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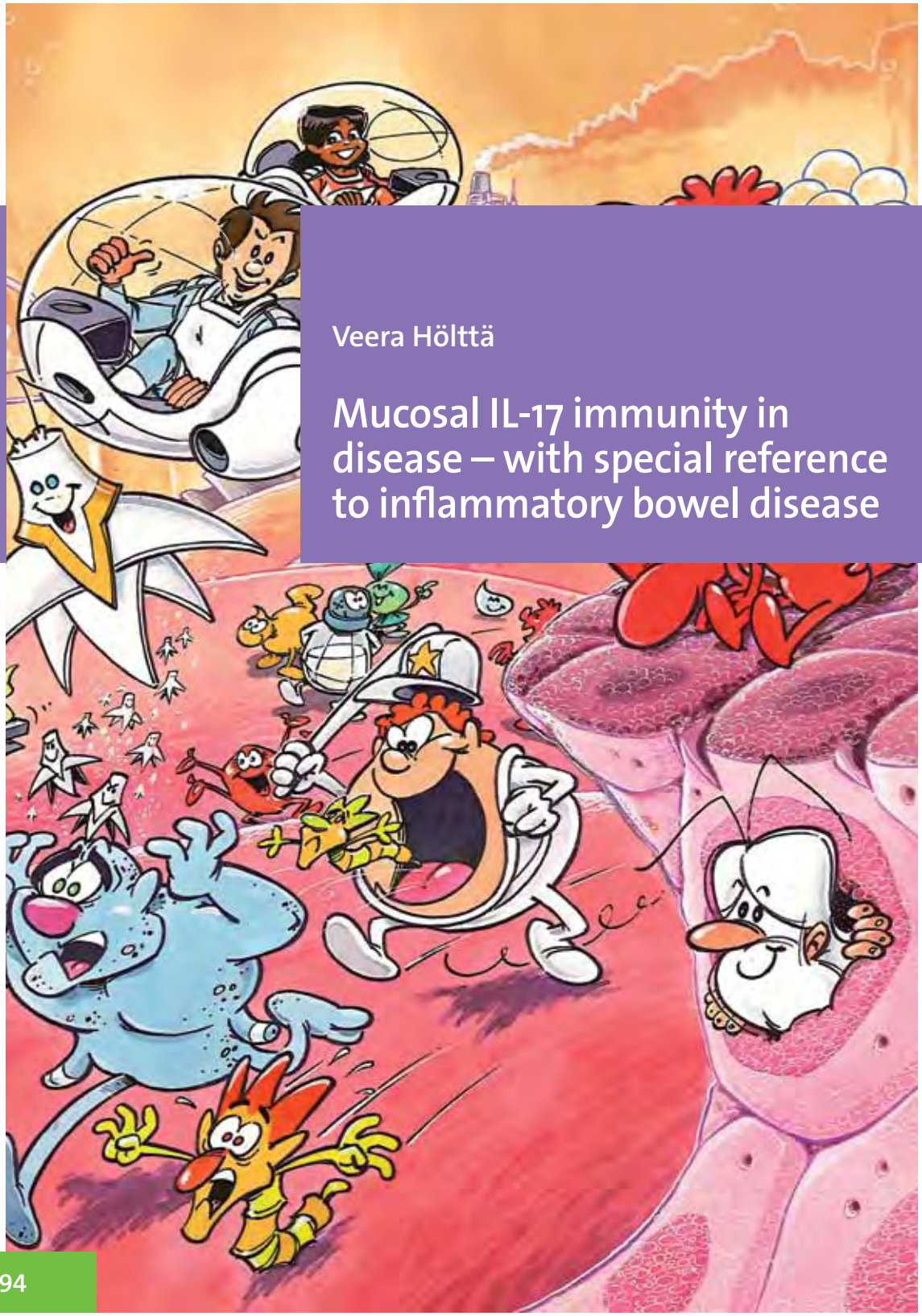

Veera Hölttä

# Mucosal IL-17 immunity in disease – with special reference to inflammatory bowel disease

RESEARCH 94/2012

Veera Hölttä

# MUCOSAL IL-17 IMMUNITY IN DISEASE

## – with special reference to inflammatory bowel disease

## ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in the Niilo Hallman Auditorium, Children's Hospital on 14th December 2012, at 12 noon.

Immune Response Unit, Department of Vaccination and Immune Protection, National Institute for Health and Welfare, Helsinki, Finland
and
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Dedicated to Albert Barillé,
the creator of “Il était une fois la vie”

## Abstract

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T cells are CD4 lymphocytes participating in cell-mediated immunity, and play a critical role in immune-mediated diseases. T cells divide further into subclasses such as Th1, Th2, and Th17 that participate in mucosal immunity responses caused by extracellular pathogens and also play a role in autoimmune diseases. Th17 cells produce cytokines called the interleukin-17 family (IL-17). Regulatory $CD4^+$ T cells marked by CD4, CD25, and forkhead box P3 (FOXP3), contribute to maintaining tolerance and regulating immune responses to antigens. This doctoral thesis studies the role of IL-17 type of immunity in intestinal inflammation in Crohn's disease, ulcerative colitis, celiac disease, and type I diabetes (T1D). In particular, the study explores the balance between effector and regulatory T cells in active and inactive Crohn's disease in adults, as well as in pediatric patients with Crohn's disease and ulcerative colitis. Furthermore, the investigation focuses on the effect of anti-TNF-α treatment on the effector and regulatory T cells in Crohn's disease in adults.

Adult patients with Crohn's disease had higher numbers of intestinal $IL\text{-}17^+$ and $FOXP3^+$ cells than did control subjects, both before and after the anti-TNF-$\alpha$ treatment. Intestinal interferon-$\gamma$ and IL-17 mRNA expression was higher in Crohn's disease and remained elevated after anti-TNF-$\alpha$ treatment, although the treatment improved intestinal balance between $IL\text{-}17^+$ effector and regulatory T cells. In Crohn's disease, mRNA expression of IL-17A, IL-6, and FOXP3 was increased. Fecal IL-17 concentration showed increase in patients with active disease. IL-17 enhanced the IL-8 and TNF-α response of the epithelial cell line to lipopolysaccharide in vitro. The findings suggest that activation of the IL-17 axis is fundamentally connected to the etiology of Crohn's disease and may represent the basis for the relapsing nature of the disease.

In pediatric patients, IL-17, IL-22, IL-6, and $FOXP3^+$ mRNA up-regulation and increase in the number of $IL\text{-}17^+$ and $FOXP3^+$ cells existed in inflammatory bowel disease. Activation of the IL-17/IL-22 axis and up-regulation of FOXP3 occurred both in pediatric Crohn's disease and ulcerative colitis, indicating shared immunological aberrancy.

In pediatric patients with untreated celiac disease, the mucosal expression of IL-17 and FOXP3 transcripts were elevated as compared with TGA-negative reference children, children with potential celiac disease, gluten-free diet-treated children with celiac disease, or children with T1D. Enhanced spontaneous secretion of IL-1β, IL-6, and IL-17 occurred in biopsy specimens from patients with untreated celiac disease. Activation of anti-apoptotic bcl-2 in IL-17-treated CaCo-2 epithelial cells suggests

that IL-17 might be involved in mucosal protection. Up-regulation of IL-17, however, could serve as a biomarker for the development of villous atrophy and active celiac disease.

Keywords: interleukin-17, regulatory T cells, inflammatory bowel disease, celiac disease, type 1 diabetes

## Tiivistelmä

Veera Hölttä. Mucosal IL-17 immunity in disease – with special reference to inflammatory bowel disease [Limakalvon IL-17-immuniteetti tautitiloissa – erityisesti tulehduksellisessa suolistosairaudessa]. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 94/2012. 135 sivua. Helsinki, Finland 2012.
ISBN 978-952-245-755-4 (painettu); ISBN 978-952-245-756-1 (pdf)

CD4-lymfosyytit ovat T-soluja, jotka osaltaan toteuttavat soluvälitteistä immuniteettia ja joilla on tärkeä rooli immuunivälitteisissä taudeissa. T-solut jaetaan alaluokkiin, kuten Th1, Th2 ja Th17, jotka osallistuvat solunulkoisten patogeenien aiheuttamaan immuunivasteeseen ja niillä on merkitystä autoimmuunitaudeissa. Th17-solut tuottavat sytokiinejä, kuten interleukiini 17:ää (IL-17). Säätelevät T-solut, joiden merkkiaineita ovat CD4, CD25 ja FOXP3, osaltaan ylläpitävät toleranssia ja säätelevät immuunivastetta.

Tässä väitöskirjatyössä tutkittiin IL-17-immuniteettia Crohnin taudissa, ulseratiivisessa koliitissa, keliakiassa ja tyypin I diabeteksessa (T1D). Tasapainoa efektori- ja säätelevien T-solujen välillä selvitettiin aikuisten aktiivisessa ja inaktiivisessa Crohnin taudissa sekä lasten tulehduksellisissa suolistosairauksissa. Lisäksi tutkittiin TNF-α-salpaajahoidon vaikutusta efektorisoluihin ja sääteleviin T-soluihin aikuisten Crohnin taudissa.

Crohnin tautia sairastavien aikuisten suolistosta löytyi verrokkeja enemmän IL-17- ja FOXP3-positiivisia soluja, myös TNF-α-salpaajahoidon jälkeen. Myös suoliston interferoni-*γ*:n ja IL-17:n lähetti-RNA-pitoisuudet olivat korkeampia kuin verrokkipotilailla. Ne pysyivät korkeina huolimatta TNF-*α*-salpaajahoidosta, vaikka hoito paransi tasapainoa IL-17-positiivisten efektorisolujen ja säätelevien T-solujen välillä. Aktiivisessa ja remissiossa olevassa Crohnin taudissa IL-17A:n, IL-6:n ja FOXP3:n lähetti-RNA-pitoisuudet olivat kohonneet. In vitro IL-17 vahvisti IL-8:n ja TNF-α:n vastetta lipopolysakkarideille epiteelisoiluissa. Tämän vuoksi on mahdollista, että IL-17-akselin aktivoituminen liittyy Crohnin taudin etiologiaan ja saattaa olla syy taudin relapsointitaipumukseen.

Tulehduksellista suolistosairautta sairastavilla lapsilla todettiin IL-17:n, IL-22:n, IL-6:n ja FOXP3:n lähetti-RNA-tasot verrokkeja korkeammiksi. Sekä Crohnin taudissa että ulseratiivisessa koliitissa oli IL-17/IL-22-akseli aktivoitunut ja FOXP3 lisääntynyt, viitaten taudeille yhteiseen immunologiseen puutteeseen.

Hoitamatonta keliakiaa sairastavien lasten suolen limakalvolla IL-17 ja FOXP3 ekspressoituivat voimakkaammin verrattaessa määrää antitransglutaminaasi vasta-ainenegatiivisiin, potentiaalista keliakiaa sairastaviin ja gluteiinittomalla dieetillä hoidettuihin lapsiin, tai lapsiin, joilla oli T1D. Hoitamatonta keliakiaa sairastavien koepaloissa oli myös enemmän spontaania IL-1β-, IL-6- ja IL-17-eritystä. IL-17:llä käsitellyissä CaCo-2-epiteelisoluissa aktivoitui anti-apoptoottinen bcl-2 viitaten

siihen, että IL-17 saattaa liittyä limakalvon suojaamiseen. IL-17:n lisääntyminen voisi kuitenkin toimia villusatrofian kehittymisen ja aktiivisen keliakian merkkinä.

Avainsanat: interleukiini-17, säätelevät T-solut, tulehduksellinen suolistosairaus, keliakia, tyypin 1 diabetes

## Contents

## List of original papers

The dissertation is based on the following original publications, which shall be referred to in the text by their Roman numerals (I-IV) and are reprinted with permission of the copyright holders.

I. Hölttä V Klemetti P, Sipponen T, Westerholm-Ormio M, Kociubinski G, Salo H, Räsänen L, Kolho K-L, Färkkilä M, Savilahti E, Vaarala O. IL-23/IL-17 immunity as a hallmark of Crohn's disease. Inflamm bowel Dis. 2008 Sep; 14(9):1175-84.
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II. Hölttä V, Sipponen T, Westerholm-Ormio M, Salo HM, Kolho K-L, Färkkilä M, Savilahti E, Vaarala O, Klemetti P. Anti-TNF-α treatment changes the balance between mucosal IL-17, FOXP3 and CD4 cells in patients with Crohn's disease. ISRN Gastroenterology. 2012; 2012:505432. Epub 2012 Jun 14.
*Copyright 2012 Veera Hölttä et al*.

III. Hölttä V, Klemetti P, Salo HM, Koivusalo A, Pakarinen M, Westerholm-Ormio M, Vaarala O, Kolho K-L. IL-17 Immunity in Pediatric Crohn's Disease and Ulcerative Colitis. Submitted.

IV. Lahdenperä A, Hölttä V, Ruohtula T, Salo H, Orivuori L, Westerholm-Ormio M, Savilahti E, Fälth-Magnusson K, Högberg L, Ludvigsson J, Vaarala O. Up-regulation of small intestinal IL-17 immunity in untreated celiac disease but not in potential celiac disease or in type 1 diabetes. Clin Exp Immunol. 2012 Feb;167(2):226-34.
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Publication II was published earlier in Sipponen T (2009): Noninvasive monitoring of activity in Crohn's disease, University of Helsinki.

## Abbreviations

| | |
|---|---|
| APC | Antigen-presenting cell |
| Caco-2 | Colon adenocarcinoma cell line |
| CCR | Chemokine receptor |
| CD | Cluster of differentiation |
| CDAI | Crohn's disease activity index |
| CDEIS | Crohn's disease endoscopic index of severity |
| CTL | Cytotoxic T cells |
| CTLA4 | Cytotoxic T-lymphocyte antigen 4 |
| DC | Dendritic cell |
| Ebi-3 | Epstein-Barr-induced gene 3 |
| EC | Epithelial cells |
| FOXP | Forkhead-winged helix transcription factor |
| GALT | Gut-associated lymphoid tissue |
| GFD | Gluten-free diet |
| GI | Gastrointestinal |
| GITR | Glucocorticoid-inducible tumor necrosis factor receptor |
| HLA | Human leucocyte antigen |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IEC | Intestinal epithelial cell |
| IBD | Inflammatory bowel disease |
| ICOS | Inducible co-stimulator |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IPEX | Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome |
| iTreg | Inducible T regulatory cell |
| LFA | Lymphocyte function-associated antigen |
| LP | Lamina propria |
| LPL | Lamina propria lymphocytes |
| M cell | Microfold cell |
| MAdCAM-1 | Mucosal addressin cell adhesion molecule-1 |
| MALT | Mucosal-associated lymphoid system |
| MHC | Major histocompatibility complex |
| MDP | Muramyl dipeptide |
| MLN | Mesenteric lymph nodes |
| mRNA | Messenger ribonucleic acid |
| NK | Natural killer |
| NKT | Natural killer T cells |

| | |
|---|---|
| NOD | Nucleotide-binding oligomerization domain |
| nTreg | Natural T regulatory cell |
| OCT | Optimal cutting temperature |
| PAMP | Pathogen-associated molecular patterns |
| pANCA | Perinuclear antineutrophil cytoplasmic antibodies |
| PBMC | Peripheral blood mononuclear cell |
| PD-1 | Programmed death-1 |
| PP | Peyer's patch |
| PRR | Pattern recognition receptor |
| ROR | Retinoic acid receptor-related orphan receptor |
| qRT-PCR | Quantative Reverse transcriptase-polymerase chain reaction |
| SASP | Sulfasalazine |
| T1D | Type 1 diabetes |
| Tconv | Conventional T cells |
| TCR | T-cell receptor |
| TGA | Transglutaminase antibodies |
| TGF | Transforming growth factor |
| Tfh | T follicular helper cell |
| Th | T-helper lymphocyte |
| TJ | Tight junction |
| TLR | Toll-like receptor |
| TL1A | Tumour necrosis factor-like ligan 1 |
| TNF | Tumour necrosis factor |
| Treg | Regulatory T cell |
| tTG | Tissue transglutaminase |
| TG-2 | Transglutaminase-2 |
| UC | Ulcerative colitis |
| 5ASA | 5-aminosalicylic acid |

# 1 Introduction

The immune system acts to protect the body from invading microbes and foreign antigens, but when the immune systems functions improperly the immune diseases arise. These diseases can be divided into:

- Systemic autoimmune diseases in which symptoms occur in many organs and often circulating autoantibodies are markers of disease, as in systemic lupus erythematous and rheumatoid arthritis.
- Tissue-specific autoimmune diseases are restricted to a single organ, as in celiac disease and type 1 diabetes (T1D).
- Immune diseases or "auto-inflammatory diseases": Inflammatory bowel disease, for example, in which no autoantigen specific immune response is detectable, but a chronic inflammatory response is maintained and results in self-tissue destruction. A hereditary predisposition associates with innate immunity, as genome-wide association studies show (NOD, CTLA-4).

Autoimmune diseases comprise a group of diseases in which an unusually strong attack towards self-antigens causes a disease. In autoimmune diseases, typically the levels of symptoms vary, and symptoms occur in phases in which active and remission periods follow. The equilibrium of the immune system varies based on the changes in regulatory and effector mechanisms. Often inflammation turns out to be the trigger point of the active phase of disease. Accumulating evidence suggests that environmental factors play an important role in the everlasting game between the perpetuating homeostasis and its breakdown. A genetic predisposition exposes, however, individuals to development of this disease. In a rising number of cases, this harmful process is ongoing. To those affected, the consequences of these diseases, like Crohn's disease, ulcerative colitis (UC), T1D and celiac disease, are often disabling, and – if untreated – can even be fatal. In order to develop better treatment options or even to postpone the beginning of the disease, better understanding of the immunological process behind the disease is necessary. The Th17 type of cells form a subclass of adaptive immune cells that plays a role in the pathogenesis of many autoimmune type of diseases.

This thesis aimed to study the intestinal interleukin (IL) 17 type of cell as the number of IL-17-positive cells in the intestine and the number of IL-17 transcripts in the intestinal biopsies in Crohn's disease, in pediatric IBD, in celiac disease, and in T1D.

# 2 Review of the literature

## 2.1 The immune system

The immune system consists of two parts, both playing a fundamental role in the immunological response: the innate and the adaptive immune systems.

The innate immune system provides the first line of defence with a fast but limited response. It presents universal and evolutionarily ancient defence mechanisms as plants and animal share the same molecular modules. Innate immunity depends on germ-line encoded receptors (Janeway, Medzhitov 2002). Broadly defined, innate immunity consists of all aspects of defence mechanisms: physical barriers, a secreted mucus layer, epithelial cilia, soluble proteins and bioactive molecules within the biological fluids (or released by activation), membrane-bound receptors, and cytoplasmic proteins that recognize structures in invading microbes (Chaplin 2010). Regarding cell lines, dendritic cells, macrophages, γδ T cells, natural killer cells (NK), natural killer T cells (NKT), mast cells, neutrophils, and eosinophils make up the innate immunity that comprises the nonspecific resistance mechanisms to infectious non-self. In addition to this, the innate immune system may also defend against non-infectious noxious insults like environmental irritants (Medzhitov 2010). Innate lamina propria (LP) leukocytes produce several inflammatory cytokines driving intestinal pathology (Harrison, Maloy 2011).

The adaptive immune system, in contrast, is the more sophisticated one: it relies on a specific response to a certain stimulus. Reaction is slow, but it has the capability to offer immunological memory for future reference. Bone marrow-originated T and B lymphocytes comprise the human adaptive immune system. T cells possess a T cell receptor (TCR) to recognize their antigen. B cells use antibodies as receptors in order to recognize antigens (Andersen et al. 2006). Naïve B cells express immunoglobulins (Ig) M and D, which switch to IgG, IgA, and IgE types of surface receptors in mature cells. Activated B cells proliferate and differentiate to plasma cells that secrete antibodies and can further differentiate into long-lived memory cells. Both the forms of adaptive immune system are necessary and cooperate in order to maintain homeostasis in the host. They have a close relation ship and are most likely cross-regulated, as genome-wide association studies of immune-mediated diseases suggest (Barrett et al. 2008).

### 2.1.1 Immunological tolerance

The function of the immune system in discriminating between self and non-self, simultaneously inhibiting autoimmune responses and allowing an effective response against microbial antigens, is the fundamental immunological dilemma. The goal of

an adequate immune response is to fight external threats - pathogens and environmental challenges - without damaging self-tissues (Chaplin 2010). In order to perform these actions, the immune system has developed various mechanisms to establish and sustain unresponsiveness to self-antigens; such unresponsiveness is called immunological self-tolerance. Central and peripheral tolerances work to maintain immunological homeostasis. Physical deletion (clonal deletion), functional inactivation (anergy), and T cell-mediated active suppression of potentially pathogenic self-reactive T cells are the mechanisms of self-tolerance (Sakaguchi et al. 2008, Zheng, Rudensky 2007). Central tolerance consists of regulation of self-reactive T cell development in the thymus (section 2.1.3). Peripheral tolerance, conversely, sustains homeostasis by regulating peripheral autoreactive T cells.

### 2.1.2 T lymphocytes

T lymphocytes comprise the key cell population of the adaptive immunity arm. Hematopoietic precursor cells originate in bone marrow and migrate to the thymus in order to go through a five-step maturation process (Takahama 2006):

1. Lymphoid progenitor cells enter the thymus.
2. At the outer cortex of the thymus, a cluster of differentiation (CD) $4^+$ and $CD8^+$ double-positive thymocytes are generated.
3. In the cortex occurs the positive and negative selection of double-positive thymocytes
4. To complete thymocyte development, positively selected thymocytes interact with medullary thymic epithelial cells. This also ensures central tolerance.
5. Mature T cells exit the thymus.

In order for elimination of autoreactive T cells to take place, major histocompability complex (MHC) classes I and II molecules present self-peptides to T cells. Sometimes this deletion process is, however, inadequate, and autoreactive T cells end up in the periphery. In healthy individuals, peripheral tolerance controls these autoreactive cells.

During this process, the arising T cells learn to discriminate between self and non-self-structures, and T cells with inappropriately high TCR affinity towards self-peptides are deleted in a process called central tolerance (Takahama 2006). On the T cell, CD3 - a marker of all T cells - associates with TCR in order to transmit intracellular activation signals. Expression of αβ or γδ TCR distinguishes T lymphocytes from other leukocytes.

In order to become activated, T cells require a three-step pathway (Andersen et al. 2006):

1. Interaction of TCR and antigen-presenting cell (APC). TCRs vary and thus ensure specific responses. Most of the human TCRs comprise a αβ hetero-

dimer; some 5% of peripheral blood TCRs are γδ. In intestinal mucosa, the proportion of γδTCRs is higher (Aljurf et al. 2002). APCs present the antigenic peptides bound to the cell surface MHC. All somatic cells express MHC class I molecules; they present peptides originating from endogenous proteins. $CD8^+$ cytotoxic T cells (CTLs) recognize these MHC I molecules. Only professional APCs express class II MHC molecules that present exogenous peptides to $CD4^+$ T helper cells. Cytokines can, however, induce MCH II in the majority of cells (Andersen et al. 2006). Human leukocyte antigen (HLA) refers to MHC in humans, and the complex comprises three loci (HLA-A, -B, and –C) in chromosome six.

2. APC gives a co-stimulatory signal to the T cell. This occurs through expression of a surface molecule B7-1 or B7-2 (CD80 or CD86) that activates receptor CD28 on the T cell.
3. Cytokines and other mediators induce activation. Cytokines are secreted proteins that contribute to the initiation and guidance of the adaptive immune response (Commins et al. 2010). Cells of the innate immune system and of the adaptive immune system secrete these cytokines.

### 2.1.3 T helper cells

$CD4^+$ T cells comprise the T helper (Th) class of T cells. Naïve Th cells produce primarily IL-2. Th cells are divided into functional subtypes according to the cytokines they secrete. The classical dichotomy of T helper cells consists of Th1 and Th2 cells, first described in the mid-1980s (Mosmann et al. 1986). Thereafter, new subclasses have appeared, and currently Th cells are divided into several subclasses: Th1, Th2, Th17, iTreg, Th3, Th9, Th22, and T follicular helper (Tfh) cells (Figure 1).

Most of these cell classes were first described in murine models and later in humans. Unlike in mice, however, CD4 cells producing categorically distinct Th cytokine profiles seldom appear in humans, but Th cells secreting, for example, both Th1 and Th17 cytokines occur in humans.

Th9 presents a unique subclass of IL-9–producing Th2 cells. Transforming growth factor (TGF) β is central to the differentiation of Th9 cells (Commins et al. 2010). T-follicular helper cells, on the other hand, were described initially as follicular B helper T cells after the finding that in the B cell follicle reside $CD4^+$ T cells expressing the chemokine receptor CXCR5. In addition, they coexpress other surface markers like programmed death-1 and inducible co-stimulator (Deenick, Ma 2011).

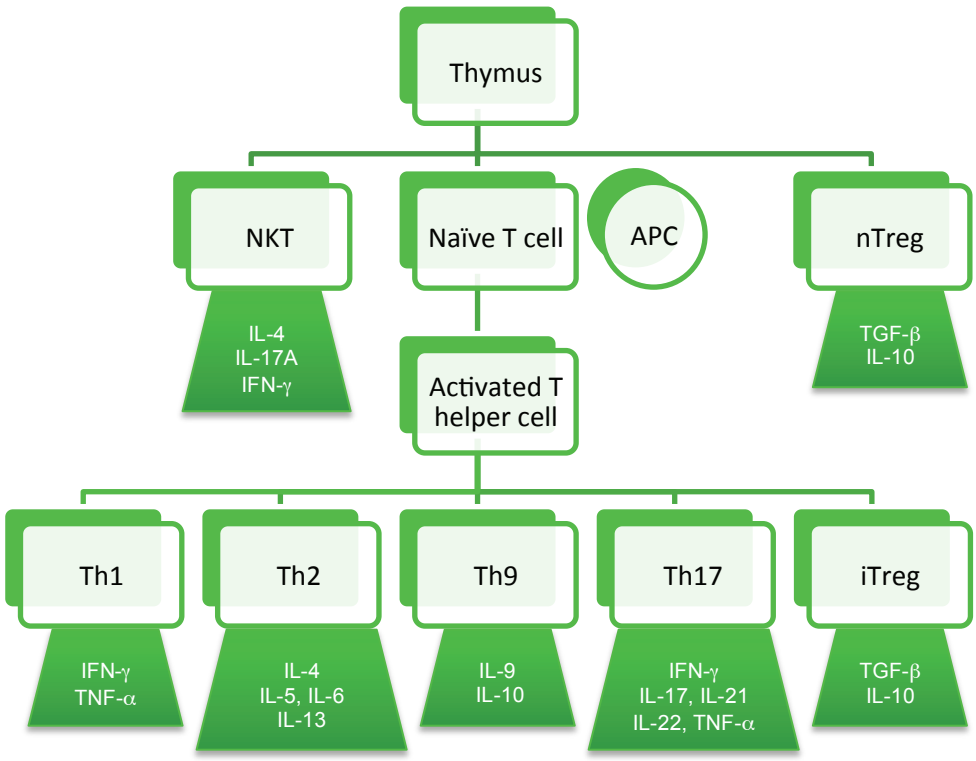


Figure 1. T helper cell differentiation.

### 2.1.4 Th1 and Th2 cells

IL-2 is a cytokine typical of both Th1 and Th2 cells. Stimulation of T cells in the presence of IL-1 and IL-6 induces IL-2 secretion and expression of IL-2R on effector T cells. Binding of secreted IL-2 to the IL-2R leads to clonal T cell proliferation (Commins et al. 2010). IL-2-specific IL-2Rα (CD25), IL-2Rβ (CD122), and the common γ chain form a receptor complex through which IL-2 signals. IL-2 is also involved in activation of NK cells, B cells, CTLs, and macrophages, thus acting both in adaptive and innate immunity (Commins et al. 2010).

Typically, Th1 –mediated immune responses arise in response to an intracellular pathogen presented by APC in the presence of IL-12. This immune response tends to localize the infectious agent at the site of inflammation and to secrete cytokines (interferon (IFN)-γ and tumor necrosis factor (TNF) β, but no IL-4 or IL-5) that promote apoptosis or induce the differentiation of cytotoxic T lymphocytes. Granuloma is the hallmark of the Th1 response (Kobayashi et al. 2001).

Cytokines IL-12, IL-18, and IL-27 contribute to the Th1 type differentiation (Manetti et al. 1993). Interaction of IL-12 and naïve T cells causes activation of STAT4 that leads to expression of transcription factor T-box expressed in T cells (T-

bet), a master regulator for Th1 differentiation. T-bet induces production of IFN-γ and IL-12R and inhibits alternative Th differentiation pathways. IL-12 is a heterodimer consisting of two subunits: IL-12p35 (homologous to the soluble IL-6 receptor) and IL-12p40 (shared also by IL-23, homologous to IL-6). IL-12 activities include stimulation of IFN-γ production, activation of NK cells (IL-12 activates and induces proliferation, cytotoxity, and cytokine production of NK cells), and proliferation of Th cells and CTLs (Commins et al. 2010). In synergy with IL-12, IL-27 induces NK and Th cells to produce IFN-γ, leading to Th1 immune deviation. IL-27 is a heterodimer consisting of IL-27B (EBV-induced gene B) and IL-27p28 (IL-30). Macrophages and DCs produce IL-27 that plays an important role in Th immunity (Commins et al. 2010).

Homodimer IFN-γ is the key cytokine for cell-mediated immunity to intracellular pathogens and is a hallmark of Th1 type immunity (Farrar, Schreiber 1993). Interferons are a class of proteins known for their ability to interfere with viral growth, and IFN-γ comprises the class of type II interferons. In addition to T cells, NK cells and to a lesser extent macrophages produce IFN-γ, suggesting it is more like an interleukin than an interferon. IFN-γ plays a role in MHC I and II expression and stimulates APCs, mononuclear phagocytic functions, and killing by NK cells and neutrophils (Commins et al. 2010).

The Th2 subunit of T cells secretes a different set of cytokines: IL-4, IL-5, and IL-13, but not IFN-γ. Through IgE responses, and eosinophil and mast cell activation, Th2 cells promote antiparasitic immune responses. IL-4, one determinant of Th2 differentiation (Seder et al. 1992), activates STAT6, which in turn promotes expression of transcription factor GATA-3, the master regulator for Th2 type differentiation. GATA-3 has a positive effect on potentiating a Th2 type response by supporting IL-4 expression, Th1 suppression, and inhibition of the Th17 type response (together with IL-4) (Commins et al. 2010). Excessive Th2 activation promotes atopy.

### 2.1.5 Regulatory T cells

Regulatory T (Treg) cells are essential in regulation of the immune response to self-antigens, allergens, commensal microbiota, infectious agents, and tumors in order to maintain immunological homeostasis. A lineage specification factor for these Treg cells is the forkhead-winged helix transcription factor FOXP3. Thymus derived natural T regulatory cells (nTregs) suppress self-reactive T cells in order to prevent autoimmunity (Sakaguchi et al. 2006). Inducible Tregs (iTregs) arise in the periphery from naïve $CD4^+$ T cells after antigen exposure, particularly in the intestine (Sun et al. 2007). $FOXP^+CD4^+$ cells constitute less than 10% of peripheral $CD4^+$ cells.

Discovery of these regulatory cells occurred in 1995 through the finding of a subset of $CD4^+$ T cells expressing CD25, which is the interleukin-2 (IL-2) receptor α-chain (IL-2Rα). These $CD4^+CD25^+$ cells appear to possess suppressive capability

(Sakaguchi et al. 1995). Treg cell development in the thymus requires IL-2 (Thornton, Shevach 1998), as it is essential for nTreg survival in the periphery (Sakaguchi et al. 2006). Similarly to activated T cells, these Treg cells also display cytotoxic T-lymphocyte antigen 4 (CTLA4), glucocorticoid-inducible tumor necrosis factor receptor (GITR), IL-2 receptor β-chain (CD122), lymphocyte function-associated antigen 1 (LFA-1/CD11a), and intercellular adhesion molecule 1 (ICAM-1) (Read et al. 2000, Yamaguchi et al. 2011). Initially these cells were considered anergic and thus unable to proliferate and produce IL-2, but later studies showed thymus-derived $CD25^{+}CD4^{+}$ Treg cells to be an example of a distinguishable long-lived cell subset with suppressor function (Rudensky 2011).

These Treg cells express transcription factor FOXP3 (Fontenot et al. 2003, Hori et al. 2003). *Foxp3* serves as the master control gene for Treg function, and mutations in FOXP3 cause severe failures in the immune system, indicating the role of Tregs as a distinct cell line. Initially, discovery of FOXP3 was done in animal studies; the X chromosomal mutation led to severe autoimmunity in "scurfy" mice (Brunkow et al. 2001). In humans, loss of Treg cell function results in immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), a severe multi-organ autoimmune and inflammation disorder (Wildin et al. 2001). The clinical picture of IPEX consists of gastrointestinal disease characterized by watery diarrhea, autoimmune endocrinopathies involving commonly the pancreas and the thyroid, and dermatitis in the form of a mild eczema. Symptoms vary between patients, and in addition to this basic triad other autoimmune alterations exist, such as hematological disorders (Blanco Quiros et al. 2009).

Tregs suggested the performance of suppressive actions by several mechanisms:

- **Modulation of APC functions**
  Activated Tregs inhibit the contact of naïve T cells to APCs by reducing the time for T cell–dendritic cell interactions to one-third of the time spent when Tregs were not present (Tadokoro et al. 2006).
- **CTLA-4-dependent mechanism**
  In order to activate and proliferate, naïve T cells need co-stimulatory signals transferred by CD28. CTLA-4 inhibits CD28-mediated co-stimulation by the following mechanism: CTLA-4 and CD28 share the ligands CD80 and CD86, but the former has higher affinity for both. Tregs express CTLA-4 and thus inhibit the conventional T cells (Tconv) (Yokosuka et al. 2010).
- **IL-2-dependent mechanism**
  All nTregs express IL-2R but produce no IL-2 and are therefore dependent on exogenous IL-2. They can also prevent activation of Tconv by depriving IL-2 of Tconv (Thornton, Shevach 1998).
- **Extracellular ATP degradation**
  Extracellularly released ATP enhances immune reactions; however, enzymes produced by Tregs can degrade it and thus inhibit T cell activation (Yip et al. 2009).

- **TGF-β**
  Numerous cell types produce TGF-β: eosinophils, monocytes, and Th cells (of them, primarily Treg cells). Having both stimulatory and inhibitory effects, TGF-β is a pleitropic cytokine (Sporn, Roberts 1992). It is inhibitory for B cells, Th cells, and CTLs. Generally, TGF-β acts to inhibit proliferation and to induce apoptosis. When expressed on Tregs, TGF- β is an immunosuppressive cytokine, and a TGF-β-dependent feedback loop enhances Treg suppression. It also affects the induction and maintenance of FOXP3 expression, differentiation of naïve T cells into FOXP3 cells in vitro, induction of peripheral FOXP3 cells in vivo (especially in the gut), and the maintenance of both nTregs and iTregs (Yamaguchi et al. 2011).
- **Cytotoxic and other molecule**s
  The cytotoxic molecules granzyme and perforin contribute to Treg-mediated suppression of NK and $CD8^+$ T cell activity (Cao et al. 2007).
- **IL-10/IL-35**
  Another essential immunoregulatory and anti-inflammatory cytokine is IL-10, a homodimer produced by several cell lines: Tregs, but especially in humans, also monocytes and B cells. IL-10 has effects that are anti-inflammatory and suppressive to most hematopoietic cells (Rudensky 2011). Among other functions, IL-10 inhibits Th1 cells from producing IFN-γ and Th2 cells which produce IL-4 and IL-5 (Del Prete et al. 1993). For Treg-dependent suppression, IL-10 is especially needed in the intestinal mucosal along with other mucosal tissues. Intestinal T cells, regardless of their FOXP3-status (+/-), secrete excessive IL-10 that inhibits both the generation and proliferation of Th17 cells possessing IL-10-receptors. IL-10 also affects tolerogenic DCs and the differentiation of $CD4^+$ naïve cells into IL-10-secreting cells (Yamaguchi et al. 2011).

One specific subset of Tregs is IL-10-secreting Tr1 cells (Cong et al. 2002). Another novel population of suppressive cells comprises Tr35 cells: $FOXP3^-$ cells that secrete IL-35, an immune-suppressive heterodimer composed of Epstein-Barr-induced gene 3 (Ebi-3) encoding IL-27β and IL-12α encoding IL-12α/p35. Murine Treg cells lacking either IL-35 dimer have reduced ability to suppress inflammation (Collison et al. 2007, 2010). IL-35 leads to Treg cell proliferation and reduces Th17 cell activity (Niedbala et al. 2007). Tregs mediate potent T cell suppression in a IL-35- and IL-10–dependent manner, suggesting that, rather than Treg function, induction of suppression is contact-dependent (Collison et al. 2009).

Th3 cells are regulatory T cells that secrete TGF- β abundantly (Santos et al. 1994). These primarily gut-derived cells play a role in mucosal tolerance, and participate in secretory IgA production (Commins et al. 2010). In addition to these, also $CD8^+$ T cells, natural killer T cells, B cells, and γδ TCR-expressing T cells suppress effector T-cell responses (MacDonald et al. 2011).

Regulatory T cell-mediated suppression acts in two distinctive ways:

1. In physiological and non-inflammatory states, suppression aims to keep T cells in a naïve state and serves to maintain natural self-tolerance by Treg-dependent deprivation of activation signals from responder T cells.
2. In inflammatory states, suppression serves to sustain local immune homeostasis by Treg-mediated killing and inactivation of responder T cells and APCs (Yamaguchi et al. 2011).

### 2.1.6 Th17 cells

The first reports of Th17 cells date to less than 10 years ago (Harrington et al. 2005); vigorous studies followed, and now the role of Th17 cells in the intestinal immunological response becomes clearer: Th17 cells mediate the T-cell immune response to extracellular mucosal pathogens.

IL-17-secretion characterizes Th17 cells, although these cells are also able to secrete other cytokines. Yao et al. (1995) were the first to describe IL-17; it shares amino acid identity with *Herpesvirus saimiri* (HSV13) and murine CTLA-8. IL-17 induces fibroblasts to produce IL-6 and IL-8, and up-regulates expression of the ICAM-1. IL-17 is a family of six cytokines (IL-17A through IL-17F) (Kawaguchi et al. 2004). In humans, IL-17A (generally referred to as IL-17) and IL-17F play a pivotal role. Th17 cells, neutrophils, eosinophils, and CD8+ T cells express IL-17A (Kawaguchi et al. 2004). IL-17A induces stromal cells, fibroblasts, endothelium, and epithelium to express a variety of cytokines and chemokines: IL-6, IL-11, GM-CSF, CXCL8, CXCL10, and TGF-β; all participating in fibroblast activation and neutrophil recruitment. IL-17F shows homology to the IL-17 motif; it regulates angiogenesis and cytokine expression in endothelial cells (Starnes et al. 2001). Th17 cells, but activated basophils and mast cells as well, express IL-17F (Kawaguchi et al. 2004).

A dichotomy between $FOXP3^+$ Tregs and Th17 cells exists. Under inflammatory conditions, IL-2 and IL-1β can convert human Tregs into Th17 cells (Deknuydt et al. 2009). In the absence of IL-6, TGF-β also induces FOXP3 and generates iTregs. IL-6, however, inhibits this TGF-β-driven FOXP3 expression and in cooperation with TGF-β induces Th17 cells (Bettelli et al. 2007). IL-6 activates STAT3, which in turn potentiates activation of transcription factor retinoic acid receptor-related orphan receptor (ROR) γt, a master regulator for Th17 cells (Commins et al. 2010). RUNT-related transcription factor *RUNX1* induces RORγt expression, and they cooperate during *Il17* transcription. RUNX1 has a dual role: interaction of RUNX1 and FOXP3 is necessary for FOXP3 to inhibit Th17 differentation (Zhang et al. 2008).

IL-6 and TGF-β activate Th17 cells, and IL-23 develops them into mature IL-17-secreting cells (Zhou et al. 2007). IL-23 is a heterodimer consisting of IL-12p40 and IL-23p19. IL-23 induces remodelling through activation of matrix metalloproteinases, increased angiogenesis, and reduced $CD8^+$ T cell infiltration (Oppmann et al.

2000). IL-21 is central to B cell immunity but is also involved in Th17 differentiation. It shares biological activities with IL-2 and IL-15: it activates NK cells and promotes proliferation of B and T lymphocytes (Parrish-Novak et al. 2000).

Th17 cells also secrete IL-22, a cytokine from the IL-10-family produced by T cells, mast cells, and activated NK cells (Dumoutier et al. 2000, Xie et al. 2000). Although Th17 cells in particular express IL-22, the immune cells are not targets of IL-22 due to a lack of IL-22R1, another part of the heterodimer forming the IL-22 receptor complex. IL-22 is instead a T-cell mediator that induces innate immunity in tissues and has many unique functions. It primarily acts on hepatocytes and epithelial cells, where it promotes antimicrobial defence, regeneration, and protection against damage and induces acute-phase reactants and some chemokines (Wolk et al. 2004). IL-22 is an ambivalent cytokine (Brand et al. 2006, Andoh et al. 2005), meaning that in chronic inflammatory disorders it may take either a protective or a pathogenic role. In the intestinal barrier, IL-22 induces the expression of protective defensins (Brand et al. 2006). $CD4^+$ cells secreting IL-22 form the subclass of Th cell called Th22 cells that produce IL-22, but no IL-17 or IFN-γ (Duhen et al. 2009, Trifari et al. 2009). Studies report IL-22 expression in the mucosa in IBD, especially in Crohn's disease, and in the plasma of Crohn's disease patients (Andoh et al. 2005, Wolk et al. 2007).

## 2.2 Gut immune system

### 2.2.1 Gut structure and function

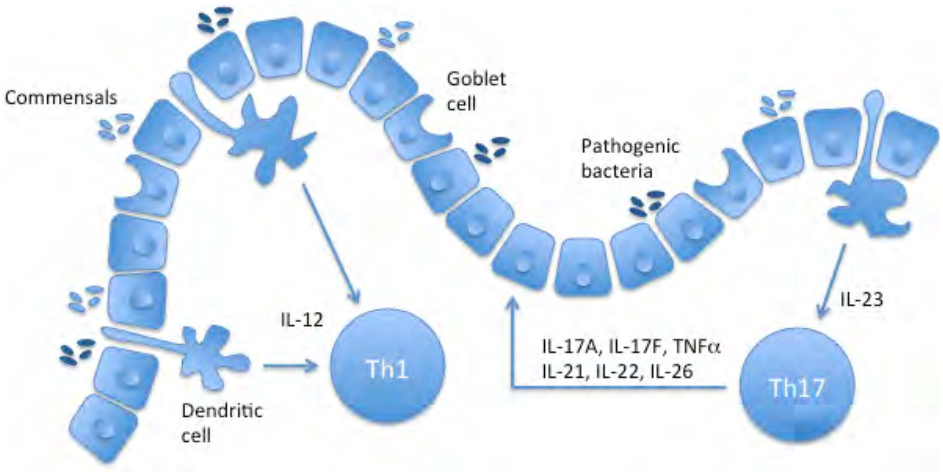


**Figure 2.** Interaction between intestinal epithelial cells, Th1, Th17, and antigen-presenting cells (dendritic cells). Th17 cells secrete proinflammatory cytokines, which in turn stimulate intestinal epithelial cells. Bacteria act on antigen-presenting cells, supporting the secretion of IL-12, a stimulus for Th1 expansion, and IL-23, a stimulus for Th17 expansion. (Adapted from Hundorfean et al. 2012)

The gut constitutes a crucial organ for communication between the body and its external environment. In an adult human being, intestinal epithelium covers an area of 300 to 400 $m^2$, thus comprising the largest organ communicating with various pathogenic and non-pathogenic micro-organisms such as infective agents, food antigens, and allergens, all of them potentially provoking a harmful immune response.

The intestinal epithelium serves as the primary protection against the external environment, and as the first line of defence, the epithelial and Paneth cells play an important role (Figure 2). The barrier against all these outsider threats is generally only one cell layer thick but yet protected by various chemical and physical innate defence mechanisms having close cooperation with the adaptive immune system (Turner 2009). Further, the high acidity of the stomach and active proteolytic enzymes secreted by the pancreas reduce the numbers of living micro-organism reaching the intestine. They also effectively digest antigenic proteins to small peptides unable to initiate immune responses.

A thick layer of mucus forms a physical and biological protective barrier, and stabilizes the antibody concentration. Intestinal villous epithelium houses goblet cells, which produce mucin 2, an intestinal mucus-formation molecule. In mice deletion of mucin 2, a major mucin component, results in intestinal inflammation (Van der Sluis et al. 2006).

Intercellular junctions between the epithelial cells called tight junctions (TJ), and also known as zonula occludens, regulate epithelial permeability. The TJ in epithelial cells includes the integral membrane proteins (occludins), junctional adhesion molecules, claudin proteins, scaffold proteins (ZO-1 and myosin IXB), and zonulin (Turner 2009). Zonulin is a 47-kDa protein involved in intestinal immunity and associated with autoimmune diseases: its upregulation occurs both in celiac disease and in T1D (Clemente et al. 2003, Sapone et al. 2006, Fasano 2011). Upregulation of zonulin is also linked to other autoimmune diseases such as ankylosing spondylitis, rheumatoid arthritis, and Crohn's disease - thus linking together the diseases studied here.

To cross the intestinal barrier, absorbed proteins mainly use a transcellular pathway during which lysosomal degradation converts the proteins to nonimmunogenic peptides. A small amount of proteins (10%) carry on as intact proteins leading to antigen-specific immune responses. This process uses the microfold (M) cell or the paracellular pathway that regulates intercellular TJ, and antigenic tolerance follows (Neutra et al. 2001). The intestinal epithelium plays an active role in both the innate and the adaptive type of mucosal immunity through the collaboration of adjacent epithelial cells (ECs), parenchymal cells, hematopoietic cells, and likely microbial components in the lumen (Paul 2003).

Underneath the surface epithelium and above the muscularis mucosa is LP, an area populated by smooth muscle cells, fibroblasts, and cells belonging to the gut-associated lymphoid tissue (GALT), discussed below.

### 2.2.2 Cells in the gut-associated lymphoid tissue

GALT is part of the mucosal-associated lymphoid system (MALT) and plays a major role in the body's delicate equilibrium. GALT consists of mesenteric lymph nodes, Peyer's patches (PPs), isolated lymph nodes, T and B lymphocytes, macrophages, dendritic cells, mast cells, neutrophils, and other granulocytes.

In a normal gastrointestinal (GI) tract, about one-third of the intestinal LP cells constitute T cells distributed into $CD4^+$ and $CD8^+$ T cells similarly as in peripheral blood. Most of these lamina propria lymphocytes (LPLs) are HLA-$DR^+$, $\alpha4\beta7^+$, $CD62^{lo}$, $CD25^{hi/lo}$, and $CR45RO^+$, similar to effector-memory cells (MacDonald et al. 2011). In equal abundance to T cells are IgA-producing plasma cells, in addition to which, macrophages and dendritic cells (DCs) reside in the intestine (Macdonald, Monteleone 2005). In the small intestine, the epithelial layer contains one T cell for every 10 epithelial cells; in the large intestine the ratio is approximately 1:20. Despite the activated immune cells, healthy individuals have no pathologic features indicating that regulatory pathways maintain immunologic homeostasis. In inflammatory diseases of the intestinal tract this homeostasis is interrupted, and intestinal inflammation is evident. Inflammation most likely arises from homeostatic disruptions and recognition of normal microbiota as pathogens (Macdonald, Monteleone 2005).

In addition to PPs, interactions between commensal bacteria, GI antigens, and immune cells take place in isolated follicles. In these follicles, M cells within the follicle-associated epithelium translocate antigens from the lumen. Immature myeloid DCs encounter and process these antigens, become differentiated into mature DCs, and migrate to PPs or mesenteric lymph nodes (MLNs) in order to activate T cells. GALT thus serves as a source of activated effector cells. Other mechanisms by which luminal bacteria enter the body are the M-cell-independent pathway consisting of LP DCs that extend dendrites into the lumen, and the columnar epithelial cells that are eligible for antigen uptake (MacDonald et al. 2011).

First, naïve T cells home to the PPs or MLNs, where they encounter antigen-loaded DCs that prime, polarize, and expand the lymphocytes to yield Th1 or Th17-effector cells (Yen et al. 2006). DCs present enteric antigens in association with MHC II. T cells proliferate during this initial priming process and enhance expression of surface molecules (such as α4β7, CCR 9, LFA-1, and CD44). Following initial priming, the effector T cells reenter the circulation and home to the intestinal interstitium. APCs present to T cells their specific antigen, resulting in a rapid and avid response of T cells that elevates the production of IFN-γ, IL-17, TNF-α, lymphotoxin-α, and IL-2. The production of these cytokines further enhances the production of Th1/Th17 and of macrophage-derived inflammatory mediators, resulting in recruitment and activation of additional intestinal leukocytes, thus causing intestinal inflammation (Koboziev et al. 2010). In LP T cells, TCR signaling is hyporesponsive compared to that of peripheral blood T cells. In addition to activated

CD4 cells, CD8 cells recognizing MHC class I molecules are also present in the LP, although they predominate in the epithelium.

### 2.2.3 Mucosal immune response

In the intestine, immunological tolerance is crucial; the intestinal immune system must defend against pathogens but at the same time coexist with resident intestinal microbiota. Mechanisms of tolerance include limiting intestinal microbial exposure and actively down-regulating the immune response (Abraham, Medzhitov 2011). Microbial signals affect intestinal immune tolerance, and inflammatory processes can be down-regulated by host-microbial interaction. These interactions may regulate pattern recognition receptor (PRR) expression and responsiveness, secrete inhibitory mediators, and modulate transcription and expression in intracellular signaling pathways (Abraham, Medzhitov 2011). The GI tract poorly tolerates any type of uncontrolled immune response, and thus intestinal inflammation easily follows (Siegmund, Zeitz 2011).

In animal models, development of oral tolerance relies on bacterial colonization of the GI tract (Sudo et al. 1997, Tsuda et al. 2010). Unlike other tissues, the intestinal mucosa experiences continuous physiologic inflammation. The intestine is exposed to a huge antigenic load ranging from luminal bacteria to toll-like receptor (TLR) ligands and potential mitogens. Intestinal innate immunity includes the epithelial barrier and phagocytic cells within the LP (macrophages, dendritic cells, and neutrophils). In addition, it encompasses several innate leukocyte and intestinal epithelial cells (IEC) populations, which cooperate to sustain a balanced immune response to the microbiota (Harrison, Maloy 2011).

### 2.2.4 Immune regulation by commensal microbiota

The intestinal tract houses microbial communities that are essential for mammalian health. These microbial communities, termed the intestinal microbiota, sustain a symbiosis with their host. The intestinal microbiota consist of about $10^{14}$ bacteria that aid the host by breaking down indigestible food, (e.g. fiber), in part into absorbable compounds, at the same time they secure for themselves an environment with a constant flow of nutrients. To maintain homeostasis, the immune system plays a dual role: it has to be tolerant to the microbiota but simultaneously respond efficiently to infection. The microbiota itself also prevents outgrowth of pathogens, but changes in the complexity and density of the microbiota can disrupt this ability (Jarchum, Pamer 2011). A variety of factors influences the microbiota composition: diet, antibiotic therapy, environmental exposure to microorganisms, and sequential microbial colonization in the neonatal period. Production of antimicrobial peptides by Paneth cells, mucus production by goblet cells, and the control of microbes by secretory IgA are all immune defence mechanisms serving to maintain immune

homeostasis. Dysregulation of these host-microbe interactions can lead to intestinal inflammation (Abraham, Medzhitov 2011).

The intestinal immune system defends against microbiota. PRRs recognize microorganisms and initiate defence action. TLRs, C-type lectins, nucleotide-binding domain and leucine-rich repeat-containing receptors, and retinoic acid-inducible gene 1-like receptors are PRRs that recognize pathogen-associated molecular patterns. TLRs participate both in innate and in adaptive immune responses and play a key role in infection defence (Rakoff-Nahoum, Medzhitov 2008). IECs mediate defence mechanisms by expressing PRRs, and by secreting cytokines and antimicrobial proteins, and by up-regulating surface molecules (Abraham, Medzhitov 2011). Phagocytosis and autophagy mediate the killing of microbes. Intestinal LP macrophages are distinct from blood monocytes and retain active phagocytic and bactericidal activity (Smythies et al. 2005). In clearance of intracellular components, autophagy is a crucial mechanism. In autophagy, response to invasive bacteria, intracellular sensors nucleotide-binding oligomerization domain (NOD) 1 and NOD2 are essential because they recruit the autophagic protein ATG16L1 to the site. Mutant NOD2 fails to recruit ATG16L1, resulting in impaired autophagy (Travassos et al. 2010). The IL-23/Th17 cell pathway defends against microbial infection, but the activated Th17 cell produces IL-23 and other cytokines that contribute to tissue inflammation (Hue et al. 2006). Mucosal responses actively regulate these cytokines. Secretion of intestinal IgA also reduces microbe penetration (Macpherson et al. 2008).

Association of IBD with variants in IL23R and genomic regions including other loci in the IL-23/Th17 pathway indicates that this pathway plays an important role in regulating intestinal immune homeostasis (Barrett et al. 2008).

## 2.3 Intestinal manifestations of disease

### 2.3.1 Crohn's disease

Inflammatory bowel disease (IBD) comprises different disease entities: Crohn's disease, ulcerative colitis (UC), and unclassified colitis. Of these, Crohn's disease was traditionally considered to be a predominantly Th1 type of disease and ulcerative colitis a Th2 type. In Crohn's disease versus UC, different T cell populations aberrantly are activated (Fuss et al. 1996). Discovery of Th17 cells made, however, this dichotomy invalid. Populations in northern Europe and North America have the highest prevalence of IBD indicating the role of the westernized lifestyle and environment in IBD pathogenesis. Factors associated with IBD are smoking, diets high in fat and sugar, use of medication, stress, and high socioeconomic status (Ahuja, Tandon 2010, Danese et al. 2004).

Crohn's disease is an intestinal disease also called terminal ileitis, regional enteritis, granulomatous ileitis, hyperplastic ileitis, chronic ulcerative ileitis, and intes-

tinal phlegmon. Crohn, Oppenheimer and Ginzburg in 1932 were the first to describe regional ileitis, later called Crohn's disease (Crohn et al. 1984). Crohn's disease is a chronic disease characterized by periods of inactive disease (remission) interrupted by acute flares of inflammation (relapses); sometimes the disease may be constantly active (chronically active disease). The site of inflammation may be anywhere from the mouth to the anus, and lesions in both sites are typical. The most prevalent site in the intestine is the terminal ileum, but inflammation may affect any part of the intestine. Typical lesions in the intestine are aphtous ulcerations. Lesions are patchy with intervening normal tissue. Under a microscope, the inflammation is transmural. Its hallmark is granulomatous inflammation.

Crohn's disease manifests in young people with its incidence highest between ages 15 to 30, and with 25 to 35% developing the disease before the age of 20 (Mestecky et al. 2005). Early-onset IBD is likely to be more extensive and aggressive than is adult-onset IBD (Turunen et al. 2009). In pediatric patients, Crohn's disease often manifests as colonic disease, with the upper GI tract also being commonly involved (Biank et al. 2007, Heyman et al. 2005).

### *Pathogenesis*

A hypothesis is that a breakdown in tolerance is central to the immune pathogenesis of IBD (MacDonald 1995). Only limited evidence confirms this, however. The pathogenesis of Crohn's disease is incompletely resolved, and currently the general hypothesis is still accepted: in genetically predisposed individuals, exposure to distinct environmental factors results in a dysregulation of the mucosal immune system. In Crohn's disease, innate immunity abnormalities associate with the gene variants of NOD2, ATG16L1, and IRGM, which encode microbial recognition mediators. NOD2, also designated CARD15, was, in western populations, the first gene associated with Crohn's disease susceptibility (Hugot et al. 2001, Ogura et al. 2001, Hampe et al. 2007, Parkes et al. 2007). Three variants of NOD2 exhibit the strongest Crohn's disease association, leading to an increased risk for developing Crohn's disease that is 2- to 4-fold (Economou et al. 2004). Muramyl dipeptide (MDP) is a peptidoglycan in the cell wall of $Gram^+$ and $Gram^-$ bacteria; NOD2 is a cytoplasmic receptor for MDP (Girardin et al. 2003).

Traditional views are of Crohn's disease as a Th1 type of disease, IL-12 being the key inducer of Th1 cells. Finding the key role of the IL-12 family in IBD pathogenesis supports this classification (Neurath et al. 1995). The factor behind the relapsing nature of the disease may be colitogenic Th17 cells (Kanai et al. 2009).

The immunological background may vary between early-onset and adult-onset IBD (Henderson et al. 2011, Nieuwenhuis, Escher 2008). Epithelial chemokine production is linked only with early-onset Crohn's disease (Damen et al. 2006), and in both pediatric and adult IBD patients, intestinal immunoregulation fluctuates (Kugathasan et al. 2007, Damen et al. 2006).

### 2.3.2 Ulcerative colitis

Continuous inflammation limited to the large intestine characterizes ulcerative colitis. Typically, the inflammation is most severe in the rectum (proctitis) and sometimes spreads to the whole large intestine. In the acute phase, lesions are continuous bleeding ulcerations and edema. Chronic disease results in fibrosis and polyps. Microscopy shows infiltration of glands by neutrophils, and their number gradually falls; the inflammation is present only in the LP.

Clinically, the hallmark of UC is bloody diarrhea with or without mucus. The first description of UC appeared long before Crohn's disease, in the mid-19$^{th}$ century, by Samuel Wilks (Danese, Fiocchi 2011). Compared to Crohn's disease, UC has a higher incidence and is more prevalent among adults. In Finland, UC continues to be more common in spite of the increase in prevalence of Crohn's disease in between 1987 and 2003 (Lehtinen et al. 2011).

Originally UC was thought to be a Th2-driven disease, but this dogma changed after observations that IL-13 and IFN-γ are both elevated in UC mucosa, and that colonic IL-4 is absent from UC. Thus, UC represents a quite superficial epithelial injury disorder (Siegmund, Zeitz 2011).

#### *Pathogenesis*

The working hypothesis for the pathogenesis of UC suggests that UC consists of a heterogeneous group of diseases with similar yet distinguishing phenotypes (Mestecky et al. 2005). Genetic, subclinical, and clinical varieties exist and play a role in the intestinal inflammation seen in UC (Mestecky et al. 2005). The genetic background is less dominant in UC than in Crohn's disease: a meta-analysis of genetic studies shows an association of 47 loci with UC, with only 19 of them specific to UC and 28 being also present in Crohn's disease (Anderson et al. 2011). These associations link to epithelial barrier dysfunction (ECM1, HNF4A, CH1, LAMB1), apoptosis and autophagy (DAP), and defects transcription regulation (IL23R, JAK2, STAT3, IL12B, PTPN2). In addition, UC shares risk loci associated with other immune-system-mediated diseases (HLA and genes participating in Th1 and Th17 differentiation, such as IL10, IL17R, IL23R, and IFN-γ).

Autoantibodies are characteristic of various autoimmune diseases; in UC, an association exists with perinuclear antineutrophil cytoplasmic antibodies (pANCA) and IgG1 antibodies against colonic epithelial antigen exists (Bhagat, Das 1994, Seibold et al. 1998). About 70% of UC patients present with the circulating autoantibody pANCA, which reacts to the mucosal pANCA-reactive antigen and is related to a mucosal immune response (Saxon et al. 1990). The T-cell response to this antigen links T cell immunity to the autoantibodies (Mestecky et al. 2005)

To maintain intestinal homeostasis requires a controlled innate immunity response to the microbiota. Epithelial and immune cells house TLRs and NOD-like receptors, which recognize microbiota and affect the recognition process leading to

tolerance, or – if deregulated – leading to inflammation (Abreu 2010). Inflammatory bowel disease, regardless of the type of disease, shows increased production of pro-inflammatory cytokines like IL1β, IL-6, TNF-α, and tumor necrosis factor-like ligan 1 (Danese, Fiocchi 2011). To date, no definite evidence exists as to specific innate immune defects in UC. However, adaptive immunity abnormalities both in humoral and cellular aspects are associated with UC. In addition to elevated IgM, IgA and IgG levels, especially IgG1 antibodies increase in UC (Takahashi, Das 1985). According to the traditional Th division, UC is a Th2-type disease characterized by the colonic non-classical IL-13-secreting NK T cells. These cells lead to cytotoxity in epithelial cells, to apoptosis, and dysfunction of the epithelial barrier (Fuss et al. 2004, Heller et al. 2005). Moreover, in UC, IL-5-secreting Th2 type T cells prevail, and Th17 cells also occur in the mucosa of IBD patients (Fujino et al. 2003).

### 2.3.3 Celiac disease

Celiac disease is an autoimmune disease characterized by enteropathy caused by gluten (composed of two main proteins: gliadins and glutenins) in the diet. The hallmark of celiac disease is an increased rate of destruction of small intestinal surface epithelial cells by inflammation. In the early phase of celiac disease, only inflammatory cells are infiltrating the epithelium; compensation for loss of epithelial cells is by an increased rate of proliferation of cells in the crypts; these are consequently elongated. When proliferation fails to compensate for the loss of epithelial cells, the villi are gradually lost, and even the remaining flat epithelium becomes made up of immature epithelial cells (Lindfors et al. 2010). Small-intestinal destruction in celiac disease manifests with malabsorption symptoms in young children under the age of two: abnormal stools, impaired growth, abdominal distension, muscle wasting and hypotonia, and poor appetite. In older children and adults, these symptoms may be absent, and other intestinal complaints, such as abdominal pain, constipation, and bloating prevail. Iron deficiency anemia is the most common nutritional deficiency (Savilahti et al. 2010). Currently the only treatment for celiac disease is a lifelong gluten-free diet (GFD) diet. The prevalence of celiac disease is rising and has doubled in Finland over the decades: 1.05% from 1978 to 1980 and 1.99% from 2000 to 2001 (Lohi et al. 2007). This increasing prevalence is not due solely to efficient detection, but most likely depends partly on unknown environmental factors.

In addition to the environmental factors, a genetic predisposition is necessary. Celiac disease occurs mainly in genetically susceptible individuals and associates with HLA II genes; most patients are either HLA-$DQ2^{+}$ or HLA-$DQ8^{+}$. The absence of these HLA types has a negative predictive value for celiac disease that is close to 100% (Wolters, Wijmenga 2008).

*Pathogenesis*

In the pathogenesis of the disease, an immunologic response to gliadin peptide modified by transglutaminase-2 (TG-2) plays a major role. Enhanced permeability challenges the usual intestinal homeostasis; for instance, the TJ system is less efficient, partly due to upregulation of zonulin. In addition, the Th1 type of inflammatory cytokines, like TNF-α and IFN-γ, enhance intestinal permeability and potentiate the access of gliadin to the LP and the subsequent intestinal damage (Lionetti, Catassi 2011). In the LP a cascade leading to celiac disease occurs: the adaptive immune response to gluten-derived peptides causes intestinal destruction. APCs housing HLA molecules (DQ2/DQ8) present gluten peptides and induce a $CD4^+$ T-cell response. Preceding deamination, tissue TG-2 converts glutamine into negatively charged glutamic acid, and deaminated gliadin peptide binds to HLA molecules with high affinity (Molberg et al. 1998). Activated $CD4^+$ T cells promote inflammatory effects by secreting pro-inflammatory cytokines, followed by secretion of matrix metalloproteinases from fibroblasts or LP mononuclear cells (Lionetti, Catassi 2011). Activated $CD4^+$ T cells also secrete Th2-type cytokines, causing activation and clonal expansion of B cells that differentiate into plasma cells and produce anti-gliadin and anti-tTG antibodies. This inflammation reduces the ability of epithelial cells to adhere to the basal membrane. At the same time, the apoptosis of epithelial cells is increased; these phenomena result in the increased destruction of villous epithelial cells.

In potential celiac disease, the normal mucosal architecture prevails, but in the intestine, elevation of the density of $\gamma\delta TCR^+$ intraepithelial lymphocytes occurs and celiac disease-associated antibodies against tissue transglutaminase (TGA) appear in the peripheral blood (Holm et al. 1992, Arranz et al. 1994, Kaukinen et al. 1998). In addition, manifestation of serum endomysium autoantibodies predicts future celiac disease (Maki, Collin 1997, Kaukinen et al. 2007).

### 2.3.4 Type 1 diabetes

Unlike the diseases already discussed, T1D is not traditionally considered an intestinal disease, but instead, is considered an endocrinal disease characterized by β-cell destruction in the pancreas resulting in failure of insulin secretion and thereby leading to high plasma-glucose levels. T1D predominantly affects young children, with half of the new cases younger than 15.

*Pathogenesis*

T1D is an immunological disease developing in genetically predisposed individuals: it associates with HLA DQB1*0302 and 02 alleles. First, during the pre-diabetic phase, circulating autoantibodies in blood are detectable. Later, T-cell-mediated immune mechanisms cause the death of insulin-producing beta cells in the pancreas to the extent that clinical diabetes ensues (Vaarala et al. 2008). In fetal development, the pancreas originates from the gut endoderm thus sharing the same biological

background as the intestine. In pancreatic islets, the endothelium expresses the mucosal homing receptor MadCAM-1 (Hanninen et al. 2007).

Animal models were the first to show the intestinal aspect of the disease: diet modulated the development of autoimmune diabetes (Elliott et al. 1988). Studies have shown that approximately half of all the children with T1D present with subclinical intestinal immune activation in their intestinal specimens (Westerholm-Ormio et al. 2003, Auricchio et al. 2004). Preceding the clinical onset of T1D, small-intestinal enteropathy already exists (Bosi et al. 2006). High gut permeability also associates with early onset autoimmune diabetes in an animal model of Bio-Breeding rats (Visser et al. 2010). Nevertheless, based on current evidence, not only does the leaking gut cause pancreatic beta-cell destruction, but intestinal inflammation also contributes to it. Evidence exists that enteric infection contributes to the destruction of the intestinal epithelial barrier leading to the activation of beta-cell-specific autoimmunity in pancreatic lymph nodes, and thus affecting the development of insulitis and beta-cell destruction (Graham et al. 2004, Watts et al. 2005, Watts et al. 2005, Neu et al. 2005). Enteric infections associate with epithelial cell destruction (Jalonen et al. 1991, Coyne, Bergelson 2006).

Control of Th17 cells occurs in the gut, especially in the small intestine. According to a recent study, the Th17 cells navigate to the duodenum to be either eliminated or re-programmed to gain regulatory and immunosuppressive features (Esplugues et al. 2011). In peripheral blood samples, activated IL-17 immunity associates with human T1D (Honkanen et al. 2010); these IL-17$^{+}$ T-cells also express CCR6, a chemokine receptor for Th17, indicating abilities for mucosal homing. In T1D, Th 17 immunity presents with plastic Th17 cells capable of secreting various cytokines such as IL-9, IFN-γ, and IL-22.

### 2.3.5 Immunomodulation by medication

#### *Immunomodulation by TNF-α-blocking agents*

TNF-α is a protein derived from mononuclear phagocytes; neutrophils, lymphocytes, NK cells, mast cells, and endothelium also produce it. When interacting with endothelial cells, TNF induces intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1, and E-selectin, thus causing migration of granulocytes to the infective site. TNF activates neutrophils and mediates adherence, chemotaxis, degranulation, and the respiratory burst. TNF share many biologic activities with IL-1, but unlike IL-1, TNF has no direct effect on lymphocyte proliferation (Commins et al. 2010).

TNF-α-blocking agents include infliximab, adalimumab, and certolizumab. Infliximab is an intravenously administered chimeric monoclonal IgG1 antibody which binds both circulating and membrane-bound human TNF-α. Infliximab induces a cell-mediated cytotoxic reaction, enhances apoptosis of activated T cells, and causes elimination of TNF-α-expressing cells (Triantafillidis et al. 2011). Ada-

limumab is a subcutaneously administered human IgG1 type anti- TNF-α monoclonal antibody. Certolizumab pegol is a subcutaneously administered humanized TNF-α-binding Fab´ fragment of an IgG antibody attached to an ethylene glycol moiety. The USA currently approves certolizumab, but in Europe only Switzerland does so (Triantafillidis et al. 2011).

Opportunistic infections, malignancies, and infusion/injection reactions comprise the most important side-effects concerning TNF-α-blocking agents (Triantafillidis et al. 2011). Formation of antibodies to infliximab (human antichimeric antibodies, called HACA) leads to infusion reactions, loss of response, and serum-sickness-like delayed reactions. Screening for latent tuberculosis is necessary. Around 30% of patients with refractory Crohn's disease are resistant to infliximab therapy. Patients with biologically active inflammation (increased CRP) are the best responders to infliximab. Smokers are less likely to respond (Rutgeerts et al. 2006).

### *Immunomodulation related to IL-17*

Immunomodulation by IL-17 or IL-17R-blocking agents is the next step in the search for new medications against autoinflammatory diseases. Ixekizumab is a subcutaneously administered humanized monoclonal IgG4 anti-IL-17 antibody. It improves clinically evident symptoms of psoriasis, a chronic skin disorder (Leonardi et al. 2012). Brodalumab, on the other hand, is an intravenously administered human anti-IL-17RA monoclonal antibody that antagonizes the IL-17 pathway and improves plaque psoriasis (Papp et al. 2012).

# 3 Aims of the study

The purpose of this study was to evaluate the role of IL-17 immunity, the expression patterns of immunological markers associated with the IL-17 type of immunity, and the role of regulatory $CD4^+$ cells in the intestine in disorders of the gut.
The intestinal markers for regulatory T cells and effector T cells (Th1, Th17) were therefore characterized:

- in adult patients with active and inactive Crohn's disease treated with conventional treatment
- in adult patients with Crohn's disease treated with TNF-α-blocking agents
- in children with inflammatory bowel disease
- in children with celiac disease or type I diabetes

# 4 Patients and methods

## 4.1 Adults with Crohn's disease

### 4.1.1 Patients receiving conventional therapy

At the Helsinki University Central Hospital, one group comprised 24 adult Crohn´s disease patients with ileal or ileocolic disease referred to ileocolonoscopy (Table 1). The Vienna classification (Gasche et al. 2000) served to classify the Crohn's disease diagnosis that was based on the clinical, endoscopic, radiologic, and histologic findings. The Crohn's disease diagnosis of four patients relied on their current endoscopic and histological findings. Pregnancy, history of an extensive bowel resection (ileosigmoideostomy, ileorectostomy), ostomy, longterm use of NSAIDs, or symptoms related to perianal fistulizing disease served as exclusion criteria. At the time of endoscopy (±2 weeks), patients completed a diary for the Crohn's disease activity index (CDAI) (Best et al. 1976), and provided a stool sample and a heparinized blood sample. The Crohn´s disease index of severity (CDEIS) served to grade the endoscopy findings (Mary, Modigliani 1989). Endoscopic signs of active ulcerative inflammation in the ileum verified by active inflammation in ileal samples in routine histology served as criteria for active disease. As criteria showing inactive disease, all 13 patients in the remission group had macroscopically normal mucosa in routine histology. In endoscopy, 10 of them had an uninflamed ileum and 3 mild inflammatory activity. Both patient groups showed either ileal or ileocolic disease. The control group comprised 14 patients referred to endoscopy for the following indications: change in bowel habits in 3, diverticular disease in 2, colorectal cancer follow-up in 2, history of polyps in 1, history of adenoma in 2, rectal bleeding in 2; exclusion of Crohn's disease for one was history of perianal abscesses and for one was abdominal pain. All control subjects, in routine histological analysis of ileal biopsies, showed normal findings. Fecal samples were available from nine patients with active and 11 patients with inactive disease. In addition, 10 healthy adults provided fecal samples.

**Table 1.** Characteristics of patients in Study I. Data as medians (with ranges).

| | References | Inactive disease | Active disease |
|---|---|---|---|
| **Gender** | 5M/9F | 5M/8F | 6M/5F |
| **Age in years** | 57.5 (22.0-74.1) | 37.3 (22.7-53.2) | 30.6 (20.0-51.3) |
| **Disease duration in years** | - | 7.5 (3.3-31.1) | 3.0 (0-26.7) |
| **CDAI** | - | 78 (2-136) | 156 (0-605) |
| **CDEIS** | - | 1.3 (0-3.2) | 9.5 (4.0-26.0) |
| **f-Calprotectin (µg/g)** | - | 62 (11-177) | 969 (53-2284) |
| **Maintenance treatment** | | | |
| **no medication** | 14 | 0 | 5 |
| **5ASA** | - | 9 | 4 |
| **SASP** | - | 1 | 2 |
| **thiopurine** | - | 10 | 5 |
| **methotrexate** | - | 0 | 1 |
| **metronidatzole** | - | 0 | 1 |
| **corticosteroids** | - | 1 | 2 |

5-aminosalicylic acid (5ASA), sulfasalazine (SASP)

### 4.1.2 Patients receiving anti-TNF-α-blocking agents

At Helsinki University Central Hospital, 13 adult Crohn's disease patients underwent ileocolonoscopy twice: a baseline endoscopy and at 12 weeks a treatment-response evaluation. CDEIS served to assess the endoscopy findings: scores < 3 suggested inactive disease, and ≥ 3 endoscopically active disease (Mary, Modigliani 1989, Sipponen et al. 2008). CDAI served to evaluate the clinical activity (Best et al. 1976). At the time of endoscopies, patients also provided blood and fecal samples (Table 2). Following the baseline endoscopy, all patients received anti-TNF-α therapy (median 7 days afterwards, range 1-38 days): infliximab infusions 5 mg/kg took place at weeks 0 and 8. At week 0, one patient received a subcutaneous induction dose of 80 mg adalimumab, followed by 4 doses of 40 mg subcutaneously every other week; thereafter, at 10 weeks she underwent the control endoscopy. Prior to the introduction of anti-TNF-α-therapy, no changes in medication occurred, but afterwards their use of corticosteroids diminished.

**Table 2.** Characteristics of patients in Study II. Data as medians (with ranges).

| | Reference patients | Crohn's disease patients before treatment | Crohn's disease patients at 3-month follow-up |
|---|---|---|---|
| **Gender** | 5M/9F | 6F/7M | |
| **Age in years** | 58 (22-74) | 23 (19-44) | |
| **Disease duration in years** | - | 5.1 (0.4-27.0) | |
| **CDEIS** | - | 14.4 (1.8-25.3)* | 4.4 (0.0-11.2) |
| **CDAI** | - | 174 (50-605)** | 64 (24-112) |
| **f-Calprotectin (µg/g)** | | 1173 (88-15326)*** | 127 (13-1419) |
| **C-reactive protein (mg/l)** | | 8 (<5-42)§ | <5 (<5-6) |
| **Inflammatory disease** | - | 6 | |
| **Stricturing disease** | - | 4 | |
| **Inflammatory and perianal disease** | - | 3 | |
| **Ileal disease** | - | 2 | |
| **Colonic disease** | - | 3 | |
| **Ileocolonic disease** | - | 8 | |
| **Medication** | - | 13 | 13 |
| **azathioprine/6-mercaptopurin** | - | 9 | 11 |
| **methotrexate** | - | 2 | 2 |
| **corticosteroids** | - | 9 | 0 |
| **mesalamine** | - | 9 | 8 |
| **salazosulfapyridine** | - | 2 | 2 |
| **metronidatzole** | - | 1 | 0 |
| **Previous anti-TNF-α-treatment** | - | 4 | |

*P=0.006, ** P=0.003, *** P=0.002, § P=0.012

## 4.2 Pediatric patients

### 4.2.1 Children with inflammatory bowel disease

At the Children's Hospital of University of Helsinki, we obtained ileal and colonic biopsy specimens from 29 pediatric patients undergoing diagnostic ileo-colonoscopy for exclusion of IBD: 8 Crohn's disease, 11 UC, and 10 control patients. The Lennard-Jones criteria (Lennard-Jones 1989) served to establish the IBD diagnoses. The endoscopy allowed taking biopsy specimens from the distal ileum and colon for routine histology and study purposes: immunohistochemical and quantitative RT-PCR studies. All children also underwent upper GI endoscopy, and all control subjects showed normal findings in routine histological analyses of biopsies (Table 3).

**Table 3.** Characteristics of patients in Study III. Data as medians (with ranges). In addition to medication, one Crohn's disease patient also took *Saccharomyces Boulardii* and one ulcerative colitis patient omepratzole.

| | Reference children | Crohn's disease | Ulcerative colitis |
|---|---|---|---|
| **Patients** | 14 | 12 | 13 |
| **Intestinal biopsy and blood samples** | 4F/6M | 2F/6M | 4F/6M |
| **Only blood sample** | 2F/2M | 1F/3M | 3M |
| **Age in years** | 7.7 (2.3-15.3) | 13.6 (9.1-18.5) | 12.3 (2.6-16.3) |
| **Disease duration in years** | - | 2.5 (0-15.6) | 0 (0-6.1) |
| **C-reactive protein (mg/l)** | <5 (<5-27) | 15 (<5-45) | 28 (<5-64) |
| **Erythrocyte sedimentation rate (mm/h)** | 7 (2-30) | 27 (7-102) | 31 (2-53) |
| **Hemoglobin (g/l)** | 133 (125-150) | 125 (109-142) | 110 (103-150) |
| **Blood leucocyte count (10E9/l)** | 6.3 (3.5-18.0) | 5.6 (3.2-17.4) | 9.3 (5.4-18.0) |
| **f-Calprotectin (µg/g)** | 31 (1-103) | 1226 (268-2010) | 952 (73-2604) |
| **No medication** | 14 | 8 | 7 |
| **Azathioprine** | - | 2 | 1 |
| **Corticosteroids** | - | 2 | 1 |
| **Mesalamine** | - | 1 | 5 |
| **Metronidatzole** | - | 1 | 2 |

### 4.2.2 Children with celiac disease

Study IV comprised 108 patients both from Sweden and from Finland (Table 4). The Finnish population comprised 71 patients undergoing capsule endoscopy at the Children's Hospital of the University of Helsinki. None of them fulfilled diagnostic criteria of celiac disease as established by the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (Troncone et al. 2004). Of the 32 patients classified with celiac disease, none had been on a gluten-free diet, and all showed adequate structural changes in the intestine. Five patients had suspected celiac disease (Ferguson et al. 1993). Reference children comprised 15 undergoing endoscopy for clinical reasons such as stomach ache. They showed no positive antibodies related to celiac disease before the endoscopy, and a pathologist classified their biopsy specimens as healthy.

**Table 4.** Celiac disease patient characteristics in Study IV. Data as means.

| | Reference | Potential celiac disease | Celiac disease | Celiac disease, gluten-free diet |
|---|---|---|---|---|
| **Patients** | 32 | 15 | 45 | 16 |
| **Gender** | 11M/21F | 6M/9F | 15M/30F | 5M/11F |
| **Age in years** | 8.7 | 11.7 | 9.0 | 6.4 |

### 4.2.3 Children with type 1 diabetes

Within Study IV, we had 19 children with T1D. Of these, 13 had previously established T1D, and their biopsies served to exclude celiac disease. In addition to T1D, 6 had also celiac disease (Table 5).

**Table 5.** Type I diabetes patient characteristics in Study IV. Data as medians (with ranges).

| | T1D | T1D+CD |
|---|---|---|
| **Patients** | 13 | 6 |
| **Gender** | 11M/2F | 3M/3F |
| **Age in years** | 9.3 (2.6-15) | 9.7 (2.3-13.8) |
| **Disease duration in years** | 4.5 (0.3-11.7) | 3.7 (1.3-9.5) |

## 4.3 Samples

Ileocolonoscopy was performed with a colonoscope to obtain specimens from the colon and distal ileum in Studies I, II, and III. In Study IV, a tissue sample from the distal duodenum or the proximal jejunum was acquired by a Watson capsule biopsy device or a gastroscope. In Studies III and IV, all the children underwent endoscopies under general anesthesia.

In the laboratory, the specimens were embedded in optimal cutting temperature (OCT) compound (Miles Laboratories), snap frozen, and stored at -80°C. For immununohistochemical staining, frozen tissue samples were cut into 7-µm sections, and thereafter coded and evaluated by a person blinded to specimen identity.

## 4.4 Immunohistochemistry

### 4.4.1 Immunoenzymatic labeling

Intestinal biopsy specimens from the ileum and the colon intended for immunohistochemistry were embedded in optimal cutting temperature compound (Miles Laboratories), and thereafter frozen and stored at -80ºC until used. Prior to immunohisto-

chemical staining, the tissue was cut into 7-µm sections. The avidin-biotin immunoperoxidase system served as the stain for the frozen sections, in which the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) was used according to the kit instructions. After acetone fixation, the slides were blocked in normal serum for 30 minutes. Mouse monoclonal antibodies and one rabbit polyclonal antibody (Table 6) served as the primary antibodies, and the slides were incubated for one hour. Methanol-0.5% hydrogen peroxide for 20 minutes quenched the endogenous peroxidase activity. Thereafter, the slides were incubated in biotinylated antibody and in ABC reagent for 30 minutes each. The chromogen we used, 9-amino-ethyl-cardatzole, and thereafter the slides were counterstained with Harris hematoxylin, and were washed in PBSbetween the staining-protocol steps. Omission of the primary antibodies created the negative controls. Prior to immunostaining of cytokines and FOXP3, the slides were incubated in 0.1% PBS-Tween20 for 10 minutes at room temperature to make the tissue permeable. 1% normal serum in 0.1% PBS-Tween20 was used to dilute the intracellular antibodies, and then the slides were incubated with the antibodies for one hour at +37$^{o}$C in a humified chamber.

**Table 6.** Antihuman antibodies used for the immunihistochemical stainings.

| Antibody | Dilution | Company | Clone | Study |
|---|---|---|---|---|
| **CD4** | 1:20 | BD Pharmingen | RPA-T4 | I, II, III, IV |
| **CD8** | 1:200 | Dakocytomation | PK25 | I, II |
| **γδ-TCR** | 1:50 | Endogen | 5A6.E9 | I, |
| **CD25** | 1:50 | Neomarkers | 143-13 | I |
| **CD19** | 1:100 | Dakocytomatin | HD37 | I |
| **IFN-γ** | 1:50 | Mabtech | 1-D1K | I, III |
| **TNF-α** | 1:50 | Biosource International | 68B6A3L1 | I |
| **CTLA-4** | 1:20 | BD Pharmingen | BNI3.1 | I |
| **FOXP3** | 1:40 | Abcam | 236A/E7 | I, II, III, IV |
| **IL-4** | 1:50 | Mabtech | IL4-II | I |
| **IL-10** | 1:100 | R&D Systems | 23738 | I |
| **IL-12p70** | 1:100 | R&D Systems | 24945 | I |
| **IL-17** | 1:200 | Santa Cruz Biotehcnology | rabbit polyclonal | I, II, III, IV |
| **IL-18** | 1:100 | MBL/ R&D systems | 125-2H | I |
| **IL-23(p19)** | 1:10 | GenWay | DCS100.1 | I |

### 4.4.2 Microscopic evaluation

Data evaluation was done blinded to the clinical data. The number of positively stained cells in the LP was counted systematically under a light microscope through a calibrated eyepiece graticule 10x. In Study I, an Olympus BX50 light microscope was used at 60x magnification. The positively stained cells were counted either in

the epithelium or residing in the LP. Epithelial staining with HLA-DR was graded from 1 to 3 according to the area of staining: 1 representing only the tips of the villi, 2 all of the villi, and 3 the crypts as well. In Studies II to IV a Leica DM4000B light microscope served for counting the cells. Positively stained cells in approximately 30 fields were counted at 100x magnification. The cell densities are expressed as a mean number of positive cells per millimetre (cells/mm) or square millimetre (cells/mm$^2$), with analysis based on this number.

## 4.5 Quantitative reverse transcriptase polymerase chain reaction

Analyses of target gene expression in the intestinal biopsy samples and the peripheral-blood mononuclear cells were performed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). In Studies I to III, biopsies were disrupted in lysis buffer containing 140 mM beta-mercaptoethanol by grinding them with a pestle in an Eppendorf tube. In Study IV, biopsies from Finnish subjects were dissected from a matrix of OCT compound and homogenized by pestle (Starlab, Ahrensburg, Germany) in a 0.5-ml Eppendorf tube containing 250 µl of lysis buffer (Sigma, St. Louis, MO, USA). In Studies I, II and IV, the GenElute Mammalian total RNA miniprep kit (Sigma) served for extracting total RNA from biopsies. In Study III, total RNA was isolated with a Qiagen RNeasy mini kit with optional QIAshredder homogenization and DNAse digestion steps (Qiagen, Hilden, Germany). RNA yield and purity was measured with a spectrophotometer (ND-1000, NanoDrop Technologies Inc, Wilmington, DE, USA). Reverse transcription was performed with TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA) (Studies I-IV), with additional treatment of 200 ng of total RNA with DNAse I (0.01U/µl) (Roche Diagnostics, Mannheim, Germany) to eliminate genomic DNA in Studies I and IV.

From the Swedish children in Study IV, the Qiagen RNeasy mini kit and DNAse treated with the RNnse-free DNase Set (Qiagen) served to isolate total RNA from cryopreserved and homogenized biopsies. A spectrophotometer (ND-1000, Nanodrop Technologies) served to measure the RNA concentration and purity. QRT-PCR was performed with predesigned FAM-labeled TaqMan Gene Expression Assay reagents (Applied Biosystems) for selected cytokines and transcription factors (Table 7). Ribosomal 18s RNA served as the endogenous control in all tests.

The quantities of target gene expression were analyzed by a comparative threshold cycle ($C_t$) method as recommended by the manufacturer (Applied Biosystems). An exogenous cDNA pool calibrator was collected from PHA-stimulated peripheral blood mononuclear cells (PBMC) and considered as an interassay standard, to which normalized samples were compared. $\Delta C_t$ stands for the difference between the $C_t$ of the marker gene and $C_t$ of the 18S gene, whereas $\Delta\Delta C_t$ is the difference between

the $\Delta C_t$ of the sample and $\Delta C_t$ of the calibrator. Calculation of $2^{-\Delta\Delta^{Ct}}$ then gives the relative amount of target gene in the sample compared with the calibrator, both normalized to an endogenous control (18S). For presentations, the relative amounts ($2^{-\Delta\Delta^{Ct}}$) of target genes were multiplied by a suitable factor and expressed as relative units. If the samples' $C_t$ value failed to reach the quantitative level, the artificial value given was half of the lowest acceptable value on a relative-unit scale.

**Table 7.** Primers in Studies I-IV.

| Primer | Applied Biosystems, Cat. no | Study |
|---|---|---|
| **IL-4** | 4327038 | I |
| **IL-6** | Hs00174131_m1 | III |
| **IL-8** | Hs00174103_m1 | I |
| **IL-17A** | Hs00174383_m1 | I, II, III, IV |
| **IL-22** | Hs00220924_m1 | III |
| **IL-23A p19** | Hs00413259_m1 | I, II |
| **FOXP3** | Hs00203958_m1 | I, II, III, IV |
| **IFN-γ** | 4327052<br>Hs0014143_m1 | I<br>IV |
| **RORc** | Hs01076112_m1 | IV |
| **TGF-β1** | Hs00171257_m1 | I |
| **TNF-α** | Hs00174128_m1 | I, II |
| **Ribosomal 18S RNA** | Hs99999901_s1 | I-IV |

## 4.6 Caco-2 cell culture and stimulation protocols

ATCC (Teddington, UK) provided the human colon adenocarcinoma cell line (Caco-2). For 6 days in a 75 $cm^2$ flask, cells were grown in Eagles's minimal essential medium (Sigma) containing 10% heat-activated and sterile-filtered fetal bovine serum supplemented with penicillin (0.1 g/l) and streptomycin (0.15 g/l) at +37°C and 5% $CO^2$. Thereafter, the cells were plated on sterile 48-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and grown for 4 days at a density of 1.5 x $10^5$ cells/well and a final volume of 0.5 ml/well. The cells were incubated for 5 hours in +37°C, 5% $CO^2$ in the combinations listed in Table 8. Thereafter, the cells were collected for analyses of target gene expression in the Caco-2 cells with qRT-PCR, in which total RNA was isolated (Sigma), reverse transcription was done with added DNAse treatment, and qPCR analyses were performed as described above for biopsy samples. Apoptosis markers were bcl-2(Hs00608023_m1) and BAX (Hs00180269_m1).

**Table 8.** Combinations in Caco-2 cell stimulation.

| | | Cat. no | Study |
|---|---|---|---|
| **S. minnesota LPS** | 10 or 100 ng/mL | | I |
| **rhIL-17** | 1 or 50 pg/mL | 11340176 | I, IV |
| **TNF-α** | | | IV |
| **S.minnesota LPS + rhIL-17** | | | I |
| **rhIL-17 + TNF- α** | | | IV |

## 4.7 IL-17 levels in plasma and feces

ELISA (Biosource, Camarillo, CA, USA) served for study of the concentration of IL-17 in plasma and feces. Fecal samples were homogenized with phosphate-buffered saline (wt/vol ratio 1:1) on a shaker for 30 minutes at 4°C. These homogenates were centrifuged for 15 minutes at 10,000 *g* at 4°C. The supernatants were collected and stored at -70°C for subsequent analysis.

## 4.8 Fecal calprotectin assays

A quantative enzyme immunoassay (PhiCal Test, Calpro AS, Oslo, Norway) served for measurement of fecal calprotectin, and the values quoted as normal were <100 µg/g of stool (Kolho et al. 2006, von Roon et al. 2007).

## 4.9 Flow cytometry methodology

Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation served to separate the PBMCs from heparinized blood as previously described (Klemetti et al. 1998). In $CD4^{+}CD25^{high}FOXP3^{+}$ Treg cell analysis, staining of the cells was according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). First, monoclonal antibodies PerCP–anti-human-CD4 (Pharmingen) and PE–anti-human-CD25 (Miltenyi Biotec) served for the cell surface staining. Then cells ($1 \times 10^6$ cells / tube) were washed twice with 1 mL permeabilization buffer. After the surface staining, the sample was mixed with 1 mL fixation/permeabilization buffer, incubated at room temperature for 30 min, and washed twice with 2 mL permeabilization buffer. After that, cells were incubated for 15 min in RT with 1% normal mouse IgG in 100 µl permeabilization buffer. For intracellular staining, 20 µL Alexa 488-conjugated FOXP3 antibody or rat immunoglobulin G2a isotype control was added to the reaction system and incubated in a 4°C freezer for 30 min and rewashed twice with 2 mL permeabilization buffer. Then the stained cells were resuspended in a 1mL flow cytometry staining buffer and analyzed by flow cytometry with FACSDiva software (BD, Piscataway, NJ, USA).

## 4.10 Cytokine secretion from in vitro cultured biopsies

Small-intestinal biopsy specimens were cultured for 72 hours in RPMI-5% human AB serum, and thereafter, a flow-cytometric bead array (Bender Medsystems) served to measure the concentration of IL-17, IL-1beta, and IL-6 secreted in the culture supernatants. To enable statistical analyses, samples below either the detection limit or below the cutoff level of the method were given half the cut-off value.

## 4.11 Statistical analysis

SPSS v 14.0 (I and II), SPAW Statistics 17.0 (III and IV) for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (San Diego, CA) served for the data analysis. The non-parametric Kruskall-Wallis test, Mann-Whitney U-test, Wilcoxon signed rank test, Spearman's rank correlation test, and Fisher's exact test served for performance of statistical analyses. P-values $< 0.05$ were considered significant.

## 4.12 Ethical Considerations

In Studies I and II, specimens from adult patients were taken for diagnostic purposes at the Helsinki University Central Hospital, Clinic for Gastroenterology. In Studies III and IV, Finnish pediatric patients underwent endoscopy for diagnostic purposes at the Children's Hospital, Helsinki University Hospital. Written informed consent was obtained from all the adults participating in Studies I or II and from the parents or care-givers of the children participating in Study III. In Study IV, oral witnessed informed consent was available from the parents and from age-appropriate patients among Finnish patients. For the Swedish patients, written informed consent came from parents and children.

The study plan for Studies I and II was approved by the Ethics Committee, Department of Medicine, Helsinki University Central Hospital, Helsinki. The Ethics Committee of the Children's Hospital, Helsinki University Central Hospital, Finland, approved the study plan for Study III and for the Finnish patients in Study IV. For the Swedish patients, the Regional Ethics Committee for Human Research at the University Hospital of Linköping, Sweden approved the study plans.

# 5 Results and discussion

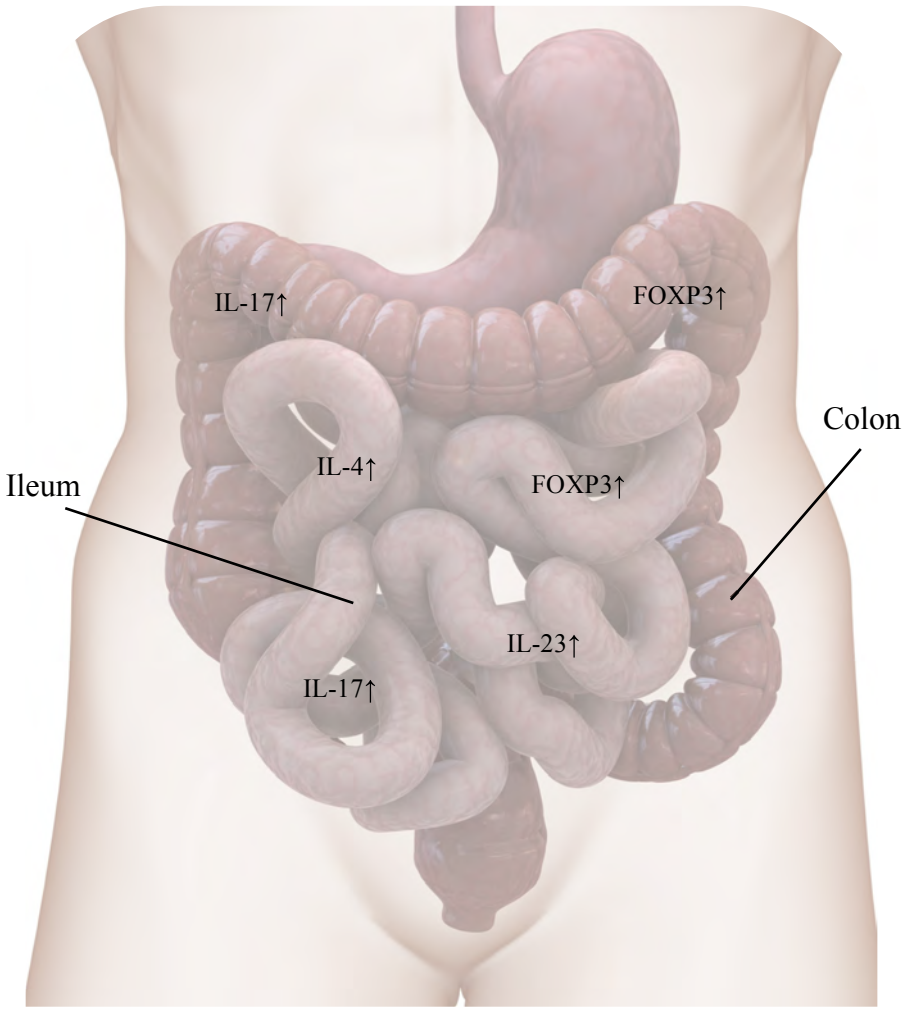


**Figure 3.** Changes in immunological markers in the intestine.

**Table 9.** Immunological changes studied in Crohn's disease.

| | Th1/Th2 type immunity | IL-17 type immunity | Regulatory T cells |
|---|---|---|---|
| **Inactive Crohn's disease (I)** | *IFN-γ$^+$ cells* ↔ IFN-γ mRNA ↔ *IL-4$^+$ cells* ↑ (all in ileum) | *IL-17$^+$ cells* ↑ IL-17 mRNA ↑ *IL-23p19$^+$ cells* ↑ IL-23 mRNA ↔ IL-6mRNA ↑ (all in ileum) | *FOXP3$^+$ cells* ↔ FOXP3 mRNA ↑ (all in ileum) |
| **Active Crohn's disease (I)** | *IFN-γ$^+$ cells* ↔ IFN-γ mRNA ↔ *IL-4$^+$ cells* ↑ (all in ileum) | *IL-17$^+$ cells* ↑ IL-17mRNA ↑ *IL-23p19$^+$ cells* ↑ IL-23 mRNA ↑ IL-6 mRNA ↑ (all in ileum) | *FOXP3$^+$ cells* ↔ FOXP3 mRNA ↑ (all in ileum) |
| **Crohn's disease before TNF-α-blocking therapy (II)** | IFN-γ mRNA ↑ (i&c) | *IL-17$^+$ cells* ↑*(i)* IL-17 mRNA ↑(c) IL-23 mRNA ↑ (i) | *FOXP3$^+$ cells* ↑ *(i)* FOXP3 mRNA ↑ (i&c) |
| **Crohn's disease after TNF-α-blocking therapy (II)** | IFN-γ mRNA ↑ (i&c) | *IL-17$^+$ cells* ↑ *(i)* IL-17 mRNA ↔ (i&c) IL-23 mRNA ↑ (i) | *FOXP3$^+$ cells* ↑ FOXP3 mRNA ↑ (all in ileum) |

i = ileum, c = colon

## 5.1 Th1 and Th2 type of immunity in Crohn's disease

Several studies show high colonic IFN-γ expression in Crohn's disease and emphasize the traditional dichotomy of Crohn's disease as a Th1-type disease. In Study II, we saw increased intestinal IFN-γ before and after TNF-α-blocking therapy. Study I, however, showed no characteristics related to the Th1 immune response, such as IFN-γ or IL-12p70 expression (Figure 3, Table 9). Of course, one explanation as to why we see less constantly increased Th1 type of immunity could be the relative small number of patients. Altogether, our results suggest that in Crohn's disease, Th17 immunity may play a more important role than does Th1 type immunity. Th1 and Th17 types of immunity have been interconnected: Annunziato et al. (2007) suggest that some intestinal Th17 cell lines derived from Crohn's disease patients are able to secrete IFN-γ. Transcription factor T-bet reciprocally regulates IL-17 and IFN-γ, supporting IFN-γ up-regulation but inhibiting the IL-17 axis by down-regulating IL-23R transcription (Gocke et al. 2007).

Most of the other studies showing, for example, a Th1 response in Crohn's disease were focused on colon samples. In Crohn's disease, tissue damage occurs most commonly in the ileum (Nikolaus, Schreiber 2007) emphasizing the need to study

the ileal changes as we did in Study I. It is possible that the balance between Th1 and Th17 differs between ileum and colon, Th17 being more prominent in the ileum.

Considering the earlier reports of IFN-γ expression in Crohn's disease, our finding of increased intestinal IL-4 response in Study I was somewhat unexpected (Table 9). The numbers of IL-$4^+$ cells in the LP differed between study groups, being higher in Crohn's disease than in the reference patients. The number of IL-$4^+$ cells was higher in active than in inactive Crohn's disease (Figure 4). Parronchi et al. (1997) reported a high number of activated $CD4^+$ T cells with no IL-4 reactivity in Crohn's disease. IL-17 activation seems to be unable to down-regulate Th2-type stimulation in the form of elevated IL-4 levels, although some studies suggest that both IL-4 and IFN-γ inhibit the development of IL-17-secreting Th cells (Steinman 2007).

A unique subset of intestinal macrophages exists that produces proinflammatory cytokines such as IL-23, TNF-α, and IL-6 (Kamada et al. 2008). In Crohn's disease, the number of these macrophages is high, and rather than IL-17 production, they contribute to IFN-γ production by LP mononuclear cells. IFN-γ triggers further abnormal macrophage differentiation, with hyperproduction of IL-23. Likewise, in Study II we showed enhanced ileal IFN-γ mRNA expression in Crohn's disease. Double positive $Th1^+Th17^+$ mucosal cells and T cell lines are able to secrete both IL-17 and IFN–γ in Crohn's disease to cause the related intestinal inflammation (Rovedatti et al. 2009, Annunziato et al. 2007). In Study II, the positive correlation between IL-17 and IFN-γ mRNA expression in ileal Crohn's disease may reflect activation of these kinds of cells. Accordingly, in Study II, ileal and colonic IFN-γ and IL-17 mRNA expression correlated positively ($r=0.636$; $P=0.026$; $r=0.539$; $P=0.021$). Possibly, $Th1^+Th17^+$ cells produced IL-17 and IFN-γ, but because we lacked material to further characterize IL-17- or IFN-γ-producing cells, this question remains open. Unfortunately, methodological problems prevented us from accomplishing double staining of IL-17 or FOXP3 and surface markers.

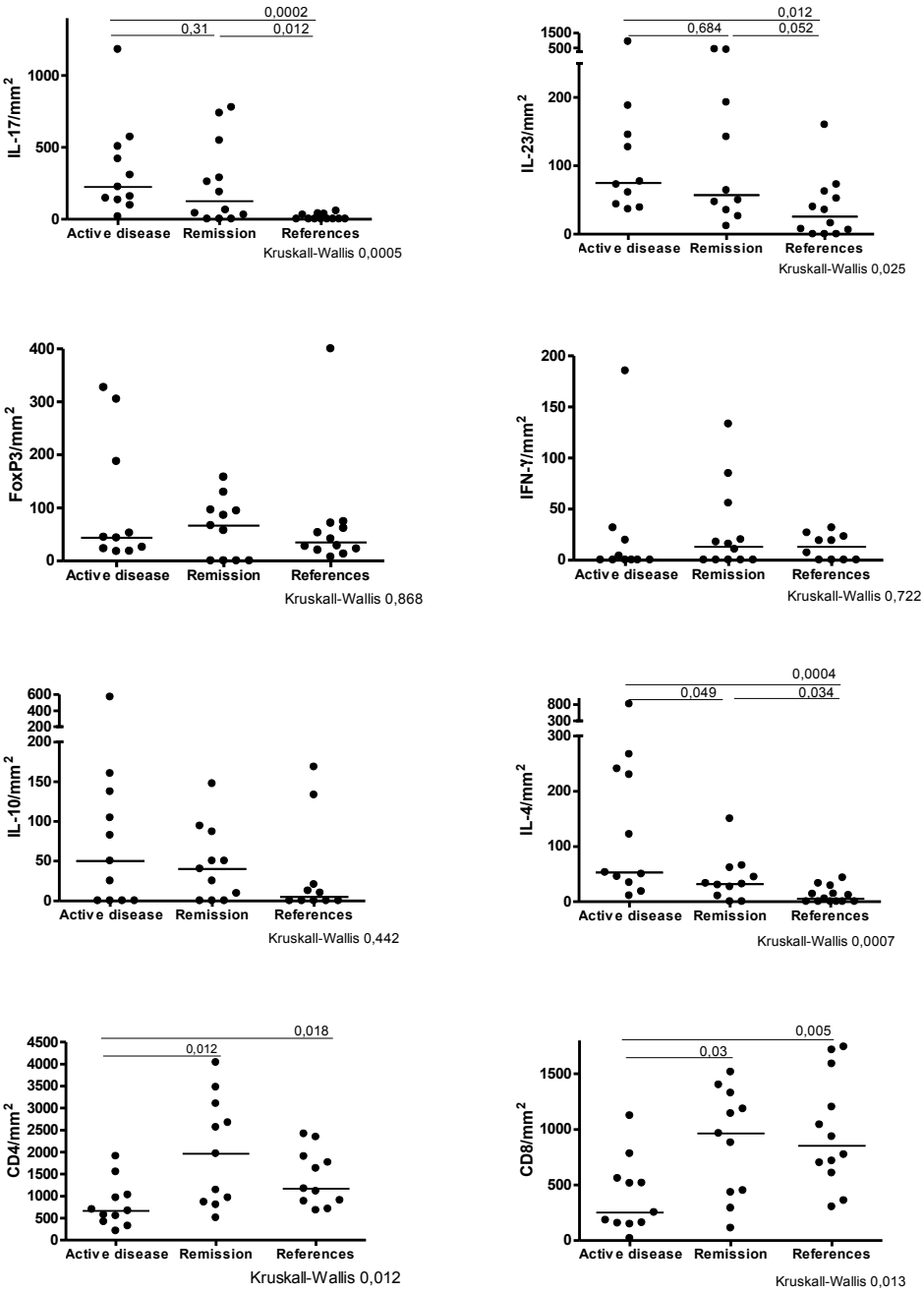


**Figure 4.** Immunohistochemical stainings in active and inactive Crohn's disease (Study I).

## 5.2 Intestinal IL-17 activation in Crohn's disease

Evidence accumulates regarding the importance of the IL-23/IL-17 axis in animal models of IBD (Maloy 2008). Our results from humans support this view and indicate IL-17 as a hallmark of Crohn's disease, both in active and inactive disease. In Studies I and II, Crohn's disease patients had increased amounts of intestinal IL-17- and IL-23-expressing cells, not only in active disease but also in clinical remission, providing a potential link to the cause of the disease and the basis for its relapsing nature.

Earlier studies of Crohn's disease report IL-17$^+$ cells and high levels of *IL-17*-specific mRNA in the colonic mucosa, but in normal intestinal mucosa, the number of IL-17-expressing cells is low (Fujino et al. 2003, Nielsen et al. 2003). Studies I and II support this finding: in Study I, the number of LP IL-17$^+$ cells was elevated in Crohn's disease, but showed no difference between the active and inactive disease (original publication I, Table 3). Likewise, the expression of IL-17A mRNA was elevated in Crohn's disease when compared to that of the reference group, but no difference existed between active and inactive Crohn's disease (original publication I, Figure 1). In Study II, the numbers of ileal and colonic IL-17$^+$ cells were higher in Crohn's disease both before and after TNF-α-blocking than in the reference patients (original publication II, Figure 2). At baseline, colonic IL-17 mRNA expression was higher in Crohn's disease than in the reference group (Table 9).

Fujino et al. (2003) reported colonic IL-17 expression to be higher in Crohn's disease than in UC, and found that in active IBD the number of IL-17$^+$ cells to be higher than in inactive IBD. Another study supports this view: inflamed colonic lesions showed elevated IL-17F mRNA expression compared to the uninflamed mucosa in Crohn's disease, whereas intestinal *IL-17F* mRNA expression was higher in UC than in Crohn's disease (Seiderer et al. 2008). Study I, however, showed no difference between patients with active or inactive Crohn's disease as to the number of ileal cells or as to the level of *IL-17*-specific mRNA. The increased numbers of IL-17$^+$ cells in both samples in Study II supports this. The difference in disease localization or the classification of activity of the disease may explain the discrepancy between Study I and the Fujino et al. results. Because we aimed to compare the active phase of the disease (defined as endoscopically confirmed signs of active ulcerative inflammation) with the remission phase (defined as endoscopically confirmed uninflamed mucosa), we classified patients according to the endoscopic finding, and with the CDEIS assessed the state of the disease.

Unfortunately, we were unable to demonstrate IL-17-specific mRNA in PBMCs or increased circulating IL-17 levels in patients' plasma, as reported earlier (Fujino et al. 2003). In Study I, plasma concentration of IL-17 was generally low, in most cases undetectable (in 12 of 14 control subjects and in 22 of 24 patients). Our findings support the view that IL-17$^+$ cells home to the gut immune system and do not

circulate in the periphery. When a specific treatment removes the detrimental effect of TNF-α, the role of IL-17 in the intestinal inflammation becomes evident. Three months after TNF-α-blocking, CDEIS correlated positively with magnitude of intestinal IL-17 immunity in the amount of ileal IL-17$^+$ cells (r=0.806, P=0.007) and IL-17 mRNA (r=0.736, P=0.038), whereas this kind of correlation was absent before TNF-α-blocking. Following the treatment, lower IL-17 activity associated with endoscopic remission. The only patient with increasing CDEIS during treatment presented also with an increase in number of IL-17$^+$ cells. Thus, TNF-α-blocking was unable to directly down-regulate the IL-23/IL-17 axis. However, changes in the immunological network, seen as a better balance between effector and regulatory T cells, mediated the beneficial effects of TNF-α-blocking (discussed in section 5.3).

In active Crohn's disease, CD4$^+$ and CD8$^+$ cells decreased, an unexpected finding in Study I (Figure 4) and in Study II were absent. In remission, the number of CD4$^+$ and CD8$^+$ cells normalized, suggesting a regulatory function for either of these cell types. The earlier study found the IL-17$^+$ cells to be CD3$^+$CD68$^+$ in Crohn's disease, suggesting that in inflamed mucosa both monocytes/macrophages and T cells produce IL-17 (Fujino et al. 2003). In Study I, the ratio of the IL-17$^+$ cells to CD4$^+$ or CD8$^+$ cells was higher in Crohn's disease than in the reference group, and the highest ratio of IL-17$^+$ cells to CD4$^+$ cells was in active disease (original publication I, Figure 2). Study II supports this phenomenon, as the ratio of ileal IL-17$^+$ cells to CD4$^+$ cells was elevated both before and after the anti-TNF-α treatment when compared to levels in the reference group (original publication II, Figure 3). Anti-TNF-α treatment, however, reduced markedly the ratio of IL-17$^+$ cells to CD4$^+$ cells (original publication II, Figure 3). The ratio of ileal IL-17$^+$ cells to CD8$^+$ cells was higher before and after TNF-α blocking in Crohn's disease than in the reference group (P=0.0001 and P=0.0031). Therefore, the normalization of the total number of CD4$^+$ and CD8$^+$ cells in inactive Crohn's disease seen in Study I suggests the regulatory function of either of these cell types, something needing further characterization. Despite the clinical and macroscopic improvement in symptoms after TNF-α-blocking, the high numbers of activated T cells in the intestine may signify a need for continuous TNF-α-blocking.

Innate immune cells such as dendritic cells produce IL-23, a cytokine that activates Th17 cells and seems to be an important factor in maintaining the production of IL-17 (Neurath 2007). IL-23 can induce intestinal inflammation without Th1, thus suggesting that IL-23 influences various aspects of tissue inflammation and damage (Hue et al. 2006, Kullberg et al. 2006, Uhlig et al. 2006, Marx 2007). This finding motivated us also to study IL-23 in Crohn's disease. In Study I, the expression of IL-23 mRNA was higher in active disease than in the controls (P=0.014). Study II confirmed this finding, in which the ileal IL-23 mRNA expression remained elevated both before and after TNF-α-blocking when compared to the levels in the reference group (Table 9). Our results show that even in the endoscopically confirmed healed mucosa, IL-17$^+$ and IL-23$^+$ cells still persisted (Figure 4). The high numbers of IL-

$17^+$ and IL-$23^+$ cells in inactive disease explain the potential for the flaming of the disease and demonstrate that this immunological phenotype of the mucosa is fundamentally linked to Crohn's disease. It also is likely linked to the pathogenesis of the disease, for example, by acting as a microbial stimulus, because IL-17 enhanced the inflammatory response of epithelial cells to LPS in vitro.

In addition to their role as the first line of defence against bacterial invasion, intraepithelial lymphocytes participate in inflammatory and immune reactions. Intestinal epithelial cells and isolated human intraepithelial lymphocytes constitutively express IL-8 mRNA and secrete low levels of IL-8 protein; even in response to LPS, Caco-2 cells expressed low amounts of IL-8 (Eckmann et al. 1993), which Study I also demonstrated. Pro-inflammatory cytokines and LPS have a synergistic effect: the effect of combinations of different cytokines (IL-1β, IL-2, IL-6, IFN-γ, TGF-β) and LPS on IL-8 secretion and the response to an individual cytokine in colonic epithelial cell lines were similar (Eckmann et al. 1993). Invasive bacterial infection induces expression and up-regulation of pro-inflammatory cytokines in IECs; for example, IL-8 and TNF-α (Jung et al. 1995). In this respect, our finding of enhanced IL-8 and TNF-α expression in LPS plus IL-17 stimulation in the intraepithelial cell line is interesting. We used Caco-2 cells, in the presence of IL-17 alone or in combination with LPS, to study the effect of IL-17 on ECs. Incubation of the Caco-2 cell line with *S. minnesota* LPS together with rhIL-17 induced higher expression of TNF-α and IL-8 mRNA than did LPS or rhIL-17 alone (original publication I, Figure 3). This could provide a link between enhanced IL-17 production, bacterial stimulus, and mucosal damage.

Whereas IL-23 serves to maintain IL-17 production, IL-6 and TGF-β induce the development of human Th17 cells (Bettelli et al. 2007). This so intrigued us that we evaluated the level of IL-6- and TGF-β-specific transcripts in Study I. Expression of IL-6 mRNA was higher both in active and inactive Crohn's disease (original publication I, Figure 1) than in the reference group. In addition, TGF-β-specific mRNA was higher in Crohn's disease than in the reference group (P=0.031, with active and inactive groups pooled). In the presence of IL-6, TGF-β supports the induction of the Th17 phenotype, whereas these cytokines together suppress generation of induced $FOXP3^+$ Tregs, as suggested by in vitro studies (Bettelli et al. 2006).

After our finding of elevated IL-17 at both protein and transcriptional levels, we were keen to learn whether the mucosal damage in Crohn's disease leads to high intestinal production of IL-17. We addressed this question by studying the fecal levels of IL-17 in Crohn's disease (Study I). IL-17 fecal levels were elevated in active disease (P=0.05), but in general were low but detectable in about half of the patients with active Crohn's disease (5 of 9), in only 1 of 11 patients with inactive disease, and in none of the healthy adults.

In the active phase of Crohn's disease, increased levels of fecal IL-17 demonstrate elevated Th17 activity, which supports the role of IL-17 in tissue damage. In addition, an increased level of IL-23 transcription further supports the idea that in

active Crohn's disease, IL-23 independently or via IL-17 causes mucosal damage. The findings of IL-23 as being a T-cell-independent inducer of mucosal inflammation (Hue et al. 2006), and of IL-23R polymorphism as being closely associated with Crohn's disease (Duerr et al. 2006), suggests the latter interpretation.

## 5.3 Regulatory T cells in Crohn's disease

Transcription factor FOXP3 characterizes Tregs, a non-comparable subset of T cells regulating immune response; a lack of FOXP3 leads to serious autoimmunity associated with poor Treg development (Fontenot et al. 2003, Hori et al. 2003, Kim, Rudensky 2006, Rudensky 2011, Yamaguchi et al. 2011). A few studies address Tregs and their role in intestinal inflammation, and most of these studies reveal a concordant expansion of intestinal Tregs and suppression of Treg activity in vitro (Maul et al. 2005, Saruta et al. 2007, Yu et al. 2007). One study in IBD patients showed elevation of $FOXP3^+$ cells in inflamed colonic mucosa, but a reduction when compared with other intestinal inflammatory diseases such as diverticulitis or infectious enteritis (Maul et al. 2005). We were, therefore, inspired to study the number of $FOXP3^+$ cells and levels of FOXP3 mRNA in Crohn's disease patients as well as in ileal samples.

Our results showed up-regulation of FOXP3 in Crohn's disease in all the subgroups: active disease, inactive disease, and treatment response after TNF-α-blocking agent (Table 9). In Study I, FOXP3 mRNA expression was elevated both in active (P=0.0005) and inactive Crohn's disease (P=0.009) compared to the expression in reference patients. This phenomenon occurred also in Study II, as the patients' ileal samples both before and after TNF-α-blocking treatment showed an elevation in FOXP3 mRNA expression (P=0.023) and in numbers of $FOXP3^+$ cells (P=0.005, original publication II, Figure 2). In addition, the number of colonic $FOXP3^+$ cells was higher in Crohn's disease in the 3-month samples than in the reference patients (Figure 5). The ratio of ileal $FOXP3^+$ cells to $CD4^+$ cells was higher in both, before (P=0.03) and after TNF-α-blocking treatment (P=0.03) than in the reference group. To conclude: in Study I, no up-regulation of FOXP3 was apparent at cellular level, but in Study II, the up-regulation existed both at cellular and mRNA levels.

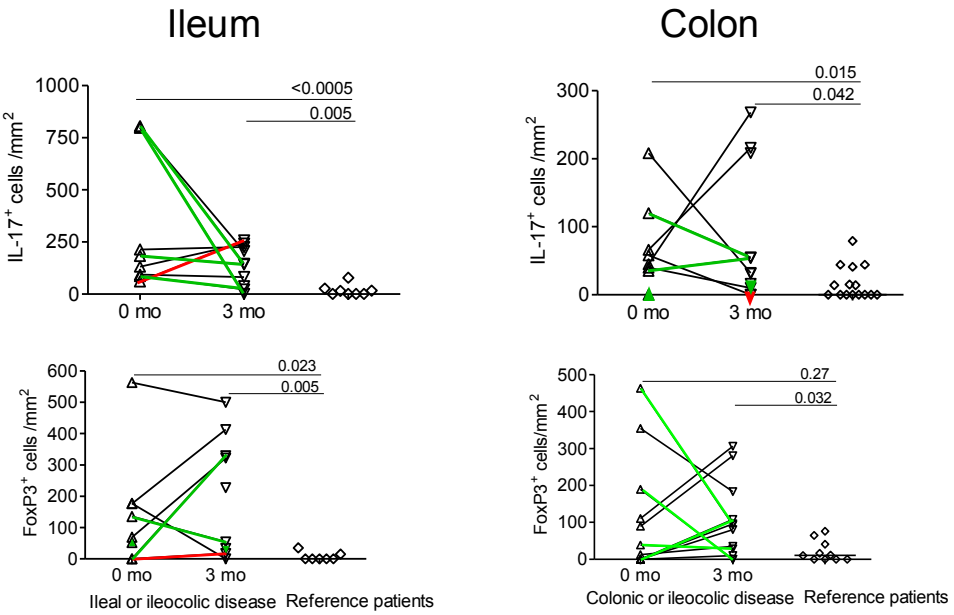


**Figure 5.** Immunohistochemical findings of IL-17- and FOXP3-positive cells in the intestine in Crohn's disease (Study II).

Reports find most FOXP3 cells to be $CD4^{+}CD25^{high}$ cells, Tregs. FOXP3 expression, however, is not restricted to Tregs, because activation in $CD4^{+}CD25^{-}$cells induces FOXP3 (Walker et al. 2003, Wang et al. 2007) leading to the impression that intestinal FOXP3-expressing cells may originate either in classical Tregs or in activated T cells. Induction of FOXP3 on activation, however, leads to development of a hyporesponsive T-cell phenotype with an impaired suppressive function. Transient FOXP3 expression may thus also exist in a nonsuppressive T-cell population (Wang et al. 2007). Our finding of up-regulated FOXP3 expression in active Crohn's disease may be a signal from FOXP3 expression induced by T-cell activation and therefore mean imperfect suppressive capability. In remission, a high number of $FOXP3^{+}$ cells may, however, reflect the number of natural, suppressive Tregs.

In the presence of IL-6, TGF-β supports the induction of the Th17 phenotype, whereas these cytokines together sudppress the generation of induced $FOXP3^{+}$ Tregs, as suggested by in vitro studies (Bettelli et al. 2006). Xu et al. (2007) suggest that, in the absence of exogenous TGF-β, FOXP3 Tregs can induce Th17 and may differentiate into Th17 cells. This shines new light on our findings of up-regulated FOXP3 together with IL-17 in Crohn's disease. Interestingly, Study I showed close correlation between expression of FOXP3 and IL-17 at mRNA level in the active disease (r=0.817, P=0.007), whereas the reference group showed no correlation between these parameters (r=0.06, P=0.86).

In IBD, the balance between Th17 and Tregs cells has an important effect (Kanai et al. 2009). One study suggests that in IBD the relative number of $FOXP3^+$ cells to effector cells is low and that no functional defect of Tregs appears (Makita et al. 2004). In Study II, the ratio of ileal or colonic IL-17 or IFN-γ mRNA expression to FOXP3 mRNA expression showed no difference before or after the treatment. However, for protein expression a difference did exist: the ratio of ileal IL-17-expressing cells to FOXP3-expressing cells was higher at baseline than in 3-month samples (P=0.038, Mann-Whitney U-test, original publication II, Figure 4). Study II thus suggests a relative decrease in IL-17 immunity in relation to total number of $FOXP3^+$ cells. The plasticity of Tregs may be one mechanism behind the beneficial change in balance between $IL\text{-}17^+$ and $FOXP3^+$ during TNF-α-blocking treatment. $IL\text{-}17^+$ cells may arise from Tregs: in the presence of IL-6, $CD4^+CD25^+FOXP3^+$ cells can differentiate to Th17 (Xu et al. 2007). TNF-α-blocking might inhibit this differentiation, because it blocks IL-6 production. This phenomenon may be reflected in the beneficial change in the balance between $IL\text{-}17^+$ and $FOXP3^+$ cells after TNF-α-blocking treatment.

More recently, Baba et al. (2010) showed that TNF-α suppresses the development of Th17 cells from naïve CD4 cells by means of mechanisms that, however, remain to fully explored. In Study II, TNF-α blocking did not manage to fully downregulate IL-17 immunity. The function of Tregs complicates this interaction, and an understanding of the complete relation between Th17 and Tregs is lacking. TNF-α-blocking led to an improved balance between Tregs and IL-17 effector T-cells.

A marked decrease in CDEIS was associated with a decreasing ratio of intestinal $IL\text{-}17^+$ cells to the numbers of $CD4^+$ cells and $FOXP3^+$ cells. After TNF-α-blocking treatment, $FOXP3^+$ or $CD4^+$ cells and CDEIS showed no correlation, but at baseline, ileal FOXP3 mRNA expression and CDEIS correlated positively ($r=0.830$; $P=0.041$). The relative enhancement of regulatory mechanisms likely explains this clinical and endoscopic response to treatment.

Considering changes in maintenance therapy is important when evaluating the effect of anti-TNF-α agents on immunological markers. Such changes may have an independent effect on these immunological markers, thus making interpretation of the results more challenging. Most patients in Study II received corticosteroid treatment at the first administration of TNF-α blocking, but at the time of the second colonoscopy, none. Corticosteroids have multiple mechanisms to inhibit T cell activation (Leung, Bloom 2003), possibly through induction of Tregs. FOXP3 mRNA expression rises in asthma patients treated with glucocorticoids, both in adults and in pediatric patients (Karagiannidis et al. 2004, Hartl et al. 2007). In Study II, corticosteroid treatment was tapered off in most patients, excluding the possibility that corticosteroid use would explain the increase in relative number of $FOXP3^+$ cells. (In active disease, but also after treatment, Study II showed increased numbers of $FOXP3^+$ cells.)

In the inflamed mucosa, IL-17 and FOXP3 were activated similarly, regardless of the activation state of the Crohn's disease. In Study II, TNF-α blocking induced mucosal healing that associated with beneficial changes in the balance between effector T cells and Tregs. Th17 cells surviving after treatment underline the need for maintenance therapy in order to avoid relapse.

## 5.4 Immunological changes in pediatric IBD

**Table 10.** Immunological changes studied in pediatric inflammatory bowel disease.

| | Th1/Th2 type immunity | IL-17 type immunity | Regulatory T cells |
|---|---|---|---|
| **Crohn's disease** | IFN-γ mRNA ↑ | *IL-17$^+$ cells ↑ (c)*<br>IL-17 mRNA ↑ (c)<br>*IL-17$^+$ cells ↔ (i)*<br>IL-17 mRNA ↔ (i)<br>IL-6 mRNA ↑ (c)<br>IL-22 mRNA ↑ (c) | *FOXP3$^+$ cells ↑ (c)*<br>FOXP3 mRNA ↑ (c)<br>*FOXP3$^+$ cells ↔ (i)*<br>FOXP3 mRNA ↔ (i) |
| **Ulcerative colitis** | IFN-γ mRNA ↑ | *IL-17$^+$ cells ↑ (c)*<br>IL-17 mRNA ↑ (c)<br>*IL-17$^+$ cells ↔ (i)*<br>IL-17 mRNA ↔ (i)<br>IL-6 mRNA ↑ (c)<br>IL-22 mRNA ↑ (c) | *FOXP3$^+$ cells ↑ (c)*<br>FOXP3 mRNA ↑ (c)<br>*FOXP3$^+$ cells ↔ (i)*<br>FOXP3 mRNA ↔ (i) |

Classification of colonic inflammation into Crohn's disease and UC can be difficult (Heyman et al. 2005). Intestinal IL-17A and IL-17F mRNA expression is increased in pediatric patients (Seiderer et al. 2008, Verdier et al. 2011). Interestingly, IL-17 elevation occurred in colonic mucosa also in UC, suggesting that pediatric IBD colitis is a Th17-related phenomenon irrespective of its clinical classification (Table 10). Crohn's disease patients with endoscopically active disease as well as children with a macroscopically healthy ileum exhibit mucosal IL-17 activation. In pediatric IBD patients, IL-17 mRNA up-regulation occurred in colonic mucosa both in Crohn's disease and in UC when compared to control levels (original publication III, Figure 1). The numbers of colonic of IL-17$^+$ cells varied: when the highest number of IL-17$^+$ cells in controls served as the cut-off, half the Crohn's disease patients showed elevated levels of IL-17$^+$ cells, as did 5 of 9 UC patients (original publication III, Figure 1). No significant difference in ileal IL-17 mRNA or IL-17$^+$ cells appeared between study groups. The ratio of ileal IL-17$^+$ cells to CD4$^+$ cells differed, however, between these groups (P=0.027) and was higher in Crohn's disease than in controls (P=0.016, Mann-Whitney U-test, original publication III, Figure 2). In agreement with Studies I and II, up-regulation of IL-17-related immunity occurred in inactive Crohn's disease, meaning that the classification separating ileal

from colonic Crohn's disease may be clinically valid but immunologically arbitrary. As the children with UC showed no comparable ileal up-regulation of IL-17, localization of the disease is likely restricted.

Natural and induced regulatory T cells need FOXP3 for their suppressor function (Chatila 2008). Increased intestinal FOXP3 has been reported in adult IBD patients (Maul et al. 2005), and was also seen in Study I. Study III reports increased intestinal FOXP3 expression in pediatric UC similar to that reported in untreated pediatric Crohn's disease patients (Reikvam et al. 2011). Colonic FOXP3 mRNA expression differed significantly between the groups and was higher both in Crohn's disease and in UC than in reference subjects (original publication III, Figure 1). Colonic $FOXP3^+$ cells differed between the three groups. Both Crohn's disease and UC showed higher amounts of positive cells than in the controls (original publication III, Figure 1). Ileal FOXP3 mRNA or $FOXP3^+$ cells showed no significant difference between the groups. In pediatric IBD, both in Crohn's disease and UC, activation of colonic IL-17 and FOXP3 occur at protein and mRNA levels, indicating shared immunological characteristics. Unfortunately, we were unable to accomplish double staining for IL-17 and other markers such as FOXP3.

FOXP3 blocks the ability of ROR-c to activate IL-17 immunity. IL-6 reverses this inhibition, but as FOXP3 mRNA is present in IL-6 treated cells, FOXP3 transcription does not halt (Zhou et al. 2008). In Study III, the numbers of IL-6 transcripts were up-regulated in the inflamed colonic mucosa both in Crohn's disease and UC (original publication III, Figure 1), thus agreeing with the previous in vitro studies. In IBD, the pro-inflammatory environment including activation of IL-6 may explain simultaneous up-regulation of mucosal FOXP3 and IL-17. Elevated expression of bifunctional cytokine IL-22 transcripts was also associated with IL-17 response in pediatric IBD (original publication III, Figure 1). The increased levels of IL-22 might not signify Th17 immunity, but instead may be produced by innate lymphoid cells, as seen in Crohn's disease (Geremia et al. 2011).

Maul et al. (2005) reported an increase in the number of regulatory $CD4^+CD25^+FOXP3^+$ cells in remission in peripheral blood but a decrease in active disease (Maul et al. 2005). As PBMC samples obtained from children in Study III were insufficient for suppression experiments, we investigated the percentage of Treg cells, evaluated as $CD25^{bright}CD4^+$ (Makita et al. 2004, Baecher-Allan et al. 2005, Maul et al. 2005, Holmen et al. 2006), and the percentage of $FOXP3^+CD25^{high}CD4^+$ cells. We found the percentages of both $CD25^{high}CD4^+$ and $CD25^{high}CD4^+FOXP3^+$ cells to be higher in the children with IBD. $CD25^{high}CD4^+FOXP3^+$ cells differed between the groups (P=0.014, Kruskall-Wallis) and to be significantly higher in Crohn's disease than in the reference patients (Original publication III, Figure 3).

Children undergoing their first endoscopy and having newly diagnosed disease, showed no difference in any of the measured variables (Table 10) from children undergoing a follow-up endoscopy; the disease duration had no impact on results.

Medication and immunological differences showed no significant association. The controls were slightly younger than the patients, but no statistically significant difference existed between the groups. Thus, in Study III, the duration of disease had no impact on results. The sample size was, however, limited and allows no firm conclusions.

As previously found in adult patients (Fujino et al. 2003), the up-regulation of IL-17 immunity existed also in pediatric IBD. This indicates that pediatric Crohn's disease and UC share the same immunological background. Thus, clinical differences between pediatric Crohn's disease and UC seem to be unrelated to IL-17 immunity.

## 5.5 Intestinal immunity in celiac disease and type I diabetes

**Table 11.** Immunological changes studied in celiac disease and T1D.

| | Th1/Th2 type immunity | IL-17 type immunity | Regulatory T cells |
|---|---|---|---|
| **Potential celiac disease** | - | IL-17 mRNA ↔<br>**IL-17A secretion ↔** | FOXP3 mRNA ↔ |
| **Celiac disease** | IFN-γ mRNA ↑ | *IL-17$^+$ cells* ↑<br>IL-17 mRNA ↑<br>**IL-17A secretion ↔** | *FOXP3$^+$ cells* ↑<br>FOXP3 mRNA ↑ |
| **GFD-celiac disease** | - | IL-17 mRNA ↓ | FOXP3 mRNA ↔ |
| **T1D** | IFN-γ mRNA ↔ | *IL-17$^+$ cells* ↔<br>IL-17 mRNA ↔ | *FOXP3$^+$ cells* ↔<br>FOXP3 mRNA ↔ |
| **T1D and celiac disease** | - | **IL-17A secretion ↑** | - |

### *Th1 and Th2 type of immunity in celiac disease*

Increased numbers of γδ T-cells and the up-regulation of the IFN-γ pathway characterize the inflamed intestinal mucosa in celiac disease, a Th1-type disease (Kontakou et al. 1994, 1995, Nilsen et al. 1998, Westerholm-Ormio et al. 2002). Here, expression of IFN-γ transcripts was elevated in the children with celiac disease when compared to levels in children with T1D or reference children (original publication IV, Figure 1). Dietary gliadin, however, may not be the only trigger to activate Th1 response. Instead Th1 response is fundamentally associated with celiac disease: mucosal up-regulation of IFN-γ pathway remained elevated even one year after GFD (Lahdenpera et al. 2011).

### *IL-17-related immunity in celiac disease and T1D*

Earlier studies have shown an association of mucosal IL-17 activation in untreated but not in GFD-treated celiac disease (Sapone et al. 2009, Monteleone et al. 2010).

In agreement is our demonstration of intestinal IL-17 up-regulation as a sign of villous atrophy and active celiac disease but as having no association with T1D (Table 11). In children with untreated celiac disease, up-regulation of IL-17 immunity occurred in the numbers of IL-17$^+$ cells and in the amounts of IL-17 transcripts (original publication IV, Figure 1). In children with untreated celiac disease, the number of IL-17$^+$ cells correlated positively with their IL-17 mRNA expression levels (R=0.444; P=0.111, Spearman), whereas no such correlation appeared in the reference group (R=-0.247; P=0.555) or in children with T1D (R=-0.104; P=0.775). In Swedish patients, the mucosal IL-17 mRNA was higher in the children with untreated celiac disease than in the other groups (original publication IV, Figure 2). In addition, the duodenal samples of untreated celiac disease patients secreted spontaneously more IL-17A in vitro than did those of reference children: of the 23 celiac disease patients, secretion of IL-17 was above the detection limit in 8, but we found none in 5 reference samples (original publication III, Figure 3).

According to one recent study, the mucosa of untreated celiac disease patients presents gliadin-specific Th17 cells (Fernandez et al. 2011). As a sign of plasticity, these cells secrete pro-inflammatory and anti-inflammatory cytokines simultaneously, indicating events such as changes in the profile of secreted cytokines. Another recent study, however, showed that T cells reactive to deaminated gliadin secrete no IL-17 (Bodd et al. 2010). Our findings of the activation of IL-17 immunity only at a late stage of the disease may explain these discrepant reports. In agreement, Study IV showed the effect of GFD on intestinal IL-17 down-regulation (Table 11). Thus, IL-17 response associated with untreated celiac disease that is characterized by villous atrophy. During villous atrophy, up-regulation of IL-17A production can occur without marked expansion of Th17 cells.

Similarly, our findings indicate that the wheat-gliadin-induced mucosal inflammation seen in potential celiac disease does not include IL-17 immunity. TGA$^+$ children showed no up-regulation of IL-17 immunity, even though they show an elevated risk for celiac disease and can be categorized as having potential celiac disease. T cells reactive to deaminated gliadin secrete no IL-17 (Bodd et al. 2010), but gliadin-specific Th17 cells do exist in the mucosa of untreated celiac disease patients (Fernandez et al. 2011). These discrepant results could be explained by our findings of activated IL-17 immunity only at a late stage of the disease, because Th17 cells show plasticity by secreting both anti- and pro-inflammatory cytokines concurrently.

An IL-1β and IL-6 cytokine environment supports the conversion of FOXP3 expressing Tregs to IL-17-secreting cells. In Study IV, the active celiac disease mucosa secreted both IL-1β and IL-6. Cultured small-intestinal specimens of patients with untreated celiac disease (with or without T1D) spontaneously secreted more IL-1β and IL-6 than did children with potential celiac disease or TGA$^-$ reference children (original publication IV, Figure 3).

IL-17 immunity plays a dual role in tissue inflammation, which depends at least partly on the response of the target tissue to IL-17. The induction of IL-17 immunity

exacerbated the effector phase of autoimmune diabetes in a murine model of autoimmune diabetes (Emamaullee et al. 2009), and in human islet cells IL-17 induced apoptotic mechanisms (Honkanen et al. 2010). In a rodent model, however, a commensal bacteria strain mediated protection from autoimmune diabetes and caused induction of mucosal IL-17 immunity (Lau et al. 2011). In Study IV, the number of IL-17$^+$ cells or transcripts exhibited no difference in T1D from those of the reference children.

Bradshaw et al. (2009) showed that the number of IL-17-secreting cells from anti-CD3-stimulated PBMCs was greater in long-term T1D (disease duration > 1 year, predominantly juvenile type of disease) than in controls, indicating that IL-17 relates to T1D. Between recent onset T1D (disease duration <1 year, predominantly adult onset) and controls, however, we found no significant difference. In T1D, up-regulation of IL-17 immunity has been evident in peripheral blood (Honkanen et al. 2010).

Similarly to Crohn's disease, IL-23 mRNA seemed to be up-regulated in celiac disease, but surprisingly, IL-23R mRNA was slightly down-regulated in celiac disease and T1D when compared to controls. The ratio of IL-23 mRNA to IL-23R mRNA showed a statistically significant elevation in celiac disease (Figure 6). This finding of elevated ratio of IL-23 to IL-23R needs further study for evaluation.

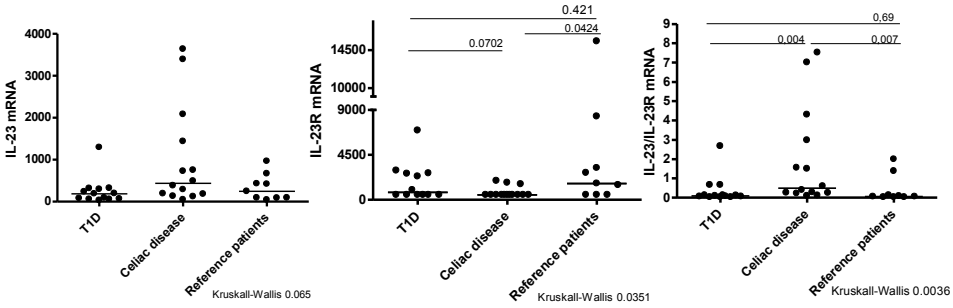


**Figure 6.** Levels of IL-23 and IL-23 transcripts in T1D and celiac disease.

Due to the difference in RNA isolation steps between the Finnish and Swedish samples, between series of samples, the levels of transcripts varied. In Finnish samples, RNA was isolated from samples embedded in OCT matrix, and IL-17A mRNA fell below the detection limit in 10 of 13 children with T1D, in 8 of 9 reference children, and in 2 of 14 children with untreated celiac disease. In Swedish samples, IL-17A was undetectable in only 2 of 17 reference children and in 1 of 8 children with potential celiac disease. The Swedish reference children were younger than their celiac disease patients, but no correlation appeared between IL-17 mRNA expression and age (r=0.193; P=0.16).

### *In Caco2-cells, IL-17-induced apoptosis*

IL-17 plays a role in the induction of epithelial cell apoptosis and villous atrophy (Mazzarella et al. 2008). Thus, to study induction of apoptosis, we treated the epithelial cell line Caco-2 with IL-17. As TNF-α is apoptotic for epithelial cells (Gitter et al. 2000), we examined the apoptotic effects of IL-17 on Caco2 cells in vitro, alone, or in combination with TNF-α. Caco2 cells show high expression of IL-17RA mRNA transcripts. Furthermore, incubation with IL-17 enhanced the transcription of the anti-apoptotic gene bcl-2 but showed a slight decrease in the expression of apoptotic-signaling gene BAX, which is active in the apoptosis (original publication IV, Figure 4). Thus, IL-17 may not contribute to the apoptosis of enterocytes, but instead it possibly activates protective anti-apoptotic mechanisms in ECs. In tissue inflammation, IL-17 immunity seems to play a dual role that may depend at least partly on the target tissue's response to IL-17.

In a murine model of autoimmune diabetes, during the effector phase of the disease the induction of IL-17 immunity exacerbated diabetes, and in human islet cells, IL-17 caused apoptosis (Emamaullee et al. 2009, Honkanen et al. 2010). A recent study showed that in a rodent model, a commensal bacterial strain protective against autoimmune diabetes caused induction of mucosal IL-17 immunity (Lau et al. 2011). As also suggested by others, our results support the view that up-regulation of intestinal IL-17 immunity is linked with the mechanisms of protection from tissue damage in the inflamed mucosa (Blaschitz, Raffatellu 2010), which could thus explain the beneficial effects of mucosal IL-17 up-regulation in autoimmune diabetes. In children with T1D, evidence exists of enhanced activation of IL-17 immunity in peripheral blood T cells (Honkanen et al. 2010). We, however, found no evidence of the up-regulation of small intestinal IL-17 immunity in children with T1D but without celiac disease.

An elevated level of IL-17 immunity existed only in children with both T1D and celiac disease. T1D patients with celiac disease spontaneously released significantly more intestinal IL-17 in vitro (P=0.0049, Kruskall-Wallis, Figure 7). When compared to patients with celiac disease alone or to reference children, they secreted an elevated amount of IL-17A (P=0.009 and 0.015, Fischer´s exact test; the detection limit served as the cut-off line). Thus, under certain conditions, T1D possibly induces IL-17 production, such as when high-grade mucosal inflammation associates with villous atrophy. Interestingly, the Langerhans islets from a newly diagnosed T1D patient showed elevation in IL-17A transcripts when compared to that of non-diabetic individuals (Arif et al. 2011). Possibly IL-17$^{+}$ cells infiltrate to the islets instead of to the intestine. Our samples were from the small intestine, but in nonobese diabetic mice, up-regulation of IL-17 immunity has occurred in the colon (Alam et al. 2010). Up-regulation of IL-17 contributes to the villous atrophy related to untreated celiac disease.

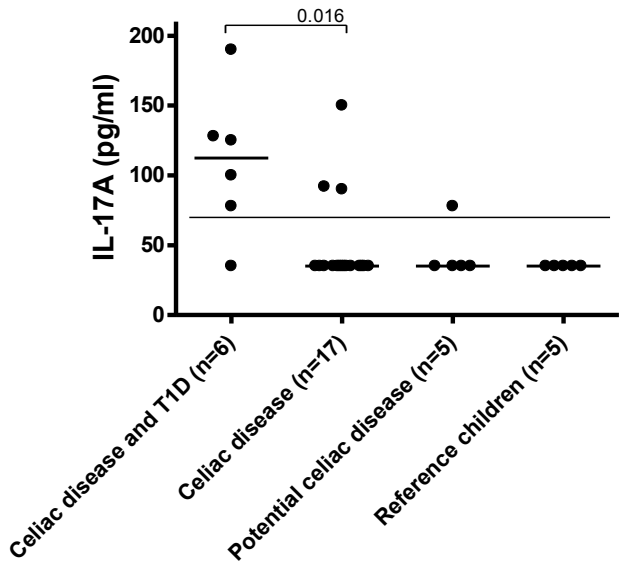


**Figure 7.** Biopsy specimens from children with both celiac disease and T1D secreted higher amounts of IL-17, also when compared to the children with only celiac disease (P=0.0156, Mann-Whitney U-test).

As IL-17 did not promote apoptotic mechanisms in the CaCo-2 epithelial cell line, it may not act as a direct trigger of villous atrophy and tissue destruction. IL-17 up-regulation implied active celiac disease; its role as a predictive biomarker of villous atrophy, and the need for small intestinal biopsy in $TGA^+$ subjects require further study.

### *Intestinal FOXP3 in celiac disease and type 1 diabetes*

Studies suggest that the number of $FOXP3^+$ cells is increased in celiac disease and especially in those with coexisting T1D (Vorobjova et al. 2008). This is in agreement with other findings implying that children with celiac disease or T1D in combination with celiac disease had higher FOXP3 mRNA expression levels in BPMC than did healthy or children with T1D (Kivling et al. 2008). In Study IV, in the Finnish children the number of $FOXP3^+$ cells and amount of FOXP3 mRNA differed significantly between the groups. When compared to T1D and reference children, an elevated number of $FOXP3^+$ cells and higher FOXP3 mRNA levels appeared in untreated celiac disease. In the Swedish children, however, the mucosal FOXP3 mRNA expression differed between the study groups, being higher in the children with untreated celiac disease than in GFD-treated children, in children with potential celiac disease, or in $TGA^-$ children. Between children with treated celiac disease and $TGA^-$ reference children, no difference existed in the FOXP3 mRNA expression levels (original publication IV, Figure 1).

In children with untreated celiac disease, we found up-regulation of intestinal FOXP3 to associate with enhanced IL-17 immunity: IL-17 mRNA correlated positively with FOXP3 mRNA in untreated celiac disease (r=0.60, P=0.03 Spearman). Studies have suggested that FOXP3-expressing Tregs show plasticity, and in inflamed tissue, may develop into Th17 cells (Ayyoub et al. 2009, Komatsu et al. 2009, Zhou et al. 2009). Similar to IL-17 immunity, the activation of intestinal FOXP3 seems to occur only in the late phase of disease progression, and potential celiac disease did not exhibit up-regulation of FOXP3. GFD treatment, however, normalized the expression of both FOXP3 and IL-17 (Figure 8).

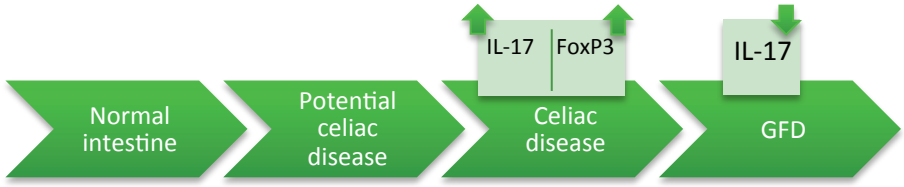


**Figure 8.** Patogenesis of celiac disease. Gluten-free diet (GFD).

The mucosal cytokine environment in celiac disease supports IL-17 differentiation, but may cause impaired suppressive function of FOXP3-expressing cells (Beriou et al. 2009). A recent study suggested that Th17 cell clones might also change their phenotype when RORc is down-regulated and FOXP3 up-regulated upon repeated TCR engagement (Ye et al. 2011). This kind of plasticity may explain the low RORc mRNA expression in association with IL-17 and FOXP3 expression that was demonstrated in the mucosa of untreated celiac disease. In Study IV, the expression of RORc mRNA showed no difference between the study groups (original publication IV, Figure 2), nor did RORc mRNA correlate with IL-17 mRNA (r=-0.24, P=n.s., Spearman).

During pathogenesis of T1D, autoreactive T cells destroy insulin-secreting pancreatic islet β-cells, causing insulin deficiency and elevated plasma glucose levels (Atkinson, Maclaren 1994). According to other reports, increased numbers of HLA class II-, CD25-, MadCAM-1-, IL-1-, and IL-4-positive cells signal promoted small intestinal immune activation in T1D (Westerholm-Ormio et al. 2003, Tiittanen et al. 2008, Auricchio et al. 2004), thus suggesting therefore intestinal inflammation as part of the disease pathogenesis (Vaarala 2008a, Vaarala et al. 2008b). Based on animal studies, alterations in the gut immune system such as increased permeability and enteropathy seem to be key regulators of autoimmune insulitis and of development of T1D (Graham et al. 2004, Neu et al. 2005). In T1D, the number of intestinal

FOXP3$^+$ Treg cells is reduced because of their defective differentiation in the intestine (Badami et al. 2011). A previous study from our group showed no infiltration of FOXP3-expressing cells in the small intestinal mucosa in T1D (Tiittanen et al. 2008), and Study IV confirms this, because no difference in the number of FOXP3$^+$ cells or transcripts emerged between T1D children and the reference children (original publication IV, Figure 1).

# 6 Conclusions

Changes in immunological balance play a crucial role in inflammatory bowel disease, in celiac disease, and in T1D. During recent years, the new types of Th cells detected have been of great importance. This thesis has focused on the IL-17 type of immunity and on the balance between Tregs and Th17 cells.

In adult patients, IL-17 immunity plays a fundamental role in active and inactive Crohn's disease. Activation of the IL-23/IL-17 axis is associated with the etiology of Crohn's disease, and by causing increased sensitivity of the epithelium to microbial LPS, it may also be the basis for the relapsing nature of the disease. Immunomodulatory treatment such as with TNF-α-blocking agents ameliorated the balance between intestinal IL-17$^{+}$ T-effector and regulatory cells, despite the fact that, even after TNF-α blocking treatment, intestinal IL-17 upregulation persisted.

In pediatric IBD patients, studies of the role of IL-17 immunity are lacking, although the need for pediatric studies is widely acknowledged among pediatricians. In our study, no difference existed between pediatric Crohn's disease and UC, because the inflamed mucosa exhibited IL-17 upregulation in both diseases.

In celiac disease, upregulation of IL-17 immunity has a link to the villous atrophy seen in the late stage of the disease, but not to early phases preceding atrophy. After GFD, the level of IL-17 type immunity and the structure of the intestine are restored. In TID, no mucosal IL-17 up-regulation existed. As IL-17 showed no promotive effect on the apoptotic mechanism in the Caco-2 epithelial cell line, IL-17, however, may not be the initial step causing villous atrophy and tissue damage.

To conclude: based on these studies, the IL-17 type of immunity and increased levels of regulatory T cells are present in IBD and celiac disease when the structure of the intestine is disrupted, and in IBD also when the mucosa has healed. Anti-TNF- α-blocking is incapable of reversing completely the immunological changes in Crohn's disease, but it seems to play a role in the balance between effector and regulatory T cells. IL-17 immunity was not upregulated in T1D, in which tissue destruction of the intestine is absent. IL-17 immunity thus appears to be one of the causes of tissue damage in IBD and celiac disease. It, therefore, seems, based on this thesis and on the current literature, that IL-17, functioning with IFN-γ, either exacerbates or promotes the tissue destruction seen in Crohn's disease and celiac disease. By itself, IL-17 may, however, play a protective role.

Due to methodological difficulties in this thesis, IL-17-positive cells could not be specified in detail, and we lack full understanding of these cells, although their upregulation in IBD and celiac disease is evident. Detailed characterization of IL-17 type immunity would be a necessary object for future studies.

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# References


Abraham, C. & Medzhitov, R. 2011, "Interactions between the host innate immune system and microbes in inflammatory bowel disease", *Gastroenterology,* vol. 140, no. 6, pp. 1729-1737.

Abreu, M.T. 2010, "Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function", *Nature reviews.Immunology,* vol. 10, no. 2, pp. 131-144.

Ahuja, V. & Tandon, R.K. 2010, "Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences", *Journal of digestive diseases,* vol. 11, no. 3, pp. 134-147.

Alam, C., Valkonen, S., Palagani, V., Jalava, J., Eerola, E. & Hanninen, A. 2010, "Inflammatory tendencies and overproduction of IL-17 in the colon of young NOD mice are counteracted with diet change", *Diabetes,* vol. 59, no. 9, pp. 2237-2246.

Aljurf, M., Ezzat, A. & O Musa, M. 2002, "Emerging role of gammadelta T-cells in health and disease", *Blood reviews,* vol. 16, no. 4, pp. 203-206.

Andersen, M.H., Schrama, D., Thor Straten, P. & Becker, J.C. 2006, "Cytotoxic T cells", *The Journal of investigative dermatology,* vol. 126, no. 1, pp. 32-41.

Anderson, C.A., Boucher, G., Lees, C.W., Franke, A., D'Amato, M., Taylor, K.D., Lee, J.C., Goyette, P., Imielinski, M., Latiano, A., Lagace, C., Scott, R., Amininejad, L., Bumpstead, S., Baidoo, L., Baldassano, R.N., Barclay, M., Bayless, T.M., Brand, S., Buning, C., Colombel, J.F., Denson, L.A., De Vos, M., Dubinsky, M., Edwards, C., Ellinghaus, D., Fehrmann, R.S., Floyd, J.A., Florin, T., Franchimont, D., Franke, L., Georges, M., Glas, J., Glazer, N.L., Guthery, S.L., Haritunians, T., Hayward, N.K., Hugot, J.P., Jobin, G., Laukens, D., Lawrance, I., Lemann, M., Levine, A., Libioulle, C., Louis, E., McGovern, D.P., Milla, M., Montgomery, G.W., Morley, K.I., Mowat, C., Ng, A., Newman, W., Ophoff, R.A., Papi, L., Palmieri, O., Peyrin-Biroulet, L., Panes, J., Phillips, A., Prescott, N.J., Proctor, D.D., Roberts, R., Russell, R., Rutgeerts, P., Sanderson, J., Sans, M., Schumm, P., Seibold, F., Sharma, Y., Simms, L.A., Seielstad, M., Steinhart, A.H., Targan, S.R., van den Berg, L.H., Vatn, M., Verspaget, H., Walters, T., Wijmenga, C., Wilson, D.C., Westra, H.J., Xavier, R.J., Zhao, Z.Z., Ponsioen, C.Y., Andersen, V., Torkvist, L., Gazouli, M., Anagnou, N.P., Karlsen, T.H., Kupcinskas, L., Sventoraityte, J., Mansfield, J.C., Kugathasan, S., Silverberg, M.S., Halfvarson, J., Rotter, J.I., Mathew, C.G., Griffiths, A.M., Gearry, R., Ahmad, T., Brant, S.R., Chamaillard, M., Satsangi, J., Cho, J.H., Schreiber, S., Daly, M.J., Barrett, J.C., Parkes, M., Annese, V., Hakonarson, H., Radford-Smith, G., Duerr, R.H., Vermeire, S., Weersma, R.K. & Rioux, J.D. 2011, "Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47", *Nature genetics,* vol. 43, no. 3, pp. 246-252.

Andoh, A., Zhang, Z., Inatomi, O., Fujino, S., Deguchi, Y., Araki, Y., Tsujikawa, T., Kitoh, K., Kim-Mitsuyama, S., Takayanagi, A., Shimizu, N. & Fujiyama, Y. 2005, "Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts", *Gastroenterology,* vol. 129, no. 3, pp. 969-84.

Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E. & Romagnani, S. 2007, "Phenotypic and functional features of human Th17 cells", *J Exp Med,* vol. 204, no. 8, pp. 1849-61.

Arif, S., Moore, F., Marks, K., Bouckenooghe, T., Dayan, C.M., Planas, R., Vives-Pi, M., Powrie, J., Tree, T., Marchetti, P., Huang, G.C., Gurzov, E.N., Pujol-Borrell, R., Eizirik, D.L. & Peakman, M. 2011, "Peripheral and islet interleukin-17 pathway activation characterizes human autoimmune diabetes and promotes cytokine-mediated beta-cell death", *Diabetes,* vol. 60, no. 8, pp. 2112-2119.

Arranz, E., Bode, J., Kingstone, K. & Ferguson, A. 1994, "Intestinal antibody pattern of coeliac disease: association with gamma/delta T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease", *Gut,* vol. 35, no. 4, pp. 476-482.

Atkinson, M.A. & Maclaren, N.K. 1994, "The pathogenesis of insulin-dependent diabetes mellitus", *N Engl J Med,* vol. 331, no. 21, pp. 1428-1436.

Auricchio, R., Paparo, F., Maglio, M., Franzese, A., Lombardi, F., Valerio, G., Nardone, G., Percopo, S., Greco, L. & Troncone, R. 2004, "In vitro-deranged intestinal immune response to gliadin in type 1 diabetes", *Diabetes,* vol. 53, no. 7, pp. 1680-1683.

Ayyoub, M., Deknuydt, F., Raimbaud, I., Dousset, C., Leveque, L., Bioley, G. & Valmori, D. 2009, "Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17

lineage-specific transcription factor RORgamma t", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 106, no. 21, pp. 8635-8640.

Baba, N., Rubio, M. & Sarfati, M. 2010, "Interplay between CD45RA+ regulatory T cells and TNF-alpha in the regulation of human Th17 differentiation", *International immunology,* vol. 22, no. 4, pp. 237-244.

Badami, E., Sorini, C., Coccia, M., Usuelli, V., Molteni, L., Bolla, A.M., Scavini, M., Mariani, A., King, C., Bosi, E. & Falcone, M. 2011, "Defective differentiation of regulatory FoxP3+ T cells by small-intestinal dendritic cells in patients with type 1 diabetes", *Diabetes,* vol. 60, no. 8, pp. 2120-2124.

Baecher-Allan, C., Wolf, E. & Hafler, D.A. 2005, "Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells", *Clinical immunology (Orlando, Fla.),* vol. 115, no. 1, pp. 10-18.

Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H., Duerr, R.H., Rioux, J.D., Brant, S.R., Silverberg, M.S., Taylor, K.D., Barmada, M.M., Bitton, A., Dassopoulos, T., Datta, L.W., Green, T., Griffiths, A.M., Kistner, E.O., Murtha, M.T., Regueiro, M.D., Rotter, J.I., Schumm, L.P., Steinhart, A.H., Targan, S.R., Xavier, R.J., Libioulle, C., Sandor, C., Lathrop, M., Belaiche, J., Dewit, O., Gut, I., Heath, S., Laukens, D., Mni, M., Rutgeerts, P., Van Gossum, A., Zelenika, D., Franchimont, D., Hugot, J.P., de Vos, M., Vermeire, S., Louis, E., Cardon, L.R., Anderson, C.A., Drummond, H., Nimmo, E., Ahmad, T., Prescott, N.J., Onnie, C.M., Fisher, S.A., Marchini, J., Ghori, J., Bumpstead, S., Gwilliam, R., Tremelling, M., Deloukas, P., Mansfield, J., Jewell, D., Satsangi, J., Mathew, C.G., Parkes, M., Georges, M. & Daly, M.J. 2008, "Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease", *Nat Genet,* vol. 40, no. 8, pp. 955-962.

Beriou, G., Costantino, C.M., Ashley, C.W., Yang, L., Kuchroo, V.K., Baecher-Allan, C. & Hafler, D.A. 2009, "IL-17-producing human peripheral regulatory T cells retain suppressive function", *Blood,* vol. 113, no. 18, pp. 4240-4249.

Best, W.R., Becktel, J.M., Singleton, J.W. & F., K.,Jr 1976, "Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study", *Gastroenterology,* vol. 70, no. 3, pp. 439-44.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L. & Kuchroo, V.K. 2006, "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells", *Nature,* vol. 441, no. 7090, pp. 235-8.

Bettelli, E., Oukka, M. & Kuchroo, V.K. 2007, "T(H)-17 cells in the circle of immunity and autoimmunity", *Nature immunology,* vol. 8, no. 4, pp. 345-50.

Bhagat, S. & Das, K.M. 1994, "A shared and unique peptide in the human colon, eye, and joint detected by a monoclonal antibody", *Gastroenterology,* vol. 107, no. 1, pp. 103-108.

Biank, V., Broeckel, U. & Kugathasan, S. 2007, "Pediatric inflammatory bowel disease: clinical and molecular genetics", *Inflamm Bowel Dis,* vol. 13, no. 11, pp. 1430-8.

Blanco Quiros, A., Arranz Sanz, E., Bernardo Ordiz, D. & Garrote Adrados, J.A. 2009, "From autoimmune enteropathy to the IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked) syndrome", *Allergologia et Immunopathologia,* vol. 37, no. 4, pp. 208-215.

Blaschitz, C. & Raffatellu, M. 2010, "Th17 cytokines and the gut mucosal barrier", *Journal of clinical immunology,* vol. 30, no. 2, pp. 196-203.

Bodd, M., Raki, M., Tollefsen, S., Fallang, L.E., Bergseng, E., Lundin, K.E. & Sollid, L.M. 2010, "HLA-DQ2-restricted gluten-reactive T cells produce IL-21 but not IL-17 or IL-22", *Mucosal immunology,* vol. 3, no. 6, pp. 594-601.

Bosi, E., Molteni, L., Radaelli, M.G., Folini, L., Fermo, I., Bazzigaluppi, E., Piemonti, L., Pastore, M.R. & Paroni, R. 2006, "Increased intestinal permeability precedes clinical onset of type 1 diabetes", *Diabetologia,* vol. 49, no. 12, pp. 2824-2827.

Bradshaw, E.M., Raddassi, K., Elyaman, W., Orban, T., Gottlieb, P.A., Kent, S.C. & Hafler, D.A. 2009, "Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells", *Journal of immunology (Baltimore, Md.: 1950),* vol. 183, no. 7, pp. 4432-4439.

Brand, S., Beigel, F., Olszak, T., Zitzmann, K., Eichhorst, S.T., Otte, J.M., Diepolder, H., Marquardt, A., Jagla, W., Popp, A., Leclair, S., Herrmann, K., Seiderer, J., Ochsenkuhn, T., Goke, B., Auernhammer, C.J. & Dambacher, J. 2006, "IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration", *American journal of physiology.Gastrointestinal and liver physiology,* vol. 290, no. 4, pp. G827-38.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F. & Ramsdell, F. 2001, "Disruption of a new forkhead/winged-

helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse", *Nature genetics,* vol. 27, no. 1, pp. 68-73.

Cao, X., Cai, S.F., Fehniger, T.A., Song, J., Collins, L.I., Piwnica-Worms, D.R. & Ley, T.J. 2007, "Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance", *Immunity,* vol. 27, no. 4, pp. 635-646.

Chaplin, D.D. 2010, "Overview of the immune response", *The Journal of allergy and clinical immunology,* vol. 125, no. 2 Suppl 2, pp. S3-23.

Chatila, T. 2008, "Molecular mechanisms of regulatory T cell development", *J Clin Immunol,* vol. 28, no. 6, pp. 625-30.

Clemente, M.G., De Virgiliis, S., Kang, J.S., Macatagney, R., Musu, M.P., Di Pierro, M.R., Drago, S., Congia, M. & Fasano, A. 2003, "Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function", *Gut,* vol. 52, no. 2, pp. 218-223.

Collison, L.W., Chaturvedi, V., Henderson, A.L., Giacomin, P.R., Guy, C., Bankoti, J., Finkelstein, D., Forbes, K., Workman, C.J., Brown, S.A., Rehg, J.E., Jones, M.L., Ni, H.T., Artis, D., Turk, M.J. & Vignali, D.A. 2010, "IL-35-mediated induction of a potent regulatory T cell population", *Nature immunology,* vol. 11, no. 12, pp. 1093-1101.

Collison, L.W., Pillai, M.R., Chaturvedi, V. & Vignali, D.A. 2009, "Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner", *Journal of immunology (Baltimore, Md.: 1950),* vol. 182, no. 10, pp. 6121-6128.

Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S. & Vignali, D.A. 2007, "The inhibitory cytokine IL-35 contributes to regulatory T-cell function", *Nature,* vol. 450, no. 7169, pp. 566-569.

Commins, S.P., Borish, L. & Steinke, J.W. 2010, "Immunologic messenger molecules: cytokines, interferons, and chemokines", *The Journal of allergy and clinical immunology,* vol. 125, no. 2 Suppl 2, pp. S53-72.

Cong, Y., Weaver, C.T., Lazenby, A. & Elson, C.O. 2002, "Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora", *Journal of immunology (Baltimore, Md.: 1950),* vol. 169, no. 11, pp. 6112-6119.

Coyne, C.B. & Bergelson, J.M. 2006, "Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions", *Cell,* vol. 124, no. 1, pp. 119-131.

Crohn, B.B., Ginzburg, L. & Oppenheimer, G.D. 1984, "Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. By Burril B. Crohn, Leon Ginzburg, and Gordon D. Oppenheimer", *JAMA : the journal of the American Medical Association,* vol. 251, no. 1, pp. 73-79.

Damen, G.M., Hol, J., de Ruiter, L., Bouquet, J., Sinaasappel, M., van der Woude, J., Laman, J.D., Hop, W.C., Buller, H.A., Escher, J.C. & Nieuwenhuis, E.E. 2006, "Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn disease", *Journal of pediatric gastroenterology and nutrition,* vol. 42, no. 2, pp. 142-149.

Danese, S. & Fiocchi, C. 2011, "Ulcerative colitis", *The New England journal of medicine,* vol. 365, no. 18, pp. 1713-1725.

Danese, S., Sans, M. & Fiocchi, C. 2004, "Inflammatory bowel disease: the role of environmental factors", *Autoimmunity reviews,* vol. 3, no. 5, pp. 394-400.

Deenick, E.K. & Ma, C.S. 2011, "The regulation and role of T follicular helper cells in immunity", *Immunology,* vol. 134, no. 4, pp. 361-367.

Deknuydt, F., Bioley, G., Valmori, D. & Ayyoub, M. 2009, "IL-1beta and IL-2 convert human Treg into T(H)17 cells", *Clinical immunology (Orlando, Fla.),* vol. 131, no. 2, pp. 298-307.

Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M.G., Biagiotti, R. & Romagnani, S. 1993, "Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production", *Journal of immunology (Baltimore, Md.: 1950),* vol. 150, no. 2, pp. 353-360.

Duerr, R.H., Taylor, K.D., Brant, S.R., Rioux, J.D., Silverberg, M.S., Daly, M.J., Steinhart, A.H., Abraham, C., Regueiro, M., Griffiths, A., Dassopoulos, T., Bitton, A., Yang, H., Targan, S., Datta, L.W., Kistner, E.O., Schumm, L.P., Lee, A.T., Gregersen, P.K., Barmada, M.M., Rotter, J.I., Nicolae, D.L. & Cho, J.H. 2006, "A genome-wide association study identifies IL23R as an inflammatory bowel disease gene", *Science,* vol. 314, no. 5804, pp. 1461-3.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. & Sallusto, F. 2009, "Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells", *Nature immunology,* vol. 10, no. 8, pp. 857-863.

Dumoutier, L., Van Roost, E., Colau, D. & Renauld, J.C. 2000, "Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-

stimulating factor", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, no. 18, pp. 10144-10149.

Eckmann, L., Jung, H.C., Schurer-Maly, C., Panja, A., Morzycka-Wroblewska, E. & Kagnoff, M.F. 1993, "Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8", *Gastroenterology,* vol. 105, no. 6, pp. 1689-97.

Economou, M., Trikalinos, T.A., Loizou, K.T., Tsianos, E.V. & Ioannidis, J.P. 2004, "Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis", *The American Journal of Gastroenterology,* vol. 99, no. 12, pp. 2393-2404.

Elliott, R.B., Reddy, S.N., Bibby, N.J. & Kida, K. 1988, "Dietary prevention of diabetes in the non-obese diabetic mouse", *Diabetologia,* vol. 31, no. 1, pp. 62-64.

Emamaullee, J.A., Davis, J., Merani, S., Toso, C., Elliott, J.F., Thiesen, A. & Shapiro, A.M. 2009, "Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice", *Diabetes,* vol. 58, no. 6, pp. 1302-1311.

Esplugues, E., Huber, S., Gagliani, N., Hauser, A.E., Town, T., Wan, Y.Y., O'Connor, W.,Jr, Rongvaux, A., Van Rooijen, N., Haberman, A.M., Iwakura, Y., Kuchroo, V.K., Kolls, J.K., Bluestone, J.A., Herold, K.C. & Flavell, R.A. 2011, "Control of TH17 cells occurs in the small intestine", *Nature,* vol. 475, no. 7357, pp. 514-518.

Farrar, M.A. & Schreiber, R.D. 1993, "The molecular cell biology of interferon-gamma and its receptor", *Annual Review of Immunology,* vol. 11, pp. 571-611.

Fasano, A. 2011, "Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer", *Physiological Reviews,* vol. 91, no. 1, pp. 151-175.

Ferguson, A., Arranz, E. & O'Mahony, S. 1993, "Clinical and pathological spectrum of coeliac disease--active, silent, latent, potential", *Gut,* vol. 34, no. 2, pp. 150-151.

Fernandez, S., Molina, I.J., Romero, P., Gonzalez, R., Pena, J., Sanchez, F., Reynoso, F.R., Perez-Navero, J.L., Estevez, O., Ortega, C. & Santamaria, M. 2011, "Characterization of gliadin-specific Th17 cells from the mucosa of celiac disease patients", *The American Journal of Gastroenterology,* vol. 106, no. 3, pp. 528-538.

Fontenot, J.D., Gavin, M.A. & Rudensky, A.Y. 2003, "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells", *Nat Immunol,* vol. 4, no. 4, pp. 330-6.

Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., Bamba, T. & Fujiyama, Y. 2003, "Increased expression of interleukin 17 in inflammatory bowel disease", *Gut,* vol. 52, no. 1, pp. 65-70.

Fuss, I.J., Heller, F., Boirivant, M., Leon, F., Yoshida, M., Fichtner-Feigl, S., Yang, Z., Exley, M., Kitani, A., Blumberg, R.S., Mannon, P. & Strober, W. 2004, "Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis", *The Journal of clinical investigation,* vol. 113, no. 10, pp. 1490-1497.

Fuss, I.J., Neurath, M., Boirivant, M., Klein, J.S., de la Motte, C., Strong, S.A., Fiocchi, C. & Strober, W. 1996, "Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5", *J Immunol,* vol. 157, no. 3, pp. 1261-70.

Gasche, C., Scholmerich, J., Brynskov, J., D'Haens, G., Hanauer, S.B., Irvine, E.J., Jewell, D.P., Rachmilewitz, D., Sachar, D.B., Sandborn, W.J. & Sutherland, L.R. 2000, "A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998", *Inflammatory bowel diseases,* vol. 6, no. 1, pp. 8-15.

Geremia, A., Arancibia-Carcamo, C.V., Fleming, M.P., Rust, N., Singh, B., Mortensen, N.J., Travis, S.P. & Powrie, F. 2011, "IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease", *The Journal of experimental medicine,* vol. 208, no. 6, pp. 1127-1133.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J. & Sansonetti, P.J. 2003, "Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection", *The Journal of biological chemistry,* vol. 278, no. 11, pp. 8869-8872.

Gitter, A.H., Bendfeldt, K., Schulzke, J.D. & Fromm, M. 2000, "Leaks in the epithelial barrier caused by spontaneous and TNF-alpha-induced single-cell apoptosis", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 14, no. 12, pp. 1749-1753.

Gocke, A.R., Cravens, P.D., Ben, L.H., Hussain, R.Z., Northrop, S.C., Racke, M.K. & Lovett-Racke, A.E. 2007, "T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity", *J Immunol,* vol. 178, no. 3, pp. 1341-8.

Graham, S., Courtois, P., Malaisse, W.J., Rozing, J., Scott, F.W. & Mowat, A.M. 2004, "Enteropathy precedes type 1 diabetes in the BB rat", *Gut,* vol. 53, no. 10, pp. 1437-1444.

Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., Albrecht, M., Mayr, G., De La Vega, F.M., Briggs, J., Gunther, S., Prescott, N.J., Onnie, C.M., Hasler, R., Sipos, B., Folsch, U.R., Lengauer, T., Platzer, M., Mathew, C.G., Krawczak, M. & Schreiber, S. 2007, "A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1", *Nature genetics,* vol. 39, no. 2, pp. 207-211.

Hanninen, A., Nurmela, R., Maksimow, M., Heino, J., Jalkanen, S. & Kurts, C. 2007, "Islet beta-cell-specific T cells can use different homing mechanisms to infiltrate and destroy pancreatic islets", *The American journal of pathology,* vol. 170, no. 1, pp. 240-250.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M. & Weaver, C.T. 2005, "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages", *Nature immunology,* vol. 6, no. 11, pp. 1123-32.

Harrison, O.J. & Maloy, K.J. 2011, "Innate immune activation in intestinal homeostasis", *Journal of innate immunity,* vol. 3, no. 6, pp. 585-593.

Hartl, D., Koller, B., Mehlhorn, A.T., Reinhardt, D., Nicolai, T., Schendel, D.J., Griese, M. & Krauss-Etschmann, S. 2007, "Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma", *The Journal of allergy and clinical immunology,* vol. 119, no. 5, pp. 1258-1266.

Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J., Gitter, A.H., Burgel, N., Fromm, M., Zeitz, M., Fuss, I., Strober, W. & Schulzke, J.D. 2005, "Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution", *Gastroenterology,* vol. 129, no. 2, pp. 550-564.

Henderson, P., van Limbergen, J.E., Wilson, D.C., Satsangi, J. & Russell, R.K. 2011, "Genetics of childhood-onset inflammatory bowel disease", *Inflammatory bowel diseases,* vol. 17, no. 1, pp. 346-361.

Heyman, M.B., Kirschner, B.S., Gold, B.D., Ferry, G., Baldassano, R., Cohen, S.A., Winter, H.S., Fain, P., King, C., Smith, T. & El-Serag, H.B. 2005, "Children with early-onset inflammatory bowel disease (IBD): analysis of a pediatric IBD consortium registry", *J Pediatr,* vol. 146, no. 1, pp. 35-40.

Holm, K., Maki, M., Savilahti, E., Lipsanen, V., Laippala, P. & Koskimies, S. 1992, "Intraepithelial gamma delta T-cell-receptor lymphocytes and genetic susceptibility to coeliac disease", *Lancet,* vol. 339, no. 8808, pp. 1500-1503.

Holmen, N., Lundgren, A., Lundin, S., Bergin, A.M., Rudin, A., Sjovall, H. & Ohman, L. 2006, "Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity", *Inflammatory bowel diseases,* vol. 12, no. 6, pp. 447-456.

Honkanen, J., Nieminen, J.K., Gao, R., Luopajarvi, K., Salo, H.M., Ilonen, J., Knip, M., Otonkoski, T. & Vaarala, O. 2010, "IL-17 immunity in human type 1 diabetes", *Journal of immunology (Baltimore, Md.: 1950),* vol. 185, no. 3, pp. 1959-1967.

Hori, S., Nomura, T. & Sakaguchi, S. 2003, "Control of regulatory T cell development by the transcription factor Foxp3", *Science,* vol. 299, no. 5609, pp. 1057-61.

Hue, S., Ahern, P., Buonocore, S., Kullberg, M.C., Cua, D.J., McKenzie, B.S., Powrie, F. & Maloy, K.J. 2006, "Interleukin-23 drives innate and T cell-mediated intestinal inflammation", *The Journal of experimental medicine,* vol. 203, no. 11, pp. 2473-83.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M. & Thomas, G. 2001, "Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease", *Nature,* vol. 411, no. 6837, pp. 599-603.

Hundorfean, G., Neurath, M.F. & Mudter, J. 2012, "Functional relevance of T helper 17 (Th17) cells and the IL-17 cytokine family in inflammatory bowel disease", *Inflammatory bowel diseases,* vol. 18, no. 1, pp. 180-186.

Jalonen, T., Isolauri, E., Heyman, M., Crain-Denoyelle, A.M., Sillanaukee, P. & Koivula, T. 1991, "Increased beta-lactoglobulin absorption during rotavirus enteritis in infants: relationship to sugar permeability", *Pediatric research,* vol. 30, no. 3, pp. 290-293.

Janeway, C.A.,Jr & Medzhitov, R. 2002, "Innate immune recognition", *Annual Review of Immunology,* vol. 20, pp. 197-216.

Jarchum, I. & Pamer, E.G. 2011, "Regulation of innate and adaptive immunity by the commensal microbiota", *Current opinion in immunology,* vol. 23, no. 3, pp. 353-360.

Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzycka-Wroblewska, E. & Kagnoff, M.F. 1995, "A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion", *J Clin Invest,* vol. 95, no. 1, pp. 55-65.

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., Sakuraba, A., Kitazume, M.T., Sugita, A., Koganei, K., Akagawa, K.S. & Hibi, T. 2008, "Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis", *J Clin Invest,* vol. 118, no. 6, pp. 2269-2280.

Kanai, T., Nemoto, Y., Kamada, N., Totsuka, T., Hisamatsu, T., Watanabe, M. & Hibi, T. 2009, "Homeostatic (IL-7) and effector (IL-17) cytokines as distinct but complementary target for an optimal therapeutic strategy in inflammatory bowel disease", *Current opinion in gastroenterology,* vol. 25, no. 4, pp. 306-313.

Karagiannidis, C., Akdis, M., Holopainen, P., Woolley, N.J., Hense, G., Ruckert, B., Mantel, P.Y., Menz, G., Akdis, C.A., Blaser, K. & Schmidt-Weber, C.B. 2004, "Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma", *J Allergy Clin Immunol,* vol. 114, no. 6, pp. 1425-33.

Kaukinen, K., Collin, P., Holm, K., Karvonen, A.L., Pikkarainen, P. & Maki, M. 1998, "Small-bowel mucosal inflammation in reticulin or gliadin antibody-positive patients without villous atrophy", *Scandinavian journal of gastroenterology,* vol. 33, no. 9, pp. 944-949.

Kaukinen, K., Collin, P. & Maki, M. 2007, "Latent coeliac disease or coeliac disease beyond villous atrophy?", *Gut,* vol. 56, no. 10, pp. 1339-1340.

Kawaguchi, M., Adachi, M., Oda, N., Kokubu, F. & Huang, S.K. 2004, "IL-17 cytokine family", *The Journal of allergy and clinical immunology,* vol. 114, no. 6, pp. 1265-73; quiz 1274.

Kim, J.M. & Rudensky, A. 2006, "The role of the transcription factor Foxp3 in the development of regulatory T cells", *Immunological reviews,* vol. 212, pp. 86-98.

Kivling, A., Nilsson, L., Falth-Magnusson, K., Sollvander, S., Johanson, C. & Faresjo, M. 2008, "Diverse foxp3 expression in children with type 1 diabetes and celiac disease", *Annals of the New York Academy of Sciences,* vol. 1150, pp. 273-277.

Klemetti, P., Savilahti, E., Ilonen, J., Akerblom, H.K. & Vaarala, O. 1998, "T-cell reactivity to wheat gluten in patients with insulin-dependent diabetes mellitus", *Scand J Immunol,* vol. 47, no. 1, pp. 48-53.

Kobayashi, K., Kaneda, K. & Kasama, T. 2001, "Immunopathogenesis of delayed-type hypersensitivity", *Microscopy research and technique,* vol. 53, no. 4, pp. 241-245.

Koboziev, I., Karlsson, F. & Grisham, M.B. 2010, "Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation", *Annals of the New York Academy of Sciences,* vol. 1207 Suppl 1, pp. E86-93.

Kolho, K.L., Raivio, T., Lindahl, H. & Savilahti, E. 2006, "Fecal calprotectin remains high during glucocorticoid therapy in children with inflammatory bowel disease", *Scandinavian journal of gastroenterology,* vol. 41, no. 6, pp. 720-5.

Komatsu, N., Mariotti-Ferrandiz, M.E., Wang, Y., Malissen, B., Waldmann, H. & Hori, S. 2009, "Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 106, no. 6, pp. 1903-1908.

Kontakou, M., Przemioslo, R.T., Sturgess, R.P., Limb, G.A., Ellis, H.J., Day, P. & Ciclitira, P.J. 1995, "Cytokine mRNA expression in the mucosa of treated coeliac patients after wheat peptide challenge", *Gut,* vol. 37, no. 1, pp. 52-57.

Kontakou, M., Sturgess, R.P., Przemioslo, R.T., Limb, G.A., Nelufer, J.M. & Ciclitira, P.J. 1994, "Detection of interferon gamma mRNA in the mucosa of patients with coeliac disease by in situ hybridisation", *Gut,* vol. 35, no. 8, pp. 1037-1041.

Kugathasan, S., Saubermann, L.J., Smith, L., Kou, D., Itoh, J., Binion, D.G., Levine, A.D., Blumberg, R.S. & Fiocchi, C. 2007, "Mucosal T-cell immunoregulation varies in early and late inflammatory bowel disease", *Gut,* vol. 56, no. 12, pp. 1696-1705.

Kullberg, M.C., Jankovic, D., Feng, C.G., Hue, S., Gorelick, P.L., McKenzie, B.S., Cua, D.J., Powrie, F., Cheever, A.W., Maloy, K.J. & Sher, A. 2006, "IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis", *The Journal of experimental medicine,* vol. 203, no. 11, pp. 2485-94.

Lahdenpera, A., Ludvigsson, J., Falth-Magnusson, K., Hogberg, L. & Vaarala, O. 2011, "The effect of gluten-free diet on Th1-Th2-Th3-associated intestinal immune responses in celiac disease", *Scandinavian Journal of Gastroenterology,* vol. 46, no. 5, pp. 538-549.

Lau, K., Benitez, P., Ardissone, A., Wilson, T.D., Collins, E.L., Lorca, G., Li, N., Sankar, D., Wasserfall, C., Neu, J., Atkinson, M.A., Shatz, D., Triplett, E.W. & Larkin, J.,3rd 2011, "Inhibition

of type 1 diabetes correlated to a Lactobacillus johnsonii N6.2-mediated Th17 bias", *Journal of immunology (Baltimore, Md.: 1950),* vol. 186, no. 6, pp. 3538-3546.

Lehtinen, P., Ashorn, M., Iltanen, S., Jauhola, R., Jauhonen, P., Kolho, K.L. & Auvinen, A. 2011, "Incidence trends of pediatric inflammatory bowel disease in Finland, 1987-2003, a nationwide study", *Inflammatory bowel diseases,* vol. 17, no. 8, pp. 1778-1783.

Lennard-Jones, J.E. 1989, "Classification of inflammatory bowel disease", *Scand J Gastroenterol Suppl,* vol. 170, pp. 2-6; discussion 16-9.

Leonardi, C., Matheson, R., Zachariae, C., Cameron, G., Li, L., Edson-Heredia, E., Braun, D. & Banerjee, S. 2012, "Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis", *The New England journal of medicine,* vol. 366, no. 13, pp. 1190-1199.

Leung, D.Y. & Bloom, J.W. 2003, "Update on glucocorticoid action and resistance", *J Allergy Clin Immunol,* vol. 111, no. 1, pp. 3-22; quiz 23.

Lindfors, K., Maki, M. & Kaukinen, K. 2010, "Transglutaminase 2-targeted autoantibodies in celiac disease: Pathogenetic players in addition to diagnostic tools?", *Autoimmunity reviews,* vol. 9, no. 11, pp. 744-749.

Lionetti, E. & Catassi, C. 2011, "New clues in celiac disease epidemiology, pathogenesis, clinical manifestations, and treatment", *International reviews of immunology,* vol. 30, no. 4, pp. 219-231.

Lohi, S., Mustalahti, K., Kaukinen, K., Laurila, K., Collin, P., Rissanen, H., Lohi, O., Bravi, E., Gasparin, M., Reunanen, A. & Maki, M. 2007, "Increasing prevalence of coeliac disease over time", *Aliment Pharmacol Ther,* vol. 26, no. 9, pp. 1217-1225.

MacDonald, T.T. 1995, "Breakdown of tolerance to the intestinal bacterial flora in inflammatory bowel disease (IBD)", *Clinical and experimental immunology,* vol. 102, no. 3, pp. 445-447.

Macdonald, T.T. & Monteleone, G. 2005, "Immunity, inflammation, and allergy in the gut", *Science,* vol. 307, no. 5717, pp. 1920-1925.

MacDonald, T.T., Monteleone, I., Fantini, M.C. & Monteleone, G. 2011, "Regulation of homeostasis and inflammation in the intestine", *Gastroenterology,* vol. 140, no. 6, pp. 1768-1775.

Macpherson, A.J., McCoy, K.D., Johansen, F.E. & Brandtzaeg, P. 2008, "The immune geography of IgA induction and function", *Mucosal immunology,* vol. 1, no. 1, pp. 11-22.

Maki, M. & Collin, P. 1997, "Coeliac disease", *Lancet,* vol. 349, no. 9067, pp. 1755-1759.

Makita, S., Kanai, T., Oshima, S., Uraushihara, K., Totsuka, T., Sawada, T., Nakamura, T., Koganei, K., Fukushima, T. & Watanabe, M. 2004, "CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells", *J Immunol,* vol. 173, no. 5, pp. 3119-30.

Maloy, K.J. 2008, "The Interleukin-23 / Interleukin-17 axis in intestinal inflammation", *J Intern Med,* vol. 263, no. 6, pp. 584-90.

Manetti, R., Parronchi, P., Giudizi, M.G., Piccinni, M.P., Maggi, E., Trinchieri, G. & Romagnani, S. 1993, "Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells", *The Journal of experimental medicine,* vol. 177, no. 4, pp. 1199-1204.

Marx, J. 2007, "Biomedicine. Puzzling out the pains in the gut", *Science,* vol. 315, no. 5808, pp. 33-5.

Mary, J.Y. & Modigliani, R. 1989, "Development and validation of an endoscopic index of the severity for Crohn's disease: a prospective multicentre study. Groupe d'Etudes Therapeutiques des Affections Inflammatoires du Tube Digestif (GETAID)", *Gut,* vol. 30, no. 7, pp. 983-9.

Maul, J., Loddenkemper, C., Mundt, P., Berg, E., Giese, T., Stallmach, A., Zeitz, M. & Duchmann, R. 2005, "Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease", *Gastroenterology,* vol. 128, no. 7, pp. 1868-78.

Mazzarella, G., Stefanile, R., Camarca, A., Giliberti, P., Cosentini, E., Marano, C., Iaquinto, G., Giardullo, N., Auricchio, S., Sette, A., Troncone, R. & Gianfrani, C. 2008, "Gliadin activates HLA class I-restricted CD8+ T cells in celiac disease intestinal mucosa and induces the enterocyte apoptosis", *Gastroenterology,* vol. 134, no. 4, pp. 1017-1027.

Medzhitov, R. 2010, "Innate immunity: quo vadis?", *Nature immunology,* vol. 11, no. 7, pp. 551-553.

Mestecky, J., Lamm, M.E., McGhee, J.R., Bienenstock, J., Mayer, L. & Strober, W. (eds) 2005, *Mucosal immunology*, Thrid Edition edn, Elsevier Academic Press, United States of America.

Molberg, O., Mcadam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K.E., Sjostrom, H. & Sollid, L.M. 1998, "Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease", *Nature medicine,* vol. 4, no. 6, pp. 713-717.

Monteleone, I., Sarra, M., Del Vecchio Blanco, G., Paoluzi, O.A., Franze, E., Fina, D., Fabrizi, A., MacDonald, T.T., Pallone, F. & Monteleone, G. 2010, "Characterization of IL-17A-producing

cells in celiac disease mucosa", *Journal of immunology (Baltimore, Md.: 1950),* vol. 184, no. 4, pp. 2211-2218.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. 1986, "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins", *J Immunol,* vol. 136, no. 7, pp. 2348-57.

Neu, J., Reverte, C.M., Mackey, A.D., Liboni, K., Tuhacek-Tenace, L.M., Hatch, M., Li, N., Caicedo, R.A., Schatz, D.A. & Atkinson, M. 2005, "Changes in intestinal morphology and permeability in the biobreeding rat before the onset of type 1 diabetes", *J Pediatr Gastroenterol Nutr,* vol. 40, no. 5, pp. 589-595.

Neurath, M.F. 2007, "IL-23: a master regulator in Crohn disease", *Nature medicine,* vol. 13, no. 1, pp. 26-8.

Neurath, M.F., Fuss, I., Kelsall, B.L., Stuber, E. & Strober, W. 1995, "Antibodies to interleukin 12 abrogate established experimental colitis in mice", *J Exp Med,* vol. 182, no. 5, pp. 1281-90.

Neutra, M.R., Mantis, N.J. & Kraehenbuhl, J.P. 2001, "Collaboration of epithelial cells with organized mucosal lymphoid tissues", *Nature immunology,* vol. 2, no. 11, pp. 1004-1009.

Niedbala, W., Wei, X.Q., Cai, B., Hueber, A.J., Leung, B.P., McInnes, I.B. & Liew, F.Y. 2007, "IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells", *European journal of immunology,* vol. 37, no. 11, pp. 3021-3029.

Nielsen, O.H., Kirman, I., Rudiger, N., Hendel, J. & Vainer, B. 2003, "Upregulation of interleukin-12 and -17 in active inflammatory bowel disease", *Scandinavian journal of gastroenterology,* vol. 38, no. 2, pp. 180-5.

Nieuwenhuis, E.E. & Escher, J.C. 2008, "Early onset IBD: what's the difference?", *Dig Liver Dis,* vol. 40, no. 1, pp. 12-5.

Nikolaus, S. & Schreiber, S. 2007, "Diagnostics of inflammatory bowel disease", *Gastroenterology,* vol. 133, no. 5, pp. 1670-89.

Nilsen, E.M., Jahnsen, F.L., Lundin, K.E., Johansen, F.E., Fausa, O., Sollid, L.M., Jahnsen, J., Scott, H. & Brandtzaeg, P. 1998, "Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease", *Gastroenterology,* vol. 115, no. 3, pp. 551-563.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G. & Cho, J.H. 2001, "A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease", *Nature,* vol. 411, no. 6837, pp. 603-606.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J.S., Moore, K.W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J.F. & Kastelein, R.A. 2000, "Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12", *Immunity,* vol. 13, no. 5, pp. 715-725.

Papp, K.A., Leonardi, C., Menter, A., Ortonne, J.P., Krueger, J.G., Kricorian, G., Aras, G., Li, J., Russell, C.B., Thompson, E.H. & Baumgartner, S. 2012, "Brodalumab, an anti-interleukin-17-receptor antibody for psoriasis", *The New England journal of medicine,* vol. 366, no. 13, pp. 1181-1189.

Parkes, M., Barrett, J.C., Prescott, N.J., Tremelling, M., Anderson, C.A., Fisher, S.A., Roberts, R.G., Nimmo, E.R., Cummings, F.R., Soars, D., Drummond, H., Lees, C.W., Khawaja, S.A., Bagnall, R., Burke, D.A., Todhunter, C.E., Ahmad, T., Onnie, C.M., McArdle, W., Strachan, D., Bethel, G., Bryan, C., Lewis, C.M., Deloukas, P., Forbes, A., Sanderson, J., Jewell, D.P., Satsangi, J., Mansfield, J.C., Wellcome Trust Case Control Consortium, Cardon, L. & Mathew, C.G. 2007, "Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility", *Nature genetics,* vol. 39, no. 7, pp. 830-832.

Parrish-Novak, J., Dillon, S.R., Nelson, A., Hammond, A., Sprecher, C., Gross, J.A., Johnston, J., Madden, K., Xu, W., West, J., Schrader, S., Burkhead, S., Heipel, M., Brandt, C., Kuijper, J.L., Kramer, J., Conklin, D., Presnell, S.R., Berry, J., Shiota, F., Bort, S., Hambly, K., Mudri, S., Clegg, C., Moore, M., Grant, F.J., Lofton-Day, C., Gilbert, T., Rayond, F., Ching, A., Yao, L., Smith, D., Webster, P., Whitmore, T., Maurer, M., Kaushansky, K., Holly, R.D. & Foster, D. 2000, "Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function", *Nature,* vol. 408, no. 6808, pp. 57-63.

Parronchi, P., Romagnani, P., Annunziato, F., Sampognaro, S., Becchio, A., Giannarini, L., Maggi, E., Pupilli, C., Tonelli, F. & Romagnani, S. 1997, "Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients

with Crohn's disease", *The American journal of pathology,* vol. 150, no. 3, pp. 823-32.

Paul, W.E. (ed) 2003, *Fundamental immunology*, Sixth edn, Lippincott Williams & Wilkins, USA.

Rakoff-Nahoum, S. & Medzhitov, R. 2008, "Innate immune recognition of the indigenous microbial flora", *Mucosal immunology,* vol. 1 Suppl 1, pp. S10-4.

Read, S., Malmstrom, V. & Powrie, F. 2000, "Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation", *J Exp Med,* vol. 192, no. 2, pp. 295-302.

Reikvam, D.H., Perminow, G., Lyckander, L.G., Gran, J.M., Brandtzaeg, P., Vatn, M. & Carlsen, H.S. 2011, "Increase of regulatory T cells in ileal mucosa of untreated pediatric Crohn's disease patients", *Scandinavian Journal of Gastroenterology,* vol. 46, no. 5, pp. 550-560.

Rovedatti, L., Kudo, T., Biancheri, P., Sarra, M., Knowles, C.H., Rampton, D.S., Corazza, G.R., Monteleone, G., Di Sabatino, A. & Macdonald, T.T. 2009, "Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease", *Gut,* vol. 58, no. 12, pp. 1629-1636.

Rudensky, A.Y. 2011, "Regulatory T cells and Foxp3", *Immunological reviews,* vol. 241, no. 1, pp. 260-268.

Rutgeerts, P., Van Assche, G. & Vermeire, S. 2006, "Review article: Infliximab therapy for inflammatory bowel disease--seven years on", *Alimentary Pharmacology & Therapeutics,* vol. 23, no. 4, pp. 451-463.

Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T. & Nomura, T. 2006, "Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease", *Immunological reviews,* vol. 212, pp. 8-27.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. 1995, "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", *Journal of immunology (Baltimore, Md.: 1950),* vol. 155, no. 3, pp. 1151-1164.

Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. 2008, "Regulatory T cells and immune tolerance", *Cell,* vol. 133, no. 5, pp. 775-787.

Santos, L.M., al-Sabbagh, A., Londono, A. & Weiner, H.L. 1994, "Oral tolerance to myelin basic protein induces regulatory TGF-beta-secreting T cells in Peyer's patches of SJL mice", *Cellular immunology,* vol. 157, no. 2, pp. 439-447.

Sapone, A., de Magistris, L., Pietzak, M., Clemente, M.G., Tripathi, A., Cucca, F., Lampis, R., Kryszak, D., Carteni, M., Generoso, M., Iafusco, D., Prisco, F., Laghi, F., Riegler, G., Carratu, R., Counts, D. & Fasano, A. 2006, "Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives", *Diabetes,* vol. 55, no. 5, pp. 1443-1449.

Sapone, A., Lammers, K.M., Mazzarella, G., Mikhailenko, I., Carteni, M., Casolaro, V. & Fasano, A. 2009, "Differential Mucosal IL-17 Expression in Two Gliadin-Induced Disorders: Gluten Sensitivity and the Autoimmune Enteropathy Celiac Disease", *International archives of allergy and immunology,* vol. 152, no. 1, pp. 75-80.

Saruta, M., Yu, Q.T., Fleshner, P.R., Mantel, P.Y., Schmidt-Weber, C.B., Banham, A.H. & Papadakis, K.A. 2007, "Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease", *Clinical immunology (Orlando, Fla.),* vol. 125, no. 3, pp. 281-290.

Savilahti, E., Kolho, K.L., Westerholm-Ormio, M. & Verkasalo, M. 2010, "Clinics of coeliac disease in children in the 2000s", *Acta Paediatrica (Oslo, Norway : 1992),* vol. 99, no. 7, pp. 1026-1030.

Saxon, A., Shanahan, F., Landers, C., Ganz, T. & Targan, S. 1990, "A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease", *The Journal of allergy and clinical immunology,* vol. 86, no. 2, pp. 202-210.

Seder, R.A., Paul, W.E., Davis, M.M. & Fazekas de St Groth, B. 1992, "The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice", *The Journal of experimental medicine,* vol. 176, no. 4, pp. 1091-1098.

Seibold, F., Brandwein, S., Simpson, S., Terhorst, C. & Elson, C.O. 1998, "pANCA represents a cross-reactivity to enteric bacterial antigens", *Journal of clinical immunology,* vol. 18, no. 2, pp. 153-160.

Seiderer, J., Elben, I., Diegelmann, J., Glas, J., Stallhofer, J., Tillack, C., Pfennig, S., Jurgens, M., Schmechel, S., Konrad, A., Goke, B., Ochsenkuhn, T., Muller-Myhsok, B., Lohse, P. & Brand, S. 2008, "Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD", *Inflammatory bowel diseases,* vol. 14, no. 4, pp. 437-445.

Siegmund, B. & Zeitz, M. 2011, "Innate and adaptive immunity in inflammatory bowel disease", *World journal of gastroenterology : WJG,* vol. 17, no. 27, pp. 3178-3183.

Sipponen, T., Savilahti, E., Kolho, K.L., Nuutinen, H., Turunen, U. & Farkkila, M. 2008, "Crohn's disease activity assessed by fecal calprotectin and lactoferrin: Correlation with Crohn's disease activity index and endoscopic findings", *Inflamm Bowel Dis,* vol. 14, no. 1, pp. 40-6.

Smythies, L.E., Sellers, M., Clements, R.H., Mostel-ler-Barnum, M., Meng, G., Benjamin, W.H., Orenstein, J.M. & Smith, P.D. 2005, "Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity", *The Journal of clinical investigation,* vol. 115, no. 1, pp. 66-75.

Sporn, M.B. & Roberts, A.B. 1992, "Transforming growth factor-beta: recent progress and new challenges", *The Journal of cell biology,* vol. 119, no. 5, pp. 1017-1021.

Starnes, T., Robertson, M.J., Sledge, G., Kelich, S., Nakshatri, H., Broxmeyer, H.E. & Hromas, R. 2001, "Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production", *Journal of immunology (Baltimore, Md.,* vol. 167, no. 8, pp. 4137-40.

Steinman, L. 2007, "A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage", *Nature medicine,* vol. 13, no. 2, pp. 139-45.

Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C. & Koga, Y. 1997, "The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction", *Journal of immunology (Baltimore, Md.: 1950),* vol. 159, no. 4, pp. 1739-1745.

Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R. & Belkaid, Y. 2007, "Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid", *The Journal of experimental medicine,* vol. 204, no. 8, pp. 1775-1785.

Tadokoro, C.E., Shakhar, G., Shen, S., Ding, Y., Lino, A.C., Maraver, A., Lafaille, J.J. & Dustin, M.L. 2006, "Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo", *The Journal of experimental medicine,* vol. 203, no. 3, pp. 505-511.

Takahama, Y. 2006, "Journey through the thymus: stromal guides for T-cell development and selection", *Nature reviews.Immunology,* vol. 6, no. 2, pp. 127-135.

Takahashi, F. & Das, K.M. 1985, "Isolation and characterization of a colonic autoantigen specifically recognized by colon tissue-bound immunoglobulin G from idiopathic ulcerative colitis", *The Journal of clinical investigation,* vol. 76, no. 1, pp. 311-318.

Thornton, A.M. & Shevach, E.M. 1998, "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production", *The Journal of experimental medicine,* vol. 188, no. 2, pp. 287-296.

Tiittanen, M., Westerholm-Ormio, M., Verkasalo, M., Savilahti, E. & Vaarala, O. 2008, "Infiltration of forkhead box P3-expressing cells in small intestinal mucosa in coeliac disease but not in type 1 diabetes", *Clin Exp Immunol,* vol. 152, no. 3, pp. 498-507.

Travassos, L.H., Carneiro, L.A., Ramjeet, M., Hussey, S., Kim, Y.G., Magalhaes, J.G., Yuan, L., Soares, F., Chea, E., Le Bourhis, L., Boneca, I.G., Allaoui, A., Jones, N.L., Nunez, G., Girardin, S.E. & Philpott, D.J. 2010, "Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry", *Nature immunology,* vol. 11, no. 1, pp. 55-62.

Triantafillidis, J.K., Merikas, E. & Georgopoulos, F. 2011, "Current and emerging drugs for the treatment of inflammatory bowel disease", *Drug design, development and therapy,* vol. 5, pp. 185-210.

Trifari, S., Kaplan, C.D., Tran, E.H., Crellin, N.K. & Spits, H. 2009, "Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells", *Nature immunology,* vol. 10, no. 8, pp. 864-871.

Troncone, R., Bhatnagar, S., Butzner, D., Cameron, D., Hill, I., Hoffenberg, E., Maki, M., Mendez, V., de Jimenez, M.Z. & European Society for Paediatric Gastroenterology, Hepatology and Nutrition 2004, "Celiac disease and other immunologically mediated disorders of the gastrointestinal tract: Working Group report of the second World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition", *Journal of pediatric gastroenterology and nutrition,* vol. 39 Suppl 2, pp. S601-10.

Tsuda, M., Hosono, A., Yanagibashi, T., Kihara-Fujioka, M., Hachimura, S., Itoh, K., Hirayama, K., Takahashi, K. & Kaminogawa, S. 2010, "Intestinal commensal bacteria promote T cell hyporesponsiveness and down-regulate the serum antibody responses induced by dietary anti-

gen", *Immunology letters,* vol. 132, no. 1-2, pp. 45-52.

Turner, J.R. 2009, "Intestinal mucosal barrier function in health and disease", *Nature reviews.Immunology,* vol. 9, no. 11, pp. 799-809.

Turunen, P., Ashorn, M., Auvinen, A., Iltanen, S., Huhtala, H. & Kolho, K.L. 2009, "Long-term health outcomes in pediatric inflammatory bowel disease: a population-based study", *Inflamm Bowel Dis,* vol. 15, no. 1, pp. 56-62.

Uhlig, H.H., McKenzie, B.S., Hue, S., Thompson, C., Joyce-Shaikh, B., Stepankova, R., Robinson, N., Buonocore, S., Tlaskalova-Hogenova, H., Cua, D.J. & Powrie, F. 2006, "Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology", *Immunity,* vol. 25, no. 2, pp. 309-18.

Vaarala, O. 2008, "Leaking gut in type 1 diabetes", *Curr Opin Gastroenterol,* vol. 24, no. 6, pp. 701-6.

Vaarala, O., Atkinson, M.A. & Neu, J. 2008, "The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity", *Diabetes,* vol. 57, no. 10, pp. 2555-62.

Van der Sluis, M., De Koning, B.A., De Bruijn, A.C., Velcich, A., Meijerink, J.P., Van Goudoever, J.B., Buller, H.A., Dekker, J., Van Seuningen, I., Renes, I.B. & Einerhand, A.W. 2006, "Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection", *Gastroenterology,* vol. 131, no. 1, pp. 117-129.

Verdier, J., Begue, B., Cerf-Bensussan, N. & Ruemmele, F.M. 2011, "Compartmentalized expression of Th1 and Th17 cytokines in pediatric inflammatory bowel diseases", *Inflammatory bowel diseases,* .

Visser, J.T., Lammers, K., Hoogendijk, A., Boer, M.W., Brugman, S., Beijer-Liefers, S., Zandvoort, A., Harmsen, H., Welling, G., Stellaard, F., Bos, N.A., Fasano, A. & Rozing, J. 2010, "Restoration of impaired intestinal barrier function by the hydrolysed casein diet contributes to the prevention of type 1 diabetes in the diabetes-prone BioBreeding rat", *Diabetologia,* vol. 53, no. 12, pp. 2621-2628.

von Roon, A.C., Karamountzos, L., Purkayastha, S., Reese, G.E., Darzi, A.W., Teare, J.P., Paraskeva, P. & Tekkis, P.P. 2007, "Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy", *The American journal of gastroenterology,* vol. 102, no. 4, pp. 803-13.

Vorobjova, T., Uibo, O., Heilman, K., Rago, T., Honkanen, J., Vaarala, O., Tillmann, V., Ojakivi, I. & Uibo, R. 2008, "Increased FOXP3 expression in small-bowel mucosa of children with coeliac disease and type I diabetes mellitus", *Scand J Gastroenterol,* , pp. 1-9.

Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Van Landeghen, M., Buckner, J.H. & Ziegler, S.F. 2003, "Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells", *J Clin Invest,* vol. 112, no. 9, pp. 1437-43.

Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W. & Toes, R.E. 2007, "Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells", *Eur J Immunol,* vol. 37, no. 1, pp. 129-38.

Watts, T., Berti, I., Sapone, A., Gerarduzzi, T., Not, T., Zielke, R. & Fasano, A. 2005, "Role of the intestinal tight junction modulator zonulin in the pathogenesis of type I diabetes in BB diabetic-prone rats", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 8, pp. 2916-2921.

Westerholm-Ormio, M., Garioch, J., Ketola, I. & Savilahti, E. 2002, "Inflammatory cytokines in small intestinal mucosa of patients with potential coeliac disease", *Clin Exp Immunol,* vol. 128, no. 1, pp. 94-101.

Westerholm-Ormio, M., Vaarala, O., Pihkala, P., Ilonen, J. & Savilahti, E. 2003, "Immunologic activity in the small intestinal mucosa of pediatric patients with type 1 diabetes", *Diabetes,* vol. 52, no. 9, pp. 2287-95.

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F.D., Byrne, G., McEuen, M., Proll, S., Appleby, M. & Brunkow, M.E. 2001, "X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy", *Nature genetics,* vol. 27, no. 1, pp. 18-20.

Wolk, K., Kunz, S., Witte, E., Friedrich, M., Asadullah, K. & Sabat, R. 2004, "IL-22 increases the innate immunity of tissues", *Immunity,* vol. 21, no. 2, pp. 241-254.

Wolk, K., Witte, E., Hoffmann, U., Doecke, W.D., Endesfelder, S., Asadullah, K., Sterry, W., Volk, H.D., Wittig, B.M. & Sabat, R. 2007, "IL-22 induces lipopolysaccharide-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease", *Journal of immunology (Baltimore, Md.: 1950),* vol. 178, no. 9, pp. 5973-5981.

Wolters, V.M. & Wijmenga, C. 2008, "Genetic background of celiac disease and its clinical im-

plications", *The American Journal of Gastroenterology,* vol. 103, no. 1, pp. 190-195.

Xie, M.H., Aggarwal, S., Ho, W.H., Foster, J., Zhang, Z., Stinson, J., Wood, W.I., Goddard, A.D. & Gurney, A.L. 2000, "Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R", *The Journal of biological chemistry,* vol. 275, no. 40, pp. 31335-31339.

Xu, L., Kitani, A., Fuss, I. & Strober, W. 2007, "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,* vol. 178, no. 11, pp. 6725-9.

Yamaguchi, T., Wing, J.B. & Sakaguchi, S. 2011, "Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions", *Seminars in immunology,* vol. 23, no. 6, pp. 424-430.

Yao, Z., Painter, S.L., Fanslow, W.C., Ulrich, D., Macduff, B.M., Spriggs, M.K. & Armitage, R.J. 1995, "Human IL-17: a novel cytokine derived from T cells", *Journal of immunology (Baltimore, Md.,* vol. 155, no. 12, pp. 5483-6.

Ye, J., Su, X., Hsueh, E.C., Zhang, Y., Koenig, J.M., Hoft, D.F. & Peng, G. 2011, "Human tumor-infiltrating Th17 cells have the capacity to differentiate into IFN-gamma+ and FOXP3+ T cells with potent suppressive function", *European journal of immunology,* vol. 41, no. 4, pp. 936-951.

Yen, D., Cheung, J., Scheerens, H., Poulet, F., McClanahan, T., McKenzie, B., Kleinschek, M.A., Owyang, A., Mattson, J., Blumenschein, W., Murphy, E., Sathe, M., Cua, D.J., Kastelein, R.A. & Rennick, D. 2006, "IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6", *J Clin Invest,* vol. 116, no. 5, pp. 1310-6.

Yip, L., Woehrle, T., Corriden, R., Hirsh, M., Chen, Y., Inoue, Y., Ferrari, V., Insel, P.A. & Junger, W.G. 2009, "Autocrine regulation of T-cell activation by ATP release and P2X7 receptors", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 23, no. 6, pp. 1685-1693.

Yokosuka, T., Kobayashi, W., Takamatsu, M., Sakata-Sogawa, K., Zeng, H., Hashimoto-Tane, A., Yagita, H., Tokunaga, M. & Saito, T. 2010, "Spatiotemporal basis of CTLA-4 costimulatory molecule-mediated negative regulation of T cell activation", *Immunity,* vol. 33, no. 3, pp. 326-339.

Yu, Q.T., Saruta, M., Avanesyan, A., Fleshner, P.R., Banham, A.H. & Papadakis, K.A. 2007, "Expression and functional characterization of FOXP3+ CD4+ regulatory T cells in ulcerative colitis", *Inflammatory bowel diseases,* vol. 13, no. 2, pp. 191-199.

Zhang, F., Meng, G. & Strober, W. 2008, "Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells", *Nature immunology,* vol. 9, no. 11, pp. 1297-1306.

Zheng, Y. & Rudensky, A.Y. 2007, "Foxp3 in control of the regulatory T cell lineage", *Nature immunology,* vol. 8, no. 5, pp. 457-462.

Zhou, L., Ivanov, I.I., Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J. & Littman, D.R. 2007, "IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways", *Nature immunology,* vol. 8, no. 9, pp. 967-974.

Zhou, L., Lopes, J.E., Chong, M.M., II, I., Min, R., Victora, G.D., Shen, Y., Du, J., Rubtsov, Y.P., Rudensky, A.Y., Ziegler, S.F. & Littman, D.R. 2008, "TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function", *Nature,* vol. 453, no. 7192, pp. 236-40.

Zhou, X., Bailey-Bucktrout, S.L., Jeker, L.T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W. & Bluestone, J.A. 2009, "Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo", *Nature immunology,* vol. 10, no. 9, pp. 1000-1007.