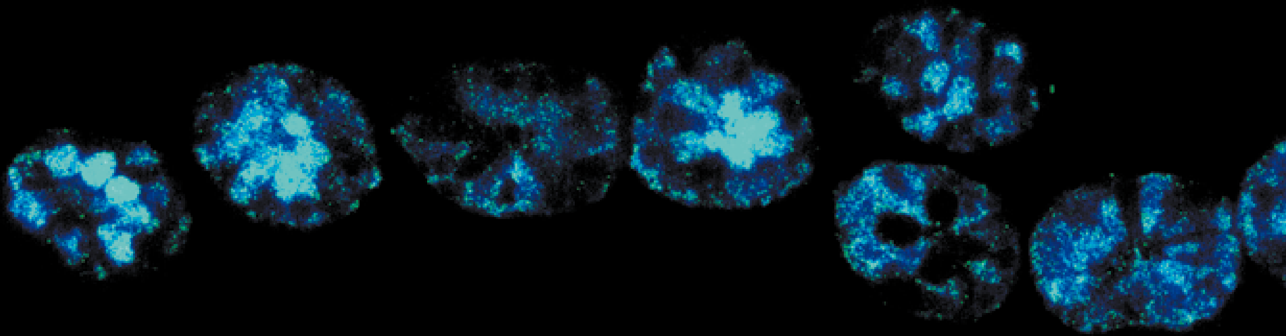
the damaged mucosal barrier in intestinal inflammation:

interaction with therapeutic bacteria and roles for metallothioneins as immunomodulators



Anouk Waeytens

FACULTY OF MEDICINE AND HEALTH SCIENCES

UNIVERSITEIT
GENT

FACULTY OF MEDICINE
AND HEALTH SCIENCES

The damaged mucosal barrier in intestinal inflammation:

interaction with therapeutic bacteria and roles for metallothioneins as immunomodulators

Anouk Waeytens

Thesis submitted in fulfilment of the requirements for the degree of
"Doctor in Medical Sciences"

February 2009

Cover picture:
Polarization light micrograph of immunogold-labelled metallothionein in intestinal crypts

© Copyright, Anouk Waeytens, 2009

"even the water dripping can wear away the hardest stone"

Chinese proverb

aan mijn ouders

mijn eindeloze steunpilaren

aan Bert

mijn grote liefde

en aan Jill en Vic

mijn hartendiefjes

Promotors

Prof. Dr. Claude Cuvelier

vakgroep Pathologische anatomie, Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent

Prof. Dr. Martine De Vos

vakgroep Inwendige ziekten, Gastroenterologie, Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent

Members of the reading and examination committee

Prof. Dr. Guy Joos (chairman)

vakgroep Inwendige ziekten, Pneumologie, Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent

Prof. Dr. Ria Cornelissen

vakgroep Anatomie, embryologie, histologie en medische fysica, Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent

Prof. Dr. Erik Cox

vakgroep Virologie, parasitologie en immunologie, Faculteit Diergeneeskunde, Universiteit Gent

Prof. Dr. Pieter Demetter

department of Pathology, Erasme University Hospital, Université Libre de Bruxelles

Prof. Dr. Dirk Elewaut

vakgroep Inwendige ziekten, Reumatologie, Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent

Prof. Dr. Michael Lynes

department of Molecular and Cell Biology, University of Connecticut, United States of America

Table of contents

table of contents		7
list of abbreviations		9
summary		11
samenvatting		13
Chapter 1	**general introduction**	**15**
	1.1 inflammatory bowel diseases	**17**
	risk factors for IBD	19
	genetic factors	19
	environmental triggers	21
	epigenetic effects in IBD	22
	pathogenesis of IBD	23
	involvement of bacteria	23
	evidence for the role of bacteria in experimental colitis	23
	evidence for the role of bacteria in human studies	24
	the damaged mucosal barrier in genetically susceptible individuals	26
	disturbances at the level of the epithelial barrier	26
	disturbances at the level of the innate immune response	33
	disturbances at the level of the adaptive immune response	40
	disturbances at the level of immune regulation	43
	disturbances at other levels	46
	conclusion	48
	1.2 roles for metallothioneins in intestinal inflammation	**67**
	metallothioneins	67
	MT regulation in inflammation	67
	MT functions relevant in inflammation and possibly in inflammatory bowel diseases	68
	protective functions of MTs in animal models of inflammation	71
	MT expression in IBD	71
	conclusion	73
Chapter 2	**research objectives**	**79**
	interaction of the mucosal barrier with therapeutic bacteria	81
	roles for metallothioneins as immunomodulators in intestinal inflammation	82

Chapter 3 **interaction of the mucosal barrier with therapeutic bacteria** **83**

Chapter 4 **roles for metallothioneins as immunomodulators in intestinal inflammation** **95**

4.1 impaired epithelial metallothionein expression as a possible aetiopathogenic mechanism in Crohn's disease 97

4.2 metallothionein expression in ulcerative colitis: inflammation-dependent regulation differs in epithelium and granulation tissue 127

4.3 human metallothionein expression under normal and pathological conditions: mechanisms of gene regulation 147

Chapter 5 **general discussion and future perspectives** **177**

interaction with therapeutic bacteria 180

roles for metallothioneins as immunomodulators 182

curriculum vitae 193

dankwoord - acknowledgements 199

List of abbreviations

APC	antigen presenting cell
DC	dendritic cell
DSS	dextran sodium sulphate
IBD	inflammatory bowel diseases
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
M cell	membranous or microfold cell
MDP	muramyl dipeptide
MLC	myosin light chain
MLCK	myosin light chain kinase
MT	metallothionein
NADPH	nicotinamide adenine dinucleotide phosphatase
NF-κB	nuclear factor-κB
NKT cell	natural killer T cell
NLR	Nod-like receptor
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NSAIDS	non-steroidal anti-inflammatory drugs
PAMP	pathogen-associated molecular pattern
PG	peptidoglycan
PRR	pattern recognition receptor
SOD	super oxide dismutase
STAT	signal transducer and activator of transcription
ROS	reactive oxygen species
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TLR	Toll-like receptor
TGF	transforming growth factor
TNBS	trinitrobenzene sulphonic acid
TNF-α	tumour necrosis factor-α
Treg	regulatory T cell
Tr1	T regulatory cell 1
VEGF	vascular endothelial growth factor

Summary

Inflammatory bowel diseases (IBD) are a group of chronic, relapsing, immunologically-mediated disorders of the intestine, the main forms being Crohn's disease and ulcerative colitis. Recent studies underscore the importance of the damaged epithelial barrier and the dysregulated innate immune system in the pathogenesis of IBD. These two components cause defects in the interaction of the host with the bacterial flora. The epithelium must maintain a physical barrier between the lumen and the underlying mucosa. Defects in the mucus layer, integrity, and permeability are found in IBD and are thought to have direct implications on disease initiation and/or progress. Furthermore, it has become clear that the intestinal epithelial cell lining exerts an important role in the identification of bacteria and the appropriate counteraction. The involvement of the innate immune system can be explained by two theories. The first one states that an exaggerated innate response and a loss of tolerance for the commensal intestinal microflora are caused by defective down-regulation of pattern recognition receptor signalling. The second theory encompasses a defective innate immunity originating from failure of recognition of bacterial threats or an ineffective response, which results in persistence of the exogenous stimulus and an exaggerated secondary immune response.

The first part of this thesis focuses on the interaction of therapeutic bacteria with the damaged epithelial barrier. Genetically modified *Lactococcus lactis* bacteria were developed in order to provide a local mucosal delivery of the anti-inflammatory cytokine interleukin (IL)-10. Since local delivery of the cytokine in the tissue seems primordial, we focused on the *in vivo* interaction of the bacteria with intestinal murine mucosa and observed differences between an intact and an inflamed (damaged) epithelial barrier. In healthy intestinal tissue, we found an occasional transcellular uptake in follicle-associated epithelium. Our data were suggestive of dendritic cell sampling, but not of sampling by M cells. In inflamed mucosal tissue, both confocal and electron microscopic analysis suggested an enhanced uptake of lactococci through the paracellular route, which is likely to be a result of the damaged mucosal barrier. We were able to show the presence of viable and IL-10-producing lactococci in ileal and colonic lamina propria. The absence of lactococci in mesenteric lymph nodes or in the spleen excluded a systemic circulation of the bacteria, being safe for administration in patients.

In the second part, the role of metallothioneins (MTs) as immunomodulators in intestinal inflammation is investigated. We found a significantly lower mRNA and protein expression in the epithelium of unaffected tissue of Crohn's disease patients. *In vitro* studies with an MT-knockdown intestinal epithelial cell line showed a defective secretion of the neutrophil chemokine IL-8 upon bacterial challenge. A time course study of dextran sodium sulphate-induced colitis revealed that MTnull mice exhibited a significantly more severe histological inflammation in the early phase of colitis compared with wild type mice, a difference which was due to epithelial MTs. Both these *in vitro* and *in vivo* studies point to an immunomodulatory role for epithelial MTs in the acute intestinal immune response.

In ulcerative colitis, we did not find this impaired basal epithelial MT expression. Here, active inflammation caused a down-regulation of epithelial MT and an up-regulation of non-epithelial MT which was also observed in other forms of intestinal inflammation. The inflammation-dependent down-regulation in the epithelium is in contrast with the known induction of MTs by proinflammatory cytokines and reactive oxygen intermediates, which are produced during inflammation. We found that transforming growth factor (TGF)-β, one of the few inhibitors identified for MT mRNA production, could decrease epithelial MT expression in *ex vivo* experiments. Contrary to the decrease in the epithelium, an increase in MT-expressing cells occurs in the lamina propria during active inflammation, predominantly caused by strongly MT-expressing fibroblasts of the granulation tissue. Since we did not find a correlation between MT expression and proliferation (a correlation which has been reported *in vitro* in the literature), we suggest that the role of MTs in these cells is a cytoprotective one, which would be beneficial for host cells in the inflammatory environment. Finally, we reviewed the MT regulatory mechanisms in health and in disease.

Samenvatting

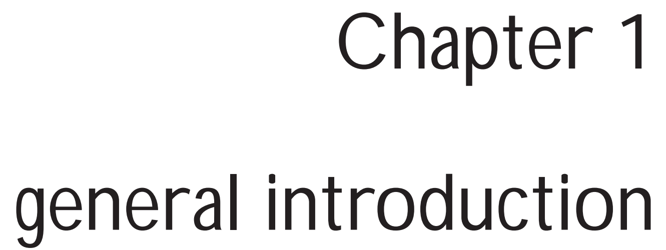
De ziekte van Crohn en colitis ulcerosa zijn de belangrijkste chronische ontstekingsziekten van de darm. Recente studies benadrukken het belang van schade aan de epitheelbarrière en defecten in het aangeboren immuunsysteem in het ontstaan van beide ziekten. Deze schade zorgt ervoor dat de interactie tussen de microbiële darmflora en de gastheer niet meer optimaal verloopt. Het darmepitheel dient een fysische barrière te vormen tussen het lumen en de onderliggende mucosa. Bij de ziekte van Crohn en colitis ulcerosa worden afwijkingen in de mucuslaag, de integriteit en de permeabiliteit van het epitheel gevonden, waarvan men denkt dat ze een directe invloed hebben op de initiatie en de progressie van de ziekte. Naast zijn rol als fysische barrière heeft het epitheel ook een belangrijke taak in de identificatie van bacteriën en de gepaste reactie hierop. Het aangeboren immuunsysteem kan op twee manieren een rol spelen in the pathogenese. Een eerste theorie stelt dat defecten in het uitschakelen van proinflammatoire signalen een overdreven aangeboren immuunantwoord veroorzaken en een verlies van tolerantie tegenover de commensale microbiota. Volgens de tweede theorie worden pathogenen niet goed herkend, waardoor er een inefficiënte opruiming volgt die ervoor zorgt dat de exogene stimulus blijft bestaan en er een overdreven secundair immuunantwoord op gang komt.

Het eerste deel van deze thesis handelt over de interactie van therapeutische bacteriën met de beschadigde epitheelbarrière. Genetisch gemodificeerde *Lactococcus lactis* bacteriën werden ontwikkeld om te zorgen voor een lokale toelevering van het anti-inflammatoir cytokine interleukine (IL)-10 in de mucosa. Aangezien deze lokale toelevering uitermate belangrijk is voor de therapeutische werking van de bacteriën, hebben we de interactie onderzocht tussen deze bacteriën en de darmmucosa. We vonden dat de bacteriën op transcellulaire wijze kunnen opgenomen worden in gezond follikelgeassocieerd epitheel. Onze data suggereerden eveneens opname door dendritische cellen, maar niet door M cellen. In ontstoken mucosa bemerkten we een verhoogde opname door paracellulair transport. Dit was waarschijnlijk veroorzaakt door de beschadigingen aanwezig in de mucosale barrière. We konden aantonen dat er levende en IL-10-producerende lactococcen in de lamina propria aanwezig waren. Uiteindelijk kon de afwezigheid van lactococcen in de mesenterische lymfeknopen en de milt uitsluiten dat deze bacteriën in de systemische circulatie terechtkomen.

In het tweede deel onderzochten we de rol van metallothioneïnes (MTs) in darmontsteking. We vonden een significante daling in de mRNA en eiwitexpressie van MTs in het epitheel van gezond darmweefsel van patiënten met de ziekte van Crohn. Wanneer we bacteriële stimulaties uitvoerden op een darmepitheel cellijn met deficiënte MT expressie zagen we dat deze deficiëntie zorgde voor een daling in de secretie van het chemokine IL-8. Een gedetailleerde studie van dextraan sodiumsulfaat-colitis gaf aan dat MTnull muizen een zwaardere histologische inflammatie vertoonden dan wild type muizen in de vroege fase van colitis, een verschil dat te wijten was aan epitheliaal MT. Beide studies wijzen in de richting van een immunomodulatorische rol voor epitheliaal MT in het acute immuunantwoord van de darm.

Deze verminderde basale epitheelexpressie vonden we niet terug bij colitis ulcerosa. In deze en andere ontstekingsziekten van de darm vonden we dat actieve inflammatie zorgde voor een daling in de epitheelexpressie van MT tegenover een stijging in de expressie van MT in de lamina propria. De ontstekingsafhankelijke daling in het epitheel komt niet overeen met de gekende inductie van MTs door proinflammatoire cytokines en reactieve zuurstofradicalen die geproduceerd worden tijdens ontsteking. Wij vonden dat transforming growth factor (TGF)-β de expressie van MT in epitheelcellen kon doen afnemen. In tegenstelling tot deze afname in het epitheel werd tijdens actieve inflammatie een toename in de expressie waargenomen in cellen van de lamina propria, voornamelijk veroorzaakt door fibroblasten van het granulatieweefsel die sterk positief waren. We suggereren dat MTs een cytoprotectieve rol hebben in deze cellen, wat een voordeel betekent in een inflammatoir milieu.

Uiteindelijk hebben we een overzicht gemaakt van de mechanismen die de expressie van MTs regelen in ziekte en gezondheid.



Chapter 1

general introduction

inflammatory bowel diseases

Crohn’s disease (1) and ulcerative colitis are chronic, relapsing, immunologically mediated disorders of the intestine that are collectively referred to as inflammatory bowel diseases (IBD) (2). What distinguishes IBD from inflammatory responses seen in the normal gut is an inability to down-regulate those responses. In healthy persons, the intestine becomes inflamed in response to a potential pathogen, then returns to a state of tolerance once the pathogen is eradicated from the gut. In individuals with IBD, however, inflammation is not down-regulated, the mucosal immune system remains chronically activated, and the intestine remains chronically inflamed (3). The main difference between Crohn's disease and ulcerative colitis is the location and nature of the inflammatory changes. Crohn's disease can affect any part of the gastrointestinal tract, from mouth to anus, although a majority of the cases starts in the terminal ileum. Regarding to disease location, Crohn’s disease is categorized into ileal, colonic, or ileocolonic disease (4). Ulcerative colitis, in contrast, is restricted to the rectum and the colon, and can be categorized based on the maximal extent of involvement into ulcerative proctitis, left sided (distal) ulcerative colitis, or extensive ulcerative colitis (pancolitis) (4).

Microscopically, chronic intestinal inflammation displays mostly epithelial alterations. These can include an increased number of goblet cells, impaired mucus secretion, an increase in Paneth cell number, displacement of Paneth cells higher up in the crypts in the ileum and even appearance in the left colon, and the presence of the ulcer-associated cell lineage (UACL). Architectural alterations regarding villous shape and size include enlargement, blunting, broadening at the top (drumstick appearance), atrophy, and irregular or diffuse shortening, while crypts might be branching, shortened, atrophic, lost, or arranged in groups. The composition of the inflammatory cell infiltrate, which is less important for diagnosis, is patchy and consists of lymphocytes, plasma cells, and polymorphonuclear cells. In active inflammation, the inflammatory cell infiltrate is more striking and goes along with dilatation and congestion of capillary blood vessels. The severity of active inflammation rises with the increasing appearance of cryptitis, crypt abscesses, and ulcers.

Crohn’s disease exhibits some characteristic features, but these are rarely all present in a single specimen and may even be entirely absent in some cases. The most characteristic microscopic features of the disease are its multifocal involvement, focal ulceration (with small

aphthoid ulcers in early disease and fissuring, serpiginous ulcers in more extensive disease), transmural inflammation in the form of lymphoid aggregates, and sarcoid granulomas (figure 1A). In ulcerative colitis inflammation is restricted to the mucosal and submucosal layer, the latter often showing intense congestion and dilatation of capillaries and veins (figure 1B). Crypt abscesses are a common feature but not specific, whereas the consequences of cycles of epithelial destruction followed by epithelial repair can be useful histological signs. These include impaired mucus secretion, Paneth cell metaplasia, and dysplasia (5).

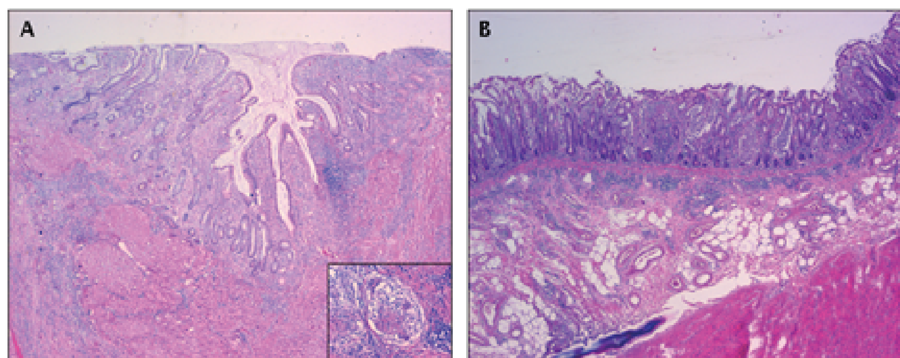


Figure 1. Histological characteristics of active Crohn's disease (A) and ulcerative colitis (B).

(A) Chronic ileitis featuring irregular, distorted villi and crypts, some of which are grouped. Chronically inflamed lamina propria with lymphoid follicles (magnification 20x). Insert: typical sarcoid granuloma (magnification 200x).

(B) Ulcerative colitis, same magnification as (A), showing an irregular atrophic mucosa with distorted and branched crypts, diffuse mucin depletion and numerous crypt abscesses. Diffuse inflammatory infiltration of the lamina propria. The inflammatory infiltrate is limited to the mucosa and superficial submucosa.

risk factors for IBD

The prevalence rate of IBD reaches up to 387 cases per 100,000 persons, the highest rates being reported in northern and western Europe as well as North America, whereas lower rates are recorded in Africa, South America and Asia (6). Although the incidence and prevalence are beginning to stabilize in high-incidence areas, they continue to rise in low-incidence areas (6;7). The peak age of onset for IBD is 15 to 30 years old, although it may occur at any age. Ulcerative colitis is slightly more common in males, whereas Crohn's disease is marginally more frequent in women (3). Epidemiologic observations indicate that both genetic and environmental factors are involved in the pathogenesis of IBD.

genetic factors

A genetic component seems to be stronger in Crohn's disease than in ulcerative colitis, since the concordance rate in identical twins is higher for Crohn's disease (~50% compared to ~16% for ulcerative colitis) (8). The increased incidence of IBD in first-degree relatives of probands with either disease, as well as associations of Crohn's disease with other genetic disorders (ankylosing spondylitis, psoriasis, and eczema) all add up to the proof that genetic factors are involved. These findings also indicate that IBD is not inherited as a Mendelian trait but rather has a complex genetic basis with many contributing genes (3;9). Racial and ethnic differences were at first also thought to arise from genetic susceptibility. However, these differences seem to be narrowing, and studies of migrant populations suggest that they may be more related to lifestyle and environmental influences than true genetic differences (10-12).

Advances in the genetics of IBD have occurred from genome wide scans and candidate gene approaches, delineating IBD susceptibility loci (*IBD1-IBD9*). A recent study combined the data of three genome wide association studies in Crohn's disease, thus creating a very large data set and improving power to discover alleles with small effect sizes. This genome-wide meta-analysis strongly confirmed 11 previously reported loci and provided significant evidence for 21 new loci (table 1) (13). Besides these confirmed loci/associations, other genes have been associated with IBD which have functions that can be related to the pathogenesis (table 2). Studies in mouse experimental colitis that used transgenic and knockout techniques have added functional data on susceptibility genes (14;15). Work in these two independent systems has yielded partially overlapping results, with genes involved in both IBD and experimental colitis. The association of polymorphisms in several of these

Table 1. Convincingly replicated CD risk loci by genome-wide meta-analysis (13)

SNP	chromo-some	no. of genes	gene of interest	odds ratios		allocated function	previous associations
				CC	TDT		
(a) Previously published loci							
rs11465804	1p31	NA	*IL23R*	2.50	2.77	interleukin-23 receptor	(16-20)
rs3828309	2q37	NA	*ATG16L1*	1.28	1.30	autophagy-related	(18-22)
rs9858542	3p21	35	*MST1*	1.20	1.20	macrophage stimulating 1 (hepatocyte growth factor-like)	(19;20)
rs4613763	5p13	0	*PTGER4*	1.32	1.28	prostaglandin receptor EP4	(17)
rs2188962	5q23	7		1.25	1.26		
rs11747270	5q31	3	*IRGM*	1.33	1.31	induces autophagy	(19;20;23)
rs4263839	9q32	2	*TNFSF15*	1.22	1.07	TNF-like factor, is induced by TNF and can activate NF-κB	(24)
rs10995271	10q21	1	*ZNF365*	1.25	1.53	Zinc-finger protein 365	
rs11190140	10q24	1	*NKX2-3*	1.20	1.28	homebox transcription factor, required for T- and B-cell localization in the spleen and mesenteric lymph nodes	(19;20)
rs2066847	16q12	NA	*NOD2*	3.99	2.57	pattern recognition receptor	(18;19;25;26)
rs2542151	18p11	1	*PTPN2*	1.35	1.14	T-cell protein tyrosine phosphatase, a negative regulator of inflammatory responses	(19;23)
(b) Novel loci							
rs2476601	1p13	7	*PTPN22*	1.31	1.17	T-cell protein tyrosine phosphatase	
rs2274910	1q23	2	*ITLN1*	1.14	1.62	intelectin-1	
rs9286879	1q24	0		1.19	1.08		
rs11584383	1q32	3		1.18	1.20		
rs10045431	5q33	1	*IL12B*	1.11	1.36	encodes IL-12p40 subunit of IL-12 and IL-23	
rs3763313	6p21	7	*BTNL2, SLC26A3, HLA-DRB1, HLA-DQA1*	1.19	1.01		
rs6908425	6p22	1	*CDKAL1*	1.21	1.09	poorly characterized; gene is a type 2 diabetes susceptibility gene	
rs7746082	6q21	0		1.17	1.19		
rs2301436	6q27	3	*CCR6*	1.21	1.16	chemokine receptor 6	
rs1456893	7p12	0		1.20	1.14		
rs1551398	8q24	0		1.08	1.25		
rs10758669	9p24	3	*JAK2*	1.12	1.21	Janus kinase 2, downstream signalling molecule of many cytokines and growth factors	
rs17582416	10p11	3		1.16	1.26		
rs7927894	11q13	1	*C11orf30*	1.16	1.07		
rs11175593	12q12	3	*LRRK2, MUC19*	1.54	1.44	leucine-rich repeat kinase 2, mutant form induces autophagy; mucin gene 19	
rs3764147	13q14	3		1.25	1.19		
rs2872507	17q21	17	*ORMDL3*	1.12	1.24	inducible T-cell co-stimulator ligand	
rs744166	17q21	4	*STAT3*	1.18	1.25	signal transducer and activator of transcription 3, downstream signalling molecule of many cytokines and growth factors	
rs4807569	19p13	2		1.02	1.26		
rs1736135	21q21	0		1.18	1.10		
rs762421	21q22	1	*ICOSLG*	1.13	1.21	inducible T-cell co-stimulator ligand	

Abbreviations: NA, not applicable; SNP, single nucleotide polymorphism; CC, Case Control; TDT, transmission disequilibrium test

genes (at least in Crohn’s disease) further highlights the complex polygenic nature of the disease. A recent study provides the first detailed illustration of the genetic relationship between Crohn’s disease and ulcerative colitis (20). It shows that several risk loci are common to ulcerative colitis and Crohn's disease (among which *IL23R*), whereas autophagy genes *ATG16L1* and *IRGM*, along with *NOD2* (also known as *CARD15*), are specific for Crohn's disease.

Table 2. Genes interesting according to function (with single association)

Gene	Chromosome	Allocated function	Reference
Crohn’s disease			
SLC22A4 & *SLC22A5*	5	solute carrier family 22 (organic cation transporter), members 4 and 5 (formerly *OCTN1* and *OCTN2*); organic cation, carnitine transporters, possibly transport xenobiotic substances	(27)
DLG5	10	discs large homolog 5 (Drosophila); epithelial scaffolding protein; CARD-containing protein	(28;29)
NCF4	22	regulatory component in superoxide production	(18)
Ulcerative colitis			
MDR1	7	multidrug resistance 1; efflux transporter for drugs and, possibly, xenobiotic compounds	(30;31)

environmental triggers

Differences in incidence of IBD across age, time, and geographic region suggest that environmental factors significantly modify the expression of Crohn’s disease and ulcerative colitis (6). Furthermore, the incomplete penetrance of IBD (e.g. in monozygotic twins) suggests that non-genetic triggers are necessary. Indeed, several studies implicate environmental factors in the pathogenesis of IBD. The strongest factor identified is smoking (32), being protective in ulcerative colitis but increasing risk in Crohn’s disease (33). Mechanisms behind the effect of smoking on IBD remain obscure, but potentially important effects of nicotine or smoking on cytokines (34;35), eicosanoids (36), mucosal microcirculation (37), small intestinal permeability (38), and colonic mucus (39;40) have been suggested, possibly involving nicotinic acetylcholine receptors in the bowel wall (41). The kind of involvement of these factors and/or processes can differ in Crohn’s disease *versus* ulcerative colitis (see further), probably accounting for the opposite effects of smoking observed in the two disorders.

Another factor with a similar differential influence on Crohn's disease and ulcerative colitis is appendectomy (42-44). A hypothesis on appendectomy is that the development of appendiceal inflammation or mesenteric adenitis might protect against ulcerative colitis and/or predispose to Crohn's disease. Alternatively, removal of the appendix might influence the mucosal immune system of the gut (6;45). Recently, preterm birth and other perinatal circumstances were associated with the development of IBD (46). Whether other factors such as the use of antibiotics and non-steroidal anti-inflammatory drugs (NSAIDS), stress, diet, oral contraceptives, perinatal/childhood infections, or atypical mycobacterial infections play a role in expression of IBD remains unclear (6;47).

epigenetic effects in IBD

Emerging evidence suggests a key role for epigenetics in human pathologies, including in inflammatory disorders. Epigenetic mechanisms result in heritable modifications in the expression of genes that are independent from DNA coding variability. These phenomena have been recognized as important permissive and suppressive factors in controlling the expressed genome via gene transcription. Two major epigenetic mechanisms are the posttranslational modification of histone proteins in chromatin and the methylation of DNA itself (48).

As discussed above, the concordance rate of IBD in monozygotic twins is maximum 50%, which might be partly due to epigenetic effects. Moreover, the epigenome is influenced by environmental factors such as diet and smoking, which are (presumed) triggers of IBD. The specific presence of epigenetic modifications in IBD has only been studied in the context of IBD-associated colorectal cancer, and their occurrence seems to be a risk factor. One study found that methylation was a frequent and early event in IBD even before the occurrence of neoplasia (49). The importance of the epigenome in the pathogenesis of IBD will probably be pointed out in the near future as it will be for other diseases.

pathogenesis of IBD

Genetic and environmental factors can play a role at different levels involved in the pathogenesis of IBD: the epithelial barrier, the innate immune response, the adaptive immune response, and regulation. In all of these components, a disturbed handling of host-bacteria interactions seems to be important.

involvement of bacteria

There is abundant evidence that bacteria are significant contributors to the development of both IBD and experimental colitis.

evidence for the role of bacteria in experimental colitis

In at least 11 different animal models, colitis and immune activation fail to develop in the absence of commensal bacteria, and multiple experimental colitis models respond to antibiotics and probiotics (table 3). Bacterial association studies show that different bacterial species can cause different disease phenotypes in a single host, e.g. monoassociation of gnotobiotic IL-10$^{-/-}$ mice with the commensal bacteria *Enterococcus faecalis* and *Escherichia coli* induced phenotypically distinct forms of colitis (*E. faecalis*: late onset, distal colonic; *E. coli*: early onset, caecal) (50). Dual-association with both bacteria induced aggressive pancolitis and duodenal inflammation, implicating the likelihood that various commensal enteric bacterial species interact to induce and perpetuate mucosal immune responses that accompany intestinal inflammation (51). On the other hand, variable responses to the same bacterial species in different models of colitis demonstrate the requirements for host specificity: *E. faecalis* and *E. coli* both induced colitis in monoassociated IL-10$^{-/-}$ mice, but not in HLA-B27 transgenic rats. By contrast, *Bacteroides vulgatus* induced colonic inflammation in HLA-B27 transgenic rats but not in IL-10$^{-/-}$ mice (52). These data show that some (but not all) endogenous bacteria can provide an antigenic stimulation in colitis, and that genetic predisposition is critical to determine the immune response to bacteria (53). In a similar way, various probiotic species have variable effects in the same host, and different hosts respond variably to the same probiotic species (54;55). A recent study sheds another light on the role of commensals in the development of IBD by showing that commensal bacteria are required for exacerbation but not development of ileitis in SAMP1/YitFc mice (56). All these data show that the occurrence of IBD depends upon a complex and specific interaction between the right micro-organism in the right host.

Table 3. The role of bacterial flora in experimental colitis models (adapted from (57), adjusted)

Model	Role of bacterial flora	Reference
HLA-B27 transgenic	bacterial flora necessary for development of intestinal inflammation	(58)
	prevention of colitis by probiotic following antibiotic treatment	(54)
IL-10 deficient	bacterial flora necessary for development of intestinal inflammation	(59)
	probiotic prevention and treatment of colitis	(55;60;61)
SAMP1/Yit	bacterial flora necessary for development of intestinal inflammation	(62)
C3H/HeJBir	bacterial flora necessary for development of intestinal inflammation	(63)
IL-2 deficient	bacterial flora necessary for development of intestinal inflammation	(64)
TCRα deficient	bacterial flora necessary for development of intestinal inflammation	(65)
$CD4^+CD45RB^{hi}$ adoptive transfer	amelioration in SCID recipients with reduced bacterial flora or treated with antibiotics	(66)
mdr1a deficient	antibiotics prevent and treat	(67)
DSS	antibiotics can prevent or treat colitis; bacterial products or probiotics can ameliorate colitis	(68-71)
	cave: germ-free mice develop colitis to the same or even more severe degree as conventional mice	(72)
STAT-4 transgenic	immunization necessary to develop colitis	(73)
	enteric bacterial antigen-reactive Th1 cells mediate the colitis	
$TGF\beta RII/IL\text{-}10R2^{DKO}$	fulminant colitis was completely inhibited by a combination of broad-spectrum antibiotics	(74)

Abbreviations: DSS, dextran sodium sulphate; IL, interleukin; mdr, multidrug resistance; STAT, signal transducer and activator of transcription; TCR, T-cell receptor, $TGF\beta RII/IL\text{-}10R2^{DKO}$, double knockout for TGF-β receptor II and interleukin-10 receptor 2

evidence for the role of bacteria in human studies

In IBD, the evidence that bacteria contribute to the pathogenesis of IBD is more indirect, but still considerable. First, luminal bacterial concentrations reach 10^7 to 10^8 organisms/g luminal contents in the terminal ileum, 10^{11}/g in the colon, and 10^{10-11}/g in ileal pouches (figure 2) (75). Lesions in IBD predominate in these areas of highest bacterial exposure (53). Further evidence for the importance of the microflora is the dysbiosis which is seen in patients with IBD. Several groups have documented a change in gut flora of IBD patients in both the faecal (or luminal) and the mucosal flora, which differ greatly (76-78). For example, in Crohn's disease, the balance in faecal flora shifts towards more *Bacteroides* species (which reduce innate immunity) (78) and less bifidobacteria (which increase innate immunity) (76).

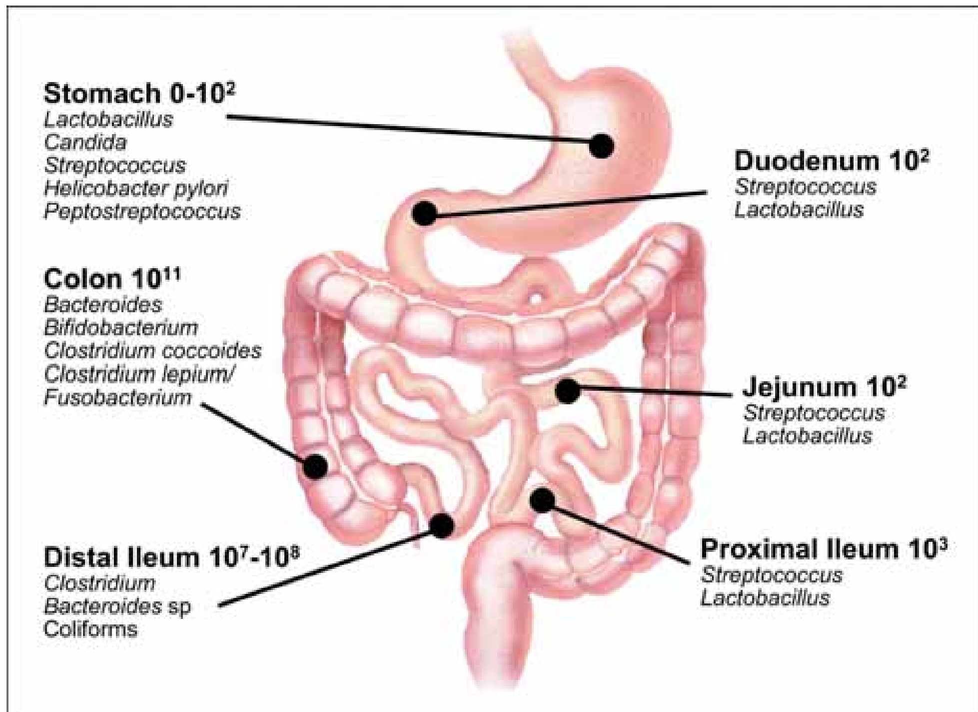


Figure 2. Human gastrointestinal bacteria: the interface between the host and the environment. (adapted from (75))

The luminal concentrations of the gastrointestinal microbiota are provided with the dominant identified species in each region.

Comparisons of faecal floras from patients with Crohn's disease, ulcerative colitis and infectious colitis showed significant differences (79), and active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the faecal flora (80). The mucosal flora also differs between IBD patients and healthy controls, containing a higher number of bacteria in patients (81-83), but being less diverse (84). It has been put forward that the mucosal microbiota may have a more important role in IBD as this ecosystem is closer to human cells (53). In tissue specimens of IBD patients, bacterial invasion of the mucosa was found as opposed to total absence of bacteria in tissue specimens of controls (81-83). Additionally, the early postoperative recurrence of Crohn's disease has led to studies on the role of the faecal stream in the inflammatory process. It was shown that diversion of the faecal stream from inflamed bowel loops induces improvement of Crohn's disease, whereas reanastomosis results in relapse (85-87). Comparative studies on reintroduction of small bowel effluent *versus* a sterile ultrafiltrate of it suggest that factors

greater than 0.22 microns in the faecal stream are responsible for the maintenance and exacerbation of inflammation in Crohn's disease (85). Finally, multiple antibiotic, probiotic and prebiotic treatment studies in IBD patients show favourable responses (reviewed in (88)), offering considerable promise for treating IBD.

the damaged mucosal barrier in genetically susceptible individuals

The host response to the microbiota can be ranked into several broad components: the epithelial barrier, the innate immune response, the adaptive immune response, and the regulatory mechanisms (figure 3). Experimental models reveal that severe impairment of a single component can result in disease, but many models demonstrate milder effects in more than one component (table 4). The current paradigm is that multiple defects (or 'hits') in these interacting components are thought to be required for development of IBD.

disturbances at the level of the epithelial barrier

The intestinal epithelial barrier must maintain a physical and immunological barrier between the underlying mucosa and luminal bacteria, at the same time distinguishing commensal bacteria and food proteins from trillions of potentially harmful organisms. Permeability can refer to the degree to which the epithelium is permissive to the passage of luminal substances or can refer more broadly to all of the protective functions of the intestinal mucosa. Factors contributing to normal permeability include an intact intestinal epithelium, surface mucus, peristalsis, and the secretion of host protective factors. The actual barrier includes the cells themselves plus the paracellular space. Tight junctions exist in the space between the cells, allowing for selective entry of fluids, nutrients, and select micro-organisms (89). Selective transport of luminal antigens or bacteria into the mucosa is performed by M (membranous or microfold) cells in the follicle associated epithelium (90), or by dendritic cells (DCs) which intercalate their dendrites between epithelial cells and form tight junctions (91). It has been shown in human ileum that follicle associated epithelium is more permeable to antigens and bacteria, not only because of the presence of M cells but also because of increased transcellular transport and increased transepithelial DC sampling (92).

Differential cDNA screening and mRNA display of control, ulcerative colitis, or Crohn's disease epithelial cells have shown differences in gene expression (93). Another study compared gene expression profiles in Crohn's disease with ulcerative colitis and found differences in epithelia-specific genes (94). Both studies found a downregulation of genes involved in colonocyte energy metabolism in ulcerative colitis samples. In the Crohn's

disease profile, the over-expression of antimicrobial defensins was dominant. Proteome analysis has also identified a differential protein expression profile in the intestinal epithelium from IBD patients, revealing significant expression changes of proteins that are associated

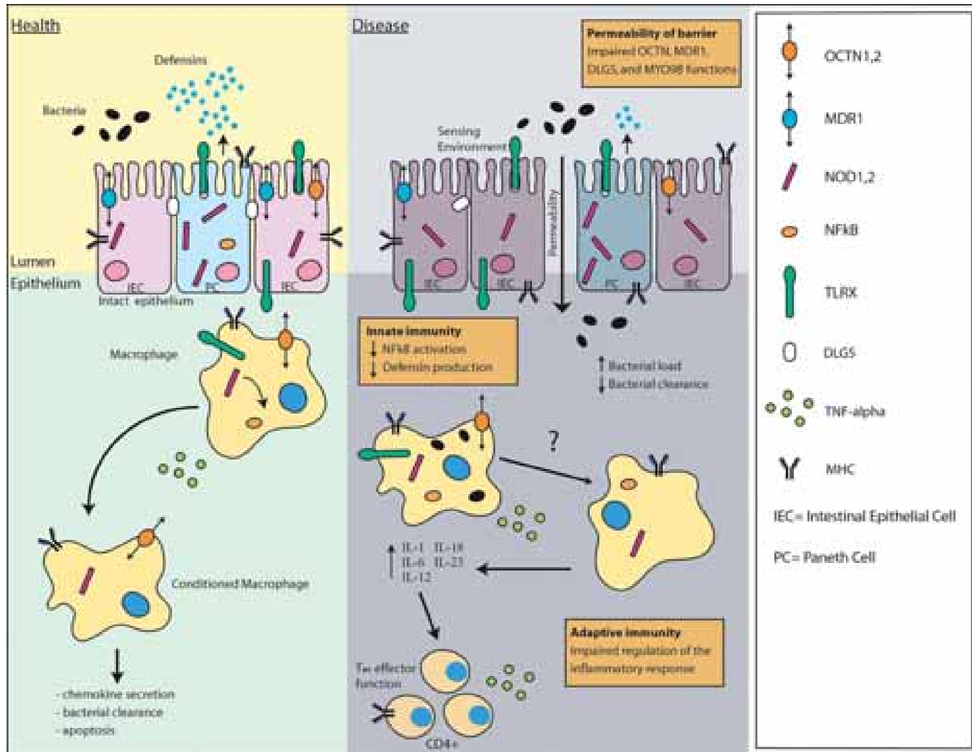


Figure 3. Potential molecular pathways involved in inflammatory bowel disease (IBD) pathogenesis. (adapted from (95), adjusted)

In healthy individuals, bacterial invasions trigger cascades of immune events leading to chemokine secretion, bacterial clearance and apoptosis. Although there is still much to be discovered, genetic defects at four different levels are suspected to lead to chronic inflammation in IBD: 1) Mutations in transporters such as MDR1, OCTN1 and OCTN2, or in genes involved in epithelial integrity (DLG5) may affect the permeability of the epithelial barrier. 2) Mutations in environmental sensors and their signalling pathways, such as TLRs and CARD15/NOD2, may impair the innate immune system and cause a decrease in NF-κB activation and defensin production. 3&4) Mutations in genes involved in adaptive immunity, such as the human leukocyte antigen (HLA) genes and tumor necrosis factor (TNF)-α within the major histocompatibility complex (MHC), as well as in genes coding for subunits of cytokines and cytokine receptors like IL-12 and IL-23R, may cause an imbalance between regulatory and effector cell immune responses important in the control of inflammatory reactions. TLR, Toll-like receptor; NF-κB, nuclear factor-κB; MDR1, multidrug resistance gene; OCTN1 and 2, organic cation transporter 1 and 2; DLG5, drosophila discs large homolog 5.

Table 4. Mouse models of intestinal inflammation with defective epithelial barrier, altered innate immune response, excessive effector T-cell response, or altered regulatory mechanisms (adapted from (57), adjusted)

Model	Defective epithelial barrier	Altered innate immune response	Excessive effector T-cell response	Altered regulatory mechanisms	Known defects	Reference
mdr1a deficient	✓				altered epithelial barrier	(30)
Gαi2 deficient	✓			✓	defective epithelial barrier; defective regulatory B cells	(96)
dn N-cadherin	✓				intestinal epithelial cell apoptosis and epithelium gaps	(97;98)
deletion of enteric glia nerves	✓				disrupted epithelium	(99)
DSS	✓				direct damage to epithelial barrier	(100)
muc2 deficient	✓				defective mucus layer	(101)
macrophage-PMN Stat-3 deficient		✓		✓	increased response to LPS; resistant to IL-10 regulation	(102)
bone marrow Stat-3 deficient		✓		✓	increased response to LPS; impairment of innate immune function	(103)
A20 deficient*		✓	✓		increased response to LPS	(104)
IL-10 deficient		✓		✓	lack Tr1 activity; lack TGFβ signalling	(105)
NF-κB $p50^{-/-}$ $p65^{+/-}$		✓			increased IL-12 production	(106)
TGFβ1 deficient*		✓		✓	decreased regulatory T cells	(107)
C3H/HeJBir		✓	✓		impaired innate responses to TLR ligands; increased bacterial reactive T cells	(63)
SAMP1/Yit	✓		✓		epithelial cell defects; expanded B-cell population; increased activated T cells	(62)
STAT-4 transgenic			✓		excessive T-cell responses to enteric bacteria	(73)
CD40L transgenic*			✓		increased activated T cells	(108)
$TNF^{\Delta ARE}$*			✓		increased TNF-α production	(109)
$CD4^{+}CD45RB^{hi}$ transfer				✓	decreased regulatory T cells	(110)
IL-2 deficient				✓	decreased $CD4^{+}CD25^{+}$ T cell	(111)
IL-2 receptor α deficient				✓	decreased $CD4^{+}CD25^{+}$ T cell	(112)
TCRα deficient				✓	loss of a regulatory B cell	(113;114)
TGFβRII deficient*				✓	decreased regulatory T cells	(115)
Smad3 deficient*				✓	decreased regulatory T cells	(116)
$Muc2/IL\text{-}10^{DKO}$	✓			✓	combined defects in epithelial and immunoregulatory factors	(117)
$TGF\beta RII/IL\text{-}10R2^{DKO}$				✓	combined multiple genetic hits in immune regulation	(74)

Abbreviations: dn, dominant negative; DSS, dextran sodium sulphate; IL, interleukin; mdr, multidrug resistance; LPS, lipopolysaccharide; muc, mucin gene; NF-κB, nuclear factor κB; PMN, polymorphonuclear cell; STAT, signal transducer and activator of transcription; TCR, T-cell receptor, $TGF\beta RII/IL\text{-}10R2^{DKO}$, double knockout for TGF-β receptor II and interleukin-10 receptor 2, TLR, Toll-like receptor; TNF, tumour necrosis factor; Tr1, T regulatory cell type 1
*multi-organ failure not limited to intestine

with signal transduction, stress response as well as energy metabolism (118). However, both expression and proteome analyses were carried out on samples from inflamed tissue, and as such several of the detected variations may reflect secondary rather than primary abnormalities. Therefore, follow-up studies with higher numbers of patients (including appropriate patient subsets) and well-chosen controls are necessary to confirm and understand the number of significantly regulated proteins (119). Thus, epithelial cell expression is altered in IBD. Defects in the epithelial barrier could lead to exposure of potentially harmful luminal contents to the mucosal immune system, resulting in its activation.

abnormal mucus composition (increased adherence of bacteria)

The epithelial cell lining is covered by a layer of glycocalyx, which is formed from mucins that bind the apical membrane of the cells. A thick layer of mucus, comprising diverse mucins, forms an additional system of protection and separates luminal bacteria from the epithelial surface throughout the colon in healthy individuals (82). The mucus is produced by goblet cells in the epithelium, and massive release of mucin granules is triggered by the presence of physical, chemical or infectious insults (120). The formation of a semi-permeable protective barrier and the repair of intestinal damage through intestinal trefoil factor (121) are the main functions of the mucus layer. Additionally, mucins interact with bacterial constituents, thereby trapping them in the mucus flow so that they are washed away by intestinal peristalsis (122). The secretion and dehydration of mucus is regulated tightly and viscosity gradients of the mucus layers exist in both vertical (surface to lumen) and longitudinal (proximal to distal colon) directions, determining a spatial organization of the intestinal microbiota and mucosal barrier function (123). Recently, it was found that secreted antimicrobial peptides are retained by the mucus and thereby provide an additional antibacterial barrier. This distribution is thought to facilitate a high local peptide concentration on vulnerable mucosal surfaces, while still allowing the presence of an enteric microflora (124).

In IBD, protection of the mucosa is thought to be reduced as a result of specific changes in the thickness or composition of the intestinal mucus layer (125;126). The layer is thinner than normal in ulcerative colitis and thicker than normal in Crohn’s disease (127). A number of important biochemical changes such as glycosylation and sulphation have also been noted in IBD (128-131). The changes described are likely to alter the viscoelastic properties of the gels formed and influence interactions of mucins with microorganisms, electrolytes, defensive proteins and dietary components, hence reducing the effectiveness of supramucosal layer function (126). In patients with ulcerative colitis, the activity of mucosal inflammation correlates significantly with a decrease in synthesis and activity of the mucin gene *MUC2*, encoding for an important structural component of the mucus layer (132;133). Mice deficient

for the Muc2 gene spontaneously develop colitis (101). In ileal Crohn's disease, a decreased expression was reported in MUC3, MUC4, and MUC5B in both healthy and involved mucosa (134). The rectal mucus of ulcerative colitis patients features a lack of phosphatidylcholine compared to normal subjects or patients with Crohn's disease, independent of the level of inflammation, suggesting primary involvement in pathogenesis (135). All these events could lead to adherence of bacteria to the mucosa and an increased exposure of the mucosal immune system to intestinal bacteria or bacterial components (136). The intestinal mucus layer from IBD patients has been shown to harbour high numbers of unspecific bacteria compared with lack of bacteria in normal mucus (81;82;137).

decreased epithelial integrity

Epithelial damage is a hallmark of IBD. The proportions of differentiated intestinal epithelial cells such as goblet cells, Paneth cells (specialized crypt enterocytes secreting antimicrobial peptides and enzymes), and mature enterocytes may be altered. Rates of epithelial proliferation and apoptosis are changed (138-140) and can lead to crypt hyperplasia, loss of villi, ulcerations, and erosion of the epithelial layer (57). Oxidative stress, originating from reactive oxygen species (ROS) produced by phagocytic leukocytes that are present in large numbers in the inflamed mucosa can also result in intestinal injury, and might be elevated in IBD due to an imbalanced mucosal antioxidant response (141-144). Lesions of the intestinal epithelium allow penetration of commensal or pathogenic bacteria. The earliest observable lesions of recurrent Crohn's disease are microscopic erosions and damaged M cells at the specialized follicle-associated epithelium (145). Recently, an increased uptake of non-pathogenic bacteria was demonstrated in follicle associated epithelium of macroscopically normal ileum from patients with longstanding Crohn's disease (146). These alterations of the intestinal epithelium often appear to be the consequence of the inflammatory response. However, it is also possible that a primary epithelial defect could be the trigger initiating the inflammatory process.

Some animal models show that impairment of epithelial integrity can result in intestinal inflammation (table 3). A number of models involve induction of acute epithelial damage via administration of chemical irritants or some other direct damage to the epithelial barrier, although in many cases a secondary immune component is also involved. Dextran sodium sulphate (DSS) (100) is the most commonly used, other models include treatment with acetic acid, oxazolone, trinitrobenzene sulphonic acid (TNBS)/ethanol, indomethacin, peptidoclycan polysaccharide, and carrageenan (57). Several lines of evidence indicate that the primary instigator of DSS colitis is damage to the epithelium, perhaps via direct toxicity (147). Early epithelial defects are also encountered in genetic models. Deletion of the *mdr1a* gene, which

encodes transmembrane transporter protein P-glycoprotein, results in spontaneous transmural colitis (30). Although this protein is also expressed in other cell types, the data suggest that the principal cause of colitis in this model is a primary epithelial defect (57;148;149). Mice deficient in Gαi2 (a signal transducer found in many cell types) develop a pancolitis at 8 to 12 weeks of age (96), and there are indications of a defective epithelial barrier prior to histological inflammation (150). Mice that transgenically express a mutant N-cadherin protein have disruption of E-cadherin homotypic interactions, resulting in intestinal epithelial cell apoptosis and gaps in the epithelium (98). These mice develop intestinal inflammation and dysplasia (97). Finally, an important argument for a primary role of epithelial cell alterations is found in the SAMP1/Yit model of spontaneous ileitis. This mouse strain bears increased numbers of Paneth cells and goblet cells that are present before the onset of ileitis (151). Although the effect of these epithelial changes on the barrier prior to inflammation needs further evaluation, the early and severe alterations of the epithelium suggest that epithelial changes may be an initiating factor in this model of ileitis.

increased permeability

Patients with Crohn's disease have an increased intestinal permeability (152;153), which is also observed in 10 to 20% of unaffected relatives (154-159). In clinically asymptomatic Crohn's disease patients, increased intestinal epithelial permeability precedes clinical relapse by as much as 1 year (160-162), indicating that a permeability defect may be an early event in disease reactivation. A case report of a healthy first-degree relative of Crohn's disease patients who exhibited increased intestinal permeability at the age of 13 showed that a permeability defect can exist long before the onset of full-blown disease 8 years later, indicating that, at least in this case, an intestinal permeability defect may have been an early event in disease pathogenesis (163). Spouses of Crohn's disease patients show a trend towards increased permeability (164), suggesting a role for environmental factors as well. Some environmental factors like smoking and non-steroidal anti-inflammatory drugs are thought to influence intestinal permeability and amplify abnormal 'leakiness' in Crohn's disease (153). Enteric bacterial and parasitic infections have also been found to directly increase permeability (165;166).

Epithelial permeability is predominantly defined by tight junction integrity. Recent studies provide evidence that the tight junction barrier is altered in active Crohn's disease, implicating the claudin family of tight junction proteins (167;168). Altered claudin isoform expression patterns are more likely to be a consequence rather than a cause of active disease, and their expression can be altered by interleukin (IL)-13, interferon (IFN)-γ and tumour necrosis factor (TNF)-α (167-169). Regardless of these differences, TNF-α and IFN-γ can increase intestinal

epithelial permeability by mechanisms not involving altered claudin isoform expression, which are independent of apoptosis (139;170-174). Incubation of intestinal epithelial cell monolayers with both IFN-γ and TNF-α leads to reorganization of many tight junction proteins, including zonula occludens-1, junctional adhesion molecule 1, and occludin, next to claudin isoforms (139;175). The changes in paracellular permeability caused by IFN-γ and TNF-α are associated with marked increases in myosin light chain (MLC) phosphorylation and can be reversed using a specific membrane permeant inhibitor of MLC kinase (MLCK), indicating that these cytokines also utilize the MLCK-driven pathway to increase tight junction permeability (170). Intestinal epithelium of IBD patients showed increased expression and enzymatic activity of MLCK, correlating positively with disease activity (176). In contrast with claudin expression, MLCK expression was modestly increased in patients with histologically inactive IBD, suggesting that increased intestinal epithelial MLCK expression may be a stable characteristic of IBD patients.

Another pathway that has been connected with tight junction regulation is signalling through TLR2, one of the Toll-like receptors (TLRs, see further) that recognize bacterial cell wall constituents and activate the innate immune response. Data from *in vitro* and *ex vivo* models of intestinal epithelial cells revealed that TLR2 stimulation effectively preserves tight junction-associated barrier assembly against stress-induced damage (177;178). Furthermore, *in vivo* studies underscored that TLR2-mediated tight junction regulation critically determines susceptibility to intestinal injury and inflammation (178). These studies provide a molecular mechanism for commensal-induced enhancement of intestinal epithelial barrier function, which has been observed by diverse groups (61;179;180). In addition, TLRs serve as a means through which bacteria regulate normal intestinal barrier function and activate the normal genetic program of the intestinal epithelium (89;179;181;182). Oxidative stress is also associated with increased permeability. Apart from causing direct cellular injury, ROS can disrupt the epithelial barrier function by destabilizing tight junctions (183).

Genetic factors that could be implicated in increased permeability include the solute carrier family 22 genes (*SLC22A4* and *SLC22A5*, formerly organic cation transporter *OCTN1* and *OCTN2*) (27), and the guanylate kinase *DLG5* (Drosophila discs large homolog 5) gene (28). Mutations of the former genes alter transcription and carnitine transport function of their gene products, while mutations of the latter impair the ability of the DLG5 protein to maintain epithelial integrity. Both gene products seem to be important in epithelial permeability, and disruption of their function could lead to inappropriate exposure of the mucosal immune system to bacterial products (89).

defects in epithelial cell immune function

Not only do intestinal epithelial cells form a physical barrier between the lumen and the mucosa, they also exert an important role in the identification of pathogens and counteraction, when necessary. As such, they are regarded as a part of the innate immune system. Defects in epithelial cell immune function that can lead to chronic inflammation will be discussed in the next chapter on the innate immune response.

disturbances at the level of the innate immune response

While most research investigating the mucosal immunology relevant to IBD during a number of years focussed on the T cell as the key player, recent advances in genetics and immunology in IBD have switched the emphasis to innate immunity as pivotal in the development of this disease. The innate immune system provides an immediate, but non-specific response based on sensing, recognition, and clearing of microbial pathogens. Innate immune recognition relies upon germline-encoded receptors that recognize a limited number of highly conserved microbial structures, so-called pathogen-associated molecular patterns (PAMPs). The receptors, referred to as pattern recognition receptors (PRRs), include Toll-like (TLR, transmembrane) and Nod (nucleotide-binding oligomerization)-like (NLR, cytoplasmic) receptors. They are expressed on epithelial cells, macrophages, DCs, and B cells. Among the structures detected by PRRs are lipopolysaccharides (LPS), peptidoglycan (PG), lipoproteins, flagellin, and nucleic acids. Ligand binding to host cell receptors induces cellular signalling events that lead to the production and secretion of molecules involved directly in defence, in addition to recruitment and activation of phagocytic cells (particularly neutrophils), and activation of DCs to promote the generation of an adaptive immune response. These molecules vary greatly in composition and activity and include antimicrobial peptides, cytokines, chemokines, and eicosanoids (184). When these molecules are generated unnecessarily or in excess, however, they can lead to damage of the host tissue or an unnecessary activation of the immune system (figure 4a). Otherwise, failure of recognition of a bacterial threat or an ineffective response can result in persistence of the exogenous stimulus and an exaggerated secondary and compensatory immune response (figure 4b) (185).

NOD proteins

The first susceptibility gene that was identified for Crohn's disease is *NOD2/CARD15* (25;26), initially thought to be expressed only in the cytoplasm of macrophages (186). At present, however, it is known that *NOD2* is also expressed in DCs (187), Paneth cells (188), and intestinal epithelial cells, with higher expression in the crypts than in the villi (187;189).

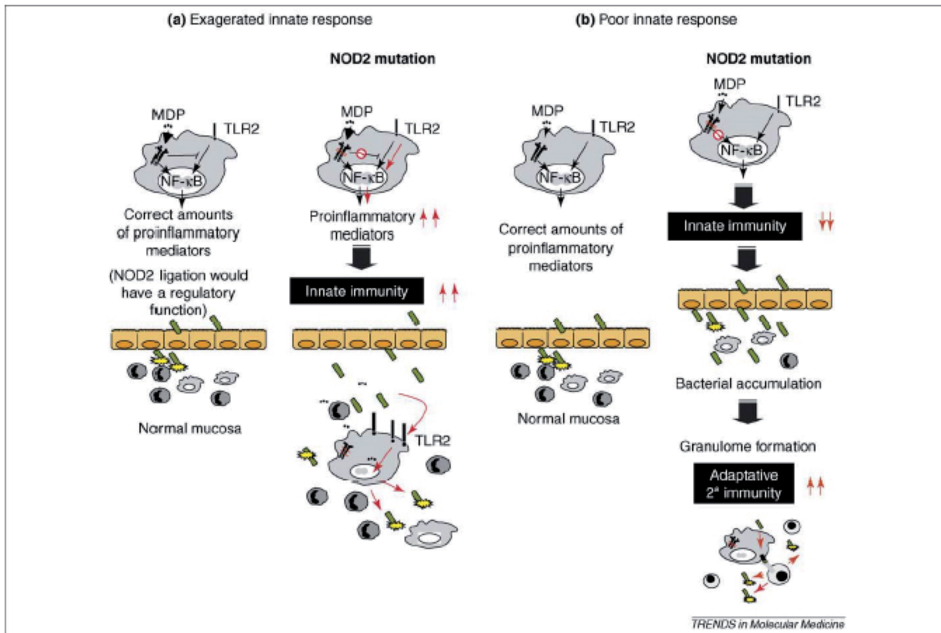


Figure 4. Two models for the involvement of the innate immune response in Crohn's disease (adapted from (185))

(a) In this model, NOD2 should down-regulate TLR signalling by an unknown mechanism. The mutated NOD2 protein loses this negative regulation of TLR signalling (as a consequence of the Crohn's disease-associated mutation), hence resulting in increased innate immunity to bacterial stimulation and predisposing to intolerance for the commensal intestinal microbiota, with intestinal inflammation as a consequence. **(b)** In the second model, both signalling pathways (NOD2 and TLR) are required for optimal bacterial defence. The mutated NOD2 receptor does not contribute to the pro-inflammatory gene transcription in response to bacteria, resulting in an inadequate innate response to bacterial invasion and enabling the accumulation of commensal bacteria. The poor innate response leads to the formation of the granulome and, thus, the activation and perpetuation of a deregulated secondary adaptive response.

Polymorphisms of *NOD2* associated with Crohn's disease are deficient in their recognition of muramyl dipeptide (190), which ensures ileal expression of antimicrobial peptides and promotes cytokine and chemokine production by enterocytes and immunocytes. Crohn's disease-associated *NOD2* polymorphisms have also been linked with impaired expression of certain Paneth-cell derived antimicrobial peptides (191-193), although the association with reduced expression of antimicrobial α-defensin has recently been contested (194). Most functional studies on mutant NOD2 have been performed *in vitro* on cell lines or on macrophages and monocytes from patients carrying NOD2 mutations and support a loss-of-function phenotype. Three common mutations (Arg702Trp, Gly908Arg, Leu1007fsinsC),

accounting for about 80% of variants associated with Crohn's disease, lead to defective PG and muramyl dipeptide (MDP) sensing, reduced nuclear factor (NF)-κB activation and impaired cytokine production (TNF-α, IL-1β, IL-8, and IL-10) upon stimulation (195-198). However, this loss-of function phenotype is not entirely consistent with the common clinical observations of elevated pro-inflammatory cytokine levels and presence of activated NF-κB in the lamina propria of Crohn's disease patients. Macrophages of *Nod2* mutant mice that carry a mutation analogous to the most common Crohn's disease susceptibility allele exhibit a gain-of-function phenotype with increased NF-κB and more efficient IL-1β secretion upon stimulation (199). The following hypotheses have been put forward to reconcile this apparent paradox: a) defective NOD2 signalling may lead to delayed clearance and uncontrolled proliferation of micro-organisms in the intestine, with a prolonged adaptive immune response; this model is compatible with the impaired expression of antimicrobial peptides by Paneth cells that has been linked with NOD2 mutations; b) defective NOD2 signalling could dampen anti-inflammatory signals, such as secretion of IL-10 or transforming growth factor (TGF)-β (197); c) some studies show that MDP activation of NOD2 negatively regulates TLR2 responses and that absence of such regulation leads to TLR2-mediated NF-κB activation and heightened T helper type 1 (Th1) cell responses (200;201). This hypothesis is further expanded by the findings that MDP activation of NOD2 regulates innate responses to intestinal microflora by downregulating multiple TLR responses, not just TLR2 (202). d) mutations have different effects in different contexts, i.e. in the mucosa *versus* the periphery (89); e) additional Crohn's disease-susceptibility genes and environmental factors affect disease expression in humans (89;203).

Current knowledge on the consequences of mutant *epithelial* NOD2 function is indirect and comes mostly from mouse models. *Nod2* mutant mice, carrying a mutation that is analogous to the most common Crohn's disease susceptibility allele, show no sign of spontaneous inflammatory lesions, but are more sensitive to DSS-induced colitis (199). *Nod2*-deficient mice neither show spontaneous inflammatory lesions, yet these animals are susceptible to bacterial infection via the oral route but not through intravenous or peritoneal delivery (192). Furthermore, host recognition of MDP was dispensable for the bacteria-induced cytokine production and bactericidal function of macrophages, suggesting a predominant role of NOD2 within the intestinal epithelial barrier. A protective, antibacterial function for epithelial NOD2 in the gut is further supported by the observation that forced overexpression of NOD2 by intestinal epithelial cell lines protects against *Salmonella* infection (189). Recent evidence proves that the subcellular localization of NOD2 in epithelial cells, i.e. cytosolic versus membrane-bound, is crucial in correctly inducing a proinflammatory response (204). An indirect influence of NOD2 on epithelial innate immune function is shown in a study on small

intestinal allograft rejection, which resembles Crohn's disease clinically and pathologically. The likelihood of allograft failure in patients was about 100-fold higher in recipients with mutant *NOD2* alleles compared to recipients with wild-type *NOD2* loci, suggesting a role for NOD2-expressing cells of haematopoietic origin in the regulation of epithelial immune function (205). This regulation may happen by regulating expression of epithelial antimicrobial peptides, since rejection in *NOD2* mutant recipients was characterised by decreased expression of these peptides prior to epithelial injury and inflammation.

Another NOD protein which has been the focus of research in Crohn's disease is NOD1, which mediates the epithelial response to invasive Gram-negative bacteria (206;207). Mutations in *NOD1* have not been found in IBD (208), although there is a study that reports an association between a polymorphism in *NOD1* and susceptibility to IBD (209).

TLRs

It is likely that innate immune PRRs are not triggered one-at-once by single ligands but rather that complex activation of multiple receptors takes place due to the many TLR and NOD ligand motifs present on a micro-organism (210;211). Different reports have shown synergy between NOD2 activation and several TLR ligands in cellular responses (198;212;213), and the synergistic effect between NOD2 stimulation and CpG DNA (TLR9 ligand) is lost in Crohn's disease patients homozygous for NOD2 mutations (214). Mammalian TLRs comprise a family of 13 individual receptors which are each selectively activated by different PAMPs (figure 5) (215).

Evidence for protective (epithelial) effects of TLR comes from mouse models, since *in vitro* studies are limited by the difficulty in culturing primary epithelial cells. Intact TLR signalling is necessary for the healing of injured intestinal epithelium and the clearance of intramucosal bacteria (216). In the absence of effective TLR signalling, there is diminished chemokine expression, diminished neutrophil recruitment, and subsequent increased translocation of bacteria to mesenteric lymph nodes (217). TLR/MyD88 deficient mice developed markedly increased histological inflammation in the DSS model of colitis, suggesting protective effects of this innate signalling cascade at the epithelial cell level (181). The latter findings are consistent with observations that TLR4 mutant C3H/HeJ mice are more sensitive to DSS-induced colitis than wild-type mice (63;218). Also TLR9 triggering resulted in protection from experimental colitis, and this protection was found to be a result of TLR9-induced activation of protective type I interferon (IFN-α/β) (219). Furthermore, apical TLR9 stimulation of intestinal epithelial cells confers intracellular tolerance to subsequent TLR challenges (220). Polymorphisms in *TLR4* and *TLR9* have been linked with Crohn's disease and/or ulcerative

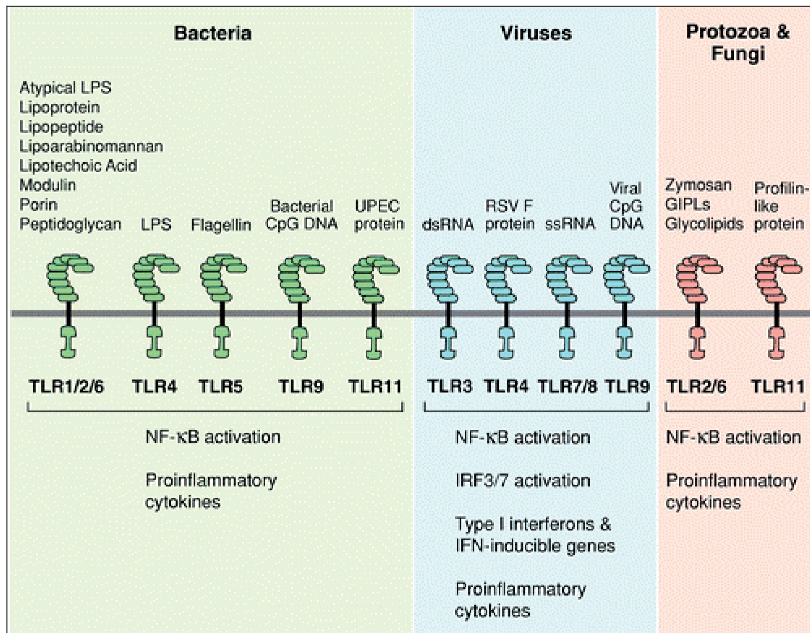


Figure 5. TLR ligand specificities (adapted from (215))

TLRs recognize a diverse array of PAMPs from bacteria, viruses, protozoa, and fungi. For detection of bacteria, heterodimeric TLR2/1 binds triacyl lipopeptides, whereas TLR2/6 dimers bind diacyl lipopeptides and lipoteichoic acid. Homodimeric TLR2 binds peptidoglycan, atypical LPS, phenol-soluble modulin from *Staphylococcus epidermidis*, and porin proteins from *Neisseria*. In addition, TLR4 binds LPS, TLR5 binds flagellin, and TLR9 binds bacterial CpG DNA. TLR11 detects an unidentified protein(s) from uropathogenic *Escherichia coli*. Viral dsRNA, RSV F protein, ssRNA, and unmethylated CpG DNA are sensed by TLRs 3, 4, 7/8, and 9, respectively. Finally, heterodimeric TLR2/6 binds fungally derived zymosan and *Trypanosoma cruzi* GIPLs, whereas TLR11 also senses a profilin-like protein from *Toxoplasma gondii*. Abbreviations: dsRNA, double-stranded RNA; LPS, lipopolysaccharide; GIPLs, glycolipids; PAMP, pathogen-associated molecular pattern; RSV F, respiratory syncytial virus fusion; ssRNA, single-stranded RNA; TLR, Toll-like receptor.

colitis, and although the association is not straightforward with different studies showing different associations (221-223), recent studies provide evidence that association with a *TLR4* polymorphism cannot be set aside (224;225). An association between a number of variants in the *TLR1*, *2*, and *6* genes and disease phenotype in ulcerative colitis and Crohn's disease was found in a Belgian study, although none of the polymorphisms was involved in disease susceptibility (226). IBD may be associated with distinctive changes in TLR expression and function in intestinal epithelial cells as well as other types of cells in the gut. Epithelial cell expression of TLR4 is strongly up-regulated in Crohn's disease and ulcerative

colitis (227). It is thought that host-derived feed-back mechanisms are critical in maintaining epithelial cell homeostasis, i.e. hypo-responsiveness of the intestinal epithelium towards bacterial signals under normal conditions. Several TLR pathway intrinsic mechanisms have been proposed for this mechanism (228-233), and lack of adequate control signals at the epithelial cell level may lead to the development of chronic intestinal inflammation.

Another way in which TLRs are involved in the regulation of tolerance to commensal bacteria is through their effects on DCs. Besides sampling luminal contents, these potent antigen-presenting cells play a key role in shaping intestinal immune responses including the generation of regulatory T cells in the gut. DCs that encounter invading microbes capture bacterial antigens and migrate to lymphoid organs, where they home to the T cell areas. While migrating to the lymph nodes, they shift from an endocytic/phagocytic immature stage to a mature stage of efficient T cell stimulation (234). Maturation of DCs can be induced by stimuli from bacteria, and TLR2 and TLR4 interaction with the appropriate ligand is essential for bacteria-induced maturation of DCs (235).

signalling defects

The various signal transduction cascades that are triggered upon stimulation of TLRs and NODs include the mitogen-activated protein kinases and the NF-κB system. The molecular mechanisms in gut epithelium that control epithelial integrity and intestinal immune homeostasis are critically regulated by NF-κB. Numerous studies have been performed on the role of NF-κB signal transduction in host defence and chronic inflammation, and the results support the hypothesis that the acute and transient activation of NF-κB may be protective for the host, while sustained and uncontrolled NF-κB signalling in the intestinal epithelium may indeed contribute to the immunopathology of experimental colitis (119). IL-10 deficient mice for instance show lack of activation of STAT (signal transducer and activator of transcription)-3 through IL-10 and subsequently enhanced NF-κB activation (236;237). Conditional STAT-3 knockout mice that are targeted to either macrophages and neutrophils or to bone-marrow derived cells that also include DCs, endothelial cells, and B cells, both develop a severe enterocolitis (102;103). A primary NF-κB signalling defect in intestinal epithelial cells was shown to disrupt immune homeostasis in the gastrointestinal tract, causing an IBD-like phenotype in mice (238). It was demonstrated that intestinal epithelial cells can directly influence responses of innate (dendritic) cells and adaptive ($CD4^+$ T) cells in the gut through the NF-κB pathway (239). Intestinal epithelial cells from IBD patients stimulated the proliferation and cytokine secretion by $CD4^+$ T cells to a significantly bigger degree than normal intestinal epithelial cells (240).

antimicrobial peptides

Reduced expression of antimicrobial peptides can compromise mucosal host defences. Patients with ileal Crohn's disease have reduced ileal expression of the α-defensins HD5 and HD6, produced by Paneth cells, in affected (inflamed) ileum compared to unaffected ileum and ileum from healthy controls (191;193). This specific decrease was independent of the degree of inflammation and was not observed in other intestinal inflammatory disorders. The functional consequence of the low α-defensin levels was a diminished antibacterial activity in ileal mucosal extracts (193). Furthermore, in a transgenic mouse model changes in HD5 expression levels had a pronounced impact on the luminal microbiota, indicating a role for Paneth-cell defensin expression in bacterial dysbiosis (see earlier). A recent study found the reduction in α-defensin expression to be dependent on the inflammatory state of the tissue and to be due to loss of surface epithelium as a consequence of secondary changes rather than being the inciting event prior to inflammation (194). As opposed to ileal Crohn's disease, colonic Crohn's disease is characterized by an attenuated induction of inducible β-defensins caused by a reduction in β-defensin gene copy number (241;242). These changes appear to be primary and rather specific for Crohn's disease since in coeliac disease the adherence of bacteria to the mucosa is actually associated with an increase in defensin expression (243).

impaired neutrophil recruitment/function

Neutrophils constitute the first line of defence to penetrating microbial and organic debris (244). A few decades ago, a reduction in the number of neutrophils migrating to the sites of skin abrasions was observed in patients with Crohn's disease, including those in complete remission (245-247). Ulcerative colitis patients demonstrate no impairment of neutrophil recruitment. The migration defect in Crohn's disease patients was not explained by abnormalities in neutrophil function, since the neutrophils themselves behaved normally during *in vitro* assays of chemotaxis (248;249), suggesting alterations in the inflammatory environment. An impaired production of IL-8, a potent neutrophil chemoattractant, was found in new inflammatory lesions in bowel biopsies and in skin of Crohn's disease (250). Direct subcutaneous injection with heat-killed *E. coli* into the forearm of Crohn's disease patients consistently failed to induce an acute inflammatory response. These findings were not present in healthy subjects or patients with other chronic inflammatory conditions, including ulcerative colitis and rheumatoid arthritis. This outcome supports the hypothesis that there is a major defect in the handling of bowel flora in Crohn's disease in the body. The theory that impaired neutrophil recruitment is a primary defect in Crohn's disease matches with its characteristic presence of granulomata, which could result from uptake and encirclement of abnormally persisting exogenous material in the intestinal mucosa by macrophages (251). It

also nicely corresponds with the observation that smoking clearly predisposes to the development of Crohn's lesions (see earlier). Smoking is both directly immunosuppressive, reducing concentrations of IL-8 within the intestinal mucosa (252), and vasoconstrictive, diminishing mucosal blood flow (253). Both IL-8 and blood flow are closely correlated to rates of neutrophils emigration into the tissues (254).

Recently, a strong association was reported between the *NCF4* gene and Crohn's disease that could implicate impaired neutrophil function (18). The *NCF4* gene encodes the p40phox protein which is a component of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. In response to bacterial infection, the neutrophil NADPH oxidase assembles on phagolysosomes to catalyze the transfer of electrons from NADPH to oxygen, forming superoxide and downstream ROS. *In vitro* studies showed that this protein has a positive role in coupling Fcγ receptor-mediated phagocytosis to activation of the NADPH oxidase (255), and depletion of endogenous p40phox reduced ROS production and impaired bacterial killing (256). Neutrophils from mice deficient for p40phox exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing (257). Furthermore, these mice were deficient in clearing an *in vivo* infection with *Staphylococcus aureus* bacteria, resembling an equally severe defect as that observed in chronic granulomatous disease.

disturbances at the level of the adaptive immune response

If the antigens are not eliminated, a second phase of the immune response takes over. The adaptive immune system allows a stronger immune response as well as immunological memory, where each pathogen is "remembered" by it's signature antigen (258). This phase involves the participation of B and T lymphocytes, which are responsible for humoral (antibody-mediated) and cell-mediated immunity, respectively. It is long-lasting and developing during several days, a period during which B lymphocytes produce and secrete antibodies and T lymphocytes either activate macrophages or directly destroy infected cells. The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during antigen presentation. Given the nature of the inflammation in IBD, research has been focussed for years on the role of the adaptive immune response and on mucosal effector T cells.

increase of Th1/Th2 infiltrate (the Th1/Th2 paradigm)

The Th1 response is characterized by the production of IFN-γ, which activates the bactericidal activities of macrophages, induces B cells to make opsonizing antibodies, and leads to "cell-mediated immunity". The Th2 response is characterized by the release of IL-4,

which results in the activation of B cells to make neutralizing antibodies, leading to "humoral immunity" (259). The inflammation in Crohn's disease is associated with a shift towards an excessive Th1 cell response in the mucosa, characterized by increased expression of Th1 cytokines such as IL-12, TNF-α and IFN-γ (260-263). Although polymorphisms in the TNF-α gene have been found in certain populations (264-266), and they may result in differences in the levels of TNF-α expression (267;268), the contributions of these polymorphisms are generally not thought to explain the prevalence of cytokine expression abnormalities found in Crohn's disease (89).

Most animal models of IBD are characterized by increased levels of Th1 cytokines (269). Practically all these models develop colonic rather than ileal inflammation, which is a more common feature of Crohn's disease (89). Two exceptions where ileitis is developed are $TNF^{\Delta ARE}$ mice and SAMP1/Yit mice, both Th1-dependent models which are currently the closest to mimicking Crohn's disease. $TNF^{\Delta ARE}$ mice produce increased concentrations of TNF-α and develop ileitis and arthritis (109). The SAMP1/Yit mouse model develops a spontaneous ileitis and, over many generations of brother-sister mating, mice also develop peri-anal disease (270;271). Transgenic models with an excessive effector T cell function that develop colitis include STAT-4 transgenic mice and CD40 ligand transgenic mice. Mice transgenic for STAT-4, a crucial transcription factor in the IL-12 signalling pathway, expressed strikingly increased nuclear STAT-4 levels in lamina propria $CD4^+$ T lymphocytes upon immunization and developed chronic transmural colitis characterized by infiltrates of mainly $CD4^+$ T lymphocytes of the Th1 phenotype (73). CD40 ligand is a member of the TNF family and is expressed primarily by activated T lymphocytes. Mice overexpressing CD40 ligand in T cells and some B cells develop multi-organ inflammation and severe colitis, with the ability to extend to ileitis (108).

Recently, Crohn's disease has also been associated with the presence of Th17 cells, a novel subset of $CD4^+$ T cells that are protective against extracellular microbes and may relate to Th1 cells regarding development and/or function (272). These cells have been linked with autoimmune disorders in mice but their properties in humans are only partially known. IL-17, characteristic for Th17 cells, has been detected in sera and colon biopsies of IBD patients (273;274). In addition, the presence of IL-17–producing $CD4^+$ T cells was demonstrated in the gut of subjects with Crohn's disease, some of which produce both IL-17 and IFN-γ (subtype Th17/Th1) (272). Differentiation of the Th17 subset is induced through activation of orphan nuclear receptor RORγt signalling, depending on the pleiotropic cytokine TGF-β, which is also linked to the development of $Foxp3^+$ regulatory T cells (see further) (275-277). A successful trial in Crohn's disease patients with anti-IL12p40 (subunit of both IL-12 and

IL-23 that drive Th1 and Th17 polarization, respectively) indicates that Crohn's disease may not be a classical Th1 T cell response but a Th1 and Th17 driven inflammation (278;279). Another T-cell derived cytokine that is overproduced in IBD is IL-21, necessary to up-regulate Th17-associated molecules during gut inflammation (280). Interestingly, IL-21-deficient mice were largely protected against both TNBS and DSS colitis.

Ulcerative colitis is not accompanied by an increase in Th1 cytokines, but has more of a Th2 character. This is insinuated by the finding of increased IL-5 secretion by lamina propria cells of ulcerative colitis patients (262). The major Th2 cytokine IL-4 was, however, not found in excess. An animal model resembling ulcerative colitis, i.e. oxazolone-induced colitis, is initially dominated by an IL-4 response and can be prevented by anti-IL-4 antibody administration (281). As colitis progresses, the IL-4 response declines and is replaced by IL-13, another Th2 cytokine. This latter cytokine is produced by natural killer T (NKT) cells, which can function as cytotoxic cells and can produce IFN-γ or IL-4/IL-13 under various circumstances (282). An increased production of IL-13 was also found in lamina propria cells isolated from inflamed tissue of ulcerative colitis patients, most likely originating from the NKT subset (283). Furthermore, lamina propria cells enriched for NKT cells from these patients could be shown to be cytotoxic for epithelial cells and such cytotoxicity was enhanced by IL-13. This cytokine was put forward as the key effector Th2 cytokine in ulcerative colitis that effects epithelial tight junctions, apoptosis, and cell restitution (169). On the basis of these data, a possible immunopathological mechanism that is thought to operate in ulcerative colitis is that antigen in the mucosal microflora activates NKT cells, that in turn cause cytolysis of epithelial cells and the characteristic ulcerations associated with the disease (284).

defective T cell apoptosis

The function of T cells is not only dependent on their activation, but also limited by apoptosis, which can be initiated by various factors. T-cell activation leads to cell proliferation, a process necessary to expand the respective immune cell population, as well as for cytokine production and cell differentiation. Lamina propria T cells from Crohn's disease patients often exhibit an increased cell proliferation and a decreased cell death (285-287). In addition, Crohn's disease T cells cycle faster, display aberrant activation of cell cycle regulatory proteins, and exhibit less caspase activity but more telomerase activity compared with normal cells (288). The resistance to apoptosis of lamina propria T cells probably occurs at the level of the mitochondria in the mitochondrial pathway to programmed cell death (286;288;289). Because apoptosis of activated T cells is an important mechanism of peripheral immune tolerance, this finding may explain why the inflammatory reaction in Crohn's disease patients

is resistant to resolution. Intervening in the process of T cell apoptosis has shown to be therapeutically effective in Crohn's disease patients (290).

In contrast, T cells from ulcerative colitis patients have a limited capacity to expand and this correlates with a strong activation induced apoptosis in these cells. Furthermore, ulcerative colitis T cells cycle slower, display more caspase activity, but have no telomerase activity (288). The caspase mediated pathway of apoptosis is believed to be impaired in ulcerative colitis lamina propria T cells, whereas the (activation induced) mitochondrial pathway is intact (289). Thus, regulation of programmed cell death seems fundamentally different in mucosal T cells from patients with Crohn's disease compared with patients with ulcerative colitis.

defects in antigen presenting cells

Antigen presenting cells (APCs) influence the decision by naïve T cells to differentiate into Th1 or Th2 cytokine-producing cells. Macrophages and DCs can do this through their own elaboration of cytokines or cell surface ligands which initiate a program of gene expression in T cells. The production of IL-12 by DCs or macrophages is required for IFN-γ expression and essential for Th1-type $CD4^+$ T cell differentiation in Crohn's disease (291). Interestingly, it appears that the IL-12p40 subunit, which is shared by the related cytokine IL-23, is constitutively expressed by DCs in the terminal ileum in mice, the area which is predominantly affected in Crohn's disease. This expression depended on the presence of luminal bacteria since it was absent in germ-free mice (292). Mice deficient for IL-23p19 (the other subunit of the IL-23 heterodimer) were highly susceptible for the development of experimental T cell-mediated TNBS colitis and showed even more severe colitis than wild-type mice by endoscopic and histologic criteria (293). Subsequent studies revealed that DCs from p19-deficient mice produce elevated levels of IL-12, and that IL-23 down-regulates IL-12 expression upon TLR ligation. Finally, *in vivo* blockade of IL-12p40 in IL-23-deficient mice rescued mice from lethal colitis. In human IBD, IL-12 expression is increased in APCs isolated from the lamina propria of Crohn's disease patients (294). IL-23, which is also produced by DCs and macrophages, is involved in the possible maintenance and/or expansion of Th17 cells (272). In a human anti-IL-12p40 trial of Crohn's disease patients, successful treatment correlated with dramatic decrease in both IL-12 and IL-23 production by macrophages (278;279).

disturbances at the level of immune regulation

Regulation of the acquired immune response is necessary to maintain immune homeostasis. Regulatory T cells (Tregs, sometimes referred to as suppressor T cells) are a specialized

subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens (295;296). Tregs can be functionally defined as T cells that produce IL-10 or TGF-β and mediate antigen-specific decreases in T-effector responses. They come in many forms, including those that express CD8 ($CD8^+$ Tregs), those that express CD4, CD25 and Foxp3 ($CD4^+CD25^+$ or naturally occurring Tregs), and other T cell types that have suppressive function. $CD4^+CD25^+$ Tregs are present not only in the spleen but also in the intestinal lamina propria (297). Next to T cells, B cells can also have regulatory effects on immune function (298). Disruption of immune homeostasis and tolerance to self-antigens results in unchecked immune responses and chronic intestinal inflammation.

regulatory T cells

In murine models, the protective role of Tregs has been absolutely shown, e.g. in the $CD4^+CD45RB^{hi}$ adoptive transfer model. Based on surface CD45RB expression, normal $CD4^+$ T cells can be separated into naïve cells, expressing high levels of CD45RB ($CD4^+CD45RB^{hi}$), and memory cells, expressing low levels of CD45RB ($CD4^+CD45RB^{lo}$). Adoptive transfer of $CD4^+CD45RB^{hi}$ cells into SCID or $RAG^{-/-}$ mice results in colitis and wasting (110;299). In this adoptive transfer model, colitis is abrogated by cotransfer of the $CD4^+CD45RB^{lo}$ T-cell subset, and administration of either anti-TGF-β or anti-IL-10 receptor antibody could reverse the prevention of colitis by these T cells (300;301). Also naturally occurring Tregs and a $CD4^+$ T-cell subset generated *in vitro* through IL-10 addition (T regulatory cells 1 or Tr1) can prevent and treat intestinal inflammation in the same colitis model (302;303). Other models that indicate involvement of a defective regulation in chronic intestinal inflammation are IL-10 deficient mice and TGF-β1 deficient mice. The mechanisms underlying the enhancement of a T cell response in IL-10 deficient mice are likely due in part to the lack of IL-10-regulatory function and in part to their innate immune defect (see earlier). These mice lack Tr1 cell activity, TGF-β/Smad signalling, and fail to inhibit proinflammatory gene expression in intestinal epithelial lymphocytes (304). TGF-β, a cytokine involved in growth and differentiation, has pleiotropic effects including inflammation, fibrosis, and immunosuppression, depending on tissue location and concentration. TGF-β1-deficient mice develop a multiorgan inflammation and die by 5 weeks of age, with or without the presence of a microbial flora (107;305;306). Blocking of TGF-β signalling in T cells resulted in the development of severe colitis with wasting and diarrhoea (115), and in the Gαi-2 model of colitis, TGF-β is also involved (307). A cytokine involved in the TGF-β-mediated induction of $Foxp3^+$ regulatory T cells is IL-2 (308;309), and IL-2-deficient as well as IL-2 receptor α deficient mice both are characterized by a decreased Treg population and develop intestinal inflammation (112;310;311). Interestingly, recent data accumulates that establishes the

existence of reciprocal developmental pathways for the generation of Foxp3^{+} Tregs and Th17 effector cells (275-277). Th17 differentiation is induced by TGF-β in the presence of proinflammatory cytokines such as IL-6 (312). In contrast, Treg cell differentiation is induced by TGF-β in the absence of IL-6 and/or presence of retinoic acid (313). Although these data show the importance of TGF-β in regulatory T cell function for the suppression of intestinal inflammation, the cytokine seems not crucial since in certain situations CD4^{+}CD25^{+} T cells are able to suppress intestinal inflammation by a mechanism independent of Treg cell-derived TGF-β1 (314).

Active IBD is associated with a decreased number of peripheral blood Treg cells and an only moderate expansion in intestinal lesions compared with acute diverticulitis (315). Since no functional defects were found in this and other studies in IBD patients (296;316), this study concluded that active IBD is characterized by a numerical defect in compensatory mechanisms. This theory is consistent with the observations that patients with IBD have defective generation of Tregs *in vivo* (317-319). This defect was also observed in a subset of unaffected family members of Crohn's disease patients but not in the control population without a family history of IBD. These results suggest that oral tolerance might be genetically regulated and that first-degree relatives of Crohn's disease patients might share the genetic defect without exhibiting the clinical symptoms. Candidates for such a genetic involvement could be IL-10 and TGF-β, given their role in the generation of the regulatory phenotype of T cells (302;303;320). Although there are signs of a diminished IL-10 synthesis in IBD (321), this is most likely not due to polymorphisms in the *IL-10* gene. Except one study reporting a lower frequency of a high IL-10 producer allele in ulcerative colitis patients (322), the majority of studies did not find any influence of *IL-10* genetic variants on the appearance of ulcerative colitis or Crohn's disease (323-325). Increased levels of TGF-β have been found in affected mucosa from IBD patients (326;327). This does however not lead to higher TGF-β signalling, because Smad7, an inhibitor of TGF-β1 signalling, is overexpressed in the gut of IBD patients and blocks this signalling pathway, resulting in a maintained NF-κB activation and a subsequent chronic production of proinflammatory cytokines (328;329). Smad7 is not transcriptionally regulated in human gut but its increase in patients with IBD is due to posttranscriptional acetylation and stabilization by a protein called p300, which prevents Smad7 ubiquitination and degradation in the proteasome (330).

regulatory B cells

Functionally characterized by their specific feature of antibody production, B cells were not considered in the past to be a major source of cytokines. This idea was revised by the demonstration that, like Th1/Th2 cells, B cells also possess the potential ability to produce

large amounts of several cytokines under certain inflammatory conditions (331). Through murine models of intestinal inflammation, the existence of a B cell subset was demonstrated that was induced in gut-associated lymphoid tissues and was characterized by CD1d up-regulation (332). This B cell subset appeared under a chronic inflammatory environment, produced IL-10, and suppressed progression of intestinal inflammation by down-regulating inflammatory cascades associated with IL-1 up-regulation and STAT-3 activation rather than by altering polarized T helper responses. This study for the first time indicated that B cells, by producing cytokines such as IL-10, can act as regulatory cells in immunologically mediated inflammatory reactions. Since this report, IL-10 producing regulatory B cells have been identified in several kinds of inflammations in humans and mice (333). Last year, a novel IL-12-producing regulatory B-cell subset was identified that develops under Th2-mediated intestinal inflammatory conditions and in the presence of IL-10 and is involved in the regulation of intestinal inflammation (334). Through the production of cytokines such as IL-10 and IL-12, B cells are believed to dampen T-cell activation (333). The involvement of regulatory B cells in ileitis and colitis has been proven in different animal models, including TCRα deficient mice, Gαi2 deficient mice, and the $CD4^{+}CD45RB^{hi}$ transfer model (335-339). Further studies are needed to define the role of regulatory B cells in human IBD.

disturbances at other levels

The discovery of new genetic factors in Crohn's disease and ulcerative colitis reveals novel possible implications of certain processes that are now beginning to be studied. With the identification of *ATG16L1* and *IRGM* as new susceptibility genes in Crohn's disease, the relevance of autophagy in IBD has gained interest (18;21-23). Autophagy is a unique process of membrane trafficking in which the membrane compartment (autophagosomes) engulfs both organelles and cytosolic macromolecules and delivers them to the lysosome for degradation (340). The appearance of bacteria in the cytoplasm of APCs triggers specialized autophagy or 'xenophagy'. This process shows a lot of agreements with the cytoplasmic recognition of bacterial antigens through intracellular PRRs such as NOD2. The TLR4 signalling pathway has already been identified as a sensor for autophagy/xenophagy associated with innate immunity (341). Alternatively, the major function of autophagy is the regulation of self-responses by clearing apoptotic cells (340;342). In this process, cytosolic proteins such as self-antigens and pathogens in host cells are constitutively and efficiently delivered for MHC class II presentation to activate the adaptive immune system (343;344). This role of autophagy in self-responses sheds a new light on the apparent autoimmune phenomena that occur in IBD (reviewed in (345)) There is reasonable evidence for a role of autoreactivity against colonic epithelial cells in ulcerative colitis, whereas immune reactivity

against intestinal flora is the prominent feature of Crohn’s disease. Taken together, autophagy may be an important process in IBD pathogenesis, inducing not only innate immune responses against exogenous antigens but also acquired immune responses against self-antigens.

Angiogenesis and inflammation are codependent processes (346). New blood vessels can maintain the chronic inflammatory state by transporting inflammatory cells to the site of inflammation and supplying nutrients and oxygen to the proliferating inflamed tissue. In turn, inflammatory mediators can also, either directly or indirectly, promote angiogenesis. Recently, angiogenesis has been described as a novel component of IBD. Morphological, phenotypic and functional evidence of potent angiogenic activity in both Crohn’s disease and ulcerative colitis mucosa was found, indicating that the local microvasculature undergoes an intense process of inflammation-dependent angiogenesis (347). Many factors are involved in this phenomenon, including growth factors, cytokines, chemokines, adhesion molecules, integrins, matrix-associated molecules, and signalling targets. These factors are produced by various vascular, inflammatory, and immune cell types that are involved in IBD pathology (348). Examination of the relationship between angiogenesis and inflammation in experimental colitis shows that initiating factors for these responses simultaneously increase as disease progresses and correlate in magnitude. Importantly, treatment with anti-angiogenic agents significantly reduced angiogenic activity and associated histological inflammation during experimental colitis (349;350). Vascular endothelial growth factor (VEGF) is an angiogenic molecule that has been found increased in Crohn's disease and ulcerative colitis mucosa and serum. A study on VEGF polymorphisms concluded that these are not implicated in susceptibility to IBD and do not predict serum VEGF levels, and thus increased serum VEGF and angiogenesis do not appear genetically determined (351). A recent study questioned whether a pro-inflammatory *E. coli* could regulate the expression of VEGF in human intestinal epithelial cells. Results demonstrated that a certain entero-adherent strain induced the up-regulation of bioactive VEGF in cultured human intestinal cells in both a time- and bacteria concentration-dependent manner. Thus, these results suggest a link between an entero-adherent, pro-inflammatory *E. coli* strain and angiogenesis (352).

conclusion

Although the pathogenesis of IBD is not completely revealed yet, extensive research on a pallet of topics has shown the involvement of an inappropriate host-microbe communication, which is likely to be the result of environmental triggers combined with defects situated at the level of the epithelial barrier, innate immune responses, adaptive immune responses, and/or immune regulation. Especially for Crohn's disease, a number of defects in mucosal immunity have been established. Environmental as well as genetic factors may permit the exposure of the lamina propria to commensal flora. Defects in innate immunity can result in the activation and accumulation of pathogenic $CD4^+$ T cells that mediates acute and chronic intestinal inflammation. Both deficient and excessive innate responses are proposed as mechanisms for this pathogenic T-cell activation, featured by persistence of the exogenous stimulus and an exaggerated secondary and compensatory immune response in the first case and unnecessary and excessive generation of proinflammatory molecules leading to damage of the host tissue or an unneeded activation of the immune system in the second case. Furthermore, dysregulated adaptive immune responses themselves or disturbed immune regulation can also take part in the pathogenesis of IBD, but a primary *versus* a secondary (inflammation-dependent) involvement of these processes needs more clarification. This last distinction needs to be made as well for new emerging processes such as autophagy and angiogenesis.

The ultimate goal of medical research is to understand disease mechanisms and to apply the knowledge of these mechanisms in therapeutic strategies. The numerical studies on IBD pathogenesis have already translated into clinical benefit for patients in the form of biologic therapy. Novel therapeutic approaches for IBD focus on manipulating enteric microflora, repairing barrier function, and targeting the different components of the immune response (353). Future studies will identify specific markers that permit the stratification of patients based on distinct pathogenic mechanisms.

references

(1) Crohn BB, Ginzburg L, Oppenheimer GD. Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. By Burril B. Crohn, Leon Ginzburg, and Gordon D. Oppenheimer. Journal of the American Medical Association 1984; 251(1):73-79.

(2) Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nature Clinical Practice Gastroenterology & Hepatology 2006; 3(7):390-407.

(3) Hanauer SB. Inflammatory bowel disease: Epidemiology, pathogenesis, and therapeutic opportunities. Inflammatory Bowel Diseases 2006; 12:S3-S9.

(4) Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut 2006; 55(6):749-753.

(5) Day DW, Jass JR, Price AB, Shepherd NA, Sloan JM, Talbot IC et al. Morson and Dawson's Gastrointestinal Pathology. 4 ed. Oxford, UK: Blackwell Science Ltd, 2003.

(6) Loftus EV. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology 2004; 126(6):1504-1517.

(7) Lakatos PL. Recent trends in the epidemiology of inflammatory bowel diseases: Up or down? World Journal of Gastroenterology 2006; 12(38):6102-6108.

(8) Annese V, Valvano MR, Palmieri O, Latiano A, Bossa F, Andriulli A. Multidrug resistance 1 gene in inflammatory bowel disease: A meta-analysis. World Journal of Gastroenterology 2006; 12(23):3636-3644.

(9) Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nature Reviews Immunology 2003; 3(7):521-533.

(10) Probert CSJ, Jayanthi V, Wicks ACB, Mayberry JF. Mortality from Crohns-Disease in Leicestershire, 1972-1989 - An Epidemiologic Community Based Study. Gut 1992; 33(9):1226-1228.

(11) Carr I, Mayberry JF. The effects of migration on ulcerative colitis: A three-year prospective study among Europeans and first- and second-generation South Asians in Leicester (1991-1994). American Journal of Gastroenterology 1999; 94(10):2918-2922.

(12) Montgomery SM, Morris DL, Pounder RE, Wakefield AJ. Asian ethnic origin and the risk of inflammatory bowel disease. European Journal of Gastroenterology & Hepatology 1999; 11(5):543-546.

(13) Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nature Genetics 2008; 40(8):955-962.

(14) Newman B, Siminovitch KA. Recent advances in the genetics of inflammatory bowel disease. Current Opinion in Gastroenterology 2005; 21(4):401-407.

(15) Schreiber S, Rosenstiel P, Albrecht M, Hampe J, Krawczak M. Genetics of Crohn disease, an archetypal inflammatory barrier disease. Nature Reviews Genetics 2005; 6(5):376-388.

(16) Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006; 314(5804):1461-1463.

(17) Libioulle C, Louis E, Hansoul S, Sandor C, Farnir F, Franchimont D et al. Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. Plos Genetics 2007; 3(4).

(18) Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nature Genetics 2007; 39(5):596-604.

(19) Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 2007; 447(7145):661-678.

(20) Fisher SA, Tremelling M, Anderson CA, Gwilliam R, Bumpstead S, Prescott NJ et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. Nature Genetics 2008; 40(6):710-712.

(21) Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nature Genetics 2007; 39(2):207-211.

(22) Prescott NJ, Fisher SA, Franke A, Hampe J, Onnie CM, Soars D et al. A nonsynonymous SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and 1BD5. Gastroenterology 2007; 132(5):1665-1671.

(23) Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nature Genetics 2007; 39(7):830-832.

(24) Yamazaki K, McGovern D, Ragoussis J, Paolucci M, Butler H, Jewell D et al. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. Human Molecular Genetics 2005; 14(22):3499-3506.

(25) Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001; 411(6837):599-603.

(26) Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001; 411(6837):603-606.

(27) Peltekova VD, Wintle RF, Rubin LA, Amos CI, Huang QQ, Gu XJ et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. Nature Genetics 2004; 36(5):471-475.

(28) Stoll M, Corneliussen B, Costello CM, Waetzig GH, Mellgard B, Koch WA et al. Genetic variation in DLG5 is associated with inflammatory bowel disease. Nature Genetics 2004; 36(5):476-480.

(29) Friedrichs F, Henckaerts L, Vermeire S, Kucharzik T, Seehafer T, Moller-Krull M et al. The Crohn's disease susceptibility gene DLG5 as a member of the CARD interaction network. Journal of Molecular Medicine 2008; 86(4):423-432.

(30) Panwala CM, Jones JC, Viney JL. A novel model of inflammatory bowel disease: Mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. Journal of Immunology 1998; 161(10):5733-5744.

(31) Schwab M, Schaeffeler E, Marx C, Fromm MF, Kaskas B, Metzler J et al. Association between the C3435T MDR1 gene polymorphism and susceptibility for ulcerative colitis. Gastroenterology 2003; 124(1):26-33.

(32) Lakatos PL, Szamosi T, Lakatos L. Smoking in inflammatory bowel diseases: good, bad or ugly? World Journal of Gastroenterology 2007; 13(46):6134-6139.

(33) Bridger S, Lee JCW, Bjarnason I, Jones JEL, Macpherson AJ. In siblings with similar genetic susceptibility for inflammatory bowel disease, smokers tend to develop Crohn's disease and non-smokers develop ulcerative colitis. Gut 2002; 51(1):21-25.

(34) van Dijk APM, Meijssen MAC, Brouwer AJBW, Hop WCJ, van Bergeijk JD, Feyerabend C et al. Transdermal nicotine inhibits interleukin 2 synthesis by mononuclear cells derived from healthy volunteers. European Journal of Clinical Investigation 1998; 28(8):664-671.

(35) Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S et al. Nicotinic acetylcholine receptor alpha 7 subunit is an essential regulator of inflammation. Nature 2003; 421(6921):384-388.

(36) Motley RJ, Rhodes J, Williams G, Tavares IA, Bennett A. Smoking, Eicosanoids and Ulcerative-Colitis. Journal of Pharmacy and Pharmacology 1990; 42(4):288-289.

(37) Danese S. Inflammation and the mucosal microcirculation in inflammatory bowel disease: the ebb and flow. Current Opinion in Gastroenterology 2007; 23(4):384-389.

(38) Suenaert P, Bulteel V, Den Hond E, Hiele M, Peeters M, Monsuur F et al. The effects of smoking and indomethacin on small intestinal permeability. Alimentary Pharmacology & Therapeutics 2000; 14(6):819-822.

(39) Zijlstra FJ, Srivastava ED, Rhodes M, Vandijk APM, Fogg F, Samson HJ et al. Effect of Nicotine on Rectal Mucus and Mucosal Eicosanoids. Gut 1994; 35(2):247-251.

(40) Finnie IA, Campbell BJ, Taylor BA, Milton JD, Sadek SK, Yu LG et al. Stimulation of colonic mucin synthesis by corticosteroids and nicotine. Clinical Science 1996; 91(3):359-364.

(41) Richardson CE, Morgan JM, Jasani B, Green JT, Rhodes J, Williams GT et al. Effect of smoking and transdermal nicotine on colonic nicotinic acetylcholine receptors in ulcerative colitis. Qjm-An International Journal of Medicine 2003; 96(1):57-65.

(42) Rutgeerts P, Dhaens G, Hiele M, Geboes K, Vantrappen G. Appendectomy Protects Against Ulcerative-Colitis. Gastroenterology 1994; 106(5):1251-1253.

(43) Russel MG, Dorant E, Brummer RJM, VandeKruijs MA, Muris JW, Bergers JM et al. Appendectomy and the risk of developing ulcerative colitis or Crohn's disease: Results of a large case-control study. Gastroenterology 1997; 113(2):377-382.

(44) Andersson RE, Olaison G, Tysk C, Ekbom A. Appendectomy is followed by increased risk of Crohn's disease. Gastroenterology 2003; 124(1):40-46.

(45) Koutroubakis IE, Vlachonikolis IG, Kouroumalis EA. Role of appendicitis and appendectomy in the pathogenesis of ulcerative colitis: A critical review. Inflammatory Bowel Diseases 2002; 8(4):277-286.

(46) Sonntag B, Stolze B, Heinecke A, Luegering A, Heidemann J, Lebiedz P et al. Preterm birth but not mode of delivery is associated with an increased risk of developing inflammatory bowel disease later in life. Inflammatory Bowel Diseases 2007; 13(11):1385-1390.

(47) Halfvarson J, Jess T, Magnuson A, Montgomery SM, Orholm M, Tysk C et al. Environmental factors in inflammatory bowel disease: A co-twin control study of a Swedish-Danish twin population. Inflammatory Bowel Diseases 2006; 12(10):925-933.

(48) Wilson AG. Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. Journal of Periodontology 2008; 79(8 Suppl):1514-1519.

(49) Dhir M, Montgomery EA, Glockner SC, Schuebel KE, Hooker CM, Herman JG et al. Epigenetic regulation of WNT signaling pathway genes in inflammatory bowel disease (IBD) associated neoplasia. Journal of Gastrointestinal Surgery 2008; 12(10):1745-1753.

(50) Kim SC, Tonkonogy SL, Albright CA, Tsang J, Balish EJ, Braun J et al. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. Gastroenterology 2005; 128(4):891-906.

(51) Kim SC, Tonkonogy SL, Karrasch T, Jobin C, Sartor RB. Dual-association of gnotobiotic IL-10-/- mice with 2 nonpathogenic commensal bacteria induces aggressive pancolitis. Inflammatory Bowel Diseases 2007; 13(12):1457-1466.

(52) Kim SC, Tonkonogy SL, Albright CA, Sartor RB. Different host genetic backgrounds determine disease phenotypes induced by selective bacterial colonization. Gastroenterology 2005; 128(4):A512.

(53) Marteau P. Relevance of bacteria in the pathogenesis of inflammatory bowel disease. FALK SYMPOSIUM 2007; 153:21-29.

(54) Dieleman LA, Goerres MS, Arends A, Sprengers D, Torrice C, Hoentjen F et al. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. Gut 2003; 52(3):370-376.

(55) Schultz M, Veltkamp C, Dieleman LA, Grenther WB, Wyrick PB, Tonkonogy SL et al. Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. Inflammatory Bowel Diseases 2002; 8(2):71-80.

(56) Bamias G, Okazawa A, Rivera-Nieves J, Arseneau KO, De la Rue SA, Pizarro TT et al. Commensal bacteria exacerbate intestinal inflammation but are not essential for the development of murine ileitis. Journal of Immunology 2007; 178(3):1809-1818.

(57) Elson CO, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG, Weaver CT. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. Immunological Reviews 2005; 206:260-276.

(58) Rath HC, Herfarth HH, Ikeda JS, Grenther WB, Hamm TE, Balish E et al. Normal luminal bacteria, especially bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta(2) microglobulin transgenic rats. Journal of Clinical Investigation 1996; 98(4):945-953.

(59) Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infection and Immunity 1998; 66(11):5224-5231.

(60) Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 1999; 116(5):1107-1114.

(61) Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 2001; 121(3):580-591.

(62) Matsumoto S, Okabe Y, Setoyama H, Takayama K, Ohtsuka J, Funahashi H et al. Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. Gut 1998; 43(1):71-78.

(63) Sundberg JP, Elson CO, Bedigian H, Birkenmeier EH. Spontaneous, Heritable Colitis in A New Substrain of C3H/Hej Mice. Gastroenterology 1994; 107(6):1726-1735.

(64) Contractor NV, Bassiri H, Reya T, Park AY, Baumgart DC, Wasik MA et al. Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnotobiotic IL-2-deficient mice. Journal of Immunology 1998; 160(1):385-394.

(65) Dianda L, Hanby AM, Wright NA, Sebesteny A, Hayday AC, Owen MJ. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. American Journal of Pathology 1997; 150(1):91-97.

(66) Aranda R, Sydora BC, McAllister PL, Binder SW, Yang HY, Targan SR et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4(+), CD45RB(high) T cells to SCID recipients. Journal of Immunology 1997; 158(7):3464-3473.

(67) Banner KH, Cattaneo C, Le Net JL, Popovic A, Collins D, Gale JD. Macroscopic, microscopic and biochemical characterisation of spontaneous colitis in a transgenic mouse, deficient in the multiple drug resistance 1a gene. British Journal of Pharmacology 2004; 143(5):590-598.

(68) Verdu EF, Bercik P, Cukrowska B, Farre-castany MA, Bouzourene H, Saraga E et al. Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice. Clinical and Experimental Immunology 2000; 120(1):46-50.

(69) Rath HC, Schultz M, Freitag R, Dieleman LA, Li F, Linde HJ et al. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. Infection and Immunity 2001; 69(4):2277-2285.

(70) Setoyama H, Imaoka A, Ishikawa H, Umesaki Y. Prevention of gut inflammation by Bifidobacterium in dextran sulfate-treated gnotobiotic mice associated with Bacteroides strains isolated from ulcerative colitis patients. Microbes and Infection 2003; 5(2):115-122.

(71) Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology 2004; 126(2):520-528.

(72) Kitajima S, Morimoto M, Sagara E, Shimizu C, Ikeda Y. Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. Experimental Animals 2001; 50(5):387-395.

(73) Wirtz S, Finotto S, Kanzler S, Lohse AW, Blessing M, Lehr HA et al. Cutting edge: Chronic intestinal inflammation in STAT-4 transgenic mice: Characterization of disease and adoptive transfer by TNF- plus IFN-gamma-producing CD4(+) T cells that respond to bacterial antigens. Journal of Immunology 1999; 162(4):1884-1888.

(74) Kang SS, Bloom SM, Norian LA, Geske MJ, Flavell RA, Stappenbeck TS et al. An antibiotic-responsive mouse model of fulminant ulcerative colitis. PLoS Medicine 2008; 5(3):e41.

(75) Sartor RB, Blumberg RS, Braun J, Elson CO, Mayer LF. CCFA microbial-host interactions workshop: highlights and key observations. Inflammatory Bowel Diseases 2007; 13(5):600-619.

(76) Favier C, Neut C, Mizon C, Cortot A, Colombel JF, Mizon J. Fecal beta-D-galactosidase production and Bifidobacteria are decreased in Crohn's disease. Digestive Diseases and Sciences 1997; 42(4):817-822.

(77) Lepage P, Seksik P, Sutren M, de la Cochetiere MF, Jian R, Marteau P et al. Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. Inflammatory Bowel Diseases 2005; 11(5):473-480.

(78) Swidsinski A, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. Journal of Clinical Microbiology 2005; 43(7):3380-3389.

(79) Sokol H, Seksik P, Rigottier-Gois L, Lay C, Lepage P, Podglajen I et al. Specificities of the fecal microbiota in inflammatory bowel disease. Inflammatory Bowel Diseases 2006; 12(2):106-111.

(80) Swidsinski A, Loening-Baucke V, Vaneechoutte M, Doerffel Y. Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. Inflammatory Bowel Diseases 2008; 14(2):147-161.

(81) Kleessen B, Kroesen AJ, Buhr HJ, Blaut M. Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls. Scandinavian Journal of Gastroenterology 2002; 37(9):1034-1041.

(82) Swidsinski A, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M et al. Mucosal flora in inflammatory bowel disease. Gastroenterology 2002; 122(1):44-54.

(83) Swidsinski A, Loening-Baucke V, Lochs H, Hale LP. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World Journal of Gastroenterology 2005; 11(8):1131-1140.

(84) Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Folsch UR et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut 2004; 53(5):685-693.

(85) Harper PH, Lee ECG, Kettlewell MGW, Bennett MK, Jewell DP. Role of the Fecal Stream in the Maintenance of Crohns Colitis. Gut 1985; 26(3):279-284.

(86) Rutgeerts P, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R et al. Effect of Fecal Stream Diversion on Recurrence of Crohns-Disease in the Neoterminal Ileum. Lancet 1991; 338(8770):771-774.

(87) Winslet MC, Allan A, Poxon V, Youngs D, Keighley MRB. Fecal Diversion for Crohns Colitis - A Model to Study the Role of the Fecal Stream in the Inflammatory Process. Gut 1994; 35(2):236-242.

(88) Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: Antibiotics, probiotics, and prebiotics. Gastroenterology 2004; 126(6):1620-1633.

(89) Cobrin GM, Abreu MT. Defects in mucosal immunity leading to Crohn's disease. Immunological Reviews 2005; 206:277-295.

(90) Owen RL, Jones AL. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology 1974; 66(2):189-203.

(91) Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nature Immunology 2001; 2(4):361-367.

(92) Keita AV, Gullberg E, Ericson AC, Salim SY, Wallon C, Kald A et al. Characterization of antigen and bacterial transport in the follicle-associated epithelium of human ileum. Laboratory Investigation 2006; 86(5):504-516.

(93) Fukushima K, Yonezawa H, Fiocchi T. Inflammatory bowel disease-associated gene expression in intestinal epithelial cells by differential cDNA screening and mRNA display. Inflammatory Bowel Diseases 2003; 9(5):290-301.

(94) Lawrance IC, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. Human Molecular Genetics 2001; 10(5):445-456.

(95) Goyette P, Labbe C, Trinh TT, Xavier RJ, Rioux JD. Molecular pathogenesis of inflammatory bowel disease: Genotypes, phenotypes and personalized medicine. Annals of Medicine 2007; 39(3):177-199.

(96) Rudolph U, Finegold MJ, Rich SS, Harriman GR, Srinivasan Y, Brabet P et al. Ulcerative-Colitis and Adenocarcinoma of the Colon in G-Alpha(I2)-Deficient Mice. Nature Genetics 1995; 10(2):143-150.

(97) Hermiston ML, Gordon JI. Inflammatory Bowel-Disease and Adenomas in Mice Expressing A Dominant-Negative N-Cadherin. Science 1995; 270(5239):1203-1207.

(98) Wong MH, Rubinfeld B, Gordon JI. Effects of forced expression of an NH2-terminal truncated beta-catenin on mouse intestinal epithelial homeostasis. Journal of Cell Biology 1998; 141(3):765-777.

(99) Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L et al. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. Cell 1998; 93(2):189-201.

(100) Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990; 98(3):694-702.

(101) Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006; 131(1):117-129.

(102) Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity 1999; 10(1):39-49.

(103) Welte T, Zhang SSM, Wang T, Zhang ZY, Hesslein DGT, Yin ZN et al. STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: A critical role of STAT3 in innate immunity. Proceedings of the National Academy of Sciences of the United States of America 2003; 100(4):1879-1884.

(104) Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP et al. Failure to regulate TNF-induced NF-kappa B and cell death responses in A20-deficient mice. Science 2000; 289(5488):2350-2354.

(105) Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993; 75(2):263-274.

(106) Tomczak MF, Erdman SE, Poutahidis T, Rogers AB, Holcombe H, Plank B et al. NF-kappa B is required within the innate immune system to inhibit microflora-induced colitis and expression of IL-12 p40(1). Journal of Immunology 2003; 171(3):1484-1492.

(107) Kulkarni AB, Ward JM, Yaswen L, Mackall CL, Bauer SR, Huh CG et al. Transforming Growth-Factor-Beta-1 Null Mice - An Animal-Model for Inflammatory Disorders. American Journal of Pathology 1995; 146(1):264-275.

(108) Clegg CH, Rulffes JT, Haugen HS, Hoggatt IH, Aruffo A, Durham SK et al. Thymus dysfunction and chronic inflammatory disease in gp39 transgenic mice. International Immunology 1997; 9(8):1111-1122.

(109) Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity 1999; 10(3):387-398.

(110) Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. Phenotypically Distinct Subsets of Cd4(+) T-Cells Induce Or Protect from Chronic Intestinal Inflammation in C - B-17 Scid Mice. International Immunology 1993; 5(11):1461-1471.

(111) Sadlack B, Lohler J, Schorle H, Klebb G, Haber H, Sickel E et al. Generalized Autoimmune-Disease in Interleukin-2-Deficient Mice Is Triggered by An Uncontrolled Activation and Proliferation of Cd4(+) T-Cells. European Journal of Immunology 1995; 25(11):3053-3059.

(112) Willerford DM, Chen JZ, Ferry JA, Davidson L, Ma A, Alt FW. Interleukin-2 Receptor-Alpha Chain Regulates the Size and Content of the Peripheral Lymphoid Compartment. Immunity 1995; 3(4):521-530.

(113) Mombaerts P, Mizoguchi E, Grusby MJ, Glimcher LH, Bhan AK, Tonegawa S. Spontaneous Development of Inflammatory Bowel-Disease in T-Cell Receptor Mutant Mice. Cell 1993; 75(2):275-282.

(114) Mizoguchi A, Mizoguchi E, Chiba C, Spiekermann GM, Tonegawa S, NaglerAnderson C et al. Cytokine imbalance and autoantibody production in T cell receptor-alpha mutant mice with inflammatory bowel disease. Journal of Experimental Medicine 1996; 183(3):847-856.

(115) Gorelik L, Flavell RA. Abrogation of TGF beta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity 2000; 12(2):171-181.

(116) Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JJ, Mizel DE et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nature Cell Biology 1999; 1(5):260-266.

(117) Van der Sluis M, Bouma J, Vincent A, Velcich A, Carraway KL, Buller HA et al. Combined defects in epithelial and immunoregulatory factors exacerbate the pathogenesis of inflammation: mucin 2-interleukin 10-deficient mice. Laboratory Investigation 2008; 88(6):634-642.

(118) Shkoda A, Werner T, Daniel H, Gunckel M, Rogler G, Haller D. Differential protein expression profile in the intestinal epithelium from patients with inflammatory bowel disease. Journal of Proteome Research 2007; 6(3):1114-1125.

(119) Werner T, Haller D. Intestinal epithelial cell signalling and chronic inflammation: From the proteome to specific molecular mechanisms. Mutation Research 2007; 622(1-2):42-57.

(120) Specian RD, Neutra MR. Mechanism of Rapid Mucus Secretion in Goblet Cells Stimulated by Acetylcholine. Journal of Cell Biology 1980; 85(3):626-640.

(121) Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky DK. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. Gastroenterology 1995; 109(2):516-523.

(122) Wadolkowski EA, Laux DC, Cohen PS. Colonization of the Streptomycin-Treated Mouse Large-Intestine by A Human Fecal Escherichia-Coli Strain - Role of Adhesion to Mucosal Receptors. Infection and Immunity 1988; 56(5):1036-1043.

(123) Swidsinski A, Sydora BC, Doerffel Y, Loening-Baucke V, Vaneechoutte M, Lupicki M et al. Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. Inflammatory Bowel Diseases 2007; 13(8):963-970.

(124) Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. Gut 2008; 57(6):764-771.

(125) McCormick DA, Horton LW, Mee AS. Mucin depletion in inflammatory bowel disease. Journal of Clinical Pathology 1990; 43(2):143-146.

(126) Shirazi T, Longman RJ, Corfield AP, Probert CSJ. Mucins and inflammatory bowel disease. Postgraduate Medical Journal 2000; 76(898):473-478.

(127) Pullan RD, Thomas GAO, Rhodes M, Newcombe RG, Williams GT, Allen A et al. Thickness of Adherent Mucus Gel on Colonic Mucosa in Humans and Its Relevance to Colitis. Gut 1994; 35(3):353-359.

(128) Clamp JR, Fraser G, Read AE. Study of the Carbohydrate Content of Mucus Glycoproteins from Normal and Diseased Colons. Clinical Science 1981; 61(2):229-234.

(129) Raouf AH, Tsai HH, Parker N, Hoffman J, Walker RJ, Rhodes JM. Sulfation of Colonic and Rectal Mucin in Inflammatory Bowel-Disease - Reduced Sulfation of Rectal Mucus in Ulcerative-Colitis. Clinical Science 1992; 83(5):623-626.

(130) Campbell BJ, Finnie IA, Hounsell EF, Rhodes JM. Direct Demonstration of Increased Expression of Thomsen-Friedenreich (Tf) Antigen in Colonic Adenocarcinoma and Ulcerative-Colitis Mucin and Its Concealment in Normal Mucin. Journal of Clinical Investigation 1995; 95(2):571-576.

(131) Parker N, Tsai HH, Ryder SD, Raouf AH, Rhodes JM. Increased Rate of Sialylation of Colonic Mucin by Cultured Ulcerative-Colitis Mucosal Explants. Digestion 1995; 56(1):52-56.

(132) Tytgat KM, van der Wal JW, Einerhand AW, Buller HA, Dekker J. Quantitative analysis of MUC2 synthesis in ulcerative colitis. Biochemical and Biophysical Research Communications 1996; 224(2):397-405.

(133) Van Klinken BJ, van der Wal JW, Einerhand AW, Buller HA, Dekker J. Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis. Gut 1999; 44(3):387-393.

(134) Buisine MP, Desreumaux P, Debailleul V, Gambiez L, Geboes K, Ectors N et al. Abnormalities in mucin gene expression in Crohn's disease. Inflammatory Bowel Diseases 1999; 5(1):24-32.

(135) Ehehalt R, Wagenblast J, Erben G, Lehmann WD, Hinz U, Merle U et al. Phosphatidylcholine and lysophosphatidylcholine in intestinal mucus of ulcerative colitis patients. A quantitative approach by nanoelectrospray-tandem mass spectrometry. Scandinavian Journal of Gastroenterology 2004; 39(8):737-742.

(136) Sartor RB. The influence of normal microbial flora on the development of chronic mucosal inflammation. Research in Immunology 1997; 148(8-9):567-576.

(137) van der Waaij LA, Harmsen HJM, Madjipour M, Kroese FGM, Zwiers M, van Dullemen HM et al. Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: Commensal bacteria live in suspension and have no direct contact with epithelial cells. Inflammatory Bowel Diseases 2005; 11(10):865-871.

(138) Bojarski C, Gitter AH, Bendfeldt K, Mankertz J, Schmitz H, Wagner S et al. Permeability of human HT-29/B6 colonic epithelium as a function of apoptosis. Journal of Physiology-London 2001; 535(2):541-552.

(139) Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. Journal of Immunology 2003; 171(11):6164-6172.

(140) Bojarski C, Weiske J, Schoneberg T, Schroder W, Mankertz J, Schulzke JD et al. The specific fates of tight junction proteins in apoptotic epithelial cells. Journal of Cell Science 2004; 117(10):2097-2107.

(141) Rozga J. The role of reactive oxygen metabolites in the pathogenesis of ulcerative colitis: a speculative synthesis. Materia Medica Polona 1989; 21(4):263-268.

(142) Yamada T, Grisham MB. Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation. Klinische Wochenschrift 1991; 69(21-23):988-994.

(143) LihBrody L, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E et al. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. Digestive Diseases and Sciences 1996; 41(10):2078-2086.

(144) Kruidenier L, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. Journal of Pathology 2003; 201(1):28-36.

(145) Cuvelier CA, Quatacker J, Mielants H, De Vos M, Veys E, Roels HJ. M-cells are damaged and increased in number in inflamed human ileal mucosa. Histopathology 1994; 24(5):417-426.

(146) Keita A, Salim S, Jiang T, Yang PC, Franzen L, Soderkvist P et al. Increased uptake of non-pathogenic E. coli via the follicle-associated epithelium in longstanding ileal Crohn's disease. Journal of Pathology 2008; 215(2):135-144.

(147) Kitajima S, Takuma S, Morimoto M. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. Experimental Animals 1999; 48(3):137-143.

(148) Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular-Localization of the Multidrug-Resistance Gene-Product P-Glycoprotein in Normal Human-Tissues. Proceedings of the National Academy of Sciences of the United States of America 1987; 84(21):7735-7738.

(149) Klimecki WT, Futscher BW, Grogan TM, Dalton WS. P-Glycoprotein Expression and Function in Circulating Blood-Cells from Normal Volunteers. Blood 1994; 83(9):2451-2458.

(150) Ohman L, Franzen L, Rudolph U, Harriman GR, Hornquist EH. Immune activation in the intestinal mucosa before the onset of colitis in G alpha i2-deficient mice. Scandinavian Journal of Immunology 2000; 52(1):80-90.

(151) Vidrich A, Buzan JM, Barnes S, Reuter BK, Skaar K, Ilo C et al. Altered epithelial cell lineage allocation and global expansion of the crypt epithelial stem cell population are associated with ileitis in SAMP1/YitFc mice. American Journal of Pathology 2005; 166(4):1055-1067.

(152) Hollander D. Crohns-Disease - A Permeability Disorder of the Tight Junction. Gut 1988; 29(12):1621-1624.

(153) Hollander D. Intestinal permeability, leaky gut, and intestinal disorders. Current Gastroenterology Reports 1999; 1(5):410-416.

(154) Katz KD, Hollander D, Vadheim CM, McElree C, Delahunty T, Dadufalza VD et al. Intestinal Permeability in Patients with Crohns-Disease and Their Healthy Relatives. Gastroenterology 1989; 97(4):927-931.

(155) Murphy MS, Eastham EJ, Nelson R, Pearson ADJ, Laker MF. Intestinal Permeability in Crohns-Disease. Archives of Disease in Childhood 1989; 64(3):321-325.

(156) Teahon K, Smethurst P, Levi AJ, Menzies IS, Bjarnason I. Intestinal Permeability in Patients with Crohns-Disease and Their 1St Degree Relatives. Gut 1992; 33(3):320-323.

(157) Hollander D. Permeability in Crohns-Disease - Altered Barrier Functions in Healthy Relatives. Gastroenterology 1993; 104(6):1848-1851.

(158) Munkholm P, Langholz E, Hollander D, Thornberg K, Orholm M, Katz KD et al. Intestinal Permeability in Patients with Crohns-Disease and Ulcerative-Colitis and Their First Degree Relatives. Gut 1994; 35(1):68-72.

(159) Peeters M, Geypens B, Claus D, Nevens H, Ghoos Y, Verbeke G et al. Clustering of increased small intestinal permeability in families with Crohn's disease. Gastroenterology 1997; 113(3):802-807.

(160) Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal Permeability and the Prediction of Relapse in Crohns-Disease. Lancet 1993; 341(8858):1437-1439.

(161) D'Inca R, Di Leo V, Corrao G, Martines D, D'Odorico A, Mestriner C et al. Intestinal permeability test as a predictor of clinical course in Crohn's disease. American Journal of Gastroenterology 1999; 94(10):2956-2960.

(162) Arnott IDR, Kingstone K, Ghosh S. Abnormal intestinal permeability predicts relapse in inactive Crohn disease. Scandinavian Journal of Gastroenterology 2000; 35(11):1163-1169.

(163) Irvine EJ, Marshall JK. Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. Gastroenterology 2000; 119(6):1740-1744.

(164) Breslin NP, Nash C, Hilsden RJ, Hershfield NB, Price LM, Meddings JB et al. Intestinal permeability is increased in a proportion of spouses of patients with Crohn's disease. American Journal of Gastroenterology 2001; 96(10):2934-2938.

(165) Yuhan R, Koutsouris A, Savkovic SD, Hecht G. Enteropathogenic Escherichia coli-induced myosin light chain phosphorylation alters intestinal epithelial permeability. Gastroenterology 1997; 113(6):1873-1882.

(166) Scott KGE, Meddings JB, Kirk DR, Lees-Miller SP, Buret AG. Intestinal infection with Giardia spp. reduces epithelial barrier function in a myosin light chain kinase-dependent fashion. Gastroenterology 2002; 123(4):1179-1190.

(167) Prasad S, Mingrino R, Kaukinen K, Hayes KL, Powell RM, MacDonald TT et al. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. Laboratory Investigation 2005; 85(9):1139-1162.

(168) Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut 2007; 56(1):61-72.

(169) Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B et al. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology 2005; 129(2):550-564.

(170) Zolotarevsky Y, Hecht G, Koutsouris A, Gonzalez DE, Quan C, Tom J et al. A membrane-permeant peptide that inhibits MLC kinase restores barrier function in in vitro models of intestinal disease. Gastroenterology 2002; 123(1):163-172.

(171) Clayburgh DR, Barrett TA, Tang YM, Meddings JB, Van Eldik LJ, Watterson DM et al. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. Journal of Clinical Investigation 2005; 115(10):2702-2715.

(172) Wang FJ, Graham WV, Wang YM, Witkowski ED, Schwarz BT, Turner JR. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. American Journal of Pathology 2005; 166(2):409-419.

(173) Clayburgh DR, Musch MW, Leitges M, Fu YX, Turner JR. Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. Journal of Clinical Investigation 2006; 116(10):2682-2694.

(174) Wang FJ, Schwarz BT, Graham WV, Wang YM, Su LP, Clayburgh DR et al. IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction. Gastroenterology 2006; 131(4):1153-1163.

(175) Clayburgh DR, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. Laboratory Investigation 2004; 84(3):282-291.

(176) Blair SA, Kane SV, Clayburgh DR, Turner JR. Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. Laboratory Investigation 2006; 86(2):191-201.

(177) Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. Gastroenterology 2004; 127(1):224-238.

(178) Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology 2007; 132(4):1359-1374.

(179) Hooper LV, Wong MH, Thelin A, Hansson L, Falk PC, Gordon JI. Molecular analysis of commensal host-microbial relations hips in the intestine. Science 2001; 291(5505):881-884.

(180) Otte JM, Podolsky DK. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. American Journal of Physiology-Gastrointestinal and Liver Physiology 2004; 286(4):G613-G626.

(181) Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004; 118(2):229-241.

(182) Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005; 307(5717):1915-1920.

(183) Rao R. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Frontiers in Bioscience 2008; 13:7210-7226.

(184) Mumy KL, McCormick BA. Events at the Host-Microbial Interface of the Gastrointestinal Tract - II. Role of the intestinal epithelium in pathogen-induced inflammation. American Journal of Physiology-Gastrointestinal and Liver Physiology 2005; 288(5):G854-G859.

(185) Comalada M, Peppelenbosch MP. Impaired innate immunity in Crohn's disease. Trends in Molecular Medicine 2006; 12(9):397-399.

(186) Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. Journal of Biological Chemistry 2001; 276(7):4812-4818.

(187) Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. Journal of Biological Chemistry 2002; 277(44):41701-41705.

(188) Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S et al. Crohn's disease and the NOD2 gene: A role for paneth cells. Gastroenterology 2003; 125(1):47-57.

(189) Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. Gastroenterology 2003; 124(4):993-1000.

(190) Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Journal of Biological Chemistry 2003; 278(8):5509-5512.

(191) Wehkamp J, Harder J, Weichenthal M, Schwab M, Schaffeler E, Schlee M et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut 2004; 53(11):1658-1664.

(192) Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005; 307(5710):731-734.

(193) Wehkamp J, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE et al. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. Proceedings of the National Academy of Sciences of the United States of America 2005; 102(50):18129-18134.

(194) Simms LA, Doecke JD, Walsh MD, Huang N, Fowler EV, Radford-Smith GL. Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. Gut 2008.

(195) Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ et al. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. Journal of Biological Chemistry 2003; 278(43):41702-41708.

(196) Li J, Moran T, Swanson E, Julian C, Harris J, Bonen DK et al. Regulation of IL-8 and IL-1 beta expression in Crohn's disease associated NOD2/CARD15 mutations. Human Molecular Genetics 2004; 13(16):1715-1725.

(197) Netea MG, Kullberg BJ, de Jong DJ, Franke B, Sprong T, Naber THJ et al. NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. European Journal of Immunology 2004; 34(7):2052-2059.

(198) van Heel DA, Ghosh S, Butler M, Hunt KA, Lundberg AMC, Ahmad T et al. Murarnyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. Lancet 2005; 365(9473):1794-1796.

(199) Maeda S, Hsu LC, Liu HJ, Bankston LA, Iimura M, Kagnoff MF et al. Nod2 mutation in Crohn's disease potentiates NF-kappa B activity and IL-10 processing. Science 2005; 307(5710):734-738.

(200) Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nature Immunology 2004; 5(8):800-808.

(201) Yang ZQ, Fuss IJ, Watanabe T, Asano N, Davey MP, Rosenbaum JT et al. NOD2 transgenic mice exhibit enhanced MDP-mediated down-regulation of TLR2 responses and resistance to colitis induction. Gastroenterology 2007; 133(5):1510-1521.

(202) Watanabe T, Asano N, Murray PJ, Ozato K, Tailor P, Fuss IJ et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. Journal of Clinical Investigation 2008; 118(2):545-559.

(203) Pauleau AL, Murray PJ. Role of Nod2 in the response of macrophages to Toll-like receptor agonists. Molecular and Cellular Biology 2003; 23(21):7531-7539.

(204) Lecine P, Esmiol S, Metais JY, Nicoletti C, Nourry C, McDonald C et al. The NOD2-RICK complex signals from the plasma membrane. Journal of Biological Chemistry 2007; 282(20):15197-15207.

(205) Fishbein T, Novitskiy G, Mishra L, Matsumoto C, Kaufman S, Goyal S et al. NOD2-expressing bone marrow-derived cells appear to regulate epithelial innate immunity of the transplanted human small intestine. Gut 2008; 57(3).

(206) Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nature Immunology 2003; 4(7):702-707.

(207) Kim JG, Lee SJ, Kagnoff MF. Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by toll-like receptors. Infection and Immunity 2004; 72(3):1487-1495.

(208) Zouali H, Lesage S, Merlin F, Cezard JP, Colombel JF, Belaiche J et al. CARD4/NOD1 is not involved in inflammatory bowel disease. Gut 2003; 52(1):71-74.

(209) McGovern DPB, Hysi P, Ahmad T, van Heel DA, Moffatt MF, Carey A et al. Association between a complex insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease. Human Molecular Genetics 2005; 14(10):1245-1250.

(210) Philpott DJ, Girardin SE. The role of Toll-like receptors and Nod proteins in bacterial infection. Molecular Immunology 2004; 41(11):1099-1108.

(211) Sansonetti PJ. War and peace at mucosal surfaces. Nature Reviews Immunology 2004; 4(12):953-964.

(212) Yang SH, Tamai R, Akashi S, Takeuchi O, Akira S, Sugawara S et al. Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. Infection and Immunity 2001; 69(4):2045-2053.

(213) Uehara A, Yang S, Fujimoto Y, Fukase K, Kusumoto S, Shibata K et al. Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. Cellular Microbiology 2005; 7(1):53-61.

(214) van Heel DA, Ghosh S, Hunt KA, Mathew CG, Forbes A, Jewell DP et al. Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. Gut 2005; 54(11):1553-1557.

(215) West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annual Review of Cell and Developmental Biology 2006; 22:409-437.

(216) Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. Journal of Immunology 2005; 174(8):4453-4460.

(217) Fukata M, Michelsen KS, Eri R, Thomas LS, Hu B, Lukasek K et al. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. American Journal of Physiology-Gastrointestinal and Liver Physiology 2005; 288(5):G1055-G1065.

(218) Mahler M, Bristol IJ, Leiter EH, Workman AE, Birkenmeier EH, Elson CO et al. Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. American Journal of Physiology-Gastrointestinal and Liver Physiology 1998; 37(3):G544-G551.

(219) Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type IIFN protects mice from experimental colitis. Journal of Clinical Investigation 2005; 115(3):695-702.

(220) Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT et al. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nature Cell Biology 2006; 8(12):1327-13U7.

(221) Arnott IDR, Nimmo ER, Drummond HE, Fennell J, Smith BRK, MacKinlay E et al. NOD2/CARD15, TLR4 and CD14 mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? Genes and Immunity 2004; 5(5):417-425.

(222) Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut 2004; 53(7):987-992.

(223) Torok HP, Glas J, Tonenchi L, Bruennler G, Folwaczny M, Folwaczny C. Crohn's disease is associated with a toll-like receptor-9 polymorphism. Gastroenterology 2004; 127(1):365-366.

(224) Browning BL, Huebner C, Petermann I, Gearry RB, Barclay ML, Shelling AN et al. Has toll-like receptor 4 been prematurely dismissed as an inflammatory bowel disease gene? Association study combined with meta-analysis shows strong evidence for association. American Journal of Gastroenterology 2007; 102(11):2504-2512.

(225) Hume GE, Fowler EV, Doecke J, Simms LA, Huang N, Palmieri O et al. Novel NOD2 haplotype strengthens the association between TLR4 Asp299Gly and Crohn's disease in an Australian population. Inflammatory Bowel Diseases 2008; 14(5):585-590.

(226) Pierik M, Joossens S, Van Steen K, Van Schuerbeek N, Vlietinck R, Rutgeerts P et al. Toll-like receptor-1,-2, and-6 polymorphisms influence disease extension in inflammatory bowel diseases. Inflammatory Bowel Diseases 2006; 12(1):1-8.

(227) Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infection and Immunity 2000; 68(12):7010-7017.

(228) Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of toll-like receptor signaling. Cell 2002; 110(2):191-202.

(229) Zhang GL, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. Journal of Biological Chemistry 2002; 277(9):7059-7065.

(230) Boone DL, Turer EE, Lee EG, Ahmad RC, Wheeler MT, Tsui C et al. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nature Immunology 2004; 5(10):1052-1060.

(231) Kelly D, Campbell JI, King TP, Grant G, Jansson EA, Coutts AGP et al. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nature Immunology 2004; 5(1):104-112.

(232) Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to toll-like receptor bacterial ligands in intestinal epithelial cells. Gastroenterology 2004; 126(4):1054-1070.

(233) Lotz M, Gutle D, Walther S, Menard S, Bogdan C, Hornef MW. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. Journal of Experimental Medicine 2006; 203(4):973-984.

(234) Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392(6673):245-252.

(235) Michelsen KS, Aicher A, Mohaupt M, Hartung T, Dimmeler S, Kirschning CJ et al. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCs) - Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. Journal of Biological Chemistry 2001; 276(28):25680-25686.

(236) Lang R, Patel D, Morris JJ, Rutschman RL, Murray PJ. Shaping gene expression in activated and resting primary macrophages by IL-10. Journal of Immunology 2002; 169(5):2253-2263.

(237) Hoentjen F, Sartor RB, Ozaki M, Jobin C. STAT3 regulates NF-kappa B recruitment to the IL-12p40 promoter in dendritic cells. Blood 2005; 105(2):689-696.

(238) Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 2007; 446(7135):557-561.

(239) Zaph C, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du YR et al. Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. Nature 2007; 446(7135):552-556.

(240) Dotan I, Allez M, Nakazawa A, Brimnes J, Schulder-Katz M, Mayer L. Intestinal epithelial cells from inflammatory bowel disease patients preferentially stimulate CD4+T cells to proliferate and secrete interferon-gamma. American Journal of Physiology-Gastrointestinal and Liver Physiology 2007; 292(6):G1630-G1640.

(241) Wehkamp J, Fellermann K, Herrlinger KR, Baxmann S, Schmidt K, Schwind B et al. Human beta-defensin 2 but not beta-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. European Journal of Gastroenterology & Hepatology 2002; 14(7):745-752.

(242) Fahlgren A, Hammarstrom S, Danielsson A, Hammarstrom ML. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. Clinical and Experimental Immunology 2003; 131(1):90-101.

(243) Forsberg G, Fahlgren A, Horstedt P, Hammarstrom S, Hernell O, Hammarstrom ML. Presence of bacteria and innate immunity of intestinal epithelium in childhood celiac disease. American Journal of Gastroenterology 2004; 99(5):894-904.

(244) Segal AW. How neutrophils kill microbes. Annual Review of Immunology 2005; 23:197-223.

(245) Segal AW, Loewi G. Neutrophil Dysfunction in Crohns-Disease. Lancet 1976; 2(7979):219-221.

(246) Worsaae N, Johansen KS, Christensen KC. Impaired Invitro Function of Neutrophils in Crohns-Disease. Scandinavian Journal of Gastroenterology 1982; 17(1):91-96.

(247) Wandall JH, Binder V. Leukocyte Function in Crohns-Disease. Gut 1982; 23(3):173-180.

(248) Morain CO, Prestage H, Harrison P, Levi AJ, Tyrrell DAJ. Cytopathic Effects in Cultures Inoculated with Material from Crohns-Disease. Gut 1981; 22(10):823-826.

(249) Wandall JH. Function of Exudative Neutrophilic Granulocytes in Patients with Crohns-Disease Or Ulcerative-Colitis. Scandinavian Journal of Gastroenterology 1985; 20(9):1151-1156.

(250) Marks DJB, Harbord MWN, MacAllister R, Rahman FZ, Young J, Al-Lazikani B et al. Defective acute inflammation in Crohn's disease: a clinical investigation. Lancet 2006; 367(9511):668-678.

(251) Harbord MWN, Marks DJB, Forbes A, Bloom SL, Day RM, Segal AW. Impaired neutrophil chemotaxis in Crohn's disease relates to reduced production of chemokines and can be augmented by granulocyte-colony stimulating factor. Alimentary Pharmacology & Therapeutics 2006; 24(4):651-660.

(252) Sher ME, Bank S, Greenberg R, Sardinha TC, Weissman S, Bailey B et al. The influence of cigarette smoking on cytokine levels in patients with inflammatory bowel disease. Inflammatory Bowel Diseases 1999; 5(2):73-78.

(253) Emmanuel AV, Kamm MA. Laser Doppler measurement of rectal mucosal blood flow. Gut 1999; 45(1):64-69.

(254) Issekutz AC. Vascular-Responses During Acute Neutrophilic Inflammation Their Relationship to Invivo Neutrophil Emigration. Laboratory Investigation 1981; 45(5):435-441.

(255) Suh CI, Stull ND, Li XJ, Tian W, Price MO, Grinstein S et al. The phosphoinositide-binding protein p40(phox) activates the NADPH oxidase during Fc gamma IIA receptor-induced phagocytosis. Journal of Experimental Medicine 2006; 203(8):1915-1925.

(256) Bissonnette SA, Glazier CM, Stewart MQ, Brown GE, Ellson CD, Yaffe MB. Phosphatidylinositol 3-phosphate-dependent and -independent functions of p40(phox) in activation of the neutrophil NADPH oxidase. Journal of Biological Chemistry 2008; 283(4):2108-2119.

(257) Ellson CD, Davidson K, Ferguson GJ, O'Connor R, Stephens LR, Hawkins PT. Neutrophils from p40(phox-/-) mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. Journal of Experimental Medicine 2006; 203(8):1927-1937.

(258) Pancer Z, Cooper MD. The evolution of adaptive immunity. Annual Review of Immunology 2006; 24:497-518.

(259) Janeway CA, Travers P, Walport M, Shlomchik MJ. Immunobiology. 5 ed. New York, USA: Garland Publishing, 2001.

(260) Breese E, Braegger CP, Corrigan CJ, Walkersmith JA, MacDonald TT. Interleukin-2-Secreting and Interferon-Gamma-Secreting T-Cells in Normal and Diseased Human Intestinal-Mucosa. Immunology 1993; 78(1):127-131.

(261) Breese EJ, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P et al. Tumor-Necrosis-Factor Alpha-Producing Cells in the Intestinal-Mucosa of Children with Inflammatory Bowel-Disease. Gastroenterology 1994; 106(6):1455-1466.

(262) Fuss IJ, Neurath M, Boirivant M, Klein JS, de la MC, Strong SA et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. Journal of Immunology 1996; 157(3):1261-1270.

(263) Plevy SE, Landers CJ, Prehn J, Carramanzana NM, Deem RL, Shealy D et al. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. Journal of Immunology 1997; 159(12):6276-6282.

(264) Louis E, Satsangi J, Roussomoustakaki M, Parkes M, Fanning G, Welsh K et al. Cytokine gene polymorphisms in inflammatory bowel disease. Gut 1996; 39(5):705-710.

(265) Negoro K, Kinouchi Y, Hiwatashi N, Takahashi S, Takagi S, Satoh J et al. Crohn's disease is associated with novel polymorphisms in the 5 '-flanking region of the tumor necrosis factor gene. Gastroenterology 1999; 117(5):1062-1068.

(266) O'Callaghan NJ, Adams KE, van Heel DA, Cavanaugh JA. Association of TNF-alpha-857C with inflammatory bowel disease in the Australian population. Scandinavian Journal of Gastroenterology 2003; 38(5):533-534.

(267) Gonzalez S, Rodrigo L, Martinez-Borra J, Lopez-Vazquez A, Fuentes D, Nino P et al. TNF-alpha-308A promoter polymorphism is associated with enhanced TNF-alpha production and inflammatory activity in Crohn's patients with fistulizing disease. American Journal of Gastroenterology 2003; 98(5):1101-1106.

(268) Levine A, Shamir R, Wine E, Weiss B, Karban A, Shaoul RR et al. TNF promoter polymorphisms and modulation of growth retardation and disease severity in pediatric Crohn's disease. American Journal of Gastroenterology 2005; 100(7):1598-1604.

(269) Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annual Review of Immunology 2002; 20:495-549.

(270) Kosiewicz MM, Nast CC, Krishnan A, Rivera-Nieves J, Moskaluk CA, Matsumoto S et al. Th1-type responses mediate spontaneous ileitis in a novel murine model of Crohn's disease. Journal of Clinical Investigation 2001; 107(6):695-702.

(271) Rivera-Nieves J, Bamias G, Vidrich A, Marini M, Pizarro TT, McDuffie MJ et al. Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis. Gastroenterology 2003; 124(4):972-982.

(272) Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B et al. Phenotypic and functional features of human Th17 cells. Journal of Experimental Medicine 2007; 204(8):1849-1861.

(273) Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y et al. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 2003; 52(1):65-70.

(274) Nielsen OH, Kirman I, Rudiger N, Hendel J, Vainer B. Upregulation of interleukin-12 and-17 in active inflammatory bowel disease. Scandinavian Journal of Gastroenterology 2003; 38(2):180-185.

(275) Bettelli E, Carrier YJ, Gao WD, Korn T, Strom TB, Oukka M et al. Reciprocal developmental pathways for the generation of pathogenic effector T(H)17 and regulatory T cells. Nature 2006; 441(7090):235-238.

(276) Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ et al. The orphan nuclear receptor ROR gamma t directs the differentiation program of proinflammatory IL-17(+) T helper cells. Cell 2006; 126(6):1121-1133.

(277) Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: An effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006; 24(6):677-688.

(278) Mannon PJ, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D et al. Anti-interleukin-12 antibody for active Crohn's disease. New England Journal of Medicine 2004; 351(20):2069-2079.

(279) Fuss IJ, Becker C, Yang ZQ, Groden C, Hornung RL, Heller F et al. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. Inflammatory Bowel Diseases 2006; 12(1):9-15.

(280) Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F et al. Regulation of gut inflammation and Th17 cell response by interleukin-21. Gastroenterology 2008; 134(4):1038-1048.

(281) Boirivant M, Fuss IJ, Chu A, Strober W. Oxazolone colitis: A murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. Journal of Experimental Medicine 1998; 188(10):1929-1939.

(282) Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity 2002; 17(5):629-638.

(283) Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. Journal of Clinical Investigation 2004; 113(10):1490-1497.

(284) Fuss IJ. The adaptive immune responses in inflammatory bowel disease. FALK SYMPOSIUM 2007; 153:12-20.

(285) Boirivant M, Marini M, Di Felice G, Pronio AM, Montesani C, Tersigni R et al. Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. Gastroenterology 1999; 116(3):557-565.

(286) Ina K, Itoh J, Fukushima K, Kusugami K, Yamaguchi T, Kyokane K et al. Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. Journal of Immunology 1999; 163(2):1081-1090.

(287) Itoh J, de la Motte C, Strong SA, Levine AD, Fiocchi C. Decreased Bax expression by mucosal T cells favours resistance to apoptosis in Crohn's disease. Gut 2001; 49(1):35-41.

(288) Sturm A, Leite AZA, Danese S, Krivacic KA, West GA, Mohr S et al. Divergent cell cycle kinetics underlie the distinct functional capacity of mucosal T cells in Crohn's disease and ulcerative colitis. Gut 2004; 53(11):1624-1631.

(289) Peppelenbosch MP, van Deventer SJH. T cell apoptosis and inflammatory bowel disease. Gut 2004; 53(11):1556-1558.

(290) Van den Brande JM, Braat H, Van Den Brink GR, Versteeg HH, Bauer CA, Hoedemaeker I et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003; 124(7):1774-1785.

(291) Becker C, Wirtz S, Neurath MF. Stepwise regulation of T(H)1 responses in autoimmunity: IL-12-related cytokines and their receptors. Inflammatory Bowel Diseases 2005; 11(8):755-764.

(292) Becker C, Wirtz S, Blessing M, Pirhonen J, Strand D, Bechthold O et al. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. Journal of Clinical Investigation 2003; 112(5):693-706.

(293) Becker C, Dornhoff H, Neufert C, Fantini MC, Wirtz S, Huebner S et al. Cutting edge: IL-23 cross-regulates IL-12 production in T cell-dependent experimental colitis. Journal of Immunology 2006; 177(5):2760-2764.

(294) Monteleone G, Biancone L, Marasco R, Morrone G, Marasco O, Luzza F et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology 1997; 112(4):1169-1178.

(295) Cong YZ, Weaver CT, Lazenby A, Elson CO. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. Journal of Immunology 2002; 169(11):6112-6119.

(296) Makita S, Kanai T, Oshima S, Uraushihara K, Totsuka T, Sawada T et al. CD4(+)CD25(bright) T cells in human intestinal lamina propria as regulatory cells. Journal of Immunology 2004; 173(5):3119-3130.

(297) Makita S, Kanai T, Nemoto Y, Totsuka T, Okamoto R, Tsuchiya K et al. Intestinal lamina propria retaining CD4(+)CD25(+) regulatory T cells is a suppressive site of intestinal inflammation. Journal of Immunology 2007; 178(8):4937-4946.

(298) Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. Current Opinion in Immunology 2008.

(299) Morrissey PJ, Charrier K, Braddy S, Liggitt D, Watson JD. Cd4+ T-Cells That Express High-Levels of Cd45Rb Induce Wasting Disease When Transferred Into Congenic Severe Combined Immunodeficient Mice - Disease Development Is Prevented by Cotransfer of Purified Cd4+ T-Cells. Journal of Experimental Medicine 1993; 178(1):237-244.

(300) Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4(+) T cells. Journal of Experimental Medicine 1996; 183(6):2669-2674.

(301) Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. Journal of Experimental Medicine 1999; 190(7):995-1003.

(302) Groux H, OGarra A, Bigler M, Rouleau M, Antonenko S, deVries JE et al. A CD4(+) T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997; 389(6652):737-742.

(303) Mottet C, Uhlig HH, Powrie F. Cutting edge: Cure of colitis by CD4(+) CD25(+) regulatory T cells. Journal of Immunology 2003; 170(8):3939-3943.

(304) Ruiz PA, Anna S, Kim SC, Sartor RB, Haller D. IL-10 gene-deficient mice lack TGF-beta/Smad signaling and fail to inhibit proinflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic Enterococcus faecalis. Journal of Immunology 2005; 174(5):2990-2999.

(305) Diebold RJ, Eis MJ, Yin M, Ormsby I, Boivin GP, Darrow BJ et al. Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. Proceedings of the National Academy of Sciences of the United States of America 1995; 92(26):12215-12219.

(306) Boivin GP, JonesCarson J, Otoole BA, Doetschman T. Germ-free and barrier-raised TGF beta 1-deficient mice have similar inflammatory lesions. Transgenic Research 1997; 6(3):197-202.

(307) Wu JY, Jin Y, Edwards RA, Zhang Y, Finegold MJ, Wu MX. Impaired TGF-beta responses in peripheral T cells of G alpha i2-/- mice. Journal of Immunology 2005; 174(10):6122-6128.

(308) Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. Journal of Experimental Medicine 2005; 201(5):723-735.

(309) Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting edge: IL-2 is essential for TGF-beta-mediated induction of Foxp(3+) T regulatory cells. Journal of Immunology 2007; 178(7):4022-4026.

(310) Kundig TM, Schorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I. Immune-Responses in Interleukin-2 Deficient Mice. Science 1993; 262(5136):1059-1061.

(311) Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Penit C. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. International Immunology 1998; 10(4):371-378.

(312) Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGF beta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006; 24(2):179-189.

(313) Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M et al. Reciprocal T(H)17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007; 317(5835):256-260.

(314) Kullberg MC, Hay V, Cheever AW, Mamura M, Sher A, Letterio JJ et al. TGF-beta 1 production by CD4(+)CD25(+) regulatory T cells is not essential for suppression of intestinal inflammation. European Journal of Immunology 2005; 35(10):2886-2895.

(315) Maul J, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A et al. Peripheral and intestinal regulatory CD4+CD25(high) T cells in inflammatory bowel disease. Gastroenterology 2005; 128(7):1868-1878.

(316) Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4(+)CD25(+) regulatory T cells in patients with multiple sclerosis. Journal of Experimental Medicine 2004; 199(7):971-979.

(317) Kraus TA, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. Gastroenterology 2004; 126(7):1771-1778.

(318) Kraus TA, Toy L, Chan L, Childs J, Cheifetz A, Mayer L. Failure to induce oral tolerance in Crohn's and ulcerative colitis patients: possible genetic risk. Annals of the New York Academy of Sciences 2004; 1029:225-238.

(319) Kraus TA, Cheifetz A, Toy L, Meddings JB, Mayer L. Evidence for a genetic defect in oral tolerance induction in inflammatory bowel disease. Inflammatory Bowel Diseases 2006; 12(2):82-88.

(320) Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4(+)CD25(-) T cells through Foxp3 induction and down-regulation of Smad7. Journal of Immunology 2004; 172(9):5149-5153.

(321) Schreiber S, Heinig T, Thiele HG, Raedler A. Immunoregulatory Role of Interleukin-10 in Patients with Inflammatory Bowel-Disease. Gastroenterology 1995; 108(5):1434-1444.

(322) Tagore A, Gonsalkorale WM, Pravica V, Hajeer AH, McMahon R, Whorwell PJ et al. Interleukin-10 (IL-10) genotypes in inflammatory bowel disease. Tissue Antigens 1999; 54(4):386-390.

(323) Klein W, Tromm A, Griga T, Fricke H, Folwaczny C, Hocke M et al. The IL-10 gene is not involved in the predisposition to inflammatory bowel disease. Electrophoresis 2000; 21(17):3578-3582.

(324) Aithal GP, Craggs A, Day CP, Welfare M, Daly AK, Mansfield JC et al. Role of polymorphisms in the interleukin-10 gene in determining disease susceptibility and phenotype in inflamatory bowel disease. Digestive Diseases and Sciences 2001; 46(7):1520-1525.

(325) Castro-Santos P, Suarez A, Lopez-Rivas L, Mozo L, Gutierrez C. TNF alpha and IL-10 gene polymorphisms in inflammatory bowel disease. Association of-1082 AA low producer IL-10 genotype with steroid dependency. American Journal of Gastroenterology 2006; 101(5):1039-1047.

(326) Babyatsky MW, Rossiter G, Podolsky DK. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. Gastroenterology 1996; 110(4):975-984.

(327) Kanazawa S, Tsunoda T, Onuma E, Majima T, Kagiyama M, Kikuchi K. VEGF, basic-FGF, and TGF-beta in Crohn's disease and ulcerative colitis: A novel mechanism of chronic intestinal inflammation. American Journal of Gastroenterology 2001; 96(3):822-828.

(328) Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-beta 1 signaling in chronic inflammatory bowel disease. Journal of Clinical Investigation 2001; 108(4):601-609.

(329) Monteleone G, Mann J, Monteleone I, Vavassori P, Bremner R, Fantini M et al. A failure of transforming growth factor-beta 1 negative regulation maintains sustained NF-kappa B activation in gut inflammation. Journal of Biological Chemistry 2004; 279(6):3925-3932.

(330) Monteleone G, Blanco GD, Monteleone I, Fina D, Caruso R, Gioia V et al. Post-transcriptional regulation of Smad7 in the gut of patients with inflammatory bowel disease. Gastroenterology 2005; 129(5):1420-1429.

(331) Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nature Immunology 2000; 1(6):475-482.

(332) Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity 2002; 16(2):219-230.

(333) Mizoguchi A, Mizoguch E. Inflammatory bowel disease, past, present and future: lessons from animal models. Journal of Gastroenterology 2008; 43(1):1-17.

(334) Sugimoto K, Ogawa A, Shimomura Y, Nagahama K, Mizoguchi A, Bhan AK. Inducible IL-12-Producing B cells regulate Th2-mediated intestinal inflammation. Gastroenterology 2007; 133(1):124-136.

(335) Mizoguchi A, Mizoguchi E, Smith RN, Preffer FI, Bhan AK. Suppressive role of B cells in chronic colitis of T cell receptor alpha mutant mice. Journal of Experimental Medicine 1997; 186(10):1749-1756.

(336) Mizoguchi E, Mizoguchi A, Preffer FI, Bhan AK. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. International Immunology 2000; 12(5):597-605.

(337) Dalwadi H, Wei B, Schrage M, Su TT, Rawlings DJ, Braun J. B cell developmental requirement for the G alpha i2 gene. Journal of Immunology 2003; 170(4):1707-1715.

(338) Wei B, Velazquez P, Turovskaya O, Spricher K, Aranda R, Kronenberg M et al. Mesenteric B cells centrally inhibit CD4(+) T cell colitis through interaction with regulatory T cell subsets. Proceedings of the National Academy of Sciences of the United States of America 2005; 102(6):2010-2015.

(339) Ostanin DV, Pavlick KP, Bharwani S, D'Souza D, Furr KL, Brown CM et al. T cell-induced inflammation of the small and large intestine in immunodeficient mice. American Journal of Physiology-Gastrointestinal and Liver Physiology 2006; 290(1):G109-G119.

(340) Yoshimori T. Autophagy: Paying Charon's toll. Cell 2007; 128(5):833-836.

(341) Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 2007; 27(1):135-144.

(342) Qu XP, Zou ZJ, Sun QH, Luby-Phelps K, Cheng PF, Hogan RN et al. Autophagy gene-dependent clearance of apoptotic cells during embryonic development. Cell 2007; 128(5):931-946.

(343) Schmid D, Munz C. Innate and adaptive immunity through autophagy. Immunity 2007; 27(1):11-21.

(344) Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 2007; 26(1):79-92.

(345) Wen Z, Fiocchi C. Inflammatory bowel disease: autoimmune or immune-mediated pathogenesis? Clinical & Developmental Immunology 2004; 11(3-4):195-204.

(346) Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. The codependence of angiogenesis and chronic inflammation. Faseb Journal 1997; 11(6):457-465.

(347) Danese S, Sans M, de la Motte C, Graziani C, West G, Phillips MH et al. Angiogenesis as a novel component of inflammatory bowel disease pathogenesis. Gastroenterology 2006; 130(7):2060-2073.

(348) Chidlow JH, Shukla D, Grisham MB, Kevil CG. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. American Journal of Physiology-Gastrointestinal and Liver Physiology 2007; 293(1):G5-G18.

(349) Chidlow JH, Langston W, Greer JJM, Ostanin D, Abdelbaqi M, Houghton J et al. Differential angiogenic regulation of experimental colitis. American Journal of Pathology 2006; 169(6):2014-2030.

(350) Punekar S, Zak S, Kalter VG, Dobransky L, Punekar I, Lawler JW et al. Thrombospondin 1 and its mimetic peptide ABT-510 decrease angiogenesis and inflammation in a murine model of inflammatory bowel disease. Pathobiology 2008; 75(1):9-21.

(351) Ferrante M, Pierik M, Henckaerts L, Joossens M, Claes K, Van Schuerbeek N et al. The role of vascular endothelial growth factor (VEGF) in inflammatory bowel disease. Inflammatory Bowel Diseases 2006; 12(9):870-878.

(352) Cane G, Moal VL, Pages G, Servin AL, Hofman P, Vouret-Craviari V. Up-regulation of intestinal vascular endothelial growth factor by Afa/Dr diffusely adhering Escherichia coli. PLoS ONE 2007; 2(12):e1359.

(353) Kucharzik T, Maaser C, Lugering A, Kagnoff M, Mayer L, Targan S et al. Recent understanding of IBD pathogenesis: implications for future therapies. Inflammatory Bowel Diseases 2006; 12(11):1068-1083.

Roles for metallothioneins in intestinal inflammation

metallothioneins

Metallothioneins (MTs) are a superfamily of small proteins that are present in virtually every living organism. A typical feature is their highly conserved number and position of cysteine residues, enabling them to incorporate monovalent and divalent metal atoms and to reduce reactive oxygen and nitrogen species. So far, 19 human isoforms have been cloned, 11 of which are known to be functional (i.e. MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, MT1X, MT2A, MT3, and MT4). The majority of these genes cluster together on a single locus on chromosome 16 (16q13) (1). Heterogeneity of isoforms results from posttranslational modifications and/or variations in metal composition. The most widely expressed isoforms are MT-1 and MT-2, of which MT-2 appears to be expressed more on the basal level. These isoforms are highly inducible, whereas the MT-3 and MT-4 proteins are constitutively expressed and are found mainly in the brain, kidney, and reproductive organs (MT-3) and in certain squamous epithelia (MT-4). Human MT isoforms are regulated independently of each other, and can be induced by metals, stress hormones, cytokines, reactive oxygen species, and chemicals. In mice, the situation is more simple. Only four functional murine MT genes are known (MT1, MT2, MT3, and MT4), and the MT1 and MT2 isoforms are co-ordinately regulated (2;3).

MT regulation in inflammation

One of the most striking observations that link MTs to inflammation is their rapid up-regulation in response to a variety of stresses including inflammation. The observation that hepatic expression of MTs is dramatically elevated in response to bacterial infection, an effect mediated by endotoxin (lipopolysaccharide-LPS), has lead to the classification of MTs as acute phase proteins. LPS induction of MT gene expression in mice occurred in each of ten organs examined (liver, kidney, pancreas, intestine, lung, heart, brain, ovary, uterus, and spleen) (4). This induction by LPS was shown to be mediated by several pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and interferon

(IFN)-γ (4), nitric oxide (NO) (5), as well as the stress hormones glucocorticoids (6). These factors have been shown to up-regulate MTs independently of LPS, but they seem to act synergistically and result in different levels of MT expression, depending on the tissue and the combination of factors (4;7-9). Reactive oxygen species (ROS), generated during the inflammatory response, may activate MT expression through multiple pathways, including directly by stimulating an antioxidant response element and specific metal response elements in the promoter region as well as indirectly by events associated with second-messenger protein kinase pathways (10;11). During radiation injury to the small intestine, which implicates ROS and leads to acute inflammation, metallothioneins were induced (12). All these data illustrate that MT regulation in inflammation is a rapid but complex process with different results in differing tissues. One constant, however, is that MT induction by inflammatory mediators seems to be conditional upon the presence of zinc (3).

MT functions relevant in inflammation and possibly in inflammatory bowel diseases

The first function of MTs that was put forward as playing a role in inflammation was their central position in zinc homeostasis, redistributing the intracellular zinc pool. In that way, MTs can have two opposite roles: they can either increase the intracellular zinc pool, thereby facilitating metabolic processes during the acute phase response, or sequester zinc to allow maximal activity of enzymes which would be otherwise inhibited by zinc (3). Besides the immense number of enzymes that use or are inhibited by zinc, clarifying these postulations is hampered by the fact that the affinity of MTs for zinc probably differs depending on the stimulus by which MTs were induced. In an *in vivo* displacement assay of cadmium (whose affinity for MT is stronger than that of zinc), it was shown that oxidative stress-induced MT displaced zinc to cadmium, whereas MT induced by fasting stress or by restraint stress did not (13). This study did not make a distinction between different MT isoforms, although metal-binding capacities of isoforms can vary (14;15), as can their gene-regulation (see chapter 4 part 3). Conformational changes of MT under certain stimuli might also explain the observed influence on zinc affinity. It has been shown that the binding of ATP (which reflects the energy state of a cell) to MT elicited conformational changes and altered zinc binding in MT (16). Zinc deficiency is a potential complication of Crohn's disease and may result from a variety of processes, including reduced dietary intake, impaired absorption, increased excretion, hypoalbuminemia, or an internal redistribution of zinc (17;18). Although the consequences of this deficiency on the pathogenesis of the disease are not clear (19), it could be implicated through the involvement of zinc in immune function, redox signalling and

wound healing (20-23). Studies where zinc was administered to rats or mice with chemically induced colitis showed a dose-dependent therapeutic effect (24-28). Furthermore, zinc was shown to induce MT synthesis in ileal and colonic mucosa of control rats and to a lesser extent in that of colitic rats (29). A placebo-controlled double-blind cross-over trial was conducted with seven Crohn's disease patients and seven ulcerative colitis patients that had inactive to moderately active disease and received oral zinc supplementation (30). Although supplementation increased plasma zinc concentrations and slightly (but not significantly) increased mucosal MT concentration, there were no changes in histological inflammation or disease activity.

MTs are reported to modulate the activation of the transcription factor nuclear factor (NF)-κB, which has a pivotal role in immune and inflammatory responses and as such also in inflammatory bowel diseases (IBD) (see previously). However, published results vary concerning the relationship between MT expression level and NF-κB activity. The modulatory effect might be based upon a direct interaction (31), regulation of zinc concentrations (32), or modulation of the redox balance through antioxidant functions (33). Data supporting a positive regulatory role for MTs on NF-κB activity are the zinc-induced inhibition of this activity, which is attenuated by MTs (32). The requirement of MTs for the zinc-induced expression of macrophage colony stimulating factor, a chemokine downstream of NF-κB, illustrates this positive regulatory role (34). On the other hand, data exist that MTs may function as a negative regulator of NF-κB, showing that MTs inhibited the activation of NF-κB by TNF-α (35;36) and that splenocytes from MTnull mice displayed elevated levels of NF-κB activity (37). Although these reports seem contradictory, explanations for this discrepancy could be found in the distinct redox regulation of NF-κB activation between the cytoplasm and the nucleus (33), a balance which may be modulated by the antioxidant capacities of MTs. Another explanation might be found in differences in the cell types used. All these studies, however, indicate that MTs are an important regulator of NF-κB activity.

As just mentioned, the antioxidant capacities of MTs might influence the inflammatory response through modulating NF-κB activity, but a more obvious role for MTs as antioxidants is the sequestration of harmful oxygen and nitrogen intermediates which are generated during the inflammatory response. In order to kill bacteria and parasites, infiltrating neutrophils and macrophages produce free oxygen radicals (hydrogen peroxide, NO, and superoxide anion) which are extremely cytotoxic to host cells (38;39). MTs could, together with other known molecules such as super oxide dismutase, vitamin E and ascorbate, provide a cytoprotection for host cells, preventing cellular damage and allowing survival and growth in an inflammatory environment (40). The increased presence of ROS, an imbalance

in antioxidant expression, and oxidative DNA and protein damage have been reported in IBD (41-44). In Crohn's disease, oxidative protein damage as measured by lipid peroxidation was associated with the concentration of MTs (42). ROS can disrupt the epithelial barrier function by destabilizing tight junctions (45), thus increasing permeability, a phenomenon which is observed in patients with Crohn's disease (46). Experiments in animal models of IBD have already confirmed the possibility to use antioxidants as therapeutic agents. In IL-10 deficient mice, local mucosal administration of the antioxidant enzyme superoxide dismutase (SOD) by genetically modified *Lactobacillus* bacteria significantly reduced the severity of inflammation (47). Mice overexpressing human SOD demonstrated attenuated inflammation when subjected to a mild form of dextran sodium sulphate (DSS)-induced colitis, and a remarkable survival benefit from severe DSS colitis (48).

In certain oxidative and inflammatory environments MTs have been shown to reduce apoptosis (49), and in cases where inflammation-dependent apoptosis is detrimental, induction of MTs might provide a benefit. Since Crohn's disease is characterized by defective T cell apoptosis, whereas T cells from ulcerative colitis patients show a strong activation induced apoptosis, the role of MTs might be different in these two diseases.

Under certain conditions such as cell proliferation, differentiation, and after cell injury, MTs are translocated from the cytosol to the nucleus. MT regulation during cell cycle progression has been demonstrated in normally cycling cells, with maximal nuclear accumulation within the S and G2 phases, whereas high cytoplasmic expression occurred during late G1 and G1/S transition and basal amounts were found in the G0 phase (50;51). Hepatocytes show a transient nuclear localization of MTs at the G1-to-S phase transition during the priming phase of liver cell regeneration after partial hepatectomy (52). Two premises for the nuclear retention of MTs have been proposed. First, it might reflect the role of MTs as chaperones to provide zinc for crucial enzymes and transcription factors involved in cell division (53). Otherwise, it has been proposed that it might protect DNA from oxidative damage (54).

Besides their intracellular functions, MTs could also be involved in inflammation and IBD through specific extracellular effects (55). For example, MTs have been shown to directly and specifically mediate leukocyte chemotaxis (56). Extracellular MTs can stimulate lymphocyte proliferation (57). MT binds to the plasma membrane of both T and B lymphocytes, but, in the absence of a costimulatory agent, MT induces lymphoproliferation only in B cells. MT also enhances the capacity of naive B lymphocytes to differentiate into plasma cells (58). On the other hand, MTs can suppress cytotoxic T cell function *in vitro* and a T-dependent humoral response *in vivo* (59-61).

protective functions of MTs in animal models of inflammation

The availability of MTnull mice (knockout for MT1 and MT2) has permitted the investigation of the involvement of MTs in animal models of inflammation. In an animal model for multiple sclerosis, a chronic inflammatory and demyelinating disease of the central nervous system in which oxidative stress plays a pathogenic role, MTs were demonstrated to be protective (62). MTnull mice were more susceptible to *Helicobacter pylori*-induced gastritis and showed more severe inflammation of the stomach than wild type mice (63). This correlates with the reported antibacterial activity that was associated with MT function. This activity might be mediated directly by MTs themselves (64) or indirectly through nitric oxide production (65). In the collagen-induced arthritis model, repeated administration of MT1 and 2 during the course of disease dramatically reduced the incidence and severity of the disease (66). MTs suppressed the disease through the generation of IL-10- and transforming growth factor (TGF)-β-producing type 1 regulatory T-like cells (67). In all of these models, MTs seem to be protective against local inflammation. In the TNF-induced lethal shock model (a model for systemic inflammatory response syndrome), however, MTs seem to sensitize (68). An explanation for this finding was not found, although it is contradictory to the reported resistance that MTs confer to the cytotoxic effects of TNF *in vitro* (69) and to the protective effects of MTs in another model of systemic inflammatory response syndrome (LPS-induced lethal shock in sensitized mice) (70).

MT expression in IBD

Given their possible functions in IBD pathogenesis, the expression of MTs has been studied in patient samples by different research groups. These studies have yielded contradictory results (table 1) and as such the role of MTs in IBD is not yet clarified. First of all, two studies report an increase in MT expression in IBD (71;72), while the rest demonstrates a downregulation. Most of the studies that used samples of both Crohn's disease patients and ulcerative colitis patients reported no differences between the two patient groups, except for two studies (both using DNA microarrays). These two studies, however, describe distinct findings, i.e. decreased MT expression in ulcerative colitis and normal expression in Crohn's disease (73) *versus* normal expression in ulcerative colitis and increased MT expression in Crohn's disease (72). The influence of medication is also not clear yet, given that one study reports an influence of steroid therapy on MT expression (74) whereas a second study contests this (75). A study describing down-regulation of MTs *in vitro* in colon epithelial cells after stimulation with azathioprine did not discuss this effect in patients, although two azathioprine-treated patients were included in the study (72). The results of this study should

Table 1. MT expression in IBD patients

study	subjects	methods	results
Clarkson *et al.* 1985 (74)	ileal resection specimens of 13 CD patients (6 had received steroid therapy; 5 had not), 2 UC patients, and 3 controls	IHC on resection specimens	less MT immunoreactivity in patients with IBD than controls; patients on steroid therapy had more immunoreactivity; immunoreactivity in enterocytes and lamina propria
Elmes *et al.* 1987 (75)	ileal resection specimens of 17 CD patients (11 had received steroid therapy; 6 had not), and 5 controls	IHC on resection specimens	decreased intestinal MT in IBD patients; no significant difference when patients had received steroid therapy; immunoreactivity in enterocytes and basement membrane region
Mulder *et al.* 1991 (79)	19 ileum and 16 colon specimens from 29 CD patients; 12 colon specimens and 1 ileum specimen from 12 UC patients; colon specimens from 18 control patients	RIA on homogenized mucosa (dissected from resection specimens)	MT content was decreased in non-inflamed IBD mucosa compared with control mucosa; further decrease was found in inflamed mucosa; no differences between UC and CD; no significant effect of medication or tissue localisation
Sturniolo *et al.* 1998 (80)	colonic biopsies of 24 UC patients and 10 controls	Ag-hem on biopsies	Reduced MT concentrations in patients with active disease as compared with controls and patients in remission; reduced MT concentrations in inflamed versus non-inflamed mucosa taken from the same patient
Brüwer *et al.* 2001 (71)	22 CD patients, 48 UC patients, 10 controls	IHC on resection specimens	MT overexpression in the fibroblasts of all ulcerative and/or fissural lesions in UC and CD; MT overexpression in intestinal epithelial cells of 40% of UC and CD lesions correlated significantly with the grade of inflammation.
Lawrance *et al.* 2001 (73)	colonic resection specimens with moderately severe histological inflammation from 12 UC and 6 CD patients (with moderately severe clinical disease); and from 6 controls	DNA microarray	decrease of MT1H and MT1G mRNA expression in UC; no difference in CD
Ioachim *et al.* 2003 (76)	ileum, colon or rectum resection specimens from 10 CD patients, 41 UC patients, 5 controls	IHC	Decreased MT expression in UC and CD compared with normal mucosa; no difference in MT expression between UC and CD; in UC, a gradually decreased expression from remission, to resolving and to active phase was observed; only epithelial MT expression
Kruidenier *et al.* 2003 (77)	resection specimens from 19 CD patients, 15 UC patients, 18 controls	RIA on tissue homogenates and IHC on resection specimens	RIA: Lower tissue MT content in inflamed CD and UC mucosa compared with non-inflamed and control mucosa; IHC: decreased MT-positive epithelial cell numbers at inflamed sites in CD and UC patients; no detection of MT in lamina propria
Dooley *et al.* 2004 (72)	2 sets of colon samples: a) control (1 uninvolved colon from CD patient), 1 CD patient, and 1 UC patient; b) control, 1 azathioprine-treated CD patient, and 1 azathioprine-treated UC patient; drug-treated CaCo-2 cells	microarray and qRT-PCR with consensus primer sequences for multiple metallothionein genes.	Microarray: up-regulation of MT1F, MT1G, MT1H in CD in tissue set a); down-regulation of MT1F, MT1H, MT1L in azathioprine-treated CaCo-2 cells; confirmed by qRT-PCR

Abbreviations: CD: Crohn's disease, UC: ulcerative colitis, IHC: immunohistochemistry, RIA: radio-immunoassay, Ag-hem: Silver-heme saturation assay, qRT-PCR: quantitative reverse transcriptase-polymerase chain reaction

be interpreted with care, considering the small data-set used. Finally, the immunohistochemical studies do not agree whether MT expression is confined to the epithelium (76;77), or whether expression in the lamina propria occurs as well (71;74;75). The study of MT protein expression in IBD is even complicated by the possible destructive influence of the oxidative environment on the immunodominant epitope of the protein. Therefore, it is not sure whether the absence of immunoreactivity equals absence of MT protein, or whether it reflects a failure of the antibody to recognize the present protein. In the DSS model of colitis, MTs were not protective in two separate studies comparing MTnull mice with wild type mice (28;78).

conclusion

Although several functional associations of MTs can confer a role for this family of proteins in the pathogenesis of IBD, the results of human and experimental colitis studies are not decisive. However, it is clear that a deviant MT expression exists in this disease, and as such it is important to meticulously clarify these aberrations in Crohn’s disease and in ulcerative colitis. Furthermore, investigating whether the regulation of MTs in these diseases is dependent or independent on inflammation will add knowledge on their involvement in IBD.

references

(1) Karin M, Eddy RL, Henry WM, Haley LL, Byers MG, Shows TB. Human metallothionein genes are clustered on chromosome 16. Proceedings of the National Academy of Sciences of the United States of America 1984; 81(17):5494-5498.

(2) Miles AT, Hawksworth GM, Beattie JH, Rodilla V. Induction, regulation, degradation, and biological significance of mammalian metallothioneins. Critical Reviews in Biochemistry & Molecular Biology 2000; 35(1):35-70.

(3) Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: The multipurpose protein. Cellular and Molecular Life Sciences 2002; 59(4):627-647.

(4) De SK, McMaster MT, Andrews GK. Endotoxin induction of murine metallothionein gene expression. Journal of Biological Chemistry 1990; 265(25):15267-15274.

(5) Arizono K, Kagawa S, Hamada H, Ariyoshi T. Nitric-Oxide Mediated Metallothionein Induction by Lipopolysaccharide. Research Communications in Molecular Pathology and Pharmacology 1995; 90(1):49-58.

(6) Itoh N, Kasutani K, Muto N, Otaki N, Kimura M, Tanaka K. Blocking effect of anti-mouse interleukin-6 monoclonal antibody and glucocorticoid receptor antagonist, RU38486, on metallothionein-inducing activity of serum from lipopolysaccharide-treated mice. Toxicology 1996; 112(1):29-36.

(7) Cousins RJ, Leinart AS. Tissue-Specific Regulation of Zinc-Metabolism and Metallothionein Genes by Interleukin-1. Faseb Journal 1988; 2(13):2884-2890.

(8) Schroeder JJ, Cousins RJ. Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proceedings of the National Academy of Sciences of the United States of America 1990; 87(8):3137-3141.

(9) Sato M, Sasaki M, Hojo H. Differential Induction of Metallothionein Synthesis by Interleukin-6 and Tumor-Necrosis-Factor-Alpha in Rat-Tissues. International Journal of Immunopharmacology 1994; 16(2):187-195.

(10) Ren YF, Smith A. Mechanism of Metallothionein Gene-Regulation by Heme-Hemopexin - Roles of Protein-Kinase-C, Reactive Oxygen Species, and Cis-Acting Elements. Journal of Biological Chemistry 1995; 270(41):23988-23995.

(11) Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochemical Pharmacology 2000; 59(1):95-104.

(12) Haton C, Francois A, Vandamme M, Wysocki J, Griffiths NM, Benderitter M. Imbalance of the antioxidant network of mouse small intestinal mucosa after radiation exposure. Radiation Research 2007; 167(4):445-453.

(13) Kondoh M, Imada N, Kamada K, Tsukahara R, Higashimoto M, Takiguchi M et al. Property of metallothionein as a Zn pool differs depending on the induced condition of metallothionein. Toxicology Letters 2003; 142(1-2):11-18.

(14) Palumaa P, Eriste E, Njunkova O, Pokras L, Jornvall H, Sillard R. Brain-specific metallothionein-3 has higher metal-binding capacity than ubiquitous metallothioneins and binds metals noncooperatively. Biochemistry 2002; 41(19):6158-6163.

(15) Tio L, Villarreal L, Atrian S, Capdevila M. Functional differentiation in the mammalian metallothionein gene family - Metal binding features of mouse MT4 and comparison with its paralog MT1. Journal of Biological Chemistry 2004; 279(23):24403-24413.

(16) Jiang LJ, Maret W, Vallee BL. The ATP-metallothionein complex. Proceedings of the National Academy of Sciences of the United States of America 1998; 95(16):9146-9149.

(17) Matsui T. Zinc deficiency in Crohn's disease. Journal of Gastroenterology 1998; 33(6):924-925.

(18) Goh J, O'Morain CA. Review article: nutrition and adult inflammatory bowel disease. Alimentary Pharmacoly & Therapeutics 2003; 17(3):307-320.

(19) Ainley C, Cason J, Slavin BM, Wolstencroft RA, Thompson RPH. The Influence of Zinc Status and Malnutrition on Immunological Function in Crohns-Disease. Gastroenterology 1991; 100(6):1616-1625.

(20) Shankar AH, Prasad AS. Zinc and immune function: the biological basis of altered resistance to infection. American Journal of Clinical Nutrition 1998; 68(2):447S-463S.

(21) Cario E, Jung S, Harder DJ, Schulte C, Sturm A, Wiedenmann B et al. Effects of exogenous zinc supplementation on intestinal epithelial repair in vitro. European Journal of Clinical Investigation 2000; 30(5):419-428.

(22) Maret W. Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxidants & Redox Signaling 2006; 8(9-10):1419-1441.

(23) Vasto S, Mocchegiani E, Malavolta M, Cuppari I, Listi F, Nuzzo D et al. Zinc and inflammatory/immune response in aging. Annals of the New York Academy of Sciences 2007; 1100:111-122.

(24) Chen BW, Wang HH, Liu JX, Liu XG. Zinc sulphate solution enema decreases inflammation in experimental colitis in rats. Journal of Gastroenterology and Hepatology 1999; 14(11):1088-1092.

(25) Luk HH, Ko JKS, Fung HS, Cho CH. Delineation of the protective action of zinc sulfate on ulcerative colitis in rats. European Journal of Pharmacology 2002; 443(1-3):197-204.

(26) Sturniolo GC, Fries W, Mazzon E, Di Leo V, Barollo M, D'Inca R. Effect of zinc supplementation on intestinal permeability in experimental colitis. Journal of Laboratory and Clinical Medicine 2002; 139(5):311-315.

(27) Ohkawara T, Takeda H, Kato K, Miyashita K, Kato M, Iwanaga T et al. Polaprezinc (N-(3-aminopropionyl)-L-histidinato zinc) ameliorates dextran sulfate sodium-induced colitis in mice. Scandinavian Journal of Gastroenterology 2005; 40(11):1321-1327.

(28) Tran CD, Ball JM, Sundar S, Coyle P, Howarth GS. The role of zinc and metallothionein in the dextran sulfate sodium-induced colitis mouse model. Digestive Diseases and Sciences 2007; 52(9):2113-2121.

(29) Di Leo V, D'Inca R, Barollo M, Tropea A, Fries W, Mazzon E et al. Effect of zinc supplementation on trace elements and intestinal metallothionein concentrations in experimental colitis in the rat. Digestive and Liver Disease 2001; 33(2):135-139.

(30) Mulder TP, van dS, V, Verspaget HW, Griffioen G, Pena AS, Janssens AR et al. Effect of oral zinc supplementation on metallothionein and superoxide dismutase concentrations in patients with inflammatory bowel disease. Journal of Gastroenterology and Hepatology 1994; 9(5):472-477.

(31) Abdel-Mageed AB, Agrawal KC. Activation of nuclear factor kappa B: Potential role in metallothionein-mediated mitogenic response. Cancer Research 1998; 58(11):2335-2338.

(32) Kim CH, Kim JH, Lee J, Ahn YS. Zinc-induced NF-kappaB inhibition can be modulated by changes in the intracellular metallothionein level. Toxicology and Applied Pharmacology 2003; 190(2):189-196.

(33) Kabe Y, Ando K, Hirao S, Yoshida M, Handa H. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. Antioxidants & Redox Signaling 2005; 7(3-4):395-403.

(34) Kanekiyo M, Itoh N, Kawasaki A, Matsuda K, Nakanishi T, Tanaka K. Metallothionein is required for zinc-induced expression of the macrophage colony stimulating factor gene. Journal of Cellular Biochemistry 2002; 86(1):145-153.

(35) Sakurai A, Hara S, Okano N, Kondo Y, Inoue J, Imura N. Regulatory role of metallothionein in NF-kappaB activation. FEBS Letters 1999; 455(1-2):55-58.

(36) Kanekiyo M, Itoh N, Kawasaki A, Matsuyama A, Matsuda K, Nakanishi T et al. Metallothionein modulates lipopolysaccharide-stimulated tumour necrosis factor expression in mouse peritoneal macrophages. Biochemical Journal 2002; 361(Pt 2):363-369.

(37) Crowthers KC, Kline V, Giardina C, Lynes MA. Augmented humoral immune function in metallothionein-null mice. Toxicology & Applied Pharmacology 2000; 166(3):161-172.

(38) Yamada T, Grisham MB. Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation. Klinische Wochenschrift 1991; 69(21-23):988-994.

(39) Naito Y, Takagi T, Yoshikawa T. Molecular fingerprints of neutrophil-dependent oxidative stress in inflammatory bowel disease. Journal of Gastroenterology 2007; 42(10):787-798.

(40) Irato P, Santovito G, Piccinni E, Albergoni V. Oxidative burst and metallothionein as a scavenger in macrophages. Immunology and Cell Biology 2001; 79(3):251-254.

(41) Singer II, Kawka DW, Scott S, Weidner JR, Mumford RA, Riehl TE et al. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 1996; 111(4):871-885.

(42) Kruidenier L, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. Journal of Pathology 2003; 201(1):28-36.

(43) Rezaie A, Ghorbani F, Eshghtork A, Zamani MJ, Dehghan G, Taghavi B et al. Alterations in salivary antioxidants, nitric oxide, and transforming growth factor-beta 1 in relation to disease activity in Crohn's disease patients. Annals of the New York Academy of Sciences 2006; 1091:110-122.

(44) Dincer Y, Erzin Y, Himmetoglu S, Gunes KN, Bal K, Akcay T. Oxidative DNA damage and antioxidant activity in patients with inflammatory bowel disease. Digestive Diseases and Sciences 2007; 52(7):1636-1641.

(45) Rao R. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Frontiers in Bioscience 2008; 13:7210-7226.

(46) Hollander D. Crohns-Disease - A Permeability Disorder of the Tight Junction. Gut 1988; 29(12):1621-1624.

(47) Carroll IM, Andrus JM, Bruno-Barcena JM, Klaenhammer TR, Hassan HM, Threadgill DS. Anti-inflammatory properties of Lactobacillus gasseri expressing manganese superoxide dismutase using the interleukin 10-deficient mouse model of colitis. American Journal of Physiology-Gastrointestinal and Liver Physiology 2007; 293(4):G729-G738.

(48) Kruidenier L, van Meeteren ME, Kuiper I, Jaarsma D, Lamers CB, Zijlstra FJ et al. Attenuated mild colonic inflammation and improved survival from severe DSS-colitis of transgenic Cu/Zn-SOD mice. Free Radical Biology and Medicine 2003; 34(6):753-765.

(49) Penkowa M, Hidalgo J. Metallothionein treatment reduces proinflammatory cytokines IL-6 and TNF-alpha and apoptotic cell death during experimental autoimmune encephalomyelitis (EAE). Experimental Neurology 2001; 170(1):1-14.

(50) Tsujikawa K, Imai T, Kakutani M, Kayamori Y, Mimura T, Otaki N et al. Localization of Metallothionein in Nuclei of Growing Primary Cultured Adult-Rat Hepatocytes. FEBS Letters 1991; 283(2):239-242.

(51) Nagel WW, Vallee BL. Cell-Cycle Regulation of Metallothionein in Human Colonic-Cancer Cells. Proceedings of the National Academy of Sciences of the United States of America 1995; 92(2):579-583.

(52) Cherian MG, Kang YJ. Metallothionein and liver cell regeneration. Experimental Biology and Medicine 2006; 231(2):138-144.

(53) Schmidt C, Beyersmann D. Transient peaks in zinc and metallothionein levels during differentiation of 3T3L1 cells. Archives of Biochemistry and Biophysics 1999; 364(1):91-98.

(54) Levadoux-Martin M, Hesketh JE, Beattie JH, Wallace HM. Influence of metallothionein-1 localization on its function. Biochemical Journal 2001; 355:473-479.

(55) Lynes MA, Zaffuto K, Unfricht DW, Marusov G, Samson JS, Yin X. The physiological roles of extracellular metallothionein. Experimental Biology and Medicine 2006; 231(9):1548-1554.

(56) Yin X, Knecht DA, Lynes MA. Metallothionein mediates leukocyte chemotaxis. BMC Immunology 2005; 6:21.

(57) Lynes MA, Garvey JS, Lawrence DA. Extracellular metallothionein effects on lymphocyte activities. Molecular Immunology 1990; 27(3):211-219.

(58) Borghesi LA, Youn J, Olson EA, Lynes MA. Interactions of metallothionein with murine lymphocytes: plasma membrane binding and proliferation. Toxicology 1996; 108(1-2):129-140.

(59) Lynes MA, Borghesi LA, Youn J, Olson EA. Immunomodulatory activities of extracellular metallothionein. I. Metallothionein effects on antibody production. Toxicology 1993; 85(2-3):161-177.

(60) Youn J, Lynes MA. Metallothionein-induced suppression of cytotoxic T lymphocyte function: an important immunoregulatory control. Toxicological Sciences 1999; 52(2):199-208.

(61) Canpolat E, Lynes MA. In vivo manipulation of endogenous metallothionein with a monoclonal antibody enhances a T-dependent humoral immune response. Toxicological Sciences 2001; 62(1):61-70.

(62) Penkowa M, Espejo C, Martinez-Caceres EM, Poulsen CB, Montalban X, Hidalgo J. Altered inflammatory response and increased neurodegeneration in metallothionein I+II deficient mice during experimental autoimmune encephalomyelitis. Journal of Neuroimmunology 2001; 119(2):248-260.

(63) Tran CD, Huynh H, van den BM, van der PM, Campbell MA, Philcox JC et al. Helicobacter-induced gastritis in mice not expressing metallothionein-I and II. Helicobacter 2003; 8(5):533-541.

(64) Jin HY, Hu Q, Jun JY, Ju AC, Sen LD, Qian DR et al. Preliminary studies on the zinc-induced metallothionein protein with antibacterial activity in housefly larvae, Musca domestica. Acta Biologica Hungarica 2005; 56(3-4):283-295.

(65) Itoh N, Shibayama H, Kanekiyo M, Namphung D, Nakanishi T, Matsuyama A et al. Reduced bactericidal activity and nitric oxide production in metallothionein-deficient macrophages in response to lipopolysaccharide stimulation. Toxicology 2005; 216(2-3):188-196.

(66) Youn J, Hwang SH, Ryoo ZY, Lynes MA, Paik DJ, Chung HS et al. Metallothionein suppresses collagen-induced arthritis via induction of TGF-beta and down-regulation of proinflammatory mediators. Clinical and Experimental Immunology 2002; 129(2):232-239.

(67) Huh S, Lee K, Yun HS, Paik DJ, Kim JM, Youn J. Functions of metallothionein generating interleukin-10-producing regulatory CD4+ T cells potentiate suppression of collagen-induced arthritis. Journal of Microbiology and Biotechnology 2007; 17(2):348-358.

(68) Waelput W, Broekaert D, Vandekerckhove J, Brouckaert P, Tavernier J, Libert C. A mediator role for metallothionein in tumor necrosis factor-induced lethal shock. Journal of Experimental Medicine 2001; 194(11):1617-1624.

(69) Leyshon-Sorland K, Morkrid L, Rugstad HE. Metallothionein: a protein conferring resistance in vitro to tumor necrosis factor. Cancer Research 1993; 53(20):4874-4880.

(70) Kimura T, Itoh N, Takehara M, Oguro I, Ishizaki JI, Nakanishi T et al. Sensitivity of metallothionein-null mice to LPS/D-galactosamine-induced lethality. Biochemical and Biophysical Research Communications 2001; 280(1):358-362.

(71) Bruwer M, Schmid KW, Metz KA, Krieglstein CF, Senninger N, Schurmann G. Increased expression of metallothionein in inflammatory bowel disease. Inflammation Research 2001; 50(6):289-293.

(72) Dooley TP, Curto EV, Reddy SP, Davis RL, Lambert GW, Wilborn TW et al. Regulation of gene expression in inflammatory bowel disease and correlation with IBD drugs: screening by DNA microarrays. Inflammatory Bowel Diseases 2004; 10(1):1-14.

(73) Lawrance IC, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. Human Molecular Genetics 2001; 10(5):445-456.

(74) Clarkson JP, Elmes ME, Jasani B, Webb M. Histological demonstration of immunoreactive zinc metallothionein in liver and ileum of rat and man. Histochemical Journal 1985; 17(3):343-352.

(75) Elmes ME, Clarkson JP, Jasani B. Histological demonstration of immunoreactive metallothionein in rat and human tissues. Experientia Suppl 1987; 52:533-537.

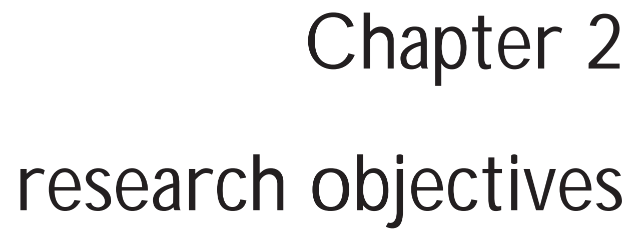
(76) Ioachim E, Michael M, Katsanos C, Demou A, Tsianos EV. The immunohistochemical expression of metallothionein in inflammatory bowel disease. Correlation with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices. Histology and Histopathology 2003; 18(1):75-82.

(77) Kruidenier L, Kuiper I, Van Duijn W, Mieremet-Ooms MA, Van Hogezand RA, Lamers CB et al. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. Journal of Pathology 2003; 201(1):17-27.

(78) Oz HS, Chen T, de Villiers WJ, McClain CJ. Metallothionein overexpression does not protect against inflammatory bowel disease in a murine colitis model. Medical Science Monitor 2005; 11(3):BR69-BR73.

(79) Mulder TP, Verspaget HW, Janssens AR, de Bruin PA, Pena AS, Lamers CB. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. Gut 1991; 32(10):1146-1150.

(80) Sturniolo GC, Mestriner C, Lecis PE, D'Odorico A, Venturi C, Irato P et al. Altered plasma and mucosal concentrations of trace elements and antioxidants in active ulcerative colitis. Scandinavian Journal of Gastroenterology 1998; 33(6):644-649.



Chapter 2

research objectives

This thesis combines the research conducted in two fields, both related to the defective mucosal barrier in IBD. The first part focuses on the interaction of therapeutic IL-10 producing bacteria with the intestinal mucosa. In the second part, we took a closer look at the expression and function of MTs, that can be considered immunomodulatory proteins, in intestinal inflammation.

Interaction of the mucosal barrier with therapeutic bacteria

Genetically modified *Lactococcus lactis* secreting IL-10 has been demonstrated to provide localized delivery of a therapeutic agent through active *in situ* synthesis in murine colitis (1). A safety study conducted at the Academic Medical Centre in Amsterdam showed that the bacteria were well tolerated and some clinical efficacy could be demonstrated (2). Further clinical trials with this potential therapeutic agent are planned.

The exact mechanism by which the beneficial effect of the IL-10 producing *L. lactis* on the mucosa is mediated is complex and many aspects remain to be unveiled. At the time we started this study, preliminary data existed that showed the presence of small amounts of IL-10, delivered by the bacteria, in intestinal tissue homogenates. Based on these findings, we formulated different hypotheses for the mechanism of action of these therapeutic bacteria. First, the lactococci could produce IL-10 at the luminal side, with a subsequent diffusion of the cytokine to responsive cells in the epithelium. Alternatively, the lactococci may be taken up by M cells or DCs with consecutive production of IL-10 *in situ* in intestinal lymphoid tissue. A third route might involve paracellular transport of intact bacteria. All three routes are known to be enhanced in inflammation.

In chapter 3, we aimed to determine the interaction of *L. lactis* with the intestinal mucosa. We were interested whether this interaction would be different in a healthy mucosa compared with an inflamed (damaged) mucosa. Therefore, we performed *in vivo* experiments in healthy mice and in two mouse models of chronic (entero)colitis. Samples were extensively examined with both electron and confocal microscopy to test the three formulated hypotheses. To test whether bacteria present in intestinal mucosa could deliver IL-10 *in situ* in the tissue, viability and activity of *L. lactis* in lamina propria homogenates were assessed. Finally, given the possible uptake of the bacteria in the mucosa, we wanted to assess their presence in systemic lymphoid tissues.

Roles for metallothioneins as immunomodulators in intestinal inflammation

In search of new candidate genes for Crohn's disease, we came upon the family of the MTs. Their location within the susceptibility locus *IBD1*, together with their presumed protective functions in inflammation made them an interesting family to study. In order to elucidate the role of MTs in Crohn's disease, we first studied their expression and compared it with the expression in normal controls in chapter 4 part 1. We analyzed mRNA levels and performed immunohistochemical stainings, making a distinction between expression in unaffected and affected tissue to clarify whether aberrant MT expression is dependent or independent on inflammation.

To study the functional consequences of aberrant MT expression in Crohn's disease, we induced a dextran sodium sulphate colitis in MT-null mice. We examined whether these mice demonstrated a different course of colitis compared with wild type mice of the same genetic background, and tried to correlate this to colonic MT expression, which we studied in detail. In addition, we created a knockdown intestinal epithelial cell line. To investigate whether a decreased MT expression could influence the response of the epithelial barrier on bacteria, we challenged this cell line with invasive bacteria.

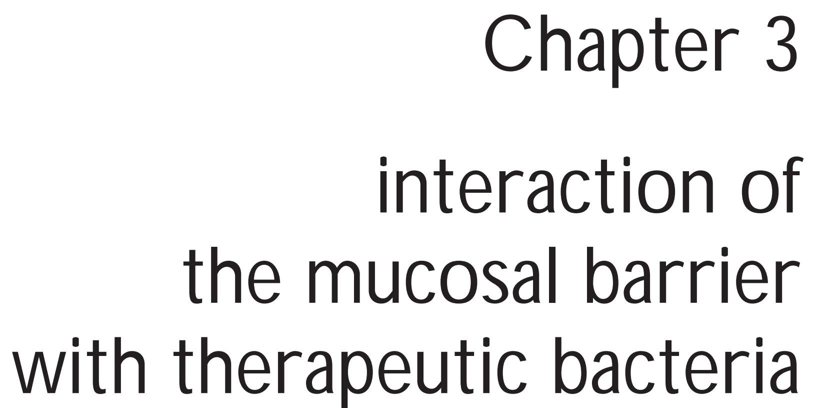
Next to studying MT protein expression in Crohn's disease, we studied this in ulcerative colitis and in two other intestinal inflammatory conditions, i.e. diverticulitis and ischaemic inflammation (chapter 4 part 2). We attempted to provide a detailed expression pattern of MTs in the intestine under normal and inflammatory conditions, describing the MT levels in different cell types during the course of inflammation.

The results of the studies in chapter 4 part 1 and part 2 revealed that an important role is reserved for MT gene regulation, regarding to expression in disease as well as to expression in different cell types. Therefore, we reviewed the regulation of MT gene expression in health and disease in chapter 4 part 3. For this review, we started from an *in silico* comparison of human and murine MT promoters.

references

(1) Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 2000 Aug 25;289(5483):1352-5.

(2) Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, et al. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol 2006 Jun;4(6):754-9.



Article published in Inflammatory Bowel Diseases 2008 Apr;14(4):471-9

Paracellular Entry of Interleukin-10 Producing *Lactococcus lactis* in Inflamed Intestinal Mucosa in Mice

Anouk Waeytens, MBE, Liesbeth Ferdinande, MD,* Sabine Neirynck, PhD,† Pieter Rottiers, PhD,‡ Martine De Vos, MD, PhD,§ Lothar Steidler, PhD,† and Claude A. Cuvelier, MD, PhD**

Background: Genetically modified *Lactococcus lactis* secreting interleukin-10 (IL-10) has been demonstrated to provide localized delivery of a therapeutic agent through active in situ synthesis in murine colitis. At present, many aspects of the exact mechanism by which the beneficial effect of the IL-10-producing *L. lactis* on the mucosa is mediated remain to be clarified.

Methods: Our aim was to determine the interaction of *L. lactis* with the intestinal mucosa. Therefore, we administered IL-10-producing *L. lactis* to healthy mice and in 2 mouse models of chronic colitis. Paraffin sections of ileum and colon samples were examined with confocal and transmission electron microscopy. Ileum and colon homogenates were prepared after flushing and after removal of mucus layer and epithelium. These homogenates and homogenates of mesenteric lymph nodes and spleen were plated on agar and immunoblotting for *L. lactis* and IL-10 was performed.

Results: Both confocal and electron microscopy showed the presence of lactococci in inflamed intestinal mucosa of mice with colitis. We recovered viable bacteria that could still produce IL-10 from homogenates of inflamed ileum and colon of which mucous and epithelial layers were removed. We did not find lactococci in mesenteric lymph nodes or in the spleen of mice with colitis.

Conclusions: This study demonstrates uptake of IL-10-secreting *L. lactis* by the paracellular route in inflamed mucosal tissue. We suggest that IL-10 production by *L. lactis* residing inside the mucosa in the vicinity of responsive cells can improve the local action of interleukin-10 in inflamed tissue and the efficiency of the treatment.

(*Inflamm Bowel Dis 2007;00:000–000)*

Received for publication June 7, 2007; Accepted October 23, 2007.

From the *Department of Pathology, Ghent University, Ghent, Belgium, †Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland, ‡Department of Molecular Biomedical Research, Ghent University – VIB, Zwijnaarde, Belgium, §Department of Internal Medicine, Ghent University Hospital, Ghent, Belgium.

Supported by a concerted action grant BOF2005/GOA/012 of Ghent University. L.F. is supported by a personal grant of the Bijzonder Onderzoeksfonds, Ghent University.

Present address for S.N., P.R., L.S.: ActoGeniX, Zwijnaarde, Belgium.

Reprints: Anouk Waeytens, Department of Pathology, Ghent University, De Pintelaan 185, B-9000 Ghent, Belgium (e-mail: Anouk.Waeytens@UGent.be).

Copyright © 2007 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1002/ibd.20346

Published online in Wiley InterScience (www.interscience.wiley.com).

Key Words: *Lactococcus lactis*, inflammatory bowel disease, interleukin-10 therapy, intestinal mucosa

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is characterized by chronic, nonspecific inflammation of the gastrointestinal tract. Conventional therapy for IBD consists of aminosalicylates and corticosteroids combined with immunosuppressive agents for maintenance. In most cases this means lifelong treatment with systemically administered medication, involving inefficient drug delivery and possible unpleasant side effects. Novel therapeutic strategies for IBD focus on targeting inflammatory cytokines, antiinflammatory cytokines, antigen processing/presentation/recognition, apoptosis, and repair/restitution of barrier function.[1] One of the biological agents under investigation is interleukin-10 (IL-10). The principal function of this cytokine is to contain and eventually terminate inflammatory responses by suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, and antigen-presenting and costimulatory molecules in monocytes/macrophages, neutrophils, and T cells.[2] The observation that IL-10-deficient mice develop chronic enterocolitis indicated that IL-10 is an essential immune modulator in the intestinal tract and formed the rationale behind IL-10 administration in animal models of IBD.[3] The results of these studies showed that IL-10 was successful in preventing but not reversing intestinal inflammation, mainly by downregulating an intestinal proinflammatory Th1 response.[4–6]

Systemic IL-10 treatment has been shown to yield rather disappointing results in multicenter trials,[7,8] probably due to low final concentrations of IL-10 in the intestine. Some years ago a new therapeutic approach to IBD was described that uses genetically modified *Lactococcus lactis* bacteria secreting IL-10.[9] The local delivery of this cytokine by food-grade bacteria seems to offer a solution to overcome the problem of systemic IL-10 treatment. Previous studies have shown that intragastric administration of IL-10-producing *L. lactis* caused a 50% reduction in colitis in the chronic dextran sodium sulfate model and prevented the onset of colitis in IL-10-deficient mice.[9] A safety study conducted at the Academic Medical Center in Amsterdam showed that the bacteria were well tolerated and some clinical efficacy could be demonstrated.[10]

The exact mechanism by which the beneficial effect of the IL-10-producing *L. lactis* on the mucosa is mediated is complex and many aspects remain to be unveiled. Previously, we could detect small amounts of IL-10 in intestinal tissue homogenates by enzyme-linked immunosorbent assay (ELISA), but not in the lumen.[9] One possibility is that the lactococci produce IL-10 at the luminal side in close proximity to responsive cells in the epithelium. Alternatively, the lactococci may be taken up by membranous (M) cells or by dendritic cells (DCs) and subsequently produce IL-10 in situ in intestinal lymphoid tissue. A third route might involve paracellular transport mechanisms. All three routes are known to be enhanced in intestinal inflammation.

To determine the interaction of *L. lactis* with the intestinal mucosa, we administered human (h)IL-10-producing *L. lactis* to healthy mice and in 2 mouse models of chronic colitis. We analyzed ileum and colon samples by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), paying special attention to the possible uptake by M cells or DCs. Plating and subsequent immunoblotting were carried out to assess viability and activity of *L. lactis* in tissue homogenates of epithelium-stripped ileum and colon, mesenteric lymph nodes (MLNs), and spleen from IL-10-deficient mice.

MATERIALS AND METHODS

Bacteria

An hIL-10-producing *L. lactis* MG1363 (*LL-hIL-10*) culture[11] was prepared by inoculating a stock suspension 1/1000 in M17 broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.5% glucose (GM17) and was grown overnight. The culture was then washed twice in sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in BM9 medium[9] to a concentration of 2×10^{10} CFU/mL.

Animals

Two mouse models of IBD were used in this study. First, we induced a chronic dextran sodium sulfate (DSS) colitis.[12] Twelve-week-old Balb/c mice (Charles River, Boston, MA) received 4 administration cycles of DSS, each cycle consisting of 7 days of 4% DSS (MW = 36,000–50,000, MP Biomedicals, Irvine, CA) in tap water followed by 10 days of tap water (DSS-Balb/c, $n = 3$; control animals: con-Balb/c, $n = 3$). After the last cycle mice received tap water for an additional 11 days. During the experiment weight loss and stool consistency were monitored. Alternatively, IL-10-deficient 129Sv/Ev (IL-$10^{-/-}$) mice (bred in-house) were used as a model for chronic enterocolitis ($n = 13$).[3] They were used at 26 weeks of age, when chronic colitis had fully developed. Healthy 129Sv/Ev (IL-10WT) mice served as control animals ($n = 6$). The animal studies were approved by the Ethics Committee for Animal Studies of the Faculty of Medicine and Health Sciences (file no. ECP 03-49).

Intestinal Loops

To study the interaction of *L. lactis* with the mucosa by means of TEM and CLSM, in vivo ligated loops were constructed in the terminal ileum of IL-$10^{-/-}$, IL-10WT, DSS-Balb/c, and con-Balb/c. Mice were deprived of food for 24 hours and then anesthetized. After laparotomy, 1 ileal loop of ≈2 cm per mouse was formed and injected with 0.2 mL of BM9 buffer containing 4×10^9 *LL-hIL-10* (2 IL-$10^{-/-}$, 2 IL-10WT, 2 DSS-Balb/c, and 2 con-Balb/c) or buffer alone (sham control, 1 IL-$10^{-/-}$, 1 IL-10WT, 1 DSS-Balb/c, and 1 con-Balb/c). The abdomen was closed and the animals were placed in a climate room of 37°C. After 60 minutes the animals were killed through cervical dislocation and the loops were removed. Tissue samples were immediately fixed in 2% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate buffer for TEM analysis or in 4% buffered formalin for CLSM analysis.

Intragastric Inoculation

In order to study the presence of lactococci in larger pieces of intestine and in systemic tissue, IL-$10^{-/-}$ ($n = 10$) and IL-10WT mice ($n = 3$) received lactococci through intragastric inoculation (IG). Following food deprivation for 24 hours, mice were given 0.1 mL of BM9 buffer containing 2×10^9 *LL-hIL-10* or buffer alone (sham control) by IG 10 times with intervals of 30 minutes. Two and 20 hours after the last inoculation mice were sacrificed by cervical dislocation and spleen, mesenteric lymph nodes (MLNs), ileum (only after 2 hours), and colon (only after 2 hours) were isolated. After 2 washes in PBS, spleen and MLNs were mixed in 1 mL BM9. At this step in the protocol, samples of ileum and colon were taken and fixed in 4% buffered formalin for CLSM analysis. The remaining ileum and colon were flushed thoroughly with Hanks' balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) and one-third of the tissue was mixed in 1 mL PBS. The rest of the tissue was incubated for 20 minutes with 1 mM 1,4-dithiothreitol (DTT, Roche Diagnostics, Basel, Switzerland)/HBSS and half of the tissue was mixed in 1 mL PBS. The remaining tissue was then incubated twice in 10 mM EDTA (Invitrogen)/HBSS for 20 minutes and mixed in 1 mL PBS. After each incubation step, pieces of ileum and colon were taken and fixed in 4% buffered formalin for histological evaluation.

Histology

Pieces of fixed mucosa were embedded in paraffin wax and hematoxylin and eosin (H&E) staining was carried out according to routine procedures. A gastrointestinal histological score ranging from 0 to 4+ was used in a blinded fashion to evaluate intestinal inflammation, as validated for IL-$10^{-/-}$ mice[13] and the DSS model.[14] Scores are expressed as medians ± interquartile range. Statistical significance between different groups were evaluated by the Mann–Whitney U-test.

Differences were considered significant at a *P*-value of <0.05.

Transmission Electron Microscopy

Glutaraldehyde/paraformaldehyde fixed loop samples were postfixed in osmium tetroxide, dehydrated in a series of alcohol, and embedded in Epon (Aurion, Wageningen, The Netherlands). Semithin 1-μm sections were stained with Toluidine blue (Roth, Karlsruhe, Germany) and were used for selection of areas where bacteria were present. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss TEM900 at 50 kV (Carl Zeiss, Oberkochen, Germany). A culture of *LL-hIL-10* was processed in the same way to serve as a morphological characterization of the *L. lactis* bacteria to identify these in our intestinal loop samples.

Confocal Laser Scanning Microscopy

Paraffin wax sections of ≈30 μm of formalin-fixed intestinal mucosa sampled from intestinal loop and IG experiments were rehydrated and then quenched with 50 mM NH_4Cl. Nonspecific binding sites were blocked with 0.4% Fish Skin Gelatin (Sigma, St. Louis, MO), after which sections were incubated for 1 hour with rabbit anti-*L. lactis* antibody (anti-MG1363, raised at the Applied Molecular Bacteriology Unit, DMBR, Ghent). Alexa Fluor 488 antirabbit (Molecular Probes, Eugene, OR) was used as secondary antibody. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Visualization was performed on a Bio-Rad (Hercules, CA) Radiance 2100 Blue Diode CLSM system (Carl Zeiss) using a 60× plan apo oil immersion objective. Images were taken with Bio-Rad Lasersharp 2000 software (Carl Zeiss) using a sequential scanning method and processed with ImageJ (W.S. Rasband, US National Institutes of Health, Bethesda, MD [http://rsb.info.nih.gov/ij/]).

Immunoblotting

IG homogenate (100 μL) from spleen, MLNs, ileum (flushed, DTT/EDTA-treated), and colon (flushed, DTT/EDTA-treated) was plated out on GM17 agar supplemented with 5 μg/mL erythromycin to selectively grow the erythromycin-resistant *LL-hIL-10*. Plate colonies were transferred to nitrocellulose membranes (Millipore, Billerica, MA) on which immunoblotting was performed using rabbit anti-MG1363 or rabbit anti-hIL-10 (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibody and horseradish peroxidase-antirabbit (Santa Cruz Biotechnology) as secondary antibody. For visualization we applied BM Chemiluminescence blotting substrate (Roche Diagnostics) and Hyperfilm ECL (Amersham Biosciences, Uppsala, Sweden). Negative controls were carried out similarly with omission of primary antibody.

RESULTS

Assessment of Inflammation in the Chronic DSS Model and IL-10$^{-/-}$ Mice

During the consecutive DSS-cycles, DSS-Balb/c mice lost weight and got loose stool after drinking DSS and regained weight during the recovery (water) phase of the cycles. Histological examination showed signs of acute ileitis, characterized by focal infiltrates of mononuclear cells and granulocytes, elongation of villi, and mucosal thickening (Fig. 1A). In the distal colon, DSS-Balb/c mice presented with chronic inflammation, featuring architectural crypt distortion, an increased number of lymphoid follicles, and an extensive inflammatory infiltrate (Fig. 1B). Con-Balb/c mice showed normal histological findings. Histological inflammatory scores for the colon were significantly different between DSS-Balb/c and Con-Balb/c (2.50 ± 2.25 versus 0.50 ± 1.25, $P = 0.014$).

IL-10$^{-/-}$ mice, which spontaneously develop enterocolitis at the age of 3 to 8 weeks,[3] were used at 26 weeks, at which timepoint 3 out of 13 mice had developed rectal prolapse. In our studies, IL-10$^{-/-}$ mice showed less severe inflammation at corresponding ages than that observed in the literature,[15] probably because of different housing.[9] Ileal sections revealed mild inflammation with inflammatory cell infiltration in the lamina propria (Fig. 1C), whereas in colon tissue we observed a more severe inflammation represented by an inflammatory infiltrate reaching to the submucosa, mucus cell depletion, and architectural crypt distortion (Fig. 1D). Neither macroscopic nor microscopic examination revealed the occurrence of tumors. Moreover, dysplasia was not seen in H&E sections. Sections from IL-10WT control mice showed normal histological findings. Histological inflammatory scores for the colon were significantly different between IL-10$^{-/-}$ mice and IL-10WT mice (2.40 ± 1.20 versus 0.00 ± 0.00, $P = 0.008$).

H&E slides of tissue obtained after DTT/EDTA treatment proved that these treatments adequately removed mucus and epithelium, respectively (Fig. 1E,F).

Interaction of *L. lactis* with Normal Mucosa

To investigate whether lactococci would be taken up by M cells in the follicle-associated epithelium (FAE), we first created terminal ileum loops in a region with Peyer's Patches in healthy con-Balb/c mice and injected *LL-hIL-10* in these loops. TEM of intestinal tissue showed numerous bacteria in the lumen, some of which adhered to the glycocalyx (data not shown). Bacteria that were abundantly present in the sections and that had morphological features identical to those of a *Lactococcus* culture were assumed to be lactococci, also because we did not detect such bacteria in sham controls that had not received *LL-hIL-10*. FAE of healthy mice showed transcellular uptake of lactococci (Fig. 2A), although this was a rare observation. We also occasionally recognized transep-

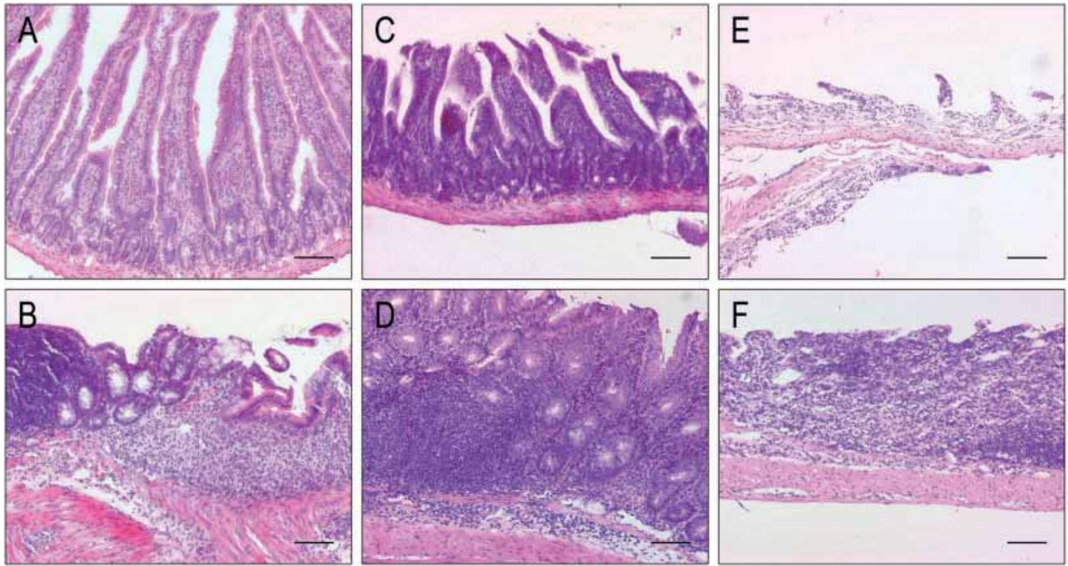


FIGURE 1. Representative micrographs of ileal (A,C,E) and colonic (B,D,F) mucosa from a DSS-Balb/c and an IL-$10^{-/-}$ mouse, H&E-stained. A: Acute inflammation in DSS-Balb/c ileum presented by elongated villi and thickened mucosa. B: Distortion of crypt architecture, an increased number of lymphoid follicles, and an extensive inflammatory infiltrate demonstrate chronic inflammation in the colon of DSS-Balb/c mice. C: Mild chronic inflammation in the ileum of an IL-$10^{-/-}$ mouse illustrated by an increased amount of inflammatory cells in the lamina propria. D: Severe chronic inflammation in the colon characterized by a diffuse infiltrate of mononuclear and polymorphonuclear cells reaching to the submucosa, atrophy and branching of crypts and depletion of mucus cells. E,F: DTT/EDTA-treatment of ileum and colon, respectively, efficiently removed mucous and epithelial layers. Scale bars = 100 μm.

ithelial dendrites close to luminal lactococci (Fig. 2B). In addition, we observed *L. lactis* in the lumen adjacent to M cells but not in contact with or inside M cells.

We wanted to confirm the ultrastructural findings on larger tissue areas by confocal analysis. For that reason we performed fluorescent anti-lactococcal staining on thick (30 μm) sections of ileal tissue originating from loop experiments. Confocal analysis of loops inoculated with hIL-10-producing *L. lactis* showed a great amount of cocci in the lumen positive for the anti-MG1363 antibody. Sham controls showed no positive staining. Lactococci were not detected in the FAE or underlying lymph follicles by confocal microscopy (data not shown).

hIL-10-Producing *L. lactis* Enters Inflamed Intestinal Mucosa

Next we examined if and how epithelial damage due to inflammation influences the interaction of the *Lactococcus* bacteria with the mucosa. Therefore, we repeated the intestinal loop experiments in 2 mouse models for colitis: Balb/c mice in which we had induced a chronic DSS colitis and IL-$10^{-/-}$ mice. In previous studies we found that mice with chronic DSS colitis showed signs of acute ileitis next to chronic colitis. In ileal samples of colitic mice (DSS-Balb/c and IL-$10^{-/-}$ mice) lactococci were seen entering acutely injured epithelium in a paracellular way. The bacteria were detected in small groups between cells and debris inside inflamed mucosal tissue, inside lymph vessels, and inside phagosomes in mucosal cells that morphologically resemble dendritic cells (Fig. 2C–F).[16] Some single bacteria, other than *L. lactis* (based on their morphology), could also be detected in inflamed tissue, between cells, and inside phagocytosing cells.

Confocal analysis of sections of inflamed ileum confirmed the data obtained with TEM: next to an abundant occurrence in the lumen, MG1363-positive *L. lactis* bacteria were present in the epithelium and the lamina propria of inflamed tissue. Collections of Z-series of 30-μm sections clearly demonstrated that the bacteria were present inside the tissue, between cells, and were not lying on top of the sections (Fig. 3A–C). The regular detection of lactococci inside inflamed ileal tissue prompted us to examine inflamed colon and to look for lactococci in MLNs and spleen. For that purpose we intragastrically administered *LL-hIL-10* to IL-

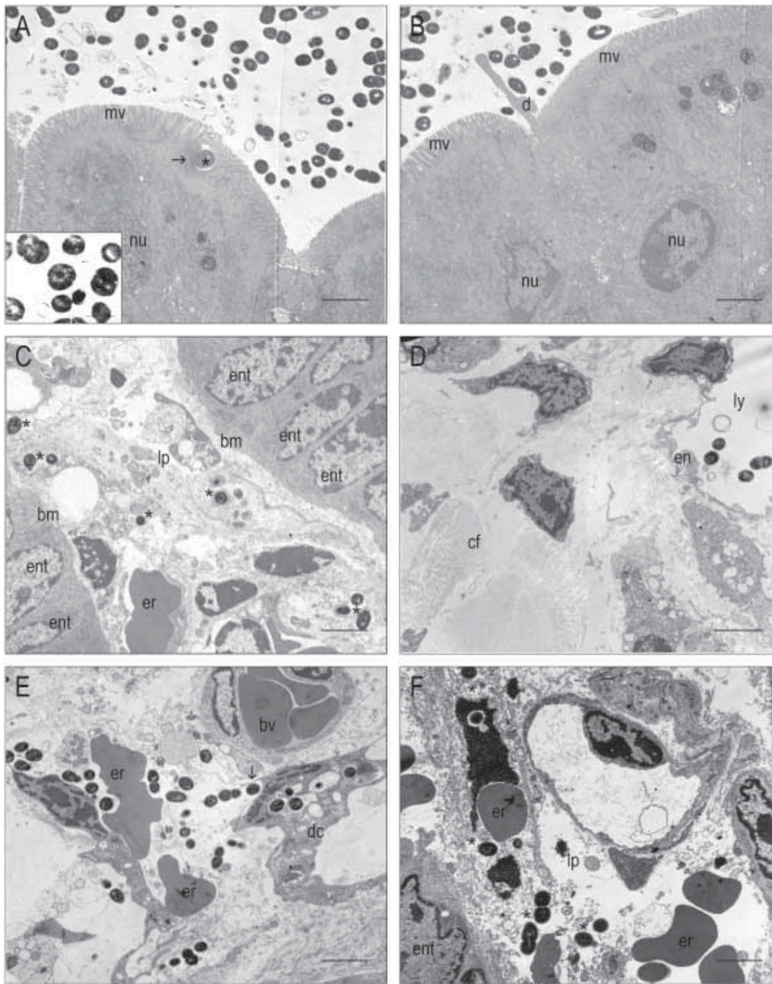


FIGURE 2. Transmission electron micrographs of intact mucosa from loops in control mice (A,B) and of inflamed mucosa originating from ileal loops in DSS-Balb/c mice (C–E) and IL-$10^{-/-}$ mice (F). A: *L. lactis* was identified as morphologically resembling bacteria of a culture of *LL-hIL-10* (insert, bar = 1 μm) and present in large quantities in the loop samples. In healthy ileal mucosa, *L. lactis* was abundantly present in the lumen. Additionally, lactococci were found to be internalized (asterisk) by enterocytes of the FAE demonstrating transcellular passage. Note the actin accumulation at the contact site (arrow) (mv: microvilli, nu: nucleus). B: A transepithelial dendrite (d) close to lactococci, suggesting DC uptake of lactococci. C–F: After 60 minutes, *L. lactis* is demonstrated inside acutely inflamed ileal mucosa. The micrographs represent focal observations. Average bacterial counts per block scanned were 12.2 (range 0–33) for IL-$10^{-/-}$ mice (n = 9) and 29.4 (range 0–106) for DSS-Balb/c mice (n = 18). C: *L. lactis* (asterisks) in a strip of lamina propria (lp) between 2 crypts (ent: enterocytes, bm: basement membrane, er: erythrocytes). D: *L. lactis* in a lymphatic vessel (ly) in the submucosa (cf, collagen fibers; en, endothelium). E: *L. lactis* inside phagosomes and being engulfed by (arrow) a presumed dendritic cell (dc) in the lamina propria (bv, blood vessel). F: *L. lactis* (asterisks) between inflammatory cells, erythrocytes and debris in the lamina propria between enterocytes in inflamed ileum. Scale bars = 3 μm.

$10^{-/-}$ mice and took samples in the ileum and the colon. We specifically chose this model to be able to study IL-10 delivery since these mice lack endogenous IL-10. With CLSM, we regularly found the bacteria in the lamina propria of colon sections from IL-$10^{-/-}$ mice, offering further evidence for the paracellular uptake of lactococci in inflamed intestinal tissue (Fig. 3D–F).

Lactococcus lactis Present in Inflamed Mucosa Are Viable and Actively Secreting hIL-10

To investigate whether the lactococci that were found in inflamed mucosa were alive, we prepared ileum and colon homogenates from IL-$10^{-/-}$ mice that received *LL-hIL-10* intragastrically. Homogenates were prepared after flushing intact tissue twice with HBSS and after removal of the mucous layer and the epithelium by DTT and EDTA. Plating of these homogenates on GM17E plates not only unveiled *LL-hIL-10* colonies but phenotypically differing colonies as well. Therefore, we performed immunoblotting with a specific antibody for *L. lactis*. In this way, we could detect $\approx$2.5–3.75 $\times$ 10^6 lactococcal CFU in flushed ileum and colon homogenates and $\approx$1.5 $\times$ 10^4 in DTT/EDTA-treated ileum homogenates (Fig. 4A). In DTT/EDTA-homogenates of colon we could show the presence of 3 $\times$ 10^4 and 138 CFU in only 2 out of 5 mice. The numbers of bacteria recovered did not correlate with histological inflammatory scores. Immuno-

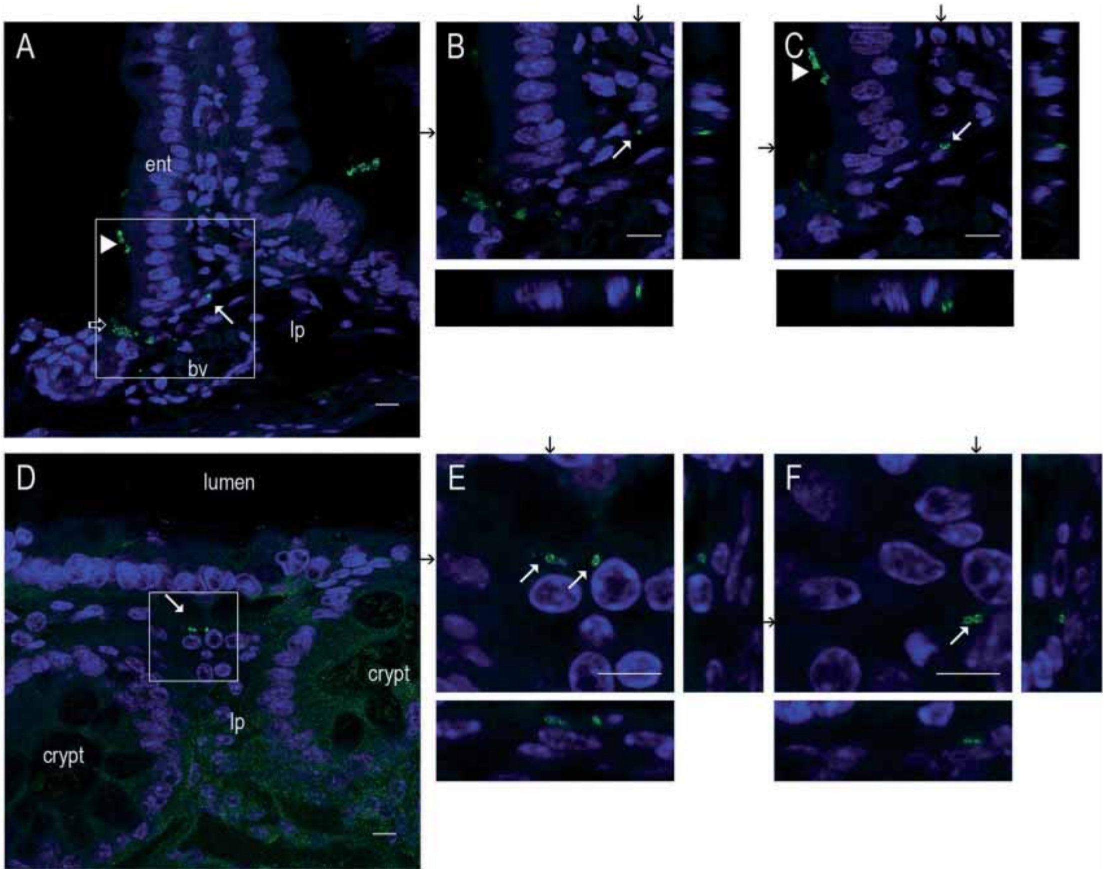


FIGURE 3. Confocal images of thick sections from ileum (A–C) and colon (D–F) from IL-$10^{-/-}$ mice that received *L. lactis* intragastrically. A: Some lactococci (green fluorescence) are adhering to (arrowhead), entering (hollow arrow) and inside (arrow) inflamed ileum (nuclei: blue fluorescence). At the place of the box a series of optical sections was taken in 0.2-μm steps, of which XY sections at 16 μm (B) and at 10.8 μm (C) from the glass slide are shown. Corresponding XZ (bottom) and YZ (right) cross-sections that were taken in the directions of the black arrows clearly show that lactococci are inside of the tissue. D: Inflamed colon shows the presence of lactococci (arrow) in the lamina propria (lp) below the surface epithelium. XY sections at 26 μm (E) and at 17.2 μm (F) with their corresponding XZ and YZ cross-sections demonstrate that the bacteria are residing inside the tissue. Enterocytes (ent), lamina propria (lp), blood vessel (bv). Scale bars = 10 μm.

blotting with an antibody against hIL-10 revealed that these lactococci could still produce IL-10 (Fig. 4B). Negative controls showed no signal.

Lactococcus lactis Do Not Enter Systemic Tissue

The presence of lactococci in mucosal tissue of inflamed samples caused us to examine the MLNs and the spleen. Therefore, we isolated MLNs and spleen from IL-$10^{-/-}$ mice that had received *LL-hIL-10* intragastrically, both 2 and 20 hours after the last dose, since MacPherson and Uhr[17] reported maximal commensal CFU about 20 hours after intragastric gavage. Plating and subsequent immunoblotting for lactococci of MLN or spleen homogenates revealed no lactococcal CFU, although CFU from other bacteria were found on the plates.

DISCUSSION

In this study we examined the penetration of lactococci in healthy versus inflamed intestinal tissue. The results we obtained with TEM show that, after administration by loop construction, *L. lactis* is occasionally taken up in a transcellular way in healthy FAE in the terminal ileum by entero-

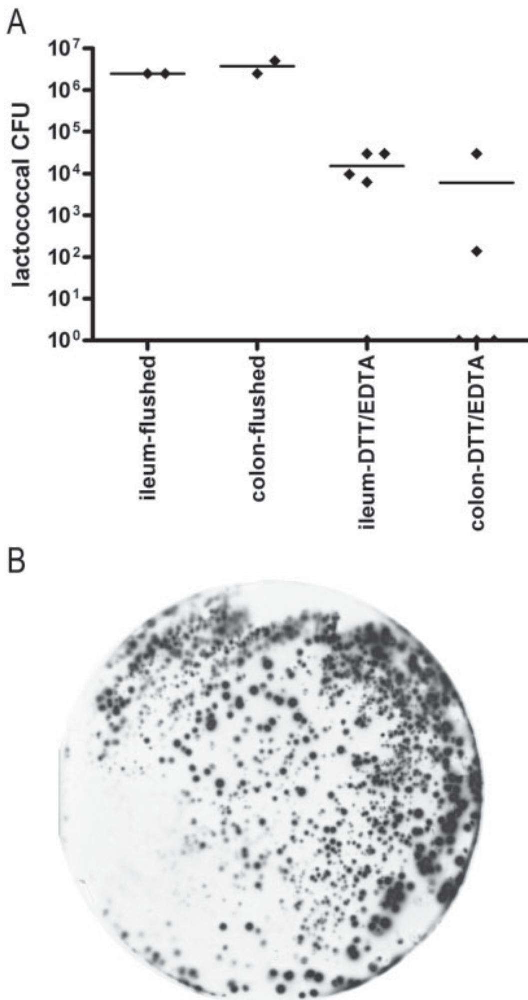


FIGURE 4. Viability and hIL-10 production of *LL-hIL-10* recovered from inflamed murine mucosa. *LL-hIL-10* was administered to IL10$^{-/-}$ mice by intragastric inoculation, and homogenates of ileum and colon were prepared after flushing and DTT/EDTA-treatment. A: The number of viable *LL-hIL-10* was determined by plating and subsequent immunoblotting with a specific anti-MG1363 antibody. B: *LL-hIL-10* recovered from DTT/EDTA-treated ileum and colon is still able to produce hIL-10, as shown by immunoblotting with an anti-hIL-10 antibody.

cytes, similar to the recently reported uptake of nonpathogenic bacteria.[18] However, this was a rare observation. We also occasionally recognized transepithelial dendrites close to luminal lactococci, suggesting DC sampling as described by Rescigno et al.[19,20] Other authors could not detect transepithelial dendrites in response to pathogen challenge in Balb/c mice in contrast to C57BL6 mice.[21] The fact that we spotted this event in Balb/c mice could be due to the nonpathogenic character of the lactococci or to the different origin of Balb/c mice used in this study. In our samples we could not detect uptake of lactococci by M cells. We did observe *L. lactis* in the lumen adjacent to M cells, suggesting this is not a major entry route for this bacterium. The fact that we could not confirm the presence of lactococci in ileal tissue of healthy mice with confocal microscopy could be because the anti-MG1363 antibody might not recognize internalized lactococci, but only lactococci that are residing free in the lumen or tissue.

Both confocal and electron microscopic analysis suggest that the uptake of bacteria is predominantly mediated by the paracellular route in inflamed mucosal tissue in mice. The entrance of the bacteria in inflamed tissue is related to the defective barrier in inflamed mucosa, allowing unrestrained uptake of antigens and proinflammatory molecules, including luminal bacteria. In IBD, protection of the mucosa is thought to be reduced as a result of specific changes in the thickness or composition of the intestinal mucus layer.[22] The mucus layer can be diminished or become weaker and the number of bacteria can be increased within the intestinal lumen or in the residual mucus layer. When these events take place, bacteria may adhere to the mucosa and increase exposure of the mucosal immune system to intestinal bacteria or bacterial components.[23] The defective mucosal barrier is also a result of dysfunctional epithelial tight junctions, which give rise to an increased paracellular permeability that seems to be independent of activity of disease.[24] Finally, loss of surface epithelial cells due to erosion allows unrestricted passage of luminal antigens and bacteria to the underlying lamina propria.

Our experimental data correspond with the findings of other groups,[25–27] in that we find *L. lactis* as well as other bacteria in inflamed mucosal tissue in small groups between the cells and inside phagocytosing cells. One group found that in IBD specimens gut bacteria, including nonpathogenic endogenous bacteria, were present in both the mucosa and the submucosa and that bacterial invasion was pronounced in the area of erosions and in crypt abscesses.[25] They observed some bacterial cells in epithelial cells or phagocytes of the lamina propria. Bacteria were seen either as cell clusters or as single cells scattered throughout the inflamed tissue. By plating and subsequent immunoblotting, we detected viable lactococci in flushed ileum and colon homogenates and in DTT/EDTA-treated ileum homogenates. In DTT/EDTA-homogenates of colon we showed the presence of viable lactococci in only 2 out of 5 mice. Three might have been false-negative due to the high bacterial background in the colon. These results match the findings of other studies that report the highest mean concentrations of mucosal bacteria in the ileum of IBD patients.[26,27] In those studies, individual

differences in concentrations of mucosal bacteria were found as well, differing markedly from patient to patient.

Data from animal models and the potent antiinflammatory properties of IL-10 suggested that this cytokine could be a new candidate for therapeutic suppression of mucosal inflammation in Crohn's disease. Moreover, data from the first study to report on the effects of the clinical application of IL-10 in human disease indicated that such treatment might be efficacious.[28] Several large multicenter trials were performed testing multiple IL-10 dosages in patients with Crohn's disease and the overall outcome was that IL-10 therapy was safe and well-tolerated.[7,8,29] However, IL-10 treatment did not result in significantly higher remission rates or clinical improvement compared with placebo treatment. One of the possible explanations for these disappointing results is the restricted bioavailability of IL-10 at the intestinal mucosa. However, increasing the dose of systemically administered IL-10 is not possible because of side effects, probably because high doses of IL-10 (20 μg/kg) induce the production of the proinflammatory cytokine interferon-γ.[30,31] The local delivery of therapeutic concentrations of IL-10 may overcome this problem. The amount of IL-10 delivered by a treatment protocol with *L. lactis* that decreased inflammation in DSS colitis with $\approx$50% was estimated to be $\approx$0.28 ng or $\approx$1 U IL-10 per mouse. This amount is several times lower than the optimized amount of systemically administered IL-10 in the same model with an equal reduction in inflammation (1.25×10^4U per mouse).[9]

So far, intestinal mucosal delivery of IL-10 has been provided by 3 approaches: 1) active synthesis in situ by *L. lactis*[9]; 2) gene transfer[6,32,33]; and 3) gelatin microspheres.[34] *L. lactis* has proven to be an effective delivery vehicle for the treatment of Crohn's disease in 2 mouse models[9] and has since been the subject of improvements regarding biological containment[11] and formulation.[35–37] A Phase I trial using the bacteria in Crohn's disease patients was carried out recently.[10] Although commensals can be taken up by DCs to be transported to MLNs[17] and we did observe lactococci being phagocytosed by DCs, we could not recover lactococci from MLNs in our experiments. Since we did not recover lactococci from murine MLN or spleen homogenates after oral challenge, we state that *L. lactis* is restricted to the mucosal compartment and is unlikely to elicit a systemic immune response, being safe for administration in humans. This is in good agreement with its GRAS (generally recognized as safe)-status.

Plating of homogenates from epithelium-stripped mucosa and subsequent immunoblotting for hIL-10 revealed that the lactococci we recovered were viable and could still produce IL-10. In previous ELISA studies, we could detect small amounts of IL-10 in intestinal tissue homogenates but not in the lumen.[9] Furthermore, evidence exists that the bacterial context of IL-10 presentation is necessary for the therapeutic action.[10] We hypothesize that an important source of the IL-10 acting as a therapeutic on the mucosa is produced by those bacteria that have entered the nutrient-rich mucosa. IL-10 produced in the lumen and subsequently diffusing to responsive cells in the epithelium or the lamina propria may not be excluded as a mechanism, but the effect of the latter may not be as considerable as IL-10 production directly in situ in intestinal tissue, in the close proximity of responsive cells, given its known paracrine ways of action. Although IL-10 can directly affect the function of T cells, many of its limiting effects on ongoing immune responses and inflammation occur indirectly through modulation of cytokine production and function of antigen-presenting cells such as DCs and macrophages.[2] Most likely, IL-10-producing *L. lactis* modulates $CD4^+$ T-cell function via downregulatory effects on DCs, since DCs exposed to *LL-hIL-10* and maturation factors showed a profound ability to suppress the proliferation of responder ($CD4^+$) T cells.[38] Further investigation on the downstream targets of IL-10 is currently being carried out to reveal the exact mechanism of the IL-10-producing *L. lactis*.

In conclusion, this study provides more insight into the interaction of IL-10-producing *L. lactis* with the intestinal mucosa. We demonstrated that *L. lactis* can enter healthy mucosa via a transcellular route and uptake is enhanced through the paracellular route in inflamed intestinal tissue. We recovered viable lactococci from DTT/EDTA-stripped inflamed mucosa that could still produce IL-10. These results suggest that IL-10 production by *L. lactis* in the mucosa nearby target cells might improve the efficacy of the IL-10 treatment.

ACKNOWLEDGMENT

We thank Inge Bruggeman, Betty De Prest, Isabelle Rottiers, and Dorothea Van Limbergen for technical assistance.

REFERENCES

1. Kucharzik T, Maaser C, Lugering A, et al. Recent understanding of IBD pathogenesis: implications for future therapies. *Inflamm Bowel Dis.* 2006;12:1068–1083.
2. Moore KW, de Waal MR, Coffman RL, et al. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol.* 2001;19:683–765.
3. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell.* 1993;75:263–274.
4. Powrie F, Leach MW, Mauze S, et al. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity.* 1994;1:553–562.
5. Herfarth HH, Mohanty SP, Rath HC, et al. Interleukin 10 suppresses experimental chronic, granulomatous inflammation induced by bacterial cell wall polymers. *Gut.* 1996;39:836–845.
6. Barbara G, Xing Z, Hogaboam CM, et al. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut.* 2000;46:344–349.
7. Fedorak RN, Gangl A, Elson CO, et al. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology.* 2000;119:1473–1482.
8. Schreiber S, Fedorak RN, Nielsen OH, et al. Safety and efficacy of

recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology.* 2000;119:1461–1472.
9. Steidler L, Hans W, Schotte L, et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science.* 2000;289:1352–1355.
10. Braat H, Rottiers P, Hommes DW, et al. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol Hepatol.* 2006;4:754–759.
11. Steidler L, Neirynck S, Huyghebaert N, et al. Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. *Nat Biotechnol.* 2003;21:785–789.
12. Okayasu I, Hatakeyama S, Yamada M, et al. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology.* 1990;98:694–702.
13. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun.* 1998;66:5224–5231.
14. Rath HC, Schultz M, Freitag R, et al. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infect Immun.* 2001;69:2277–2285.
15. Berg DJ, Davidson N, Kuhn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest.* 1996;98:1010–1020.
16. Cruickshank SM, English NR, Felsburg PJ, et al. Characterization of colonic dendritic cells in normal and colitic mice. *World J Gastroenterol.* 2005;11:6338–6347.
17. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science.* 2004;303:1662–1665.
18. Keita AV, Gullberg E, Ericson AC, et al. Characterization of antigen and bacterial transport in the follicle-associated epithelium of human ileum. *Lab Invest.* 2006;86:504–516.
19. Rescigno M, Urbano M, Valzasina B, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol.* 2001;2:361–367.
20. Rescigno M, Rotta G, Valzasina B, et al. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology.* 2001;204:572–581.
21. Vallon-Eberhard A, Landsman L, Yogev N, et al. Transepithelial pathogen uptake into the small intestinal lamina propria. *Journal of Immunology.* 2006;176:2465–2469.
22. McCormick DA, Horton LW, Mee AS. Mucin depletion in inflammatory bowel disease. *J Clin Pathol.* 1990;43:143–146.
23. Sartor RB. The influence of normal microbial flora on the development of chronic mucosal inflammation. *Res Immunol.* 1997;148:567–576.
24. Clayburgh DR, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. *Laboratory Investigation.* 2004;84:282–291.
25. Kleessen B, Kroesen AJ, Buhr HJ, et al. Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls. *Scand J Gastroenterol.* 2002;37:1034–1041.
26. Swidsinski A, Ladhoff A, Pernthaler A, et al. Mucosal flora in inflammatory bowel disease. *Gastroenterology.* 2002;122:44–54.
27. Swidsinski A, Loening-Baucke V, Lochs H, et al. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. *World J Gastroenterol.* 2005;11:1131–1140.
28. van Deventer SJ, Elson CO, Fedorak RN. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology.* 1997;113:383–389.
29. Colombel JF, Rutgeerts P, Malchow H, et al. Interleukin 10 (Tenovil) in the prevention of postoperative recurrence of Crohn's disease. *Gut.* 2001;49:42–46.
30. Lauw FN, Pajkrt D, Hack CE, et al. Proinflammatory effects of IL-10 during human endotoxemia. *J Immunol.* 2000;165:2783–2789.
31. Tilg H, Van Montfrans C, van den Ende A, et al. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut.* 2002;50:191–195.
32. Lindsay JO, Ciesielski CJ, Scheinin T, et al. Local delivery of adenoviral vectors encoding murine interleukin 10 induces colonic interleukin 10 production and is therapeutic for murine colitis. *Gut.* 2003;52:981–987.
33. Van Montfrans C, Rodriguez Pena MS, et al. Prevention of colitis by interleukin 10-transduced T lymphocytes in the SCID mice transfer model. *Gastroenterology.* 2002;123:1865–1876.
34. Nakase H, Okazaki K, Tabata Y, et al. New cytokine delivery system using gelatin microspheres containing interleukin-10 for experimental inflammatory bowel disease. *J Pharmacol Exp Ther.* 2002;301:59–65.
35. Huyghebaert N, Vermeire A, Neirynck S, et al. Evaluation of extrusion/spheronisation, layering and compaction for the preparation of an oral, multi-particulate formulation of viable, hIL-10 producing Lactococcus lactis. *Eur J Pharm Biopharm.* 2005;59:9–15.
36. Huyghebaert N, Vermeire A, Neirynck S, et al. Development of an enteric-coated formulation containing freeze-dried, viable recombinant Lactococcus lactis for the ileal mucosal delivery of human interleukin-10. *Eur J Pharm Biopharm.* 2005;60:349–359.
37. Termont S, Vandenbroucke K, Iserentant D, et al. Intracellular accumulation of trehalose protects Lactococcus lactis from freeze-drying damage and bile toxicity and increases gastric acid resistance. *Appl Environ Microbiol.* 2006;72:7694–700.
38. Braat H, Steidler L, Neirynck S, et al. Turbo-probiotics as a tool for cell-based (mucosal) delivery of interleukin-10. *Gastroenterology.* 2005; 128:A5.

Chapter 4

roles for metallothioneins as immunomodulators in intestinal inflammation

Chapter 4.1

impaired epithelial metallothionein expression as a possible aetiopathogenic mechanism in Crohn's disease

Article submitted

Impaired epithelial metallothionein expression as a possible aetiopathogenic mechanism in Crohn's Disease

Condensed title: Metallothionein deficiency in Crohn's Disease

Debby Laukens[1,2,3*], Anouk Waeytens[4*], Harald Peeters[3], Sara Bogaert[3], Charlotte Aelbrecht[3], Jasmien Taildeman[4], Liesbeth Ferdinande[4], Ellen Van Belle[4], Kenny Knecht[5], Paul Van Hummelen[6], Wim Waelput[4], Dirk Elewaut[7], Erik Remaut[1,2], Pieter Rottiers[1,2], Claude Cuvelier[4] and Martine De Vos[3].

*Both authors contributed equally to this work.

[1]Department for Molecular Biomedical Research, Flanders Institute for Biotechnology (VIB), Gent, Belgium

[2]Department of Molecular Biology, Ghent University, B-9000 Gent, Belgium.

[3]Department of Gastroenterology, Ghent University Hospital, B-9000 Gent, Belgium

[4]Department of Pathology, Ghent University Hospital, B-9000 Gent, Belgium

[5]Applied Maths BVBA, B-9830 Sint-Martens-Latem, Belgium

[6]MicroArray Facility, VIB, B-3000 Leuven, Belgium

[7]Department of Rheumatology, Ghent University Hospital, B-9000 Gent, Belgium

Corresponding author:

Debby Laukens, Department of Gastroenterology, Ghent University Hospital, De Pintelaan 185 3K12IE, B-9000 Gent, Belgium, Tel: +3293322064, Fax: +3293324984, E-mail: debby.laukens@ugent.be

Key words: Metallothionein, Crohn's disease, gene expression

Word count: 3,948

ABSTRACT

Background: This study sought to identify new candidate genes for Crohn's disease (CD) by integrating known disease loci with gene expression in endoscopically normal colon biopsies of CD patients. The expression and functional role of metallothionein (MT) was studied in healthy, CD, and inflammatory conditions.

Methods: Colon biopsies of CD patients and healthy controls were subjected to microarray analysis. Detailed expression of MT in the intestine and blood were studied using quantitative PCR, ELISA, immunohistochemistry and immunogold electron microscopy. Dextran sodium sulphate (DSS)-induced colitis in $MT^{-/-}$ mice and bacterial challenge of MT-knockdown HT29 cells were used as *in vivo* and *in vitro* models, respectively, for functional analyses of MT.

Results: Microarray analysis identified 18 differentially expressed genes located near a previously identified locus for CD. We focused on the reduced expression of MT in the colon, ileum and blood of CD patients. In inflamed tissue, reduced MT expression was seen in the epithelium, while granulation tissue was highly positive. Reduced epithelial MT in non-inflamed tissue was only observed in CD patients. Surprisingly, $MT^{-/-}$ mice were less susceptible to DSS-induced mortality, although they suffered more severe histological changes in the colon at an early stage of the disease, coincident with the highest epithelial MT expression in wild type mice. Bacterial challenge of MT-knockdown HT29 cells resulted in reduced IL-8 secretion as compared to control HT29 cells.

Conclusions: We suggest that deficient epithelial MT expression may be a mechanism of aetiopathogenesis in CD patients. A role for epithelial MT during the process of colitis and bacterial challenge was demonstrated.

A new approach to identify potential susceptibility genes for multifactorial diseases like Crohn's disease (CD) is the integration of the variation in gene expression in unaffected tissue samples with previously documented susceptibility loci (1). Two of the candidate genes that were identified in this way are located near the *IBD1* locus and belong to a family of highly conserved proteins with immunomodulating properties, the metallothioneins (MTs) (2). These intracellular proteins can be rapidly induced by a variety of stimuli, and are considered acute stress proteins. They are involved in zinc homeostasis and help to protect cells against toxic levels of metal ions and radicals by binding and sequestering these molecules. In addition, MTs can be secreted through as yet unknown mechanisms (2). These extracellular MTs were shown to have antibacterial, immunomodulating and chemotactic properties (3-5). In humans, 18 MT genes have been identified (MT1A-MT1X, MT2A, MT3 and MT4). Metallothionein 3 and MT4 are constitutively expressed in the brain and squamous epithelium, respectively, while MT1 and MT2 are ubiquitously expressed and highly inducible by cytokines, hormones, metals and general stress (6).

While two previous studies have shown an up-regulation of MT in CD tissue, others found a down-regulation (7-12). MT levels were also decreased in CD tissue when quantified by radioimmunoassay, microarray and silver-saturation assay (13-15). These contradictory findings could have resulted from the differential regulation of MT in different cell fractions or from diverging grades of inflammation of the samples used in the different studies. We wondered whether abnormal MT expression in CD is an aetiopathogenic event or secondary to inflammation. We studied the mRNA expression of different MT isoforms, as well as the cellular localization of MT proteins in various inflammatory conditions. The functional consequences of decreased MT expression were studied in two models: $MT^{-/-}$ mice and HT29 epithelial cells expressing siRNA targeted to endogenous MT.

MATERIALS AND METHODS

Patients. This study was approved by the local ethics committee (EC UZG 2004/242 and 2006/362). All individuals who participated in this study were Caucasians. CD and acute infectious enterocolitis were diagnosed according to clinical, endoscopic and histological criteria. CD patients were classified according to the Vienna classification (16). Disease location was defined as the maximal spread of inflammation before the first resection. Control tissue was obtained from patients who underwent ileocolonoscopy for follow up of polyp detection or screening for colorectal carcinoma. Biopsies were collected during colonoscopy and immediately placed in RNAlater (Ambion, Foster City, CA) for RNA extraction or in formalin for immunohistochemical analysis. Archived, formalin-fixed, and paraffin-embedded tissue was also processed for immunohistochemistry.

RNA extraction. Total RNA was extracted from biopsies and whole blood using the Qiagen RNeasy Mini and Midi Kit, respectively. Concentration and purity of the extracted RNA were determined spectrophotometrically. The quality of RNA used for microarray analysis was checked on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarrays. Microarray analyses were performed by the VIB MicroArray Facility (www.microarrays.be). The microarray (VIB Crohn 7K2) was specifically designed for the study of colonic gene expression (GEO accession number GPL5134) (17). Hybridization and scanning protocols are detailed on the aforementioned website. GeneMaths® XT (Applied Maths, St-Martens-Latem, Belgium) was used to perform all subsequent analyses.

qPCR. One µg of RNA was converted to cDNA with oligodT priming (Gibco, Carlsbad, CA). One tenth of the cDNA was used for real-time quantification with SYBR green (Eurogentec, Seraing, Belgium). A two-step program was run on the iCycler (Bio-Rad, Hercules, CA), and reactions were run in duplicate and normalized to GAPDH levels. Primer sequences are listed in Supplementary Table 1.

Induction assays. Ficoll-paque (Amersham Biosciences, Buckinghamshire, UK) isolated PBMCs were seeded in 6-well plates at 10^6 cells per well and treated the next day with H_2O_2, $ZnSO_4$, dexamethasone (all Sigma, St. Louis, MO), human IL-6 (Peprotech, Germany), human TNF (produced in-house) or human IFN-γ (Biosource, Belgium). Cells were lysed after 2, 4 or 6 hrs and MT1M mRNA expression was assessed by qPCR. Normalized Ct values were corrected for background at each time point, and plotted against time. Induction

was calculated as the area under the curve (AUC) for the three time points: AUC = $\int_0^3 (m_1.x)dx + \int_3^6 (m_2.x + b)dx + \int_6^{24} (m_3.x + c)dx$.

Immunohistochemistry. Immunohistochemical staining of human samples was carried out on the NexES IHC automated staining system (Ventana Medical Systems, Tucson, AZ) with mouse monoclonal anti-metallothionein clone E9 (Zymed, San Francisco, CA) or mouse IgG_1 negative control (Dako, Glostrup, Denmark) as an isotype specific control. Murine tissue sections were stained manually. Relative proportions of positively-staining epithelial cells and lamina propria cells were measured separately using a semi-quantitative grading system: 0: no MT-positive cells; 1: single MT-positive cells or foci (1 to 5%); 2: moderate number of foci of MT-positive cells (6 to 40%); 3: large number of cells are MT-positive (40 to 70%); 4: majority of cells are MT-positive (>70%). Sections were scored by two observers who were blinded for diagnosis and clinical data. Cohen's kappa between observers was 0.65 (18). Histological evaluations of inflammation for all samples are summarised in Supplementary Table 2.

Immunofluorescence. Double immunostaining for Ki67 (rabbit monoclonal anti-Ki67 clone SP6, Lab Vision, Fremont, CA) or CD68 (FITC-conjugated mouse monoclonal anti-CD68 clone KP1, Dako) and MT was performed on paraffin sections, together with the appropriate negative controls.

Immunogold electron microscopy. Tissue samples were immediately fixed in 4% formaldehyde and embedded in Lowicryl K4M (Agar Scientific, Essex, UK). Ultra thin sections were stained with anti-MT or the matched isotype control antibody. Sections were examined in a Zeiss TEM900 at 50kV (Carl Zeiss, Oberkochen, Germany).

Acute dextran sodium sulphate (DSS) colitis. Animal experiments were approved by the Ethics Committee for Animal Studies of the Faculty of Medicine and Health Sciences (ECP 03/10). Colitis was induced in $MT^{-/-}$ mice (129S7/SvEvBrd-$Mt1^{tm1Bri}$ $Mt2^{tm1Bri}$/J, strain 002211, The Jackson Laboratory, Bar Harbor, MA) versus wild type (WT) mice (129S1/SvImJ, strain 002448, The Jackson Laboratory). Five percent DSS (M.W. 36,000-50,000; MP Biomedicals, Irvine, CA) was administered in the drinking water of 9 to 18 week-old mice. Parameters used to calculate the disease activity index (DAI) were monitored daily (19). One, 2, 3, 5, and 7 days after DSS administration, mice were sacrificed by cervical dislocation. Samples from anus to caecum were taken for histology and myeloperoxidase (MPO) measurements.

Epithelial damage and inflammatory infiltration was scored semi-quantitatively (20). MPO activity was determined as described (21).
For survival experiments, colitis was induced by administration of DSS for 8 or 10 days. In one experiment, mice received 2 mg/kg or 30 mg/kg $ZnSO_4$ twice a day by gavage starting two days before the induction of colitis.

Small interfering RNA. Short hairpin RNA (shRNA) fused to an H1 promoter was synthesized by PCR on the pSUPER vector (Tronolab, Lausanne, Switzerland). The PCR fragments were cloned into pLVTH-siGFP (Tronolab). To produce virus for delivery of the shRNA, HEK293T cells (ATCC CRL1573) were transfected by the calcium phosphate method with pCMV-d8.91 (Tronolab), PMDG2 (Tronolab) and the shRNA construct. After 48 hrs, supernatant was harvested and filtered. One day before viral transduction, HT29 cells (ATTC HTB38) were seeded at $5x10^4$ cells per well in a 24-well plate. They were overlaid with viral supernatant and centrifuged for 1 hr at 32°C. This procedure was repeated with the 72-hr viral supernatant. Cells expressing high levels of GFP were sorted with the EPICS altra cell sorter (Beckman Coulter, Mijdrecht, The Netherlands).

ELISA. Cells were infected with *adherent-invasive E. coli LF82* (MOI100) for 1 hr, after which bacteria were killed using 100 µg/ml gentamycin. After 8, 24 and 48 hrs, IL-8 secretion in supernatant was measured using sandwich ELISA (BD Biosciences, Franklin Lakes, NJ). For each time point, values were calculated by extracting the mean IL-8 secretion by uninfected cells from the mean IL-8 secretion by infected cells. For the determination of MT in sera, a competition ELISA protocol was used (22).

Statistics. Statistical analyses were performed using SPSS. Differences between groups in qPCR experiments, ELISA, immunohistochemical scores (mean of two observers) and induction AUCs were calculated using the Mann-Whitney U test. Median ± SD is given in the text. Correlations between metric data were calculated using Pearson's Rho, and those between rank data with Spearman's Rho. For comparison of normally distributed variables in the DSS experiment (colon length and MPO activity), the *t*-test or one-way ANOVA was performed. Significance of differences in DAI and ELISA IL-8 concentrations between time-series was calculated using a general linear model. Two-tailed probabilities were calculated, and *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Identification of potential candidate genes for CD

Gene expression in normal colon biopsies of 16 CD patients (Supplementary Table 3, median age: 33 yrs, range 16-53, 31% male) and 11 healthy controls (median age: 53 yrs, range 21-76, 27% male) was analyzed on a microarray chip containing 6,779 expressed sequence tags (data are accessible at the MIAME database http://www.mged.org/Workgroups/MIAME/miame.html). We characterized genes that were differentially expressed between CD patients and controls (P<0.01, n=240) and selected for further analysis the genes that were located near a chromosomal region that has been linked to CD in at least two independent studies or in a Flemish cohort of patients living in the same area of Belgium (Supplementary Table 4) (23-51). We employed an arbitrary distance of five megabases around markers that showed the highest linkage. In this way, 18 genes were selected (Table 1), and all clones of the respective genes were sequence verified and annotated correctly.

Table 1. Differentially expressed genes in colon biopsies of CD patients located near a locus for CD

unigene	gene symbol	gene description	chromosomal location	expression in CD	P-value
Hs.486246	PHTF1	putative homeodomain transcription factor 1	1p13	↑	0.000099
Hs.77955	MEF2D	MADS box transcription enhancer factor 2, polypeptide D	1q22	↓	0.0035
Hs.106674	BAP1	BRCA1 associated protein	3p21.31-p21.2	↓	0.0017
Hs.438691	GMPPB	GDP-mannose pyrophosphorylase B	3p21.31	↓	0.0013
Hs.302047	PLCL3	phospholipase C-like 3	3q25.31	↓	0.0048
Hs.318567	NDRG1	N-myc downstream regulated gene 1	8q24.3	↓	0.00038
Hs.84072	TM4SF3	transmembrane 4 superfamily member 3	12q12.1	↑	0.0046
Hs.85951	XPOT	exportin, tRNA (nuclear export receptor for tRNAs)	12q14.1	↑	0.0021
Hs.419776	NAP1L1	nucleosome assembly protein 1-like 1	12q21.1	↓	0.0088
Hs.159481	GALGT	UDP-N-acetyl-alpha-D-galactosamine	12q13.3	↓	0.00054
Hs.438737	MT1F	metallothionein 1F	16q12.2	↓	0.001
Hs.188518	MT1M	metallothionein 1M	16q12.2	↓	0.00058
Hs.100914	CEP192	Centrosomal protein 192kDa	18p11.21	↑	0.000042
Hs.512640	PRKCSH	protein kinase C substrate 80K-H	19p13.2	↓	0.00042
Hs.134074	SLC35E1	solute carrier family 35, member E1	19p13.11	↑	0.006
Hs.437	TCF15	transcription factor 15 (basic helix-loop-helix)	20p13	↓	0.0024
Hs.102336	ARHGAP8	Rho GTPase activating protein 8	22q13.31	↓	0.0093
Hs.28491	SAT	spermidine/spermine N1-acetyltransferase	Xp22.1	↑	0.0035

Metallothionein mRNA is down-regulated in colon tissue and blood leukocytes of CD patients

In the microarray screen of CD tissue, we found a down-regulation of two closely related metallothionein (MT) transcripts, MT1F and MT1M. A detailed expression study of inducible MT isoforms using quantitative real-time PCR (qPCR) was performed on colon biopsies and blood leukocytes of 46 CD patients (median age: 36 yrs, range 10-71, 37% male) and 19 controls (median age: 61 yrs, range 32-76, 37% male). Primers for MT isoforms were designed and evaluated for specificity by direct sequencing and agarose gel electrophoresis. Additionally, the presence of a single melting peak after denaturation of the amplicons following qPCR confirmed the specificity of the reaction in each sample. We obtained specific primer sets for MT1M, MT1F, MT1E, MT1J and MT2A.

The highest mRNA expression in colon biopsies was observed for MT1E, followed by MT2A>MT1J>MT1F>MT1M. A significant correlation was found between the expression of MT1M mRNA and the other isoforms tested (Table 2). A significantly lower expression of MT1M was observed in biopsies taken from endoscopically normal colon tissue of CD patients as compared to healthy control tissue, predominantly in patients with colonic disease (L2 and L3, P=0.0076, fig 1A). No correlation was found with drug intake, including azathioprine (n=8). A similar down-regulation of MT1F mRNA was measured in peripheral blood leukocytes (P=0.026, fig 1B). MT1M mRNA levels in blood were too low for reliable quantitative measurement. CRP levels were normal in CD patients as well as in controls.

Table 2. Correlations of MT isoform expression in colon biopsies of CD patients and controls

		MT1F	MT1J	MT1M	MT2A
MT1E		0.631	0.496	0.500	0.499
	P value	<0.01	<0.01	<0.01	0.058
	N	65	55	66	15
MT1F			0.589	0.378	0.784
	P value		<0.01	<0.01	<0.01
	N		55	62	15
MT1J				0.609	0.805
	P value			<0.01	<0.01
	N			53	13
MT1M					0.615
	P value				<0.05
	N				15

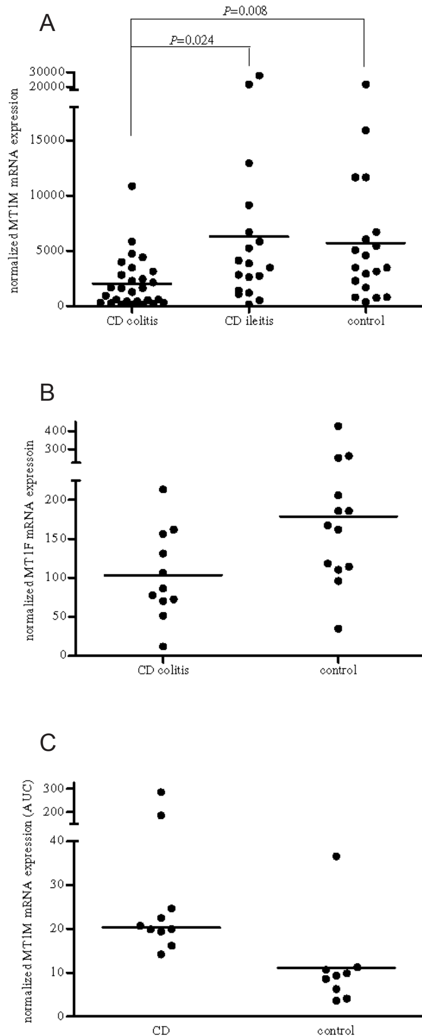


Figure 1. Metallothionein mRNA expression is down-regulated in CD patients with colonic involvement.

Quantitative real-time PCR was used to measure (A) MT1M expression in unaffected colon biopsies from CD patients and healthy controls and (B) MT1F expression in blood leukocytes of CD patients with colonic involvement and healthy controls (P=0.026). CD colitis: L3 + L2 (n=28 in A, n=11 in B), healthy controls (n=19 in A, n=13 in B), CD ileitis: L1 (n=18). (C) MT1M mRNA induction in response to oxidative stress is higher in CD patients than in controls irrespective of the disease location (P=0.0015). The mean value is shown as a bar. CD patients n=10, healthy controls n=9.

Metallothionein mRNA is inducible in blood leukocytes of CD patients

As MT mRNA expression was decreased in the colon, ileum and blood leukocytes of CD patients with colonic involvement, we examined whether there was a defect in MT induction. Therefore, peripheral blood mononuclear cells (PBMCs) isolated from ten CD patients (L1: 4, L2: 2, L3: 4) and nine controls were stimulated with known MT inducers (10 ng/ml IL6, 1000 U/ml TNF, 200 μM $ZnSO_4$, 200 U/ml IFN-γ, 100 μM H_2O_2 and 2 μM dexamethasone)

and resulting changes in MT1M mRNA expression were measured by qPCR (52). No difference in induction was found between CD patients and controls except following exposure to H_2O_2, which resulted in a significantly higher induction of MT1M in CD patients (*P*=0.0015, fig 1C).

Reduced epithelial metallothionein expression is independent of inflammation in CD but not in acute enterocolitis

In biopsies of healthy controls, MT expression was almost totally confined to the ileal epithelium, predominantly the crypt epithelium (Table 3, fig 2A). Some Paneth cells were positive, and showed cytosolic localization of the MT signal (fig 2B). Double staining of MT with the proliferation marker Ki67 revealed that nearly all Ki67 positive epithelial cells showed MT expression, whereas not all MT positive cells were Ki67 positive (fig 2A). At the subcellular level, immunogold electron microscopy showed reactivity of epithelial MT in the nucleus, cytosol, and endoplasmic reticulum (fig 2C-D). Mitochondria and Paneth cell granules were negative.

Table 3. Semi-quantitative score for epithelial expression in non-inflamed biopsies

	Ileum		Colon	
	Score	P-value	Score	P-value
healthy control	2.500 ± 1.4[§]		0.700 ± 1.6[§]	
CD	0.750 ± 2.1	0.007**	0.313 ± 1.0	0.401
L1	1.000 ± 2.4	0.084	0.875 ± 1.7	0.808
L2 + L3	0.750 ± 2.1	0.015*	0.063 ± 0.7	0.128
acute enterocolitis	2.500 ± 2.9	0.923	ND	

Scores are expressed as medians ± interquartile range. L1: ileal involvement only, L2: colonic involvement only, L3: ileal and colonic involvement. [§]*P*<0.001 between colon and ileum. *P*-values are compared to healthy controls. ND = not determined.

Biopsy samples of normal tissue of CD patients showed a reduced MT expression in the ileal epithelium as compared to healthy control tissue (Table 3, fig 3A-B), predominantly in patients with colonic disease (L2+L3). This result confirms the qPCR data. This difference in MT expression in non-inflamed tissue was not seen in acute enterocolitis, where the reduced epithelial MT expression was only found in inflamed tissue. In these samples, a negative correlation was found between the grade of inflammation and epithelial MT expression (R: -0.668, *P*<0.001, fig 3C-E). This was not the case in CD samples, where moderately active inflammation seemed to slightly induce MT expression (fig 3F).

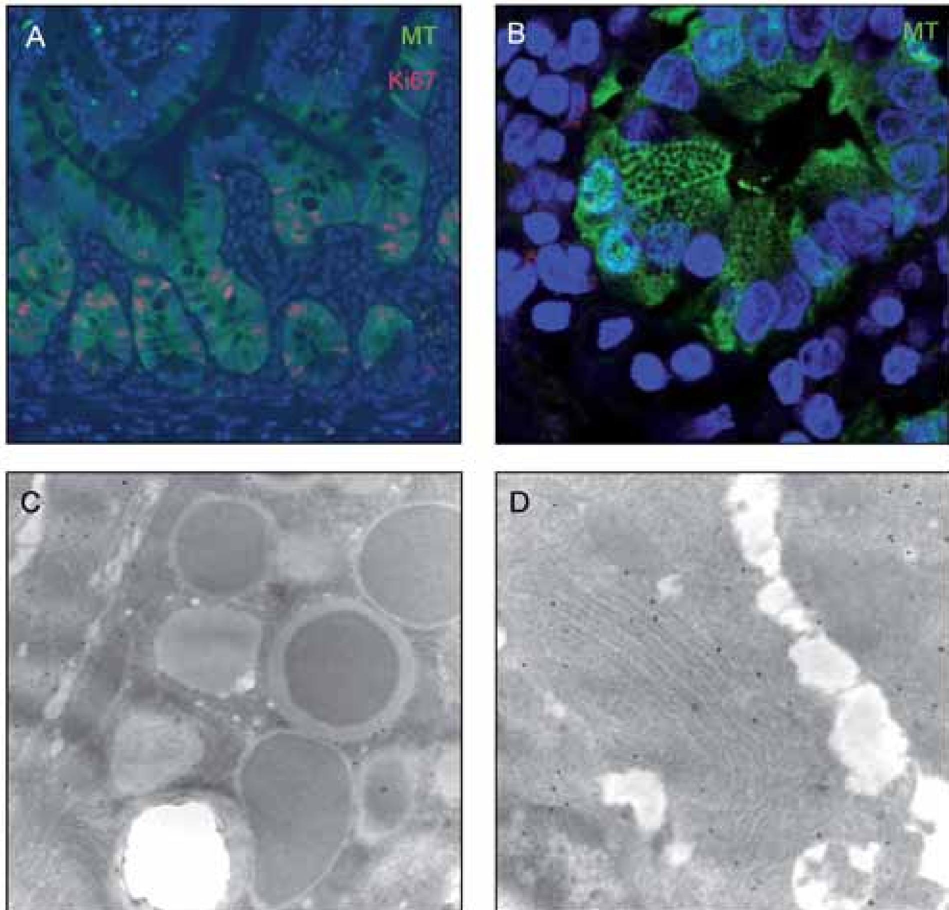


Figure 2. Normal intestinal expression of MT protein.

(A) In biopsies of healthy controls, MT (green) is predominantly expressed in the crypt epithelium. Double staining with Ki67 (red) did not reveal a correlation with proliferating cells. (B) Immunofluorescence and (C, D) immunogold labelling of MT shows cytosolic MT in Paneth cells and in the rough endoplasmic reticulum but no signal was detected in the granules. Magnification: A=200x; B=600x, C-D=12000x

Extracellular metallothionein is reduced in sera of CD patients

To test whether extracellular MT is also reduced in CD patients, we measured the protein content in sera by ELISA. A significantly reduced MT concentration was seen in sera from CD patients (17.6±10.4 µg/µl, *N*=46) as compared to healthy controls (26.7±13.9 µg/µl, *N*=15, *P*=0.009). No correlation was found between MT expression and sex, age or CRP level.

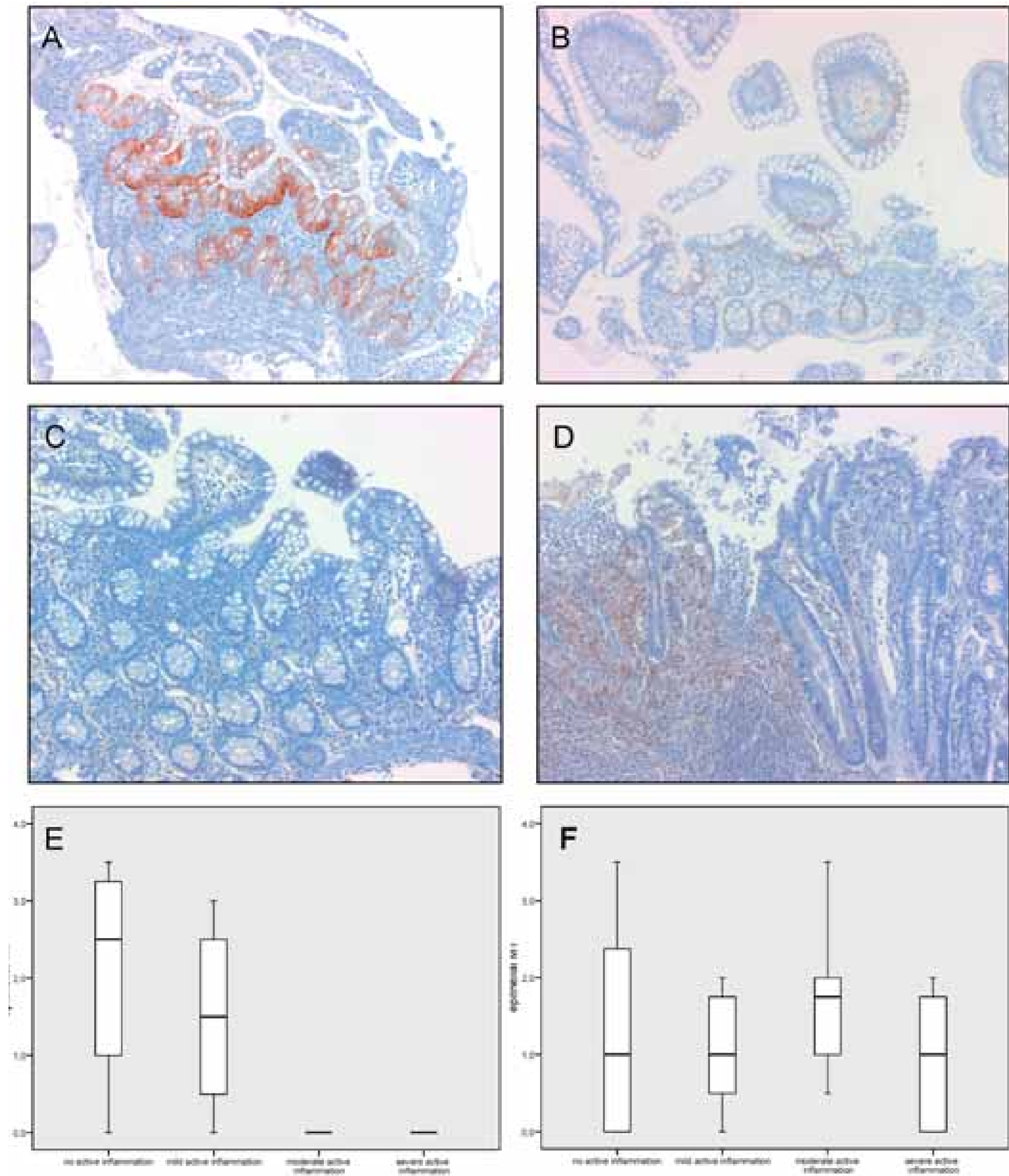


Figure 3. Epithelial MT expression is basally reduced in CD and decreases during inflammation.

MT expression in ileum from (A) healthy control and (B) normal tissue from CD patient. Decreased epithelial MT in (C) mild and (D) severe acute ileitis. (E,F) Boxplots showing the IHC score for epithelial MT in function of the grade of inflammation. Epithelial MT is negatively correlated with grade of inflammation in (E) acute enterocolitis but (F) not in CD. Magnification=100x

Metallothioneins are predominantly expressed in fibroblasts of granulation tissue

With increasing inflammatory activity, more MT-positive cells arose in the lamina propria (R:0.636, *P*<0.001, fig 4A-B). Those cells were spindle cells (fibroblasts) and polygonal cells belonging to the inflammatory infiltrate and/ or granulation tissue. Double immunofluorescence for MT and the macrophage marker CD68 revealed that (polygonal) macrophages were weakly positive (fig 4C). Granulation tissue was almost always positive (62 out of 77 cases), and no correlation was found between MT and Ki-67 staining (fig 4D).

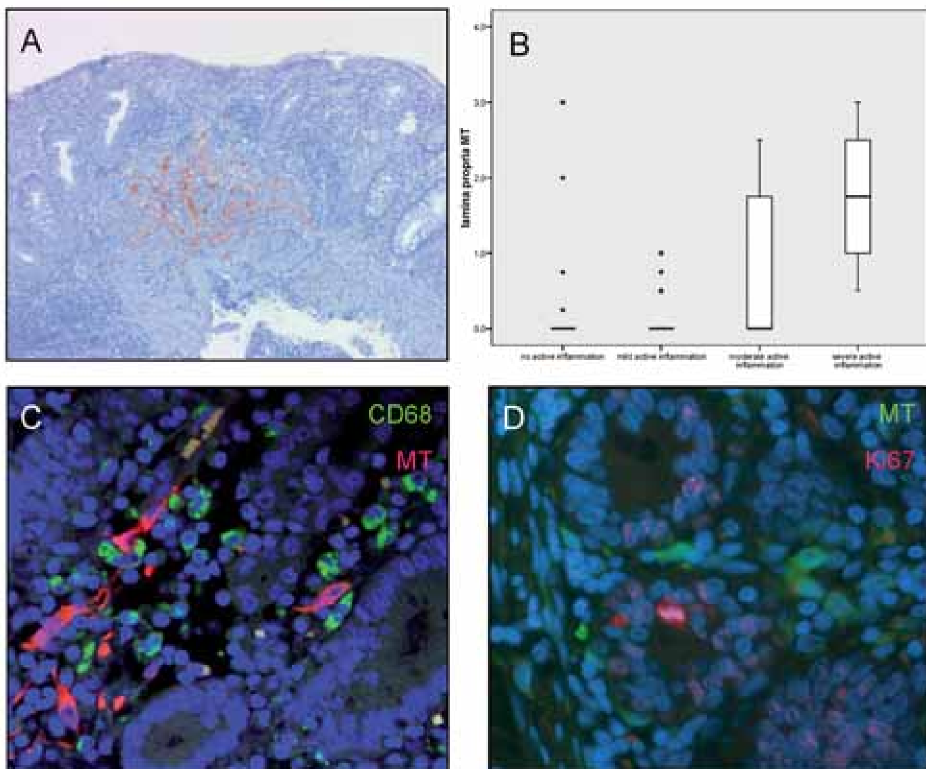


Figure 4. MT expression in granulation tissue.

MT expression in granulation tissue (A) was almost always positive for MT and (B) showed a positive correlation with grade of inflammation in both CD and acute enterocolitis (boxplots of the IHC score for lamina propria MT in function of the grade of inflammation, * represent extremes). (C) Double immunofluorescence with CD68 shows that some macrophages are weakly positive for MT (yellow/orange), whereas strong MT staining (red) was noticed in spindle cells (fibroblasts). (D) No correlation was found between Ki-67 and MT. Magnification: A=100x; C-D=600x

Epithelial metallothionein expression is induced in the early phase of experimental colitis

To determine the role of MT in experimental colitis, we first evaluated its colonic expression in a time course after induction with dextran sodium sulphate (DSS). After one day of DSS administration, a mild up-regulation of MT in epithelial cells was observed, which diminished from day 2 and was completely absent at days 5 and 7. In contrast, MT-positive inflammatory infiltrate/granulation tissue was present in the mucosa and submucosa from day 5 onward (fig 5A-B).

Absence of metallothionein influences the course of experimental colitis

To investigate whether the absence of MT influences the initiation or progression of inflammation, we induced acute DSS colitis in $MT^{-/-}$ and wild type (WT) mice. In basal conditions, $MT^{-/-}$ mice had shorter colons (P=0.006), and showed higher MPO activity (P=0.003) without microscopic inflammation. After DSS treatment, the disease activity index (DAI) significantly increased in both $MT^{-/-}$ and WT mice (P<0.001), but progressed slower in $MT^{-/-}$ mice (P=0.017).
Histologically, inflammation was more severe in $MT^{-/-}$ mice on day 3 (P=0.0148, fig 5C), which is the time-point where in WT mice MT expression in intact epithelium is inhibited and before its expression in lamina propria begins to increase (fig 5A-B). No significant difference was observed between MTnull and WT mice for MPO and colon length.

The presence of metallothioneins reduces the survival of severe DSS colitis

WT mice subjected to DSS treatment died from day 7 onward, whereas $MT^{-/-}$ mice showed an extended survival (P=0.0017, fig 6A), suggesting that the absence of MT conferred some type of protection against mortality. To determine whether elevated levels of MT would lead to the reverse case, or a reduction in survival, zinc was administered to the mice before the induction of colitis. In WT mice, a low dose of zinc (2 mg/kg) slightly reduced survival (P=0.1001), whereas a high dose of zinc (30 mg/kg) resulted in a significant drop in survival rate (P=0.0002, fig 6B). $MT^{-/-}$ mice were not susceptible to the combined effect of DSS and zinc administration.

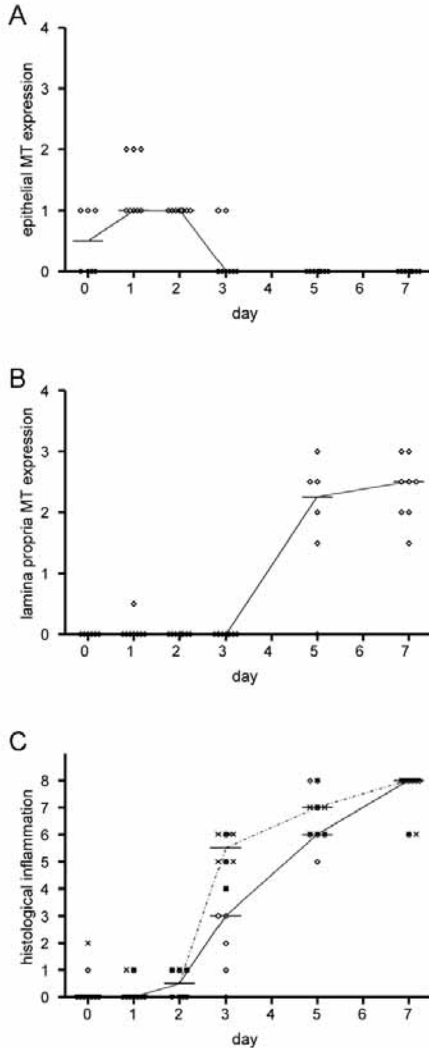


Figure 5. Epithelial MT expression is important in the early phase of acute DSS colitis.

Immunohistochemical staining of MT showed single positive epithelial cells in control colon, while $MT^{-/-}$ tissue was negative. (A+B) Semi-quantitative scores for immunohistochemical staining. (A) Epithelial MT was present at day 0 to 3. From day 5 on, epithelial expression diminished, and (B) a positive MT signal was seen in the infiltrate and granulation tissue in the (sub)mucosa. (C) On day 3, histological inflammation was significantly higher in $MT^{-/-}$ mice (P=0.0148), coincident with the highest epithelial MT expression. n=8 per genotype per day.

Colonic epithelial cells defective in metallothionein expression secrete less IL-8 in response to *adherent-invasive E. coli*

Small interfering RNA (siRNA) was used to create a MT-deficient HT29 colonic epithelial cell line (HT29MTkd). The siRNA target sequence was present in MT1B, MT1E, MT1J and MT1M, while it differed by two nucleotides in MT1F, MT1A and MT2A. The mRNA expression levels of MT1A, MT1B, MT1E, MT1M and MT2A were significantly reduced in the HT29MTkd cell line as compared to a siRNA control cell line expressing a non-sense RNA sequence with the same base pair composition (fold reduction: 1.7±0.26, 2.8±0.41, 3.7±0.12, 1.8±0.53 and

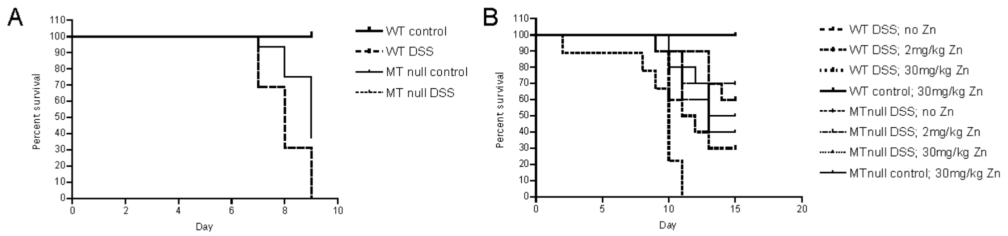


Figure 6. The presence of MT decreases survival.

Mortality was seen from day 7 on in a survival experiment of 8 days of DSS administration (*n*=16 mice per genotype in DSS groups; *n*=8 per genotype in control groups). (A) Kaplan Meier analysis shows that $MT^{-/-}$ mice are less sensitive to mortality (*P*=0.0017). (B) DSS was given for 10 days (*n*=10 per genotype in mice receiving DSS, *n*=5 per genotype in control mice). When zinc was administered to induce intestinal MT, mortality in WT mice was increased in a dose dependent manner (*P*=0.1001 for 2 mg/kg zinc, *P*=0.0002 for 30 mg/kg zinc).

3.8±0.19, respectively). MT1J and MT1F were not significantly reduced (fold reduction: 0.5±0.14 and 1.1±0.13, respectively). The specificity and lack of off target effects of the siRNA were evaluated through an expression analysis of the interferon-inducible gene MX1, which is frequently found to be induced in such systems (53). The expression of MX1 was unaffected in the knockdown cells (fold change 0.4±0.2). Diminished protein expression was shown by immunofluorescence using the monoclonal anti-MT antibody (Additional fig. 1). Interleukin 8 (IL-8) secretion in response to stimulation with *adherent-invasive E. coli* (*AIEC LF82*) was measured. The secretion of this cytokine was significantly lower in HT29MTkd cells than in the siRNA control cell line (*P*<0.001, fig 7).

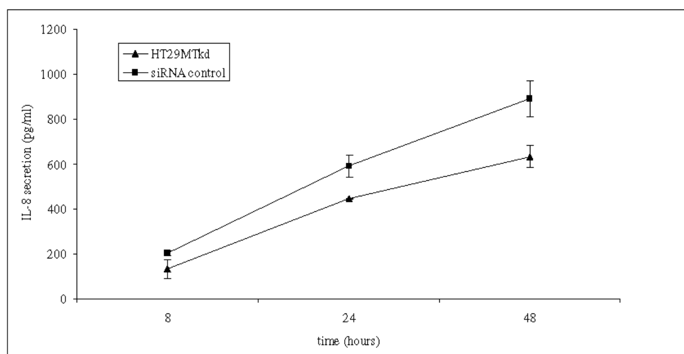


Figure 7. IL-8 secretion in response to bacterial challenge is reduced in HT29 cells expressing low MT levels.

Each value is the mean of three dilutions ± SD. HT29MTkd: HT29 cells expressing shRNA targeted against MT; siRNA control: HT29 cells expressing shRNA as an irrelevant scrambled sequence with the same base pair composition. *P*<0.001. This experiment was repeated five times.

DISCUSSION

In this study, we identified 18 novel potential candidate genes for CD by the integration of transcriptome analysis of macroscopically normal colon biopsies with previously determined CD-associated genomic loci. We performed a microarray analysis of unaffected tissues to target basal differences in gene expression due to genetic variation rather than inflammation-related events. A similar study was performed by Lawrance and colleagues, with the exception that they used moderately inflamed resected colonic tissue (14).

Two of the candidate genes belong to the family of closely related metallothioneins (MT). They are located within the *IBD1* locus in a tandem arrangement and arose by non-processed gene duplications (54). There are several possible mechanisms by which MT might play a role in CD. Deregulated MT levels in CD patients might indicate a hampered maintenance of free radicals produced during the inflammatory process. Anti-bacterial properties of MT could be important in the process of CD progression, as bacteria are considered to be involved in initiating and propagating the inflammatory process (55). In addition, extracellular MT has been found to promote tissue repair, a process which is disturbed in CD (56;57).

This study showed a down-regulation of MT mRNA and protein expression in serum and in unaffected tissue of CD patients as compared to healthy controls. We further demonstrated that although basal MT levels were reduced in CD patients, they were still inducible by conventional stimuli in PBMCs. Unexpectedly, MT induction by oxidative stress was higher in CD patients. This relative overreaction in CD patients might reflect the fact that lower basal levels of MT result in a higher concentration of unscavenged free hydroxide radicals when hydrogen peroxide is applied. This would lead to the activation of the MT transcription factor, MTF1, which in turn would result in an overall increased transcription of MT (58). The physiological relevance of this observation in the intestine of CD patients is not clear.

Detailed immunohistochemical analysis showed that the reduction of MT expression in CD patients was due to impaired epithelial expression. In healthy tissue, MT expression was predominantly epithelial and higher in the ileum than in the colon. Epithelial cells were only positive in the base of the crypts, where proliferation is highest. MT expression *in vitro* is cell cycle dependent (59). Nevertheless, we could not find a correlation between the proliferation marker Ki67 and MT, indicating that expression in the crypts is not solely explained by proliferation.

Paneth cells, specialized crypt enterocytes secreting antimicrobial peptides and enzymes, were positive for MT, an observation that has also been described in rats (60). Since MT has been accredited antimicrobial properties *in vitro* (61), it seemed likely that the protein would be secreted by the Paneth cells. However, immunofluorescence and immunogold electron microscopy showed an absence of granular enrichment in Paneth cells. Moreover, the expression in these cells was similar to the expression in surrounding crypt cells, suggesting a similar role for MT in both cell types. Localization of MT in the endoplasmic reticulum suggests the possibility of MT secretion via this pathway. However, secretion via this pathway seems unlikely since 1) the protein does not contain any (known) signal peptide and 2) the majority of mRNA is translated on free polysomes (2).

Active inflammation in acute enterocolitis patients resulted in a decrease in epithelial MT expression, and an influx of MT positive inflammatory infiltrate and granulation tissue. Mainly fibroblasts were moderately to strongly positive for MT, independent of the grade of inflammation. MT expression in fibroblasts surrounding mucosal ulcerations was previously reported in human chronic gastric and small intestinal ulcers (62).

Down-regulation of MT in inflammatory bowel disease (IBD) has been reported and implicated in the pathogenesis of both ulcerative colitis (UC) and CD (9-15). In the present study, the significant reduction of MT expression in the epithelium of non-inflamed tissue of CD patients suggests that impaired epithelial function may be an aetiopathogenic mechanism in CD.

The exact cause of the down-regulation of epithelial MT during inflammation remains unknown and is not consistent with data indicating that MTs are up-regulated by pro-inflammatory cytokines. To further investigate this inconsistency, we studied the time course of MT expression during DSS-induced colitis in WT mice. A very rapid up-regulation of MT was seen in colon epithelia, which was inhibited after three days. Following this event, the inflammatory infiltrate and granulation tissue appeared and was shown to express MT. These results suggest different roles of MT in epithelium and fibroblasts.

The role of epithelial MT during induction of colitis was further clarified by the more severe histological inflammation on day 3 in $MT^{-/-}$ mice as compared to WT mice. Since colonic MT expression is restricted to the epithelium until day 3, we hypothesize that epithelial MT has an immunomodulating role in the early phase of colitis. A DSS study in $MT^{-/-}$ mice performed by Oz and colleagues noted no histological differences between the knockouts and WT mice; however, they examined the mice at day 8 (63). The more deleterious role of MT during the

late phase of induction was suggested by the lower survival rate of WT mice. Especially mice that were treated with zinc, which induces endogenous MT in the intestine, were at risk.

In order to clarify the role of epithelial MT, we generated MT-knockdown HT29 cells using siRNA. In response to bacterial challenge with a CD-associated *E. coli* strain, reduced expression of MT in epithelial cells correlated with reduced IL-8 secretion (64). This observation is in accordance with the recent finding that IL-8 up-regulation is impaired in response to acute trauma to the colon or skin of CD patients (65). IL-8 is a potent chemoattractant and activator of neutrophils, which respond rapidly to different types of infections, and play an essential role in the inflammatory response. They release antimicrobial peptides as well as reactive oxygen intermediates that may cause tissue damage. We hypothesize that the intestinal epithelial cell lining of CD patients expresses less MT and initially secretes less IL-8 in response to bacteria. A delay in neutrophil accumulation might lead to the persistence of the exogenous stimulus and an exaggerated secondary and compensatory immune response.

In conclusion, we propose deficient epithelial MT expression in CD patients as a possible aetiopathogenic mechanism. Our *in vivo* and *in vitro* studies point to an immunomodulatory role for epithelial MT in early intestinal inflammation. However, the dual roles of MT during the complete inflammatory cascade and especially the events accompanying MT expression in the epithelium and fibroblasts need to be clarified.

ACKNOWLEDGEMENTS

We wish to thank Dorothy Van Limbergen, Isabelle Rottiers and Kim Olievier for technical assistance, and Darryn W. Unfricht for his kind help with the MT ELISA protocol.

REFERENCES

(1) Cheung VG, Spielman RS. The genetics of variation in gene expression. Nature Genetics 2002; 32:522-525.

(2) Lynes MA, Zaffuto K, Unfricht DW, Marusov G, Samson JS, Yin X. The physiological roles of extracellular metallothionein. Experimental Biology and Medicine 2006; 231(9):1548-1554.

(3) Jin HY, Hu Q, Jun JY, Ju AC, Sen LD, Qian DR et al. Preliminary studies on the zinc-induced metallothionein protein with antibacterial activity in housefly larvae, Musca domestica. Acta Biologica Hungarica 2005; 56(3-4):283-295.

(4) Tran CD, Huynh H, van den BM, van der PM, Campbell MA, Philcox JC et al. Helicobacter-induced gastritis in mice not expressing metallothionein-I and II. Helicobacter 2003; 8(5):533-541.

(5) Yin X, Knecht DA, Lynes MA. Metallothionein mediates leukocyte chemotaxis. BMC Immunology 2005; 6:21.

(6) Haq F, Mahoney M, Koropatnick J. Signaling events for metallothionein induction. Mutation Research 2003; 533(1-2):211-226.

(7) Bruwer M, Schmid KW, Metz KA, Krieglstein CF, Senninger N, Schurmann G. Increased expression of metallothionein in inflammatory bowel disease. Inflammation Research 2001; 50(6):289-293.

(8) Dooley TP, Curto EV, Reddy SP, Davis RL, Lambert GW, Wilborn TW et al. Regulation of gene expression in inflammatory bowel disease and correlation with IBD drugs: screening by DNA microarrays. Inflammatory Bowel Diseases 2004; 10(1):1-14.

(9) Elmes ME, Clarkson JP, Jasani B. The Immunocytochemical Demonstration of Metallothionein in Human-Liver and Small-Intestine. Acta Pharmacologica et Toxicologica 1986; 59:510-513.

(10) Clarkson JP, Elmes ME, Jasani B, Webb M. Histological demonstration of immunoreactive zinc metallothionein in liver and ileum of rat and man. Histochemical Journal 1985; 17(3):343-352.

(11) Ioachim E, Michael M, Katsanos C, Demou A, Tsianos EV. The immunohistochemical expression of metallothionein in inflammatory bowel disease. Correlation with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices. Histology and Histopathology 2003; 18(1):75-82.

(12) Kruidenier L, Kuiper I, Van Duijn W, Mieremet-Ooms MA, Van Hogezand RA, Lamers CB et al. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. Journal of Pathology 2003; 201(1):17-27.

(13) Mulder TP, Verspaget HW, Janssens AR, de Bruin PA, Pena AS, Lamers CB. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. Gut 1991; 32(10):1146-1150.

(14) Lawrance IC, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. Human Molecular Genetics 2001; 10(5):445-456.

(15) Sturniolo GC, Mestriner C, Lecis PE, D'Odorico A, Venturi C, Irato P et al. Altered plasma and mucosal concentrations of trace elements and antioxidants in active ulcerative colitis. Scandinavian Journal of Gastroenterology 1998; 33(6):644-649.

(16) Gasche C, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ et al. A simple classification of Crohn's disease: Report of the Working Party for the world congresses of gastroenterology, Vienna 1998. Inflammatory Bowel Diseases 2000; 6(1):8-15.

(17) Laukens D, Peeters H, Cruyssen BV, Boonefaes T, Elewaut D, De Keyser F et al. Altered gut transcriptome in spondyloarthropathy. Annals of the Rheumatic Diseases 2006; 65(10):1293-1300.

(18) Cross SS. Grading and scoring in histopathology. Histopathology 1998; 33(2):99-106.

(19) Cooper HS, Murthy SNS, Shah RS, Sedergran DJ. Clinicopathological Study of Dextran Sulfate Sodium Experimental Murine Colitis. Laboratory Investigation 1993; 69(2):238-249.

(20) Kojouharoff G, Hans W, Obermeier F, Mannel DN, Andus T, Scholmerich J et al. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. Clinical and Experimental Immunology 1997; 107(2):353-358.

(21) Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of Cutaneous Inflammation - Estimation of Neutrophil Content with An Enzyme Marker. Journal of Investigative Dermatology 1982; 78(3):206-209.

(22) Borghesi LA, Lynes MA. Nonprotective effects of extracellular metallothionein. Toxicology and Applied Pharmacology 1996; 139(1):6-14.

(23) Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Mcleod RS, Griffiths AM et al. Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. American Journal of Human Genetics 2000; 66(6):1863-1870.

(24) Vermeire S, Rutgeerts P, Van Steen K, Joossens S, Claessens G, Pierik M et al. Genome wide scan in a Flemish inflammatory bowel disease population: support for the IBD4 locus, population heterogeneity, and epistasis. Gut 2004; 53(7):980-986.

(25) Satsangi J, Parkes M, Louis E, Hashimoto L, Kato N, Welsh K et al. Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. Nature Genetics 1996; 14(2):199-202.

(26) Hampe J, Lynch NJ, Daniels S, Bridger S, Macpherson AJS, Stokkers P et al. Fine mapping of the chromosome 3p susceptibility locus in inflammatory bowel disease. Gut 2001; 48(2):191-197.

(27) Duerr RH, Barmada MM, Zhang LL, Achkar JP, Cho JH, Hanauer SB et al. Evidence for an inflammatory bowel disease locus on chromosome 3p26: linkage, transmission/disequilibrium and partitioning of linkage. Human Molecular Genetics 2002; 11(21):2599-2606.

(28) van Heel DA, Dechairo BM, McGovern DPB, Negoro K, Carey AH, Cardon LR et al. The IBD6 Crohn's disease locus demonstrates complex interactions with CARD15 and IBD5 disease-associated variants. Human Molecular Genetics 2003; 12(20):2569-2575.

(29) Paavola-Sakki P, Ollikainen V, Helio T, Halme L, Turunen U, Lahermo P et al. Genome-wide search in Finnish families with inflammatory bowel disease provides evidence for novel susceptibility loci. European Journal of Human Genetics 2003; 11(2):112-120.

(30) Ma YH, Ohmen JD, Li ZM, Bentley L, McElree C, Pressman S et al. A genome-wide search identifies potential new susceptibility loci for Crohn's disease. Inflammatory Bowel Diseases 1999; 5(4):271-278.

(31) Shaw SH, Hampe J, White R, Mathew CG, Curran ME, Schreiber S. Stratification by CARD15 variant genotype in a genome-wide search for inflammatory bowel disease susceptibility loci. Human Genetics 2003; 113(6):514-521.

(32) Dechairo B, Dimon C, van Heel D, Mackay I, Edwards M, Scambler P et al. Replication and extension studies of inflammatory bowel disease susceptibility regions confirm linkage to chromosome 6p (IBD3). European Journal of Human Genetics 2001; 9(8):627-633.

(33) Barmada MM, Brant SR, Nicolae DL, Achkar JP, Panhuysen CI, Bayless TM et al. A genome scan in 260 inflammatory bowel disease-affected relative pairs. Inflammatory Bowel Diseases 2004; 10(5):513-520.

(34) Hampe J, Shaw SH, Saiz R, Leysens N, Lantermann A, Mascheretti S et al. Linkage of inflammatory bowel disease to human chromosome 6p. American Journal of Human Genetics 1999; 65(6):1647-1655.

(35) Hampe J, Schreiber S, Shaw SH, Lau KF, Bridger S, Macpherson AJS et al. A genomewide analysis provides evidence for novel linkages in inflammatory bowel disease in a large European cohort. American Journal of Human Genetics 1999; 64(3):808-816.

(36) Curran ME, Lau KF, Hampe J, Schreiber S, Bridger S, Macpherson AJS et al. Genetic analysis of inflammatory bowel disease in a large European cohort supports linkage to chromosomes 12 and 16. Gastroenterology 1998; 115(5):1066-1071.

(37) Pierik M, Yang HY, Barmada MM, Cavanaugh JA, Annese V, Brant SR et al. The IBD international genetics consortium provides further evidence for linkage to IBD4 and shows gene-environment interaction. Inflammatory Bowel Diseases 2005; 11(1):1-7.

(38) Cavanaugh JA, Callen DF, Wilson SR, Stanford PM, Sraml ME, Gorska M et al. Analysis of Australian Crohn's disease pedigrees refines the localization for susceptibility to inflammatory bowel disease on chromosome 16. Annals of Human Genetics 1998; 62:291-298.

(39) Brant SR, Fu YF, Fields CT, Baltazar R, Ravenhill G, Pickles MR et al. American families with Crohn's disease have strong evidence for linkage to chromosome 16 but not chromosome 12. Gastroenterology 1998; 115(5):1056-1061.

(40) Parkes M, Satsangi J, Lathrop GM, Bell JI, Jewell DP. Susceptibility loci in inflammatory bowel disease. Lancet 1996; 348(9041):1588.

(41) Akolkar PN, Gulwani-Akolkar B, Lin XY, Zhou ZF, Daly M, Katz S et al. The IBD1 locus for susceptibility to Crohn's disease has a greater impact in Ashkenazi Jews with early onset disease. American Journal of Gastroenterology 2001; 96(4):1127-1132.

(42) Forabosco P, Collins A, Latiano A, Annese V, Clementi M, Andriulli A et al. Combined segregation and linkage analysis of inflammatory bowel disease in the IBD1 region using severity to characterise Crohn's disease and ulcerative colitis. European Journal of Human Genetics 2000; 8(11):846-852.

(43) Ohmen JD, Yang HY, Yamamoto KK, Zhao HY, Ma YH, Bentley LG et al. Susceptibility locus for inflammatory bowel disease on chromosome 16 has a role in Crohn's disease, but not in ulcerative colitis. Human Molecular Genetics 1996; 5(10):1679-1683.

(44) Zouali H, Chamaillard M, Lesage S, Cezard JP, Colombel JF, Belaiche J et al. Genetic refinement and physical mapping of a chromosome 16q candidate region for inflammatory bowel disease. European Journal of Human Genetics 2001; 9(10):731-742.

(45) Hugot JP, LaurentPuig P, GowerRousseau C, Olson JM, Lee JC, Beaugerie L et al. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 1996; 379(6568):821-823.

(46) Low JH, Williams FA, Yang XS, Cullen S, Colley J, Ling KL et al. Inflammatory bowel disease is linked to 19p13 and associated with ICAM-1. Inflammatory Bowel Diseases 2004; 10(3):173-181.

(47) Cho JH, Nicolae DL, Gold LH, Fields CT, Labuda MC, Rohal PM et al. Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1q, 3q, and 4q: Evidence for epistasis between 1p and IBD1. Proceedings of the National Academy of Sciences of the United States of America 1998; 95(13):7502-7507.

(48) Annese V, Latiano A, Bovio P, Forabosco P, Piepoli A, Lombardi G et al. Genetic analysis in Italian families with inflammatory bowel disease supports linkage to the IBD1 locus - A GISC study. European Journal of Human Genetics 1999; 7(5):567-573.

(49) Vermeire S, Satsangi J, Peeters M, Parkes M, Jewell DP, Vlietinck R et al. Evidence for inflammatory bowel disease of a susceptibility locus on the X chromosome. Gastroenterology 2001; 120(4):834-840.

(50) Hampe J, Frenzel H, Mirza MM, Croucher PJP, Cuthbert A, Mascheretti S et al. Evidence for a NOD2-independent susceptibility locus for inflammatory bowel disease on chromosome 16p. Proceedings of the National Academy of Sciences of the United States of America 2002; 99(1):321-326.

(51) Duerr RH, Barmada MM, Zhang LL, Pfutzer R, Weeks DE. High-density genome scan in Crohn disease shows confirmed linkage to chromosome 14q11-12. American Journal of Human Genetics 2000; 66(6):1857-1862.

(52) Borghesi LA, Lynes MA. Stress proteins as agents of immunological change: some lessons from metallothionein. Cell Stress & Chaperones 1996; 1(2):99-108.

(53) Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. Nature Genetics 2003; 34(3):263-264.

(54) Karin M, Eddy RL, Henry WM, Haley LL, Byers MG, Shows TB. Human metallothionein genes are clustered on chromosome 16. Proceedings of the National Academy of Sciences of the United States of America 1984; 81(17):5494-5498.

(55) Eckburg PB, Relman DA. The role of microbes in Crohn's disease. Clinical Infectious Diseases 2007; 44(2):256-262.

(56) Lansdown ABG. Metallothioneins: potential therapeutic aids for wound healing in the skin. Wound Repair and Regeneration 2002; 10(3):130-132.

(57) Okamoto R, Watanabe M. Cellular and molecular mechanisms of the epithelial repair in IBD. Digestive Diseases and Sciences 2005; 50:S34-S38.

(58) Zhang B, Georgiev O, Hagmann M, Gunes C, Cramer M, Faller P et al. Activity of metal-responsive transcription factor 1 by toxic heavy metals and H2O2 in vitro is modulated by metallothionein. Molecular and Cellular Biology 2003; 23(23):8471-8485.

(59) Nagel WW, Vallee BL. Cell-Cycle Regulation of Metallothionein in Human Colonic-Cancer Cells. Proceedings of the National Academy of Sciences of the United States of America 1995; 92(2):579-583.

(60) Nishimura H, Nishimura N, Tohyama C. Immunohistochemical Localization of Metallothionein in Developing Rat-Tissues. Journal of Histochemistry & Cytochemistry 1989; 37(5):715-722.

(61) Itoh N, Shibayama H, Kanekiyo M, Namphung D, Nakanishi T, Matsuyama A et al. Reduced bactericidal activity and nitric oxide production in metallothionein-deficient macrophages in response to lipopolysaccharide stimulation. Toxicology 2005; 216(2-3):188-196.

(62) Douglas-Jones AG, Thomas ND, Elmes ME, Jasani B. Immunocytochemically detectable metallothionein in granulation tissue surrounding mucosal ulceration. Histochem J 1992; 24(1):40-50.

(63) Oz HS, Chen T, de Villiers WJ, McClain CJ. Metallothionein overexpression does not protect against inflammatory bowel disease in a murine colitis model. Medical Science Monitor 2005; 11(3):BR69-BR73.

(64) Darfeuille-Michaud A, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N et al. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. Gastroenterology 2004; 127(2):412-421.

(65) Marks DJB, Harbord MWN, MacAllister R, Rahman FZ, Young J, Al-Lazikani B et al. Defective acute inflammation in Crohn's disease: a clinical investigation. Lancet 2006; 367(9511):668-678.

APPENDICES

Supplementary Table 1. Sequences of qPCR primer sets

Gene symbol	Reference sequence	Forward primer	Reverse primer
GAPDH	NM_002046	TGC ACC ACC AAC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG
MT1A	NM_005946	GCA AAG GGG CAT CAG AGA AGT G	AAA TAC AGT AAA TGG GTC AGG GTT G
MT1B	NM_005947	AAG TGC TGC TGC TCT TGC TG	TGG TTG CTC TAT TTA TGT CTG GGA G
MT1E	NM_175617	GCA TCC TCT GGG TCT GGG TTC	AAC AGC AGC CTG GGG AAG AAG
MT1F	NM_005949	GCG ACT GAT GCC AGG ACA AC	CAC AGG AAA AGG AAT GTA GCA AAT G
MT1J	AF348994	GCT GTG CCT GAT GTG GGA AC	AAA TGC AGC AAA TGG CTC AGT ATT G
MT1M	NM_176870	CTG CAA AGG GAC GTT GGA GAA C	CAG CAA ATG GCT CAG TAT CGT ATT
MT2A	NM_005953	AAA GGG GCG TCG GAC AAG TG	GAA TAT AGC AAA CGG TCA CGG TCA G
MX1	NM_002462	TCC GAA GTG GAC ATC GCA AAA	GGG AAG GGC AAC TCC TGA C
MTF1	NM_005955	TGT TGC TTC TCT TCT GTG TCT G	GCT GCC CGC TAG GAC TAA G

Supplementary Table 2. Histological evaluation of inflammation

Group	Normal	Mild active	Moderate active	Severe active	Chronic quiescent	Total
control	32	0	0	0	0	32
CD	39	27	20	23	16	125
acute enterocolitis	5	7	6	3	0	21
Total	76	34	26	26	16	178

Active inflammation was subdivided in mild (1 or 2 crypt abscesses, small number of infiltrated granulocytes), moderate (multiple crypt abscesses, moderate number of infiltrated granulocytes) and severe (presence of ulcers, large granulocytic infiltrate). Chronic quiescent inflammation was based upon structural changes only.

Supplementary Table 3. Clinical characteristics of CD patients analyzed by microarrays

Patient	Familial CD	Age of onset	Behaviour	Disease location	CARD15 status	Operation	Medication
1	no	A1	B2	L3	WT	no	immunosuppressives
2	no	A1	B3	L3	ND	no	no
3	no	A2	B3	L3	mutant	no	5-ASA
4	yes	A1	B3	L3	WT	resection	corticosteroids
5	no	A1	B3	L3	mutant	fistula	5-ASA
6	no	A1	B3	L2	mutant	resection	remicade
7	no	A1	B2	L1	WT	resection	5-ASA
8	no	A1	B3	L3	WT	resection	immunosuppressives
9	yes	A1	B3	L3	WT	fistula	immunosuppressives
10	yes	A2	B2	L1	WT	resection	immunosuppressives
11	no	A1	B3	L3	mutant	fistula	immunosuppressives
12	no	A1	B1	L2	WT	resection	5-ASA
13	no	A1	B3	L3	WT	no	immunosuppressives
14	no	A1	B1	L3	WT	no	5-ASA
15	no	A1	B3	L3	WT	fistula, resection	corticosteroids
16	no	A1	B3	L3	WT	fistula, resection	5-ASA

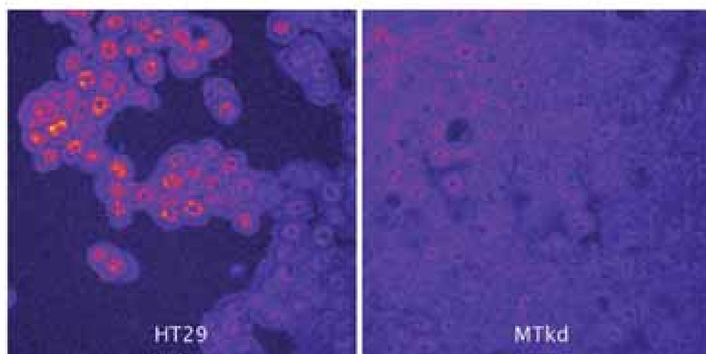
A1: <40; A2: ≥40; B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating; disease location is defined as maximal extension of inflammation before first resection of patients with L1: ileal involvement only, L2: colonic involvement only, L3: ileal and colonic involvement; mutant *CARD15*: carriage of at least one mutant allele for rs.2066844, rs.2066845 or rs.5743293; WT *CARD15*: not carrying a mutant allele for rs.2066844, rs.2066845 or rs.5743293; 5-ASA: 5-aminosalicylates; ND: not determined.

Supplementary Table 4. Chromosomal regions with repeated significant linkage to CD

IBD locus	chromosomal position	marker at highest lod score	population	reference
IBD7	1p13.1	D1S252	Flemish IBD	24
IBD7	1p33-32.3	D1S197	Flemish IBD	24
IBD7	1p36.13-36.11	D1S552-D1S234	Non-Jewish American IBD	46
	1q21.3	D1S305	Flemish IBD	24
IBD9	3p14.2-14.1	D3S1766-D3S1285	Canadian IBD	23
IBD9	3p21.31	D3S1573	North European IBD	25
IBD9	3p24.1-22.3	D3S1266-D3S1298	North European IBD	26
IBD9	3p26.3	D3S1297	American IBD	28
IBD9	3p26.1-25.3	D3S3591	North European IBD	26
IBD9	3p26.2-26.1-25.3	D3S3706-D3S3591	European IBD	26
	3q25.1-26.31	D3S1279-D3S3725	European CD	28
	3q26.31	D3S3053-D3S2427	American IBD	46
	4q25	D4S406	Finnish IBD	29
	4q25	D4S406	Flemish IBD	24
	4q25	D4S2623	American IBD	47
IBD5	5q31.1-31.3	D5S816-D5S1480	Canadian CD	23
IBD5	5q33-35		Jewish American CD	30
IBD3	6p	D6S197	North European CD	31
IBD3	6p21.31	D6S291	European IBD	32
IBD3	6p22.2	D6S2439	Caucasian CD	16
IBD3	6p22.2-21.2	D6S1281-D6S1019	Canadian IBD	23
IBD3	6p23-22.3-22.2	D6S289-D6S276	North European IBD	33
IBD3	6p22.3	D6S461	North European IBD	34
	6q24.1	D6S314	Flemish IBD	24
	6q25.2-26	D6S2436-D6S305	Caucasian IBD	16
	7q21.11	D7S669	North European IBD	25
	7q32.1-31.33	D7S40-D7S648	American IBD	50
	8q12.1-13.1	D8S1113-D8S1136	Caucasian IBD	16
	8q24.13	D8S198	Flemish IBD	24
	10p12.1	D10S197	Flemish IBD	24
	10p12.1	D10S197	North European CD	31
	10p12.31-12.32-12.1	D10S548-D10S197	North European CD	33
	11q22.1-22.3	D11S35-D11S927	Flemish IBD	24
IBD2	12q12	GATA91H06	Caucasian IBD	16
IBD2	12q14.1	D12S83	North European IBD	25
IBD2	12q21.1-21.2	D12S303-D12S326	North European CD	33
IBD2	12q21.1-21.33	D12S303-D12S351	European CD	35
IBD2	12q23.3	D12S78	Finnish CD	29

IBD4	14q11.2		American CD	30
IBD4	14q11.2	D14S261	American CD	50
IBD4	14q11.2	D14S50	Flemish IBD	24
IBD4	14q11.2	D14S261	multicenter (IBDIGC)	36
IBD4	14q12	D14S80	Flemish IBD	24
IBD4	14q13.1	D14S49	Flemish IBD	24
IBD8	16p11.2-12.1	D16S409-D16S753	Australian CD	37
IBD8	16p12.1	D16S3145	European CD	49
IBD8	16p12.1	D16S769	American CD	38
IBD8	16p13.13	D16S748	American CD	46
IBD1	16q		English IBD	39
IBD1	16q		American CD	38
IBD1	16q12.2	D16S306	European CD, IBD	49
IBD1	16q12.1	D16S409	Ashkenazi Jews CD	40
IBD1	16q12.2	D16S408	Italian CD, IBD	47
IBD1	16q12.2	D16S408	Italian IBD	41
IBD1	16q12.1	D16S411	American non-Jewish CD	42
IBD1	16q12.1	D16S3136, D16S3117, D16S770	European CD	43
IBD1	16q12.1	D16S409-D16S411	European CD	34
IBD1	16q12.1-12.2	D16S409, D16S419	Caucasian CD	44
IBD1	16q12.1-12.2	D16S409-D16S408	European CD	35
	18p11.31	D18S62	Flemish IBD	24
IBD6	19p13		UK Caucasian	45
IBD6	19p13.3	D19S591-GATA21G05	Canadian CD	23
IBD6	19p13.3-13.2	D19S1034-D19S586	American IBD	46
	20p12.3	D20S192	Flemish IBD	24
	22q11.23-12.1	D22S315-D22S421	European IBD	34
	22q12.1	D22S689	Caucasian IBD	16
	22q13.31	D22S274	Flemish IBD	24
	Xp22.11-21.2	DXS1226-DXS1214	North European CD	28
	Xp21.2-21.3	DXS1202-DXS1214	European IBD	34
	Xq21.32	DXS1203	Flemish IBD	48

Additional figure 1



Metallothionein is repressed in MT-knockdown cells. Immunofluorescent staining of MT in HT29 cells and in the siNRA MT-knockdown cells (MTkd).

Chapter 4.2

metallothionein expression in ulcerative colitis: inflammation-dependent regulation differs in epithelium and granulation tissue

Article in preparation

Metallothionein expression in ulcerative colitis: inflammation-dependent regulation differs in epithelium and granulation tissue

Anouk Waeytens[1,2], Martine De Vos[2], Isabelle Rottiers[1], Debby Laukens[2], Claude Cuvelier[1]

[1] Department of Pathology, Ghent University, Belgium

[2] Department of Gastroenterology, Ghent University Hospital, Belgium

Corresponding author:
Anouk Waeytens
Dept. of Pathology
De Pintelaan 185
B-9000 Gent
Belgium
tel +32 9 332 36 86
fax +32 9 332 49 65
e-mail: Anouk.Waeytens@UGent.be

ABSTRACT

Introduction: Metallothioneins (MTs) are small proteins that are involved in zinc metabolism and have antioxidant features. They are rapidly induced upon stress and inflammation and are thought to possess a cytoprotective potential. Different studies report aberrant expression in inflammatory bowel diseases although some findings seem contradictory. Furthermore, these studies have not investigated whether aberrant MT expression is a primary aetiopathogenic event or secondary to inflammation. We have recently reported a basal down-regulation of MTs in Crohn's disease compared to normal patients. The aim of this study was to clarify the expression pattern of MT in ulcerative colitis and to compare it with its expression pattern in normal patients and in other inflammatory conditions.

Materials and methods: Immunohistochemistry for MT was performed on 128 biopsy and surgical specimens from control, ulcerative colitis, diverticulitis, ischaemia, and acute enterocolitis samples. Each sample was given a semi-quantitative score for MT expression and this was correlated with microscopic inflammation. Double immunofluorescent stainings for MT and markers for proliferation, macrophages (CD68), and fibroblasts (vimentin and smooth muscle actin/SMA) were performed. *Ex vivo* intestinal cultures were stimulated with zinc and transforming growth factor (TGF)-β.

Results: In histologically normal intestinal tissue from ulcerative colitis patients, MTs are predominantly expressed in epithelium, which corresponds with MT expression in control tissue. In all inflammatory disorders studied, active inflammation causes a shift towards a weak to absent epithelial expression and a moderate to strong expression in the lamina propria. This latter positivity originated mainly from cells of the inflammatory infiltrate and the granulation tissue. Double stainings with cell markers revealed that the MT-positive cell population in the lamina propria consisted of some weakly positive macrophages and a majority of vimentin-positive fibroblasts, part of which were SMA-positive. The expression pattern in these cells was independent of grade of activity and no relationship was found with mitotic activity. *Ex vivo* stimulation showed that TGF-β down-regulated epithelial MT expression in a dose-dependent manner.

Conclusion: The MT expression pattern in ulcerative colitis corresponds with that observed in a 'normal' inflammatory reaction in the intestine, and as such MT defects are not specific for this disease. MT up-regulation in the lamina propria fits with their expected induction upon inflammation. The associated down-regulation of MT in epithelium reveals a different regulation. Epithelial MT might be down-regulated by TGF-β, which is reported to be increased in inflamed intestinal mucosa, especially near epithelial cells.

INTRODUCTION

Metallothioneins (MTs) are a family of ubiquitously expressed, highly conserved, small proteins that can bind divalent metal ions. Although they are not essential for life in mammals, the sequence and structural homology between MT proteins in different species highlights their important biological role (for review, see (1)). Initially, the biological function of MTs was mainly linked to their metal-binding capacity, i.e. protection from acute heavy metal toxicity and involvement in Zn^{2+} metabolism. However, their amino acid composition also provides them the ability to sequester reactive oxygen species (ROS), thus granting them an antioxidant function (2). They are rapidly induced by stress and inflammatory mediators, such as metals, cytokines, stress hormones, ROS, and chemicals. The MT promoter contains responsive sequences to glucocorticoids (3), interleukin-6 (4), phorbol esters (5) and hydrogen peroxide (6). Other known inducers and inhibitors of MT expression are reactive oxygen intermediates (7), nitrogen monoxide (8), epidermal growth factor and transforming growth factor (TGF)-β (9), interleukin-1 (10), interferon-α (11) and tumor necrosis factor (TNF)-α (12). The regulation of MT synthesis in inflammation is complex and is the result of a combination of factors that act synergistically in different tissues.

In the intestine, aberrant MT expression has been described in patients with ulcerative colitis (UC) and Crohn's disease (CD). Two studies report elevated MT levels in CD and UC (13;14), but most studies report decreased MT expression in CD as well as UC, using immunohistochemical techniques (15-18), radioimmunoassay (18;19), and a silver-saturation assay (20). None of these studies addresses the issue whether these changes in MT expression are inflammation-dependent or specific for CD and/or UC. In a previous study, we were able to show a decreased MT expression at basal levels in colon and ileum biopsies and in whole blood samples of CD patients (Laukens *et al* submitted). This lowered mRNA expression correlated with lowered epithelial protein expression in biopsies taken at another time point, suggesting a genetic involvement. The aim of this study was to elucidate the expression pattern of MT in UC and to compare it with its expression pattern in healthy conditions and in other intestinal inflammatory conditions (acute and ischaemic enterocolitis, and diverticulitis).

MATERIAL AND METHODS

Tissue specimens

Formalin fixed, paraffin embedded tissue from the archive of the clinical department of Pathology of the Ghent University Hospital was used. A total of 128 biopsy and surgical specimens was studied, including 31 control samples, 39 UC samples, 11 diverticulitis samples, 26 ischaemia samples and 21 samples of acute enterocolitis. Sample characteristics are summarised in Table 1. UC samples were chosen from patients that were diagnosed with disease according to clinical, endoscopic and histological criteria. Six of the UC patients did not receive medication before biopsies were taken or surgery was performed, five received aminosalicylates (5-ASA), two received corticosteroids, two received aminosalicylates and immunosuppressive agents, four received aminosalicylates and corticosteroids, four received immunosuppressive agents and corticosteroids, and one patient received aminosalicylates, immunosuppressive agents and corticosteroids. Control tissue was obtained from patients who underwent ileocolonoscopy for follow up of polyp detection or screening for colorectal carcinoma. All other samples were chosen on the basis of clinical and histopathological features and controlled for absence of clinical features suggestive of CD or UC. Patient characteristics are summarised in Table 2. This study was approved by the local ethics committee (EC UZG 2006/362).

Table 1. Characteristics of samples

		type of specimen		
tissue	group	biopsy	surgical	Total
ileum	control	16	0	16
	UC	9	0	9
	ischaemia	0	16	16
	acute enterocolitis	12	1	13
	Total	37	17	54
colon	control	15	0	15
	UC	27	3	30
	diverticulitis	0	11	11
	ischaemia	0	10	10
	acute enterocolitis	8	0	8
	Total	50	24	74

Table 2. Characteristics of patients

group	gender		median age (range)
	female	male	
control	10	11	41 (24-65)
UC	13	15	34 (13-68)
diverticulitis	3	6	60 (44-82)
ischaemia	9	9	68 (0-79)
acute enterocolitis	6	8	42 (6-71)

Histological assessment

Haematoxylin and eosin staining was carried out according to routine procedures. The histological evaluation of inflammation of all samples is summarised in Table 3. Active inflammation was subdivided in mild (1 or 2 crypt abscesses, small number of infiltrated granulocytes), moderate (multiple crypt abscesses, moderate number of infiltrated granulocytes) and severe (presence of ulcers, large granulocytic infiltrate). Chronic quiescent inflammation was based upon structural changes only.

Table 3. Histological evaluation of samples

		histological evaluation					
	group	normal	mild active inflammation	moderate active inflammation	severe active inflammation	chronic quiescent inflammation	Total
	control	31	0	0	0	0	31
	UC	8	10	6	10	5	39
	diverticulitis	3	1	1	6	0	11
	ischaemia	5	3	1	17	0	26
	acute enterocolitis	5	7	6	3	0	21
Total		53	21	14	36	5	128

Immunohistochemistry

Paraffin embedded sections were rehydrated in serial immersion in xylene and ethanol. Immunohistochemical staining was carried out on the NexES IHC automated staining system (Ventana Medical Systems, Tucson, AZ) with the mouse monoclonal anti-metallothionein clone E9 (Zymed Laboratories, San Francisco, CA). An isotype-specific irrelevant antibody (mouse

IgG_1 negative control, DakoCytomation, Belgium) was used in a matched concentration to control for non-specific binding of the primary antibody. Stained sections were coded and analyzed by two independent observers who were blinded for diagnosis and clinical data. Relative proportions of positive staining epithelial cells and non-epithelial cells were separately measured using the following semi-quantitative grading system: 0: no MT-positive cells; 1: single MT-positive cells or foci (1 to 5%); 2: moderate number of foci of MT-positive cells (6 to 40%); 3: large number of cells are MT-positive (40 to 70%); 4: majority of cells are MT-positive (>70%). In case of active inflammation, granulation tissue was scored using the same grading system. Cohen's kappa was calculated for the scores obtained by the two observers and showed excellent agreement with kappa = 0.65 (21).

Immunofluorescence

Paraffin embedded sections were rehydrated in serial immersion in xylene and ethanol. After heat induced epitope retrieval with 0.01M citrate buffer pH 6.0 (sodium citrate tribasic dehydrate, Sigma, St Louis, MO), autofluorescence was quenched with ammoniumchloride (50mM, UCB, Drogenbos, Belgium) and non-specific binding sites were blocked with 1% bovine serum albumine (Sigma). Sections were incubated with a mixture of two primary antibodies (mouse monoclonal IgG_1 anti-MT clone E9, Invitrogen, Carlsbad, CA; rabbit monoclonal anti-Ki67 clone SP6, Lab Vision, Fremont, CA; mouse monoclonal IgG_{2a} anti-vimentin clone Vim3B4, DakoCytomation; mouse monoclonal IgG_{2a} anti-SMA clone 1A4, BioGenex, San Ramon, CA), and secondary antibodies applied were Alexa 488 anti-mouse IgG_1, Alexa 594 anti-rabbit, Alexa 594 anti-mouse IgG_{2a}, and Alexa 594 anti-mouse antibodies (Molecular Probes, Eugene, OR). Double-staining with anti-CD68 included a tertiary step using FITC-anti-CD68 (clone KP1, DakoCytomation). Appropriate negative controls were included to verify that no cross-reactions occured.

***Ex vivo* stimulation**

For *ex vivo* stimulation, macroscopically normal rest tissue (taken at areas distant from diseased tissue) was obtained from ileal resection specimens that were sent to the pathology department for diagnostic investigation. Immediately upon arrival, tissue specimens of approximately $1cm^2$ were placed in RPMI medium (Invitrogen) containing antibiotics (Invitrogen), foetal calf serum (PAA Laboratories, Pasching, Austria), and 100µg/ml gentamycin (Schering-Plough, Brussels, Belgium). After two rinses in this medium followed by pre-incubation in medium containing

50µg/ml gentamycin, tissue cultures were stimulated with 1, 5 or 10ng/ml recombinant human TGF-β1 (R&D Systems), or 200µM $ZnSO_4.7H_2O$ (VWR, West Chester, PA) supplemented with 10ng/ml TGF-β1 for 15 hours.

Statistical analysis

The means of the scores from the two observers were used to compare different subsets with the Mann-Whitney U test. Scores are expressed as medians ± interquartile range. Differences were considered significant at a *P* value of < 0.05. Since MT positivity was significantly higher in ileal epithelium than in colonic epithelium ($P < 0.001$), all comparisons for epithelial scores were split according to type of tissue. Since scores for MT positivity in non-epithelial cells (cells from inflammatory infiltrate and granulation tissue) was significantly influenced by the type of specimen (biopsy versus resection specimen, $P = 0.001$), all comparisons for scores of non-epithelial cells were split according to specimen type. Correlations were calculated with the Spearman's rho test.

RESULTS

Metallothionein expression in normal intestine

In samples of control biopsies, MT was moderately to highly expressed in the epithelium. This expression was stronger in the ileum than in the colon (semi-quantitative score ileum: 2.5 ± 1.4 *versus* colon: 0.7 ± 1.6; $P < 0.001$). More cells from the villus base and crypt cells were positive than cells in the upper part of the villi or superficial epithelium (Fig. 1a,b). Sometimes Paneth cells were positive. Some samples showed strong positivity in isolated epithelial cells, reminiscent of enteroendocrine cells. When positivity was encountered in the lamina propria, it originated from macrophages, as was shown by double immunofluorescence with CD68 (Fig. 1c). Double staining of MT with the proliferation marker Ki67 revealed that almost all Ki67 positive epithelial cells showed MT expression, whereas not all MT positive cells were Ki67 positive or proliferating (Fig. 1d).

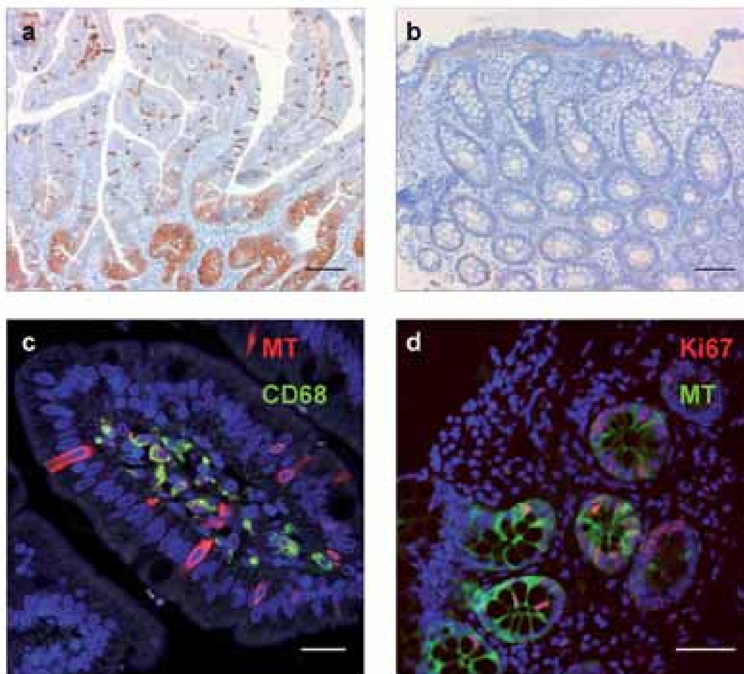


Figure 1. MT expression in normal intestine.

MT is strongly expressed in ileum (a) and moderately to weakly in colon (b). Expression is higher in crypt and basal part of the villus than in the top of the villi (ileum). Double staining with CD68 (c, ileum; MT = red, CD68 = green, nuclei = blue) showed that positive cells in the lamina propria were macrophages. Double staining with Ki67 (d, ileum; Ki67 = red, MT = green, nuclei = blue) did not reveal 100% correlation. Scale bars: a, b: 100μm; c: 25μm; d: 50μm.

Tissue of UC patients that shows no active inflammation displays normal MT expression

Since we previously found a decreased MT expression in normal ileal tissue of CD patients, even in patients with no ileal involvement, (Laukens *et al.* submitted) we wondered if UC would show a similar decrease. Biopsy samples of normal ileal tissue of UC patients showed normal MT expression. Semi-quantitative scores for ileal expression were 2.5 ± 1.4 for healthy control tissue, 2.5 ± 1.0 for tissue from UC patients (P = 0.431). The expression pattern of MT was similar in all samples and corresponded with the pattern in healthy controls (see earlier).

Active inflammation causes down-regulation of epithelial MT expression

Next, we investigated the influence of mucosal inflammation on epithelial MT expression. We found that epithelial MT expression diminished as histological inflammation progressed (Fig. 2a,b). When epithelial MT expression was plotted against the degree of inflammation, a negative correlation was found in these samples (Fig. 2c; Spearman's rho = -0.455, P = 0.004 for ileum; Spearman's rho = -0.184, P = 0.167 for colon). Surgical specimens containing both healthy and inflamed areas elegantly illustrated this inverse association: areas with normal histology showed (normal) moderate to high epithelial expression, whereas epithelium covering inflamed areas was only weakly positive or negative. In the case of ischaemic inflammation, epithelium bordering necrotic regions was even completely negative in all cases (n=23).

After the disappearance of active inflammation, epithelial MT immunoreactivity reappeared. Colon biopsies of UC patients that showed chronic quiescent but no active inflammation were compared with colon biopsies without inflammation from controls. UC tissue demonstrated a trend towards higher expression ($score_{control}$ = 0.5 ± 1.5, $score_{UC}$ = 2.0 ± 1.8, P = 0.142).

Active inflammation positively correlates with non-epithelial MT expression

With increasing activity of inflammation, more positive cells emerged in the lamina propria (Fig. 2a,b,d). Positive cells were mostly cells of the inflammatory infiltrate and granulation tissue, judging by the morphology fibroblasts and macrophages (Fig. 2e). This appearance was positively correlated with the degree of active inflammation (Fig. 2f; Spearman's rho = 0.493, $P < 0.001$). Lymphocytes, granulocytes and neutrophils appeared to be negative. Double immunofluorescence with cell markers for macrophages and fibroblasts confirmed that the positive cell fraction was composed of some mildly positive macrophages and a majority of strongly positive fibroblasts. These fibroblasts were positive for vimentin and a minority was also

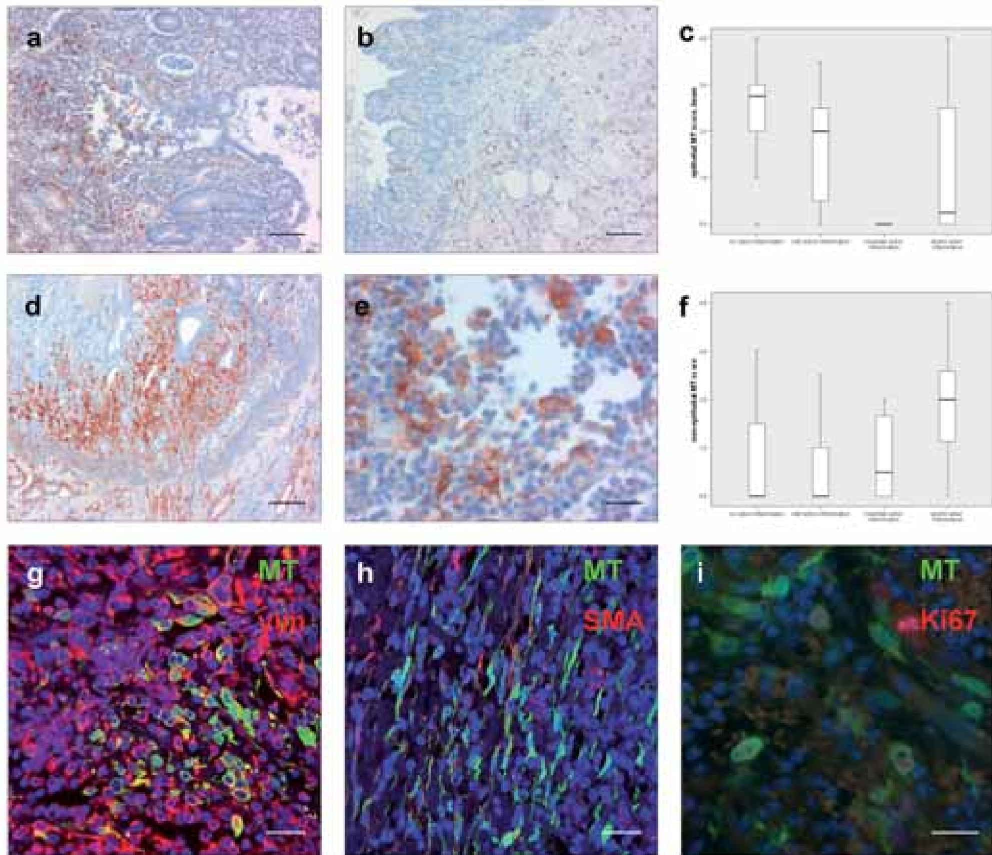


Figure 2: MT expression in inflamed intestine.

Epithelial MT expression decreases following inflammation, while positive cells emerge in the lamina propria. (a) active UC, (b) ischaemic inflammation, (d) active diverticulitis. In ischaemia, there was a moderate expression in fibroblasts, macrophages, pericytes and nerve tissue regardless of presence of inflammation. (c) Boxplots of semi-quantitative scores of epithelial MT expression in the ileum during inflammation showing decreased epithelial MT expression. (e) Magnification of (a) showing MT positive spindle and polygonal cells. (f) Boxplots of semi-quantitative scores of MT expression in the lamina propria during inflammation showing increased MT expression. Double stainings showed that fibroblasts (spindle cells) were positive for vimentin (g; MT = green, vim = vimentin = red, nuclei = blue), while a small proportion was also positive for SMA (h; MT = green, SMA = red, nuclei = blue). Inflammatory infiltrate consists of moderately to strongly positive MT cells, independent of proliferative status as illustrated by doublestaining with Ki67 (i, Ki67 = red, MT = green, nuclei = blue). Scale bars: a, b, d: 100µm, e, g, h, i: 25µm.

positive for SMA (Fig. 2g,h). When granulation tissue was present, it was almost always positive (62 out of 77 cases). The degree of expression in the granulation tissue itself did not differ in mild, moderate or severe inflammation. MT expression was not dependent on the proliferative status of these cells, since we found no relationship between MT and Ki-67 (Fig. 2i). In surgical samples from patients with ischaemic enterocolitis, there was a moderate expression in stromal cells regardless of presence of inflammation (2 out of 6 cases without inflammation, 11 out of 23 cases with inflammation). These cells were fibroblasts, macrophages, pericytes and nerve tissue (Fig. 2b).

Stimulation with TGF-β down-regulates epithelial MT expression

Ex vivo cultures of small intestinal tissue showed moderate to strong epithelial MT expression without stimulation (Fig. 3a). Stimulation with zinc resulted in an increase in MT staining (Fig. 3b). Administration of TGF-β1 caused a decrease in epithelial MT expression which was most clearly visible with the highest dose (Fig. 3c-e). The highest dose of TGF-β1 neutralized the MT induction by zinc (Fig. 3f). Stimulation did not result in signal in the lamina propria.

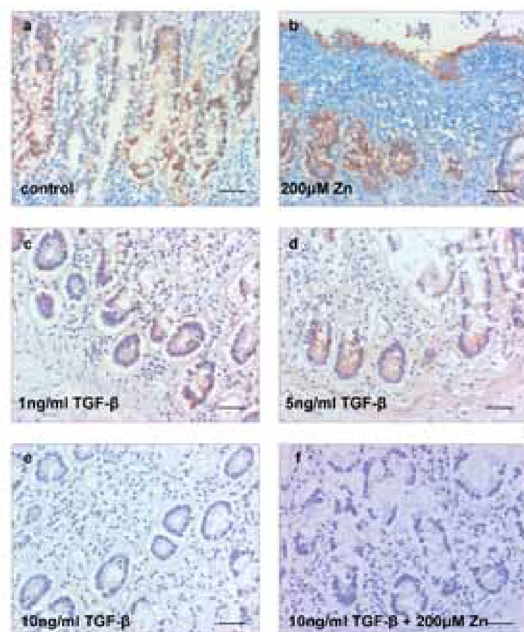


Figure 3: *Ex vivo* stimulation.

MT staining on stimulated intestinal tissue showing normal expression in unstimulated (control) tissue (a) and higher signal after stimulation with 200μM zinc (b). Administration of TGF-β could decrease MT expression in a dose-dependent manner (c: 1ng/ml, d: 5ng/ml, e: 10ng/ml) and even counter MT induction by zinc (f: 10ng/ml TGF-β + 200μM zinc). Scale bars: 50μm.

DISCUSSION

MT expression in normal conditions was predominantly epithelial and was highest in the ileum and in crypts. In these sites of the intestine, antibacterial effects are most critical, supporting an antibacterial role of MT in these regions (22). However, the proliferative status of crypt cells might also explain high MT expression, since MT is known to be cell-cycle dependent and up-regulated in proliferating cells *in vitro* (23). Double staining for MT and the proliferation marker Ki67 could not confirm this correlation in intestinal epithelial cells. A lot of MT positive cells were Ki67 negative, which indicates that expression in the crypts cannot be (solely) explained by proliferation.

In contrast to Crohn's disease, MT expression was not reduced in samples of UC patients that showed no active inflammation. In all tested conditions, active histological inflammation caused a decrease in the epithelial expression of MT. Simultaneously, an up-regulation was noticed in infiltrating cells in the lamina propria. Our findings could explain why some conflicting conclusions regarding MT expression in IBD were drawn in previous studies. First, it is important to recognize that MT expression varies in epithelium and lamina propria cells. Therefore, analyses of whole tissue lysates will yield different results than analyses of different cell fractions. Secondly, MT expression is dependent on the severity of active inflammation. The sampling during an inflammatory course is in that regard equally important. Our results demonstrate that the inflammation-dependent MT expression pattern is normal in ulcerative colitis, and comparable with that in diverticulitis, ischaemic and acute enterocolitis. In contrast, it differs from that in Crohn's disease, where we found decreased epithelial MT expression in spite of absence of inflammation in a previous study (Laukens *et al.* submitted).

An inflammation-dependent decrease in MT expression is not consistent with the accepted knowledge that MTs are rapidly up-regulated in conditions of stress and inflammation. Most molecules that are present during inflammation induce MTs, such as IL-1, TNF-α, IL-6, nitrogen monoxide, reactive oxygen radicals, and others (7;8;10;12). One of the few molecules that has been shown to be able to inhibit MT expression is transforming growth factor (TGF)-β. This pleiotropic cytokine inhibited induction of MT by epidermal growth factor *in vitro* in rat hepatocytes (9). TGF-β is a good candidate for epithelial regulation in inflammation, since an increase is found in mucosa with active intestinal inflammation, with the highest concentration in inflammatory cells closest to the luminal surface, near epithelial cells (24). When we stimulated human intestinal tissue with TGF-β1 this resulted in a dose-dependent decrease in epithelial MT

expression. These results suggest that TGF-β1 might be a regulator for epithelial MT expression during inflammation. TGF-β1 inhibits enterocyte proliferation (25) and can induce apoptosis (26), two processes where MTs have an opposite association. In addition, the induction of TGF-β by ROS (27) and by MTs in T lymphocytes (28) stresses the need to further investigate the relationship between MTs and TGF-β.

Contrary to the decrease in the epithelium, an increase in MT-positive cells occurred in the lamina propria during inflammation. This positive cell fraction was shown to consist of some weakly positive macrophages (that were also present in non-inflamed tissue) and a majority of fibroblasts. These fibroblasts, present in the granulation tissue, were vimentin-positive and a small proportion was SMA-positive. They probably represent a subset of fibroblasts, a cell-type which is known to have different possible phenotypes during physiologic and pathologic conditions (29). MT expression in granulation tissue surrounding mucosal ulceration has previously been reported in human chronic gastric and small intestinal ulcers (30). The expression level in the infiltrate itself is not dependent on the activity of inflammation and the higher scores for MT expression in the lamina propria in actively inflamed tissue are due to the elevated proportion of infiltrating inflammatory cells and fibroblasts. The fact that the majority of these cells is moderately to strongly positive for MT, independent of proliferative status, denotes a specific function for MT in inflammation and not only a reflection of mitotic activity. A possible function might be found in the antioxidant features of MT. Inflammatory cells (neutrophils and macrophages) produce free oxygen radicals upon stimulation by locally produced cytokines (interleukin-1 and interferon-γ) to kill bacteria and parasites. These free radicals are also extremely cytotoxic and are capable of causing local host bystander tissue necrosis (31). Given the antioxidant properties of MT, it is possible that, together with other known molecules such as super oxide dismutase, vitamin E and ascorbate, MTs provide a cytoprotection for host cells, preventing cellular damage and allowing survival and growth in an inflammatory environment and protection against chemical or ischaemic injury (30). Interestingly, in non-affected surgical specimens of patients with ischaemic enterocolitis, an up-regulation of MT was found in fibroblasts in the submucosa, a pattern that was maintained during active inflammation. Also pericytes and nerve fibres were found more positive in ischaemic conditions, suggesting that MTs are rapidly up-regulated in these cell types in stress conditions and have a cytoprotective effect against ischaemic injury.

The expression profile of MTs in normal ileum and colon hint at a cytoprotective/antibacterial role in epithelium, maybe indirectly through its antioxidative and/or Zn-binding capacities, but does not explain a decrease in epithelial MT expression induced by inflammation. The explanation might lie in the capacities of MTs to modulate the activation of the transcription factor nuclear factor (NF)-κB, which has a fundamental role in inflammation. This modulation might be regulated directly (32) or indirectly through modification of the redox balance (33;34) or regulation of zinc concentrations (35). Since zinc is a structural and/or catalytic element in many other transcription factors and enzymes as well, it can be implicated in metabolic regulation on many levels. A decrease in MT would increase zinc ion availability against stressor agents and inflammation (for review see (36)).

In conclusion, we showed that the MT expression pattern in ulcerative colitis corresponds with the pattern encountered in other intestinal inflammatory disorders. Intestinal expression of MT shifts from mainly epithelial in normal circumstances towards a reduced epithelial expression and a pronounced expression in cells of the granulation tissue during progression of active inflammation. These results indicate a difference in regulation in the epithelium versus the granulation tissue during inflammation.

References

(1) Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: The multipurpose protein. Cellular and Molecular Life Sciences 2002; 59(4):627-647.

(2) Kumari MVR, Hiramatsu M, Ebadi M. Free radical scavenging actions of metallothionein isoforms I and II. Free Radical Research 1998; 29(2):93-101.

(3) Kelly EJ, Sandgren EP, Brinster RL, Palmiter RD. A pair of adjacent glucocorticoid response elements regulate expression of two mouse metallothionein genes. Proceedings of the National Academy of Sciences of the United States of America 1997; 94(19):10045-10050.

(4) Lee DK, Carrasco J, Hidalgo J, Andrews GK. Identification of a signal transducer and activator of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression. Biochemical Journal 1999; 337:59-65.

(5) Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ et al. Phorbol Ester Inducible Genes Contain A Common Cis Element Recognized by A Tpa-Modulated Trans-Acting Factor. Cell 1987; 49(6):729-739.

(6) Dalton TP, Li Q, Bittel D, Liang L, Andrews GK. Oxidative stress activates metal-responsive transcription factor-1 binding activity. Occupancy in vivo of metal response elements in the metallothionein-I gene promoter. Journal of Biological Chemistry 1996; 271(42):26233-26241.

(7) Ren YF, Smith A. Mechanism of Metallothionein Gene-Regulation by Heme-Hemopexin - Roles of Protein-Kinase-C, Reactive Oxygen Species, and Cis-Acting Elements. Journal of Biological Chemistry 1995; 270(41):23988-23995.

(8) Arizono K, Kagawa S, Hamada H, Ariyoshi T. Nitric-Oxide Mediated Metallothionein Induction by Lipopolysaccharide. Research Communications in Molecular Pathology and Pharmacology 1995; 90(1):49-58.

(9) Moffatt P, Plaa GL, Denizeau F. Induction of metallothionein gene expression by epidermal growth factor and its inhibition by transforming growth factor-beta and dexamethasone in rat hepatocytes. Hepatology 1995; 21(4):1038-1044.

(10) Cousins RJ, Leinart AS. Tissue-Specific Regulation of Zinc-Metabolism and Metallothionein Genes by Interleukin-1. Faseb Journal 1988; 2(13):2884-2890.

(11) Guevara-Ortiz JM, Omar-Castellanos V, Leon-Chavez BA, Achanzar WE, Brambila E. Interferon alpha induction of metallothionein in rat liver is not linked to interleukin-1, interleukin-6, or tumor necrosis factor alpha. Experimental and Molecular Pathology 2005; 79(1):33-38.

(12) Sato M, Sasaki M, Hojo H. Differential Induction of Metallothionein Synthesis by Interleukin-6 and Tumor-Necrosis-Factor-Alpha in Rat-Tissues. International Journal of Immunopharmacology 1994; 16(2):187-195.

(13) Bruwer M, Schmid KW, Metz KA, Krieglstein CF, Senninger N, Schurmann G. Increased expression of metallothionein in inflammatory bowel disease. Inflammation Research 2001; 50(6):289-293.

(14) Dooley TP, Curto EV, Reddy SP, Davis RL, Lambert GW, Wilborn TW et al. Regulation of gene expression in inflammatory bowel disease and correlation with IBD drugs: screening by DNA microarrays. Inflammatory Bowel Diseases 2004; 10(1):1-14.

(15) Clarkson JP, Elmes ME, Jasani B, Webb M. Histological demonstration of immunoreactive zinc metallothionein in liver and ileum of rat and man. Histochemical Journal 1985; 17(3):343-352.

(16) Elmes ME, Clarkson JP, Jasani B. Histological demonstration of immunoreactive metallothionein in rat and human tissues. Experientia Suppl 1987; 52:533-537.

(17) Ioachim E, Michael M, Katsanos C, Demou A, Tsianos EV. The immunohistochemical expression of metallothionein in inflammatory bowel disease. Correlation with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices. Histology and Histopathology 2003; 18(1):75-82.

(18) Kruidenier L, Kuiper I, Van Duijn W, Mieremet-Ooms MA, Van Hogezand RA, Lamers CB et al. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. Journal of Pathology 2003; 201(1):17-27.

(19) Mulder TP, Verspaget HW, Janssens AR, de Bruin PA, Pena AS, Lamers CB. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. Gut 1991; 32(10):1146-1150.

(20) Sturniolo GC, Mestriner C, Lecis PE, D'Odorico A, Venturi C, Irato P et al. Altered plasma and mucosal concentrations of trace elements and antioxidants in active ulcerative colitis. Scandinavian Journal of Gastroenterology 1998; 33(6):644-649.

(21) Cross SS. Grading and scoring in histopathology. Histopathology 1998; 33(2):99-106.

(22) Itoh N, Shibayama H, Kanekiyo M, Namphung D, Nakanishi T, Matsuyama A et al. Reduced bactericidal activity and nitric oxide production in metallothionein-deficient macrophages in response to lipopolysaccharide stimulation. Toxicology 2005; 216(2-3):188-196.

(23) Nagel WW, Vallee BL. Cell-Cycle Regulation of Metallothionein in Human Colonic-Cancer Cells. Proceedings of the National Academy of Sciences of the United States of America 1995; 92(2):579-583.

(24) Babyatsky MW, Rossiter G, Podolsky DK. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. Gastroenterology 1996; 110(4):975-984.

(25) Murphy MS. Growth factors and the gastrointestinal tract. Nutrition 1998; 14(10):771-774.

(26) Black D, Lyman S, Qian T, Lemasters JJ, Rippe RA, Nitta T et al. Transforming growth factor beta mediates hepatocyte apoptosis through Smad3 generation of reactive oxygen species. Biochimie 2007; 89(12):1464-1473.

(27) Kinnula VL, Myllarniemi M. Oxidant-antioxidant imbalance as a potential contributor to the progression of human pulmonary fibrosis. Antioxidants & Redox Signaling 2008; 10(4):727-738.

(28) Huh S, Lee K, Yun HS, Paik DJ, Kim JM, Youn J. Functions of metallothionein generating interleukin-10-producing regulatory CD4+ T cells potentiate suppression of collagen-induced arthritis. Journal of Microbiology and Biotechnology 2007; 17(2):348-358.

(29) Sappino AP, Schurch W, Gabbiani G. Differentiation Repertoire of Fibroblastic Cells - Expression of Cytoskeletal Proteins As Marker of Phenotypic Modulations. Laboratory Investigation 1990; 63(2):144-161.

(30) Douglas-Jones AG, Thomas ND, Elmes ME, Jasani B. Immunocytochemically detectable metallothionein in granulation tissue surrounding mucosal ulceration. Histochemical Journal 1992; 24(1):40-50.

(31) Yamada T, Grisham MB. Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation. Klinische Wochenschrift 1991; 69(21-23):988-994.

(32) Abdel-Mageed AB, Agrawal KC. Activation of nuclear factor kappa B: Potential role in metallothionein-mediated mitogenic response. Cancer Research 1998; 58(11):2335-2338.

(33) Schreck R, Rieber P, Baeuerle PA. Reactive Oxygen Intermediates As Apparently Widely Used Messengers in the Activation of the Nf-Kappa-B Transcription Factor and Hiv-1. Embo Journal 1991; 10(8):2247-2258.

(34) Kabe Y, Ando K, Hirao S, Yoshida M, Handa H. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. Antioxidants & Redox Signaling 2005; 7(3-4):395-403.

(35) Kim CH, Kim JH, Lee J, Ahn YS. Zinc-induced NF-kappaB inhibition can be modulated by changes in the intracellular metallothionein level. Toxicology and Applied Pharmacology 2003; 190(2):189-196.

(36) Vasto S, Mocchegiani E, Malavolta M, Cuppari I, Listi F, Nuzzo D et al. Zinc and inflammatory/immune response in aging. Biogerontology: Mechanisms and Interventions 2007; 1100:111-122.

Chapter 4.3

human metallothionein expression under normal and pathological conditions: mechanisms of gene regulation

Review in preparation

Human metallothionein expression under normal and pathological conditions: mechanisms of gene-regulation

Debby Laukens[1*], Anouk Waeytens[1,2*], Pieter De Bleser[3,4], Claude Cuvelier[2], Martine De Vos[1]

*Both authors contributed equally to this work.

[1]Department of Gastroenterology, Ghent University Hospital, Gent, Belgium
[2]Department of Pathology, Ghent University Hospital, Gent, Belgium
[3]Department for Molecular Biomedical Research, Flanders Institute for Biotechnology (VIB), Gent, Belgium
[4]Department of Molecular Biology, Ghent University, Gent, Belgium.

Correspondence:
Debby Laukens, Department of Gastroenterology, Ghent University Hospital, De Pintelaan 185 3K12IE, B-9000 Gent, Belgium, Tel: +3293322064, Fax: +3293324984, E-mail: debby.laukens@ugent.be

ABSTRACT

Metallothioneins (MT) are ubiquitous metal-binding proteins that are highly conserved throughout evolution. Although the exact physiological function is not completely understood, they are involved in a variety of processes including metal homeostasis, heavy metal detoxification, free radical scavenging, inflammation and cell proliferation. The human MT gene family consists of at least 19 isoforms, containing pseudogenes as well as genes encoding functional proteins. Most of these genes can be induced by a wide variety of substances, such as metals, cytokines and hormones. In addition, different cell types express discrete MT isoforms, reflecting the specifically adapted functions of MT isoforms, and hence a divergence in their regulation. Aberrant expression of MT has been described in a number of apparently diverse diseases, including Crohn's disease, cancer, Alzheimer's disease, amyotrophic lateral sclerosis, Menkes disease and diabetes. Therefore, a thorough understanding of the regulation of MT expression is imperative. To date, the regulation of transcription of these genes has primarily been studied in mice. However, the situation in mice is somehow less complicated, since only four isoforms are expressed. Nevertheless, the high homology between mouse and human MTs allows us to evaluate regulatory regions in their respective promoters. In this article, we review the aberrant expression of human MT in disease, and the mechanisms that regulate MT expression.

INTRODUCTION

Metallothioneins (MTs) are a family of small, highly conserved proteins with the specific capacity to bind metal ions. The MT protein was first purified from the equine renal cortex in 1960 (1). Since, they were described in a wide variety of species, including vertebrates, invertebrates, plants, fungi and some prokaryotes. A great deal of sequence and structural homology exists between MT proteins in different species, underlining their important biological role. Mammalian MT proteins typically consist of 61 to 68 amino acids, with a high content of polar, highly catalytic cysteine residues. These cysteines are strictly conserved and arranged in motifs that form the framework of two distinct metal-binding domains, linked by a short peptide (Figure 1). Eleven cysteine residues are located in the C-terminal α domain, nine cysteine residues are located in the N-terminal β domain, and the two domains can incorporate four and three divalent metal atoms, respectively. These two domains most probably account for distinct functional structures, given the marked differences in metal-mobilities found for the two domains. The α domain tightly binds metals and is involved most likely in detoxification, while the more labile metal binding in the β domain is probably implicated in metal homeostasis (2). Attempts to elucidate the exact order of metal binding were deterred by the observation of domain specific metallation such that only M_4-α or M_7-αβ species were observed and not the intermediate sequentially metallated species. However, based upon the crystal structure of rat MT and studies with a peptide fragment containing residues 49-61 of rabbit MT2, the C-terminus of the protein encompassing four cysteine residues is put forward as the site of nucleation during the folding of MT upon metal sequestration (3).

Originally, reports investigating the biological role of MTs were centralized to their exceptional metal-binding capacity. This characteristic can establish a function for MTs in two ways. First, they can provide homeostasis of metal ions within the cell, and are able to protect them from acute heavy metal toxicity. Indeed, in normal conditions, excessive concentrations of essential and nonessential metal ions like cadmium, mercury, and lead can be toxic. Therefore, most organisms use a redundant array of cellular mechanisms to limit toxicity of metal ions (4), one of which is sequestration by MT. Secondly, through participating in zinc metabolism, MTs can regulate the activity of fastly exchanging metalloproteins, such as NF-κB (5;6), the zinc finger-containing factor Sp1 (7), and the tumour suppressor gene p53 (8). Zinc is a necessary component of NF-κB for its DNA binding activity, thus regulating its activity (9). Nevertheless, it is now clear that the function of MTs is not restricted to this metal-binding activity. Because they are rapidly induced by various stimuli such as metals, hormones and cytokines, MTs are believed to participate in different protective functions. In

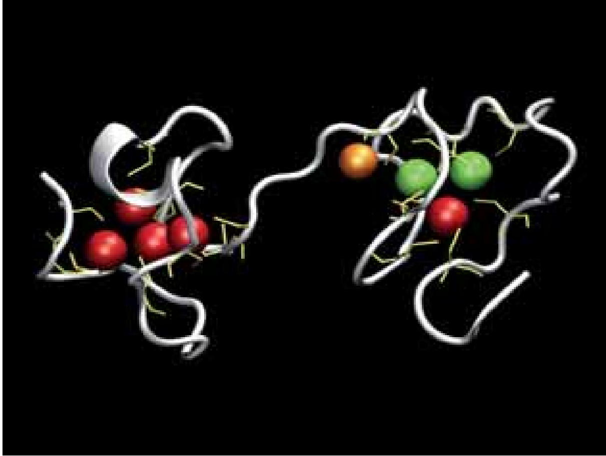


Figure 1 Homology model of human metallothionein 1A.

Cysteine residues are shown as yellow sticks, metal ions are shown in red (Cd^{2+}), green (Zn^{2+}) and orange (Na^{+}). The model was constructed using the Swiss Model web server (Schwede *et al.* 2003) with the crystal structure of the rat metallothionein 1A protein (PDB ID 4MT2) as template structure.

addition, MTs are capable of scavenging free radicals and thus play a role in protection of tissues against oxidative injury, including radiation, lipid peroxidation and inflammation. Although MTs are believed to be active intracellularly, extracellular MT has been shown in brain tissue (10), and similarly, they are found in serum and urine samples of cadmium-exposed individuals (11). However, the importance of the exact physiological role of extracellular MT is only recently emerging (12). It was shown that MTs in the extracellular environment may act as a "danger-signal" promoting movement of leukocytes to the site of inflammation (13), and thus act as a kind of chemokine. A receptor for MT has not been identified yet, but is a work of great interest. Interestingly, various immune functions have been attributed to extracellular MT. Therefore, they might be involved during the process of inflammation, which is further supported by their up-regulation by inflammatory cytokines such as interleukin 1 and tumour necrosis factor alpha (TNF). Moreover, MTs are thought to down-regulate the inflammatory response by interfering with nitric oxide (NO) synthesis, an important "end" mediator of inflammation (14).

The significance of a strictly controlled regulation of MT in both growth of cells and in their response towards several stimuli is obvious. It is therefore plausible that defects in one of these restricted mechanisms can lead to pathological situations, for example tumour growth or excessive inflammation. Good knowledge of their regulation is thus crucial in understanding their role in pathogenesis.

THE HUMAN METALLOTHIONEIN FAMILY

The classification of metallothioneins has been revised in 1999 and is based on phylogenetic relationships between protein and nucleotide sequences of the isoforms (15). Rodents express four MT isoforms, MT1 to MT4, while all primates examined so far contain multiple copies of the MT1 isoform. The situation is most complex in humans: a total of 19 MT isoforms and 5 MT-like genes have been cloned so far, many of which only differ in distinct amino acids (Table 1). So far, eight of the MT isoforms are classified as non-processed pseudogenes. It is, however, not clear whether some of these genes are nonetheless functional: MT1JP and MT1DP contain a promoter, are transcribed, but contain a premature stop codon. Fifteen out of 19 isoforms cluster together on chromosome 16q13 (Table 1) (16). Apparently, chromosome 16 is one of the most enriched chromosomes for intrachromosomal duplications (17). Similarly, the cadherin gene cluster is also located on this chromosome.

Table 1. Annotated human metallothionein genes and their characteristics

Symbol	Chromosomal location	Aliases	Protein length	Refseq status	Expression
MT:					
MT4	16q13		62	provisional	squamous epithelium
MT3	16q13	GIF	68	validated	brain, kidney, reproductive system
MT2A	16q13	MT2	61	provisional	ubiquitous
MT1A	16q13	MT1S, MTC	61	validated	ubiquitous
MT1B	16q13	MT1Q, MTP	61	provisional	ubiquitous
MT1E	16q13	MTD	61	validated	ubiquitous
MT1F	16q13		61	provisional	ubiquitous
MT1G	16q13		61	provisional	ubiquitous
MT1H	16q13	MT-0	61	validated	ubiquitous
MT1M	16q13	MT1K, MT1Y	61	validated	ubiquitous
MT1X	16q13	MT1I	61	provisional	ubiquitous
MT pseudogenes:					
MT1JP	16q13	MT1IP, MT1NP	-	provisional	-
MT1L	16q13	MT1R, MTF	-	validated	-
MT1CP	16q13		-	inferred	-
MT1DP	16q13	MTM	-	provisional	-
MT2P1	4q13		-	inferred	-
MT1P1	9q22.32			inferred	-
MT1P2	1q43			inferred	-
MT1P3	20q11.2			inferred	-
MT-like:					
MTL1	1p		?		?
MTL2	1p22		?		?
MTL3	18		?		?
MTL4	20		?		?
MTL5	11q13	TESMIN	178	reviewed	testis

The various MT isoforms differ mainly in their expression pattern (see also Table 1): MT3 and MT4 are constitutively expressed in specific cell types, while MT1 isoforms, and to a lesser extend MT2, are highly inducible. The MT1 and MT2 genes are ubiquitously expressed. By comparing the coding sequences of rodent and primate MT genes, it was shown that human MT1 isoforms show less divergence from human MT2 than from mouse MT1, suggesting that the MT1 and MT2 isoforms arose after the emergence of primates (18). On the other hand, a phylogenetic analysis including mouse and human MT3 and MT4 proteins shows that these isoforms probably diverged prior to the primate/rodent divergence (Figure 2). In addition, human as well as mouse MT1 and MT2 isoforms are clearly separated from the MT3 and MT4 clusters, most likely reflecting the distinct regulation of expression of these isoforms in both organisms. Interestingly, this could signify that MT1, MT2, MT3 and MT4 proteins have similar functions in mice and men.

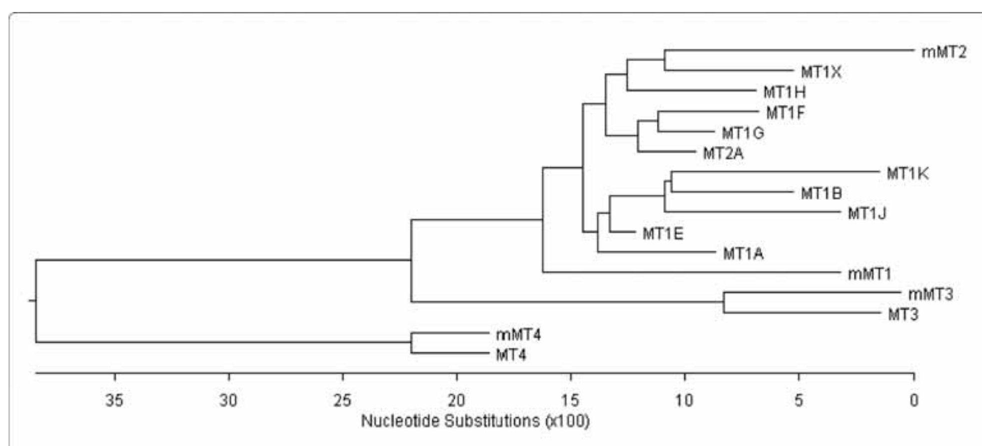


Figure 2. Phylogenetic tree of human and mouse metallothionein protein isoforms.

Metallothionein 3 and 4 probably arose prior to the divergence of primates and rodents, while MT1 and MT2 genes arose later. Mouse MT genes are designated as mMT1, mMT2, mMT3 and mMT4.

METALLOTHIONEIN IN DISEASE

Since cellular zinc homeostasis is extremely important for normal functioning, it is not surprising that MT deregulation has been implicated in natural processes such as ageing (19), but also in pathological conditions. Unfortunately, the role of these proteins in disease aetiology is not clear as a result of at times conflicting reports. Incompatible data can partly be explained by the fact that expression data obtained by immunohistochemistry, semi-quantitative PCR or more recent techniques such as quantitative PCR (qPCR) are not strictly comparable. The early studies on MT expression relied on immunohistochemistry using antibodies, which cannot distinguish between MT isoforms. Only for MT3 a specific antibody has been generated against a unique eight amino acid sequence not shared by any other MT isoform (20). Using non-distinguishing antibodies, strong staining can be found in pathological tissues, especially in tumors (21). However, in normal tissues, MT expression is usually undetectable by immunohistochemistry except in myoepithelial (22), renal (23), ileal epithelial cells (24), pancreas (25) and fetal liver (26). Real-time qPCR analysis is much more sensitive than staining procedures. Furthermore, highly specific primers can be designed to study the expression of every MT isoform individually. Another issue which complicates the interpretation of MT in disease between studies is the gathering of tissues for analysis (e.g. resection versus biopsy material, delay time between sampling and storage). Since MTs are stress proteins, their expression is largely determined by the handling of tissues. This also applies for *in vitro* analysis of MT expression, because cultured cells are very sensitive to changes in pH, temperature and addition of serum (27). Here, we summarize data of abnormal MT expression in several diseases.

Metallothionein and Crohn's disease

Crohn's disease (CD) is a chronic inflammatory disease of the gastro-intestinal tract. The complete bowel wall of the ileum and/or colon is affected. In the normal intestine, we found that MT mRNA and protein expression is highest in the ileum as compared with the colon (24). Metallothionein is localized in the enterocytes, predominantly at the base of crypts. Although the high proliferative state of these cells might explain their high MT expression, we could not find a correlation between MT expression and the proliferation marker Ki67 (28). We propose that MT might have an antibacterial role in these cells, given that antibacterial effects are most critical in these sites of the intestine. Focal staining is often observed in the intestine, as a result of the uniform staining in distinct single crypts. Based on a mutagen-induced mouse model it was suggested that this staining pattern is the result of somatic mutations in stem cells, leading to strong clonal expression of MT in the entire crypt (29;30). There is thus far no evidence that this process exists in the human intestine. Aberrant mRNA

and protein expression was found in tissue samples originating from CD patients. Down-regulation as well as up-regulation have been reported. Two papers reported an up-regulation (31;32), while we and others found a down-regulation of MT in CD (24;33-36). Moreover, MT quantification in CD based on radioimmunoassay (36;37) and microarray (24) also showed a down-regulation. Using qPCR, we provided evidence for a decreased expression of a large proportion of MT1 isoforms and MT2 at basal level in colon biopsies, ileum biopsies and whole blood samples of CD patients (24). This lowered mRNA expression level correlated with protein expression, even though biopsies were not acquired at the same time. These results suggested that the deficient MT expression in CD patients with colonic disease is at least partly genetically determined. Interestingly, the MT gene cluster is located in the *IBD1* chromosomal region, which was significantly associated to CD in many genome scans (38).

There are several possible mechanisms by which MT might play a role in CD. Deregulated MT levels in CD patients might indicate a hampered maintenance of free radicals produced during the inflammatory process. Anti-bacterial properties of MT could be important in the process of CD progression, as bacteria are considered to be involved in initiating and propagating the inflammatory process (39). We found evidence that the decreased epithelial MT level correlated with reduced secretion of the neutrophil chemokine interleukin-8 *in vitro* upon stimulation (40). This observation was in accordance with the recent finding that IL-8 up-regulation is impaired in response to acute trauma to the colon or skin of CD patients (41). We hypothesize that the intestinal epithelial cell lining of CD patients expresses less MT and initially secretes less IL-8 in response to bacteria. A delay in neutrophil accumulation might lead to the persistence of the exogenous stimulus and an exaggerated secondary and compensatory immune response. In a murine model for acute colitis, based on the oral administration of dextran sodium sulphate, the role of epithelial MT was further clarified by the more severe histological inflammation on day 3 in MT deficient mice as compared to wild type mice (24). Since colonic MT expression was restricted to the epithelium until day 3, this corresponds with an immunomodulating role for MT in the early phase of colitis. A more deleterious role of MT during the late phase of induction was suggested by the lower survival rate of wild type mice. Especially mice that were treated with zinc, which induces endogenous MT in the intestine, were at risk.

Serum zinc concentrations are often decreased in CD patients. This element plays an important role in the prevention of free radical formation and in protection of biological structures from damage (42). It was shown that dietary zinc causes an MT increase in all gut regions in rats (43;44). On the other hand, CD patients receiving a zinc supplementation

during 4 weeks did not change the concentration of MT in their plasma and erythrocytes (45), and the MT concentration in both inflamed and non-inflamed intestinal mucosa was only slightly higher. Histological inflammation scores of intestinal biopsies, plasma albumin levels, and the disease activity index of the patients did not change during the trial. Notably, in this study, only disease-inactive to moderately active patients were included.

Next to Crohn's disease, the inflammatory bowel diseases comprise ulcerative colitis. As opposed to Crohn's disease, MT expression was not reduced in samples of ulcerative colitis patients without active inflammation. Active inflammation caused a decrease in epithelial MT expression and an increase in non-epithelial MT expression that was also noted in other intestinal inflammatory conditions (28). These findings emphasize that MT regulation is inflammation-dependent and future studies will be directed on the regulation and role of MT in inflammation.

Metallothionein and cancer

Immunocytochemically detectable MT overexpression was described in a variety of human tumours (21). However, the expression of MT is not universal to all human tumours, but may depend on their differentiation status and proliferative index. Metallothionein overexpression is associated with resistance to anticancer drugs and is combined with a poor prognosis. However, its use as a marker of tumour differentiation, cell proliferation and prognosis predictor remains unclear. On the other hand, gastric carcinomas and colorectal adenomas are apparently accompanied by a decreased expression of MT, however, those with a relatively high level seem to have an increased malignant potential (46).

Cell-type specific differential regulation of human MT genes was found in different cancer cell lines, correlating with DNA methylation and chromatin structure (see later) (47). Tumour cell-lines arising from paraxial mesoderm and endoderm have MT2A and MT1E genes in fully inducible form and the MT1F in the refractory state (18). On the other hand, tumours originating from ectoderm, intermediate and lateral mesoderm exhibit MT2A and MT1F genes in inducible form and the MT1E gene in a refractory form.

Using 53 adherent cell lines of the National Cancer Institute (NCI) tumour panel, Woo et al. (48) studied the basal MT content and subcellular localization in human tumour cells with immunofluorescence and tried to correlate this basal MT phenotype to responsiveness to anticancer drugs. Among the cell types, they found a 400-fold range in the basal MT levels and a tenfold range in the ratio of nuclear to cytoplasmic (N/C) staining that was independent

of basal MT content. Ovarian- and breast-derived cell lines all had mean MT levels lower than the overall mean MT level. Approximately half of the cell lines had a karyophilic (N/C > 1) phenotype, and tissue specificity of MT localization was observed with breast cancer cell lines, which were all seven cytoplasmophilic (N/C < 1), whereas the two tested prostate cell lines were karyophilic. Both basal MT levels and subcellular distribution appeared to be determinants of resistance to metal-containing compounds.

Metallothionein, Alzheimer's disease and amyotrophic lateral sclerosis

Metallothionein 3 was first cloned as growth inhibitory factor (GIF), which showed a decreased RNA expression in Alzheimer's disease (AD). Metallothionein 3 suppresses the neurotrophic activity present in the normal human brain (49). The down-regulation of MT3 in AD has been confirmed by two recent studies. In the first study, MT3 expression was determined in a large number of AD cases by qPCR as well as by immunohistochemistry and Western blotting (50). In the second study, DNA microarrays were used to compare RNA levels from control and AD hippocampal regions and found, amongst others, MT3 down-regulation (51). However, Erickson and colleagues (52) disputed that neuronal changes in AD are related to a decrease in MT3, since they could not find a significant down-regulation in neither RNA nor protein expression in their AD population. There is more consensus on the overexpression of MT1 and MT2 in the astrocytes from AD as well as other neurological disorders (53-56). In the brain, astrocytes are the main source of MT1 and MT2, although other cell types, such as choroid plexus epithelia, endothelium and meningeal cells may also express these isoforms (57). In neurodegenerative diseases such as AD, astrocytes become abundant and activated in the affected areas. While in other organs, the main function of MT is related to zinc metabolism and protection against heavy metal and/or oxidative damage, the key role of MTs in the brain seems to be a protection in the cellular response to neuronal injury. It was suggested that the specific increase in MTs was associated with the initial stages of the disease process (54). The precise mechanisms downstream of MT have not been fully established, but convincing data showed that they are essential in dealing with neuropathology and for brain recovery in AD as well as other brain pathologies. MTs might even be used as therapeutic and/or preventive drugs for a range of brain disorders (57).

Amyotrophic lateral sclerosis (ALS) is a progressive, invariably fatal neurological disease due to degeneration of the nerve cells responsible for controlling voluntary muscles. In ALS, motor neurons in the brain stem, spinal cord and motor cortex degenerate or die, ceasing to send messages to muscles. Consequently, the muscles gradually weaken, waste away, and twitch. Fifteen to 20% of cases of familial ALS are associated with mutations in the

superoxide dismutase 1 gene (*SOD1*), an enzyme that converts harmful superoxide radicals into oxygen and hydrogen peroxide.

Elevated levels of MT have been found in spinal cord, kidney and liver of patients with ALS (58-60), but not in serum (60). A detailed study on MT isoform expression revealed no evidence for either the induction of a specific MT repertoire, or for the inability of glia to express any MT gene (61). Probably, the enhanced expression of MT in ALS reflects an early protective function. This was also concluded from a study on MT expression in mice carrying the *SOD1* mutation (62). These mice were backcrossed with MT-knockout mice. The offspring reached the onset of clinical signs significantly earlier in response to the reduction of protein expression. These results indicated that the copper-mediated free radical generation derived from mutant *SOD1* might be related to the degeneration of motor neurons in ALS and that MT might play a protective role against the expression of the disease (63). Recently, MT3 was screened for mutations in 20 patients with ALS, but no functionally relevant polymorphism could be associated with the disease (64).

Metallothionein, Menkes disease and Wilson disease

Menkes disease is an X-linked, recessive disorder of the copper metabolism that occurs in less than 1 in 200,000 live births. The condition is characterized by early retardation in growth, peculiar hair, focal cerebral and cerebellar degeneration, skeletal abnormalities, and patient mortality in early childhood (65). Three independent research groups cloned the Menkes gene, a copper transporting ATPase, *ATP7A*, to the long arm of the X chromosome (66-68). A spectrum of mutations adversely affecting protein expression have been observed in severely affected Menkes patients (69). The genetic defect in Menkes syndrome leads to a progressive copper deficiency and copper-dependent enzymes fail in most tissues.

Using cultured fibroblasts, it was shown that low extracellular copper concentrations induce synthesis of MT in Menkes' cells but not in normal cells (70). Therefore, in the early studies on tissue abnormalities in Menkes disease, MT were believed to be involved in pathogenesis (71;72). Now, it is clear that a defect in efflux and consequently intracellular accumulation of copper, due to mutations in *ATP7A*, is responsible for this up-regulation in Menkes’ cells. As a result of accumulation of copper in the cell MTs are up-regulated, but they are as such not causative for pathogenesis in Menkes disease. In affected cells, copper accumulates bound to MT in the cytosol, while its transport to the organelles, as well as copper efflux, is disturbed. The low activity of metalloenzymes is believed to contribute significantly to the pathogenesis of this condition.

Wilson disease is an autosomal recessive disorder characterized by dramatic build-up of intracellular hepatic copper with subsequent hepatic and neurologic abnormalities. Copper toxicity occurs when the liver is overloaded and non-ceruloplasmin-bound copper is released into the bloodstream, from where it can diffuse into the brain. Today, the treatment of Wilson disease is no longer aimed at 'decoppering', the removal of accumulated copper, but at the normalization of the free copper concentration in blood, to reverse the copper poisoning. Therefore, new therapy is aimed at administration of zinc to these patients, because this increases MT expression and sequesters the excess of copper in the blood (73).

HUMAN METALOTHIONEIN GENE REGULATION

Because MT expression is involved in a number of pathological conditions, the transcriptional control of MT has become a major topic. Metallothioneins are induced by a wide variety of physiological and chemical agents like cytokines, metals, hormones, and stress in general (74). Furthermore, they are transiently induced after tissue injury caused by e.g. inflammation or irradiation (75). It is generally accepted that *MT1* and *MT2* genes are inducible, while *MT3* and *MT4* are constitutively expressed. Metallothionein 1 isoforms have a restricted and transient role, perhaps in the process of the cell cycle, in stress or during infection. On the other hand, MT2A is ubiquitously expressed, and plays a general role in cellular physiology, possibly in zinc metabolism. Metallothionein 3 is predominantly expressed in the central nervous system (49;61), but also in the kidney (76), prostate (77), retina (78), salivary glands (79) and reproductive system (80). The mouse MT4 is expressed in stratified squamous epithelium of the tongue (81), but there are so far no data on human MT4 expression.

Most of the functional studies on MT transcription were performed in mice. However, in mice, MT1 and MT2 isoforms are co-ordinately regulated (82), while the human MT isoforms are regulated in a cell-type specific manner (24;83;84). This probably resulted in the adaptation of MT genes to more specific functions throughout evolution. Phylogenetic tree analysis of the promoter region of mouse and human MT isoforms shows that MT2, MT3 and MT4 promoters are highly homologous between mouse and human (Figure 3), which might reflect their more strictly regulated expression pattern. Human MT1 isoforms are dispersed into two groups. Interestingly, these two clusters correlate to the physical location of the genes on chromosome 16. On the other hand, the mouse MT1 promoter region is relatively unrelated to the human MT1 group (Figure 3). It is thus plausible that regulatory regions in the mouse and human promoter have adapted differently. Therefore, care must be taken when evaluating MT expression and maybe even MT function in the mouse, when addressing

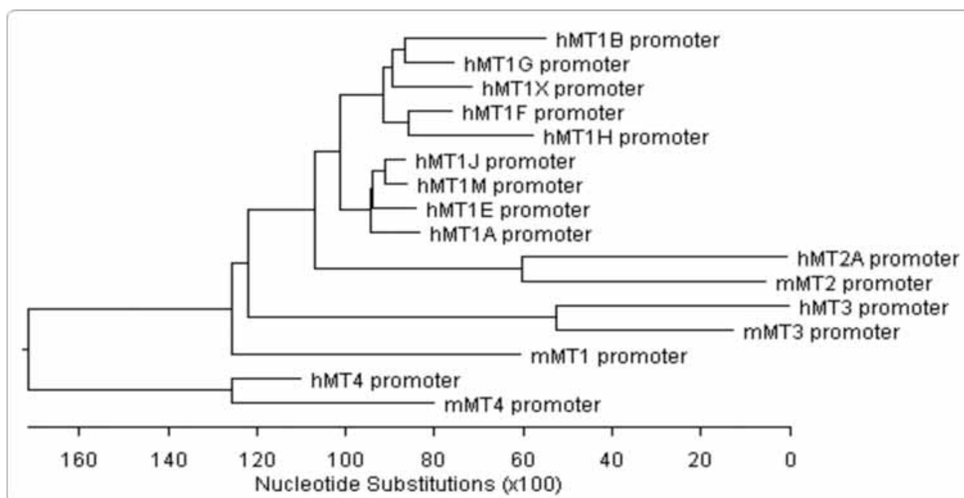


Figure 3. Phylogenetic tree analysis of the promoter regions of human and mouse MT isoforms.

Promoter regions are defined from nucleotides -800 to -1 relative to the transcriptional start site.

questions related to human circumstances. Nevertheless, if common transcription factor binding sites are found in human and mouse promoters, extrapolation of the experimental data on mouse MT regulation is likely.

We performed a comparative study on the transcriptional control of MT in mice and humans, using *in silico* data of promoter transcription factor binding sites. Based on these common regulatory sites, we review the MT regulatory mechanisms.

In silico analysis of human and mouse MT1 promoters

Although marked sequence conservation exists, the RNA level for each MT isoform is unique. This is largely due to inherent differences in promoter regulatory sequences. Promoters are organized with a variety of elements that contribute to promoter function. The elements found in any promoter differ in number, location and orientation.

A difference distance matrix approach (85) was used to identify a set of transcription factor binding sites that are specifically present in following MT isoforms: MT1A, MT1B, MT1E, MT1F, MT1G, MT1E, MT1J, MT1M and MT1X (Table 2). Many of these binding sites and their transcription factors have been described in MT regulation, however, we found a number of unexplored regulatory regions. We next searched for these transcription factor

binding sites in the mouse MT1 promoter. In addition, well-known mouse MT1 promoter binding sites (USF, MLTF) were explored in the promoters of human MT1 isoforms. Below, we describe the involvement of these transcriptional regulators in more detail.

Table 2. Transcription factor binding sites in 800 bp of the promoter of human and mouse metallothionein 1 isoforms

	Transcription factor	MT1A	MT1B	MT1E	MT1F	MT1G	MT1H	MT1J	MT1M	MT1X	mMT1
basal	TBP	+	+	-	-	+	-	-	-	+	+
	TFII-I	2	2	1	3	1	2	2	4	0	1
	Sp1	5	1	1	8	5	2	2	4	3	3
	AP2	0	1	1	1	1	3	1	0	0	0
	USF/Nrf2	0	0	0	0	1	0	0	0	0	1
induction	ChCh	9	3	6	8	6	5	8	9	5	3
	HELIOS	2	8	2	6	6	7	4	3	9	3
	E2F	3	0	4	4	3	2	6	5	3	3
	Spz1	4	4	5	4	2	5	3	6	1	2
	Egr_1	4	0	3	5	0	0	3	8	4	1
	GR	1	1	6	0	0	3	1	2	2	3
	STAT	1	0	4	2	1	1	1	1	2	0
	MTF1	2	4	1	5	2	5	2	2	3	3
	RAR-a	2	5	1	5	3	3	3	2	3	3
repression	ETF	6	2	3	9	6	2	6	6	6	4
	NFI	1	0	1	3	4	2	1	2	2	4

The number of hits as found by Match™ (TRANSFAC PRO 8.4) using a core matrix match of 100% and a matrix match of 85%. Exceptions are IL6RE and MLTF for which consensus sequences were used in combination with Patch™ (TRANSFAC PRO 8.4). The Match procedure introduces false positives; therefore, the number of hits listed is an overestimation of the real hits. The MT isoforms with TBP consensus sequences located close tot the transcription start site are denoted with a plus (+). Those that do not contain a TBP close to the transcription start site are denoted with a minus (-). mMT1= mouse MT1

Basal expression

RNA polymerase II is responsible for transcribing genes coding for messenger RNA (mRNA). The first step in transcription is the binding of the TFIID complex to a region upstream of a sequence called the TATA box. The TFIID complex consists of the TATA-binding protein (TBP) and TBP-associated factors (TAF). The location of the TATA box with respect to the start point is relatively fixed, usually located ~25 bp upstream of the transcriptional start site. Therefore, TATA box consensus sequences located further upstream are probably not functional.

Metallothionein 1E, MT1F, MT1H, MT1J and MT1M contain TATA-less promoters. This could partly explain the observation that the MT1G promoter is five times more active than the MT1F promoter in transfection studies (86;87). GC boxes, common promoter components involved in basal transcription, are frequently found in MT promoters, often clustered together close to the start site. It has been proposed that the Sp1 factor binds to multiple GC boxes, resulting in an interaction of GC box-bound Sp1 factors with each other to synergistically stimulate transcription. Furthermore, basal level enhancer sequences, binding activator protein-2 (AP2), are found in some human MTs, but not in the mouse MT1. TFII-I binds specifically to initiator elements (Inr), supporting basal transcription. An E box, binding the upstream stimulatory factor (USF) was only found in the mouse MT1 and the human MT1G. It was shown that TFII-I also binds to upstream E box, and that TFII-I and USF interact co-operatively at both Inr and E box sites (88). The USF sequence overlaps with an antioxidant response element (ARE). These AREs are usually found in genes responsive to free radicals, through interaction of ARE with NF-E2-related factor 2 (Nrf2). However, this sequence is only found in the mouse MT1 and human MT1G, suggesting that they might be more responsive to free radical exposure. Nevertheless, response to oxidative stress can also be mediated by metal responsive elements (MRE) in the promoter (89). MREs are recognized by the MRE-binding transcription factor 1 (MTF1), and are classically required for metal induction of MTs (see below). Nevertheless, they also participate in basal transcription. Indeed, the basal expression of MT is highly correlated to the activity of MTF1 (90). Furthermore, we have recently shown that basal MT levels in whole blood vary considerably between individuals, but they correlate well with MTF1 expression (24).

Inducible expression

Metal induction. Metallothionein 1 and MT2 isoforms are highly inducible by many metal ions, including zinc, cadmium, bismuth, mercury, copper, nickel and cobalt. The concentration of metal ions to induce MTs depends on the type of ion and MT isoform. Metallothioneins are capable of binding most of these elements, however, they do not bind nickel and cobalt. The transcriptional regulation responsible for this metal induced expression, is controlled by MREs, present in the promoter of MT as multiple, non-identical copies. The mouse and human transcription factor that binds to these MRE elements is the MRE-binding transcription factor 1 or MTF1. When this factor was first cloned (91), it was shown that the human MTF1 was more effective than the mouse equivalent. MTF1 is absolutely necessary for both basal and metal inducible MT expression (92). Moreover, although MTF1 is activated by a number of metals, it absolutely requires zinc for its activity. It was hypothesized that inducing non-zinc metals can displace zinc from its storage proteins, resulting in a pool of

free zinc available for activation of MTF1 (93). These storage proteins could be MT itself, leading to a complex feedback interaction between MTF1 and MTs.

Interestingly, treatment of cells with cadmium increases MT expression, although it does not influence the DNA binding activity of MTF1 to the MRE. This suggests that additional mechanisms play a role in metal induction. For example, cadmium induces oxidative stress, which could activate binding of USF to the E box. Alternatively, as stated before, cadmium might replace the intracellular zinc in storage proteins, which results in more free zinc to activate MTF1. Recently, it was shown that cadmium induction of MT1 and MT2A mRNA isoforms in human thyroid carcinoma cells is mediated through ERK signalling, and is dependent on intracellular calcium rise (94). The responses of each individual MT isoform is not identical, reflecting the different transcriptional regulation of isoforms.

In addition, posttranslational modification of MTF1 has been shown. Phosphorylation of MTF1 plays a critical role in its activation by zinc and cadmium (95). Several phosphorylation sites are present throughout the complete MTF1 protein. This was thought to be mediated through a complex pathway involving protein kinase C, tyrosine kinase, and casein kinase II.

Stress and inflammation mediated induction. Similar to acute phase proteins, MT is induced by inflammation, bacterial infection and stress. Stress in general often results in the synthesis of glucocorticoid hormones, resulting in the suppression of inflammation and an increase in blood sugar levels. These steroid hormones are synthesized by the adrenal gland, and can enter the cell by simple diffusion. Within the cell, it binds to its receptor, the glucocorticoid receptor (GR), which in turn gets activated and translocates to the nucleus. There, it has high affinity for a consensus sequence called the glucocorticoid response element (GRE), and activates transcription from MTs and other GRE containing genes. Glucocorticoid hormones have been known for a long time as inducers of MT (96;97). The human MT2 gene contains one GRE, while the mouse MT1 and MT2 genes contain two tandem copies ~1kb upstream of the MT2 gene and ~7 kb of the MT1 gene (98). In humans, MT2A is significantly more inducible by glucocorticoids as compared with MT1 isoforms (83). It was shown that dexamethasone, a synthetic glucocorticoid agonist, appears to have no significant effect on the expression of MT1F (84). This can be explained by the fact that there is no GRE sequence in the promoter of MT1F.

During acute inflammation, such as after tissue damage or during infection, macrophages are recruited and activated to the site of inflammation. They secrete pro-inflammatory cytokines such as IL6, TNF, IL1α and IL1β. These cytokines are able to induce MT (99). Fast MT up-

regulation after challenge with IL1 is probably mediated through glucocorticoids (100). Interleukin 6 is one of the most potent inducers of MT (101). Metallothioneins have IL6 response elements (IL6RE) in their promoter. These DNA stretches bind STAT transcription factors. A synergistic effect was demonstrated between IL6 and glucocorticoid in MT induction (102), which is possibly mediated by the close proximity of the GRE and the IL6RE in the MT promoters.
Interestingly, it was reported that MTs inhibit the release of pro-inflammatory cytokines (103;104). This could be explained by the regulatory role of MT in NF-κB activation (5).

Cell cycle. Metallothionein expression appears to be cell cycle dependent, and is used as a marker for cell proliferation (21). A tenfold rise of MT synthesis was described in exponentially growing human hepatocytes (105;106), and peaks of MT expression were found in late G1 and G1/S transition in HT29 epithelial cells (105). Similarly, in placental tissue, positive immunostaining for MT was found only in trophoblast and proliferating cells (107). We have recently described that MT expression in the intestine was most apparent in the rapidly proliferating cells of the crypts (24). Moreover, it was suggested that MT transcription is altered by the differentiation process. Indeed, it was shown that the differentiation of teratocarcinoma cells using retinoic acid is associated with a rise in MT expression. Retinoic acid receptors (RAR) are nuclear receptors related to the steroid and thyroid hormone receptors, a family of proteins that functions as ligand-dependent transcription factors. Retinoic acid is a regulator of differentiation at various stages of vertebrate embryogenesis. In accordance, multiple RAR receptor binding sites are found in human and mouse MT1. However, whether this codependence is initiated by MTs is not known: is the overexpression of MT in many tumors the cause of cell cycle progression, or is a rapid cell growth causing MT to be highly expressed?

Although MT expression is generally cytosolic, nuclear translocation has been observed at G0/G1 to early S-phase (108). This nuclear and cytoplasmic localization of MT was also observed in several tumours, especially in regions of high proliferation. Moreover, antisense down-regulation of MT1 in endothelial cells resulted in the cell cycle arrest at the G1 phase (109), and cell growth was inhibited in MT1 antisense tumor cells (110). Hesketh and colleagues have shown that the nuclear translocation is determined by the 3' untranslated region of MT1 (111). Recently, they identified a 11 nucleotide sequence in the 3'UTR, containing a CACC repeat, that is necessary for the nuclear translocation of MT1 (112).

In the human and mouse MT1 promoters, potential binding sites for the E2F transcription factor are present. This transcription factor is a critical determinant of the G1/S-phase

transition during the mammalian cell cycle, serving to activate the transcription of a group of genes that encode proteins necessary for DNA replication. In addition, E2F activity appears to be directly regulated by the action of retinoblastoma protein (Rb). Human DP-1 and E2F-1 associate both *in vivo* and *in vitro*, and this interaction leads to enhanced binding to E2F DNA-binding sites (113). The association of E2F-1 and DP-1 leads to co-operative activation of an E2F-responsive promoter. It was also demonstrated that trans-activation by E2F-1/DP-1 heterodimers is inhibited by RB. Nevertheless, the actual binding of E2F to MT promoters needs to be determined.

The nuclear need for MT at specific stages of the cell cycle might point towards a critical function of MT in regulating metalloproteins, or protection from DNA damage and apoptosis (114).

Development. Metallothionein expression is tightly regulated and activated during mammalian embryonic development, with high levels of MT1 and MT2 mRNA in both maternal and fetal liver, and the visceral yolk sac (115). During early development of the mouse embryo, expression of MT1 is induced specifically in the endoderm cells of the visceral yolk sac (116). It was shown that MTF1 is absolutely essential for up-regulation of MT1 gene expression in visceral endoderm cells and that optimal expression also involves the binding of USF to the promoter. Only the human MT1G promoter contains an E box, therefore, it is not known whether the human USF binding is necessary in development.

Inhibition of transcription

Contrary to the activation of MT expression, their down-regulation by *cis*-acting events has not been extensively studied. However, a reduced expression has been frequently found in many types of cancers and in CD. Three factors are probably involved in suppression of MT: nuclear factor 1 (NFI), ZBTB11 and EGFR-specific transcription factor (ETF). Overexpression of NFI in human hepatoma cells suppressed both constitutive and metal induced activation of the MT1 promoter (117). NFI binds to an MRE-c' sequence in the mouse promoter (118). NFI sites are present in the mouse and all human promoters except MT1B. One study reports a 120 kDa zinc finger protein (PZ120) repressing the transcription of the human MT2A gene by binding to its transcription initiation site (119). The PZ120 gene is now replaced in the NCBI database as the zinc finger and BTB domain containing 11 (ZBTB11). So far, no additional reports describing this transcription factor were published since. ETF is present in all MT isoforms, and has not been described in the context of MT inhibition. Overexpression of this factor in primate kidney CV1 cells showed that it represses expression originating from both the EGFR and beta-actin gene promoters (120).

The influence of environmental factors on MT expression was illustrated by the inhibition of zinc induced MT induction by chromium, a major environmental carcinogen (121). This inhibition was working through interfering with MTF1. Treatment of mammalian cells with cycloheximide, a protein synthesis inhibitor, resulted in increased MT1 transcription (122). Recently, the presence of a labile inhibitor of MT1 expression was suggested (123). This repressor negatively controls agonist-induced turnover of the MTF1 protein.

Promoter methylation

Methylation of cytosine residues in promoter sequences is generally associated with a low transcriptional level of the respective gene. In mammals, methylation is mainly found within so-called CpG islands, regions of high CG content, thought to be involved in transcriptional regulation. The general rule is that CpG islands are not methylated, except for genes on the inactive X chromosome and at imprinted loci. Generally, to study the methylation status of a gene in a cell line or in tissue, its expression is correlated to the amount of methylated CG dinucleotides in genomic DNA isolated from the same source. If a correlation is found, e.g. low gene expression and a hypermethylation of the CpG island, the cells or tissue specimens are subjected to a demethylation agent, such as 5-azacytidine. If the demethylation results in an increased expression of the gene, a role for methylation in expression regulation of the gene is established.

Rodent and human MT genes contain a CpG island in their promoter. Tissue specific methylation of MT1B has been demonstrated (124). The MT1B gene is only expressed in human hepatoma and renal carcinoma cell lines, and not in HeLa cells, where the 5' flanking region of MT1B is highly methylated. MT3 hypermethylation has been shown in gastric cancer (125) and in oesophagal squamous carcinoma (126).

CONCLUSION

The persistent differential expression of MTs in stress reaction and in pathological conditions suggests a strict regulation of these proteins. It is plausible that a change in e.g. cell cycle regulation of MT could influence cancer development. In addition, cell-type specific expression of MTs may indicate a divergence in functions within cell types or organs. For instance, disability of the tightly controlled proliferation of stem cells in the intestinal crypts may render individuals more susceptible for developing colon cancers. Similarly, a disturbed immune balance in the intestine due to changes in MT expression could be a prerequisite for CD. Our *in silico* search for transcriptional regulatory regions in the MT promoters revealed new potential targets that could help to unravel some of the MT regulatory mechanisms. Future studies on the expression and regulation of MT genes are likely to provide insights to their role in both health and disease. Ultimately, novel strategies for manipulating intracellular MT levels could lead to new therapies, and are already being investigated for the treatment of skin wounds (127), rheumatoid arthritis (128), central nervous system injury and disease (129), and diabetes-induced organ dysfunction (130).

REFERENCES

(1) Kagi JHR, Vallee BL. Metallothionein - Cadmium-Containing and Zinc-Containing Protein from Equine Renal Cortex. Journal of Biological Chemistry 1960; 235(12):3460-3465.

(2) Zangger K, Armitage IM. Dynamics of interdomain and intermolecular interactions in mammalian metallothioneins. Journal of Inorganic Biochemistry 2002; 88(2):135-143.

(3) Munoz A, Laib F, Petering DH, Shaw CF. Characterization of the cadmium complex of peptide 49-61: a putative nucleation center for cadmium-induced folding in rabbit liver metallothionein IIA. Journal of Biological Inorganic Chemistry 1999; 4(4):495-507.

(4) Dameron CT, Harrison MD. Mechanisms for protection against copper toxicity. American Journal of Clinical Nutrition 1998; 67(5):1091S-1097S.

(5) Sakurai A, Hara S, Okano N, Kondo Y, Inoue J, Imura N. Regulatory role of metallothionein in NF-kappaB activation. FEBS Letters 1999; 455(1-2):55-58.

(6) Kim CH, Kim JH, Lee J, Ahn YS. Zinc-induced NF-kappaB inhibition can be modulated by changes in the intracellular metallothionein level. Toxicology and Applied Pharmacology 2003; 190(2):189-196.

(7) Zeng J, Heuchel R, Schaffner W, Kagi JHR. Thionein (Apometallothionein) Can Modulate Dna-Binding and Transcription Activation by Zinc Finger Containing Factor-Sp1. FEBS Letters 1991; 279(2):310-312.

(8) Ostrakhovitch EA, Olsson PE, Jiang S, Cherian MG. Interaction of metallothionein with tumor suppressor p53 protein. FEBS Letters 2006; 580(5):1235-1238.

(9) Zabel U, Schreck R, Baeuerle PA. DNA Binding of Purified Transcription Factor NF-KappaB - Affinity, Specificity, Zn2+ Dependence, and Differential Half-Site Recognition. Journal of Biological Chemistry 1991; 266(1):252-260.

(10) Chung RS, West AK. A role for extracellular metallothioneins in CNS injury and repair. Neuroscience 2004; 123(3):595-599.

(11) Falck FY, Fine LJ, Smith RG, Garvey J, Schork A, England B et al. Metallothionein and Occupational Exposure to Cadmium. British Journal of Industrial Medicine 1983; 40(3):305-313.

(12) Lynes MA, Zaffuto K, Unfricht DW, Marusov G, Samson JS, Yin X. The physiological roles of extracellular metallothionein. Experimental Biology and Medicine 2006; 231(9):1548-1554.

(13) Yin X, Knecht DA, Lynes MA. Metallothionein mediates leukocyte chemotaxis. BMC Immunology 2005; 6:21.

(14) Zangger K, Oz G, Haslinger E, Kunert O, Armitage IM. Nitric oxide selectively releases metals from the amino-terminal domain of metallothioneins: potential role at inflammatory sites. Faseb Journal 2001; 15(7):1303-1305.

(15) Binz PA, Kägi JHR. Metallothionein: molecular evolution and classification. In: Klaassen C, editor. Metallothionein IV. Basel: Birkhäuser Verlag, 1999: 7-13.

(16) Karin M, Eddy RL, Henry WM, Haley LL, Byers MG, Shows TB. Human metallothionein genes are clustered on chromosome 16. Proceedings of the National Academy of Sciences of the United States of America 1984; 81(17):5494-5498.

(17) Martin J, Han C, Gordon LA, Terry A, Prabhakar S, She XW et al. The sequence and analysis of duplication-rich human chromosome 16. Nature 2004; 432(7020):988-994.

(18) Schmidt CJ, Jubier MF, Hamer DH. Structure and Expression of 2 Human Metallothionein-I Isoform Genes and A Related Pseudogene. Journal of Biological Chemistry 1985; 260(12):7731-7737.

(19) Mocchegiani E, Giacconi R, Cipriano C, Costarelli L, Muti E, Tesei S et al. Zinc, metallothioneins, and longevity--effect of zinc supplementation: zincage study. Annals of the New York Academy of Sciences 2007; 1119:129-146.

(20) Garrett SH, Sens MA, Todd JH, Somji S, Sens DA. Expression of MT-3 protein in the human kidney. Toxicology Letters 1999; 105(3):207-214.

(21) Theocharis SE, Margeli AP, Klijanienko JT, Kouraklis GP. Metallothionein expression in human neoplasia. Histopathology 2004; 45(2):103-118.

(22) van den Oord JJ, De Ley M. Distribution of Metallothionein in Normal and Pathological Human Skin. Archives of Dermatological Research 1994; 286(1):62-68.

(23) Mitropoulos D, Kyroudi-Voulgari A, Theocharis S, Serafetinides E, Moraitis E, Zervas A et al. Prognostic significance of metallothionein expression in renal cell carcinoma. World Journal of Surgical Oncology 2005; 3(1):5.

(24) Laukens D, Waeytens A, Peeters H, Bogaert S, Aelbrecht C, Taildeman J et al. Impaired epithelial metallothionein expression as a possible aetiopathogenic mechanism in Crohn's disease. submitted 2008.

(25) Tomita T, Matsubara O. Immunocytochemical localization of metallothionein in human pancreatic islets. Pancreas 2000; 20(1):21-24.

(26) Fuller CE, Elmes ME, Jasani B. Age-Related-Changes in Metallothionein, Copper, Copper-Associated Protein, and Lipofuscin in Human Liver - A Histochemical and Immunohistochemical Study. Journal of Pathology 1990; 161(2):167-172.

(27) Kuppens K, De Ley M. Serum-induced expression of metallothionein isoforms in K-562 cells. Biochemical and Biophysical Research Communications 2006; 342(3):979-983.

(28) Waeytens A, De Vos M, Rottiers I, Laukens D, Cuvelier C. Metallothionein expression in ulcerative colitis: inflammation-dependent regulation differs in epithelium and granulation tissue. In preparation 2008.

(29) Jasani B, Campbell F, Navabi H, Schmid KW, Williams GT. Clonal overexpression of metallothionein is induced by somatic mutation in morphologically normal colonic mucosa. Journal of Pathology 1998; 184(2):144-147.

(30) Cook HA, Williams D, Thomas GA. Crypt-restricted metallothionein immunopositivity in murine colon: validation of a model for studies of somatic stem cell mutation. Journal of Pathology 2000; 191(3):306-312.

(31) Bruwer M, Schmid KW, Metz KA, Krieglstein CF, Senninger N, Schurmann G. Increased expression of metallothionein in inflammatory bowel disease. Inflammation Research 2001; 50(6):289-293.

(32) Dooley TP, Curto EV, Reddy SP, Davis RL, Lambert GW, Wilborn TW et al. Regulation of gene expression in inflammatory bowel disease and correlation with IBD drugs: screening by DNA microarrays. Inflammatory Bowel Diseases 2004; 10(1):1-14.

(33) Clarkson JP, Elmes ME, Jasani B, Webb M. Histological demonstration of immunoreactive zinc metallothionein in liver and ileum of rat and man. Histochemical Journal 1985; 17(3):343-352.

(34) Elmes ME, Clarkson JP, Jasani B. The Immunocytochemical Demonstration of Metallothionein in Human-Liver and Small-Intestine. Acta Pharmacologica et Toxicologica 1986; 59:510-513.

(35) Ioachim E, Michael M, Katsanos C, Demou A, Tsianos EV. The immunohistochemical expression of metallothionein in inflammatory bowel disease. Correlation with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices. Histology and Histopathology 2003; 18(1):75-82.

(36) Kruidenier L, Kuiper I, Van Duijn W, Mieremet-Ooms MA, Van Hogezand RA, Lamers CB et al. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. Journal of Pathology 2003; 201(1):17-27.

(37) Mulder TP, Verspaget HW, Janssens AR, de Bruin PA, Pena AS, Lamers CB. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. Gut 1991; 32(10):1146-1150.

(38) Mathew CG, Lewis CM. Genetics of inflammatory bowel disease: progress and prospects. Human Molecular Genetics 2004; 13:R161-R168.

(39) Eckburg PB, Relman DA. The role of microbes in Crohn's disease. Clinical Infectious Diseases 2007; 44(2):256-262.

(40) Waeytens A, Laukens D, Janssens S, Peeters H, Rottiers P, Cuvelier CA et al. Downregulation of metallothioneins (MTS) in intestinal epithelial cells (IECs) is associated with impaired chemoattractant activity. Gastroenterology 2007; 132(4):A236-A237.

(41) Marks DJB, Harbord MWN, MacAllister R, Rahman FZ, Young J, Al-Lazikani B et al. Defective acute inflammation in Crohn's disease: a clinical investigation. Lancet 2006; 367(9511):668-678.

(42) Stefanidou M, Maravelias C, Dona A, Spiliopoulou C. Zinc: a multipurpose trace element. Archives of Toxicology 2006; 80(1):1-9.

(43) Tran CD, Butler RN, Howarth GS, Philcox JC, Rofe AM, Coyle P. Regional distribution and localization of zinc and metallothionein in the intestine of rats fed diets differing in zinc content. Scandinavian Journal of Gastroenterology 1999; 34(7):689-695.

(44) Szczurek EI, Bjornsson CS, Taylor CG. Dietary zinc deficiency and repletion modulate metallothionein immunolocalization and concentration in small intestine and liver of rats. J Nutr 2001; 131(8):2132-2138.

(45) Mulder TP, van dS, V, Verspaget HW, Griffioen G, Pena AS, Janssens AR et al. Effect of oral zinc supplementation on metallothionein and superoxide dismutase concentrations in patients with inflammatory bowel disease. Journal of Gastroenterology and Hepatology 1994; 9(5):472-477.

(46) Janssen AML, Van Duijn W, Oostendorp-van de Ruit MM, Kruidenier L, Bosman CB, Griffioen G et al. Metallothionein in human gastrointestinal cancer. Journal of Pathology 2000; 192(3):293-300.

(47) Jahroudi N, Foster R, Pricehaughey J, Beitel G, Gedamu L. Cell-Type Specific and Differential Regulation of the Human Metallothionein Genes - Correlation with Dna Methylation and Chromatin Structure. Journal of Biological Chemistry 1990; 265(11):6506-6511.

(48) Woo ES, Monks A, Watkins SC, Wang AS, Lazo JS. Diversity of metallothionein content and subcellular localization in the National Cancer Institute tumor panel. Cancer Chemotherapy and Pharmacology 1997; 41(1):61-68.

(49) Tsuji S, Kobayashi H, Uchida Y, Ihara Y, Miyatake T. Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. Embo Journal 1992; 11(13):4843-4850.

(50) Yu WH, Lukiw WJ, Bergeron C, Niznik HB, Fraser PE. Metallothionein III is reduced in Alzheimer's disease. Brain Research 2001; 894(1):37-45.

(51) Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG, Lukiw WJ. Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. Journal of Neuroscience Research 2002; 70(3):462-473.

(52) Erickson JC, Sewell AK, Jensen LT, Winge DR, Palmiter RD. Enhanced neurotrophic activity in Alzheimer's disease cortex is not associated with down-regulation of metallothionein-III (GIF). Brain Research 1994; 649(1-2):297-304.

(53) Duguid JR, Bohmont CW, Liu NG, Tourtellotte WW. Changes in brain gene expression shared by scrapie and Alzheimer disease. Proceedings of the National Academy of Sciences of the United States of America 1989; 86(18):7260-7264.

(54) Adlard PA, West AK, Vickers JC. Increased density of metallothionein I/II-immunopositive cortical glial cells in the early stages of Alzheimer's disease. Neurobiology of Disease 1998; 5(5):349-356.

(55) Zambenedetti P, Giordano R, Zatta P. Metallothioneins are highly expressed in astrocytes and microcapillaries in Alzheimer's disease. Journal of Chemical Neuroanatomy 1998; 15(1):21-26.

(56) Chung RS, Adlard PA, Dittmann J, Vickers JC, Chuah MI, West AK. Neuron-glia communication: metallothionein expression is specifically up-regulated by astrocytes in response to neuronal injury. Journal of Neurochemistry 2004; 88(2):454-461.

(57) Penkowa M. Metallothioneins are multipurpose neuroprotectants during brain pathology. FEBS Journal 2006; 273(9):1857-1870.

(58) Sillevis Smitt PA, Blaauwgeers HGT, Troost D, de Jong JM. Metallothionein Immunoreactivity Is Increased in the Spinal-Cord of Patients with Amyotrophic-Lateral-Sclerosis. Neuroscience Letters 1992; 144(1-2):107-110.

(59) Sillevis Smitt PA, Vanbeek H, Baars AJ, Troost D, Louwerse ES, Kropshermus ACM et al. Increased Metallothionein in the Liver and Kidney of Patients with Amyotrophic-Lateral-Sclerosis. Archives of Neurology 1992; 49(7):721-724.

(60) Sillevis Smitt PA, Mulder TP, Verspaget HW, Blaauwgeers HG, Troost D, de Jong JM. Metallothionein in amyotrophic lateral sclerosis. Biological Signals 1994; 3(4):193-197.

(61) Blaauwgeers HGT, Chand MA, vandenBerg FM, Dejong JMBV, Troost D. Expression of different metallothionein messenger ribonucleic acids in motor cortex, spinal cord and liver from patients with amyotrophic lateral sclerosis. Journal of the Neurological Sciences 1996; 142(1-2):39-44.

(62) Gong YH, Elliott JL. Metallothionein expression is altered in a transgenic murine model of familial amyotrophic lateral sclerosis. Experimental Neurology 2000; 162(1):27-36.

(63) Nagano S, Satoh M, Sumi H, Fujimura H, Tohyama C, Yanagihara T et al. Reduction of metallothioneins promotes the disease expression of familial amyotrophic lateral sclerosis mice in a dose-dependent manner. European Journal of Neuroscience 2001; 13(7):1363-1370.

(64) Morahan JM, Yu B, Trent RJ, Pamphlett R. Screening the metallothionein III gene in sporadic amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders 2005; 6(2):115-117.

(65) Bankier A. Menkes Disease. Journal of Medical Genetics 1995; 32(3):213-215.

(66) Chelly J, Tumer Z, Tonnesen T, Petterson A, Ishikawabrush Y, Tommerup N et al. Isolation of A Candidate Gene for Menkes Disease That Encodes A Potential Heavy-Metal Binding-Protein. Nature Genetics 1993; 3(1):14-19.

(67) Mercer JFB, Livingston J, Hall B, Paynter JA, Begy C, Chandrasekharappa S et al. Isolation of A Partial Candidate Gene for Menkes Disease by Positional Cloning. Nature Genetics 1993; 3(1):20-25.

(68) Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J. Isolation of A Candidate Gene for Menkes Disease and Evidence That It Encodes A Copper-Transporting ATPase. Nature Genetics 1993; 3(1):7-13.

(69) Kodama K, Murata Y. Molecular genetics and pathophysiology of Menkes disease. Pediatrics International 1999; 41(4):430-435.

(70) Hamer DH. Metallothionein Gene-Regulation in Menkes Syndrome. Archives of Dermatology 1987; 123(10):A1384-&.

(71) Garnica AD, Chan WY, Rennert OM. Role of Metallothioneins in Copper Transport in Patients with Menkes Syndrome. Annals of Clinical and Laboratory Science 1978; 8(4):302-309.

(72) Schmidt CJ, Hamer DH, Mcbride OW. Chromosomal Location of Human Metallothionein Genes - Implications for Menkes Disease. Science 1984; 224(4653):1104-1106.

(73) Hoogenraad TU. Paradigm shift in treatment of Wilson's disease: Zinc therapy now treatment of choice. Brain & Development 2006; 28(3):141-146.

(74) Borghesi LA, Lynes MA. Stress proteins as agents of immunological change: some lessons from metallothionein. Cell Stress & Chaperones 1996; 1(2):99-108.

(75) Manuel Y, Thomas Y, Pellegrini O. Metallothionein and tissue damage. IARC Scientific Publications 1992;(118):231-237.

(76) Hoey JG, Garrett SH, Sens MA, Todd JH, Sens DA. Expression of MT-3 mRNA in human kidney, proximal tubule cell cultures, and renal cell carcinoma. Toxicology Letters 1997; 92(2):149-160.

(77) Dutta R, Sens DA, Somji S, Sens MA, Garrett SH. Metallothionein isoform 3 expression inhibits cell growth and increases drug resistance of PC-3 prostate cancer cells. Prostate 2002; 52(2):89-97.

(78) Tate DJ, Miceli MV, Newsome DA. Expression of metallothionein isoforms in human chorioretinal complex. Current Eye Research 2002; 24(1):12-25.

(79) Irie Y, Mori F, Keung WM, Mizushima Y, Wakabayashi K. Expression of neuronal growth inhibitory factor (Metallothionein-III) in the salivary gland. Physiological Research 2004; 53(6):719-723.

(80) Moffatt P, Seguin C. Expression of the gene encoding metallothionein-3 in organs of the reproductive system. Dna and Cell Biology 1998; 17(6):501-510.

(81) Quaife CJ, Findley SD, Erickson JC, Froelick GJ, Kelly EJ, Zambrowicz BP et al. Induction of A New Metallothionein Isoform (Mt-Iv) Occurs During Differentiation of Stratified Squamous Epithelia. Biochemistry 1994; 33(23):7250-7259.

(82) Searle PF, Davison BL, Stuart GW, Wilkie TM, Norstedt G, Palmiter RD. Regulation, Linkage, and Sequence of Mouse Metallothionein-I and Metallothionein-II Genes. Molecular and Cellular Biology 1984; 4(7):1221-1230.

(83) Schmidt CJ, Hamer DH. Cell Specificity and An Effect of Ras on Human Metallothionein Gene-Expression. Proceedings of the National Academy of Sciences of the United States of America 1986; 83(10):3346-3350.

(84) Varshney U, Jahroudi N, Foster R, Gedamu L. Structure, Organization, and Regulation of Human Metallothionein If Gene - Differential and Cell-Type-Specific Expression in Response to Heavy-Metals and Glucocorticoids. Molecular and Cellular Biology 1986; 6(1):26-37.

(85) De Bleser PJ, Hooghe B, Vlieghe D, van Roy F. A distance difference matrix approach to identifying transcription factors that regulate differential gene expression. Genome Biology 2007; 8(5).

(86) Gedamu L, Varshney U, Jahroudi N, Foster R, Shworak NW. Structure and expression of the human metallothionein genes. Experientia Suppl 1987; 52:361-372.

(87) Shworak NW, Oconnor T, Wong NCW, Gedamu L. Distinct TATA Motifs Regulate Differential Expression of Human Metallothionein-I Genes Mt-I(F) and Mt-I(G). Journal of Biological Chemistry 1993; 268(32):24460-24466.

(88) Roy AL, Meisterernst M, Pognonec P, Roeder RG. Cooperative Interaction of An Initiator-Binding Transcription Initiation-Factor and the Helix Loop Helix Activator Usf. Nature 1991; 354(6350):245-248.

(89) Dalton T, Palmiter RD, Andrews GK. Transcriptional Induction of the Mouse Metallothionein-I Gene in Hydrogen Peroxide-Treated Hepa Cells Involves A Composite Major Late Transcription Factor Antioxidant Response Element and Metal Response Promoter Elements. Nucleic Acids Research 1994; 22(23):5016-5023.

(90) Ghoshal K, Jacob ST. Regulation of metallothionein gene expression. Progress in Nucleic Acid Research and Molelucar Biology 2001; 66:357-384.

(91) Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR et al. Cloning, Chromosomal Mapping and Characterization of the Human Metal-Regulatory Transcription Factor Mtf-1. Nucleic Acids Research 1994; 22(15):3167-3173.

(92) Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M, Schaffner W. The Transcription Factor Mtf-1 Is Essential for Basal and Heavy Metal-Induced Metallothionein Gene-Expression. Embo Journal 1994; 13(12):2870-2875.

(93) Jacob C, Maret W, Vallee BL. Control of zinc transfer between thionein, metallothionein, and zinc proteins. Proceedings of the National Academy of Sciences of the United States of America 1998; 95(7):3489-3494.

(94) Liu ZM, Chen GG, Shum CKY, Vlantis AC, Cherlan MG, Koropatnick J et al. Induction of functional MT1 and MT2 isoforms by calcium in anaplastic thyroid carcinoma cells. FEBS Letters 2007; 581(13):2465-2472.

(95) Saydam N, Adams TK, Steiner F, Schaffner W, Freedman JH. Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1 - Identification of signal transduction cascades that control metal-inducible transcription. Journal of Biological Chemistry 2002; 277(23):20438-20445.

(96) Karin M, Herschman HR. Dexamethasone Stimulation of Metallothionein Synthesis in Hela-Cell Cultures. Science 1979; 204(4389):176-177.

(97) Jacob ST, Ghoshal K, Sheridan JF. Induction of metallothionein by stress and its molecular mechanisms. Gene Expression 1999; 7(4-6):301-310.

(98) Kelly EJ, Sandgren EP, Brinster RL, Palmiter RD. A pair of adjacent glucocorticoid response elements regulate expression of two mouse metallothionein genes. Proceedings of the National Academy of Sciences of the United States of America 1997; 94(19):10045-10050.

(99) De SK, McMaster MT, Andrews GK. Endotoxin induction of murine metallothionein gene expression. Journal of Biological Chemistry 1990; 265(25):15267-15274.

(100) Coto JA, Hadden EM, Sauro M, Zorn N, Hadden JW. Interleukin-1 Regulates Secretion of Zinc-Thymulin by Human Thymic Epithelial-Cells and Its Action on Lymphocyte-T Proliferation and Nuclear-Protein Kinase-C. Proceedings of the National Academy of Sciences of the United States of America 1992; 89(16):7752-7756.

(101) Schroeder JJ, Cousins RJ. Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proceedings of the National Academy of Sciences of the United States of America 1990; 87(8):3137-3141.

(102) Kasutani K, Itoh N, Kanekiyo M, Muto N, Tanaka K. Requirement for cooperative interaction of interleukin-6 responsive element type 2 and glucocorticoid responsive element in the synergistic activation of mouse metallothionein-1 gene by interleukin-6 and glucocorticoid. Toxicology and Applied Pharmacology 1998; 151(1):143-151.

(103) Kanekiyo M, Itoh N, Kawasaki A, Matsuyama A, Matsuda K, Nakanishi T et al. Metallothionein modulates lipopolysaccharide-stimulated tumour necrosis factor expression in mouse peritoneal macrophages. Biochemical Journal 2002; 361(Pt 2):363-369.

(104) Inoue K, Takano H, Shimada A, Wada E, Yanagisawa R, Sakurai M et al. Role of metallothionein in coagulatory disturbance and systemic inflammation induced by lipopolysaccharide in mice. Faseb Journal 2005; 19(14):533-+.

(105) Nagel WW, Vallee BL. Cell-Cycle Regulation of Metallothionein in Human Colonic-Cancer Cells. Proceedings of the National Academy of Sciences of the United States of America 1995; 92(2):579-583.

(106) Studer R, Vogt CP, Cavigelli M, Hunziker PE, Kagi JHR. Metallothionein accretion in human hepatic cells is linked to cellular proliferation. Biochemical Journal 1997; 328:63-67.

(107) Haerslev T, Jacobsen GK, Horn N, Damsgaard E. Metallothionein Expression in Placental Tissue in Menkes Disease - An Immunohistochemical Study. Apmis 1995; 103(7-8):568-573.

(108) Cherian MG, Apostolova MD. Nuclear localization of metallothionein during cell proliferation and differentiation. Cellular and Molecular Biology 2000; 46(2):347-356.

(109) Miyashita H, Sato Y. Metallothionein 1 is a downstream target of vascular endothelial zinc finger 1 (VEZF1) in endothelial cells and participates in the regulation of angiogenesis. Endothelium-Journal of Endothelial Cell Research 2005; 12(4):163-170.

(110) Takeda A, Hisada H, Okada S, Mata JE, Ebadi M, Iversen PL. Tumor cell growth is inhibited by suppressing metallothionein-I synthesis. Cancer Letters 1997; 116(2):145-149.

(111) Hesketh J. 3 '-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. Biochemical Society Transactions 2004; 32:990-993.

(112) Nury D, Chabanon H, Levadoux-Martin M, Hesketh J. An eleven nucleotide section of the 3 '-untranslated region is required for perinuclear localization of rat metallothionein-1 mRNA. Biochemical Journal 2005; 387:419-428.

(113) Helin K, Wu CL, Fattaey AR, Lees JA, Dynlacht BD, Ngwu C et al. Heterodimerization of the Transcription Factors E2F-1 and Dp-1 Leads to Cooperative Transactivation. Genes & Development 1993; 7(10):1850-1861.

(114) Meplan C, Mann K, Hainaut P. Cadmium induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. Journal of Biological Chemistry 1999; 274(44):31663-31670.

(115) Andrews GK, Adamson ED, Gedamu L. The Ontogeny of Expression of Murine Metallothionein - Comparison with the Alpha-Fetoprotein Gene. Developmental Biology 1984; 103(2):294-303.

(116) Andrews GK, Lee DK, Ravindra R, Lichtlen P, Sirito M, Sawadogo M et al. The transcription factors MTF-1 and USF1 cooperate to regulate mouse metallothionein-1 expression in response to the essential metal zinc in visceral endoderm cells during early development. Embo Journal 2001; 20(5):1114-1122.

(117) Majumder S, Ghoshal K, Gronostajski RM, Jacob ST. Downregulation of constitutive and heavy metal-induced metallothionein-I expression by nuclear factor I. Gene Expression 2001; 9(4-5):203-215.

(118) Datta PK, Jacob ST. Identification of a sequence within the mouse metallothionein-I gene promoter mediating its basal transcription and of a protein interacting with this element. Cellular & Molecular Biology Research 1993; 39(5):439-449.

(119) Tang CM, Westling J, Seto E. trans repression of the human metallothionein IIA gene promoter by PZ120, a novel 120-kilodalton zinc finger protein. Molecular and Cellular Biology 1999; 19(1):680-689.

(120) Kageyama R, Pastan I. Molecular-Cloning and Characterization of A Human Dna-Binding Factor That Represses Transcription. Cell 1989; 59(5):815-825.

(121) Majumder S, Ghoshal K, Summers D, Bai SM, Datta J, Jacob ST. Chromium(VI) down-regulates heavy metal-induced metallothionein gene transcription by modifying transactivation potential of the key transcription factor, metal-responsive transcription factor 1. Journal of Biological Chemistry 2003; 278(28):26216-26226.

(122) Mccormick CC, Salati LM, Goodridge AG. Abundance of Hepatic Metallothionein Messenger-Rna Is Increased by Protein-Synthesis Inhibitors - Evidence for Transcriptional Activation and Posttranscriptional Regulation. Biochemical Journal 1991; 273:185-188.

(123) Bi YY, Lin GX, Millecchia L, Ma Q. Superinduction of metallothionein I by inhibition of protein synthesis: Role of a labile repressor in MTF-1 mediated gene transcription. Journal of Biochemical and Molecular Toxicology 2006; 20(2):57-68.

(124) Heguy A, West A, Richards RI, Karin M. Structure and Tissue-Specific Expression of the Human Metallothionein-Ib Gene. Molecular and Cellular Biology 1986; 6(6):2149-2157.

(125) Deng DJ, El Rifai W, Ji JF, Zhu BD, Trampont P, Li JY et al. Hypermethylation of metallothionein-3 CpG island in gastric carcinoma. Carcinogenesis 2003; 24(1):25-29.

(126) Smith E, Drew PA, Tian ZQ, De Young NJ, Liu JF, Mayne GC et al. Metallothionien 3 expression is frequently down-regulated in oesophageal squamous cell carcinoma by DNA methylation. Molecular Cancer 2005; 4.

(127) Lansdown ABG. Metallothioneins: potential therapeutic aids for wound healing in the skin. Wound Repair and Regeneration 2002; 10(3):130-132.

(128) Youn J, Hwang SH, Ryoo ZY, Lynes MA, Paik DJ, Chung HS et al. Metallothionein suppresses collagen-induced arthritis via induction of TGF-beta and down-regulation of proinflammatory mediators. Clinical and Experimental Immunology 2002; 129(2):232-239.

(129) Chung RS, Hidalgo J, West AK. New insight into the molecular pathways of metallothionein-mediated neuroprotection and regeneration. Journal of Neurochemistry 2008; 104(1):14-20.

(130) Li XK, Cai L, Feng WK. Diabetes and metallothionein. Mini-Reviews in Medicinal Chemistry 2007; 7(7):761-768.

Chapter 5

general discussion and future perspectives

Inflammatory bowel diseases (IBD), comprising Crohn's disease and ulcerative colitis, are characterised by chronic, non-specific inflammation of the gastrointestinal tract. Conventional therapy for IBD mostly entails lifelong treatment with systemically administered medications, which exhibit possible side effects. Much research has been conducted on the pathogenesis of IBD in order to design new therapeutic strategies (**chapter 1**). Although a clear involvement of environmental triggers exists, especially bacteria, the most important progress has been made in the field of genetic risk factors. During the last few years, polymorphisms in several genes have been associated with IBD, especially with Crohn's disease where the genetic component is the strongest. The relevance of most of these polymorphisms is just starting to be explored, but has already provided a critical turn of the focus on IBD research.

For years, studies have been concentrating on the role of the adaptive immune system in the pathogenesis of both Crohn's disease and ulcerative colitis. However, with the identification of *NOD2/CARD15* as the first susceptibility gene for Crohn's disease, much interest has been dedicated to the role of the innate immune system. More recently discovered polymorphisms highlight the importance of innate immunity in the dysregulated host-bacterial interactions implicated in the pathogenesis of IBD. In this regard, two theories have evolved. The first one states that an exaggerated innate response and a loss of tolerance for the commensal intestinal microflora are caused by defective down-regulation of pattern recognition receptor (PRR) signalling. The second theory encompasses a defective innate immunity originating from failure of recognition of bacterial threats or an ineffective response, which results in persistence of the exogenous stimulus and an exaggerated secondary immune response (1). In the innate immune system, the intestinal epithelial cell lining has received more and more interest as it became clear that these cells exert an important role in the identification of bacteria and the appropriate counteraction. Furthermore, the epithelium must also maintain a physical barrier between the lumen and the underlying mucosa. Defects in the mucus layer, integrity, and permeability are found in IBD and are thought to have direct implications on disease initiation and/or progress. Many of these findings emanate from animal models of IBD that, although never representing human disease entirely, can shed light on several aspects of the disease and thus help to uncover its pathogenesis, piece by piece.

The research that was conducted in this thesis focussed on two topics that were related to the defective mucosal barrier in IBD. The first topic focussed on the mechanism of action of therapeutic interleukin (IL)-10 producing bacteria related to their interaction with the

damaged barrier. In the second part, the metallothioneins, which can be considered immunomodulatory proteins, were studied in different human and animal inflammatory conditions.

Interaction with therapeutic bacteria

Genetically modified *Lactococcus lactis* bacteria secreting IL-10 were developed in order to provide a local mucosal delivery of IL-10. The principal function of this cytokine is to contain and eventually terminate inflammatory responses by suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, and antigen-presenting and co-stimulatory molecules in monocytes/macrophages, dendritic cells, neutrophils, and T cells (2). The fact that IL-10 is an essential immune modulator in the intestinal tract was supported by the observation that IL-10-deficient mice spontaneously develop a chronic enterocolitis, histologically characterised by infiltration with inflammatory cells, mucus cell depletion, and architectural crypt distortion (3). Studies in animal models of IBD showed that systemic IL-10 administration was successful in preventing intestinal inflammation and established the base of its use as a therapeutic agent in IBD (4-6). However, these positive results could not be achieved in human trials (7-9). This was likely due to the low bioavailability of injected IL-10 in the gastrointestinal tissues and in its short half-life in the circulatory system. Although safe and well-tolerated at multiple tested dosages (10), increasing the dose of systemically administered IL-10 was not possible because of side effects probably mediated by interferon-γ induction (11;12). Attempts to provide a local mucosal delivery of IL-10, including gene transfer (6;13), gelatine microspheres (14), and active synthesis *in situ* by *Lactococcus lactis* bacteria (15), proved to be more efficacious. Most advances have been made with the last approach, which is currently the subject of further development and commercialisation in the VIB-UGent spin-off company ActoGeniX as its lead product AG011. After being successfully tested in a phase 1 clinical trial with Crohn´s disease patients (16), clinical-grade AG011 has recently been produced and formulated. ActoGeniX has initiated a multicentre phase 2A study in ulcerative colitis patients in Europe and North America and also plans a phase 2 study with the same product in Crohn´s disease patients in 2009 (17;18).

The exact mechanism by which IL-10-producing *L. lactis* mediates a beneficial response on the mucosa is mostly unknown. Since local delivery of the cytokine in the tissue seems primordial, we focused on the *in vivo* interaction of the bacteria with intestinal murine mucosa and observed differences between an intact and an inflamed (damaged) epithelial barrier (**chapter 3, figure 1**). In healthy intestinal tissue, transmission electron microscopy showed an occasional transcellular uptake in follicle-associated epithelium, similar to the recently

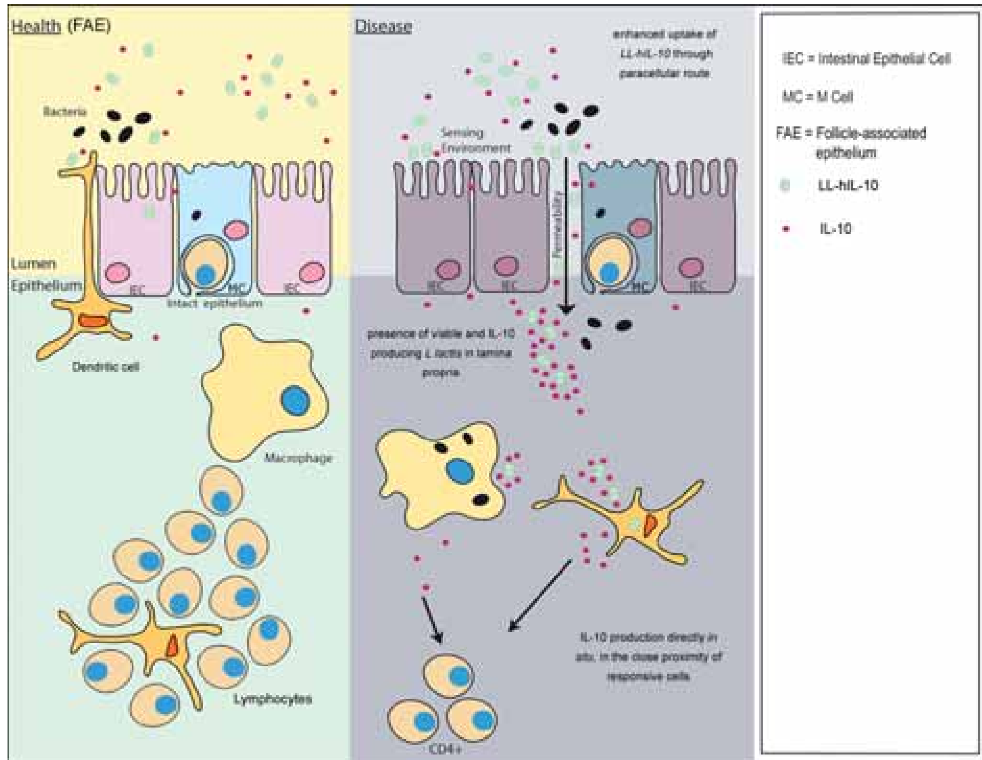


Figure 1. Interaction of the mucosal barrier with therapeutic bacteria. (adapted and adjusted from (19))

In healthy intestinal tissue, IL-10 producing *L. lactis* only occasionally enter the mucosa through transcellular uptake in follicle-associated epithelium and possibly through dendritic cell sampling (left). In inflamed mucosal tissue, the damaged mucosal barrier causes an enhanced uptake of bacteria, resulting in the presence of viable and IL-10 producing *L. lactis* in the lamina propria, close to responsive immune cells (right). IL-10-producing *L. lactis* probably modulate $CD4^+$ T cell function indirectly via down-regulatory effects on DCs.

reported uptake of non-pathogenic bacteria (20). We also recognised transepithelial dendrites close to luminal lactococci, suggestive of dendritic cell (DC) sampling (21), but we found no arguments for sampling by M cells. In inflamed mucosal tissue, both confocal and electron microscopic analysis suggested an enhanced uptake of lactococci through the paracellular route leading to an accumulation in the lamina propria. This enhanced uptake is likely to be a result of the damaged mucosal barrier, a feature that, as pointed out earlier (**chapter 1**), is also characteristic of human IBD. We were able to show the presence of viable and IL-10 producing lactococci in ileal and colonic tissue of which the epithelial layer was removed, thus providing evidence that these bacteria are capable of entering inflamed intestinal mucosa and delivering IL-10 in the lamina propria. The absence of lactococci in

mesenteric lymph nodes or in the spleen exclude a systemic circulation of the bacteria, being safe for administration in patients.

The results of our study correspond with earlier studies that detected small amounts of IL-10 in intestinal tissue homogenates (15). No IL-10 was detected in the lumen in those studies. Together with arguments that the bacterial context of IL-10 presentation seems necessary for the therapeutic action of the IL-10 producing lactococci (16), our data prompted us to formulate the hypothesis that an important source of the IL-10 acting as a therapeutic on the mucosa is produced by those bacteria that have entered the nutrient-rich mucosa. The production of IL-10 in the lumen followed by diffusion to responsive cells in the epithelium or the lamina propria may not be excluded as a mechanism, but the effect of the latter may not be as considerable as IL-10 production directly *in situ* in intestinal tissue, in the close proximity of responsive cells.

Future perspectives

Our study only addresses a small aspect of the therapeutic mechanism of IL-10-producing *L. lactis*. Further investigation on the downstream targets of IL-10 in the mucosa is/was carried out by the group that designed the bacteria. They found that DCs that were exposed to IL-10 producing *L. lactis* and maturation factors could strongly suppress the proliferation of responder $CD4^{+}$ T cells (22). Therefore, it is reasonable to presume that However, other cellular targets may not be excluded. It is well-known that IL-10 suppresses the expression of proinflammatory cytokines and chemokines in monocytes/macrophages and neutrophils as well. Furthermore, IL-10 has direct effects on T cells through its regulatory functions, down-regulating antigen-specificity in T-effector responses (2). All these pathways could lead to the down-regulation of an intestinal proinflammatory T-cell response, which was observed in experimental colitis (4-6).

Roles for metallothioneins as immunomodulators (figure 2)

Genetically-determined abnormalities at the mucosal level are a critical hallmark of gut inflammation in IBD, particularly in Crohn's disease. By integration of transcriptome analysis of unaffected colon biopsies with previously-documented susceptibility loci, we identified 18 potential new candidate genes for Crohn's disease (**chapter 4.1**). Two of these genes, *MT1F* and *MT1M*, belong to the metallothioneins (MTs). With attributed antioxidant and antibacterial functions and as keepers of zinc homeostasis, this family of highly-conserved proteins promised to be possibly relevant in the pathogenesis of Crohn's disease. Although some studies have already examined the expression of MTs in IBD over the last 20 years, no

consensus has been reached on their expression pattern. The majority of studies report a down-regulation in either Crohn's disease, ulcerative colitis, or both (23-28), but others described elevated MT levels (29;30). Furthermore, all of these studies used affected tissue, from mildly to severely inflamed, and as such give no information about whether aberrant MT expression is a primary, aetiopathogenic event, or an inflammation-dependent event.

In order to clarify these two issues, we first studied both mRNA and protein expression of MTs in Crohn's disease in detail. We performed a detailed analysis of the inducible mRNA expression of MT1 and MT2 isoforms on endoscopically normal colon biopsies and blood leukocytes of patients with Crohn's disease and healthy controls. We found a significantly lower expression of MT1M in Crohn's disease colon tissue and of MT1F in Crohn's disease blood leukocytes, predominantly in patients with colonic disease involvement. The significant correlation between the expression of MT1M mRNA and the other isoforms tested implies that the inducible MTs are basally down-regulated in Crohn's disease patients as a group. This makes the chance that polymorphisms are the cause of MT down-regulation unlikely, since finding polymorphisms in all of these MT genes is not likely. Furthermore, we found no defective MT induction in peripheral blood mononuclear cells from Crohn's disease patients. This suggests the involvement of an upstream regulator of MT expression, which might be found in the presence of a transcription factor since the promoter region of MT isoforms is duplicated during evolution. The unaffected MT expression in other cell types, such as fibroblasts and macrophages (see next), suggests that this upstream regulator acts differently in different cell types.

An immunohistochemical study of ileal and colonic tissue samples demonstrated a reduced expression of MT in epithelium of unaffected Crohn's disease samples as compared to normal controls. To study the functional consequences of this reduction, we created an MT-deficient HT-29 colonic cell line with small interfering RNA. Challenges with invasive bacteria resulted in a significantly lower secretion of IL-8, a potent chemokine for neutrophils, compared with control cells. This observation is in accordance with the recent finding that IL-8 up-regulation is impaired in response to acute trauma to colon and skin of Crohn's disease patients (31). These results suggest that a decreased MT expression causes disturbed cell migration, which could lead to a defective acute inflammatory response in Crohn's disease. We have preliminary data showing that a decreased epithelial MT level is also associated with the decreased secretion of another chemokine (granulocyte-macrophage colony-stimulating factor) and with impaired chemoattraction of neutrophils (32).

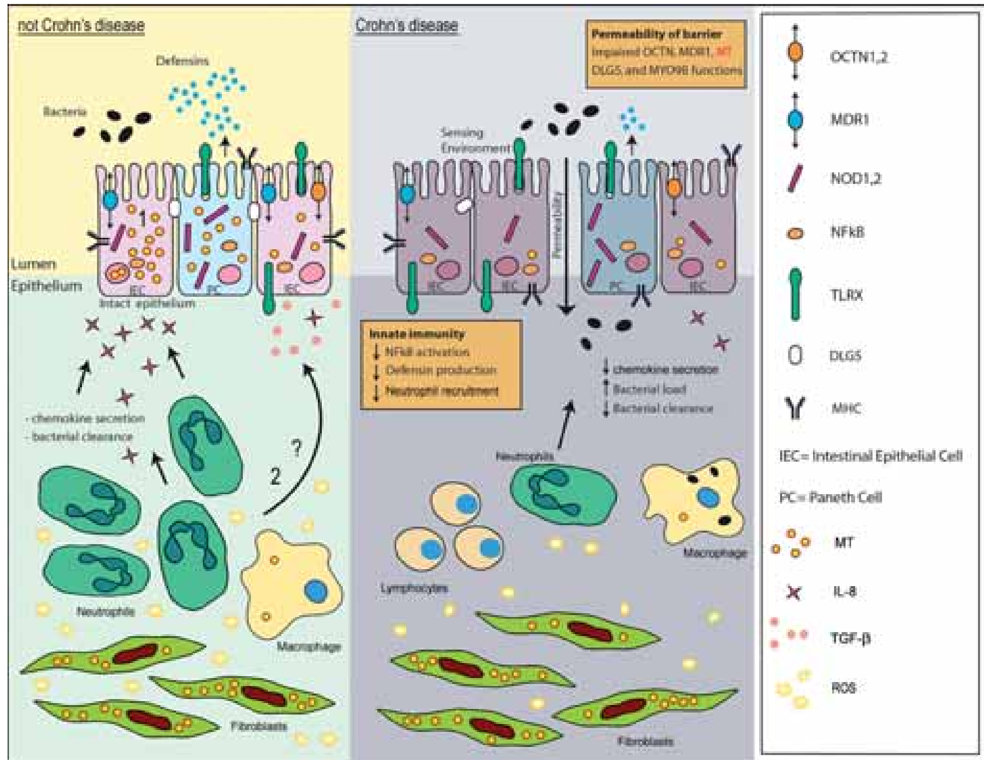


Figure 2. Roles for metallothioneins as immunomodulators in intestinal inflammation. (adapted and adjusted from (19))

The intestinal mucosa of a patient not suffering from Crohn's disease (left) shows a moderate to high expression of MTs in the epithelium (1). Epithelial MT might have an antibacterial function or might be involved in the chemotaxis of neutrophils. During the course of inflammation, MT-positive fibroblasts appear in the lamina propria, where MT possibly protects the cells against the oxidative inflammatory environment. An inflammation-dependent decrease in epithelial MT is observed, which could be mediated by TGF-β, found to be increased in inflammatory cells near the epithelium in actively inflamed mucosa (2). The intestinal mucosal barrier of a Crohn's disease patient (right) is found to be damaged as a result of multiple defects (discussed in chapter 1). Deficient epithelial MT expression can be added to these defects. This deficient expression could result in a reduced antibacterial function or a reduced chemotaxis-modulating function of MTs, further impairing bacterial clearance and the acute immune response. These defects in innate immunity can result in activation and accumulation of pathogenic $CD4^+$ T cells that mediate chronic intestinal inflammation.

Further evidence for the involvement of inducible MTs in the acute immune response was found in *in vivo* experiments with MTnull mice, which lack MT1 and MT2 expression. A time course study of DSS-induced colitis taught us that MTnull mice showed a significantly more severe histological inflammation in the early phase of colitis compared with wild type mice. We attribute this difference to epithelial MT, since colonic MT expression is restricted to and even mildly up-regulated in the epithelium the first days after the start of colitis induction.

Thus, we hypothesize that MTs have an immunomodulatory role in the early intestinal immune response.

The down-regulation in the epithelium observed later in the course of experimental colitis, when the inflammatory infiltrate and the granulation tissue appeared, was also noticed in human samples of ulcerative colitis, acute infectious enterocolitis, diverticulitis, and ischaemic intestinal inflammation (**chapter 4.2**). In those samples, as opposed to samples of Crohn's disease patients, histologically normal regions showed an epithelial MT expression comparable with normal control tissue. This expression was highest in the ileum and in epithelium of crypts and the base of the villi, which are the regions where antibacterial properties are most critical. Therefore, a possible function of MTs in epithelial cells might be related to their antibacterial properties (33). Support for a non-cell-cycle related function is the fact that this MT expression pattern did not correlate with proliferation.

The inflammation-dependent down-regulation in the epithelium is probably a reflection of the different events occurring during an immune response. It is, however, surprising that (epithelial) MTs are *down*-regulated during inflammation, since it is known that proinflammatory cytokines and reactive oxygen intermediates, which are produced during inflammation, rapidly induce MT expression (34). We found that transforming growth factor (TGF)-β, one of the few inhibitors identified for MTs (35), could decrease epithelial MT expression in a dose-dependent manner in *ex vivo* experiments. TGF-β is a good candidate for epithelial regulation in inflammation, since this pleiotropic cytokine was increased in affected mucosa from patients with active intestinal inflammation. The highest concentration was found in inflammatory cells near epithelial cells (36). Furthermore, epithelial cells bear the TGF-β receptor (37).

Contrary to the decrease in the epithelium, an increase in MT-expressing cells occurs in the lamina propria during inflammation. While in normal conditions and during inflammation some mildly positive macrophages are present in the lamina propria, the inflammation-related increase is predominantly caused by strongly MT-expressing fibroblasts of the granulation tissue. The granulation tissue consists at least partially of activated myofibroblasts. Double stainings with the myofibroblast marker SMA showed that the majority of MT positive spindle cells were negative for this marker. These MT positive fibroblasts probably present a subset of this cell type which is known to have different possible phenotypes during physiologic and pathologic conditions (38). MT positive fibroblasts have also been noticed by other research groups in human chronic gastric and small intestinal ulcers (39). The allocated functions for MTs in these cells were either a

cytoprotective or a proliferation-dependent role. Since we did not find a correlation with proliferation in our study, we are inclined towards a cytoprotective role that would be beneficial for host cells in the inflammatory environment which is rich in free radicals.

Taken together, the results presented in chapters 4.1 and 4.2 reveal two major findings. First, a genetically-determined impaired epithelial expression of MTs could provide a possible aetiopathogenic mechanism in Crohn's disease. Second, MT expression is differentially regulated in intestinal epithelium and granulation tissue during a 'normal' inflammatory reaction. In **chapter 4.3**, we reviewed the regulation of MT expression and gave an overview of known aberrant MT expression in diseases. The existence of several MT isoforms complicates MT research, especially on the protein level since these different isoforms are indistinguishable by the antibodies currently available. Their high homology makes it even hard to design specific primers or probes for the detection of different mRNA isoforms. Nevertheless, it is at this level that differential expression and regulation of MT isoforms are recently being elucidated. Metallothioneins are induced by many cytokines, metals, and hormones, and their promoters contain several regulatory sequences. We performed an *in silico* analysis of transcription factor binding sites of the mouse and human MT1 promotors. Based on these common regulatory sites, we reviewed the MT regulatory mechanisms.

As noticed earlier, the down-regulation of several MT isoforms indicate that an upstream regulator of MT expression is responsible for the impaired expression in Crohn's disease. Hence, transcription factors of MTs are good candidates to study in this disease. Actually, our group has preliminary data that a polymorphism in the MTF1 gene, located within the susceptibility locus *IBD7* and coding for an important transcription factor of MTs, is associated with disease location in Crohn's disease. Furthermore, this polymorphism showed less transactivation potential in an *in vitro* reporter assay (40).

Future perspectives

Revealing the cell-type specific regulation of MT expression in the inflammatory course and in Crohn's disease will be an interesting challenge for our future research. First of all, further analysis of MTF1 and other upstream regulators of MT expression will hopefully reveal the cause of genetically-determined impairment of epithelial MT expression in Crohn's disease. Examination of the methylation status of the MT promoters is an alternative mechanism we will investigate, given the appearance of tissue specific MT methylation and the hereditary properties of these modifications. On the other hand, we will attempt to find the regulator that causes the inflammation-dependent epithelial MT decrease. A parallel inflammation-dependent decrease of a transcription factor (such as MTF1) might be a possible

mechanism. Recognized inhibitors of MT induction are possible candidates, but this regulator may presently be unknown to influence MT expression. Besides further exploring the role of TGF-β, we will test other regulatory cytokines or molecules that are expressed during inflammation. Especially those that are known to be expressed near or by the epithelium are attractive. Parallel induction studies in fibroblasts will have to be carried out to find out whether the regulation of MTs is cell-type specific or the reflection of a site-specific distribution of MT regulator(s) in the inflamed mucosa. In addition, examining MT expression in inflammatory disorders in other organs might shed more light on their regulation in inflammation in general. It would be interesting to know if the pattern we observed is specific for the intestine (which harbours numerous bacteria in its lumen) or not. For this purpose, immunohistochemical stainings for patient groups with inflammatory disorders in other organs will be repeated, and if a group shows inflammation-dependent MT immunoreactivity, we could then try to induce a corresponding experimental model in MTnull mice.

A weakness in the immunohistochemical studies is that we cannot with full certainty state that the immunoreactivity of the antibody used is a reliable measure for MT protein expression, given the possibility that the immunodominant epitope of MT is destroyed by oxidation in the local oxidative/inflammatory environment. We will test the susceptibility of the epitope to oxidation by subjecting recombinant MT protein to oxidative reagents and subsequently analyzing antibody-binding in ELISA.

The function of MTs in the epithelium and in fibroblasts is very intriguing. While the normal high expression of MTs in ileal epithelium might reflect a cytoprotective/antibacterial role in this part of the intestine that tries to keep bacterial numbers low as opposed to the colon, these same cytoprotective functions should provide a benefit in an inflammatory environment, and thus an up-regulation is expected. The observed down-regulation insinuates that epithelial MTs could possess a different function. Again, this function might be explained by the observed influence of the epithelial MT level on the secretion of IL-8 (and maybe other chemokines). In the acute anti-bacterial response, a quick and sufficient recruitment of innate immune cells, especially neutrophils, is necessary. Later on, the immune response is down-regulated and thus a decrease in MTs could help to reduce the chemoattractant activity of the epithelium and the influx of neutrophils. The involvement of MTs in chemotaxis will be further elaborated *in vivo* in an infectious colitis model. The expression of MTs will be examined by immunohistochemistry and quantitative PCR during the course of the infection-induced inflammation. We will assess disease activity, histological inflammation, bacterial colonization, neutrophil activity, and chemokine production in MTnull mice and compare these parameters with those of wild type mice. We will also try to

establish the role of MTs in fibroblasts. Although a cytoprotective role seems obvious in these cells, studies on the relation between the MT-level and a possible anti-oxidant capacity of fibroblasts are necessary to prove this hypothesis. Furthermore, we should examine whether different MT levels could have effects on the chemokine release of fibroblasts. We will study chemokine secretion of both epithelial cells and fibroblasts upon stimulation *ex vivo* in isolated intestinal epithelial cells and fibroblasts of MTnull and WT mice. The ultimate model to study the role of MTs in these different cell types would be conditional and tissue-specific gene-knockdown mice, which we could then subject to experimental colitis.

The mechanism of the modulating effects of MTs on IL-8 secretion is an interesting issue and certainly material for future investigations. Could it be found in the anti-oxidative and/or zinc-binding capacities of MTs? By quickly modulating small changes in the concentrations of reactive oxygen species or zinc levels in the epithelial cells, MTs could serve as immunomodulators in intestinal inflammation. Small, non-toxic concentrations of reactive oxygen species have been found to act as second messengers on e.g. NF-κB, a central immune modulator (41). The first way to investigate this is to search for a correlation with localized increased oxidation, i.e. via staining for malondialdehyde (MDA, reflecting lipid peroxidation), and nitrotyrosine (NITT, reflecting protein tyrosine nitration). This will be performed on serial sections of the samples used for the immunohistochemical study on human intestinal inflammation and on samples of the DSS model. A greater challenge will be to try to correlate inflammation-dependent MT expression in intestinal epithelial cells with the activity of NF-κB, given the complex composition of this transcription factor. An indication of this activity might be its nuclear localization and DNA binding. This could be checked in stimulated MT-deficient cells *versus* stimulated siRNA control cells. Next to modulating reactive oxygen species concentrations, the purpose for down-regulation might also be the release of zinc. Zinc is a structural and/or catalytic element in over 300 enzymes and in that way is strongly implicated in metabolic regulation (42). Zinc indicators exist that can measure intracellular free zinc concentrations in the range of 1 to 100nm (Molecular Probes, Invitrogen, Carlsbad, CA). Of course, all of these speculations need thorough investigation, but could form the basis of future research objectives.

References

(1) Comalada M, Peppelenbosch MP. Impaired innate immunity in Crohn's disease. Trends in Molecular Medicine 2006; 12(9):397-399.

(2) Moore KW, de Waal MR, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annual Review of Immunology 2001; 19:683-765.

(3) Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993; 75(2):263-274.

(4) Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity 1994; 1(7):553-562.

(5) Herfarth HH, Mohanty SP, Rath HC, Tonkonogy S, Sartor RB. Interleukin 10 suppresses experimental chronic, granulomatous inflammation induced by bacterial cell wall polymers. Gut 1996; 39(96):836-845.

(6) Barbara G, Xing Z, Hogaboam CM, Gauldie J, Collins SM. Interleukin 10 gene transfer prevents experimental colitis in rats. Gut 2000; 46(3):344-349.

(7) Fedorak RN, Gangl A, Elson CO, Rutgeerts P, Schreiber S, Wild G et al. Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. Gastroenterology 2000; 119(6):1473-1482.

(8) Schreiber S, Fedorak RN, Nielsen OH, Wild G, Williams CN, Nikolaus S et al. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. Gastroenterology 2000; 119(6):1461-1472.

(9) Colombel JF, Rutgeerts P, Malchow H, Jacyna M, Nielsen OH, Rask-Madsen J et al. Interleukin 10 (Tenovil) in the prevention of postoperative recurrence of Crohn's disease. Gut 2001; 49(1):42-46.

(10) van Deventer SJ, Elson CO, Fedorak RN. Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. Gastroenterology 1997; 113(2):383-389.

(11) Lauw FN, Pajkrt D, Hack CE, Kurimoto M, van Deventer SJ, van der PT. Proinflammatory effects of IL-10 during human endotoxemia. Journal of Immunology 2000; 165(5):2783-2789.

(12) Tilg H, Van Montfrans C, van den Ende A, Kaser A, van Deventer SJH, Schreiber S et al. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. Gut 2002; 50(2):191-195.

(13) Lindsay JO, Ciesielski CJ, Scheinin T, Brennan FM, Hodgson HJ. Local delivery of adenoviral vectors encoding murine interleukin 10 induces colonic interleukin 10 production and is therapeutic for murine colitis. Gut 2003; 52(7):981-987.

(14) Nakase H, Okazaki K, Tabata Y, Ozeki M, Watanabe N, Ohana M et al. New cytokine delivery system using gelatin microspheres containing interleukin-10 for experimental inflammatory bowel disease. Journal of Pharmacology and Experimental Therapeutics 2002; 301(1):59-65.

(15) Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 2000; 289(5483):1352-1355.

(16) Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP et al. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clinical gastroenterology and hepatology 2006; 4(6):754-759.

(17) ActoGeniX. Press release 14-5-2008. http://www.actogenix.com/eng/documents/news/ActoGeniX%20Press%20Release%2014%20May%202008%20ENG_final.pdf. 2008.

(18) ActoGeniX. Press release 11-9-2008. http://www.actogenix.com/eng/documents/news/ActoGeniX%20Press%20Release_11Sept2008_E.pdf. 2008.

(19) Goyette P, Labbe C, Trinh TT, Xavier RJ, Rioux JD. Molecular pathogenesis of inflammatory bowel disease: Genotypes, phenotypes and personalized medicine. Annals of Medicine 2007; 39(3):177-199.

(20) Keita AV, Gullberg E, Ericson AC, Salim SY, Wallon C, Kald A et al. Characterization of antigen and bacterial transport in the follicle-associated epithelium of human ileum. Laboratory Investigation 2006; 86(5):504-516.

(21) Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nature Immunology 2001; 2(4):361-367.

(22) Braat H, Steidler L, Neirynck S, Kapsenberg ML, van Deventer SJH, de Jong EC et al. Turbo-probiotics as a tool for cell-based (mucosal) delivery of interleukin-10. Gastroenterology 2005; 128(4):A5.

(23) Clarkson JP, Elmes ME, Jasani B, Webb M. Histological demonstration of immunoreactive zinc metallothionein in liver and ileum of rat and man. Histochemical Journal 1985; 17(3):343-352.

(24) Elmes ME, Clarkson JP, Jasani B. Histological demonstration of immunoreactive metallothionein in rat and human tissues. Experientia Suppl 1987; 52:533-537.

(25) Mulder TP, Verspaget HW, Janssens AR, de Bruin PA, Pena AS, Lamers CB. Decrease in two intestinal copper/zinc containing proteins with antioxidant function in inflammatory bowel disease. Gut 1991; 32(10):1146-1150.

(26) Sturniolo GC, Mestriner C, Lecis PE, D'Odorico A, Venturi C, Irato P et al. Altered plasma and mucosal concentrations of trace elements and antioxidants in active ulcerative colitis. Scandinavian Journal of Gastroenterology 1998; 33(6):644-649.

(27) Ioachim E, Michael M, Katsanos C, Demou A, Tsianos EV. The immunohistochemical expression of metallothionein in inflammatory bowel disease. Correlation with HLA-DR antigen expression, lymphocyte subpopulations and proliferation-associated indices. Histology and Histopathology 2003; 18(1):75-82.

(28) Kruidenier L, Kuiper I, Van Duijn W, Mieremet-Ooms MA, Van Hogezand RA, Lamers CB et al. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. Journal of Pathology 2003; 201(1):17-27.

(29) Bruwer M, Schmid KW, Metz KA, Krieglstein CF, Senninger N, Schurmann G. Increased expression of metallothionein in inflammatory bowel disease. Inflammation Research 2001; 50(6):289-293.

(30) Dooley TP, Curto EV, Reddy SP, Davis RL, Lambert GW, Wilborn TW et al. Regulation of gene expression in inflammatory bowel disease and correlation with IBD drugs: screening by DNA microarrays. Inflammatory Bowel Diseases 2004; 10(1):1-14.

(31) Marks DJB, Harbord MWN, MacAllister R, Rahman FZ, Young J, Al-Lazikani B et al. Defective acute inflammation in Crohn's disease: a clinical investigation. Lancet 2006; 367(9511):668-678.

(32) Waeytens A, Laukens D, Janssens S, Peeters H, Rottiers P, Cuvelier CA et al. Downregulation of metallothioneins (MTS) in intestinal epithelial cells (IECs) is associated with impaired chemoattractant activity. Gastroenterology 2007; 132(4):A236-A237.

(33) Itoh N, Shibayama H, Kanekiyo M, Namphung D, Nakanishi T, Matsuyama A et al. Reduced bactericidal activity and nitric oxide production in metallothionein-deficient macrophages in response to lipopolysaccharide stimulation. Toxicology 2005; 216(2-3):188-196.

(34) Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: The multipurpose protein. Cellular and Molecular Life Sciences 2002; 59(4):627-647.

(35) Moffatt P, Plaa GL, Denizeau F. Induction of metallothionein gene expression by epidermal growth factor and its inhibition by transforming growth factor-beta and dexamethasone in rat hepatocytes. Hepatology 1995; 21(4):1038-1044.

(36) Babyatsky MW, Rossiter G, Podolsky DK. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. Gastroenterology 1996; 110(4):975-984.

(37) Ohtani H, Kagaya H, Nagura H. Immunohistochemical localization of transforming growth factor-beta receptors I and II in inflammatory bowel disease. Journal of Gastroenterology 1995; 30 Suppl 8:76-77.

(38) Sappino AP, Schurch W, Gabbiani G. Differentiation Repertoire of Fibroblastic Cells - Expression of Cytoskeletal Proteins As Marker of Phenotypic Modulations. Laboratory Investigation 1990; 63(2):144-161.

(39) Douglas-Jones AG, Thomas ND, Elmes ME, Jasani B. Immunocytochemically detectable metallothionein in granulation tissue surrounding mucosal ulceration. Histochemical Journal 1992; 24(1):40-50.

(40) Laukens D, Peeters H, Waeytens A, Bogaert S, Vander Cruyssen B, Elewaut D et al. Reduced metallothionein expression in colonic Crohn's disease: Evidence for a new disease-modifying gene. Gastroenterology 2008; 134(4):A457.

(41) Schreck R, Rieber P, Baeuerle PA. Reactive Oxygen Intermediates As Apparently Widely Used Messengers in the Activation of the Nf-Kappa-B Transcription Factor and Hiv-1. Embo Journal 1991; 10(8):2247-2258.

(42) Vasto S, Mocchegiani E, Malavolta M, Cuppari I, Listi F, Nuzzo D et al. Zinc and inflammatory/immune response in aging. Biogerontology: Mechanisms and Interventions 2007; 1100:111-122.

CURRICULUM VITAE

Anouk Waeytens was born on January 26th 1977 in Dendermonde, Belgium. She obtained her degree of Bio-Engineer, cell and gene biotechnology, with great distinction in 2000 at the Ghent University. She performed her master thesis on the breast cancer susceptibility gene *BRCA1* under the guidance of Prof. Dr. Christian De Potter in the department of Pathology, Ghent University. In August 2000, she started in this department as assistant academic staff where she provided service for ultrastructural microscopy (diagnostic and scientific) and confocal microscopy. She performed scientific research under the promotorship of Prof. Dr. Claude Cuvelier (pathology, since 2001) and Prof. Dr. Martine De Vos (gastroenterology, since 2006). Anouk Waeytens is a fellow at the department of gastroenterology since August 2007.

STUDIES ABROAD

August 16-25, 2000: stay at the Sir William Dunn School of Pathology, South Parks Road, Oxford, UK in the lab of David Vaux (learning techniques for confocal and electron microscopy).

August 14-16, 2002: Bio-Rad Laser Scanning Microscopy Course (organised and run by Nick White); University of Oxford, Oxford, UK.

July 3-12, 2003: The joint EMBO/EU Practical Course on Electron Microscopy and Stereology in Molecular Biology of Cells and Tissues; Ceske Budejovice, Czech Republic.

May 1-28, 2004: stay at the European Molecular Biology Laboratory (EMBL), Gareth Griffiths' Group, Heidelberg, Germany (collaboration)

November 7-9, 2006: Workshop Aurion: Immuno gold silver staining; Wageningen, The Netherlands.

SEMINARS

4de Wetenschappelijke vergadering rond moleculaire technieken, RUG FGG, June 21, 2002
Confocale laser scanning microscopie. A. Waeytens.

Vorming UZ Gent, March 11, 2004
Confocale microscopie. A. Waeytens.

Séminaires Service d'anatomie pathologique, Hôpital Erasme, January 9, 2008.
The damaged mucosal barrier in IBD: interaction with therapeutic bacteria and roles for metallothionein as immunomodulator. A. Waeytens

TRAVEL GRANTS

11th United European Gastroenterology Week "UEGW 2003" Madrid, Spain.

CONGRESS ORGANISATION

XIth EuroCellPath Course: Molecular Mechanisms in Chronic Inflammatory Disease. Het Pand, Ghent, Belgium, June 13-18, 2001. Local Organizing Committee: C. Cuvelier (chairman), P. Demetter, V. Schelfhout, J. Van Huysse, A. Waeytens and G. Maes (secretary)

a1 PUBLICATIONS

Schelfhout V.R.J., Coene E.D., Delaey B., Waeytens A.A.T., De Rycke L., Deleu M., and De Potter C.R.
The role of heregulin-α as a motility factor and amphiregulin as a growth factor in wound healing.
The Journal of Pathology, 2002; 198: 523-533

Cauwels A., Janssen B., Waeytens A., Cuvelier C. and Brouckaert P.
Caspase inhibition causes hyperacute tumor necrosis factor-induced shock *via* oxidative stress and phospholipase A2.
Nature Immunology, 2003; 4: 387-393

Cauwels A., Janssen B., Waeytens A., Cuvelier C. and Brouckaert P.
Caspase inhibition causes hyperacute tumor necrosis factor-induced shock *via* oxidative stress and phospholipase A2.
Inflammation Research, 2003; 52: S178-S182

Geerts A, Lagae E, Dhaene K, Peeters M, Waeytens A, Demetter P, Cuvelier C, Defreyne L, De Vos M, Pattyn P.
Metastatic follicular dendritic cell sarcoma of the stomach: a case report and review of the literature.
Acta Gastro-enterologica Belgica, 2004;67:223-227

Coene E.D., Hollinshead M.S., Waeytens A.A., Schelfhout V.R., Eechaute W.P., Shaw M.K., Van Oostveldt, P.M. and Vaux D.J.
Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria.
Molecular Biology of the Cell, 2005; 16: 997-1010

Verbrugghe P., Waelput W., Dieriks B., Waeytens A., Vandesompele J. and Cuvelier C.
Murine M cells express annexin V specifically.
The Journal of Pathology, 2006; 209: 240-249

Raedt R., Pinxteren J., Van Dycke A., Waeytens A., Craeye D., Timmermans F., Vonck K., Vandekerckhove B., Plum J., and Boon P.
Differentiation assays of bone marrow derived Multipotent Adult Progenitor Cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers.
Experimental Neurology, 2007; 203: 542-554

Van Herrewege Y., Michiels J., Waeytens A., De Boeck G., Salden E, Heyndrickx L, van den Mooter G, de Béthune MP, Andries K, Lewi P, Praet M and Vanham G.
A dual chamber model of female cervical mucosa for the study of HIV transmission and for the evaluation of candidate HIV microbicides.
Antiviral Research, 2007; 74: 111-124

Waeytens A., Ferdinande L., Neirynck S., Rottiers P., De Vos M, Steidler L. and Cuvelier C.
Paracellular entry of interleukin-10 producing *Lactococcus lactis* in inflamed intestinal mucosa in mice.
Inflammatory Bowel Diseases, 2008; 14: 471-479

Pérez-Novo C.A., Waeytens A., Claeys C., Van Cauwenberge P., and Bachert C.
Staphylococcus aureus enterotoxin B regulates prostaglandin E2 synthesis, growth, and migration in nasal tissue fibroblasts.
The Journal of Infectious Diseases, 2008; 197: 1036-1043

Ferdinande L., Demetter P., Pérez-Novo C., Waeytens A., Taildeman J., Rottiers I., Rottiers P., De Vos M., and Cuvelier C.
Inflamed intestinal mucosa features a specific epithelial expression pattern of indoleamine 2,3-dioxygenase.
The International Journal of Immunopathology and Pharmacology, 2008; 21: 289-295

De Baere, T., Hollants J., Waeytens A., Huyghe J., Cuvelier C., Verhelst R., Deschaght P., Vaneechoutte M., and Dhooge I.
Culture, PCR and confocal laser scanning microscopy of middle ear biopsies and middle ear mucosal swabs for microbial analysis of otitis media.
Accepted, B.ENT (2008-12-15).

Taildeman, J., Pérez-Novo, C.A., Rottiers, I., Ferdinande, L., Waeytens, A., De Colvenaer, V., Bachert, C., Demetter, P., Waelput, W., Braet, K., and Cuvelier, C.A.
Human mast cells express leptin and leptin receptors.
Accepted, Histochemistry and Cell Biology (2009-02-01).

ABSTRACTS

E.D. Coene, V.R.J. Schelfhout, C.R. De Potter, M. Hollinshead, A. Waeytens, P. Van Oostveldt, W. Eechaute and D.J. Vaux. Phosphorylated BRCA1 in Nucleus & Mitochondrion. Keystone Symposia on Molecular and Cellular Biology, 2000. Lake Tahoo, Nevada, USA. Abstract book.

A. Waeytens, S. Neirynck, W. Waelput, L. Steidler, C. Cuvelier. Morphological study of interleukin-10 producing Lactococcus Lactis in mouse ileal loops. 11th United European Gastroenterology Week “UEGW 2003” Madrid, Spain. Gut, 2003; 52 (suppl VI): A13.

J. Pinxteren, A. Waeytens, P. Verdonck, R. Raedt, C. Van Geyt, D. Craeye, J. Plum and B. Vandekerckhove. A straightforward method to generate multi-potent adult progenitor cells (MAPC) from human bone marrow. Keystone Symposia on Stem Cells (B2), 2004, Keystone, Co, USA. Abstract book.

J. Pinxteren, D. Craeye, R. Raedt, A. Waeytens, J. Plum and B. Vandekerckhove. The Isolation of multi-potent adult progenitor cells (MAPC) from human bone marrow: comparison of methods. BioScience 2004 – from molecules to organisms, SECC, Glasgow, UK, 2004. BioScience 2004.

A. Waeytens, S. Neirynck, W. Waelput, P. Rottiers, L. Steidler and C. Cuvelier. Morphological study of interleukin-10 producing Lactococcus lactis in mouse ileal loops. 13th European Microscopy Congress, Antwerp, Belgium, 2004. Abstract in Congress proceedings
A. Waeytens, K. Narine, R. Forsyth, M. Cornelissen, F. Roels, M. Praet and G. Van Nooten. Kangaroo aortic valves are histologically more similar to human aortic valves: a comparative study of kangaroo and porcine aortic valves. 13th European Microscopy Congress, Antwerp, Belgium, 2004. Abstract in Congress proceedings

A Waeytens, S. Neirynck, W. Waelput, P. Rottiers, L. Steidler, C. Cuvelier. Morphological study of interleukin-10 producing Lactococcus Lactis in mouse ileal loops. Belgian Society of Pathology: Wintermeeting, Antwerp, Belgium, 2005. Abstract in Proceedings of the Meeting

A. Waeytens, S. Neirynck, W. Waelput, P. Rottiers, L. Steidler and C. Cuvelier. Morphological study of interleukin-10 producing *Lactococcus lactis* in mouse ileal loops. XVIIth Belgian Week of Gastroenterology: Acta Gastro-enterologica Belgica, Fasc. 1, 2005: A58

Wyckhuys TMF, R Raedt, P Claeys, T De Smedt, J Pinxteren, D Craeye, K Smits, K Vonek, S Dedeurwaerdere, A Waeytens, C Van Den Broecke, B Van De Kerckhove, J Plum, P Boon. Towards a cell therapy for temporal lobe epilepsy (TLE)? Epilepsia 46: 355-355 Suppl. 6, 2005

A Waeytens, E Van Belle, L Ferdinande, J Taildeman, W Waelput, M De Vos, C Cuvelier. Zinc supplementation increases dextran sodium sulphate induced mortality in wild type mice but not in metallothionein null mice. 5th Meeting of the European Mucosal Immunology Group, Prague, Czech Republic, 2006. Abstract in Proceedings of the meeting

A Waeytens, Y Van Herrewege, G De Boeck , J Michiels, M Praet and G Vanham. A dual chamber model of female cervical mucosa for the study of HIV transmission and for the evaluation of candidate HIV microbicides. Belgian Society for Cell and Developmental Biology: autumn meeting 2006: “cytoskeleton and cellular imaging”, Ghent, Belgium, 2006. Abstract in Proceedings of the Meeting

D Laukens, H Peeters, S Bogaert, A Waeytens, B Vander Cruyssen, D Elewaut, F De Keyser, H Mielants, C Cuvelier, EM Veys, K Knecht, P Van Hummelen, J Del-Favero, E Remaut, M De Vos, P Rottiers. Reduced metallothionein expression in colonic Crohn’s disease: evidence for MTF1 as a new disease-modifying gene. 14th United European Gastroenterology Week “UEGW 2006”, Berlin, Germany, 21-25 October 2006. Gut Suppl. Mon-G-162

A Waeytens, D. Laukens, S. Janssens, M. De Vos, C. Cuvelier. Metallothioneins are downregulated in colonic Crohn’s disease and are possibly involved in chemotaxis of neutrophils. Belgian Society of Pathology: Wintermeeting, Brussels, Belgium, 18 November 2006. Abstract in Proceedings of the Meeting.

A Waeytens, D Laukens, S Janssens, H Peeters, P Rottiers, C Cuvelier, M De Vos. Metallothionein silencing in intestinal epithelial cells results in impaired chemoattractant activity. Inflammatory Bowel Diseases, Congress of the European Crohn’s and Colitis Organisation (ECCO) 2007. Abstract book, P158

A Waeytens, D Laukens, S Janssens, H Peeters, P Rottiers, C Cuvelier, M De Vos. Downregulation of metallothioneins in intestinal epithelial cells is associated with impaired chemoattractant activity. XIXth Belgian Week of Gastroenterology. Acta Gastroenterologica Belgica, Vol 70, Fasc. 1, 2007: L03.

J Taildeman, C Perez-Novo, L Ferdinande, A Waeytens, W Waelput, P Pauwels, C Cuvelier. Human gastro-intestinal mast cells express signalling leptin receptor. XIXth Belgian Week of Gastroenterology. Acta Gastroenterologica Belgica, Vol 70, Fasc. 1, 2007: P05.

A Waeytens, D Laukens, S Janssens, H Peeters, P Rottiers, C Cuvelier, M De Vos. Downregulation of metallothioneins (MTs) in intestinal epithelial cells (IECs) is associated with impaired chemoattractant activity. Digestive Disease Week, Washington DC, USA, 2007. Gastroenterology 132 (4): A236-A237 Suppl 2 April 2007.

Van Dycke A., Raedt R., Waeytens A., Vonck K., Wadman W., and Boon P. Neural progenitor cells demonstrate long term survival and differentiate mainly towards astrocytes upon transplantation in a sclerotic hippocampus. European Journal of Neurology 2007, 14 (Suppl. 1), 315-316.

Waeytens A., De Vos M., Laukens D., and Cuvelier C. Intestinal metallothionein features a different expression pattern in Crohn’s disease versus inflammatory disorders. XXth Belgian Week of Gastroenterology: Acta Gastroenterologica Belgica, Vol 7, Fasc. 1, 2008.

Laukens D., Peeters H. Waeytens A., Bogaert S., Vander Cruyssen B, Elewaut D., De Keyser F., Mielants H., Cuvelier C., Knecht K., Van Hummelen P., Del-Favero J., De Vos M., and Rottiers P. Reduced metallothionein expression in colonic Crohn’s disease: evidence for a new disease-modifying gene. XXth Belgian Week of Gastroenterology: Acta Gastroenterologica Belgica, Vol 7, Fasc. 1, 2008.

Laukens D., Peeters H. Waeytens A., Bogaert S., Vander Cruyssen B, Elewaut D., De Keyser F., Mielants H., Cuvelier C., Knecht K., Van Hummelen P., Del-Favero J., Remaut E., De Vos M., and Rottiers P. Reduced metallothionein expression in colonic Crohn’s disease: evidence for a new disease-modifying gene. Proceedings of the 3rd Congress of ECCO, P188.

Laukens D., Peeters H. Waeytens A., Bogaert S., Vander Cruyssen B, Elewaut D., De Keyser F., Mielants H., Cuvelier C., Knecht K., Van Hummelen P., Del-Favero J., Remaut E., De Vos M., and Rottiers P. Reduced metallothionein expression in colonic Crohn’s disease: evidence for a new disease-modifying gene. Digestive Disease Week, San Diego, USA. Gastroenterology 2008, 134 (4): A457

DANKWOORD

Dit is een moment waarnaar ik lang heb uitgekeken, en waarvan ik me vaak heb afgevraagd of het er wel zou komen… En dat het nu toch zover is, heb ik te danken aan vele mensen, die ik hierbij graag oprecht wil bedanken.

PROFESSOR CUVELIER, bedankt om me destijds, als 'weesje', op te vangen in uw lecb-groep en me te introduceren in de wondere wereld van het darmkanaal en, uw stokpaardje, de M cel. In mijn thesis zijn er uiteindelijk amper M-cellen te vinden, maar ik hoop dat hij u toch kan bekoren. Via uw samenwerkingen en contacten heb ik vele professoren en onderzoekers leren kennen die cruciaal gebleken zijn in mijn werk, merci!
PROFESSOR DE VOS, dankzij zowel uw wetenschappelijke als uw financiële inbreng heb ik deze thesis kunnen afwerken tot een werk waar ik tevreden over ben en in geloof. Uw inzichten en beschouwingen hebben me tijdens al mijn onderzoeksjaren geholpen om gevonden resultaten beter te begrijpen en om nieuwe doelstellingen te vinden en proeven op te stellen. Vooral de laatste jaren bent u de drijvende kracht achter mijn werk. Mijn enorme waardering en dank daarvoor.

Ik wil ook graag PROF. LOTHAR STEIDLER, SABINE NEIRYNCK en PIETER ROTTIERS bedanken om me bij te staan tijdens de lactococcen-studie. Ook INGE BRUGGEMAN en MICHAELA LOOS voor de praktische tips over het bacteriewerk. PROF. DIRK ELEWAUT, door uw uitgebreide kennis van de immunologie, zowel qua literatuur als qua bench-werk, gaven discussies met u over onze resultaten en nieuwe experimenten steeds een geruststellend gevoel achteraf, en verminderde de drang tot piekeren. PROF. DEMETTER, Pieter, door jouw carrièrekansen aan de ULB hebben we niet zoveel overleg gehad de laatste jaren. Voor mij lig jij echter wel aan de basis van het 'lecb-*labo*': jij bent als eerste begonnen met de differentiatie van de caco's, en zovele andere toepassingen waar heel het labo nu nog van profiteert. WIM (Waelput), bedankt om muizen te introduceren in ons labo; *in vivo* proeven blijken een grote meerwaarde te zijn in ons onderzoek. DEBBY (Laukens), door jouw onderzoek heeft het mijne een serieuze 'boost' gekregen. Je hebt reeds een ruime kennis opgebouwd en het is prettig om met iemand over de MTs te kunnen doorbomen. Ik heb veel respect voor je werkijver en ambitie.

Spijtig genoeg betekent het afleggen van deze thesis ook het afscheid van het pathologie-labo. Ik ben hier verzeild geraakt (via PROF. PATRIC VAN OOSTVELDT) door PROF. CHRISTIAN DE POTTER. Chris De Potter was een zeer competente promotor en gedreven onderzoeker, die me door zijn enthousiasme en bezieling al snel aanstak met de liefde voor de wetenschap. LIESBETH (Coene), ik heb mijn eerste stappen in het labo gezet onder jouw deskundige begeleiding, en je vastberadenheid om je hypothesen te bewijzen is steeds een voorbeeld voor mij geweest. Jij hebt me geleerd om experimenten nauwgezet en kritisch uit te voeren. VERA, wat zou ik geweest zijn zonder jou? Je steun, luisterend oor en wijze raad hebben me door moeilijke perioden geholpen ❀.. Ik ben zo ontzettend blij met de vriendschap van je gezin. GRIET, samen met Vera waren wij de ‘onafhankelijken’ op de pathologie. Ondanks je eigen problemen was je steeds bereid om naar de mijne te luisteren. Bedankt, en ik hoop dat we over de middag zullen blijven lunchen op het UZ 🍽.

Een speciaal woordje van genegenheid aan al mijn ‘partners in crime’, die vele frustraties met mij gedeeld hebben. JACQUES, PHEBE, LIESBETH (FERDINANDE), JASMIEN, ELLEN, STEPHANIE, het was prettig om met jullie samen te werken. Als de sfeer niet zo goed geweest was,… Ondanks veel miserie hebben we toch heel wat afgelachen, en wat we allemaal zeker opgedaan hebben is assertiviteit en mensenkennis. Ik denk dat we veel aan elkaar gehad hebben: steun, vriendschap, nuttige discussies over experimenten en hypothesen… DIKKE ZOENEN 💋 💋 💋!

DOROTHY, BETTY, ISABELLE, bedankt om zoveel praktisch werk over te nemen. Zonder jullie enthousiaste en nauwgezette hulp zou ik dit werk niet kunnen combineren hebben met mijn gezin. Dodo, jij hebt me de praktische beginselen van de EM bijgebracht, en je bent een wandelende encyclopedie wat betreft protocols voor fixeren, snijden, inbedden, enz. van alle mogelijke weefsels. Ik heb veel van jou geleerd. Betty, je had steeds een klare kijk op de zaken, en maar een half woord nodig om me te verstaan. Je bereidheid om samen kleuringen te blijven uitvoeren, tot we alles geprobeerd hadden en ze eindelijk op punt stonden, gaf me moed om niet op te geven. Isabelle, naast experimenten doe jij zoveel meer voor het labo, allemaal zaken die niemand graag doet en iedereen vanzelfsprekend vindt (bestellingen, onderhoud, veiligheid, audits, SAP). Ik kan me de tijd niet meer voorstellen dat jij er nog niet was… Je bent een harde en competente werker, ik hoop dat je een boeiende carrière mag hebben! Alledrie bedankt, jullie verdienen een medaille 🎖 🎖 🎖!

PROF PRAET, u bent me steeds blijven steunen en hebt ervoor gezorgd dat ik steeds mijn werk kon blijven doen. We hebben in het begin vele EM-foto's bekeken en besproken, en op die manier heb ik veel bijgeleerd over zowel normale als pathologische ultrastructuur. Ook de rest van de dienst wil ik bedanken voor de ontspannende babbels en interesse in zowel mijn werk als mijn gezin. PROF PAUWELS, PROF ROELS, CAROLINE, RAMSES en GITTE (bedankt voor de antilichamen!), KATLEEN B, LIES (ik wens je een wolk van een baby!), KATHLEEN L (ik zal je rake opmerkingen missen), AMÉLIE, WIM, ALEXANDRA, WINNIE; secretariaat- CINDY, GODELIEVE, JOSINE, KATHY, NATALIE, WENDY; en ex-UZ'ers PASCALE, BART, LIEVE, KARL, KRIS.

De laboranten ben ik extra dankbaar om me, ondanks de overvolle bezetting, toch op de Ventana te laten kleuren tijdens de uren, en de toestellen te laten gebruiken. We hebben in de lunchroom menig verhaal uitgewisseld, wat vaak opluchtte. Bedankt ANJA, ANN, ANNEKE, ANNE-MARIE, BART, CAROLINE, CHRIS, FRANCINE, HILDE, LEEN, LILIANE, LINDA, MADELEINE, MARIJKE, MIEKE, MONIQUE, NICOLE, ROOS, SOFIE, SÜKRÜ, THOMAS, VANESSA, VÉRONIQUE, YOLANDE 🎭. We zullen elkaar nog wel tegenkomen als ik in blok A proeven kom doen…

Ik ben zeer blij dat ik op de gastro kan verderwerken aan dit onderzoek. Sommigen van jullie ken ik al langer, anderen heb ik beter leren kennen sinds ongeveer anderhalf jaar. Ondanks dat ik nog veel in blok A zat, voel ik me toch al een beetje thuis bij jullie. Ik kijk ernaar uit om naar de K12 te komen. Bedankt HARALD, SARA, PIETER, KIM, NANCY, ELIEN, CHRISTOPHE, en co. Ook ANNETTE en KATRIEN (secretariaat), bedankt voor de hulp bij veel papierwerk en de eeuwige glimlach ☀!

CLAUDINA en JERINA, bedankt voor jullie interesse en vriendschap. Dank aan INGRID en co van het animalarium om zo goed voor mijn muisjes te zorgen. Daarnaast ook bedankt aan alle onderzoekers die ik via de microscopie tegengekomen ben. Eens in andere topics ondergedompeld worden, werkt verruimend… Bedankt voor de leuke babbels… En RIET, bedankt voor je vriendschap en je begrip en advies voor vele EM-zaken. Wie weet worden we ooit nog collega's (maar nu wil ik toch me toch nog wat verdiepen in de darmen…)

Ik wil ook mijn familie en vrienden bedanken. Een leuke uitstap ☃, bezoekjes, even sporten, telefoontjes ☎,… allemaal dingen waardoor ik stoom kon aflaten, zomaar of als het me allemaal even te hectisch werd. Bedankt FILIP, JANNIK, neefje FERRE en nichtje MANOU, ALEX,

KATRIEN en GUNTHER, EVA, STEVEN en kids, KATRIJN, OMA, PABON, MARTINE, RAF, MAREN en LANDER, KRISTIEN, GUY en JELLE, ELS en ALAIN, ELKE en FREDERIK, ANNELIES en STIJN, tantes en nonkels…

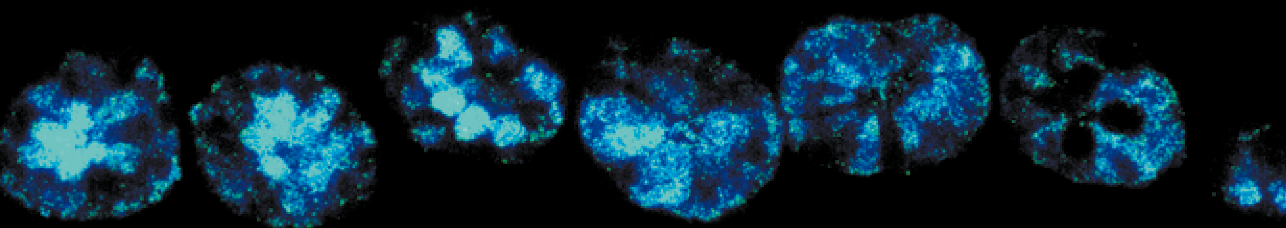
MAMA en PAPA, dankzij jullie ben ik opgegroeid in een warm nest, en ben ik nooit iets tekort gekomen ❀. Jullie eeuwige en onvoorwaardelijke steun, op alle gebied, doet me zoveel deugd. Bedankt daarvoor, en ook om zo goed voor mijn kindjes te zorgen als het nodig is! Ik hoop dat ik voor hen een even goeie mama zal zijn als jij voor mij bent, mama.

En dan… BERT, hoewel je voor dit boek zelf geen letter op papier gezet hebt en er volgens eigen zeggen amper een woord uit begrijpt, zit er toch ook een deel van jou in. Ik wil je bedanken, niet alleen voor je liefde en steun, maar ook voor je hulp en advies om te overleven ‘op de werkvloer’, en voor zoveel hulp in het huishouden, ondanks je drukke en veeleisende job. Je verdient een beloning voor moed en zelfopoffering: 🏆.

JILL en VIC, mijn prinsesje en mijn superman, ik zie jullie ontzettend graag en zou jullie nooit meer kunnen missen. Het is zo fijn om thuis te komen en jullie lachende gezichtjes te zien… dan verdwijnen alle problemen in het niets… Een dikke knuffel ♥.

Een woord van dank ook aan de firma’s AstraZeneca en Schering-Plough voor de ondersteuning van mijn fellowship. Dank aan de volgende firma’s die een deel van het drukwerk en de receptie gesponsord hebben: Carl Zeiss, Nikon, Roche, MLS en Resolution Technology. Deze laatste wil ik ook bedanken voor de ondersteuning bij de digitale camera die ik gebruikt heb voor het maken van de foto’s.

allemaal ontzettend bedankt,
Anouk



thesis submitted in partial fulfilment of the requirements for the degree of

"Doctor in Medical Sciences"