the FINDIA group (n = 95), 0.79 (0.62-1.00; $P = 0.05$), and in the group not exposed to study formula (n = 190), 0.56 (0.45-0.68; $P < 0.001$). At 6 months, the corresponding ratios in comparison with the CMF group (n = 67) were in the WHF group (n = 61), 0.43 (95% CI, 0.30-0.62; $P <$ 0 .001), in the FINDIA group (n = 63), 0.70 (0.51-0.97; $P = 0.03$) and in the group not exposed to study formula (n = 70) 0.51 (0.36-0.72; $P < 0.001$).

We could not observe any reduction in signs of beta-cell autoimmunity among children who received the WHF, which was in line with the outcome of the TRIGR study which concluded that weaning to a hydrolyzed casein formula did not reduce the cumulative incidence of T1D after a median follow-up of children for 11.5 years [266]. This outcome is consistent with the report of that trial that showed no difference between the study groups in the appearance of islet autoantibodies [267] but is not consistent with data from the pilot study [268]. Both FINDIA and WHF are whey-based formulas, but the reduction of beta-cell autoimmunity was demonstrated only with the FINDIA formula, which questions the role of caseins in the development of T1D [260]. The peptide composition, insulin, and other derived whey proteins, in extensively hydrolyzed formulas, may differ between formulas resulting from differences in the hydrolysis process. Several groups have shown that the composition of the peptides in casein-based formulas affect the

development of intestinal microbiota and permeability, which are regulators of autoimmune diabetes [366, 367].

In NOD mice studies, the role of altered peptides in the activation of autoantigen-specific T-cell reactivity and loss of tolerance to primary autoantigens has been implicated [368-370]. Eisenbarth's group showed that native proinsulin/insulin molecules have a sequence (B chain amino acids 9-23 (insulin B:9-23)), that is a primary target of the autoimmunity that causes diabetes, by creating a knockout NOD (B16:A-dKO) mouse model with a mutated proinsulin transgene (Tyrosine to Alanine (Y16A) on the B chain). This mutation abrogated the T-cell stimulation of the major insulin autoreactive NOD T-cell clones. Female mice with only the altered insulin gene did not develop insulin autoantibodies, insulitis, or autoimmune diabetes, in contrast with mice containing at least one copy of the native insulin gene [370, 371]. Also, transplantation of NOD islets, but not bone marrow, expressing native insulin sequences B:9-23 into these mice rapidly restored development of insulin autoantibodies and insulitis, despite the recipients' pancreatic islets lacking native insulin sequences. Splenocytes from mice immunized with native insulin peptide-induced rapid diabetes upon transfer only in recipients expressing the native insulin B:9-23 peptide in their pancreata [369]. In a BALB/c mouse model, Polyinosinic-polycytidylic acid (PolyIC), a "mimic" of double-stranded viral RNA, was used to immunization of the mice with insulin B:9-23 peptide resulting in the rapid induction of insulin autoantibodies. Simultaneous administration of PolyIC and B:9-23 peptide to BALB/c mice (but with neither alone) induced insulitis. CD4 T lymphocytes predominated within islets, and the mice did not progress to hyperglycemia [368]. These studies demonstrate that "normal" mice have autoreactive T lymphocytes able to rapidly target islets and insulin given appropriate MHC alleles and that a peripherally administered insulin peptide can enhance specific anti-islet autoimmunity. These phenomena are yet to be shown in diabetes in humans.

6 CONCLUSIONS

I. In the first publication, a two-step maturation process was observed in the circulating Treg cell population in the first two years of life. First, during the first year, we detected a high proportion of circulating Treg cells, followed by a decline in their percentage, and a simultaneous increase in the highly activated Treg cell ($Treg^{FOXP3high}$) subpopulation, characterized by high FOXP3, TGF-beta1, and Helios expression, during the second year of life. This maturation process coincides with the development of the microbiota from a *Bifidobacteria* dominated to an adult-like microbiota dominated by butyrate-producing species. This switch in the microbiota happened earlier in Estonian children compared to Finnish children. Also, the ratio of *B. longum* vs. *B. breve* at three months of age, which was associated with the numbers of $Treg^{FOXP3high}$ cells, was higher in Estonian children. In addition, Finnish children showed a higher abundance of *Bacteroides* than Estonian children at 12 and 18 months of age. Atopic sensitization later in life is associated inversely with the abundance of Bifidobacteria at three months of age. Furthermore, the relative abundance of *B. longum* alone at three months of age shows an inverse association with atopic sensitization, number of allergen-specific IgEs and development of allergy suggesting, that richness of *B. longum* among Bifidobacteria is an important factor protecting from atopic sensitization. These results suggest earlier maturation of the Treg population with cells having an enhanced suppression capacity in Estonian children with a lower risk of allergic diseases.

II. The second publication showed that enterovirus infected infants had a decreased FOXP3 expression in Treg cells, particularly in highly activated $Treg^{FOXP3high}$ cells, and this effect lasted up to 60 days after the infection. We could also show a cytokine profile with increased activation of Th1 and Th17 response and decreased activation of CCL22, which is a DC-derived chemokine associated with Th2 deviation. These findings provide evidence of the potential of immunopathology induced by enterovirus infections (or lack of immunotolerance). This immunopathology might be a sign of an early event in the breaking of tolerance in these infants who all carried T1D-associated HLA risk genotypes.

III. The third publication showed that weaning to the FINDIA-formula, a whey-based formula virtually free of bovine insulin, reduced the incidence of at least one autoantibody, and for repeated autoantibody positivity or positivity for multiple autoantibodies compared with the use of CMF by the age of 3 years. This result may reflect a dysfunctional immune system, where bovine insulin peptides from the infant formula may activate autoreactivity against the primary autoantigen, i.e., human insulin and result in nontolerogenic T cells. In theory, these T cells could potentially activate autoreactivity against human insulin expressed in the islets of Langerhans.

The study shows that environmental factors, such as the development, the timing of the development, and the diversity of the gut microbiota, or the viruses encountered can influence the development of regulatory T cells and immunotolerance. Also, the nutrition, i.e., breast milk or type of infant formula in early infancy, can influence the development of the immune system, or autoantibodies to T1D in infants genetically at risk. The new-born infant encounters all of these environmental factors simultaneously when the immune system is maturing. Most likely, the interplay of all of these factors, or some of them, might be needed for the induction of autoimmunity in T1D or atopic sensitization.

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