Tuomo Kokkonen

ENDOTHELIAL FasL IN LYMPH NODES AND IN INTESTINAL LYMPHATIC TISSUE
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Abstract
The function of the transmembrane protein FasL is to complex with the Fas receptor in a target cell and induce target cell apoptosis. Fas/FasL-mediated apoptosis plays important role in immunoregulation. FasL expression is mostly seen in activated lymphocytes. We have characterized endothelial FasL expression in different functional compartments of lymph nodes and gut-associated lymphoid tissue. Furthermore, we have explored the functional role of endothelial FasL expression by analyzing correlation with apoptosis of lymphocyte subpopulations in lymph nodes and by assessing endothelial expression under different conditions by activation of immune functions in gastrointestinal mucosa.

Immunohistochemical stainings (Fas, FasL, CD3, CD20, CD19, CD23, CD56, FVIII) were performed on 20 reactive lymph node tissues (I and II), 60 pediatric endoscopy biopsy samples (III) or 60 samples from gut resections (IV). A double-staining method combining apoptosis detection with the TUNEL-method and lymphocyte classification with FasL, Fas and cell lineage markers was optimized. Patient groups included non-pathological lymph nodes, pediatric cow’s milk-sensitive enteropathy, pediatric celiac disease, appendicitis, ulcerative colitis and Crohn’s disease. Control groups included normal biopsy samples from pediatric patients and non-pathological resectate samples from the appendix, colon or ileum to correspond to patient groups. Quantitative analysis (positive vessels or cells per mm²) was performed thoroughly for each anatomical region. In a subset of patients, soluble FasL in the serum was quantified with standard enzyme-linked immunosorbent assay.

In reactive lymph nodes FasL expression was predominantly present in high endothelial venules located in the paracortical area, where apoptotic T and B lymphocytes, some expressing Fas, were subsequently found. In the gut wall vascular FasL expression was seen in high endothelial vessels near lymphoid follicles. Serum FasL was elevated in children with an abundance of mucosal lymphoid follicles. In IBD, vascular FasL was upregulated in ulcers and in the submucosa of colons affected by Crohn’s disease.

The results indicate that endothelial FasL is characteristically present in high endothelial venules of lymphoid tissues. Detection of apoptotic Fas expressing lymphocytes adjacent to such vessels supports the idea that endothelial FasL functions as a selective gatekeeper by inducing apoptosis of Fas+ lymphocytes entering from the blood stream.

Keywords: Activation-induced cell death, Apoptosis, CD95L, Celiac disease, CMSE, Crohn’s disease, Double-stain, Enteropathy, FasL, High Endothelial Venules, IEL, Immune privilege, Immunohistochemistry, Immunology, Lymph node, Lymphocytes, Milk allergy, Ulcerative Colitis
Kokkonen, Tuomo, Endoteelin FasL imusolmukkeissa ja suoliston imukudoksissa.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala
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Tiivistelmä
Solukalvon läpäisevän proteiinin, FasL:n, tehtävä on sitoutua kohdesolun Fas-reseptoriin ja indusoida kohdesolun apoptoosia. Fas/FasL-välitteinen apoptoosi on merkittävä tekijä immunologisessa säätelyssä. FasL ilmentyy pääsääntöisesti aktivoituneissa lymfossytyissä. Olemme kuvanneet tutkimuksessamme FasL:n endoteelistä ilmentymistä imukudoksen eri toiminnallisia alueissa ja suoliston lymfattisessa kudoksessa. Lisäksi kartoitimme endoteelin FasL:n toiminnallista merkitystä analysoimalla sen yhteyttä lymfossytyten alaryhmien apootoosiin imusolmukkeissa ja arvioimalla FasL:n endoteelistä ilmentymistä suoliston limakalvon immunologiisesti erilaisissa sairauksissa.

Teimme immunohistokemiallisia värjäyksiä (Fas, FasL, CD3, CD20, CD19, CD23, CD56 ja FVIII) 20 reaktiiviselle imusolmukkeelle (I ja II), 60 lapsen endoskooppielle biopsianäytteelle (III) sekä 60 soluresekaattinäytteelle (IV). Optiimoimme kaksoisvärjäysmenetelmann, missä yhdistettiin apootoosiin havainnointimenetelmiä TUNEL ja Fas-, Fas- tai solulinjamarkkeri. Potilasryhmien kuului potilaita, joilla oli normaali imusolmukkeet, sekä potilaita, jotka sairastivat lasten viivästynyttä lehmänmaaitoallergiaa, lasten keliakia, umpilisäketulehdusta, haavaista paksusuolitulehdusta tai Crohnin tautia. Verrokkiryhmien kuului normaalit najbyoopettajat sekä terveitä resekaattinäytteitä umpilisäkkeestä sekä paksu- tai sykkyräsuolasta potilasryhmien mukaisesti. Jokaiselle anatomiselle alueelle suoritimme perusteellisen määrällisen analyysin (positiivista suomenta tai solua per mm²). Osalle ryhmistä suoritimme seerumin liukoinen FasL:n määrityksen entsymivalitettisellä immunosorbenetehdöllytyksellä.

Reaktiivisissa imusolmukkeissa FasL:n ilmentymenä näkyi pääsääntöisesti parakortikaalialueen korkeaendoteelissä venuleissa, missä myös apootootiset T- ja B-lymfossytyt (joista osa ilmeni Fasaa) sittenkin näkyivät. Suoliston seinässä havaitsimme verisuoniperäistä FasL:n ilmentymistä korkeaendoteelissä suonissa lymfaattisten itakeskusten läheisyydällä. Niillä lapsipotentilaita, joilla havaitsimme limakalvon lymfaattisten itakeskuksen lisääntymistä, oli myös seerumin FasL-pitoisuus koholla. Tulehduseläinissä suolistosairauksissa verisuoniperäinen FasL oli lisääntynyt limakalvon haavaruissa sekä Crohnin tautia sairastavien potilaiden submukosaossa.

Tulokset osoittivat verisuoniperäisenä FasL:n tyypillisiä ilmentyvän imukudoksen korkeaendoteelissä suonissa. Apootoosiin havaittavissa Fasia ilmentävissä lymfossytyissä näiden suojan läheisyydessä tukka ajatusta siitä, kuinka verisuoniperäinen FasL toimii valikoivana portinvarittajan ja aiheuttaa Fas-positiivisten lymfossytyten apootoosiin estämällä niiden pääsyn verenkerrosta.

Asiasanat: aktivaation aiheuttama solukuolema, apootoosi, CD95L, CMSE, Crohnin tauti, FasL, haavanainen, FasL, ilmalaajittuminen, immunologia, immunologinen erityisöikeus, imusolmu, kaksoisvääräys, keliakia, korkeaendoteeliset venulet, lymfossytyt, maaltoallergia, suolistosairaus
To my late Father
Acknowledgements

In 2001 a young medical student was recruited to Department of Pathology to test a novel antibody for the Fas ligand, the clone G247-4, in human pancreas samples. Without any knowledge about immunohistochemistry, immunology or science, he started training in the laboratory. Under the guidance of a few of the best laboratory masters, a kind colleague and a professor he gained more insight in immunohistochemistry and soon the first test slides were completed and being observed under the microscope together with one of the best gastrointestinal pathologist in northern Finland. What they found out was that one of the slides of pancreas samples happened to have a lymph node where FasL was seen in a few endothelical cells. At this point they had no knowledge of previous FasL expression in endothelium, even after a thorough search of PubMed. This random finding was the beginning of this Doctoral Thesis.

This thesis would not have been possible unless my late father had pushed me forward and made me contact Professor Tuomo Karttunen. It has been an honor for me to work with Professor Tuomo Karttunen, Doctor Merja Augustin, Doctor Miika Arvonen, Mr. Manu Tuovinen, Mrs. Erja Tomperi, Mrs. Mirja Vahera and Mrs. Riitta Vuento. These people made this thesis possible, and I owe my deepest gratitude to all of you!

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Oulu, February 2015

Tuomo Kokkonen
Abbreviations

AICD  activation-induced cell death
ALPS  autoimmune lymphoproliferative syndrome
APC   antigen presenting cells
CAD   caspase-activated DNAases
CD    Crohn’s disease
CeD   celiac disease
c-FLIP cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CMSE  cow’s milk-sensitive enteropathy
DAB   3,3’-diaminobenzidine
DCs   dendritic cells
DISC  death inducing signaling complex
EC    endothelial cell
ELISA enzyme-linked immunoabsorbant assay
FADD  Fas-associated death domain
FasL  Fas ligand
FLICE FADD-like IL-1β-converting enzyme
FLIP  Fas-associated death domain protein-like interleukin-1β-converting enzyme-like inhibitory protein
GALT  gut-associated lymphoid tissue
GI    gastrointestinal
Gr    granzyme
GrA   granzyme A
GrB   granzyme B
HEV   high endothelial venule
HLA   human leukocyte antigen
HUVECs human umbilical vein endothelial cells
IBD   inflammatory bowel disease
IEL   intraepithelial lymphocyte
IFN-α interferon-alpha
IFN-γ interferon-gamma
IHC   immunohistochemistry
IL    interleukin
LNH   lymphoid nodular hyperplasia
LP    lamina propria
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MAAdCAM-1</td>
<td>mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>MALT</td>
<td>mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>M-cells</td>
<td>microfold-cells</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>NK-cells</td>
<td>natural killer-cells</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline with Tween</td>
</tr>
<tr>
<td>PNAd</td>
<td>peripheral node addressin</td>
</tr>
<tr>
<td>PPs</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>RAST</td>
<td>radioallergoimmunosorbent test</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TECK</td>
<td>thymus-expressed chemokine</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell-restricted intracellular antigen type 1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>tdt-mediated dUTP nick-end labeling</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
</tbody>
</table>
List of original papers

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


IV  Kokkonen TS, Karttunen TJ. Endothelial Fas-ligand in inflammatory bowel diseases and in acute appendicitis. J Histochem Cytochem. 2015 Dec;63(12):931-42
# Contents

Abstract

Tiivistelmä

Acknowledgements 9

Abbreviations 11

List of original papers 13

Contents 15

1 Introduction 17

2 Review of literature 19
    2.1 Development of mucosal immune system .............................................. 19
    2.2 Anatomical structure of mucosal immune system ........................................ 19
    2.3 Lymph node ............................................................................................ 20
    2.4 Circulation of gut-homing lymphocytes ................................................. 20
    2.5 High endothelial venules ......................................................................... 21
    2.6 Regulation of lymph node homeostasis .................................................. 23
    2.7 Apoptosis ................................................................................................ 23
        2.7.1 Fas receptor .................................................................................. 23
        2.7.2 Fas ligand ..................................................................................... 24
        2.7.3 Fas signaling ............................................................................... 25
        2.7.4 Activation induced cell death ....................................................... 26
        2.7.5 Abnormalities of Fas or Fas ligand and associated diseases and conditions .............................................................................. 27
        2.7.6 Concept of immune privilege via FasL expression ...................... 28
        2.7.7 Endothelial FasL expression ......................................................... 28
    2.8 Pediatric cow’s milk-sensitive enteropathy .............................................. 29
    2.9 Celiac disease .......................................................................................... 30
        2.9.1 Immunopathology of celiac disease ............................................. 30
    2.10 Inflammatory bowel diseases .................................................................. 31
        2.10.1 Role of vascularization and lymphocyte traffic in IBD ............. 31
        2.10.2 Fas Ligand in IBD .......................................................................... 32

3 Aims of this thesis 33

4 Patients & Methods 35
    4.1 Patients .................................................................................................... 35
    4.2 Methods ................................................................................................... 36
        4.2.1 Standard immunohistochemistry .................................................. 36
        4.2.2 Double immunohistochemistry of apoptotic cells ....................... 37
4.2.3 Serum analysis with ELISA ......................................................... 38
4.2.4 Assessment of immunohistochemical stainings ...................... 38
4.2.5 Statistical analysis ........................................................................ 39
4.2.6 Ethical comments ......................................................................... 40

5 Results 41
5.1 FasL expression in the endothelium......................................................... 41
  5.1.1 Reactive lymph nodes............................................................... 41
  5.1.2 Small intestinal mucosa .............................................................. 42
  5.1.3 Appendical mucosa ................................................................. 44
  5.1.4 Colorectal mucosa ................................................................. 44
  5.1.5 Correlation of endothelial FasL expression with PNAd
      and MAdCAM-1 ......................................................................... 44
  5.2 Endothelial FasL and apoptosis in lymph nodes ......................... 48
  5.3 Serum FasL in CeD and CMSE ..................................................... 50
  5.4 Endothelial FasL expression in IBD and in acute appendicitis......... 52

6 Discussion 55
  6.1 Methodological aspects................................................................. 55
  6.2 Vascular FasL expression in normal lymph nodes and intestinal
      wall .......................................................................................................... 57
  6.3 Vascular FasL expression in inflamed gut ...................................... 58
  6.4 Function of vascular FasL ............................................................... 59
  6.5 Future aspects .................................................................................. 61

7 Conclusions 63
References 65
Original articles 73
1 Introduction

Immunological reactions are dynamic, based on the need to confront threats. There are specialized cells that can recognize and report threats, while other cells carry this report further on for activation of immune functions against this threat. Finally, there are cells that downregulate the whole process, pushing the system back into a steady state. One of the most important factors in the downregulation process is the Fas/Fas ligand system. We observed Fas ligand (FasL) expression in a specific pattern in lymph node vasculature and started to explore this novel phenomenon more thoroughly (Strasser et al. 2009).

Lymphocytes circulate freely within the bloodstream throughout the body. Whenever tissues are in need of help from lymphocytes, they can migrate through endothelium into the tissue. Endothelium differentiates and grows into a morphologically taller endothelium and these vessels are referred to as high endothelial venules (HEV). HEVs produce specialized addressin molecules to aid in lymphocyte landing on the surface of the endothelium. Furthermore, tissues signal lymphocytes with chemical messengers known as chemokines to lure lymphocytes (Salmi & Jalkanen 2005).

Despite all these precise measures to aid lymphocyte migration to targets, not all lymphocytes are able to migrate into tissue. Some tissues, like the eyes and testes, have an ability to tolerate the introduction of antigens without eliciting an inflammatory immune response. These tissues are named ‘immune privileged’ sites because of this ability. However, the reason for this has been unknown (Ferguson & Griffith 2006).

What are the mechanism suppressing inflammatory response in tissues with immune privilege ability? Fas is a membrane protein, the activation of which induces apoptosis of the cell. Fas is activated by its natural ligand, the Fas ligand, on the surface of another cell next to a Fas-bearing cell. FasL expression is mostly restricted to activated T-lymphocytes, but in certain tissues, e.g. the eyes and testes, also certain non-lymphatic cells express it. This non-lymphatic expression of FasL is assumed to be the secret behind immune privilege status (Ferguson & Griffith 2006). FasL expression in epithelial cells has been proposed as an immune privilege mediator. There are several other factors like physical structures surrounding privilege sites that cause a lack of lymphatic drainage limiting lymphocyte trafficking. Further local production of immunosuppressive cytokines (e.g. TGF-B) suppresses NK-cell activity, promotes generation of regulatory T-lymphocytes and inhibits complement formation or T-cell activity (Forrester et al. 2008).
This study was designed to chart expression of vascular FasL in different immunologically active organs, to search for clues about the function of endothelial FasL and to assess whether serum assays could be used as diagnostic markers of intestinal immune activation. For this purpose we studied human-reactive lymph nodes, biopsies and serum from pediatric patients with gastrointestinal symptoms, and resected colon and ileum samples of patients with severe inflammatory bowel disease.
2 Review of literature

2.1 Development of mucosal immune system

Maturation of the fetal gut is structurally complete from week 19 of gestation. By this time the immune system of the gut and its cellular components, Peyer’s patches (PPs), mesenteric lymph nodes (MLN), lamina propria (LP) and epithelium, are presented. Lymphocytes, macrophages and dendritic cells infiltrate gut-associated lymphoid tissue (GALT) between the 10th to 16th weeks of gestation. Full maturation of gut immune system takes place after birth after antigen stimulation of the commensal gut bacteria. Maturation is seen in isolated lymphoid follicles (ILF), containing single B-cell follicle, dendritic cells and few T-cells, developing germinal centers (GC) after bacterial challenge at birth (Blumer et al. 2007).

2.2 Anatomical structure of mucosal immune system

GALT consists of PPs, mucosal MLN and mucosal epithelium. Antigens from intestinal lumen are collected through specialized epithelial cells, the microfold cells (M-cells) (McGhee et al. 2007). Antigens are transferred via M-cells to dendritic cells (DC), which process and present antigens to naïve lymphoid cells in PPs activating and imprinting these cells to home back to mucosa LP and epithelium. Activated cells continue from PPs via lymphatic vessels to MLN (Eberl & Lochner 2009). Efferent lymphatic vessels continue from MLN via the thoracic duct to circulation (Salmi & Jalkanen 2005).

Lymphatic vascularization begins from most superficial tunica mucosa with lacteal vessels draining in the subepithelial network. From the surface these vessels continue between follicle domes into vessels of deeper mucosa forming a lymphatic network and forming a basket-like structure at the middle and basal portions of lymphoid follicles. These vessels continue draining into larger lymphatic vessels and form a submucosal network and continue through tunica muscularis via collectors of mesenteric arches to MLNs (Azzali 2003). In the colon, lymph vessels are only present in the deepest parts of the mucosa (Rahier et al. 2011).
2.3 Lymph node

T-lymphocyte development originates as the common lymphoid progenitor (CLP) in the bone marrow from hematopoiesis. CLP cells that migrate to the thymus differentiate into mature T cells. After a maturation process in the thymus, T-lymphocytes move freely in bloodstream and are there referred to naïve T-lymphocytes. These naïve T-lymphocytes recirculate from the blood, through lymph nodes into lymphatics and back to the blood in search of foreign antigens.

Lymph nodes are small encapsulated organs located along the pathway of lymphatic vessels. In healthy persons lymph node size varies from about 1mm to 20mm in diameter. They are widely distributed throughout the body concentrating in areas where lymph vessels converge. Lymph nodes are anatomically complicated structures with several functionally different compartments such as the paracortex, cortex, medulla, and the sinusoidal system. They are also dynamic structures reacting to various stimuli by changing their size and cellular composition by influx of lymphocytes and other cells, and tightly regulated proliferation and apoptosis of these cells (van der Valk & Meijer 1987, Pereira et al. 2015).

2.4 Circulation of gut-homing lymphocytes

Naïve lymphocytes migrate through endothelium into tissues quite randomly and not in a tissue-specific manner. In organized lymphoid tissues specialized post capillary high endothelial venules (HEVs) offer an environment for extravasation. Tissue migration includes sequentially tethering, rolling, adhesion and transmigration through endothelium into tissue. Tethering decreases the velocity of lymphocytes bringing the cell closer to the endothelial cell (EC) line allowing rolling. These first two steps involve help from selectins and mucins. Guided by chemokines and with the help of integrins, lymphocytes are able to be in closer contact with endothelial lining allowing firm adhesion and transmigration into tissue. In tissues lymphocytes move in the direction of increasing chemokine gradient to the effector site (Salmi & Jalkanen 2005, McGhee et al. 2007).

Migration into tissues requires both guidance of chemokines and addressins from ECs. Lymphocytes homing to the gut contain C-C chemokine receptor type 9 (CCR9) and α4β7 integrin which counter CCL25 (also known as Thymus-expressed chemokine, TECK) and mucosal addressin cell adhesion molecule MAdCAM-1, respectively, expressed by mucosal HEVs. Another mucosa-specific chemokine receptor, C-C chemokine receptor type 10 (CCR10), is expressed in IgA
immunoblasts, and its ligand is CCL28 chemokine. Homing during inflammation is altered in relation to hemodynamics and permeability after activation of local microvascular ECs. In EC, expression of many adhesion molecules like E-selectin, P-selectin, MAdCAM-1, ICAM-1, VAP-1 and VCAM-1, is induced (Williams 2004).

Peripheral lymph nodes have different addressin and chemokine profiles than MALT. Peripheral node addressin (PNAd) is expressed in HEVs and are widely recognized by the MECA-79 antibody that is a common marker for HEVs (Streeter et al. 1988). PNAd bind to L-selectin, expressed in lymphocytes, and interaction contributes to the initial step of lymphocyte homing (tethering and rolling) into tissue. Lymphoid cells imprinted to home to PLNs have either CCR7 (mostly T-cells) or CXCR5 (mostly B-cells). CCR7 ligands CCL19 and CC21 (also known as ELC and SLC, respectively) are constitutively presented in the T-cell zone of PLN and CXCR5 ligand CXCL13 (also known as BCA-1) in the B-cell zone. CCR7+ cells are characteristically naïve and central memory T-cells whereas CXCR5+ cells are T follicular helper cells in germinal centers (Salmi & Jalkanen 2005).

2.5 High endothelial venules

HEVs are small post-capillary veins that occur in the lymph node paracortical area and MALT in the gut (von Andrian & M'Rini 1998, Kobayashi et al. 2012). HEVs are lined by tall cuboidal rather than the usual flat ECs. On their surface HEVs also expresses specific lymphocyte binding molecules known as addressins, which allow lymphocytes to bind to the endothelium as the first step of migrating across into the tissue (Mebius et al. 1991). Through HEVs lymphocytes are able to migrate from bloodstream into LN (Mackay 1991). Another route to the lymph node is through afferent lymphatics from where about 10% of all lymphocytes reach the lymph node (Tohya et al. 2010, Young 1999, Girard & Springer 1995, Girard et al. 2012). In the gut, HEVs are mostly seen in interfollicular areas. In inflammatory conditions HEVs are increased in numbers (Kobayashi et al. 2012).

Circulating lymphocytes are able to migrate through HEV. The migrating process requires addressins on the surface of HEV and their ligand. In extra-intestinal secondary lymphoid organs the migrating process is mediated by L-selectin/PNAd interaction. The migration process in GALT, on the other hand, is mediated by α4β7/MAdCAM-1 interaction differing from other secondary lymphoid organs. The first step of migration requires interaction between the addressin and its ligand at the surface of migrating lymphocytes. The second step
is triggered by chemokine-dependent activation of integrins which leads to firm attachment of lymphocytes to the endothelium. Third step is that the lymphocytes migrate through HEV into tissue. The migration process is illustrated in Figure 1 (Salmi & Jalkanen 2005, Kobayashi et al. 2012).
2.6 Regulation of lymph node homeostasis

Lymphoid cell activation and extinction are complex processes. The key to activation is that the dendritic cells or macrophages process the antigens foreign to the body and present them to a large number of different T- and B-lymphocytes in the lymph nodes (LN) or PPs. The so-called naïve lymphocytes, cells that have not encountered antigens, circulate in the blood and enter lymph nodes through HEVs. Encountered by a suitable antigen in lymph nodes or PPs, the lymphocytes are activated, multiplied (monoclonal expansion) and are transferred into the blood stream via thoracic duct. Activated cells extravasate from the blood stream to where the foreign antigen was first encountered.

2.7 Apoptosis

Apoptosis, programmed cell death, is a mechanism whereby the organism seeks to get rid of surplus or damaged cells and in some organs is part of the renewal cycle. Apoptosis is abundant during fetal development where apoptosis is involved in the development of the nervous system, digestive system, extremities, and the immunological system. Later in adulthood apoptosis occurs in many physiological situations, such as immunological reactions. Changes in apoptosis are also found as a pathophysiological mechanism in many disease states, for example in neurodegenerative, inflammatory, infectious, immunological and autoimmune diseases, and in cancer development. Many external factors can trigger apoptosis, e.g. UV- and X-ray radiation, various chemicals, such as cytostatic drugs, variations in temperature, osmotic stress, and nitric oxide. In physiological situations, the body is able to induce apoptosis by many mechanisms, one of which is the Fas-FasL system (Krammer 1999, Kaufmann & Hengartner 2001). Differing from another form of cell death, necrosis, apoptosis is “programmed” via a set of molecular mechanisms like the caspase cascade (below). However, in some contexts necrotic cell death can be stimulated via cell internal molecules and in this situation it is called necroptosis (Linkermann & Green 2014).

2.7.1 Fas receptor

Cellular homeostasis maintains a balance between new and dying cells. When it is time to die, one of the best known systems of signaling a cell to die is Fas/FasL-mediated apoptosis. In lymphoid cells Fas-mediated apoptosis has a crucial role in
T- and B-cell maturation in thymus and bone marrow, respectively, and downregulation process of both lymphoid cell types (Strasser et al. 2009).

Fas (also known as CD95, APO-1) is a type I transmembrane glycoprotein receptor on the cell membrane. It is a member of the tumor necrosis factor receptor (TNFR) superfamily. Activation of Fas triggers apoptosis of a Fas+ cell in a complex intracellular mechanism (below). Fas is expressed widely in epithelial cells and myeloid cells and after activation also in lymphoid cells, such as B- and T-cells and natural killer cells (NK cells) (Itoh et al. 1991, Iwai et al. 1994). Among blood cells Fas is expressed only in a minority of resting peripheral blood lymphocytes (Yoshino et al. 1994). T-lymphocytes express Fas after activation, for example, when bacterial antigens are presented to T-cell, and T-cell clonal expansion begins. At the same time, paradoxically, certain T-cells begin also expression of the natural ligand of Fas, FasL (Pinkoski et al. 2000, Wajant 2002, Lee & Ferguson 2003).

2.7.2 Fas ligand

FasL is a type II transmembrane protein, related to the TNF-superfamily (Cohen & Eisenberg 1991, Lettau et al. 2011). Its function is to complex with the Fas receptor and thereby induce target cell apoptosis. Therefore Fas-FasL interaction causes apoptosis of the Fas-expressing cell (Krammer 1999, Nagata & Golstein 1995). In the immune system FasL is present in T-cells, NK cells, macrophages (Badley et al. 1996) and neutrophils (Suda et al. 1993, Liles et al. 1996). FasL expression is not restricted to lymphocytes; it is also expressed by Sertoli cells in the testes (Bellgrau et al. 1995) and by epithelial cells of the eye (Griffith et al. 1995). There are controversial studies on FasL expression in different organs and cells. D’alessio et al. (2001) showed testicular FasL expression might not be in Sertoli cells, but in stem cells. In the past there has been discussion about FasL antibodies used in research. Sträter et al. (2001) tested twelve antibodies commonly used for FasL research and noted that only one (clone G247-4) was reliable enough. This finding suggests, that many reported findings about FasL expression may be biased.

In the lymph nodes FasL has been reported to be expressed in germinal centers, the interfollicular zone and the mantle zone. The cell population seems to be composed of lymphocytes and dendritic cells (Hur et al. 2000, Verbeke et al. 1999, Strater et al. 1999). No vascular FasL has been reported in these studies.

FasL has been also reported in the gastrointestinal (GI) tract and is mostly noted in LP inflammatory cells, including cytotoxic T-cells (Pinkoski et al. 2000), NK-
cells (Nagata & Suda 1995), plasma cells (Strater et al. 1999), macrophages and neutrophils (Griffith et al. 1995), and in non-lymphoid cells, in Paneth cells of the epithelial crypts (Moller et al. 1996). No vascular expression has been reported in these studies. In the gut, villus epithelium does not normally express FasL, but does express Fas. Suzuki et al. (Suzuki et al. 2006) showed that mouse villus epithelial cells express FasL in similar quantities as ECs after exposure to oxidative stress. The significance of this FasL expression is hypothesized to be auto-activation of pro-apoptotic measures against oxidative stress.

2.7.3 Fas signaling

Fas receptor is completely formed from three homotrimeric sections after attachment of FasL. The intrinsic component is formed after activation containing Fas-Associated Death Domain (FADD) and procaspase-8. This complex is called the Death Inducing Signaling Complex (DISC). Activation of Fas by its ligand activates cleavage of caspase-8 from FADD. Activated caspase-8 affects procaspases -3, -6 and -7 by cleaving and activating these caspases. Active caspase-3 further activates Caspase-activated DNases (CADs) in the nucleus. CADs starts DNA splicing and fragmentation leading to apoptosis of the whole cell. Fas signaling is illustrated in Figure 2 (Wilson et al. 2007).

This simplified pathway of Fas activation provides a clue about how Fas activation is signaled in cells. However, as shown in Figure 2 signaling is very widespread, has two different pathways and can be regulated by other environmental factors or internally. Pathway type I is more associated with lymphoid cells and type II with non-lymphoid cells (Brint et al. 2013). In the type II Fas signaling chain, caspase-8 affects a bcl-2 family member bid in the mitochondrial pathway, further activating caspase-3 and finally resulting in the induction of apoptosis. Cleavage of caspase-8 can be inhibited by high concentrations of cellular FADD-like IL-1β-converting enzyme (FLICE) inhibitory protein (c-FLIP), which at low concentrations promotes activation of caspase-8 (Strasser et al. 2009).
2.7.4 Activation induced cell death

During activation process the cells start to produce Fas proteins on the cell membrane. If the cell surface Fas protein is attached by soluble or cell surface FasL, the cell starts a chain of events leading to apoptosis. This phenomenon is known as Activation-Induced Cell Death (AICD) and is important in regulating the immunological reaction (Krammer et al. 2007, Strasser et al. 2009).

Apoptosis mediated by Fas/FasL interaction is an important mechanism in the maintenance of lymph node homeostasis including regulation of both T- and B-lymphocyte responses (Strasser et al. 2009). T-cells can regulate the extent of their populations by killing each other with the Fas-FasL-mediated system or even self-destructing when co-expressing both Fas and FasL (Lynch et al. 1995). In the germinal centers, low-affinity or self-reacting B-cells are eliminated via Fas-FasL interaction, and high-affinity cells receive a survival stimulus via CD40 - CD40L blocking Fas-mediated apoptosis (van Eijk et al. 2001).
2.7.5 Abnormalities of Fas or Fas ligand and associated diseases and conditions

Cohen and Eisenberg (1991) presented two different inbred mouse lines with the following disorders: generalized lymphoproliferative disease (GLD), and lymphoproliferation (LPR). The symptoms of lymphoproliferation and autoimmune-type disorders are almost identical. In the first strain, a genetic defect was found in the FasL gene and the latter in the Fas receptor gene. In both cases the mutations resulted in abnormal accumulation of autoreactive lymphocytes. These genetic defects are also linked to excess production of lymphatic cells, which may present almost like a malignant lymphoma (Cohen & Eisenberg 1991). These abnormal cells are CD3⁺CD4⁻CD8⁻ that have not been eliminated in the maturation selection process in the thymus.

Autoimmune lymphoproliferative syndrome (ALPS) is a rare condition in humans. 70% of ALPS patients have a mutation in the gene coding Fas, and some have a mutation in the gene coding FasL or caspase 10. Others might even acquire a somatic mutation of the Fas receptor (Shah et al. 2014). New entities are found to cause ALPS-like symptoms, like RAS-associated ALPS-like disease (RALD) (Takagi et al. 2011).

Recent reviews underline changes in ALPS, mainly lymphadenopathy and splenomegaly, and these patients have a 20-fold increased risk for lymphomas and autoimmune diseases. Similarly to mice of GLD / LPR lines, ALPS patients show double negative T-cell clones (CD4⁻CD8⁻) that in normal conditions are deleted in the thymus via Fas-mediated apoptosis. In contrast to what would be expected by a defective function of Fas-mediated apoptosis in extinguishing immunological activation, there are no reports of abnormal inflammation of the eyes or testes or excess tissue damage at these sites (Sneller et al. 1997, Lim et al. 1998, Turbyville & Rao 2010, Teachey 2011). ALPS patients show circulating autoantibodies explicit to autoimmune diseases. The most common autoimmune diseases are hemolytic anemia and idiopathic thrombocytopenic purpura. Nonhematologic autoimmune diseases can occur involving different organ systems in a single patient. These might be, for example, Guillain-Barré syndrome, glomerulonephritis, coagulopathy associated with anti-Factor VIII antibodies, acute disseminated encephalomyelitis and other conditions (Sneller et al. 2003).

Autoimmune lymphoproliferative syndrome arises early in childhood in people who inherit mutations in genes that mediate lymphocyte apoptosis, or programmed cell death. In the immune system, antigen-induced lymphocyte apoptosis maintains
immune homeostasis by limiting lymphocyte accumulation and minimizing reactions against self-antigens. In autoimmune lymphoproliferative syndrome, defective lymphocyte apoptosis manifests as chronic, nonmalignant adenopathy and splenomegaly; the expansion of an unusual population of CD4-CD8- T cells; and the development of autoimmune disease. Most cases of autoimmune lymphoproliferative syndrome involve heterozygous mutations in the lymphocyte surface protein Fas (CD95, Apo1) that impair a major apoptotic pathway. Prospective evaluations of patients and their families have revealed an ever-expanding spectrum of autoimmune lymphoproliferative syndrome and its major complications.

### 2.7.6 Concept of immune privilege via FasL expression

A system’s own immune reaction can cause extensive tissue destruction, e.g. in autoimmune diseases or strong inflammation processes. However, there are organs in which the immunological reaction does not start, because cells of the immune system do not reach the tissue. In humans, for example, the eye and testes are the places where widespread immunological reaction is seldom found. This is considered to be due to FasL expression in epithelial cells lining the eye and in Sertoli cells in the testes, which trigger apoptosis of tissue infiltrating immunologically active cells before they can affect the actual tissue. Therefore FasL expression of epithelial cells of the eye and Sertoli cells of the testes is considered as a defense mechanism against an immunological reaction, which could have serious consequences for the tissue structure and physiological function. Eyes and testicles are called ‘Immune privileged’ sites (Ferguson & Griffith 2006). However, this concept has been challenged. Using pancreatic islets genetically engineered to express FasL, it was shown that FasL may not be critical in the establishment of immune privilege for protection of transplanted islets. (Allison et al. 1997, Strasser et al. 2009).

### 2.7.7 Endothelial FasL expression

Endothelial FasL has been reported by Imanishi et al. (Imanishi et al. 2001), with the FasL clone G247-4 antibody and ECs expressing FasL at the sites of atherosclerosis plaques. Also Suzuki et al. (2006) reported evidence of FasL expression induced after oxidative stress in ECs. A more recent review by Stoneman et al. (Stoneman & Bennett 2009) summarizes the role of FasL in
vascular cell apoptosis, but no endothelial FasL expression was mentioned. In lymph nodes several studies (above) have not reported vascular FasL expression. A few studies showed only some endothelial expression without discussing this phenomenon (Strater et al. 1999). Motz et al. (2014) showed endothelial FasL in tumor vasculature but none in normal samples. Further, cultured HUVECs express FasL after induction with nicotine (Cheng et al. 2010).

2.8 Pediatric cow’s milk-sensitive enteropathy

Food allergy is understood as the body's immunological response against proteins contained in food. Food allergies can be roughly divided into the direct IgE-mediated and slower non-IgE-mediated reactions. Immunological responses activate simultaneously and the symptoms vary. Immediate hypersensitivity is characterized by rapid appearance of skin or intestinal symptoms after ingestion of a specific food antigen. In small children it should be noted that the key symptom may only be a negative impact on child growth (Sicherer 2002, Host et al. 2002). In this thesis we focus only on the delayed type non-IgE-mediated GI-symptom milk allergy referred to as cow’s milk-sensitive enteropathy (CMSE).

Food allergy in infancy is usually mediated by immediate hypersensitivity, whereas in older children delayed responses on the GI mucosa are increasingly activated and are involved in association with downregulation of IgE-mediated immune responses. CMSE has recently been described as the most familiar manifestation of this clinical entity (Sicherer 2002, Kokkonen et al. 2001). The symptoms of the delayed food hypersensitivity are variable and include recurrent abdominal pains, diarrhea or constipation, blood in stools, anemia and even failure to thrive (Kokkonen 2001, Bellanti et al. 2003, Turunen et al. 2004, Iacono et al. 2007).

Diagnosis of CMSE includes investigations such as elimination challenge tests and clinical, endoscopic and histological examinations. The skin PRICK tests and radioallergoimmunosorbent (RAST) tests usually remain negative (Kokkonen et al. 2001). Endoscopically, a major finding is lymphoid nodular hyperplasia (LNH) without villous atrophy or other histopathological abnormalities. However, depending on how extensively patients are examined, LNH is seen in CMSE as patchy lesions at any level of the GI tract from the duodenum down to the anal region; it is present only in 40–75% of cases (Kokkonen 2004). A typical histological finding of CMSE is a slight increase of intraepithelial (IEL) gamma-delta (γδ) T-cell receptor (TCR)-bearing T-lymphocytes, although diagnostic
specificity and sensitivity are weak due to overlap with healthy subjects and patients with latent celiac disease (Kokkonen et al. 1999, Kokkonen et al. 2000, Kokkonen et al. 2001, Bellanti et al. 2003, Iacono et al. 2007).

The immunopathogenesis of CMSE is currently unknown. We and others have recently shown that CMSE and celiac disease (CeD) share some features of the functional aberration of the mucosal immune system. These include an increase of the duodenal γδ-IELs (Kokkonen et al. 2000), the cytotoxic lymphocytes expressing T-cell-restricted intracellular antigen type 1 (TIA-1), (Augustin et al. 2001), granzymes A and B (GrA and GrB, respectively), and perforin (Augustin et al. 2005a). We have shown that serum concentrations of GrA and GrB are increased in both CMSE and CeD, providing evidence of actual cytotoxic activation in CMSE (Augustin et al. 2005a). Further, Paajanen et al. showed an increase of Th1-prone interferon-gamma (IFN-γ) together with Th2 cytokine interleukin (IL)-6 in CMSE (Paajanen et al. 2005, Paajanen et al. 2006). In CMSE lymphocyte cytotoxicity does not seem to target enterocytes, like in CeD. In our previous studies there was no evidence of enterocyte apoptosis, but an increased apoptosis rate of IEL lymphocytes was observed (Augustin et al. 2005b).

2.9 Celiac disease

Celiac disease is an autoimmune disorder triggered by ingestion of gluten found in wheat, barley and rye. As a consequence of autoimmunological activation (below) in intestine, mucosal villi are flattened causing nutrient malabsorption (Briani et al. 2008). In children common symptoms are chronic diarrhea, vomiting, abdominal distension, muscle wasting, weight loss, irritability and even failure to thrive (Troncone & Jabri 2011). Genetically, celiac disease is strongly associated with haplotypes of the human leukocyte antigen (HLA)-DQ8 and/or HLA-DQ2 (Troncone & Jabri 2011). Diagnosis of celiac disease is based on clinical, serological and histological findings (Di Sabatino & Corazza 2009). The prevalence of CeD among Finnish schoolchildren is estimated to be at least 1 case in 99 children (Maki et al. 2003).

2.9.1 Immunopathology of celiac disease

Gluten peptides are digested and proteolytically processed for easier transport through villus epithelium. Several sections of gluten are resistant against proteolysis. One of the most immunologically interesting findings has been the
amino acid peptide that is 33 residues long (residues 57–89), which contains many prolines and glutamines. Prolines give this peptide increased resistance to proteolysis and further strengthen binding with HLA-DQ2 and HLA-DQ8 in antigen presenting cells (APC) in mucosa LP. Deamidated gluten recognized with HLA-DQ2/8 are presented to CD4⁺ helper T-cells by dendritic cells of LP. In the presence of interferon-alpha (IFN-α), CD4⁺ helper T-cells are activated and produce several proinflammatory cytokines, mainly IFN-γ. Increased IFN-γ promotes activation of cytotoxic IELs of the villus. Cytotoxic IEL cause enterocytes to undergo apoptosis via Fas/FasL interaction and raises secretion of tissue damaging matrix metalloproteinases (MMP). IL-15 prompts cytotoxicity of NK-cells and IELs causing activation of perforin/Gr pathways in these cells causing apoptosis of enterocytes. IL-15 production is also induced by a non-immunodominant fragment of α-gliadin (p31-43/49) (Di Sabatino & Corazza 2009).

2.10 Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is a disease category which mostly consists of Crohn’s disease (CD) and ulcerative colitis (UC). CD is characterized by granulomatous inflammation, ulcers and often transmural inflammation. CD localization can extend from the colon to the ileum and include extra-colonic inflammation in any part of the gastrointestinal tract, and is often seen as a discontinuous or patchy disease. UC is located superficially within the mucosa, and granulomas are only seen in relation to ruptured crypts. UC often involves the rectum, disease severity is increased distally, and inflammation is diffuse and continuous (Yantiss & Odze 2006). The etiology of both CD and UC is still unknown but several aspects in pathophysiology have been identified. These include genetic factors, the host immune system and gut microbiota (Maloy & Powrie 2011).

2.10.1 Role of vascularization and lymphocyte traffic in IBD

In IBD, induction of the peripheral node addressins (PNAd, recognized by MECA-79 antibody) results in increased density of L-selectin⁺ cells in LP. Further changes in chemokine profile in CD result in decreased numbers of CCR9⁺ LP lymphocytes and a 30-fold increase in numbers of CCR2⁺CD4⁺ cells (Connor et al. 2004). In active UC, MECA-79 vessels are seen more in numbers and as a proportion of
CD34+ vessels, as compared to UC in remission (Kobayashi et al. 2009). MAdCAM-1 overexpression was reported by Briskin et al. (1997) in both UC and CD. Similarly, Arihiro et al. (2002) showed abundant MAdCAM-1 vessels in inflamed mucosa both in CD and UC but, in a different pattern, MAdCAM-1 expression was increased more in CD than UC.

2.10.2 Fas Ligand in IBD

FasL has been studied extensively in gut-associated diseases in the past and results have been controversial. Souza et al. found increased numbers of FasL-positive cells in LP of patients with IBD but this did not correlate to rate of apoptosis in LP or epithelium (Souza et al. 2005). Di Sabattino et al. (2003) did not find a significant increase of FasL LP cells. Melgar et al. (2004) showed that frequency of FasL-expressing cells is increased in UC patients but not in ileum affected by CD. Ueyama et al. (1998) showed increased FasL mRNA levels in active lesions of UC but CD and inactive colitis showed levels similar to controls. None of these studies reported vascular expression of FasL or FasL mRNA in endothelium.

TNF-α analogs (Infliximab) are used in IBD and in Crohn’s disease and 70–80% of patients respond to treatment (Hlavaty et al. 2005). One study has shown that FasL gene polymorphisms respond differently to this treatment (Hlavaty et al. 2005). There is polymorphism of TNF receptors in different subgroups of CD (Waschke et al. 2005). This indicates that the Fas/FasL system seems to be involved more in pathogenesis of CD than was previously understood.
3 Aims of this thesis

Cell surface protein Fas is widely expressed in both lymphoid and non-lymphoid cells. Interaction of Fas with its cognate ligand FasL triggers a signaling cascade leading to apoptosis of the Fas+ cell. Expression of FasL in endothelial cells has not been widely noted and its role is unknown.

The main aim of this study is to describe expression patterns of FasL in endothelium of lymph node vessels and vessels of gastrointestinal lymphatic tissue both in normal and immunologically active intestine. In addition, we sought to survey functional significance of this endothelial expression by assessing correlation with lymphocyte apoptosis and with functional markers of endothelial cells.

Detailed aims of study were:

1. To describe FasL expression in lymph node endothelium in functionally different vessel types, and in cells lining the marginal sinus of lymph nodes.
2. To explore the functional role of endothelial FasL by assessing location and cell lineage of apoptotic cells in different anatomical compartments of lymph node. For this purpose a novel immunohistochemical double-stain method for detecting TUNEL and a lymphocyte marker in the same cell using permanent DAB-coloring was optimized.
3. To describe endothelial FasL expression in normal ileum, colon and appendix and to compare expression with that of functional endothelial markers MECA-79, MAdCAM-1 and podoplanin.
4. To characterize alterations in FasL tissue expression in CeD, CMSE, acute appendicitis and IBD, and detect associations of serum sFasL levels and pediatric intestinal diseases and structural features of intestinal mucosa.
4 Patients & Methods

Patients and methods are described in more detail in the original articles (I-IV).

4.1 Patients

(I, II) Lymph node samples were selected to represent different types of reactive alterations, and included specimens from 12 patients (aged 1–73 years, mean 38 years; 8 males). All lymph nodes had been clinically enlarged and removed for diagnostic purposes. Anatomic distribution of these lymph nodes varied, including neck, axilla, abdominal cavity and inguinal region. Reaction types included six cases with paracortical hyperplasia, one sinus histiocytosis, three follicular hyperplasia cases, and two cases with a mixed reaction.

(III) An unselected series of 57 children with a mean age of 10.2 years (range 4–15 years; 22 males) were referred to the Oulu University Hospital, Finland, for pediatric gastroenterological consultation because of refractory GI complaints. Of the patients (mean age 9.2; range 4–15; 11 males) 23 were diagnosed as CMSE cases. Of the patients with CMSE, 18 (mean age 9.3 years; range 4–15; 9 males) were studied before confirmed diagnosis, while 5 (mean age 9 years; range 6–14; 2 males) were on the elimination diet during the examinations (treated CMSE). The other group consisted of 20 patients with CeD (mean age 10.5, range 5–15 years; 5 males) diagnosed according to the acknowledged criteria (13). Colonoscopy had been performed in 10 children with CeD as indicated by clinical symptoms and by the lack of a definite diagnosis of malabsorption at the time of planning the set of clinical investigations. The control group consisted of 14 subjects (mean age 11.4; range 6–15; 6 males) referred and examined for exclusion of any definite GI diseases.

(IV) Tissue samples representing normal and inflamed ileum, colon and appendix were selected for evaluation from the files of the Department of Pathology, Oulu University Hospital. Patients had a partial resection of gut because of severe inflammation or intestinal cancer, or they had had an appendectomy. For each diagnostic group and anatomical region, 10 specimens were selected. Diagnosis of either Crohn’s disease or ulcerative colitis was confirmed by both the clinician and pathologist. Specimens for normal groups, of ileum and colon, had been resected due to intestinal cancer and a sample from a healthy area, distant from the tumor, was used. Patients for both appendicitis and normal appendix had had symptoms
of appendicitis and their appendices had been surgically removed and diagnosis had been made by a pathologist.

4.2 Methods

The main methods used in this thesis are immunohistochemistry (IHC), for analyzing tissue samples, and enzyme-linked immunosorbent assay (ELISA) for serum analysis. Immunohistochemically stained samples were analyzed under a light microscope.

4.2.1 Standard immunohistochemistry

Formalin-fixed and paraffin-embedded tissue samples were cut into 4-µm sections and immunohistochemical stainings were performed using the DAKO EnVision™ (DAKO A/S, Glostrup, Denmark) detection system kit using 3,3’-diaminobenzidine (DAB) as a chromogen. After deparafinization and rehydration, slides were pre-treated, treated with peroxidase blocking solution for 15 minutes and a primary antibody was applied. Primary antibodies used and pre-treatments for each antibody are summarized in Table 1. Visualization was performed using an EnVision solution for 30 minutes and washed extensively for 15 minutes with three changes in Phosphate Buffered Saline with Tween (PBST). DAB was incubated for 5 min. and counterstaining was performed with either Methyl Green or Mayer’s hematoxylin (Reagena Oy, Helsinki, Finland) and blued in 2% NH₃-water. After every step explained above, slides were washed in PBST for 10 min unless otherwise stated. Finally, slides were dehydrated in a standard xylene-alcohol series and a cover slide was placed on top of slide.

For negative control stainings, the primary antibody was omitted or an irrelevant antibody was used (I-IV). Specimens from testes were used as a positive control for FasL (I and II). As internal positive controls, lymphocytes in B- and T-lymphocyte regions of lymph nodes, germinal centers and HEV vessels in the lymph nodes served as positive controls for CD20, CD3, Fas and FasL stainings, respectively (I and II).
Table 1. Antibodies in detail used in this thesis.

<table>
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<tr>
<th>Antibody</th>
<th>Diluent</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Temperature</th>
<th>Manufacturer</th>
<th>Manufacturer location</th>
</tr>
</thead>
<tbody>
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<td>PBS</td>
<td>1:1000 - 1:1500</td>
<td>overnight</td>
<td>4°C</td>
<td>Pharmingen</td>
<td>San Diego, California</td>
</tr>
<tr>
<td>mouse monoclonal G247-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>60 min.</td>
<td>37°C</td>
<td>Dako</td>
<td>Glostrup, Denmark</td>
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<tr>
<td>rabbit code No. A 0082</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fas</td>
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<td>60 min.</td>
<td>RT</td>
<td>Santa-Cruz Biotech,</td>
<td>California</td>
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<tr>
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<td>Envision Ab diluent</td>
<td>1:100</td>
<td>60 min.</td>
<td>RT</td>
<td>Dako</td>
<td>Glostrup, Denmark</td>
</tr>
<tr>
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<td>Envision Ab diluent</td>
<td>1:1000</td>
<td>60 min.</td>
<td>RT</td>
<td>Dako</td>
<td>Glostrup, Denmark</td>
</tr>
<tr>
<td>monoclonal F7.2.38</td>
<td>Envision Ab diluent</td>
<td>1:100</td>
<td>60 min.</td>
<td>RT</td>
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</tr>
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<td>RT</td>
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</tr>
<tr>
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</tr>
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</tbody>
</table>

4.2.2 Double immunohistochemistry of apoptotic cells

Double IHC was evaluated and performed with Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and the standard primary antibody described in the original Article II. For double-stainings, the 4-µm sections in slides were first incubated in 70°C for 10 minutes and then deparaffinized and rehydrated. Pre-treatment was performed with Tris-EDTA (pH 9.0) in a microwave for 15 minutes. After cooling in room temperature (RT), slides
were moved to 3 % H2O2 (diluted to PBS) for 5 minutes. Staining continued according to the manufacturer’s instructions of the TUNEL kit (Apoptag® peroxidase In Situ, Cat. N:o S7100, Chemicon, Temecula, California) until addition of chromogen. The chromogen used was DAB (Cat Nr. D8001, Sigma-Aldrich) 3g/l in 0.5 mol/l Tris-Buffer (pH 7.6) with 2.5% CoCl2 added (Hsu & Soban 1982) after filtering and incubated for 10 minutes at RT. Then 0.8 µl / ml 30% H2O2 was added to DAB-black solution and incubated for a further 10 min. This gives TUNEL-positive cells a black color. Sections were then stained as in single staining described above. For counterstaining, Nuclear Fast Red (0.05% Karcherot, E Merck Ag, Darmstadt, Germany), diluted in 5% aluminiumsulphate-18-hydrate, was used (incubation time was 2 minutes).

4.2.3 Serum analysis with ELISA

Serum samples were evaluated by the ELISA method (III) for sFasL (Medical and Biological Laboratories Co., Ltd., Japan), sCD23 (R&D Quantikine, Minneapolis, Minnesota, USA) and sIL-15 (R&D Quantikine, Minneapolis, Minnesota, USA). Analyses of all samples were performed simultaneously according to the manufacturer’s instructions.

4.2.4 Assessment of immunohistochemical stainings

FasL and Factor-8 (FVIII)-stained vessel density in LN (I) was counted using x400 magnification with a 0.25 * 0.25 mm (ocular graticules with a 10 * 10 grid) at least three different areas from each of the four anatomical areas. Positive vessels were classified either as HEVs or non-HEVs based on their morphological structure. HEV was identified by the cuboidal shape of their ECs.

For evaluation of TUNEL and the lymphocyte marker in double-stained specimens (II), TUNEL-positive cells in different regions and reaction types of LN were assessed using an oil immersion objective (x1000). The proportion of TUNEL double-positive cells (TUNEL and CD marker) was assessed for each double-stain combination and each anatomical region by analyzing a minimum of 10 TUNEL+ cells. Several anatomical regions of each type were analyzed for each lymph node specimen. Classification as a double-positive was made if a cell had a black nucleus (TUNEL) and brown (CD marker) rim around the nucleus staining the cytoplasm or cell membrane. In some cells without visible cytoplasm, some brown color was seen within the black nucleus, which was often fragmented. Such a brownish
staining pattern was seen in single immunohistochemical stainings in some morphologically apoptotic cells, but it was absent from single TUNEL-stained sections and this kind of staining was considered as double-positive staining. In cases where a TUNEL-positive cell was surrounded by a group of cells positive to some antibody (e.g. CD20-positive cells in GC) the TUNEL cell was classified as single-positive if the black nucleus was surrounded by a clear halo separating it from brown-positive cells. Cells with no halo surrounding the black nucleus and brown staining in contact with the nucleus were classified as a double-positive.

For biopsy samples from children who had undergone endoscopy (III), we analyzed the expression of CD23, FasL and IL-15 separately in the epithelium and LP. The extent of expression in the epithelium was assessed using a six-point scale: 0= negative; 1=1–20%; 2=21–40%; 3=41–60%; 4=61–80%; 5=81–100%. The intensity of epithelial staining was evaluated using a three-point scale: 0=negative, 1= weak; 2= moderate and 3=strong staining. The proportion of IL-15 and FasL expressing cells among the mononuclear inflammatory cells in the villus lamina propria was evaluated by counting positive and negative cells in 1–2 villi by using x400 magnification. Positive cells in the intercryptal LP were similarly counted. To get an estimate of the relative areal density of the positive cells, we first assessed the density of mononuclear inflammatory cells in the LP by using a visual analogue scale. Based on the proportion of cells expressing IL-15 or FasL, their relative areal density in the LP was calculated. The mean proportion of villous and intercryptal LP was used in these calculations.

Adult gut tissue samples (IV) were stained for FVIII, FasL, MECA-79, and MAdCAM-1 and densities of positive vessels of HEV and non-HEV types were counted. Vessel counting was performed with a microscope using a 10x magnifying objective and 10x magnifying oculars, one with a 10x10 grid. At 100x magnification the total area of the whole 10x10 grid was 1 mm². Depending on the size of tissue regions in the section, at maximum ten fields (maximum counting area per slide was 10 mm²) were separately counted in each of the following anatomical areas of the bowel wall: mucosa, mucosal lymphoid follicles, submucosa, submucosa lymphoid follicles, subserosa, and subserosa lymphoid follicles.

4.2.5 Statistical analysis

Statistical analyses were performed using statistical software IBM SPSS (versions 10.1, 12.0, 16.0 and 18.0, SPSS Inc., Chicago, USA). Normality of distribution was
assessed with the Kolmogorov-Smirnov test and Shapiro-Wilk test as well as with histograms and normal Q-Q Plots. If criteria for normality of distribution were not met, non-parametrical tests were used. Correlation analysis was performed using Spearman’s rank correlation test or Pearson correlation test depending on normality of data. A p value of <0.05 was considered significant. Box-Plot charts were made using Origin 7.0 or 8.0 (Origin Lab Corp., Massachusetts, USA). Microphotographs were taken with a Leica DFC320 (Leica Microsystems Ltd), and composed with Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, California, USA).

4.2.6 Ethical comments

The protocol was approved by the Ethical Committee for Clinical Science of Oulu University Hospital. The National Authority for Medicolegal Affairs (TEO) has approved use of tissue samples in this research. For the first two articles (I and II) work was started before the year 2001 when the Finnish law regulating the use of tissue specimens taken for diagnostic purposes came into effect. According to former legislation, no such approval by state authorities was necessary for the use of the specimens in this research.
5 Results

5.1 FasL expression in the endothelium

Endothelial FasL expression in lymph nodes was mainly seen in HEVs (Article I, Table 2). In biopsy samples of children with GI symptoms, endothelial expression was restricted to areas in close proximity to lymphoid follicles. In normal intestine, endothelial FasL was seen in HEV-type vessels close to lymphoid follicles. Endothelial FasL expression was more abundant in inflamed intestine, especially near ulcers.

5.1.1 Reactive lymph nodes

FasL was expressed in EC in all twelve patients’ LNs. FasL+ vessels appeared mostly in the paracortical region and had the morphology of HEVs. The FasL staining in ECs was diffuse in the cytoplasm and in some EC accentuation on luminal plasma membrane was noted. Subcellular distribution of FasL expression could not be fully confirmed and requires further study. In cells with a small amount of cytoplasm the resolution of light microscopy makes it difficult to identify membrane-associated staining when cytoplasmic expression is present. Generally, localization in the cell membrane of FasL is considered necessary for induction of apoptosis (Suda et al. 1997) and the observed location of apoptotic Fas+ cells close to vessels with FasL indirectly supports occurrence of membrane-associated localization for FasL.

FasL expression was occasionally present in cells lining the inner wall of the marginal sinus. These cells did not show any FVIII positivity, but their distribution corresponded to CD68-positive cells seen in serial sections, suggesting that these cells are of mononuclear phagocyte lineage. There were no FasL-expressing vessels either in the GC or in the perifollicular areas, and only a minority of extracapsular vessels showed expression. Figure 3 shows FasL+ vessel location in different parts of the lymph node.

The areal density of FasL-positive vessels was significantly higher in the paracortex (median 82.6 vessels /mm2; mean 90.7 /mm2; min 53; max 176) than in the medulla (median 16.0 vessels /mm2; mean 20.9 /mm2; min 0; max 69). Similarly, the density of FasL-positive HEVs was much higher in the paracortical zone (median 77.3 vessels /mm2; mean 77.3 /mm2; min 32; max 144) than in the
medullary zone (median 5.3 vessels /mm²; mean 4.4 /mm²; min 0; max 11; p=0.003). The density of non-HEV vessels expressing FasL was equally low in the paracortex (median 13.3 vessels /mm²; mean 13.3 /mm²; min 0; max 32) and the medulla (median 5.3 vessels /mm²; mean 16.5 /mm²; min 0; max 59).

The densities of FasL and FVIII-positive vessels were separately compared in the paracortex and in the medulla. In the paracortex, areal densities of FVIII and FasL-positive HEVs did not show significant difference (p=0.553), indicating that most of paracortical HEVs express FasL. Of the paracortical non-HEV vessels only 11% were FasL-positive. In the medulla, the areal density of FasL-positive HEVs was only about 25 % of FVIII-positive HEV, indicating that only a minority of medullary HEVs express FasL. Table 2 summarizes FasL and FVIII areal densities in reactive lymph nodes. No significant differences were seen between the different reaction types.

Table 2. Areal densities (vessels/mm²) of FasL-positive vessels (all) and FasL-positive HEV-type vessels and their proportions of FVIII-positive vessel counts in different anatomical areas of reactive lymph nodes.

<table>
<thead>
<tr>
<th>Lymph node</th>
<th>Areal density /mm²</th>
<th>Proportion % (FasL of FVIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (SD) N</td>
<td>mean (SD) N</td>
</tr>
<tr>
<td></td>
<td>FasL⁺ HEV</td>
<td>FasL⁺ all vessels</td>
</tr>
<tr>
<td>paracortex</td>
<td>66.1 (34) 13</td>
<td>81.2 (40.5) 13</td>
</tr>
<tr>
<td>medulla</td>
<td>4 (4) 12</td>
<td>19.1 (21.7) 12</td>
</tr>
</tbody>
</table>

5.1.2 Small intestinal mucosa

According to the study of endoscopical biopsy samples of pediatric patients with GI symptoms, we found ECs of the HEVs present close to mucosal GC showing a constant expression of FasL (II).

As studied from resected intestines, FasL expression in endothelial cells was rare in normal ileum. Only 13% (1 of 12) of subjects with normal ileum showed any endothelial FasL expression in mucosa and none (0 of 12) in submucosa. Mucosal expression was mainly present in HEV-type vessels around lymphoid follicles. However in subserosa 43% (4 of 9) of subjects with normal ileum showed some endothelial FasL expression, but densities remained low (Table 3) (IV); positive vessels mainly had flat endothelium.
Fig. 3. FasL staining of a lymph node with follicular hyperplasia. Figure A shows an overall view of the lymph node. The cells lining the marginal sinus (MS) show FasL positivity (arrows). The medulla (M) contains the medullary sinuses (S). The paracortex (P) lies around the cortex with the germinal centers (GC). FasL+ vessels are seen mainly in the paracortex and in the medulla, whereas no such vessels can be seen in the germinal centers. Most of the FasL vessels are HEVs. Figure B shows a detailed view of a HEV vessel with a high endothelium showing intensive FasL positivity. For comparison, Figure C shows a non-HEV vessel with flat endothelium and weak FasL positivity.
5.1.3 Appendical mucosa

Vascular FasL expression was significantly more common in normal appendix than in the ileum or colon in the submucosa (70% [7 of 10]; p=0.012 vs ileum and p=0.011 vs colon). In the mucosa and subserosa there was a similar, but non-significant trend. Vascular densities and proportion of vessels positive for FasL are summarized in Table 3. Proportions of FasL+ vessels were significantly higher in appendical mucosa (median 2.4%) and submucosa (median 1.7%), than in the corresponding regions of ileum (median values 0%, 0%; p<0.005) or colon (0%, 0%; p<0.05; Table 3). FasL was mostly seen in HEVs but not solely, as some non-HEV expression was seen (Table 3). The proportion of FasL-positive HEVs of those marked by FVIII was quite high in normal appendix tissue (mucosa 10.7% and submucosa 3.12%).

5.1.4 Colorectal mucosa

FasL-positive vessels were rare in normal colon subjects, as in mucosa 17% (2 of 12), in submucosa 8% (1 of 12) and in subserosa 11% (1 of 9) showed endothelial FasL. Areal densities and proportions of all vessels were low (Table 3). Vascular FasL expression was mainly associated with non-HEV vessels.

5.1.5 Correlation of endothelial FasL expression with PNAd and MAdCAM-1

We analyzed MECA-79 and MAdCAM-1 (Figure 4) markers in the gut samples to show whether endothelial FasL is connected with any specific expression of these addressins. We found areal density of MECA-79 HEVs to correlate in all areas with FasL HEVs (Spearman's correlation; mucosa p<0.001, ulcers p=0.001, submucosa p=0.006, subserosa p=0.006). In contrast, MECA-79 non-HEVs correlated with FasL non-HEVs only in the mucosa (p<0.001). MAdCAM-1 areal density correlated with FasL only in the mucosa (HEVs p=0.001; non-HEVs p=0.004).

We further analyzed correlations on combined HEV and non-HEV calculations (all positive vessels) between FasL and FVIII, MECA-79 or MAdCAM-1. These correlations in different anatomical areas are shown in Figure 5.
Fig. 4. Immunohistochemical stainings of FasL (A), MECA-79 (B), MAdCAM-1 (C) and Podoplanin (D) of a sample of histologically normal colon. In FasL staining, HEVs (A; arrows) at the outer aspect of a lymphoid follicle show weak positive reaction while other vessels are negative. In MECA-79 staining, HEVs in the outer parts of a lymphoid follicle, corresponding to vessels marked by FasL positivity in A, show a positive reaction (B; arrows). In addition, vessels with flat endothelium at the inferior (submucosal) aspect of the lymphoid follicle show a positive reaction for MECA-79 mainly at the side against the lymphoid follicle (B; arrowheads). In MAdCAM-1 staining a positive reaction is present in HEVs (C; arrows) and in mucosal vessels with flat endothelium (C; arrowheads). Podoplanin antibodies (D) only label vessels with flat endothelium in the very basal part of mucosa, (D; arrow) and submucosa (D; arrowheads), consistent with lymphatic vessels. Bars: 50 µm. Modified from IV.
Table 3. Areal densities (vessels/mm²) of FasL-positive vessels (all) and FasL-positive HEV-type vessels and their proportions of FVIII-positive vessel counts in different anatomical areas of normal intestinal wall.

<table>
<thead>
<tr>
<th>Anatomical area</th>
<th>Areal density /mm² (range)</th>
<th>Proportion % (FasL of FVIII) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median</td>
<td>median</td>
</tr>
<tr>
<td></td>
<td>FasL⁺ HEV</td>
<td>FasL⁺ all vessels</td>
</tr>
<tr>
<td>Normal ileum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mucosa</td>
<td>(0 - 0.2)</td>
<td>(0 - 0.2)</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 0)</td>
<td>(0 - 0)</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 1.43)</td>
<td>(0 - 12.26)</td>
</tr>
<tr>
<td>Normal colon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mucosa</td>
<td>(0 - 0)</td>
<td>(0 - 0.16)</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 0)</td>
<td>(0 - 0.54)</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 0)</td>
<td>(0 - 12)</td>
</tr>
<tr>
<td>Normal Appendix</td>
<td>0.62</td>
<td>0.91</td>
</tr>
<tr>
<td>mucosa</td>
<td>(0 - 7.36)</td>
<td>(0 - 7.87)</td>
</tr>
<tr>
<td>Normal Appendix</td>
<td>5.24</td>
<td>5.56</td>
</tr>
<tr>
<td>Ulcers</td>
<td>(0 - 13.32)</td>
<td>(0 - 15.58)</td>
</tr>
<tr>
<td>Normal Appendix</td>
<td>0.2</td>
<td>0.43</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 1.29)</td>
<td>(0 - 4.84)</td>
</tr>
<tr>
<td>Normal Appendix</td>
<td>0.0</td>
<td>0.16</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 0.74)</td>
<td>(0 - 1.85)</td>
</tr>
</tbody>
</table>
Table 4. Areal densities (per square millimeter) of FasL-positive vessels (all) and FasL-positive HEV-type vessels and their proportions of FVIII-positive vessel counts. Different anatomical areas of inflamed intestinal wall were studied. *p=>0.05; **p>0.001; t=trend compared to normal.

<table>
<thead>
<tr>
<th>Anatomical area</th>
<th>Areal density</th>
<th>Proportion (FasL of FVIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (range)</td>
<td>median (range)</td>
</tr>
<tr>
<td></td>
<td>FasL+ HEV</td>
<td>FasL+ all vessels</td>
</tr>
<tr>
<td>Crohn's ileum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mucosa</td>
<td>0,26</td>
<td>0,48</td>
</tr>
<tr>
<td>Ulcers</td>
<td>(0 - 1,8)</td>
<td>(0 - 3,44)</td>
</tr>
<tr>
<td>Crohn's ileum</td>
<td>5,54</td>
<td>10,81</td>
</tr>
<tr>
<td>Ulcers</td>
<td>(0 - 8,7)</td>
<td>(0 - 18,7)</td>
</tr>
<tr>
<td>Crohn's ileum</td>
<td>0</td>
<td>0,7</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 8,37)</td>
<td>(0 - 12,93)</td>
</tr>
<tr>
<td>Crohn's ileum</td>
<td>0,56</td>
<td>0,87</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 3,51)</td>
<td>(0 - 10,88)</td>
</tr>
<tr>
<td>Crohn's colon</td>
<td>0,15</td>
<td>0,15</td>
</tr>
<tr>
<td>mucosa</td>
<td>(0 - 2,78)t</td>
<td>(0 - 36,11)</td>
</tr>
<tr>
<td>Crohn's colon</td>
<td>4,63</td>
<td>5,91</td>
</tr>
<tr>
<td>Ulcers</td>
<td>(1,55 - 4,67)</td>
<td>(5,69 - 29,33)</td>
</tr>
<tr>
<td>Crohn's colon</td>
<td>0,12</td>
<td>0,53</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 1,79)t</td>
<td>(0 - 7,14)**</td>
</tr>
<tr>
<td>Crohn's colon</td>
<td>0</td>
<td>1,53</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 1,79)</td>
<td>(0 - 15,18)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mucosa</td>
<td>(0 - 0)</td>
<td>(0 - 4,4)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>1,56</td>
<td>9,17</td>
</tr>
<tr>
<td>Ulcers</td>
<td>(0 - 2,69)</td>
<td>(0 - 16,92)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>0</td>
<td>0,15</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 4,56)</td>
<td>(0 - 13,78)t</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 0,56)</td>
<td>(0 - 8,81)</td>
</tr>
<tr>
<td>Appendicitis</td>
<td>0,65</td>
<td>1,2</td>
</tr>
<tr>
<td>mucosa</td>
<td>(0 - 5,19)</td>
<td>(0 - 6,4)</td>
</tr>
<tr>
<td>Appendicitis</td>
<td>0,38</td>
<td>1,46</td>
</tr>
<tr>
<td>Ulcers</td>
<td>(0,16 - 4,76)</td>
<td>(0,16 - 5,35)</td>
</tr>
<tr>
<td>Appendicitis</td>
<td>0,52</td>
<td>0,61</td>
</tr>
<tr>
<td>submucosa</td>
<td>(0 - 6,74)</td>
<td>(0 - 7,77)</td>
</tr>
<tr>
<td>Appendicitis</td>
<td>0</td>
<td>0,33</td>
</tr>
<tr>
<td>subserosa</td>
<td>(0 - 0,83)</td>
<td>(0 - 7,08)</td>
</tr>
</tbody>
</table>
5.2 Endothelial FasL and apoptosis in lymph nodes

A novel double-staining method combining TUNEL and standard immunohistochemical staining was used to gain insight into localization and type of cells undergoing apoptosis in LN. Evaluation of double-stains are shown in Figure 6. In the paracortical area a majority of TUNEL$^+$ cells (about 70%) expressed Fas and/or FasL. About 40% and 30% of TUNEL$^+$ cells were positive for CD3 or CD20, respectively (Figure 7).
Fig. 6. The figure shows three different combinations of TUNEL and Fas immunostainings of the same sample. Counterstaining was performed with Nuclear Fast Red. (A, B) Double-staining with TUNEL and Fas showing double-positive cells with a black nucleus and brown cytoplasm (arrows). C and D show control stainings where either the primary antibody (anti-Fas; C) or TdT-Enzyme of the TUNEL reaction (D) has been omitted. After omission of the Fas primary antibody (C), there is no brown coloration of the immunoreaction, and only TUNEL-positive nuclei (arrows) are labelled. (D) After omission of the TdT-enzyme in a double-staining (sinus) only Fas-positive brown cells (arrows) are visible. Scale bars are 50µm for A-B and 200µm for C-D. Modified from II.

In GCs about half of TUNEL+ cells showed Fas expression. Double positivity showed a wide variation in the GC, with about 50% of TUNEL+ also showing FasL. CD3+TUNEL+ cells were absent in the GC. About half of the TUNEL-positive cells showed CD20 expression. Since about a half of the TUNEL-positive cells in the GCs were negative for both CD3 and CD20, we further investigated GC cells by combining TUNEL with CD19, CD23 and CD68. The proportion of TUNEL+/CD19+ cells was lower than TUNEL+/CD20+. TUNEL-CD68 staining indicated that the other half of apoptotic cells are macrophages and a similar
proportion of TUNEL+/CD23+ cells indicated that a major portion of the apoptotic cells of the macrophage lineage are follicular dendritic cells.

In the sinuses, Fas expression among apoptotic cells showed the most variation between the cases. FasL and CD3 were found only in a few TUNEL+ cells and CD20 was found in less than half of the cells.

In the medullary zone a minority of apoptotic cells were T- or B-cells. Fas and FasL were found in the medulla, but less than in the other regions of the lymph nodes.

Fig. 7. A. Boxplot showing distribution of proportions of different types of double-positive cells of all TUNEL-positive cells in the paracortex of human reactive lymph nodes. Each double-staining combined TUNEL with either Fas, FasL, or lymphocyte lineage markers CD3 or CD20. Box plots include median (horizontal line), quartiles, 5–95% interval (line with whiskers) and mean (small square). B. Scatter plot showing distributions of areal densities (vessels/mm²) of HEV and non-HEV blood vessels expressing FVIII or FasL in lymph node paracortex. Modified from I and II.

5.3 Serum FasL in CeD and CMSE

Serum levels of sFasL in pediatric patients with GI symptoms were assessed with ELISA analysis. CMSE and CeD did not show any significant differences compared with controls. Serum sFasL correlated with IEL counts in CMSE (untreated) as the numbers of CD3 IELs, αβ and γδ expressing IELs all showed a significant positive correlation with sFasL. This was not evident in the CeD group.
(Figure 8). Similarly, in the untreated CMSE group, sFasL was higher in patients with LNH than in those without (p=0.025; Mann-Whitney; Figure 9).

There was no correlation between estimated FasL$^+$ cells’ areal densities in the mucosa with serum levels of sFasL. Further, sFasL concentrations did not correlate with villus or crypt measurements. Endothelial expression of FasL in biopsy specimens was rare and did not show a correlation with the serum levels.

Fig. 8. Correlation of serum sFasL concentration and the numbers of CD3, $\alpha\beta$ and $\gamma\delta$ expressing intraepithelial lymphocytes in children with CMSE. Significance of the correlation was assessed with a non-parametric Spearman's test. Modified from III.
Fig. 9. Distribution of serum soluble FasL concentration in children with and without endoscopical diagnosis of LNH in either in the small or large intestine in the controls (CTRL), in untreated cow’s milk-sensitive enteropathy (CMSE), and in celiac disease (CD). Horizontal line indicates median. Significant difference in the CMSE group is shown (Mann-Whitney). Modified from III.

5.4 Endothelial FasL expression in IBD and in acute appendicitis

In the colon mucosa (not including the follicles) HEVs FasL expression was seen significantly more often in subjects with CD than in the controls (60% vs. 0%; p=0.015, Fisher’s exact test). In the submucosa, CD patients showed elevated non-HEV FasL expression compared with the controls both as areal density (p=0.027) and proportional percentages (p=0.027) (Table 4). Similarly, in colon affected by CD the density of FasL-expressing HEVs in both mucosa and submucosa showed a trend of increment compared to normal colon (p=0.064, p=0.064, respectively; Table 3).

In the ileum mucosa there were no differences in areal densities or percentages of FasL expression between CD and normal ileum (Table 4). However, in the submucosa, FasL non-HEVs were present more often in subjects with CD (60%)
than in the normal ileum (0%; p=0.035). Subjects with UC tended to express FasL more commonly in non-HEVs compared to normal colon (50% vs 0%; p=0.056). There was also significant elevation of FasL densities (both HEVs p=0.009 and non-HEVs p=0.009) in submucosa compared to normal colon submucosa (Table 4).

In subjects with appendicitis, vascular FasL density, proportion of all vessels and number of subjects that had any evidence of vascular FasL did not differ from subjects with a normal appendix (Table 3 and 4).

Vascular density in the ulcer base was compared with the anatomically corresponding layer of the intestinal wall, i.e., submucosa. Ulcers in CD of the colon showed an increment in the density of both FasL-expressing HEVs (p=0.004) and non-HEVs (p=0.004) and FasL expression was seen significantly more commonly in both HEVs (100% vs 8%; p=0.002) and non-HEVs (100% vs 8%; p=0.009) compared to normal colon submucosa (Table 4).

Similarly, in ulcers of the subjects with CD in the ileum, FasL was seen more often in both HEVs and non-HEVs (both 60%), than in normal ileum submucosa (both 0%; both p=0.035).

Also, in the ulcer bases in UC, both FasL HEVs (80%; p=0.002) and FasL non-HEVs (80%; p=0.010) were more commonly present than in the normal colon submucosa (8% both).

In appendiceal ulcers, FasL expression was similar in both HEVs and non-HEVs (100% both) as in the submucosa of normal appendix (70%).
6 Discussion

The present study has shown that endothelial FasL is expressed by endothelial cells in human reactive lymph nodes and in immunologically active gut mucosa, submucosa and subserosa. The functional significance of this expression remains speculative. Fas/FasL-mediated apoptosis is one critical factor in down regulation of immunological activation (Krammer 1999). Accordingly, it is suggested that function of endothelial FasL could be a key point in the down regulation of immunological activation by removing previously activated cells. Such a role would be analogous to that suggested for FasL in protection of immune privileged sites, such as eyes and testes (Billingham & Boswell 1953), where epithelial FasL expression has been considered as a key factor in gaining immune privilege status (Ferguson & Griffith 2006).

6.1 Methodological aspects

There have been some reports of FasL expression in endothelium and more studies that have not reported endothelial FasL in the same organs (Griffith et al. 1995, Pinkoski et al. 2000). These discrepancies may be due to the antibody properties or differences in the pre-treatment or detection methods used, or both. In an evaluation of different anti-FasL antibodies, Sträter et al. (2001) showed that several of the commercial antibodies were not specific enough. According to their study only one monoclonal antibody (clone G247-4), used in all our studies, showed a high specificity to detect FasL. Sträter et al. (1999) showed FasL expression in human normal lymphoid organs with FasL antibody clone G247-4, but they did not report vascular expression of FasL. The immunohistochemical detection method we used was polymeric-based (EnVision), which has a higher sensitivity than the avidin-biotin-based or streptavidin-based methods and does not have specificity problems related to endogenous biotin (Sabattini et al. 1998). Moreover, as a pre-treatment method we used Tris-EDTA (pH 9.0) instead of the commonly used citrate buffer (pH 6.0), which may relieve more epitopes for the antibodies to attach to. Accordingly, both the pre-treatment and detection methods used likely explain our more constant expression in EC and the HEVs in particular, as compared with the negative results of Sträter et al. (1999). Finally, our control stainings were negative, excluding method-related false positive staining.

Generally, a drawback of IHC is the specificity of the antibody (Saper 2009). FasL antibody clone G247-4 was evaluated by Sträter et al. (2001) to be only one
of twelve tested FasL antibodies that is recommendable for IHC. They stained FasL transgenic cells and control CV-1/EBNA cells. For more precise results they compared staining patterns in localization of CD95L+ cells in in situ hybridization with immunohistochemically labeled tissue sections. G247-4 was the only antibody giving satisfying signals in tissue sections perfectly matching the distribution of CD95L+ cells by in situ hybridization.

In our study we tested FasL G247-4 antibody in a positive control (testis) where known (sertoli cells) FasL-positive cells were detected. Further in mucosa samples we showed positive staining in known FasL positive cells (Paneth cells in crypts and lymphocytes in LP) corresponding to the findings of Möller et al. (1996) who found FasL mRNA in Paneth cells. For negative control stainings a primary antibody was omitted or an irrelevant antibody was used. Based on these findings we can rely on specificity of the G247-4 antibody.

Immunohistochemical double-stain methods have been commonly used in research. However, it is difficult to evaluate two antigens with the same cellular or subcellular location. For simultaneous characterization of two antigens in the same cell, other methods are usually preferred, such as immunofluorescence detection or flow cytometry. However, a drawback of the latter method is that it lacks information about anatomical organization and therefore it does not provide a complete picture of reactions in vivo. Our novel immunohistochemical double-staining method was able to simultaneously show if a cell is undergoing apoptosis and expression of the epitope recognized by the primary antibody in use. Using a five-step control procedure, we assessed staining reliability as good. First we confirmed that the double staining protocols for lymphocyte lineage markers did not compromise specificity or sensitivity of detection of either DNA degradation by TUNEL (Figure 6). Omission of TdT in double stainings resulted in a similar staining pattern as the single immunostainings (Figure 6D). Similarly, omission of the primary antibody did not affect TUNEL staining in terms of intensity or the number of positive cells (Figure 6C). A drawback of this procedure is that it requires high magnification (x1000) for evaluation, experience in pathology and even then careful evaluation to see whether a cell is double-positive or not.

There were two major reasons justifying the use of the TUNEL protocol, which shows a late phase of apoptosis, over markers of an earlier phase of apoptosis. First, TUNEL was the most commonly used method in visualization apoptosis (Watanabe et al. 2002). Second, when using Annexin V mechanical damage to cells may make non-apoptotic cells Annexin V-positive (Valavanis et al. 2001). On the other hand, comparing TUNEL with caspase-3, also used as an indicator of apoptosis, caspase-
3 is cytoplasmic marker and interpretation of double-staining with other intracellular markers (e.g. Fas or FasL) might have been more difficult.

For evaluation of reproducibility of vessel density counts, a few cases in each series were assessed twice. The correlation between two counts indicated a reasonable reproducibility ($c=0.802 \ p=0.86$, Spearman’s correlation).

### 6.2 Vascular FasL expression in normal lymph nodes and intestinal wall

FasL in blood vessel endothelium was found in both LNs (I-II) and in normal gut (III-IV). FasL expression in lymph nodes has been a matter of controversy (see 6.1.). In the intestine, FasL has been reported to have been found in mononuclear cells in LP (Pinkoski et al. 2000), Paneth cells at the bottom of the crypts (Moller et al. 1996) and in cytotoxic IELs (Lin et al. 1998). However none of these studies reported vascular FasL. Indeed, our report (II) was the first reporting endothelial FasL in the intestine.

In LNs we found vascular FasL in all paracortical HEVs and in smaller proportion in other vessels than HEVs. We did not notice vascular FasL in larger vessels than post capillary venules. In the gut wall, FasL expression was seen in HEV-type vessels close to lymphoid follicles, mostly in the submucosal, but also in the mucosal, aspect. In the ileum and colon we only rarely found vascular FasL elsewhere in the mucosa. In the appendix FasL$^+$ ECs tended to be more common, but the difference was not statistically significant. The appendix tissue showed increased vascular FasL density in submucosa compared to mucosa and this was even more increased in appendix tissue compared to ileum and colon (70%; $p=0.012$ vs ileum and $p=0.011$ vs colon). In submucosa and subserosa vascular FasL expression did not restrict only to postcapillary venules but was also seen in some larger veins and even in some cases in arterioles and arteries (Figure 4).

Previous studies have reported only very limited vascular FasL expression in humans (see above) (Imanishi et al. 2001), and recently, normal tissue samples were reported to be negative for FasL expression, although carcinoma-associated endothelium was positive (Motz et al. 2014). However, cultured human umbilical endothelial cells (HUVECs) express FasL after exposure to oxidative stress (Suzuki et al. 2006). FasL expression studies in the intestine have failed to show vascular FasL expression (Ueyama et al. 1998, Souza et al. 2005, Ciccocioppo et al. 2001, Di Sabatino et al. 2003). These discrepancies might be due to methodological
aspects (see above). Alternatively, weak endothelial reactions may have remained unnoticed or may have been ignored due to the focus on immune cells in the studies.

Taken together, we found vascular FasL in reactive lymph nodes, normal duodenum, ileum, appendix, and colon mainly in the HEV-type vessels adjacent to lymphoid follicles; all these locations being related with specific components of the immune system. In addition, serosal vessel commonly expressed FasL. These findings showing association of FasL expression with anatomical location of vessel and specific vessel types suggest that endothelial FasL expression is tightly regulated and associated with specific functions.

Transcriptional regulation of FasL is complex and several transcription regulators have been identified, including nuclear factor kappa B, involved in inflammatory/immune reactions and TGF-β in constitutive expression in some of the supposed immune privileged locations (Kavurma & Khachigian 2003). Several cytokines including IL-2 induce FasL expression in lymphocytes while IL-6 is an important downregulating factor.

There are only a few studies addressing regulation of HEV formation and expression of FasL in EC. In mouse, lymphotoxin-β receptor signaling by dendritic cells around the vessels regulates formation HEVs (Browning et al. 2005) and VEGF is involved in mediating the effect to the endothelial cells (Chyou et al. 2008). In the mouse tumor model, VEGF-A, prostaglandin E2 and IL-10 were recently shown to upregulate endothelial FasL expression in tumor-associated HEVs, but factors regulating FasL expression in HEVs of the lymphatic tissues and in human tissue in particular are not known. However, being dynamic structures and reacting as a part of the immune response, it is likely that FasL expression is linked with overall regulation of the response.

6.3 Vascular FasL expression in inflamed gut

The present study showed (IV), for the first time that FasL endothelial expression is increased in inflammatory bowel disease. The increase was most evident in ulcers of both UC and CD. In addition, vascular FasL was increased significantly in submucosa of subjects with CD. Increase of vascular FasL expression did not seem to be specific to IBD as it was seen in acute inflammatory reactions, such as appendicitis. We found vascular FasL to correlate significantly with the numbers of single FasL+ cells in all areas except for ulcers. In addition, vascular FasL correlated with increased numbers of lymphoid follicles. These observations suggest that
activation of inflammatory/immune response induces both increase of endothelial FasL expression and accumulation of FasL-expressing lymphoid cells.

Smoking has been noted to exacerbate the course of CD increasing the risk of developing strictures and fistulas and further need of surgery (Inamdar et al. 2014). Interestingly, cultured HUVECs treated with nicotine expressed both Fas and FasL (Cheng et al. 2010). However, the role of possible endothelial effect of nicotine with possible increase of vascular FasL expression in the development of complications of IBD is unknown.

Lately, biological treatments have altered treatment of IBDs. Infliximab is a TNF-α blocker used widely in IBDs, even though its effective mechanism is not clearly understood. Some patients do gain remission after treatment but others do not. Further, for some of those who gain remission, effectiveness of infliximab decreases over time.

Genetic factors have been hypothesized to play a role in differences in effectiveness of infliximab treatment. Hlavaty et al. (2005) showed that among patients with FasL gene polymorphism (-843 CC/CT) 75% responded to infliximab treatment and of patients with TT polymorphism, only 38% responded (Hlavaty et al. 2005). It is tempting to speculate that vascular FasL could have a role in the effectiveness of infliximab treatment. Hypothetically, the level of vascular FasL could indicate whether the patient would benefit from infliximab treatment or not.

6.4 Function of vascular FasL

Vascular FasL reported by us in LNs (I, II) and in the gut was seen in areas of high immunological activation (III, IV). Initially, after the first report in lymph nodes, we hypothesized that vascular FasL could serve as a gateway to lymph nodes by inducing apoptosis of activated, cytotoxic cells that could be hazardous if they reach lymph node parenchyma. We showed in lymph nodes in vivo that in the area near FasL-positive HEVs, 60–80% of apoptotic cells are Fas-positive (II). In our study on human inflammatory bowel disease (IV), vascular FasL was mostly noted, and increased as compared with normal intestine, in ulcers of CD, UC and appendicitis subjects. This could be explained by the fact that ulcers seen here were all in the healing process and the effector phase in these sites was already downregulated. Otherwise, if FasL would function as an apoptosis-inducing factor, it would not make sense. In concordance with our concept, Motz et al. (2014) show that regulatory T-lymphocytes (Treg) are able to migrate freely via HEV. Treg cells are this way able to limit inflammatory reaction.
Studied in intestinal samples, vascular FasL expression correlated with MECA-79 in HEVs, and expression of both of them was most constantly seen in the HEVs near the lymphoid follicles (IV). The MECA-79 antibody recognizes PNAd and is a common marker for HEVs (Streeter et al. 1988). This addressin binds to L-selectin in lymphocytes and is essential in the first step of lymphocyte homing (see Figure 1). Concurrent expression with MECA-79 in HEV-type vessels suggests that functionally, endothelial FasL is related with regulation of lymphocyte homing, and could share factors regulating the expression. However, in contrast with HEV-type vessels, in non-HEVs FasL was seen more frequently than MECA-79, and in ulcers MECA-79 expression was not elevated similarly to FasL (Figure 4 and 5). This indicates presence of different regulatory elements for endothelial expression of FasL and MECA-79. It also suggests that lymphocyte populations in which FasL induces apoptosis, as suggested below, are heterogeneous for expression of addressin ligands.

Our observations in the LNs paracortex indicated that apoptotic Fas+ lymphocytes predominantly locate around HEVs expressing FasL. LN GC area remained negative of vascular FasL. This finding provides indirect support to the concept that endothelial FasL induces apoptosis of Fas-expressing lymphocytes arriving to lymph nodes via HEVs. According to our findings this apoptotic Fas+ population includes both T and B lymphocytes (II). We suggest that the physiological rationale could be to eliminate possibly harmful activated and cytotoxic lymphocytes. Could freshly activated lymphocytes, those intended to have an effect in tissue, pass this barrier? According to Strasser et al. (2009), FasL is perhaps exclusively expressed by cytotoxic T-cells and NK-cells (Strasser et al. 2009). These active cells would also express Fas but after activation cytotoxic T-lymphocytes are resistant to Fas-mediated apoptosis for at least 48 hours unless sensitized with IL-2 (Peter et al. 1997). As described earlier, cells activated in lymph nodes travel via the thoracic duct into circulation from where these cells spread to the whole body. Some of these lymphocytes just happen to drift to the site of inflammation in the gut where they migrate through the vascular wall into tissue guided by chemokines and addressins. This raises the question of whether vascular FasL in inflammation sites could serve as a proinflammatory factor for cytotoxic cells activated within 48 hours and an apoptosis promoting factor for cytotoxic cells older than 48 hours.

Early reports of Fas stimulation causing apoptosis came from studies where a Fas antibody was used to activate a Fas-receptor (Strasser et al. 2009). Further, FasL immune privilege theory raised a question of whether transplants expressing
FasL could prevent a rejection reaction. These studies showed that transplanted FasL did not only fail to protect grafts from rejection but accelerated infiltration of granulocytes that coalesced in transplants into abscesses (Restifo 2000). Buonocore et al. (2002) showed that viral FasL transducted dendritic cells did not show evidence of allostolerance in vivo. B-cells seem to escape Fas-mediated apoptosis after co-stimulation with CD40 (van Eijk et al. 2001). Similarly, Maksimow et al. (2003) showed that about half of naïve T-cells undergo apoptosis after Fas stimulation with FasL but surprisingly among surviving T cells it accelerated cell division and interferon-gamma (IFN-gamma) production. Surviving was associated with expression of c-FLIP, Bcl-2 and Bcl-X (L) (Maksimow et al. 2003) and is controlled by NF-kappaB signaling and caspase activity (Maksimow et al. 2006). Further Fas stimulation with soluble sFasL seems to block Fas antibody stimulated apoptosis (Cheng et al. 1994).

In our study serum levels of sFasL did not show association with mucosal expression of FasL with disease type, and accordingly, sFasL does not seem to be a useful diagnostic marker. Interestingly however, increase of serum-soluble FasL was associated with endoscopical LNH and increased numbers of γδ IEL in the intestine. However, it is not clear if either of these conditions directly contribute to serum sFasL levels. Soluble FasL (sFasL) can be formed by differential splicing, proteolytical cleavage (Janssen et al. 2003) or released in the form of microvesicles (Brint et al. 2013). FasL in microvesicles differs from cleaved, truncated, sFasL being similar to membrane-bound FasL (mFasL). sFasL has been reported to have the ability to induce apoptosis, proliferation or decrease mFasL activation of Fas via competition. The function of sFasL remains unclear (Brint et al. 2013). Soluble FasL is increased in patient’s sera in several blood cell cancers, colon cancer, immunological diseases and viral infections (Janssen et al. 2003). More studies are needed to dissect factors affecting sFasL levels and the role of endothelial FasL expression.

Taken together, with this study we can only state that there is vascular FasL in lymph nodes and the gut, but understanding its function will require further research.

6.5 Future aspects

Our study was a preliminary study describing immunohistochemical expression of FasL in reactive LNs, GI-symptomatic pediatric patients and patients with severe inflammatory bowel disease. The function of vascular FasL remains unclear. We
speculate vascular FasL to have a role in controlling inflammation in tissue. In theory, vascular FasL can both activate and cause apoptosis of Fas+ cells depending on the inner state of target cells.

In this thesis we only focused on lymph nodes and the gut. FasL in EC has also been reported in atherosclerosis at the site of plaque. We should also expand our research to gain more insight into where and how FasL is expressed in ECs. It seems that vascular FasL is somehow induced and it is associated with immunological reactions.

In IBDs FasL polymorphism does seem to affect infliximab treatment (Hlavaty et al. 2005), however the functional mechanism of infliximab is still unclear. Does vascular FasL play a role here or could we use a simple FasL immunohistochemical staining method to predict whether the patient would benefit from infliximab treatment or not? Infliximab is a blocker of the proinflammatory TNF-α. TNF-α plays a major role in inflammatory induction but also blocks activity of cysteine uptake by blocking EAAT-3 transporter activity (Pradillo et al. 2006). Cysteine is also required for glutathione metabolism, the depletion of which disturbs dendritic cell maturation (Kim et al. 2007). Cysteine is also an important building block of the Fas receptor that has many cysteine-rich domains.

Fas and FasL have a major role in removing possibly harmful lymphocytes, which could cause autoimmunity, in the selection process in the thymus. Vascular FasL is now seen in immunologically active sites positioning as a gatekeeper and could function also as the final step eliminating autoreactive cells. However, the function of vascular FasL is still unknown and more research is required to confirm it.

Recently, endothelial FasL expression has been shown to regulate access of lymphocytes into tumor tissue and thereby protect tumors (Motz et al. 2014). Besides supporting the importance of endothelial FasL expression as an immunoregulatory mechanism, the findings indicate presence of analogous mechanisms in physiology of lymph nodes and the mucosal immune system and in tumor immunity.
7 Conclusions

This thesis describes vascular FasL expression in reactive lymph nodes and in both normal and inflamed intestine. Vascular FasL is associated with increased immunological activation. However, its function remains unclear and requires more detailed research. The main conclusions of this thesis are:

1. Endothelial FasL was seen in lymph node vessels especially in high endothelial venules in the paracortical area. This is a novel finding with unknown functional significance. Cells of monocyte macrophage lineage expressing FasL form the interface at the parenchymal side of the marginal sinus.

2. In the paracortical area of lymph node, where FasL expression was mostly seen, about 70% of lymphocytes undergoing apoptosis were Fas positive. This finding favors the idea that endothelial FasL induces apoptosis in Fas expressing lymphocytes entering lymph node parenchyma, and might explain why only naïve lymphocytes can survive after extravasation into lymph nodes from the bloodstream.

3. In normal ileum, colon and appendix tissues, vascular FasL was expressed in high endothelial vessels close to lymphoid follicles, and in these locations correlated with expression of MECA-79. Such co-expression suggests that endothelial FasL has a specific role in the regulation of access of circulating lymphocytes to mucosal lymphatic tissues. However, occasional expression of FasL was seen in other blood vessels including some without MECA-79 or MAdCAM-1 expression. This suggests functional heterogeneity of blood vessels in the intestinal wall.

4. In pediatric cow’s milk-sensitive enteropathy and celiac disease, biopsies showed vascular FasL in HEVs only near lymphoid follicles. Increase of serum soluble FasL was not associated with mucosal expression or specific disease types, but was associated with mucosal lymphoid nodular hyperplasia and increased levels of γδ IELs, suggesting linkage with immunological activation. In IBD, increase of vascular FasL expression was seen predominantly in submucosa in CD and in ulcers, suggesting a possible role in pathogenesis.
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