Expression of regulatory T cell –associated genes in white blood cells

Master's Thesis

Institute of Biomedical Technology

University of Tampere

March 2011

Laura Kummola

ACKNOWLEDGEMENTS

This research project was carried out in the Virology Group in the University of Tampere. First, I would like to thank my supervisor, professor Heikki Hyöty for his guidance and advice during the project. I would also like to thank my supervisor M.Sc Hanna Honkanen and MD Maria Lönnrot for their help. Without them, this work would not have been possible. I warmly thank the personnel of the Virology laboratory for their assistance and advice with the laboratory work. I am grateful for M.Sc Kirsikka Kaipainen for helping me with the statistical work. I would also like to thank my family and friends for their support during this study.

Tampere, 2011

Laura Kummola

PRO GRADU –TUTKIELMA

Paikka:	TAMPEREEN YLIOPISTO Biolääketieteellisen teknologian tieteenalayksikkö
Tekijä:	KUMMOLA, LAURA MIRJAMI
Otsikko:	Regulatorisiin T-soluihin liittyvien geenien ilmentyminen veren valkosoluissa
Sivumäärä:	96 s.
Ohjaajat:	Professori Heikki Hyöty (LT), Hanna Honkanen (FM) ja Maria Lönnrot (LT)
Tarkastajat:	Professorit Markku Kulomaa ja Heikki Hyöty (LT)

TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Viime vuosikymmeninä allergia sekä erilaiset autoimmuunisairaudet ovat yleistyneet länsimaissa ja länsimaistuneissa maissa. Näissä sairauksissa immuunijärjestelmän toiminta on muuttunut epänormaaliksi. Yhdeksi syyksi arvellaan parantunutta hygieniaa ja siitä seurannutta infektioiden vähenemistä. Immuunijärjestelmän häiriöt ilmenevät mm. lymfosyyttien tiettyjen geenien ilmentymisen muutoksina sekä säätelijä-T-solujen toiminnan ja määrän poikkeamina. Tämän tutkimuksen tavoitteena oli optimoida reaaliaikainen PCR-menetelmä immunosuppressiivisten sytokiinien, IL-10:n ja TGF-β:n havaitsemiseksi ihmisen mononukleaarisista soluista. Lisäksi PCR-menetelmä kehitettiin keskeisen immuunijärjestelmän säätelijägeenin, Foxp3:n, ilmentymisen tutkimiseksi. Pitkän tähtäimen suunnitelmana oli luoda menetelmä, jolla voitaisiin tutkia näiden geenien ilmentymistä immunologisissa häiriöissä sekä infektioissa. Tavoitteena oli myös testata tätä menetelmää DIPP-tutkimukseen osallistuneiden lasten näytteillä sekä *in vitro* –valkosolustimulaatiokokeella.

Tutkimusmenetelmät: Tutkimuksessa käytettiin terveiltä vapaaehtoisilta sekä DIPP-tutkimukseen osallistuneilta lapsilta saatuja mononukleaaristen leukosyyttien näytteitä. Valkosolustimulaatiokokeessa vapaaehtoisilta saatuja soluja stimuloitiin tunnetuilla immuunijärjestelmää stimuloivilla aineilla. Soluista eristettiin RNA, joka RT-reaktiossa käännettiin cDNA:ksi. Saatua cDNA:ta käytettiin PCR-ajossa templaattina, jotta saatiin määritettyä relatiivisella kvantifikaatiolla tutkittavien geenien ilmentymisen määrä ko. näytteissä.

Tutkimustulokset: Reaaliaikainen PCR-menetelmä optimoitiin onnistuneesti immunosuppressiivisille sytokiineille sekä Foxp3:lle. IL-10:n ilmentyminen oli hieman voimistunut lapsilla, joilla oli ollut useita enterovirusinfektioita. Valkosolustimulaatiokokeessa useat stimulantit aikaansaivat lisääntynyttä IL-10:n sekä Foxp3:n ilmentämistä. Vastaavaa ei havaittu TGF-β:n kohdalla.

Johtopäätökset: Tutkimuksessa kehitettiin reaaliaikainen PCR-menetelmä, jota voi käyttää luotettavasti IL-10:n, TGF-β:n sekä Foxp3:n ilmentymisen tutkimiseen veren mononukleaarisissa soluissa sekä *in vitro* –valkosolustimulaatiokokeissa. Menetelmää voidaan käyttää näiden geenien ilmentymisen määrittämiseen erilaisten immunologisten häiriöiden yhteydessä.

MASTER'S THESIS

Place:	UNIVERSITY OF TAMPERE Institute of Biomedical Technology (IBT)
Author:	KUMMOLA, LAURA MIRJAMI
Title:	Expression of regulatory T cell –associated genes in white blood cells
Pages:	96 pp.
Supervisors:	Professor Heikki Hyöty (MD), Hanna Honkanen (M.Sc) and Maria Lönnrot (MD)
Reviewers:	Professors Markku Kulomaa and Heikki Hyöty (MD)

ABSTRACT

Background and Aims: In the past few decades, the incidence of many allergic and autoimmune diseases has increased rapidly. These diseases are characterized by the immune system's inability to function normally. Improved hygiene and related decrease in infectious burden have been proposed as one of the possible explanations (hygiene hypothesis). Immunological disorders are often accompanied by changes in the expression of certain genes, as well as changes in the functions and amounts of regulatory T cells. The aim of this study was to optimize a real-time RT-PCR protocol in order to determine the expression of immunosuppressive cytokines, IL-10 and TGF-β in human peripheral blood mononuclear cells. Additionally, an RT-PCR protocol was developed for the analyses of the expression of Foxp3, the master regulator of the immune system. The final goal of the study was to develop a protocol that could be used for analyses of changes in the expression of these genes in immune-mediated and infectious diseases. The method was tested using the samples of children participating in the DIPP study and in an *in vitro* –leukocyte stimulation experiment.

Methods: Peripheral blood mononuclear cell (PBMC) samples were obtained from healthy volunteers and children participating in the DIPP study. In the *in vitro* –PBMC stimulation experiment, cells obtained from healthy volunteers were stimulated by different stimulants of the immune system. Total RNA was isolated from the cells and reverse transcribed into cDNA. This cDNA was used in a real-time PCR reaction as a template, and the expression levels of IL-10, TGF-β and Foxp3 were determined by relative quantification.

Results: The real-time PCR protocol was successfully optimized for these cytokines and for Foxp3. IL-10 was upregulated in children who had experienced several enterovirus infections. In the *in vitro* –PBMC stimulation experiment several stimulants increased the expression of IL-10 and Foxp3 while they did not change the expression of TGF-β.

Conclusions: In this study a new real-time PCR assay was developed for the detection of IL-10, TGF-β and Foxp3 expression in PBMC samples. The assay can be used to determine possible changes in the expression of these genes in different immunological disorders.

TABLE OF CONTENTS

ABBREVIATIONS

AHR	Airway hypersensitivity reaction
ALK	Activin receptor like kinase
AMP	Adenosine monophosphate
APC	Antigen presenting cell
CD(-4+ etc.)	Cluster of differentiation (4+ etc.)
CIA	Collagen-induced arthritis
cki	Cyclin-dependent kinase inhibitor
Ct	Threshold cycle
CTL	Cytotoxic lymphocyte
DC	Dendritic cell
DIPP	Diabetes Prediction and Prevention project
Foxp3	Forkhead box protein P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICOS	Inducible costimulator
IDO	Indoleamine 2,3-dioxygenase
IFN(-γ etc.)	Interferon (γ etc.)
Ig(G etc.)	Immunoglobulin (G etc.)
IL(-10 etc.)	Interleukin (10 etc.)
IPEX	Immune dysfunction, polyendocrinopathy, enteropathy, X-linked
IPV	Inactivated polio vaccine
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Induced regulatory T cell
Lag	Lymphocyte activation gene
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK cell	Natural killer cell
NOD mice	Non-obese diabetic mice
nTreg	Natural regulatory T cell
OPV	Oral polio vaccine
PBMC	Peripheral blood mononuclear cells
ROR	Retinoic acid related orphan receptor
SLE	System lupus erythematosus
STAT(4 etc.)	Signaling transducer and activation of transcription (4 etc.)
T1D	Type 1 diabetes
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th(1 etc.)	T helper (1 etc.) cell
TLR(2 etc.)	Toll-like receptor (2 etc.)
T_m	Melting temperature
Tr1 cell	Type 1 regulatory T cell
TRAIL-DR	Tumor-necrosis factor-related apoptosis inducing ligand–death receptor
Treg	Regulatory T cell

1. INTRODUCTION

In the past few decades, the incidence of atopic and autoimmune diseases has increased in western countries and more recently, in developing countries. Genetic and environmental factors, such as the decreasing infectious burden, have been connected with this ongoing epidemiological change (the hygiene hypothesis). For example, the incidence of atopic dermatitis has doubled or tripled in industrialized countries during the last 30 years, affecting 15-30% of children and 2-10% of adults. (Okada et al., 2010) In Finland, the prevalence of type 1 diabetes is especially high, with an increasing number of children of 0-4 years of age affected. (Harjutsalo et al., 2008) This makes Finland a good area to study these kinds of immunological diseases. The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) project screens newborns for HLA-genes which confer susceptibility for type 1 diabetes, and recruits those with increased genetic risk to longitudinal follow-up starting from birth. (Kupila et al., 2001) This large, population-based cohort provided a unique opportunity to study the molecular mechanisms of ontogenesis of immune system and its deviations in immune-mediated diseases.

In autoimmune and allergic diseases the normal function of the immune system is compromised. In physiological conditions both inflammatory and anti-inflammatory responses need to act in a balanced, fine-tuned manner. Atopic and autoimmune diseases are characterized by abnormalities in helper T cell and regulatory T cell functions. (Ozdemir et al., 2009) Regulatory T cells act by suppressing inappropriate and exacerbated responses against self and foreign antigens. The immunosuppressive cytokines IL-10 and TGF-β are signature cytokines for regulatory T cells and in many cases, important for their differentiation and suppressive action. Foxp3 is a transcription factor expressed by most regulatory T cells, bearing the title of “master regulator” for regulatory T cells. It has been shown, that in many immune-mediated diseases the expression of these genes is altered, along with the functional decline of regulatory T cells. (Workman et al., 2009) Along this, the distortion of the balance between T helper 1 (cell mediated immunity) and T helper 2 (humoral immunity) is contributing to failures of the immune regulation. (Ozdemir et al., 2009)

The hygiene hypothesis suggests that the decreasing incidence of infections is the main reason for increasing atopy (Strachan, 1989) and recently this hypothesis has been proposed to explain also the paralleling increase in autoimmune diseases. Underlying mechanisms are multiple and complex. Decreased immunoregulation and homeostatic factors, including various regulatory T cell subsets and Toll-like receptor stimulation, are probably involved. (Okada et al., 2010)

In this study, real time PCR is used to quantitatively analyze the expression of IL-10, TGF-β and Foxp3 in white blood cell samples in varying conditions. Special focus is placed on the development and optimization of the method in the results section.

2. REVIEW OF THE LITERATURE

2.1 The human immune system and T cells

The immune system is a complex and interactive network that protects the host from invading pathogens while maintaining a tolerance to self and avoiding exacerbated responses to foreign antigens. The innate and adaptive branches of the immune system employ numerous types of cells, such as lymphocytes, phagocytes and other cells that work in cooperation.

T lymphocytes comprise the cell-mediated arm of adaptive immunity. Mature T cells can be divided into two main classes according to the surface proteins they express. Cytotoxic CD8+ cells express glycoprotein CD8+ and CD4+ cells express CD4 on their surface. CD8+ cells mainly function by destroying cells infected by viruses and other intracellular pathogens. (Male et al., 2006) CD4+ T cells have multiple functions. They enable antibody production by B cells, help CD8+ T cells in maintaining and enhancing responses, regulate macrophage function and the magnitude and persistence of immune responses during invasions of pathogens, and suppress or control autoimmunity. (Zhu et al., 2010).

This literature review focuses on CD4+ T cells.

2.1.1. CD4+ T cells: development and function

CD4+ T cells can be divided further into lineages, all with distinct biological functions. The lineages include T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and regulatory T (Treg) cells. In addition to these, new possible lineages have been identified recently, including T helper 9 (Th9) and follicular helper T cells. (Wan & Flavell 2009)

T cells are a target for a highly selective, multi-checkpoint process which ensures that the cells recognize self from non-self and the attacks of the immune system will be

directed only against potentially harmful invaders. This selection starts in the thymus, where antigen presenting cells (APCs) present self-peptides to immature T cells with their major histocompatibility complex (MHC) receptors. T cells recognize the peptides with their T cell receptors (TCRs). Depending on the affinity of the MHC-self-peptide complex and the TCR, the T cell receives a signal of varying intensity. This signal, together with signals from different co-stimulatory molecules, can result either in deletion or maturation and differentiation of the corresponding T cell. (Workman et al., 2009)

In T cell development and function, cytokines are important signal molecules that affect immune responses. All cytokines are proteins or glycoproteins, and they are categorized in several groups, such as interleukins (IL), interferons (IFN) and chemokines. Cytokines are produced by lymphocytes, phagocytes and other cells of the body, and they cause cells to divide, they mediate inflammation and cytotoxic reactions and direct the movement of leukocytes around the body. (Male et al., 2006) During the TCR-mediated activation process of T cells, cytokine milieu is the predominant factor in determining, what lineage a T cell will commit into.

Master transcription factors (such as GATA3, T-bet and Foxp3) and signaling transducer and activation of transcription (STAT) proteins are an imperative for Th cell lineage determination and cytokine production. The expression levels of the master transcription factors are the main determinants of their activities, whereas the actions of the STATs are regulated by cytokine-mediated posttranslational modification, such as tyrosine and/or serine/threonine phosphorylation. The STAT proteins regulate cytokine production of Th cells and are also involved in inducing the master transcription factors. In addition to these, transcription factors that are constitutively expressed or induced by TCR and/or cytokine-mediated signaling, also contribute to the fine-tuning of Th cell functions. As a whole, these molecular interactions form a complex network, essential for Th cell differentiation, expansion and function. (Zhu et al., 2010)

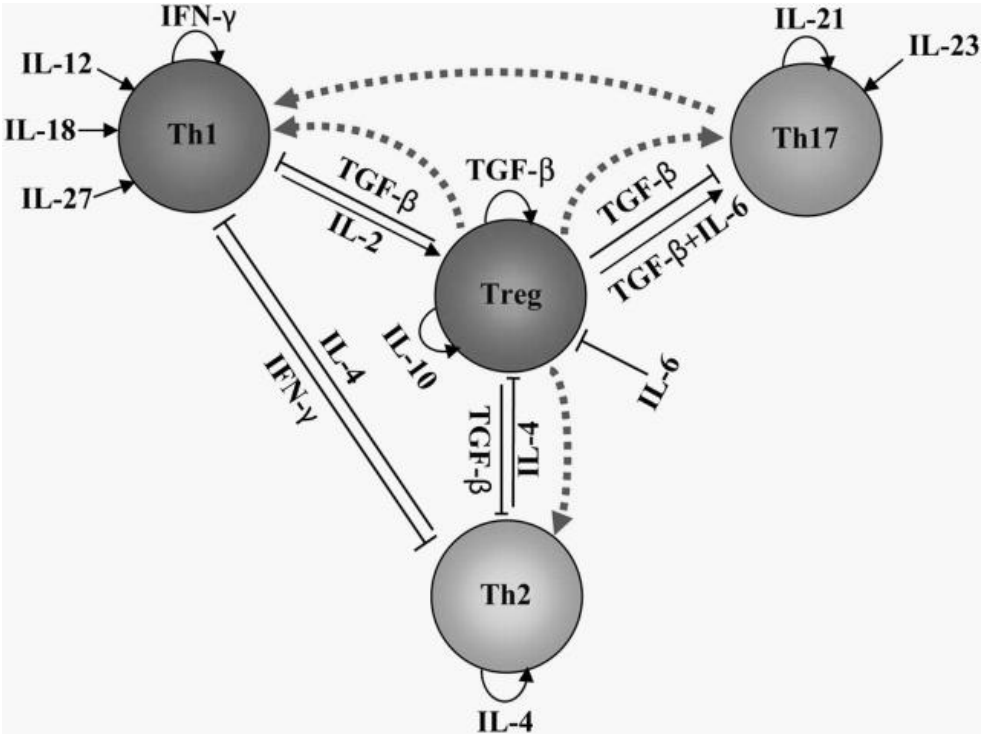

Figure 1. Summary of T cell differentiation in humans. Modified from Wan & Flavell, 2009.

2.1.1.1. Th1, Th2 and Th17 cells

The initial understanding of different CD4+ T cell populations came in 1986, when it was discovered that Th1 and Th1 cell types differ from each other by their cytokine production and surface protein expression. (Mosmann et al., 1986, Coffman & Carty 1986) Th1 cells are characterized by their IFN-γ and lymphotoxin production. They also tend to be good IL-2 producers, and many Th1 cells make tumor necrosis factor alpha (TNF-α) as well. The signature cytokines of Th2 cells are IL-4, IL-5 and IL-13. TNF-α is also produced by them, and some make IL-9. (Zhu et al., 2010)

For Th2 cells, their signature cytokine IL-4 (along with IL-2) is a crucial determinant of differentiation (Le Gros et al., 1990). It also appears, that the same is true for Th1 cells: IFN-γ seems to be important for Th1 differentiation (Lighvani et al., 2001), as is IL-12 (Hsieh et al., 1993). IFN-γ is produced by NK, CD8 and CD4 Th1 cells to skew T cell differentiation and fight invading pathogens. Naïve CD4 T cells and B cells were also shown to produce IFN-γ. It remains controversial, whether macrophages and DCs make IFN-γ. (Wan & Flavell 2009)

A third major population of CD4+ T cells was shown to exist in 2003 (Cua et al., 2003, Aggarwal et al., 2003). These cells were designated Th17 cells and their lineage was shown to be distinct from that of other Th cells (Harrington et al., 2005). The production of IL-17A, IL-17F and IL-22 characterizes these cells and they are also good producers of IL-21, although this also applies to other Th cells (Nurieva et al., 2007).

2.1.1.1.1. Th1 cell differentiation and function

IL-12 is one of the most important cytokines in Th1 differentiation during the initial encounter of a naïve T cell with an antigen. Its receptor is composed of two subunits, designated IL-12Rβ1 and IL-12Rβ2. Of the expression of these subunits, β2 is restricted to Th1 cells and the regulation of its expression is likely to be a central mechanism by which IL-12 responsiveness is controlled. IL-12 is mainly produced by antigen presenting cells as a response for pathogen invasions. In differentiated Th1 cells, IL-12 serves as a costimulus needed for IFN-γ production when the T cells encounter their specific antigens. When bound to its receptor, IL-12 activates Jak2/Tyk2-STAT1, 3 and 4 signaling pathways. (Gately et al., 1998)

A novel cytokine, IL-27, acts in the same manner as IL-12. It induces differentiation from naïve CD4+ T cells and synergizes with IL-12 to induce IFN-γ production. (Pflanz et al., 2002) IL-27 also activates STAT1 and promotes the expression of IL-12Rβ2 (Takeda et al., 2003).

T-bet is the master transcription factor specific for Th1 cells, also affecting IFN-γ production (Szabo et al., 2000). Its expression is controlled by both TCR and IFN-γR-STAT1 signals. IFN-γR-STAT1 signaling maintains high T-bet expression in developing Th1 cells. (Lighvani et al., 2001) T-bet upregulates IL-12Rβ2 expression and remodels the *Ifng* gene, which leads to IFN-γ expression and selective Th1 cell expansion in response to IL-12. (Mullen et al., 2001) T-bet synergizes with STAT4 in order to induce many Th1 specific genes, including IFN-γ and IL-12Rβ2 (Thieu et al., 2008). Both STAT4 and T-bet bind to the IFN-γ promoter and the optimal binding of one factor requires the presence of the other. It appears, that both are required for the

chromatin remodeling of the IFN-γ locus. (Zhu et al., 2010) It is likely, that IFN-γ regulates the expression of STAT4 positively and IL-4 and GATA3 regulate it negatively (Frucht et al., 2000). STAT4 is activated mainly by IL-12, and its expression is higher in Th1 cells than Th2 cells (Usui et al., 2003).

Th1 function is characterized by the production of pro-inflammatory cytokines IFN-γ, TNF-α and TNF-β to stimulate innate and T cell immune responses. They promote IgG antibody production in B cells. Cell mediated forms of immunity, manifesting mainly in cytolytic activity, tend to be the consequence of the Th1 response. Protection of the body from obligate intacellular parasites is the major function of Th1 cells. Th1 cells are also involved in tumor immune rejection. (Wan & Flavell 2009)

2.1.1.1.2. Th2 cell differentiation and function

IL-4 is essential for Th2 differentiation. It is possibly the most dominant cytokine known to have the greatest influence in Th2 lineage selection. (Le Gros et al., 1990) It is secreted by naïve T cells, mast cells, basophils, eosinophils, natural killer T cells, and previously differentiated Th2 cells (Ansel et al., 2006). IL-4 binds to its receptor IL-4Rα and triggers STAT6 activation and Th2 differentiation (Kaplan et al., 1996). One of the mechanisms by which STAT6 promotes the differentiation, is by inducing high transcription levels of the Th2 master transcription factor, GATA3 (Kurata et al., 1999). However, Th2 GATA3 dependent responses can occur also independently of STAT6, and STAT6 is likely to play a role in enhancing primary IL-4 responses (Finkelman et al., 2000). Strong STAT5 signaling is also required for Th2 differentiation. It binds to the DNase I hypersensitive sites II and III in the *Il4* locus in Th2 but not Th1 cells. (Zhu et al., 2003)

GATA3 binds to the IL-4 locus that encompasses the IL-4, IL-5 and IL-13 genes and regulates the Th2 cytokine production (Zheng & Flavell 1997). Its expression is downregulated in Th1 cells and upregulated in Th2 cells, although naïve CD4+ cells express it at detectable levels (Ouyang et al., 1998). It binds to the promoters of IL-5 and IL-13, but only to the IL-4 enhancer (Zhu et al., 2004).

Th2 cells promote IgG1 and IgE class-switching and eosinophil recruitment. They are involved in humoral responses, characterized by pathogen-specific antibody production in order to neutralize invading organisms. The Th2 response is especially important when defending the body against extracellular pathogens, such as nematodes and helminths. Th2 cells also contribute to the mucosal immunity in the lung. It is thought that Th2 immunity evolved in vertebrates after Th1 immunity, because the Th2 response is used to combat pathogens of more recent origins. (Wan & Flavell 2009)

2.1.1.1.3. Th17 cell differentiation and function

IL-6 acts cooperatively and non-redundantly with TGF-β to promote Th17 differentiation (Bettelli et al., 2006). IL-21 can substitute many of the function of IL-6 to induce Th17 cells with TGF-β (Nurieva et al., 2007). In the survival and proliferation of these cells, IL-23 is thought to play a role. IL-23 binds to the IL-23R complex composed of IL-12Rβ1 chain and a novel receptor chain (IL-23R) related to IL-12Rβ2. Jak2/Tyk2-STAT1, 3, 4, and 5 are involved in its intracellular signal transduction. (Parham et al., 2002) IL-6, IL-21 and IL-23 mediate their functions through the activation of STAT3. STAT3 is responsible for the induction of RORγt and the IL-23R. (Zhou et al., 2007) IL-6, -21, or -23, through the activation of STAT3, together with IL-1, an NF-κB activator, induce TCR-independent, cyclosporine A–independent IL-17A production (Guo et al., 2009).

Retinoic acid related orphan receptor ROR-γt is the master regulator of Th17 cells. It is likely to act synergistically with RORα, which is induced by IL-6 and TGF-β, in IL-17 production and Th17 differentiation. (Yang et al., 2008). ROR-γt mRNA is upregulated in response to IL-23 and it highly correlates with IL-17 expression. (Ivanov et al., 2006) The target genes directly regulated by ROR-γt have not yet been identified (Wan & Flavell 2009).

The function of the Th17 cells is to induce a massive inflammatory response in order to destroy pathogens that are not adequately dealt by Th1 or Th2 immunity. Different pathogens including bacteria, fungi and yeast are all successful in eliciting a Th17

response. Th17 cells communicate with a variety of immune and non-immune cells via the cytokines they produce. They can induce the production of proinflammatory cytokines and chemokines, which attract neutrophils to the site of inflammation, and antimicrobial peptides. (Korn et al., 2009)

2.1.2. Regulation of the immune system

When the immune system defends the host against pathogens, it constantly receives stimuli from external sources. It also receives stimuli from itself, from internal sources. Strong reactions against the body's own tissues or harmless antigens found, for example, in food, would not be beneficial to the host. Also, when an infection has been cleared, the immune system must know when to suppress the actions of the innate and adaptive cells. Otherwise unwanted tissue damage might occur. It is of utmost importance that the system can recognize self and non-self, and maintain a tolerance to its own cells and tissues, as well as to innocuous antigens. (Tang & Bluestone 2008) In the strictest sense, tolerance can be defined as complete absence of any antigen-specific immunity. This absence can be achieved by antigen inaccessibility and T cell deletion or non-responsiveness due to lack of sufficient activation signals. However, considering the whole of the immune system and the multiple functions of T cells, it is better to define tolerance as the absence of pathogenic autoimmunity. (von Herrath & Harrison 2003) Maintaining the fine balance between suppression and promotion of inflammatory responses requires immune regulation, orchestrated by complex interactions of cells and signaling.

2.1.2.1. Regulatory T cells and their development

The key mechanism by which the immune system suppresses inappropriate or excessive responses and maintains tolerance is the existence of regulatory T cells (Tregs). The concept of suppressor cells is several decades old, but it was not until about fifteen years ago, when cells with unique regulatory properties were described (Sakaguchi et al., 1995). Tregs are CD4+ T cells and there are different subclasses of them defined by their origin and cytokine profile. Natural Tregs (nTregs) develop in the thymus, and

induced Tregs (iTregs) develop in the periphery from conventional CD4+ cells as a consequence of antigenic stimulation during varying conditions. Both subsets of Tregs balance between suppressing potential autoimmunity and controlling responses to infections. (Workman et al., 2009) Other regulatory cell types include $CD8^+$ T cells, γδT cells, $CD4^-CD8^-$ T cells, IL-10-producing B cells, IL-10-producing natural killer cells, IL-10-producing dendritic cells and some macrophage subsets (Palomares et al., 2010).

2.1.2.1.1. nTregs

As all T cells, nTregs originate from progenitor cells in the bone marrow, after which they undergo their differentiation and maturation in the thymus. Of peripheral CD4+ T cells, nTregs comprise only a small portion: about 5-10%. (Sakaguchi 2004) There is no cell surface marker that has uniquely been connected with Tregs, but there are a number of cell surface proteins that are preferentially expressed on nTregs (Workman et al., 2009). The first one to be identified was the IL-2 receptor α chain, CD25 (Sakaguchi et al., 1995). Purification of human nTregs is more efficient, if cells expressing CD127 are excluded. It has been shown, that CD127 expression is inversely correlated with Foxp3 expression and suppressive capacity of nTregs. Forkhead box protein P3 (Foxp3) cannot be used as a Treg marker since it is expressed in the nucleus. (Liu et al., 2006) Foxp3 expression is limited mainly to thymus-derived nTregs, although certain suppressive peripheral iTreg populations also express it.

It has not been entirely discovered, which mechanisms influence nTreg development *in vivo*. It is known, that contributing factors include TCR and antigen affinity, the location and context in which the antigen is encountered in the thymus, and co-stimulatory molecules and cytokines. Tregs undergo selection in the thymus in the same manner, as conventional T cells: by the peptides presented to them by APCs. (Workman et al., 2009) For Tregs, co-stimulatory molecules such as CD28, CD80/86 (B7) (Keir & Sharpe 2005) and IL2Rβ (Malek et al., 2002) seem to be of special importance. In murine models, IL-2 has proven to be crucial for Treg development and survival. nTregs are dependent on paracrine IL-2, as they do not produce it themselves due to chromatin inaccessibility of the IL-2 locus. (Su et al., 2004) In T cells, IL-2 activates the

JAK3-STAT5 pathway (Lin & Leonard 1997). CD28 interacts with CD80/86. Depletion of CD28 expression leads to reduction in IL-2 expression, which in turn leads to insufficient IL-2R signaling and reduction in Foxp3 expression. In Treg precursors, CD28 costimulation is needed, as even in the presence of exogenous IL-2, Foxp3 expressing Treg number is reduced in $CD28^{-/-}$ hematopoietic stem cells. (Tai et al., 2005) For peripheral nTregs, TGF-β signaling is required. It maintains Foxp3 expression and suppressor function. It is not known, if a similar role exists in the thymus. (Marie et al., 2005)

When T cells are differentiating in the thymus, the strength of the signal received through the interaction between the TCR and MHC-peptide complexes determines the outcome: non-existent or very low signal results in cell death by neglect, while strong signals, due to strong interactions, lead to negative selection. Positive selection takes place, when the signal is intermediate or weak. (Workman ym. 2009) There is a suggestion, that in Treg differentiation, the signal leading to positive selection is between those required for the positive and negative selection for other T cells (Kronenberg & Rudensky 2005). However, there is no consensus over this matter. An alternative theory suggests that nTregs have a lower activation threshold than conventional T cells. (Workman et al., 2009) It has been shown, that human Tregs are responsive to TCR stimulation at 10- to 100-fold lower antigen concentrations than conventional T cells (Takahashi et al., 2006). It might be, that Treg differentiation is driven by the TCR affinity for self, coupled with the sensitivity to antigen stimulation which mediates a functional outcome (Workman et al., 2009). It has also been proposed, that Treg lineage commitment occurs prior to TCR-mediated selection and that even weak TCR-self-peptide interactions might allow cell survival in the thymus (Pennington et al., 2006). It is also possible, that the context within the thymus where antigen is encountered may have an impact on thymocyte selection, deletion and Treg differentiation. A study quantitatively altering the thymic expression of a T cell epitope showed that the absolute number of nTregs remained independent of different degrees of epitope expression. However, the number of conventional, deleted T cells increased with increasing thymic antigen, while the number of nTregs was unchanged. This raises the suggestion, that the affinity for self-peptides is not the most important determinant in nTreg selection, but rather the context or niche in which the T cells encounter their antigen. (van Santen et al., 2004)

2.1.2.1.2. iTregs

Probably the main difference between nTregs and iTregs is their origin of development: thymus versus periphery. Regulatory T cells can be induced from conventional, naïve CD4+ cells both *in vitro* and *in vivo*. It is currently under study, what are the relative contributions of both subsets *in vivo*. (Workman et al., 2009) There is a theory, that iTregs do not differ from nTregs by their origin, but rather as a consequence of different antigen exposure and other factors that are differently expressed in distinct circumstances. (Bluestone & Abbas 2003)

There are two main subsets of iTregs, that have been described: type 1 regulatory T cells (Tr1), and T helper 3 cells (Th3). Tr1 cells are induced by IL-10 (Vieira et al., 2004), and Th3 cells by TGF-β (Weiner 2001). Both subsets express their suppressive activities through the secretion of the same cytokine responsible for their induction (Vieira et al., 2004, Weiner 2001). Although Tr1 and Th3 subsets are distinct, they have similar phenotypes and they use overlapping mechanisms in suppression of immune responses (Workman et al., 2009). Foxp3 is expressed by Th3 cells following induction, but not by Tr1 cells. (Vieira et al., 2004, Weiner 2001).

An essential role for IL-4 and IL-13 has been proposed in iTreg development (Skapenko et al., 2005). Both cytokines are able to induce the development of Foxp3 expressing Tregs from $Foxp3^-$ naïve T cells independently of IL-10 or TGF-β. IL-4 and IL-13 both signal through the IL-4Rα chain, implying an important role for the receptor in peripheral Treg generation. Note that this is also the pathway of Th2-mediated regulation of Th1-responses.

Both nTregs and iTregs express the same surface markers of an activated T cell, such as CD25, CTLA-4 and GITR. (Workman et al., 2009). According to studies with mice, nTregs and iTregs seem to differ in their requirements for development. iTregs seem to develop in response to a weaker TCR stimulation and exogenous antigen exposure in the periphery. Even though it is not impossible that the iTreg repertoire may include high affinity TCRs for self-antigens, these cells are primarily generated during

inflammation in the presence of anti-inflammatory cytokines. Also, while co-stimulation of CD28 is necessary for nTregs, iTregs are able to develop in its absence. (Kretschmer et al., 2005)

In addition to the factors discussed above, there are other factors influencing iTreg differentiation. These include antigen presenting cells, cytokines, tissue specific factors and the route of antigen exposure. (Workman et al., 2009) Dendritic cells (DCs) play an especially important role and they are able to induce Treg differentiation, particularly in the gut-associated lymphoid tissue (GALT). Tregs in turn can induce dendritic cells to become tolerogenic, which in the GALT creates a positive feedback loop that in the presence of TGF-β leads to reinforcement of iTreg formation. (Min et al., 2003, Sun et al., 2007) Furthermore, Treg cells can be induced in a microenvironment of tumors and chronic infections by local DCs. This is the mechanism by which the limitation of collateral tissue damage is controlled, along with maintenance of immune tolerance to tumor antigens and microbial persistence. (Akdis & Akdis 2009)

A number of studies have shown plasticity of iTregs in the periphery. It is possible to convert the lineage commitment of naïve CD4+ cells from iTreg (in the presence of TGF-β) to Th17 by adding IL-6 and IL-21. (Bettelli et al., 2006) Even activated Tregs can convert into Th17 in the presence of IL-6 (Xu ym. 2007). In contrast, retinoic acid causes the induction of Tregs and inhibits Th17 differentiation, probably by enhancing TGFβ signaling and inhibiting IL-6 signaling (Mucida et al., 2007).

2.1.2.2. The master switch of Tregs: Foxp3

As mentioned above, forkhead box protein P3 (Foxp3), which is a transcription factor of the forkhead/winged helix family, acts as a master regulator for regulatory T cells being expressed by most Tregs. (Hori et al., 2003) The members of this family are characterized by their FKH-domain, which has been shown to be sufficient and necessary for DNA binding (Kaufmann & Knochel 1996). DNA-binding analyses from a number of Fox family proteins have defined a core DNA sequence (5′-A(A/T)TRTT(G/T)R-3″, where R=pyrimidine) surrounded by less conserved sequences (Kaufmann et al., 1995). It has been shown experimentally, that Foxp3 is capable of specific binding to a FKH DNA binding site.

It appears that the Fox family members act both as transcriptional activators and repressors. It has become clear, that cytokine genes are a major target for transcriptional regulation by Foxp3. For example, IL-2 production was markedly reduced after ectopic expression of Foxp3 in Jurkat cells. (Schubert et al., 2001) Additionally, transgenic Foxp3 mice were unable to produce IL-2, IL-4, or IFN-γ following TCR-mediated stimulation *in vitro* and they could not produce cytokines efficiently *in vivo* after immunization (Khattri et al., 2001). It is known, that Foxp3 contains at least three distinct structural domains: FKH, C2H2 zinc finger and leucine zipper. The FKH domain is important for nuclear localization and DNA binding, as mentioned above. The function of the rest of the protein is not so well understood. However, studies of the other Foxp-family may be relevant to the analysis of Foxp3 function. (Ziegler 2006) In Foxp1 and Foxp2 the leucine zipper is critical for homo- and heterodimer formation. The deletion of the leucine zipper domain abrogated their ability to act as transcriptional repressors. (Li et al., 2004) It is likely, that this domain is also involved in dimerization in Foxp3. The amino terminal half of Foxp3 contains at least two functional domains, both involved in transcriptional repression (Ziegler 2006).

In humans, the mutation of Foxp3 is linked with a syndrome known as IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked). It was first characterized in 1982 and mapped to the X chromosome. Affected individuals typically develop symptoms, including diarrhea, dermatitis and autoimmune endocrinopathies early in infancy and in most cases they die in the first two years of life. (Bennett & Ochs 2001) The mutation in the Foxp3 gene leads to depletion of functional Tregs (Chen et al., 2003).

Foxp3 can be induced in iTreg cells by TGF-β following antigenic stimulation. Over-expression of Foxp3 in conventional T cells converts them to a Treg phenotype endowed with suppressive activity and anergy in mice. (Fontenot et al., 2003) In humans, the expression of Foxp3 is not sufficient for a regulatory phenotype, as a significant percentage of human activated T cells express it and yet do not mediate regulatory functions. Additionally, induction of human T cells by TGF-β does not lead to a regulatory phenotype, in contrast to murine cells. (Tran et al., 2007) Reduced Foxp3 expression has been shown to lead to abrogated suppressive function and a

conversion of Treg cells into Th2 phenotype. (Wan & Flavell 2007) Cells expressing Foxp3 have been shown to develop even in the absence of Foxp3, so it is likely, that Foxp3 is not absolutely required for the development of Tregs, but rather is critical for the establishment and stabilization of the suppressive function of these cells. (Gavin et al., 2007)

2.1.2.3. Immunoregulatory cytokines: IL-10 and TGF-beta

2.1.2.3.1 IL-10

IL-10 is a key immunosuppressive cytokine that limits inflammatory responses. It also has an important role in immune homeostasis especially in the gastrointestinal tract. (Sanjabi et al., 2009) It has multiple biologic effects on different cell types. Tregs are the primary T cell source for it, but monocytes and B cells are the major source in human subjects. There is growing evidence of association of IL-10 in human allergic disease. Constitutive expression of IL-10 by APCs in the respiratory tract of healthy subjects has a crucial role in the induction and maintenance of tolerance to allergens, whereas allergic rhinitis and asthma are connected with decreased IL-10 expression in the allergic airway (Borish et al., 1996). In mice, the lack of IL-10 leads to spontaneous enterocolitis and other symptoms resembling Crohn's disease (Kuhn et al., 1993). Deletion of the IL-10 gene results in increased Th1 responses, which in turn lead to susceptibility to toxoplasmic, fungal and bacterial infection. Asthmatic and allergic responses are also exaggerated in these mice.

The IL-10 gene locus is about 2 kb in length in humans. Comprising of five exons, the gene encodes six helices and two loops. (Pestka et al., 2004) IL-10 forms a homodimer and functions through binding into the IL-10R1 and IL-10R2 receptor complex. Binding is followed by the subsequent activation of Jak 1 and tyrosine kinase 2, STAT1 and STAT3. IL-10 inhibits the production of IFN-γ and IL-2 by Th1 cells and IL-4 and IL-5 by Th2 cells. It also inhibits the expression of MHC class II molecules and CD80 and CD86, which are important co-stimulatory molecules in dendritic cells and other APCs. This eliminates their ability to provide signals necessary for T helper cell activation. Thus, the production of Th1 and Th2 associated cytokines is inhibited. (Commins et al.,

2008) Recent studies have also indicated that IL-10 is able to function directly on T cells in order to inhibit their cytokine production by suppressing co-stimulatory molecules CD2, CD28 and inducible costimulator (ICOS), thereby regulating their threshold of activation (Taylor et al., 2007). It has also been shown, that in mice and humans, IL-10 promotes the differentiation of certain dendritic cells that induce the differentiation of Tr1 cells both *in vitro* and *in vivo* (Roncarolo et al., 2006)

Several IL-10 homologs have been found in viruses. Although the role of these homologs in viral life cycle and survival remain to be established, it is sensible to expect that the viral homologs of IL-10 play an important role in suppressing the host immune system, allowing the survival of viruses in the host and growth of virus-induced tumors. (Pestka et al., 2004)

2.1.2.3.2. TGF-β

The TGF-β-superfamily is a large group of extracellular growth factors, consisting of over 35 members in vertebrates. They include bone morphogenetic proteins, activins, and growth differentiation factors and they are concerned in many aspects of development. (Chang et al., 2002) In the immune system, TGF-β has a pivotal role in maintaining tolerance through the regulation of lymphocyte proliferation, differentiation, and survival. Additionally, TGF-β is involved in the initiation and resolution of inflammatory responses. This is achieved via the regulation of chemotaxis, activation, and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes. In mammals, there are three homologous isoforms: TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is the predominant isoform in the immune system. (Li et al., 2006)

TGF-β is synthesized as a precursor that undergoes processing in the Golgi apparatus. It subsequently forms a complex with mature TGF-β and associates with latent-TGF-β-binding protein (LTBP). This LTBP plays a pivotal role in targeting TGF-β to the extracellular matrix. TGF-β is unable to bind to its receptor in the latent form, and it needs to be activated by freeing it from the constraints of the complex. (Annes et al., 2003). *In vivo*, the mechanisms for activation are not clear, but several models have

been suggested, such as proteolytic activation by transglutaminase and mechanical traction by αvβ6 integrins on epithelial cells (Annes et al., 2004, Yehualaeshet et al., 1999).

The transcriptional regulation of TGF-β1 is not well understood. It is likely, that activator protein (AP-1), for which there is a binding site in the TGF-β1 promoter, acts as both an activator and inhibitor of TGF-β1. Cytokines have also an important role in controlling TGF-β1 expression, although in many cases, the mechanisms are uncharacterized. (Li et al., 2006) It is typical that TGF-β1 mRNA levels do not correlate with the amount of secreted protein. This suggests post-transcriptional and/or post-translational regulation. (Kim et al., 1992)

The biological functions of TGF-β are mediated through binding to type I and II transmembrane serine/threonine kinase receptors. There are several types of each receptor type. (Chang et al., 2002) TGF-β mostly functions via binding ALK5 (activin receptor like kinase) and TGF-β receptor II (TGF-βRII). ALK5 is not necessary for the initial binding, but is needed for the signaling. Activated TGF-β binds as a dimer to tetrameric ALK5 and TGF-βRII complex. This leads to subsequent phosphorylation of intracellular Smad proteins. The transcriptional regulation of target genes requires nuclear localization of Smads. (Massague 1998) There are eight different types of Smad proteins, and they work in co-operation with multiple transcriptional factors in controlling gene expression (Li et al.,. 2006) In addition, TGF-β responses seem to be modulated by cross-talk between Smads and MAP kinases (Blanchette yet al., 2001).

One of the important functions of TGF-β is its antiproliferation activity on various cell types, including T cells. It inhibits the expression of the T cell mitogenic cytokine IL-2, probably via suppression of IL-2 transcription. Other antiproliferatory mechanisms include direct targeting of cell cycle regulators, such as upregulation of cyclin-dependent kinase inhibitors (cki) and downregulation of c-myc. (Li et al., 2006) The differentiation status and integrated signals from cytokines and costimulatory molecules of T cells affects the proliferational inhibition by TGF-β. Although TGF-β inhibits naïve T cell proliferation, it has minimal effect on activated T cells, correlating with reduced TGF-βRII expression. However, IL-10 is able to enhance TGF-βRII expression and restore the responsiveness of activated T cells to TGF-β. (Cottrez & Groux 2001) CD28

costimulation weakens the inhibition of TCR-mediated naïve CD4+ T cell proliferation by TGF-β. The expression of IL-2 is strongly inhibited by TGF-β even in the presence of CD28 stimulation, but IL-2 is nevertheless required for the proliferation of TCR/CD28/ TGF-β-stimulated naïve T cells. (Sung ym. 2003) It is likely, that CD28 synergizes with low IL-2 to enable T cell proliferation, and TGF-β inhibitory effects are overridden. It is also sensible to speculate, that in the absence of costimulation, the inhibition of naïve T cells by TGF-β blunts the T cell responses to self-antigens under steady state. Reversal of inhibition by strong costimulatory signals, often connected with infection, could limit the suppressive actions of TGF-β during normal immune responses.

TGF-β has also a role in controlling T cell differentiation. By inhibiting the expression of GATA-3, it prevents the development of Th2 cells. (Gorelik et al., 2000) In addition, it has been shown that that TGF-β interferes with TCR and costimulatory receptor signaling pathways in CD4+ cells. It inhibits the activation of the Tec kinase Itk and the calcium influx elicited by TCR-CD28 stimulation. Following this, the nuclear translocation of nuclear factor of activated T cells (NFAT) is impaired along with expression of GATA-3. (Chen et al., 2003)

TGF-β blocks Th1 differentiation by reducing T-bet and IL-12 receptor β2 (IL-12Rβ2) expression. It is likely, that the inhibition of the receptor is due to inhibiting T-bet expression, as IL-12Rβ2 expression is induced by T-bet, which in turn is dependent on IFN-γ. (Afkarian et al., 2002) STAT4, which is activated by IL-12, is also inhibited by TGF-β. The downregulation of STAT4 inhibits IFN-γ expression at priming, while reduced T-bet expression blocks IFN-γ at recall responses. (Lin et al., 2005)

It is known that TGF-β1 is perhaps the most dominant factor in the generation of iTregs (Th3 cells) from naïve CD4+ cells, along with increased Foxp3 expression after TCR stimulation in mouse and human cells (Workman et al., 2009). In mice, nTreg development and peripheral maintenance is dependent on TGF-β (Marie et al., 2005).

Multiple autoimmune and atopic diseases have been connected with the altered expression of TGF-β. It seems that TGF-β plays a role in controlling these diseases, often site and context dependently. (Li et al., 2006) For example, mice lacking TGF-β1

develop symptoms resembling those of system lupus erythematosus (SLE) (Shull et al., 1992). Consistent with this, decreased levels of active TGF-β1 were found in some of SLE patients (Ohtsuka et al., 1998). Another example is collagen-induced arthritis (CIA), which is a mouse model for rheumatoid arthritis (RA). It has been shown to be inhibited by systemic administration of TGF-β1 (Thorbecke et al., 1992), while local injections to joints aggravate the symptoms (Allen et al., 1990).

2.1.2.4. Mechanisms of Treg suppression

Four basic "modes of action" can be described as mechanisms of Treg suppression: suppression by inhibitory cytokines, cytolysis, metabolic disruption, and suppression by modulation of APC maturation or function. (Vignali et al., 2008)

2.1.2.4.1. Suppression by inhibitory cytokines

Inhibitory cytokines, such as IL-10 or TGF-β are known for their general suppressive functions and their importance in iTreg induction, but their contribution to nTregs is still under debate (Shevach 2006). This is partly due to the controversy over the possible contact-dependent manner of Treg function. *In vitro* studies using T cells that are unable to produce or respond to IL-10 and TGF-β or neutralizing antibodies have suggested that these cytokines may not play an essential role for Treg function. (Vignali et al., 2008) *In vivo* studies have, however, led to different conclusions (Annacker et al., 2003, Hawrylowicz & O'Garra 2005). Indeed, controversy remains over the matter of soluble factors mediating Treg suppression. There is evidence of disease control being more or less dependent on IL-10 (and TGF-β in some cases) in asthma and allergy models, with these cytokines being produced either by Tregs themselves or other cells, but yet under Treg control. It seems that the relative importance of Treg-cell-derived IL-10 is very dependent on the experimental system and the target organism and disease. Controversial results have been shown concerning TGF-β also. (Vignali et al., 2008) Some studies have indicated that Treg cell surface bound TGF-β might have an important role for Treg function. This membrane-tethered TGF-β could mediate suppression by cell-cell contact-dependent manner. (Nakamura et al., 2001) TGF-β may act by directly participating in T cell suppression. TGF-β-resistant T cells cannot be

controlled by Tregs in an inflammatory bowel disease model (Fahlen et al., 2005a), and TGF-β produced by Tregs has been shown to be important in the control of immune responses to *M. tuberculosis* (Kursar et al., 2007) and suppression of allergic responses (Joetham et al., 2007). It appears that TGF-β is able to limit the anti-tumour activity of cytokine-induced killer cells by rendering T cells unresponsive to the tumor (Li et al., 2007).

It has been recently suggested, that a new inhibitory cytokine, IL-35, would be required for Treg maximal suppressive activity. IL-35 is formed by the pairing of Epstein–Barr virus-induced gene 3 (*Ebi3*), and p35 (also known as *Il12a*). Both of these genes are preferentially expressed in murine Foxp3-positive Treg cells, but not in active or resting effector T cells. They are strongly upregulated in actively suppressing Treg cells. *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ Treg cells showed *in vitro* reduced regulatory activity and they did not succeed in curing IBD nor controlling homeostatic proliferation *in vivo*. An important notion is that IL-35 was not only required for maximal Treg suppression, but also sufficient for it. Regulatory activity of naïve T cells was conferred by ectopic expression of IL-35, and recombinant IL-35 suppressed T cell proliferation *in vitro*. (Collison et al., 2007a)

However, much remains to be defined about IL-35, IL-10 and TGF-β, although it is now clear that they are the key mediators of Treg function. The extent to which they are utilized in different homeostatic and pathogenic settings differs, and this strongly hints a non-overlapping function. (Vignali et al., 2008)

2.1.2.4.2. Suppression by cytolysis

Although granzyme-mediated cytolysis has been considered a weapon of cytotoxic $CD8^+$ T lymphocytes (CTLs) and natural killer cells, many human CD4+ cells too exhibit cytotoxic activity. It has been shown that human nTregs express granzyme A and their target cell killing was achieved by granzyme A and perforin adhering to CD18. (Grossman et al., 2004) However, murine CD4+ cells show no cytolytic activity and therefore it was surprising to find that granzyme B was upregulated in murine Treg cells. It was also discovered, that granzyme B deficient murine Tregs had reduced

suppressive activity *in vitro*, but this cell-induced apoptosis seemed to be perforin-independent. (Gondek et al., 2005) Further studies showed that Treg cells can kill B cells in a granzyme B-dependent and partially perforin-dependent manner. The result is a suppression of B cell activity (Zhao et al., 2006). Tregs have recently also been shown to be able to prevent NK cells and CTLs from clearing tumours by killing these cells in a granzyme-B- and perforin-dependent manner (Cao et al., 2007). Some new studies have suggested that Tregs can also induce cytolysis through the TRAIL–DR5 (tumor-necrosis factor-related apoptosis inducing ligand–death receptor 5) pathway (Ren et al., 2007) and that galectins can mediate cytolysis in a granzyme- and perforin-independent manner (Toscano et al., 2007).

2.1.2.4.3. Suppression by metabolic disruption

There are a collection of suppressive mechanisms, by which Tregs affect effector T cells, and these mechanisms are called "metabolic disruption". One of these mechanisms is the debated ability of Tregs, empowered by high expression of CD25, to consume local IL-12 and thus starve actively dividing effector T cells as they need IL-12 to survive. There are research results in favor and against this theory. (Vignali et al.,. 2008)

Two other mechanisms of metabolic disruption have recently been suggested. It has been shown, that concordant expression of ectoenzymes CD39 and CD73 generates pericellular adenosine. By activating the adenosine A2A receptor, pericellular adenosine suppresses effector T cell function. In addition to this, binding of adenosine to the A2A receptor also enhances adaptive Treg generation by inhibiting IL-6 expression and promoting TGF-β secretion. (Zarek et al., 2008) Treg differentiation and Foxp3 are induced by TGF-β, and IL-6 inhibits them and promotes the development of pro-inflammatory Th17 cells. Inhibiting IL-6 is thus important in Treg maintenance. (Oukka 2007) Effector T cell function can also be directly suppressed by Tregs transferring the potent inhibitory second messenger cyclic AMP onto target cells through membrabe gap junctions (Bopp et al., 2007). However, much study is still needed in this area of Treg function.

2.1.2.4.4. Suppression by modulation of APC maturation or function

It is an attractive idea, that Tregs could be able to modulate the maturation and/or function of dendritic cells, which are needed for effector T cell activation. However, there is only little evidence to support this. (Bluestone & Tang 2005) Direct interactions between DCs and Tregs *in vivo* have been revealed by intravital microscopy. It was proposed that the contact was to attenuate T cell activation by DCs. (Tang et al., 2006) Treg cells constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA4), and it has been shown that in the absence of functional CTLA-4, the ability of Tregs to suppress effector T cells via DCs is reduced (Oderup et al., 2006). It seems that Tregs can program DCs to express indoleamine 2,3-dioxygenase (IDO). IDO is a potent regulatory molecule and it induces the catabolism of tryptophan into pro-apoptotic metabolites that in turn cause the suppression of effector T cells. This programming of DCs is accomplished by a mechanism dependent on the interactions of CTLA4 and CD80 and/or CD86. (Fallarino et al., 2003)

Tregs have been reported to have the ability to downmodulate the capacity of DCs to activate effector T cells by downregulating the expression of co-stimulatory molecules CD80 and CD86 *in vitro* (Cederbom et al., 2000). Additionally, there are studies suggesting that Tregs modulate the DC maturation and function (Kryczek et al., 2006), as well as the function of monocytes and macrophages (Tiemessen et al., 2007). The precise mechanism through which the modulation is achieved still remains to be described, but it might be mediated through cell-surface molecules such as CTLA4 and/or cytokines such IL-10 and TGF-β (Vignali et al., 2008).

There are studies suggesting that lymphocyte activation gene 3 (Lag3, also known as CD223) might be involved in blocking DC maturation. Lag3 binds MHC II molecules with high affinity and it is required for maximal Treg suppression. Immature DCs express MHC II molecules to which Lag3 binds, inducing an immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory signaling pathway. This pathway involves the recruitment of SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), which suppresses DC maturation and immunostimulatory functions. (Liang et al., 2008)

2.1.3. The role of Tregs in infection

It has become evident, that regulatory T cells participate in responses to infection as well as the clearance of the pathogen. Some infectious agents are difficult to control, and normal responses to them might result in tissue damage. It is possible, that Tregs help control the damage. However, in some situations, they may affect the outcome negatively by preventing effector T cells from adequately clearing the infection, promoting enhanced pathogen survival. The fine balance between regulatory and effector cells is once again of the essence. Factors influencing the end result include the dose of the pathogen, the stage of the infection and genotype and immunological status of the host as well as the presence of other infections or concurrent diseases.

One of the main functions of nTregs may be to minimize collateral tissue damage by responding to tissue destruction signals. The gastrointestinal homeostasis and the involvement of nTregs in maintaining it is a well studied example. Sometimes harmful inflammatory diseases are triggered by commensal gut bacteria. In murine models of colitis nTregs have been shown to work as chief regulators of such lesions. By transferring naïve T cell populations (lacking nTregs) a massive gut inflammation is caused in T cell deficient mice. Disease development can be suppressed by transferring natural regulatory cells together with those naïve T cells.

Similar involvement of nTregs is seen in other infection models also. Often the site of the infection requires more control in these cases. Sites like this include the eye, lung, skin and liver. (Belkaid ja Rouse 2005) For example, when $CD4^{+}CD25^{-}$ T cells were transferred to immunodeficient mice infected with *Pneumocystis carinii*, the result was a better control of the infection but a lethal florid pneumonitis was also triggered. The transfer of nTregs prevented this outcome. (Hori et al., 2002) In another example, in *Candida albicans* –infected mice, reducing the numbers of nTregs resulted again in better control of the infection, but it was encompanied by an enhanced gastrointestinal pathology (Montagnoli et al., 2002).

In some diseases, nTregs have been shown to have a suppressing effect on the inflammation. For example, in *Schistosoma mansoni* –infected mice, nTregs promote Th2 polarization by suppressing Th1 responses, which protects the host from lethal inflammatory pathology. This is achieved by preventing the production of IL-12 by dendritic cells, an action mediated by IL-10. (McKee & Pearce 2004). In human hepatitis C, a major complication is a massive liver damage often requiring organ transplant. Liver biopsies show, that there is an inverse correlation between the inflammatory score and the number of nTregs. (Cabrera et al., 2004)

Suppression of the immune response during infection may have controversial consequences. Even though suppressive nTregs limit local tissue damage, they might prevent strerilizing immunity against the invader and allowing for persistent infection. An example of this is provided by malaria. It takes several years of natural immunity to occur against it and the mechanism of it is not yet fully understood. The participation of nTregs might be one factor. In mouse models, nTreg depletion prevents death caused by the lethal strain of *Plasmodium yoelii* by restoring a vigorous effector cell immune response and control of the infection. (Hisaeda et al., 2004)

However, the persistent infection results in protective immunity against following attacks of the same pathogen. In a *Leishmania major* model, depletion of Tregs leads to the clearance of the parasite. (Belkaid et al., 2002) Yet, Treg depletion may also have detrimental effects on the host. It seems that Tregs have a critical role in the early stages of HSV infection and depletion of them caused an increase in the viral loads and accelerated the time to death. (Lund et al., 2008)

It is not clear how Tregs actually alter or suppress the immune response against bacterial, fungal, viral and parasitic infections. This is mainly due to the fact that when the immune system is being challenged by foreign antigens, a milieu of cytokines and inhibitory factors, especially IL-10 and TGF-β, is produced. (Belkaid 2008) It has been shown, that although most of the inhibition of the immune responses raised against certain pathogens is probably mediated by IL-10, Tregs may not be the producers of it (Anderson et al., 2007).

2.1.4. When the regulatory system fails: autoimmunity and allergy

Massive evidence links the failures in Treg function to abnormalities such as autoimmune and allergic diseases. Several diseases connected to the altered Treg function have been studied, including examples such as inflammatory bowel disease, type 1 diabetes, multiple sclerosis, asthma and atopic dermatitis. Some of these diseases are discussed below.

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract, connected with Crohn's disease and ulcerative colitis. Both environmental and genetic factors contribute to the individual's susceptibility to the disease. In IBD, an aberrant inflammation towards the normal commensal bacterial flora in the gut is exhibited. Murine models utilizing T cell –induced colitis have been studied in detail in order to determine the etiology and underlying mechanims of IBD. In these model organisms, the role of Tregs as protectors from the disease has been dissected. (Workman et al., 2009)

In the murine colitis model, a small number of naïve $CD4^{+}CD45RB^{hi}$ T cells are transferred into immunodeficient *Rag*$^{-/-}$ or scid mice which leads to Th1-mediated colitis in a few weeks. Co-transfer or adoptive transfer of Tregs prevents or reverses the disease. (Maloy et al., 2003, Mottet et al., 2003) IL-35 has also been shown to have a role in regulating it (Collison et al., 2007), along with IL-10 and TGF-β. *Il10*$^{-/-}$ mice develop enterocolitis, but not in germ-free or Helicobacter-free environments. This suggests that IL-10 has a special role in controlling inflammatory responses against certain normal flora bacteria. (Workman et al., 2009) Scid mice can be protected from colitis by administrating rIL-10 following transfer of $CD4^{+}CD45RB^{hi}$ T cells in the absence of Tregs (Powrie et al., 1994). $CD4^{+}CD45RB^{lo}$ cells isolated from *Il10*$^{-/-}$ mice were not able to prevent IBD when transferred together with $CD4^{+}CD45RB^{hi}$ cells and $CD4^{+}CD45RB^{lo}$ cells from *Il10*$^{-/-}$ mice caused inflammation in *Rag*$^{-/-}$ mice when transferred alone. It is likely that Treg derived IL-10 is critical in regulating IBD. (Asseman et al., 1999) In humans, Crohn's disease patients have intestinal CD4+ cells that are not able to produce IL-10 normally (Hvas et al., 2007).

Mice deficient of TGF-β develop a lethal multifocal inflammatory disease which leads to an early death in 3-5 weeks of age (Kulkarni et al., 1993). Mice develop colitis in the absence of T cell specific TGF-β, and Tregs from these mice do not protect from IBD but rather potentiate disease (Fahlen et al., 2005). In addition, anti-TGF-β antibodies prevent wild type Tregs from mediating protection (Powrie et al., 1996). TGF-β is clearly involved in regulating colitis, but evidence suggests that TGF-β originating from other cells contributes too to the disease control (Workman et al., 2009)

Type 1 diabetes (T1D), also known as insulin-dependent diabetes mellitus, is an autoimmune disease resulting from the selective aggression of autoreactive T cells towards insulin-secreting β cells of the islets of Langerhans. The disease pathogenesis involves both CD4 and CD8 cells. It is not quite clear, what the exact nature of the triggering and target autoantigens is, although several candidates have been described, including insulin or proinsulin, glutamic acid decarboxylase and heat shock protein 60. (Bach & Chatenoud 2001) The mouse model for T1D, the non-obese diabetic (NOD) mouse, is one of the most extensively studied examples of a spontaneous autoimmune disease. T1D appears in mice by the age of 12-16 weeks, preceded by a phase of asymptomatic pre-diabetes. T1D has been shown to be induced by diabetogenic, autoreactive T cells and a protective role for Tregs in preventing spontaneous disease has been established. (You et al., 2008)

It seems that the reduction of the functional capacity of Tregs over time is correlated with the occurrence of T1D in NOD mice (Bach & Chatenoud 2001). In diabetic humans, conventional T cell susceptibility to Treg suppression has been reduced (Lawson et al., 2008). The same applies to the NOD mice (You et al., 2008). Co-injection of Tregs from young, pre-diabetic mice or islet-specific iTregs with diabetogenic T cells into NOD-scid mice prevents the disease (You et al., 2007). It is also noted, that the absence of Tregs in young NOD mice with anti-CD25 mAb accelerates the disease onset (Billiard et al., 2006). The Treg protection can work in an antigen-specific or non-specific manner, but the suppression by islet antigen-specific Tregs is more effective than suppression by polyclonal Tregs as fewer are needed in order to suppress the disease (Tarbell et al., 2004).

Based on the NOD model, it is likely that Tregs mediate their protection against T1D via CTLA-4 and TGF-β. The loss of CTLA-4 and TGF-β activity accelerates the disease progression and raises the level of incidence. (You et al., 2007) Additionally, treating *Cd28*$^{-/-}$ NOD mice lacking Tregs with anti-CTLA-4 and anti-TGF-β results in earlier T1D onset (Salomon et al., 2000).

The discoveries in Treg involvement in T1D have raised a strong interest for utilizing them in the treatment of T1D. The induction of Tregs *in vivo* and the adoptive transfer of *in vitro* cultured Tregs have resulted in promising results both in mice and humans. (Workman et al., 2009)

The involvement of Tregs in asthma, allergy and atopic dermatitis has been demonstrated in several examples in both mice and humans. In allergen-sensitized mice, the depletion of Tregs led to increased levels of Th2 cytokines, IgE and airway hypersensitivity reaction (AHR) (Lewkowich et al., 2005). In allergic human patients, Tregs were unable to suppress the IL-5 secretion and proliferation of allergen-stimulated CD4+ cells, while Tregs from healthy donors were not (Ling et al., 2004). At least in patients with atopic dermatitis, the functional defect is not reflected as a reduction in number of Tregs (Ou et al., 2004). The opposite holds true for children with asthma (Hartl et al., 2007).

From human studies with allergic patients it has become evident, that IL-10 has an important role in protecting against allergy. Non-allergic individuals seem to have a higher number of IL-10-producing activated T cells in response to IL-10. (Akdis et al., 1998) When antigen-specific Tregs were transferred to mice before they were challenged with an allergen, allergic symptoms and Th2 cytokine production were inhibited. The effect was mediated via IL-10. (Kearley et al., 2005)

Not surprisingly, TGF-β has also been connected with allergic reactions. Tolerized CD4+ cells express membrane-tethered TGF-β as a response to respiratory antigens (Ostroukhova et al., 2004). In a murine asthma model, AHR was suppressed by the adoptive transfer of Tregs overexpressing TGF-β. Yet, if the cells did not express IL-10 in addition to TGF-β, complete protection was not achieved. (Presser et al., 2008).

It seems that human individuals with high doses of allergen exposure tend to demonstrate a switch towards IL-10-sectering Tr1 cells (Meiler et al., 2008). It might be also possible, that nTregs and Tr1 cells are able to induce IgG4 and suppress IgE production, thus indirectly contributing to allergy suppression (Meiler et al., 2008).

Recently, Tregs and Treg-derived IL-10 with TGF-β have presented an attracting target for therapeutic manipulation of allergic diseases, with some promising results already achieved in reducing the severity of allergen-induced inflammation of the lung by administrating recombinant IL-12 with anti-IL-12 mAb. The effect is due to the increase in Treg numbers. (Wilson et al., 2008)

It is worth noticing, that IL-10 and TGF-β may not be the only contributors in Tregs-mediated protection against atopic diseases, as other mechanisms might also play a role in it (Workman et al., 2009).

2.1.5. Regulatory T cells and tumors

Perhaps the most important function of Tregs is the maintenance of peripheral tolerance, especially suppressing autoimmune T cell responses. This becomes a problem, when tumors are concerned, as Tregs also appear to control anti-tumor activity and tumors are seen a part of self. This results in ineffective clearance of the tumors by other T cells. (Yang et al., 2006) Consistent with this, Tregs are indeed found in many tumor environments, including cancers such as melanoma, lymphoma, ovarian, pancreatic and breast cancer (Workman et al., 2009). There is, however, still debate over the issue whether the presence of Tregs in the tumor environment indicates a poor prognosis or a positive prognosis (Curiel et al., 2004, Alvaro et al., 2005). It is, of course, important to take notice of these discrepancies, but it should be taken into account that these studies were performed by staining Foxp3, which does not necessarily correlate with human Treg presence as it is also expressed by conventional T cells following their activation (Morgan et al., 2005). There is also a study showing by staining and functional analysis, that Foxp3-positive T cells found in the tumor environment were indeed Tregs and that their presence was connected with an unfavorable prognosis (Curiel et al., 2004). Additionally, murine studies suggest that by depleting Tregs *in vivo* with anti-CD25

antibodies, an anti-tumor response can be mounted and the tumor cleared (Onizuka et al., 1999).

The question of the mechanisms by which Tregs inhibit the anti-tumor response has received much attention. Again, the inhibitory cytokines IL-10 and TGF-β have been under the main focus. It has been demonstrated, that in both mice and humans, Tregs are able to suppress the adaptive and innate branches if the anti-tumor response by inhibiting CD8+ cells and NK cells in a TGF-β-dependent manner (Chen et al., 2005, Ghiringhelli et al., 2005). In a study concerning head and neck squamous cell carcinoma, it was evident that Tregs suppressed the anti-tumor response via IL-10 (Strauss et al., 2007).

It seems that Tregs are also able to prevent tumor clearance by turning APCs immunosuppressive. Treg-derived IL-10 causes the upregulation of B7H4 in APCs which results in augmented APC function. (Kryczek et al., 2006) Another study demonstrated that that B7-H4^{+} tumor infiltrating macrophages showed immunosuppressive activities in human ovarian carcinoma (Kryczek et al., 2006).

Tregs additionally suppress APC function by direct contact in the tumor microenvironment. CTLA-4 on Tregs mediate the downregulation of CD80 and CD86, thus rendering APCs unable to activate other T cells via CD28 co-stimulation. This mechanism of suppression was clearly important, as a massive anti-tumor response was mounted in the presence of CTLA-4 deficient Tregs. (Wing et al., 2008)

CD8+ and NK cells primarily utilize granzyme B –dependent cytolosis during tumor clearance. Tregs are able to kill conventional T cells in a granzyme B-dependent, perforin-independent manner. (Gondek et al., 2005) It has been shown, that granzyme B was upregulated in the tumor environment Tregs and in a setting with granzyme B –deficient Tregs, an anti-tumor response was generated and the tumor destroyed. Based on these data, it seems that Tregs can work via cytolysis in order to suppress the anti-tumor response. (Cao et al., 2007b)

2.1.6. The hygiene hypothesis

The hygiene hypothesis is a theory first proposed by Strachan in 1989, when a study including over 17 000 British children demonstrated an inverse correlation between hay fever and the number of older siblings in a family. The hypothesis stated that the decreased incidence of infections might be the underlying cause. (Strachan 1989) Autoimmune diseases, such as multiple sclerosis or type 1 diabetes have also been included in the theory, as many epidemiologic and experimental studies have shown them to be connected to the lack of infections and improved sanitation. The leading idea of the theory is that some infectious agents somehow mediate protection against numerous immunological disorders.

2.1.6.1. The rise of immunological disorders and decreasing infectious burden

During the last twenty years, allergic diseases such as atopic dermatitis, asthma and allergic rhinitis have become somewhat of an epidemic in industrialized countries. (Bach 2002) The incidence of asthma is marked in developed countries - over 15% in United Kingdom, New Zealand and Australia – but also in developing countries: more than 10% in Peru, Costa Rica and Brazil. In Africa, South Africa has the greatest incidence (8%). (Eder et al., 2006) In the last three decades, the prevalence of atopic dermatitis has doubled or tripled in developed countries, affecting 15-30% of children and 2-10% of adults (Bieber 2008). At the same time, the prevalence of autoimmune diseases has also peaked. Especially T1D has become a serious public health problem in European countries and particularly in Finland, where children aged 0-4 years are becoming progressively affected (Harjutsalo et al., 2008). Likewise, the incidence of inflammatory bowel disease (Bach 2002) and primary biliary cirrhosis (Rautiainen et al., 2007) is rising. The rise in the incidence of these disorders may partly be due to better means of diagnosis or improved access to medical care, but these facts cannot explain the obvious increase in disease prevalence that has occurred over such a short period of time (Okada et al., 2010).

Simultaneously, the infectious burden has gone down in industrialized countries. After the industrial revolution, public health measures were taken in order to limit the spread

of infections. These measures included vaccinations, antibiotics, decontamination of the water supply, pasteurization and sterilization of milk and food and respect of the cold chain procedure. The result was a clear and spectacular decline of parasitic infections, such as filariasis, onchocercosis, schistosomiasis or other soil-transmitted helminthiasis, childhood diarrhea and hepatitis A. (Zaccone et al., 2006) The incidence of minor parasitic infections, such as *Enterobius vermicularis*, has also decreased (Gale 2002). People are still chronically infected by various pathogens causing those diseases in countries, where poor health standards exist. In those countries, the incidence of allergy and autoimmunity is low. (Zaccone et al., 2006) Additionally, the emergence of immunological disorders is seen in several countries that have eradicated common infections. (Okada et al., 2010).

An overall North-South gradient for immune disorders can be seen in North America (Wallin et al., 2004), Europe (Bach 2002) and China (Yang et al., 1998). It seems that the geographical distribution of allergy and autoimmunity is a mirror image of the geographical distribution of different infectious diseases. However, puzzling exceptions exist, such as high incidence of asthma in South America and T1D and MS in Sardinia. (Okada et al., 2010) In Europe, a West-East gradient can be seen. In Bulgaria and Romania the incidence of T1D is low, but a fast increase is obvious (Green et al., 2001). Genetic differences cannot fully explain the observed phenomenon. For example, the incidence of diabetes is six times higher in Finland than in the adjacent Karelian republic of Russia, even though people there have similar genetic background. (Kondrashova et al., 2005) In addition, migration studies demonstrate, that the offspring of immigrants coming from a country with a low incidence of T1D and MS, acquire the same incidence as the host country in the first generation (Bodansky et al., 1992, Hammond et al., 2000).

There are several possible explaining factors to the incidence of allergy and autoimmunity according to geographical distribution and time. Socio-economic status is one factor. A positive correlation exists between gross national product and the prevalence of asthma, MS and T1D in Europe. (Bach 2002) The same is true at the level of smaller regions, such as in Northern Ireland, where the low average socio-economic level is connected with the low incidence of T1D (Patterson et al., 1996). Family income has also been correlated with the incidence of atopic dermatitis (Werner et al.,

2002). The mere correlation of immunological diseases and income does not yet, however, pinpoint the actual causative agent of the disorders. There are several epidemiological studies showing a positive correlation between sanitary conditions and T1D (Patterson et al., 1996) and MS (Leibowitz et al., 1966), which suggests a possible role for infections. Some studies show that exposure to farming and cowsheds early in life or during the mother's pregnancy prevent atopic diseases (Riedler et al., 2001). Also, it seems that prolonged exposure to high levels of endotoxins during the first years of life prevents asthma and atopy (Braun-Fahrlander et al., 2002). There are, however, other data contradictory to this, demonstrating that the incidence of asthma and atopy is correlated with high level of endotoxins in urban housing (Thorne et al., 2005). There are epidemiological data of different parasitic infections, such as schistosoma and *Necator americanus* protecting against atopy. Contrasting, *Ascaris lumbricoides* and *Trichuris trichiura* seem to have no significant effect on the disease. (Okada et al., 2010)

2.1.6.2. Proof of causality

It is evident that there is a strong correlation between lifestyle changes and hygiene and the prevalence of immunological disorders, but this does not necessarily mean that a causal relationship exists. Numerous other factors, unrelated to infections, are also a consequence of the change in lifestyle. Animal models and clinical intervention studies provide an answer to this dilemma.

The non-obese diabetic mouse has a very low diabetes incidence or it is completely absent in normal conditions, but when bred in specific pathogen-free conditions, spontaneous diabetes occurs in nearly 100% of the female mice. Infection of NOD mice with viruses, bacteria or parasites protects the "clean" mice completely from diabetes. (Bach 2002) Likewise, complete Freund's adjuvant (e.g. mycobacteria) prevents the onset of experimental autoimmune encephalomyelitis (Hempel et al., 1985) and allergic asthma induced by ovalbumin (Hopfenspirger et al., 2001).

Helminth eradication has been shown to increase atopic skin sensitization in Venezuela, Gabon and Vietnam, but also induce clinical improvement of asthma in Venezuela, whereas similar anti-helminth treatment showed no effect in Equador. The results are hard to explain. (Okada et al., 2010) It is noteworthy, that an increase in atopy was

observed in South Africa after vaccination with *Streptococcus pneumoniae.* (Klugman et al., 2003) When the ova of swine-derived parasite *Trichuris suis* was deliberately administered to patients with active Crohn's disease, symptoms improved in 21 of 29 patients (Summers et al., 2005). Significant improvement was also noticed, when the ova were given to patients with ulcerative colitis (43% improvement vs. 17% placebo) (Summers et al., 2005).

More than 10^{14} bacteria of more than thousand different species occupy the human gut (Gill et al., 2006). Different stains of bacteria colonize the gut immediately after birth, and this commensal microbiota shapes the immune system and affects other physiological functions (Hooper et al., 2001) and the integrity of the intestinal barrier (Rakoff-Nahoum et al., 2004). In a small study of allergic Estonian and Swedish children, a difference in the intestinal flora was observed, when compared to the control group. The allergic children had a higher count of aerobic bacteria, such as coliforms and *Staphylococcus aureus.* The number of *Lactobacilli* and anaerobes, such as *Bifidobacterium* or *Bactereoides* was decreased. (Bjorksten et al., 1999) Yet, in a larger birth cohort study comparing three European baby populations, such difference was not shown. However, this study demonstrated that typical faecal bacteria, such as *Escherichia coli*, were acquired slower in children delivered by caesarean section or with no siblings. (Adlerberth et al., 2007) It is noteworthy, that these studies were performed by analysing only culturable bacteria. In addition, only atopic dermatitis and skin prick tests were evaluated.

It seems that the biodiversity of the faecal microbiota of patients with Crohn's disease is diminished. The metagenomic approach reveals that this is especially true for the Firmicutes phylum. (Manichanh et al., 2006) In a study of the murine model of the disease, *Faecalibacterium prautsnitzii* was shown to improve IBD. This bacterium was one of the Firmicutes that was depleted. The protective effect was also achieved using only the supernatant of the *F. prautsnitzii* culture. (Sokol et al., 2008) In addition, *Bacteroides fragilis* has been shown to prevent experimental colitis in animals. The effect was linked to polysaccharide A molecule. (Mazmanian et al., 2008) However, except for IBD, the evidence is still light for the role of microbiota changes in the immunomodulation of allergy and autoimmunity.

2.1.7. Mechanisms of microbial influence on tolerance

Th1-Th2 deviation is the oldest suggested mechanism for explaining how microbes and parasites might induce prevention of atopic and autoimmune diseases. As discussed above, Th1 cells mediate their functions through the production of inflammatory cytokines, such as IL-2, IFN-γ and TNF-α. These molecules are also operational in cell-mediated immunity and autoimmunity. Th2 cells produce IL-4, IL-5, IL-6 and IL-13 and control the IgE production and allergic responses. It was first suggested that the lack of exposure to microbes in early childhood directs the immune system to a Th2-dominated immunity, when normally the microbial influence would cause the host to favor Th1-biased immune responses. The problem of this theory is that autoimmune diseases – which are usually Th1-mediated – can be prevented by infection leading to a Th1 response and that parasites that induce a Th2 response may protect the host from Th2-mediated atopy. Also, contrary to initial reports (Decreased prevalence of atopic diseases in children with diabetes, 2000), it seems that there is association between autoimmunity and allergy in individual patients: rheumatoid arthritis and diabetes are sometimes connected with atopy (Kero et al., 2001). However, this relationship is far from clear. In a recent study of Finnish and Russian Karelian children, a connection was found between T1D and allergen-specific IgE in the Karelian children, but not among Finnish children, despite similar genetic background. It seems that in countries with low income (and lower prevalence of diabetes and higher incidence of microbial infections), a positive association between T1D and allergy exists, but the same does not hold true for countries with higher income. (Seiskari et al., 2010) Considering these observations, a common mechanism underlying the protective effect against immune disorders is likely.

Antigenic competition is a phenomenon known for decades: when two different antigens both cause an immune response, the responses tend to inhibit each other. It has been proposed, that strong responses against antigens from pathogens could inhibit responses to weaker signals from allergens and autoantigens. Recently, lymphocyte competition for cytokines, growth factors affecting the differentiation and proliferation of B and T cells during immune responses and recognition of MHC-self peptide – complexes have been under special attention. (Okada et al., 2010)

Bystander suppression is another mechanism suggested. Tregs are able to suppress immune responses distinct from responses against the antigen in question – infectious agents in this context. Tregs become stimulated at sites where they recognize cognate autoantigen. Once activated locally in an MHC class-II-restricted manner by antigen presenting cells, Tregs can suppress other T cells that are specific for other antigens, if such antigens are expressed by the same APC or target cell, or if the cells are in close proximity. However, there are numerous regulatory T lymphocytes and diverse cytokines involved in Treg differentiation and regulatory actions, which makes the theory problematic to prove. (von Herrath & Harrison 2003) Foxp3-positive CD4+CD25+ cells have been proposed by transfer experiments performed in a murine parasite model (Belkaid et al., 2002b). One study reported that Foxp3 was upregulated in the cord blood of newborns whose mothers had been exposed to farming (Schaub et al., 2009). This result should, however, be interpreted with caution as human markers of these cells are still uncertain. There is other data showing a possible role for IL-10-producing B cells (Fillatreau et al., 2002) and natural killer T cells (Wu & Van Kaer 2009) and more generally to any cell types producing IL-10 (Moore et al., 2001) and TGF-β (Gorelik & Flavell 2002).

The stimulation of non-antigen specific receptors might be a mechanism by which infectious agents may promote protection from atopic diseases, without their constitutive antigens. Toll-like receptors (TLRs) provide a good example of this. TLRs are receptors for various bacterial components, such as peptidoglycan and bacterial lipoproteins (TLR2), LPS of gram-negative bacteria (TLR4), flagellin (TLR5) and CpG motif of bacterial DNA (TLR9). Eleven TLRs are known in human and most of them are extracellular, but TLR-3, -7, -8 and 9 recognize intracellular signals. The binding of these molecules to TLRs stimulates mononuclear cells to produce cytokines, including those with immunoregulatory functions. (Akira et al., 2001) These receptors act in the early rapid innate immune responses and they affect the development of adaptive responses. When the immune system encounters an invader, APCs are stimulated through their TLRs and they present microbial antigens. It is possible, that activated cells processing microbial antigens and apoptotic host cells might present self-antigens too and cause bystander activation during infection. It has been suggested that in these situations, microbial antigens might be preferentially presented by MHC II molecules and that TLRs thus control the selection of antigens to be presented. It has been shown

under experimental conditions that the antigens from host cells were not presented by MHC II unless they also contained microbial ligands that could stimulate TLRs. (Blander & Medzhitov 2006)

Naturally, TLRs also stimulate regulatory T cells. This is important in terminating the response to the infection and limiting immunopathology. These effects on Tregs might have an important role in protecting against autoimmunity. Experiments with RT-PCR have shown, that TLRs 1, 2, 4, 5, 6, 7, and 8 were detected on regulatory $CD4^+CD25^+$ T cells, with TLRs 4, 5, 7, and 8 selectively expressed on a $CD4^+CD45RB^{lo}$ population. This study suggested that stimulating Tregs with LPS could enhance their suppressive actions. (Caramalho et al., 2003) Another study demonstrated that endogenous heat-shock protein 60 stimulated TLR2 on Tregs, making them inhibitory towards CD8+ cells through IL-10 and TGF-β production (Zanin-Zhorov et al., 2006). A different mechanism for TLR2-dependent activation of suppression was proposed in a study using the synthetic TLR2 ligand PAM_3Cys, as well as natural ligands, peptidoglycan and heat-killed bacteria. During these experiments, in the absence of antigen presenting cells, upregulation of CD25 was induced together with proliferation of $CD4^+CD25^+$ T cells. (Sutmuller et al., 2006) It has also been shown, that TLR-2, -3, -4, -7 and -9 stimulation may prevent the onset of spontaneous autoimmune diseases such as type 1 diabetes in NOD mice. In this model, the disease progression is completely stopped if mice are treated with TLR agonists before disease onset. The mechanism may be dependent on immunoregulatory cytokines, Tregs and NK T cells. (Wong & Wen 2008) In addition, there are some experiments suggesting that TLR stimulation in fact exacerbates allergic and autoimmune responses, as TLRs have powerful capacity to provoke cytokine production and immune responses (Lang et al., 2005).

Last but not least, genetics are an important factor to be taken into account when considering atopic and autoimmune diseases. Interestingly, an association between some polymorphisms of genes implicated in the control of infection and atopic diseases have been found. For example, genes polymorphisms in genes of the innate immune system such as TLR2, TLR4, TLR6, TLR10 and CD14 appear to have a role in the development of allergy and asthma. (Vercelli 2008)

2.2. Real-time PCR

Real-time polymerase chain reaction (PCR) is a method based on the principle of the traditional PCR, which was invented by Kary Mullis in 1980's. With PCR, it is possible to amplify specific DNA sequences more than billion-fold and in real-time PCR the amplification is monitored at the same time the reaction proceeds, which makes it possible to better determine the starting amount of DNA in the sample. The term "quantitative PCR" refers to this ability. In the early 1990's, Roche Molecular Systems and Chiron had their first demonstration of real-time PCR, when they added a common fluorescent dye called ethidium bromide (EtBr) in the PCR run and videotaped the reaction under ultraviolet light. As EtBr is fluorescent under UV-light, they were able to visualize and record the accumulation of DNA. In recent years, real-time polymerase chain reaction (PCR) has become the most sensitive and accurate method for detecting specific nucleic acids and thus it is widely used in biological investigation. With real-time PCR, it is possible to rapidly and accurately determine changes in gene expression, for example, as a result of experimental stimuli or physiological, pathophysiological and developmental phenomena. In clinical molecular diagnostics, real-time PCR can be used to measure viral and bacterial loads or evaluate cancer status. (Valasek & Repa 2005)

2.2.1. The principle of real time PCR

The basic principle underlying real-time PCR is the same as in conventional PCR. The nucleotide sequence of every living organism is uniquely and specifically present only in its own species and complex organisms possess sequences specific for particular individuals. Unique variations make it possible to trace DNA (or RNA) back to its origin, at least on the level of species and sometimes on the level of individuals. PCR can be used to amplify very small amounts of genetic material. (Powledge 2004) It requires a template of the DNA that is to be copied, and two sequence-specific, single stranded oligonucleotides that act as primers and start the amplification. The primers each attach to separate strands of the DNA molecule (forward and reverse primer) and during every cycle of the PCR, double the amount of DNA in the sample. (Valasek & Repa 2005) Additionally, dNTPs (four nucleotide triphosphates), a heat-stable DNA polymerase and magnesium ions in the buffer are needed. First, the temperature is raised to about 95°C, which separates the DNA strands. The temperature is then

lowered to allow for primer annealing, and finally the temperature is set around 72°C, which is optimal for the polymerase to extend the primers using the dNTPs according to the template. The cycle is repeated several times. The first melting step must be sufficiently long to separate the strands completely, so that partially melted strands would not re-anneal when the temperature is dropped. The exact melting temperature and duration of the step depends on the template length and sometimes the sequence, and also, the instrument and reaction container used. Some hot-start polymerases might need a long activation time in the first cycle. The annealing temperature is primer-dependent. It is optimally a few degrees below the melting temperature of the primers, which should have about the same melting temperature. This helps to avoid the problem of unstable complexes with the template and complexes with other sequences. The elongation temperature is set to match the optimum for the polymerase in use, and for Taq polymerase it is about 72°C. However, some protocols use very different elongation temperatures and it does not seem to be very important. The elevated temperature is probably needed more for the melting of any secondary structures that may form in the template and prevent extension. (Kubista et al., 2006)

As the PCR reactants are consumed over the cycles, the reaction will eventually reach a plateau. Additionally, self-annealing of the accumulating product will also contribute to the effect. This in why real-time PCR technology is so important. Because the plateau effect is inevitable after certain amount of amplification, there is no way to reliably determine how much DNA there was before the cycles, as starting and finishing amounts of the product do not correlate. Real-time PCR solves this problem by measuring product formation early in the reaction process, when the amplification is exponential. This measurement correlates to the amount of starting DNA and quantification is possible by utilizing fluorogenic probes that “light up” to show the amount of DNA present during each PCR cycle.

The problem of DNA polymerases is that they generally use DNA as their template. That is why it would be impossible to measure gene expression values as a function of mRNA levels in a certain sample. However, reverse transcriptase (RT) real time PCR solves this dilemma. The enzyme reverse transcriptase generates complementary DNA (cDNA) from a RNA template. Several different reverse transcriptases are commonly used, including avian myeloblastosis virus reverse transcriptase and Moloney murine

leukemia virus reverse transcriptase. Under right reaction conditions, the relative amount of generated cDNA is proportional to the relative amount of its RNA template. This cDNA can be used a raw material for real time PCR, thereby providing a sensible and accurate means to measure, for example, mRNA levels in a given sample. RT real-time PCR has become the most widely used method of quantifying steady-state mRNA levels. (Valasek & Repa 2005)

The foundation of the real-time analysis is the binding of a fluorescent reporter to the PCR product. The fluorescence is measurable and reflects the amount of the product formed. During the first cycles the fluorescent signal is weak and indistinguishable from the background. When the product accumulates exponentially, the signal also amplifies. In a typical real-time PCR run all response curves reach plateau (saturate) at the same level, and thus end-point analysis tells nothing about the starting amount of DNA. However, the response curves are separated in the exponential phase, which reflects the difference in the initial amount of the template. The difference can be quantified by comparing the number of cycles needed for each sample to reach a threshold fluorescence signal level. This threshold cycle value is called the Ct value (Figure 2). The amplification curves are parallel in the exponential phase of the reaction, and thus the setting of the threshold level is not critical. The exact level is selected by the software by varying algorithms, and often PCR instruments let the user manually set the threshold. The setting does affect individual Cts, but not the differences between them, which are far more important. If the PCR reaction would be 100% efficient, then the ratio of the initial numbers of the template copies in the sample is given by

$$\frac{[N_0]_A}{[N_0]_R} = 2^{(CT_B - CT_A)}$$

$[N_0]_A$ and $[N_0]_B$ are the initial numbers of template molecules in samples A and B, and Ct_A and Ct_B are the corresponding Ct values. In most cases the PCR efficiency is not perfect, and the efficiency must be corrected into the equation:

$$\frac{[N_0]_A}{[N_0]_B} = (1+E)^{(CT_B - CT_A)}$$

The efficiency can be estimated by a series of dilutions that are used to form a standard curve. The standard curve can be constructed, for example, from a diluted test sample. The Ct values of the standards are plotted versus the logarithm of the samples' concentration, number of template copies or dilution factor. The PCR efficiency is calculated from the slope according to equation:

$E = 10^{-1/\text{slope}} - 1$

where 10 is the dilution coefficient. (Kubista et al., 2006) In this study, dilution series were based on dilution factor of 2, so the formula used was $E = 2^{-1/\text{slope}} - 1$.

There are two basic ways to quantify the DNA amounts in the samples: the absolute and relative quantification. In absolute quantification, the absolute amount of DNA or RNA molecules per cell, per total DNA or RNA, or per unit mass of tissue is determined. To achieve this, it is necessary to construct a standard curve in which the absolute DNA amount is known. (Cikos & Koppel 2009) The discussion on absolute quantification is, however, beyond the scope of this literature review, so only relative quantification will be discussed here in detail.

In relative quantification, the amount of DNA in the target sample is determined in relation to another sample, which is chosen to be the reference (control) sample. Quantity of target nucleic acid in all target samples is then expressed relative to the control sample. To compensate for sample to sample variation, The Ct value of the target is usually normalized to one or more reference sequences, for example some commonly known housekeeping genes. (Cikos & Koppel 2009) Relative quantification and normalization will be discussed further below.

2.2.2. Quantitative analysis techniques

Techniques based on fluorescence reporters are exclusively used in real-time PCR today. There are both sequence specific probes and non-sequence specific labels available. Especially asymmetric cyanine dyes, such as SYBR Green I and BEBO have become popular. (Zipper et al., 2004) These asymmetric cyanines have two aromatic systems containing nitrogen. One of these is positively charged and connected by a

methine bridge. When free in a solution, the dyes do not have fluorescence, but when bound to DNA minor groove, they become highly fluorescent. During the PCR cycles,

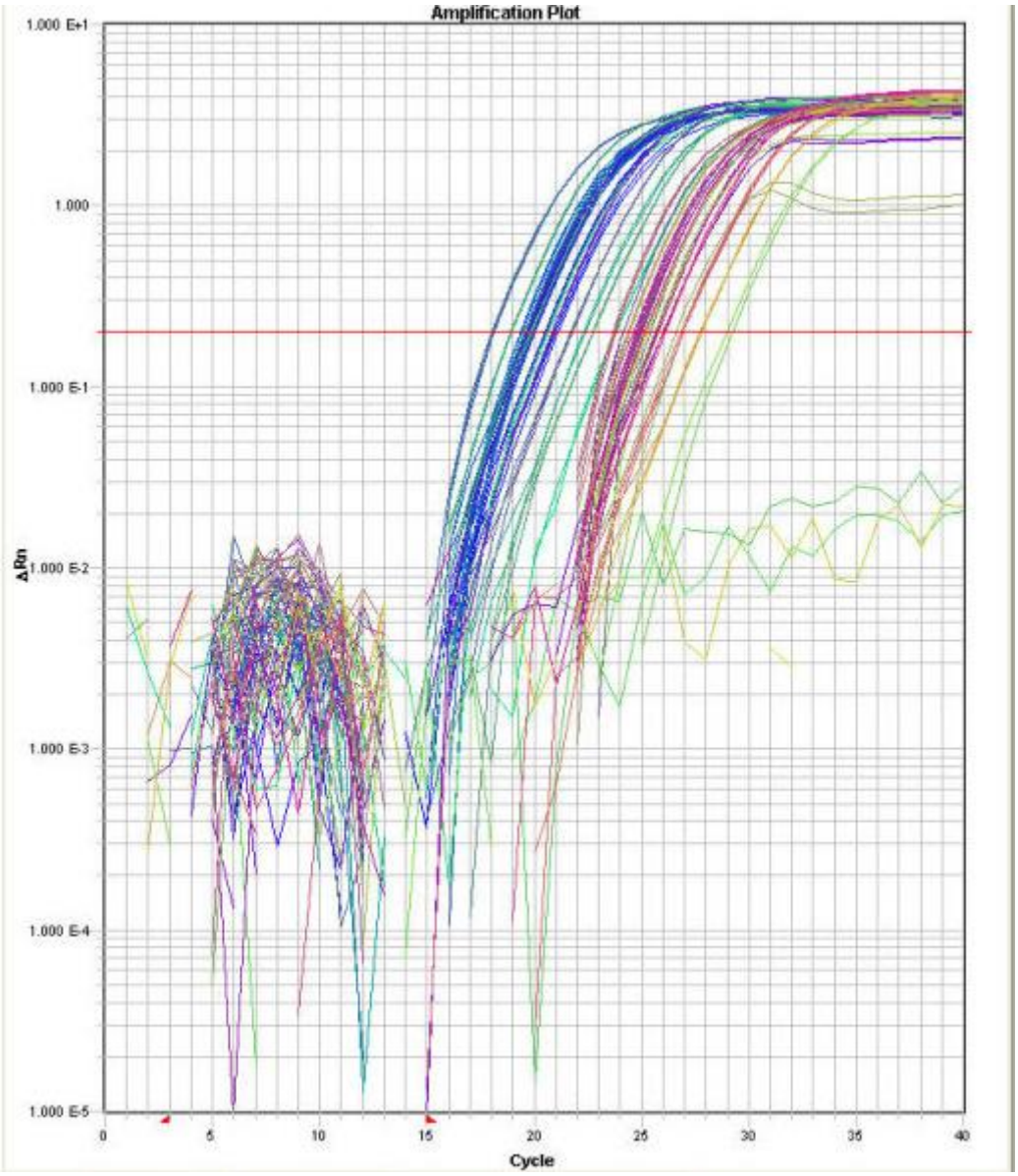

Figure 2. A real-time PCR amplification plot. The red horizontal line is the threshold level and determines the Ct values for different samples. The picture shows the exponential amplification phase and the following plateau.

the fluorescence increases with the amount of double stranded product formed. The fluorescence is not, however, strictly proportional as the dye fluorescence depends on the dye-base binding ration, which decreases during the course of the reaction. The

DNA sequence also has some effect on the fluorescence. Asymmetric dyes are yet considered non-sequence specific.

Labeled primers and probes comprise the second class of fluorescence reporters. They are based on nucleic acids or their synthetic analogues. There are two kinds of dye labels: fluorophores that have intrisically strong fluorescence, such as rhodamine and fluorescein derivatives utilizing a quencher molecule, and fluorophores that change their fluorescence properties when binding DNA. Examples of dyes with a quencher molecule are the hydrolysis probes (Taqman probes) and Molecular Beacons. (Kubista et al., 2006) The probes are sequence-specific dually fluorophore-labeled DNA oligonucleotides that base their function on resonance energy donor-acceptor principle. One fluorophore is called the quencher and the other is the reporter. When attached to the same short oligonucleotide, the quencher and reporter are close to each and the quencher absorbs the signal from the reporter. During amplification, the oligonucleotide is broken apart by the action of DNA polymerase and the reporter and quencher separate. The result is the liberation of the fluorescence signal. Thus, when DNA is amplified, the reporter signal increases corresponding to the destruction or hydrolysis of the oligonucleotide. Examples of common quenchers include fluorophores such as TAMRA, DABCYL, and BHQ. Reporter molecules include examples like FAM, VIC, and NED. (Valasek & Repa 2005)

Single-dye probes that change their fluorescence upon binding DNA include the LightUp probes, AllGlo probes and Displacement probes. In some probes, the oligonucleotide backbone is altered to allow for better binding to the target template. This makes it possible to use shorter probes, even as short as 10-12 bases, which is useful especially for detecting targets that have short conserved regions, such as retroviruses.

When there is only one sample per tube to detect, it makes not much difference, whether the reporter of choice is a dye or a probe. Dyes are cheaper, but probes are advantageous in multiplexing (amplifying and detecting several samples in one tube at the same time). Probes distinguish between products, but hydrolysis probes require two-step PCR, which is not optimal for the polymerase reaction. They also need short amplicons to ensure amplification efficiency. (Kubista et al., 2006

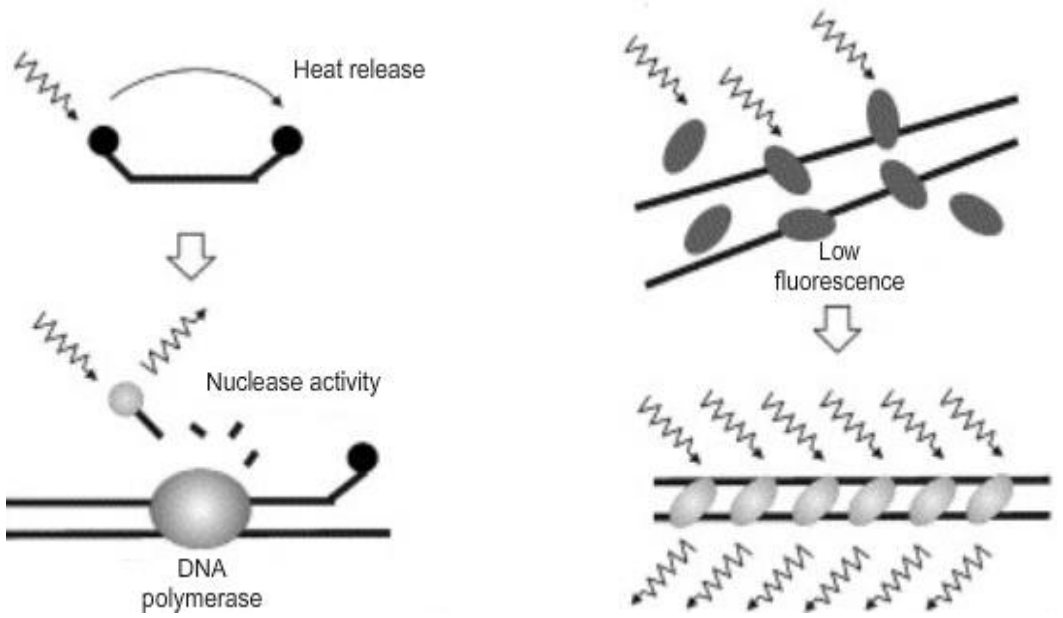

Figure 3. The picture on the left represents the principle of the Taqman hydrolysis probe. When in proximity with the fluorophore, the quencher quenches the fluorescent signal. During DNA extension, the fluorophore is released and its fluorescence can be detected. The right picture shows the fuorescence of dyes such as SYBR Green by the binding of double-stranded DNA.

2.2.3. Relative quantification, normalization and the Pfaffl equation

Because there are differences in the amount of biological material in the samples to be amplified, normalization is necessary in order to compensate for this. Most popular ways for normalization are normalization to total RNA amount, to ribosomal RNA, to externally added RNA standards or to internal reference genes. Reference genes are the most widely used method. (Kubista ym. 2006) Mainly non-regulated reference genes or housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), albumin, actins, tubulins and cyclophilin are used as references. They are used as endogenous standards to compensate for inter-PCR variations between runs. (Pfaffl 2001)

The ΔΔCt method is perhaps the most well-known formula for calculating relative gene expression ratios. The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene, the expression of which is assumed to be constant. In

the ΔΔCt method, it is also assumed, that the PCR efficiency is 100% in both samples. Thus, the ratio of the target sample vs. control sample is given by the equation

$$ratio = \frac{2^{CT_{B1} - CT_{B2}}}{2^{CT_{A1} - CT_{A2}}} = 2^{(CT_{B1} - CT_{B2}) - (CT_{A1} - CT_{A2})} = 2^{\Delta\Delta CT}$$

Where A and B are the target and reference gene in the sample, respectively. The subscript numbers (1 and 2) refer to target and control samples, respectively. However, as this method does not account for differences in the PCR efficiencies, it should only be used as an approximation method. (Kubista et al., 2006, Pfaffl 2001)

More accurate calculations need to include the efficiencies (E) of the target and reference gene amplification. This is considered in the equation

$$ratio = \frac{(E_{target})^{\Delta CT_{target}(control-sample)}}{(E_{ref})^{\Delta CT_{ref}(control-sample)}}$$

also called the Pfaffl method. The efficiencies are determined as described above (section 2.2.1.), by calculating the slope of a standard curve. The correlation coefficient should be $r > 0.95$. (Pfaffl 2001)

2.2.4. Problems associated with real time PCR

Several points in the real-time PCR run are prone for problems that either inhibit proper reaction mechanism or distort the results.

The nucleotide sequence of the target template and primers is the first possible error source. Sequential runs of guanines, perhaps only 2-3 bases long, may lead into tetraplex folding of the template. This folding can be avoided in the presence of K+ ions. Intra-sequence complementarity is another issue, as these regions in the template can fold into hairpins and other structures that may block strands extension by the polymerase. The template should thus be carefully chosen. Same is true for primer sequences. Primer design is very important, as complementary between primers, especially in their 3'-ends, is a potent source of aberrant PCR results. The formation of

primer-dimers might distort completely quantitative results as asymmetric cyaninine dyes such as SYBR Green make no difference to what double-stranded DNA they bind to. In addition, primer-dimers compete with the target product for reagents.

Sometimes even careful selection of the primers is not enough to inhibit primer-dimer formation. It is, however, possible to check if they are indeed present. This is achieved through a melting curve analysis at the end of the PCR run. The melting curve is seen when the temperature of the sample is gradually increased, which leads to the simultaneous decrease of fluorescence due to the increased thermal motion which allows for more internal rotation in the DNA-bound dye. When the target sequence's melting temperature, $T_{m,}$ is reached and the strands separate, an abrupt drop in the fluorescence is seen as the dye releases the DNA molecule. The maximum of the first derivative of the melting curve demonstrates this point most clearly as a sharp peak on the curve (Figure 4). Because primer-dimers typically are shorter than the amplified product, their T_m is lower and their presence can be easily recognized on the first derivative curve as a separate peak before the peak formed by the actual product. (Kubista et al., 2006)

In biological samples, there are several possible PCR inhibitors present. For example, certain body fluids contain inhibitors such as heme, heparin, IgG, lipids and urea. (Kubista et al., 2006) Organic and phenolic inhibitors may become a problem in food microbiological applications. Using alternative DNA polymerases, such as *Tfl, Pwo* or *Tth* might help overcome this issue.

It is also worth considering, what problems might arise from the fact that RNA step is included in the PCR protocol. Compared to DNA, RNA is unstable and its isolation must be conducted carefully. RNA integrity must be ensured, along with the removal of contaminating nucleases, genomic DNA and RT or PCR inhibitors. specially clinical samples are prone to inconsistencies in sample size and collection, storage, and transport related problems that might lead to compromises in RNA quality.

The reverse transcriptase step is an additional subject to variability as there are multiple RT enzymes with different characteristics, and the RT reaction can be primed using different kinds of oligonucleotides, including random hexamers, poly-dT and sequence-specific primers. (Valasek & Repa 2005)

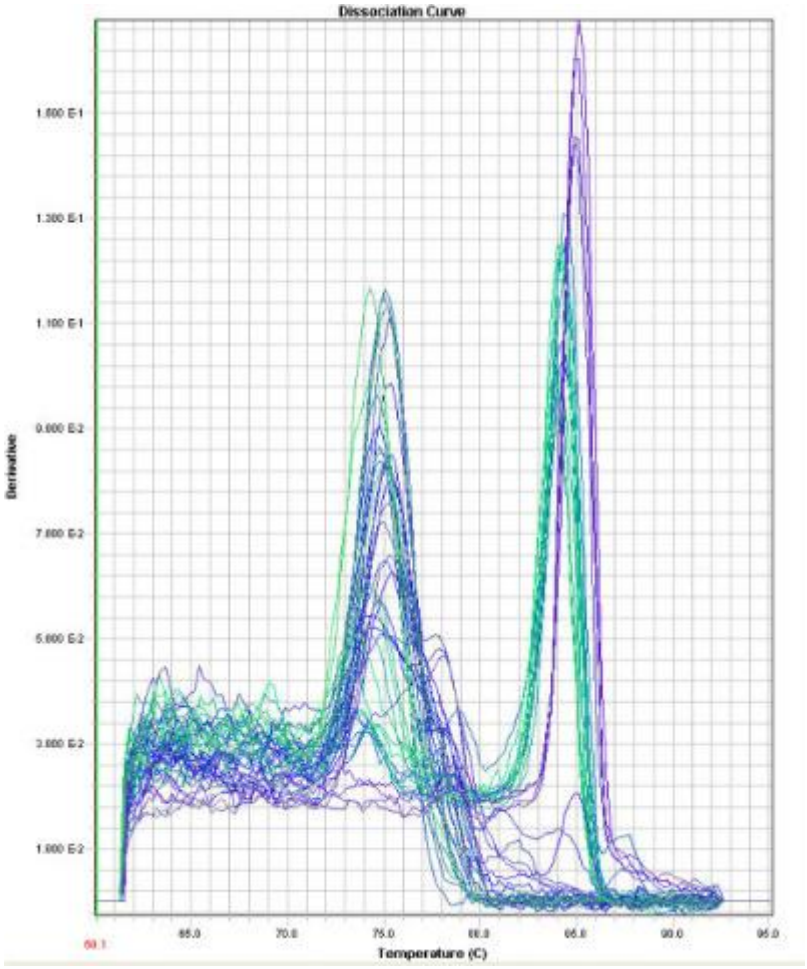

Figure 4. A real-time PCR dissociation curve. Two spikes are clearly visible, showing the melting of primer dimers (peak on the left side) and the actual product (peak on the right side).

3. AIMS OF THE RESEARCH

The specific aims of this study were:

1. To develop primers for IL-10, TGF-β and Foxp3 and optimize quantitative real time RT-PCR in order to analyze their expression in peripheral blood mononuclear cells.

2. To apply the RT-PCR method for PBMC samples of the DIPP study and determine, if samples from children with viral infections differ from control samples in the expression of IL-10, TGF-β and Foxp3.

3. To apply the RT-PCR method for PBMC samples, cultured *in vitro* under different stimulative agents, in order to determine the effect of the stimuli on the expression of IL-10, TGF-β and Foxp3.

4. MATERIALS AND METHODS

4.1. Study subjects

4.1.1. DIPP samples

For the expression study of FOXP3, IL-10 and TGF-β and their connection to viral infections, PBMC-samples from children were obtained. These samples have been taken as a part of the DIPP (Diabetes Prediction and Prevention) project, which is a prospective type 1 diabetes study launched in 1994 in Finland. In this project, general population children are screened at birth for increased risk of type 1 diabetes. Individuals with genetic risk factors are invited to a follow-up study during which blood and stool samples are collected at regular intervals. Local institutional review boards and ethics committees have approved the DIPP study protocols and written informed consent has been given by the parents of the individuals participating in the study. (Kupila et al., 2001) Eighteen children were chosen for this expression study. They were divided into ten case subjects and eight control subjects. The case subjects had been given the oral polio vaccine (OPV), which is a live-attenuated vaccine, while the control subjects had received the inactivated polio vaccine (IPV), which contains inactivated polio virus. This OPV vaccine was given as a part of ENVAC trial which was carried out in context of DIPP study in 2000 and was based on separate informed consent and ethical approvals. In this trial children received OPV vaccines at the age of 2, 3, 6 and 12 months while control group received IPV at the age of 6 and 12 months. As a live attenuated vaccine OPV led to replication of polio vaccine virus strains in the recipients while IPV, as a killed vaccine, did not increase the exposure to live viruses. Several stool samples from all the children in the case group had been confirmed enterovirus-positive: (number of positive samples/number of samples taken) 9/20, 7/9, 5/17, 5/11, 5/18, 7/18, 5/19, 5/18, 8/11 and 9/18. Respective positive numbers for the children in the control group were the following: (number of positive samples/number of samples taken) 1/20, 1/18 and 2/18 and five children had no confirmed infections at all. From each child, 2-4 PBMC-samples were chosen, comprising a total of 56 samples.

The samples were taken at the age of 6, 9, 12 and 18 months, although for most children the samples from all four different sample collection points were not available.

4.1.2. Healthy volunteers

For the *in vitro* PBMC stimulation experiment and some control sample purposes, PBMC samples from healthy laboratory personnel were obtained.

4.1.3. Sample preparations

Peripheral blood mononuclear cells (PBMCs) were purified using the BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate (BD, NJ, USA) according to manufacturer's instructions.

The Vacutainer® CPT™ tube is an evacuated blood collection tube, in which a gel forms a barrier between the light plasma and mononuclear cells and the heavier blood components during centrifugation. (BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate handbook, BD).

Collected whole blood (4 ml for the DIPP study children and 8 ml for the *In vitro* PBMC stimulation experiment) was let to cool down for 15-20 minutes in the blood collection tube at room temperature. The blood sample was centrifuged at 2800 rpm for 20 minutes, after which the layer of mononuclear cells was suspended into the plasma and the suspension was transferred to a new tube. The sample was centrifuged again at 1700 rpm for 15 minutes and the plasma was removed almost entirely. The remaining plasma was used for resuspending the cell pellet. The DIPP study samples were divided in two cryotubes and snap frozen in liquid nitrogen. The tubes were kept in liquid nitrogen overnight and stored then at -70^{o}C. The *in vitro* PBMC stimulation experiment samples were not snap frozen but used freshly after washing the cells twice with RPMI 1640 (Gibco, Invitrogen, CA, USA). After this, further handling and stimulation followed immediately.

4.1.4. Cell culture and stimulation

For the *in vitro* PBMC stimulation experiment, cells were cultured for 48 hours or 5 days at +37 °C (5% CO_2) in round-bottom microtitre plate wells (Costar 96 well cell culture cluster, Corning Inc., NY, USA) using 200.000 cells per 100 µl per well. Culture medium contained 10% human serum, 1% penicillin, 1% streptomycin and 1% L-glutamin in RPMI 1640 and one of the following stimulants: Medium (control), infective enterovirus (coxsackievirus B4) (3 PFU/cell), highly purified and heat-treated CBV4 in 1,0 µg/ml concentation, dsRNA analog (TLR-3 agonist) poly(I:C) in 5µg/ml concentration (Alexis Biochemicals, Lausen), TLR7/8 agonist Resiquimod in 5 µg/ml concentration (Alexis Biochemicals), LPS from E.coli serotype J5 (Rc) as TLR4 agonist in 100 µg/ml concentration (Alexis Biochemicals) as well as combination of soluble anti-CD3 and anti-CD28 antibodies (R&D Systems, MN, USA) as polyclonal T-cell activator. Each of the different stimulations was performed in quadruplicate. After 48 hours or 5 days, cells were harvested and stored in buffer RLT, supplied with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) at -70°C.

4.1.5. RNA isolation

For the isolation of total mRNA from the samples, the RNeasy Mini Kit (Qiagen) was used according to manufacturer's instructions. With the DIPP samples, the lysis buffer (supplied with the RNeasy Mini Kit) was added directly to frozen samples in order to minimize RNA loss due to RNase activation. Lysate homogenization was carried out using Qiashredder spin columns (Qiagen). An additional on-column DNase digestion step was included in the RNA isolation protocol using the RNase-Free DNase Set (Qiagen) according to manufacturer's instructions.

4.1.6. Reverse transcription

The RNA was reverse transcribed to complementary DNA (cDNA) using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Promega, Madison, WI, USA). The reaction was primed by random hexamers (TAG Copenhagen A/S, Denmark). 10 µl of RNA eluate was incubated at 70°C for 5 min with 1,4 µl of random

hexamers. The mixture was cooled on ice and spinned briefly. To reach a final reaction volume of 25 µl, the following reagents were added to the incubation mixture: 5 µl of 5x enzyme buffer (Promega), 1,25 µl of 10 µM dNTP (Qiagen), 0,6 µl of Recombinant RNasin® Ribonuclease inhibitor (Promega), 1 µl of M-MLV reverse transcriptase and 5,75 µl of sterile H_2O. The reaction mixture was incubated at 37°C for 1 hour. The resulting cDNA was stored at -70°C or -20°C.

4.1.7. Quantitative real time PCR: optimization and protocol

For real time PCR assays, custom primers for Foxp3, IL-10 and TGF-β were designed with the help of a oligonucleotide properties calculator (available at <http://www.basic.northwestern.edu/biotools/oligocalc.html> 1.2.2008). Primers for the housekeeping gene TATA box binding protein (TBP) were from a previous publication (Isomäki et al., 2007) with a slight modification in the forward primer. Primer melting temperatures (T_m) were designed to be around 65°C, calculated by Finnzymes' T_m-calculator (available at <https://www.finnzymes.fi/tm_determination.html> 1.2.2008). However, the final melting temperature was usually lower, according to the manufacturer of the primers (TAG Copenhagen A/S, Denmark). The accepted product length was 200-300 bp. Whenever possible, a maximum of three cytosine or guanine nucleotides were included in the 3'-end of the primers. All primers were blasted (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> 1.2.2008). Each primer set had one primer spanning the exon-exon border, so that the risk of unwanted genomic DNA amplification would be minimized.

The PCR amplification was performed with the 7900 HT Fast real time PCR system (Applied Biosystems, Foster City, CA, USA). The 50 µl reactions were carried out using the DyNAmo Flash SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). The reaction mixture contained 25 µl of the master mix, 2 µl or 1,5 µl of forward and reverse primers (for 0,4 or 0,3 µM primer concentration, respectively), 1 µl of passive reference dye ROX and 15 µl or 16 µl of sterile water (for 0,4 or 0,3 µM primer concentration, respectively).

Table 1. Primer information for FOXP3, IL-10, TGF-β and TBP. T_m according to the primer manufacturer (TAG Copenhagen A/S, Denmark).

Name	Forward primer	T_m (^oC)	Product size (bp)
Foxp3 fwd	5'-ACA GCA CAT TCC CAG AGT TCC-3'	59,8	281
Foxp3 rev	5'-GAA CTC CAG CTC ATC CAC G-3'	58,8	
IL-10 fwd	5'-CAG TTT TAC CTG GAG GAG GTG-3'	59,8	236
IL-10 rev	5'-AGA TGC CTT TCT CTT GGA GCT TAT-3'	59,3	
TGF-β fwd	5'-ACG TGG AGC TGT ACC AGA AAT AC-3'	55,9	223
TGF-β rev	5'-GTA GTG AAC CCG TTG ATG TCC-3'	59,4	
TBP fwd	5'-CGA ATA TAA TCC CAA GCG GTT-3'	60,6	202
TBP rev	5'-ACT TCA CAT CAC AGC TCC CC-3'	59,8	

A 0,4 μM primer concentration was used for Foxp3 and IL-10, while a 0,3 μM concentration was adequate for TGF-β and TBP, based on primer concentration optimization trials, in which each primer pair was tested for optimal concentration (between 0,1-0,5 μM with 0,1 μM intervals) . The choice of optimal annealing temperature was based on trials with temperatures between 58-62^oC with intervals of two degrees. Temperature of 60 ^oC was chosen for all primers. Each sample was run in duplicate.

Thermal cycling conditions were the following:

1. Initial enzyme activation 95^oC 7 min
2. Denaturation 95^oC 10 s
3. Annealing/extension 60 ^oC 30s
4. Data acquisition 78 ^oC 30s

   - steps 2-4 repeated 40 times
5. Final extension 60 ^oC 1 min
6. Dissociation curve 60^oC-95^oC

The purpose of the data acquisition step was to nullify the fluorescent signal of possible primer dimers by raising the temperature above their T_m, so that the primer dimer signal would not interfere with the signal detection of the desired gene product. For data

analysis, the threshold cycle (Ct) values of Foxp3, IL-10 and TGF-β were normalized to the Ct –values of the endogenous control gene TBP. Relative expression values were calculated using the Pfaffl method as previously described (Pfaffl, 2001). For calculating the PCR efficiencies, six-point standard curves using two-fold serial dilutions of a pooled sample from healthy volunteers were included in each run. The standard curve samples were run in triplicate. The purity of the PCR products was monitored by analyzing the dissociation curves and by determining their size with agarose gel electrophoresis.

4.1.8. Statistical analysis

A two-tailed one-sample T-test was used to test whether the relative expression values of FOXP3, IL-10 or TGF-β in the DIPP samples differed from value 1 (value 1 means no difference in expression compared to the control samples). When comparing different age groups, oneway ANOVA test was used.

5. RESULTS

5.1 Real-time PCR optimization

5.1.1 Selection of primers

Primers for IL-10, TGF-β and Foxp3 were designed as described above. For IL-10, the first tested primer pair proved to work adequately. For TGF-β, seven pairs were tested and for Foxp3, four primer pairs were tested and the best one was chosen based on the lowest Ct-values and the dissociation curves with minimal primer dimer formation. The sequences of the chosen primers are shown in table 1. After the primer selection, optimization trials for best concentration and annealing temperature followed. The final reaction mixture and PCR protocol are discussed above.

5.1.2 Optimization of primer concentration and annealing temperature

Each primer pair concentration was tested for 0,1-0,5 μM concentrations with 0,1 μM intervals. The best concentration was chosen based on the lowest Ct-values and the lack of formation of primer dimers: Higher concentration often gives better Ct-values, but sometimes comes with unwanted primer dimer formation. The Ct-values of these experiments are shown in table 2. Three samples from healthy volunteers were used for each concentration, and each sample run in duplicate. For Foxp3, only two samples per concentration were taken into account, because of a PCR failure. Samples were, nevertheless, run in duplicate.

When Ct-values and dissociation curve analysis were taken into account, a concentration of 0,4 μM was chosen for Foxp3 and IL-10. Concentration of 0,3 μM was best for TBP and TGF-β.

Table 2. Average Ct-values of concentration optimization trial runs.

Gene	0,1 µM	0,2 µM	0,3 µM	0,4 µM	0,5 µM
Foxp3	30,2	27,2	26,7	26,4	26,0
IL-10	31,3	28,4	28,9	27,4	27,6
TGF-β	20,3	17,7	16,8	16,5	16,3
TBP	29,3	25,3	24,2	23,8	23,7

After the optimization of concentrations, optimal annealing temperature was determined. For all primers, temperatures of 58 °C, 60 °C and 62 °C were tested to see, if the temperature had any effect on the Ct-values. The results are shown in table 3. Samples used in these trials were obtained from healthy volunteers, and their number varied between 1-5 (2 samples for Foxp3 at all three temperatures, 3 samples for TGF-β at all temperatures and IL-10 and TBP at 60°C, 2 samples for IL-10 and TBP at 58 °C , 5 samples for TBP at 62°C and 1 sample for IL-10 at 62°C). All samples were run in duplicate.

Table 3. Average Ct-values of annealing temperature optimization trial runs.

Gene	Annealing temperature 58 °C	Annealing temperature 60 °C	Annealing temperature 62 °C
Foxp3	25,4	26,4	25,7
IL-10	28,1	27,4	28,7
TGF-β	16,9	16,8	16,6
TBP	23,9	24,2	24,2

The recommendation of the DyNAmo Flash SYBR® Green qPCR Kit manufacturer (Finnzymes, Espoo, Finland) was to use 60 °C annealing/extension temperature, and as the different temperatures all gave quite good results, this temperature was chosen for the final protocol.

5.1.3. Evaluation of PCR efficiency

To calculate PCR efficiency for each primer pair, it is necessary to include a dilution series of a standard sample in each run. Standard samples in this study were obtained from healthy volunteers. The efficiencies were first tested by using a six-point dilution series of 10^{-n}, and plotting the dilution factor against the Ct-values. The resulting line should be straight with $r > 0.95$. This dilution factor, however, did not result in a straight line for the Ct-values of Foxp3 and IL-10, as the Ct-values plateued after a couple of dilutions, implying that the assay was not sensitive enough to detect very small amounts of DNA. Because of this, it was decided to use 2^{-n} dilution series for all primer pairs, which gave appropriate straight lines of Ct-values with correlation coefficients of 0.95 or greater.

5.1.4 Genomic DNA contamination

The possible amplification of genomic DNA was tested by using RNA samples in the PCR run with and without DNase treatment during the RNA isolation (see section 4.1.5.). However, DNase treatment was decided to be used as a precaution in every run, despite the negative results of genomic DNA amplification.

5.2. DIPP study

Foxp3 and immunoregulatory cytokines such as IL-10 and TGF-β are expressed by regulatory T-cells, which play a major role in immune regulation. It is possible that the activation of these cells could be mediated by viral infections. Therefore, it was interesting to study, whether enterovirus infections had any effect on the expression of these genes at mRNA level. For each different analysis, case subjects with several enterovirus-positive stool samples were compared to control subjects with only a few or no observed infections. In order to obtain relative expression values, for each case subject selected for the comparison, a control subject was chosen from the same PCR run and age group.

5.2.1. An enterovirus infection less than 45 days before sample collection does not induce Foxp3, IL-10 or TGF-β expression

Nine samples from case subjects had been collected within 45 (n=2) or 30 days (n=7) after the observed enterovirus-positive stool sample. For IL-10, only eight samples could be taken into the study because of a failure at some point of the sample preparation or PCR. No significant increase or decrease in the expression levels of Foxp3, IL-10 or TGF-β was observed in these samples when compared control subjects in the same PCR run and age group (Figures 5-7). The difference of relative expression values from value 1 were not statistically significant ($p>0,05$) – value 1 means no difference in expression compared to the control samples – in a two-tailed one-sample T-test.

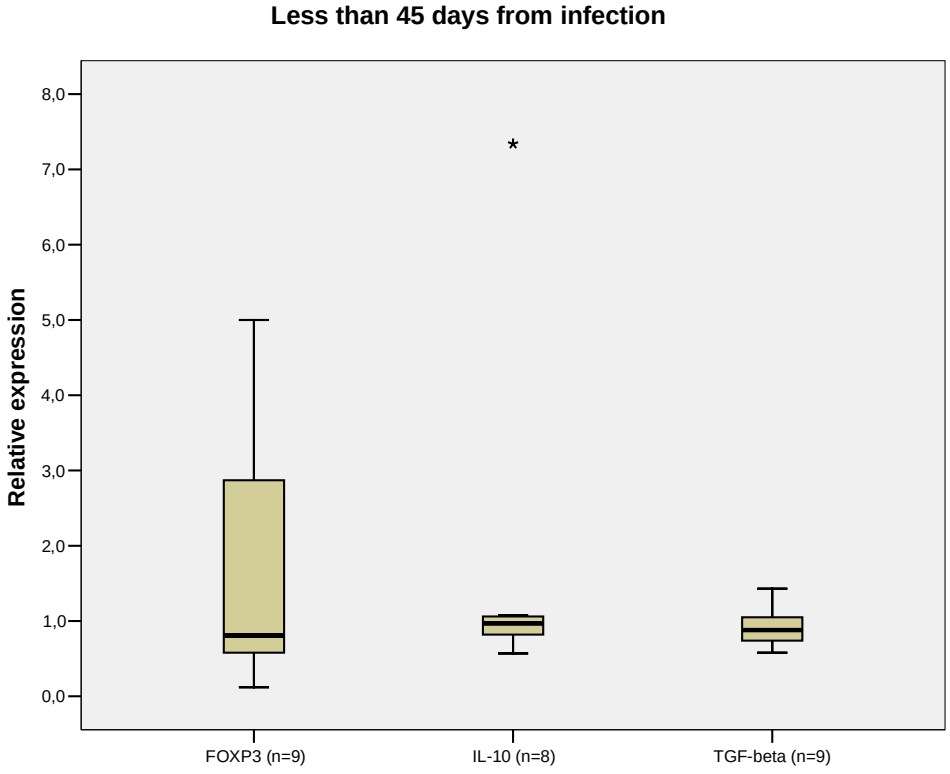

Figure 5. Relative expression values of Foxp3, IL-10 and TGF-β, less than 45 days from infection. Each case subject compared to a control subject in the same PCR run and age group. No significant increase or decrease in expression is observed.

5.2.2. The expression of FOXP3, IL-10 and TGF-β is not different between age groups

Older children tend to have had more enterovirus infections than younger children. It was studied, if children at different age groups (9, 12 and 18 months of age at the time of sample collection) would express Foxp3, IL-10 and TGF-β differently. Again, for each case subject with several enterovirus infectios a control subject with no infections was matched from the same age group and PCR run. Due to some problems with sample preparation, the number of samples included in this study was not equal for each gene. For Foxp3 and age group of 9 months, n=4, 12 months n=6 and for 18 months n=7. For IL-10 and age group of 9 months, n=4, 12 months n=6 and for 18 months n=8, and for TGF-β and age group of 9 months, n=5, 12 months n=6 and for 18 months n=7. The relative expression of the genes in different age groups was compared using oneway ANOVA test. No difference of statistical significance was observed between different age groups (p>0,05) (Figures 6-8). However, a larger group of observations might have resulted in statistical significance with IL-10, the expression of which decreased with increasing age (p=0,089).

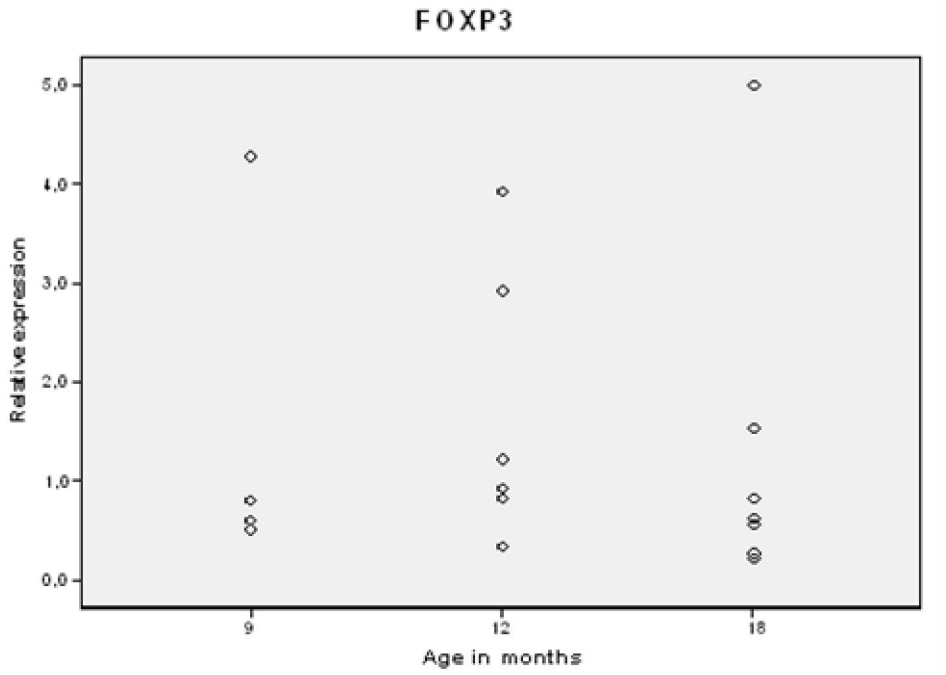

Figure 6. Relative expression values of Foxp3. Subjects divided into three age groups (9, 12 and 18 months). No statistically significant difference between groups. Mean value for 9-month group was 1,56; for 12-month group 1,70 and for 18-month group 1,29.

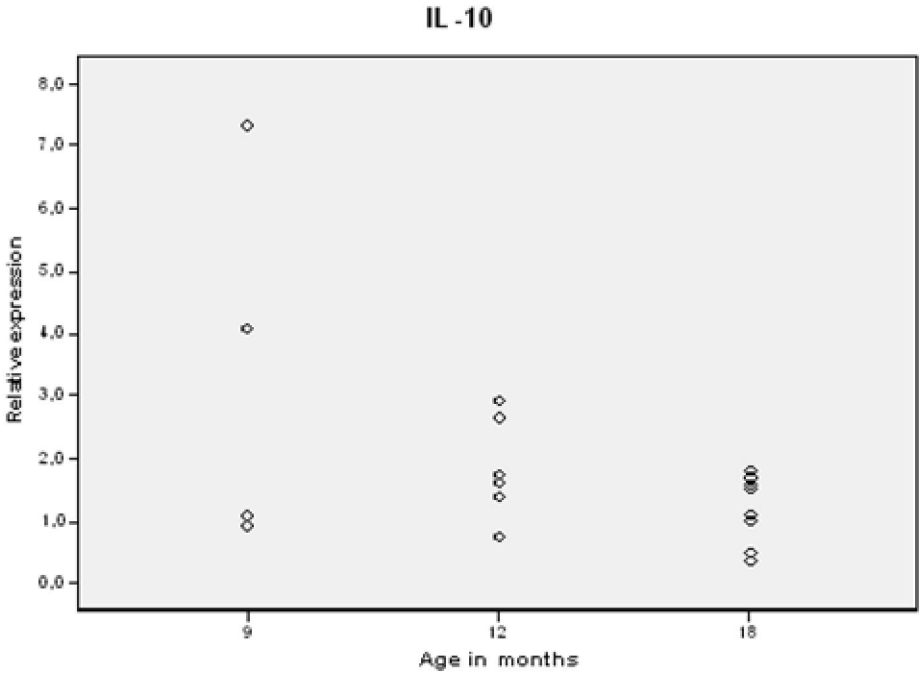

Figure 7. Relative expression values of IL-10. Subjects divided into three age groups (9, 12 and 18 months). No statistically significant difference between groups. Mean value for 9-month group was 3,35; for 12-month group 1,84 and for 18-month group 1,19.

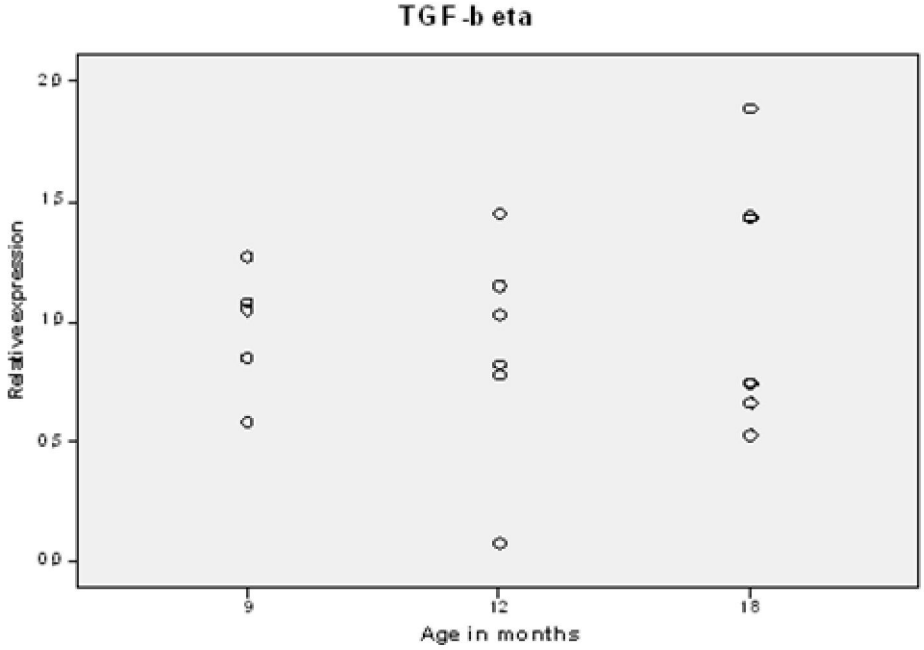

Figure 8. Relative expression values TGF-β. Subjects divided into three age groups (9, 12 and 18 months). No statistically significant difference between groups. Mean value for 9-month group was 0,97; for 12-month group 0,89 and for 18-month group 1,06.

5.2.3. IL-10 is upregulated in children with several enterovirus infections

It was also interesting to see, if there was any difference in the overall expression of Foxp3, IL-10 and TGF-β between the case group (with several enterovirus infections) and the control group (with no infections), when all subjects were taken into account without taking into consideration the timing of infections (recent or not) or age groups. Altogether, 18, 20 and 19 samples were taken into the study for Foxp3, IL-10 and TGF-β, respectively, and each case sample was matched with a suitable control sample from a child of the same age in the same PCR run. A two-tailed one-sample T-test was used to determine, whether the relative expression values of Foxp3, IL-10 or TGF-β in the samples differed from value 1 (value 1 means no difference in expression compared to the control samples). The expression of Foxp3 and TGF-β was not significantly up- or downregulated ($p>0,05$) while IL-10 was found to be upregulated ($p=0,031$) (Figure 9). This suggests that several enterovirus infections could affect IL-10 expression.

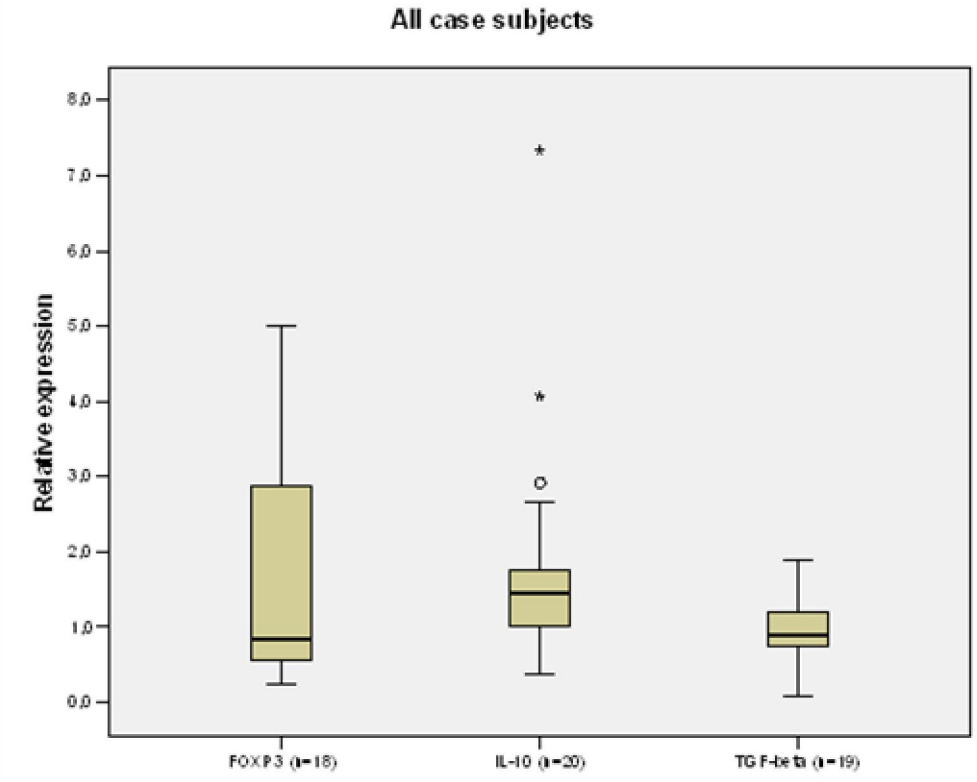

Figure 9. Relative expression of Foxp3, IL-10 and TGF-β, all case subjects. IL-10 is upregulated, (p=0,031). Mean value for Foxp3was 1,57 and SD 1,52; for IL-10 mean value was 1,82 and SD 1,56 and for TGF-β mean value was 0,97 and SD 0,41.

It was also interesting to see, if there was any difference in the overall expression of Foxp3, IL-10 and TGF-β between the case group (with several enterovirus infections) and the control group (with no infections), when all subjects were taken into account without taking into consideration the timing of infections (recent or not) or age groups. Altogether, 18, 20 and 19 samples were taken into the study for Foxp3, IL-10 and TGF-β, respectively, and each case sample was matched with a suitable control sample from a child of the same age in the same PCR run. A two-tailed one-sample T-test was used to determine, whether the relative expression values of Foxp3, IL-10 or TGF-β in the samples differed from value 1 (value 1 means no difference in expression compared to the control samples). The expression of Foxp3 and TGF-β was not significantly up- or downregulated ($p>0,05$) while IL-10 was found to be upregulated ($p=0,031$) (Figure 9). This suggests that several enterovirus infections could affect IL-10 expression.

5.2.4. When compared to the first sample of an individual subject, the expression of Foxp3, IL-10 or TGF-β shows no clear pattern between case and control subjects

The last comparison of Foxp3, IL-10 and TGF-β expression was done by using the first sample taken from each study subject as a control sample. The relative expression values are given as multiples of the first sample, which always receives the value 1. All case subjects (n=10) and control subjects (n=8) were included, except for Foxp3, for which one control subject had to be excluded because of a PCR failure.

The relative expression of Foxp3 in case subjects did not follow any clear pattern between samples taken at a younger or older age. In some children, the expression increased and in some children it decreased. However, in the control group, the expression values were all lower in the samples taken at an older age (Figures 10 and 11, table 4).

IL-10 relative expression values show no clear difference between case and control subjects. In both groups, there are children with increasing and decreasing values. It seems that the expression decreases with age more often than increases (Figures 12 and 13, table 5).

TGF-β expression seems to stay quite the same, regardless of age. There are no obvious differences between case and control groups. In both groups, the 18 month sample seems to have a slightly increased expression in many subjects, but there are subjects whose 18 month shows a decreased expression as well. (Figures 14 and 15, table 6).

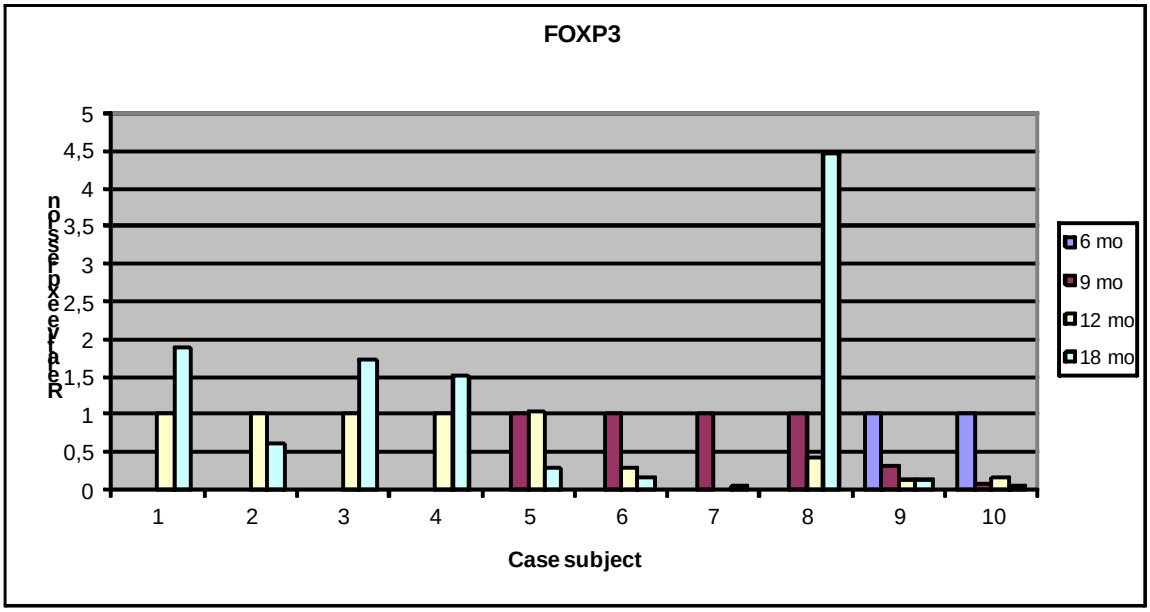

Figure 10. The variation of Foxp3 relative expression by age, first sample of a subject used as a control. The case subjects.

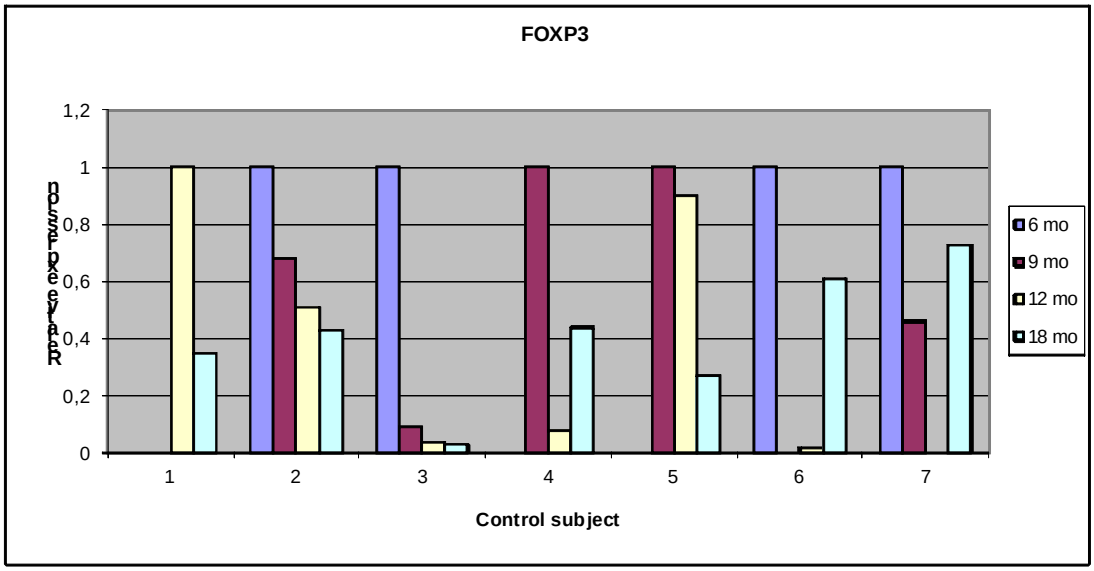

Figure 11. The variation of Foxp3 relative expression by age, first sample of a subject used as a control. The control subjects.

Table 4. Mean and standard deviation values of Foxp3 expression by age, first sample of a subject used as a control.

Gene/group	6 mo mean/SD	9 mo mean/SD	12 mo mean/SD	18 mo mean/SD
Foxp3/Case	-	0,21/0,16 (n=2)	0,41/0,37 (n=5)	1,09/1,39 (n=10)
Foxp3/Control	-	0,41/0,30 (n=3)	0,31/0,39 (n=5)	0,41/0,23 (n=7)

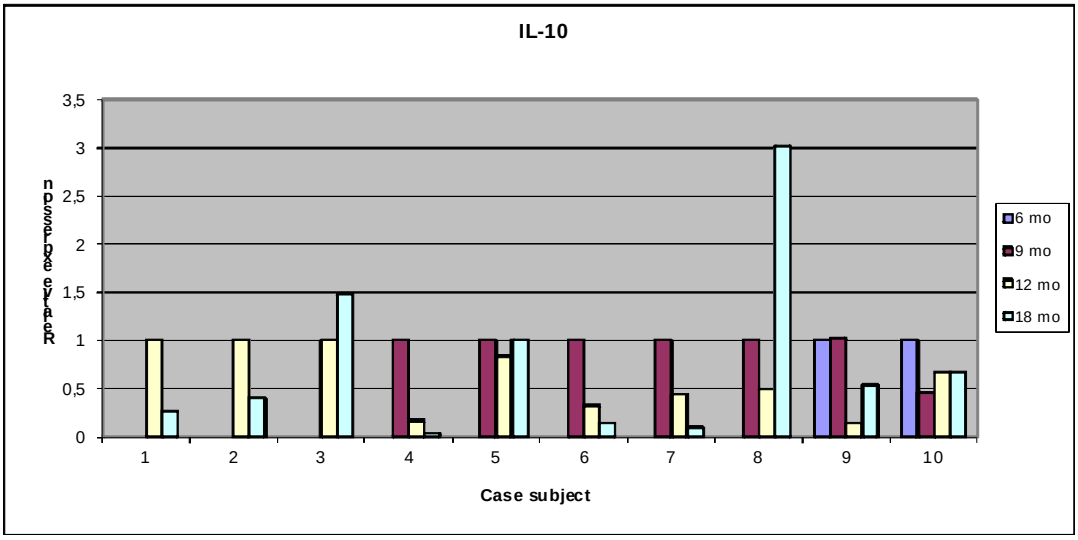

Figure 12. The variation of IL-10 relative expression by age, first sample of a subject used as a control. The case subjects.

Table 5. Mean and standard deviation values of IL-10 expression by age, first sample of a subject used as a control.

Gene/group	6 mo mean/SD	9 mo mean/SD	12 mo mean/SD	18 mo mean/SD
IL-10/Case	-	0,75/0,40 (n=2)	0,44/0,26 (n=7)	0,77/0,91 (n=10)
IL-10/Control	-	0,64/0,38 (n=3)	0,47/0,52 (n=5)	1,13/1,52 (n=8)

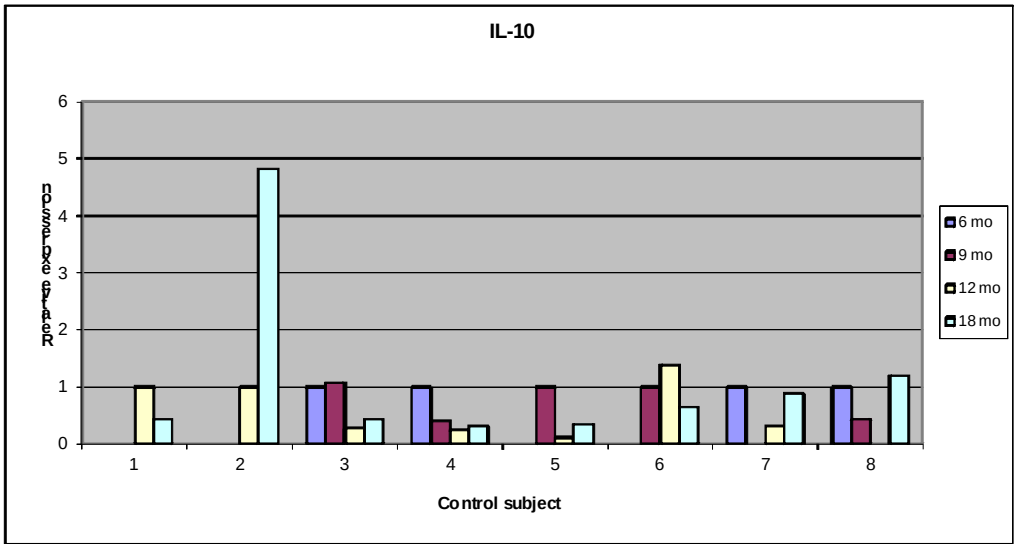

Figure 13. The variation of IL-10 relative expression by age, first sample of a subject used as a control. The control subjects.

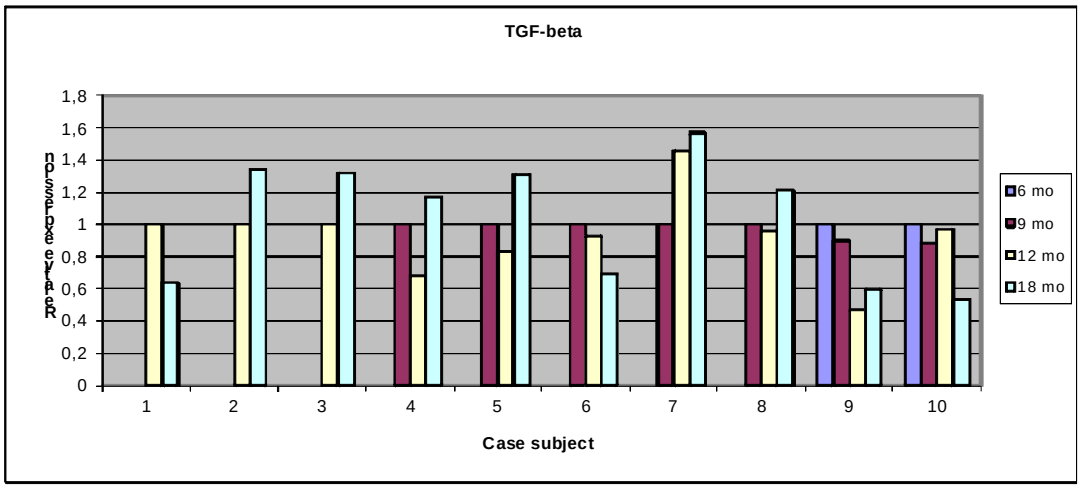

Figure 14. The variation of TGF- β relative expression by age, first sample of a subject used as a control. The case subjects.

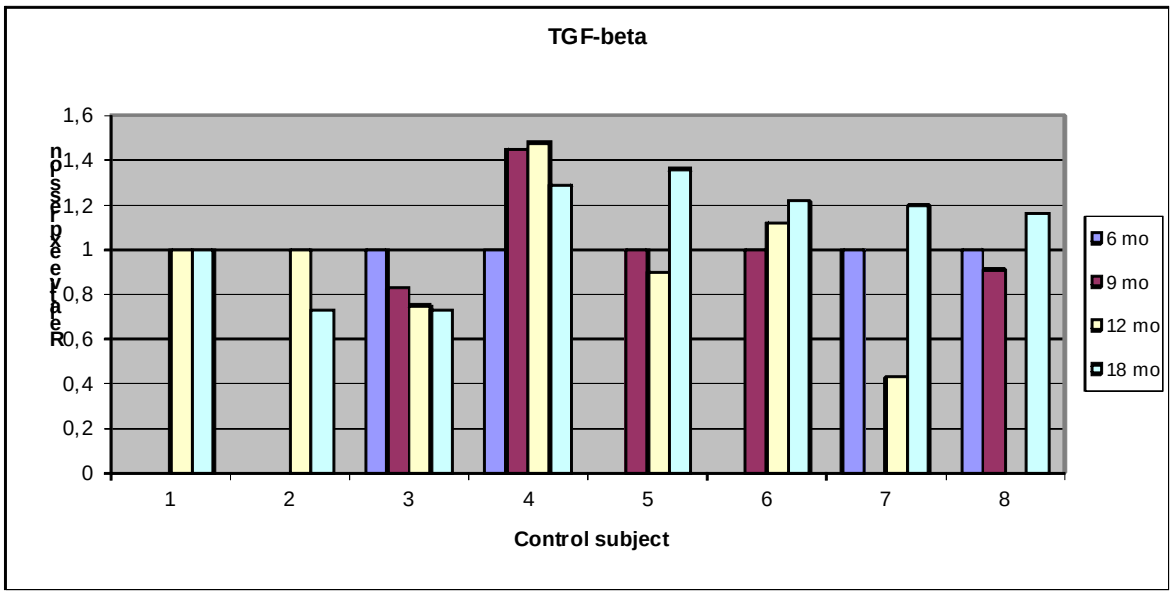

Figure 15. The variation of TGF-β relative expression by age, first sample of a subject used as a control. The control subjects.

Table 6. Mean and standard deviation values of TGF-β expression by age, first sample of a subject used as a control.

Gene/group	6 mo mean/SD	9 mo mean/SD	12 mo mean/SD	18 mo mean/SD
TGF-β /Case	-	0,89/0,01 (n=2)	0,9/0,31 (n=7)	1,04/0,38 (n=10)
TGF-β /Control	-	1,06/0,34 (n=3)	0,94/0,40 (n=5)	1,09/0,24 (n=8)

5.3. The *in vitro* –PBMC stimulation experiment

The purpose of this experiment was to analyse if enterovirus can stimulate these peripheral blood mononuclear cells *in vitro* by exposing these cells to infective enterovirus or heat-treated purified enteroviruses. Virus-induced response was also compared to that obtained with various TLR-agonists (dsRNA analog poly(I:C) which mimics viral RNA, Resiquimod, LPS from E.coli) and polyclonal T-cell activator (mixture of monoclonal anti-CD3 and anti-CD28 antibodies). The cells were incubated in the presence of these stimulants for 48 hours or 5 days. After this, the cells were harvested and the relative expression values of Foxp3, IL-10 and TGF-β were obtained.

5.3.1. The *in vitro* –PBMC stimulation experiment results

Exposure of mononuclear cells to infective CBV4 led to a clear (3,3 fold) increase in the expression of Foxp3 specific mRNA (Figure 16) and a 3,6 fold increase in IL-10 specific mRNA compared to mock-treated cultures (Figure 17) during the 48 hour stimulation. Similarly, heat-treated CBV4 led to a 1,9 fold increase in Foxp3 mRNA and a 3,4 fold-increase in IL-10 mRNA. Poly(I:C) did not induce either Foxp3 or IL-10 mRNA while anti-CD28/anti-CD3 combination induced the expression of both Foxp3 and IL-10 (8,1 and 2,61 fold increases, respectively). Resiquimod and LPS did not induce Foxp3 expression but caused a clear increase in IL-10 expression. During the 5-day experiment, only the anti-CD28/anti-CD3 combination was able to induce Foxp3 expression. IL-10 expression, however, showed a decrease under the influence of every stimulant, when the stimulation time was extended. In the 48-hour experiment, TGF-β expression in the cells exposed to different stimulants was roughly half of that of untreated cells, with the only exception being poly(I:C), which only caused a 0,8-fold decrease in the expression (Figure 18). The results were similar during the incubation of five days, but poly(I:C) and anti-CD28/anti-CD3 did not influence the TGF-β expression any more.

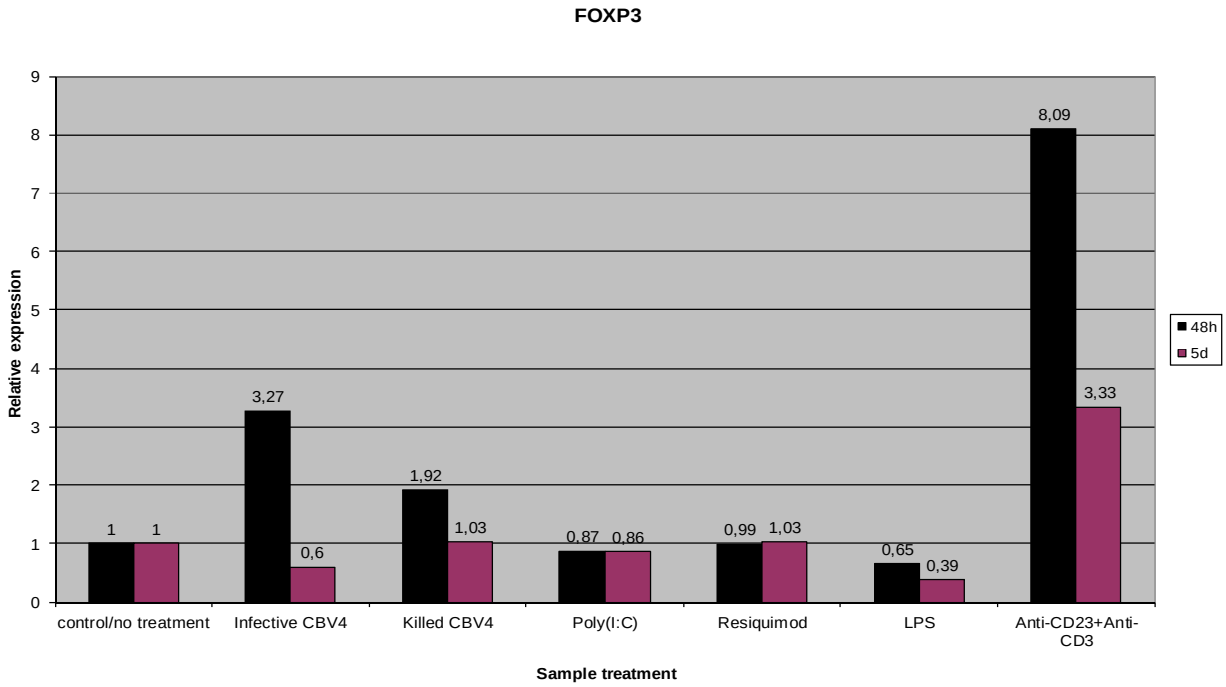

Figure 16. The relative expression of Foxp3, compared to control samples.

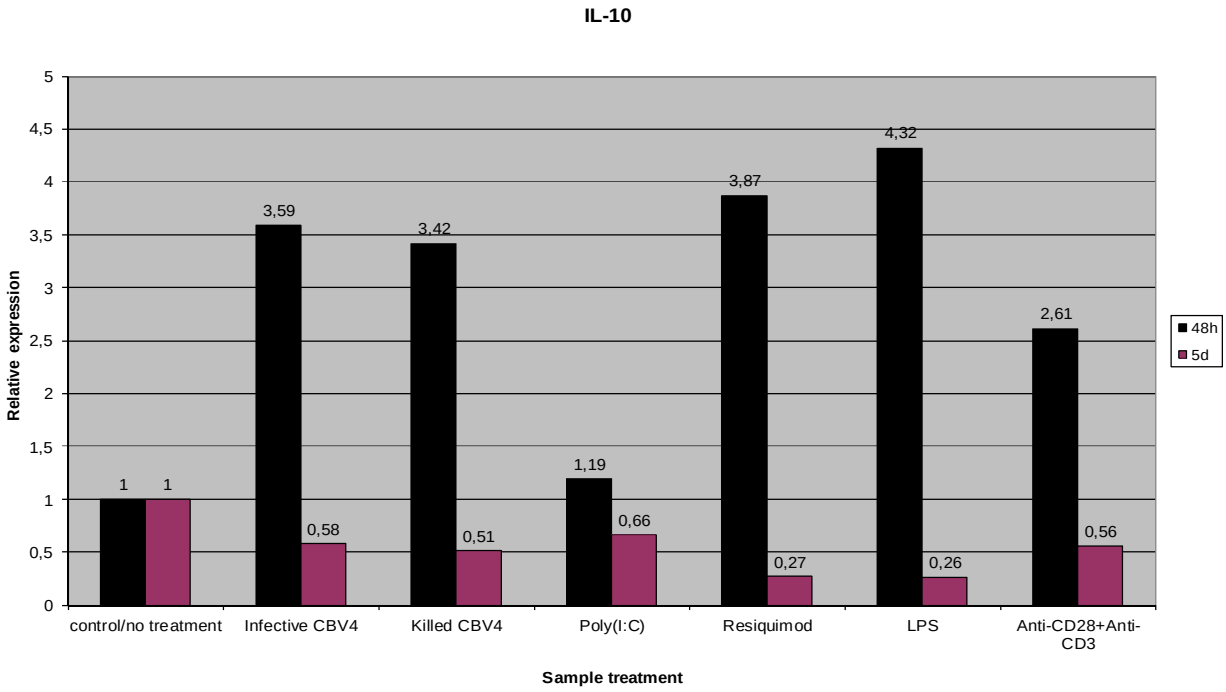

Figure 17. The relative expression of IL-10, compared to control samples.

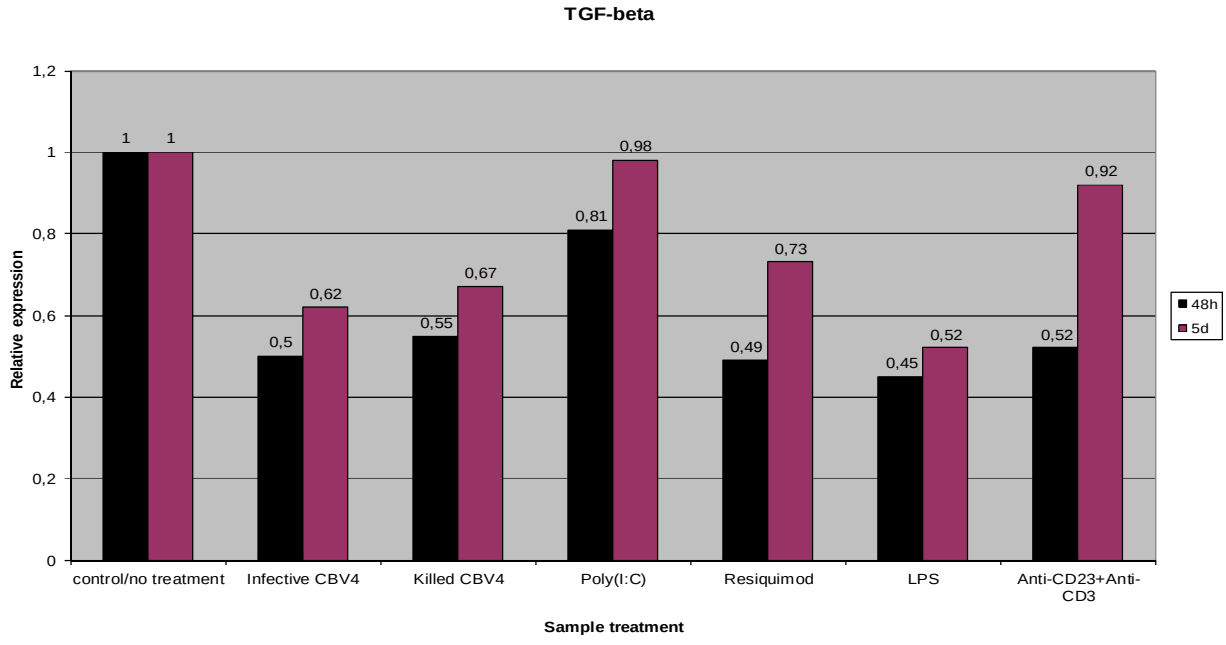

Figure 18. The relative expression of TGF-β, compared to control sample.

6. DISCUSSION

The most important goal of this study was to develop a real-time PCR assay for the detection of Foxp3, IL-10 and TGF-β expression in peripheral blood mononuclear cells. The assay was tested in a small-scale study of the expression of these genes in the DIPP study children and in an *in vitro* –PBMC stimulation experiment. In this chapter, the success of the development process will be evaluated, and the DIPP study and PBMC stimulation experiment results discussed.

6.1. The real-time PCR assay

Primers for Foxp3, IL-10 and TGF-β were designed and tested as described in preceding sections. Because it was known that possible primer-dimer formation could distort the PCR results, special attention was paid to design the primers so that there would be no complementary regions between the reverse and forward primers. The optimization of primer concentration was also performed keeping this in mind. However, primer dimer formation was nevertheless often observed in water control samples of Foxp3, IL-10 and TBP. This can be expected, as the amplification of primer-dimers and the actual product is a competing process. Indeed, in the cDNA samples primer-dimer formation was not usually seen on the dissociation curve. An additional data acquisition step in a temperature above the melting temperature of the primer-dimers was included in every PCR cycle to ensure that the possible primer-dimers were not affecting the results.

The reverse and forward primers were designed so that at least one of them spanned an exon-exon border. As genomic DNA contains introns between the exons, it would be unlikely, that exon-exon border –spanning primers amplified genomic DNA. To make sure that there was absolutely no contamination from genomic DNA at all, a DNase digestion step was routinely performed during RNA isolation, even though test PCR runs with RNA samples were usually negative for the amplification of genomic DNA. Considering these precautions, it is reasonable to assume the PCR assay to be reliable in terms of amplifying only the product of interest.

All primer pairs were able to amplify DNA product from cDNA samples obtained from peripheral blood mononuclear cells. However, especially Foxp3 and IL-10 assays were not sensitive enough to properly amplify target sequences in dilute samples (dilution factors of 10^{-n}), but in undiluted samples the sensitivity was adequate. PCR efficiencies were determined using 2^{-n} dilution series, and this enabled proper construction of standard curves.

The efficiency of PCR amplification is usually not ideal, because there are variations in the reagents and biological samples often contain PCR inhibitors that negatively affect the efficiency (Kubista et al., 2006). That is why it is important to use the Pfaffl method that takes into account the variation in the efficiencies in both reference and target genes, as well as the possible differences in the expression of the reference gene in different samples. However, not even this method is perfect as it is very impractical to determine the efficiency of amplification for every sample individually. Thus, the efficiency determination can be considered a mere approximation.

6.2 Effect of enterovirus on immune regulation in prospectively followed children

The greatest limitation of the DIPP study was its small number of samples, which made it very hard to see any patterns in the expression of the genes of interest. Some samples also showed great variation. Overall, there did not seem to be any correlation between recent infections and gene expression of Foxp3, IL-10 and TGF-β, nor was the expression different between age groups. However, IL-10 was slightly upregulated in children who had had several infections, when compared to the control group with no infections. This is in line with the literature, as microbial and parasitical infections are known to induce IL-10 secretion (Belkaid 2008). However, when the expression of each gene was studied as a function of age by setting the sample taken at the youngest age as a control, there was no clear pattern of increasing or decreasing expression. Foxp3 expression seemed to decrease by age in the group of children with no infections, which would be logical for the same reason as IL-10 expression would be expected to increase in children with infections – infectious agents possibly modulate the maturation of the

immune system by affecting the cytokine expression profile of leukocytes (Bach 2002). As Foxp3 is connected with regulatory T cell differentiation and function (Hori et al., 2003), it would not be surprising if the number of infections affected its expression too.

The interpretation of TGF-β mRNA levels is not straightforward, as TGF-β is a subject to post-transcriptional and/or post-translational regulation and mRNA levels do not necessarily correlate with protein secretion. (Li et al., 2006) Therefore, the results of TGF-β expression in this study may not tell much about the actual biological activity of TGF-β as a function of exposure to viral infections. During the time this study was made, the lack of correlation of mRNA and secreted protein levels was unclear, and therefore protein secretion measurements were not included in the study.

6.3 The *in vitro* –PBMC stimulation experiment

In this experiment, peripheral blood mononuclear cells were exposed to infective and heat-killed enteroviruses, TLR-agonists (dsRNA analog poly(I:C) which mimics viral RNA, Resiquimod, which is a TLR7/8 agonist, and LPS from E.coli) and polyclonal T-cell activator (mixture of monoclonal anti-CD3 and anti-CD28 antibodies) during a stimulation period of 48 hours or 5 days.

The results show that enteroviruses are able to activate regulatory T-cells. Enterovirus-induced activation was comparable to that obtained using a strong polyclonal T-cell activator (anti-CD28/anti-CD3 mixture) supporting the biological relevance of this phenomenon. The induced expression of Foxp3 and IL-10, which are involved in the induction of regulatory and suppressive Treg activity, can be seen as a positive evidence of the hygiene hypothesis. Enterovirus was able to induce stronger Foxp3 expression than the three classical TLR agonists, which may reflect the complexity of Treg activation. Enterovirus-induced IL-10 expression was as strong as TLR4 and TLR7/8 agonists (LPS and Resiquimod, respectively). During the five-day incubation in the presence of stimulants, the expression of IL-10 decreased below the level of the mock-treated sample. In the case of TLR-agonist-stimulated samples, this may be due to the nature of toll-like receptors, as the act in the early innate immune responses.

The expression of TGF-β decreased as a result of the stimulation, but unlike IL-10 expression, when the incubation time was extended, the expression of TGF-β increased when compared to the 48-hour experiment. However, as the TGF-β mRNA levels may not correlate with the amount of active protein secreted, the relevance of these results remains unclear and further studies are needed to evaluate the significance of this pehenomenon.

6.4 Future aspects

The real-time PCR assays developed in this study can be used in the future to determine Foxp3, IL-10 and TGF-β mRNA expression in peripheral blood mononuclear cell samples. The assays may be used in the future in the analysis of DIPP samples and in different studies that aim at identifying the role and actions of regulatory T cells in infections, allergy and autoimmune diseases.

7. CONCLUSIONS

The aim of this study was to develope a real-time PCR method suitable for detecting immunoregulatory cytokines IL-10 and TGF-β and transcription factor Foxp3 from human peripheral blood mononuclear cells. The assays were to be tested in a small study involving samples from children of the DIPP study and in an *in vitro* -PBMC stimulation experiment. The final goal of the study was to create reliable assays for future studies of the roles of these genes in infections and immunological disorders, as they often are accompanied by an altered expression of these genes. This study was succesful at developing the real-time PCR assays and they are ready for future applications.

8. REFERENCES

Decreased prevalence of atopic diseases in children with diabetes. The EURODIAB Substudy 2 Study Group. J Pediatr 2000;137:470-4.

Adlerberth I, Strachan DP, Matricardi PM, et al. Gut microbiota and development of atopic eczema in 3 European birth cohorts. J Allergy Clin Immunol 2007;120:343-50.

Afkarian M, Sedy JR, Yang J, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. Nat Immunol 2002;3:549-57.

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ and Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003;278:1910-4.

Akdis CA and Akdis M. Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. J Allergy Clin Immunol 2009;123:735,46; quiz 747-8.

Akdis CA, Blesken T, Akdis M, Wuthrich B and Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102:98-106.

Akira S, Takeda K and Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675-80.

Allen JB, Manthey CL, Hand AR, Ohura K, Ellingsworth L and Wahl SM. Rapid onset synovial inflammation and hyperplasia induced by transforming growth factor beta. J Exp Med 1990;171:231-47.

Alvaro T, Lejeune M, Salvado MT, et al. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 2005;11:1467-73.

Anderson CF, Oukka M, Kuchroo VJ and Sacks D. CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. J Exp Med 2007;204:285-97.

Annacker O, Asseman C, Read S and Powrie F. Interleukin-10 in the regulation of T cell-induced colitis. J Autoimmun 2003;20:277-9.

Annes JP, Chen Y, Munger JS and Rifkin DB. Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. J Cell Biol 2004;165:723-34.

Annes JP, Munger JS and Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci 2003;116:217-24.

Ansel KM, Djuretic I, Tanasa B and Rao A. Regulation of Th2 differentiation and Il4 locus accessibility. Annu Rev Immunol 2006;24:607-56.

Asseman C, Mauze S, Leach MW, Coffman RL and Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 1999;190:995-1004.

Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-20.

Bach JF and Chatenoud L. Tolerance to islet autoantigens in type 1 diabetes. Annu Rev Immunol 2001;19:131-61.

BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate For the Separation of Mononuclear Cells from Whole Blood, handbook from BD, pp. 1-2, 2001

Belkaid Y. Role of Foxp3-positive regulatory T cells during infection. Eur J Immunol 2008;38:918-21

Belkaid Y, Piccirillo CA, Mendez S, Shevach EM and Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature 2002;420:502-7.

Belkaid Y and Rouse BT. Natural regulatory T cells in infectious disease. Nat Immunol 2005;6:353-60.

Bennett CL and Ochs HD. IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. Curr Opin Pediatr 2001;13:533-8.

Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235-8.

Bieber T. Atopic dermatitis. N Engl J Med 2008;358:1483-94.

Billiard F, Litvinova E, Saadoun D, et al. Regulatory and effector T cell activation levels are prime determinants of in vivo immune regulation. J Immunol 2006;177:2167-74.

Bjorksten B, Naaber P, Sepp E and Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy 1999;29:342-6.

Blanchette F, Rivard N, Rudd P, Grondin F, Attisano L and Dubois CM. Cross-talk between the p42/p44 MAP kinase and Smad pathways in transforming growth factor beta 1-induced furin gene transactivation. J Biol Chem 2001;276:33986-94.

Blander JM and Medzhitov R. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature 2006;440:808-12.

Bluestone JA and Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003;3:253-7.

Bluestone JA and Tang Q. How do CD4+CD25+ regulatory T cells control autoimmunity?. Curr Opin Immunol 2005;17:638-42.

Bodansky HJ, Staines A, Stephenson C, Haigh D and Cartwright R. Evidence for an environmental effect in the aetiology of insulin dependent diabetes in a transmigratory population. BMJ 1992;304:1020-2.

Bopp T, Becker C, Klein M, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J Exp Med 2007;204:1303-10.

Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J and Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. J Allergy Clin Immunol 1996;97:1288-96.

Braun-Fahrlander C, Riedler J, Herz U, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;347:869-77.

Cabrera R, Tu Z, Xu Y, et al. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. Hepatology 2004;40:1062-71.

Cao X, Cai SF, Fehniger TA, et al. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. Immunity 2007;27:635-46.

Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M and Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med 2003;197:403-11.

Cederbom L, Hall H and Ivars F. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. Eur J Immunol 2000;30:1538-43.

Chang H, Brown CW and Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 2002;23:787-823.

Chen CH, Seguin-Devaux C, Burke NA, et al. Transforming growth factor beta blocks Tec kinase phosphorylation, Ca2+ influx, and NFATc translocation causing inhibition of T cell differentiation. J Exp Med 2003;197:1689-99.

Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. Proc Natl Acad Sci U S A 2005;102:419-24.

Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003;198:1875-86.

Cikos S and Koppel J. Transformation of real-time PCR fluorescence data to target gene quantity. Anal Biochem 2009;384:1-10.

Coffman RL and Carty J. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. J Immunol 1986;136:949-54.

Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007;450:566-9.

Commins S, Steinke JW and Borish L. The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. J Allergy Clin Immunol 2008;121:1108-11.

Cottrez F and Groux H. Regulation of TGF-beta response during T cell activation is modulated by IL-10. J Immunol 2001;167:773-8.

Cua DJ, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003;421:744-8.

Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942-9.

Eder W, Ege MJ and von Mutius E. The asthma epidemic. N Engl J Med 2006;355:2226-35.

Fahlen L, Read S, Gorelik L, et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J Exp Med 2005;201:737-46.

Fallarino F, Grohmann U, Hwang KW, et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 2003;4:1206-12.

Fillatreau S, Sweenie CH, McGeachy MJ, Gray D and Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol 2002;3:944-50.

Finkelman FD, Morris SC, Orekhova T, et al. Stat6 regulation of in vivo IL-4 responses. J Immunol 2000;164:2303-10.

Fontenot JD, Gavin MA and Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-6.

Frucht DM, Aringer M, Galon J, et al. Stat4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. J Immunol 2000;164:4659-64.

Gale EA. A missing link in the hygiene hypothesis?. Diabetologia 2002;45:588-94.

Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 1998;16:495-521.

Gavin MA, Rasmussen JP, Fontenot JD, et al. Foxp3-dependent programme of regulatory T-cell differentiation. Nature 2007;445:771-5.

Ghiringhelli F, Menard C, Terme M, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med 2005;202:1075-85.

Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. Science 2006;312:1355-9.

Gondek DC, Lu LF, Quezada SA, Sakaguchi S and Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol 2005;174:1783-6.

Gorelik L, Fields PE and Flavell RA. Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. J Immunol 2000;165:4773-7.

Gorelik L and Flavell RA. Transforming growth factor-beta in T-cell biology. Nat Rev Immunol 2002;2:46-53.

Green A, Patterson CC and EURODIAB TIGER Study Group. Europe and Diabetes. Trends in the incidence of childhood-onset diabetes in Europe 1989-1998. Diabetologia 2001;44 Suppl 3:B3-8.

Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP and Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood 2004;104:2840-8.

Guo L, Wei G, Zhu J, et al. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. Proc Natl Acad Sci U S A 2009;106:13463-8.

Hammond SR, English DR and McLeod JG. The age-range of risk of developing multiple sclerosis: evidence from a migrant population in Australia. Brain 2000;123 (Pt 5):968-74.

Harjutsalo V, Sjoberg L and Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet 2008;371:1777-82.

Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123-32.

Hartl D, Koller B, Mehlhorn AT, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol 2007;119:1258-66.

Hawrylowicz CM and O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol 2005;5:271-83.

Hempel K, Freitag A, Freitag B, Endres B, Mai B and Liebaldt G. Unresponsiveness to experimental allergic encephalomyelitis in Lewis rats pretreated with complete Freund's adjuvant. Int Arch Allergy Appl Immunol 1985;76:193-9.

Hisaeda H, Maekawa Y, Iwakawa D, et al. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat Med 2004;10:29-30.

Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG and Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001;291:881-4.

Hopfenspirger MT, Parr SK, Hopp RJ, Townley RG and Agrawal DK. Mycobacterial antigens attenuate late phase response, airway hyperresponsiveness, and bronchoalveolar lavage eosinophilia in a mouse model of bronchial asthma. Int Immunopharmacol 2001;1:1743-51.

Hori S, Carvalho TL and Demengeot J. CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice. Eur J Immunol 2002;32:1282-91.

Hori S, Nomura T and Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A and Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 1993;260:547-9.

Hvas CL, Kelsen J, Agnholt J, et al. Crohn's disease intestinal CD4+ T cells have impaired interleukin-10 production which is not restored by probiotic bacteria. Scand J Gastroenterol 2007;42:592-601.

Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121-33.

Isomäki P et al., The expression of SOCS is altered in rheumatoid arthritis, Rheumatology 2007: 46:1538-46

Joetham A, Takeda K, Taube C, et al. Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. J Immunol 2007;178:1433-42.

Kaplan MH, Schindler U, Smiley ST and Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity 1996;4:313-9.

Kaufmann E and Knochel W. Five years on the wings of fork head. Mech Dev 1996;57:3-20.

Kaufmann E, Muller D ja Knochel W. DNA recognition site analysis of Xenopus winged helix proteins. J Mol Biol 1995;248:239-54.

Kearley J, Barker JE, Robinson DS and Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. J Exp Med 2005;202:1539-47.

Keir ME and Sharpe AH. The B7/CD28 costimulatory family in autoimmunity. Immunol Rev 2005;204:128-43.

Kero J, Gissler M, Hemminki E and Isolauri E. Could TH1 and TH2 diseases coexist? Evaluation of asthma incidence in children with coeliac disease, type 1 diabetes, or rheumatoid arthritis: a register study. J Allergy Clin Immunol 2001;108:781-3.

Khattri R, Kasprowicz D, Cox T, et al. The amount of scurfin protein determines peripheral T cell number and responsiveness. J Immunol 2001;167:6312-20.

Kim SJ, Park K, Koeller D, et al. Post-transcriptional regulation of the human transforming growth factor-beta 1 gene. J Biol Chem 1992;267:13702-7.

Klugman KP, Madhi SA, Huebner RE, et al. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Engl J Med 2003;349:1341-8.

Kondrashova A, Reunanen A, Romanov A, et al. A six-fold gradient in the incidence of type 1 diabetes at the eastern border of Finland. Ann Med 2005;37:67-72.

Korn T, Bettelli E, Oukka M and Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol 2009;27:485-517.

Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC and von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol 2005;6:1219-27.

Kronenberg M and Rudensky A. Regulation of immunity by self-reactive T cells. Nature 2005;435:598-604.

Kryczek I, Wei S, Zou L, et al. Cutting edge: induction of B7-H4 on APCs through IL-10: novel suppressive mode for regulatory T cells. J Immunol 2006;177:40-4.

Kryczek I, Zou L, Rodriguez P, et al. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. J Exp Med 2006;203:871-81.

Kubista M, Andrade JM, Bengtsson M, et al. The real-time polymerase chain reaction. Mol Aspects Med 2006;27:95-125.

Kuhn R, Lohler J, Rennick D, Rajewsky K and Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993;75:263-74.

Kulkarni AB, Huh CG, Becker D, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A 1993;90:770-4.

Kupila A, Muona P, Simell T, et al. Feasibility of genetic and immunological prediction of type I diabetes in a population-based birth cohort. Diabetologia 2001;44:290-7.

Kurata H, Lee HJ, O'Garra A and Arai N. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. Immunity 1999;11:677-88.

Kursar M, Koch M, Mittrucker HW, et al. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol 2007;178:2661-5.

Lang KS, Recher M, Junt T, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 2005;11:138-45.

Lawson JM, Tremble J, Dayan C, et al. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. Clin Exp Immunol 2008;154:353-9.

Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD and Paul WE. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. J Exp Med 1990;172:921-9.

Leibowitz U, Antonovsky A, Medalie JM, Smith HA, Halpern L and Alter M. Epidemiological study of multiple sclerosis in Israel. II. Multiple sclerosis and level of sanitation. J Neurol Neurosurg Psychiatry 1966;29:60-8.

Lewkowich IP, Herman NS, Schleifer KW, et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. J Exp Med 2005;202:1549-61.

Li H, Yu JP, Cao S, et al. CD4 +CD25 + regulatory T cells decreased the antitumor activity of cytokine-induced killer (CIK) cells of lung cancer patients. J Clin Immunol 2007;27:317-26.

Li MO, Wan YY, Sanjabi S, Robertson AK and Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99-146.

Li S, Weidenfeld J and Morrisey EE. Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. Mol Cell Biol 2004;24:809-22.

Liang B, Workman C, Lee J, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol 2008;180:5916-26.

Lighvani AA, Frucht DM, Jankovic D, et al. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci U S A 2001;98:15137-42.

Lin JT, Martin SL, Xia L and Gorham JD. TGF-beta 1 uses distinct mechanisms to inhibit IFN-gamma expression in CD4+ T cells at priming and at recall: differential involvement of Stat4 and T-bet. J Immunol 2005;174:5950-8.

Lin JX and Leonard WJ. Signaling from the IL-2 receptor to the nucleus. Cytokine Growth Factor Rev 1997;8:313-32.

Ling EM, Smith T, Nguyen XD, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 2004;363:608-15.

Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203:1701-11.

Lund JM, Hsing L, Pham TT and Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. Science 2008;320:1220-4.

Male D, Brostoff J, Roth DB, Roitt I. Immunology. Elsevier Limited, 2006, pp. 1-5

Malek TR, Yu A, Vincek V, Scibelli P and Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. Immunity 2002;17:167-78.

Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ and Powrie F. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 2003;197:111-9.

Manichanh C, Rigottier-Gois L, Bonnaud E, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 2006;55:205-11.

Marie JC, Letterio JJ, Gavin M and Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J Exp Med 2005;201:1061-7.

Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998;67:753-91.

Mazmanian SK, Round JL and Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 2008;453:620-5.

McKee AS and Pearce EJ. CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. J Immunol 2004;173:1224-31.

Meiler F, Klunker S, Zimmermann M, Akdis CA and Akdis M. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. Allergy 2008;63:1455-63.

Min WP, Zhou D, Ichim TE, et al. Inhibitory feedback loop between tolerogenic dendritic cells and regulatory T cells in transplant tolerance. J Immunol 2003;170:1304-12.

Montagnoli C, Bacci A, Bozza S, et al. B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to Candida albicans. J Immunol 2002;169:6298-308.

Moore KW, de Waal Malefyt R, Coffman RL and O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

Morgan ME, van Bilsen JH, Bakker AM, et al. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum Immunol 2005;66:13-20.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA and Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-57.

Mottet C, Uhlig HH and Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol 2003;170:3939-43.

Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256-60.

Mullen AC, High FA, Hutchins AS, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science 2001;292:1907-10.

Nakamura K, Kitani A and Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001;194:629-44.

Nurieva R, Yang XO, Martinez G, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 2007;448:480-3.

Oderup C, Cederbom L, Makowska A, Cilio CM and Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology 2006;118:240-9.

Ohtsuka K, Gray JD, Stimmler MM, Toro B and Horwitz DA. Decreased production of TGF-beta by lymphocytes from patients with systemic lupus erythematosus. J Immunol 1998;160:2539-45.

Okada H, Kuhn C, Feillet H and Bach JF. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clin Exp Immunol 2010;160:1-9.

Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T and Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 1999;59:3128-33.

Ostroukhova M, Seguin-Devaux C, Oriss TB, et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. J Clin Invest 2004;114:28-38.

Ou LS, Goleva E, Hall C and Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. J Allergy Clin Immunol 2004;113:756-63.

Oukka M. Interplay between pathogenic Th17 and regulatory T cells. Ann Rheum Dis 2007;66 Suppl 3:iii87-90.

Ouyang W, Ranganath SH, Weindel K, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 1998;9:745-55.

Ozdemir C, Akdis M and Akdis CA. T regulatory cells and their counterparts: masters of immune regulation. Clin Exp Allergy 2009;39:626-39.

Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M and Akdis CA. Role of Treg in immune regulation of allergic diseases. Eur J Immunol 2010;40:1232-40.

Parham C, Chirica M, Timans J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. J Immunol 2002;168:5699-708.

Patterson CC, Carson DJ and Hadden DR. Epidemiology of childhood IDDM in Northern Ireland 1989-1994: low incidence in areas with highest population density and most household crowding. Northern Ireland Diabetes Study Group. Diabetologia 1996;39:1063-9.

Pennington DJ, Silva-Santos B, Silberzahn T, et al. Early events in the thymus affect the balance of effector and regulatory T cells. Nature 2006;444:1073-7.

Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y and Fisher PB. Interleukin-10 and related cytokines and receptors. Annu Rev Immunol 2004;22:929-79.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 2002;16:779-90.

Powledge TM. The polymerase chain reaction. Adv Physiol Educ 2004;28:44-50.

Powrie F, Carlino J, Leach MW, Mauze S and Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med 1996;183:2669-74.

Powrie F, Correa-Oliveira R, Mauze S and Coffman RL. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. J Exp Med 1994;179:589-600.

Presser K, Schwinge D, Wegmann M, et al. Coexpression of TGF-beta1 and IL-10 enables regulatory T cells to completely suppress airway hyperreactivity. J Immunol 2008;181:7751-8.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S and Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004;118:229-41.

Rautiainen H, Salomaa V, Niemela S, et al. Prevalence and incidence of primary biliary cirrhosis are increasing in Finland. Scand J Gastroenterol 2007;42:1347-53.

Ren X, Ye F, Jiang Z, Chu Y, Xiong S and Wang Y. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. Cell Death Differ 2007;14:2076-84.

Riedler J, Braun-Fahrlander C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-33.

Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K and Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006;212:28-50.

Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531-62.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M and Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151-64.

Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000;12:431-40.

Sanjabi S, Zenewicz LA, Kamanaka M and Flavell RA. Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. Curr Opin Pharmacol 2009;9:447-53.

Schaub B, Liu J, Hoppler S, et al. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. J Allergy Clin Immunol 2009;123:774,82.e5.

Schubert LA, Jeffery E, Zhang Y, Ramsdell F and Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem 2001;276:37672-9.

Seiskari T, Viskari H, Kondrashova A, Haapala AM, Ilonen J, Knip M and Hyöty H. Co-occurrence of allergic sensitization and type 1 diabetes. Annals of Medicine 2010:42:352-359.

Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity 2006;25:195-201.

Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 1992;359:693-9.

Skapenko A, Kalden JR, Lipsky PE and Schulze-Koops H. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. J Immunol 2005;175:6107-16.

Sokol H, Pigneur B, Watterlot L, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A 2008;105:16731-6.

Strachan DP. Hay fever, hygiene, and household size. BMJ 1989;299:1259-60.

Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT and Whiteside TL. A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. Clin Cancer Res 2007;13:4345-54.

Su L, Creusot RJ, Gallo EM, et al. Murine CD4+CD25+ regulatory T cells fail to undergo chromatin remodeling across the proximal promoter region of the IL-2 gene. J Immunol 2004;173:4994-5001.

Summers RW, Elliott DE, Urban JF,Jr, Thompson R and Weinstock JV. Trichuris suis therapy in Crohn's disease. Gut 2005;54:87-90.

Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-85.

Sung JL, Lin JT ja Gorham JD. CD28 co-stimulation regulates the effect of transforming growth factor-beta1 on the proliferation of naive CD4+ T cells. Int Immunopharmacol 2003;3:233-45.

Sutmuller RP, den Brok MH, Kramer M, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 2006;116:485-94.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG and Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100:655-69.

Tai X, Cowan M, Feigenbaum L and Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol 2005;6:152-62.

Takahashi M, Nakamura K, Honda K, et al. An inverse correlation of human peripheral blood regulatory T cell frequency with the disease activity of ulcerative colitis. Dig Dis Sci 2006;51:677-86.

Takeda A, Hamano S, Yamanaka A, et al. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. J Immunol 2003;170:4886-90.

Tang Q, Adams JY, Tooley AJ, et al. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol 2006;7:83-92.

Tang Q and Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 2008;9:239-44.

Tarbell KV, Yamazaki S, Olson K, Toy P and Steinman RM. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. J Exp Med 2004;199:1467-77.

Taylor A, Akdis M, Joss A, et al. IL-10 inhibits CD28 and ICOS costimulations of T cells via src homology 2 domain-containing protein tyrosine phosphatase 1. J Allergy Clin Immunol 2007;120:76-83.

Thieu VT, Yu Q, Chang HC, et al. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. Immunity 2008;29:679-90.

Thorbecke GJ, Shah R, Leu CH, Kuruvilla AP, Hardison AM and Palladino MA. Involvement of endogenous tumor necrosis factor alpha and transforming growth factor beta during induction of collagen type II arthritis in mice. Proc Natl Acad Sci U S A 1992;89:7375-9.

Thorne PS, Kulhankova K, Yin M, Cohn R, Arbes SJ,Jr and Zeldin DC. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. Am J Respir Crit Care Med 2005;172:1371-7.

Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S and Taams LS. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. Proc Natl Acad Sci U S A 2007;104:19446-51.

Toscano MA, Bianco GA, Ilarregui JM, et al. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. Nat Immunol 2007;8:825-34.

Tran DQ, Ramsey H and Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-90.

Usui T, Nishikomori R, Kitani A and Strober W. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. Immunity 2003;18:415-28.

Valasek MA and Repa JJ. The power of real-time PCR. Adv Physiol Educ 2005;29:151-9.

van Santen HM, Benoist C and Mathis D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. J Exp Med 2004;200:1221-30.

Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat Rev Immunol 2008;8:169-82.

Vieira PL, Christensen JR, Minaee S, et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol 2004;172:5986-93.

Vignali DA, Collison LW and Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:523-32.

von Herrath MG and Harrison LC. Antigen-induced regulatory T cells in autoimmunity. Nat Rev Immunol 2003;3:223-32.

Wallin MT, Page WF and Kurtzke JF. Multiple sclerosis in US veterans of the Vietnam era and later military service: race, sex, and geography. Ann Neurol 2004;55:65-71.

Wan YY and Flavell RA. How diverse--CD4 effector T cells and their functions. J Mol Cell Biol 2009;1:20-36.

Wan YY and Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 2007;445:766-70.

Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev 2001;182:207-14.

Werner S, Buser K, Kapp A and Werfel T. The incidence of atopic dermatitis in school entrants is associated with individual life-style factors but not with local environmental factors in Hannover, Germany. Br J Dermatol 2002;147:95-104.

Wilson MS, Pesce JT, Ramalingam TR, Thompson RW, Cheever A and Wynn TA. Suppression of murine allergic airway disease by IL-2:anti-IL-2 monoclonal antibody-induced regulatory T cells. J Immunol 2008;181:6942-54.

Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271-5.

Wong FS and Wen L. Toll-like receptors and diabetes. Ann N Y Acad Sci 2008;1150:123-32.
Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR ja Vignali DA. The development and function of regulatory T cells. Cell Mol Life Sci 2009;66:2603-22.

Wu L and Van Kaer L. Natural killer T cells and autoimmune disease. Curr Mol Med 2009;9:4-14.

Xu L, Kitani A, Fuss I and Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. J Immunol 2007;178:6725-9.

Yang XO, Pappu BP, Nurieva R, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity 2008;28:29-39.

Yang Z, Wang K, Li T, et al. Childhood diabetes in China. Enormous variation by place and ethnic group. Diabetes Care 1998;21:525-9.

Yang ZZ, Novak AJ, Stenson MJ, Witzig TE and Ansell SM. Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+ T cells in B-cell non-Hodgkin lymphoma. Blood 2006;107:3639-46.

Yehualaeshet T, O'Connor R, Green-Johnson J, et al. Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. Am J Pathol 1999;155:841-51.

You S, Alyanakian MA, Segovia B, et al. Immunoregulatory pathways controlling progression of autoimmunity in NOD mice. Ann N Y Acad Sci 2008;1150:300-10.

You S, Leforban B, Garcia C, Bach JF, Bluestone JA and Chatenoud L. Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. Proc Natl Acad Sci U S A 2007;104:6335-40.

Zaccone P, Fehervari Z, Phillips JM, Dunne DW and Cooke A. Parasitic worms and inflammatory diseases. Parasite Immunol 2006;28:515-23.

Zanin-Zhorov A, Cahalon L, Tal G, Margalit R, Lider O and Cohen IR. Heat shock protein 60 enhances CD4+ CD25+ regulatory T cell function via innate TLR2 signaling. J Clin Invest 2006;116:2022-32.

Zarek PE, Huang CT, Lutz ER, et al. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. Blood 2008;111:251-9.

Zhao DM, Thornton AM, DiPaolo RJ and Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood 2006;107:3925-32.

Zheng W and Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89:587-96.

Zhou L, Ivanov II, Spolski R, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007;8:967-74.

Zhu J, Cote-Sierra J, Guo L and Paul WE. Stat5 activation plays a critical role in Th2 differentiation. Immunity 2003;19:739-48.

Zhu J, Min B, Hu-Li J, et al. Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol 2004;5:1157-65.

Zhu J, Yamane H and Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol 2010;28:445-89.

Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006;24:209-26.

Zipper H, Brunner H, Bernhagen J and Vitzthum F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Res 2004;32:e103.