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Innate and Adaptive Immunity in Childhood Celiac Disease

by

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Cover: Scanning electron micrographs
Front page: Rod shaped bacteria in jejunal biopsies in small intestine.
Back page: Normal small intestine and subtotal villous atrophy in small intestinal mucosa.
All micrograhps kindly provided by Per Hörstedt
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Till Mina Barn

Det spricker upp….

ABSTRACT

Celiac disease (CD) is an inflammatory small-bowel enteropathy caused by a permanent intolerance to wheat gluten and related proteins in rye and barley. Even though the disease originate from the small intestine the clinical symptoms varies in affected individuals and are often different in small children compared to adolescents and adults. Susceptibility to develop the disease is strongly associated with certain genetic factors i.e. HLA-DQ2/DQ8 but it is undoubtedly that additional inherited and environmental factors are involved.

As specific T-lymphocyte reactions are central in the pathogenesis of CD, six key cytokine messenger RNA levels in intestinal intraepithelial and lamina propria T lymphocytes (IEL, LPL), retrieved from small intestinal biopsies, were determined by using quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). Levels of cytokines, small secreted proteins which mediate and regulate immunity, in children with active disease were compared with that of treated children and controls. Interferon (IFN)-γ and interleukin (IL)-10 were also determined at the protein level by immunohistochemistry. Active celiac disease was characterized by distortions in cytokine expression, with highly significant increases of IFN-γ and IL-10 but no concomitant increases in tumor necrosis factor α (TNF-α), transforming growth factor β1 (TGFβ1), or IL-2 and no induction of IL-4. A marked shift of IFN-γ and IL-10 production from LPLs to IELs was characteristic of active celiac disease, and as many as one fourth of the IELs expressed IFN-γ. IELs in treated, symptom-free celiac patients still had increased IFN-γ levels compared with controls. In CD, gluten intake seems to cause an overreaction in IELs, with uncontrolled production of IFN-γ and IL-10 which may cause both recruitment of more IELs and a leaky epithelium, leading to a vicious circle with amplified immune activity and establishment of the intestinal lesion. In order to determine different IEL subsets contribution of the produced cytokines, γδIELs, $CD4^+$αβIELs, and $CD8^+$αβIELs as well as $CD94^+CD8^+$αβIELs and $CD94^-CD8^+$αβIELs of children with active CD and children with no food-intolerance were analyzed for cytokine mRNA expression levels by RT-PCR. In active CD, $CD8^+$αβIELs had the highest expression levels of IFN-γ- and IL-10 mRNA and constituted the cellular source for almost all IFN-γ and a large fraction of the IL-10. Expression levels of these two cytokines correlated and were higher in $CD94^-CD8^+$αβIELs than $CD94^+CD8^+$αβIELs. $CD4^+$αβIELs had the highest expression levels of TNF-α and despite the small number of this cell subset they contributed with half of the small amounts of this cytokine. Interestingly, TNF-α levels correlated with IL-10 in $CD4^+$αβIELs. γδIELs had the lowest expression levels of IFN-γ, TNF-α, IL-10, and TGF-β1. Essentially no IL-2 mRNA was detected in the three IEL subpopulations. "Classical" $CD8^+CD94^-$αβT cells in the epithelial compartment are responsible for most of the excessive production of proinflammatory IFN-γ. The question whether an impaired extrathymic T cell maturation and/or capacity for secondary T cell receptor (TCR) gene recombination in iIELs is a contributing factor to CD was addressed. Expression levels of recombination activating gene-1 (RAG1) and the pre T α-chain (preTα) mRNAs were determined in IEL T cell lineage subsets of children with CD and controls. In controls, RAG1 was expressed in both mature ($TCR\gamma\delta^+$ and $TCR\alpha\beta^+$) and immature ($CD2^+CD7^+TCR^-$) IELs while preTα was expressed preferentially in immature IELs. The RAG1 splice form selectively expressed outside thymus (RAG1 1A/2) as well as preTα were significantly decreased in CD patients both in active and inactive disease suggesting a deteriorated capacity of *de novo* TCR gene rearrangement in local T cell development and / or of secondary TCR gene rearrangement during editing or antigen-driven revision. This may lead to an imbalance between thymus- and gut derived T lymphocytes in the intestinal mucosa with consequent inefficient regulation of T cell responses against food antigens.

Innate or nonspecific immunity is the first line, immediate defense against pathogens mediated by the epithelial cells in the intestine (IECs). As certain adaptive immune reaction in CD mimics that of intestinal infections, aberrant innate immune reaction could be a contributing factor to CD. Therefore jejunal biopsies were screened for bacteria and the innate immune status of the epithelium was investigated. Bacteria were freqently (40%) associated with the mucosa of children with active but also treated disease (20%) compared to controls (2%).

Lack of antimicrobial factors such as mucins, proteins forming protective biofilm on the IECs, defensins and lysozym, peptides and enzymes with antibacterial effects, could not explain the presence of bacteria. If anything, mucin-2 (MUC2), α-defensins, HD-5, HD-6, and lysozyme mRNA levels were increased in epithelial cells in active CD, returning to normal levels in treated CD. Their expression levels correlated to the IFN-γ mRNA levels in IELs. Analysis of beta defensins, hBD-1 and hBD-2 as well as carcinoembryonic antigen (CEA) cell adhesion molecule 1a (CECAM1a), glycoproteins in the glycocalyx with ability to bind micro organisms, were not affected by the disease. Lectin staining by histochemistry revealed that goblet cells were stained by UEA1 in CD both active and treated but not in controls. The opposite pattern was seen for the lectin PNA where staining was seen in controls in the glycocalyx layer but not in CD. Thus altered glycocalyx/mucous layer may promote bacterial adhesion in CD.

ABBREVIATION LIST

AGA	anti-gliadin antibodies
APC	antigen presenting cell
BCR	B cell receptor
CD	celiac disease
DC	dendritic cell
EMA	anti-endomysium antibodies
FAE	follicle-associated epithelium
Fas	Fast apoptosis stimulating protein
GALT	gut associated lymphoid tissue
GFD	gluten-free diet
hBD	human beta defensin
HD	human alpha defensin
HNP	human neutrophil peptide
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
kDa	kilo Dalton
IL	interleukin
LP	lamina propria
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
MHC	major histocompatibility complex
NK	natural killer cell
NKT	natural killer T cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PP	Peyer's patch
PRR	pattern recognition receptor
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RAG	recombination activating gene
Tc	cytotoxic T lymphocyte
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TGF	transforming growth factor
Th	helper T cell
TNF	tumor necrosis factor
Treg	regulatory T cell
tTG	tissue transglutaminase
UTR	untranslated region

PAPERS IN THIS THESIS

The thesis is based on the following papers:

I. **Göte Forsberg**, Olle Hernell, Silvia Melgar, Anne Israelsson, Sten Hammarström, and Marie-Louise Hammarström**.** Paradoxical coexpression of proinflammatory and down-regulatory cytokines in intestinal T cells in childhood celiac disease. *Gastroenterology* 2002, 123:667-678.

II. **Göte Forsberg**[1], Anna Fahlgren[1], Per Hörstedt, Sten G. Hammarström, Olle Hernell, and Marie-Louise K. C. Hammarström. Presence of bacteria and innate immunity of intestinal epithelium in childhood celiac disease. *Am J Gastroenterol* 2004, 99: 894-904.

III. **Göte Forsberg,** Olle Hernell, Sten Hammarström, and Marie-Louise Hammarström. Concomitant increase of IL-10 and proinflammatory cytokines in intraepithelial lymphocyte subsets in celiac disease. *Submitted*

IV. Anna Bas**, Göte Forsberg**, Olle Hernell, Sten Hammarström, and Marie-Louise Hammarström. Aberrant extrathymic T cell receptor gene rearrangement in the small intestinal mucosa - a risk factor for celiac disease? *Submitted*

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INTRODUCTION

CELIAC DISEASE

Celiac disease (CD) is a chronic small intestinal enteropathy, in its classical form in infants and young children resulting in a typical malabsorption syndrome with diarrhoea and failure to thrive. It is partly genetic and is caused by an inappropriate immune response to gluten, a storage protein in wheat, and related proteins in barley and rye. It affects at least 1% of the population in the Western world, but there is evidence to suggest that the majority of affected individuals are undiagnosed since they have mild and diffuse symptoms, which do not primarily evoke suspicion on CD. Life long adherence to a gluten free diet (free of wheat, barley and rye) is the only effective treatment so far. Both innate and adaptive immune reactions seem to take place in the small intestinal mucosa in active CD.

Historic background

Dr Samuel Gee's presentation "On the Coeliac affection" in 1888, where he described a distinct type of diarrhoea and malnutrition in children is considered to be the first relevant description of CD. However the Swedish professor, Nils Rosén von Rosenstein, often considered the founder of modern paediatrics already 1764 in his paediatric textbook, "The diseases of Children and Their Remedies", described a variant of childhood diarrhoea "Fluxus coeliacus" which seems to fulfil the criteria for classical CD. The Dutch paediatrician Willem Karel Dicke noticed that during the Dutch famine in 1944-1945 patients with CD did better when wheat products were replaced by non-cereal food. In his thesis Dicke proposed wheat gluten to be the cause of the disease (1). The first report of a small intestinal biopsy showing the characteristic subtotal villous atrophy, or flat mucosa in a child with CD was published in the Lancet 1957 (2).

Clinical features

Even though the disease originates from the small intestine the clinical symptoms varies in affected individuals and are often different in infants and young children compared to adolescents and adults (3-5). In infants and young children the classical symptoms *i.e.* chronic diarrhoea, malabsorption, retarded growth, poor appetite, abdominal distension and unhappy

behaviour dominate. In older children CD more often presents itself as recurrent abdominal pain, diarrhoea or constipation, anaemia due to iron deficiency and pubertal delay (4) In adult CD, osteoporosis is a complication even in "silent" CD, *i.e.* CD without obvious or even absence of clinical symptoms, but with elevated serological markers and intestinal lesion when on a gluten-containing diet (6, 7). Unfavourable outcome of pregnancy due to untreated CD has also been reported (8-10). Fatigue is a common finding in adults with CD (11) and psychiatric symptoms are also overrepresented in these patients (12). There is a widely spread misunderstanding that CD always results in poor weight gain but in an adult population with CD in the USA obesity was seen in 27% of the patients (13). With increasing awareness of the disease and more patients detected by screening the typical presentation of CD has changed from the majority of diagnosed cases being infants and young children with classical symptoms, to the majority now being older children and adults with non-gastrointestinal, "atypic" disease (14).

Epidemiology

In 1973 the highest reported incidence of CD was 1/650 in western Irland (15). In the mid 1980-ies CD suddenly became one of the most commonly chronic diagnosed diseases in Swedish children with a cumulative incidence of around 4 per 1000 live births, which was the highest incidence reported thus far in the world. Most of the cases were children < 2 years of age with classical symptoms. After a little more than a decade the incidence rate rapidly declined to the previous level (16-18). Thus, the CD incidence rate curve resembled a typical pattern for an epidemic rather than a pattern seen for other immunological diseases which also have increased during the last decades, *e.g.* type 1 diabetes mellitus (19) and allergic diseases (20). A similar rise and fall in incidence was not seen in other countries. For instance, despite the geographic location close to Sweden an average incidence rate of less than 0.1 per 1000 live birth was reported in Denmark during the peak of the Swedish "epidemic" (21, 22).

In 1994 the first study from USA revealed that the incidence rate of CD was 1.2 per 100,000 person-years and the estimated prevalence in 1991 was 21.8 per 100,000 (0.02%) (23). Recent screening studies have now shown that CD is far more common than previously thought and the prevalence is believed to be close to 1% in the Western population including the U.S.A (24-29). However in the general population the majority of adults with CD are believed to be undiagnosed (30).

There seems to be some ethnical or cultural differences influencing the prevalence of CD. In a screening of an Estonian population of 1461 individuals not a single case of CD was found.

(31), while as many as 55 of 989 of Saharawi children (5.6%) had CD as suggested by elevated levels of IgA antibodies against endomysium (EMA) in their serum. Sixteen of these children underwent a small intestinal biopsy, verifying the diagnosis in all of them (32). It also seems that the early feeding pattern may affect the risk of contracting CD (17, 33).

Genetic predisposition

The risk of CD among relatives has been estimated to about 10% in several studies (34). However in a study from the U.S.A. the prevalence among offspring and first- or second-degree relatives was found to be as high as 15-21% (35). The concordance rate of CD in monozygotic twins is approximately 70% (34). In 1972 it was first reported that the risk of contracting CD is associated with carrying the major histocompatibility complex (MHC) class II alleles for HLA-DQ8 (DQA1*0301/DQB1*0302) (36). Four years later the stronger linkage to HLA-DQ2 (DQA1*05/DQB1*2) was reported (37, 38). More than 90 % of patients with CD carry HLA-DQ2, and the rest with few exceptions DQ8 (39, 40). In a European population, only 61/1008 (6%) of patients with CD carried neither the DQ2 nor the DQ8 heterodimer but of these, 57/61 (93%) carried one of the alleles for the DQ2 heterodimer and only 4/1008 CD did not carry any DQ8 or DQ2 allele (41).

There are two variants of the HLA-DQ2 heterodimer that bind gluten peptides and consequently can present them to T cells, *i.e.* (DQA1*0**5**01, DQB1*0**2**01) named HLA-DQ2.5 and (DQA1*0201, DQB1*0201) named HLA-DQ 2.2 (42, 43). Being homozygous for HLA-DQ2.5 or having HLA-DQ2.5/2.2 increases the risk for contracting CD while having HLA-DQ2.5/non 2.2 results in only slight risk to contract CD and having HLA DQ2.2 without DQ2.5 does not result in any increased risk for CD (39, 44-46). The HLA-DQ2.5 molecule is able to bind a large number of different gliadin peptides while HLA-DQ 2.2 only binds a subset of these (42). These genetic differences may support a dose-dependency, i.e. that the dose of gluten used when gluten is first introduced into infants´ diet is a risk factor for contracting the disease (17, 33). However, not all individuals with these HLA-DQ alleles develop CD if exposed to gluten. Additional genetic factors [susceptibility regions 5q31-33, 11q and 19p13.1 have been proposed (47, 48)] and environmental factors are required (33). Interestingly, the MICA-A5.1 allele is also associated with susceptibility for CD (49). This allele produces a soluble form of the ligand for the NKG2D-receptor MICA, which is also suggested as a target molecule for mucosal γδ T cells. Recent studies have demonstrated that the 19p13.1 association involves a myosin IXB variant pointing towards a primary intestinal

barrier defect (50). There is also an unexplained gender difference in the risk of contracting the disease, where twice as many girls are affected compared to boys (51).

Diagnostic procedure

An inflammatory lesion in the upper small intestinal mucosa characterized by increased frequency of intraepithelial lymphocytes (IELs), together with various degrees of small intestinal villous atrophy and crypt hyperplasia is a hallmark of untreated CD (52). However, immunological reactions can also be found in the lower intestine and extra-intestinal tissues and organs (53). Biopsies from the terminal ileum of CD patients have exhibited significantly higher frequencies of IEL compared to controls and rectal challenge with gluten shows a rise in mucosal IEL within hours (54). IgA and IgM antibodies are normally produced in the small intestinal mucosa and secreted into the lumen in order to exclude antigen from penetrating the mucosal surface, but are also secreted into the blood. In the celiac mucosa activated B-cells (plasma cells) produce increased amounts of antibodies of the IgA, IgM and IgG isotypes. The use of elevated serum levels of anti-gliadin antibodies (AGA) for identification of the disease was first described in 1958 (55) and for decades (AGA) of IgA isotype became the golden standard in "screening" for CD. However, AGA ELISA tests, particulary for AGA of IgG class have varying sensitivity and specificity depending on the exact method used (30, 56-59).

A more reliable blood test, the anti-endomysial antibody (EMA) test was launched by Chorzelski et al in 1983 (60) and tests were developed for detection of IgA antibodies in serum by immunofluorescence, which now has become the golden standard. In 1997 the enzyme tissue transglutaminase (tTG) was identified as the previously uncharacterized endomysial autoantigen and various ELISA-tests were developed for detection of IgA antibodies to this enzyme. A systematic review reveals fairly high sensitivity (93%) for both the IgA EMA and the IgA anti-tTG tests, particularly when using the human antigen (*rht*TG), and specificities around 98-99% (61). The anti-*rht*TG test has practical and economical advantages when compared to the EMA test (61). A complicating factor in testing for CD is that IgA deficiency prevalence is high and even increased in the celiac population, and both anti-*rht*TG and EMA tests are based on IgA antibodies. In a recently published study from Australia with 254 patients with CD, 3.5 % of children and 10% of adults had partial or total IgA deficiency (62). In cases of IgA deficiency tests based on IgG antibodies can be of value but it is probably safer to exclude IgA deficiency by assessing total serum IgA concentration at the same time as screening for CD by use of serological markers (30).

The European Society for Paediatric Gastroenterology, Hepatolgy, and Nutrition, (ESPGHAN), recommended in 1970 that a three-biopsy procedure should be the standard for the diagnosis of CD; 1) One initial biopsy on suspicion of CD during active disease showing abnormal intestinal mucosa, 2) a second biopsy on gluten-free diet (GDF) showing normalization of the gut mucosa and 3) a third biopsy showing deterioration of the gut mucosa during challenge with gluten-containing diet (63). In 1990 these criteria were revised by ESPGHAN when a large retrospective study on more than 3000 patients showed that the initial diagnosis of CD using multiple biopsies would have been incorrect in just a few per cent had the diagnosis been based only on the first biopsy. The misdiagnosed cases would have been mainly young children with a final diagnose of cow's milk protein enteropathy (64). When an initial diagnose in children was based upon gastrointestinal symptoms alone more than half of the cases turned out to be incorrect by diagnosed when compared to the outcome of small intestinal biopsy (65, 66). Thus, to diagnose CD in typical cases still requires at least one small intestinal biopsy (64). Biopsies can be obtained by duodenal intubation with a capsule, *e.g* a Watson capsule under fluoroscopic control or via upper endoscopic procedure, both being regarded safe without any major risk of complications (67-70). The villous atrophy may be patchy both in children and adults, and more pronounced in the proximal than the distal duodenum why multiple biopsies are now performed in some clinics (71, 72). Video capsule endoscopy, which is a new technique for investigation of the small intestinal mucosa has been proposed but so far the specificity is insufficient to diagnose CD (63.6%) and biopsies can not be secured (73).

The classification of the histological pathology of the small intestinal mucosa has changed with time. The Alexander score (Table 1) is now widely used. An alternative classification by Marsh grades the celiac mucosa into four stages similar to the Alexander grading: 0) Normal mucosa; I) Only increased density of IELs; II) Addition of hyperplasia of the crypts; III) villous shortening with mild, marked or total villous atrophy (52).

Grade 1	Normal villous length and crypt depth (villous/crypt ratio ≥ 2). No sign of inflammation
Grade 2	Normal villous length and crypt depth (villous/crypt ratio ≥ 2). Increased frequency of IELs and inflammation in LP
Grade 3	Partial villous atrophy, elongation of crypts (villous/crypt ratio <2). Increased frequency of IELs and inflammation in LP
Grade 4	Total villous atrophy, elongation of crypts (villous/crypt ratio <2). Increased frequency of IELs and inflammation in LP

Table 1. Modified Alexander score for small intestinal histology. IELs, Intraepithelial lymphocytes. LP, Lamina propria.

Associated diseases

Today CD is considered to be an autoimmune disease (47, 53). There is an association between CD and Down's syndrome and several studies from Europe and USA have revealed that the prevalence of CD among patients with Down's syndrome ranges between 3.2 – 10.3 %. The symptom of CD in patients with Down's syndrome is often misunderstood as the syndrome itself often causes gastrointestinal problems (74-76). There seems to be a subgroup of patients with Down's syndrome who have subtotal villous atrophy, not carrying HLA-DQ2 and without elevated levels of anti-tTG and EMA antibodies (77). Whether this is a variant of CD or another immune enteropahty has to be further scrutinized.

CD is relatively frequent among children suffering from type 1 diabetes mellitus (T1D) and three studies from European centres involving more than 500 children with T1D found a prevalence of CD between 6.2 – 10.4 % (78-80). In a Brazilian study of 354 children with T1D 10.5% had elevated levels of IgA anti-tTG (81). The risk to contract both diseases seems to be linked to HLA-DQ2 (DQA1*05/DQB1*02) (78-81).The risk to contract both CD and T1D or even other autoimmune diseases like thyroid disorders seems to be linked to HLA-DQ2 (DQA1*05/DQB1*02) (82, 83)

There also seems to be a link between CD and Dermatitis Herpetiformis, in which an itchy blistering skin rash is associated with an increased density of IELs in response to gluten challenges (84-86). Refractory sprue/celiac disease has been defined as small intestinal villous atrophy, sometimes with severe malabsorption mimicking CD but without any improvement on strict (GFD), although many cases of failure may be caused by poor adherence to a strict GFD. A subgroup of patients with refractory sprue, however have abnormal IEL phenotypes and some may develop Enteropathy-associated T cell lymphoma (EATL) (87). Refractory sprue has not been reported in children. Autism has often been suggested to be associated to CD but there is no present evidence for this association (88). In one study dental enamel defects was found in 20% of CD patients (89), although this was not the case in a later study from Sweden (90). Thirty percent of patients suffering from recurrent aphthous stomatitis seem to benefit from a gluten-free diet (89)

Treatment

CD relapses if gluten is reintroduced into the diet after first having been withdrawn. The treatment is a lifelong diet free from gluten, *i.e.* wheat, barley and rye. Patients with CD generally tolerate oat provided that it is free from contamination by wheat, barley and rye. Gluten-free, wheat starch-containing food still contains traces of gluten but is considered safe for the majority of patients in many countries as it contributes less than 100 mg gluten/day (91). Even though a GFD is well tolerated by most patients, there is a considerable inconvenience of being forced to adhering to a lifelong restricted diet (92). Hence, there is intensive research aiming at future alternative treatments such as feeding the enzyme propyl endopeptidase with the potential to cleave the "toxic" gluten peptides before these reach the mucosa and raise an immune response (93), tTG inhibitors or substances blocking the binding of gliadin peptides to HLA-DQ2/HLA-DQ8 (94, 95).

Gluten and its interplay with tissue transglutaminase

Wheat consists of 15% protein and 75% starch. Gluten is a major protein fraction in wheat. In the baking process yeast produces carbon dioxide which is trapped by a net of fibres made up by wheat gluten, making gluten vital for baking properties. Wild relatives of wheat, first grown in the Middle East, were probably the first plants cultivated about 11,000 years ago. Around 4,000 B.C. wheat farming had spread to Asia, Europe and North Africa and settlements were established when crops provided people with a stable food supply. The primary species cultivated were einkorn wheat and emmer wheat with hulled grains. They were later replaced by wheat with naked grains, mainly durum wheat during the Bronze Age. Bread wheat was introduced 4,000 years BC. It was produced by crossing the cultivated *Triticum tugidum* with the wild grass *Aegilops squarrosa*. In contrast to the other wheat species the modern wheat, *Triticum aestivum* has no wild progenitor. The first "wild" species of wheat contained low levels of protein with poor baking properties but cultivation through the years has increased the levels of gluten in order to increase the nutritional and baking properties.

Gluten is a complex mixture of heterogeneous wheat proteins. Soon after Dicke found that wheat protein was responsible for the development of CD (1) it was thought that gliadin, which is the gluten fraction soluble in alcohol, was the toxic agent to CD patients, whereas the insoluble fraction glutenin was non-toxic or "possibly toxic". However it has recently been shown that also tG2-deamidated high molecular weight glutenins can evoke response in T cells within the celiac mucosa (96). Gliadin belongs to a group of proteins named prolamines. Related cereal proteins are zein, (corn), secalin (rye), hordein (barley) and avenin (oats). These

proteins have the general characteristic of being insoluble in water but soluble in 70% alcohol. Despite the close relationship with other cereals, corn has never been associated with CD and oat has been judged safe (97) even if common commercialy grown oat often is contaminated with gluten (98, 99). Gliadin consists of α–, β–, γ– and ω-gliadins, which collectively are characterized by high percentage of the two amino acids prolamine and glutamine (some 20% and 38%, respectively). Gliadin is poorly digested in the small intestine and generates long peptides that contain most of the known immunodominant T cell epitopes (100). Many different gliadin peptides have been shown to express epitopes for gliadin-specific T-cell clones and also to be active when intestinal biopsies or isolated cells from intestinal biopsies are challenged with them *ex vivo* (101-106). Tisue transglutaminase (tTG) is a Ca^{2+} dependent enzyme, present in most human cells in the body and important in differentiation, proliferation and apoptosis and thus for control of cell and tissue homeostasis (107). tTG activity was demonstrated in human small intestinal mucosa already in 1985. Increased tTG activity was also demonstrated in small intestinal biopsies from patients with untreated CD (108). tTG has the capacity to crosslink and also deamidate gluten peptides, thereby transforming certain glutamines to glutamic acid, which increases their affinity to HLA-DQ2 and HLA-DQ8 as these two MHC class II molecules preferably bind peptides with multiple negatively charged residues (102, 109, 110). Small-bowel mucosal deposits of anti-tTG-specific IgA has been found along the surface and crypt basement membranes and around mucosal vessels in untreated CD, patients without villous atrophy (111).

A few years ago a deamidated peptide of α-gliadin (p57-73), QLQPFPQPELPYPQPQS was identified as a dominant epitope for *in vitro* stimulated peripheral T cells of CD patients (112). There may be an alternative role for tTG where enzymaticaly inactive tTG located on the apical surface of the epithelial cells binds to different gliadin peptides causing activation and proliferation of IELs (113, 114). Gliadins can be harmful not only in CD. IgE antibodies to α-gliadins have been found in all sera tested from 24 patients with baker's asthma (115).

THE HUMAN INTESTINE

The luminal surface of the lower gastrointestinal tract constitutes an area of around 400 m^2 in adults, which is 250 times larger than the area of the skin. It is lined by a single layer of epithelial cells. The small intestine, a convoluted tube, is the longest section of the gastrointestinal tract, around 6-7meters in length in an adult and located between the stomach and the large intestine (Figure 1a). In the first short section, the duodenum which is around 25 cm in length (as long as twelve fingers breadth) the partially digested food from the stomach is mixed with digestive enzymes secreted from exocrine pancreas and bile secreted from the liver to continue digestion. The small intestine continues with its longest segment, the jejunum, followed by ileum connecting to the large intestine. While the primary function of the small intestine is to digest and absorb dietary nutrients and the main task for the large intestine is to resorb water and absorb some nutrients such as short chain fatty acids, the intestine is also a major endocrine organ and constitutes a dominating part in the immune system of the body. The intestinal wall is composed of four layers: the mucosa, the submucosa, the muscularis mucosae, and the serosa (Figure 1b). The large area of the intestinal mucosal surface is acchieved by millions of finger-like projections called villi. Each villi is 0.5 -1.5 mm in height. The area is even further enlarged because each enterocyte, the dominating epithelial cell type, has 3000 cylindrical protrusions of 1 μm height, microvilli, at its luminal surface. In the small intestine there are a number of different types of epithelial cells all originating from stem cells located within the lower part of the crypts. Each new cell will undergo 4-6 rounds of cell division and differentiate as it rapidly migrates to the mucosal surface or the base of the crypt. The dominating epithelial cell type is the enterocyte (Figure 1b). These absorptive cells are vital for active as well as passive transport of nutrients. Goblet cells are large mucus-secreting cells interspersed between the enterocytes, both in the crypts and at the villus surface. Interspersed between the enterocytes are also endocrine cells that secrete neuropeptides as polypeptide YY and substance P. Paneth cells are only found in the base of the crypts. They contain granules with antimicrobial peptides, antibacterial enzymes and antibacterial glycoproteins and may have a role in regulating the intestinal flora. One specialized epithelial cell is the M-cell located in the epithelium covering lymphoid follicles. The M-cell seems to have an antigen sampling function by transporting antigens from the lumen to the immune cells in the follicles. The small intestinal epithelium also harbours lymphocytes, so called IELs, which are all of the T cell-lineage (116). Several types of immune cells are present in the lamina propria, *i.e.* T cells, B cells, plasma cells, macrophages and dendritic cells (Figure 1b).

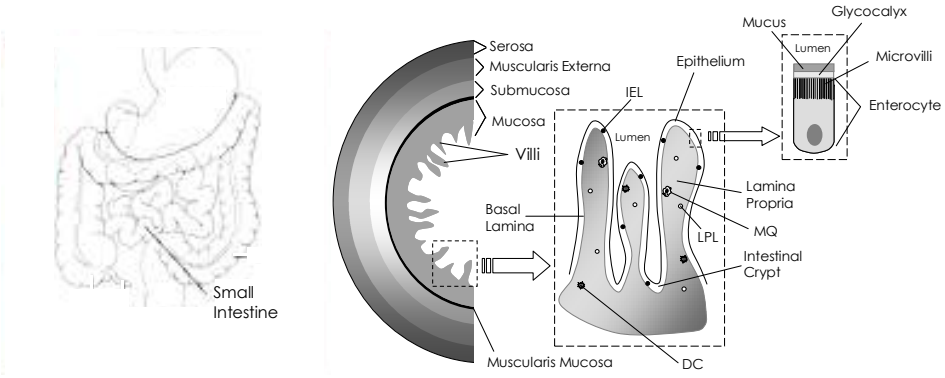

Figure 1. **a**) Lower gastrointestinal tract. **b**) Cross-section of the small intestinal wall and close-ups of the mucosa and one enterocyte. IEL: Intraepithelial Lymphocyte; LPL: Lamina Propria Lymphocyte; MQ: Macrophage; DC: Dendritic cell.

Intestinal microbiota

After birth, the human gastrointestinal tract is colonized with a microbiota within a few days (117-119). Under normal condition, just a few bacteria are present in the small intestine while the large intestine are hosting a large number (10^{14}) bacteria with a total weight of around 1.5 kg, divided on some 500 species, of which most have not yet been properly characterized (120, 121). In the upper small intestine the number of microorganisms range from 10^3 to 10^4 /ml of intestinal content, while in the distal ileum there are 10^7-10^8 bacteria/ml luminal. The normal microbiota colonizing the gut, the commensal biota, is essential for the well-being of the individual, *i.e.* they reside in a non harmful coexistence with the host. In recent years there has been a growing interest in the interplay between our intestinal microflora and the influence it might have in our health and the crosstalk with the immune system. Prebiotics, could be defined as short-chain carbohydrates supplements that alter the composition or metabolism of the intestinal microflora in a beneficial way (122) while probiotics is defined as live bacteria potentially beneficial to the host. Probiotics has been used as treatments in inflammatory bowel diseases with varying results (123). In atopic children it has been shown to decrease atopic symptoms, prevent atopic diseases in high-risk children and even diminished production of inflammatory cytokines (124).

THE IMMUNE SYSTEM

The immune system has evolved to defend the organism. One important mission is surveillances to defend the host against potentially dangerous agents and invading organisms that could cause infections, *i.e.* bacterial-, fungal-, viral- or parasite infections. Another equally important task is the defence against transformed autologous cells, which by uncontrolled growth can endanger the organism. The immune system in man can be divided into two parts, the *innate* and the *adaptive*, determined by the speed and specificity of the reaction. Whereas the innate response is unspecific and rapid, the adaptive response is precise but takes several days to develop. The innate immune system responds in a stereotype action without memory, while the response from the adaptive immune system increases in strength every time it is activated. The combined action of innate and adaptive immunity constitutes an efficient system for protection of the host against foreign potentially hazardous substances including microbes causing infectious diseases.

Innate Immunity

Innate immunity refers to the first line of defence that serves to prevent microbes from entery into the body tissues and to limit infection in the early phase. Innate immunity includes mechanical barriers like skin and mucosal membranes, physiological barriers like low pH and biochemical barriers comprising antimicrobial agents as NO and peroxidase. Most importantly, it encompasses the elements of the immune system, both cellular such as neutrophils, natural killer (NK) cells, macrophages, and humoral such as the complement system, lactoferrin, lysozyme, and defensins. The immune system in vertebrates is based on the capacity to recognize "microbial nonself" and "missing self".

The strategy of microbial nonself recognition is based on the detection of conserved structures present in large groups of microorganisms, referred to as pathogen-associated molecular patterns (PAMPs) (125). The best-known examples of PAMPs are lipopolysaccharide (LPS) of gram-negative bacteria, peptidoglycan of gram-positive bacteria, glycolipids of mycobacteria, and mannans (126). PAMPs are recognized by germline encoded, non-clonal pattern recognition receptors (PRRs). Functionally, these can be divided into three classes: secreted, endocytic, and signalling (127). Secreted PRRs are involved in opsonization of bacteria and viruses for enhanced phagocytosis or activation of complement via the lectin pathway of complement system. The best-characterized receptor of this class is the mannan-binding lectin, a member of the collectin family, which binds to microbial carbohydrates (126). Endocytic PRRs are expressed on the

surface of dendrtic- and phagocytic cells. Upon recognition of PAMPs, the endocytic PRRs mediate the uptake and delivery of the pathogen into lysosomes. The pathogen derived proteins can thereafter be processed and presented by MHC class II molecules on the surface of dendritic cells and monocytes/macrophages and consequently be recognized by T lymphocytes with the appropriate specificity (127). Toll-like receptors comprise the class of signalling receptors. On recognition of PAMPs, they trigger signalling pathways that result in the induction of transcription of a variety of immune response genes, such as antimicrobial peptides and inflammatory cytokines (127).

NK cells are capable of killing tumour cells and virus infected cells. NK cells express cell surface receptors, killer-cell immunoglobulin-like receptors (KIRs) that deliver either activating or inhibitory signals and the relative balance of these signals regulates the cytotoxic activity of the NK cell. NKG2D is an activating receptor that binds to a number of target ligands. Examples of NKG2D ligands are MICA and MICB which are closely related molecules expressed during cell stress and during viral infections and up-regulated in tumor cells (128, 129). The NKG2D-MICA/MICB interaction leads to NK cell activation and killing of the target cell (128).

Mucins

The small intestinal mucosa is coated with a mucous layer secreted by specialized epithelial cells, the goblet cells (figure 1b), which are packed with numerous secretory granulae containing mucins (130). Mucin was purified for the first time from human small intestinal goblet cells in 1976 (131). For a long time the only function of mucins was thought to be to protect and lubricate the epithelial surface (130). Mucins can be divided in three groups; gel-forming, soluble and membrane-bound mucins. Oligosaccharide constitutes up to 80% of the mucin molecule (132). The mucus gel could be useful to enteric bacteria in offering ecological advantages for resident over pathogenic bacteria (133). Mucins can also provide nutrients *i.e.* saccharides used for growth by bacteria. The mucous layer also creates a physical barrier against enteric microbial pathogens (134). There are several mucins characterized and four of them, MUC 1-4 are expressed by intestinal epithelial cells (IECs) in the small intestine. MUC2 is secreted by goblet cells in the crypts and villi while MUC3 exists in both a secreted and a membrane-bound form and is expressed both in goblet cells and enterocytes of the villous epithelium. MUC1 and MUC4 are expressed in ileum and are membrane associated in both goblet cells and enterocytes (135).

Antimicrobial peptides

Production of antimicrobial peptides is an important mechanism of innate immunity. Antimicrobial peptides are amphiphatic cationic molecules, frequently in α-helical or β-sheet conformation, which penetrate into the phospholipid bilayers of microorganisms and form lytic pores (136). Their preferential activity against certain microorganisms depends on differences in phospholipid composition of the surface membranes of microorganisms and eukaryotes. In humans, the two main families of antimicrobial peptides are the defensins and cathelicidin (137). The defensins are 29-40 amino acid residues long peptides containing six disulfide-linked cysteines (138). They are divided into two classes, α- and β-defensins, based on structural characteristics *i.e.* they differ in length of peptide segments between the six cysteines and the pairing of the cysteines (139). Defensins can exhibit strong antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, mycobacteria, and viruses (138). They are widely distributed in mammalian epithelial cells and phagocytes and are often present at high (up to millimolar) concentrations (139). They are encoded by single genes as prepropeptides, which are then processed to their mature, active forms (140). To date, six α-defensins have been identified in humans (140). Four of these, designed human neutrophil peptide (HNP) 1-4, are stored in the azurophil granules of neutrophils and form part of the armoury of these cells. The remaining two, α-defensins, human defensin 5 (HD5) and HD6, are expressed in cytoplasmic granules of small intestinal Paneth cells which are located at the bottom of the Lieberkühn crypts of the small intestine (140). HD5 and probably HD6, is stored in cytoplasmatic granules as the precursor form which, upon granule secretion, is cleaved into a mature form by trypsin, co-expressed in the human Paneth cells (141). In addition to enteric α-defensins, the neutrophil α-defensins HNP 1-3 are also expressed in intestinal epithelial cells, but not Paneth cells, in inflammatory bowel disease (142). Under normal conditions α-defensins provide antimicrobial host defence throughout the small intestine through their expression in Paneth cells and systemically through their expression in granulocytes. β-defensins is mainly expressed in epithelial cells (143, 144) but are also expressed by monocytes/macrophages (hBD1 and hBD2) and dendrictic cells (hBD1) (145). Seven members of the human β-defensin (hBD) sub-family have been cloned from various mucosal sites (144). The best characterized are hBD1-4. hBD1 is expressed in the epithelial cells of the urinary tract, the keratinocytes in the skin, the epithelial cells in the gastrointestinal tract and in the kidney (137-139). In contrast to hBD1, which is constitutively expressed, hBD2-4 are inducible and produced by keratinocytes and epithelial cells in response to proinflammatory stimuli such as interleukin-1(Il-1), tumor necrosis factor-α, (TNF-α) and LPS for hBD2 (139). In

the gastrointestinal tract, hBD2 is not present in healthy conditions but is induced during the course of inflammation or infection (146). hBD3 is expressed in the epithelium of many organs and in non-epithelial tissues. It is induced by microorganisms or TNF-α and interferon-γ (IFN-γ) (147, 148). hBD4 is expressed in gastric antrum and testis (135, 149). Both hBD3 and hBD4 have recently been found to be expressed in small and large intestine and are up regulated in ulcerative colitis (147, 149). In addition to their antibacterial role, hBD1, hBD2 and hBD3 also act as linkers to the adaptive immune system. All three bind to chemokine receptor CCR6 expressing cells, including immature dendritic cells, memory T cells and some $CD8^+$T cells thereby recruiting these cells to sites of infection by chemotaxis (150). Also HNP1-3 have been reported to be chemotactic for monocytes, naïve T cells and immature dendritic cells (151).

Cathelicidins are linear α-helical peptides without cystein residues (137). Human cathelicidin (LL-37) is a 37 amino acids long peptide that was first described in circulating neutrophils (152). It is both stored in neutrophil granules and is also an inducible product of epithelial cells in a variety of organs, T cells and monocytes (152, 153). As in the case of defensins, LL-37 is a chemoattractant for neutrophils, monocytes and T lymphocytes and may, in addition to being antimicrobial, play a role in stimulating and orientating the adaptive immune response (153).

The carcinoembryonic antigen family

In humans, the carcinoembryonic antigen (CEA) family comprises 29 genes; of which 18 are expressed and 11 are pseudogenes (154). The CEA family consists of seven CEA-related cell adhesion molecules, CEACAMs, (CEACAM1, CEACAM3-CEACAM8) and 11 pregnancy-specific glycoproteins (PSG1-PSG11). CEACAMs are highly glycosylated membrane-bound cell surface molecules, while PSGs are secreted (155). The proteins in the CEA family belong to the immunoglobulin (Ig) superfamily and are composed of one Ig variable-like (IgV) and a varying number (0-6) of Ig constant-like (IgC) domains (154). Release into the blood and possible over-expression of CEA (CEACAM5) in tumors of epithelial origin is the basis of its wide-spread use as a tumor marker. The function(s) of CEACAM family members varies widely and is still not fully understood for several family members but functions as cell adhesion molecules, tumor suppressors, regulators of signal transduction, receptors of Neisseria species and other bacteria have been described (154, 156). Four CEACAMs are expressed in epithelial cells of human intestine, *i.e.* CEACAM1 (four splice-forms differing in length of the cytoplasmic tail and/or number of extracellular domains), CEA (CEACAM5), CEACAM6 and CEACAM7 (two splice-forms differing in the number of extracellular domains) (154, 157). CEA, CEACAM1, and

CEACAM6 constitute the major components of the glycocalyx in the large intestine and hence have a proposed function in innate immunity (158).

Lectins

Lectins are carbohydrate-interacting proteins found in a variety of species from plants to insects to man. They recognize and bind specifically to monosaccharides, disaccharides or even oligosaccharides and are classified by which sugar residues they recognize. Most lectins recognize either N-acetylneuraminic acid, N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, or fucose. Lectins are used for microbial adhesion to tissue glycoproteins, *e.g. Escherichia coli* and *Salmonella typhimurium* adhere to mannose residues on host cells via their type 1 fimbriae. The carbohydrate moieties of glycoproteins and lipids not only serve as receptors for binding of bacterial surface proteins, toxins, etc but also serve as nutrients for the commensal microflora.

Adaptive Immunity

Adaptive immunity is the hallmark of the immune system of higher animals. It is characterized by memory of previously encountered antigens, diversity and specificity generated by the high number of different, clonally distributed T cell receptors (TCRs) and B cell receptors (BCRs). The potential repertoire of these antigen specific receptors is the result of random rearrangement gene segments in of the genes encoding the TCR and BCR/immunoglobulin (Ig), respectively. The central cellular elements of the adaptive response are the T- and B lymphocytes. **B cells** are phenotypically defined by their cell surface expression of transmembrane bound Ig that can bind antigen in its native, unprocessed form independently of antigen presenting cells (APCs). The basic structure of Ig is two identical heavy chains and two identical light chains held together by disulfide linkages to form an approximately 160 kDa glycoprotein with two identical binding-sites for the antigen. Igs are either carried on the surface of B cells where they act as receptors for specific antigens or secreted either systemically into the blood and lymph or into mucus and saliva at the mucosal surfaces. Igs in the soluble form are commonly referred to as antibodies. The unique antigen specificity of an Ig is determined by the variable region of the heavy and light chain, respectively that together form the antigen binding site. The constant region of the heavy chain determines the Ig class and consequently the biological function of the secreted antibodies. Contact between B cells and antigen is needed to cause the B cell to develop into antibody secreting cells, called plasma cells, which secrete large amounts of antibodies. The antibody produced by the mature plasma cell has the same binding specificity as the membrane

bound Ig on the B cell it developed from. Most protein and glycoprotein antigens require T cell help for B cell activation. In response to such so called T cell dependent antigens, the B cell may exchange the constant part of its Ig (isotype switching), increase the antibody affinity due to accumulation of mutations in the variable regions (affinity maturation) and differentiate into long lived memory B cells. If the B cell becomes activated by a T cell independent antigen, *e.g.* a LPS, the B cell differentiates into IgM secreting plasma cells with no further isotype switching, no induction of affinity maturation or establishment of B cell memory.

T lymphocytes are defined by their cell surface expression of TCR a heterodimeric antigen receptor. TCRs occur in two forms. One consists of one α– and one β–chain, each with a constant and a variable domain. This receptor is carried by most T cells and binds antigen that has been broken down and displayed on MHC class I or II molecules. The other form has one γ– and one δ– chain (159). In contrast to αβ T cells, γδ T cells can recognize antigens without presentation on MHC molecules. During T cell maturation the TCR associates with several signal-transducing transmembrane molecules known as the CD3 complex. As compared to B cells, T cells have a wider range of activities. Some are involved in the control of B lymphocyte development and antibody production. Others interact with phagocytic cells and help them destroy pathogens they have taken up. Yet another set of T lymphocytes recognize cells infected with virus and destroy them. Mature αβ T cells are phenotypically composed of two major subpopulations defined by cell surface expression of the CD4 or CD8 transmembrane glycoproteins. $CD4^+CD8^-$ T cells recognize antigen presented in the context of MHC class II molecules and $CD4^-CD8^+$ T cells recognize antigen presented in the context of MHC class I molecules.

Before a T cell encounters an antigen it lacks most effector functions and is said to be naïve (identified by a marker denominated CD45RA). Activation through the TCR results in proliferation and acquisition of a variety of effector functions and thus an array of effector and memory cell types identified by the CD450 marker. T effector cells can be divided into two major types, T helper (Th) and T cytotoxic (Tc), bearing either CD4 or CD8 molecules on their surface, respectively. $CD4^+$ lymphocytes are the orchestrating cells of the immune response. They recognize foreign antigens and activate other cells of the immune response to eradicate the pathogen. They are also the major players in activation of B cells. Th cells are subdivided functionally by the pattern of cytokines they produce (160, 161). On stimulation, naïve T cells (Th0) become either Th1 or Th2 cells. Th1 cells mainly produce IL-2 and IFN-γ and participate mainly in cell-mediated immune response (160). Th2 cells predominantly produce IL-4 and IL-5 generating mainly antibody responses (162). Cells producing high amounts of transforming

growth factor β (TGF-β) have been termed Th3 and are associated with down-regulatory functions (161).

$CD8^+$ cytotoxic cells are involved in antiviral activity. After binding to the target cell bearing its specific antigen presented on MHC class I, the Tc inserts perforin into the cell membrane. Perforin causes pore formation in the plasma membrane, which allows granzymes to enter the target cell. This activates caspase enzymes that induce DNA fragmentation and cell apoptosis. Tc can also bind target cell surface Fas molecules by their Fas-ligand (FasL), which also induces apoptosis (163). Similarly to Th cells, Tc lymphocytes can be divided into Tc1 and Tc2 based on their cytokine secretion (164). Through cytokine secretion, but also through cell-cell interaction, Tc cells play a role in the regulation of activation and differentiation of CD4 cells (165).

Regulatory T cells (Treg) play an important role in down-regulation of immune responses. Among $CD4^+$ T cells, two subsets of Treg exist, natural and adaptive (166). Natural Treg develop in the thymus and are believed to function mainly to prevent autoreactive T cell responses through direct interaction with responding T cells or APCs (166). Adaptive Treg are generated from mature T cells and their development in the periphery is believed to be triggered by low-affinity antigen (166). Adaptive Treg suppress immune responses during inflammatory reactions to microbes or transplanted tissue, mainly by producing immunosuppressive cytokines, such as TGF-β and IL-10 (166). Besides among $CD4^+$ T lymphocytes, Treg cells can be found among other lymphocyte populations such as $CD8^+$ T cells, γδ T cells and natural killer T (NKT) cells (167).

NKT cells constitute a lymphocyte subpopulation that co-express an TCR **αβ** and molecular markers typically associated with NK cells, such as NK1.1. In mice, NKT cells are typically defined as NK1.1TCR$\alpha\beta^+$ cells (168). Human NKT cells express the CD3/TCR complex together with NK associated markers such as CD56, CD57, CD161 and CD122 (168). NKT cells have a highly restricted TCR repertoire, with the vast majority of human NKT cells expressing an invariant α-chain (Vα24Jα15) in conjugation with Vβ11, TCR homologs of those used by mouse NKT cells (169). NKT cells recognize glycolipid antigens in the context of the non-classical MHC class I molecule CD1d (170). When stimulated, NKT cells particularly produce high levels of IL-4 and IFN-γ and are thus considered to be important for the initiation and regulation of immune responses (170).

Although 90-95% of circulating T cells in human use the TCRαβ, ~5-10% use the alternative TCRγδ. T cells are frequent within the human epithelia even though αβ T cells are the dominant celltype also at these sites (116). On average, 30% of IELs in the small intestinal epithelium are γδ

T cells (116) while γδ T cells are rare in lamina propria (LP) (116, 171). The majority of circulating human γδ T cells utilize the Vδ2-and Vγ9-segments in their TCRs whereas γδ T cells of the human intestine predominantly use Vδ1 together with Vγ8 (172, 173).

Major histocompatibility complex (MHC)

In humans them major histocompatibility complex (MHC) covers large genomic region of 3.6 x106 base pairs in 140 genes (174) The MHC complex is divided into MHC class I, MHC class II and MHC class III. Almost all nucleated cell in the body expresses MHC class I molecules, which are heterodimers, consisting of a single transmembrane polypeptide chain (the α-chain) and a β_2 microglobulin peptide. The α-chain has two polymorphic domains, α_1, α_2, which bind peptides derived from cytoplasmatic proteins of the cells expressing MHC class I molecules. Proteins in infected or transformed cells will be presented on MHC class I molecules at the cellular surface to specific $CD8^+$ cytotoxic T cells (CTLs) that can then attack and kill the target cells which in most cases lead to apoptosis of the affected cell. In man, MHC class II molecules are expressed on APCs; macrophages, dendritic cells, B cells and activated T cells. MHC class II molecules are cell surface glycoproteins consisting of two homologous peptides, one α- and one β-chain. The peptides presented by MHC class II molecules are derived from extracellular proteins mainly from pathogens and will be presented to $CD4^+$ Th cells. The peptides presented by MHC class II molecules are longer than peptides presented by MHC class I molecules, generally between 15 and 24 amino acid residues long because the peptide-binding groove of MHC class II molecules is open at both ends while the groove on class I molecules is closed at each end. The MHC III region encodes for other immune components, such as complement factors and some cytokines, e.g. TNF-α.

Cytokines

Cytokines are bioactive polypeptides that are secreted by one cell to modify the behaviour of it self or another cell. They are produced by virtually all cells in the body, although immune cells constitute the main source and play an important role in many physiological responses particular in immune responses and inflammation. Cytokines influence the behaviour of the target cell by sending intracellular signals when binding to specific cell-surface receptors. Responses to cytokines include mainly activation, proliferation, and secretion of effector molecules including the same or other cytokines. Cytokines have a wide variety of names; these include lymphokine (cytokine secreted by lymphocytes), monokine (cytokine secreted by monocytes), chemokine

(cytokine with chemoattractant activity), interleukin (IL; cytokine produced by one leukocyte and acting on other leukocytes), colony-stimulating factor (cytokine that stimulates differentiation and proliferation of stem cells), TNF and IFN; cytokine that interferes with viral replication in infected cells) (159, 175). As described earlier, T cells are subdivided functionally by the pattern of cytokines they produce. T cells are initially activated as Th0 cells, which produce IL-2, IL-4 and IFN-γ (176). The nearby cytokine environment then directs differentiation into either Th1 or Th2 cells. IL-4 stimulates Th2 activity and suppresses Th1 activity, while IL-12 promotes Th1 activity. Human Th1 cells are involved in cell-mediated inflammatory responses, typically secrete IFN-γ, which activates macrophages, and IL-2, which stimulates proliferation of antigen-activated T and B cells (175, 177). By contrast, Th2 lymphocytes are typified by the production of IL-4 and IL-5, which stimulate proliferation and differentiation of B cells. IL-6, IL-9 and IL-13 are also commonly produced by Th2 cells (178). Treg mediate their inhibitory activities by producing immunosuppressive cytokines such as TGF-β and IL-10, which inhibit both Th1- and Th2 cell responses (167).

Cytokines act on their target cells by binding to specific membrane receptors. The receptors and their corresponding cytokines are divided into several families based on their structure and activities. The main cytokine receptor families are: hematopoietin family, which include receptors for IL-2 through IL-7, IL-9, IL-12, IL-15 and GM-CSF; IFN family receptors which include the receptors for IFN-α, IFN-β, IFN-γ and IL-10; TNF family receptors which include receptors for soluble TNF-α and TNF-β as well as membrane-bound CD40 (important for B cell and macrophage activation) and Fas (which signals the cell to undergo apoptosis); and TGF-β receptor family which includes receptors for TGF-β (176).

T CELL MATURATION

Intrathymic T cell maturation

T lymphocytes develop from progenitor cells in the bone marrow but migrate to the thymus at an early stage (as thymocytes) for further maturation and education. To acquire clonally distributed antigen specific TCRs is a vital part of the maturation process. This is achieved by a process of random rearrangement of multiple DNA segments generating functional genes for the α- and β-chains or the γ- and δ-chains, respectively. There are four segments of genes involved in T cell receptor formation called the variable (V), diversity (D), joining (J), and constant (C) regions; thereof the name V(D)J recombination. The progression from progenitor cell to mature T lymphocyte involves several groups of genes and their products.

Two proteins vital for the initiation of the assembly of TCR genes are the lymphoid-specific recombination activating gene 1(RAG1) and 2 (RAG2). The human RAG1 and RAG2 genes lie 8 kilobases apart on chromosome 11 and their molecular weights are 119 and 58 kDa respectively (179-181). The RAG1 and RAG2 proteins act synergistically to activate V(D)J recombination by introducing a sequence specific cleavage in the DNA (182). This generates two hairpin-shaped coding ends which are subsequently opened, subjected to addition or deletion of nucleotides and finally joined to form a coding exon for the variable, specificity determining domain in the TCR-chain (182). This forms the final gene sequence from which protein will be transcribed to form the receptor molecule.

When the gene segments of the β-chain have been successfully rearranged in the process of generating TCRαβ, the β-chain is expressed on the cell surface together with an invariant preTCR-α (pTα) chain, another key molecule required for the proper development of T lymphocytes, and a CD3 subunit to form the preTCR complex (Fig. 2) (183). The expression of the pre-TCR complex coincides with temporary down-regulation of RAG expression and triggers proliferative signals, termination of TCRβ locus rearrangement and induction of CD4 and CD8 expression (184). The pTα–chain exists in two forms: a long form, $pT\alpha^a$, including all four exons and associates with the TCRβ-chain during TCRa-chain rearrangement forming a preTCR, and a short form, $pT\alpha^b$, where exon 2 which encodes an Ig-like domain composing most of the extracellular portion, is spliced out (185, 186).

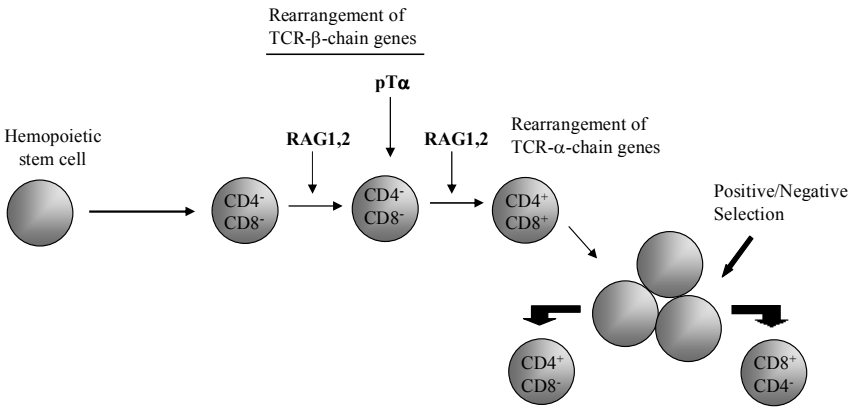

Figure 2. A simplified model of intrathymic development of αβ T cells.

V(D)J recombination is the main source of antigen receptor diversity. Rearrangement of TCR genes leads the production of a repertoire of over 10^8 specificities, all clonally distributed (159). There are several ways in which this large diversity occurs. First, there is a multiplicity of the gene segments involved in the reaction and they can be assembled in various combinations. Second, the processing of the often asymmetrically opened coding-end hairpins results in palindromic nucleotides or loss of nucleotides. In addition, the lymphoid cell specific polymerase terminal deoxynucleotidyl transferase can insert non-templated nucleotides at the opened ends of DNA during recombination to further alter the sequence (182).

Once TCRαβ receptor rearrangement has occurred, the T cell undergoes positive selection for self-MHC class I or II restriction and starts to loose one of its two co-receptors, the CD4 or CD8 molecules. Before leaving the thymus, the T lymphocyte must also undergo negative selection in which potentially self-reactive cells are deleted by apoptosis. However, experiments in transgenic mice have shown that developing thymocytes encountering an antigen do not necessarily die at this stage (187). Instead, the autoreactive TCR is internalized providing the cell a second possibility to make a secondary TCR-gene rearrangement and "edit" its receptor specificity. Only ~ 2% of the thymocytes survive these selection processes and exit to the periphery as self-tolerant T cells able to recognize foreign antigens when presented on self MHC (176). The majority of thymocytes develop into αβ T cells and only about 0.5-1% become γδ T cells. The intrathymic developing pathway of γδ T cells is still enigmatic but a recent study suggests that, similarly to αβ T cells, γδ T cells go through positive selection before the transport to periphery (188). During the T cell maturation several signals in the thymic microenvironment are important for a proper development. IL-7 has shown to be particularly important for the development and survival of γδ T cells (189).

Extrathymic TCR gene rearrangement

Even though the development of most T lymphocytes occurs in the thymus, T cell lymphopoiesis can also take place in the absence of a thymus, as has been observed in athymic mice (190). This observation led to the conclusion that T cell differentiation also occurs extrathymically, in mice in particular in the gut and in the liver (190-193). In the murine intestine, small lymphoid aggregates, called cryptopatches, have been found along the gut mucosa between the crypts in the LP (194). These structures contain small progenitor lymphocytes that express Thy1, the receptor for stem cell factor (c-kit) and the receptor for IL-7 (IL-7R), and have been suggested as essential sites for extrathymic T cell development of precursor T cells destined to become thymus-independent IELs (194-196). Other studies in mice have indicated that immature, early thymic emigrants can continue their development into mature T cells in the small intestinal mucosa (197). Also in humans, small intestinal mucosa has been proposed as a site of extrathymic TCR gene rearrangement (116, 198-200). In these studies, expression of RAG1 (116, 198, 200), RAG2 (198, 200) as well as pTα (199, 200) has been found among small intestinal IELs and/or LPLs. In addition, two new 5' untranslated region (5'UTR) exons were identified in the human RAG1 gene isolated from small intestinal lymphocytes (200). The new 5'UTR exons 1A and 1B could be utilized in three different combinations (1A/2, 1A/1B/2 and 1B/2), and all three were shown to be expressed in jejunal IELs- and LPLs of the T cell lineage (200). Furthermore, splice-forms containing the 1A exon were found to be expressed exclusively outside thymus. In contrast, RAG1 mRNA with the 5'UTR-splice-form dominating in thymus is not detected in jejunal T cell lineage lymphocytes (200). Identification of cells expressing markers associated with early stages of T cell development, *e.g.* c-kit, IL-7R, CD1a, CD2 and/or CD7 without the expression of CD3, in the human small intestine further support the notion of extrathymic T cell development in the human small intestinal mucosa (200). The authors suggest that in addition to a local *de novo* T cell development, there also is a possibility of an antigen driven secondary TCR gene rearrangement in this organ.

Recent studies shows that T cell maturation continues in children who had been partially or completely thymectomized during heart surgery (201, 202). Interestingly, these children had significantly higher proportions of circulating unconventional T lymphocytes (TCRαβ CD8αα$^+$ and TCRγδ CD8αα$^+$). In patients with a chromosome 22q11.2 deletion, (DiGeorge syndrome) thymic hypoplasia is a prominent feature. A group of 15 children with just a trace of, or a small thymus, was followed up at a median age of 4 years. At birth they were all prone to infection but

at a follow up they were free from serious infections and there was no difference compared to controls regarding gene expression of IFN-γ, IL-10, TGF-β, CTLA-4 or FoxP3 (203).

Gut Associated Lymphoid Tissue (GALT)

The immune system in the intestine has a difficult task. From the weaning period onwards the human infant is exposed to an increasing number of antigens, to which it has not been exposed previously. The ingested antigens, most proteins are digested by enzymes into smaller fragments (peptides) a process initiated as soon as they reach the oral cavity, and continuing in the stomach and intestine. Some larger peptides are not fully degraded and reaches the proximal small intestine. Oligopeptides below a length of 8 aminoacids are nonreactive in antigen recognition or presentation and, thus, are immunologically ignored (204). Larger peptides or proteins that have escaped or been protected from digestion have the potential to be immunogenic and thus cause harm especially to infants and young children whose digestive ……and the secretory IgA system is not fully developed (205). Besides food antigens the small intestine is also exposed to potential harmful pathogens in contaminated food or from other sources as well as swallowed (mostly harmless) bacteria from the oral cavity. In the small intestine a single layer of epithelial cells only 40 μm in height, joined by tight junctions separates the luminal contents from the competent immune system into the underlaying tissues. The epithelial cells are active participants in the mucosal defence as they recognize dangerous microbial components through pattern-recognition receptors such as Toll-like receptors (TLRs). They are also responding by producing cytokines and chemokines affecting cells in lamina propria, such as dendriric cells and macrophages, to trigger an innate defence and promote adaptive immune response (206, 207). Epithelial cells, together with IELs and underlying phagocytic/antigen presenting cells, can modulate and dampen these signals to prevent undesirable responses to non-threatening nutrients and the normal intestinal flora that could lead to inflammation (208-210). The intestinal cells in the immune system are in a constant state of alert, set to tolerate peptides, such as food peptides (oral tolerance). In analogy, protein vaccines that produce vigorous immune responses if injected into a muscle will in most cases be "ignored" by the mucosa immune system in the small intestine(211). Food allergy, defined as adverse immune response to food proteins affects as many as 6–8% of children below 3 years of age and 3-4 % of adults (212, 213), mainly milk, egg and peanuts (214). Apart from milk hypersensitisation where the immunological mechanism is still poorly understood, other types of allergic response to food are mainly mediated by undesired

IgE responses. This is however not the case in CD. In CD, gliadin rich in proline is poorly digested during its passage to the small intestine (100) and generates fairly long peptides which contain immunodominant epitopes (215). Gluten is in this respect different from other peptides derived from food proteins and the adverse immune response different from seen in other food proteins (215). In addition to the lymphoid tissue within the lymph nodes and spleen, lymphoid tissue is also located to at mucosal sites like the gastrointestinal tract, the respiratory tract and the urogenital tract. In the gastrointestinal, it is called Gut associated lymphoid tissue (GALT). It consists of solitary and aggregated lympoid nodules (Peyer's patches), and the appendix. GALT is especially rich in B-cells and is responsible for localized production of IgA against pathogens such as bacteria, viruses, and parasites (216, 217). The most prominent aggregates of lymphoid nodules is called Peyer's Patches located in the terminal ileum (seen grossly as protrusions of the mucosa). The epithelium covering the lymphoid nodules/follicles contains special epithelial cells that lack microvilli but instead have a microfold surface and therefore denominated "M cells". These specialized cells seem to sample the luminar content and transport antigen to the immune cells in the underlying follicles (218). IgA is the Ig class produced in large amount in the body per day. It is mostly secreted into mucous, saliva and milk where its main defence action seems to be prevention of microbes and othr antigen from binding to epithelial cells at the mucosal surfaces. In the small intestinal mucosa there are numerous IgA producing plasma cells in lamina propria. The epithelial cells have a receptor for the J-chain of dimeric IgA at their basolateral surface to which the IgA binds and is transported by transcytosis to the luminal surface where it is secreted as secretory IgA (217).

Intraepithelial Lymphocytes (IELs)

IELs are located between epithelial cells in the epithelial lining, the thin outermost compartment in contact with the intestinal lumen. They are in intimate contact with the epithelial cells and commonly in contact with the basal lamina, but in CD they are seen closer to the apical part of the epithelium (paper I). There are approximately 10-20 IELs per 100 IECs in healthy human small intestine but fewer in the large intestine (116). The upper limit of IELs in normal proximal small intestine is considered to be 25 IELs per 100 IECs (116, 219). The vast majority of the IELs are T cells of different subsets, some of them containing cytoplasmic granules (171). Most of the IELs are $CD45R0^+$ indicating an activated/ memory phenotype. More than 70% of IELs are $CD8^+$ and most of these have the $CD8\alpha\beta$ heterodimer but there are also a significant proportion with an $CD8\alpha\alpha$ homodimer which is essentially absent from circulation (163) and studies in mice suggest that the latter are of extrathymic origin (220).

Less than 20 % of the IELs are $CD4^+$ $TCR\alpha\beta^+$ and there are no B cells present(116). On average ≈5% of the IELs carry the TCRγδ in humans and almost all of these are $CD4^-CD8^-$ even though some can express CD8 (116). Furthermore, there are IELs with a thymocyte-like phenotype, *i.e.* expressing CD1a, CD2 without CD3 and CD7 witout CD3 (116, 200, 221, 222) and CD4 and CD8 simultaneously (223). Possibly there are also NKT cells among the human small intestinal IELs since some of them express NK-receptors. These include IELs with mature T cell phenotype and immature cells (116, 222) *e.g.* a subpopulation of $CD56^+CD3^+$ (116)and a minor population of $CD94^+CD8^+$ IELs (224). Interestingly the $TCR\gamma\delta^+$ IELs preferentially utilise Vδ1 in the δ-chain, often in combination with the Vγ8 in the γ-chain, in contrast to the small population of γδ T cells in the peripheral blood that often express Vδ2 in combination with Vγ9 (116, 173, 225). Even though the immunological roll of intraepithelial γδ T cells is still unknown experiments with TCR gene knock out mice suggest that they have a key role in the surveillance of epithelial cells (226, 227). The specificity of $V\delta1^+$ γδ T cells is poorly understood. $V\delta1^+$ clones have been shown to react with the MHC class I-like CD1 molecule (228) and $V\delta1^+$ T cells were shown to kill carcinoma cells expressing the stress induced MHC class I related MICA molecule (229) hence γδ T cells are considered to be able to kill stressed, transformed, and malignant epithelial cells (220, 229, 230). Moreover, the stimulatory NK cell receptor NKG2D seems to act as a co-stimulatory receptor when expressed on γδ T cells and $CD8^+$ αβ T cells. MICA was the first described ligand for NKG2D and it was reported that MICA reactive $V\delta1^+$ T cells exhibit dual recognition of MICA utilizing both the TCRγδ- and NKG2D (231). Additional ligands for NKG2D are the MHC class I related, so called UL16-binding proteins (ULBPs). One study showed that NKG2D expressing $V\delta1^+$ T cells kill ULBP3 expressing B cell leukaemia cells in an NKG2D dependent manner (232). γδ T cells have also been reported to play a role in maintaining epithelial integrity by producing growth factors and by secreting chemokines such as lymphotactin and RANTES, thus recruiting peripheral αβ T cells and inflammatory cell types to sites of epithelial damage (233, 234).

AIMS OF THE THESIS

The over-all goal of this thesis was to understand the mechanisms of the immunopathology in the small intestinal mucosa of patients with CD, in particular the reaction in the epithelial lining, and to explore the possibility of identifying risk factors for contraction of CD. Emphasis was put on analyses of small intestinal intraepithelial T cells and epithelial cells upon exposure to gluten in the diet. Specifically I aimed to:

- Understand the role of T lymphocytes in CD.
- Delineate the cytokine production by jejunal T cells in CD patients in response to exposure to gluten *in vivo*.
- Investigate how expression of antimicrobial agents, such as α- and β-defensins and lysozyme, and components of mucous and glycocalyx, such as mucins and CEA family members, are affected in children with CD.
- Investigate whether there are particular glycosylation patterns of cell surface components in epithelial cells that could explain the common occurrence of bacteria adhered to the epithelial surface in the small intestine of CD patients
- Explore the possibility that aberrant extrathymic TCR gene rearrangement in the small intestinal mucosa is a contributing factor to the adverse T lymphocyte reactions in CD.

RESULTS AND GENERAL DISCUSSION

Throughout the evolution during millions of years, humans have struggled against enteric pathogens and exposure to other foreign antigens. An effective immune response in the intestine has been vital for survival of the human race. The immune system has been "tuned" to discriminate potential dangerous pathogens from harmless or rather useful microbes *e.g*, the normal microbiota or commensal bacteria. In contrast to the exposure to enteric pathogens, man was first exposed to gluten only a few thousands years ago.

When the aetiology of CD was discovered in the 1950-ies mainly children with classical gastrointestinal symptoms and total villous atrophy were diagnosed (2). In 1960, Anderson could show that the histological changes in the small intestine caused by gluten were reversed by GFD (235) and in 1977 Ferguson published the important histological finding that an increased number IELs was a prominent feature of the celiac lesion (236). In 1989 it was discovered that many of these IELs had a TCRγδ and that the increased proportion of these γδT cells seemed to remain despite a GFD. The proportion of both αβ- and γδIELs are increased in active CD, however while the αβIELs vary in frequency with disease activity it seems that the γδ IELs stay elevated even for years on GFD (237, 238). The role of γδIELs is still poorly understood in general and in the pathogenesis of CD in partcular. The fact that they remain in an increased proportion in the celiac small intestinal mucosal epithelial compartment even in patients where the antigen has been removed for years, could point to an inherent immune deviation. γδT cells seem to play an important role in the protection of the epithelial barrier and also in destruction of stressed epithelial cells (220, 229, 234). An intriguing question is why the epithelium is stressed also in treated CD.

In the 1970-ies the strong linkage to the MHC class II alleles of HLA-DQ2/DQ8 was recognized (36-38, 239). Antigen presenting cells with their MHC class II molecules on their cell surface present extracellular peptides to $CD4^+$ T cells. In 1993 HLA-DQ2 restricted, gluten specific $CD4^+CD25^+$ T cells were isolated from small intestinal biopsies of CD patients after *ex vivo* challenge with gluten peptides (240). These T cell clones produced IFN-γ upon challenge with gluten. Increased levels of IFN-γ mRNA were also seen in small intestinal biopsies of CD patients with active disease and in biopsies of treated CD patients after *ex vivo* challenge with gluten (241). Taken together these results led to the conclusion that $CD4^+$ T

cells in lamina propria are the key-players in the immunopathogenesis of CD causing the intestinal lesion by producing high amounts of IFN-γ (242).

Cytokines expressed in T cells in celiac and normal small intestinal mucosa (Paper I and III)

At the onset of these studies there were accumulating data suggesting a crucial role for intestinal T cells in the pathogenesis of CD. Although several models had been proposed, little was known about the role of these cells in the local immune reactions in the CD lesion. Therefore we set out to analyze the T cell cytokine response to *in vivo* exposure to gluten. Initially intraepithelial T cells ($CD3^+$IELs) and lamina propria T cells ($CD3^+$LPLs) were compared. Two groups of CD patients with active disease were studied; newly diagnosed children who responded promptly to withdrawal of gluten from the diet, and children who responded adversely to challenge with gluten-containing diet after a symptom-free period on gluten-free diet. These were compared to symptom-free CD patients on a gluten-free diet and to children with no known food-intolerance (paper I). The results from these studies indicated that the most vigorous reaction was by the $CD3^+$IELs particularly in the group of untreated CD patients, which prompted us to analyze the γδIEL-, $CD4^+$αβIEL-, and $CD8^+$αβIEL subsets of untreated CD patients (paper III). Ongoing immune responses were estimated by determination of cytokine mRNA levels in freshly isolated T cells and T cell subsets. Selection of cytokines for quantitative mRNA analysis was based on an initial screen for IL-2, the Th2 cytokines IL-4 and IL-5, the proinflammatory cytokines IL-6, IFN-γ and TNF-α, and the down-regulatory cytokine TGF-β1 using qualitative RT-PCR (paper I). IL-4, IL-5 and IL-6 mRNAs were not detected in any sample in contrast to the remaning RNAs and in particular an increased frequency of IFN-γ mRNA positive samples in active CD compared to controls. Based on the screening we constructed real-time quantitative RT-PCR assays with RNA copy standards for IL-2, IFN-γ, TNF-α, TGF-β1, and also IL-10 as an additional down-regulatory cytokine. A qRT-PCR assay for IL-4 mRNA was also constructed to analyze the expression of this Th2 cytokine with higher sensitivity than qualitative RT-PCR. Frequencies of IFN-γ expressing IELs were determined by immunomorphometry (paper I). Altogether samples from 101 children were analysed in paper I.

Th2 cytokines

IL-4 and IL-5 mRNAs were not detected in any $CD3^+$IEL or $CD3^+$LPL sample from either active CD, treated CD or controls by qualitative RT-PCR. Sensitive real-time quantitative RT-PCR analysis also failed to detect IL-4 mRNA in intestinal $CD3^+$IELs and $CD3^+$LPLs (paper I) or purified IEL subsets (paper III). Low or undetectable levels of IL-4 and IL-5 in controls agrees with previous studies on IELs and LPLs in adults. However, since the number of plasma cells is increased in lamina propria in CD (243) it is somewhat unexpected that Th2 cytokines expression was not been induced in $CD3^+$LPLs in CD. Interestingly, no induction of IL-4 or IL-5 production by colonic lamina propria T cells could be seen in ulcerative colitis (244), a chronic inflammatory bowel disease that resembles CD in the increased numbers of plasma cells and local autoantibody production. Thus, activation of B cells to antibody production in human intestinal mucosa may involve other cytokines than classical Th2 cytokines.

IL-2

Since IL-2 is normally produced during an immune response and IL-2 seems necessary for the development of T cell immunologic memory (245, 246) we expected increased levels of expression of IL-2 mRNA in $CD3^+$IELs and $CD3^+$LPLs in active CD. However, this was not the case and in fact in untreated CD the expression was even lower compared to treated CD and controls, while there was no statistically significant difference between challenged CD, treated CD and controls. The expression levels of IL-2 mRNA in T cell subsets in active CD were generally low and several samples from children with active CD did not even have detectable amounts of IL-2 mRNA (7/12, 6/12, and 2/12 for γδIEL, $CD4^+$IEL and $CD8^+$IEL samples, respectively). Expression levels of IL-2 were markedly lower in γδIELs compared to $CD4^+$IELs and $CD8^+$IELs in both CD patients and controls. The difference between untreated and challenged CD is interesting and could result from longstanding inflammation since IL-2 mRNA levels were decreased in intestinal T cells isolated from patients with ulcerous colitis and Crohn's disease as well as patients with chronic inflammation in gingiva (244, 247). Recent studies have suggested a role for IL-15 in CD (248-250). Possibly IL-15 substitutes for IL-2, as a T cell growth factor in inflammation. IL-15 was first discovered due to its IL-2-like stimulatory actions on T cells (251, 252). IL-2 and IL-15 share receptor subunits, which most likely explain the similar properties of the two cytokines. Enterocytes produce and respond to IL-15 (253) and IL-15 potently stimulates IELs (254).

The expression of IL-15 is increased in the intestinal epithelium and present at the enterocyte cell surface as well as in the lamina propria in untreated CD (255).

Recent work has suggested that a wheat gliadin peptide (A-gliadin p31–43 or p31–49), different to one that recognized by T cells, might act directly to induce IL-15 production in the lamina propria and initiate epithelial apoptosis. This peptide induces expression of the stress molecule MICA on enterocytes, an effect mediated by IL-15. IL-15 also activates intraepithelial lymphocytes, including upregulation of the NKG2D receptor, which can interact with MICA thus enabling direct lymphocyte mediated cytotoxicity to enterocytes (249, 256).

IFN-γ

Interferons are divided into alpha(α), beta(β), and gamma(γ) interferons and were the first group of cytokines to be discovered already in 1957 (257). IFN-γ is produced by $CD4^+$ T helper 1 (Th1) cells, $CD8^+$ CTLs and NK cells and has a central role in both innate and adaptive immunity, especially in response to virus infections. It has been known for more than a decade that IFN-γ could play an importat role in CD but previous studies had been performed by gluten challenge of peripheral blood lymphocytes, T cells clones or whole biopsies (241, 258). By using real-time quantitative qRT-PCR in analysis of $CD3^+$ IELs and LPLs we could demonstrate that in normal controls only 5% of the IFN-γ mRNA was derived from IELs while 60% of the IFN-γ mRNA in children with untreated CD was expressed by IELs. This was confirmed by immunohistochemistry staining where the frequency of IFN-γ^+IELs was 10 times higher in the epithelial compartment of active disease compared to controls. In analyzing the subsets of IELs (paper III) it became obvious that $CD8^+CD94^-\alpha\beta$ T cells were responsible for most of the excessive production of IFN-γ in untreated CD, implying that approximately half of all IFN-γ is derived from $CD8^+$T cells in the epithelium underscoring the importance of the epithelial reaction in the disease. The average IFN-γ mRNA content per cell in fact exceeded that of polyclonally activated blood T lymphocytes (259). IFN-γ induces increased cell surface expression of both MHC class I and II molecules in APCs but also in intestinal epithelial cells and it has even been suggested that IFN-γ can cause a phenotypic switch for enterocytes to became immune accessory cells (260).

The nominal antigen for the $CD8^+$IELs has not yet been determined. However, short term cultures of MHC class I restricted, α-gliadin specific $CD8^+$ cells have been established from small intestinal biopsies of CD patients (261). Interestingly, these cells produced IFN-γ upon *in vitro* challenge with gliadin peptides. Non-specific mechanisms may also be operating in the

celiac lesion as a certain gliadin peptide that apparently is not a T cell antigen can induce rapid expression of IL-15 in the intestinal mucosa of CD patients (262) and recent studies suggest that IL-15 can stimulate IELs to cytotoxicity by mechanisms that over-ride restriction by TCR recognition (249, 263). The frequency of $CD8^+$IELs expressing the NK-receptor CD94 is increased in active CD (224) and IL-15 was also reported to induce CD94 expression and IFN-γ production by IELs (264). However, from the present study it became evident that "classical" $CD8^+$IELs contribute the most to the IFN-γ production in active CD with $CD94^+CD8^+$IELs also contributing significantly. Thus gluten intake might cause parallel adaptive and innate reactions, both involving IFN-γ production by $CD8^+$IELs in CD patients or there might be a gradual transition from specific to TCR-unrestricted activation of $CD8^+$IELs.

IL-10

IL-10 is an anti-inflammatory cytokine, capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, and TNF-α. Infections can induce the expansion of interleukin-10-producing regulatory T cells (265). Due to its anti inflammatory action recombinant IL-10 has even been proposed as a novel treatment in inflammatory bowel diseases (266). IL-10 is significantly increased in $CD3^+$ IELs in active CD compared to controls and is most pronounced in challenged CD. The result was confirmed by immunhistochemistry where IL-10^+ IELs were detected in active CD small intestinal biopsies but could not be detected in IELs from normal control. In all three IEL subsets studied active disease induced drastic changes in the expression levels of IL-10. This was most marked in $CD8^+$IELs and γδIELs, *i.e.* from a mean of a few mRNA copies/18S rRNA unit in controls up to ≈ 100 copies/18S rRNA unit, which is equivalent to approximately 1 copy/cell, in CD patients compared to controls. Although the expression levels of IL-10 mRNA were similar in all three IEL subsets in active CD $CD8^+$IELs were the main contributors (62±15%), followed by γδIELs (23±14%) and CD4+IELs (15±14%). Since the $CD94^+CD8^+$ subset of IELs is expanded in active CD (224) these cells might subsequently constitute the cellular source of the increased amounts IL-10 mRNAs expressed in active CD. In opposite to what was expected the expression levels of IL-10 were higher in most $CD94^-CD8^+$IEL samples than in the corresponding $CD94^+CD8^+$IEL samples with the average expression level was 5.2-fold higher in $CD94^-CD8^+$IELs. One very intriguing finding from this study is the fact that active CD is associated with significantly elevated expression levels of the down-regulatory cytokine

IL-10 in all three major IEL subsets analyzed. Furthermore expression levels of IL-10 correlated with a "marker cytokine" of the respective IEL subtype. In the case of $CD8^+$IELs there was a significant correlation between expression levels of the IL-10 and IFN-γ mRNAs. Interestingly, this simultaneous expression of IL-10 and IFN-γ in human small intestinal IELs has previously been shown after prolonged stimulation with IL-15 *in vitro* (264)

In the case of $CD4^+$IELs the IL-10 mRNA levels correlated to TNF-α mRNA levels. I am not aware of any report of $CD4^+$ T cells simultaneously secreting these two cytokines. In γδIELs the IL-10 levels correlated with TGF-β1 levels, a common combination in regulatory cells. Several types of regulatory T cells exert their function in the intestinal mucosa by secretion of IL-10 (267) and addition of IL-10 significantly inhibited responses to gliadin challenge in organ cultures of intestinal mucosa of CD patients (268) Thus, the expression of IL-10 in the IEL subsets is indeed suggestive of a regulatory function and an attempt to counteract the proinflammatory features in the mucosal gluten response apparently in an autocrine fashion.

It is shown that IL-10 can promote Fas-Ligand mediated cytotoxicity (264). Thus it is likely that local immune reactions in the small intestinal mucosa normally are regulated by a delayed onset of IL-10 production in the activated IEL that shuts down the immune reaction by killing of activated IELs by, activation induced cell death (AICD) and that the IL-10 production seen in active CD is a reflection of an AICD that is not sufficiently strong to turn off the inflammatory process.

TNF-α

A role for TNF-α, a proinflammatory cytokine, has been proposed in the pathogenesis of CD (242). Previous studies with intact biopsies or frequencies of TNF-α^+ cells have revealed a slight increased expression of TNFα or increased frequencies of TNFα^+ cells (269-271). However no increase in TNFα mRNA levels were seen in $CD3^+$ cells of children with active CD as compared to controls. If anything the level of TNFα mRNA in IELs was lower in untreated CD than in controls (Paper 1). Macrophages are the likely to be the source of TNFα in these previous studies. Among the subpopulations of T cells, only $CD4^+$IELs showed a slight increase in TNFα mRNA in active CD and, hence, these cells were the source of the small amount TNFα mRNA expressed. A small population of $CD4^+$IELs with a Th1 cytokine profile is normally present in the small intestinal mucosa of man (116, 272). Their function is not yet settled but it is conceivable that they regulate immune responses within the epithelial compartment and they may

have a role in initiating the reactivity towards gluten peptides executed by $CD8^+$IELs. Whether these cells are gluten-specific in CD patients is not known. However HLA-DQ is expressed on a small fraction of the iECs of the small intestine (116) and IFN-γ can upregulate the HLA-DQ expression in intestinal epithelial cell lines (273) giving the opportunity for presentation of immunogenic gliadin T cell epitopes on iECs in individuals carrying the predisposing HLA-DQ alleles.

Interestingly, the pronounced increased expression of the proinflammatory cytokine IFN-γ in CD is not paralleled by an increased TNFα expression, which is in contrast to Cohn's disease, an chronic inflammatory bowel disease, in which both these cytokines are increased and an chimeric antibody against TNFα is successfully used in the treatment of Cohn's disease (274).

TGF-β1

For the down-regulatory cytokine TGF-β1 mRNA, the expression level of $CD3^+$ was similar in all 3 groups of CD children and did not differ significantly from controls. (paper I)In the $CD3^+$ cells isolated from sequential biopsies taken after the reintroduction of gluten to treated CD children gluten challenge did not cause any increase in TGF-β1 mRNA expression with time. Most of the TGF-β1 was expressed by $CD3^+$ LPLs. Among the IEL subsets TGF-β1 mRNA levels were moderately increased only in $CD4^+$IEL subsets in CD children as compared to controls. However again $CD8^+$IELs contributed with significantly more TGF-β1 mRNA than $CD4^+$IELs and γδIELs which could be explained by their relatively high expression level and large proportion of total IELs (paper III). TGF-β1 mRNA levels were high compared to the other four cytokines analyzed in both CD patients and controls and its mRNA levels were ≈30 times higher than those of IL-10 in $CD4^+$IELs of both groups.

Taken together this point to a pronounced epithelial reaction in active CD with all three major IEL subsets activated. Apparently both $CD8^+$IELs and $CD4^+$IELs contain cell populations that simultaneously secrete a proinflammatory cytokine, IFN-γ and TNF-α respectively, and a down-regulatory cytokine, IL-10 while the γδIELs seem to have a more conventional regulatory role with simultaneous production of IL-10 and TGF-β1. The strong inflammatory reaction, most pronounced in the $CD8^+$IELs, is compatible with the notion that gluten is

mistaken for a pathogen at the epithelial surface in CD patients yielding innate and adaptive immune responses perhaps triggered or enhanced by bacterial adhesion to the epithelium

This high degree of correlation between expression levels of two cytokines in the subfractionated IEL populations could mean that the same cell produces the cytokines. With this assumption the data indicate that IEL contains at least five functionally different T cell populations (Figure 3)

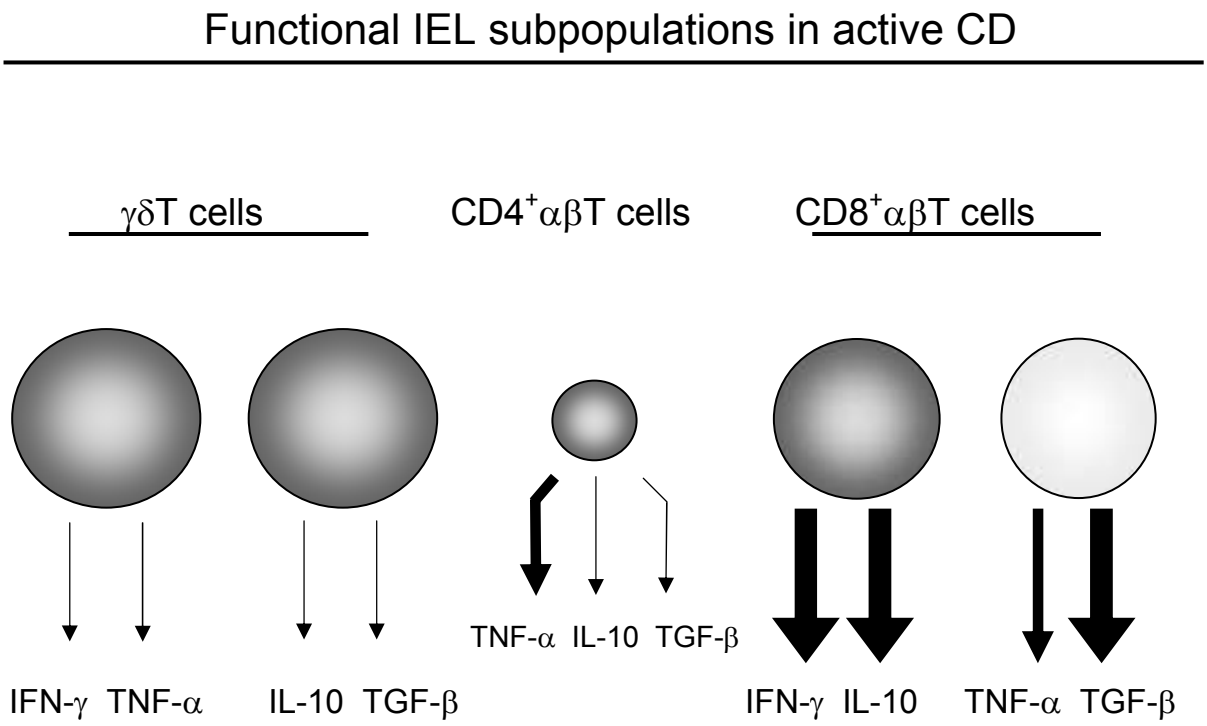

Figure 3. Schematic drawing depicting the cytokine profile of the five functionally different IEL subpopulations suggested by the results in the present study. Filled circle indicates that the cell type is activated and/or expanded in active CD. Arrow indicates production of the indicated cytokine. Thick arrow indicates prominent production.

Bacterial aherence in the small intestinal mucosa and innate immunity in Celiac disese. Paper II

When small intestinal samples from 153 children with CD and 59 controls were analyzed by scanning electron microscopy (SEM), as many as 37 % of the patients with untreated CD revealed bacteria adhering to the epithelial surface, whereas only 2% of the patients in the control group showed signs of adhering bacteria. Most intriguing was that 19% of the children

with treated CD, despite normal small intestinal histology, AEA concentration in serum and clinical recovery, still had signs of adhering bacteria. Most of the bacteria were rod-shaped but in some samples coccoid bacteria were also seen. The rod-shaped bacteria were often adhering in small groups like bouquets. The fact that the difference in the proportion of samples with adhering bacteria between untreated and treated CD patients was not statistical significant raised the question whether a primary defect in the innate status in the small intestine of children with CD could predispose for the bacterial adhesion and even be a contributing factor to the disease. Therefore different aspects of the innate status of children with celiac disease and controls were investigated , *i.e.*:

* Production of antimicrobial peptides and protein, *i.e.* defensins and lyzosyme.
* Changes in production of the major mucous component MUC2.
* Changes in glycokalyx composition.

The mRNA expression levels of mRNA for the α-defensins HD5 and HD6, the β-defensins hBD1 and hBD2 and lyzosyme were determined in RNA from villous epithelial cells (*v*-IEC) and epithelial cells from the crypt (*c*-IEC) using qRT-PCR. The expression levels of lysozyme, HD5 and HD6 mRNAs were up-regulated in active CD compared to controls. The elevated expression was linked to disease activity since the expression levels in children with treated CD were the same as in controls. The most pronounced changes in expression levels were seen in the c-IECs probably due to increased synthesis in the Paneth cells. Immunohistochemistry staining for HD5 and lysozyme in biopsies from active disease revealed cells expressing the proteins in the bottom of the crypts as expected, but also in the mid crypt region indicating metaplastic Paneth cell differentiation. Low levels of hBD1 and hBD2 mRNAs were detected in IECs in active and treated CD. If anything, hBD1 was slightly down regulated in active disease compared to treated disease, which is in line with what has been found in the large intestinal IECs in ulcerative colitis (275). This indicates that inflammation in the intestine results in decreased expression of this defensin. Interestingly, the disease did not affect the expression level of hBD2 although it has previously been shown that this defensin is upregulated *in vitro* by several bacterial species (144, 146). When comparing the IFN-γ expression levels in $CD3^+$ IELs from the same samples (Paper I) with the expression levels of defensins and lysozyme mRNAs in IECs we found that there was a significant correlation between the expression levels of HD5, HD6, and lysozyme in IECs with IFN-γ level in the $CD3^+$ IELs. Thus, a complex picture emerges for the production of antimicrobial peptides/proteins by IECs in CD with an unexpected increase in production of the constitutively expressed α-defensins and lysozyme, a slight decrease in the constitutively

expressed β-defensin hBD1 and no induction of the β-defensin hBD2. The low levels of β-defensins in CD may contribute to the bacterial adherence observed providing the bacteria are resistant to α-defensins. It is also possible that the conditions for generating an active peptide of HD5 and HD6 are defective in CD. In order to understand the possible role of glycocalyx and/or mucus composition in the pathogenesis of CD and the occurrence of bacteria in the intestinal mucosa, MUC2, MUC3, CEA (CEACAM5) and CEACAM1 mRNA expression levels were analysed in the same set of IECs by qRT-PCR and the protein expression by immunohistochemistry. MUC3 which is produced both by epithelial cells and goblet cells was expressed at similar levels by c-IECs and these levels were not affected by the disease. The expression level of MUC2 mRNA was increased in active CD compared to controls but returned to normal in treated CD. Furthermore, staining by immunohistochemistry of MUC2 in active disease revealed MUC2 in enterocytes in the crypts, in addition to the expected staining for MUC2 in goblet cells, which could indicate metaplastic goblet cells in active CD. Moreover the expression level of IFN-γ mRNA in $CD3^+$ IELs and the expression levels of MUC2 mRNA in the crypt epithelial cells of the same sample correlated which could reflect an altered epithelial differentiation as a consequence of IFN-γ exposure or a direct response to the adhering bacteria. CEA and CECAM1 are two cell surface glycoproteins localized to the glycocalyx of the epithelial cells in large intestine. CEA is also expressed in goblet cells. Their localization and high degree of glycosylation suggest that they may have a role in the innate immunity protecting the epithelium from microbial invasion (158). There were no differences in expression levels of CEA and CEACAM1 mRNAs or staining pattern at protein level between active CD, treated CD or controls, although these molecules are known to be up-regulated by proinflammatory cytokines in intestinal epithelial cells *in vitro* (158). To investigate if alteration in glycosylation of glycocalyx components and other epithelial cell surface molecules could be a predisposing factor for bacterial adherence in CD we used a panel of 21 biotinylated lectins to stain small intestinal biopsies from patients with active CD and treated CD, as well as from controls. Lectins are carbohydrate-binding proteins isolated from plants, prokaryotes, and animals that have been proven useful for localization of glycoconjugates *in vitro* and *in vivo* (276-279). The lectin panel consisted of Concanavalin A (Con A), *Datura stramonium* lectin (DSL), *Dolichos biflorus* agglutinin (DBA), *Erythina cristagalli* lectin (ECL), *Griffonia simplicifolia*lectin I (GSL-I), *Griffonia simplicifolia* lectin II (GSL-II), Jacalin, *Lens culinaris* agglutinin (LCA), *Lycopersicon esculentum* lectin (LEL), peanut agglutinin (PNA), *Phaseolus vulgaris* leucoagglutinin (PVL), *Phaseolus vulgaris* erythroagglutinin (PVE), *Pisum sativum* agglutinin (PSA), *Ricinus communis* agglutinin I (RCA-I), *Solanum tuberosum* lectin (STL), *Sophora japonica*

agglutinin (SJA), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA-I), *Vicia villosa* lectin (VVL), wheat germ agglutinin (WGA), and succinylated wheat germ agglutinin (sWGA). Absorptive cells were evaluated for staining of Golgi complex, cytosol, and glycocalyx, and goblet cells for mucin staining. Five lectins showed a difference in staining pattern between CD patients and controls and were therefore chosen for an enlarged study. They were: GSLI, PNA, SBA, UEA-I, and sWGA. Three interesting patterns could be seen: 1) UEA-I, which is an L-fucose-binding lectin, displayed intensive goblet cell staining in small intestinal mucosa from 15/16 CD patients but no staining in controls. Glycocalyx of absorptive cells of CD patients was also more intensely stained by UEA-I than those of controls. 2) PNA with main specificity for galactosyl (-1, 3) N-acetylgalactosamine showed staining of glycocalyx of controls but not of CD patients. 3) Golgi-complex staining of absorptive cells with GSLI, SBA (GalNAc specificity), and sWGA (GlcNAc specificity) was weak or absent in jejunum from patients with active CD in comparison to treated CD and controls. Similar observations were previously reported for WGA and SBA brush border staining in CD compared to controls (280). The staining patterns in UEA-I and PNA in CD were the same in active and treated disease. As CD children on GFD, (treated CD) had significantly more bacteria than controls, it is possible that children who develop CD have abnormal glycosylation of the mucous/glycocalyx layers allowing bacterial binding to the epithelium.

Lectins are used by bacteria for adhernce to tissue glycoproteins, *e.g. Escherichia coli* and *Salmonella typhimurium* adhere to mannose residues on host cells via their type 1 fimbriae. The carbohydrate moieties of glycoproteins and lipids do not only serve as receptors for binding of bacterial microflora. There are several examples where microorganisms are able to induce the production of certain required glycoconjugates or a change in the glycosylation of luminal membranes and cytoplasmic glycoconjugates of small intestinal IECs (277, 279, 281-286). Studies of germ-free mice compared to mice monocolonized with *Bacteroides thetaiotamicron* have shown that bacteria can have the capacity to metabolize fucose and thereby induce the expression of a host -1, 2-fucosyltransferase in small IECs (281). Whether an altered glycosylation is an inherited property in children contracting CD or that bacteria themselves influence the glycosylation has to be further scrutinized. But the glycosylation differences may facilitate bacterial adhesion. Gluten could maintain the inflammatory process by continued stimulation of immunocompetent cells in the mucosa. However, resident bacteria could also play an important role in maintenance of the inflammation, *i.e.* pathogenic bacteria could affect the disease or the changed milieu in CD mucosa which could offer a new niche for commensal bacteria that in turn modulates glycosylation. Another possibility is that children

who develop CD primarily have an inherited altered glycosylation pattern, which allows binding of bacteria. An interesting parallel is that UEA-I does not stain small intestinal goblet cells of germ-free mice but strongly stains goblet cells in conventional mice (279)

Aberrant extrathymic T cell receptor gene rearrangement in the small intestinal mucosa - a risk factor for celiac disease? Paper IV

Expression of RAG1 and RAG2 is crucial for the TCR gene rearrangement (182). T cell development and the rearrangement of the TCR take place mainly in the thymus but can also take place outside this organ. Ongoing extrathymic T cell maturation in the small intestinal mucosa of man was previously suggested by RAG1 mRNA expression in IELs of the T cell lineage (116) and the demonstration of RAG1 and RAG2 mRNA in RNA extracted from crude preparations of small intestinal epithelium and lamina propria (198). In addition, the expression of RAG1- and pTα mRNAs has been demonstrated in IELs and LPLs of fetal intestine and in young children (199, 200). Recently, two novel 5'UTR exons in the human RAG1 gene have been identified (200). One of these, exon 1A, was shown to be utilized by lymphocytes of the T cell lineage selectively outside thymus, and particularly in the small intestinal mucosa. Even though the function of extra-thymically derived T cells in the human small intestine is so far not known, our results in this study suggest that they may be implicated in the maintenance of "oral tolerance". Their regulatory or "silence" properties upon antigen encounter may be achieved by either of two parallel processes; extrathymic T cell development resulting in Treg cells and peripheral antigen driven T cell receptor editing leading to down-regulation of the reactive TCR.

Others and our studies pointed toward a central role of T cells in the pathogenesis of CD. Hence abrogated or decreased TCR recombination may negatively influence tolerance to food antigens and thereby contribute to the adverse T cell reactions seen in CD. RAG1 mRNA exists in four splice-forms 1A/2, 1B/2, 1A/1B/2, and 1C/2 that all code for the same protein. Therefore, the expression levels of the four RAG1 mRNA splice forms (1A/2, 1B/2, 1A/1B/2, and 1C/2) and pTα mRNA were determined in T cell-lineage subsets of IEL and compared in children with active CD with controls without any food intolerance (paper IV). Both IELs with a

mature TCR, *i.e.* γδIELs and αβIELs, and immature, thymocyte-like IELs not expressing either type of TCR but the T cell markers CD2 and CD7 ($CD2^+CD7^+$IELs) were analyzed.

In controls, RAG1 mRNA was detected in all three IEL subsets analyzed and the RAG1 1A/2 splice-form dominated, while much lower but still detectable amounts were seen for the RAG1 1B/2 splice-form. Only occasional samples contained detectable amounts of the 1A/1B/2 splice-form and in agreement with previous result RAG1 1C/2 mRNA was detected in IELs. The expression level of the RAG1-1A/2 mRNA splice-form was significantly reduced in CD patients both with active and inactive disease compared to controls and in all three studied IEL subsets (γδIELs, αβIELs, and $CD2^+CD7^+$ IELs) (paper IV). The RAG1 1B/2 splice-form showed no significant difference between CD patients and controls in any of the three IEL subsets. The RAG1 1C/2 splice form was not detected in any of the T cell subsets indicating that this splice variant is not induced in CD. γδIELs, αβIELs, and $CD2^+CD7^+$IELs were further analyzed for the expression of preTα mRNA. In controls, virtually all pTα mRNA was expressed in the immature $CD2^+CD7^+$IELs suggesting ongoig extrathymic T cell maturation. As was the case for RAG1 mRNA the pTα mRNA expression levels were significantly lower in CD patients, both in active and inactive disease, as compared to controls.The expression levels of pTα mRNA in γδIELs and αβIELs were low with no difference between any of the CD groups and controls (paper IV).

The simultaneous expression of RAG1 and pTα mRNAs in IELs with the phenotype of immature T cell lineage cells seen in this study confirms previous results (200) that extrathymic T cell maturation is taking place in the small intestinal epithelium of young children. Furthermore, to our knowledge we have for the first time demonstrated expression of RAG1 and pTα mRNAs in IELs with a mature phenotype, *i.e.* αβIELs and γδIELs. The expression of RAG1 mRNA in IELs with already rearranged and displayed TCR could reflect TCR editing in the later stages of local T cell maturation, or TCR revision in thymus derived T cells upon encounter of foreign antigens in the small intestinal epithelium. Thus one way to avoid over-reaction against food antigens may be to initiate revision of the TCRs in IELs that bind these antigens. The significantly decreased RAG1 1A/2- and pTα mRNA levels in children with CD suggests reduced extrathymic T cell maturation accompanied by decreased TCR editing and/or revision and thus, a decrease in production of intestinal Treg cells important for the maintenance of oral tolerance. One study in mice showed that an impairment of intraintestinal T lymphopoiesis and the presence of thymus derived T cells associates with intestinal inflammation (287). Thus, the intestine-derived T lymphocytes seem to be important for the regulation of the function of

thymus-derived T cells within the intestinal mucosa, and consequently, a balance of thymus and gut derived T lymphocytes in the intestinal mucosa could be important for the maintenance of gut integrity.

Finally, our study shows that the decreased expression of RAG1 and pTα mRNAs in CD was independent of disease activity suggesting that decreased T cell maturation in the intestinal mucosa is an inherent property of CD patients. Thus, failure to adequately adapt the T cell repertoire to the milieu at the intestinal mucosal surface and its exposure to dietary proteins and the microbial flora might be an important factor in the pathogenesis of CD and even be a contributing factor for contraction of the disease.

CONCLUSIONS

- There is a pronounced epithelial reaction in active CD where all three major IEL subsets (γδIELs, $CD4^+$αβIELs, and $CD8^+$αβIELs) are activated.
- Classical" $CD8^+CD94^-$αβT cells in the epithelial compartment are responsible for most of the excessive production of proinflammatory IFN–γ in active CD
- $CD8^+$IELs and $CD4^+$IELs contain cell populations that simultaneously secrete a proinflammatory cytokine, IFN–γ and TNF-α respectively, and a down-regulatory cytokine, IL-10 while the γδIELs seem to have a more conventional regulatory role with simultaneous production of IL-10 and TGF-β1
- The strong inflammatory reaction, most pronounced in the $CD8^+$IELs, is compatible with the notion that gluten is mistaken for a pathogen at the epithelial surface in CD patients yielding innate and adaptive immune responses perhaps triggered or enhanced by bacterial adhesion to the epithelium
- This high degree of correlation between expression levels of two cytokines in the subfractionated IEL populations could mean that the same cell produces the cytokines. With this assumption the data indicate that IEL contains at least five functionally functionally different T cell populations
- Scanning electron microscopy from small intestinal samples in 212 children revealed presences of rodshaped bacteria in the intestinal mucosa in 40 % of children with active CD and 20% of treated CD compared to only less than 2% of controls. Presence of bacteria seems to be a trait of CD in children.
- mRNAs in enterocytes for MUC2, HD-5 and HD-6 and lysozyme are increased in children with active CD but returns to normal levels in treated CD. The increased expression is a consequence of goblet- and Paneth cell metaplasia, which in turn correlated to the increased IFN-γ in CD
- A difference in glycosylation of certain glycocalyx/mucin proteins exposes unique carbohydrate structures that is predisposing for the binding of the bacteria seen.
- A decreased expression of RAG1 and pTα mRNAs in CD is independent of disease activity suggesting that decreased T cell maturation in the intestinal mucosa is an inherent property of CD patients. Thus, failure to adequately adapt the T cell repertoire to the milieu at the intestinal mucosal surface and its exposure to dietary proteins and the microbial flora might

be an important factor in the pathogenesis of CD and even be a contributing factor for contraction of the disease.

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”Pappa, vad är det nu den heter den där sjukdomen?”

Slutligen:

♥ ***Anna*****,** utan dina kritiska synpunkter, din kärlek,
ditt oreserverade stöd skulle inte det här……
Hur skall jag någonsin kunna…… ??

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