Immune response of the small intestinal mucosa in children with celiac disease

- Impact of two environmental factors, resident microbiota and oats

Veronika Sjöberg

Department of Clinical Microbiology, Immunology
Department of Clinical Sciences, Pediatrics
Umeå 2013
To my family
Table of Contents

Abstract iii
Populärvetenskaplig sammanfattning på svenska v
Abbreviations list viii
List of papers in the thesis ix

1  Introduction 1
   1.1 General overview of the immune system 1
   1.2 Lymphocytes 2
   1.2.1 B-lymphocytes 2
   1.2.2 T-lymphocytes 2
      1.2.2.1 Extrathymic T-cell maturation 4
   1.2.3 T-helper cells 5
   1.2.4 Regulatory T-cells 6
   1.2.5 Natural killer T-cells 7
   1.2.6 γδT-cells 7
   1.2.7 Cytotoxic T-cells 8
   1.3 Lymphocytes of the innate immune system 9
      1.3.1 NK-cells 9
      1.3.2 Innate lymphoid cells 11
   1.4 Antigen presenting cells 11
   1.5 Cytokines and chemokines 12
   1.6 The human intestine 16
      1.6.1 Mucosal immunology 16
      1.6.2 Intraepithelial lymphocytes 18
      1.6.3 Lamina propria Lymphocytes 19
      1.6.4 Intestinal epithelial cells 19
      1.6.5 The human gut microbiota 20
   1.7 Celiac disease 22
      1.7.1 Clinical features 22
      1.7.2 Diagnosis 23
      1.7.3 Genetic predisposition 24
      1.7.4 Environmental factors 25
         1.7.4.1 The Swedish epidemic of celiac disease 26
      1.7.5 Gluten and the immune response 27
      1.7.6 Treatment 28
   2  Aims 31
   3  Clinical material and methods 32
      3.1 Patient characteristics 32
      3.2 Biopsies 32
      3.3 Cell isolation 32
Abstract

Background. Much remains to be learned about the immune status of the human intestinal mucosa, especially how tolerance to food antigens is established and maintained, and how the gut microbiota affects this as well as other aspects of the local immune system. Celiac disease (CD) is an inflammatory small-bowel enteropathy caused by permanent intolerance to dietary gliadin in wheat gluten and related prolamines in barley and rye, collectively often referred to as gluten. Whether CD patients tolerate oats is still under dispute. Prerequisites for developing CD are that the individual carries the MHC class II alleles for HLA-DQ2 and/or HLA-DQ8 and is exposed to dietary gluten. However, most individuals fulfilling these criteria do not develop CD. Additional genetic and environmental risk factors must therefore also contribute. Dysbiosis in the resident microbiota could be one risk factor. In CD patients, ingestion of gluten provokes a lesion in the small intestine characterized by various degrees of villous atrophy and crypt hyperplasia and infiltration of immune cells, particularly T cells, into the epithelium, so called intraepithelial lymphocytes (IELs). In active CD there is production of the pro-inflammatory cytokine interferon (IFN)-γ and the down-regulatory cytokine interleukin (IL)-10 by T cells. There is also production of autoantibodies. Presently, the only treatment for CD is a lifelong, strict gluten-free diet (GFD).

Aim. To delineate the pathology of CD from an immunological perspective with special emphasis on the role of T cells in exacerbation and down-regulation of the intestinal inflammation; investigate the possible influence of CD-associated bacteria on T cell activity in the mucosa; and determine whether oats in the diet elicits an inflammatory response in the intestinal mucosa of CD patients. The long-term goal of the project, of which this thesis is one part, is to identify risk factors that can be avoided to reduce the risk of developing CD and to understand the immunological mechanisms of CD to identify a possibility to cure.

Methods. In this translational study, we used small intestinal biopsies and immune and epithelial cell subsets isolated from the biopsies to determine changes in mRNA and protein expression of several markers for immune- and epithelial cell function in inflammation as well as extrathymic T cell maturation (ETCM). Biopsies were collected from children with active, newly diagnosed untreated CD (active CD), symptom-free CD patients on a GFD (treated CD), and controls with no known food intolerances. Biopsies from CD patients enrolled in a double-blinded randomized oats intervention study were also analyzed. In this case, a first biopsy was collected when the patient presented with active CD and a second biopsy was collected after one year on a GFD with or without oats.

Results. The major findings were: First, indicators of ETCM, i.e., the RAG1 enzyme required for recombination of the T cell receptor genes and the surrogate α-chain in the immature T cell receptor preTα, were both
expressed at lower levels in CD patients compared to controls. In addition, IELs expressing RAG1 were less abundant in CD patients compared to controls. The levels of these two indicators stayed low in treated CD patients, suggesting that impaired capacity of ETCM is an inherent feature of CD. Second, IL-17A, a cytokine involved in both inflammation and anti-bacterial responses, and IFN-γ were both increased in active CD, but normalized in treated CD. The major cellular source was CD8+IELs. Furthermore, ex vivo challenge of biopsies from treated CD patients with gluten and with CD-associated bacteria induced an IL-17A response. Interestingly, the CD-associated bacteria also influenced the magnitude of the IL-17A response to gluten. Third, we investigated the effect of dietary oats on local immune status in the intestinal mucosa by comparing CD patients receiving GFD with and without oats. A total of 22 different mRNAs for immunity effector molecules and tight junction proteins were analyzed. We found that expression of two down-regulatory cytokines, two activating NK-receptors and the tight-junction protein claudin-4 normalized in virtually all patients on a standard GFD while they did not normalize in several patients on a GFD with oats. Fourth, we analyzed the expression level of mRNAs for chemokines, cytotoxic effector molecules, NK-receptors and their ligands in IELs and epithelial cells and could show that expression levels of several of these genes follow disease activity, suggesting massive recruitment of immune cells by both cell types accompanied by increased IEL-mediated cytotoxicity in the epithelium of inflamed mucosa.

Conclusions. The etiology of CD is still unknown. In the present study we have identified three potential risk factors for development of CD: 1) an inherent lower level of extrathymic T cell maturation in the small intestinal mucosa than in controls. This could lead to impaired secondary rearrangement of intestinal T cells which in turn could lead to decreased generation of regulatory T cells (Tregs) and thus less capacity to tolerate gluten and adapt to the local milieu in the mucosa. 2) Dysbiosis of the resident microbiota with CD associated bacteria that increase antimicrobial responses by inducing IL-17A production and also influence IL-17A responses to gluten thereby promoting local inflammation and immune cell infiltration. 3) Dietary oats, which may provoke a local immune response in a sub-population of CD patients. These patients should probably avoid oats in their GFD. Larger studies are needed.
Celiaki är en livslång inflammatorisk tarmsjukdom som kan debutera när som helst i livet. Sjukdomen orsakas av en intolerans till vissa glutenproteiner i vete, och andra besläktade proteiner i råg och korn. Intag av glutenthaligt kost, hos patienter med sjukdomen, ger upphov till en inflammation i tunntarmens slemhinna. Långvarig inflammation i slemhinna leder till nedbrytning av tarmvävnad, villi, samt ökat antal, så kallade intraepiteliala lymfocyter (IEL). Inflammationen i tarmvävnaden försvinner först då gluten tas bort från kosten. Idag är en livslång strikt glutenfri diet (GFD) den enda behandling som finns för celiakipatienter, d.v.s. en kost fri från vete, råg och korn. Om havre kan ingå i GFD hos alla patienter med celiaki är oklart och en av de frågor som studerades i denna avhandling.

Vad som orsakar att en individ drabbas av celiaki är ännu okänt. Det finns vissa krav som måste uppfyllas; Personen måste vara bärare av de gener som ger upphov till endera av transplantationsantigenerna HLA-DQ2 eller HLA-DQ8. Att exponeras för gluten i födan är en nödvändig omgivningsfaktor för att insjukna i celiaki. De personer som uppfyller dessa två krav är betydligt fler än de som drabbas av sjukdomen–alltså måste det finnas fler ärtfria faktorer eller omgivningsfaktorer faktorer som har betydelse för om man utvecklar sjukdomen. Föreslagna omgivningsfaktorer/miljöfaktorer är bl.a. infektioner i barnåren och en obalanserad tarmflora.


Syftet med denna avhandling var att dels identifiera några möjliga riskfaktorer för celiaki, samt förstå de immunologiska mekanismer som pågår i epitelet vid uppmosten av sjukdomen.

Den viktigaste funktionen för immunsystemet i tunntarmen är att förmedla en tolerans mot främmande ämnen, antigener, i mat och normal bakteriefloran och samtidigt reagera snabbt och skyddande mot sjukdomsframkallande bakterier och virus. T-cell har en T-cells receptor (TCR) som den känner igen främmande ämnen med. Varje T-cell känner endast igen ett antigen, d.v.s. är specifik för ett visst antigen. Denna TCR får
T-cellen när den bildas men i tarmen kan den förändras med hjälp av ett enzym, recombination activating gene-1 (RAG-1), beroende på vilka antigener som finns i miljön där T-cellen befinner sig. I artikel I såg vi att barn med celiaki, både behandlade som inte hade inflammation i tarmen och nydiagnosterade patienter med aktiv tarmsjukdom, hade ett lägre uttryck av enzymet RAG-1 i sina T-celler i tunntarmen. Detta medför att T-cellerna i tunntarmen hos barn med celiaki inte kan förändras till den rådande miljön, vilket kan vara en bidragande orsak till att de inte tolererar gluten. Eftersom fenomenet var oberoende av om sjukdomen var aktiv eller inte verkar det vara en medfödd riskfaktor för celiaki.

Det är sedan länge känt att IELs hos celiakipatienter producerar förhöjda halter av cytokinerna, IFN-γ och IL-10. I två av studierna undersökte vi hur immunförsvaret reagerar när patienter med celiaki åter slut. I artikel II visade vi att cytokinet IL-17A var överuttryckt i tarmen på celiakipatienter när deras sjukdom är aktiv. IL-17A har sedan tidigare visats vara överuttryckt i andra inflammatoriska sjukdomar och även i vissa autoimunnas sjukdomar. Vi visade också att det framförallt var cytokinerna, IFN-γ och IL-10, som stod för den mesta utsöndringen av IL-17A. I artikel IV undersökte vi hur ett stort antal cytokiner, kemokiner och cytokiniska effektormolekyler och receptorer som startar cytokiniska reaktioner, s.k. NK-receptorer, uttrycks i de två celltyperna som bygger upp epitelen i tunntarmen nämligen, T-lymfocyter och epitceller. Vi jämförde uttrycket hos barn med celiaki och kontrollpatienter. Resultatet visade att flera av dessa markörer var överuttryckta i tarmen hos celiakipatienter. Det sammantagna uttrycksmönsteret och skillnaderna mot kontrollpatienterna visade att i aktiv celiaki så utsöndras cytokiner/kemokiner vilket resulterar i ett stort antal immunceller rekryteras till tarmen och flera av dessa celler är cytokiniska, som kan medverka i nedbrytningen av slimmhinnan.

I artikel II och III undersökte vi även hur immunförsvaret i tarmen på celiakipatienter, reagerar på de två miljöfaktorerna, tarmflora och havre. I artikel II gjorde vi provokationer av biopsier från behandlade celiakipatienter med gluten och tarmbakterier som odlats fram från celiakipatienters tarmflora. Det visade sig att uttrycket av cytokinet IL-17A påverkas av dels gluten men även av tarmbakterierna. Detta innebär att en möjlig orsak till den höga nivån av IL-17A i celiaki kan vara en obalanserad tarmflora, dysbios och att dysbios i tarmfloran skulle kunna vara en riskfaktor för celiaki. I artikel IV undersökte vi immunförsvaret hos barn som ätit GFD med eller utan havre. Resultat visade att barn som ätit havre tillsammans med sin GFD hade inte normaliserats i sin tarm vilket däremot barnen som ätit vanlig GFD hade gjort. Många av celiakipatienterna som ätit havre hade förhöjda nivåer av de molekyler som T-cellerna använder när de dödar andra celler, d.v.s. utövar cytotoxicitet, men även höga nivåer av cytokiner som T-cellerna utsöndrar när de stänger av immunreaktioner. Detta innebär att en del av celiakipatienterna fortfarande har en inflammation i tarmen även efter att de ätit GFD som inte tål havre.
Sammanfattningsvis har vi visat tre olika potentiella riskfaktorer för celiaki. Den första är att de inte verkar kunna anpassa sina T-celler till den rådande miljön i tarmen, d.v.s. de har låg grad av extratymisk T-cells utmognad. Den andra är att en obalans i tarmfloran ger ett förhöjt uttryck av IL-17A som kan underblåsa inflammationen och öka risken att individen utvecklar celiaki. IL-17A kan i sin tur rekrytera nya immunceller som bryter ned tarmsemhinnan. De tredje är att havre påverkar det immunologiska svaret negativt och att ett flertal av celiakipatienterna inte tål havre.
## Abbreviations list

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>CD</td>
<td>Celiac disease</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CTLs</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EMA</td>
<td>Endomysium</td>
</tr>
<tr>
<td>ETCM</td>
<td>Extrathymic T-cells maturation</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FAE</td>
<td>Follicle-associated epithelium</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GFD</td>
<td>Gluten-free diet</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3 dioxygenase</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iTreg</td>
<td>Induced T regulatory cells</td>
</tr>
<tr>
<td>LI</td>
<td>Large intestine</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LPL</td>
<td>Lamina propria lymphocytes</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cells</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural T regulatory cells</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>SFB</td>
<td>Segmented filamentous bacteria</td>
</tr>
<tr>
<td>SI</td>
<td>Small intestine</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper cells</td>
</tr>
<tr>
<td>Tc</td>
<td>T cytotoxic cells</td>
</tr>
<tr>
<td>tTG</td>
<td>Tissue transglutaminase 2</td>
</tr>
<tr>
<td>qRT</td>
<td>PCR-Real-time Quantitative Reverse Transcriptase-PCR</td>
</tr>
<tr>
<td>ULBPs</td>
<td>UL16-binding proteins</td>
</tr>
</tbody>
</table>
List of papers in the thesis

I.

Anna Bas, Göte Forsberg, Veronika Sjöberg, Sten Hammarström, Olle Hernell, Marie-Louise Hammarström. Aberrant extrathymic T cell receptor gene rearrangement in the small intestinal mucosa: a risk factor for coeliac disease? *Gut* 2009, **58:**189-195

II.

Veronika Sjöberg, Olof Sandström, Maria Hedberg, Sten Hammarström, Olle Hernell, Marie-Louise Hammarström. Intestinal T-cell responses in celiac disease – impact of celiac disease associated bacteria. *PloS ONE* 2013, **8:** e53414

III.


IV.


* The authors contribute equally to this work
* Shared senior authorship

The published papers are reprinted with permission from the relevant publishers.
1 Introduction

1.1 General overview of the immune system

In man, the immune system has evolved to protect the body from potential dangers, and at the same time avoid excessive and/or undesired immune responses and retain self-tolerance. The human body is surrounded by a variety of pathogens, allergens, toxic agents and beneficial agents such as food and a commensal flora that lives in symbiosis and homeostasis with the human body under normal circumstances. A disruption in the balance provokes a response from the immune system to mobilize and eliminate potential dangers and establish immunological memory. The immune system in humans and other mammals is divided into two branches, the innate and adaptive immune systems that are connected to each other in order to protect the host. The innate immune system is the first line of defense. It is unspecific and rapid in its protective response. The main function of the innate immune system is to limit early infections, by preventing microbes from entering the body and limiting their spread in tissues, using both molecular and cellular defense mechanisms. The unspecific defense against external invaders consists of a physical barrier, such as the skin and mucus membranes, with epithelial cells that are sealed and connected by tight junction proteins. The innate immune response also includes a chemical barrier that consists of secreted cytokines, complement proteins, antimicrobial peptides, such as defensins, and enzymes such as lysozyme. Lastly, the innate immune system also consists of a cellular barrier, composed of neutrophils, macrophages, dendritic cells (DCs), and NK-cells, that are unspecific in their recognition. They recognize a number of conserved structures that are present on most pathogens, referred to as pathogen-associated molecular patterns (PAMPs). PAMPs can either be detected by soluble factors that initiate the complement system or by cell-membrane receptors, e.g., Toll-like receptors (TLRs). Interaction of TLRs and PAMPs initiates a response that stimulates the immune cells to produce signal molecules, like the cytokines that recruit cells of the adaptive immune system. Adaptive immunity is present in vertebrates and is, in contrast to innate immunity, specific. It is characterized by antigen specificity, diversity, immunological memory and self-nonself recognition. The adaptive immune system is the second line of defense and the major cellular component is the lymphocytes. Lymphocytes recognize microbes by their antigen-specific receptors—the T-cell receptor (TCR) expressed on T-lymphocytes, and the B-cell receptor (BCR) expressed on B-lymphocytes. Upon activation, T-lymphocytes start to proliferate and differentiate into either effector cells that secrete signal molecules, like chemokines and cytokines, or into memory
cells that remember the antigen and can confer lifelong immunity. Activated B-lymphocytes proliferate and thereafter differentiate into plasma cells that secrete specific antibodies and memory B cells.

1.2 Lymphocytes

Lymphocytes are the main cell type involved in humoral and cell-mediated adaptive immunity. They are derived from lymphoid progenitors in the bone marrow by the process of hematopoiesis. The two main lymphocyte subtypes in the adaptive immune system are T-cells and B-cells. NK-cells constitute the major lymphocyte subset in the innate immune system. Certain T-cell subsets, e.g., NKT cells and γδT-cells can be classified as belonging both to the adaptive and innate immune systems. The different lymphocytes can be distinguished from each other by different functions and surface phenotypes (1, 2).

1.2.1 B-lymphocytes

B-lymphocytes mature from lymphoid precursor cells and maturation takes place in the bone marrow, where the cells undergo rearrangement of the BCR gene fragments thereby generating a mature, antigen-specific BCR expressed on their cell membrane. The BCR is membrane-bound on B-cells, and secreted in fluid or mucosa, as antibodies when the B-cell has matured into a plasma cell. The naive B-cells express BCRs of both IgM and IgD immunoglobulin (Ig) classes on their membranes. Upon antigen activation in the secondary lymphoid tissue, the B-cell will undergo Ig class-switch, which results in production of antibodies of isotypes IgG, IgE and IgA, with the same antigenic specificity. Raising an antibody response to protein and glycoprotein antigens as well as making an Ig class-switch is dependent on collaboration with a T-lymphocyte subtype called the T helper (Th) cell (3).

1.2.2 T-lymphocytes

T-lymphocytes are derived from the same lymphoid precursor cell as B-cells in the bone marrow, but unlike the B-cells, they mature in the thymus. The thymus contains three compartments, the subcellular zone, the cortex and the medulla. Pre-T lymphoid cells are attracted to thymus by the expression of chemokines, e.g., CCL12, CCL25 and CXCL12, by thymic epithelial cells (TEC). The Progenitors in the thymus are exposed to the cytokine interleukin (IL)-7 and the notch ligand DL4, expressed by cortex TECs, which induce
proliferation and differentiation of each thymocyte. These ligands also induce the expression of CD25, the α chain for IL-2 receptor, on the thymocytes (4).

The maturation process in the thymus also involves rearrangements of TCR gene segments, in order to generate either genes for a functional αβ- or γδ-TCR. The TCR is generated by random rearrangement of the four gene segments—variable (V), diversity (D), joining (J) and constant (C)—for the α- and β-chains and the three gene segments—V, J and C—for the γ- and δ-chains. V(D)J recombination of the TCR is generated to produce a receptor specific to a certain target antigen, and to generate a tremendous variety of specificities of the TCR repertoire. V(D)J recombination in the thymus starts with rearrangement of the β-chain, in double negative thymocytes (CD4-CD8-), catalyzed and directed by the two enzymes, recombination activating gene 1 (RAG1) and RAG2. RAG1 and RAG2 are enzymes that initiate the recombination of the V, D and J segments by recognizing and cleaving specific DNA sites flanked by sequences, called recombination signal sequences (RSSs), at the end of each segment. In order to get a TCR repertoire with high diversity, the enzyme terminal deoxynucleotidyl transferase (TdT) adds nucleotides to the junction and thereby further increases the variation of the TCRs. The complete rearranged β-chain is then expressed on the surface of the thymocytes together with a surrogate α-chain, pre-Tα, which are associated with the CD3 subunit that forms a complex, named preTCR (5). The thymocytes with the preTCR start to proliferate and differentiate into double positive (DP) thymocytes (CD4+CD8+) that start to rearrange the α-chain. The recombination of the α-chain is also catalyzed and directed by the two enzymes, RAG1 and RAG2 (6, 7).

During intrathymic maturation, the immature T-cells will undergo two selection processes, one positive selection and one negative selection. The positive selection takes place in the cortex of the thymus. DP immature T-cells that are unable to recognize self-MHC or have low affinity for self-MHC molecules are deleted, due to lack of TCR signaling, “death by neglect”. These positively selected cells are then induced to survive and differentiate into single positive cells (SP), e.g., CD4+CD8- or CD4CD8+. The negative selection takes place in the medulla, an area to which SP thymocytes migrate by chemotaxis, mediated by the chemokines CCL19 and CCL21 produced by medulla TECs. SP cells with high affinity for self-antigen presented by both DC and medulla TEC are deleted. This process contributes to establishing self-tolerance and the production of naturally occurring regulatory T-cells (nTregs) (4). In humans, Hassall’s corpuscles, a microanatomical structure formed by medulla TECs, secrete thymic stromal lymphopoietin (TSLP),
which can further activate and instruct the medullary DCs to induce development of nTreg (2, 8).

The αβ T-cells are further divided by the single expression of the co-receptors, CD4 or CD8, into T-helper cells (Th) (CD4+ T-cells) and cytotoxic T cells (CD8+ T-cells). Generally, the T-helper cells recognize antigen presented on MHC class II molecules and cytotoxic cells recognize antigen presented on MHC class I molecules.

After the selection processes in the thymus, the mature, naïve T-cells are transported into the blood and lymphatic systems to reside in the secondary lymphoid organs—lymph nodes, spleen and tonsils—where antigen-driven activation of the T-cells occurs. The T-cells require two different signals in order to be activated and to expand into effector cells. The first signal is generated by interaction of the TCR-CD3 complex with an MHC molecule, presenting an antigenic peptide to which the T-cell is specific. The second signal is an antigen-nonspecific co-stimulatory signal provided by the interaction of the glycoproteins CD28, CTLA-4 or CD40 ligand on T-cells and B7-1, B7-2 or CD40 on antigen-presenting cells (APCs). Signalling through CD28 delivers a positive stimulatory signal to the T-cells whereas signaling through CTLA-4 delivers an inhibitory signal and down-regulation of the action of the T-cell. The lack of a second signal promotes inability to proliferate and results in clonal anergy or apoptosis of the T-cell. These two signals trigger the T-cell to induce the production and expression of IL-2 and its receptor. IL-2 binds to the IL-2 receptor in an autocrine fashion, leading to proliferation and clonal expansion of T-cells with identical specificity. Activated T helper cells can differentiate into different Th subclasses, depending of the local environment. Some of the activated T-cells will remain in the lymph nodes as memory cells.

1.2.2.1 Extrathymic T-cell maturation

Most of the T-cell maturation takes place in the thymus, where the TCRs are rearranged and the autoreactive T-cells are eliminated. In humans, the thymus regresses at puberty while T-cell maturation seems to be ongoing throughout life. Instead of the thymus, other sites for T-cells maturation in humans has been suggested, like the small intestine and the liver (9, 10) and recently also the nasopharyngeal tonsil (11). Evidence of extrathymic T-cells maturation (ETCM) was first discovered in thymectomized mice, where T-cells were observed in the epithelium of the gut and in the liver. In mice, the extrathymically developed T-cells are phenotypically different from the thymus-derived T-cells, in that it has a higher frequency of double positive
(CD4^+CD8^+) and double negative (CD4^-CD8^-) αβ T-cells, as well as γδ T-cells. Many of the ETCM-derived cells are also positive for the homodimer, CD8αα (12). Other valuable evidence of ETCM is the simultaneous expression of the two enzymes involved in the recombination of the TCR, RAG-1 and RAG-2, and preTα (9). The human-derived RAG1 protein has three different 5’ untranslated region exons that generate four different splice forms, RAG1 1A/2, 1B/2, 1A/B/2 and 1C/2. Rag1 1C/2 and 1B/2 are expressed in the thymus while 1A/2, 1A/B/2 and 1B/2 are expressed exclusively in the small intestine, by both thymocyte-like cells (CD2^-CD7^+CD3^-) and mature T-cells (CD3^+), in the epithelium and lamina propria. The preTα, which are associated with the TCR β-chain during rearrangement of the α-chain, are also expressed at high levels in the small intestine by thymocyte-like cells CD2^-CD7^+CD3^- and other T-cells (CD3^+). These findings suggest that both editing of TCR in mature cells and TCR rearrangement of immature cells are occurring preferentially in the intestinal epithelium of man (13). The most possible role for ETCM in the intestine is in adaptation of the TCR to the local antigen milieu, where a tremendous variety of antigens—food, commensal flora and also pathogens—are introduced, throughout life.

1.2.3 T-helper cells

Th cells are a subpopulation of effector cells that express the co-receptor CD4 and bind to extracellular antigens presented on MHC class II molecules. Th cells have multiple immune functions, like secretion of cytokines to mobilize immune cells to active sites, and activation of B- and T-cells. The activated Th cells can further be polarized into different subclasses, depending on the cytokine milieu at the present site in which they are activated. At least four subclasses of Th cells are described—Th1, Th2, Th9 and Th17 (14, 15) (Figure 1). All of them secrete different cytokines to promote different biological functions. Th1 cells are involved in cell-mediated immunity by activation of cytotoxic T cells (CTLs) and secretion of the cytokine IFN-γ that activates macrophages to eliminate intracellular microbes, stimulate CTLs and NK-cells to a higher level of cytotoxic activity and also induce inflammation that can cause tissue damage (14, 16, 17). Th2 cells are involved in allergic reactions; in sustaining help to B-cells for activation and production of antibodies; in particular, in switching to the IgE isotype; and in clearing extracellular parasitic infections (14, 16, 17). Th2 cells produce the cytokines IL-4, IL-5, and IL-13. The cytokine IL-4 also inhibits Th1 cells while the cytokine IFN-γ inhibits Th2 cells (16-18). Th9 cells are involved in allergic inflammation as well as promotion of intestinal inflammation. These cells produce the cytokines IL-9, IL-21 and IL-10 (15).
Th17 cells were first described in 2006 as a new subset of Th cells, distinct from the Th cells Th1 and Th2, which were discovered first. Th17 cells produce the pro-inflammatory cytokines IL-17A, IL-17F, IL-22 and IL-26, and play a crucial role in immune responses. Increased amounts of the cytokine IL-17A are seen in many autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) and inflammatory bowel diseases like, Crohn’s disease. Th17 cells are also implicated in host defense against bacterial and fungal infections (17, 19, 20). Th1 and Th17 cells are considered inflammatory mediators that induce inflammation in the host, at the same time as the Treg cells actively work to suppress immune responses against self and foreign antigens as well as to maintain homeostasis (17).

1.2.4 Regulatory T-cells

Tregs are a subpopulation of T-cells that have regulatory and inhibitory properties (Figure 1). They can be divided into several different types of regulatory cells, CD25⁺Foxp3⁺ natural Tregs (nTreg), CD4⁺CD25⁺Foxp3⁺ induced or adaptive Tregs (iTreg), IL-10-dependent Tr1 cells Tregs, TGF-β₁ dependent Th3 Tregs and CD8⁺ Tregs (21). The CD25⁺Foxp3⁺ nTreg are developed in the thymus during the T-cell maturation process and are believed to be specific for autoantigens and thereby preventive against autoimmunity. The CD4⁺CD25⁺Foxp3⁺ iTregs are generated in the periphery, in secondary lymphoid tissues. The main functions are to suppress immune responses from external antigens in the microbiota and food, thereby controlling potential inflammation (22, 23). Tr1 Tregs are distinct from conventional Th1 and Th2 cells, by their production of IL-10 as well as the suppressive function to Th1-mediated autoimmune disease. Th3 Tregs are located in the peripheral tissue and produce the cytokine TGF-β₁ upon triggering by orally administered antigens. TGF-β₁ also has regulatory properties via suppression of autoimmune and inflammatory responses, as well as providing help to CD4⁺CD25⁺Foxp3⁺ iTregs for suppression of Th1 and Th2 cells and for synergies with IL-6, to induce Th17 cells. Th3 Tregs can also induce Foxp3 expression. CD8⁺ Tregs have been found in inflammatory bowel disease (IBD) and are implicated in both oral tolerance and the suppression of Th1 and Th17 cells (21). The mechanism by which Tregs suppress the immune response are not yet fully resolved (24).
CD4+ T-cells differentiate into several different subsets depending on the cytokine milieu at the present site in which they are activated. Each subset produces characteristic cytokine profiles to promote different biological functions.

1.2.5 **Natural killer T-cells**

Natural killer T-cells (NKT) are a distinct subpopulation of T-cells that express both the αβ TCR and the surface antigens, associated with NK-cells. NKT cells can further be classified into two main subsets, type I and type II NKT. The human type I NKT expresses an invariant α-chain (Vα24Jα18) together with a limited number of β-chains (Vβ11) that recognize glycolipid antigens that are presented to the non-classical MHC class I molecule CD1d, on restricted APCs (25). The human type II NKT expresses a more diverse TCR repertoire than the type I NKT. The type II NKT is, as well as the type I, CD1 restricted. Upon activation, both types produce a number of cytokines, like IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-21 and TNF-α, that are involved in many different immune responses, such as response to infections and autoimmune diseases (26).

1.2.6 **γδT-cells**

γδT-cells are a subpopulation of T-cells that are defined by the γδ-TCR. Their TCRs are obtained by random V(D)J rearrangement of the γ- and δ-chains
during their maturation in the thymus and represent 1-5% of the circulating T-cells. Human γδT-cells can further be divided into two subsets, Vδ1+ and Vδ2+ cells. The Vδ1+ T-cells reside mostly at mucosal surfaces and epithelia. Vδ2-T cells often utilize the Vγ9 gene segment and exist in the blood and lymphatic system (27). The γδT-cells have been implicated in innate immune responses that control intracellular infections. They are also suggested to be the first line of defense against pathogens. The mechanisms these cells use to defend the host comprises cytotoxic activity, by expression of granzyme A, granzyme B; Fas ligand (FasL); as well as expression of cytokine/chemokines such as IFN-γ, IL-2 and IL-10 (25, 28).

Unlike the somatically rearranged antigen receptors on αβ T-cells, γδ-cells do not require presentation of antigen on restricted MHC class I and II molecules. The γδIELs exert cytotoxic killing by binding to the MHC class Ib molecules MICA and MICB on stressed epithelial cells. Some γδT-cells recognize phospholipids presented by CD1, another MHC class Ib molecule. (29).

1.2.7 Cytotoxic T-cells

Cytotoxic T-cells (CTLs) are a subpopulation of effector cells that express the co-receptor CD8 and recognize intracellular antigen presented on MHC class I molecules. CTLs play a crucial role in the defense against intracellular pathogens, virus-infected cells and tumor cells. The elimination of infected target cells by CTLs is mediated by two different pathways, granzyme/perforin-mediated apoptosis and FasL/Fas-mediated apoptosis (18). Similarly as Th cells, activated CD8+ T-cells can be polarized into different types of cells, Tc1, Tc2 and Tc17, depending on the cytokine milieu at the present site and the cytokines they produce themselves (30) (Figure 2). Tc1 cells produce the cytokine IFN-γ and Tc2 cells produce IL-4 and IL-5 (31). Recognition of an antigen presented on an MHC class I molecule triggers the Tc cell to differentiate into a CTL effector cell.

Tc17 cells are found in intestinal mucosa and the lungs of both humans and mice, and are shown to be expanded during infections, cancer and several autoimmune diseases, like psoriasis, and, as shown in this thesis, in celiac disease (CD) (32, 33) (Paper 2). These cells have been shown to be highly plastic and co-express the Th17 cytokine IL-17A and the Tc1 signature cytokines IFN-γ and TNF-α (33, 34). These cells have also been shown to make a transition from Tc17 to Tc1 cells by the production of first IL-17A and then IFN-γ and TNF-α (34). There is also evidence that these cells can escape Treg activity since the proliferation of Tc17 cells is not down-regulated by
expression of TGF-β1 (33). Tc17 cells have low cytolytic capability and a lower expression of granzyme and perforin than CTLs (32-34).

Figure 2 CD8+ T-cells can be polarized into different types of cells, Tc1, Tc2 and Tc17. Each subset produces different cytokine profiles to promote different biological functions. Tc1 cells can further be differentiated into cytotoxic T cells upon activation and antigen triggering.

1.3 Lymphocytes of the innate immune system

1.3.1 NK-cells

Natural killer (NK) cells constitute a subpopulation of lymphocytes that belong to the innate immune system. They play an important role in host defense by killing some types of malign and virus-infected target cells, mainly those recognized due to low levels or absence of MHC class I molecules on the cell surface. As is the case for CTLs, these cells mediate apoptosis of the infected cells by two different pathways: 1) by granzyme/perforin or FasL/Fas, which can be triggered by antibody dependent cellular cytotoxicity (ADCC) or 2) by activating NK-receptors (35). Unlike T- and B-cells, NK-cells lack expression of specific germline rearranged antigen receptors and uses instead the repertoire of different inhibitory and activating receptors (36, 37) (Table 1). Activating NK-receptor can also be expressed as co-stimulatory receptors on both αβ T-cells and γδ T-cells. Under normal circumstances in humans, NK cells express inhibitory receptors, like killer immunoglobulin-like receptors (KIR) and CD94/NKG2A, that inhibit an activating signal and thereby prevent the NK-cells from killing the target cell (38). During an infection, the NK-cells will bind to target cells and up-regulate the expression of activating receptors; if they have stronger signaling than the inhibitory receptors, the NK-cells will enforce the target cell to undergo apoptosis. The NKG2 family belongs to C-type lectins that are expressed on NK-cells and deliver activating and
inhibitory signals. NKG2/A/C/E receptors form a heterodimer together with CD94 and deliver activating signal by binding to the non-classic HLA-E molecule on target cells. Another C-type lectin, NKG2D, forms a homodimer that recognizes MICA/B and glycoprotein UL16-binding proteins (ULBPs) on target cells and delivers cytotoxicity activating signals to the receptor-bearing cell (35, 36).

NK-cells are a heterogeneous population of lymphocytes that in humans express CD56 and represent 15% of the peripheral blood lymphocytes. CD56+ NK-cells can further be divided into either CD56^{dim} or CD56^{bright} cells. Both cell types function differently in their disposal of infected cells. CD56^{dim} cells lyse infected cells spontaneously and lack the ability to produce chemokines and cytokines in order to activate cells. CD56^{bright} cells have the opposite function, with the ability to produce chemokines and cytokines but lack the ability to lyse infected cells spontaneously (36).

Table 1 NK-receptors and their ligands

<table>
<thead>
<tr>
<th>Receptor family</th>
<th>Receptor</th>
<th>Synonym</th>
<th>Function</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-type lectin</td>
<td>KLRB1</td>
<td>CD161</td>
<td>Inhibitory</td>
<td>αGal(1-3)Gal-/N-acetyllactosamine</td>
</tr>
<tr>
<td></td>
<td>KLRC1</td>
<td>NKG2A</td>
<td>Inhibitory</td>
<td>HLA-E</td>
</tr>
<tr>
<td></td>
<td>KLRC2</td>
<td>NKG2C</td>
<td>Activating</td>
<td>HLA-E</td>
</tr>
<tr>
<td></td>
<td>KLRC3</td>
<td>NKG2E</td>
<td>Activating</td>
<td>HLA-E</td>
</tr>
<tr>
<td></td>
<td>KLRD1</td>
<td>CD94</td>
<td>Activating</td>
<td>HLA-E</td>
</tr>
<tr>
<td></td>
<td>KLRK1</td>
<td>NKG2D</td>
<td>Activating</td>
<td>MICA/B, ULBPs</td>
</tr>
<tr>
<td>KIR -Killer Cell Immunoglobulin-Like Receptor</td>
<td>KIR2DL1</td>
<td>Inhibitory</td>
<td>HLA-Cw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DL2</td>
<td>Inhibitory</td>
<td>HLA-Cw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DL3</td>
<td>Inhibitory</td>
<td>HLA-Cw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DL4</td>
<td>Activating</td>
<td>HLA-G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DL5</td>
<td>Inhibitory</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DS1</td>
<td>Activating</td>
<td>HLA-Cw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DS2</td>
<td>Activating</td>
<td>HLA-Cw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DS3</td>
<td>Activating</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DS4</td>
<td>Activating</td>
<td>HLA-Cw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR2DS5</td>
<td>Activating</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR3DL1</td>
<td>Inhibitory</td>
<td>HLA-Bw4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR3DL2</td>
<td>Inhibitory</td>
<td>HLA-A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KIR3DS1</td>
<td>Activating</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>
1.3.2 **Innate lymphoid cells**

Innate lymphoid cells (ILCs) are a recently described heterogeneous population of innate immune cells that are implicated in inflammation of the gut, formation of lymphoid tissues, wound healing and mucosal immunity against pathogens. The ILCs are derived from the same lymphoid progenitor as T- and B-lymphocytes and share some features with lymphoid cells, such as cytokine secretion and dependence of the IL-2 receptor γ chain (IL-2γ). In addition, ILCs lack the rearranged antigen receptor as well as other essential markers of the adaptive immunity. This heterogeneous population of innate cells was recently classified into three different groups, group 1 (ILCs 1), 2 (ILCs 2) and 3 (ILCs 3) (39, 40). ILCs 1 include NK-cells and produce the Th1 signature cytokines, IFN-γ and TNF-α, and express the chemokine receptor CXCR3 and its ligand CCL3. Most of the ILCs 1 possess capacity for granule-mediated cytotoxicity, by production of perforin and granzymes. Some ILCs 1 were shown to be distinct in their characteristics by lacking cytotoxic molecules and the NK markers NKp44, NKp46 and CD56 (40, 41). ILCs 1 cells are also significantly increased in lamina propria of inflamed tissue from patients with Crohn’s disease (39, 41). ILCs 2 cells share some properties with Th2 cells, via production of the signature cytokines IL-4, IL-5, IL-9 and IL-13. These cells can be found in the gut (adult and fetal), lungs and mesenteric lymph nodes. ILCs 3 are similar to Th17 cells in expression of the cytokines IL-17A and IL-22 as well as and the transcriptional marker RORγt. ILC3 cells also lack the cytotoxic effector molecules, perforin and granzymes, and cannot mediate cytolysis of target cells. Most ILC 3 cells reside in the mucosa of the intestine where they mediate a key role in homeostasis and immunity against extracellular bacteria (40, 41).

1.4 **Antigen presenting cells**

One of the hallmarks for adaptive immunity is recognition and response to foreign antigens displayed by MHC molecules on the surface of APCs. MHC molecules can be classified as either MHC class I or MHC class II. MHC class I are expressed on almost all nucleated cells and present endogenous antigens, such as viral and tumor antigens, to Tc cells. MHC class II molecules present exogenous antigen to Th cells and are expressed by professional APCs, which include DCs, monocytes/macrophages and B-cells. The professional APCs migrate from the periphery to secondary tissues to present antigens to T-cells. APCs are also important for activation of T-cells through their interaction with the co-stimulatory molecule B7, for which T-cells have a ligand. DCs are the most potent of the APCs because of the
constitutive high expression of MHC class II molecules on their surface. Both macrophages and B-cells must be activated before they express the MHC class II molecules.

Mucosal DCs are distributed along the small and large intestine and are found in lamina propria (LP), isolated lymphoid follicles, Peyer's patches and mesentric lymph nodes. In mice, two major populations of DCs are found in the mucosa that can be distinguished by the expression of either the chemokine receptor CX3CR1 or the integrin CD103. The mucosal DCs can produce the cytokines IL-10 and TGF-β1 as well as retinoic acid and indoleamine 2,3 dioxygenase (IDO), which promotes the conversion of Tregs and inhibition of Th17 cells. The DCs can also regulate the induction of isotype class-switch to IgA (42).

### 1.5 Cytokines and chemokines

Cytokines are soluble factors that are secreted by immune cells in order to modify their own biological activities or those of nearby cells. They are involved in almost all immune responses in both innate and adaptive immunity as well as the communication between cells of the immune system. Target cells, with a specific membrane receptor, can bind the secreted cytokines, which results in intracellular signaling that alters the gene expression and function of the target cell. The secreted cytokine binds to the receptor with high affinity and mediates immune responses at very low concentrations. Some cytokines are secreted in the circulation and execute their activity to distant cells, in an endocrine fashion. The target cell is often located in a milieu where they are exposed to several different cytokines at the same time that either mediates a synergistic or antagonistic effect. A synergistic effect occurs when two or more cytokines enhance biological activity, and the antagonistic effect occurs when the combined action of the cytokines inhibits the activity, or the cytokines themselves. In addition, the cytokines also have the ability to provoke the target cells to synthesize more cytokines, which signals other nearby cells to produce more or other cytokines. This results in a cascade of new cytokines that affects more cells that can mobilize the immune system. Among the biological activities that are influenced by the cytokine signaling are proliferation, differentiation, activation of effector cells, and development of humoral and cell-mediated immunity. Cytokines can further be classified into several different structural families, the hematopoietic family, the interferon family, the chemokine family and the tumor necrosis factor family, depending on different biological functions. Almost all immune cells can produce cytokines, but among the most common producers are Th cells, DCs and macrophages. As mentioned earlier, T-helper cells can be polarized into different subclasses.
depending on the cytokine milieu, at the site where they are primed when they encounter their nominal antigen for the first time, and these are also divided into several different subclasses depending on their cytokine profile.

During an infection, a cascade of several different cytokines is produced, in order to mobilize the immune system and to eliminate the infectious invader. After the elimination, the expression of the cytokines is down-regulated and returned to normal. In many autoimmune and inflammatory diseases, the secretion of pro-inflammatory cytokines is sometimes constantly enhanced, together with ongoing infiltration of immune cells to the site of inflammation. A long period of inappropriate expression of cytokines from activated immune cells may lead to allergies and chronic inflammation in the tissue, where autoreactive cells fail to shut down their activity (43).

IFN-γ is a pro-inflammatory cytokine, essential for host defense for clearance of intracellular bacteria and viruses. The cytokine is produced by a number of different cell types, such as Th1, CTL, NK-cells, NKT-cells and ILCs 1. IFN-γ plays a key role in bridging the innate and adaptive immune systems and is also involved in many different biological activities. The cytokine influences naïve Th-cell differentiation, causes up-regulation of MHC class I and II molecules in immune and epithelial cells, and regulates leukocyte-endothelial interactions by directing these cells to express chemoattractants and adhesion molecules (44, 45). IFN-γ has also been implicated in several different autoimmune and inflammatory diseases, like RA, Crohn’s disease and CD (46, 47).

IL-17A is a pro-inflammatory cytokine involved in host defense against extracellular bacteria and fungi, by stimulating epithelial cells and fibroblast to express chemokines and inflammatory mediators, which recruit neutrophils to the site of infection. The cytokine was first described in Th cells and these cells are also the dominating cellular source of IL-17A. Later on, other cellular sources of IL-17A were described, such as Tc cells, γδ-cells, NK-cells, NKT-cells (33, 48). IL-17A has also been implicated in the pathogenesis of several autoimmune and inflammatory diseases, like CD, MS, RA, Crohn’s disease and psoriasis (20, 49, 50)(Paper 2).

IL-10 is a cytokine that can be produced by almost all cell types and plays a key role by preventing immune-mediated injury to the host. IL-10 is produced in order to maintain homeostasis in the immune system by inhibiting the production of pro-inflammatory cytokines and chemokines from Th1 and Th17 cells and also down-regulates the expression of MHC class molecules. IL-10 is also a signature cytokine produced by Tregs that promotes the survival of these cells. Under normal circumstances, the expression of IL-10 is low and only up-regulated during inflammation and after triggering by commensal flora or pathogens (51).
TGF-β1 is a multipotent cytokine that is involved in immune regulation, by controlling T cell differentiation, promotion of B-cell class-switch to IgA as well as inflammatory responses and tolerance. In contrast to IL-10, TGF-β1 is constantly expressed in normal tissue and is important for differentiation of Treg and Th17. TGF-β1 is produced as an inactive protein complex that is activated by proteolytic cleavage in the environment (51).

Chemokines are cytokines with chemotactic activity. They are divided into four different subclasses, C, CC, CXC and CX3C, depending of the positions of cysteine residues in the peptide (Table 2). They are involved in recruitment of immune cells and play a critical role in regulation of the immune responses. Some of the chemokines are constitutively expressed in the thymus and lymphoid tissue and function as homing and non-inflammatory chemokines. Others are expressed as inflammatory mediators in recruitment of immune cells to the site of inflammation and infection. Cells with chemokine binding receptors migrate by chemotaxis towards a chemokine concentration gradient. The different subclasses attract different populations of cells. C chemokines attract T-cells towards the thymus, while monocytes, basophils, DCs, macrophages, NK-cells and T-cells migrate by chemotaxis towards CC chemokines. The CXC chemokines attract neutrophils, T-cells and B-cells and the CX3C chemokine is an important adhesion molecule during infiltration of T-cells and NK-cells. Increased expression of chemokines and their receptors will enhance the protective reaction but immune cell infiltration in tissues may also result in tissue injury and is seen in several different immune-related diseases, like IBD, RA, MS (52-54).

In IBD, levels of CXCL9, CXCL10 and CXCL11 mRNA and their receptor CXCR3 are all overexpressed in inflamed tissue, which suggests a migration of T-cells into the intestine (52, 55). The receptors, CXCR3 and CCR6, are both identified in the inflamed intestinal mucosa in IBD and are expressed on Th17 and Th1 cells, which suggest infiltration of T-cell expressing the pro-inflammatory cytokine IL-17A and IFN-γ in inflamed tissue (52, 55).
<table>
<thead>
<tr>
<th>Subclasses</th>
<th>Chemokines</th>
<th>Chemokine receptors</th>
<th>Synonym</th>
<th>Cell attracted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL4/ XCL2</td>
<td>XCR1/ XCR2</td>
<td>Lymphotactin/SCM-1β</td>
<td></td>
<td>T-cells, NK-cells</td>
</tr>
<tr>
<td><strong>CC-Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>CCR1, CCR5</td>
<td>MIP-1α</td>
<td>T-cells, NK-cells, monocytes, macrophages, immature dendritic cells</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>CCR5, CCR8</td>
<td>MIP-1 β</td>
<td>T-cells, monocytes and macrophages, immature dendritic cells</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>CCR1, CCR3, CCR5</td>
<td>Rantes</td>
<td>T-cells, NK-cells, monocytes, macrophages, immature dendritic cells, eosinophils</td>
<td></td>
</tr>
<tr>
<td>CCL8</td>
<td>CCR3</td>
<td>MCP-2</td>
<td>T-cells, Eosinophils, basophils, mast cells</td>
<td></td>
</tr>
<tr>
<td>CCL13</td>
<td>CCR2, CCR3</td>
<td>MCP-4</td>
<td>T-cells, NK-cells, monocytes, Eosinophils, basophils, mast cells, immature dendritic cells</td>
<td></td>
</tr>
<tr>
<td>CCL20</td>
<td>CCR6</td>
<td>MIP-3α/LARC</td>
<td>T-cells, B-cells, immature dendritic cells</td>
<td></td>
</tr>
<tr>
<td>CCL25</td>
<td>CCR9</td>
<td>TECK</td>
<td>Thymocytes, T-cells</td>
<td></td>
</tr>
<tr>
<td><strong>CX-Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL1</td>
<td>CXCR1, CXCR2</td>
<td>GROα</td>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td>CXCL2</td>
<td>CXCR2</td>
<td>GROβ</td>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td>CXCL8</td>
<td>CXCR1, CXCR2</td>
<td>IL-8</td>
<td>Neutrophils, macrophages</td>
<td></td>
</tr>
<tr>
<td>CXCL9</td>
<td>CXCR3</td>
<td>Mig</td>
<td>T-cells, NK-cells</td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td>CXCR3</td>
<td>IP-10</td>
<td>T-cells, NK-cells</td>
<td></td>
</tr>
<tr>
<td>CXCL11</td>
<td>CXCR3, CXCR7</td>
<td>I-TAC</td>
<td>T-cells, NK-cells</td>
<td></td>
</tr>
<tr>
<td>CXCL12</td>
<td>CXCR4, CXCR7</td>
<td>SDF-1</td>
<td>Progenitors cell, hematopoietic cells</td>
<td></td>
</tr>
<tr>
<td>CXCL13</td>
<td>CXCR5</td>
<td>BCA-1</td>
<td>Naïve B-cells, CD4+ T-cells</td>
<td></td>
</tr>
<tr>
<td>CXCL14</td>
<td>Unknown</td>
<td>BRAK</td>
<td>NK-cells, monocytes, immature dendritic cells</td>
<td></td>
</tr>
<tr>
<td>CXCL16</td>
<td>CXCR6</td>
<td>SR-PSOX</td>
<td>Naïve CD8+ T-cells, activated CD4+ T-cells, NKT-cells</td>
<td></td>
</tr>
<tr>
<td><strong>CX3-C Chemokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX3CL1</td>
<td>CX3CR1</td>
<td>Fractalkine</td>
<td>T-cells, monocytes, neutrophils</td>
<td></td>
</tr>
</tbody>
</table>
1.6 The human intestine

The human intestine is part of the gastrointestinal tract and consists of two segments, the small intestine (SI) and the large intestine (LI). The LI is divided into the cecum, appendix, ascending colon, transverse colon, sigmoid colon, rectum and anal canal. The SI is further divided into three anatomic segments, duodenum, jejunum and ileum. The primary function of the small intestine is digestion and absorption of nutrients from food, while the large intestine mainly absorbs water. A cross-section of the anatomic structure of both the small and the large intestines reveals four layers from the lumen and inwards, mucosa, submucosa, muscularis and serosa. The mucosa is the innermost layer towards the lumen and it in turn is composed of the layers of the glandular epithelium, lamina propria and muscularis mucosa from the lumen and inwards. The glandular epithelium forms crypts and villi, i.e., finger-like protrusions composed of a single layer of epithelial cells covering the lamina propria. The villi considerably increase the luminal surface area for absorption of nutrients. The SI has a mucosa rich in villi and with shallow crypts, while the LI has an almost flat mucosa with deep crypts. The muscularis mucosa layer is composed of a thin layer of smooth muscles bound to mucosa and submucosa and is responsible for sequential contraction of the intestine, resulting in peristalsis. The submucosa consists of connective tissue that contains fibroblasts, mast cells and blood- and lymph-vessels. The outermost layer is serosa, i.e., connective tissue covered by mesothelial cells, facing the peritoneal cavity.

1.6.1 Mucosal immunology

The mucosal surface in the human body constitutes the lining of the gastrointestinal, respiratory and urogenital tracts and is the first line of defense for prevention of pathogen entry into bodily tissues from the lumina. The intestinal mucosa separates the external environment in the lumen from the sterile internal environment, by the epithelium, a single, one-cell thick layer containing epithelial cells and IELs. The main mechanical function of the epithelium is physically preventing penetration of pathogens. The apical side of the epithelium is covered by a protective mucus layer, comprised of mucins, soluble glycoproteins produced by goblet cells (56). Beneath the mucus layer is the glycocalax composed of carbohydrate-rich glycoproteins that are released soluble and vesicle-bound from the microvilli of the enterocytes, collectively named the brush border (57). The mucus protects the epithelium physically by preventing bacteria from reaching the epithelium. The mucins also contain carbohydrate moieties that can bind bacteria, and with synchronized movement, by the villi, and constitutive
production of mucins, the mucus layer is refreshed and the entrapped bacteria are cleared from the mucosal surface. The commensal flora can also provide extensive help by occupying binding sites or secreting bacteriocins as inhibitory compounds. The microbial flora in the gastrointestinal tract differs in composition and bacterial species along the intestine, with a much more abundant and probably also more diverse flora in LI.

The mucosal immune system is in close proximity to the epithelium in the gastrointestinal tract and is organized in gut-associated lymphoid tissue (GALT). GALT in the intestine contains immune cells, both organized in solitary and aggregated follicles, as the Peyer’s patches (PP) in the SI and the appendix in the LI, and as diffusely disseminated, unorganized immune cells within the lamina propria, such as lamina propria lymphocytes (LPLs), macrophages and plasma cells, and IELs within in the epithelium. The organized tissues serve as inductive sites where lymphocytes are primed for locally transported antigens from the lumen and the unorganized tissue represents effector sites, to which the primed cells arrive. The follicles consist of a germinal center with B-cells and follicular DCs that is surrounded by a T-cell-rich area. The GALT is separated from the lumen by a follicle-associated epithelium (FAE) that contains M-cells, which are specialized epithelial cells that lack microvilli and play an important role in controlling the delivery of foreign antigens and macromolecules to the follicles (58). M-cells are plastic and form a pocket beneath the cell that creates a site for DCs, macrophages, T-cells and B-cells to encounter and obtain the transported antigens (59, 60). After antigen sampling locally and in the nearest draining lymph node, the APC initiates an immune response both in the follicle and in the draining lymph nodes, where naive T-cells are activated and release chemotactic molecules, such as cytokines and chemokines, and B-cells produce specific neutralizing secretory antibodies of IgA class (60).

An alternative route for sampling of bacterial antigens, independent of M-cells, has been suggested for CD18+ DCs in murine studies. DCs in the lamina propria are able to incorporate and establish contact to adjacent epithelial cells through their own production of tight junction proteins. The incorporated DCs can then face the luminal side and sample bacterial contents and transport the antigen to the underlying lymphoid tissue to induce an immune response (61). The lamina propria resident CX3CR1+ monocytes, which are recruited by the expression of CX3CL1 on epithelial cells, and CD103+ DCs are also found to sense bacterial uptake by the alternative route and transport them over the epithelium (62, 63).
The most demanding and essential task in mucosal immunity is oral tolerance and distinguishing between harmful pathogens and harmless commensal flora and food antigens. Different mechanisms have been suggested for induction and maintenance of tolerance in the intestine. One suggestion is that when the dose of the antigen delivered by M-cells is lower than the amount of toxins and pathogens, then the limited amount can initiate an immune response from DCs to produce IL-10 and TGF-β1 (58). The delivery pathway, via M-cells, via antigen sampling DCs and via enterocytes, is also thought to influence the outcome of active immune protection response versus tolerance.

1.6.2 Intraepithelial lymphocytes

Intestinal intraepithelial lymphocytes (IELs) are located above the basal lamina within the epithelium layer in close proximity to adjacent enterocytes, in both the SI and the colon (9, 57). The main function of IELs is to protect the epithelium from external invaders by eliminating pathogens that cross the epithelial barrier, to maintain epithelial integrity by elimination of infected and stressed epithelial cells and to prevent damage from excessive inflammatory reactions (64). IELs are more abundant in the SI than in the colon, with approximately 20 IELs per 100 intraepithelial cells (IEC) and nine IELs per 100 IEC respectively (9). The different proportions of IELs in the epithelium along the intestine probably reflect the distinct functions for the cells. In the SI, the epithelium is more exposed to food antigens while the colon is more exposed to bacterial antigens. IELs in the SI are one of the most abundant lymphocyte populations in humans and comprise a heterogeneous population of T lymphocytes. Most of them are CD3⁺T-cells that express either αβ⁺- or γδ⁺-TCR and the memory marker CD45RO. A small proportion of the IELs are thymocyte-like lymphocytes that have the marker profile CD1a⁺, CD2⁺CD3⁻ and CD4⁺CD8⁻(9). In addition, a small population of IELs has been demonstrated to be CD3⁺CD7⁺ cells with phenotypic resemblance to NK-cells, and are expressed at lower frequency in CD patients (9, 65). Approximately 60% of the cells in the jejunum are TCRαβ cells and among these the most abundant population is TCRαβ⁺CD8⁻ IELs. Most of them are TCRαβ⁺CD8⁺ cells that express the heterodimer CD8αβ, but a small fraction of the IELs express the CD8αα homodimer, which prevent them from systemic circulation (66). The γδ⁺TCR IELs stands for around 30% of the IELs and most of them are TCR γδ⁺CD4⁺CD8⁻ IELs (9). All IELs, regardless of other cell surface molecules, express the integrin αβ1, αδ/CD103, which forms a heterodimer with β2, that anchors the IELs to epithelial cells by binding to the ligand E-cadherin. In addition, the IELs are also a part of ETCM in the small intestine through
expression of the enzymes RAG-1 and RAG2 as well as the invariant TCR surrogate chain preTα (9, 13).

Both αβ+IELs and γδ+IELs provide cytolytic activity and express genes that are involved in the cytotoxic mediated pathways like, granzyme A, granzyme B, perforin and FasL. (67, 68). IEL can rapidly respond with cytotoxic activity and eliminate dysregulated epithelial cells. Most of the cells also express the cytokines, IFN-γ, IL-17A, and the NK-receptors, which induce either activating or inhibitory signals (47, 67). Furthermore, the IELs express the inhibitory NK receptors NKG2A-CD94 and KLRB1 that mediate down-regulatory signals in the target cells (38, 69). During inflammation, as in CD, expression of the activating NK-receptors NKG2C and NKG2D is increased and can lead to cytolyis, by binding to their ligands on epithelial cells (69-71).

1.6.3 Lamina propria Lymphocytes

The LP is a loose connective tissue lying between the epithelium and muscularis mucosa. It is heavily populated with several types of immune cells, e.g., LPLs, DCs, macrophages B-cells and plasma cells that receive signals from the epithelial cell. LPLs constitute one of the largest cell populations in LP, and most of them have a phenotype of activated/memory cells expressing CD45RO. The majority of LPLs express αβ TCR together with either CD4 or CD8 while the γδ T cells are rare. In addition, the CD8+ LPLs also have cytotoxic capability and express FasL, granzyme B and perforin (68).

1.6.4 Intestinal epithelial cells

The main function of intestinal epithelial cells (IECs) is to digest and absorb nutrients and regulate the secretion of macromolecules. All IECs in the intestine derive from a pluripotent stem cell in the base of the crypts (53). The stem cells differentiate into four different lineages—1) enterocytes; 2) goblet cells and 3) enteroendocrine cells, on the villi and at the crypt surface; and 4) Paneth cells in the crypt base in the small intestine. Enterocytes are the main absorptive cell that control the uptake of antigens and transport IgA over the epithelium to the apical side. Goblet cells and Paneth cells both contribute to innate immunity by secretion of mucin, lysozymes and antimicrobial peptides (60). Recent studies showed that the Paneth cells, in addition, are essential for niche signals to the nearby stem cell during the cell proliferation (72, 73). Enteroendocrine cells secrete neurohumoral factors,
such as substance P. The paracellular space between the cells is sealed by tight junction proteins, such as occludin, claudins, and zonula occludens, which also regulate the transport of antigens over the epithelial barrier (74). Cytokines can further regulate the paracellular space between the IECs. IL-10 and TGF-β1 can enhance the tight junction and diminish paracellular transport while inflammatory cytokines such as IFN-γ, TNF-α degrade the tight junction and enhance permeability. IECs are the first lining toward the external environment in the lumen and are responsible for barrier function and regulation. The IECs are in close contact and interact with immune cells in the lamina propria as well as IELs via soluble mediators. The IECs are also capable of presenting antigens to the surrounding IELs via both MHC class I and II molecules as well as the MHC class Ib molecules MICA, MICB, ULBP16 and HLA-E.

1.6.5 The human gut microbiota

The human microbiota consists of bacteria, archaea, viruses and unicellular eukaryotes that colonize every surface exposed to the environment, such as skin and the genitourinary, gastrointestinal and respiratory tracts. The colonization starts immediately at birth, and maybe even before birth, and is influenced by the mother’s microbiota, the infant’s diet, including breast milk, environmental factors and antibiotic treatments (75). During the first year of life, the microbiota composition becomes successively more diversified (76). The number of bacteria in the human microbiota is estimated to be $10^{14}$ and the colon is the most colonized area in the human body and is inhabited by approximately 500-1000 different species, dominated by the phyla Bacteroidetes, Firmicutes and Proteobacteria (77, 78). The bacterial density as well as the composition of bacterial species varies along the intestine, with approximately $10^{11}$ to $10^{12}$ colony forming units (CFUs) /ml luminal content in the colon. The population in the colon is dominated by the Bacteroidetes and Firmicutes, while the luminal content in SI contains $10^4$ to $10^7$ CFUs/ml dominated by the phyla Firmicutes, Bacteroides, Proteobacteria, and Actinobacteria (77-79).

The SI also has an adherent microbiota dominated by the genera Streptococcus and Neisseria (77). The gut microbiome in humans seems to be plastic and the composition of the bacteria can be changed with the influence of diet, environment and antibiotic treatments. In recent years, dysbiosis, a change in the microbiota composition in an unfavourable direction, has been implicated in the progression of several inflammatory and autoimmune diseases, such as Crohn’s disease, ulcerative colitis and multiple sclerosis (80-82).
The intestinal microbiota can also influence the mucosal immune system by stimulating the innate immune system via TLRs and secretion of antimicrobial peptides. The commensal microbiota, which consists of a large number of diverse microorganisms that possess beneficial functions for the host, have been observed to influence adaptive immunity by recruiting CD4⁺ T-cells as well as inducing organization of secondary lymphoid tissue. The composition of the microbiota is also important in the induction and differentiation of Th17 cells and the regulation of the balance between Tregs and Th17 cells (83). Mice with different origin and composition of the microbiota have been shown to have different numbers of Th17 cells in the SI. Germ-free mice (lacking bacteria and fungi) completely lacked Th17 cells but, in addition, the numbers of Tregs cells were increased (83). A further study revealed that a single member of the commensal microbiota known as segmented filamentous bacteria (SFB), belonging to the genus Candidactus, can induce Th17 cells to migrate into the SI of mice. SFB are a Gram-positive spore-forming bacteria closely related to the genus Clostridium (84, 85)

It has also been shown that a component of the intestinal microbiota, a cluster of species belonging to the XIVa and IV of the genus Clostridium can induce the appearance of colonic Tregs in mice. The Clostridium activated IECs to produce TGF-β1 and IDO that are implicated in the induction of Tregs (86).
1.7 Celiac disease

Celiac disease (CD) is defined as a chronic small intestinal immune-mediated enteropathy caused by failure to establish and/or maintain tolerance to the food antigen gluten in wheat and related prolamines in barley and rye. The development of CD is a complex process influenced by both genetic and environmental factors. Virtually all patients with CD carry the MHC class II alleles HLA-DQ2 and/or HLA-DQ8, and gluten in the diet is a necessary environmental factor to develop the disease. Ingestion of gluten, in genetically susceptible individuals with CD, provokes an immune response in the small intestine leading to villus atrophy, crypt hyperplasia and infiltration of immune cells, particularly αβ T-cells into the epithelium and lamina propria. In many respects, it has features of an autoimmune disease and might also affect tissues other than the SI.

The immune system and in particular the intestinal T-cells has long been recognized in the etiology of CD, but still, the exact pathogenesis of the disease is unknown. One possible risk factor might be dysbiosis during the establishment of the gut microbiota, which may contribute to the etiology of CD. Presently, the only treatment for CD is lifelong avoidance of gluten.

1.7.1 Clinical features

CD is the most common, chronic gastrointestinal disease in children but it may also have its onset in adulthood and affects people at all ages. The prevalence of developing CD is of approximately 1% in the Western population. The definition of CD was defined in Oslo 2012 as “a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals” (87). According to the Olso definition, classic CD is defined as “CD presenting with signs and symptoms of malabsorption. Diarrhoea, streatorroea, weight loss or growth failure is required”. However, not all CD patients have the classic symptoms and the outcome can vary among patients. In some individuals, the disease can even be asymptomatic. Among the definitions of CD are also non-classic CD, without signs and symptoms of malabsorption, and potential CD, referred to people with normal histology who are at increased risk of developing CD indicated by a positive serology (87, 88).
1.7.2 Diagnosis

The diagnostic procedure for CD is often based on clear symptoms and positive serological markers, which have high positive predictive value for CD. In the normal SI, plasma cells produce IgA antibodies, and to a lesser extent IgM and IgG. Antibodies of the IgA and IgM isotypes are secreted into the intestinal lumen, in order to neutralize and prevent antigen from penetrating the epithelium, but these antibodies also reach the bloodstream as do the antibodies of the IgG isotype (89, 90). During the progression of CD, specific antibodies of IgA and IgG isotypes are produced against the food antigen, both the native form of gliadin (AGA) and the deamidated form of gliadin peptides (DGP), and the autoantigen tissue transglutaminase (tTG) and endomysium (EMA). Increased serum levels of IgA antibodies to these antigens are used as a serological test for diagnosis on suspicion of CD. Antibodies to tTg and gliadin, both native and deamidated, are detected by enzyme-linked immunosorbent assay (ELISA) while EMA antibodies are detected by immunofluorescence staining of monkey esophagus or human umbilical cord tissue sections. Elevated AGA titers was the first serological marker for CD, but due to low specificity and sensitivity (70-80%), the standard serological marker now is an elevated serum titer of IgA antibodies to tTg (88, 91). Even though AGA has low specificity, it still can be used as a serological marker in children under 18 months of age (92). The measurement of EMA in serum of patients with CD is highly sensitive (90%) and specific (95%) (93). However, the test for EMA is time-consuming and expensive, and it is presently only used as confirmation test to IgA-tTg (94). In 1997, the enzyme tissue transglutaminase 2 (tTG) was identified as an autoantigen in CD (95). The serological test for tTG that was developed has high specificity (95%) and sensitivity (95%) (93).

Around 2% of CD patients have selective IgA deficiency, with no or low production of IgA antibodies, which is more common in CD patients than in the general population. In patients with selective IgA deficiency, serology is performed using tests for antibodies of the IgG isotype to tTg and EMA (96). Besides IgA deficiency, a diet with low gluten content might give a lower titer of IgA antibodies to the serological markers than expected, and hence a missed diagnosis.

Even though the serological test for tTg has high sensitivity and specificity, a small intestinal biopsy is still often required, to confirm the diagnosis of CD in most situations. The biopsies are captured with gastroscopy in the proximal SI. Multiple biopsies are recommended due to the fact that many patients have patchy villous atrophy (91). The histological pathology of the small intestinal mucosa is classified according to Marsh, which is based on a
four graded scale (97). Type 0 indicates normal mucosa; type 1, normal villous architecture, normal height of the crypt and increased number of IEL; type 2, normal villous architecture, crypt hyperplasia and increased number of IEL; and type 3 is divided into three subgroups according to their degree of villous atrophy. All type 3 pathologies have crypt hyperplasia and increased IEL together with either mild villous flattening (type 3a), marked villous flattening (type 3b) or total villous atrophy (type 3c). Type 4 has flat mucosa, normal crypt height and normal IEL count (98).

The diagnostic criteria for CD was established by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) in 1970, revised in 1990 and revised again more recently in 2012. The first criteria were based on a three-biopsy procedure with an initial biopsy on suspicion of CD that showed abnormal histology, a second biopsy after challenge with a gluten-containing diet showing normal histology and a third biopsy after gluten challenge showing abnormal histology again. When revised in 1990, after the introduction of serology tests, only two prerequisites remained as mandatory. The first was that a first biopsy should show villous atrophy, crypt hyperplasia with increased IEL together with detectable serological markers in the serum, while the patient ingested adequate amounts of gluten. The second criterion was full remission of histology and serology markers after exclusion of gluten from the diet. A control biopsy was only needed in a few cases, to verify the outcome of the gluten-free diet (GFD) (99). In 2012, the criteria for CD was revised again due to better understanding of the pathological process and the introduction of the serological test for tTG. The new guidelines established by ESPGHAN defined two groups of CD patients: group 1, children with symptoms and a suggestive CD, and group 2, children with asymptomatic CD with an increased risk of developing the disease. The diagnostic approaches are different for the two groups. Group 1 is diagnosed based on positive serology and histology, consistent with CD. In cases of high levels of anti-tTG (>10 times the upper limit of normal) the requirement of a biopsy is not mandatory. In group 2, the diagnosis is based on positive serology, histology and in some cases HLA-DQ2 and HLA-DQ8 testing (88).

### 1.7.3 Genetic predisposition

CD is a multifactorial disease with strong genetic associations. An indication of the genetic influence, in developing the disease, is the increased incidence among relatives of CD patients. The total risk of developing CD is as high as 11-13% in siblings and the concordance rate in monozygotic twins is approximately 75-83% and 11-17% in dizygotic twins (100, 101). In the
beginning of 1970, the first report on genetic susceptibility to CD was published, demonstrating the discovery of association with certain HLA class alleles. The HLA class II complex is a collection of genes located on chromosome 6, consisting of one α chain and one β chain, expressed mainly on APCs. Later on, specifically the HLA class II molecules, HLA-DQ2 and HLA-DQ8, were found to be strongly associated with CD. Ninety percent of CD patients carry the HLA-DQ2:5 heterodimer, encoded by the alleles DQA1*05 (α chain) and DQB1*02 (β chain) (69, 102, 103). The alleles can either be on the same chromosome, in a cis position (DR3DQ2:5 haplotype), or located on opposite chromosomes, in a trans position, (DR3DQ7/DR7DQ2 haplotypes). The remaining CD patients carry the HLA-DQ8 molecule, encoded by the alleles DQA1*03:01 and DQB1*03:02 for the haplotype DR4DQ8 (102, 103). In addition, around 30% of the general population also expresses HLA-DQ2 and HLA-DQ8, but only 1-3% of them develop CD. Thus, this genetic predisposition in combination with a gluten-containing diet cannot fully explain why an individual contracts CD.

It has been estimated that the HLA genotype only explains half of the genetic susceptibility and therefore it is believed that other genetic factors must also be associated with the disease. In a large genome-wide association study (GWAS), non-HLA regions were analyzed and 13 new regions could be associated with CD (104). Many of the new regions are involved in immunological functions, like T- and B-cell co-stimulation (CTLA4-ICOS-CD28, TNFRSF14, CD80, ICOSLG, TNFRSF9, TNFSF4), cytokines, chemokines and their receptors (TNFSF18 and CCR4) and T-cell development in the thymus (THEMIS) (104). To identify the precise genes that are involved in the pathogenesis of CD, more functional studies and more sensitive techniques are required.

1.7.4 Environmental factors

Genetic predisposition in CD patients is necessary but not sufficient for developing CD. Several different environmental risk factors, besides gluten, have also been suggested. In a population-based incident case-referent study, the incidence of developing CD after early infections was studied. This suggested that, repeated early infections, together with large amounts of gluten during the introduction of gluten-containing foods into an infant’s diet conferred a risk (105). More importantly, introduction of gluten during continued breastfeeding reduces the risk of CD before two years of age compared to when the mother stopped breastfeeding before introduction of gluten (105). All of these environmental factors have an impact on the composition of the microbiome; in infants, and dysbiosis has also been
suggested as a risk factor for CD (106). In adult CD patients, the composition and the diversity of duodenal microbiota were altered, with more abundant bacteria of the phyla *Proteobacteria* and less abundant bacteria of the phyla *Firmicutes*, than in controls (79). In pediatric CD patients, the microbiota of both feces and duodenal biopsies was altered, with the composition having fewer *Bifidobacterium* and *B. longum* compared to controls (107). Moreover, rod-shaped bacteria of the *Lachnoanareobaculum*, *Prevotella* and *Actinomyces* genera were detected by scanning electron microscopy and isolated from the mucosa of CD patients (77, 108, 109). In addition, CD patients have increased levels of α- and β-defensins and lysozyme together with an altered composition of the glycocalyx/mucus layer that facilitates an enhanced capacity for bacterial adhesion to the epithelial cells (108).

### 1.7.4.1 The Swedish epidemic of celiac disease

During the years 1984 through 1996, Sweden experienced an epidemic with a four-fold increase in the incidence of symptomatic CD in children below two years of age. This period was later termed “the Swedish epidemic of celiac disease”. The epidemic started with an abrupt increase of annually diagnosed cases in the mid-1980s that coincided with new national recommendations for infant feeding modes. The recommendations were to postpone the introduction of gluten-containing foods, from 4 to 6 months of age, and coincidentally at the same time the gluten content of the commercially available milk cereal drinks used by the Swedish infants was increased (110, 111). This resulted more infants who were first exposed to gluten when the mother had stopped breastfeeding and they were exposed to comparatively high amounts of gluten quite abruptly. When a case referent study had revealed introduction during breastfeeding as a protective factor and larger compared to smaller amounts of gluten during the introduction as a risk factor, the national recommendations were changed again in 1996. Since then the recommendation is to introduce gluten-containing products between 4-6 months, in small successively increasing amounts and preferentially while the mother is still breastfeeding. The new recommendations coincided with a steep decrease in annual incidence rate of CD in infants. In a population-based incident case referent study in children, below two years of age, the potential risk of changes in dietary pattern was studied. The authors suggested that small amounts of gluten-containing foods under the protection of breastfeeding reduced the risk of developing CD in early childhood (112). Still, the feeding modes for introduction of gluten could not fully explain the epidemic.
1.7.5 Gluten and the immune response

Gluten can be divided into two fractions, prolamines and gluteins. The toxic agent in gluten is prolamines, an alcohol-soluble protein fraction with high content of the amino acids proline and glutamine. The prolamine in wheat is gliadins, in barley hordeins and in rye secalins. Wheat, barley and rye all belong to the same tribe, Triticeae. Another, more distantly related prolamine is avenin in oats that belong to the Aveneae tribe. Avenin has a lower amount of proline, than gliadin, hordein and secalin. These prolamine-rich peptides are resistant to degradation by gastric and pancreatic enzymes, and will remain as positively charged long fragments in the SI (113). The gliadin peptides can affect both the epithelium and the LP. One manner in which this occurs is that poorly digested peptides can be transported over the epithelium to the LP. In LP, the gliadin fragment serves as a good substrate for the ubiquitously expressed enzyme tissue transglutaminase, which modifies the positively charged amino acid glutamine into a negatively charged glutamic acid, by a process called deamidation. The deamidated peptide increases the binding affinity to the HLA-DQ2 and HLA-DQ8 molecules. The interaction of the MHC molecule and the gluten peptide initiates an activation of specific gluten-reactive CD4+ T-cells in the LP, which triggers an immune response that results in increased expression of pro-inflammatory cytokines, like IFN-γ and IL-21, and activation of B-cells to produce tTg antibodies (Figure 3) (69, 114, 115). Most of the reactive CD4+ T-cells are highly specific in their recognition of gliadin, hordein or secalin peptides, while some T-cells can cross-react with any of the peptides from these three grains. Thus, these cross-reactive T-cells can be harmful for patients ingesting any of these three grains, and possibly avenin from oats as well (116). The second alternative route for gliadin peptides is that they can be recognized by CD8+ T-cells in the epithelium, through the HLA class I/TCR complex, which mediates apoptosis of epithelial cells (117, 118). The activated CD8+ T-cells also secrete the pro-inflammatory cytokine IFN-γ, and the cytotoxic molecule FasL (47, 117, 118).

The pro-inflammatory cytokine environment, produced by the specific gluten-reactive T-cells, will promote infiltration of immune cells, and local inflammation in the mucosa (69). At the same time, the number of IELs is increased during active CD, and most of them express the activating NK-receptors, NKG2D and NKG2C. These receptors mediate signaling through their ligands, MICA and HLA-E on IEC, which induces cytolysis and enhancement of IFN-γ and IL-15 production (70, 71, 119). In addition, gliadin peptides can also mediate an innate immune response, in a TCR-independent manner, by inducing up-regulation of the stress molecules MICA/MICB on IEC (70, 120, 121).
Figure 3 Schematic overview of the immune situation in the small intestine of celiac disease patients upon ingestion of gluten. Gliadin peptides cross the epithelium to the lamina propria where they are deamidated by the enzyme tissue transglutaminase (tTg). The deamidated peptides bind more effectively to the HLA-DQ2 and HLA-DQ8 molecules that activate gluten-reactive T-cells. The activated T-cells produce pro-inflammatory cytokines and provide help to B-cells for antibody production, which results in tissue damage and increased inflammation. Reproduced with permission from (N Engl J Med, (114)), Copyright Massachusetts Medical Society.

1.7.6 Treatment

Thus far, the only treatment for CD patients is lifelong exclusion of gluten from the diet. It is still uncertain whether or not CD patients tolerate oats. Even a small amount of gluten can cause lesions in the mucosa of CD patients. One hundred mg of gliadin or 2.5 g of wheat flour is enough to
cause morphological changes in the small intestinal mucosa (122), although the susceptibility between individuals may vary. The highest recommended amount of ingested gluten without any harm to the mucosa is 10-50 mg per day (123). It is crucial for CD patients to adhere to a strict GFD. It may take 6-24 months before there is recovery of the mucosa and normalization of antibody titers as well as numbers of IEL (Figure 4). Adhering to a GFD is also of importance to reduce the risk of developing associated diseases and nutritional deficiencies due to the chronic inflammation in the small intestine, like small bowel lymphomas and osteoporosis (94, 124).

Figure 4 Scanning electron micrograph of the small intestine. a) showing a subtotal villus atrophy of the mucosa in a CD patient and b) normal mucosa from a treated CD patient.

The previous recommendation for CD patients was to also exclude oats from the diet when beginning a GFD. In some countries, the commercially available gluten-free products are allowed to contain pure (free from contamination of wheat, barley and rye) oats. Strictly adhering to a GFD is problematic, particularly when switching from a new diet after the first year of life. From a nutritional point of view, the GFD is low in fiber, as well as some vitamins and minerals (125, 126). In recent years several studies on GFD containing oats have been investigated with partly contradictory results. Some studies have reported that an oat-containing GFD in adult CD patients is harmless, both for newly diagnosed patients and patients in remission. These patients have normal mucosa and serum titers of serological markers as well as decreased infiltration of IELs, even after five years (124, 125, 127, 128). In a randomized double-blinded study in children, the addition of moderate amount of oats was found to be safe, as it did not prevent clinical and mucosal recovery or normalization of serological marker titers (129). However, anti-avenin antibodies are detected in serum of CD patients regardless of the diet (130). On the other hand, other studies have reported elevated levels of IEL and more gastrointestinal symptoms, including diarrhea in CD patients consuming oats (125, 131). In addition, there is a case report on a CD patient consuming oats that had mucosal
damage and high levels of IFN-γ as well as avenin-reactive T-cells in the mucosa. These avenin-reactive T-cells were also isolated from the patient (132, 133).
2 Aims

- Understand the mechanisms behind oral tolerance and the reasons behind the failure to establish and maintain tolerance to food antigens and microbiota, as exemplified by intolerance to dietary gluten in patients with CD, by investigation of the cytokine network of effector T-cells and the balance and the interplay with Tregs in the small intestinal mucosa.

- To deliniate the pathology of CD from an immunological perspective with special emphasis on the role of T cells in exaberation and down-regulation of the intestinal inflammation.

- Investigate the possible influence of CD associated bacteria on T cell activity in the mucosa and determine whether oats in the diet elicits an inflammatory response in the intestinal mucosa of CD patients or not.

- To better understand the immune defence and the cross-talk between epithelial cells and IELs at the epithelial lining of the normal small intestine and in the inflammatory reaction against gluten in CD patients.
3 Clinical material and methods

3.1 Patient characteristics

All biopsies included in this thesis were taken as a part of clinical examination with the suspicion of CD or with the intention to exclude the diagnosis. Written informed consent was obtained from the parents of participating children for permission to use the biological material in the present study. The local Research Ethics Committee of the Faculty of Medicine, Umeå University approved the studies, including the consent form. Paper 3 was also approved by the local Research Ethics Committee of the Faculty of Health Science, University of Linköping.

3.2 Biopsies

Biopsies were taken from the distal duodenum/proximal jejunum using an endoscopic procedure or by Watson pediatric capsule. Part of the biopsy was used for routine pathology examination and the rest was used in these studies for gene expression analyses at the mRNA level, either in RNA extracted from whole biopsies or from isolated IELs, LPLs and T-cell subsets thereof, or at the protein level for immunohistochemistry. The biopsies were also challenged ex vivo with gluten digest (trypsin treated gluten), a mixture of CD-associated bacteria, or with a combination of gluten digest and the CD-associated bacteria. Patients belonged to one of the three diagnostic groups: untreated CD, children on a gluten-containing diet with active disease and later confirmed to have CD; treated CD, children with CD who had been on a GFD for more than 11 months; and controls, children with no known food intolerance. The histology of the biopsies used in these studies were classified according Marsh score or Alexander score (Table 3).

3.3 Cell isolation

Intestinal IELs and LPLs were isolated from fresh small intestinal biopsies by incubation with 0.1 mM dithiothreitol (DTT) under vigorous shaking. The free cells and pieces of biopsy tissue were resuspended in DTT-free medium and vortexed. The biopsy tissue pieces were allowed to sediment and supernatant containing the IELs were collected. The sedimiented tissue pieces were then treated with collagenase type IV and then passed through a stainless-steel filter to collect LPLs. IELs together with LPLs were subjected to negative selection to remove myeloid cells and inherently sticky cells. Cell
<table>
<thead>
<tr>
<th>Study</th>
<th>Patients group</th>
<th>Number of patients</th>
<th>Sex (F/M)</th>
<th>Age (years, median and range)</th>
<th>Serum antibody titers (TTg)</th>
<th>Serum antibody titers (EMA)</th>
<th>Histology at inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Active CD</td>
<td>19</td>
<td>13/6</td>
<td>4.3 (1.3-17.0)</td>
<td>≥20</td>
<td>≥20</td>
<td>3-4 #</td>
</tr>
<tr>
<td></td>
<td>Treated CD</td>
<td>13</td>
<td>5/8</td>
<td>10.2 (4.5-19.0)</td>
<td>≥20</td>
<td>≥20</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>20</td>
<td>9/1</td>
<td>3.5 (1.1-18.2)</td>
<td>≥20</td>
<td>≥20</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Active CD</td>
<td>42</td>
<td>28/14</td>
<td>7.5 (4.0-12.0)</td>
<td>&gt;5 U/ml</td>
<td></td>
<td>IIIa-c</td>
</tr>
<tr>
<td></td>
<td>Treated CD</td>
<td>29</td>
<td>18/11</td>
<td>7.5 (5.3-14.5)</td>
<td>≤4 U/ml</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>15/14</td>
<td></td>
<td>5.5 (2.0-11.5)</td>
<td>≤4 U/ml</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Active CD</td>
<td>28</td>
<td>16/12</td>
<td>Paired samples with treated CD</td>
<td>&gt;3 U/ml</td>
<td>≥20</td>
<td>IIIa-c</td>
</tr>
<tr>
<td></td>
<td>Treated CD GFD-oats</td>
<td>15</td>
<td>8/7</td>
<td>4.2 (1.7-11.1)</td>
<td>≤3 U/ml*</td>
<td>≤20 $</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Treated CD GFD-std</td>
<td>13</td>
<td>8/5</td>
<td>3.3 (1.5-6.5)</td>
<td>≤3 U/ml*</td>
<td>≤20 #</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>13</td>
<td>6/7</td>
<td>7.4 (2.3-10.6)</td>
<td>≤4 U/ml</td>
<td>≥20</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Active CD</td>
<td>15</td>
<td>11/4</td>
<td>12.7 (9.8-13.1)</td>
<td>&gt;5 U/ml</td>
<td></td>
<td>IIIa-c</td>
</tr>
<tr>
<td></td>
<td>Treated CD</td>
<td>5</td>
<td>4/1</td>
<td>14.5 (14.2-14.6)</td>
<td>≤4 U/ml</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>13</td>
<td>10/3</td>
<td>8.6 (4.5-16.5)</td>
<td>≤4 U/ml</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

# One child Alexander score 2
* Not specified for 1 patient
* Five >3 U/ml
§ Four patients >20
≠Four >3 U/ml
≠Two patients ≥20
suspensions were treated with magnetic beads coated with anti-CD11b monoclonal antibody (mAb) (OKM1) and bead-bound cells were removed with a magnet. Unbound cells were subjected to positive selection (119). In Paper 1, the IELs were retrieved by sequentially positive selection using magnetic beads coated with anti-TCRγδ mAbs (γδIELs) followed by anti-TCRαβ mAbs (αβIELSs) and finally by a mixture of beads coated with an anti-CD2 mAb and an anti-CD7 mAb (CD2^−CD7^−IELs). In Paper 2, both IELs and LPLs were subjected to sequential positive selection using magnetic beads coated with anti-TCRγδ mAbs (γδ^+IELs, γδ^+LPLs), then beads coated with anti-CD4 mAbs (CD4^−IELs, CD4^−LPLs) followed by beads coated with anti-CD8 mAbs (CD8^−IELs, CD8^−LPLs). In Paper 4, IELs were directly subjected to positive selection by magnetic beads coated with anti-CD3 (CD3^−IELs) without pretreatment with OKM-1-mAb-coated magnetic beads to remove myeloid cells and inherently sticky cells.

IECs were isolated from fresh small intestinal biopsies by incubation with 0.1 mM dithiothreitol (DTT) under vigorous shaking. Biopsy tissue pieces were thereafter treated with collagenase type IV and then passed through a stainless-steel mesh to collect IECs. IECs were subjected to negative selection to remove leukocytes and sticky cells. Cell suspensions were treated with magnetic beads coated with anti-CD45 mAb and bead-bound cells were removed with a magnet. Unbound cells were subjected to positive selection using magnetic beads coated with the anti-epithelial antigen mAb BerEP4.

The methods used for isolation of IELs and LPLs yielded more than 98% pure preparations of the positive markers (13). In Paper 4, negative selection was excluded in the isolation procedure of IELs in order to obtain sufficient amounts of RNA for Illumina bead array and real-time qRT-PCR array. The cross-contamination of IECs in the IELs population was calculated using genes specially expressed by IECs obtained from Illumina genome-wide gene expression array, i.e., CEACAM5, DEF5, DEF6, MUC1 and MUC2. Reversely, the cross-contamination of CD3^+IELs in the IECs population was calculated using genes specially expressed by CD3^+IELs, i.e., CD3D, CD3G, CD247/CD3Z, CD2, CD7 and CD8A. The mean degree of contamination of CD3^+IELs in IECs was 6.4% and the corresponding value for IEC contamination in CD3^+ IEL preparations was 22.8%.
3.4 Gene expression analysis at the mRNA and protein levels

Several different techniques were used to determine and verify the expression of genes at the mRNA and protein levels for molecules involved in the inflammatory reaction in the small intestine of CD patients. Comparative analysis of the expression levels of different mRNAs were performed by real-time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR), including real-time quantitative PCR array and Illumina genome-wide gene expression bead array technology. Two different qRT-PCR assays were used in the studies, i.e., Taqman gene Expression Assays and EZ-technology assays. Taqman gene Expression Assay amplifies the target gene of interest in a pre-prepared cDNA library by real-time quantitative PCR. In the EZ-technology the mRNA of interest is specifically transcribed to cDNA from the total RNA sample in the reaction mixture and thereafter directly amplified by real-time quantitative PCR. In both techniques, the PCR primers anneal to different exons and the reporter dye-marked probe anneals to the exon boundary in the amplicon in order to eliminate signals from contaminating DNA. qRT-PCR assays constructed in the laboratory are based on the EZ-technology and also include the use of an RNA copy standard, thereby giving the actual quantity of mRNA copies and allowing for comparison of mRNA levels between different RNA species. All mRNA concentrations determined by qRT-PCR were normalized to the concentration of 18S rRNA in the same sample, by calculating mRNA copies/18S rRNA U for mRNAs analyzed by EZ-technology assays and by calculating the ΔCT between the CT for the mRNA species and the CT for 18S rRNA (CT_{sample} - CT_{18S rRNA}) for mRNAs analyzed by commercial Taqman gene Expression Assays. The results for genes analyzed by Taqman gene Expression Assays are given both as ΔCT and as relative quantity (RQ), calculated as $2^{(-\Delta \Delta CT)}$ where $\Delta \Delta CT$ is $\Delta CT$ for the sample minus the median of the control sample $\Delta CT$-values. Illumina gene expression bead array technology was used in Paper 4 for a genome-wide screening of expression of genes involved in the inflammatory reaction in the epithelia of CD patients with active disease. The Illumina HumanRef-8_v3 expression Bead chip, which covers more than 18,000 genes, was used. For the protein analysis, two different techniques were used, immunohistochemistry and double immunofluorescence. Immunohistochemistry was used in Papers 1, 2 and 4 to verify the expression of mRNAs detected in the studies and to evaluate the localization of cells expressing these genes in mucosal tissue. Morphometry was also performed to determine the number of positively stained cells. Results are expressed either as positively stained cells per length of epithelium (positive cells/mm) (Paper 1) or as positively stained cells per area of epithelium and LP
(cells/mm²) (Paper 2). Immunohistochemistry sections were fixed in paraformaldehyde to prevent soluble proteins from being washed away during the staining procedure. The procedure also included treatment with both saponin and Triton-X100 to also allow intracellular staining in the nucleus. In Paper 2, double immunofluorescence was used to verify the presence of cells staining for both IL-17A and CD8, i.e., Tc17.
4 Results and Discussion

A critical role of the mucosal immune system is to mediate oral tolerance to food antigens and commensal flora, and at the same time responding quickly to pathogens. Oral tolerance involves many complex immunological mechanisms including suppression of immune responses by Tregs and release of suppressive cytokines such as IL-10 and TGF-β1 to down-regulate inflammatory responses caused by pro-inflammatory mediators. One important part of oral tolerance might be adaptation of the TCR specificity to the local antigen milieu in the intestine. In CD, failure to establish and/or maintain tolerance to gliadin provokes an immune response in the small intestine. Immune cells and in particular the T lymphocytes have long been recognized in the pathogenesis of CD. An extended period of inappropriate expression of cytokines may lead to chronic inflammation in the tissue, where Tregs fail to shut down the activity of the other immune cells. In this thesis, we investigated the immune status and the interplay of T-cells and Tregs in the mucosa of children with CD and controls, as well as the immune status in treated CD patients, provoked by either dietary oats, CD associated bacteria or gluten.

4.1 Pediatric CD patients have an impaired capacity for ETCM and T-cell receptor editing in the small intestine

In children, T-cell maturation mostly takes place in the thymus where exposure to foreign antigens is minimal. Previous studies in our group showed that jejunal IELs and LPLs express RAG-1 mRNA and up to 6% (3.3± 0.6%) of the IELs stained positive for the RAG-1 protein (9, 13). RAG-1 mRNA was also expressed in both CD3- and CD3+ lymphocytes of T-cell lineage, while the mRNA for preTα was only expressed in thymocyte-like cells (TCR-CD2-CD7+), both in the epithelium and in LP (13). In addition, lymphocytes expressing the stem cell marker c-kit are present in the jejunal mucosa (13). These findings suggest ongoing TCR rearrangement both in TCR editing and in ETCM in the small intestine. As mentioned earlier, the human-derived RAG1 protein has three different 5’untranslated region exons that generate four different mRNA splice forms, RAG1 1A/2, 1B/2, 1A/B/2 and 1C/2. Only the Rag1 1C/2 and 1B/2 splice forms are expressed in the thymus while the 1A/2, 1A/B/2 and 1B/2 mRNA splice forms, but not the 1C/2 splice form, are expressed in the SI (13). Therefore, the SI seems to be a site, outside of the thymus, for editing of mature thymus-derived T-cells as well as generation of new T-cells locally. Rearrangement of the TCR a second time, in the periphery, could be important in a demanding milieu such as the
intestine when it encounters new, beneficial antigens from food, to which tolerance is required. The SI is also a site for de novo rearrangement of the TCR on immature T-cells. Both phenomena are likely to take place in the SI for generation of Tregs against antigens that are not encountered in the thymus, but for which tolerance is desired.

In paper 1 we investigated whether impaired ETCM and/or secondary TCR editing occur in the small intestines of CD patients. This might explain the failure to establish tolerance to gluten. Since both phenomena have been shown to be most pronounced within the epithelium (13), we focused our analyses on IELs. The four different splice forms of RAG1 mRNA were analyzed in γδIELs, αβIELs and thymocyte-like IELs (CD2\(^+\)CD7\(^+\)CD3\(^-\)) isolated from small intestinal biopsies of CD patients and controls. In controls, the 1A/2 splice form was detected at high expression levels in all three IEL subsets. This confirms that RAG1 is expressed in both mature cells, for possible TCR editing, and in immature cells, for possible generation of new T cells. Only occasional samples expressed 1B/2 and 1A/1B/2 splice forms in the IEL subsets, while the thymus-derived splice form 1C/2 was not detected. Thus, in normal epithelia of children, the mRNA 1A/2 splice form is preferentially expressed in all IEL subsets, which is in agreement with our previous results in adults (13). By immunohistochemistry, the RAG1 protein was detected in the IELs within small clusters and as scattered single cells. There were also occasional LPLs expressing RAG-1. The mRNA expression of the invariant preT\(\alpha\) chain was also analyzed in γδIELs, αβIELs and thymocyte-like IELs and was, as expected, almost exclusively detected in the thymocyte-like IELs, suggesting that they indeed are immature T cells. This suggests that ETCM occurs in the small intestinal epithelia of both adults and children (9, 134) (Paper 1). The expression of RAG1 in both mature and immature T-cells suggests that both editing of TCR and ETCM are normal parallel events in the SI throughout life, even in young children in whom the thymus has not yet regressed.

Next, we addressed the question of whether ETCM and/or TCR editing is also a normal event in CD patients. The possible impaired capacity of ETCM and editing of the TCR repertoire according to the local antigen milieu in the SI might contribute to failure to induce and maintain tolerance to gluten. The expression of the RAG1 mRNA splice forms were analyzed in γδIELs, αβIELs and thymocyte-like cells (CD2\(^+\)CD7\(^+\)CD3\(^+\)) of children with active and inactive CD compared to controls. The results revealed that the RAG1 mRNA splice forms, and in particular the 1A/2 splice form, was expressed at significantly lower levels in all IEL subsets, compared to control children. Furthermore, the number of IELs expressing the RAG1 proteins was significantly lower in CD patients. The levels of mRNA for preT\(\alpha\) were also
significantly lower in thymocyte-like cells of CD patients compared to controls. Levels of the markers for ETCM were significantly lower regardless of the disease activity, suggesting of an inherent property that children with CD have reduced capacity to adopt their TCR to the local milieu.

We showed in this study that children with CD have significantly lower mRNA levels of both RAG1 and preTα in their IELs as well as lower numbers of IELs expressing RAG1. This suggests that CD patients have a reduced capacity for secondary rearrangement of mature T-cells as well as de novo rearrangement in the thymocyte-like cells and consequent generation of extrathymically mature T cells. These phenomena might be important for the establishment of tolerance and for adaptation of a T-cell repertoire to the local milieu in the small intestine, and therefore could be a contributing factor to developing CD. The finding that TCR rearrangement is low in CD patients compared to controls suggests an impaired capacity in CD patients to generate gluten-reactive Tregs or failure to eliminate gluten-reactive T-cells. The finding that most of the TCR gene rearrangement seems to take place within the epithelium suggests that intestinal epithelial cells are involved in the ETCM selection processes, possibly by presenting antigen to the maturing T cells.

Interestingly, the thymocyte-like IELs and LPLs (CD2⁺CD7⁺CD3⁻) have phenotypic similarities with the newly described ILCs. ILCs are present in the human intestine and have been implicated in inflammation of the gut (40). Both cell types lack TCR and express the T cell-lineage marker CD7. ILC3 cells are reported to express IL-7R/CD127 and c-kit/CD117. In a previous study, our group showed that as much as 45% of the IELs expressed IL-7R and 5% expressed c-kit (13). ILC3 cells were shown to be able to secrete the cytokine IL-17A although it is not yet known whether thymocyte-like cells can do the same. It is still unclear whether the ILCs are a true subpopulation of innate cells or if they are immature T cells that can differentiate into effector cells upon antigen activation. One possibility is that ILC3 cells might be the same cell population as thymocyte-like cells, which express RAG-1 and preTα, and in CD patients have reduced capacity for de novo rearrangement as well as editing of the TCR.

4.2 Overexpression of immune modulating molecules in the small intestine of CD patients

In two of the studies (Paper 2 and 4), we investigated the immune status of the small intestine of both untreated and treated CD patients compared to
controls. As mentioned above, IL-17A is involved in antibacterial defense by recruiting immune cells to sites of infection but also acts as a pro-inflammatory cytokine in autoimmune and inflammatory diseases. That IL-17A might play a role in CD was suggested by increased levels of IL-17A in small intestinal biopsies of CD patients with active disease and after ex vivo challenge of biopsies of treated CD patients with gliadin (50). However, studies on the cellular source of IL-17A in the small intestine have been contradictory. Two groups have demonstrated Th17 cells in the small intestinal mucosa of CD patients (135, 136) while one other group failed to demonstrate IL-17A production in established gliadin-specific CD4+ T-helper cell lines (137). In Paper 2, we demonstrated elevated levels of IL-17A mRNA in an untreated CD patient that had returned to the level of controls in patients on a GFD. The mRNA expression levels of IL-17A also correlated with the expression of Foxp3 and IFN-γ mRNA for CD patients with active disease. Immunohistochemistry was used to further verify the presence and distribution of IL-17A-producing cells in the mucosa. The results revealed that the number of IL-17A-producing IELs of untreated CD patients was 10 times higher than the number of IL-17A-positive LPLs and six times higher compared to controls. This suggests that the epithelium is the major compartment for production of elevated levels of IL-17A. To identify the cellular source in the epithelium, we determined the expression levels of IL-17A mRNA in isolated γδ+IELs, CD4+IELs (Th17) and CD8+IELs (Tc17) from intestinal biopsies of both untreated CD patients and controls. The results revealed that Tc17 contributed the most to the IL-17A response, and Th17 contributed only to a lesser degree in the epithelia of untreated CD patients. In controls, the low level of IL-17A was produced mostly by Th17 cells, and in equal proportion in the epithelium and the LP. The presence of Tc17 cells was further demonstrated by two-color immunofluorescence staining of an small intestinal mucosa of an untreated CD patient, showing double-stained CD8+IL-17A+IELs. Our finding suggests that Tc17 cells are the major cellular source of the elevated level of IL-17A seen in untreated CD patients.
Elevated levels IL-17A mRNA are expressed by Tc17 cells in the small intestine of CD patients. (a) Expression levels of IL-17A mRNA in freshly taken biopsies from small intestine of Untreated CD patients, Treated CD patients and Controls. (b) Expression level of IL-17A mRNA in isolated γδ-IEL, CD4+IELs and CD8+IELs from Untreated CD patients. (c-d) Two-color immunofluorescence staining of the small intestinal mucosa of one Untreated CD patient. (c) CD8+ cells stained in red, (d) IL-17A+ cells stained in green and (e) overlay of (c) and (d) showing yellow IL-17A+CD8+ cells (Tc17). Adapted from Paper 2.

Tregs are recognized to have inhibitory properties that function to balance the immune system and suppress immune responses against external antigens, thereby controlling inflammation. We analyzed the expression of Foxp3, IL-10 and TGF-β1 mRNAs and the distribution of Foxp3+ cells in the mucosa of CD patients and controls. The expression level of Foxp3, IL-10 and TGF-β1 mRNAs showed a similar pattern with higher levels in untreated CD patients compared to treated CD patients, but no difference compared to controls. The number of Foxp3+ cells was equal in untreated CD patients and controls, with the highest distribution in the epithelium by CD4+ cells and to a lesser degree the CD4+ in LP. These results suggest that CD patients normally have reduced Treg activity and difficulty establishing tolerance to
antigens. The increased Treg activity in CD patients probably reflects an attempt to control inflammation.

Tc17 cells were previously reported to be present in intestinal mucosa and the lungs of both humans and mice and have been implicated in autoimmune diseases, such as psoriasis (32). Murine Tc17 cells were shown to have functional plasticity, i.e., they can produce both IL-17A and IFN-γ and Tc17 cells can transition into Tc1 cells (34). There is also evidence that these cells are resistant to inhibition by Tregs (33). Taken together, our results are support the notion that CD8+IELs have functional plasticity and produce both IL-17A and, from a previous finding, IFN-γ, in the inflamed epithelium of untreated CD patients (47). In addition, the high level of IL-17A together with the high Treg activity seen in untreated CD patients might be explained by the fact that Tc17 cells can be resistant to inhibition by Tregs. We hypothesize that Tc17 are not down-regulated by Tregs and that they transition into Tc1 cells during the progression of the disease into CTLs with lost antigen specificity restriction, as shown by Meresse (71). A hypothesis, based on our results, about the immune state in inflamed mucosa of CD patients with active disease is shown in (Figure 4). In the study by Meresse et al., they showed that CTLs can undergo clonal expansion and proliferation in a TCR-independent manner that involves genetic reprogramming into cells that exhibit NK-mediated functions. They suggest that the indiscriminate mode of defense by the CTLs with lost antigen specificity may lead to destruction of epithelial cells in CD.

Figure 6 Hypothetical scenario of the immune situation in the small intestinal mucosa during the inflammatory reaction. The reaction at the mucosal lining with gluten peptides and CD associated bacteria induces a process which the intraepithelial Tc17 cells (red) become activated and transform into hyperactivated cytotoxic T lymphocytes with lost antigen specificity. The transformation is driven by autocrine secretion of IFN-γ. Parallel activation of Tregs (violet) that try to down-regulate the activity of T cells. IL-17A secreted by Tc17 and Th17 cells upon activation by gluten peptides and/or bacterial components promotes inflammation. Adapted from Paper 2.
In Paper 4, we tried to get a better understanding of the epithelial reaction seen in CD patients with active disease and we tried to delineate the complex network of immune modulating molecules that are involved in the inflammatory reaction against gluten seen in CD patients. mRNA expression levels of 88 different genes, such as chemokines, chemokine receptors, cytotoxic effector molecules, cytotoxicity triggering and inhibiting receptors and MHC class molecules were analysed in CD3+IELs and IECs, retrieved from small intestinal biopsies of both CD patients and control children. Among the 88 different genes analysed, 42 were significantly up-regulated and 2 were downregulated in CD3+IELs of untreated CD patients compared to controls. The largest differences were seen for the cytokines IFNγ and IL-17A (128 times and 96 times, respectively). In IEC, there were fewer genes that were expressed at a significantly higher level in untreated CD patients compared to control, i.e. 10 of the 88 genes and no cytokines were detected. This suggests that the arsenal of immune modulatory molecules seen in the epithelial reaction of CD patients is expressed mostly by CD3+IELs and at significantly higher level than controls.

One of the features of many inflammatory and autoimmune diseases is an inappropriate expression of cytokines and chemokines that may lead to a chronic inflammation in the tissue as well as recruitment of immune cells to the site of inflammation. To estimate the involvement of chemokines in the inflammation of CD, we analysed 22 chemokines and related factors together with 16 chemokine-receptors. The results showed that 10 chemokines, i.e., CCL5, CCL4, CCL20, CCL13, CRLF3, CRLF1, CXCL11, CXCL13, CX3CL1, and XCL2/XCL1, were significantly up-regulated in CD3+IELs of untreated CD patients compared to controls. Of these CXCL11 is most probably due to cross-contamination by IECs. Among the receptors, 5 were significantly up-regulated in CD3+IELs, i.e., CCR5, CCR3, CCR6, CXCR6, and CXCR5. In contrast, the results from IECs showed significantly up-regulation of 3 chemokines, i.e., CXCL11, CXCL10, and CX3CL1, and one chemokine, CXCL14, was down-regulated. No receptors showed up-regulation in IECs and one, CXCR7, showed down-regulation. Thus, many chemokines and their corresponding receptors were both expressed at high level in CD3+IELs and IEC of CD patients, which suggest communication between T-cells and epithelial cells and also possibly act as chemoattractant to other immune cells. One of the hallmarks for CD is an increased numbers of IELs. One possibility is that the increased levels of chemokine receptors in CD3+IELs might reflect increased proportion of newly recruited IELs in the epithelium. The chemokine CX3CL1 were expressed at significantly higher levels in both CD3+IELs and IECs of CD patients (P=0.016 and P=0.004), but neither cell fraction expressed the receptor, CX3CR1. By immunohistochemistry, we showed that cells in the lamina propria positioned near the basal lamina
express the receptor. One possibility is that CX3CR1+ monocyte-lineage cells that are shown to incorporate with the adjacent epithelial cells and send protrusions to the luminal side, which in turn might lead to increased sampling of luminal antigens is also involved in the epithelial reaction of CD patients.

Another hallmark of active CD is increased cytotoxic activity of IELs and increased number of cells that express the NK-receptors, KLRD1 and KLRK1. It has also been suggested that they might mediate killing of epithelial cells (9, 68-71). The NK-receptors mediate either inhibitory or activating signals upon binding to the corresponding ligand on the target cells. Strong signaling of the activating receptor can lead to release of cytotoxic effector molecules from the cytoplasmic granule and consequent enforcement of apoptosis of the target cells. To estimate the cytotoxicity and the NK-receptors involved in the epithelial reaction of CD, we analysed 13 cytotoxicity related gens in CD3+IELs and IECs of CD patients and controls. The results showed that 11 cytotoxic genes were significantly up-regulated in CD3+IELs of CD patients compared to controls. Of these were 5 cytotoxic effector molecules, i.e., GZMA, NKG7, PRF1, GZMB, GXMH, 3 cytotoxicity activating receptors, i.e., KLRK1, KLRD1, FASL, and 2 receptors that inhibit cytotoxicity, i.e., KIR3DL1 and KLRB1. Notably, 6 ligands were also up-regulated in CD3+IELs, i.e. MICB, HLA-E, FAS, HLA-B, and HLA-A, and HLA-G. In contrast, the results from IECs showed significantly up-regulation of only 2 ligands, i.e., HLA-E and HLA-C. Unexpectedly, IECs expressed and showed significant up-regulation of one cytotoxicity activating receptor, i.e., KLRC2. These findings underscore and markedly extend the previous findings regarding the cytotoxicity in IELs of CD patients with active disease. Particularly interesting is the finding that IELs rather than IECs seem to be the main targets. The IELs expressed high levels of mRNA for the molecules involved in both of the two major cytotoxicity pathways, i.e., granzyme/perforin mediated and Fas/FasL mediated killing. In addition the CD3+IELs seem to express both activating and inhibitory NK-receptors. Activating NK-receptors probably act as a co-receptors that enhance the TCR-dependent cytotoxicity in activated CTLs and γδIELs. The simultaneous expression of inhibitory NK-receptors is intriguing. It might reflect an attempt to limit cytotoxic activity of IELs or to direct the cytotoxic activity towards defined IEL-subsets. Interestingly, the IELs also expressed both FAS and FASL, suggesting on-going ”activation induced cell death (AICD)” that is one of the mechanisms by which immune responses are shut-down. This result probably reflects that CD3+IELs mediate apoptosis of other CD3+IELs most likely to limit the immune reaction of cells producing pro-inflammatory cytokines, such as IL-17A and IFN-γ.
Taken together, in Paper 4, we identified several of the immune modulating molecules that might be involved in the pathogenesis of CD. Several of the chemokines, chemokine receptors as well as cytotoxicity genes and ligands were expressed at significantly higher levels in CD3+IELs and IECs of CD patients compared to controls. This suggests that parallel activation and communication between cells within the epithelium that either enforce apoptosis and/or recruits more immune cell to the site of inflammation.

4.3 Impact of environmental factors

The pathogenesis of CD is still unknown and it is now considered a multifactorial disease. The genetic association to the HLA-DQ2/DQ8 alleles and gluten consumption are necessary for the contraction of CD but not sufficient to explain the whole etiology. Several different environmental factors have been associated with CD in addition to a gluten-containing diet, such as dysbiosis of the microflora (77, 79), early repeated infections (105), and altered components of the epithelial lining (108). One important period in infants is during weaning and the introduction of gluten-containing food. Introduction of gluten before six months of age and under the protection of breastfeeding is considered a factor for decreased risk of developing CD (112).

High levels of cytokines and chemokines in the small intestinal mucosa as a consequence of an adverse immune response against gluten but also other environmental factors might be a risk, e.g., the microflora or cross-reactivity to similar prolamine-rich peptides similar to gliadin in the diet.

4.3.1 Risk factors of the resident microbiota in CD patients

In Paper 2, we tried to delineate which component caused the increased IL-17A response in CD patients and compared the expression to that of IL-10. An ex vivo challenge of biopsies from treated CD patients was performed. The biopsies were challenged with either gluten digest, a mixture of Prevotellas, L. umeaense and A. graevenitzii bacteria isolated from the proximal jejunum of CD patients, or a combination of both gluten digest and CD-associated bacteria. The result showed that for the majority of patients, cytokine levels of both IL-17A and IL-10 mRNA were increased in the biopsy tissue challenged with gluten digest (Table 4). This simultaneous increase, of both IL-17A and IL-10 seen in the majority of cases, probably reflects a compensatory reaction to the inflammatory response in the mucosa, whereby Tregs work to balance the immune system. In addition, the
challenge with the CD-associated bacteria alone caused up-regulation of IL-17A in half the cases and up-regulation of IL-10 in the majority of cases (Table 4). This indicates that the CD-associated bacteria alone are sufficient to induce both IL-10 and IL-17A in CD patients without the influence of gluten. The challenge experiment with the combination of gluten and the mixture of CD-associated bacteria caused up-regulation of IL-17A in the majority of cases and of IL-10 in half the cases (Table 4). The response pattern in the biopsies challenged with the combination of gluten and CD-associated bacteria showed two different patterns. Either there was enhanced IL-17A response together with a suppressed IL-10 response, or the opposite occurred, with a suppressed IL-17A response together with an enhanced IL-10 response. The pattern revealed two groups based on the response of IL-17A to gluten. This grouping of patients showed that children with an enhanced IL-17A response toward gluten alone had a poor IL-17A response to the combination of gluten and CD-associated bacteria. Interestingly, these patients were all born during the Swedish CD epidemic. The other patients that had a poor IL-17A response to gluten and an enhanced IL-17A response to the combination of gluten and CD-associated bacteria were all born after the Swedish CD epidemic. One possibility might be that the children born during the epidemic has an altered microflora with more adherent rod-shaped bacteria (77, 108, 109). The fact that the bacteria used in the mixture were isolated from the mucosa of CD patients born during the Swedish CD epidemic is interesting in this respect. The suppressed IL-17A response against the combination of gluten and CD-associated bacteria seen in patients born during the epidemic might be due to the fact that these patients developed a tolerance against the bacteria.

Table 4. Ex vivo challenge of small intestinal biopsies of treated CD patients with gluten digest and CD associated bacteria caused increased expression of IL-17A and IL-10 mRNAs

<table>
<thead>
<tr>
<th>Stimulant:</th>
<th>IL-17A</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluten digest</td>
<td>6/8†</td>
<td>6/8</td>
</tr>
<tr>
<td>CD associated bacteria</td>
<td>4/8</td>
<td>6/8</td>
</tr>
<tr>
<td>Gluten digest plus CD associated bacteria</td>
<td>6/8</td>
<td>4/8</td>
</tr>
</tbody>
</table>

† Number of biopsies giving an mRNA level above the sham-treated medium control upon challenge with the indicated stimulant over the total number of biopsies subjected to the indicated challenge. Adapted from paper 2.
This indicates that both gluten and the CD-associated bacteria play important roles in the induction and secretion of IL-17A, seen in patients with active CD (Paper 2) (50). The increased level of IL-10 is probably due to a compensatory response to the increased IL-17A in order to mediate homeostasis and establish tolerance either against the gluten or the microflora.

In Paper 2, we also analyzed the cellular source of IL-17A from biopsies challenged with the combination of gluten and CD-associated bacteria. The expression levels of IL-17A in isolated γδ+IELs, CD4+IELs (Th17) and CD8+IELs (Tc17) revealed that Th17 cells are responsible for most of the IL-17A produced in challenged biopsy tissue. This indicates that the increased population of Th17 cells seen in patients with active CD might be involved in the pathogenesis of CD, since IL-17A is produced in the challenge experiments. One possibility is that the Th17 cells are the initial source of IL-17A at each gluten challenge and the Tc17 cells are activated thereafter.

One of the bacteria in the mixture was a L. umeaense isolate. L. umeaense is a spore-forming, SFB adherent to the intestinal epithelia of children with CD. The L. umeaense are related to the previously known SFB, Candidatus arthromitus, of the commensal microbiota that was shown to induce Th17 in the small intestines of mice. The two SFBs belong to the same family, Lacnospiraceae, but different genera and species. It is possible that L. umeaense is the human counterpart to the mouse Candidatus arthromitus, as a potent inducer of Th17 cells in the small intestinal mucosa (85).

Interestingly, previous studies suggest an important role for the composition of microbiota in establishing both homeostasis and regulation of the immune system as well as establishing the T-cell populations in the LP of the small intestine. In mice with a SFB-containing microbiota, the numbers of Th17 and Foxp3+ cells are in equal proportion and the immune system is in balance. On the other hand, in germ-free mice, in which Th17 cells are absent, Foxp3+ cells are increased (83). This indicates the importance of the composition of the microbiota, both in regulation of the T-cell populations as well as in influencing the immune system and tolerance. Another study in mice showed that Tregs can be induced by the presence of species belonging to clades XIVa and IV of the genus Clostridium. The Clostridium activated IECs to produce TGF-β1 and IDO that are implicated in the induction of Tregs in the large intestine (86). Interestingly, L. umeaense is also related to the Clostridium XIV clade (109). This suggests that this bacterium might play a role in the establishment of the immune system in the intestine.
4.3.2 Risk factor of supplementing a GFD with oats

Today, there is no cure for CD and the only treatment is a lifelong adherence to a strict GFD. The exclusion of wheat, barley and rye in the diet is sometimes difficult to achieve, and patients are at risk of inadequate intake of fiber, vitamins and minerals. Several studies have investigated the possibility to supplementing the standard GFD with oats, since the grain has a desirable nutrient content. Until now, studies regarding oats as a complement to a GFD have shown contradictory results, in both adults and children, regarding clinical parameters and histology. The overall conclusion from the previous studies is that most of the CD patients tolerate oats in their GFD. Although a small subgroup of CD patients consuming oats have shown inadequate normalization of clinical parameters and histology during the study period. In addition, most of the previous studies estimated clinical parameters and mucosal recovery and were not designed to gain a deeper understanding of the immune system of CD patients consuming oats.

In Paper 3, to understand the local immune status in the small intestines of CD patients consuming oats, we investigated the immune response in a subgroup of children in a randomized, double-blinded study that indicated tolerance to oats based on autoantibody titers and small intestinal histology (129). Paired small intestinal biopsies, before and after > 11 months on a GFD, were collected from children with CD enrolled in the trial to either of two study-groups, standard GFD (GFD-std) and oat-containing GFD (GFD-oats). A total of 22 different mRNAs for immune effector molecules and tight junction proteins used as indicators of ongoing mucosal inflammation were determined by quantitative RT-PCR.

First, we investigated some of the known cytokines involved in the pathogenesis of CD, i.e., IFN-γ, IL-17A, IL-10 and TGF-β1, as an estimation of Th1, Th17, Tc17 and Treg activity in both study groups. The results showed that both IL-17A and IFN-γ mRNA correlated with the disease activity and were elevated in untreated CD patients and declined after GFD. This indicates that the Tc17, Tc1 and Th17 activity in untreated CD patients was normal and that decline regardless of having oats in the GFD. This idea is in line with our results showing that the increased IL-17A level is caused by gluten alone or in combination with resident, CD-associated bacteria, as well as the study by Ivanov et al. (83) showing increased IL-17A in mice after colonization with Candidatus arthromitus. The expression of IL-10 and TGF-β1 mRNA were both higher than in controls before GFD in both groups. Expression of both mRNAs were reduced only in patients belonging to the study group on standard GFD and not in patients receiving an oats-containing GFD for one year. This probably reflects the fact that patient
consuming oats still have immune activity in the mucosa, and that the Tregs are fairly successful at suppressing inflammatory responses.

As an estimation of the chemokines involved in the inflammation of CD, we analyzed CXCL8, CXCL9, CXCL10, CXCL11 and CX3CL1 as well as the CXCL9-11 receptor CXCR3. The results showed that all chemokines followed the disease activity, i.e., were increased before GFD and were fully normalized in both study groups after one year. The receptor CXCR3 was expressed at low levels, the same as controls. Together, these results indicate that the recruitment of immune cells by the chemokines is disrupted after the introduction of GFD, regardless of supplementation with oats.

To estimate the potential activity of cytotoxic cells in CD patients on an oat-containing GFD, the activating receptors, KLRC2 and KLRC3, were analyzed in the two study groups. The results showed that the mRNA expression of both KLRC2 and KLRC3 were increased during active CD. After one year on GFD, the expression levels had declined significantly in the study group that consumed standard GFD, but not in the GFD-oats group. Interestingly, the levels of KLRC3 were still significantly higher in CD patients after one year of GFD than in the controls in both groups (\( P=0.04 \) and \( P=0.03 \) for GFD-oats and GFD-std, respectively). In addition, the expression of KLRC3 was reduced in all but one patient in the GFD-std group, while only half the patients in the GFD-oats group showed reduced expression after a year on a GFD. This indicates that KLRC3 participates in the active stage of the disease and starts to decline slowly after introduction of a GFD. However, there were fewer patients who consumed oats compared to those who consumed the standard GFD whose immune status normalized; they still had a high level of activity of cytotoxic T-cells, in the small intestine, which can cause cytotoxicity of stress-induced epithelial cells in CD patients.

One feature of CD is increased permeability of the epithelium, which permits increased transport of gluten peptides over the epithelium (138). Increased amounts of tight junction proteins in CD suggest there might be structural changes due to the increased IFN-\( \gamma \) that weakens the tight junction structure. Levels of mRNAs for the tight junction proteins claudin-4 as well as occludin were compared in the two study groups, i.e., standard GFD and GFD supplemented with oats. The results showed that claudin-4 was expressed on average at a higher level in active CD than in controls, and declined significantly after one year with a standard GFD (\( P=0.0002 \)), but not after one year of a GFD supplemented with oats (\( P=0.65 \)). In addition, the claudin-4 level in all patients in the GFD-std group had normalized, while the same was true in only 30% of the patients in the GFD-oats group.
The expression level of occludin did not increase significantly in active CD and the two study groups showed similar results at the end of the diet period. This indicates that the epithelium is altered in patients consuming oats. These changes could be confined to the epithelial cells. However, there is also a possibility that DCs in the lamina propria, known to have the capacity to express tight junction proteins, can extend through the epithelium. The incorporated DCs can then face the luminal side and sample antigen and possibly induce an inappropriate immune response (61).

Overall, the immune status of the GFD-std and GFD-oats study groups, assessed by the number of normalized mRNA species, differed significantly after one year on a GFD ($P=0.03$). Almost all patients who consumed GFD supplemented with oats still had two or more mRNA species that were unchanged or even elevated after one year. In contrast, only half of the patients in the GFD-std group had two or more mRNA species that were unchanged or even elevated after one year. Of these, one had clinical parameters indicating active disease, i.e., not fully compliant to the GFD. The most pronounced differences were seen in the mRNA expression levels of claudin-4 and KLRC3. All patients, except for one in the GFD-std had normalized levels of claudin-4 and KLRC3. In contrast, seven patients remained high for both claudin-4 and KLRC3 mRNAs in the GFD-oats group and one patient in this group remained high for claudin-4 only ($P=0.04$ and 0.003 for KLRC3 and claudin-4, respectively).

Several studies have reported that patients consuming an oat-containing GFD had normal mucosal histology and normal titers of tTG and EMA antibodies, and that they had recovered symptomatically (125, 128, 129). However, others have reported that subgroups of CD patients did not seem to tolerate oats with regard to increased IEL counts, unrecovered mucosal histology and demonstration of avenin-reactive T-cells (124, 132, 133). This study reveals that a subgroup of CD patients who consumed oats still had an increased level of activating NK-receptors (KLRC2 and KLRC3) that may possess cytotoxic activity to stressed target cells in the epithelium. These patients also had high levels of IL-10 and TGF-β1, suggesting that Treg activity is required to dampen ongoing inflammation. One possibility is that cross-reactive T-cells against gliadin, hordein or secalin peptides are also reactive against the more distantly related prolamin avenin in oats. This suggests that in a subgroup of CD patients consuming oats, these immune markers do not normalize; these patients may not tolerate oats due to cross-reactive T-cells. It may be possible to discriminate between CD patients who tolerate oats and those who do not by determining the levels of mRNAs from claudin-4, KLRC3, and IL-10—in both the first biopsy on suspicion of CD and in the second biopsy after one year on a GFD with oats.
4.3.3 Immune response of the small intestinal mucosa in children with celiac disease and the impact of two environmental factors, resident microbiota and oats

In this thesis, the immune status and the interplay of T-cells and Tregs in the mucosa of children with active CD was investigated. The conclusions from these studies revealed that CD patients with active disease have increased levels of pro-inflammatory cytokines, chemokines and cytotoxicity genes, both cytotoxic effector molecules and receptors that trigger cytotoxicity, that together promote inflammation in the epithelium. In contrast, Treg activity seems to be normal in CD patients, but not sufficient to down-regulate the inflammatory reaction. This might be explained by the findings that CD patients showed impaired capacity to edit and rearrange their TCR in the epithelium, with possibly incorrect TCR specificity to the local antigen milieu.

The immune status in treated CD patients provoked with either dietary oats or microbiota or gluten were also investigated. The conclusions from these studies revealed that CD patients responded with increased levels of IL-17A to both gluten and CD-associated bacteria. In addition, a subgroup of CD patients consuming oats, immune markers do not normalize; these patients may not tolerate oats due to cross-reactive T-cells.
5 Conclusions

- Simultaneous expression of RAG1 and preTα mRNAs in IELs with the phenotype of immature T cell lineage cells (CD2⁺CD7⁻TCR⁻) in controls suggests that extrathymic T cell maturation normally occurs in the small intestinal epithelium of children.

- We found that the IELs with a more mature phenotype, ie, αβIELs and γδIELs also expressed RAG1 mRNA suggesting ongoing TCR gene rearrangement and TCR specificity editing.

- Children with CD, both in active and treated disease, have significantly decreased expression of both RAG1 in all three IEL-subtypes and of preTα mRNA in immature T cells, suggesting an inherited reduced extrathymic T cell maturation accompanied by decreased TCR editing and/or revision. Taken together these results suggest that the reduced ETCM maybe a risk factor for CD by inadequate generation of T-cells important for tolerance and homeostasis.

- IL-17A response was shown to be a characteristic feature of the inflamed small intestinal mucosa of patients with active CD. The IL-17A response follows the disease activity and returns to the level of controls in CD patients on a GFD. Both Tc17 and Th17 cells contributed significantly to the IL-17A response in patients with active CD. Tc17 cells are mainly present in the epithelium and we showed that they are responsible for most of the IL-17A production in this compartment.

- Both gluten and CD associated bacteria provoked an IL-17A response in the intestinal mucosa of CD patients with inactive disease. The CD associated bacteria influenced the magnitude of the IL-17A response to gluten, either enhancing or suppressing. Suggesting that CD associated bacteria play an important role in both breaking the tolerance and determining the severity of the reaction against gluten, with the consequence that the individual might develop CD. This notion was supported by the finding that children born during the "Swedish CD epidemic", when CD associated bacteria were commonly seen adhering to the small intestinal mucosa of CD patients, showed a very high IL-17A.
response against gluten whereas children born after the epidemic showed a weak response

- The IL-17A response to gluten was suppressed by the addition of CD associated bacteria in children born during the epidemic. While addition of the CD associated bacteria during challenge with gluten showed an enhanced IL-17A response in children born after the epidemic. This phenomenon might be explained by the presence of CD associated bacteria in the resident microflora of children born during the epidemic, who might have of generated Tregs directed against CD associated bacteria

- In a fraction of CD patients the immune status in their small intestinal mucosa did not normalize after consumption of an oat-containing GFD for one year

- Marked immune status alterations in CD patients on an oat-containing GFD were elevated levels of mRNA for the anti-inflammatory IL-10 and TGF-β1, the cytotoxicity-activating NK-receptors KLRC2 and KLRC3, and the tight junction protein claudin-4

- KLRC3 and claudin-4 appear to be good markers for identification of CD patients sensitive to dietary oats since levels of these mRNAs normalized in all but one patient on standard a GFD but only in approximately 50% of those on a GFD supplemented with oats

- The inflammatory reaction in the epithelium of CD patients was accompanied by increased expression of an arsenal of chemokines and corresponding receptors, produced mostly by the CD3+IELs, although the IECs expressed additional chemokines. This findings suggest that IELs are actively participating in the recruitment of immune cells, including IELs, to the inflamed mucosa

- Parallel activation of several immune activating and inhibitory molecules in the mucosa of CD patients suggests for a role of immune protection by CD3+IELs that try to down-regulate the activation of immune cells while IECs attempts to enforce these by recruitment of more IELs
6 Acknowledgements

Det är många personer som varit delaktiga och hjälpt och stöttat mig under de år jag doktorerat. Till er vill jag rikta ett stort tack och speciellt till:


Professor Olle Hernell, min bihandledare. Tack för ditt intresse och stöd i projektet. Jag uppskatar verkligen ditt engagemang och de många goda idéer under tiden jag doktorerat.

Professor Sten Hammarström, medförfattare. Tack för ditt engagemang och intresse i mitt projekt. Jag uppskatar verkligen dina många goda råd och idéer som hjälpt mig många gånger.

Olof Sandström, medförfattare. Tack för ett bra samarbete och för den stora hjälpden den kliniska delen av mitt projekt.

Anne och Marianne. Tack för er ovärderliga hjälp om alla metoder. Min tid som doktorand hade varit mycket jobbigare utan er hjälp.

Lena. Tack för din hjälp under dessa år och för din alltid så vänliga inställning.


Mia och Lina. Det har varit väldigt roligt att lära känna er som vänner och som medarbetare. Tack för många roliga stunder på och utanför jobbet.

Alla kollegor på Klinisk Immunologi. Tack Ann-Christin, för trevliga stunder på och utanför labbet. Thank you Lucia, Eva och Vladimir for good discussions and for your knowledge in science.
Tack till alla i forskarskolan forum GIMIICum. Det har varit mycket givande forskningsmässigt men även privat att lära känna er. Tack för bra samarbeten och roliga träffar.

Tack till mina vänner i Umeå, för roliga stunder och vänskap.


Sist men inte minst min ”lilla familj”, Tomas, min bästa vän, tack för ditt oändliga stöd och för att du alltid trott på mig. Elsa, tack för att du finns och att du fick mig att tänka på annat under tiden jag skrivit denna avhandling.

This work was financially supported by grants from Swedish Research Council, Natural Sciences and Engineering Sciences, the TORNADO-project within the 7th framework program theme, the Fund for Biotechnology-oriented Basic Science at Umeå University, the Oskar’s Fund, the Kempe Foundation, ”Insamlingsstiftelserna” at the Medical Faculty of Umeå University, and PhD-study support to V. Sjöberg from the Medical Faculty of Umeå University.
References


122. Catassi, C., M. Rossini, I. M. Ratsch, I. Bearzi, A. Santinelli, R.
Castagnani, E. Piani, G. V. Coppa, and P. L. Giorgi. 1993. Dose
dependent effects of protracted ingestion of small amounts of gliadin
in coeliac disease children: a clinical and jejunal morphometric

123. Catassi, C., E. Fabiani, G. Iacono, C. D’Agate, R. Francavilla, F. Biagi,
U. Volta, S. Accomando, A. Picarelli, I. De Vitis, G. Pianelli, R.
Gesuita, F. Carle, A. Mandolesi, I. Bearzi, and A. Fasano. 2007. A
prospective, double-blind, placebo-controlled trial to establish a safe
gluten threshold for patients with celiac disease. *Am J Clin Nutr* 85:
160-166.


126. Kurppa, K., O. Lauronen, P. Collin, A. Ukkola, K. Laurila, H.
Huhtala, M. Maki, and K. Kaukinen. 2012. Factors associated with
dietary adherence in celiac disease: a nationwide study. *Digestion*

127. Janatuinen, E. K., T. A. Kemppainen, R. J. Julkunen, V. M. Kosma,
M. Maki, M. Heikkiläin, and M. I. Uusitupa. 2002. No harm from

comparison of diets with and without oats in adults with celiac

129. Hogberg, L., P. Laurin, K. Falth-Magnusson, C. Grant, E.
Grodzinsky, G. Jansson, H. Ascher, L. Browaldh, J. A. Hammersjo,
with newly diagnosed coeliac disease: a randomised double blind

130. Hollen, E., K. Holmgren Peterson, T. Sundqvist, E. Grodzinsky, L.
Hogberg, P. Laurin, L. Stenhammar, K. Falth-Magnusson, and K. E.
Magnusson. 2006. Coeliac children on a gluten-free diet with or
without oats display equal anti-avenin antibody titres. *Scand J
Gastroenterol* 41: 42-47.


