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# Basic and clinical aspects of mucosal inflammation and healing in Crohn's disease

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# Chapter 1

# Introduction

### Inflammatory Bowel disease

Inflammatory bowel disease (IBD) is a recurrent chronic idiopathic inflammatory disease of the gastrointestinal tract, and consists of ulcerative colitis (UC) and Crohn's disease (CD). In comparison with UC, CD is characterized by: 1) the formation of fistulae, the result of burrowing ulcers, and stricture formation in the gastrointestinal tract; 2) more frequent systemic manifestations and extraintestinal complications, which include fever, weight loss, malaise and rheumatologic diseases; and 3) the presence of granulomas in the pathological presentation [1-3].

The precise aetiology of IBD still remains unknown. Ample evidence implicate genetic susceptibility, immune abnormalities and the lumenal microflora as contributors to the aetio-pathophysiology of IBD. The damage and healing processes of intestinal tissue occur as consequences of an aberrant immune reaction. The injury of the mucosal barrier by various agents, such as genetic variation in determinant molecules, drugs, dietary agents and lumenal bacteria or bacterial products, results in bacterial antigen uptake across specialized epithelial M cells to initiate the immune response in the lamina propria or in mesenteric lymph nodes. Antigen-presenting cells (APCs), i.e., drendritic cells, macrophages, and intestinal epithelial cells, process the antigens and pass them through to the $CD4^+$ T lymphocytes. Then in CD activation of the Th1 phenotype cytokine pathway occurs. Interleukin (IL)-2 and interferon (IFN)-γ, produced by Th1 type $CD4^+$ T lymphoctes, mediate the subsequent cellular immune responses. IL-2, -12, -18, interferon (IFN)-γ, tumor necrosis factor (TNF)-α produced by T lymphocytes and macrophages stimulate macrophages in a self-sustaining cycle to produce more proinflammatory cytokines, like TNF-α, IFN-γ, IL-1 and -6. These cytokines, joined by other inflammatory mediators, like reactive oxygen/nitrogen intermediates, proteolytic enzymes, finally lead to the tissue injury in CD. In UC, however, Th2 phenotype cytokines, such as IL-4 and transforming growth factor-β, predominate and regulate the pathogenesis which is considered to be of a humoral immune response phenotype [4]. In the mucosal inflammation of the intestine, the intestinal epithelial cell not only processes the antigens but also actively participates, together with other non-immune cells such as mesenchymae and endothelial cells, in the immune reaction by the release a variety of inflammatory mediators, including reactive oxygen/nitrogen intermediates, enzymes, cytokines, and growth factors [4-8].

## Chimeric processes of inflammation: tissue destruction and healing

Inflammation is a reaction of the host to a variety of stimuli such as infection and tissue injury. The early stage of the inflammatory response is also referred to as the initiation of the wound healing. This latter event evolves simultaneously with the destruction of tissue. The destruction and healing form a continuum in inflammation, and there is no distinguishable time border between these counteracting processes. In the acute inflammation neutrophils and monocytes/macrophages are recruited to the local sites by a battery of signals, including chemokines, growth factors, cytokines, fibrin degrading products, which are released by tissue leucocytes, mast cells, blood platelets and microorganisms. Neutrophils are the predominant inflammatory cells in the primary acute response. The interaction of leucocytes and endothelial cells plays a critical role in the recruitment of these leucocytes. The reactive oxygen/nitrogen intermediates and proteolytic enzymes, including matrix metalloproteinases (MMPs), produced/released from activated leucocytes execute the primary defense against various pathogens. However, their improper activity might also cause destruction of host tissue. Proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, are multifunctional regulatory peptides in inflammation, which, on the one hand, are responsible for the tissue injury and, on the other hand, are mitogens and chemoattractants for fibroblasts, endothelial cells and epithelial cells to initiate tissue reconstruction and wound healing [9]. Chronic inflammation is considered to be the abnormal result of wound healing in which the process is arrested in an early stage.

During the acute response of inflammation recruitment of large numbers of monocytes from the blood, differentiation of monocytes to macrophages and migration of macrophages to local sites occur where they play a central role in the tissue healing. Macrophages not only remove debris by phagocytosis but also produce a variety of immunomediators which have a tremendous influence on every step of the tissue repair process. Inhibition of macrophage infiltration into tissues, therefore, essentially impairs and delays healing [10-13].

After the acute phase of inflammation, granulation tissue is formed under the stimulation of various factors. Granulation tissue is comprised of new microvessels, proliferating fibroblasts, endothelial cells, as well as macrophages and granulocytes. Neo-angiogenesis, the formation of new vessels, and the formation of the granulation tissue are affected by many factors, such as basic fibroblast growth factor (bFGF, FGF-2), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietins, IL-1 and TNF-α. This formation of granulation tissue is followed by the reconstruction of extracellular matrix (ECM) in tissue repair process. The remodeling of this ECM is a pivotal process in tissue repair and has an important impact on the quality of the tissue healing. The activity of MMPs obviously affects the process of ECM remodeling [14]. In wound healing of the gastrointestinal tract re-epithelialization occurs under the cooperative influence of different factors. The aggressive immune reaction is downregulated by inhibitory elements, including IL-10. Epidermal growth factor (EGF), transforming growth factor (TGF)-β, FGFs promote the restitution of enteric epithelial cells and stimulate their proliferation and differentiation [13;15;16] (Figure 1). The epithelial cells themselves secrete defensive proteins, such as trefoil factor and defensins, to prevent the injury [17;18]. Thus, acute inflammation, proliferation and remodeling of tissue, which are three eminent phases of wound healing, are characterized by a considerable overlap in regulatory mechanisms.

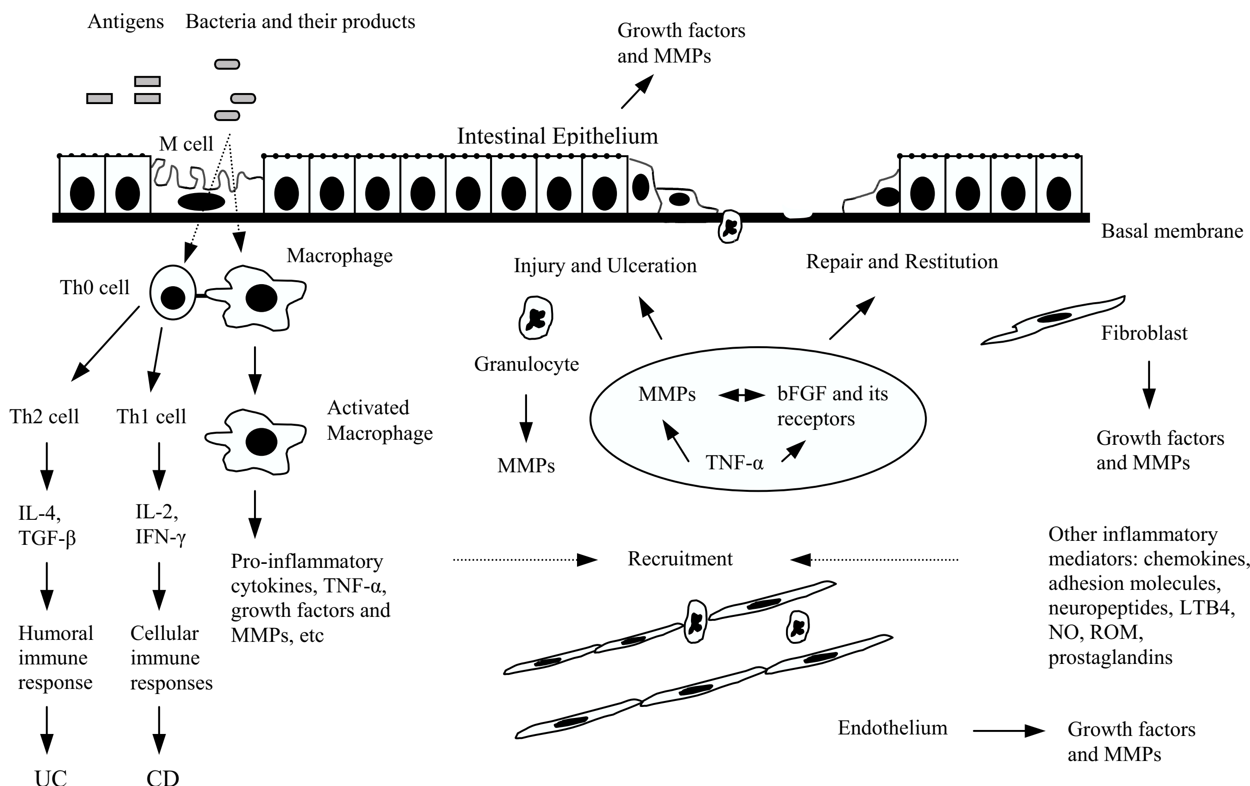


Figure 1. Activation pathways leading to cellular TNF-α, MMP and bFGF production. Each activation pathway may vary depending on the cell type and the type of extracellular stimulus. The relationship of these three factors (within circle) is the main focus of this thesis.

## bFGF, FGF receptor-1 and syndecan-1 and IBD

Fibroblast growth factors (FGFs) form a regulatory peptide family that is composed of 22 members with pleiotropic functions [19-21]. FGFs affect the embryonic development and fundamental cellular activities in the adult body, such as cell survival, apoptosis, cell-matrix and cell-cell interaction, cell motility, and differentiation, even carcinogenesis [22-25]. bFGF is one of the most well-characterized members in this family. bFGF has diverse effects, including proliferation and differentiation, on endothelial cells, smooth muscle cells, fibroblasts, epithelial cells and neural cells. bFGF is not only a potential stimulatory peptide, playing a role in angiogenesis, but also a mitogen affecting various (patho)physiological processes, e.g., tissue regeneration, would healing, tissue repair, hematopoiesis, tumor growth and metastasis [26;27].

The gene of bFGF is located on chromosome 4 and the activity of the gene is cell-type specific and conditionally regulated. bFGF is expressed in many cells and tissues, which include fibroblasts, endothelial cells, and macrophages. Different molecular forms of bFGF have been identified, e.g., the low molecular weight bFGF form (18 kDa), which is predominantly localized in the cytoplasm, and the high molecular weight form of bFGF (22, 22.5, 24 and 34 kDa), present in the nucleus [26;28]. Overexpression of the growth factor and its receptors has been implicated in transformation and malignant progression, and the nuclear localization of bFGF is thought to be essential for its mitogenic activity [27;29]. The precise mechanism of bFGF release from cells remains unclear because bFGF lacks a consensus signal sequence for the secretion. Released bFGF is usually found to be bound to heparan sulphate proteoglycans (HSPGs) of the ECM and basement membrane (BM), which act as storage depots for the regulation of bFGF binding to the receptors. Separation of bFGF from its receptors is considered to be an important regulatory mechanism for the bioactivity of

bFGF [27;30]. In the normal gastrointestinal tissue bFGF was immunohistochemically found in epithelial cells, endothelial cells, fibroblasts and smooth muscle cells, although diverse localization sites of bFGF have been described in several studies [31;32].

FGFs bind and activate their receptors to exert their biological functions. There are three kinds of receptors which are responsible for the signal transduction of FGFs: FGF receptors (FGFRs), HSPGs and a cysteine-rich FGFR (CFR) [20]. FGFR-1 is the main receptor for bFGF-initiated signal transduction, which can be facilitated by syndecan-1, a member of the HSPGs. FGFR-1 is member of the FGF receptor family which consists of four members with receptor tyrosine kinase functions [33]. FGFRs consist of an extracellular region, an intracellular region and a transmembrane domain. The extracellular region contains three consensus immunoglobulin-like loops, with between loop one and two a heparin-binding region, and a cell adhesion molecule (CAM) homology domain. The extracellular region can be cleaved by MMP-2, and the released part of the receptor may retain its FGF-binding capacity [34]. The juxtamembrane region, two tyrosine kinase domains, and the C terminal tail make up the intracellular region of the FGFR. The kinase domains and the phosphorylation sites in the intracellular region are required for FGF signal transduction [35]. A characteristic feature of the FGFR family is that numerous FGFR isoforms are generated from alternatively spliced mRNA transcripts, in both the extracellular and intracellular regions. The diversity of cell responses to FGFs partly results from the existence of multiple receptor isoforms. The function of the secreted and enzyme-truncated isoforms of FGFR-1 is that of antagonists to modulate the activities of bFGF [34;36].

Syndecan-1 is a member of the family of four transmembrane HSPGs. The structure of syndecans consists of a core protein and long unbranched carbohydrate polymers (glycosaminoglycans). At the extracellular region of the core protein the glycosaminoglycans are attached. Syndecan-1 is almost exclusively expressed by epithelial cells, in addition to pro-B lymphocytes in the bone marrow and plasma cells [37]. However, the distribution pattern of syndecan-1 in adult tissue differs from that in embryogenesis, wound healing, cell culture and carcinogenesis. It was reported that bFGF, TGF-β and PDGF induced the expression of syndecan-1 in certain of cell lines, like fibroblasts and vascular smooth muscle cells, and that TNF-α and IL-1 had a suppressive effect on the syndecan expression in intestinal epithelial cell lines and endothelial cells [37-40].

Heparan-sulphate chains of syndecans are essential for its binding of extracellular ligands. The cytoplasmic domain of syndecan is a short c-terminal tail. Recently it was demonstrated that syndecan-1 and -4 act as initial receptors to mediate intracellular signaling [41;42]. Syndecan-1, for instance, can bind a variety of extracellular molecules, including growth factors, collagen I, III, V, cell-cell adhesion receptor, blood coagulation factors, and enzymes. Therefore, syndecan-1 has a functional influence on the signaling of growth factors, on the adhesion and migration of cells, on morphology maintenance of epithelium and on the suppression of tumor growth [43;44]. As a co-receptor for bFGF syndecan-1 can bind bFGF and FGFR-1 to form a ternary complex facilitating bFGF signal transduction [45;46]. The binding of syndecan and heparin-binding growth factor family members, e.g. FGFs, VEGF and HGF, results in the increased binding affinity of growth factors with their receptor. Like other transmembrane proteins, syndecan-1 can also be proteolytically released from the plasma membrane by enzymes, including MMP-14. The released syndecan-1 fragments retain the ability to bind bFGF and other ligands, and like for secreted or truncated FGFR-1 the effect of this kind of binding on the bioactivity of bFGF is suppressive [47].

A few studies were done to investigate the role of bFGF, FGFR-1 and sydecan-1 in IBD. The contribution of the ternary complex activity in the aetiopathogenesis of IBD is still unclear. bFGF was found to be massively deposited in the ECM of inflammatory areas, closely related to increased vascular permeability and active angiogenesis [48]. In pediatric CD patients elevated bFGF levels in serum were reported to reflect the activity of the disease. The sources of this serum bFGF were proposed to be inflammatory cells and injured tissues

[49]. In adult IBD patients there were consistent results on the increased bFGF levels in serum but not so whether this was related to the activity of the disease [50;51]. In UC, the intestinal mucosal secretion of bFGF was found to be strongly correlated to the severity of inflammation [52]. No major changes were found regarding FGFR-1 in intestinal tissue of IBD patients [53].

Studies to the expression of syndecan-1 in intestinal tissue of IBD patients showed that this was decreased, particularly in reparative epithelium [54]. Therefore, heparin was proposed as a therapeutic option for IBD because, as a member of the HSPG family, it could substitute for the loss of syndecan-1 as a co-receptor for bFGF [55].

**MMPs and IBD**

MMPs, also known as "matrixins", are comprised of a subfamily of the metzincin superfamily [56]. Other metalloproteinase members in the metzincin superfamily are astacins, reprolysins (ADAMs) and serralysins [57]. The MMP family constitutes over 20 members which are $Zn^{2+}$-containing, $Ca^{2+}$-dependent neutral proteinases participating in ECM remodeling. According to their structure and substrate specificity MMPs are divided into several major subgroups: collagenases, stromelysins, gelatinases, and membrane type (MT) MMPs, the latter anchored to the cell's plasma membrane [58-60] (Table 1). Four tissue inhibitors of matrix metalloproteinases (TIMPs) are known to affect the activity of MMPs. In general, MMPs require the activation for their proteolytic activity as they are secreted in an inactive proenzyme form, with the exception of the MT-MMPs, which were demonstrated to be activated by intracellular cleavage. The zymogen activation and enzyme inhibition are two mechanisms by which the activity of MMPs is precisely controlled. Not all mechanisms of MMP activation have been clearly defined. Plasmin, urokinase plasminogen acticator (uPA), furin-like proteinase, TIMPs, and MMPs themselves have been shown to participate in the activation of MMPs. The activity of MMPs may be inhibited by endogenous and synthetic inhibitors, and by α-macroglobulins [56;61-63].

The constitutive expression of most MMPs is minimal, with the exception of a few. For example, Europhile-derived MMP-8 and MMP-9 are stored in secretory granules of neutrophils and eosinophils [64], and MMP-2, a fibroblast-derived gelatinase A, is the most commonly expressed MMP and can be isolated in large amounts from normal quiescent tissue [65;66]. In general, MMPs are synthesized on the demand by the tissues and most of the MMP genes are inducible. Growth factors, cytokines, hormones and oncogenes usually up-regulate the transcription of MMP genes. Growth factors, such as bFGF, PDGF and EGF can induce the proto-oncogene proteins c-Fos and c-Jun to form transcription factor activator protein-1 (AP-1), which can bind to the TRE (TPA-responsive element) or AP-1 binding-site, and are important factors in the message pathway leading to the activation of MMP gene transcription [62;66;67]. Pro-inflammatory cytokines like IL-1 and TNF-α are able to induce the gene transcription of MMP-1, -3 and -9 by the activation of the transcrptional factors NF-κB and AP-1 [68-72]. TGF-β, retinoic acid and glucocorticoids have been demonstrated to suppress the induction of MMP gene transcription [66;73-75]. Moreover, the stability of some MMP transcripts like MMP-1 mRNA is related to the AU-rich sequences in the 3' untranslated region, like that of cytokine genes and proto-oncogenes [76]. The gene regulation of MMPs is not uniform and there is cell-type-specific gene regulation for different MMPs. Unlike most MMPs, post-transcritional regulation of MMP-2 expression is more important than transcriptional regulation because the promotor region of MMP-2 lacks an AP-1 binding site, which exists in other MMP genes [77].

**Table 1. The main matrix metalloproteinases (MMPs) (Modified from references [14;56;62;78;94-96])**

| Sub-groups | MMP family member (Descriptive name) | Substrates | |
|---|---|---|---|
| | | Matrix proteins | Non-matrix proteins |
| Collagenases | MMP-1 (Collagenase-1, Interstitial collagenase)<br>MMP-8 (Collagenase-2, Neutrophil collagenase)<br>MMP-13 (Collagenase-3)<br>MMP-18 (Collagenase-4) | Fibrilar collagen I, II, III,VI, VII, X, gelatins, aggrecan, entactin | L-selectin, perlecan, IGFBP-2,3, α1-A, α1- PI, pro-MMP-1,-2,-8,-9, and -13 |
| Stromelysins | MMP-3 (Stromelysin-1)<br>MMP-10 (Stromelysin-2)<br>MMP-11(Stromelysin-3) | Aggrecan, gelatins, fibronectin, laminin, collagen III, IV, IX, X, VII, large tenascin C, vitronectin | Decorin, perlecan, pro-IL-1β, pro-HB-EGF, plasminogen, E-cadherin, IGFBP-1, and -3, α1-A, α1- PI, pro-MMP-1,-3,-7,-8,-9,-10, and –13 |
| Gelatinases | MMP-2 (Gelatinase A, 72 kDa Gelatinase)<br>MMP-9 (Gelatinase B,92 kDa Gelatinase) | Gelatins, collagen I, IV, V, VII, X, XI, and XIV, fibronectin, laminin, aggrecan, elastin, large tenascin C, β-amyloid protein precursor | Decorin, Pro-TGF-β2, pro-IL-1β, MCP-3, IGFBP-3, FGFR-1, pro-MMP-1,-2, and -13, cell surface bound IL-2Rα, plasminogen, α1- PI |
| MT-MMPs | MMP-14 (MT1-MMP)<br>MMP-15 (MT2-MMP)<br>MMP-16 (MT3-MMP)<br>MMP-17 (MT4-MMP)<br>MMP-25 (MT5-MMP)<br>MMP-26 (MT6-MMP) | Collagen I, II, III, gelatins, fibronectin, laminin-1, -5, nidogen, tenascin, chondroitin sulfate, vitronectin, proteoglycan, aggrecan, perlecan, dermatan sulfate fibrin/fibrinogen | Pro-MMP-2,-13, cell surface bound CD44, cell surface bound tTG, myelin-inhibitory protein, α1- PI, α2M |
| Others | MMP-7 (Matrilysin)<br>MMP-26 (Matrilysin-2) | Aggrecan, fibronectin, laminin, collagen IIV, elastin, small tenascin C, vitronectin | Decorin, E-cadherin, pro-α-defensin, cell surface bound Fas-L, β4-intergrin, plasminogen, pro-MMP-2, and -7 |
| | MMP-12 (Macrophage elastase, Metalloelastase) | Elastin, fibronectin, laminin, proteoglycan | Plasminogen |

MT-MMP: Membrane-type-MMP; tTG: tissue transglutaminase; α2M: α2-macroglobulin; α1- PI: α1-roteinase inhibitor, α1-A: α1-antichymotrypsin.

Each MMP is able to degrade one or more protein components of the ECM. Collectively, MMPs can degrade all structural proteins of the ECM. However, the view that the function of MMPs is limited to the degradation of components of the ECM is obviously being changed. The substrates of MMPs have expanded from the various structural ECM components to other extracellular proteins, i.e, other proteinases, proteinase inhibitors, coagulation factors, chemotactic molecules, growth factors, cell surface receptors, and adhesion molecules [78]. The consequence of degradation by MMPs is associated with an array of normal and pathological conditions. They have been shown, for instance, to be

involved in the processes of the migration of cells, cell-cell communication, apoptosis, angiogenesis, wound healing, embryonic development and organ morphogenesis, ovulation, cervical dilatation, postpartum uterine involution, endometrial cycling, nerve growth, hair follicle cycling, bone absorption, etc [56;58;60]. Furthermore, MMPs play a major role in tumor invasion and metastasis, cardiovascular disease, neurological disease, breakdown of blood brain barrier, liver fibrosis and infection or non-infection inflammatory processes, such as rheumatoid arthritis, nephritis, periodontal disease, skin, gastric and corneal ulceration, pulmonary emphysema and fibrotic lung disease [61;64;79;80].

Several studies in IBD provided strong evidence that MMPs are not only involved in the intestinal tissue destruction by the degradation of ECM components but that they also actively participate in the wound healing of that tissue. Particularly in the acute phase of tissue injury MMP-1, -3 and -9 are considered to be the proteolytic enzymes contributing to the IBD pathogenesis. At both the mRNA and protein level overexpression of MMP-1 and -3 were found in intestinal tissues of IBD patients [81-83]. The genes of MMP-1, -3 -9 -10 and -12 were found to be upregulated in T-cell mediated intestinal tissue injury as assessed by gene array and/or in situ hybridisation analysis [84]. Immunohistochemical evaluation showed that MMP-3 was mainly present in the matrix of regions with smooth muscle cell proliferation and mucosal damage. In addition, the distribution of MMP-3 was believed to be associated with the formation of strictures in CD [85;86]. The proposed mechanism related to the activity of MMPs in pathophysiological process is thought to be the imbalance of MMPs and TIMPs within the local tissue [81;82;85]. Stallmach et al. [87] reported increased MMP-1 and -2 mRNA and protein in the intestinal tissue of UC patients. In one study, MMP-9 was found to be increased in the neutrophils in inflamed regions, however, in the same study no MMP-2 expression was found in normal or CD affected intestinal tissue by immunofluorescence [86]. Elevated levels of MMP-9, in both quantity and activity were found in the inflamed intestinal IBD tissue [85;88]. In this context, MMP-9 is concluded to be a major factor in promoting neutrophil migration across the BM. MMP-9 also affects the production or activation of c-x-c and other neutrophil chemokines, which attract neutrophils migration across the BM of capillaries to inflammatory sites [89]. Recently, Kirkegaard et al. [90] suggested that the upregulated expression of MMP-3 and -9 may be relevant to the formation of fistulae in CD patients and that blocking the activity of MMPs could be a therapeutic option in the treatment of IBD [88;91]. In addition, MMP-2 and -14 mRNAs were shown to be notably increased in ulcerated colonic IBD mucosa, rather than in mild inflammatory regions [83]. Furthermore, MMP-14 is known to be an important factor promoting neoangiogenesis in wound healing [92]. Also in animal models of IBD, MMP-2 and MMP-8 (neutrophil collagenase) in large intestinal epithelial cells were found to participate actively in the immunopathogenic process by cleaving type I and IV collagen and the laminin-5 γ2- chain of the ECM and MB [93].

## TNF-α and IBD

TNF-α (cachectin) is a member of TNF/TNF receptor(R)-related superfamily proteins, which consist of more than twenty members [97]. The activity of these proteins affects the host defense, inflammation, apoptosis, organogenesis and autoimmunity. Monocytes/macrophages are believed to the principal source of TNF-α production, although many other cells are capable of releasing TNF-α. These cells include T- and B-lymphocytes, mast cells, neutrophils, NK cells, astrocytes, Paneth cells, intestinal mesenchymal cells, and tumor cells [98;99]. In response to bacterial products, the intestinal epithelial cells are also able to produce TNF-α [100;101].

The gene for human TNF-α is located on chromosome 6, between the HLA class I and II loci [102]. Like for other proinflammatory cytokines, within the untranslated region of the TNF-α mRNA a conserved sequence UUAUUUAU exists which confers an RNAse target

and reduces the rate of TNF-α translation. As a result, the TNF-α mRNA is highly unstable: in monocytes/macrophages the half life of TNF-α mRNA is about 15 minutes and in T-lymphocytes it is approximately 40 minutes [103-105]. The stimuli for synthesis of TNF-α include bacterial products, superantigens, x-ray irradiation, inflammatory mediators, and TNF-α itself. With a feedback inhibition mechanism, TNF-α simultaneously induces negative regulatory elements to strictly control its own production. The other suppressive factors for TNF-α production include IL-10, $PGE_2$, cAMP, activator of protein kinase C, glucocorticoids and cyclosporin A [106;107].

After translation the precursor TNF-α, a 26 kDa protein containing an unusual long signal peptide, anchors at the plasma membrane, also called membrane TNF-α (mTNF-α). The intracellular portion of mTNF-α consists of a N-terminal 30 aminoacids long residue, followed by a 26 aminoacids long transmembrane domain residue. The extracellualr portion of mTNF-α contains a 177 aminoacids residue, which is cleaved by an enzyme from the metalloproteinase-disintegrin family, i.e., TNF-α converting enzyme (TACE, ADAM-17), to release a 157 aminoacids residue as a soluble TNF-α 17 kDa protein, which aggregates into a 51 kDa soluble homotrimeric active form of TNF-α. mTNF-α is also able to directly exert its biological function via engaging with the TNFR [108-110].

The signal transduction of TNF-α is generated by two TNF-α receptors, TNFR1 (55 kDa, p55, CD120a) and TNFR2 (75 kDa, p75, CD120b). TNFR1 is located on many cell types, whereas TNFR2 is predominantly expressed on leucocytes and endothelial cells. TNFR2 is also a receptor for lymphotoxin (TNF-β). The binding of TNF-α to both TNFRs leads to activation of the apoptosis pathway or to the induction of NF-κB activity, protecting from TNF-α inducing apoptosis, activating transcription of genes associated with inflammation [111]. TNFR1 can either directly transduce the signal of TNF-α to activate NF-κB in lymphocytes or receive TNF-α delivered by TNFR2. The mechanism that decides whether the signal of TNF-α results in either apoptosis or activation of inflammatory genes depends on the distribution of the TNFRs in a specific cell type and various recruitments of signaling proteins in the specific cell to the TNFR which binds TNF-α [112-114]. Besides the two receptors mTNF-α can also be engaged with ligands to reversely tranduce the signals into cells it anchors in [115-117].

Many acute pathophysiological changes are the result of the body's systemic exposure to high levels of TNF-α. They include the release of other proinflammatory cytokines, the activation of neutrophils and macrophages, the release of catabolic hormones, acute respiratory distress syndrome, gastrointestinal necrosis, and cardiovascular collapse. Chronic low levels of TNF-α leads to fever, insulin resistance, lipid depletion, protein catabolism, and wasting. TNF-α is a significant contributor to septic shock, cachexia of cancer, and some chronic inflammatory diseases, such as CD and rheumatoid arthritis [99;118;119].

There is increasing evidence suggesting that TNF-α plays a pivotal role in CD pathogenesis. Increased TNF-α expression in the intestinal mucosa rather than in serum of CD patients is thought to be of importance to the process of inflammation in CD [6;120-123]. TNF-α augments the Th1 cell function in the intestinal mucosal immunoreactivity against intestinal bacterial or cross-reactive mucosal antigens [3]. The effects of TNF-α include the activation of neutrophils and macrophages, the induction of proinflammatory cytokines (e.g. IL-1, -6,-8), chemokines and nitric oxide synthesis in inflammatory cells, up-regulation of a variety of adhesion molecules, e.g, E-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecules (VCAM)-1 and mucosal addressin cell adhesion molecule (MAdCAM)-1. These factors promote the recruitment of lymphocytes, neutrophils and monocytes to the inflammatory site, and the up-regulation of the production of chemokines, such as monocyte chemoattractant protein (MCP)-1 [124]. TNF-α also augments the IFN-γ production by mucosal T cell, activates CD44 in T cells and stimulates lymphocyte proliferation and migration. Furthermore, TNF-α is relevant to the secretion of chemokines and chloride from intestinal epithelial cells and the disruption of the epithelial barrier because

of its affects on the epithelial permeability [125-128]. TNF-α also participates in the granuloma formation, which is a histopathological hallmark of CD [129;130]. The influence of TNF-α on angiogenesis is believed to be exerted through the promotion of angiogenic factors, such as bFGF, VEGF and IL-8 [131]. Finally it has been proposed that the tissue damage caused by MMPs is related to TNF-α, particularly since TNF-α is able to activate MMPs [127;132]. In both animal models and clinical trials, amelioration of intestinal inflammation occurs following the neutralization of TNF-α with specific antibodies [133;134].

**Bacterial components/Lipopolysaccharide (LPS)**

LPS (endotoxin) is an outer membrane component of gram-negative bacteria. The LPS molecule consists of a hydrophilic polysaccharide part and lipid A. The polysaccharide part is composed of an oligosaccharide-core region and repeat units of identical polysaccharides (O-specific chain). The oligosaccharide-core region is a conserved part of LPS, which is the epitope to be recognized by monoclonal antibodies. The O-specific chain varies in length, and a number of bacteria produce LPS even without this part. The O-specific chain can be used to identify the bacterial origin of LPS. Lipid A, a covalently bound hydrophobic lipid component, is the essential portion for the bioactivity of LPS [135].

The biological effects of LPS on the host are the result of the activity of LPS-responsive cells which include monocytes/macrophages, neutrophils, lymphocytes, and vascular cells (endothelial and smooth muscle cells). In general, the response to low amounts of LPS is beneficial for the host via the enhancement of the resistance to infection and malignancy [136]. Large amounts of LPS, however, cause severe responses of the host, including fever, sepsis, disseminated intravascular coagulation, and multiorgan failure. These inflammatory responses are mediated by immune modulation molecules, e.g., TNF-α, IL-1, -6, -8, -12, nitric oxide, thromboxanes and leukotrienes, produced by LPS responsive cells. Each responsive cell type reacts to LPS in an individual way. For instance, the main reaction of monocytes/macrophages is to produce a battery of inflammatory mediators, in particular TNF-α. Monocytes/macrophages are the most sensitive cell type to the stimulation of LPS. At about 1 ng/ml LPS is already able to activate a strong response in monocytes/macrophages [137]. The phagocytosis by neutrophils is enhanced by LPS. Furthermore, LPS is one of main priming agents for neutrophils and monocytes, which in response promote the secretion of inflammatory lipid mediators and oxygen radicals and the synthesis of proteins [138]. However, the bioactivity of LPS is also neutralized by PMNL through degradation by enzymes [139].

The recognition of the LPS signal is carried out by an array of macromolecules, such as LPS-binding protein (LBP), soluble and membrane CD14, TLRs, and members of the β-integrin family [140]. The number of molecules responsible for the recognition and transduction of the LPS signal is still increasing. $CD14^+$ cells, monocytic cells and neutrophils, are very sensitive to stimulation with LPS and anti-CD14 antibodies block the release of cytokines from LPS-stimulated myeloid cells. $CD14^-$ cells, e.g., endothelial cells, fibroblasts and smooth muscle cells can be activated by the LPS-soluble CD14 complex [141-143]. CD14, as LPS receptor, plays an important but not essential role in the signal transduction because CD14 lacks a transmembrane domain. In addition, TLR-4 is recently reported to be a LPS receptor as well [144], and various other membrane molecules form the LPS-action cluster are also involved [145]. The intracellular transduction of the LPS signal results in the activation of transcription factors, like NF-κB, AP-1, and Ets which subsequently give rise to inflammatory cytokine gene transcription [140;146].

There are several lines of evidence suggesting that the bacterial flora also plays a role in the pathogenesis of IBD. Products of bacteria, such as LPS, *N*-formyl-methionyl-

leucyl-phenylalanine (fMLP) and peptidoglycan-polysaccharide (PG-PS), not only interact with immune and non-immune cells in the intestinal tissue, triggering a mucosal immunoreaction and inflammation, but also affect the activities and functions of the immune cells in the circulation [5;147;148]. Lamina propria mononuclear cells (LPMCs), activated by LPS and other bacterial products, can diminish the transport and barrier functions of epithelial cells [149]. It has been reported that systemic endotoxaemia was correlated with the activity and extent of inflammatory bowel disease [150]. Recent reports identified more bacterial components, such as staphylococcal enterotoxin A (SEA) and muramyl dipeptide, a peptidoglycan which frequently contaminates preparations of LPS, to be associated with IBD [151;152].

**The immunobiological treatment of IBD**

Anti-inflammatory and antimicrobial medication and immuno-modulation are the most relevant approaches in the treatment of IBD. Due to a better the understanding of the pathogenesis of IBD, biological treatment strategies become increasingly promising. Biological therapy is defined as the administration of various native or recombinant molecules which targets at specific sites of the pathophysiological complex. Several biological therapies emerged against different immunological stages with potential clinical application for IBD patients. The lymphocyte is one of the major targets for biological therapy because the presentation of antigens and bacterial products by drendritic cells to $CD4^+$ T lymphocyte is believed to be the initial step of the immune reaction in IBD. Anti-$CD4^+$ antibodies were developed to block this activation of the $CD4^+$ T lymphocytes [153;154]. Anti-IL-2 and anti-IL-18 receptor antibodies were designed to interfere with the effect of these cytokines on the function of Th1 cells [155;156]. Recombinant IL-10 (rHuIL-10) has been used, also in trials, to suppress the production of IL-2 and IFN-γ by Th1 cells [157;158]. Primary clinical trials with ISIS 2302, an antisense molecule to ICAM-1, failed to demonstrate that this component could increase the remission rate of CD in a low dose, two phase 3 trials at a high dose in patients with active CD are presently underway [159]. Growth factors play also an important role in mucosal immuno-modulation, and in proliferation and differentiation of epithelium, with epidermal growth factor already applied in clinical trials [160]. Inhibitors for TNF-α are at the stage of routine clinical application or efficacy assessment in clinical trials. These agents include anti-TNF-α antibodies (infliximab and CDP571), recombinant TNFR (Etanercept and Onercept), and agents affecting TNF-α gene expression (CNI-1493) and synthesis (Thalidomide) [119;161]. TACE inhibitors were proposed to be useful to block the production of soluble bio-active TNF-α, however, it is still at an early stage. In addition, a series of benzodiazepine MMP/TACE inhibitors has been synthesized to impair LPS-stimulated TNF production by human monocytes [162;163]. NF-κB, an important transcription factor, is responsible for the transcription of a variety of inflammatory mediators, and has been speculated to be a target for IBD therapy [164;165].

Up to date, clinical trials have demonstrated that inhibition of the selective adhesion molecules α4-integrin and α4β7-integrin and inhibition of TNF-α are effective in the treatment of various forms of UC and CD [159]. Infliximab (Remicade®, formerly named cA2) is the most successful biological agent in the treatment of CD patients and is approved for clinical application of patients with CD and rheumatoid arthritis (RA) [166-169]. Infliximab is a genetically constructed chimeric anti-TNF-α antibody with a 75% portion of human protein, the human Ig G1 κ light chain, linked with a 25% murine protein portion, the variable or recognition region of the mouse antihuman TNF-α monoclonal antibody (A2) [170]. The clinical trials of infliximab in CD showed that 50 to 80% of patients responded very well to a single infusion of 5-20mg/kg infliximab with a 4 week follow up [171;172]. Nowadays, infliximab is an important treatment option for patients with moderate to severely active

and/or fistulizing CD [173]. Infliximab is also one of the most intensively studied biological agents. However, mechanisms underlying the effects of infliximab are not fully understood. It is suggested that infliximab not only neutralizes the bioactivity of TNF-α, by binding soluble and membrane TNF-α, but is also a immune modulator which directly affects immune cells, e.g. by inducing apoptosis of TNF-α-producing cells [174;175]. There are controversies as to whether infliximab lyses the TNF-α-producing cells via complement-and antibody-dependent path ways [176;177]. After treatment with infliximab a reduction of activated macrophages and T lymphocytes in the intestinal mucosa of CD patients was found [175;178]. In peripheral blood, the percentage of neutrophils was decreased and the relative numbers of lymphocytes and monocytes was increased after infusion with infliximab. Although it is still not completely clear whether infliximab affects the capability of peripheral blood mononuclear cells to produce TNF-α [179;180], downregulation of epithelial cell apoptosis and barrier repair in active Crohn's disease by infliximab has been observed [181].

## Studies described in this thesis

The expression of the bFGF, FGFR-1 and syndecan-1 ternary molecular complex, at the mRNA and protein level, in the intestinal tissues from IBD patients was evaluated in relation to the presence of inflammation. The role of this complex in the inflammatory and healing processes in IBD is discussed in **Chapter 2**. Changes of the bFGF levels in serum and intestinal tissue of fistulizing and active CD patients during treatment with infliximab, from 2 clinical trials, were assessed to further evaluate the role of bFGF in intestinal healing (**Chapters 3** and **4**). In additional experiments, the regulation of bFGF expression at the protein and mRNA level in LPS-stimulated leucocytes from CD patients, with and without the presence of infliximab, was explored (**Chapter 4**). These experiments would provide insight into bFGF function and efficacy mechanisms of infliximab therapy in CD.

In **Chapter 5**, investigations of the regulation of TNF-α protein and mRNA expression in leucocytes from CD patients are described to explore the immunological impact of infliximab therapy for these patients. Furthermore, the value of the determination of TNF-α by immunosorbent assays under infliximab treatment was assessed.

The expression and activity of the gelatinase-type of matrix metalloproteinases MMP-2 and MMP-9 in intestinal tissues of IBD patients, to explore the role of these MMPs in the pathophysiological processes of mucosal inflammation, was the focus of the studies described in **Chapter 6**. The effect of immunoneutralization of TNF-α by infliximab, either *in vivo* or *in vitro*, on the expression and production of MMP-2 and MMP-9 by leucocytes, in serum and in tissues of patients with CD is described in **Chapter 7**. The functional interactions and pathophysiological consequences for CD are discussed.

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