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Chapter 1.

Introduction:

Intestinal interactions between the epithelial barrier, the immune system and the nervous system

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Inflammatory bowel disease (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases of the intestine of unknown aetiology. Although the pathogenesis of these diseases is not well understood, several components of the bacterial flora, the epithelial barrier, the immune system, the nervous system and mutations in genes that are a part of these components have been shown to play an essential role in mucosal inflammation. It has been demonstrated in animal models that the bacterial flora of the intestines is involved in pathological processes of IBD; several mouse models that are treated with antibiotics or that are housed in a germ-free environment do not develop colitis \(^1\)-\(^5\). Moreover, IBD patients show increased mucosal secretion of IgG antibodies against commensal bacteria of the resident flora \(^6\),\(^7\). A functional intestinal barrier is important to prevent commensal bacteria to gain access to the lamina propria (LP) where they can induce an inflammatory response.

We have investigated several aspects of the pathogenesis of IBD, which are outlined in this thesis.
The epithelial barrier function

To prevent access of luminal contents to the LP, the epithelial layer has developed specific barrier mechanisms, including adherens junctions, desmosomes, gap junctions and tight junctions (TJs). TJs or zonula occludens are the most apical components of these intercellular junctions. They prevent the diffusion of membrane proteins and lipids between the basolateral and apical membranes so that cell polarity is preserved (fence function) and a selective barrier to paracellular transport (barrier function) is formed. In contrast to transcellular transport, which is highly selective because of ion channels and active transport systems, paracellular transport is a rather passive process. It depends on ion and molecular gradients at the basolateral and apical side and does not distinguish between different ions and molecules. However, the barrier function of TJs restricts this paracellular transport since TJs are selectively permeable for cations, water and small uncharged molecules, whereas the passage of macromolecules is obstructed. Selectivity for ion size and strength is different between tissues and is related to the composition of TJs.

TJ complexes are composed of a network of proteins that are coupled to actin filaments of the cytoskeleton. The proteins occludin (62-82 kDa), several members of the claudin family (20-27 kDa) and junctional adhesion molecule (JAM) (36-41 kDa) make up the membrane part of the TJ. Although occludin and claudin demonstrate no significant sequence similarity, they are both tetraspan proteins with two extracellular and one intracellular loop and an intracellular N- and C-terminus. To integrate in the TJ, it is essential that occludin is phosphorylated, whereas dephosphorylation redirects occludin to intracellular pools decreasing transepithelial electrical resistance (TEER). Nevertheless, occludin-deficient mice are still able to form well-developed TJ strands and retain normal intestinal barrier integrity. Consequently, occludin may increase the strength of TJs, but other TJ proteins are obviously more essential. It seems that occludin is more involved in cell signalling than in maintaining the epithelial barrier. Claudins, which consist of a family of at least 24 members, are probably the main barrier-forming proteins. Since different types of claudins are expressed in a restricted number of cell types or during periods of development, claudins are expected to contribute to tissue-specific
functions of TJs. Intestinal epithelial cells express several claudins. It is assumed that claudin-2 has the potential to form aqueous channels, whereas the permeability of macromolecules is not increased \(^{22}\). Overexpression of claudin-1 and -4 results in increased TEER, indicating that these proteins are involved in tightening the paracellular barrier \(^{23,24}\). In CD patients it has been demonstrated that the pore-forming claudin-2 is upregulated and that the sealing claudins 5 and 8 are downregulated \(^{25}\). JAM and occludin have been implicated in the transmigration of leukocytes through the endothelial and epithelial barriers, respectively \(^{14,26}\). Mice that are JAM deficient are more susceptible for DSS-induced colitis \(^{27,28}\).

Members of the zonula occludens (ZO) family are proteins that form a bridge between these membrane proteins and actin filaments, which are connected to the perijunctional ring, a component of the cellular cytoskeleton \(^{29-31}\). ZO-1 proteins belong to the membrane-associated guanylate kinase (MAGuK) homologue family, containing three PDZ [postsynaptic density-95 (PSD-95)/Discs large (Dig)/ZO-1] domains, an SH3 domain and a guanylate kinase (GuK) domain \(^{32}\). These and some other domains are essential in the bridge function of the ZO proteins. ZO-1 interacts with ZO-2 and -3 by PDZ domains. The PDZ-1 domain is necessary to interact with the PDZ regions at the C-terminus of claudins \(^{29-31}\). The GuK region of ZO-1 mediates binding to the C-terminus of occludin \(^{29-31,33}\). Besides, the SH3 region of ZO-1 mediates binding to G proteins, like G\(\alpha_i2\), and the C-terminus of ZO proteins interacts to F-actin \(^{29,31,34}\). The function of ZO-1 is not exclusively restricted to the organisation of TJs, as it is also detected in the nucleus where it regulates cell growth and differentiation \(^{35,36}\). The expression of ZO-1 in colonic epithelium is lost in DSS-induced colitis in mice \(^{37}\). Also in colonic tissues of UC patients, the expression of occludin, ZO-1, JAM and claudin-1 is downregulated \(^{38}\).

G\(\alpha_i2\) proteins are localised within the TJs and have an important function in the maintenance and development of TJs, probably through the protein kinase C (PKC) pathway that regulates the phosphorylation of the myosin light chain (MLC) \(^{39-41}\). Mice that are G\(\alpha_i2\) deficient spontaneously develop colitis similar to that of human patients with UC, clinically manifested by diarrhoea and bloody stools \(^{42-46}\). Phosphorylation of MLC causes
contraction of the perijunctional ring, which is a component of the cellular cytoskeleton, so that the permeability of TJs is increased 47-49. MLC is phosphorylated by myosin light chain kinase (MLCK), which is regulated by PKC. The activation of PKC is in turn regulated by seven-membrane-helix receptors that are coupled to G proteins. G proteins are activated following the binding of a ligand to its receptor. Thereafter the $\alpha$ subunit of the G protein activates phospholipase C (PLC)-$\gamma$ that upregulates the second messengers diacylglycerol (DAG) and IP$_3$, so that Ca$^{2+}$ is released from the endoplasmatic reticulum to the cytoplasm. Ca$^{2+}$ and DAG activate PKC and consequently MLCK is phosphorylated so that its activity decreases MLC phosphorylation. In conclusion, activation of PKC proceeds to a decrease in transcellular permeability and an increase in TEER.

Enteric pathogens have developed several mechanisms to disrupt TJs of epithelial cells. This occurs mainly by modulating the perijunctional actomyosin ring or by interfering with TJ proteins directly 50-53. Bacterial products degenerate or (de)phosphorylate specific TJ proteins or use them as a receptor so that these proteins become dysfunctional resulting in a decrease of the efficacy of the TJ. The latter is manifested as a decrease of TEER and as an increase of the paracellular flux of macromolecules like mannitol, often clinically resulting in diarrhoea.

In IBD the epithelial layer is inflamed without obvious exogenous factors like a (bacterial) infection. Nevertheless, colonic biopsies from CD patients contain decreased numbers of Lactobacillus and Bifidobacteria, whereas the mucosa and probably even the intraepithelial layer contain an increased population of adherent bacteria 54-56. Increasing evidence suggests that the immune system itself modulates TJs and intestinal permeability. IBD patients have increased concentrations of pro-inflammatory cytokines, like tumour necrosis factor-$\alpha$ (TNF-$\alpha$), interferon-$\gamma$ (IFN-$\gamma$), interleukin (IL)-8 and IL-1$\beta$ 57-59. In vitro studies have demonstrated that these cytokines decrease the barrier function of intestinal epithelial monolayers and induce reorganisation of several TJ-associated proteins, including ZO-1, JAM-1, occludin and claudin-1, and -4 60-64. An increase of the intestinal permeability caused by an activated IFN-$\gamma$ receptor complex is also associated with a
decrease in the PLC-γ activity resulting in MLC phosphorylation. It seems that IL-1β increases intestinal permeability by the induction of MLCK gene transcription and consequently increases MLCK protein activity, probably mediated by a rapid activation of the transcription factor nuclear factor (NF)-κB. IL-1β-mediated increased intestinal permeability leads to an increased paracellular transport of luminal antigens. Also TNF-α-mediated increased intestinal permeability seems to be NF-κB dependent and leads to a downregulation of ZO-1 proteins and alteration in junctional localisation. In IBD, the intestinal permeability could be increased because of the effects on the epithelial barrier by these pro-inflammatory cytokines, which are increased in IBD patients. In chapter 8 we demonstrate that the neurotransmitter acetylcholine (Ach) and muscarine decrease NF-κB activation and decrease IL-1β- and TNF-α-induced production of IL-8 by epithelial cells. Moreover, Ach and muscarine protect against cytokine-induced enhanced permeability.
Antigen interaction with dendritic cells

Specialised epithelial cells termed M (microfold) cells, which are scattered among the epithelial cells (ECs) in the follicle-associated epithelium above the Peyer’s patches (PPs) are able to absorb, transport, process and present (microbial) antigens to dendritic cells (DCs) in the subepithelial dome (SED) of the PP. In humans, CD11c+ DCs are concentrated in the SED and T cell zones and are particularly involved in the activation of T cells that support IgA-class switching by B cells and in the induction of oral tolerance. Besides, DCs are located in the LP just below the basement membrane, where they interact with antigens that have gained access to the LP following disruption of the epithelial barrier because of infection and/or inflammation, as presumably appears in IBD. In addition, DCs sample antigen directly by expanding dendrites among ECs into the lumen. These DCs are capable to open TJ between enterocytes, since they modulate different TJ proteins, including JAM-1, claudin-1 and ZO-1. The chemokine receptor fractalkine (CX3CR1) on LP DCs enables them to sample luminal antigens directly by transepithelial dendrite interaction with epithelial CX3CL1. The authors suggest that the interaction between CX3CR1 and CX3CL1 is responsible for the accumulation of DCs and T cells in the LP of IBD patients. Rimoldi et al. have shown that the intestinal homeostasis is regulated by the interaction between ECs and DCs: EC-conditioned DCs produce IL-10 and IL-6, but not IL-12 and induce a Th2 response, even in the presence of pathogens that normally promote a Th1 response. It is possible that CD patients lack an adequate interaction between ECs and DCs resulting in a Th1-mediated inflammation. Moreover antigens are taken up by DCs indirectly by internalising apoptotic ECs and by taking up antigen-containing exosomes shed from ECs. Exosomes are small membrane-bound vesicles, which are not only secreted by ECs, but also by haematopoietic cells, including DCs and other certain cell types. It has been shown that immunosuppressive DC-derived exosomes are capable to suppress inflammatory responses in rheumatic arthritis. The exact mechanism is not clear, but it is likely that DC-derived exosomes are internalised by endogenous or follicular DCs to transfer molecules like MHC class II molecules so that antigen-specific T cell responses are induced.
Dendritic cell populations

Human DCs express high levels of human leukocyte antigen (HLA)-DR and are lineage negative, which indicate that they do not express specific markers of B and T cells, monocytes and natural killer cells. In peripheral blood five distinct subsets of DCs can be distinguished, namely CD1c⁺ (BDCA1), CD16⁺, BDCA3⁺, CD123⁺ and CD34⁺ DCs 74. Myeloid precursor DCs express high levels of CD11c and can be distinguished in DCs that express CD1b/CD1c, CD16 or BDCA3 75. These DC populations produce IL-12 in response to bacterial compounds or CD40L and are GM-CSF-dependent for survival. In contrast, plasmacytoid DCs are CD11c negative and express CD123, BDCA2 and BDCA4 76,77. These IL-3 dependent DCs respond especially in case of viral infections by the production of type I interferon (IFN), including IFN-α and IFN-β.

In the gut, DCs are present in the primary sites for the induction of intestinal T and B cell responses which include the PPs located in the small intestine, isolated lymphoid follicles in the colon and mesenteric lymph nodes (MLNs), all structures that are part of the gut-associated lymphoid tissue (GALT). Te Velde et al. had demonstrated two distinct DC subpopulations in IBD patients: an ICAM-3 grabbing non-integrin (DC-SIGN)+ population that was present scattered throughout the mucosa and a CD83⁺ population that was present in aggregated lymphoid nodules and as single cells in the LP 78. Only DC-SIGN⁺ DCs produce the pro-inflammatory cytokines IL-12 and IL-18. Interestingly, in CD patients the expression of both populations was increased compared to healthy controls. In chapter 4 we demonstrate that the myeloid DC populations positive for CD1a and BDCA-1 are absent in colonic mucosa and MLNs, whereas BDCA3⁺ DCs are highly expressed throughout the LP and around (sub)capsular and medullary sinuses, blood vessels and B-cell follicles in the MLN 79. In MLNs and lymph follicles in the colon the expression of s-100⁺ DCs is increased in CD patients.
Antigen recognition by DCs

Since the gut contains massive numbers of microbes, it necessary that the immune system is able to discriminate between commensal and pathogenic microbes. Therefore DCs and other immune cells such as macrophages are able to recognise pathogen-associated molecular patterns (PAMPs) through binding to pattern recognition receptors (PRRs) on their membrane. Most important PRRs are the Toll-like receptors (TLRs), the nucleotide oligomerisation domain (NOD)-like receptors (NLRs) and the C-type lectins.

Toll-like receptors

TLRs are highly conserved proteins and so far, eleven members of the TLR family have been identified in mammals. Toll was first identified as a protein involved in the controlled dorsoventral formation during the (embryonic) development of the Drosophila melanogaster. Drosophila that are Toll deficient are not able to clear infections caused by fungi, since some antimicrobial products will not be produced. Because Drosophila does not have an adaptive immune system, TLRs are involved in an evolutionary conserved signal pathway that induces innate immune responses. TLRs are characterised by amino-terminal leucine-rich repeats that are responsible for the recognition of PAMPs and they possess a carboxy-terminal Toll-IL-1 receptor (TIR) domain of which the sequence is homologous to that of interleukin receptor-1 (IL-R1) family proteins. Each TLR recognises different PAMPs and the first human TLR member to be discovered was TLR4, which is a transmembrane protein that has to be demonstrated the receptor for lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria. Recognition of LPS by TLR4 is a complex process and several accessory molecules are necessary. First LPS has to bind to the plasma protein LBP (LPS-binding protein) so that it is able to interact with the soluble or GPI-anchored protein CD14, which is produced by monocyte-derived cells and this complex binds to TLR4. Mice that are TLR4 or CD14 deficient are hyporesponsive to LPS. Individuals with a mutation in TLR4 have a slightly increased risk to develop CD.
Chapter 1

TLR2 recognises different components of mainly bacteria and yeast, such as peptidoglycan from gram-positive bacteria, lipoteichoid acid, zymosan from yeast and lipoproteins. TLR2 forms heterodimers with TLR1 when activated by bacterial lipoproteins, whereas mycoplasma-derived lipoprotein triggers TLR2 to form heterodimers with TLR6.

TLR5 recognises flagellin, which is a protein that forms bacterial flagella. Intestinal ECs express TLR5 at the basolateral side where they can sense flagellin from pathogenic bacteria such as Salmonella. Mice lacking TLR5 develop colitis spontaneously. Interestingly mice that are both TLR5 and TLR4 deficient have elevated bacterial loads in the colon; however they do not develop colitis. Serum IgG to flagellin is elevated in CD and UC patients and a dominant-negative TLR5 polymorphism reduces adaptive immune responses to flagellin and in some ethnicities heterozygous carriage is associated with a protection from CD.

TLR3, TLR7, TLR8 and TLR9 are intracellular receptors present in endosomal compartments and are specialised in the recognition of nucleic acids. TLR3 recognises double stranded (ds)RNA generated during the replication of viruses. It has been shown that mice that are TLR3 deficient are more susceptible for infections with cytomegalovirus and West Nile virus. DSS-induced colitis in mice is ameliorated by systemic, but not oral administration of synthetic viral RNA that activates TLR3. TLR7 and TLR8 are involved in the recognition of single stranded (ss)RNA rich in guanosine or uridine derived from RNA viruses. Mice that are TLR7 deficient are not capable to induce inflammatory cytokines, type I IFN and plasmacytoid DC maturation. Although TLR7 and TLR8 recognise both ssRNA, TLR7 activation is characterised by a strong induction of type I IFNs, whereas TLR8 activation results in the induction of pro-inflammatory cytokines as TNF-α. Unmethylated 2′-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs found in bacteria and several viruses are recognised by TLR9. TLR9-dependent activation by DNA-containing immune complexes seems to be mediated by the high-mobility group box-1 protein (HMGB-1) and the receptor for advanced glycosylated end products (RAGE). DSS-induced colitis is exacerbated in TLR9-deficient mice,
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probably because of a disturbed homeostasis. It has been shown that TLR9 at the apical site of the epithelial barrier does not give an immune reaction as a result of binding its ligand CpG DNA. However, micro-organisms that pass the epithelial barrier are recognised by TLR9 at the basolateral site resulting in activation of the NF-κB pathway.

Most TLRs activate the transcription factor NF-κB through a myeloid differentiation factor 88 (MyD88)-dependent pathway, resulting in the expression of genes encoding for inflammatory cytokines, including TNF-α, IL-6 and IL-1β. All TLRs, with the exception of TLR3, recruit the intracellular protein MyD88 through TIR domain interactions. These interactions result in the recruitment of IL-R1 associated kinase (IRAK)-1 and -4 to arrange a complex. Mice that are MyD88 deficient are more susceptible for DSS-induced colitis. Recognition of commensal bacteria seems to be important for maintaining the integrity of the epithelial barrier. Macrophages of MyD88-deficient mice are not able to activate IRAK-1 after exposure of LPS and the production of TNF-α, IL-6 and IL-1β is inhibited. IRAK-1 is a serine-threonine kinase of which the N-terminal region contains a death domain that interacts with the death domain of MyD88. Mice that are deficient for IRAK-1 confirm an insufficient response to LPS. The adaptor protein TNF receptor associated factor 6 (TRAF-6) is also recruited to the complex by association to phosphorylated IRAK-1. TRAF-6-deficient mice have osteoporosis and macrophages derived from the bone marrow of these animals are insufficient in the production of nitric oxide in response to LPS. Phosphorylated IRAK-1 and TRAF-6 dissociate from this complex to form a complex with transforming growthfactor activated kinase (TAK)-1, TAK-1 binding protein (TAB1) and TAB2 at the plasmamembrane resulting in the phosphorylation of TAB2 and TAK1. IRAK-1 is degraded and ubiquitylation of TRAF-6 leads to the activation of TAK1, which phosphorylates both mitogen-activated protein (MAP) kinases and the inhibitor of nuclear factor IκB kinase (IKK) complex. The IKK complex consists of two catalytic subunits, IKKα and IKKβ and one regulatory subunit, IKKγ or NF-κB essential modulator (NEMO). Activation of this complex results in the phosphorylation of IκBs so that NF-κB translocates from the cytosol into the nucleus where it induces the expression of its target genes. Although NF-κB is
thought to induce the transcription of mainly pro-inflammatory genes, mice that are NEMO deficient and consequently do not signal via the NF-κB pathway develop colitis spontaneously \(^{112}\). This indicates that NF-κB signalling regulates epithelial integrity and intestinal immune homeostasis.

**Nucleotide oligomerisation domain-like receptors**

NLRs are intracellular recognition proteins that contain similar to TLRs C-terminal leucine-rich repeats and an N-terminus consisting of protein-protein interaction domains, such as caspase recruitment domains (CARD) or pyrin domains. NOD1 is involved in the recognition of \(\gamma\)-D-glutamyl-*meso*-diaminopimelic acid (ie-DAP), which is a cell-wall derivate of gram-negative bacteria, whereas muramyl dipeptide (MDP), a component of both gram-negative and -positive bacterial peptidoglycan (PGN), is a ligand for NOD2 \(^{113,114}\). In theory, mutations in NOD2 will result in a decreased activation of the NF-κB pathway and consequently in a decreased production of pro-inflammatory cytokines. However, mouse models and family studies have revealed that mutations in NOD2 are associated with the development of CD accompanied with an increased production of pro-inflammatory cytokines including TNF-\(\alpha\) and IL-12 \(^{87,115-117}\). It is still not clear what causes this discrepancy and whether a mutation in NOD2 will result in a gain \(^{116}\) or a loss of function \(^{115,117}\). Different mutations in NOD2 have been described in which the substitutions R702W and G908R and the C-insertion mutation at nucleotide 3020 (3020inC) are most common in humans \(^{118,119}\). Mutations associated with CD are located in the leucine-rich repeats, whereas mutations in the NACHT domain results in Blau syndrome \(^{120}\).

MDP is not only recognised by NOD2, but also another member of the NLR family, NALP3/CIAS1/cryopyrin/NLRP3, is activated by MDP \(^{121}\). NALP3 has a similar structure of NOD2, but contains a pyrin domain instead of a CARD domain. Gain-of-functions mutations in the NACHT domain of NALP3 are associated with three auto-inflammatory diseases, Muckle-wells syndrome (MWS), familial cold auto-inflammatory syndrome (FCAS) and chronic infantile neurological cutaneous and articular syndrome...
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(CINCA), which are characterised by periodic fever syndromes. Recently is discovered that a polymorphism in NALP3 is also associated with an increased risk of CD. NALP3 is involved in the activation of the pro-inflammatory cytokines IL-1β and IL-18 through the activation of caspase-1 that cleaves the inactive cytoplasmic precursors pro-IL-1β and pro-IL18 into its mature active forms. Activated NALP3 forms together with two adapter molecules ASC and CARDINAL the so-called ‘inflammasone’ resulting in the recruitment of two caspase-1 molecules and consequently in the induction of active IL-1β and IL-18. These cytokines seem to be important in the pathogenesis of CD since IL-1β production is increased in morphological normal intestinal biopsies from patients with CD and in mice it has been shown that neutralisation of IL-18 ameliorates TNBS-induced colitis.

C-type lectins

C-type lectins are transmembrane proteins that recognise carbohydrate structures in a calcium-dependent manner using highly conserved carbohydrate recognition domains (CRDs). Glycosylated molecules and micro-organisms that bind to C-type lectins expressed by DCs will be internalised, processed in endosomes and finally presented to T cells, together with MHC class I and II molecules. Since in the cytoplasmic regions of C-type lectins immunoreceptor tyrosine based activation (or inhibitory) motifs (ITAMs or ITIMs) are present, activation of C-type lectins may result in pro- or anti-inflammatory responses. In contrast to TLRs and NOD proteins, C-type lectins recognise not only foreign, but also self-proteins, so that activation of C-type lectins will not always result in DC maturation and T cell activation. The balance between the activation of C-type lectins and TLRs regulates the outcome of the innate immune response.

Several micro-organisms such as HIV, dengue virus, hepatitis C virus, Mycobacterium tuberculosis and Candida albicans interact with the C-type lectin DC-SIGN. Nevertheless, activation of DC-SIGN alone will not result in sufficient innate immune responses; however DC-SIGN ligation on DCs after TLR4 activation increases cytokine production dramatically. Nagaoka et al. demonstrated that DC-SIGN associates with the TLR4-MD-2 complex and that signal transduction by the recognition of
LPS in gram-negative bacteria is enhanced. Interaction of the C-type lectin dectin-1 with TLR2 results in the generation of pro-inflammatory responses to fungal pathogens.

In CD patients, the expression of a subpopulation DC-SIGN$^+$IL-12$^+$IL18$^+$ DCs is increased in colonic mucosa compared to healthy controls. In Chapter 5 we demonstrate that polymorphisms in the C-type lectins DC-SIGN, dendritic cell immuno receptor (DCIR) and macrophage galactose-like lectin (MGL) are not associated with IBD. However, polymorphisms in lectin-like transcript 1 (LLT1) seems to be associated with a slightly increased risk of CD.
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Antigen presentation by DCs

Upon antigen encounter, immature DCs are activated and undergo a differentiation process in which they fully mature into highly stimulatory antigen presenting cells. During this process they lose their endocytotic capacity that was necessary for the uptake and processing of antigens in the periphery. In addition, they upregulate the chemokine receptor CCR7 so that they are able to migrate to the draining lymph node where they encounter naïve populations of T cells. Moreover, mature DCs upregulate co-stimulatory molecules, including CD80 (B7.1), CD86 (B7.2) and CD40 and adhesion molecules such as ICAM-1 and LFA-1. In the lymph node, mature DCs present the processed antigen in association with MHC class I and II molecules to naïve T cells so that these T cells become activated and differentiate into effector T cells. Naïve T cells that recognise the antigen, but that are not co-stimulated become anergic in which T cells become unresponsive. Depending on which PRRs are activated by the captured antigens, DCs will direct naïve T cells to differentiate into T helper (Th) 1, Th2, Th17 or regulatory T cells. In conclusion, to activate T cells, three signals of DCs are necessary: 1) a peptide/MHC complex that is recognised by the T cell receptor, 2) sufficient co-stimulation to prevent anergy and 3) T cell polarisation signals to adapt the effector phenotype.

Depending on the interaction between DCs and different micro-organisms, DCs produce high or low concentrations of IL-12, which is an important determinant of the direction of the immune response. High concentrations of IL-12 will direct T cells to develop into Th1 cells, whereas low concentrations allow for the production of IL-4 by the T cell pool itself which, in turn, will accelerate the development of Th2 cells. IL-10 production by (regulatory) DCs may facilitate the generation of regulatory T cells, which are important in the induction of tolerance to self and harmless foreign antigens.

It has been demonstrated that an exaggerated immune response against the endogenous microflora by Th1 and Th17 lymphocytes plays an important role in the pathogenesis of CD. This immune response is characterised by an increase of pro-inflammatory cytokines, including TNF-α, IL-1β, transforming growth factor (TGF)-β,
IFN-γ and IL-17 in the inflamed mucosa of CD patients. High concentrations of TNF-α can also be detected in the stool of CD patients\textsuperscript{145,146}. Overexpression of TNF-α in mice results in the development of chronic inflammatory arthritis and Crohn’s like IBD\textsuperscript{147}. TNF-α is present in two forms, namely as a transmembrane and as a soluble protein. Since TNF-α seems to be a key player in the pathogenesis of CD, pharmaceutical industries developed TNF-α inhibitors. However, these drugs have side-effects which include immunoreactivity. In \textbf{chapter 3} we investigated TNF-α inhibitors based on the light chains of camel antibodies in an acute and a chronic colitis model. Unfortunately, these TNF-α inhibitors did not ameliorate colitis, probably since only the soluble form of TNF-α is blocked and not the transmembrane form. Blocking of only soluble TNF-α may lead to an impaired apoptosis in IBD patients resulting in survival of reactive T cells which can maintenance inflammatory processes. Moreover, the transmembrane form of TNF-α seems to be more involved in cell survival processes instead of cell death\textsuperscript{148,149}. In \textbf{chapter 2} we discuss apoptotic mechanisms and their association to IBD. In addition, we will review how specific therapeutic approaches interact at different levels with the apoptotic pathway.

\textbf{Tolerance and regulatory T cells}

Tolerance is achieved by different mechanisms, both thymic and peripheral, to prevent accumulation and activation of auto-reactive T cells. In the thymus auto-reactive T cells are eliminated by negative selection, i.e. T cells with specificity for self antigens become apoptotic and are deleted\textsuperscript{150}. Nevertheless, a population of low-affinity self-reactive T cells will escape this thymic selection process and enter the circulation to the periphery. Here peripheral tolerance mechanisms take over to prevent auto-reactivity. Auto-reactive T cells are inhibited by regulatory (suppressor) T cells, a diverse subset of CD4\textsuperscript{+} T cells, including CD4\textsuperscript{+}CD25\textsuperscript{+} cells, Tr1 and Th3 cells\textsuperscript{151,152}. Failure of one of these mechanisms may result in allergies, rejection of transplanted organs or autoimmune diseases, such as type I diabetes and multiple sclerosis, but also IBD is probably caused by impaired regulatory mechanisms, so that overwhelming Th1 responses can be developed.
Regulatory T cells can be distinguished in naturally occurring regulatory T cells and adaptive regulatory T cells \(^{153}\). Naturally occurring T cells acquire their regulatory function in the thymus during early neonatal development and migrate into peripheral tissue where they suppress the proliferation and cytokine production of self-reactive T cells in a mainly contact dependent manner to maintain tolerance to especially auto-antigens \(^{151}\). Since they express high levels of the IL-2 receptor (CD25) they are referred as CD4\(^+\)CD25\(^+\) cells, although also activated CD4\(^+\) T cells express CD25. Moreover they are characterised by high membrane expression of CD38, CD62L, CD103 and glucocorticoid-induced TNF receptor (GITR), cytotoxic T lymphocyte antigen 4 (CTLA-4 or CD152) and by the expression of FoxP3.

On the contrary, adaptive regulatory T cells, including Tr1 and Th3, are dependent on antigen presentation of DCs and are mainly involved in the mucosal tolerance to widespread antigens and commensal microflora, predominantly by the production of anti-inflammatory cytokines, including IL-10 and TGF-\(\beta\) \(^{154,155}\). Th3 cells produce high levels of TGF-\(\beta\) and were first identified in oral tolerance studies \(^{156,157}\), whereas Tr1 cells produce high levels of IL-10 \(^{158}\). Immature DCs were shown to induce the development of Tr1 cells through the production of TGF-\(\beta\) and IL-10, both \textit{in vitro} \(^{159}\) and \textit{in vivo} \(^{160}\). However, also mature DCs can induce regulatory T cells \(^{161}\), dependent on culture conditions and the priming antigen.

Immature regulatory DCs can be induced by the hepatitis C virus and \textit{Mycobacterium tuberculosis}. In contrast, schistosoma-derived lysophosphatidylserine, filamentous haemagglutinin of \textit{Bordetella pertussis}, cholera toxin B and fungus-derived cordycepin induce mature regulatory DCs that produce variable amounts of IL-10, but all induce Tr1 cells \(^{162-165}\). Filamentous haemagglutinin of \textit{Bordetella pertussis} has been shown to ameliorate the disease activity in a chronic T cell dependent colitis model by the induction of anti-inflammatory cytokines \(^{166}\). Furthermore also commensal microbes such as lactobacilli, which act through DC-SIGN and \textit{Mycobacterium vaccae} have been associated with the induction of mature regulatory DCs and the generation of regulatory T cells \(^{161,167,168}\). Characteristically, mainly pathogens that induce chronic diseases are able to
suppress the immune response by the activation of (IL-10-producing) regulatory DCs, either immature or mature.

Since several commensal bacteria (i.e. probiotics) and helminths like *Trichuris suis* decrease the production of the Th1-associated cytokine IL-12 by DCs and increase the production of IL-10, resulting in an inhibition of the generation of Th1 cells \(^{169,170}\), they could be a potential treatment of IBD. Oral intake of genetically modified probiotic *Lactococcus lactis* that produce IL-10 has been shown to decrease disease activity in CD patients in a phase I clinical trial \(^{171}\). It is likely that in IBD patients the balance between regulatory T cells and Th1 cells is disturbed, so that mainly Th1 responses are activated. Probably by the generation of regulatory DCs, the fate of T cells can be changed in T cells that gain a regulatory function instead of T cells that induce inflammation. When we know more about responses of DCs and the fate of T cells reacting to probiotica, commensal bacteria and pathogens, we will understand more how DCs discriminate between different micro-organisms. This could be a rationale for DC immunotherapy, which is also one of the therapeutic approaches in other autoimmune diseases, such as diabetes and multiple sclerosis, cancer and allergies.

**Th17 cells**

A novel T helper cell lineage, Th17 that exclusively produces the pro-inflammatory cytokine Th17 has been reported to play an important role in many inflammatory diseases, including IBD. The IL-12 family member IL-23 is produced by DCs and promotes the differentiation of CD4\(^+\) T cells that produce IL-17 and seems to play an important role in regulating the Th1/Th17 balance in IBD \(^{172-174}\). A genome-wide association study showed that a polymorphism in the receptor for IL-23 confers strong protection against CD \(^{175}\). Moreover, anti-IL-23 therapy was effective in the prevention as well as the treatment of active experimental colitis \(^{176}\). IL-17 expression and Th17 differentiation is downregulated by IFN-\(\alpha\) in experimental colitis and UC patients that receive IFN-\(\alpha\) therapy \(^{177}\). Besides the production of IL-17, Th17 cells produce other pro-inflammatory cytokines including IL-21, IL-22, TNF-\(\alpha\) and IL-6 \(^{174,178}\). IL17 and IL-21 are overexpressed in colonic samples
from IBD patients and neutralisation of IL-21 reduces the secretion of IL-17 by LP T lymphocytes derived from CD patients \(^{179}\). Moreover, both TNBS- and DSS-induced colitis are ameliorated in IL-21-deficient mice, probably since naïve T cells from these mice failed to differentiate into Th17 cells \(^{179}\). In experimental colitis, IL-21 prevents TGF-\(\beta\)-dependent expression of FoxP3 resulting in a reduction of regulatory T cells \(^{180}\). TGF-\(\beta\)1 is able to differentiate naïve T cells into regulatory T cells, which prevent autoimmunity \(^{180}\). However, in the presence of IL-6, TGF-\(\beta\)1 has been shown to converts naïve T cells into Th17 cells \(^{180}\). It seems that the vitamin A metabolite retinoic acid plays an important role in the regulation of TGF-\(\beta\)1-dependent immune responses in which retinoic acid inhibits the IL-6- and IL-23-driven induction of Th17 cells and promotes FoxP3\(^+\) regulatory T cells differentiation by enhancing TGF-\(\beta\)-driven Smad-3 signalling \(^{181,182}\). In chapter 4 we show that polymorphisms in LLT1 are slightly associated with CD. Interestingly, LLT1 is a ligand for CD161, which is a new surface marker for human IL-17 producing Th17 cells \(^{183,184}\). It has been shown that CD161\(^+\)CD4\(^+\) T cells are a resting Th17 pool that can be activated by IL-23 and mediate destructive tissue inflammation in the intestines of CD patients \(^{184}\).
The cholinergic pathway in immune regulation and intestinal epithelial barrier function

Besides the intestinal epithelial barrier function and the intestinal immune system, the nervous system plays an important role in the homeostasis of the gut. The intestinal tract is innervated by the vagus nerve, which is part of the parasympathetic nervous system known to regulate heart rate, hormone secretion, gut motility, respiratory rate, blood pressure and other vital processes of the body. The two vagus nerves originate in the medulla oblongata and preganglionic fibres travel uninterrupted to the organs they innervate. There the preganglionic fibres synapse with short postsynaptic fibres that are distributed throughout the organ. Ach is the principal neurotransmitter of the vagus nerve and plays a key role in the anti-inflammatory pathway.

The enteric nervous system (ENS) is an integrated network of neurons and enteric glial cells (EGCs) and is organised in a submucosal plexus or Meissner’s plexus located between the mucosa and the circular muscle layer, and a myenteric plexus or Auerbach’s plexus located between the circular and longitudinal muscle layers. The ENS is regulated by the central nervous system, but is in contrast to other organs also able to function independently. In general, the Meissner’s plexus regulates secretory responses of the mucosa, whereas the Auerbach’s plexus is involved in the regulation of gastrointestinal motility. It has been shown that besides neurones also EGCs modulate gastrointestinal functions indirect or directly.

Interactions between the nervous system and the immune system

It has been described that cholinergic activation has anti-inflammatory effects in several diseases. Vagotomy and cholinergic antagonists have been shown to worsen inflammation in animal colitis models, whereas stimulation of the vagus nerve results in an amelioration of postoperative ileus in part through its anti-inflammatory effects. Most effects of the vagus nerve have been based on the effects of Ach, which signals through either muscarinic receptors or nicotinic receptors. Macrophages and also other...
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immune cells like DCs express several subunits of the nicotinic acetylcholine receptors (nAChRs) such as the α4, β2 and α7 subunit \(^{195,196,\text{own data}}\). Selective α7 nAChR agonists have been shown to ameliorate pancreatitis, DSS-induced colitis and postoperative ileus \(^{188,191,193,194}\). Nicotine, which acts through the α7 homopentamer, inhibits the production of pro-inflammatory cytokines and chemokines in macrophages and inhibits the NF-κB pathway and HMGB1 secretion \(^{187,197}\). Interestingly, nicotine has also beneficial effects in several subgroups of patients with UC, but not in CD patients \(^{194,198,199}\). Also Ach itself inhibits the release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-18 by macrophages, but stimulated with endotoxin the production of the anti-inflammatory cytokine IL-10 is not affected \(^{195}\). In chapter 7 we tested two new selective α7 nAChR agonists in two different mouse models. Although earlier research demonstrated that activation of the vagus nerve ameliorate intestinal inflammation, we show that both α7 nAChR agonists worsen colitis or are ineffective.

Besides Ach also other neuropeptides have been implicated to be anti-inflammatory. Cholecystokinin (CCK) is responsible for the activation of digestion of dietary fat and it is indicated that CCK reduces TNF-α and IL-6 release in haemorrhagic shock by the intake of high-fat nutrition \(^{189}\). Vagotomy abrogates this anti-inflammatory effect of both high-fat intake and CCK, indicating that the vagus nerve is responsible for CCK-reduced inflammation. Vasoactive intestinal peptide (VIP) regulates the secretion of water and electrolytes and the dilation of the smooth muscles of the gut to increase gut motility. In TNBS-induced colitis, VIP ameliorates clinical symptoms and microscopic inflammation by regulating the balance between Th1, Th2 and Th17 differentiation \(^{200}\).

**Interactions between the nervous system and the epithelial barrier**

Although in general activation of the cholinergic anti-inflammatory pathway and the release of VIP and Ach leads to decreased inflammation in several diseases, it also results in an increase of intestinal permeability \(^{201}\). Both VIP and Ach increase paracellular permeability in the gut \(^{202,203}\). Moreover, it has been demonstrated that the release of VIP inhibits
proliferation of epithelial cells and that it is necessary to maintain intestinal epithelial barrier integrity \textsuperscript{203,204}. Besides paracellular transport, Ach is also able to increase transcellular transport via muscarinic Ach receptor activation \textsuperscript{205}. These results seem to be contradictory since an increased intestinal epithelial barrier leads to an increased influx of antigens into the intestinal mucosa where they can induce an immune reaction.

In contrast to the cholinergic pathway, EGCs seem to decrease intestinal permeability since ablation of EGCs in transgenic mice causes an increase of intestinal permeability and causes intestinal inflammation \textsuperscript{206}. Furthermore, in vitro co-culture models of EGCs and intestinal epithelial cell lines demonstrate that EGCs decrease the permeability, probably via the release of S-nitrosoglutathione and the regulation of ZO-1 and occludin expression \textsuperscript{206}. S-nitrosoglutathione is able to restore mucosal barrier function in colonic biopsies from CD patients \textsuperscript{206}. Moreover, EGCs inhibit proliferation of intestinal epithelial cells which is partly TGF-β1 dependent \textsuperscript{207}. Mice that are deficient for EGCs show an increased uptake of thymidine in intestinal ECs and crypt hyperplasia \textsuperscript{207}. Probably an interaction between enteric neurones and EGCs is necessary to maintain epithelial barrier function homeostasis, since in general the cholinergic pathway increases intestinal permeability, whereas EGCs do the opposite. In chapter 6 we give an overview of how neurotransmitters influences epithelial barrier function. In chapter 8 we show that intestinal permeability is mainly decreased through the activation of muscarinic receptors under inflamed conditions.
Thesis outline

Since many components are involved in the pathogenesis of IBD, it is important to understand how the environment (gut flora and food antigens), epithelial barrier, immune system, nervous system and genetic make-up interact with each other. In this thesis we have investigated different parts of the pathogenesis of IBD. The first part of this thesis describes how apoptosis plays a role IBD (Chapter 2) and how we investigated a new TNF-α inhibitor in two different colitis models (Chapter 3). In the second part of this thesis we investigated which DC populations are present in the colon and MLNs of CD patients (Chapter 4) and whether mutations in genes that encodes several C-type lectins are associated with IBD (Chapter 5). The last section of this thesis describes how the ENS influences barrier function of the intestine (Chapter 6 and 8) and how we investigated two new α7 nAchR agonists in two different experimental mouse models (Chapter 7).
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