IMMUNOLOGIC INFLAMMATION IN THE SMALL INTESTINE OF CHILDREN

Cytokine Profiles and Immunologic Markers in Potential Coeliac Disease, Type 1 Diabetes, Graft-versus-Host Disease, and Food Allergy

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in the Niilo Hallman Auditorium of the Hospital for Children and Adolescents, on September 17th, 2004, at 12 noon

Helsinki 2004

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ISBN 952-91-7611-2 (paperback)
ISBN 952-10-2007-5 (PDF)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2004

To Professor Erkki Savilahti

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ABBREVIATIONS

APC	Antigen-presenting cell
bp	Base pairs
CCR	Chemokine receptor, CC
CD	Coeliac disease
DH	Dermatitis herpetiformis
EMA	Endomysium antibodies
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony stimulating factor
GvHD	Graft-versus-host disease
ICAM-1	Intercellular adhesion molecule-1
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISEL	In situ DNA 3'-end labelling
LFA	Lymphocyte function-associated antigen
LT	Lymphotoxin
mAb	Monoclonal antibody
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
M cell	Microfold cell
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK cell	Natural killer cell
NOD	Nonobese diabetic
PBMC	Peripheral blood mononuclear cell
PP	Peyer's patches
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCT	Stem cell transplantation
T1D	Type 1 diabetes
TCR	T-cell receptor
Th	T-helper lymphocyte
TGF	Transforming growth factor
TNF	Tumour necrosis factor
tTG	Tissue transglutaminase
tTGA	Tissue transglutaminase antibodies
VCAM-1	Vascular cell adhesion molecule-1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Westerholm-Ormio M**, Garioch J, Ketola I and Savilahti E. Inflammatory cytokines in small intestinal mucosa of patients with potential coeliac disease. *Clin Exp Immunol* 128:94-101, 2002

II **Westerholm-Ormio M**, Vaarala O, Pihkala P, Ilonen J and Savilahti E. Immunologic activity in the small intestinal mucosa of pediatric patents with type 1 diabetes. *Diabetes* 52:2287-2295, 2003

III Taskinen M*, **Westerholm-Ormio M***, Karikoski R, Lindahl H, Veres G, Savilahti E and Saarinen-Pihkala UM. Increased cell turnover, but no signs of increased T-cell infiltration or inflammatory cytokines in the duodenum of pediatric patients after allogeneic stem cell transplantation. *Bone Marrow Transplant* 34:221-228, 2004

IV Veres G*, **Westerholm-Ormio M***, Kokkonen J, Arato A and Savilahti E. Cytokines and adhesion molecules in duodenal mucosa of children with delayed-type food allergy. *J Pediatr Gastroenterol Nutr* 37:27-34, 2003

* These authors have contributed equally.

In addition, some previously unpublished data are presented.

ABSTRACT

Immunologically mediated enteropathies consist of a group of different diseases that are characterised by varying degree of villous destruction in the small intestine. Examples of these are coeliac disease (CD), graft-versus-host disease (GvHD), and food allergy enteropathy. In addition, immunologic changes have been observed in the small intestine of patients with type 1 diabetes (T1D). Characteristics of these disorders are excessive T-cell activation and production of proinflammatory cytokines. However, the knowledge of intestinal expression of cytokines is mainly based on studies on overt CD. Knowledge of the intestinal expression of cytokines in T1D and GvHD was, prior to this study, based only on experimental studies. Furthermore, few studies have investigated the role of cytokines in the early and mild forms of these intestinal inflammations.

The aim of this series of studies was to characterise the cytokine profiles and immunologic markers in mild inflammatory disorders of the small intestine, in order to investigate the immune activation and the role of cytokines in these diseases.

We evaluated the expression of cytokines and other immunologic markers by immunohistochemistry, *in situ* hybridisation, and RT-PCR in small intestine biopsy specimens of children 6 weeks (n=15) and 3 months (n=11) after stem cell transplantation (SCT), and of patients with potential CD (n=10), T1D (n=31) or food allergy (n=14). We compared the results with those of normal controls (n=18) and patients with coeliac disease (n=13). We also investigated apoptosis of epithelial cells by *in situ* DNA 3'-end labelling in biopsies of patients undergoing SCT.

We found increased densities of intraepithelial lymphocytes and IFN-γ and TNF-α positive cells in specimens of patients with potential CD. The densities of IL-2, IFN-γ, and TNF-α positive cells were increased in CD patients, regardless of whether they had T1D or not. In addition, patients with T1D demonstrated increased densities of intestinal IL-4 and IL-1α positive cells, regardless of duodenal morphology, duration of T1D or HLA-genotype. We found a markedly increased proliferation of crypt epithelial cells in specimens of patients undergoing SCT, who also demonstrated an increased rate of epithelial cells undergoing apoptosis both in the crypts and in the villous surface. Additionally, SCT patients had fewer T-cells in the lamina propria than controls, and the numbers of IFN-γ and TNF-α positive

cells were, surprisingly, as low as in the controls. Patients with untreated food allergy, on the other hand, showed increased number of IFN-γ positive cells.

In conclusion, we could detect inflammatory markers in the intestines of potential CD patients long before alterations in the villous structure occurred. The increased intestinal expression of IFN-γ and TNF-α positive cells in these patients indicates a skewing towards a Th1-type response already at this stage. The immunologic activation, with an unexpected Th2-dominance, in the small intestine of all patients with T1D supports the hypothesis that a link exists between the gut immune system and type 1 diabetes. Our results on increased cell turnover rate in SCT patients without symptomatic clinical or histological GvHD, may represent an early stage of intestinal GvHD in humans similar to animal models. The proposed cytokine expression in murine GvHD was not observed in our patients undergoing SCT, which may reflect the immunosuppressive medication in SCT patients. Finally, increased expression of IFN-γ in patients with untreated food allergy also implies a Th1-type immune reaction in food allergy enteropathy.

REVIEW OF THE LITERATURE

1. THE IMMUNE SYSTEM

The human body is continually exposed to infectious agents. Whether these manage to penetrate and cause disease is dependent both on the pathogenicity of the organism and on the body's immune system. The immune system is a complex organisation of lymphoid organs, cells, humoral factors, and cytokines, all with specific roles in defending against infections. There are two different types of immune responses: innate and adaptive. The innate response is immediate and includes physical and chemical barriers, as well as neutrophils, monocytes, macrophages, natural killer cells, complement, cytokines, and acute phase proteins. The innate response remains the same, whereas the adaptive response mediated by antigen-specific T- and B-lymphocytes has memory and enhances on repeated exposure to the same microbe. In contrast to innate response, the adaptive response takes several days or weeks to develop. Adaptive immune responses are generated in secondary lymphoid tissues, i.e. lymph nodes, spleen and mucosa-associated lymphoid tissue.

The most important feature of the immune system is its ability to distinguish between self and nonself. The essential function of the immune system is best demonstrated when it does not function appropriately; hypoactivity results in severe infections and hyperactivity in autoimmune diseases and allergy (reviewed by Delves and Roitt 2000a).

2. GUT IMMUNE SYSTEM

2.1 Structure and function

In the gut there is a delicate balance between the need to recognise pathogens and to prevent unwanted immune responses to food antigens or the normal intestinal flora, yet allowing adequate nutrient uptake at the same time. Considering the large area of the gastrointestinal tract, comprising almost a 400 m^2 surface in man, it is not surprising that it has developed both immunologic and non-immunologic ways of protection. The mucosal barrier consists of intestinal epithelial cells connected by tight junctions and of non-immunologic defence mechanisms such as low pH, peristalsis, and mucus coat, all together protecting the intestine from invading antigens (reviewed by Sanderson and Walker 1999).

The intestinal lymphoid tissue is the largest compartment of the immune system in the body and referred to as the gut-associated lymphoid tissue (GALT). It consists of mesenteric lymph nodes, Peyer's patches, isolated lymph follicles, and large numbers of lymphocytes

scattered throughout the lamina propria and epithelium of the intestine (Mowat and Viney 1997, MacDonald 2003). Peyer's patches (PP) are organised lymphoid aggregates within the mucosa and submucosa of the small intestine. They act as the primary inductive sites where the interaction between luminal antigens and circulating lymphocytes occurs. The PP are separated from the intestinal lumen by a single layer of epithelial cells, which contains specialised epithelial cells called the microfold cells (M cells). The M cells are specialised in antigen uptake from the intestinal lumen and transport them across the mucosal surface to the subepithelial dome area (Neutra et al. 1996). The antigens can be taken up by several antigen-presenting cells (APC), in which they are processed for presentation to T-cells in the PP (Figure 1). The T-cells are activated upon antigen presentation and differentiate and mature in the germinal centres of the follicles. They then migrate via efferent lymphatics to the mesenteric lymph nodes before reaching the thoracic duct, and eventually the systemic circulation. From the blood circulation these activated T-cells migrate with the help of adhesion molecules back to the main effector sites of the intestinal immune responses; the lamina propria and intraepithelial compartment. Here they act as effector cells, secreting cytokines and mediating specific adaptive immune defence (Mowat and Viney 1997, MacDonald 2003).

2.2 Cells in the gut-associated lymphoid tissue

Lamina propria is the region between the surface epithelium and the muscularis mucosa. The majority of the cells belonging to the gut-associated lymphoid tissue are T-lymphocytes, B-lymphocytes, and macrophages, in addition to dendritic cells, neutrophils, and low numbers of other granulocytes and mast cells. The lamina propria is also populated by smooth muscle cells and fibroblasts.

T-lymphocytes

T-lymphocytes are derived from pluripotent stem cells that migrate from the bone marrow to the thymus where they mature into T-cells. In the thymus the T-cells go through a process of positive and negative selection, that abolishes autoreactive T-cells (central tolerance) (reviewed in Delves and Roitt 2000a, Alam and Gorska 2003). Fewer than 5% of the developing T-cells survive this process, and T-cells with only a weak affinity for self major histocompatibility complex (MHC) molecules leave the thymus as single positive $CD4^+$ (helper) or $CD8^+$ (cytotoxic) T-cells. Approximately two thirds of peripheral blood T-cells are $CD4^+$ and one third are $CD8^+$.

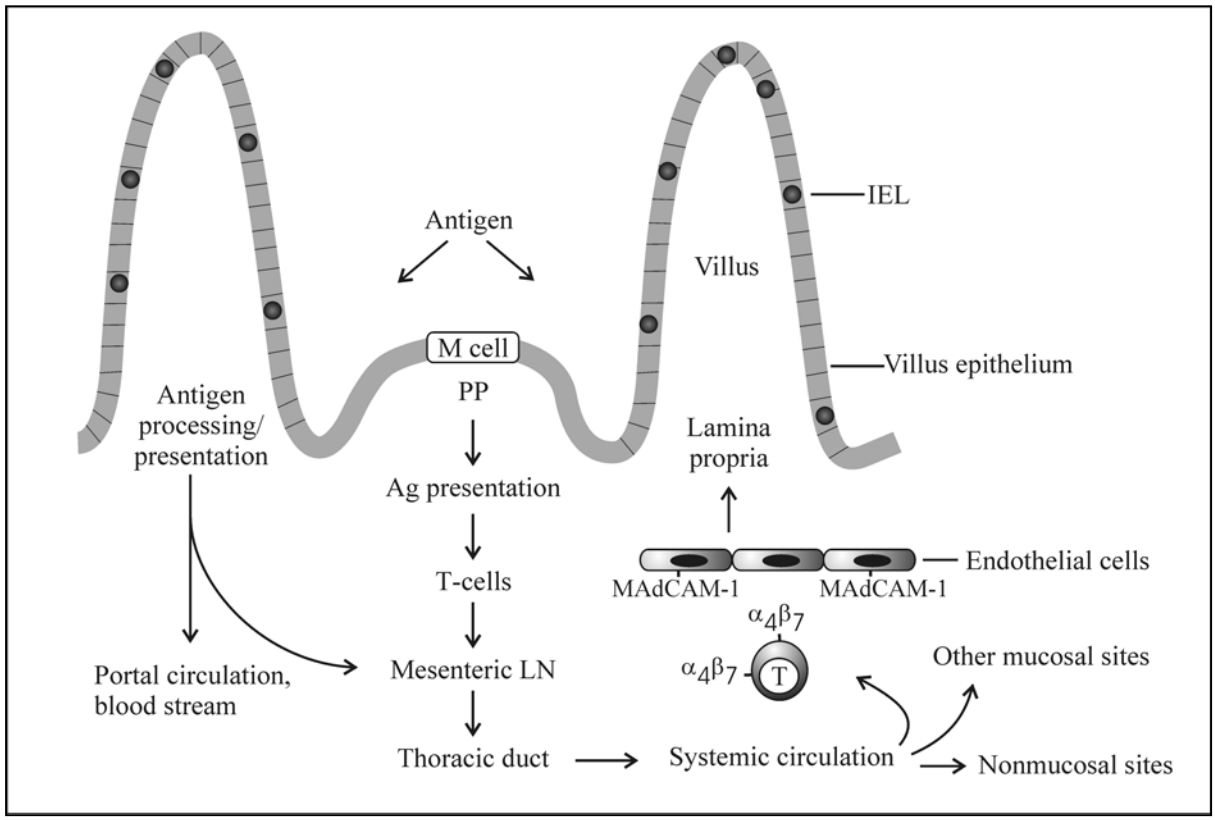

Figure 1. Antigen and T-cell traffic in the gut-associated lymphoid tissue (GALT). Antigen (Ag) is taken up either via M cells or epithelial cells. Antigen processing and presentation to T-cells in the Peyer's patch (PP) leads to the activation of T-cells, and subsequently of B-cells. The activated cells migrate via efferent lymphatics to mesenteric lymph nodes (LN) and, after passing into the thoracic duct, gain access to the systemic circulation. They then home back to the lamina propria and to other mucosal and nonmucosal sites. The return of T-cells back to the gut is mediated by the interaction between $\alpha_4\beta_7$-integrin on the surface of T-cells and mucosal addressin cell adhesion molecules (MAdCAM-1) on intestinal endothelial cells. Some soluble antigens taken up by epithelial cells also passes through the GALT directly into the portal circulation and bloodstream. The villous epithelium contains lymphocytes (IEL), most of which are activated $CD8^+$ T-cells, unique for the gut. (Modified from MacDonald and Monteleone 2001, Mowat 2003).

T-cells carry unique receptors on their cell surfaces, known as the T-cell receptors (TCR). This receptor recognises short peptides of antigens presented in association with MHC molecules, referred to as human leukocyte antigen (HLA) in humans, on antigen-presenting cells. MHC class I molecules are expressed on all somatic cells in the body and present peptides that are derived from endogenous proteins. These complexes are recognised by $CD8^+$ cytotoxic T-cells. MHC class II molecules on antigen-presenting cells present exogenous peptides to $CD4^+$ helper T-cells (Doyle and Strominger 1987). The class II proteins are expressed on professional APCs, i.e. B-cells, neutrophils, M-cells, macrophages, monocytes, eosinophils and basophils (Lanzavecchia 1996), but the expression can also be induced by interferon-γ (IFN-γ) on many other cell types, including epithelial and endothelial

cells (Skoskiewicz et al. 1985).

In humans, TCR heterodimer consists of either αβ- or γδ-chains (Brenner et al. 1986). 90-95% of circulating T-cells express the αβ form of the antigen receptor, whereas 5-10% express TCR composed of γ and δ chains. T-cell receptors are associated on the surface of T-cells with the CD3 complex (a marker of all T-cells) that transmit activation signals into the cell when the T-cell receptor binds antigen. Signalling by TCR alone in the absence of costimulatory signals, does not lead to activation of the T-cell, but to anergy or apoptosis. The requested additional signals come from various costimulatory molecules, e.g. costimulatory signals from the CD28-B7 interaction or CD40-CD40 ligand and LFA-1/ICAM-1 receptor-ligand interaction, and soluble mediators such as cytokines (Noble 2000).

T-cell populations in normal human small intestine

The intestinal lamina propria and epithelium form the largest single T-cell site in the human body. Almost all of the normal intestinal lamina propria T-cells are αβTCR positive, only 1% being positive for γδTCR. The proportion of $CD4^+$ and $CD8^+$ T-cells is similar to that in the peripheral blood. Lamina propria $CD4^+$ T-cells express markers like CD45R0 and $\alpha_4\beta_7$-integrin, and low levels of CD25 (IL-2 receptor) and HLA-DR, indicating that they have recently been activated by antigen (reviewed in James and Kiyono 1999). $CD4^+$ T-cells are considered to be the key players of the local immune regulation in the lamina propria. They proliferate poorly when stimulated with mitogen or specific antigens, but produce high levels of cytokines upon stimulation (Mowat 2003). Some of these activated $CD4^+$ cells are true effector cells that help B-cells to produce IgA, while others are “effector memory” cells. A fraction of $CD4^+$ T-cells are regulatory cells that maintain local tolerance to the load of harmless lumenal antigens (Sakaguchi 2000).

Intraepithelial lymphocytes

The epithelium contains a unique population of lymphocytes, the so called intraepithelial lymphocytes (IEL), which are separated from the intestinal lumen only by the tight junctions of the epithelial cells (Ferguson and Murray 1971). The majority of IELs are T-cells, of which 80-90% are $CD8^+$, and there are very few B-cells or natural killer (NK) cells in the epithelium. Of the T-cells 90% express the αβTCR, and about 50% express CD45R0, suggesting that they are antigen-primed memory cells (Brandtzaeg et al. 1989a). Approximately 10% of the IELs in the normal small intestine are γδTCR positive (Spencer et al. 1989), of which a large proportion are double negative cells ($CD4^-CD8^-$). It has been

suggested that a portion of the γδTCR$^+$ T-cells mature extrathymically in the gut mucosa, resulting in the relative large amount of these unique cells in the gut mucosa (Guy-Grand and Vassalli 2002).

T-helper 1 and T-helper 2 cells

Almost two decades ago Mosmann et al. discovered that naïve mouse CD4$^+$ T-helper lymphocytes differentiated into two distinct subsets with different functions and cytokine profiles upon antigenic stimulus (Mosmann et al. 1986). Later, this was also described in humans (Wierenga et al. 1991). Murine T-helper 1 (Th1) cells produce in addition to IFN-γ, also interleukin-2 (IL-2), tumour necrosis factor-α (TNF-α) and lymphotoxin (LT, also known as TNF-β), that mediate delayed type hypersensitivity responses and activation of macrophages. The main Th2 cytokine is IL-4, but Th2 cells also secrete IL-5, IL-9, IL-10 and IL-13. These cytokines provide help for B-cells and are critical in allergic responses (Mosmann and Sad 1996). The secretion of IFN-γ by Th1 cells inhibits Th2 cells, whereas the secretion of IL-4 by Th2 cells reciprocally inhibits Th1 cells (Figure 2) (reviewed by Ho and Glimcher 2002).

Although a distinct Th1/Th2 cytokine profile is not as clear in humans as in animal cells, an inverse relation remains between the tendency of T-cells to produce IFN-γ, as opposed to IL-4 and IL-5 (MacDonald and Monteleone 2001). The synthesis of IL-2, IL-6, IL-10, and IL-13 is not as tightly restricted to a single subset as in mouse T-cells, and both Th classes produce granulocyte-macrophage colony stimulating factor (GM-CSF), TNF-α, and IL-3 (Mosmann and Sad 1996). Even though the simultaneous production of IL-2, IL-4, and IFN-γ has also been observed in human T-helper cells (Paliard et al. 1988), the majority of T-cell clones and *in vivo* immune responses show a clear dichotomy between IL-2, IFN-γ, and TNF-β vs. IL-4 and IL-5. Therefore, the Th1/Th2 dichotomy is still considered an important functional division in the immune system.

Recently, two additional subsets of *in vitro*-derived regulatory T-cell types were identified and named Th3 and Tr1 (T-regulatory 1 cells). Th3 cells secrete mainly transforming growth factor-β (TGF-β) and to a lesser extent IL-4 and IL-10 (Chen et al. 1994, Fukaura et al. 1996), while Tr1 cells secrete IL-10, low levels of TGF-β, but no IL-4 (Groux et al. 1997) (Table 1). These cells may be important in actively suppressing or terminating immune responses and are thus linked to the development of oral tolerance.

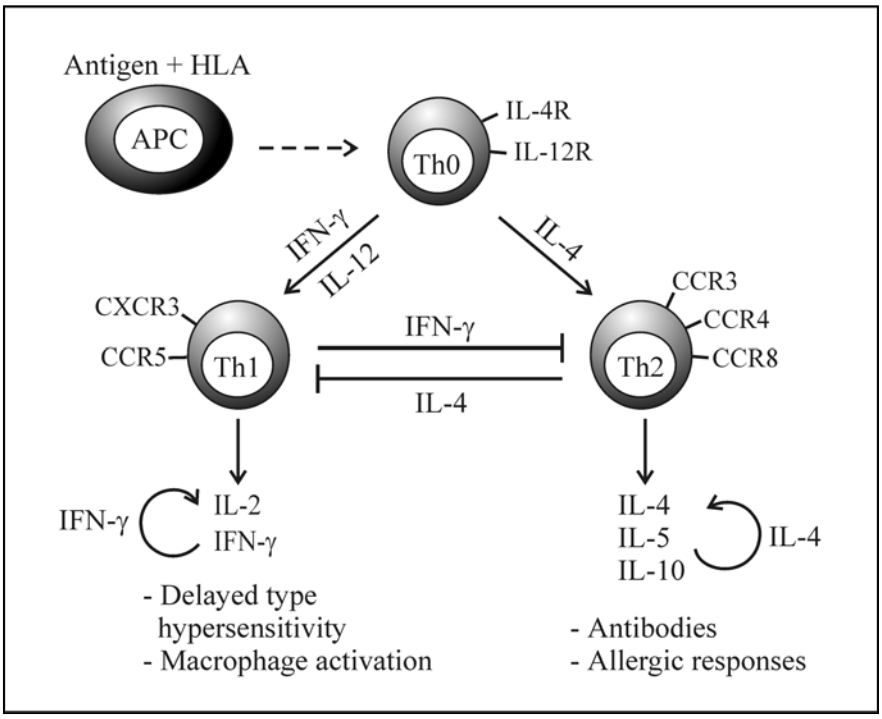

Figure 2. Overview of Th1 and Th2 cell differentiation. Naïve $CD4^+$ T-cells are activated via the TCR upon antigen encountering. When activated, IL-4 receptor (IL-4R) positive Th0 cell starts to proliferate, secrete IL-2 and express the IL-12 receptor (IL-12R). Signals that influence the differentiation in a positive way are indicated by arrows, and inhibitory signals are indicated by blunt arrows. The characteristic chemokine receptors, secreted cytokines, and type of action of the two cell types are indicated on the bottom of the cartoon. (Modified from Ho and Glimcher 2002, Alam and Gorska 2003).

T-cells that are resting do not transcribe cytokine genes, but when the T-cell is activated upon stimulation through the TCR and costimulatory receptors, the transcription of cytokine genes is rapidly induced (reviewed in Ho and Glimcher 2002). Th1 and Th2 cells appear to derive from a common precursor Th0 cell that expresses the IL-4 gene (Kamogawa et al. 1993). Naïve Th0 cells produce primarily IL-2, but may also produce cytokines characteristic of both Th1 and Th2 lymphocytes. The dose of the antigen, strength of the signal through the TCR, and costimulation all influence on the Th differentiation, but the most potent determinant is the cytokine milieu itself around the cell (Figure 2) (Ho and Glimcher 2002). IL-12 promotes IFN-γ production and Th1 development via signalling pathways that lead to activation of STAT-4 (signal transducer and activator of transcription 4) and the induction of T-bet, which promotes the Th1 lineage commitment. Ligation of the IL-4 receptor (IL-4R) expressed on naïve T-cells by IL-4 activates STAT-6 and initiates the Th2 differentiation program through the transcription factor GATA-3 (reviewed by Ho and Glimcher 2002).

Table 1. T-helper cell subtypes classified by their cytokine production

Th subset	Cytokines	Differentiation factors	Suppresses
Th0	IL-2		
Th1	IFN-γ, TNF-β, TNF-α, GM-CSF, IL-2, IL-3, IL-10, IL-13	IL-12, IL-18, IFN-γ T-bet, STAT-4	Th2
Th2	IL-4, IL-5, IL-9, IL-25 TNF-α, GM-CSF, IL-2, IL-3, IL-10, IL-13	IL-4, IL-13 GATA-3, STAT-6	Th1
Th3	TGF-β, IL-10, IL-4		Th1/Th2
Tr1	IL-10, TGF-β		Th1

Abbreviations: GATA, transcriptional factor binding to the nucleotide sequence element GATA; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; T-bet, T-box transcription factor expressed in T-cells; TGF, transforming growth factor; TNF, tumour necrosis factor; STAT, signal transducer and activator of transcription. (Modified from Alam and Gorska 2003, Borish and Steinke 2003).

B-lymphocytes

B-lymphocytes develop from the same pluripotent stem cells as T-cells, but they mature in the bone marrow. B-cell recognises antigen with antibody molecules as its receptor. Before they encounter antigen, B-cells coexpress immunoglobulin M (IgM) and IgD antibodies on their cell surface, but as they mature they switch to the use of IgG, IgA or IgE surface receptors. When the B-cells are activated they proliferate and differentiate into plasma cells that secrete antibody which has the same specificity as that of the epitope bound cell-surface receptor. The plasma cells have a half-life of only a few days, but part of them further differentiate into long-lived memory cells (Brandtzaeg et al. 1989b, Farstad et al. 2000).

In the Peyer's patch the antigen is presented by B-cells, dendritic cells or macrophages to T-cells and the induction of IgA occurs. When CD40 ligand on activated T-cell is bound to its receptor, CD40, on the surface of the B-cell, an activation signal is mediated in the B-cell and immunoglobulin class switching starts. Various cytokines secreted by the T-cell also help in the activation of the B-cell. IL-4 induces B-cell switch to IgE and IgG4 (Pene et al. 1988), whereas TGF-β in combination with IL-10 induces switch to IgA1 and IgA2 (Defrance et al. 1992). Following IgA switch the B-cells migrate from the PP into the lamina propria, similarly to T-cells, where the final maturation of IgA B-cells into plasma cells occurs. It has been shown that production of IgA correlates with the presence of antigen-specific Th2 cells in the gut mucosa. Thus, mucosal IgA responses probably require Th2, but not Th1

responses. B-cells can, on the other hand, also influence the differentiation of Th1 vs. Th2 cells by their own cytokine secretion (Harris et al. 2000).

The humoral immune response in the GALT is characterised by the production of secretory IgA (Mestecky et al. 1991). B-cells comprise about 15-40% of mononuclear cells in the normal small intestinal lamina propria. Most of them are IgA producing, but low numbers of IgG and IgM plasma cells are also present (Savilahti 1972, Farstad et al. 2000). Two distinct IgA subclasses exist, IgA1 and IgA2. IgA1 predominates in the proximal and IgA2 in the distal part of the intestine (Brandtzaeg et al. 1989b).

Epithelial cells

The mucosal surface is covered with a single layer of polarised epithelial cells, with undifferentiated, actively proliferating cells at the crypt bottom and mature, absorptive villous enterocytes at the villous surface. In addition to its barrier function and nutrient uptake, the intestinal epithelial cell has a variety of immunologic functions (reviewed in Pitman and Blumberg 2000). The epithelial cells play a crucial role in the uptake and transport of secretory IgA into the lumen (Mestecky et al. 1991), and they have been shown to express a variety of cytokines, such as IL-1β, TNF-α, IL-8, IL-10 (Lundqvist et al. 1996, Autschbach et al. 1998, Daig et al. 2000). Epithelial cells also express receptors for a variety of cytokines, e.g. IL-1R, IL-2R, IL-4R, IL-7R, IL-9R and IL-15R.

The epithelial cells express MHC class II molecules and have also been shown to be able to process and present antigens to primed T-cells (reviewed in Hershberg and Mayer 2000). Epithelial cells express E-cadherin, which is an important ligand for the mucosal integrin $\alpha_E\beta_7$ expressed on lymphocytes (Cepek et al. 1994). Furthermore, epithelial cells express or can be induced to express intercellular adhesion molecule-1 (ICAM-1, also known as CD54), lymphocyte function-associated antigen-3 (LFA-3), and B7-2 (CD86).

Macrophages

Monocytes and macrophages are the major APCs present in blood and peripheral lymph nodes. They are derived from myeloid progenitor cells in the bone marrow. After migration into tissues, monocytes differentiate into resident macrophages that may remain in the tissue for a period of days to months. The gastrointestinal tract contains also the largest number of macrophages of any tissue of the body (Stumbles et al. 1999). In addition to their antigen-presenting capability they activate T-cells through their production of accessory cytokines. The macrophages in the gut are quite heterogeneous and are more abundant in the small

intestine than in the large intestine. By immunohistochemical studies they have been shown to locate in the villous core, the subepithelial space in the crypts, under the dome epithelium overlying Peyer's patches and in the patch itself (Golder and Doe 1983). Macrophages secrete cytokines like IL-1, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and TGF-β and express receptors for IFN-γ, IL-4, IL-10, and TNF-α. They also express various adhesion molecules, as well as HLA class II molecules (reviewed in Stumbles et al. 1999).

2.3 Mucosal immune responses and oral tolerance

Oral exposure to dietary antigens by the immune system normally leads to antigen specific tolerance. This phenomenon has been termed oral tolerance and can be induced by the majority of soluble antigens (Mowat 2003). Substances that do not induce oral tolerance are bacterial polysaccharides and toxins that induce active immunity. The immunogenic immune response leads to the activation of Th1 or Th2 cells and is necessary for host defence against mucosal pathogens. The uptake of harmless antigens by APC and their presentation to T-cells is thought to lead to a short-lived Th1-type response, which is superseded by the induction of Th3 cells producing suppressor cytokines such as TGF-β, IL-4 and IL-10 (Figure 3). The Th3 cells migrate to the lamina propria, where they regulate the potentially injurious Th1 response (reviewed in Strobel 2002, Mowat 2003). The release of suppressive cytokines may also induce “bystander” effects; their release into the microenvironment may suppress an ongoing immune response to an unrelated, but anatomically colocalised antigen. It has been postulated that suppressor cells are the major players in the development and maintenance of oral tolerance to food antigens as well as to normal gut flora (Sakaguchi 2000). A unique population of naturally occurring suppressive $CD4^+CD25^+$ T-cells was originally identified in mice (Sakaguchi et al. 1995), and later also in humans (Jonuleit et al. 2001). $CD4^+CD25^+$ T-cells require cell to cell contact, and it was recently suggested that they block the proliferation of the responder cell by their surface membrane-bound TGF-β (reviewed in Chen and Wahl 2003). It is still unknown how these $CD4^+CD25^+$ cells relate to *in vitro*-derived Tr1 and Th3 cells.

The above is however, a simplified picture of oral responsiveness and nonresponsiveness. Based on animal studies the nature and dose of the antigen, the route of entry, and the timing of the antigen encounter are critical in determining the immune reaction. In addition, the age of the host, genetic background, and pre-existing inflammation in the GALT further influence the outcome (Strobel 2002). Feeding low-doses of an antigen favours

active suppression caused by regulatory T-cells in the PP, while feeding high-doses favours clonal deletion or anergy (Strobel 2002). Clonal anergy (i.e. inactivation) of antigen specific T-cells develops when APCs do not provide a costimulatory signal. The anergic T-cells remain intact but are incapable of proliferating or secreting IL-2 (Figure 3).

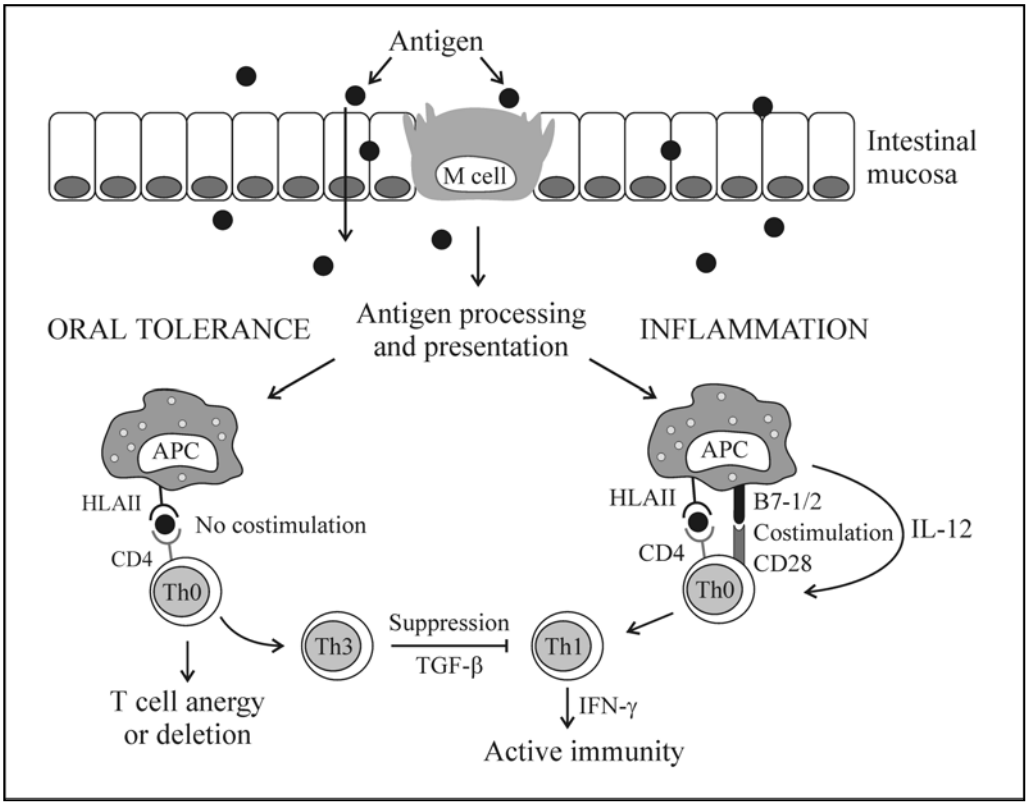

Figure 3. Immunoregulation after orally administered antigen; induction of tolerance versus inflammation. In the physiological situation, mucosally encountered antigens induce oral tolerance of lamina propria and intraepithelial lymphocytes as there is no activating costimulatory signal on MHC class II positive antigen-presenting cells (APC). This leads to anergy or deletion of $CD4^+$ T-cells, or to suppression of other antigen-reactive cells via the production of inhibitory cytokines (e.g. TGF-β) by Th3 regulatory cells. In contrast, in inflammation the antigen presentation in association with HLA class II antigens leads to activation of $CD4^+$ Th0 cells and the induction of Th1 and memory cell subtypes. (Modified from Strobel 2002).

Oral tolerance has been demonstrated to develop in humans to keyhole limpet hemocyanin (Husby et al. 1994), but otherwise the development of oral tolerance in humans is poorly documented. The normal response in Peyer's patches to food antigens (bovine β-lactoglobulin) in humans was shown to be predominated by Th1-type cytokine secreting cells, which is contradictory to the findings in rodents that typically show a local Th2/Th3 response following oral antigen feeding (Nagata et al. 2000). Multiple sclerosis patients fed

myelin basic protein had increased numbers of short lived T-cells producing TGF-β in the peripheral blood than patients who received placebo, suggesting suppressor activity (Fukaura et al. 1996). Another study with healthy volunteers found no expression of IL-2 mRNA in peripheral blood mononuclear cells (PBMC) when stimulated with ovalbumin or bovine γ-globulin, suggesting that anergy is the major mechanism for oral tolerance in humans (Zivny et al. 2001). This points to the fact that only little is known about the factors that influence the induction of oral tolerance in humans and that the results from animal studies can not directly be translated into human studies.

2.4 Oral tolerance in autoimmune diseases

Autoimmune diseases result from a combination of genetic, immunologic, hormonal, and environmental factors. Studies over the past years have suggested that there is an association between a preceding inflammation and autoimmune disease. The factors that trigger inflammation could include viruses, bacterial infection and mechanical injury (reviewed by Wucherpfennig 2001, Bach 2003). Infectious agents may induce the breakdown of immunological tolerance and the appearance of autoreactivity. However, the specific relationship between infection and autoimmunity is still unclear. One of the mechanisms responsible could be molecular mimicry, i.e. the presence of shared epitopes between infectious and self antigens, whereby epitopes on microbial agents stimulate the production of antibodies and the proliferation of T-cells that react with self antigens (reviewed by Karlsen and Dyrberg 1998).

The responses in autoimmune disorders have features of a Th1-type response, suggesting that defective T-cell tolerance underlies the disorder (recently reviewed by Hill and Sarvetnick 2002). Tissue destruction in autoimmune diseases has been associated with the presence of pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-1. However, these cytokines have also shown ability to suppress inflammation by homeostatic mechanisms, indicating that the original distinction between Th1 cells as pathology producing and Th2 cells as therapeutic is not as clear cut as previously believed (Hill and Sarvetnick 2002). Allergies and atopic diseases represent an overly aggressive Th2-type response leading to hypersensitivity to a broad spectrum of normally encountered antigens. Previously it was thought that a Th2-type response protected against development of autoimmune diseases, but two recent epidemiological studies suggest that patients with a Th1 illness are more likely to

have a Th2 illness, suggesting that they have a common underlying aetiology (Kero et al. 2001, Simpson et al. 2002).

The therapeutic efficiency of oral tolerance induction in various animal models of human diseases is well documented in models of multiple sclerosis, diabetes, arthritis, myasthenia gravis, thyroidits, and colitis (reviewed by Krause et al. 2000, Spiekermann and Walker 2001). In addition, a recent study in an animal model of human coeliac disease showed that intranasal administration of recombinant alpha-gliadin downregulated the immune response to wheat gliadin in DQ8 transgenic mice (Senger et al. 2003). Initial studies of treatment of autoimmune diseases in humans have already been reported, but the results have been equivocal at best (Spiekermann and Walker 2001).

3. ADHESION MOLECULES

Trafficking of lymphocytes in the intestinal mucosa is a complex multi-step process. It is mediated by adhesion molecules, the homing receptors on lymphocytes and their counterreceptors, termed vascular addressins, on the endothelial cells in specialised postcapillary high endothelial venules. Different combinations of these adhesion molecules ensure tissue-specific migration. The selective recruitment of lymphocytes makes the immune response more efficient by directing the cells back to the location where they first encountered their antigen and where they thus are more likely to meet their specific antigen again (Picker 1994).

Cells can express adhesion molecules constitutively or they can be upregulated by cytokines, chemokines or other proinflammatory molecules such as complement activation products or microbial metabolites. In addition to mediating adhesion, some of these molecules are also costimulatory during intercellular signalling. According to their structure and function, adhesion molecules can be divided into three different families: selectins, integrins, and Ig superfamily adhesion molecules. The selectins participate in the process of leukocyte rolling along vascular endothelium, whereas the integrins and Ig superfamily adhesion molecules are important for stopping leukocyte rolling and mediating transendothelial migration (Springer 1994).

3.1 Homing receptors in the gut

A number of cell adhesion molecules have been implicated in the selective recruitment of lymphocytes in the intestine, of which the $\alpha_4\beta_7$-MAdCAM-1 integrin-addressin pair is the most important (Salmi and Jalkanen 1999).

$\alpha_4\beta_7$- and $\alpha_E\beta_7$-integrins

The β_7-integrin chain can be expressed as a heterodimer with α_4 or α_E. $\alpha_4\beta_7$-integrin is thought to be a key lymphocyte homing receptor in flat venules in lamina propria (Berlin et al. 1993). This integrin is thought to be the primary ligand for the MAdCAM-1 vascular receptor, which normally is expressed exclusively in the gastrointestinal mucosa (Berlin et al. 1993). $\alpha_4\beta_7$-integrin also binds to vascular cell adhesion molecule-1 (VCAM-1). $\alpha_4\beta_7$-integrin is expressed on most resting lymphocytes and can be activated by a variety of stimuli, e.g. by chemokines. Both L-selectin and $\alpha_4\beta_7$ participate in activation-independent rolling of lymphocytes on MAdCAM-1 (Parker et al. 1992, Erle et al. 1994, Rott et al. 1997). As many as 70% of human lamina propria lymphocytes and 30-50% of IELs are $\alpha_4\beta_7$-integrin positive (Farstad et al. 1996).

The $\alpha_E\beta_7$-integrin (HML-1, CD103) is found on up to 90% of IELs, and on 30-50% of lamina propria lymphocytes (Farstad et al. 1996). It binds to E-cadherin on the epithelial cells (Cepek et al. 1994) and may be critical in the epithelial localisation of IELs (Parker et al. 1992).

Mucosal addressin cell adhesion molecule-1 (MAdCAM-1)

MAdCAM-1 is a key addressin for intestinal tissues (Streeter et al. 1988). It belongs to the immunoglobulin family and is related to other vascular adhesion molecules such as VCAM-1 and ICAM-1. MAdCAM-1 is selectively expressed on postcapillary venules in the Peyer's patches and in mesenteric lymph nodes, directing lymphocyte migration to intestinal lamina propria and into intestine-associated lymphoid tissues (Briskin et al. 1997). It has been shown to be involved e.g. in the lymphocyte migration into the inflamed pancreas in NOD mice (Yang et al. 1997).

Lymphocyte function-associated antigen 1 (LFA-1)

LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) is a multifunctional adhesion molecule involved in the antigen presentation process and T-cell mediated killing (Fischer et al. 1986). It is expressed on most

circulating leukocytes, and is the only integrin that is significantly expressed on lymphocytes (Fischer et al. 1986). It is involved in the later steps of the adhesion cascade, which allows the cell to arrest on the endothelial cell and transmigrate into mucosal sites (Springer 1994). It binds to the intercellular adhesion molecules (ICAM-1, ICAM-2, and ICAM-3) on endothelial cells.

Intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 (CD54) is a member of the immunoglobulin superfamily and widely expressed on vascular endothelial cells throughout the body (Boyd et al. 1988). The expression of ICAM-1 can be up-regulated by inflammatory mediators on a variety of cells, including intestinal epithelial cells and APCs. It mediates the later steps in the adhesion cascade by binding to LFA-1 (Springer 1994). The ICAM-1/LFA-1 interaction is critical for establishing a cell to cell contact between APC and T-cells, leading to T-cell activation (Springer 1994).

3.2 Alterations in lymphocyte recruitment during intestinal inflammation

During inflammation the same principles apply to lymphocyte migration as during physiological circulation. Cytokines and other mediators affect the expression of adhesion molecules at site of inflammation, thus controlling the traffic of leukocytes to tissue. As some of the molecules are up-regulated fast, whereas the appearance of others may take hours or even days, it is thought that this determines the cell composition seen at sites of inflammation at different time points. Immunohistological studies of lymphocyte trafficking indicate that gut inflammation induces dramatic changes in the extent of lymphocyte recruitment to the intestinal mucosa. Severe inflammation seems to induce additional recruitment mechanisms, as well as a superinduction of MAdCAM-1 and ICAM-1 expression. However, recruitment of T-cells to the gastrointestinal tract remains largely selective for $\alpha_4\beta_7^+$ cells also during inflammation (Briskin et al. 1997, Salmi and Jalkanen 1999).

4. MEDIATORS OF INFLAMMATION

Mediators of inflammation include cytokines, chemokines, complement activation products, immunoglobulins and acute-phase proteins.

4.1 Cytokines

Cytokines are a large group of low-molecular-weight soluble proteins that act as messengers both within the local immune system and between the immune system and other systems in the body (Table 2). Which cytokines are produced in response to an immune insult determines initially whether the response is cytotoxic, humoral, cell-mediated, or allergic (reviewed in Borish and Steinke 2003). Cytokines mediate their effects through binding to their receptors, which activates intracellular signals (reviewed by Ho and Glimcher 2002). Any cytokine may have many different biological effects depending on the target cell, and different cytokines may have similar effects. Cytokines produced by leukocytes that affect mainly other white cells are termed interleukins. Cytokines are usually divided into proinflammatory (IL-1, IL-2, IL-6, IL-12, IL-18, IFN-γ, and TNF-α), anti-inflammatory (IL-4 and IL-13), and immunosuppressive (IL-10, TGF-β), based on their activity.

IL-1α

Interleukin-1α is a proinflammatory cytokine. It belongs to the IL-1 family, which consists of four peptides; IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), and IL-18 (Dinarello 2002). IL-1α and IL-1β have similar biological effects and they bind with similar affinities to the two IL-1 receptors (Dinarello 1988). Type I receptor transduces the biological effects of IL-1, while the type II receptor, which is expressed on B-cells, neutrophils and bone marrow cells, has an anti-inflammatory function and is therefore referred to as a decoy receptor (Sims et al. 1993). Both receptors exist in membrane-bound and soluble forms. The naturally occurring IL-1Ra produced by monocytes can bind to IL-1 type I receptor with similar affinity as IL-1α and IL-1β, without transducing the biological activity and hence functions as a cytokine antagonist (Dinarello 2002). IL-1Ra is secreted in inflammatory processes and is thought to modulate the harmful effects of IL-1 in the natural course of inflammation. The production of IL-1Ra is upregulated by many cytokines such as IL-4, IL-6, IL-13 and TGF-β.

IL-1α is mainly produced by monocytes and macrophages, but also by T-cells, B-cells, neutrophils, and endothelial cells. The production may be stimulated by endotoxins, other cytokines, microorganisms, and antigens. IL-1α, as well as IL-1β, are synthesised as inactive precursors of 31 kDa (Dinarello 1988). The mature and active 17.5 kDa form is obtained through cleavage by a serine protease, termed interleukin converting enzyme (Cerretti et al. 1992).

Table 2. Major cytokines and their activities

Cytokine	Main cell source	Major activities
IL-1	Macrophages	Activates T-cells and macrophages, induces inflammatory response
IL-2	T-cells	Activates T-cells, NK cells and macrophages
IL-4	Th2 cells	Activates Th2 cells and IgE class switching, inhibits Th1 cells
IL-5	Th2 and mast cells	Induces differentiation of B-cells and eosinophils
IL-6	Th2 cells, macrophages, fibroblasts	Activates lymphocytes and antibody production, induces acute phase responses
IL-10	$CD4^+$ T-cells	Inhibits Th1 cells and production of proinflammatory cytokines, stops antigen presentation
IL-12	Macrophages, B-cells, and dendritic cells	Induces Th1 cells and stimulates production of IFN-γ
IL-18	Macrophages	Activates Th1 cells
IFN-γ	Th1 and NK cells	Activates Th1 cells, macrophages, class II MHC and adhesion molecules. Inhibits Th2 cells
IFN-β	Fibroblasts, virally infected cells	Induces resistance of cells to viral infections
TNF-α	Macrophages and lymphocytes	Activates macrophages, PMN cells, and endothelial cells, promotes inflammation
TGF-β	T-, B- and mast cells, macrophages	Immunosuppression, stimulates collagen formation
GM-CSF	Lymphocytes and macrophages	Stimulates granulocytes and monocytes

Abbreviations: GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer; PMN, polymorphonuclear; TGF, transforming growth factor; TNF, tumour necrosis factor. (Modified from Delves and Roitt 2000b, Borish and Steinke 2003).

IL-1α has many biological functions. It activates T-cells by inducing the production of IL-2 and expression of IL-2 receptors. It also enhances the expression of LFA-1 on T-cells and stimulates the adherence of leukocytes to endothelial cells by upregulating ICAM-1, VCAM-1 and E-selectin. IL-1α acts also as a chemoattractant and acts on macrophages in an autocrine way to induce the synthesis of other proinflammatory products. IL-1α is responsible for many of the symptoms associated with being ill. It interacts with the central nervous system to cause fever, lethargy, sleepiness and anorexia. It activates the synthesis of acute phase proteins, such as C-reactive protein and complement components by the liver. It also contributes to the hypotension of septic chock (reviewed by Dinarello 2002).

IL-2

IL-2 was originally designated as the "T-cell growth factor" because of its ability to stimulate proliferation of naïve T-cells (Morgan et al. 1976). IL-2 is produced by T-cells, mainly $CD4^+$, but also by $CD8^+$ T-cells, and natural killer (NK) cells (Malek 2003). It is the most powerful growth factor and activator for T-cells and promotes their maturation and the production of proinflammatory cytokines such as IFN-γ and TNF-α. It also induces growth and differentiation of B-cells and activates macrophages, NK cells, $\gamma\delta TCR^+$ IELs and cytotoxic T-cells (Schimpl et al. 2002, Malek 2003). IL-2 has recently been suggested to have immunosuppressive effects as well by promoting the development of $CD4^+CD25^+$ regulatory cells (Sakaguchi et al. 1995).

IL-2 receptor (CD25)

The IL-2 receptor is a multichain receptor composed of three subunits: β and γ-chains which are members of the haematopoietic cytokine receptor family with Ig-like domains, and the structurally unrelated α-chain (Waldmann 1991). The hematopoietin receptor superfamily includes receptors for erythropoietin, IL-3, IL-4, IL-7, GM-CSF, growth hormone and prolactin. The stimulation of T-cells by antigen in the presence of accessory signals it leads to the simultaneous secretion of IL-2 and the expression of high-affinity IL-2 receptors. The receptor is upregulated by antigen stimulation or by IL-2 on T-cells and IFN-γ on macrophages. Therefore, CD25, which is the α-chain of the IL-2 receptor, is widely used as an activation marker for mature lymphocytes.

IL-4

IL-4 is the major Th2 cytokine and produced by Th2 cells, NK cells, basophils, eosinophils and possibly mast cells (Paul and Ohara 1987, Lorentz and Bischoff 2001). IL-4 drives the initial differentiation of naïve Th0 cells toward a Th2-type and inhibits the proliferation of Th1 cells. It acts on B-cells to induce activation and differentiation, leading to the production of IgG_1 and IgE. As a result of its ability to stimulate IgE production it takes part in mast cell sensitisation and thus in allergy and in the defence against helminthie infections (Del Prete et al. 1988).

IL-4 has pleiotropic effects. IL-4 shares with IL-10 the anti-inflammatory features; they inhibit macrophage activation, T-cell proliferation, the production of IL-1α, IL-1β, TNF-α, and IL-6 by monocytes, and production of TNF-α and TNF-β by T-cells. Therefore, IL-4 may play a role in suppressing inflammatory processes mediated by these cytokines. IL-4

increases the expression of MHC class II molecules, IL-1Ra and TNF-R. IL-4 production can be induced by IL-2 and IL-4, and suppressed by TGF-β and IFN-γ (Lorentz and Bischoff 2001). The IL-4 receptor is a heterodimer, of which one chain is shared with IL-2R and IL-13R.

IFN-γ

IFN-γ is produced by Th1 cells and NK cells and is the major positive regulator of Th1-type response (reviewed in Farrar and Schreiber 1993). It belongs to the interferon family, also including IFN-α and IFN-β, which are produced by a variety of cells upon virus infection (reviewed in Farrar and Schreiber 1993). Type I interferons (IFN-α and IFN-β) bind to a common receptor, which is distinct from the one used by the type II interferon, IFN-γ. The receptor for IFN-γ is present on almost all cell types except mature erythrocytes (Farrar and Schreiber 1993).

IFN-γ stimulates antigen presentation, cytokine production and other effector functions of monocytes. This results in the accumulation of macrophages at the site of cellular immune responses and their activation to kill intracellular pathogens. IFN-γ also stimulates killing by NK cells and neutrophils. Production of IFN-γ is mainly induced by IL-12 and IL-18, which are secreted by monocytes and macrophages. IFN-γ induces the expression of MHC class II molecules (Skoskiewicz et al. 1985) and ICAM-1. IFN-γ stimulates the production of IgG2 and IgG3 subclass antibodies by B-cells. It also stimulates the production of IFN-β, IL-1α, IL-1β, TNF-α, and IL-12 (Mühl and Pfeilschifter 2003). It suppresses Th2 responses by inhibiting IL-4 and IL-10 production by macrophages (Pene et al. 1988).

TNF-α

Tumour necrosis factor was originally described as a factor that could kill tumour cells *in vitro* (Old 1985). A mature 17 kDa form is cleaved from the membrane associated TNF-α form by TNF-α converting enzyme. In the circulation TNF-α is mostly detected in a trimeric form (reviewed in Papadakis and Targan 2000). TNF-α acts via two different receptors TNFRI (p55) and TNFRII (p75), which are encoded by separate genes. The receptors are shared by TNF-β (LT, lymphotoxin), and the receptors have similar affinities for both TNF factors. The receptors belong to a family of proteins called the TNF and nerve growth factor receptor family, that includes the B-cell antigen CD40, CD27, and Fas antigen (reviewed in Papadakis and Targan 2000). Binding of Fas ligand to Fas initiates programmed cell death, apoptosis (Nagata and Golstein 1995).

TNF-α is produced mainly by macrophages and monocytes, and to a lesser extent by NK cells, T-cells, B-cells, and mast cells. Its major effect is promotion of inflammation and its effects overlap with the effects of IL-1. The responses include fever, shock, tissue injury, tumour necrosis, synthesis of collagen and proliferation of fibroblasts, and apoptosis. TNF-α also induces the production of IL-1 and IL-6. TNF-α induces ICAM-1 and VCAM-1 expression on endothelial cells, which attract circulating leukocytes to the inflammatory loci (Papadakis and Targan 2000).

The pathogenic role of TNF-α has been implicated in many chronic inflammatory processes such as coeliac disease, rheumatoid arthritis, graft-versus-host disease, and Crohn's disease (reviewed in Andreakos et al. 2002). Treatment with monoclonal antibodies against TNF-α (e.g. infliximab) has been beneficial in randomised, double-blinded clinical trials in rheumatoid arthritis and in severe cases of Crohn's disease (Andreakos et al. 2002). Recently, a successful treatment of a patient with refractory coeliac disease with TNF-α antibodies was also reported (Gillett et al. 2002).

4.2 Chemokines and chemokine receptors

Chemokines are a group of small (8-14 kD) chemotactic cytokines that regulate the migration of leukocytes from the blood into tissues. Today there are some 50 chemokines and over 20 chemokine receptors identified (recently reviewed by Ono et al. 2003). Chemokines are classified according to the position of two cysteine (C) residues compared with the other amino acids (X) near the NH_2-terminal. The four chemokine subgroups are CXC (α-chemokines), CC (β-chemokines), C, and CX_3C (Zlotnik and Yoshie 2000). Generally, CXC chemokines attract neutrophils, whereas the CC chemokines are less selective and attract lymphocytes, monocytes, basophils, and eosinophils.

Chemokines are produced by virtually all cells upon activation with proinflammatory cytokines or bacterial products. Chemokine receptors are found on all leukocytes. Each type of leukocyte bears chemokine receptors that guide it to particular chemokines in the tissue. Naïve T-cells express receptors CXCR4 and CCR7, whereas memory and effector cells express CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CXCR5, and CCR9 (reviewed in Rossi and Zlotnik 2000). Chemokine receptors are G-protein coupled, seven-transmembrane receptors. Chemokines and their receptors are not only involved in the control of hematopoietic cell migration and Th1 and Th2 development, but also in a wide variety of other physiological and pathological processes, such as lymphoid organ development, wound

healing, angiogenesis, and metastasis (Rossi and Zlotnik 2000). Recently it was discovered that Th1 and Th2 cells have a diverse expression of surface antigens; Th1 cells preferentially express the CXCR3 and CCR5 chemokine receptors, while Th2 cells express chemokine receptors CCR3, CCR4, and CCR8 (Annunziato et al. 1999).

CCR4

The chemokine receptor CCR4 was originally reported to be a selective marker for Th2 cells (Bonecchi et al. 1998, Sallusto et al. 1998), but recently it was shown to be expressed also on skin homing Th1 and Th2 cells (Campbell et al. 1999). Thereby, its role in Th2 responses still needs to be clarified. It is possible that whereas CCR3 may be a marker for IL-4 producing Th2 cell, CCR4 and CCR8 may be expressed more widely in Th2 polarised populations.

CCR-4 is a receptor for ligands CCL17 (TARC, thymus- and activation-regulated chemokine) and CCL22 (MDC, macrophage derived chemokine). CCR-4 is also expressed on NK cells and immature dendritic cells, and is involved in dendritic cell trafficking and T-cell migration from tissue to lymph nodes (Campbell et al. 1999). CCR-4 expressing cells are not found in the normal small intestine, but CCR-4 expressing cells have been described in inflammatory bowel disease patients (Agace et al. 2000).

CCR5

The chemokine receptor CCR5 is the receptor for ligands CCL3 (MIP-1α, macrophage inflammatory protein 1α), CCL4 (MIP-1β, macrophage inflammatory protein 1β) and CCL5 (RANTES, regulated on activation normal T-expressed and presumably secreted). The expression of CCR5 is biased to Th1 cells (Bonecchi et al. 1998, Loetscher et al. 1998, Sallusto et al. 1998). It is also expressed on monocytes and dendritic cells, and involved in the migration of Th1 cells and macrophages to sites of inflammation.

Normal small intestinal lymphocytes both in the epithelium and in the lamina propria have been shown to express CCR5 and CXCR3, but not CCR4 (Agace et al. 2000). CCR-5 was also expressed on peripheral blood lymphocytes expressing the β_7-integrin, suggesting a role for CCR-5 in the selective migration of lymphocytes to the intestine (Agace et al. 2000).

5. CLINICAL ASPECTS OF IMMUNOLOGIC INFLAMMATION IN THE SMALL INTESTINE

Immunologically mediated enteropathies consists of a group of different diseases that are characterised by a varying degree of villous destruction in the small intestine. Although the underlying immune mechanisms differ in the various conditions, they share a final effector pathway: activated T-cells producing cytokines that ultimately affect the villous architecture. In agreement with this, activated T-cells have been shown to cause villous atrophy in cultured explants of human foetal small intestine (MacDonald and Spencer 1988). The prototypic disorder in this group is coeliac disease (CD).

5.1 Coeliac disease

Coeliac disease is a chronic autoimmune disease in the small intestine that is triggered by dietary gluten (Farrell and Kelly 2002). Previously the prevalence of coeliac disease was estimated to be 1:1000, but screening studies identifying asymptomatic CD patients report prevalences of 1:99-1:300 in Europe (Catassi et al. 1994, Collin et al. 1997, Johnston et al. 1997, Mäki et al. 2003). Recent screening studies suggest that the rate may be the same in the USA (Not et al. 1998, Fasano et al. 2003). CD has a strong genetic predisposition. Approximately 90% of CD patients carry the HLA class II HLA-DQ2 heterodimer (DQA1*05-DQB1*02), and most of the remaining patients carry HLA-DQ8 (DQA1*0301-DQB1*0302) (Sollid 2002). However, other genetic factors are involved because up to 35% of the Caucasian population express HLA-DQ2. The disease risk is approximately 10% in healthy first-degree relatives of CD patients (Mäki et al. 1991, Mustalahti et al. 2002) and the concordance between monozygotic twins has been suggested to be as high as 75-80% (Hervonen et al. 2000, Greco et al. 2002).

Dermatitis herpetiformis (DH), a blistering skin disease with pathognomic granular IgA deposits in the skin, is one manifestation of CD (Fry et al. 1973). In most of the patients with DH, villous atrophy identical with that of CD is found, although it is mostly less severe and affects a shorter length of intestine than CD (Fry et al. 1973, Reunala et al. 1984).

Symptoms and diagnosis

The spectrum of coeliac disease has widened from the classical form of steatorrhoea and malabsorption in infants, towards milder forms in older children and adults. Paediatric patients may present with impaired growth, anaemia, or pubertial delay and totally lack

gastrointestinal symptoms. The diagnosis is increasingly made in adults, who may present with diarrhoea, flatulence, weight loss, or only with minor abdominal discomfort.

A variety of diseases has been reported to occur in association with CD. There is a well-established association between CD and type 1 diabetes (T1D) (Savilahti et al. 1986) and selective IgA deficiency (Savilahti et al. 1985, Collin et al. 1992). Other diseases associated with CD and DH are autoimmune thyroiditis, pernicious anaemia, Sjögren's syndrome, Addison's disease, rheumatoid arthritis, lupus erythematosus, sarcoidosis, vitiligo, and alopecia areata (Reunala and Collin 1997, Kaukinen et al. 1999). CD may also be associated with neurological symptoms, of which the most common were neuropathy, memory impairment and cerebellar ataxia in a Finnish adult cohort (Luostarinen et al. 1999). Patients with these associated conditions are often symptomless (called the silent form of CD) and are found only through screening. Untreated CD has also been associated with an increased risk for gastrointestinal carcinoma or lymphoma, which can be avoided with a strict gluten-free diet (Swinson et al. 1983).

The revised diagnostic criteria for childhood CD according to ESPGHAN are characteristic villous atrophy with crypt hyperplasia and lymphocytic intraepithelial infiltration in a small intestinal biopsy, and clear improvement or normalisation of serology and symptoms when on a gluten-free diet (Walker-Smith et al. 1990). CD patients on a gluten containing diet have increased levels of serum antibodies specific for various antigens, including gluten and the autoantigen tissue transglutaminase (tTG) (reviewed by Farrell and Kelly 2002). IgA antibodies against tTG are highly specific and sensitive for coeliac disease, and thereby greatly assist in the diagnosis of CD, as well as in monitoring the response to a gluten-free diet (Dieterich et al. 1997, Sulkanen et al. 1998).

Patients with DH have the same association to HLA-DQ and the same autoantibodies as CD patients. The rash in DH is located most typically to the elbows, the knees and the buttock. The diagnosis is based on the presence of granular IgA deposits at the dermal-epidermal junction in the skin by immunofluorescence staining. Like the enteropathy, the skin lesions respond to a gluten-free diet, although it usually takes months of strict diet before the symptoms are resolved, and in many patients the gluten-free diet is accompanied by treatment with the anti-inflammatory agent diaminodiphenylsulfone (dapsone) (Fry et al. 1973, Reunala et al. 1984).

Potential coeliac disease

Coeliac disease can be symptomless, or clinically silent (Ferguson et al. 1993). It has also been shown that coeliac disease can develop on previously normal mucosa (Weinstein 1974, Mäki et al. 1990, Collin et al. 1993, Troncone 1995, Corazza et al. 1996, Troncone et al. 1996b). Patients with such a latent form of CD express a normal jejunal mucosal architecture while on normal gluten-containing diet, but will later be found to have a flat mucosa, which heals on a gluten-free diet (Mäki et al. 1990, Corazza et al. 1996, Troncone et al. 1996b). Latent CD can be suspected in patients with positive coeliac autoantibodies and increased density of $\gamma\delta TCR^+$ IELs (Holm et al. 1992, Arranz et al. 1994, Kaukinen et al. 1998). Additional histological changes compatible with overt CD have also been shown to develop in these individuals when challenged with gliadin orally or rectally (Troncone et al. 1996a).

Since the term latent CD can be given only retrospectively it has been suggested that this "precoeliac" state should instead be named potential CD (Ferguson et al. 1993). Potential CD can be found in healthy family members of patients with CD or DH (Mäki et al. 1990, Mustalahti et al. 2002), and in patients with type 1 diabetes or other CD associated diseases. The presence of autoantibodies seems to predict the development of CD (Collin et al. 1993, Troncone 1995, Kaukinen et al. 1998). In follow-up studies it has been observed that potential CD progresses to overt CD in up to half of the patients (Collin et al. 1993, Kaukinen et al. 1998).

Molecular pathogenesis

Current concepts of the pathogenesis of CD include activation of $\alpha\beta TCR^+CD4^+$ T-cells in the lamina propria and the production of antibodies against a complex of gliadin peptide and tissue transglutaminase. A key observation was that the T-cells recognise modified, deamidated gluten peptides that are presented by DQ2 or DQ8 molecules, and that it is the enzyme tissue transglutaminase that performs the deamidation (Molberg et al. 1998). Tissue transglutaminase is an intracellular enzyme that is released from fibroblasts, endothelial cells, and inflammatory cells upon mechanical injury or inflammation. When secreted, tTG can crosslink glutamine-rich proteins, particularly gluten from wheat. At acidic pH tTG can deamidate the glutamine residues in gluten to glutamic acid. This posttranslational modification enhances binding of gluten epitopes to HLA-DQ2 and -DQ8 and potentiates their ability to stimulate T-cells (Molberg et al. 1998). Recently, it was shown that a 33-mer peptide fragment from gluten is resistant to digestive processing by proteases and that this

fragment is an extremely potent antigen for T-cell stimulation. The same fragment was shown to react with tTG with higher affinity than any other peptide so far (Shan et al. 2002).

The mechanisms that produce the lesions in CD are so far not well known. IELs might be involved in the lesion formation, but to date, there are no reports of IELs that recognise gluten in CD. The villous atrophy is instead thought to arise because of activation of IFN-γ secreting Th1 cells, that initiates production of inflammatory cytokines (Nilsen et al. 1995, Nilsen et al. 1998). Paradoxically, the major Th1 inducing cytokine, IL-12, has not been detected in the CD mucosa (Nilsen et al. 1998). IFN-α or IL-18 dependent Th1 activation has been suggested in recent studies (Monteleone et al. 2001, Salvati et al. 2002), and IFN-α has also been implicated in the disease process as it has been documented that patients receiving IFN-α therapy spontaneously develop CD (Monteleone et al. 2001). Th1 cells and macrophages have also been shown to secrete increased amounts of TNF-α in the coeliac mucosa (Kontakou et al. 1995a, Nilsen et al. 1998), which also plays a role in the upregulation of the expression of matrix metalloproteinases and epithelial cell death (Pender et al. 1997). Increased synthesis of matrix metalloproteinases, which degrade the lamina propria matrix, is postulated to be one of the factors causing villous atrophy. Another suggested factor involved in the mucosal remodelling and crypt hyperplasia is keratinocyte growth factor, which was shown to be overexpressed in CD mucosa (Salvati et al. 2001). However, the expression of cytokines has not been determined in the potential form of CD, which could be more informative on the factors causing villous atrophy since the mucosa is still intact at this stage.

5.2 The gut immune system in type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune disease that results from the destruction of insulin-secreting pancreatic islet β-cells by autoreactive T-cells and their mediators (Atkinson and Maclaren 1994). Although the exact sequence of events leading to the autoimmune destruction of islet β-cells is currently not completely known, it is well established that genetic, environmental, and immunological factors contribute to the pathogenesis (reviewed in Åkerblom et al. 2002). Finland has the highest incidence of T1D in the world with over 50 new cases / 100 000 children / year. In the Finnish population, the susceptibility for T1D is strongly associated with the HLA-DQB1*0302 allele (DQ8), and weakly with the DQB1*02 allele (DQ2) (Ilonen et al. 1996). Strong protection from the disease has been associated with the DQB1*0602 and weak protection with the DQA1*0301 allele (Nejentsev et al. 1999).

Although the susceptibility is linked to the MHC locus, MHC-identical siblings are only 13% concordant for T1D. T1D and CD share the same high-risk HLA-DQ2 genotype (DQA1*05 and DQB1*02). Screening of patients with T1D has shown that the prevalence of CD among children and adults with T1D is as high as 2-8% (Saukkonen et al. 1996, Carlsson et al. 1999, Hansen et al. 2001, Barera et al. 2002). T1D was on the other hand found in 1.0% of DH patients and in 5.5% of CD patients in a Finnish cohort (Reunala and Collin 1997).

Activation of the gut immune system in T1D

In addition to the association with CD, recent data suggest that the gut immune system plays a role in the development of T1D. Several serological studies on patients with newly diagnosed T1D have reported enhanced immune responses to cow's milk proteins (Savilahti et al. 1988, Karjalainen et al. 1992, Cavallo et al. 1996, Vaarala et al. 1996), and studies on infants at genetic risk of T1D indicate that primary immunisation to insulin occurs by exposure to dietary cow's milk insulin (Vaarala et al. 1998, Vaarala et al. 1999, Paronen et al. 2000). In one study glutamic acid decarboxylase-specific T-cells in patients with T1D were shown to express the gut-associated homing receptor $\alpha_4\beta_7$-integrin (Paronen et al. 1997), which suggests that autoreactive T-cells in type 1 diabetes may recirculate between the gut and the pancreas. Accordingly, T-cells derived from the human diabetic pancreas have been demonstrated to express the gut-associated homing receptor β_7-integrin (Hänninen et al. 1993). In addition, enterovirus and rotavirus infections have been associated with the development of islet cell autoimmunity (Honeyman et al. 2000, Lönnrot et al. 2000). Both types of viruses replicate in the gut and cause stimulation of the gut immune system.

Further evidence for a link between the gut immune system and type 1 diabetes comes from experimental studies. Diet has been shown to modify the development of autoimmune diabetes in biobreeding rats and nonobese diabetic (NOD) mice, which are animal models of human T1D (Elliott et al. 1988, Scott et al. 1997, Scott et al. 2002). T-cells infiltrating the pancreatic islets have been revealed to express the gut-associated homing receptor β_7-integrin, and antibodies that block this receptor or its endothelial ligand MAdCAM-1 inhibit the development of autoimmune diabetes in NOD mice (Hänninen et al. 1996, Yang et al. 1997). Moreover, autoimmune diabetes has been transferred to the recipients by mesenterial lymphocytes from young NOD mice, indicating that diabetogenic T-cells are present in the gut immune system (Hänninen et al. 1998). Finally, feeding autoantigen induces the development of autoreactive cytotoxic lymphocytes and the acceleration of autoimmune diabetes (Blanas et al. 1996, Bellmann et al. 1998).

However, it is not clear how an inflammation in the gut is associated with the process that destroys β-cells in the pancreas, and only one study has investigated the immunologic markers in the intestine of T1D patients so far. In this study an increased expression of HLA class II antigens in the villous epithelium and an increased density of $\alpha_4\beta_7$ expressing cells were found in the lamina propria, reflecting a stage of inflammation even in structurally normal small intestine of paediatric T1D patients when compared to controls (Savilahti et al. 1999). The role of these inflammatory changes in the intestinal mucosa of patients with T1D is unclear and further evidence for the involvement of the gut immune system in T1D is needed.

Th1/Th2 paradigm in T1D

Th1 cells have been suggested to be responsible for the initiation of the destruction of β-cells in the pancreas. The paradigm of T1D as a Th1 cell mediated autoimmune disease is mainly based on data from the animal models of the disease. In NOD mice and biobreeding rats the islet infiltrating lymphocytes show IFN-γ expression, which is reduced when the development of autoimmune diabetes is prevented by different interventions. IL-4 on the other hand protected against insulitis and diabetes in young NOD mice (reviewed by Rabinovitch and Suarez-Pinzon 2003). It is unclear if Th1 cells mediate β-cell destruction in human T1D as well. Systemic deviation to Th1-type response has, however, been suggested in human studies. Increased secretion of IFN-γ and TNF-α and low IL-4 production by mitogen stimulated PBMCs has been reported in patients with T1D (Berman et al. 1996, Kallmann et al. 1997).

5.3 Intestinal graft-versus-host disease

Allogeneic stem cell transplantation (SCT) is increasingly used to treat both malignant and nonmalignant diseases, including acute and chronic leukaemias, lymphomas, aplastic anaemia, and severe immunodeficiencies (Ferrara and Deeg 1991, Gratwohl et al. 2001). Among the complications of SCT, graft-versus-host disease (GvHD) is one of the major sources of morbidity and mortality (Ferrara and Deeg 1991). Approximately 10-50% of patients with sibling (Klingebiel and Schlegel 1998, Saarinen-Pihkala et al. 2001) and over 60% with matched unrelated donor transplantation (Saarinen-Pihkala et al. 2001) have signs of acute GvHD. The main target organs are the intestine, skin and liver. Symptoms of

intestinal GvHD include diarrhoea, abdominal pain, nausea, and intestinal bleeding (Klingebiel and Schlegel 1998, Iqbal et al. 2000).

The histology of acute intestinal GvHD progresses in experimental studies from proliferation of epithelial cells to villous atrophy, with single crypt epithelial cell apoptosis being the hallmark of GvHD (Mowat et al. 1988). Thus, the intestinal damage in murine acute GvHD resembles the histological changes in human CD. The knowledge of the histopathology in human intestinal acute GvHD is mostly based on studies on patients with a severe disease or end-stage enteropathy (Snover et al. 1985, Mowat 1997, Iqbal et al. 2000). Little is known about the histological picture during the early stages of human intestinal GvHD. The histology has been prospectively documented in one clinical study, which only evaluated rectal biopsies (Epstein et al. 1980). In this study the effect of cytoreductive treatment on the rectal mucosa resolved within 3 weeks after SCT (Epstein et al. 1980).

Immunopathogenesis of GvHD

Donor T-cells are critical in the induction of acute GvHD, because depletion of T-cell from the bone marrow graft prevents GvHD, but also increases the risk of leukaemic relapse (Ferrara and Deeg 1991). The donor T-cells do not just encounter a foreign environment in the host, but also one that has been altered by the underlying disease and the treatment; all these promote activation of inflammatory cells as described below.

Most of the information on the pathophysiology of GvHD comes from animal studies. Based on these studies immunopathogenesis of GvHD can be divided into three phases: 1) host conditioning, 2) donor T-cell activation, and 3) inflammatory effectors (Hill and Ferrara 2000). In phase 1, host conditioning including chemotherapy with or without total body irradiation leads to damage of host tissue. This leads to activation of host cells and secretion of proinflammatory cytokines, such as TNF-α and IL-1, which increase the expression of adhesion molecules, MHC antigens, and co-stimulatory molecules. Irradiation also induces apoptosis of endothelial cells and damages epithelial cells in the gastrointestinal tract, which allows microbial products to enter into the blood circulation. This further amplifies the GvHD reaction (Mowat et al. 1988). In phase 2, host alloantigens activate donor T-cells that differentiate into Th1- and/or Th2-type subsets. Th1 cytokines are preferentially produced in acute GvHD and have been implicated to be responsible for GvHD by inducing cytotoxic $CD8^+$ T-cells and NK cell responses, as well as by activating monocytes to produce IL-1 and TNF-α. Phase 3 of acute GvHD culminates in the host-cell destruction by cytolytic effects of these activated cells and inflammatory mediators like IL-1, TNF-α and NO. The damage in

the target organs includes apoptosis of epithelial cells and infiltration of immune cells (Hill and Ferrara 2000).

In humans, the relevance of the data from experimental studies is, however, far from clear. The early phases of the process are most likely clinically silent and the clinical diagnosis is made at the late phase of the target-organ damage. Evidence from clinical studies supports the concept that inflammatory cytokines, preferably TNF-α, and gastrointestinal tract damage are important mediators of clinical GvHD as well. Increased serum levels of TNF-α preceded severe acute GvHD with a latency of 25-54 days in adult SCT patients (Holler et al. 1990), and application of a monoclonal antibody neutralising TNF-α (infliximab) postponed the onset of acute GvHD after SCT in a clinical trial (Holler et al. 1995). However, the expression of cytokines in acute intestinal GvHD at the tissue level has not been characterised in humans. Clearly, studies on human tissues are needed to verify the proposed immunopathogenesis of GvHD.

5.4 Food allergy

The prevalence of food allergy is estimated as high as 8% in children less than 3 years of age in the Western world. The prevalence has increased during the last three decades. The majority of young children have a classic type I, IgE-mediated immediate immune response. In delayed-type food allergy, intestinal symptoms appear over several hours or even days after exposure to the allergen, and the reaction is non-IgE mediated (reviewed in Helm and Burks 2000). Immune mechanisms causing delayed food allergy are not well defined, but at least in the chronic entropathy T-cells play a role. Food allergy is most commonly caused by proteins of cow's milk, egg, peanuts, fish, and wheat (Sampson 1999).

Enteropathy associated with allergy to cow's milk, causing chronic diarrhoea and malabsorption was described 40 years ago (reviewed by Savilahti 2000). This form of food hypersensitivity resembles clinically and pathologically CD, except that there is no genetic determinant known so far. It seems to develop because of the immaturity of mucosal immune system on early exposure to cow's milk and ameliorates as the patient's immune system develops. The syndrome is cured by elimination of cow's milk from the diet.

Th1/Th2 paradigm in food allergy

In early infancy many of the mechanisms involved in the development of oral tolerance are impaired. The permeability of the gut to macromolecules is increased (Kuitunen et al. 1994),

enabling antigens to pass the mucosal barrier and impairing the normal antigen handling (Sanderson and Walker 1993). The immune system is immature, with few IgA-producing cells and a skewing of the Th1/Th2 ratio towards a Th2 dominance (reviewed by Holt and Jones 2000). The Th2 dominance continue in infants during the first year of life, whereafter a Th1 dominance develops. However, several studies have shown that children with atopy or cow's milk allergy continues to show a Th2 skewing with a high production of IL-4 and IL-5, and low production of IFN-γ by the peripheral blood leukocytes (Hill et al. 1993, Suomalainen et al. 1993, André et al. 1996, Prescott et al. 1998). IL-4 is the main cytokine that promotes IgE production (Del Prete et al. 1988), suggesting that aberrant cytokine profiles play a central role in the pathogenesis of IgE-mediated food allergy.

Although cytokine imbalance has also been proposed to be important in the pathogenesis of delayed-type food allergy (Hill et al. 1993, Sütas et al. 1997), only one study had addressed this in the gut prior to our study. Hauer et al. showed an elevation in the number of cells secreting IFN-γ and, to a lesser extent, IL-4, isolated from duodenal biopsies of 14 infants with cow's milk sensitive enteropathy by enzyme-linked immunoabsorbant spot technique (Hauer et al. 1997). Cells secreting IFN-γ were ten times more abundant than cells secreting IL-4, indicating a Th1 dominance. However, by RT-PCR they found higher expression of IL-4 and IL-10 mRNA than of IFN-γ mRNA (Hauer et al. 1997).

Symptoms and diagnosis of food allergy

The evidence of IgE mediation in acute food allergy is well established. It most commonly manifests as erythematous and urticarial skin symptoms, but acute gastrointestinal symptoms often associate these or may occur alone. The gastrointestinal symptoms of delayed food allergy are chronic diarrhoea, more rarely vomiting or constipation, and the associating skin symptom is chronic eczema. Food allergy may also induce respiratory symptoms such as allergic rhinitis, wheezing or cough.

The diagnosis of food allergy is based on the disappearance of symptoms when on an elimination diet for at least 2 weeks, followed by their reappearance during a challenge test and clinical response to a renewed elimination diet (diagnostic criteria by ESPGHAN (ESPGHAN 1992). The food challenge should be double-blind placebo-controlled in order to not cause any bias. No clinical symptoms or laboratory test can predict the challenge outcome.

6. MICROSCOPICAL FINDINGS IN IMMUNOLOGIC INFLAMMATIONS

6.1 Morphology of the small intestine

Histopathology

The mucosal atrophy in coeliac disease develops through various stages of severity, which according to the classification by Marsh can be graded from type 1 to type 3 lesions (Marsh 1992). In the infiltrative (type 1) lesion, the mucosal architecture is still normal, but there is an increased number of intraepithelial lymphocytes, especially γδTCR bearing IELs. In the hyperplastic stage (type 2), the villi are still of normal height, but the crypts are elongated due to increased generation of immature epithelial cells in the crypts in order to compensate for the increased epithelial cell loss on the villi. This stage progresses to the destructive (type 3) lesions, which show the typical flat mucosa with crypt hyperplasia. The same histopathology as for type 1-type 3 has also been documented in graft-versus-host disease (Mowat 1997), and in cow's milk (Walker-Smith et al. 1978, McCalla et al. 1980, Kuitunen et al. 1982) or soy allergy (Perkkiö et al. 1981). The absence of ulcerations and transmural thickening distinguishes the cellular infiltrate in these enteropathies from that in Crohn's disease.

Intraepithelial lymphocytes

In coeliac disease the density of $CD3^+$ IELs is increased (Marsh 1992, Järvinen et al. 2003). The increase is even more pronounced in the population of intraepithelial cells bearing the γδTCR, which constitutes up to one third of the IELs in coeliac lesions (Savilahti et al. 1990). In normal small intestine, there are 10-20 IELs per 100 epithelial cells (Ferguson and Murray 1971), which correlates with a density of 10-50 IELs per mm of surface epithelium (Savilahti et al. 1990). The increased density of $\gamma\delta TCR^+$ IELs is considered the hallmark of CD (Halstensen et al. 1989, Spencer et al. 1989). The density of $\alpha\beta TCR^+$ cells is dependent on gluten, while the density of $\gamma\delta TCR^+$ cells has been reported to remain increased even during a gluten-free diet (Savilahti et al. 1990, Savilahti et al. 1992, Savilahti et al. 1997, Järvinen et al. 2003). However, the frequency of $\gamma\delta TCR^+$ IELs has also been reported to decrease on gluten-free diet (Spencer et al. 1989, Iltanen et al. 1999).

The increased density of IELs is not specific for CD. IELs are also increased in food allergy enteropathies in humans (Perkkiö et al. 1981, Kuitunen et al. 1982, Kokkonen et al. 2000) and in murine GvHD (Mowat et al. 1988). In patients with severe food allergy enteropathy studies have also shown a moderately increased density of $\gamma\delta TCR^+$ intraepithelial

lymphocytes (Spencer et al. 1991, Kokkonen et al. 2000). An increase of γδTCR$^+$ IELs was also documented in school-aged children with slow developing mild gastrointestinal symptoms upon cow's milk challenge (Kokkonen et al. 2001a).

Lamina propria infiltration

The volume of the lamina propria is enlarged in the jejunal specimens of patients with untreated CD (Marsh 1992). The mature lesions of CD contain a mixture of T- and B-cells and macrophages. There is a marked accumulation of IgA-, IgM and IgG-producing plasma cells (Savilahti 1972). The absolute number of T-cells is increased in coeliac lamina propria (Beckett et al. 1996, Arató et al. 1998), with twice as many T-cells bearing the γδTCR than observed in normal mucosa (Savilahti et al. 1990). Most of the T-cells are still αβTCR$^+$ (Halstensen et al. 1989, Spencer et al. 1991), and there is no change in the CD4/CD8 ratio. A larger proportion of T-cells, especially in the CD4$^+$ cell population, show activation markers (CD45R0, various integrins and CD25). Nevertheless, few bear markers associated with cell proliferation (reviewed by MacDonald and Spencer 1990).

In food allergy, the numbers of lymphocytes, plasma cells and eosinophils are increased in the lamina propria. Early studies on immunoglobulins in the intestines of patients with this syndrome showed a strong infiltration of IgA and IgM containing cells at the time of clinical reaction either to cow's milk or soy (reviewed by Savilahti 2000). CD4$^+$ T-cells predominate in the lamina propria, and many CD4$^+$ cells are also HLA-DR$^+$, suggesting that they are activated. The number of these cells diminished during a cow's milk elimination diet (Nagata et al. 1995).

6.2 Production of cytokines

Since the discovery of cytokines they have been the focus of extensive research in various inflammatory conditions of the intestine. Still, there is no comprehensive data on cytokine expression in the normal human small intestine. Probably the best indication of the cytokine secretion by normal lamina propria T-cells so far comes from three studies where freshly isolated human lamina propria T-cells were shown to spontaneously secrete excessive IFN-γ, followed by weaker secretion of IL-4, IL-5, and IL-10 (Hauer et al. 1997, Carol et al. 1998, Hauer et al. 1998). Freshly isolated PP T-cells also contained more IFN-γ than IL-4 secreting cells, but the expression of IL-4 and IL-10 mRNA was higher than the expression of IFN-γ mRNA (Hauer et al. 1998). Lamina propria cells, but not epithelial cells, secrete IL-

1 (Youngman et al. 1993), whereas freshly isolated IEL secrete IFN-γ (Lundqvist et al. 1996, Carol et al. 1998). Thus, lamina propria T-cells secrete spontaneously far more cytokines than peripheral blood T-cells.

An early study on cytokine production by lamina propria lymphocytes in coeliac disease did not detect increased IFN-γ (al-Dawoud et al. 1992), whereas more recent studies all suggest a Th1 dominated response. Studies with intact jejunal biopsies from CD patients have shown increased expression of IFN-γ mRNA (Kontakou et al. 1994, Lahat et al. 1999), and increased IFN-γ mRNA production by gluten-specific intestinal T-cell clones (Nilsen et al. 1995, Troncone et al. 1998). After a wheat peptide challenge an increase in the number of cells expressing mRNA for IFN-γ and IL-6 was observed in all four investigated patients, whereas mRNA for TNF-α and IL-2 was increased in two out of four patients (Kontakou et al. 1995b). In addition, increased IFN-γ mRNA is seen after *ex vivo* gliadin challenge of small intestinal biopsies of CD patients (Nilsen et al. 1998, Troncone et al. 1998). Furthermore, anti-IFN-γ treatment in small intestinal organ cultures of CD patients *in vitro* inhibits the histological changes induced by gliadin sensitive T-cells (Przemioslo et al. 1995).

Some gluten specific T-cell clones from coeliac intestinal lesions secrete TNF-α, IL-4, IL-5, IL-6, IL-10, and TGF-β, although much less than IFN-γ (Nilsen et al. 1995, Troncone et al. 1998). Studies with intact biopsies have also reported a somewhat higher frequency of TNF-α mRNA positive specimens in active CD (Lahat et al. 1999), increased frequencies of TNF-α positive cells (Przemioslo et al. 1994, Kontakou et al. 1995a, Kontakou et al. 1995b), and sometimes increased mRNA levels after *ex vivo* challenge (Nilsen et al. 1998). Additionally, other macrophage-derived cytokines, such as IL-6 have been found in biopsy specimens from CD patients (Przemioslo et al. 1994, Kontakou et al. 1995a, Kontakou et al. 1995b).

Studies on IL-4 and IL-10 are contradictory. Increased mRNA production in CD biopsies was reported by one group for IL-10 but not for IL-4 (Lahat et al. 1999), while Nilsen et al. found increased IL-10 mRNA and protein, but no IL-4 (Nilsen et al. 1998), and Beckett et al. found no changes in either IL-4 or IL-10 by immunohistochemistry and in situ hybridisation (Beckett et al. 1996).

6.3 Expression of HLA class II antigens

MHC class II molecules are present in the epithelium of normal small intestine. Staining with antibodies against HLA-DR antigens has shown that positive expression in the normal small

intestine is restricted to the apical cytoplasm of the epithelial cells and that the intensity decreases from the top of the villous, leaving the crypts negative (Scott et al. 1980, Arnaud-Battandier et al. 1986, Ciclitira et al. 1986). In addition, capillary walls and mononuclear cells in the lamina propria stain positively (Scott et al. 1980). Staining with HLA-DP antibodies show a similar pattern, but the epithelial cells show a weaker staining than mononuclear cells (Marley et al. 1987, Mayer et al. 1991).

The expression of HLA-DR and –DP is increased in a number of diseases. In CD, the whole cytoplasm, as well as the basal surface of epithelial cells in the surface and crypt epithelium stain positively with HLA-DR (Arnaud-Battandier et al. 1986, Ciclitira et al. 1986, Kelly et al. 1988). HLA-DP stainings have shown an increased positive staining in both surface and crypt epithelium compared with control subjects (Marley et al. 1987, Kelly et al. 1988). The numbers of HLA-DR and -DP positive mononuclear cells in the lamina propria are also increased (Kelly et al. 1988). When on a gluten-free diet the staining of MHC class II molecules does not differ from that seen in controls (Arnaud-Battandier et al. 1986, Ciclitira et al. 1986, Arato et al. 1987). Increased staining has also been documented in first-degree relatives of CD patients (Holm et al. 1994). In addition to CD, increased expression of HLA-DR and –DP has been documented in the epithelium in Crohn's disease (Mayer et al. 1991), autoimmune enteropathy (Hill et al. 1991), in rat GvHD (Mason et al. 1981), and in jejunal biopsies of patients with T1D (Savilahti et al. 1999).

Despite numerous studies, positive staining for HLA-DQ in the normal (Marley et al. 1987, Kelly et al. 1988) or diseased small intestinal epithelium has not been shown (Kelly et al. 1988, Mayer et al. 1991, Schweizer et al. 1991, Holm et al. 1994, Klemola et al. 1995, Savilahti et al. 1999). The expression of HLA-DQ antigens has been confined solely to cells within the lamina propria, without any difference between control patients and patients with inflammatory diseases. The reason for absent HLA-DQ expression is unknown. Alternatives, like a too low sensitivity of the methods and antibodies used, as well as a posttranscriptional defect in HLA-DQ gene expression, have been suggested (Kelly et al. 1988, Mayer et al. 1991).

6.4 Proliferation and apoptosis of epithelial cells

The epithelial cells are produced in the crypts of Lieberkühn from progenitor cells and mature as they migrate up the crypt-villous axis. At the end of their 3-4 day-long life-span (Podolsky 1993), they undergo apoptosis and are shed into the gut lumen from the tip of the

villous (Ruemmele et al. 2002). Approximately 5-15% of the epithelial cells in the crypts are proliferating in the normal small intestine, as shown by immunohistochemical stainings with the antibody Ki-67 (Moss et al. 1996, Maiuri et al. 2001). Ki-67 is an antibody reacting with a protein in the nucleus of cells undergoing proliferation (Gerdes et al. 1983). In inflammatory states, the number of cells undergoing mitosis in the crypts is increased to compensate for the increased loss of surface epithelial cells. In CD, the epithelial cell renewal rate is increased as the result of both an increased mitosis rate and larger crypt cell compartment (Wright et al. 1973). In food allergy, an increased mitotic activity in the crypts, though of a moderate degree, has been documented as well (reviewed in Savilahti 2000).

Apoptosis is a programmed, noninflammatory form of cell death leading to the clearance of injured cells without disruption of tissue structure or function. The process leads to shrinkage and fragmentation of the cell and its nucleus and degradation of chromosomal DNA. Apoptosis is induced by diverse factors such as Fas-ligand, TNF, irradiation and genotoxic agents, such as anti-cancer drugs. In disease, apoptosis is an important mechanism for promoting resolution of inflammation and protecting against connective tissue formation by eliminating infiltrating leukocytes and proliferating cells (reviewed by Reed 2000).

Staining of apoptotic cells by in situ DNA 3'-end labelling (ISEL) (Gavrieli et al. 1992) shows that approximately 1-2% of the surface epithelial cells and 2-3% of crypt epithelial cells are undergoing apoptosis in the normal small intestine (Maiuri et al. 1997, Ciccocioppo et al. 2001, Maiuri et al. 2001). In CD the proportion of crypt and surface epithelial cells undergoing apoptosis is as high as 30-40%, while the percentage is normalised during gluten-free diet (Moss et al. 1996, Ciccocioppo et al. 2001, Maiuri et al. 2001).

AIMS OF THE STUDY

The objective of this study was to evaluate the expression patterns of cytokines and immunologic markers in inflammatory disorders of the small intestine *in vivo*.

Immunologically mediated enteropathies consist of a group of different diseases that are characterised by varying degree of villous destruction in the small intestine. Characteristics of these diseases are excessive T-cell activation and production of proinflammatory cytokines, such as IFN-γ and TNF-α. The increased production of proinflammatory cytokines has been suggested to be one of the factors causing villous atrophy. However, the knowledge of intestinal expression of cytokines is mainly based on studies on overt CD. Knowledge of the intestinal expression of cytokines in GvHD was, prior to this study, based only on experimental studies. Furthermore, the expression of cytokines has not been determined in the potential form of CD, nor in other early and mild forms of these intestinal inflammations, such as mild forms of food allergy enteropathy. Since the mucosa is still intact in these disorders, it can be postulated that studying cytokines at this stage could be more informative on the role of cytokines in mediating chronical inflammation and villous atrophy.

Immunologic inflammation in the intestine may also play a central role in the development of autoimmune diseases. Based on experimental studies, the gut immune system seems to play a role in the pathogenesis of type 1 diabetes. In addition, immunologic changes have been observed in the small intestine of patients with T1D, which implies a role for the gut immune system in human T1D as well.

Therefore, the specific aims of this study were:

1) To characterise the possible T-cell activation and the cytokine expression in the small intestine of patients with potential CD
2) to analyse the activation of the immune system in the small intestine of patients with T1D
3) to characterise cytokine profiles and T-cell markers in the intestinal mucosa of patients undergoing SCT in order to identify changes associated with human GvHD
4) to characterise the expression of cytokines and adhesion molecules in the small intestine of patients with food allergy

PATIENTS AND METHODS

1. Patients with potential coeliac disease (I)

Duodenal biopsies were obtained from five paediatric family members of CD patients and from four adult family members of DH patients. In addition, one sample from a paediatric patient with overt CD developing 11 months later was included in this potential CD study group. Seven paediatric patients and one adult patient with untreated CD served as a positive reference group. The diagnosis of CD was set according to the ESPGHAN criteria (Walker-Smith et al. 1990). As a negative reference group eight paediatric control patients undergoing upper gastroscopy because of growth retardation, gastrointestinal symptoms, positive antigliadin antibodies or any combinations of these were also included in the study. The control patients had no history of CD or DH in the family. They were not on medication, and did not have any chronic diseases. The morphology of the jejunum was normal, and the endomysium (EMA) and tissue transglutaminase (tTGA) antibodies were negative in all control children. The clinical data are summarised in Table 3.

Table 3. Characteristics of patients in study I

	Normal controls	Paediatric potential CD patients	Adult potential CD patients	CD patients
n	8	6	4	8 (including 1 adult)
F/M	4/4	3/3	3/1	5/3
Age in years, median (range)	6.9 (2.6-13.7)	6.1 (3.7-14.5)	53.5 (38-75)	1 adult, 31 years 7 paed., 6.6 (2.5-17.3)
Pos EMA[1]	0/4 (4 NT)	4/6	0/4	8/8
Pos tTGA[2]	0/3 (5 NT)	3/6 (1 NT)	0/0 (4 NT)	6/8 (1 NT)
Pos Gliad-IgA[3]	1/8	1/6	0/4	3/8
Pos Gliad-IgG [3]	7/8	3/6	0/4	3/8
Morphology	Normal 8/8	Normal 6/6	Normal 4/4	1 PVA, 7 SVA

[1] Class IgA endomysium antibodies, upper normal limit 1:5 (Kolho and Savilahti 1997), [2] Class IgA tissue transglutaminase antibodies, normal limit 8% (Stern 2000), [3] Class IgA and IgG gliadin antibodies, upper normal limit 20% for both (Kolho and Savilahti 1997). NT, not tested; PVA, partial villous atrophy; SVA, subtotal villous atrophy.

2. Patients with type 1 diabetes (II)

In study II 32 small intestinal biopsy specimens were obtained from 31 paediatric patients with type 1 diabetes. The patients were scheduled for biopsy because of positive antibodies

associated with coeliac disease in the annual CD screening tests, or because of some gastrointestinal complaints or growth retardation. The patients were divided into three groups; T1D patients with normal villous structure and low titre of CD autoantibodies, T1D patients with normal villous structure and positive EMA or tTGA, referred to as potential CD, and T1D patients with untreated CD. Two of the patients with normal mucosa had a low titre of EMA and negative tTGA antibody tests. These patients were included in the group with negative CD autoantibody tests. The diagnosis of CD was set according to the criteria by ESPGHAN (Walker-Smith et al. 1990).

Additionally, twelve age-matched control paediatric patients were included in the study, seven of which were the same as those in study I and the other five control patients were new for this study. These twelve control patients also served as controls in study III. The exclusion criteria for the controls were the same as in study I. The clinical data of the patients are summarised in Table 4.

Table 4. Characteristics of patients in study II

	Normal controls	T1D patients with normal mucosa	T1D patients with potential CD	T1D patients with CD
n	12	16	8	8
F/M	8/4	8/8	3/5	5/3
Age in years, median (range)	6.7 (1.6-13.7)	9.8 (2.9-18.2)	9.7 (6.0-13.2)	8.7 (3.9-15.8)
Age at onset of T1D; years, median (range)		3.3 (1.2-12.5)	8.0 (2.2-11.8)	5.8 (1.7-9.3)
Duration of T1D at biopsy; years, median (range)		6.6 (0.3-13)	1.3 (0.1-4.4)	2.7 (0.3-6.5)
Pos EMA[1]	0/8 (4 NT)	2/13 (3 NT)	8/8	7/7 (1 NT) [5]
Pos tTGA[2]	0/7 (5 NT)	0/13 (3 NT)	6/7 (1 NT)	5/5 (3 NT)
Pos Gliad-IgA[3]	3/12	7/16	1/8	6/8
Pos Gliad-IgG [3]	10/12	9/16	3/8	4/8
Morphology	Normal 12/12	Normal 16/16	Normal 7/8 [4]	3 PVA, 5 SVA
HLA-DQ2	NT	3/15 (1 NT)	6/8	4/8
HLA-DQ8	NT	2/15 (1 NT)	2/8	2/8

[1] Class IgA endomysium antibodies, upper normal limit 1:5, <1:50 weak positivity (Kolho and Savilahti 1997), [2] Class IgA tissue transglutaminase antibodies, normal limit 8% (Stern 2000), [3] Class IgA and IgG gliadin antibodies, upper normal limit 20% for both (Kolho and Savilahti 1997), [4] Slight changes in one biopsy, villous height was still normal, [5] The patient not tested had positive IgA reticulin antibodies. NT, not tested; PVA, partial villous atrophy; SVA, subtotal villous atrophy.

3. Patients undergoing stem cell transplantation (III)

Duodenal biopsies from patients undergoing allogeneic stem cell transplantation, who were enrolled in a sodium chromoglycate prophylaxis study against gastrointestinal graft-versus-host disease, were investigated in this study. The prospective study was run at the Hospital for Children and Adolescents, University of Helsinki, Finland, between November 1995 and October 1998. Initially, 44 patients over 1 year of age were invited prior to allogeneic SCT, and 20 were willing to take part in the study. All patients received cyclosporin from day -1 on and methotrexate on days 1, 3, 6 and 11 as conventional GvHD prophylaxis. Signs and symptoms of GvHD were recorded and graded according to conventional criteria (Thomas et al. 1975). Sodium chromoglycate did not alter the clinical, histological or immunological picture of the patients. Therefore, we combined the data from both the sodium chromoglycate and placebo groups.

Gastroscopy was prospectively scheduled at 6-7 and 12 weeks after SCT. The endoscopy at 6 weeks was performed to 17 study patients, and the follow-up endoscopy at 12 weeks was performed to 12 patients. Of these, 12 and 8 duodenal biopsies, respectively, were available for the studies presented here. In addition, three SCT patients with grade III-IV clinical and histologically proven gastrointestinal GvHD, not taking part in the chromoglycate study, were included as clinical GvHD patients. The biopsies from these clinical GvHD control patients were obtained at the time of intestinal symptoms at 3, 6 and 11 weeks, and the follow-up biopsies at 10-16 weeks after SCT, respectively. The characteristics of only the patients whose duodenal biopsies were investigated are presented in Table 5. The same 12 patients included and presented in study II served as normal paediatric controls.

Table 5. Characteristics of SCT patients in study III

	SCT study patients	Clinical GvHD patients
n	12	3
F/M	4/8	0/3
Age at SCT in years, median (range)	7.1 (1.8-13.8)	6.4 (2.8-6.6)
Time since SCT at biopsy in days, median (range)	48 (40-96)	43 (21-78)
Time since SCT at the second biopsy in days, median (range)	111 (71-231)	99 (71-112)
Donor; URD / sibling	7 / 5	2 / 1
TBI	8/10/12/14 Gy (1/9/1/1 patients)	12 Gy (3 patients)
Clinical intestinal GvHD		
- at 6 weeks (grI-IV)	2 grI, 2 grIV	3 grIV
- at 3 months	1 grIII	3 grIV
Small intestinal histology GvHD		
- at 6 weeks (grI-IV)	-	1 grII, 2 grIII
- at 3 months	1 gr III	1 grII, 2 grIII
Diagnosis		
- ALL / AML	6 / 1	3 / 0
- Other malignancy	2	-
- Non-malignant disease	3	-
Chromoglycate / placebo	6 / 6	-

Abbreviations: ALL, acute lymphoid leukaemia; AML, acute myeloid leukaemia; gr, grade; TBI, total body irradiation; URD, unrelated registered donor.

4. Patients with food allergy (IV)

Specimens from the bulb of the duodenum were taken from 14 paediatric patients with food allergy. Seven were on a normal diet and seven were on a restricted diet, but still had some clinical symptoms as an indication for gastroscopy, at the Oulu University Hospital. Diagnosis of food allergy was based on an open 6-week elimination/challenge test with the triggering food protein according to the diagnostic criteria by ESPGHAN (ESPGHAN 1992). Before the challenge, the patients were on a food elimination diet involving the suspected food for at least 2 weeks. Open oral challenges were performed with an increasing daily dose, starting from 1 ml of cow's milk or 1 g of cereals (gluten-containing cereals and oats, separately). Each period was continued for 2 weeks if symptoms did not manifest earlier.

Abdominal pain, diarrhoea or loose mucous stools, or exacerbation of dermatitis were each considered a positive result. In all cases symptoms appeared at earliest after 24 hours from the beginning of the challenge.

The study also included five paediatric control patients and five paediatric patients with untreated CD. The controls were studied for diagnostic purposes, they all had normal endoscopic and histologic results and no history of allergy. Furthermore, CD was excluded by serological testing and lactose intolerance was ruled out. The criteria for CD diagnosis was the same as in study I. The characteristics of the patients are summarised in Table 6.

Table 6. Characteristics of patients in study IV

	Normal controls	FA patients on normal diet	FA patients with restricted diet	CD patients
n	5	7	7	5
F/M	0/5	4/3	5/2	2/3
Age in years, median (range)	12.9 (4.5-16.1)	6.8 (2.0-12.9)	11.3 (0.9-14.2)	6.4 (2.5-15.2)
Allergen: milk		6	5	
Allergen: cereals		3	3	
Duration of diet; years, median (range)			3 (1-13)	
Pos EMA[1]	0/5	0/7	0/7	5/5
Morphology, LNH	Normal 5/5	LNH 6/7	LNH 7/7	5 SVA
Specific IgE >0.7 IU/l	-	2/5 (2 NT)	2/2 (5 NT)	-
Positive skin prick test	-	-	1/1 (6 NT)	-

[1] Class IgA endomysium antibodies, upper normal limit 1:5 (Kolho and Savilahti 1997). FA, food allergy; LNH, lymphonodular hyperplasia; NT, not tested; SVA, subtotal villous atrophy.

5. Samples (I-IV)

A Watson capsule biopsy device or a gastroscope was used to take a specimen from the distal duodenum or the proximal jejunum in study I-III, and from the bulb of the duodenum in study IV. Endoscopies were performed under general anaesthesia.

The specimens were embedded in optimal cutting temperature (OCT) compound (Miles Laboratories), snap frozen in liquid nitrogen, and stored at -70°C. Frozen tissue samples were cut into 8-μm sections for immunohistochemistry and ISEL analysis, and into 8- or 10-μm sections for *in situ* hybridisation. The sections were coded and evaluated without knowledge of the specimen identity.

6. Ethical considerations (I-IV)

Specimens from paediatric patients in study I and II were taken for diagnostic purposes at the Hospital for Children and Adolescents, Helsinki University Central Hospital. The St. Mary's Hospital ethics committee approved biopsies taken from the adult siblings of patients with DH in study II. The specimens from SCT patients in study III were taken because of the sodium chromoglycate prophylaxis study, and the study protocol was approved by the Institutional Review Board. Oral witnessed informed consent was obtained from the parents and age-appropriate patients. Use of the biopsy specimens in study I-III was approved by the ethics committee of the Hospital for Children and Adolescents, Helsinki University Central Hospital. Gastroscopy of the patients in study IV were performed for diagnostic purposes at the Department of Paediatrics, University of Oulu. For an additional biopsy sample, a written parental consent was obtained from the parents of all children. The protocol was approved by the ethics committee at Oulu University Hospital.

7. Immunohistochemistry (I-IV)

Immunohistochemistry was performed using a commercially available avidin-biotin immunoperoxidase system (Vectastain Elite ABC kit) according to the manufacturer's instructions. Cryostat sections were fixed in acetone for 10 min at +4°C, washed with PBS, and blocked for 30 minutes at room temperature with normal horse serum. Following blocking, the sections were incubated for one hour at room temperature with the primary antibodies described in Table 7. The slides were then treated with 0.5% hydrogen peroxide in methanol for 20 minutes at room temperature to block endogenous peroxidase activity. Subsequently, secondary biotinylated antibody was applied, followed by avidin-biotin-peroxidase complex. 3-amino-9-ethylcarbazole (Sigma) was used as chromogen, and Harris haematoxylin was used for counterstaining.

Permeabilisation was performed prior to immunostaining of cytokines by incubating the slides in 0.1% PBS-Tween20 for 10 minutes in room temperature. The cytokine antibodies were diluted in 1% normal horse serum in 0.1% PBS-Tween20 and the slides incubated with the mAbs for one hour at +37°C in a humidified chamber. As controls for specific staining, one additional mAb for IL-4 (clone 82.2, Mabtech) and IFN-γ (clone7-B6-1, Mabtech) were used, and similar staining patterns obtained.

Nonimmune mouse IgG1 (DAKO) was used as a negative primary antibody control and incubation with 1% normal horse serum on additional sections as the negative control for the reagents in the avidin-biotin immunoperoxidase system.

Table 7. Monoclonal mouse-antihuman antibodies used in immunohistochemistry

Antibody	Dilution	Company; clone	Study
CD3	1:200 (1:400 IV)	Becton-Dickinson	I-IV
γδTCR	1:200 (1:400 IV)	Endogen; clone 6A6.E9	I-IV
αβTCR	1:40 (1:50 I, 1:100 IV)	Endogen; clone 8A3	I-IV
CD4	1.20	Coulter Immunology	IV
CD8	1:400	DAKO; clone DK25	IV
ICAM-1	1:1500 (I,II) 1:2000 (III,IV)	Endogen; clone VF27	I-IV
HLA-DR	1:500 (II,III) 1:2000 (I,IV)	Becton-Dickinson	I-IV
HLA-DP	1:40	Becton-Dickinson	I-IV
Ki-67	1:100	DAKO	I-IV
CD25 (IL-2 receptor)	1:10	DAKO; clone ACT-1	IV
CD11a (LFA-1)	1:100	Immunotech; clone 25.3.1	IV
ACT-1 ($\alpha_4\beta_7$-integrin)	1:200	Leukosite	IV
IL-1α	1:50 (1:100 I)	Biosource International; clone 20B8	I-III
IL-2	1:50	Biosource International; clone 7A3	I-III
IL-4	1:50	Mabtech; clone 12.1*	I-IV
IFN-γ	1:50	Mabtech; clone 1-D1K	I-IV
TNF-α	1:50	Biosource International;clone 68B6A3	I-III
Nonimmune mouse IgG	1:100	DAKO	I-IV

* In study IV, IL-4 clone 82.4 was used at a dilution of 1:75

Microscopic evaluation

The numbers of positively stained mononuclear cells in the biopsies were counted systematically under a light microscope through a calibrated eyepiece graticule at x1000 magnification. In the same specimen, the positively cells in at least 30 fields both in the surface epithelium and the lamina propria were counted. The results were expressed as the mean number of positive staining cells/mm of epithelium and as cells/mm^2 in the lamina propria, respectively. Proliferating cells were stained with Ki-67, and positively staining crypt epithelial cells were calculated as percentages of all the crypt cells, with at least 200 cells counted in each specimen.

Epithelial staining with HLA-DR and HLA-DP antibodies was graded in studies I-III from 2 to 6 according to their intensity and cellular distribution (1=positive staining only on the apical surface of the epithelial cell, 2=in addition, positive staining of the basal surface of

epithelial cell, 3=in addition, positive staining throughout the cytoplasm of the epithelial cell), and area of staining (1=only the tip of the villous positive, 2=the whole villous, no positive crypts, 3=also positive staining in the crypts) (Klemola et al. 1995). The positive staining for ICAM-1 in the lamina propria was estimated and graded from 1 to 3 according to intensity of staining (1=weak positivity, 2=moderate positivity, 3=intense positivity). In study IV proportions of positive epithelial cell staining with HLA-DR and HLA-DP, and positive lamina propria cell staining with ICAM-1 were measured by Weibel's point counting technique, using an integration ocular with a grid with 100 points. At least 1500 points falling on epithelial cells (HLA-DR and HLA-DP) or lamina propria cells (ICAM-1) were counted in each specimen. Each point was documented as positive or negative and the percentage of positive points calculated.

8. Radioactive *in situ* hybridisation (I-IV)

Serial cryostat sections were processed for *in situ* hybridisation and immunohistochemistry for IL-4 and IFN-γ. Cryostat sections were fixed in 4% paraformaldehyde in PBS, and subjected to *in situ* hybridisation for human IL-4 and IFN-γ riboprobes obtained from the cDNAs described below. Tissue sections were hybridised with 1.2 x 10^6 cpm of [^{33}P]-labelled (1000-3000 Ci/mmol; Amersham) antisense or sense riboprobes overnight at +60°C in a total volume of 80 μl following the *in situ* protocol described in detail previously (Wilkinson 1992). The sections were treated with RNAse A to remove unhybridised probes, before applying photographic emulsion. The slides were developed after 1 and 2 weeks of autoradiography, and counterstained with Harris haematoxylin.

Human IL-4 and IFN-γ were used for preparing the riboprobes. Briefly, a 462-bp human IL-4 cDNA and a 501-bp human IFN-γ cDNA were synthesised by PCR, enclosing the whole coding region for both cytokines, and additionally the signal peptide for IFN-γ (Sareneva et al. 1994). The PCR products were cloned into pGEM®-3ZF vectors (Promega) and the sequences of the cloned PCR products were verified with an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Applied Biosystems). The lengths of the [^{33}P]-labelled riboprobes, both antisense and sense, were checked by SDS PAGE analysis.

Microscopic evaluation

Positive mRNA expression for IL-4 and IFN-γ was in study I and IV counted in two representative areas in the lamina propria, as well as in the background staining adjacent to

the tissue at x400 magnification. The relative intensity of positive mRNA signal was determined by division of the positivity in the tissue by the positivity of the background. In study II and III, positively staining cells were counted through a calibrated graticule at x400 magnification and expressed as the mean number of positive cells/area of epithelium or lamina propria. A cell was considered positive if expressing 7 or more positive cytoplasmic grains, which always corresponded to more than twice the background level. For the specific probes, a minimum of two sections were prepared for each patient investigated.

9. Taqman real time PCR (II)

The part of the mucosal specimens from T1D patients still left after immunohistochemistry and *in situ* hybridisation was used for RT-PCR. Total RNA was isolated from the mucosal samples with GenElute™ Mammalian total RNA kit (Sigma) following the manufacturer's instructions. Reverse transcription of RNA to cDNA was performed with TaqMan® Reverse Transcription Reagents (Applied Biosystems) in a reaction volume of 75 µl following the manufacturer's instructions. The reaction mixture contained 10 ng/µl template RNA, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 0.4 IU/µl RNase Inhibitor, and 2.5 µM Random Hexamers as primers. The solution was treated with 2 IU of DNAse at 37°C for 30 minutes prior to 5 minutes inactivation at 75°C. After addition of 25 IU of MultiScribe® Reverse Transcriptase, the RT reaction was carried out at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The samples were stored at -20°C until used.

Real-time quantitative PCR was performed using specific TaqMan® PDAR (Pre-Developed assay reagents) primers/probes and the ABI-Prism 7700 Sequence Detection System (Applied Biosystems) as described earlier (Heid et al. 1996). TaqMan® primers/probes for cytokines IL-2 (PN 4309882P), IL-4 (PN 4309883P), IFN-γ (PN 4309890P) and TNF-α (PN 4309891P), and for chemokine receptors CCR-4 (PN 4312819P) and CCR-5 (PN 4316064P) were used. To normalise differences in sample sizes and the amount of RNA degradation, primers/probe for ribosomal RNA 18S (PN 4310893E) was also applied as an endogenous control.

Total reaction volume used in the PCR amplification was 25 µl, containing 50 ng (for target gene) or 5 ng (for endogenous control) of template cDNA. PCR was performed under the following conditions: 50°C for 2 min, 95°C for 10 min; 50 cycles of 95°C for 15 sec and 60°C for 1 min. Analysis of a calibrator cDNA sample derived form PHA stimulated peripheral blood lymphocytes, and controls without template were also performed on each

PCR plate. Each measurement was set up in triplicate. The quantitative analysis was performed using the comparative C_T (ΔC_T) method as described in detail previously (Gibson et al. 1996).

10. HLA genotyping (II)

HLA genotyping was performed in 11 T1D patients from peripheral venous blood sample and in 19 T1D patients from duodenal paraffin blocks. HLA-DQB1, HLA-DQA1, and HLA-DRB1 analysis were performed by a technique developed for screening T1D susceptibility based on the presence of alleles associated with a risk for or with protection against T1D. This two-step screening technique is based on the hybridisation of relevant PCR products with lanthanide-labelled probes detected by time-resolved fluorometry (Nejentsev et al. 1999).

11. *In situ* detection of DNA fragmentation (ISEL) (III)

To detect cells with DNA fragmentation terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin in situ 3-end labelling (ISEL) method was used as described previously (Surh and Sprent 1994). Briefly, eight-µm sections were fixed in acetone, washed with PBS and incubated with 0.3% H_2O_2 in methanol to eliminate endogenous peroxidase activity. The sections were preincubated with 1 x terminal transferase reaction buffer (Roche Diagnostics) containing 1mM $CoCl_2$ for 10 min at room temperature. The apoptotic DNA fragments were 3'-end labelled with digoxigenin-11-ddUTP (0.5µM, Roche Diagnostics) by TdT reaction (TdT enzyme 375 U/ml) in 1 x reaction buffer containing 45µM ddATP (Amersham Pharmacia) and 1mM $CoCl_2$ (Roche Diagnostics) at 37°C for 1 hour. Following washes and blocking of nonspecific binding with 2% blocking reagent, the sections were incubated with horseradish peroxidase-conjugated anti-digoxigenin antibody (1:200, Roche Diagnostics) overnight at +4°C. The colour was developed with chromogen diaminobenzidine (Sigma) and counterstaining was performed with Harris haematoxylin. For negative controls the TdT enzyme or labelled nucleotides were replaced by distilled water, and for positive controls the sections were incubated with DNAse (1-10 IU/ml) at 37°C for 15 min prior to the TdT reaction.

The sections of the patients were always processed in parallel with the controls and a total of three sections per patient were analysed. The number of enterocytes with DNA fragmentation was calculated through a calibrated graticule as percentages of positive cells

along the surface epithelium and in the crypt epithelium, with a minimum of 200 cells counted in each compartment.

12. Histopathological analysis (III)

The intestinal biopsy samples were stained routinely by haematoxylin and eosin and analysed by one pathologist (Riitta Karikoski) blinded for the clinical data. The histological grading of intestinal GvHD ranged from signs of apoptosis and infiltration of intraepithelial lymphocytes (grade 1) through exploding crypts (grade 2) and loss of crypts (grade 3), to total sloughing off of the epithelium (grade 4).

13. Statistical analysis (I-IV)

Differences in the parameters studied were revealed by the Kruskall-Wallis analysis of variance when more than two groups were compared, and by the Mann-Whitney U-test when two groups were compared (I-IV). The correlation between proliferation and apoptosis markers in the SCT patients was analysed with the Spearman correlation (III). Calculations were done on a PC using SPSS software (SPSS Inc, Chigaco, IL, USA). Cell densities for each group were expressed as median values, and because of the small sizes of the study groups, values outside the 25 to 75 percentiles were considered abnormal for the group. A P-value < 0.05 was considered significant.

RESULTS

To study immunologic markers and expression patterns of cytokines in immunologically mediated inflammation, small intestinal biopsies were obtained from patients with potential CD (I), T1D (II), patients undergoing SCT (III), and patients with food allergy (IV). The intestinal specimens were studied using immunohistochemistry, *in situ* hybridisation, RT-PCR and *in situ* 3'-end labelling (ISEL).

The results from immunohistochemical stainings and *in situ* hybridisation of studies I-IV are combined in this section and presented separately for each parameter. In the control group, the normal control patients from study I (n=8) are pooled with the five additional control patients from study II (total n=13), if not mentioned otherwise. The results of SCT study patients (n=12) and clinical GvHD control patients (n=3) are presented in the figures in the same group at 6 weeks (n=15) and at 3 months (n=11).

1. Densities of intraepithelial T-cells (I-IV)

Intraepithelial T-cells were stained with mAbs to CD3, γδTCR, and αβTCR in all the specimens. From previous studies it is known that the numbers of IELs, especially of $\gamma\delta TCR^+$ cells, are highly increased in CD and to a lesser extent in family members of CD patients, as well as in patients with delayed type food allergy. We found increased densities of $CD3^+$, $\gamma\delta TCR^+$, and $\alpha\beta TCR^+$ IELs in potential CD and CD patients in studies I-II, regardless of associated T1D (Figure 4). The IEL infiltration was more pronounced in clinical CD than in potential CD. We also found increased densities of $CD3^+$ (P=0.008) and $\gamma\delta TCR^+$ (P=0.008), but not of $\alpha\beta TCR^+$ IELs in the bulb of duodenum from CD patients in study IV when compared to controls. The densities were somewhat lower than in the duodenal biopsies from patients with CD in studies I and II (Figure 4). Furthermore, the patients with untreated food allergy had a higher density of $\gamma\delta TCR^+$ IELs than the controls in study IV (P=0.01). The densities of all three IEL subtypes were as low in SCT patients as in normal controls. The density of $\alpha\beta TCR^+$ IELs was significantly lower in SCT patients than in controls at both timepoints (P=0.001 and 0.007, respectively) (Figure 4C).

We investigated the IELs staining positively for IFN-γ in studies II and III. Although the densities of IELs stained with T-cell markers were not increased in SCT patients, the density of IFN-γ^+ IELs was higher in SCT study patients and clinical GvHD control patients at both 6 weeks and 3 months than in controls ($P<0.01$ in all comparisons). We also found a

higher density of IFN-γ^+ IELs in T1D patients with CD or potential CD than in controls or T1D patients with normal mucosa (P<0.006 in all four cases) (Figure 4D).

We further examined the proportion of $CD4^+$ and $CD8^+$ T-cells in the epithelium and lamina propria in study IV. We found no difference in the density of $CD4^+$ IELs between the study groups (medians 0.8-1.7 cells/mm, corresponding to 2.8-8.4% of $CD3^+$ IELs), whereas the density of $CD8^+$ IELs was significantly increased in CD patients when compared to controls (P=0.032), patients with untreated food allergy (P=0.005), or patients with treated food allergy (P=0.018).

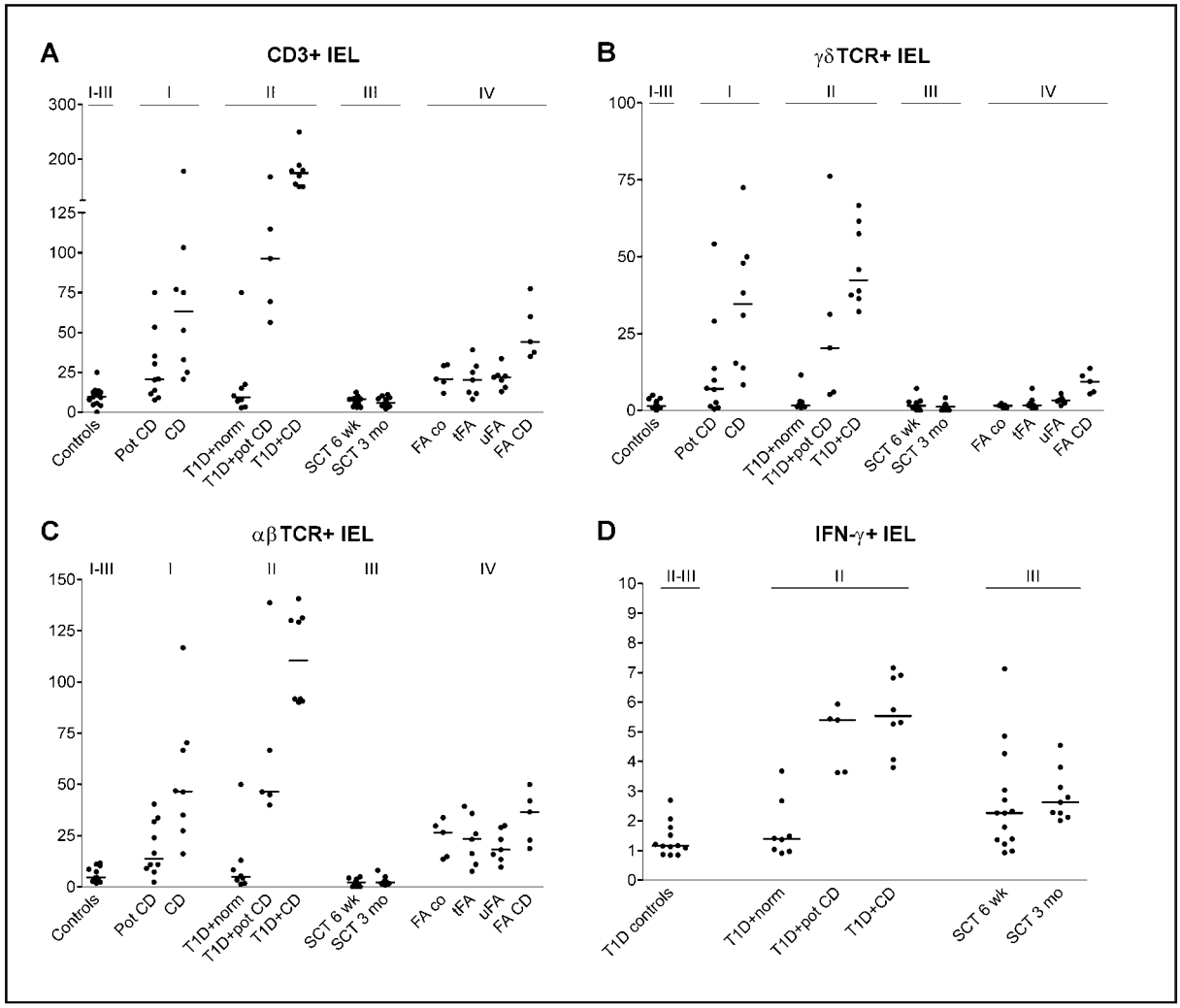

Figure 4. Densities of $CD3^+$, $\gamma\delta TCR^+$, $\alpha\beta TCR^+$, and IFN-γ^+ intraepithelial lymphocytes (IELs) in the different study groups. The individual results are shown as positively staining cells/mm of surface epithelium. The Roman numerals at the top of the figures indicate the number of the study. The medians are indicated by horisontal lines for each study group. Study groups: Pot CD, potential CD; T1D+norm, T1D patients with normal duodenal mucosa; T1D+pot CD, T1D patients with potential CD; T1D+CD, T1D patients with CD; SCT 6 wk, patients 6 weeks after SCT; SCT 3 mo, patients 3 months after SCT; FA co, control patients included in food allergy study; tFA, patients with treated food allergy; uFA, patients with untreated food allergy; FA CD, CD patients included in food allergy study.

2. Densities of T-cells in the lamina propria (I-IV)

We counted the densities of lamina propria $CD3^+$, $\gamma\delta TCR^+$ and $\alpha\beta TCR^+$ T-cells in the same microscopical slides as the IELs. Generally, the lamina propria T-cells did not show as large a variation between the study groups as IELs did (Figure 5). T1D patients with potential CD or CD had the highest densities of all T-cell subtypes. In study I we found an increased density of $\gamma\delta TCR^+$ cells in CD patients when compared to controls (P=0.038), whereas in study II all three cell subtypes were increased in T1D patients with CD (P<0.001 in all cases) or potential CD (P=0.009, P=0.006, and P=0.048, respectively) when compared to normal controls (Figure 5). In addition, the numbers of all three cell subtypes were higher in T1D patients with CD than in T1D patients with normal mucosa (P=0.015, P=0.0028 and P=0.015, respectively).

We found a significantly lower density of $\gamma\delta TCR^+$ cells in biopsies of SCT patients at 6 weeks than in controls (P=0.013) (Figure 5B). By the time of the second endoscopy, the median density of lamina propria $\gamma\delta TCR^+$ cells had increased from 22.1 (range 13.6-58.6) to 42.5 (range 21.6-141.1) positive cells/mm^2 (p=0.017) in SCT patients. The clinical GvHD patients showed a higher density of $\gamma\delta TCR^+$ cells at 6 weeks than SCT study patients (P=0.009) (Figure 5B).

In study IV we observed an increased density of $CD3^+$ cells in CD patients when compared to patients with treated food allergy (P=0.048), but not when compared to patients with untreated food allergy or normal controls (Figure 5A). The density of $CD4^+$ or $CD8^+$ cells in the lamina propria did not differ between the study groups in study IV.

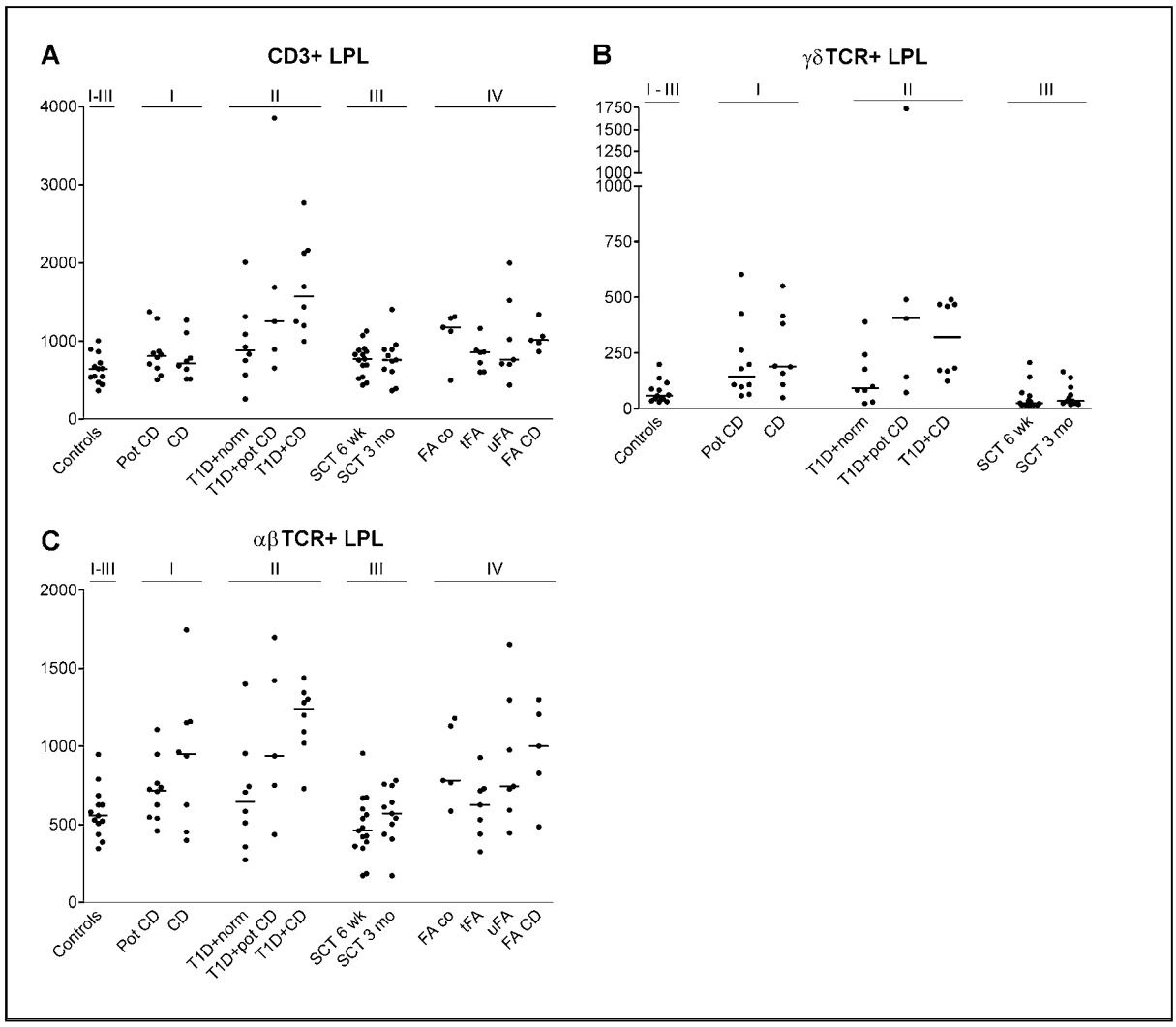

Figure 5. Densities of CD3$^+$, γδTCR$^+$, and αβTCR$^+$ lamina propria lymphocytes (LPL) in the different study groups. The individual results are shown as positive cells/mm^2 of lamina propria. The Roman numerals at the top of the figures indicate the number of the study. The medians are indicated by horisontal lines for each study group. Study groups: Pot CD, potential CD; T1D+norm, T1D patients with normal duodenal mucosa; T1D+pot CD, T1D patients with potential CD; T1D+CD, T1D patients with CD; SCT 6 wk, patients 6 weeks after SCT; SCT 3 mo, patients 3 months after SCT; FA co, control patients included in food allergy study; tFA, patients with treated food allergy; uFA, patients with untreated food allergy; FA CD, CD patients included in food allergy study.

3. Expression of cytokines detected by immunohistochemistry (I-IV)

The expression of cytokines were studied using monoclonal antibodies against IL-1α, IL-2, IL-4, IFN-γ, and TNF-α. We considered only typical paranuclear stainings as positive. In study IV only IL-4 and IFN-γ were investigated. Because of different batches used in study I vs. the other studies for all cytokines but IL-2, the data from study I can not directly be compared with the data from other studies. This is represented by the dotted line in Figures 6 and 7. Furthermore, the results of potential CD and CD patients are compared with the results of the original normal control group of study I.

Th1- and Th2-type cytokines

The densities of IFN-γ^+ cells were generally higher, while the densities of IL-4^+ cells were generally lower in study I than in the other three studies, one of the explanations probably being the different antibody batches used. When looking at the Th1 vs. Th2 balance in the studies, the general finding is that CD patients show higher densities of IFN-γ^+, IL-2^+, and IL-4^+ cells, regardless of associated T1D (Figure 6).

In study I the potential CD and CD patients expressed higher densities of IL-2^+ (P=0.043 and P=0.028), IFN-γ^+ (P=0.009 and P<0.001) and IL-4^+ cells (P=0.034 and P=0.021) than the controls (Figure 6). When potential CD patients were divided into two groups according to normal (n=4) or increased numbers of IELs (n=6), the potential CD patients with high numbers of IELs showed a higher density of IFN-γ positive cells, but the difference did not reach statistical significance (P=0.088).

In study II the T1D patients with CD had higher densities of IFN-γ^+, IL-2^+, and IL-4^+ cells than both the normal controls and the T1D patients with normal mucosa (P<0.002 in all cases), and higher density of IL-4^+ cells than T1D patients with potential CD (P=0.002). Furthermore, the T1D patients with normal mucosa or potential CD showed increased IL-4 densities when compared to controls (P=0.031 and P=0.027) (Figure 6).

The density of IL-2^+, IL-4^+ and IFN-γ^+ cells did not differ between the biopsies obtained at 6 weeks from SCT patients and normal controls in study III. However, at the time of the second biopsy 3 months after SCT, the density of IFN-γ^+ cells was lower than in the normal controls (P=0.001, SCT 3 months vs. controls), and the density of IL-4 cells was greater (P=0.001, SCT 3 months vs. controls) (Figure 6). The clinical GvHD patients did not differ from the SCT study patients with respect to any of the assessed cytokines, but showed a lower density of IL-2^+ cells than the controls (P=0.002).

We found an increased density of IFN-γ^+ cells in patients with untreated food allergy when compared to normal controls (P=0.018). Although the cell density tended to be higher in untreated than in treated food allergy patients, the difference was not significant (P=0.053).

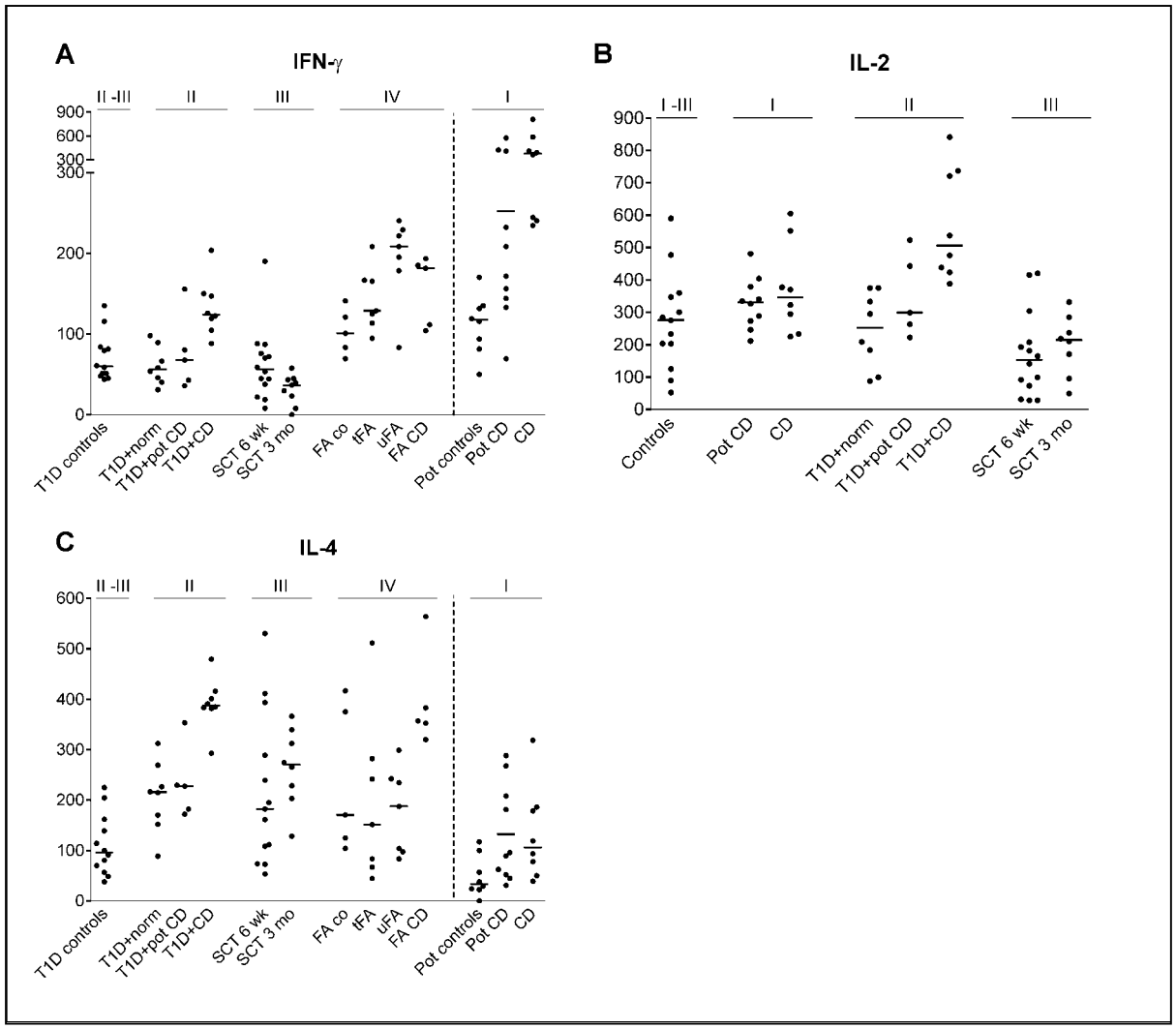

Figure 6. Densities of IFN-γ^+, IL-2^+, and IL-4^+ cells in the lamina propria detected by immunohistochemistry. The individual results are shown as positive cells/mm^2 of lamina propria. The Roman numerals at the top of the figures indicate the number of the study. The dotted vertical lines indicate that different batches of antibodies for IFN-γ and IL-4 were used in study I vs. the other studies. The medians are indicated by horisontal lines for each study group. Study groups: T1D controls, control patients included in study II; T1D+norm, T1D patients with normal duodenal mucosa; T1D+pot CD, T1D patients with potential CD; T1D+CD, T1D patients with CD; SCT 6 wk, patients 6 weeks after SCT; SCT 3 mo, patients 3 months after SCT; FA co, control patients included in food allergy study; tFA, patients with treated food allergy; uFA, patients with untreated food allergy; FA CD, CD patients included in food allergy study; Pot controls, control patients in study I; Pot CD, potential CD.

Proinflammatory cytokines

The densities of the proinflammatory cytokines IL-1α and TNF-α are shown in Figure 7. We did not find any difference in the density of IL-1α$^+$ cells between the groups in study I, whereas we found higher numbers of TNF-α$^+$ cells in CD patients than in controls or potential CD patients (P=0.001 and P=0.012). Although the potential CD patients with increased IELs expressed a higher density of TNF-α$^+$ cells than those with normal density did, the difference failed to reach statistical significance (P=0.088).

We found higher densities of IL-1α and TNF-α positive cells in T1D patients with CD than in controls (P<0.001 and P=0.050) or T1D patients with normal mucosa (P=0.002 and P<0.001). In addition, the T1D patients with normal mucosa or potential CD showed increased densities of IL-1α$^+$ cells when compared to controls (P=0.031 and P=0.006) (Figure 7).

We observed greater densities of IL-1α$^+$ cells in SCT specimens at 6 weeks and 3 months than in controls (P=0.005 and P=0.002), whereas the densities of TNF-α$^+$ cells were lower than in controls (P=0.010 and P=0.031) (Figure 7). The numbers of IL-1α$^+$ and TNF-α$^+$ cells did not differ between the study SCT study patients and clinical GvHD patients (data not shown).

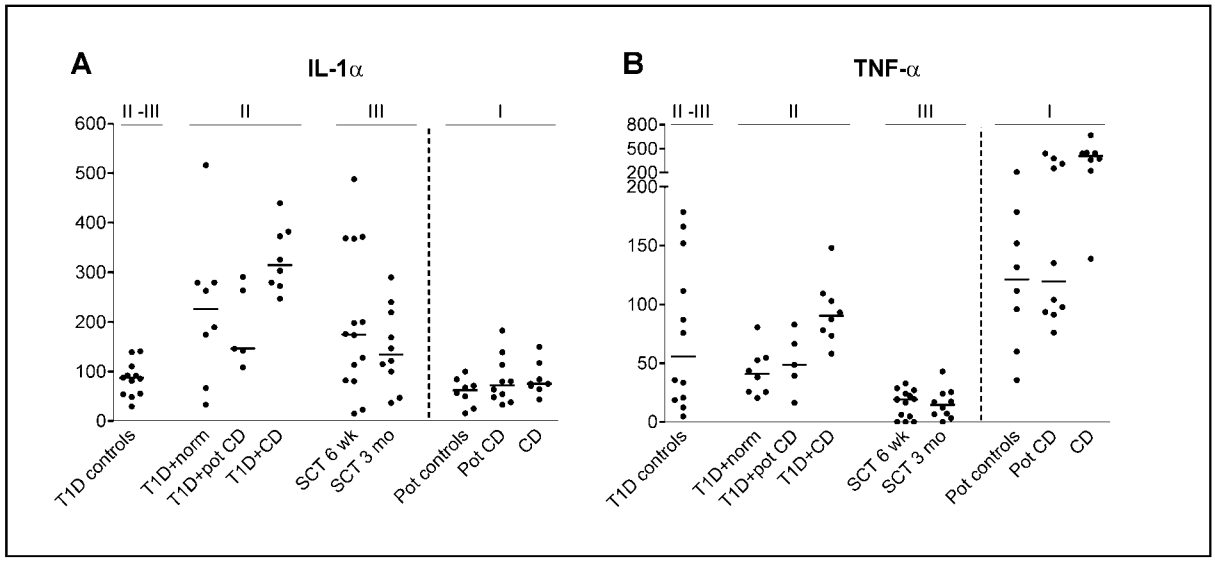

Figure 7. Densities of IL-1α$^+$ and TNF-α$^+$ cells in the lamina propria detected by immunohistochemistry. The individual results are shown as positive cells/mm^2 of lamina propria. The Roman numerals at the top of the figures indicate the number of the study. The dotted vertical lines indicate that different batches of antibodies were used in study I vs. the other studies. The medians are indicated by horisontal lines for each study group. Abbreviations of study groups are the same as in Figure 6.

4. RNA *in situ* hybridisation for IL-4 and IFN-γ mRNA detection (I-IV)

We performed radioactive RNA in situ hybridisation for IL-4 and IFN-γ in serial sections from the same samples that were used for immunohistochemical stainings. The method was the same in study I-IV, but two different interpretations were used (see page 52).

We found positive IL-4 mRNA hybridisation signals mainly in the lamina propria, with little or no signal in the surface epithelium. Mononuclear cells expressing IL-4 mRNA were present in all samples, although more sparsely distributed than IFN-γ mRNA expressing cells. In situ hybridisation with the antisense probe for IFN-γ revealed that positive cells were found mainly in the superficial lamina propria, and occasionally in the deeper parts, regardless of the underlying disease. IFN-γ mRNA expressing cells were also found in the intraepithelial compartment throughout the surface and the crypt epithelium, but the median was only 1 cell/10 areas of surface epithelium in normal controls.

In study I the potential CD patients showed a lower relative intensity of IL-4, but higher of IFN-γ when compared to controls (P=0.008 and P=0.009). In addition, CD patients showed a higher intensity of IFN-γ than controls (P=0.035) (Figure 8).

The density of IL-4 mRNA expressing cells was significantly greater in all three T1D study groups when compared to normal controls in study II (P<0.001 for all comparisons). In addition, T1D patients with CD or potential CD also showed a stronger expression of IL-4 mRNA than T1D patients with normal mucosa (P=0.010 and P=0.030) (Figure 8D). Densities of IFN-γ mRNA expressing cells in the lamina propria were significantly greater in all T1D groups than in controls. However, among the T1D patients IFN-γ mRNA positive cells were 5 and 2.7 times more abundant in T1D patients with CD or potential CD than in those with a normal intestine. Further, the densities of IFN-γ mRNA positive cells were significantly increased in T1D patients with CD compared to the other two T1D study groups (P<0.001 and P=0.019). We also found a significantly increased density of IFN-γ mRNA positive IELs in T1D specimens with CD or potential CD when compared to normal T1D specimens (P<0.001 and P=0.006) or controls (P<0.001 for both comparisons) (data not shown).

In study III both SCT groups had significantly lower densities of IL-4 mRNA expressing cells than controls did (P<0.001 for both comparisons), whereas the densities of IFN-γ mRNA expressing cells did not differ in the groups in the lamina propria or in the epithelial compartment (Figure 8).

CD patients in study IV had an increased intensity of IL-4 and IFN-γ when compared to treated food allergy patients (P=0.010 and P=0.017) and controls (P=0.029 and P=0.016), but

when compared to untreated food allergy patients only the intensity of IL-4 was higher (P=0.006) (Figure 8A and 8C).

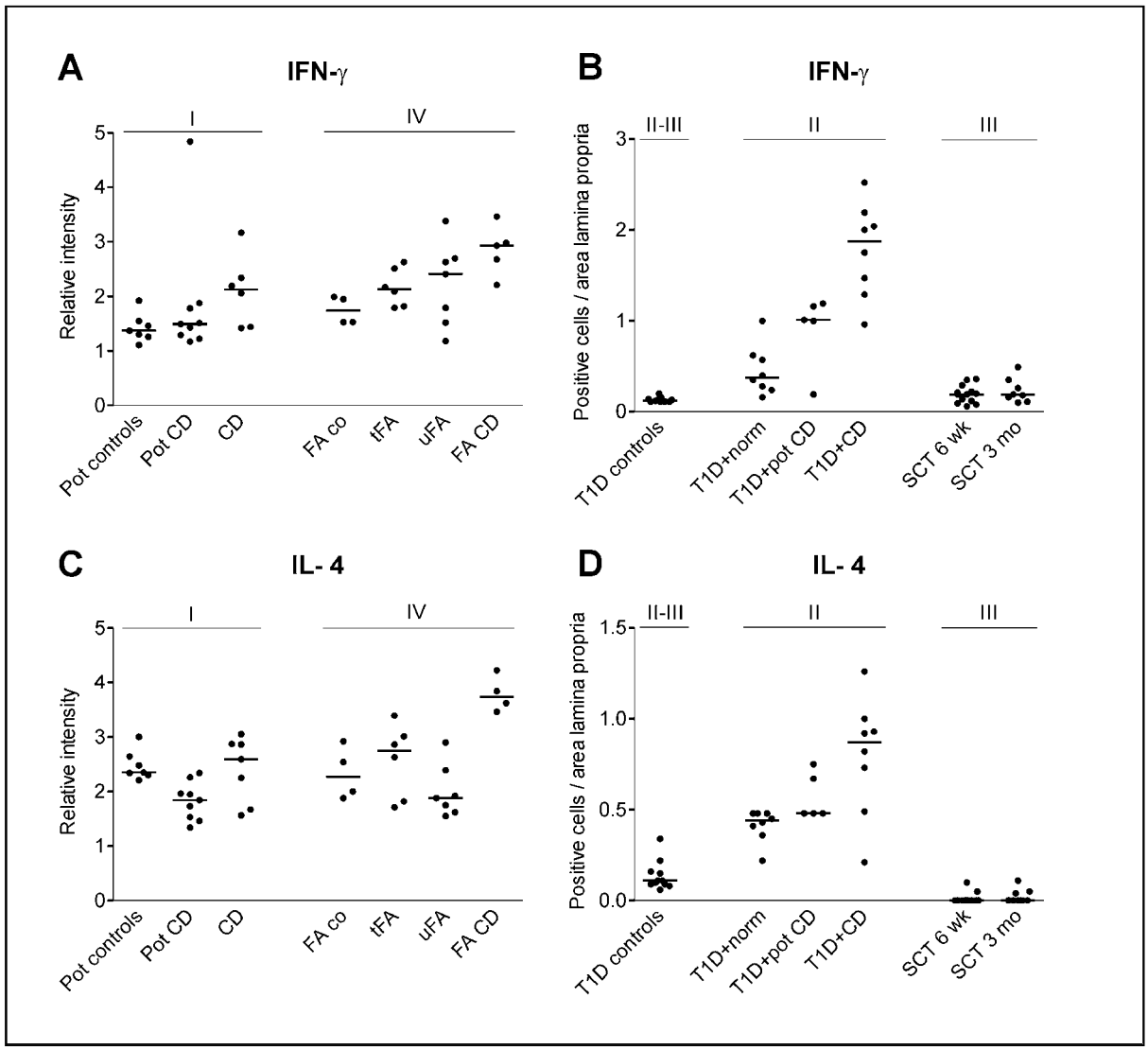

Figure 8. Expression of IFN-γ and IL-4 mRNA detected by in situ hybridisation. The individual results are shown as relative intensity in A and C, and as the number of positive cells/area of lamina propria in B and D. The Roman numerals at the top of the figures indicate the number of the study. The medians are indicated by horisontal lines for each study group. The study groups are: Pot controls, control patients in study I; Pot CD, potential CD; FA co, control patients included in food allergy study; tFA, patients with treated food allergy; uFA, patients with untreated food allergy; FA CD, CD patients included in food allergy study; T1D controls, control patients included in study II; T1D+norm, T1D patients with normal duodenal mucosa; T1D+pot CD, T1D patients with potential CD; T1D+CD, T1D patients with CD; SCT 6 wk, patients 6 weeks after SCT; SCT 3 mo, patients 3 months after SCT.

5. Taqman real time PCR analysis of cytokines, CCR-4 and CCR-5 (II)

Signals of IL-2, IFN-γ, TNF-α, CCR-4 and CCR-5 mRNA were detectable by RT-PCR in all duodenal specimens. IL-4 mRNA was, however, not detectable in the biopsies of normal controls, whereas we could detect IL-4 in 3 of 16 of the T1D specimens with normal mucosa, in 3 of 8 potential CD, and in 5 of 8 of the T1D patients with CD. The amount of IFN-γ mRNA was again significantly greater in T1D patients with CD or potential CD compared to T1D patients with normal mucosa ($P<0.001$ and $P=0.001$) and controls ($P<0.001$ and $P=0.002$), correlating with the degree of CD. Additionally, CCR-4 was expressed in significantly higher amounts in T1D patients with CD than in T1D patients with normal duodenal mucosa ($P=0.010$). We found expression of CCR5 in all investigated specimens, but we did not find any difference between the study groups. The individual results of CCR4 and CCR5 mRNA expression are shown in Figure 9.

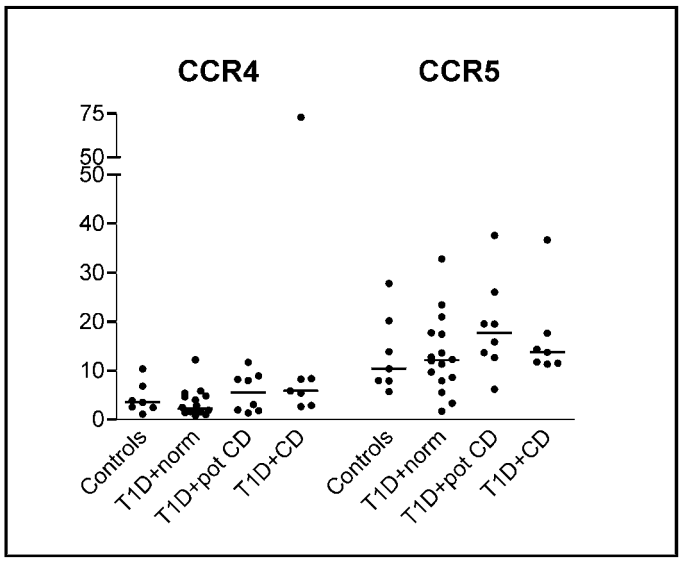

Figure 9. Expression of CCR4 and CCR5. Chemokine receptor 4 and -5 specific mRNA detected by RT-PCR in duodenal specimens of healthy controls (n=7), type 1 diabetes patients with normal duodenal mucosa (T1D+norm, n=16), with potential coeliac disease (T1D+pot CD, n=8), or with coeliac disease (T1D+CD, n=7).
Individual results are shown as relative amount ($2^{-\Delta\Delta CT}$) of target compared to the calibrator, both normalised to an endogenous reference (18S). Horisontal lines show medians for each study group.

6. Proliferation of epithelial cells (I-IV)

Proliferating cells were stained for Ki-67 and the percentage of positive crypt epithelial cells were counted in at least 200 crypt cells. The typical stainings are shown in Figure 10A and the individual results in Figure 10B. We found increased proliferation rates in CD patients, T1D patients with potential CD or CD, SCT patients and in untreated food allergy patients. We found a higher percentage of Ki-67 positive crypt cells in biopsies of patients both after 6 weeks and 3 months after SCT than in normal controls ($P=0.002$ and $P<0.001$) (Figure 10B). Specimens from SCT study patients and clinical GvHD patients showed very high rates of

proliferation, with median values of 42% and 48% at 6 weeks, and 37% and 54% at 3 months. CD patients in study I had a significantly higher expression of Ki-67 than potential CD patients (P=0.012) or controls (P=0.001) (Figure 10). T1D patients with CD and potential CD showed a higher percentage of Ki-67$^+$ cells than controls (P=0.001 and P=0.019) or T1D patients with normal mucosa did (P=0.003 and P=0.011). Finally, we also found higher positive expression in CD patients (P=0.030) and patients with untreated food allergy (P=0.026) than in treated food allergy patients in study IV.

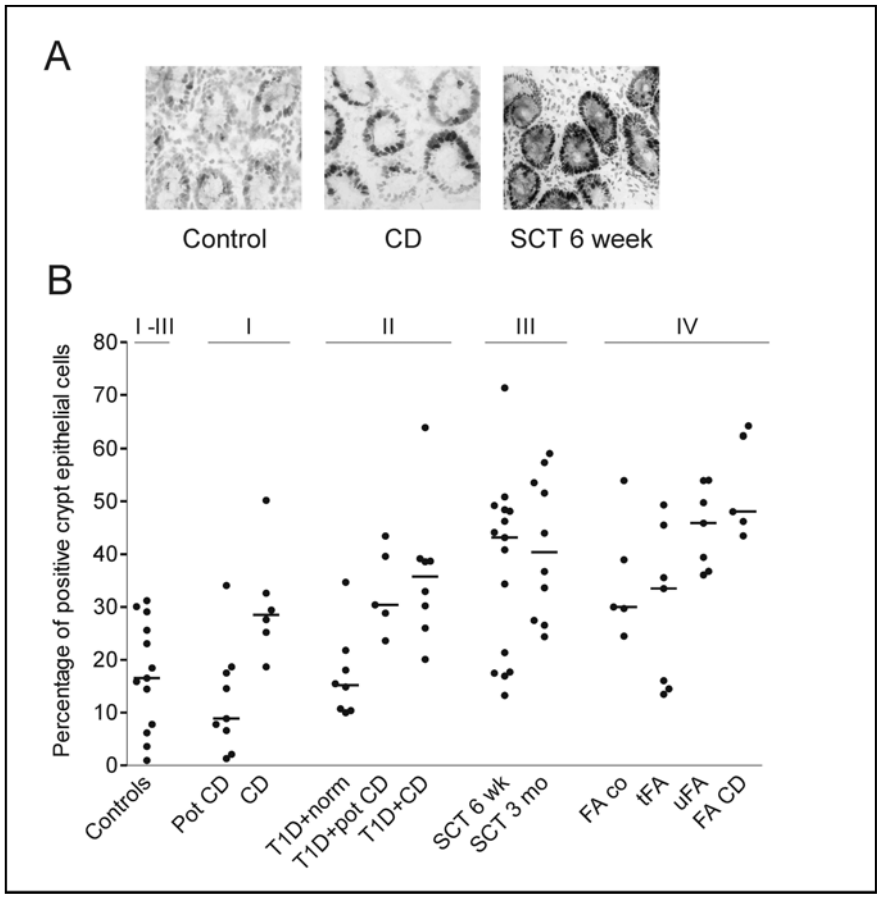

Figure 10. Proliferation of crypt epithelial cells. A. Proliferation of crypt epithelial cells was detected by immunohistochemical staining with Ki-67. The typical staining in specimens of a control patient (study I), a patient with CD (study I), and a SCT patient 6 weeks after transplantation (study III) is shown. Increased positive expression can be seen in the crypts of CD and SCT specimens when compared to the normal control (carbatzole-haematoxylin stain, original magnification x100). B. The percentages of Ki-67 positive crypt epithelial cells are shown as individual results in the different study groups. The medians are indicated by horisontal lines. Study groups: Pot CD, potential CD; T1D+norm, T1D patients with normal duodenal mucosa; T1D+pot CD, T1D patients with potential CD; T1D+CD, T1D patients with CD; SCT 6 wk, patients 6 weeks after SCT; SCT 3 mo, patients 3 months after SCT; FA co, control patients included in food allergy study; tFA, patients with treated food allergy; uFA, patients with untreated food allergy; FA CD, CD patients included in food allergy study.

7. Apoptosis of epithelial cells (III)

As we found a high proliferation rate of crypt eptihelial cells in specimens from SCT patients, we also performed apoptosis staining, in order to determine if the high rate of renewal was due to increased apoptosis. We used the ISEL staining method, which detects fragmented DNA, to detect apoptosis. Representative positive staining is shown in Figure 11A. We counted the percentage of ISEL positive epithelial cells in both the crypt and surface epithelium. The individual results are shown in Figure 11B.

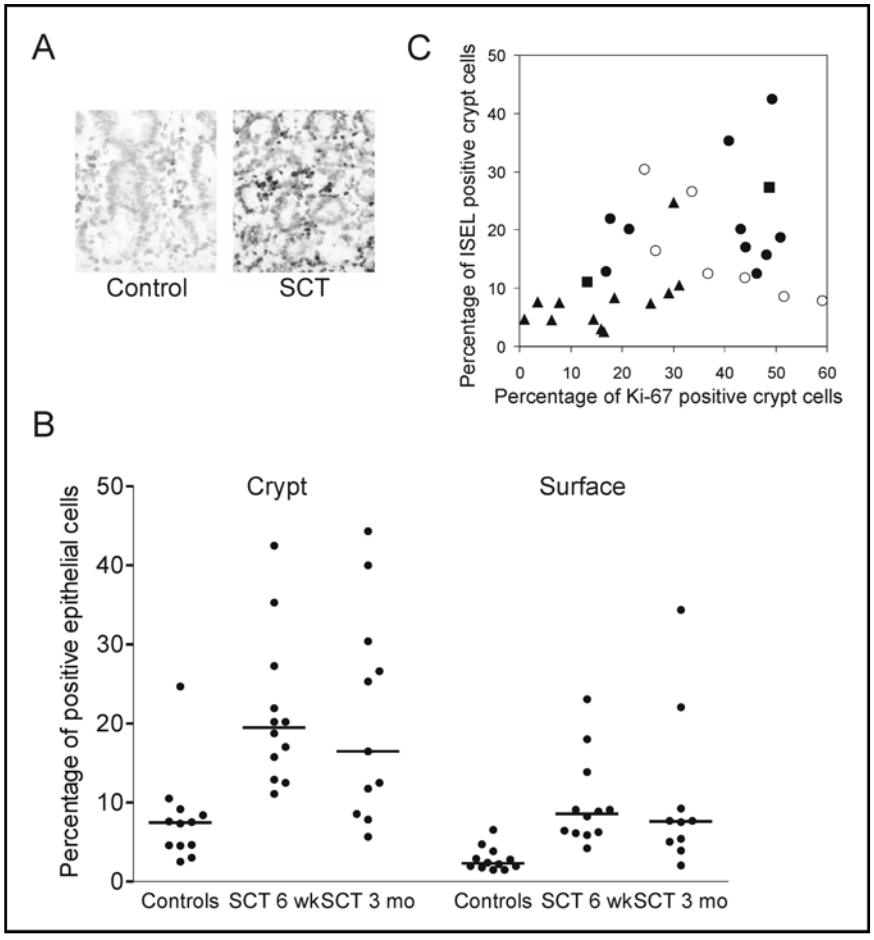

Figure 11. Apoptosis of epithelial cells after stem cell transplantation (SCT). A. Apoptosis was detected by in situ 3'-end labelling (ISEL) of apoptotic DNA in duodenal biopsy specimens. ISEL analysis revealed increased numbers of epithelial cells undergoing apoptosis both in the crypts and surface epithelium in specimens from SCT study patients (SCT) than in specimens from normal controls (control) (diaminobenzidine-haematoxylin stain, original magnification x200). B. Percentage of apoptotic crypt and surface epithelial cells detected by ISEL in duodenal specimens of normal controls, SCT patients at 6 weeks, and SCT patients at 3 months. Horisontal lines show medians for each study group. C. Proliferation detected by Ki-67 and apoptosis of crypt epithelial cells detected by ISEL was present in the same duodenal specimens of normal controls (r=0.44, P=0.01 Spearman correlation) (black triangles, n=12), SCT patients at 6 weeks (black circles, n=10), SCT patients at 3 months (open circles, n=7), and clinical GvHD patients at 6 weeks (black boxes, n=2).

We found a higher percentage of apoptotic cells both in the crypts (P<0.001 and P=0.002) and in the surface epithelium (P<0.001 and P=0.001) in SCT patient groups when compared to controls (Figure 11B). In the villi, we found apoptotic cells only in the tips in normal controls (median 2.3% of the epithelial cells in the whole villous), while the apoptotic cells were scattered along the whole villous height in SCT specimens (median 8.6% at 6 weeks and 7.6% at 3 months). The same samples showed an increased turnover rate detected by Ki-67 and increased apoptosis of the crypt cells, as shown in Figure 11C (P=0.01, Spearman correlation).

8. Expression of HLA-DR, HLA–DP, and ICAM-1 (I-IV)

We found a significantly increased expression of HLA-DR in the epithelium of CD patients, in all T1D patients, in SCT patients, and in food allergy patients. We also found an increased staining of HLA-DP in CD patients, T1D patients, SCT patients at 6 weeks, and in treated food allergy patients when compared to normal controls. The expression of ICAM-1 was increased in the lamina propria of all T1D patients and in CD patients of study I and IV.

In studies I-III the expression of HLA-DR and HLA-DP antigens was graded from 2 to 6 according to their intensity and cellular distribution, while the staining was determined by point-counting in study IV (see page 51). Only the data from studies I-III are presented in Table 8.

Table 8. Expression of HLA-DR, HLA-DP, and ICAM-1 in studies I-III.

Study	Study group	HLA-DR	HLA-DP	ICAM-1
I-III	Normal controls	2 (2-6)	3 (2-5)	2 (1-3)
I	Potential CD	2 (2-5)	2 (2-6)	2 (1-3)
I	CD	6 (2-6)	6 (5-6)	3 (3-3)
II	T1D + normal mucosa	5.5 (5-6)	5 (4-5)	3 (2-3)
II	T1D + potential CD	6 (5-6)	5 (5-5)	3 (3-3)
II	T1D + CD	6 (5-6)	6 (5-6)	3 (3-3)
III	SCT 6 weeks	5 (2-6)	5 (2-6)	2 (2-3)
III	SCT 3 months	4 (2-6)	4 (2-6)	2 (2-3)

Expression was analysed by immunohistochemistry. Values are given as median (range) for the different groups.

9. HLA-genotyping (II)

We found the DQB1*0201 allele in 6/16 T1D patients with normal mucosa (37.5%), of whom three also were positive for DQB1*0302. Four of the patients were positive for DQB1*0302 alone without protective alleles. Of the remaining patients, one was positive for DQB1*0301, one for DQB1*0602, one was not genotyped, and two were negative for all the alleles tested. Of the eight T1D patients with newly diagnosed CD, five were positive for DQB1*0201 (63%), of which two also were positive for DQB1*0302. One of the T1D patients with CD was positive for DQB1*0602 and the last two were negative for all the alleles tested. Six of the eight T1D patients with potential CD were positive for DQB1*0201 (75%), of whom two also were positive for DQB1*0302. The remaining two patients were positive for DQB1*0302 alone. The heterodimer HLA-DQ2 (DQB1*0201/DQA1*0501) associated with CD was carried by 4/8 T1D patients with CD and by 6/8 T1D patients with potential CD.

HLA-genotype associations

T1D patients carrying both the HLA-DR3 and HLA-DQ2 antigens (13 patients; 4/8 with CD, 6/8 with potential CD, and 3/16 with normal mucosa) showed higher densities of $CD3^+$ and $\gamma\delta TCR^+$ cells in the lamina propria ($P=0.041$ and $P<0.001$) and of $\gamma\delta TCR^+$ cells in the surface epithelium ($P=0.023$) than those negative for the alleles. The density of IL-4^+ cells, both protein and mRNA expressing, was equal in both DR4 positive and negative patients. The density of IL-2 positive cells was somewhat lower in DR4 positive than in negative patients ($P=0.049$).

10. Expression of adhesion molecules and CD25 (IV)

The densities of $\alpha_4\beta_7$ and CD11a positive cells were determined both in the epithelial compartment and in the lamina propria in study IV. We could not find any difference in the densities of $\alpha_4\beta_7^+$ IELs between the study groups, whereas the density of $CD11a^+$ IELs was significantly higher in CD patients than in patients with treated food allergy ($P=0.048$) (Figure 12A). The density of $\alpha_4\beta_7^+$ cells in the lamina propria was increased in CD patients when compared to the two food allergy study groups ($P=0.048$ and $P=0.005$, respectively), but not when compared to controls. Furthermore, the density of $CD11a^+$ cells was higher in CD patients than in treated food allergy patients ($P=0.048$) and controls ($P=0.032$) (Figure

12B). Surprisingly, the numbers of CD25 (IL-2R) expressing cells in the lamina propria did not differ between the study groups (Figure 12B).

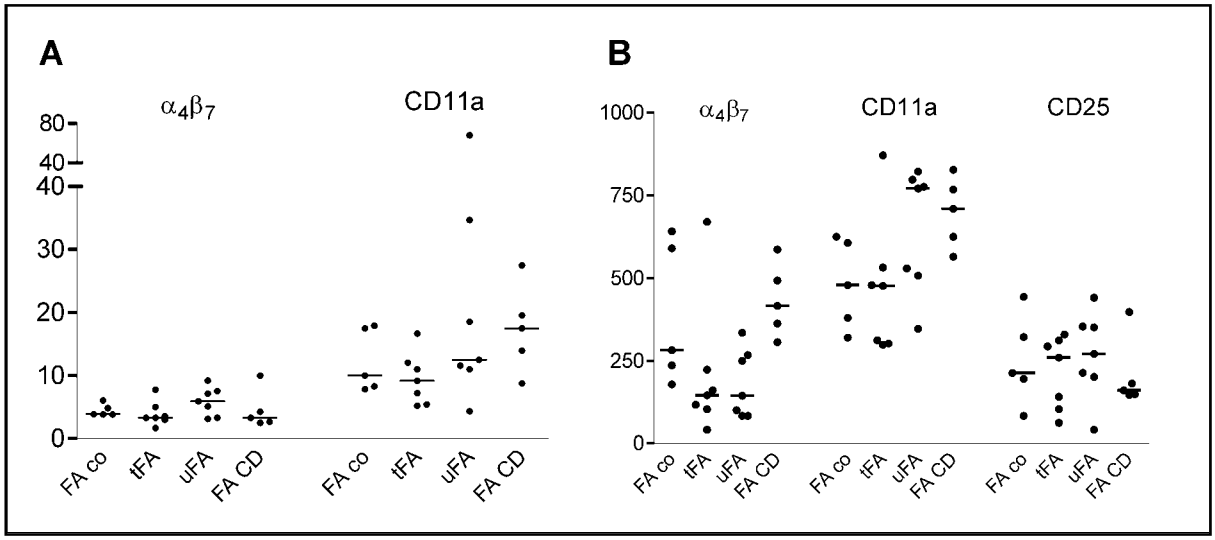

Figure 12. Expression of adhesion molecules and CD25 in food allergy.
A. Densities of $\alpha_4\beta_7$ and CD11a positive cells in the surface epithelium are expressed as positive cells/mm epithelium. B. Densities of $\alpha_4\beta_7$, CD11a, and CD25 positive cells in the lamina propria are expressed as positive cells/mm^2 lamina propria. The medians are indicated by horisontal lines for each study group; FA co, control patients; tFA, patients with treated food allergy; uFA, patients with untreated food allergy; FA CD, CD patients.

DISCUSSION

1. Methodological aspects (I-IV)

Patients and controls

Studies using human tissues have their limitations both because of ethical and practical reasons. The children included in the normal control group all had clinical indications for investigation, which may affect the immunologic markers studied. However, as the controls were chosen according to the following criteria they represent as normal available controls as possible. The exclusion criteria for the controls were: no history of CD or DH in the family, no medication and generally healthy, i.e., no chronic disease, and negative endomysium and tissue transglutaminase antibody tests. In addition, they all had normal morphology and normal density of intraepithelial lymphocytes in the small intestinal biopsy specimen taken for routine histology, which was examined independently by a specialist in pathology, as well as in the biopsy specimen used in our study. At the end of April 2004, none of the controls still affiliated to the Hospital for Children and Adolescents, Helsinki University Central Hospital, has developed CD.

The intestinal biopsy specimens obtained during gastroscopy from paediatric patients are small, which limits the number of markers that can be investigated. We used frozen tissue samples, in which the antigenic epitopes should be preserved in their native forms. The delay before the tissue was snap frozen in liquid nitrogen or the duration of sample storage before use may affect the variables measured, but we did not observe any correlation between the duration of sample storage at -70°C and the levels of markers investigated.

The biopsies in study IV were obtained from the duodenal bulb, which differs from the rest of the duodenum as well as from the duodenal mucosa. Therefore, the results from this study can not be directly compared with the results from studies I-III.

Methods

We have described cytokine positive cells in small intestinal biopsies by immunohistochemistry and radioactive *in situ* hybridisation. The methods have their limitations, not allowing recognition of the cell types involved in the cytokine expression. Double staining methods could, thus, be more informative. Difficulties with cytokine staining are well described and are due to both technical difficulties and interpretation (Arend and Dayer 1990, Hoefakker et al. 1995, van der Loos et al. 2001). We tested two antibodies against TGF-β (clone 1D11, R&D systems and T9429, Sigma) and three against IL-10

(clones JES3-9D7, JES3-12G8 and 19F1, Mabtech). Due to inconsistency and extensive background staining, which made interpretation unreliable, we did not proceed with these cytokines. The surprisingly different staining of the various batches used for IL-1α, IL-4, IFN-γ and TNF-α in our study, furthermore raises concern about the reproducibility of immunohistochemical stainings for cytokines. Consequently, results from cytokine immunohistochemistry must be interpreted with considerable caution.

Radioactive *in situ* hybridisation has its limitations as well: it is time consuming and as with immunohistochemical techniques, similar difficulties in interpretation exist. In order to overcome the subjectivity in interpretation, we developed a semiquantitative evaluation system used in studies I and IV. Still, interpretations of both methods are subjective. Although the interpretation of the slides was performed blinded with regard to the clinical data, the morphology of CD or GvHD is obvious, making the interpretation semi-blinded.

2. Densities of intraepithelial T-cells (I-IV)

We observed an increased density of IELs in potential CD and CD specimens, also in those of patients with additional T1D, which is in agreement with previous studies (Savilahti et al. 1990, Spencer et al. 1991, Kaukinen et al. 1998, Iltanen et al. 1999, Järvinen et al. 2003). We also found a moderate increase of $\gamma\delta TCR^+$ IELs in specimens from patients with untreated food allergy, which is in agreement with earlier studies (Kokkonen et al. 2000, Savilahti 2000).

While on a gluten containing diet, family members in study I and T1D patients with potential CD in study II, had almost as high densities of $\gamma\delta TCR^+$ IELs as CD patients. Still, the intestinal biopsies of potential CD cases showed a normal duodenal architecture. The presence of gluten in the diet combined with high numbers of $\gamma\delta TCR^+$ cells in the epithelium do thus, not necessarily lead to villous atrophy, which indicates that genetic factors and additional environmental agents play a further role in the manifestation of the disease. The increase of IELs in duodenal biopsies of patients with T1D is probably associated with the risk for developing CD, rather than related to T1D, as the T1D patients with increased IELs also had circulating tissue transglutaminase and/or endomysium antibodies. Moreover, the T1D patients without positive CD autoantibodies did not differ from the controls regarding the number of IELs, indicating that an increase in the IEL count is not associated with T1D.

Notably, we also found increased densities of IFN-γ positive IELs in patients with T1D and potential CD or CD, which is in agreement with recent studies on CD patients (Forsberg

et al. 2002, Olaussen et al. 2002). Paediatric CD patients were also shown to have an increased IFN-γ production intraepithelially when they were on a gluten-free diet, symptom free and their villous atrophy had resolved (Forsberg et al. 2002). This indicates continued activation of IELs in CD. Whether it is a predisposing factor for CD or a consequence of previous episodes of active CD, or due to a failure to totally eliminate gluten from the diet is not known. Recently, IFN-γ was shown to increase the production of chemoattractants for IELs by intestinal epithelial cells (Dwinell et al. 2001, Shibahara et al. 2001). Consequently, the increased amounts of IFN-γ in the epithelial compartment might be due to both increased recruitment and activation of IELs. IFN-γ is likely to cause changes in the epithelial cells. It can induce in them the ability to function as APC (Hershberg and Mayer 2000) and, secondly, increase the permeability of the epithelial monolayer (Madara and Stafford 1989).

The density of IELs in our patients study SCT patients was surprisingly low. The density of $\gamma\delta TCR^+$ IELs in clinical GvHD patients was increased, indicating that increased IEL count is associated with the pathogenesis of acute GvHD and not with the general condition in the intestine after allogeneic SCT. Our finding in GvHD patients is consistent with murine GvHD studies. Increased IELs have been a characteristic component of intestinal GvHD, where an increased IEL count is one of the earliest detectable signs, occurring within 24 hours after inducing GvHD in a neonate or in irradiated mice (Mowat et al. 1988). Despite of normal density of IELs the numbers of IFN-γ^+ IELs was increased in SCT study patients at both 6 weeks and 3 months after SCT when compared to normal controls. This indicates that the IELs, although of a normal density, are activated either because of the conditioning regimen, preceding immunosuppression, or an accompanying infection.

The function of intraepithelial $\gamma\delta TCR^+$ cells is not fully understood, but there are many postulated ones (Hayday 2000, Guy-Grand and Vassalli 2002). First, they are thought to act as cytotoxic cells against microbial pathogens via lysis of infected epithelial cells, and removal of damaged or transformed epithelial cells (Lundqvist et al. 1996). Second, they have been shown to support the growth of epithelial cells and the maintenance of the epithelial barrier integrity through their production of keratinocyte growth factor (Boismenu and Havran 1994). Thirdly, they have been suggested to have immunoregulatory functions through their production of cytokines, including IL-2, IFN-γ, TNF-α, IL-10, and TGF-β (Boismenu et al. 1996). Furthermore, it has been shown that depletion of $\gamma\delta TCR^+$ IELs improves the enteropathy of murine GvHD (Sakai et al. 1995), but abrogates oral tolerance (Fujihashi et al. 1992). Our results on increased densities of IELs in patients with CD, GvHD and untreated food allergy are based on descriptive studies and we can therefore only

hypothesise on the functions of the cells. The increase of IELs may reflect different functions of the IELs in the investigated diseases. The increase in IELs in GvHD may reflect their cytotoxic functions against transformed or otherwise stressed epithelial and stromal cells due to the conditioning regimen. The IELs may, on the other hand, protect the intestinal mucosa from chronic exposure to damaging agents such as gluten in CD and allergenic food antigens in food allergy.

3. Expression of HLA-DR and HLA-DP (I-IV)

Our data on increased expression of MHC class II HLA-DR and HLA-DP molecules in CD patients is in line with earlier reports (Arnaud-Battandier et al. 1986, Ciclitira et al. 1986, Arato et al. 1987, Marley et al. 1987, Kelly et al. 1988). We observed increased expression of these molecules in those family members of CD patients that had an increased IEL count, whereas the expression was normal in family members with a normal IEL count. Increased staining was documented in approximately half of the first-degree relatives of CD patients investigated in one previous report (Holm et al. 1994), which is in agreement with our results. We also found increased expression of HLA-DR and HLA-DP antigens in the duodenal mucosa of patients with T1D in study III, regardless of the mucosal architecture, HLA genotype, and duration of T1D. The increased expression in our T1D patients confirms the findings of an earlier study (Savilahti et al. 1999). Additionally, we observed an increased expression HLA-DR in the crypts of untreated food allergy patients.

The increased expression of these molecule in our SCT patients, is in accordance with an earlier report describing up-regulation of HLA-DR in enterocytes and keratinocytes after allogeneic SCT (Sviland et al. 1988). Notably, we also found increased expression of HLA-DR in patients without histologically proven GvHD and the increase was still present 3 months after SCT.

The increased expression of MHC class II molecules is one of the first signs of immune activation in the intestine of CD patients. Installation of gluten into the jejunum of treated CD patients induced HLA-DR expression in crypt epithelial cells within an hour in one study (Ciclitira et al. 1986), whereas stimulation of coeliac small intestine biopsies with gluten showed induced expression of HLA-DR and ICAM-1 molecules on lamina propria macrophages and epithelial cells before T-cells were seen to migrate to the subepithelial compartment (Maiuri et al. 1996). This expression is probably induced by the secretion of IFN-γ by activated Th1 cells (Skoskiewicz et al. 1985), but also IL-4 has been postulated to

increase the expression of MHC class II molecules. The increase in HLA-DR and HLA-DP staining in our studies was associated with increased expression of either IFN-γ (in patients with potential CD, CD or untreated food allergy) or IL-4 (in patients with T1D or patients undergoing SCT), supporting the regulatory function of these cytokines in the intestinal immune response.

The expression of MHC class II molecules on normal epithelial cells has been considered to indicate their ability to function as APCs. These functions are more limited than those of professional APCs, but the epithelial cells could be able to activate $CD4^+$ T-cells via classical class II mediated pathways. If class II molecules present antigens from the gut lumen, then their increased expression in disease states observed in this study would lead to an increase in the presentation of the antigens to the gastrointestinal immune system. This may cause further inflammation and even greater presentation of luminal antigens, leading to a vicious circle. How the expression of MHC class II molecules relates to the pathogenesis of the diseases investigated is out of scope for this study, but the increased expression of these molecules certainly indicates that a degree of immune activation is present.

4. Cytokine expression in potential CD (I)

We have demonstrated that positive CD autoantibodies and a high density of γδTCR positive cells in the epithelium were associated with the presence of increased densities of TNF-α and IFN-γ positive cells in the lamina propria of family members of CD or DH patients with normal duodenal structure. To the best of our knowledge no other report has been published confirming our results, but the involvement of IFN-γ and TNF-α in overt CD is well established. Recent reports have further confirmed the involvement of a Th1 deviation in CD. IL-15 has been suggested to promote Th1 activation (Maiuri et al. 2000, Maiuri et al. 2003), whereas increased expression of IL-18 and other markers of Th1 polarisation was documented in another study (Salvati et al. 2002). According to a recent report, T-cells from active coeliac mucosa did not show increased levels of TNF-α mRNA, suggesting that macrophages are the likely source of TNF-α in CD (Forsberg et al. 2002). This is in agreement with our immunohistochemical stainings showing that TNF-α^+ cells have a large cytoplasm.

Our data on IL-2 expression in samples of CD and potential CD patients, not showing any difference between the study groups, may be a sign of long-standing immune activation.

In agreement with our results, Forsberg et al. found no increase of IL-2 mRNA in paediatric CD patients (Forsberg et al. 2002).

Expression of cytokines in family members of patients with CD vs. DH

We observed higher densities of IL-4 positive cells in the lamina propria of family members of DH patients compared to family members of CD patients, whereas it was the opposite for IFN-γ and TNF-α positive cells. Diverse factors could explain our findings. The studied family members were few and individual variation may thus affect the results considerably. The family members of DH patients were all adults, and as IL-4 has been found to downregulate the inflammatory response, it could be hypothesised that in adult family members the higher expression of IL-4 may indicate the protective effects of this cytokine. Thirdly, genetic predisposition could promote the development of T-cell response towards a Th2-type. Very few studies have evaluated the cytokine profiles in patients with CD and DH, none of them including family members. A higher expression of IL-4 and lower expression of IFN-γ mRNA was reported in small intestinal biopsies of DH patients than in those of CD patients (Smith et al. 1999). The same group reported an increased production of IL-4 by intestinal T-cell lines from patients with DH compared to patients with isolated CD (Hall et al. 2000). These findings have not yet been confirmed by other reports, and the DH patients studied were furthermore all on dapsone medication, which could influence the inflammation response. Nevertheless, the difference in cytokine pattern may influence the clinical manifestation of these two forms of gluten sensitivity.

Development of overt CD in potential CD patients and cytokine expression

The follow-up of potential CD patients since their small intestinal biopsies were included in our study is now on the average 4.9 years at the end of April 2004. During this time two additional patients have developed clinical CD, one 4 years (patient no 9 in the original study), and the other almost 5 years (patient no 14) after the study biopsy was obtained. Both of these patients showed increased IEL counts and both had endomysium antibodies at the time of the original biopsy. Both patients also expressed high densities of IFN-γ and TNF-α positive cells in the lamina propria, similarly as the patient with latent CD (patient no 11). Our results support the theory that overt CD can develop on a previously normal mucosa and that the development may be a long process. The findings on immune activation described in our study were observable years before clinical CD developed. Our finding raises several concerns. Firstly, should patients with potential CD already start a gluten-free diet. This is a

well debated question but there is still no official guideline (Kaukinen et al. 2001, Mahadeva et al. 2002, Valletta et al. 2002). A gluten-free diet has a significant impact on the everyday-life, and as the patients are symptom-free the compliance with the gluten-free diet may be poorer than in symptomatic CD patients. However, the continuous gluten intake in CD has been associated with the development of other autoimmune diseases (Ventura et al. 1999, Not et al. 2001), pointing to the importance of gluten-free diet. Increased risk for lymphoma has not been established in the latent form of CD. Clearly, there is a need for a marker that could predict which patients will develop overt CD in the future. None of the markers investigated here could alone predict the progression to clinical CD. To date the presence of endomysium antibodies has been the best positive prognostic factor, as suggested also by other reports (Troncone 1995, Corazza et al. 1996, Troncone et al. 1996b).

5. Activation of the gut immune system in type 1 diabetes (II)

Th1/Th2 paradigm in the intestine of T1D patients

We demonstrated an increased density of IL-4 positive cells in the small intestines of T1D patients, regardless of the duodenal architecture, the duration of T1D, or the HLA-genotype. We had expected to find increased expression of IFN-γ in the small intestine of T1D patients, as a systemic deviation to Th1-type immunity has been suggested both by experimental animal studies and by studies on peripheral blood cells from T1D patients. To the best of our knowledge no other study has investigated the cytokine expression in the intestines of T1D patients. Intestinal cytokine profiles have been studied in experimental animal models, demonstrating a Th1 skewing (Scott et al. 2002), but as IL-4 predominates in the murine gut under normal conditions in opposite to the IFN-γ dominance in humans (Nagata et al. 2000, MacDonald and Monteleone 2001), the results from animal models can not directly be transcribed into human studies. Therefore, we can only compare our results with studies on peripheral blood cells from T1D patients, which do not directly correlate with the cytokine profile in the intestine or in the pancreas. However, the Th1 paradigm in T1D has been questioned also by other studies recently. A decreased IFN-γ secretion by PBMC in newly diagnosed T1D patients was reported in two recent studies (Mayer et al. 1999, Kukreja et al. 2002). In addition, Karlsson et al. demonstrated increased numbers of PBMC spontaneously secreting IFN-γ in high risk relatives of T1D patients, whereas they did not find any difference between newly diagnosed T1D patients and controls (Karlsson et al. 2000).

Furthermore, the relatives who developed T1D displayed lower numbers of IFN-γ secreting cells than high risk relatives that did not develop the disease during the follow-up did.

Although IL-4 has a potent inhibitory effect on the autoimmune process in the pancreas of NOD mice (Rabinovitch and Suarez-Pinzon 2003), the mechanism by which IL-4 ameliorates autoimmunity is not understood. Recently, one group showed that IL-4 acts upon antigen-presenting dendritic cells to decrease the cytolytic T-cell response, which prevented autoimmunity in transgenic mice that express IL-4 via the human insulin promoter (King et al. 2001). It has, on the other hand, also been proposed that high secretion of IL-4 contributes to the development of beta-cell autoimmunity by enhancing self-antigen presentation (Falcone et al. 2001).

Inflammation in the gut and insulin treatment are factors that may affect the cytokine balance in the gut. The fact that the cytokine profiles did not differ along the progression of T1D does not support the view that insulin treatment would explain our findings. In agreement with our results, Vaarala et al. found increased IL-4 secretion by $\alpha_4\beta_7$ expressing PBMCs in T1D patients, 5 of whom were diagnosed only one day before sampling. The cytokine profile did not differ among these five children when compared to the other patients in whom blood sampling was performed later, also confirming our results (Vaarala et al., unpublished observations).

We did not perform any double stainings, and can therefore only hypothesise on the origin of IL-4^+ cells. According to their morphology, a proportion of the cells could be macrophages or neutrophils rather than lymphocytes. T-cells have generally been believed to be the main cellular source of IL-4, but recent reports have also demonstrated IL-4 production by intestinal mast cells (Maiuri et al. 1996) and by peripheral blood neutrophils (Brandt et al. 2000). Also the appearance of IL-4^+ neutrophils in the normal human skin is induced by ultraviolet radiation (Teunissen et al. 2002), which supports our observation that IL-4^+ cells could include also other cells than T-cells.

Expression of IL-1α and ICAM-1 in the intestine of T1D patients

Our data on IL-1α in T1D duodenal mucosa imply a general inflammatory process in the intestines of these patients. IL-1α is a proinflammatory cytokine, suggested to be a key mediator in inflammatory bowel disease (Youngman et al. 1993). It is produced by mononuclear but not epithelial cells in inflammatory bowel disease (Youngman et al. 1993). This is also true for T1D patients as we did not observe any positive staining in the epithelium. IL-1α increases the expression of ICAM-1 (Dinarello 2002), which we also

observed in the duodenal mucosa of our T1D patients. The increased ICAM-1 expression may lead to the infiltration of inflammatory cells into the inflamed tissue. This may be one explanation for the higher density of $CD3^+$ lamina propria lymphocytes observed in our T1D patients, as compared with the other patient groups investigated in this thesis. ICAM-1 is also critical for establishing cell-to-cell contacts between APCs and T-cells, leading to T-cell activation (Springer 1994). Furthermore, IL-1 participates in the regulation of dendritic cells and thus also in antigen presentation. Taken together, the increase in ICAM-1 and IL-1α in the duodenal mucosa of our T1D patients imply that the innate immune system is also activated in the inflammation process.

Coeliac disease in type 1 diabetes

T1D and CD share the same high-risk HLA-DQ2 and DQ8 haplotypes, but this does not alone explain the increase of CD in T1D patients. Wheat gluten is associated with the risk of diabetes in NOD mice and biobreeding rats (Elliott and Martin 1984, Scott 1996). Furthermore, enhanced response to gluten was demonstrated in PBMCs of newly diagnosed T1D patients without CD (Klemetti et al. 1998), and by rectal gluten challenge in another study (Troncone et al. 2003). The enhanced responsiveness to wheat and other dietary proteins in T1D may be a consequence of broken oral tolerance to antigens in general. This dysregulation of oral tolerance could play a critical role in the process leading to the destruction of β-cells and T1D.

In one small series of adolescents with newly diagnosed CD and diabetes autoantibodies, treatment with a gluten-free diet led to the disappearance of the diabetes-associated autoantibodies (Not et al. 2001), suggesting that intestinal inflammation may stimulate beta cell specific immune response.

Our patients with T1D and normal mucosa had suffered from T1D for a longer period of time than the other two T1D groups, which probably reflects the fact that most cases of CD in T1D patients are identified within the first years from the diagnosis of T1D (Barera et al. 2002). Although CD is occasionally identified before diagnosis of T1D, in most cases CD autoantibodies are present at the time of or within two years of T1D diagnosis (Saukkonen et al. 1996). However, we can not conclude what impact the difference in duration of T1D has on the cytokine profiles in the intestine. Our results on IL-1α, IL-4, HLA, and ICAM-1 expression indicate that the duration of T1D does not affect the inflammation response. The findings were not restricted to CD associated HLA-DQ2 risk alleles, suggesting that activation of the gut immune system is associated with T1D as such. None of the T1D

patients with potential CD or with normal mucosa has developed clinical CD during the follow-up, which on the average is 4.8 and 5.5 years, respectively, by the end of April 2004. This further confirms that our results are not caused by the higher risk of CD in T1D patients. The densities of IL-2, IFN-γ, and TNF-α positive cells were, nonetheless, associated with the degree of CD, confirming our results from CD patients in study I. Taken together, our results suggest that the gut immune system has an important role in T1D.

6. Intestinal cytokine expression after stem cell transplantation (SCT) (III)

Our results on increased apoptosis and proliferation of intestinal crypt epithelial cells following allogeneic SCT in children is in agreement with other reports on adult patients (Epstein et al. 1980, Socié et al. 2004). We also demonstrated an increased cell turnover rate in patients without symptomatic clinical or histological GvHD. The persistence of apoptotic and proliferative activity still at 3 months may imply an ongoing process, such as infection or GvHD. However, it is not clear whether the increased cell turnover represents an early stage of intestinal GvHD in humans similar to animal models. The increased epithelial cell turnover may also reflect the injury caused by the conditioning regimen during SCT.

Th1/Th2 paradigm in GvHD

Along with the expression of the class II HLA-molecules, cytokines, such as IFN-γ, IL-1α and TNF-α, are needed for the development of murine GvHD (Hill and Ferrara 2000). In our patients, the densities of IFN-γ positive cells in the intraepithelial compartment and IL-1α positive cells in the lamina propria, but not the expression of TNF-α, were increased in the SCT patients as compared to the normal controls. The densities of IFN-γ and TNF-α positive cells in the lamina propria of SCT patients were surprisingly low, much lower than in patients with coeliac disease. On the other hand we found increased densities of IL-4 positive cells, especially in the biopsies taken at 3 months, which is in conflict with the proposed murine model of GvHD. Although a Th1 dominance has been implicated in this animal model (Mowat 1997), the role of Th1/Th2 polarisation is incompletely understood and controversial. Early polarisation of donor T-cells by administration of IFN-γ, IL-2, or IL-12 to SCT recipients can prevent acute GvHD. Furthermore, the use of Th1 cytokine-deficient mice as SCT donors still results in GvHD, and some studies have failed to show a beneficial effect of Th2 stimulation (reviewed by Reddy 2003). One study suggested that Th1- and Th2-type cytokines mediate acute GvHD with distinct end-organ targets, and that both responses are

necessary for the development of GvHD (Nikolic et al. 2000). However, to the best of our knowledge, no data on Th1- and Th2-type responses in human intestinal GvHD is yet available.

Expression of TNF-α and IL-1α

We demonstrated low densities of TNF-α positive cells in all SCT specimens, also in those with histological GvHD. This is in conflict with data from animal models, that support the role of TNF-α in the pathogenesis of intestinal GvHD (Hill and Ferrara 2000). The majority of data from GvHD derives from murine models that use inbred strains with often limited genetic disparity compared to clinical settings. The conditions in the animal models are highly controlled and the environment is pathogen-free. The use of intensive treatment schedules and immunosuppressive medications in clinical SCT is the biggest difference between the clinical SCT in patients and the experimental SCT in animal models. This certainly has its own impact on the development of GvHD, which might explain differences between our results and those from experimental models.

Nevertheless, our finding is also in conflict with recent human studies. The role of TNF-α in human GvHD was suggested by the fact that monoclonal anti-TNF-α antibody provided some benefit to patients with steroid resistant acute and chronic GvHD (Kobbe et al. 2001, Chiang et al. 2002). An increase in TNF-α levels was also detected in the sera of 84 adult SCT patients during the first 30 posttransplantation days, and an increase in TNF-α levels during the first 11-30 days correlated with the development of major transplantation-related complications (Schots et al. 2003). The proinflammatory cytokine release was common to all types of complications and no particular pattern was specific for acute GvHD. Another study with serum samples from 13 adult patients found increased levels of TNF-α after transplantation, but no correlation could be observed when TNF-α levels were compared between patients with and without acute GvHD (Visentainer et al. 2003). However, the only study so far to detect TNF-α *in situ* in duodenal biopsies reported increased expression of TNF-α positive cells in all 95 adult patients with symptoms of acute GvHD (Socié et al. 2004). Of these, 71 (73%) had histological GvHD, and the duodenal biopsies were taken within 100 days of SCT from 78 patients. Our paediatric patients were enrolled in a prospective study and most of the patients did not have symptoms of intestinal GvHD, nor did they have histologically proven GvHD as the above mentioned studies (Schots et al. 2003, Visentainer et al. 2003, Socié et al. 2004).

Still, we found increased densities of IL-1α^+ cells in the SCT patients. The increase was higher at 6 weeks compared to 3 months after SCT, suggesting that the initial damage produced by the conditioning regimen has resolved if no GvHD is developing. Mice receiving IL-1 after allogeneic SCT have increased mortality, probably due to an accelerated form of the disease (Reddy 2003). Although administration of IL-1 receptor antagonist (IL-1Ra) reduces GvHD mortality in animal models, a recent randomised human study failed to demonstrate any benefit against acute GvHD (Antin et al. 2002). This suggests that IL-1 has a pleiotropic role in the disease and may be synergistic with TNF-α.

Taken together, our results confirm the fact that only little is known about the normal course of intestinal immunopathology during the early phases after stem cell transplantation. It is unlikely that the mechanisms of murine and human GvHD differ completely, but the impact of the different cytokines and their suggested contribution to the target organ damage may differ. This might be the reason why clinical trials with specific cytokine antibodies have not yielded as good results as would have been predicted from the murine models (Bruner and Farag 2003).

7. Th1 dominance in food allergy (IV)

Our results on increased numbers of IFN-γ expressing cells in the lamina propria of children with delayed-type food allergy indicates a Th1-dominant response. The increased IFN-γ is in agreement with an earlier study (Hauer et al. 1997). However, since the start of our study, there have been a few reports on intestinal cytokines in food allergy, all suggesting other Th-type responses. Beyer et al. showed that cow's milk protein specific T-cells from the duodenum of patients with cow's milk gastroenteritis with eosinophilia expressed a Th2 cytokine profile (Beyer et al. 2002). Importantly, in that study only 2 of the 10 allergic children had elevated specific IgE levels. In another study, reduction of both TGF-β and its type 1 receptor was detected in the mucosa of 28 infants with food protein-induced enterocolitis (Chung et al. 2002). Unfortunately, the authors did not examine the expression of IL-4 or IFN-γ. Recently, Perez-Machado et al. did not find any significant Th1/Th2 skewing by flow cytometry amongst duodenal lymphocytes of 30 children with food allergy when compared to controls (Perez-Machado et al. 2003). The study included both IgE- (8 patients) and non-IgE mediated reactions (22 patients). In agreement with the previous study, they found reduced amounts of TGF-β1 lymphocytes both in the intraepithelial compartment and in the lamina propria. Thus, recent studies suggest that the dominant mucosal

abnormality in food allergy is not a Th1 or Th2 deviation, but impaired generation of Th3 cells. Regulatory Th3 cells (see Table 1) may be deficient at the site of antigen entry and thus allow an uncontrolled immune reaction to food antigens to take place.

Morphology of the duodenal bulb

The patchy villous atrophy has been considered one of the characteristics of food protein-induced enteropathy (Sampson 1999). However, the intestinal damage and the intensity of gastrointestinal symptoms have been shown to be in close association (McCalla et al. 1980). When intestinal symptoms are mild, like in the children in the present study, the intestinal changes have been shown to be minimal. Severity of mucosal deterioration in patients with food allergy has also been shown to decrease with age. Studies showing patchy lesions or partial villous atrophy have usually involved younger patients, e.g. Hauer et al. studied children aged 3-21 months (Hauer et al. 1997), and Chung et al. studied infants aged 24 days-8 months with cow's milk-sensitive enteropathy (Chung et al. 1999).

Although all but one of the patients with food allergy in this study, and one third of the patients in a previous study (Kokkonen et al. 2001b), presented with lymphonodular hyperplasia of the duodenal bulb, it can not be considered specific for this condition. In the earlier work lymphonodular hyperplasia was also found occasionally among healthy controls and in one patient with coeliac disease (Kokkonen et al. 2001b). It may be speculated that in conditions with lymphonodular hyperplasia, the mucosa has failed to downregulate or suppress the immune responses against luminal antigens, such as food proteins.

CONCLUSIONS

The present study evaluated the expression of cytokines and immunologic markers in immunologically mediated inflammations in human small intestine *in vivo*. Based on the results presented in this thesis, the main conclusions are:

1) Inflammatory markers can be detected in potential CD long before alterations in the villous structure take place. The increased numbers of IFN-γ and TNF-α expressing cells in latent CD patients indicate that a skewing towards a Th1-type response manifests already at the infiltrative stage of CD.

2) Increased densities of IL-2, IFN-γ, and TNF-α positive cells were associated with CD, also in T1D patients with CD or potential CD. In this respect T1D patients do not differ from other CD patients.

3) Signs of immune activation in the small intestine of all patients with T1D supports the hypothesis that a link exists between the gut immune system and type 1 diabetes. The increased density of IL-4 positive cells in T1D patients suggests that the immune response has an unexpected Th2 dominance.

4) The increased cell turnover rate in SCT patients without symptomatic clinical or histological GvHD, may represent an early stage of intestinal GvHD in humans similar to animal models. The proposed cytokine expression of animal GvHD was not observed in our patients undergoing SCT, which may reflect the immunosuppressive medication in SCT patients.

5) Increased expression of IFN-γ in patients with untreated food allergy suggest an association of Th1 delayed type immune reaction with food allergy enteropathy.

ACKNOWLEDGEMENTS

This study was carried out at the Research Laboratory of the Hospital for Children and Adolescents at the University of Helsinki. I wish to express my sincere gratitude to Emeritus Professor Jaakko Perheentupa and to Professor Martti Siimes, the former and current Heads of the Hospital for Children and Adolescents, University of Helsinki, for providing excellent research facilities.

I am most grateful to the patients who participated in these studies and made this work possible.

I owe my deepest gratitude to my supervisor, Professor Erkki Savilahti, for introducing me to the world of gastrointestinal immunology and for teaching me the basic principles of scientific reasoning and writing. His vast knowledge and experience on both clinical and scientific matters and crisp logical thinking have been the driving force of this study. In addition, his generosity, patience and encouragement have been invaluable to me. I also want to thank him, as the Head of the Hospital Research Laboratory, for providing excellent research facilities at my disposal.

I am most grateful to Professor Seppo Meri and Docent Juhani Grönlund for critically reviewing the manuscript of this thesis and for their valuable comments. Professor Seppo Meri is also thanked for his devoted time and constructive advises during the years as a member of my thesis committee, as well as for the unexpected linguistic revision of this thesis. I am grateful to Docent Sture Anderssson, the other member of my thesis committee, for his interest in my research project and everlasting enthusiasm.

Professor Markku Heinkinheimo is cordially thanked for collaboration on intestinal GATA expression, and more importantly, for his continuous support and encouragement toward young scientists, and for providing a forum for research and education, the Paediatric Graduate School at the Hospital for Children and Adolescents.

This work would not have been possible without collaboration with several distinguished scientists. Andras Arató, Jennifer Garioch, Jorma Ilonen, Riitta Karikoski, Ilkka Ketola, Professor Jorma Kokkonen, Harry Lindahl, Päivi Pihkala, Professor Ulla Saarinen-Pihkala, and Gabor Veres are gratefully acknowledged for pleasant and fruitful collaboration. Professor Outi Vaarala is warmly thanked for collaboration, endless enthusiasm and excellent help in scientific writing. Finally, I highly appreciate the collaboration with Mervi Taskinen, who, despite her duties as a researcher, clinician, and mother of three, always found the time for me and my endless questions.

I am grateful to Anu and Jussi Kantele for enthusiastic guidance into the world of scientific research during my early years as a student. Jussi is also warmly thanked for the most enjoyable company during meetings abroad.

Ilkka Julkunen, National Public Health Institute, Helsinki, is acknowledged for the kind gift of IL-4 and IFN-γ cDNAs.

Carol Norris is warmly thanked for linguistic revision of the original publications I and IV.

I wish to thank the personnel at the Research Laboratory for all the help during these years. Special thanks are due to Annikki Sarnesto for continuous support, Terttu Louhio for teaching me the practical skills in laboratory work in general, Sirkku Kristiansen for teaching me the basics in immunohistochemistry and for cutting hundreds of slides, Erja Karjalainen for practical help in various matters, Ritva Löfman for teaching me the basic methods in molecular biology, and Virpi Ahokas for the valuable help with the ISEL method. Harry Lybeck is warmly thanked for the skilful RT-PCR measurements.

I owe my warmest thanks to my past and present colleagues and friends at the Research Laboratory for unselfish help in everyday research matters, and for making the work enjoyable even at times of frustration. Thank you Marjut Otala, Krista Erkkilä, Virve Pentikäinen, Minna Eriksson, Ilkka Ketola, Otto Helve, Terhi Ahola, Taina Härkönen, Susanna Mannisto, Nina Linder, Henrikka Aito, Jaakko Patrakka, and Matti Ronkainen! Special thanks are due to my room-mates Emma Pohjavuori and Annaleena Anttila for friendship and endless discussions on everything and anything, to Eeva Martelin, Tiina Asikainen, Laura Suomalainen and Sanne Kiiveri for friendship and enjoyable activities outside the lab, and to Anna-Liisa Levonen and Mika Saksela for help with issues on molecular biology.

I want to thank my friends, especially Maria Blomqvist and Ulla Wartiovaara-Kautto, for all the support and enjoyable moments spent together.

My parents Christina and Rune Westerholm are sincerely thanked for their love, care and belief in me. I also want to thank them for all the practical help during this last year, especially for taking care of Oskar. My dearest sister Ann and brother Thomas are thanked for their love and for being there for me. My sister-in-law Karen and brother-in-law Lasse and my nieces and nephews Nadja, Wilhelm, Fredrik and Linn are warmly thanked for bringing such much joy into our family's life. My parents-in-law Leena and Kalevi Ormio are thanked for their continuos support, interest in my work, and especially for taking care of Oskar.

Finally, my deepest thanks are due to my dear husband Antti for unconditional love and support, and especially for taking such good care of our beloved son Oskar and me during the last phase of this project. Thank you, Antti and Oskar, for bringing so much joy and happiness into my life. Sine qua non.

The financial support of the Finnish Medical Association, the Sigrid Jusélius Foundation, the Foundation of the Friends of the University Children's Hospitals in Finland, the Finnish Medical Society Duodecim, the University of Helsinki, and the Research Funds of Helsinki University Central Hospital, is gratefully acknowledged.

Helsingfors, 17th August 2004

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