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Prolyl hydroxylase domain-containing protein 1 as potential therapeutic target for inflammatory bowel disease

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LIST OF ABBREVIATIONS

5- ASA	5-aminosalicylic acid
ACHP	2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile
APC	antigen-presenting cell
BAX	BCL-2-associated X protein
BM	bone marrow
BMDM	bone-marrow derived macrophages
BSA	bovine serum albumin
CAC	colitis-associated cancer
CD	Crohn's disease
CRE	cyclization recombination
CXCL	C-X-C motif ligand
DC	dendritic cells
DCE-MRI	dynamic contrast-enhanced magnetic resonance imaging
DMEM	Dulbecco's Modified Eagle Medium
DMOG	dimethyloxalylglycine
DSS	dextran sulfate sodium
DT	diphtheria toxin
EC	endothelial cells
ELISA	enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
EPO	erythropoietin
FOXP3	forkhead box P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIF	hypoxia-inducible factor
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule 1
IEC	intestinal epithelial cell
IL	interleukin
IFN	interferon
LP	lamina propria
LPS	lipopolysaccharide

MAdCAM-1	mucosal addressin cell adhesion molecule 1
MAMPs	microbe-associated molecular patterns
MCP1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MPO	myeloperoxidase
NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
NO	nitric oxide
PBS	phosphate buffered saline
PECAM-1	platelet and endothelial cell adhesion molecule 1
PHD	proly hydroxylase domain-containing protein
PRR	pathogen recognition receptors
qRT-PCR	quantitative real-time polymerase chain reaction
RA	retinoic acid
SDHA	succinate dehydrogenase complex A subunit
SEM	standard error of the mean
TBS-T	Tris buffered saline with 0.1% Tween-20
TCR	T cell receptor
TGF-β	transforming growth factor β
Th	T helper
TNF	tumor necrosis factor
TLR	toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
Treg	regulatory T-cell
UC	ulcerative colitis
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VWF	von Willebrand factor
WT	wild-type

SUMMARY

Inflammatory bowel diseases (IBD) are a group of inflammatory intestinal disorders comprising ulcerative colitis (UC) and Crohn's disease (CD) as the principal subtypes. Although the exact pathophysiology of IBD is largely unknown, it is generally accepted that the disease results from a dysregulated immune response towards intraluminal antigens in a genetically predisposed host, which is triggered by environmental factors. Active disease is associated with severe mucosal hypoxia due to increased oxygen consumption of inflammatory cells and decreased oxygen supply by dysfunctional blood vessels which leads to increased epithelial cell death and a compromised intestinal epithelial barrier. This inflammation-induced hypoxia in its turn can induce a pro-inflammatory response and hereby contribute to the perpetuation of the inflammatory process. On the other hand, cellular adaptation to reduced oxygen concentrations will protect cells by promoting their survival. The latter involves oxygen-sensitive signalling pathways which include the activation of hypoxia-inducible factors (HIFs) and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) under the tight control by prolyl hydroxylase domain-containing proteins (PHD1-3). Boosting these adaptive mechanisms via pan-hydroxylase inhibitors is protective in experimental models for IBD. However, potential side-effects of non-selective PHD inhibition such as increased risk of carcinogenesis, cardiovascular events (due to increased erythropoiesis) and fibrosis may hamper their clinical application. A potential strategy to circumvent these issues is to selectively target specific PHD isoforms. Therefore, the purpose of this thesis was to better define the role of PHD1, 2 and 3 in the pathogenesis of IBD to identify the main isoform(s) that will be most eligible for therapeutic inhibition.

Chapter I gives a general overview of the main differences between UC and CD, epidemiology, diagnosis and current therapeutic options. In addition, the different factors (i.e. genetics, environmental factors and the gut microbiome) involved in the pathogenesis of IBD are described, elaborating in more detail on the role of immune and endothelial cells. Following, we extensively review the involvement of hypoxia and hypoxia-induced signalling in cellular players contributing to IBD and provide an update on the development of PHD inhibitors for therapeutic use. Finally, the last part of chapter I describes the methodology used in this dissertation to study immune and endothelial cell-mediated effects in experimental IBD.

In the **first part** of **chapter III**, we examined the expression and cellular location of PHD1, 2 and 3 in colonic biopsies collected from healthy controls and patients with UC, CD and infectious colitis. We observed that *PHD1* was significantly increased in inflamed IBD biopsies both at the mRNA and the protein level. The mRNA and protein expression of PHD2 was comparable between all groups. *PHD3* mRNA expression was significantly elevated in inflamed colonic UC biopsies, but was absent at the

protein level in severely inflamed UC biopsies. Furthermore, *PHD1* mRNA levels, not *PHD2* and to a much lesser extent *PHD3*, exhibited a strong positive correlation with *TNF* and *IL-8*. Besides these pro-inflammatory cytokines, *PHD1* and to a lesser extent *PHD2*, showed a positive correlation with the apoptosis marker caspase 3. Immunohistochemical analysis revealed that the highest PHD1 expression was found in epithelial cells and mononuclear cells (e.g. dendritic cells, macrophages) of the lamina propria. The same cell types were immunopositive for PHD2 in addition to lymphocytes and smooth muscle cells in the muscularis mucosae. PHD3 staining was only present in the endothelium of blood vessels.

In the **second part** of **chapter III**, we investigated the role of PHD1, 2 and 3 in endothelial and haematopoietic cells during the course of colitis using *Phd1-3*$^{f/f}$Tie2:cre knockout mice. First, we showed that deletion of *Phd1* in this conditional model and not *Phd2* or *Phd3* was protective in dextran sulfate sodium (DSS)-induced colitis and resulted in a preservation of epithelial integrity, reduced infiltration of inflammatory cells and diminished microvascular dysfunction. Next, we performed bone marrow irradiation experiments to unravel the contribution of on the one hand endothelial and on the other hand haematopoietic *Phd1*-deficiency during colitogenesis. We found that *Phd1*-deficiency in the haematopoietic compartment is both necessary and sufficient to suppress the clinical symptoms during DSS-induced colitis. These results were confirmed in *Phd1*$^{f/f}$Flk-1:cre mice (for endothelial *Phd1*-deletion) and *Phd1*$^{f/f}$Vav:cre mice (for haematopoietic *Phd1*-deficiency). In addition, we demonstrated that these beneficial effects relied, at least in part, on the skewing of *Phd1*-deficient macrophages towards an anti-inflammatory M2 phenotype, with a diminished NFκB-dependent response to LPS and by a diminished interleukin (IL)-1β release by LPS-stimulated *Phd1*-deficient dendritic cells.

SAMENVATTING

Inflammatoir darmlijden (IBD) zijn chronische ontstekingsziekten van de darm en omvatten colitis ulcerosa (UC) en de ziekte van Crohn (CD). De exacte etiologie is nog niet volledig opgehelderd, maar het is algemeen aanvaard dat deze ziekten ontstaan door een abnormale immuunrespons tegen luminale antigenen in genetisch belaste personen en wordt waarschijnlijk getriggerd door omgevingsfactoren. Actieve ziekte wordt gekenmerkt door ernstige mucosale hypoxie die enerzijds ontstaat ten gevolge van een verhoogd zuurstofverbruik door de aanwezige dysfunctionele inflammatoire cellen en anderzijds door verminderde zuurstoftoevoer omdat de bloedvaten dysfunctioneel zijn. Dit leidt tot toegenomen epitheelceldood en een beschadigde epitheelbarrière. Deze inflammatie-geïnduceerde hypoxie gaat op zijn beurt enerzijds een pro-inflammatoire respons uitlokken wat het inflammatoir proces verder onderhoudt, maar anderzijds gaan de cellen zich aanpassen om zichzelf te beschermen tegen deze lage zuurstofomstandigheden. Hierbij worden zuurstofgevoelige signalisatiemechanismen in gang gezet waarbij *hypoxia-inducible factors* (HIFs) en *nuclear factor k light chain enhancer of activated B cells* (NFκB) geactiveerd worden. Deze transcriptiefactoren worden op hun beurt gereguleerd door prolyl hydroxylase domein-bevattende eiwitten (PHD1-3). Activatie van deze adaptatiemechanismen door middel van pan-hydroxylase-inhibitoren is protectief in verschillende experimentele modellen voor IBD. Deze aanpak is echter niet aan te raden omwille van mogelijke ongewenste bijwerkingen zoals een verhoogd risico op tumorontwikkeling, cardiovasculaire aandoeningen (omwille van verhoogde erythropoiese) en fibrose. Een mogelijke manier om deze bijwerkingen te omzeilen is isovorm-specifieke targeting. Daarom is het doel van deze thesis om de rol van de verschillende PHD-isovormen in inflammatoir darmlijden beter te begrijpen zodat de belangrijkste isovorm(en) kan/kunnen geïdentificeerd worden die het beste therapeutisch potentieel heeft/hebben.

Hoofdstuk I geeft een algemeen overzicht van de voornaamste verschillen tussen UC en CD, de epidemiologie, diagnose en de huidige therapeutica. Daarnaast worden de verschillende factoren besproken die betrokken zijn in the pathogenese van IBD waarbij in detail de rol van immuun-en endotheelcellen wordt toegelicht. Vervolgens wordt uitgebreid alle gepubliceerde data besproken over de betrokkenheid van hypoxie en hypoxie-geïnduceerde signalisatie in verschillende celtypes die bijdragen tot IBD en er wordt een update gegeven over de ontwikkeling van PHD-inhibitoren voor therapeutisch gebruik. Het laatste deel van dit hoofdstuk beschrijft de methoden die gebruikt werden tijdens deze thesis om immuun-en endotheelcelspecifieke effecten te bestuderen in experimenteel IBD.

In het **eerste deel** van **hoofdstuk III** hebben we de expressie onderzocht van PHD1, 2 en 3 in biopten uit het colon van gezonde controles en patiënten met UC, CD en infectieuze colitis. We vonden dat PHD1 significant op-gereguleerd was in geïnflammeerde IBD biopten zowel op mRNA als eiwit niveau. De mRNA en eiwitexpressie van PHD2 was vergelijkbaar tussen alle groepen. *PHD3* mRNA expressie was op-gereguleerd in ontstoken UC biopten terwijl het PHD3 eiwit afwezig was in sterk geïnflammeerde UC biopten. Verder konden we aantonen dat de *PHD1* mRNA niveaus, niet *PHD2* en in veel mindere mate *PHD3*, sterk positief correleerden met *TNF* en *IL-8*. Daarnaast vertoonde *PHD1* en in mindere mate *PHD2* een positieve correlatie met de apoptose merker caspase 3. Uit immunohistochemische analyse is gebleken dat de sterkste PHD1 expressie terug te vinden is in epitheelcellen en mononucleaire cellen (zoals dendritische cellen en macrofagen) van de lamina propria. Dezelfde celtypes waren ook immunopositief voor PHD2 naast lymfocyten en gladde spiercellen in de muscularis mucosa. PHD3 aankleuring kon enkel in endotheelcellen van de bloedvaten waargenomen worden.

In het **tweede deel** van **hoofdstuk III** hebben we de rol onderzocht van PHD1, 2 en 3 in hematopoietische en endotheelcellen op het verloop van colitis door gebruik te maken van *Phd1-3*$^{f/f}$Tie2:cre knockout muizen. In eerste instantie konden we aantonen dat genetische deletie van PHD1 en niet PHD2 of PHD3 in dit conditioneel model, beschermend was in dextraan sulfaat sodium (DSS)-geïnduceerde colitis en resulteerde in het behoud van epitheelintegriteit, verminderde infiltratie van immuuncellen en gereduceerde microvasculaire dysfunctie. Vervolgens werden beenmergbestralingsexperimenten uitgevoerd om enerzijds de specifieke bijdrage van endotheel en anderzijds hematopoietische *Phd1* knockout te achterhalen. *Phd1*-deficiëntie in het hematopoietisch compartiment bleek nodig, maar ook voldoende om de ziekteactiviteit in DSS-geïnduceerde colitis te onderdrukken. Deze resultaten werden bevestigd in *Phd1*$^{f/f}$Flk:cre voor endotheliale *Phd1*-deletie en *Phd1*$^{f/f}$Vav:cre voor hematopoietische *Phd1* knockout. Verder vonden we dat dit beschermend effect op zijn minst deels aangestuurd wordt door *Phd1*-deficiënte macrofagen die omgeschakeld zijn naar een anti-inflammatoir M2 fenotype en een verminderde NFκB-afhankelijke response vertonen na lipopolysaccharide (LPS) stimulatie wat resulteert in verminderde secretie van pro-inflammatoire cytokines. Anderzijds, berust het therapeutisch effect op de verminderde interleukine (IL)-1β productie van LPS-gestimuleerde *Phd1*-deficiënte dendritische cellen.

CHAPTER I: INTRODUCTION

1 Inflammatory bowel diseases: general introduction

1.1 Ulcerative colitis and Crohn's disease

Inflammatory bowel diseases (IBD) are a group of chronic and relapsing inflammatory disorders comprising ulcerative colitis (UC) and Crohn's disease (CD). In UC patients, inflammation is limited to the colon in which disease typically starts in the rectum and spreads proximally in a continuous fashion with sharp delineation between involved and non-involved mucosa. In some cases, limited distal ileitis is observed which is called "backwash ileitis". Microscopic features in UC are inflammatory changes limited to the mucosa and submucosa which are characterized by infiltration of polymorphonuclear and mononuclear cells, crypt abscesses and goblet cell depletion (Figure 1). In contrast, CD can affect any part of the gastrointestinal tract, from mouth to anus, in a non-continuous way which means that diseased segments are alternated by healthy bowel and is referred to as "skip areas". The main affected location is the terminal ileum. Histologically, CD is characterized by a thickened submucosa and transmural inflammation with granulomas[1] (Figure 1). Patients with UC or CD suffer from similar symptoms including abdominal cramping and pain, recurring and/or bloody diarrhea, weight loss and extreme tiredness. Some IBD patients develop extraintestinal manifestations (25-40%) like skin rash, arthritis, uveitis or liver disease (i.e. primary sclerosing cholangitis)[2]. Approximately 80% of CD patients develop complications such as stricture and penetration of the bowel wall with obstruction, fistulae and abscesses. In addition, patients with colonic involvement have an increased risk of colitis-associated cancer[3]. Besides UC and CD, 10%-15% of patients exhibit indeterminate features between UC and CD and are therefore categorized as "indeterminate colitis"[4].

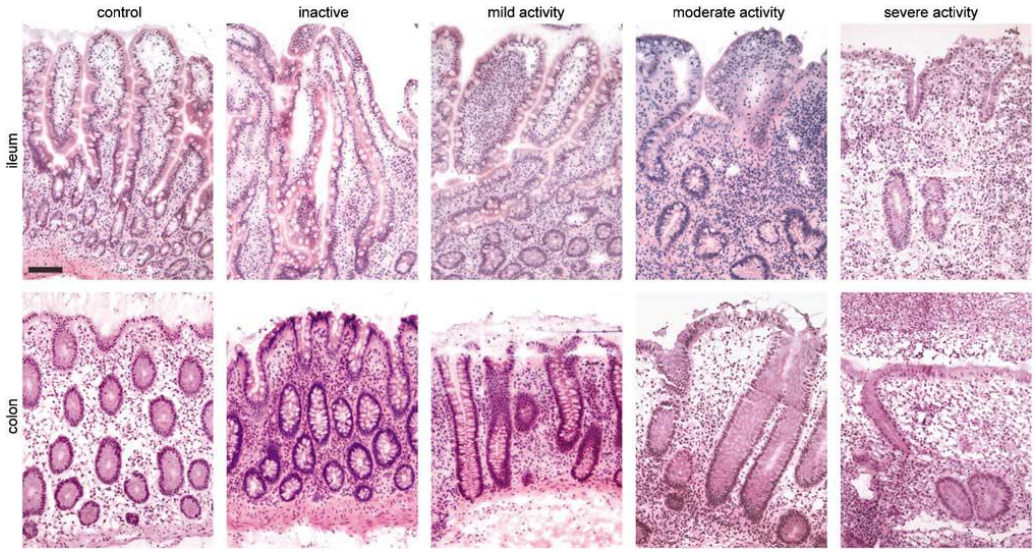

Figure 1. *Histological grading of disease activity. Biopsy and resection specimens were graded for histologically evident disease activity using frozen sections. Examples of ileal and colonic mucosa in each category are given. Bar = 100 µm. Adapted from Blair et al.*[5]

1.2 Epidemiology

The highest incidence and prevalence rates of IBD have been reported in Northern Europe and North America and declines from north to south and from west to east. In Europe, the annual incidence of CD varies from 0.5 to 10.6 cases per 100,000 persons, while the rate for UC range from 0.9 to 24.3 per 100,000 persons[6]. However, during the last decades, IBD occurrence is increasing in formerly low-incidence areas such as Asia, South America and Africa[7]. Besides geographical differences, heterogeneity among ethnic groups has also been reported. For example, higher rates of IBD have been reported in Jews than in non-Jews[8, 9]. Also, IBD is more common in Caucasians compared with blacks, Asians, Hispanics and American Indians[10]. The peak age of onset for both UC and CD is usually between 20 and 40 years although it can occur at any age[7].

1.3 Etiopathogenesis

Although the exact cause of IBD is largely unknown, it is generally accepted that the interaction of environmental factors and microbial flora in genetically predisposed individuals leads to an exaggerated immune response, an impaired intestinal epithelial barrier and vascular dysfunction. In the following paragraphs, I will briefly touch upon the role of genetics, environmental triggers and microbiota in IBD. Given the central theme of the thesis, I will elaborate in more detail on the role of immune and endothelial cell dysfunction in IBD pathogenesis.

1.3.1 Genetics and environmental triggers

Convincing evidence for a genetic predisposition came from family and especially twin studies. In particular, 5-23% of IBD patients have an affected first-degree relative[11]. Tysk and co-workers were the first to show that monozygotic twin pairs have a higher concordance rate than dizygotic twins (58,3% vs 3,9% in CD and 6,3% vs 0% in UC), suggesting a major genetic predisposition for the disease[12]. Two groups independently identified the first susceptibility gene for CD, nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*)[13, 14]. In the following years, many other IBD-associated genes were identified and led to a total of more than 163 susceptibility loci today[15]. These include polymorphisms in genes involved in autophagic killing of intracellular bacteria such as *ATG16L1*, immune cell response like *IL-23R* and epithelial barrier function such as *CDH1* to numerate the major other affected pathways. However, these susceptibility loci only explain 13.6% of the total disease variance in CD and 7.5% in UC. These findings combined with the short timeframe in which IBD occurrence has increased in previous low-incidence regions and a concordance rate in monozygotic twins below 100% further highlight the importance of external triggers in the pathogenesis of these diseases. Nowadays, air and water pollution, smoking, appendectomy, drugs, infections, stress, diet

and lifestyle have all been shown to associate with IBD, although the results are often inconsistent[16, 17].

1.3.2 The gut microbiome

Several lines of evidence suggest that the microbiota are essential for the initiation and perpetuation of intestinal inflammation in IBD. More specifically, germ-free animals do not or only mildly develop experimental colitis (e.g. IL10$^{-/-}$ and IL2$^{-/-}$ knockout mice)[18, 19] and ileitis (e.g. TNF$^{\Delta are}$ mice)[20] with the exception of germ-free mice subjected to dextran sulfate sodium (DSS, ICN Biomedicals)-induced colitis where inflammation is actually worse[21, 22]. The reason for the increased susceptibility is not immediately clear at this moment. One hypothesis goes as follows: DSS induces epithelial erosions (see section 3) which allows the penetration of bacterial antigens to enter the mucosa. Bacterial toll-like receptor (TLR) ligands subsequently trigger the proliferation of epithelial cells to restore barrier integrity, but this response is compromised due to the lack of bacteria in these mice leading to the increased severity[22]. In human IBD, inflammation is present where the bacterial concentrations are highest and it has been demonstrated that mucosal IgG antibodies against intestinal bacterial antigens are present in these patients[23]. Also, diversion of the fecal stream away from the ileocolonic anastomosis prevents recurrence of CD[24]. Finally, although antibiotics do not cure the disease, antibiotic treatment is effective for anal lesions and the prevention of postoperative recurrence of CD[25, 26]. Several microbes have been implicated in the pathogenesis of IBD, but no single organism has been identified as an IBD-causing pathogen. Instead, the microbial composition in IBD patients is severely altered compared with healthy controls which is termed dysbiosis. In particular, there is an increase in *Proteobacteria* and a reduction in *Firmicutes*. In addition, there is a decrease in bacterial diversity largely due to a decline in the diversity of *Firmicutes*[27]. More specifically, there is primarily a reduction in the abundance of species that belong to Clostridium clusters XIV and IV of which the butyrate-producing bacteria *Roseburia hominis* and *Faecalibacterium prausnitzii* (*F. prausnitzii*) are particularly well-studied. It has been documented that both species are depleted in the stool of UC patients, while *F. prausnitzii* is also reduced in the mucosa-associated microbiota of IBD patients[28]. Moreover, low levels of *F. prausnitzii* on resected ileal CD mucosa associates with a higher risk of postoperative recurrence[29]. Also, higher numbers of invasive *Escherichia coli* (*E. coli*) have been found in the ileal mucosa of CD patients which correlates with the severity of the disease[30]. Interestingly, the dysbiotic signature persists during clinical remission[31, 32] and has even been reported to be present in unaffected relatives of IBD patients[33, 34]. Therefore, it seems that IBD-associated dysbiosis might only contribute to the onset of IBD when genetic and environmental factors are present. However, it is currently still unknown if dysbiosis is in fact the cause of the disease.

1.3.3 The gut-associated immune system

In the following part, an overview is given of the different members of the innate and adaptive immune system that contribute to gut homeostasis (Figure 2) followed by a description of the dysfunctional immune response in IBD patients which has been associated with the perpetuation of inflammation.

1.3.3.1 The innate immune cells and intestinal homeostasis

Epithelial cells, intraepithelial cells and innate lymphoid cells

Intestinal epithelial cells (IECs) form a tight single layer that represents a physical and functional barrier between the lumen and the underlying mucosa. In this way, they restrict translocation of luminal antigens, but concomitantly allow the passage of water, electrolytes and nutrients[35]. In addition to their well-known barrier function, they can participate in the defence against intruders and ensure intestinal homeostasis through their pattern recognition receptors (PRRs) that recognize microbe-associated molecular patterns (MAMPs). Under normal circumstances, recognition of MAMPs does not elicit an inflammatory response, but is essential to 1) ensure epithelial barrier integrity because it promotes survival, the production of anti-microbial peptides and mucus, epithelial turnover and tight junction expression and 2) educate the mucosal immune system towards oral tolerance[36]. In addition to the classical antigen presenting cells (APCs) (i.e macrophages, dendritic cells (DCs) and B lymphocytes), IEC also act as non-professional APC as they constitutively express major histocompatibility complex (MHC) class II molecules on their basolateral membrane[37]. When bacterial invasion occurs, an acute pro-inflammatory response is provoked that aims to clear the invaded bacteria and restore epithelial barrier integrity. IEC can discriminate between commensals at the luminal side and invaded bacteria due to the presence of specific TLRs at the basolateral side of IECs such as TLR5[38]. In particular, apical stimulation with bacterial ligands induces tolerance, while basolateral stimulation elicits a pro-inflammatory response. Another member of the innate immune system is located in the epithelial layer, the so called intraepithelial lymphocytes. They represent a population of T cells, but they are separated from the lamina propria (LP) lymphocytes (see further) due to their localisation. They mainly consist of T cell receptor (TCR) $\gamma\delta^+$ cells and $CD8^+$ cells. Although they are ideally situated to receive signals from both bacteria as epithelial cells, their functions are not completely understood. What we do know is that they promote epithelial barrier functions, have cytotoxic activity to clear infected or damaged epithelial cells and induce epithelial antimicrobial peptide production[36]. In addition to influencing epithelial cell function, they also exert regulatory functions and suppress intestinal inflammation through their production of IL-10[39]. Finally, innate lymphoid cells (ILCs) have emerged during the last decade as a novel family that belongs to the innate immune compartment. They play a central role in mucosal immunity, lymphoid tissue formation and

epithelial barrier protection. In response to cytokines from epithelial and myeloid cells, they secrete pro-inflammatory and anti-inflammatory cytokines and largely resemble herein the T helper (Th) family (see further). According to their phenotype and cytokine secretion profile, they are classified into 1) ILC1 which express T-box transcription factor (T-bet) and produce interferon (IFN)-γ, 2) ILC2 which express GATA-binding protein 3 (GATA3) and produce IL-4, IL-5 and IL-13 and 3) ILC3 which exhibit retinoic acid orphan receptor (ROR)γt expression and release IL-17 and IL-22[40]. The majority of ILCs present in the healthy intestine are IL-22-producing ILC3s that stimulate epithelial cells to produce mucus and anti-microbial peptides[41] and are critical in the protection against the pathogen *Citrobacter rodentium*[42]. ILC3s also secrete GMCSF which triggers macrophages to produce retinoic acid (RA), required for the induction of regulatory T cell differentiation (Treg)[43].

Macrophages

The intestinal mucosa is characterized by a heterogeneous population of macrophages. Resident macrophages are present with phagocytic and intracellular killing capacity to eliminate invading pathogens without inducing a local inflammatory response. They are, in contrast to peripheral monocytes, tolerant towards luminal antigens through their down-regulated expression of PRR such as TLRs which results in low cytokine release, but strong bactericidal activity. In addition, they present processed antigens in the absence of co-stimulatory signals which again demonstrates their tolerating function. However, in case of bacterial invasion, the resident macrophages produce chemoattractants such as IL-8 and transforming growth factor beta (TGF-β) that recruit peripheral blood monocytes to migrate into the infected mucosa. Here, they polarize into pro-inflammatory M1 macrophages after recognition of MAMPs such as lipopolysaccharide (LPS) and in the presence of IFN-γ and TNF. This leads to phagocytosis and subsequent eradication of invaded bacteria. Upon activation, these infiltrated macrophages secrete high amounts of IL-1β, IL-6, IL-8, IL-12 and TNF that subsequently influence the activation of many other cell types. Of particular importance, they promote the differentiation and activation of Th1 and Th17 cells (see further), deregulate the localisation of tight junction proteins within the epithelial barrier and induce epithelial apoptosis leading to tissue damage and increased epithelial barrier permeability[44]. Additionally, these macrophages also produce TGF-β and monocyte chemoattractant protein (MCP)-1 to attract neutrophils and other macrophages leading to their extravasation into the inflamed tissue. By doing so, this cell type can promote the perpetuation of inflammation and therefore its actions need to be controlled as soon as the invaded bacteria are cleared. This is done by M2 macrophages which exert a regulatory, wound healing function and are regulated by IL-4 and IL-13. They typically express low levels of pro-inflammatory cytokines, but high levels of arginase 1 (ARG-I), chitinase-3 like 3 (CHI3L3 or YM-1), FIZZ-1 and IL-10[45, 46].

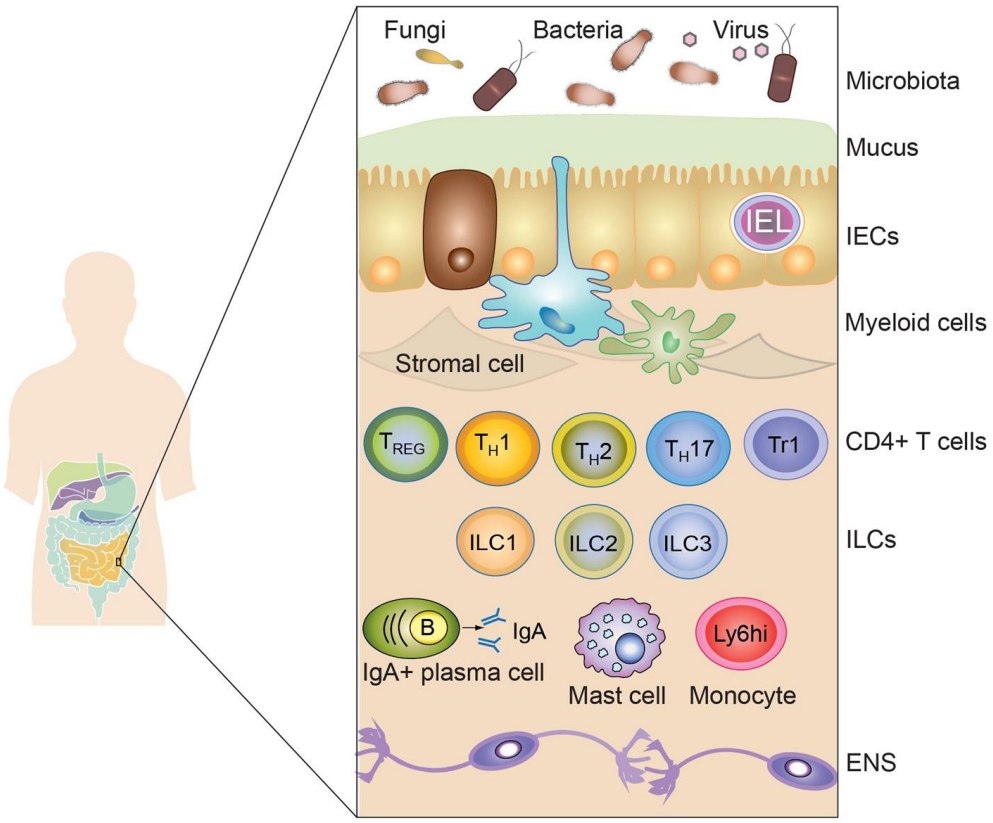

Figure 2. *The intestinal immune system. The intestinal immune system is physically separated from the microbiota and dietary compounds by a single layer of intestinal epithelial cells (IECs). Intraepithelial lymphocytes (IEL), residing in the paracellular space between epithelial cells, contribute to the maintenance of the mucosal barrier and to the protection against pathogens. The lamina propria is connective tissue constituted by stromal cells, blood vessels, nerves, and immune cells. Macrophages (Myeloid cells depicted in blue) and dendritic cells (DCs) (Myeloid cells depicted in green) are strategically located adjacent to the epithelial layer, sampling luminal antigens and orchestrating the innate and adaptive immune response. Other innate immune cells are also present in the lamina propria, including mast cells, monocytes, neutrophils, and eosinophils (not shown). T and B cells (mainly IgA-producing plasma cells) also accumulate in the lamina propria after being primed in the draining lymphoid tissues. Different subsets of CD4+ T cells are found in the lamina propria, such as regulatory T cells (Foxp3-expressing Treg and Tr1) and effector cells (Th1, Th2, and Th17). Finally, innate lymphoid cells (ILC), divided in three main subsets (ILC1, ILC2, and ILC3), are vastly enriched in the gastrointestinal mucosa participating in the protection against pathogens and in the maintenance of intestinal homeostasis. Surrounding the lamina propria and the muscularis mucosa (not shown), the submucosa and the muscularis externa contain nerves belonging to the enteric nervous system (ENS). Adapted from Parigi et al. 2015*[47]

DCs

Together with macrophages, DCs are professional APCs and therefore key mediators between innate and adaptive immunity. They represent a very heterogeneous population characterized based on phenotypic markers. At present, there is still active debate about the classification and ontogeny of LP macrophages and LPDCs. Mucosal DCs constitutively express high MHCII levels and the integrin CD11c. However, LP macrophages cannot be distinguished from LPDCs based on these markers since they are both expressed by these cell types. The expression of CX3CR1 (the receptor for the chemokine

fractalkine, CX_3CL1) and CD103 ($\alpha_E\beta_7$ integrin) allows a more reliable discrimination between these two cell types ($CD103^-CXCR1^{hi}$ and $CD103^+CX3CR1^{lo}$ are characterized as LP macrophages and LPDCs, respectively)[48]. These $CD103^+$ cells are considered to be *bona fide* DCs which can migrate to the mesenteric lymph nodes (MLN) and induce an immune cell response. They can be further subdivided based on CD11b expression. Although these findings are valid for mouse DCs, whether these DC subpopulations are equivalent in humans needs to be further studied. So far, the human equivalents to mouse $CD103^+CD11b^-$ DCs are $CD103^+Sirp\alpha^-$ in the intestine and $CD141^+$ DCs in the blood. Mouse myeloid $CD103^+CD11b^+$ DCs correspond to $CD103^+Sirp\alpha^+$ DCs in the intestine and $CD1c^+$ DCs in the blood. Similar to resident macrophages, intestinal *bona fide* DCs are in an immature or tolerogenic state that continuously patrol the mucosa and sample antigens 1) directly from the lumen or 2) shuttled through microfold cells (epithelial cells specialized in sampling of luminal antigens). After acquisition of the antigen, they process it intracellular and present it onto MHCII molecules on its cell surface. Subsequently, they migrate to the MLN, present it to T cells with a TCR specific for the presented antigen and secrete RA and TGF-β to induce on the one hand gut-homing receptors $\alpha4\beta7$ integrin and the chemokine receptor CCR9 on T cells and on the other hand Treg development producing anti-inflammatory cytokines (see further).

1.3.3.2 The adaptive immune cells and gut homeostasis

Members of the adaptive immune system include T and B lymphocytes and induce specific memory responses to certain luminal antigens. Naïve T and B cells are primed in the gut-associated lymphoid tissues (GALT) i.e. Peyer's patches, MLN and smaller lymphoid follicles. Naïve T cells differentiate into specific T cell subtypes when antigens, specific for their TCR, are presented to them by APCs through MHCII in the presence of co-stimulatory signals. Subsequent interactions of specific cytokines with signal transducer and activator of transcription (STAT) factors, results in the formation of different Th subpopulations (Figure 3). In particular, a specific subset of T cells exhibit anti-inflammatory or immunoregulatory features and are termed Tregs. They are derived from naïve T cells in the presence of TGF-β and the absence of IL-6 which induces the expression of the transcription factor forkhead box P3 (FOXP3). RA and IL-2 are further required to promote Treg development and once fully differentiated they typically secrete IL-10 and TGF-β that inhibit effector functions of other immune cells and hereby maintain immune tolerance. Th1 differentiation is induced by IFN-γ and IL-12, secreted by APCs like macrophages and DCs, which in turn activate STAT4 and T-bet, respectively. Subsequently, these cells produce predominantly IFN-γ to control intracellular pathogens. In addition, IL-4 activates STAT6 which induces the transcription factor GATA-3 to promote Th2 cell development that secrete IL-4, IL-5 and IL-13, important in the defence against helminthes[49]. In 2005, a new subtype Th cell was

described, Th17. Its differentiation is mediated by IL-6 and TGF-β which induce IL-21 that activates in turn STAT3 and the Th17 lineage specific RORγt and RORα transcription factors[50]. As a consequence, these cells selectively secrete high levels of IL-17A, IL17F, IL-21 and IL-22. Another cytokine is also involved, namely IL-23 that promotes the maintenance, but not the commitment to the Th17 lineage[51]. Afterwards, they migrate to the intestinal mucosa via their expression of CCR9 and α4β7 and distribute throughout the LP. Most of the T cells in the LP of the gut express the surface markers CD4 ("helper" cell) and CD8 ("cytotoxic or effector" cell) although $CD8^+$ T cells preferentially migrate to the epithelium. After priming of B cells in the GALT, they undergo immunoglobulin class switching from IgM to IgA-producing plasma cells in response to TGF-β and IL-10. Once they arrive in the LP mediated by the gut-homing receptors, they complete their differentiation and secrete IgA into the gut lumen[52]. The latter are specific to the sampled antigen and prevent microbial pathogens and antigens from attaching to the epithelium by a process called immune exclusion which involves antigen agglutination, entrapment in mucus and clearance through peristalsis[53].

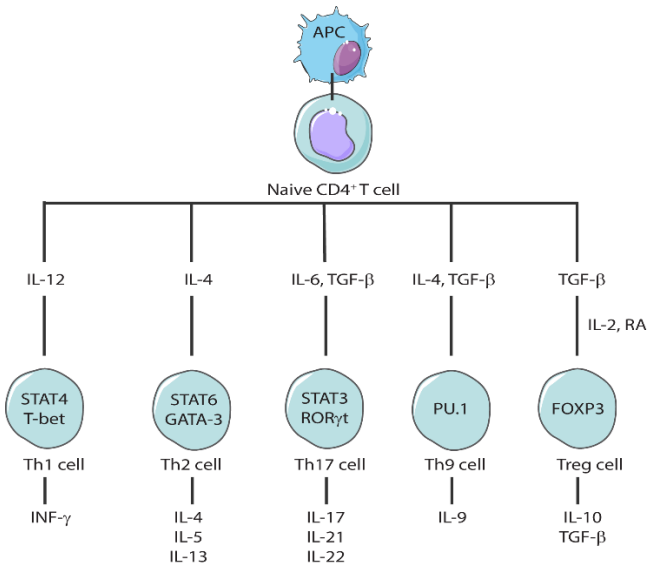

Figure 3: *Differentiation of helper $CD4^+$ T cells. When an APC presents an antigen to a naïve T cell, differentiation of that naïve T cell occurs into a Th subtype which is determined by the cytokine milieu. In the presence of IFN-γ and IL-12, naïve T cells differentiate into Th1 cells after activation of STAT4 and T-bet and they typically secrete INF-γ. Th2 cells develop when IL-4 is present and leads to the activation of STAT6 and GATA-3. Subsequently, these Th cells produce IL-4, IL-5 and IL-13. The combination of IL-4 and TGF-β on the other hand results in the differentiation of Th9 cells which can be discriminated from the other Th subtypes through their secretion of IL-9 under the control of PU.1. Absence of IL-4, but presence of IL-6 together with TGF-β gives rise to Th17 cells. This subtype exhibits STAT3 and RORγt activation and releases high amounts of IL-17, IL-21 and IL-22. Besides pro-inflammatory, also anti-inflammatory Th cells exist which are termed Tregs. They develop in the presence of TGF-β, but importantly the absence of IL-4 and IL-6 and secrete IL-10 and TGF-β after activation of FOXP3.*

1.3.3.3 Aberrant immune response in IBD patients

In IBD, the inflammatory reaction cannot be resolved and becomes chronic due to aberrant immune functioning. Therefore, a dysfunctional immune cell response may very well be the primary driver of intestinal inflammation. Hereafter, I will elaborate on the aberrant functions of macrophages, DCs and LP T lymphocytes in IBD, which are of particular relevance for this thesis.

Macrophages

In the case of IBD, there is an increased proportion of macrophages expressing CD14, a co-receptor for LPS signalling, in the inflamed, but not in the normal mucosa which may account for the exaggerated production of inflammatory mediators, leading to the perpetuation of mucosal inflammation[54]. Indeed, it was demonstrated that this $CD14^+$ subset of macrophages produced larger amounts of pro-inflammatory cytokines than resident intestinal macrophages, especially in CD patients[55]. Likewise, macrophages from UC patients display an exuberant expression of pro-inflammatory cytokines and chemokines like IL-6 and C-X-C motif ligand (CXCL) 10 in response to heat-killed *E. coli* when compared with healthy controls. Although counterintuitive, CD patients exhibit an impaired primary response to heat-killed *E.coli* due to abnormally low cytokine secretion from monocyte-derived macrophages[56] which indicates the existence of different macrophage subpopulations of which the function and contribution to IBD pathogenesis is far from being fully understood. The LP from IBD patients, predominantly CD patients, exhibit increased numbers of M2 macrophages, but also massive accumulation of M1 macrophages resulting in the dominance of the latter subtype[44]. However, adoptive transfer of M2 macrophages or molecules that skew their polarization to the M2 phenotype are effective in reducing the severity of experimental colitis[57-60]. In addition, both M1 and M2 macrophages have been implicated in the promotion of fibrosis, a major cause of intestinal strictures in CD patients, especially when M1/M2 activity persists due to unresolved inflammation. Not only M1, but mainly M2 macrophages that appear early in the wound-healing process are the predominant source of TGF-β. The latter is the key driver of fibrogenesis by inducing the differentiation of fibroblasts into collagen-producing myofibroblasts. In this context, it has been demonstrated *in vitro* that alternatively activated M2, in contrast to M1, macrophages increase the proliferation and collagen synthesis of human fibroblasts[61]. Furthermore, ARG-1 expressed by M2 macrophages activates the synthesis of proline, necessary for collagen synthesis by activated myofibroblasts and can hereby promote fibrosis[62]. Finally, pro-inflammatory macrophages produce high levels of TNF and IL-1β. These two cytokines have been demonstrated to promote epithelial-mesenchymal transition, myofibroblast activation through a TGF-β1-mediated mechanism and even promote myofibroblast proliferation[63, 64].

They also increase IL-6 release which induces Th1 cell responses and hereby drives fibrosis in unresolved inflammation[65].

DCs

The functions of the human DC subtypes remain largely elusive. Active IBD patients exhibit a decrease in the total LP CD103$^+$ DCs which also have a reduced expression of the ALDH enzymes only in UC patients with active disease and those in remission. In addition, the total number of ALDH$^+$ cells is reduced in these patients regardless of inflammation[66]. ALDH enzymes are involved in the production of RA which has many immunomodulatory properties including the promotion of Treg differentiation. The reduced abundance of ALDH$^+$ cells may therefore contribute to the reduced numbers of Treg in these patients. In contrast to myeloid DCs from the healthy LP which are hyporesponsive to TLR ligands due to low TLR2 and TLR4 expression, myeloid DCs from CD and UC patients express enhanced levels of TLR4 and higher numbers of activated DCs (more CD40$^+$ DCs) are observed.[67]. Only DCs from active UC patients and patients in remission express increased levels of TLR2[67]. Furthermore, higher frequencies of myeloid DCs have been found in IBD patients, but they also produce significantly more IL-8 and TNF after LPS stimulation compared with the levels from healthy subjects[67]. Unique for active CD patients is the elevated numbers of IL-23 secreting macrophages and DCs, but also IL-6 and IL-12 producing DCs, while this was not observed in the inflamed mucosa of UC patients nor in healthy controls[68, 69].

T lymphocytes

IBD is characterized by a dysregulated immunologic response against commensal bacterial antigens which is reflected by an imbalance in cytokines production and may therefore contribute to disease pathogenesis. In the past, CD was believed to be a Th1 disorder as LP T cells from the inflamed CD mucosa secrete increased amounts of IFN-γ compared with control LP T cells[70]. Also, defective mucosal T cell apoptosis is observed in CD patients leading to the persistence of a hyperactive T cell population[71]. In addition, IBD is characterized by a decrease in Tregs due to increased apoptosis[72, 73]. Moreover, the administration of Tregs is able to prevent, but also cure established experimental colitis[74, 75] and a clinical response after Treg treatment has been demonstrated in 40% of refractory CD patients[76]. UC patients on the other hand were thought to be mainly associated with a Th2 response because of the lack of elevated IFN-γ and elevated secretion of IL-5 and IL-13 from LP T cells and natural killer T cells, respectively[70, 77]. Besides Th1 and Th2 cytokines, there is increased expression of IL-17 in the sera and the inflamed mucosa of both UC and CD patients due to an elevated number of IL-17$^+$ cells including T cells and macrophages, indicating an important role of the Th17 subtype in IBD pathogenesis[78]. Recently, a crucial role for Th9 cells in the pathogenesis of UC has been described. This

T cell subtype develops in the presence of IL-4 and TGF-β to produce the cytokine IL-9 under the transcriptional control of PU.1 (Figure 2). Gerlach and co-workers demonstrated that the inflamed mucosa of UC, and not CD patients showed higher expression of IL-9 due to enhanced numbers of IL-9 producing mononuclear cells and $CD4^+$ T cells. Moreover, IL-9-deficient mice and mice treated with an anti-IL-9 antibody were protected in experimental models of colitis through the preservation of barrier function by suppressing the expression of the epithelial pore-forming tight junction protein claudin-2[79].

1.3.4 The microvasculature

Another factor that may exert a causative role in the development IBD is vascular dysfunction, mainly driven by the presence of pro-inflammatory cytokines and the vascular endothelial growth factor A (VEGF-A). The intestinal mucosa from IBD patients exhibits an increase in blood vessel density (angiogenesis) which directly correlates with the severity of the disease[80]. Moreover, several lines of evidence indicate that these newly formed blood vessels in IBD patients are dysfunctional and sustain inflammation, but also precede and lead to a decrease in the local mucosal oxygen levels (Figure 4).

1) Enhanced vascular permeability

The presence of tissue edema in IBD patients and in experimental IBD provides indirect evidence that the blood vessels are leaky. Cytokines and the pro-angiogenic growth factor VEGF-A play a major role in the regulation of vascular permeability. In particular, Oshima and colleagues demonstrated that IFN-γ disrupts the endothelial barrier by reducing the expression of the tight junction protein occludin which was reversed by pretreatment with IL-10. In accordance, both $CD45RB^{high}$ SCID and $IL10^{-/-}$ mice, models for chronic intestinal inflammation, exhibit increased IFN-γ expression and colonic microvascular leakage[81]. Tolstanova and co-workers demonstrated that anti-VEGF-A antibody treatment attenuates the severity of experimental UC at least in part by diminishing colonic vascular permeability[82].

2) Increased expression of cell adhesion molecules

Endothelial cells from IBD patients exhibit increased levels of endothelial cell adhesion molecules (ECAMs) which allow enhanced binding with recruited leukocytes and hence extravasation into the inflamed tissues. ECAM up-regulation has also been demonstrated in animal models of IBD and is linked to the severity of the disease[83-85]. These findings have initiated the development and evaluation of anti-adhesion therapeutics for IBD and led to the successful use of vedolizumab (anti-α4β7 integrin monoclonal antibody) in the clinic today. Up-regulated ECAMs include intercellular adhesion molecule 1 (ICAM-1), VCAM-1 and MAdCAM-1, but also selectins including E-and P-selectin[86-88]. ECAM

expression increases in response to pro-inflammatory cytokines such as IL-1β and TNF,[89] while the addition of VEGF-A has a potentiating effect on the TNF-induced increase of ICAM-1 and the selectins[90, 91]. Another ECAM involved in IBD pathogenesis is platelet and endothelial cell adhesion molecule 1 (PECAM-1). This protein is not up-regulated by pro-inflammatory cytokines, but is redistributed from the endothelial intercellular junctions to the endothelial surface in response to pro-inflammatory mediators[92]. Moreover, its inhibition reduces leukocyte adhesion and attenuates the severity of experimental colitis[93]. Given the success of blocking the α4β7 integrin, MAdCAM-1 targeting is now also under investigation. Phase 2 clinical trials with an anti-MAdCAM-1 antibody (PF-00547659) in UC and CD have now been completed and demonstrated preliminary safety and efficacy, but the results are not published yet.

3) Impaired vasodilation

Chronically inflamed IBD arterioles have diminished nitric oxide (NO)-mediated vasodilatory capacity in response to the vasodilator acetylcholine compared with non-IBD arterioles[94]. In addition, Binion and colleagues were the first to isolate and culture pure populations of human intestinal microvascular endothelial cells (HIMECs) and showed that inflamed IBD HIMECs produce less NO. This is caused on the one hand by decreased inducible NO synthase (iNOS) expression which subsequently results in leukocyte hyperadhesion[95-97] and on the other hand due to the induction of the NOS competitors ARG-I and ARG-II by inflammatory cytokines like IL-1β and TNF[98]. Reduced endothelium-dependent vasodilation in IBD may also result from the decreased endothelial NOS activity, the predominant source of vascular NO[99]. Moreover, eNOS-deficiency aggravates the severity of experimental IBD[100]. Besides decreased expression of a vasodilator, elevated levels of the vasoconstrictor peptides endothelin (ET)-1 and ET-2 were found in the colonic mucosa of UC and CD patients[101].

4) Increased platelet aggregation and coagulation

Spontaneous platelet aggregation is a feature in peripheral blood of IBD patients[102] and may contribute to the increased risk of systemic thromboembolisms seen in these patients. In this regard, Bernstein and colleagues demonstrated that IBD patients have a 3-fold increased risk for deep vein thrombosis and pulmonary emboli compared with the general population[103]. Also, microinfarctions are often detected by histology in inflamed biopsies from IBD patients [104]. Moreover, IBD seems to be a risk factor for thromboembolic events as it confers a 3.6-fold increased risk compared with other chronic inflammatory diseases like rheumatoid arthritis[105]. Indirect evidence for a role of coagulation in the pathogenesis of IBD is for example that both UC and CD patients have a decreased risk of developing hemophilia and von Willebrand's disease[106]. In addition, increased expression of markers of blood coagulation have been described in IBD patients including prothrombin fragment 1 and 2, thrombin

anti-thrombin complex, fibrinopeptide A/B and von Willebrand factor (vWF)[107, 108]. Besides proteins in the coagulation cascade, increased levels of soluble CD40 ligand, secreted by activated platelets, have been reported in IBD patients and leads to binding of platelets and immune cells to HIMEC[109]. Furthermore, the decreased NO production by IBD HIMEC may also contribute to the increased platelet aggregation since endogenous NO is able to inhibit adhesion of platelets to vascular endothelium[110].

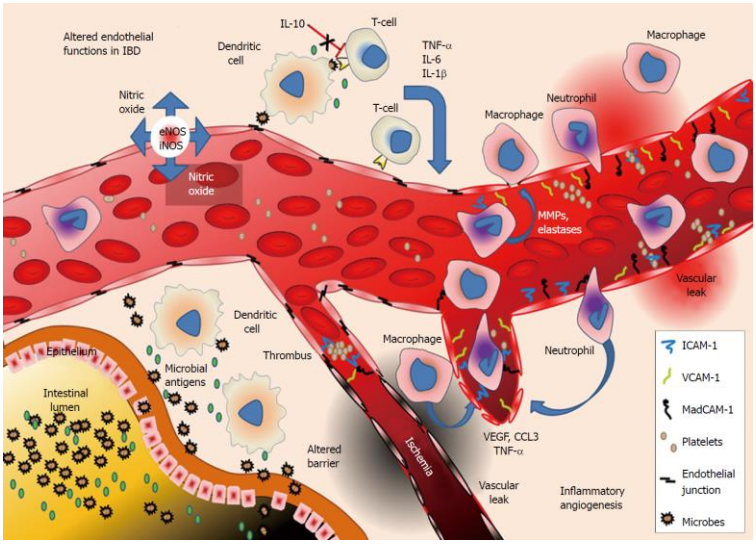

Figure 4. *Inflammation-induced vascular dysfunction. Inflammation triggers a change in the endothelium of the intestinal vasculature in response to the cytokines, chemokines and growth factors released by immune cells leading to increased angiogenesis, adhesion molecule expression, leukocyte extravasation, impaired vasodilation, decreased endothelial barrier function and increased coagulation. Adapted from Cromer et al. 2011[111].*

1.4 Diagnosis of IBD and current therapeutics

To date, the final diagnosis of UC or CD is based on combined information from the patient history, endoscopic, radiological and histological findings and negative stool cultures for infectious agents. When a diagnosis of CD is made, patients are further subdivided using the Montreal classification to aid clinicians in assessing disease prognosis and particularly to choose the most appropriate therapy [112]. Patients are herein characterized based on the age of onset (A), the location (L), the behavior (B) and the severity (S) of the disease.

There is no treatment to cure UC or CD. Current treatment strategies aim to dampen the inflammatory process in order to achieve symptom resolution and mucosal healing. Treatment options include 5-aminosalicylates (for UC only), corticosteroids, immunosuppressives such as thiopurines, methotrexate and cyclosporine, but also biologic drugs are used like TNF antagonists, vedolizumab which binds the integrin α4β7 on a subpopulation of $CD4^+$ T lymphocytes and ustekinumab (for CD only), an antibody directed against the p40 subunit shared by IL-12 and IL-23. Enhanced secretion of these cytokines has been demonstrated in LP mononuclear cells specifically in CD and not in UC patients[113, 114]. Although, anti-TNF therapy revolutionized the management of IBD, 10%-30% of patients

do not respond to the initial treatment and 23%-46% of patients lose response over time[115]. Surgery is therefore the last resort for approximately 20%-30% of UC and 30%-40% of CD patients at some point in their life[116]. Hence, there is a great need for new therapies to induce and maintain remission.

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2 Hypoxia and hypoxia-induced signalling as therapeutic targets for inflammatory bowel diseases

The following part of this introduction is written as a review paper and elaborates on the involvement of hypoxia and hypoxia-induced signalling in the pathogenesis of IBD, the subject of this dissertation.

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Abstract

Tissue hypoxia occurs when local oxygen demand exceeds oxygen supply. In chronic inflammatory conditions such as inflammatory bowel disease (IBD), the increased oxygen demand by resident and gut-infiltrating immune cells coupled with vascular dysfunction brings about a severe reduction in mucosal oxygen concentrations which is a life-threatening situation for all mucosal members. In order to counter the hypoxic challenge and ensure their survival, mucosal cells induce adaptive responses, including the activation of hypoxia-inducible factors (HIFs) and modulation of nuclear factor kappa B (NFκB). Both pathways are tightly regulated by oxygen-sensitive prolyl hydroxylases (PHDs), which therefore represent promising therapeutic targets for IBD. In this review, we discuss the involvement of mucosal hypoxia and hypoxia-induced signalling in the pathogenesis of IBD and elaborate in detail on the role of HIFs, NFκB and PHDs in different cell types during intestinal inflammation. We also provide an update on the development of PHD inhibitors and discuss their therapeutic potential in IBD.

Keypoints:

- Mucosal hypoxia is an integral component of inflammatory bowel disease (IBD)
- Hypoxia-induced signalling by hypoxia-inducible factors and nuclear factor kappa B can promote or counteract the intestinal inflammatory response, depending on the context and cell type studied.
- Oxygen-sensitive prolyl hydroxylases (PHDs) tightly regulate hypoxia-induced signalling pathways and have been identified as promising therapeutic targets in IBD.
- Pan-hydroxylase inhibitors are in an advanced stage of development for the treatment of chronic kidney disease-related anaemia and are in phase 1 for the treatment of ulcerative colitis.
- The use of orally administered and isotype-specific PHD inhibitors may reduce systemic exposure and the risk of unwanted side-effects.

Evidence for a role of hypoxia in the pathogenesis of human IBD

IBD, comprising ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders that result from a dysregulated immune response against luminal antigens in genetically predisposed individuals. Over the past decade, the presence of mucosal hypoxia during intestinal inflammation has become evident. Hypoxic staining using 2-nitroimidazole compounds revealed physiologic hypoxia in epithelial cells while progressive staining into the mucosa is observed during murine colitis[1]. -In addition, increased levels of hypoxia-induced transcription factors are detected in inflamed colonic samples from IBD patients[2, 3] and a positive correlation with the severity of UC has been demonstrated[4]. Although these findings indicate that mucosal hypoxia is an integral component of IBD, the exact consequences on disease pathogenesis and evolution are currently unknown. However, increasing new insights have revealed that hypoxia and its induced signalling can evoke both a pro- and an anti-inflammatory response depending on the context and cell type studied which will be further elaborated on in the following paragraphs.

Physiological and pathological hypoxia in the intestinal mucosa

Under physiological conditions, the gastrointestinal tract is characterized by a steep oxygen gradient from the anaerobic lumen towards the highly vascularized submucosa[1] with the oxygen concentration ranging from less than 2% in the intestinal lumen to 3% at the villus tip while arteries in the submucosa exhibit a 80-100% oxygen concentrations[5, 6]. During active inflammation, resident macrophages and dendritic cells (DCs) become activated and produce pro-inflammatory cytokines and chemokines that trigger T cell differentiation and the recruitment of inflammatory cells from the peripheral blood into the mucosa. The infiltrated leukocytes consume a large amount of the local oxygen in the mucosa and submucosa[7]. At the same time, the oxygen supply from the blood stream is decreased during inflammation as a result of microvascular occlusion and thrombosis[8]. The resulting imbalance between oxygen consumption and supply renders the inflamed intestinal mucosa severely hypoxic.

The hypoxia-induced transcriptional machinery

Mammalian cells possess evolutionary conserved endogenous mechanisms that allow them to respond to low-oxygen conditions. This response is centrally regulated by oxygen-sensitive hydroxylases, which control the activity of the oxygen-sensitive transcription factors hypoxia-inducible factors (HIFs) 1-3 and nuclear factor kappa B (NFκB) (Figure 1).

HIFs

HIFs are heterodimeric beta helix-loop-helix proteins composed of an unstable oxygen-sensitive α-subunit (HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed stable β-subunit (aryl hydrocarbon receptor nuclear translocator, ARNT or HIF-1β). Under normal oxygen conditions, HIFα subunits are regulated in two ways. Firstly, through transcriptional inactivation caused by asparaginyl (Asn^{803}) hydroxylation of HIFα by the factor inhibiting HIF (FIH). This hydroxylation event inhibits the interaction of HIFα with the transcriptional coactivators cAMP-response element binding (CREB) binding protein (CBP) and p300. Secondly, via prolyl-hydroxylase domain-containing proteins (PHD1-PHD3). The PHD enzymes hydroxylate 2 highly conserved prolyl residues within the LXXLAP motif of the oxygen-dependent degradation domains (ODDDs) of HIFα. Hydroxylation of either of these proline residue generates a binding site for the von Hippel-Lindau (pVHL) E3 ubiquitin ligase. Subsequently, pVHL chaperones HIFα subunits for ubiquitination and eventual proteasomal degradation. PHDs and FIH belong to a family of oxygenases that utilize oxygen and 2-oxoglutarate (2-OG, also known as α-ketoglutarate (KG)) as co-substrates and iron (Fe^{2+}) and ascorbic acid (vitamin C) as cofactors. As oxygen is the most ubiquitous factor controlling the PHD and FIH-catalysed hydroxylation reactions, hypoxia leads to the stabilization of HIFα. Once stabilised, HIFα translocates to the nucleus where it binds to its β subunit and form the active transcription factor HIF. The transcription factor complexes HIF1 and HIF2 recruit cofactors such as CBP and p300 and attach to hypoxia-responsive elements (HRE) within target genes involved in cell survival, angiogenesis and metabolism[9-13]. HIFs can also regulate the transcription of PHD2 and PHD3. Although hypoxic conditions inhibit their enzymatic activity, PHD2 and PHD3 proteins are present and as soon as oxygen is reintroduced, they initiate the signaling for HIF degradation and hereby promote a negative feedback mechanism[14].

NFκB

Apart from HIF, hypoxia also activates NFκB through the phosphorylation of its inhibitor IκBα [15]. Later on, it was demonstrated that only the canonical (and not the non-canonical) arm is oxygen-sensitive [16, 17]. However, the hypoxia-induced HIF-1 DNA binding activity is higher than for NFκB, especially at 1% O_2[18]. PHDs inhibit NFκB through their interaction with and inactivation of IκB kinase (IKK)β[18-20]. Although PHD2 mediates this event through its hydroxylase activity, this has yet to be demonstrated for PHD1. PHD3 inhibits both IKKβ and IKKγ independent of its hydroxylase activity but by blocking the interaction between IKKβ and heat shock protein 90 and between IKKγ and cellular inhibitor of apoptosis (cIAP)1[20, 21]. The PHD-dependent inactivation of IKKβ/IKKγ prevents phosphorylation of the NFκB inhibitor IκBα, which masks NFκB's nuclear localization signal (NLS). During hypoxia, the IκB

kinase (IKK) complex becomes active and subsequently phosphorylates IκBα[15]. Following ubiquitination, phosphorylated IκBα is degraded by the proteasome. Dissociation of IκBα from NFκB heterodimers p50/p65 during hypoxia reveals the NLS, which enables nuclear translocation and subsequent transcription of target genes involved in inflammation and cell survival[22-25]. FIH has also been demonstrated to hydroxylate members of the NFκB-pathway, namely p105 (the precursor of p50) and IκBα. However, the impact of these hydroxylation reactions still remains speculative[26].

HIF-NFκB crosstalk

Although HIF and NFκB can regulate the expression of genes in response to hypoxia independent of each other, there is a considerable degree of crosstalk between these transcription factors. It has been reported that NFκB activation is involved in the control of basal HIF-1α mRNA levels through binding of the p50 and p65 NFκB subunits to kB binding sites in the HIF-1α promotor [27]. Also, overexpression of NFκB results in HIF-1α protein expression under normoxic conditions [28]. *In vivo*, IKKβ-deficient mice exhibit decreased HIF protein levels and HIF-dependent gene expression [29]. In addition, IKKγ induces the activation of NFκB but also promotes the binding of HIF-2α with CBP and p300 and subsequently increases HIF-2 transcriptional activity [30]. Besides being induced by hypoxia, other non-hypoxic stimuli like lipopolysaccharide (LPS) and pro-inflammatory cytokines evoke a NFκB -dependent up-regulation of HIF-1 mRNA levels [31-33]. Conversely, it has been demonstrated that HIF can also promote NFκB activation in neutrophils and keratinocytes[25, 34].

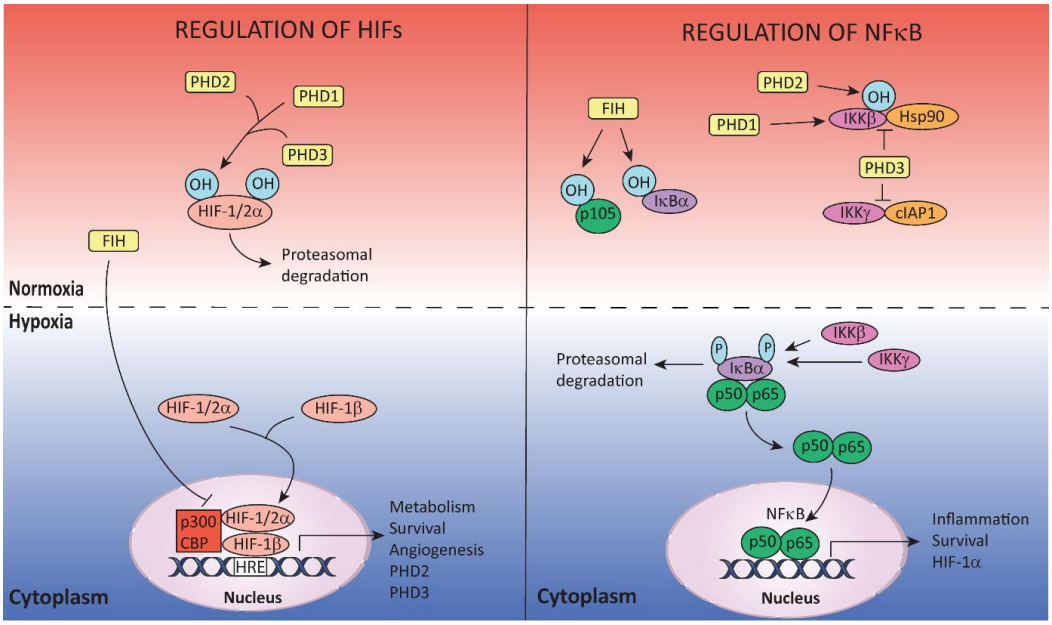

Figure 1. *Regulation of HIF and the canonical arm of the NFκB pathway through PHDs and FIH. Left: Regulation of HIF through PHDs and FIH. Under normoxia, PHDs and FIH hydroxylate the HIF-1α and HIF-2α subunits. Prolyl hydroxylation leads to proteasomal degradation of the HIFα subunits while asparaginyl hydroxylation inhibits the interaction of HIFα with the coactivators CBP and p300. During hypoxia, the enzymatic activity of the PHDs and FIH is inhibited leading to the stabilization of the HIFα subunits. After translocation to the nucleus, they complex with their β subunit, recruit p300 and CBP and bind to the HRE in the promotors of their target genes to initiate transcription. Right: Regulation of the canonical arm of the NFκB pathway through PHDs and FIH. PHDs prevent the activation of NFκB through their interaction with and inactivation of IKKs. In particular, PHD2 hydroxylates IKKβ but this is yet to be confirmed for PHD1 while PHD3 blocks the interaction between IKKβ and Hsp90 and of IKKγ with cIAP1, independent of its hydroxylase activity. FIH has been shown to hydroxylate IκBa and the p50 precursor p105 but these reactions do not impact the NFκB activation status. During hypoxia IKK proteins phosphorylate the NFκB inhibitor IκBa which results in its ubiquitination and subsequent proteasomal degradation. Dissociation of IκBa allows the nuclear translocation of the NFκB heterodimer followed by the transcription of its target genes. HIF: hypoxia-inducible factor; Hsp: heat shock protein; cIAP: cellular inhibitor of apoptosis; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; IKK: IkB kinase; PHD: prolyl hydroxylase; FIH: factor inhibiting HIF; HRE: hypoxia responsive elements; CBP: cAMP-response element binding protein.*

The pro-inflammatory response to hypoxia

Several cellular mechanisms explain how hypoxia can induce an inflammatory response in the gut and hence be an environmental risk factor for IBD patients. When the partial oxygen pressure drops below the physiologic level, intestinal epithelial cells release pro-inflammatory cytokines such as TNF that potentiate the increase in epithelial permeability during inflammatory conditions[35, 36]. In addition, enhanced epithelial hypoxia induces epithelial cell apoptosis[37] which further promotes disruption of the epithelial barrier.

Inflammation-induced hypoxia also affects the innate immune cells within the lamina propria. Hypoxia induces the expression of β2 integrin on leukocytes, which enhances adhesion to endothelial cells and extravasation[38]. Hypoxic neutrophils exhibit a delayed apoptosis rate resulting in impaired resolution

of inflammation[39, 40]. A similar resistance to apoptosis is seen in a subset of macrophages after repeated hypoxic exposure[41]. It has been shown that these hypoxia-tolerant macrophages carry a pro-inflammatory phenotype and produce more TNF than hypoxia-sensitive macrophages[41]. Likewise, hypoxia-exposed dendritic cells show increased expression of pro-inflammatory cytokines and chemokines such as IL-22, CXCL2 and CCL20[42, 43]. In addition, hypoxia induces the expression of triggering receptor expressed on myeloid cells (TREM-1) in mononuclear phagocytes, this signalling has been associated with IBD and promotes the release of pro-inflammatory cytokines and chemokines[44-46].

Finally, hypoxia-exposed endothelial cells release prostaglandins (PG) which attract neutrophils[47]. In addition, they increase their adhesiveness for neutrophils through the up-regulation of platelet-activating factor (PAF) and P-selectin[48]. Furthermore, they exhibit a perturbed anticoagulant function through the decrease of thrombomodulin, a disrupted endothelial barrier integrity[49] and an enhanced expression of pro-inflammatory cytokines[48]. Hypoxia also drives the formation of new blood vessels (angiogenesis) through the up-regulation of vascular endothelial growth factor (VEGF-A)[50], an angiogenic growth factor known to worsen the outcome of experimental colitis[51].

The anti-inflammatory response to hypoxia

Prolonged hypoxia would lead to uncontrolled excessive intestinal epithelial cell death and severe inflammation, if it were not for the fact that mammalian cells recognize hypoxia as a danger signal and initiate adaptive mechanisms.

Interestingly, the low-grade hypoxia of intestinal epithelial cells during physiological conditions offers them unique barrier-preservative properties as compared with other epithelia[52, 53]. In particular, hypoxia-exposed intestinal epithelial cells exhibit an altered gene expression pattern in order to preserve barrier function of the gut and reduce the inflammatory burden. These cells up-regulate barrier protective genes such as intestinal trefoil factor (ITF), mucin-3, MDR1, ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73)[52, 54-56]. The latter two increase extracellular adenosine production, which is known to enhance barrier function and reduce leukocyte activation and accumulation. Hypoxia further potentiates the anti-inflammatory effect of extracellular adenosine by inducing the epithelial expression of adenosine A2B receptor[57] and repressing its transporters[58, 59] on epithelial cells, hereby protecting against experimental colitis[60, 61]. In addition, the intestinal microbiota also promotes the epithelial basal hypoxic state. In particular, bacterial short-chain fatty acids like butyrate have been shown to enhance epithelial oxygen consumption and subsequently stabilize HIF-1α levels, induce target gene expression and reinforce the

epithelial barrier[62]. Hypoxia also induces epithelial expression of creatine kinases which participate in the assembly of tight and adherens junction proteins to preserve epithelial barrier integrity[63]. In addition, prolonged hypoxia represses the expression of FADD, a positive regulator of TNF-mediated apoptosis, and hereby dampens inflammation-induced barrier dysfunction[64]. Besides barrier protective genes, physiological hypoxia in intestinal epithelial cells can lead to the constitutive expression of the antimicrobial peptide β defensin-1[65]. In addition, during acute inflammation transmigrating neutrophils rapidly deplete the local oxygen levels which in turn induces the epithelial expression of hypoxia-dependent barrier protective genes aiming to resolve the local inflammation[7]. In addition, physiological hypoxia induces the expression of epithelial netrin-1, which attenuates neutrophil migration into the colon and through epithelial cells[66]. Yet physiological hypoxia also induces the epithelial expression of CD55, which increases the disengagement of neutrophils from the epithelial apical surface[67].

In addition to epithelial cells, hypoxic blood vessels exhibit the same up-regulation of CD39, CD73 and adenosine receptor A2bR and the concomitant repression of the equilibrative nucleoside transporters for the promotion of endothelial barrier[58, 68, 69]. Although these studies made use of human microvascular endothelial cells, they were isolated from the dermis. Therefore, it needs to be further investigated if these findings also hold for intestinal endothelial cells. Increased expression of CD73 has also been reported in hypoxia-exposed DCs[42].

Taken together, a dual response to hypoxia is generated which is context and cell type dependent (Figure 2).

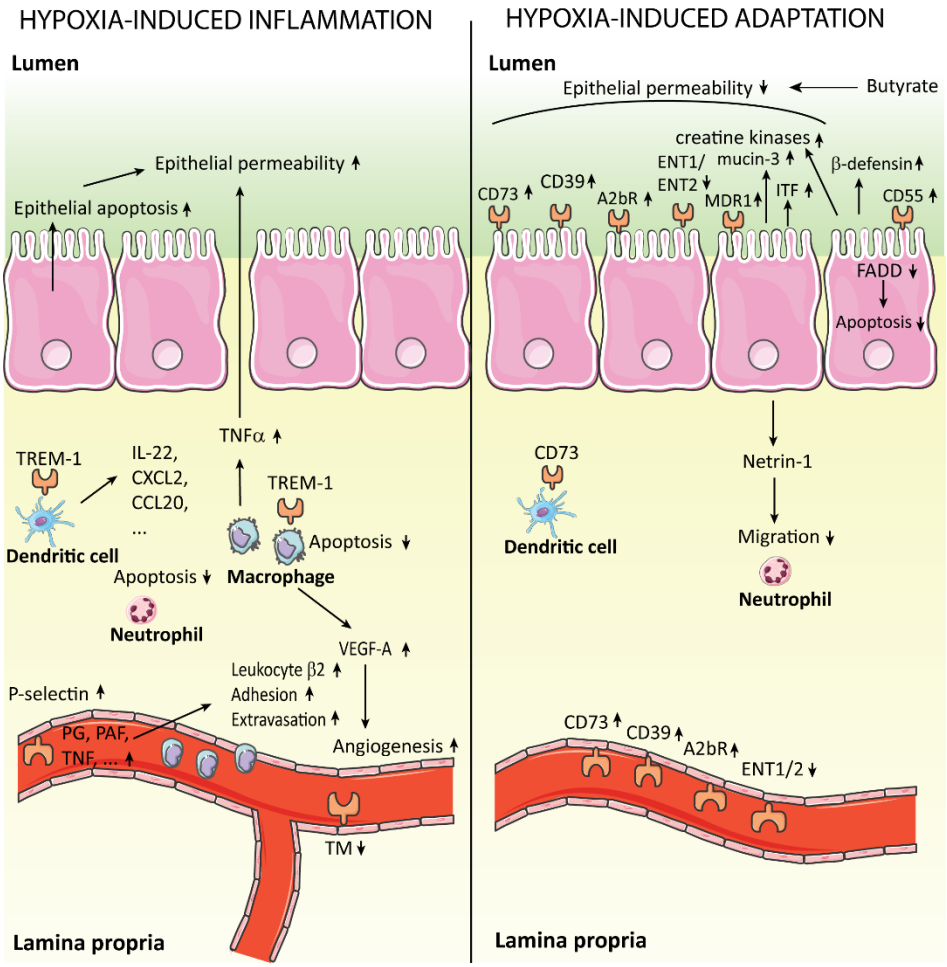

Figure 2. *Overview on the hypoxia-induced pro-inflammatory (left) versus adaptive response (right) in various cell types. Hypoxia increases the survival of myeloid cells and their expression of pro-inflammatory cytokines. An important cytokine is TNF which in turn increases epithelial permeability through the deregulation of TJ proteins. In addition, epithelial cells become apoptotic as hypoxia persists which further contributes to the disruption of barrier integrity. Besides pro-inflammatory cytokines, macrophages release the pro-angiogenic factor VEGF-A under hypoxic conditions. Also, hypoxia-exposed endothelial cells are pro-thrombogenic, up-regulate cell-adhesion molecule expression and secrete pro-inflammatory mediators which attract immune cells, increase their adhesion and extravasation. Right. Intestinal epithelial cells are well-equipped against the local physiological hypoxic state through the increased expression of barrier protective proteins, receptors involved in adenosine signalling while concomitantly down-regulating the expression of adenosine transporters. These proteins can also all be induced by butyrate, released by gut bacteria, and hence increase barrier integrity. In addition, they prevent neutrophil migration into the mucosa via the release of netrin-1 and enhance the apical neutrophil shedding by up-regulating the expression of CD55. Also, anti-microbial peptides are produced. In addition to the epithelium, endothelial cells and DCs also exhibit increased expression of the adenosine receptors. A down-regulation of adenosine transporters has also been demonstrated on endothelial cells. TJ: tight junction; TM: thrombomodulin; VEGF-A: vascular endothelial growth factor; PG: prostaglandins; PAF: platelet activating factor; TNF: tumor necrosis factor; TREM-1: triggering receptor expressed on myeloid cells; MDR1: multidrug resistance protein 1; IL: interleukin; CXCL: (C-X-C) motif ligand; CCL20: (C-C motif) ligand; ENT: equilibrative nucleoside transporter; ITF: intestinal trefoil factor; A2bR: adenosine 2b receptor; CD: cluster of differentiation.*

Hypoxia-signalling pathways in cellular players during intestinal inflammation

The fact that hypoxia-induced signalling is a prominent feature of active IBD has initiated extensive research to unravel the contribution of HIFs, NFκB and PHDs in IBD pathogenesis and explore their

potential as therapeutic targets. In the next section, we will elaborate on the role of the hypoxia-induced transcription factors and PHDs in various cell types that are involved in intestinal inflammation.

HIFs

Epithelial cells

The involvement of epithelial HIF-1 and HIF-2 has been particularly well-studied in experimental models of IBD (Table 1). Karhausen and co-workers were the first to prove that epithelial *Hif1α*-deficiency renders mice more susceptible to 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis [70]. Similarly, the same authors reported that constitutively active epithelial HIF-1 via disruption of the von Hippel-Lindau tumor suppressor gene (Vhlh) using $Vhlh^{2lox/2lox}$ Fabp1:cre ameliorates both oxazolone and TNBS-induced colitis [70]. The primary mechanism through which epithelial HIF-1 confers protection during colitis seems to be by preserving intestinal barrier function. HIF-1 induces the expression of the previously mentioned barrier-protective genes such as ITF, MDR1, mucin-3, CD73, CD55 and netrin-1[52, 54, 55, 66, 67, 70], enhances extracellular adenosine signalling while repressing adenosine transporters and genes involved in epithelial apoptosis such as FADD[58, 59, 64, 66].

While the available literature consistently points towards a protective role of HIF-1 in intestinal epithelial cells, most studies describe an opposite role for HIF-2, indicating that HIF-1 and HIF-2 have different target genes.

A chronic increase in colon epithelial HIF-2α signaling by epithelial-specific Vhlh ablation in mice resulted in a hyper-inflammatory response[71]. In agreement, intestinal epithelial-specific deletion of *Hif-2α* protects mice from acute colitis by reducing pro-inflammatory cytokine expression[72]. HIF-2α also induces the expression of caveolin-1 which promotes the degradation of occludin and in turn leads to increased colonic permeability[73]. Rather conflicting findings were reported in epithelial-specific HIF-1β deficient mice. These mice are more prone to TNBS-induced colitis through enhanced barrier defects and proposed a HIF-2α, and not HIF-1α, dependent loss of creatine kinases as the underlying mechanism[63]. However, Saeedi *et al.* latterly demonstrated that through deletion of HIF-1β, the HIF-1α-dependent induction of the tight junction protein claudin-1 was inhibited, hereby providing an additional contributing factor for the disrupted epithelial barrier in epithelial-specific HIF-1β deficient mice[74]. Both HIF-1α and HIF-2α are increased in intestinal epithelia from active UC and CD patients[72].

Table 1. Effect of intestinal epithelial-specific genetic HIF modulation on the course of experimental IBD

Isoform subunit	Modulation approach	IBD model	Outcome	Identified mechanism(s)	Ref.
HIF-1α	Overexpression	Oxazolone and 2,4,6-trinitrobenzene sulphonic acid TNBS-induced colitis	Beneficial	Induction of several barrier-protective genes	[70]
HIF-2α	Overexpression	Dextran sulfate sodium (DSS)-induced colitis	Detrimental	Increased infiltration of inflammatory cells and pro-inflammatory cytokine release	[71]
HIF-2α	Deletion	Citrobacter rodentium and DSS-induced colitis	Beneficial	Reduction of pro-inflammatory cytokine release	[72]
HIF-2α	Deletion	DSS-induced colitis	Beneficial	Induction of the tight junction protein occludin	[73]
HIF-1β	Deletion	DSS- and TNBS-induced colitis	Detrimental	Loss of creatine kinases expression	[63]
HIF-1β	Deletion	TNBS-induced colitis	Detrimental	Loss of the tight junction protein claudin-1	[74]

Endothelial cells

A role for HIF-1 and HIF-2 in endothelial cells has mainly been studied in the context of cancer, while little is known about the involvement of HIF-1 and HIF-2 in the hypoxic responses of intestinal endothelial cells. Furthermore, the consequences of intestinal endothelial-specific loss of HIF-1α or HIF-2α have not been explored in experimental IBD thus far. What we do know that could be relevant in the context of intestinal inflammation is that HIF-1 ensures endothelial barrier integrity under hypoxic conditions through its repression of equilibrative nucleoside transporter 1[58]. HIF-2 on the other hand also participates in the preservation of endothelial barrier but under normoxic and not hypoxic conditions, via activation of the transcription of the junctional protein VE-cadherin[75]. In addition, Coulet *et al.* demonstrated that hypoxia induces the expression of endothelial nitric-oxide synthase (eNOS) in a HIF-2α-dependent manner[76]. Decreased eNOS expression is observed in IBD and reduces endothelium-dependent vasodilation, a hallmark of dysfunctional blood vessels.

Taken together, HIF-1 and HIF-2 have the potential to normalize dysfunctional microvessels which could beneficially alter the course of colitis.

Fibroblasts

A role of HIF-1α and HIF-2α has also been described in the context of fibroblasts. In particular, HIF-1 activates the expression of collagen and lysyl hydroxylases, which are required for collagen deposition and extracellular matrix (ECM) stiffening. These are 2 important events in the promotion of

fibrogenesis[77]. In addition, hypoxia and HIF-1α up-regulate the synthesis of TGF-β, an important growth factor that initiates the differentiation of fibroblast into myofibroblasts which subsequently produce ECM components [78]. Also, lactic acid which accumulates as a result of HIF-1 mediated glycolytic metabolism, represents another trigger for TGF-β activation[79]. Conversely, it has also been reported that TGF-β can induce the expression of HIF-1α[80], which may therefore have a potentiating effect on the development of intestinal fibrosis. On the other hand, HIF-1 and HIF-2 activation is also able to cease fibroblast proliferation by inducing cell-cycle arrest independent from hypoxia[81]. As was previously discussed for endothelial HIF-1 and HIF-2, the involvement of fibroblast-specific HIF-1 and HIF-2 on intestinal fibrosis development remains to be conclusively determined.

The role of HIFs in innate immune cells

Neutrophils

In the following part, we discuss the role of HIFs in the different migratory cell types within the innate immune system in the gut and summarize the findings in Table 2.

Hypoxia promotes the survival of neutrophils and enhances glycolysis for their ATP production. Both events are mediated by HIF-1α as deletion of HIF1-α profoundly impairs these processes[25, 82]. Moreover, this disturbance in energy generation by loss of HIF-1α diminishes their main immune properties such as migration, invasion and bacterial killing[82-84]. The *in vivo* consequences of HIF-1 deletion in neutrophils during colitogenesis is currently unknown and not easy to predict, given the fact that proper functioning neutrophils are both necessary for induction and resolution of intestinal inflammation[85]. In CD, and not UC patients, it has been demonstrated that there is less neutrophil recruitment into the intestine due to decreased IL-8 release by resident macrophages, which results in delayed bacterial clearance[86, 87].

Information on the role of HIF-2α in neutrophils is scarce. So far, only one study reported that *Hif-2α*-deficient neutrophils exhibit enhanced apoptosis *in vivo* which reduces neutrophilic inflammation and tissue injury without an effect on neutrophil chemotaxis, phagocytosis or respiratory burst (a chemical reaction which generates hypochlorous acid and reactive oxygen species leading to the lysis of microbes)[84].

Macrophages

Like neutrophils, macrophages mainly rely on glycolysis to produce energy and this process is specifically controlled by HIF-1α[88]. Cramer *et al.* demonstrated that *Hif-1α*-deleted macrophages fail to produce enough ATP levels, which in turn compromises their survival, aggregation, motility, invasion and bacterial killing capability[82]. Macrophages can be generally divided into classical M1 macrophages and regulatory M2 macrophages. M1 macrophages are activated by IFN-γ and Toll-like receptor (TLR) ligands such as LPS to produce inducible NOS, pro-inflammatory cytokines, chemokines and cell adhesion molecules. They are essential for eradicating invading microorganisms but can cause bystander tissue damage in doing so, and therefore need to be controlled by M2 macrophages. M2 macrophages exhibit anti-inflammatory, pro-angiogenic, wound healing properties and are regulated by IL-4 and IL-13. M2 macrophages typically express low levels of pro-inflammatory cytokines but high levels of arginase 1 (Arg-1), chitinase-3 like 3, Fizz-1 and IL-10[89, 90]. While M1 macrophages rely on glycolysis for energy production, M2 macrophages utilize oxidative phosphorylation[91]. Since HIF-1α regulates the transcription of glycolytic enzymes, it has been suggested that HIF-1α activation promotes M1 polarization[91]. In support, hypoxia induces the expression of TLR4 through HIF-1 which results in an enhanced pro-inflammatory response after LPS stimulation[92]. In contrast, HIF-2α induction regulates the expression of Arg-1 and therefore causes a switch to the M2 phenotype[93]. However, the involvement of both HIF-1 and HIF-2 in macrophage plasticity is far from being fully understood. Especially for HIF-2α, it is still unclear if its activation really drives M2 polarization because it has also been reported that deletion of *Hif-2α* in macrophages attenuates their pro-inflammatory cytokine response following M1 stimulation which makes *Hif-2α*-deficient mice more resistant to LPS-induced endotoxemia[94].

DCs

HIF-1 activation in DCs is required for IFN, IL-22 and IL-10 production, promotes apoptosis and enhances differentiation and migration[42, 95, 96]. Dendritic cell-specific *Hif-1α* knockout mice are more susceptible to DSS-induced colitis, which is associated with an impaired Treg development through the reduced expression of DC-induced CCR9, a gut-homing T cell marker and aldehyde dehydrogenase-1a2, involved in Treg induction[97]. No published data is available on the role of HIF-2 in DCs.

Eosinophils, mast cells and basophils

Ablation of *Hif-1α* in eosinophils diminishes their chemotactic properties while the opposite is true for *Hif-2α*-deficient eosinophils[98]. *Hif-1α* knockdown in mast cells reduces their cytokine production such

as IL-8 and TNF following stimulation with TLR ligands while HIF-1α in basophils does not play an apparent role in TLR ligands-mediated cytokine release[99]. However, whether or not *Hif-1α* deficiency in the latter three cell types has a beneficial contribution on the course of colitis is currently unknown.

The role of HIF in adaptive immune cells

Besides their role in the innate immune compartment, HIFs have also been implicated in the regulation of T cell, B-cell and natural killer (NK) T cell functioning.

T cells

Constitutive activation of HIF-1 and HIF-2 through deletion of pVHL impairs thymocyte development with enhanced apoptosis of $CD4^+CD8^+$ T cells[100]. Furthermore, *Hif-1α* deficient T cells produce increased levels of IFN-γ and IL-2 while in contrast *Hif-1α*-deficient Th1 polarized T cells lose their capacity to produce IFN-γ[101]. In addition to its role in effector T cell responses, HIF-1 is also involved in Treg differentiation albeit with conflicting results. Ben-Shoshan *et al.* reported that HIF-1 induces the expression of FoxP3 which results in enhanced abundance of Treg[102] while Dang *et al.* showed that HIF-1 targets FoxP3 for proteasomal degradation and concomitantly induces the expression of RORyt which results in subsequent attenuation of Treg but enhanced Th17 development [103]. In the context of colitis however, HIF-1 seems to favor Treg differentiation since T cell specific *Hif-1α* knockout mice exhibit more severe colonic inflammation with increased Th1 and Th17 cells when subjected to DSS-induced colitis[104]. Also, *Hif-1α*-deficient Tregs fail to control T cell-mediated colitis[105].

NKT cells

HIF-2 limits the cytotoxicity of NKT cells by controlling their Fas ligand expression which suggests a rather anti-inflammatory role for HIF-2 in this cell type[106]. A role for HIF-1 in NKT cells is currently unknown.

B cells

Finally, HIFs have also been studied in B cells but to a much more limited extent. First of all, abnormal B cell development and autoimmunity occurs when *Hif-1α* is deleted in $Rag2^{-/-}$ mice[107]. In addition, it was reported that *Hif-1α*-deficiency abolishes hypoxia-induced cell cycle arrest[108]. Recently, it was demonstrated that stabilization of HIF through B cell specific deletion of pVHL decreases the proliferation and increases B cell death, impairs the generation of high-affinity IgG but switches to the pro-inflammatory IgG2c antibody isotype[109]. However, data on a B-cell specific role of HIFs in intestinal inflammation is lacking.

Table 2. Overview of the role of HIFs in migratory immune cells.

Cell type	Effect of HIF-1 modulation	Effect of HIF-2 modulation	Ref.
Neutrophils	Activation: - Increased survival - Increased migration - Increased invasion - Increased bacterial killing	Deletion: Increased apoptosis	25, 82-84
Macrophages	Deletion: - Decreased energy production - Decreased survival - Decreased aggregation - Decreased motility - Decreased invasion - Decreased bacterial killing Activation: - M1 polarization - Increased TLR4 expression	M2 polarization?	82, 88, 91-94
DCs	Deletion: Decreased Treg development Activation: - Essential for IFN, IL-22, IL-10 production - Increased apoptosis - Increased differentiation - Increased migration	Unknown	42, 95-97
Eosinophils	Deletion: Decreased chemotaxis	Deletion: Increased chemotaxis	98
Basophils	No role in TLR-ligands mediated cytokine release	Unknown	99
Mast cells	Deletion: Decreased IL-8, TNF	Unknown	99
T cells	Deletion: - Increased T cell IL-2 and IFN-γ - Decreased Th1 IFN-γ Activation: - Decreased thymocyte development -Treg development?	Deletion: Unknown Activation: Decreased thymocyte development	100-105
NKT cells	Unknown	Deletion: Increased cytotoxicity	106
B cells	Deletion: - Abnormal B-cell development -Decreased cell cycle arrest Activation: -Decreased proliferation and cell death -Enhanced IgG2c production	Unknown Activation: -Decreased proliferation and cell death -Enhanced IgG2c production	107-109

NFκB

The role of NFκB in inflammation has been extensively studied over the past 25 years and its crucial involvement in immune cell functioning has been reviewed elsewhere[110]. In this part of the review, we will focus on the role of the ~~of~~ canonical NFκB pathway during colitogenesis since only this branch of the pathway is oxygen sensitive.

Intravenous and rectal administration of p65 antisense oligonucleotides is effective in abolishing intestinal inflammation in TNBS-induced colitis and in IL10-deficient mice[111]. Likewise, the administration of a pharmacologic inhibitor of IkB destruction (BMS-345541) ameliorates DSS-induced colitis[112]. Also, IL10-/- mice with a deletion of IKKβ in myeloid cells exhibit an attenuation of spontaneous chronic colitis[113]. These findings suggest an adverse effect of NFκB activation on intestinal inflammation and it is generally accepted that this is caused by the promotion of leukocyte survival and the induction of pro-inflammatory cytokines, chemokines and reactive oxygen species such as iNOS[114].

However, the situation may be less straightforward than expected as NFκB activation is also needed for the resolution of inflammation by inducing immune cell apoptosis and the expression of anti-inflammatory cytokines such as IL-10[115]. This apparent paradox of an anti-inflammatory action of NFκB in immune cells was further strengthened by a study from Greten *et al.*, showing that myeloid IKKβ-deficient mice are more susceptible to endotoxic shock due to enhanced IL-1β secretion of IKKβ-deficient neutrophils and macrophages but also via reduced neutrophil apoptosis[116]. It seems likely that this is also true during experimental IBD. In agreement, one study reported that NFκB-deficient (p50-/-; p65+/-) mice are more susceptible to *Helicobacter hepaticus* (*Hh*)-induced colitis[117] mediated by the p50/p105 subunit from haematopoietic cells[118].

In the intestinal epithelium, NFκB activation seems to have an important role for the maintenance of immune homeostasis. Mice with intestinal epithelial specific ablation of NEMO (also called IκB kinase-γ (IKKγ)) or both IKKα and IKKβ, all essential mediators of NFκB activation, spontaneously develop severe chronic intestinal inflammation[119]. Likewise, pharmacological inhibition or epithelial-specific deletion of IKKβ resulted in more severe intestinal inflammation with more pronounced ulcerations in acute DSS-induced colitis[113].

In general, it is believed that increased and sustained activation of NFκB in immune and non-immune cells provoked by pro-inflammatory mediators promotes intestinal inflammation while baseline presence and activity is essential for the maintenance of immune homeostasis which has been particularly demonstrated in intestinal epithelial cells.

PHDs

In the following section, we elaborate on the role of the different hydroxylases in different cell types and their effect on intestinal inflammation in a similar fashion as we did for the HIFs (Table 3). This part will be restricted to the PHDs since no cell-type specific effects of FIH have been reported thus far. The

involvement of the different PHD isoforms in immune cell responses has mainly been deduced from whole animal or conditional knock-out of PHD1-3 and is limited to epithelial cells, endothelial cells, fibroblasts, neutrophils, macrophages, dendritic cells and T-lymphocytes.

Epithelial cells

The role of all three PHD isoforms in epithelial cells has been studied. Tambuwala and colleagues demonstrated that PHD1 serves as a positive regulator of epithelial apoptosis as full PHD1$^{-/-}$ knockout mice exhibit diminished DSS-induced epithelial apoptosis and hence epithelial barrier disruption[120]. The authors hypothesized that NFκB mediates these effects although this was not demonstrated. In addition, Chen *et al.* found a protective role of PHD3 in intestinal epithelial barrier integrity by the stabilization of the tight junction protein occludin which does not rely on its hydroxylase activity[121]. Moreover, they demonstrated that epithelial *Phd3*-deficiency causes spontaneous colitis by disturbing barrier function due to decreased occludin expression. In this set-up, the involvement of the HIFs or NFκB was not investigated, but the authors identified that PHD3 binds to the E3 ubiquitin ligase Itch, hereby preventing the proteasomal degradation of occludin. A role for epithelial PHD1 and PHD2 during experimental IBD is yet to be determined. Outside the intestinal epithelium, it has been reported that *Phd2*-deletion in skin epithelium accelerates wound healing and keratinocyte migration which relies on the HIF-1 dependent induction of β3 integrin[122]. Furthermore, silencing of *Phd2* in renal epithelial cells attenuates $CoCl_2$-induced apoptosis, mediated by the HIF-1 dependent up-regulation of the anti-apoptotic protein BCL-XL and the concomitant down-regulation of the pro-apoptotic protein Bax[123].

Endothelial cells

Takeda *et al.* demonstrated that *Phd2*-deletion enhances the hypoxia-induced proliferation in a mouse endothelial cell line[124]. In addition, it was reported that haplodeficient deletion of *Phd2* normalizes dysfunctional blood vessels in tumors and thereby inhibits metastasis[125], while also augmenting chemotherapy delivery and these events were HIF-1α and HIF-2α dependent[126]. Besides a beneficial role in tumorigenesis, loss of *Phd2* in endothelial and haematopoietic cells leads to HIF-2α dependent pulmonary vascular remodelling and arterial hypertension which relied on the increased release of CXCL12 by *Phd2*-deleted lung endothelial cells[127]. In accordance, endothelial-specific deletion of *Phd2* is responsible for this event[128, 129]. Recently, it was reported that endothelial ablation of *Phd2* also leads to renal vascular remodelling due to excessive pericyte coverage and results in impaired renal function and fibrosis[130]. This phenotype was associated with increased HIF-1α and HIF-2α levels. We found that *Phd1*-deletion, and not *Phd2* or *Phd3*, in endothelial and haematopoietic cells protects against colitis

and diminishes microvascular dysfunction (i.e. leakage and expression of adhesion molecules). However, using bone marrow transplantation and cell-specific knock-out mice we demonstrated that this effect was solely attributable to *Phd1*-deletion in the haematopoietic compartment while endothelial-specific *Phd1*-deficient mice responded similar to their WT littermate controls[131]. Despite the lack of an effect during intestinal inflammation, we demonstrated that *Phd1*-deletion in mouse endothelial MS1 cells was able to diminish the TNF-induced expression of VCAM-1, MAdCAM-1, IL-6 and the chemokine MCP-1 (unpublished data). The dependence on HIFs, NFκB or other factors was not studied in this context.

Fibroblasts

To our knowledge, there are only two papers that have studied the effect of the PHD isoforms and more specifically PHD2, in fibroblasts. Silencing of *Phd2* in a mouse fibroblast cell line leads to HIF-1 stabilization and increased the expression of angiogenic growth factors like VEGF, fibroblast growth factor 2 and angiopoietin-1, which in turn enhanced their proliferation. Moreover, these *Phd2*-deficient fibroblasts promoted endothelial proliferation *in vitro* and enhanced angiogenesis *in vivo*[132]. Recently, Manresa *et al.* reported that heterozygous *Phd2*-deleted mouse embryonic fibroblasts exhibit an increased expression of α-smooth muscle actin (SMA) and connective tissue growth factor after TGF-β stimulation which suggests that *Phd2*-deficiency might promote intestinal fibrosis. However, the same authors showed that *Phd2*$^{+/-}$ mice were equally prone to the development of fibrosis compared with WT in the DSS-induced colitis model[133].

Neutrophils

Walsmley and colleagues reported that the protein expression of PHD3 in neutrophils is up-regulated after hypoxic treatment while the expression levels of PHD1 and PHD2 remained unaltered[134]. In addition, the authors reported that *Phd3*-deficient neutrophils display an enhanced apoptosis rate under hypoxia than WT neutrophils which was independent of HIF-1α and HIF-2α but associated with increased expression of the pro-apoptotic protein SIVA1 and a concomitant down-regulation of the anti-apoptotic protein BCL-XL. While *Phd3*-deficient mice showed reduced neutrophilic infiltration during DSS colitis, this had no dampening effect on the disease course.

Macrophages

The role of the different PHD isoforms was more extensively studied in macrophages. Takeda *et al.* reported that *Phd1*-deletion in a mouse macrophage cell line (RAW264.7) results in diminished *Tnf* mRNA levels and secretion after LPS stimulation. Our group further showed that besides TNF, other

pro-inflammatory cytokines and chemokines were markedly diminished in LPS-treated *Phd1*-deficient BMDM which was associated with diminished NFκB activation[131]. In addition, we demonstrated that *Phd1* deletion facilitates M2 macrophage polarization at steady state and after IL-4 stimulation which is partially dependent on NFκB activation while expression levels of HIFs were not analysed in this set-up. However, we showed that deletion of *Phd1* solely in macrophages using *Phd1*$^{f/f}$LysM:cre mice was not able to ameliorate the course of DSS-induced colitis (unpublished data). A similar reduction in TNF expression was observed in *Phd2*-deleted cells, albeit to a lesser extent than in the *Phd1*-deficient cells[135]. In agreement, haplodeficiency of *Phd2* in macrophages diminishes the expression of pro-inflammatory cytokines and M1 markers with a concomitant switch to the M2 phenotype. These phenotypic alterations were dependent on NFκB activation while HIF-1α and HIF-2α protein levels were unchanged. These macrophages are subsequently responsible for the prevention of ischemia by inducing arteriogenesis[19]. Macrophage migration is suppressed after homozygous *Phd2*-deletion and results, together with the attenuated M1 phenotype, in an amelioration of hypertensive cardiovascular hypertrophy and fibrosis but these effects were mediated by accumulated HIF-1α and HIF-2α levels[136]. In line with these results, Guentsch and colleagues recently confirmed the decrease in migration of *Phd2*-deficient BMDM and RAW264.7[137]. They also exhibited a diminished phagocytic capacity while no clear impact on macrophage polarization was observed. These effects were due to the metabolic reprogramming to glycolysis caused by *Phd2* deletion and was dependent on HIF-1α. These results are surprising given the fact that HIF-1α deficient macrophages also have an impaired migratory and phagocytic capacity. Concerning PHD3, high numbers of PHD3^{+} macrophages are present in the inflamed gut of CD and UC patients while they are barely detectable in the normal gut[138]. In addition, the intensity of PHD3 in these PHD3^{+} macrophages is higher within the inflamed environment. The authors identified these PHD3^{+} cells as M1, and not M2, macrophages which led to their hypothesis that loss of *Phd3* would associate with an anti-inflammatory macrophage phenotype. However, murine *Phd3*-deleted macrophages rather exhibit a pro-inflammatory phenotype with enhanced pro-inflammatory cytokine release, migration, phagocytic capacity, maturation and M1 marker expression. This phenotype could be reversed either after HIF-1α or NFκB p65 knockdown while silencing of HIF-2α had no effect. Swain *et al.* reported that these *Phd3*-deficient macrophages are refractory to apoptotic cell death[139]. In contrast to the previous publication, these authors reported no obvious differences in NFκB activity nor HIF-1α and HIF-2α protein levels in *Phd3*-deficient macrophages compared with WT macrophages. Moreover, they mediate the increased susceptibility of *Phd3*$^{-/-}$ mice to sepsis-induced death while the response of *Phd1*$^{-/-}$ and *Phd2*$^{+/-}$ mice is comparable with the WT mice[140].

DCs

The role of PHD1, 2 or 3 in DC functioning or responses has not been thoroughly investigated. Our lab group was the first to show that LPS-stimulated *Phd1*-deficient BMDC produce less IL-1β in response to LPS while other pro-inflammatory cytokines such as IL-6 and TNF remain unaltered[131]. Alterations in the expression of HIFs and NFκB activation was not analysed.

T-lymphocytes

In contrast to the innate immune cells, isoform-specific roles of the PHDs in T cells are largely unknown. Evidence for a specific role of PHD1 in T-lymphocytes is non-existing. Our lab group made a first attempt to explore a role of T cell-specific PHD1 during experimental colitis but *Phd1*$^{f/f}$CD4:cre mice were equally susceptible to DSS-induced colitis compared with their colitic WT counterparts (unpublished data). In addition, 2 papers demonstrated the involvement of individual PHD2 and PHD3, respectively. In particular, Mamlouk *et al.* reported that the combined targeting of *Phd2* in myeloid and T cells delays tumor growth in mice which is at least in part due to an altered cytokine profile caused by loss of *Phd2* in these cells[141]. Also, these results were partially HIF-1α dependent. Deletion of *Phd1* and *Phd3* was not studied in this context. In addition, PHD3 expression is up-regulated in Tregs and its overexpression increases the numbers of Tregs while concomitantly inhibiting Th17 differentiation[142]. The involvement of HIFs, NFκB or other factors in these effects was not addressed. Recently, it has been demonstrated that T cell specific deletion of all 3 isoforms increases the numbers of $CD4^+$ and $CD8^+$ T cells in the lungs of these mice which additionally produce increased levels of IFN-γ. Also, the same authors reported that the PHD proteins promote Treg differentiation while restraining the differentiation of Th1 cells and this effect relies partially on the PHD-mediated inhibition of HIF-1[143].

Overall, the different PHD enzymes control diverse functions of both immune and non-immune cells which makes them interesting targets for therapeutic intervention. However, it is currently largely unknown if and how their cell-specific modulation influences the course of intestinal inflammation.

Table 3. Overview of the cell-specific effect after *Phd1-3*-deletion.

Cell type	*Phd1*-deficiency	*Phd2*-deficiency	*Phd3*- deficiency	Ref.
Epithelial cells	Reduction of apoptosis*	Induction of B3-integrin which in turn promotes wound healing	Reduction TJ occludin which increases epithelial permeability	120-123
Endothelial cells	Unknown	-Enhanced proliferation -Vessel normalization which diminishes metastasis and augments chemotherapy delivery -Increase in CXCL12 which induces SM cell proliferation	Unknown	124-127
Fibroblasts	Unknown	-Increase in VEGF, FGF2, ANG-1 which enhances proliferation and hence angiogenesis -Increase in α-SMA and connective tissue growth factor	Unknown	132, 133
Haematopoietic cells	Impact on macrophages, DCs and T cells studied (see below)	Impact on macrophages and T cells studied (see below)	Unknown	131
Neutrophils	Unknown	Unknown	Increased expression of SIVA1 and reduced expression of BCL-XL which results in increased apoptosis	134
Macrophages	- Decreased TNF, IL-1β, IL-6, MCP-1 release following LPS exposure* - M2 polarization*	- Decreased expression of pro-inflammatory cytokines - M2 polarization - Decreased migration	- Increased migration - Increased expression pro-inflammatory cytokines - Increased phagocytosis - Increased maturation - M1 polarization - Reduced apoptosis	19, 131, 135, 136, 139, 140
DCs	Decreased IL-1β release following LPS exposure*	Unknown	Unknown	131
T cells	No effect on cytokine secretion after *in vitro* T cell activation	+ deletion in myeloid cells: Diminished expression of pro-and anti-inflammatory cytokines	Overexpression of *Phd3:* Increased numbers of Treg and decreased numbers of Th17	141-143
	- Increased numbers of $CD4^+CD8^+$ T cells - Enhanced production of IFN-γ - Reduction of Treg and promotion of Th1			
Eosinophils, basophils, mast cells	Unknown	Unknown	Unknown	Not available

*Mechanisms involved in the protective effects mediated by PHD inhibitors are underlined.

Pharmacological PHD inhibitors in experimental models of IBD

The protective role of HIF-1 during colitis and the involvement of PHDs in immune and non-immune cells has initiated the investigation of hydroxylase inhibition as a potential therapeutic strategy for intestinal inflammation (Table 4). In 2008, two studies were published that simultaneously reported on the protective effects of the pan-hydroxylase inhibitors dimethyloxalylglycine (DMOG) and FG-4497 in chemically induced colitis[144, 145]. Two years later, it was reported that DMOG treatment elicited the same amelioration in a TNF-driven model for ileitis mediated by the HIF-1-dependent repression of FADD[64]. DMOG is a non-selective pharmacological compound that structurally mimics 2-OG and

thereby targets the catalytic domain of all PHDs but also FIH by blocking the entry of the co-substrate[146]. FG-4497 blocks the active site of PHDs similar to DMOG but its ability to inhibit FIH has not been demonstrated[146]. Subsequently, the use of a predominant HIF-1-specific PHD inhibitor, AKB-4924, was reported to result in systemic protection in both TNBS-induced colitis as ileitis while no improvements could be observed in epithelial HIF-1α-deficient mice[147]. Oral delivery of AKB-4924 reduced colonic inflammation, while minimally affecting HIF stabilization and HIF target genes in extraintestinal organs, thereby limiting potential off-target effects[148]. Finally, in 2014, Gupta and co-workers reported on the beneficial effects of an orally administered PHD inhibitor (TRC160334) in TNBS- and DSS-induced colitis[149]. Although this compound has been previously shown to effectively activate HIF-1α in Hep3B cells[150], it was not demonstrated whether the protected effects were HIF-1, HIF-2 or NFκB-dependent since it is a PHD and FIH inhibitor. Last year, Jeong and co-workers demonstrated that Rosmarinic acid methyl ester also exhibits pan-hydroxylase inhibition properties and is able to ameliorate TNBS-induced colitis in rats which is associated with increased colonic HIF-1 activation[151]. Besides IBD models, DMOG treatment has also proven effectiveness in the protection from ischemia/reperfusion, radiation-and *Clostridium difficile*-induced intestinal injury which was HIF-1 dependent except for radiation-induced gastrointestinal toxicity where the authors demonstrated the necessity for HIF-2 activation[152-154]. Together, these findings convincingly demonstrate the therapeutic effect of pan-hydroxylase inhibitors in experimental models of intestinal inflammation. Of note, most of these studies demonstrated the stabilization of HIF-1α and attribute the observed protective effects to its activation. However, at this moment it has not always been conclusively demonstrated that HIF-1 is in fact the primary driver of these effects. The compounds used are all PHD or combined PHD and FIH inhibitors and inhibition of PHD1, 2 and 3 is able to activate NFκB as mentioned earlier. NFκB in its turn is able to induce the transcription of HIF-1 and could therefore be the key mediator of the reported beneficial effects. In support of this hypothesis, epithelial NFκB activity is required to ensure immune homeostasis since its ablation causes severe chronic intestinal inflammation[119]. Furthermore, we demonstrated that the promotion of M2 conversion in *Phd1*-deleted macrophages is at least partially mediated by NFκB[131].

Table 4. Overview of pre-clinical animal studies using HIF-prolyl hydroxylase inhibitors during gut inflammation

Inhibitor (class)	Target	IBD model	Animal species	Administration route	Set-up	Ref.
DMOG (2-OG mimetic)	PHD + FIH	DSS-induced colitis	Mouse	Intraperitoneal and oral	Preventive	[144, 184]
		$TNF^{\Delta ARE}$ ileïtis	Mouse	Intraperitoneal	Therapeutic	[64]
FG-4497 (PHD active site blocker)	PHD + FIH (inhibition not directly demonstrated)	TNBS-induced colitis	Mouse	Intraperitoneal	Preventive	[145]
AKB-4924 (Fe2+ chelator)	PHD + FIH	TNBS-induced colitis	Mouse	Subcutaneous	Preventive and therapeutic	[7, 147]
				Oral	Preventive	[148]
		$TNF^{\Delta ARE}$ ileïtis		Intraperitoneal	Therapeutic	[147]
TRC160334 (unknown)	PHD + FIH	TNBS-induced colitis	Mouse	Oral	Preventive	[149]
		DSS-induced colitis		Oral	Therapeutic	
Rosmarinic acid methyl ester (Fe2+ chelator)	PHD + FIH	TNBS-induced colitis	Rat	Intrarectal	Therapeutic	[151]
DMOG (2OG mimetic)	PHD + FIH	Ischemia/reperfusion gut injury	Mouse	Intraperitoneal	Preventative	[152]
DMOG (2OG mimetic)	PHD + FIH	Clostridium difficile-induced gut injury	Mouse	intraperitoneal	Preventative	[154]
DMOG (2OG mimetic)	PHD + FIH	Radiation-induced gut toxicity	Mouse	intraperitoneal	Preventative	[153]

Overview on the current development of PHD inhibitors for clinical use

Several companies have developed PHD inhibitors that are currently being tested for the treatment of various diseases (Table 5). Vadadustat and Roxadustat are nonselective PHD inhibitors that are being evaluated in phase 3 trials for the treatment of chronic kidney disease (CKD)-related anemia. The preceding phase 2 studies reported that both PHD inhibitors had comparable serious adverse events compared with placebo[155, 156]. Daprodustat is another pan-hydroxylase inhibitor that induces an effective EPO response in CKD-patients[157]. In addition, AKB-6899 has entered phase I as anti-cancer drug after it was found to inhibit VEGF and phosphoglycerate kinase while stimulating the production of soluble VEGF receptor and hereby inhibiting tumor growth[158]. So far, only one company is developing a PHD inhibitor (AKB-4924, Aerpio) for the use in IBD. The first results of single and multiple ascending dose studies with AKB-4924 are expected soon.

Table 5: Overview of the PHD inhibitors in clinical trials.

Company	Compound (class)	Target	Indication	Stage of clinical development	Cinical Trials.gov. Number (Status)
Akebia Therapeutics	AKB-6548, Vadadustat (PHD active site blocker)	PHD + FIH	CKD-related anemia	Phase III	NCT02892149; NCT02865850; NCT02680574; NCT02648347 (all recruiting)
Glaxo-Smith-Kline	GSK1278863, Daprodustat (PHD active site blocker)	PHD + FIH	CKD-related anemia	Phase III	NCT02876835; NCT02879305; NCT02969655 (all recruiting)
Aerpio Therapeutics	AKB-4924 (Fe2+ chelator)	PHD + FIH	IBD	Phase I	NCT02914262 (completed)
Fibrogen	FG-4592, Roxadustat (PHD active site blocker)	PHD + FIH	CKD-related anemia	Phase III	NCT01750190 (recruiting); NCT02021318 (recruiting); NCT02174627 (recruiting); NCT02652806 (active, not recruiting); NCT02652819 (active, not recruiting); NCT02273726 (recruiting); NCT01887600 (active, not recruiting)
Akebia Therapeutics	AKB-6899 (PHD active site blocker)	PHD + FIH	Cancer	Phase I	Not registered
Bayer	BAY85-3934, Molidustat (PHD active site blocker)	PHD + FIH	CKD-related anemia	Phase II	NCT02055482 (completed); NCT01975818 (completed); NCT02064426 (completed)

Potential limitations of prolyl hydroxylase inhibitors

Although no major safety signal was observed during phase 2 studies in CKD-patients[155, 156], long-term clinical data with PHD inhibitors are not yet available. Considerable concern regarding the long-term use of these agents have been raised. In the following section we discuss the most relevant issues that may hamper their clinical utility as a maintenance therapy in IBD.

Promotion of cancer

The potential of HIF-activating therapies to promote tumor development is subject of debate. Many cancerous lesions highly express both HIF-1α and HIF-2α and their expression has been positively linked with cancer aggressiveness and mortality[159, 160]. The increased HIF-1 dependent extracellular adenosine signalling may play an important contributing role in these events. Although enhanced expression of adenosine receptors and CD73 due to HIF-1 activation protects against intestinal inflammation, increased adenosine signalling concomitantly suppresses immune cell activation (i.e. pro-inflammatory cytokine release, chemotaxis, effector T cells, …). The lack of anti-tumor immunity allows the promotion of cancer progression and metastasis[161, 162]. Moreover, anti-CD73 and A2a antagonists have proven their antitumor effects and even synergize with antineoplastic agents in preclinical animal models which has subsequently led to the evaluation of their ongoing evaluation in clinical trials (NCT02503774 and NCT02403193, respectively). Activation of HIF-2 through disruption of *Vhl* promotes colon tumor number, size and progression in an experimental colorectal cancer model[163]. The HIF-inhibitor YC-1 has been proven to be effective in blocking angiogenesis, inhibiting the growth of various tumors (all non-colon) and inhibiting metastasis in mice[164, 165]. However, these anti-cancer properties of YC-1 may be attributed to its ability to inhibit HIF-2 instead of HIF-1. In support, activation of HIF-1α does not seem to increase carcinogenesis or the progression of colon cancer[166]. Therefore, local and specific activation of HIF-1 could be safe for therapeutic use in IBD. In addition, no carcinogenic effects of PHD inhibitors have been reported thus far. Even more, haplodeletion of endothelial *Phd2* normalizes the endothelial cell layer in a HIF-2α-dependent manner with less metastasis and improved chemotherapy delivery as a consequence[125, 126]. As mentioned earlier, T cell specific loss of all three PHD isoforms promotes Th1 responses, restrains $CD8^+$ T cell effector function and limits Treg induction which is partly attributable to HIF-1α stabilization. Moreover, these T cell alterations protect these mice from metastatic tumor colonization in the lung. Of note, the risk for cancer development in IBD patients is greatly increased by the chronicity of inflammation[167]. Since pan-hydroxylase inhibitors protect against intestinal injury, the risk of inflammation-induced tumor development may therefore decrease.

Erythropoiesis

The stabilization of HIF can promote EPO release and subsequently increases erythrocyte production. Although this is desirable for the treatment of several diseases including renal anemia or cancer-related and chemotherapy-induced anemia, EPO stimulating agents have been associated with an increased risk of thromboembolic events. This might be particularly relevant for IBD patients. While anaemia is a frequent systemic complication in IBD patients with a prevalence in CD patients of 27% and 21% in UC patients,[168] the main cause is iron deficiency. The latter in addition to other conditions such as cancer, absence of a spleen or infections, can lead to secondary thrombocytosis [169, 170] and hence contribute to the increased risk of thromboembolic events. Furthermore, human IBD is associated with activation of tissue factor, impairment of the protein C pathway and enhanced generation of thrombin making the blood of these patients already hypercoagulant and more prone to the appearance of thrombi[171]. Indeed, microinfarctions are often detected in histology of inflamed biopsies from IBD patients[172] and clinical studies indicate an increased risk for systemic thromboembolisms in IBD patients[173]. However, it is reassuring that the trials with pan-hydroxylase inhibitors to treat anemia in CKD patients show no increased incidence of hypertension and cardiovascular events.[155-157].

Fibrosis

Another important limitation for the use of pan-hydroxylase inhibitors is the risk of fibrosis. Higgins and colleagues have demonstrated that epithelial activation of HIF-1α during hypoxia increases the epithelial-to-mesenchymal transition and epithelial cell migration through up-regulation of lysyl oxidases, enzymes involved in extracellular matrix degradation, while deletion of epithelial HIF-1α inhibited kidney fibrosis[174]. In addition to kidney fibrosis, it has been demonstrated that hepatocyte-specific HIF-1α deletion reduced fibrosis in several models of liver fibrosis through the down-regulation of pro-fibrotic proteins[175, 176]. Similar, myeloid-specific HIF-1α knockout mice also exhibited reduced signs of liver fibrosis associated with reduced expressions of the pro-fibrotic proteins α-SMA, type I collagen and platelet derived growth factor B[177]. Furthermore, hypoxia via HIF-1 also enhances the production of TGF-β1 and collagen synthesis through TGF-β1 in fibroblasts, two key mediators of intestinal fibrosis[78]. Therefore, the long-term use of HIF-1 activators could be problematic for CD patients where recurrent episodes of inflammation and healing leads to fibrostenotic or penetrating disease in 60% of patients[178]. Although DMOG, in addition to its anti-inflammatory effect, is also able to reduce intestinal fibrosis, this effect relied on the suppression of TGF-ERK signalling and was independent of its known HIF-1 activator function[133].

Disturbance of biochemical processes

A final word of caution on the use of pan-hydroxylase inhibitors involves the alteration of biochemical pathways. This concern specifically applies to pan-hydroxylase inhibitors that are 2-OG analogues like DMOG or Fe^{2+} chelators like AKB-4924[179]. In humans, there are at least 80 2-OG-dependent dioxygenases besides PHDs[180]. They are known to hydroxylate C-H bonds on a variety of targets involved in collagen and hormone synthesis, fatty acid metabolism and perform demethylations of N-methyl groups on histones and nucleic acids[181]. Therefore, the use of 2-OG competitors may detrimentally influence these reactions and cause disease. Indeed, Loenarz *et al.* summarizes a range of severe disorders such as growth retardation and osteogenesis imperfect type VIII, caused by impaired function of 2-OG oxygenases[181]. Concerning the HIF-1 stabilizers that are Fe^{2+} chelators, they are non-selective because they probably also inhibit the activity of other enzymes requiring Fe^{2+} and hence result in unwanted side effects. Indeed, it has been reported that iron chelation interferes with oxidative phosphorylation and arachidonic acid signalling[182, 183].

Future directions

Next-generation pan-hydroxylase inhibitors

Most of the above described potential side-effects of PHD inhibitors rely on a systemic exposure of these compounds. Recently however, the oral delivery of the prolyl hydroxylase inhibitor AKB-4924 has been shown to reduce colonic inflammation while minimally affecting HIF stabilization and HIF target genes in extraintestinal organs, thereby limiting off-target effects[148]. Oral administration of the pan-hydroxylase inhibitor DMOG is not effective against colitis which may be due to acidic and enzymatic drug degradation in the stomach and small intestine[184]. However, Tambuwala and colleagues found that orally administrated coated DMOG minispheres resulted in local release throughout the colon and protection against colitis with a lower EPO response as compared to intraperitoneal DMOG administration[184].

Isoform-specific PHD targeting

Another way to circumvent unwanted side effects is the selective targeting of the key PHD isoform involved in colitogenesis. We have demonstrated that PHD1 expression is increased in inflamed IBD biopsies, especially UC[185]. In agreement, Tambuwala and co-workers reported that only $PHD1^{-/-}$ mice,

but not PHD2 or PHD3, were highly protected in a mouse model of ulcerative colitis which is associated with reduced epithelial apoptosis and enhanced barrier function[120]. We further unravelled that in addition to an anti-apoptotic effect of *Phd1*-deletion on epithelial cells, haematopoietic *Phd1*-deficiency drives the protection during DSS-induced colitis at least in part by promoting M2 macrophage polarization and diminishing their pro-inflammatory cytokine and chemokine release[131].

PHD2 is the critical isoform that determines HIF1-α levels under normoxia[186]. As HIF-1 activation has been proven to be protective in experimental models of colitis, it is surprising that *Phd2*-deficient mice showed no protection against colitogenesis. It has been hypothesized that the heterozygous *Phd2*-deficient mice used may perhaps not sufficiently elevate HIF-1 levels to exert a therapeutic effect. Therefore, cell type specific *Phd2* targeting or PHD2 inhibitors could resolve this problem but data regarding the therapeutic effect of these approaches on intestinal inflammation is lacking.

Although loss of PHD3 in neutrophils decreased their survival under hypoxic conditions which results in reduced neutrophilic infiltration during DSS-induced colitis, it did not alter the disease course[134]. Likewise, we and others reported no improved clinical outcome in *Phd3*-deleted mice after DSS exposure [120, 131]. In addition, it has been reported that epithelial *Phd3*-deficiency causes spontaneous colitis by disturbing barrier function due to decreased occludin expression[121].

Taken together, these results highly suggest that pan-hydroxylase inhibitors exert their protective effects primarily through inhibition of PHD1. Unfortunately, there are currently no PHD1-specific inhibitors available to test its application in humans. If the development of a small molecule inhibitor with high selectivity for PHD1 is not feasible, a PHD1 antisense oligonucleotide may represent a valuable alternative. Thus far, Quaegebeur *et al.* demonstrated their effectiveness in reducing cerebral infarct size and neurological deficits following stroke[187].

Of note, it would be interesting to explore the effect of FIH-specific deletion during experimental colitis. Inhibition of both FIH and PHDs in kidney epithelial cells results in a 6-fold higher induction of HIF-1α activity than PHD inhibition alone, indicating that FIH is in fact accountable for the majority of HIF-1 activity in these cells[188]. Moreover, DMOG diminishes IL-1β-induced NFκB activation and subsequent target gene expression probably through the combinatorial inhibition of PHD1 and FIH[189]. Single knockdown of PHD1 and FIH was able to reduce the IL-1β-induced NFκB activation, but deletion of both had an additive effect. Since IL-β plays an important role in the pathogenesis of experimental IBD[190], it is possible that the therapeutic action of PHD and FIH hydroxylase inhibitors like DMOG includes its inhibition of FIH. The mechanisms by which hydroxylase inhibitors exert beneficial effects during experimental IBD are underlined in Table 3.

Summary

During the last decade intensive research efforts have been dedicated towards unravelling the role of hypoxia-induced signalling in intestinal inflammation. The oxygen-sensitive prolyl hydroxylases tightly control the activity of the transcription factors HIF-1, HIF-2 and NFκB which in turn orchestrate both a pro-inflammatory and adaptive response during hypoxic conditions such as IBD. Accumulating evidence from animal studies supports the development of strategies that boost the hypoxia-induced adaptive response in IBD. In particular, pharmacological PHD inhibitors have proven their therapeutic efficacy in several mouse models of UC and CD. The potential hazards of systemic PHD inhibition combined with the novel insights on the role of the different PHD isotypes during intestinal inflammation indicate that orally delivered PHD1-specific inhibitors are the best drug candidates for the treatment of human IBD.

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3 Methodology to study immune and endothelial cell-mediated effects in experimental IBD

In the final part of this introduction, I will elaborate on the experimental approaches that were used during this thesis to study the cell-specific role of the PHD isoforms during IBD-like inflammation.

3.1 Cell-specific transgenic mice

To study effects that are specifically mediated by immune and endothelial cells, transgenic mice can be employed such as Tie2:cre mice. In these mice the Tie2 promoter drives the expression of cyclization recombination (Cre)-recombinase. The latter is an enzyme derived from the bacteriophage P1 that catalyses sequence-specific DNA recombination between 34bp '*loxP'* sites flanking the gene of interest. Deletion of a genomic region occurs when the *loxP* sites are placed *in cis* and in the same directional orientation. More specifically, Tie2:cre mice when bred with mice containing a *loxP*-flanked gene (referred to as "floxed" mice) will result in deletion of that gene in all Tie2 expression cells, i.e endothelial and haematopoietic cells (Figure 1). In addition, these mice can also be used to study the specific effect of immune cells. The latter originate from haematopoietic stem cells that reside in the bone marrow (BM) and can be broadly divided into myeloid and lymphoid lineages. So when whole-body irradiation is performed on wild-type (WT) mice followed by transplantation with BM from floxed Tie2:cre mice, the autologous immune cells will be ablated and reconstituted with immune cells from the donor that carry the deleted gene. Besides BM transplantation, the transgenic Vav:cre mice represent a valid alternative to assess immune-mediated effects since the Vav promoter is expressed in all haematopoietic stem cells (Figure 1). Furthermore, the Tie2:cre mice can also be employed to investigate the effect(s) of endothelial-specific deletion of a *loxP*-flanked gene. This requires the irradiation of floxed Tie2:cre mice and the subsequent transplantation of WT BM. Another option that avoids BM transplantation is the transgenic Flk-1:cre mice where the Flk-1 promotor drives the expression of cre specifically in endothelial cells.

To explore the effects of macrophage-specific gene deletion, different transgenic mice have been developed, but LysM:cre mice are probably the most widely used. Although M lysozyme (LysM) is expressed by all myeloid cells, effective excision of the gene of interest mainly occurs in macrophages, but also, to a lower extent, in neutrophils. MRP8:cre mice represent a better approach to drive sole recombination in neutrophils[1, 2]. Other cells of the myeloid lineage like basophils, eosinophils, DCs and mast cells exhibit only around 10% deletion of the floxed gene mediated by the LysM promotor[3, 4].

To investigate the impact of a DC-specific deleted gene, CD11c:cre mice are commonly used. The only issue with this transgenic mouse is that DC-mediated effects cannot be discriminated from macrophage effects as mentioned earlier due to their shared CD11c expression. In support, several reports have demonstrated that CD11cDTR:cre mice effectively eliminate DCs, but also intestinal macrophages. Administration of diphtheria toxin (DT) in this mouse abolishes every CD11c expressing cell type because only these cells express the DT receptor (DTR). Therefore, breeding CD11c:cre mice with a floxed mice will result in DC, but also macrophage deletion of the *loxP*-flanked gene (Figure 1).

Lee and co-workers developed a transgenic mouse in which the Cre-recombinase is under the control of the CD4 promoter. This mouse generates as expected deletion of a floxed gene in $CD4^+$ T cells, but also $CD8^+$ T cells[5]. In this way, T cell-mediated effects can be studied.

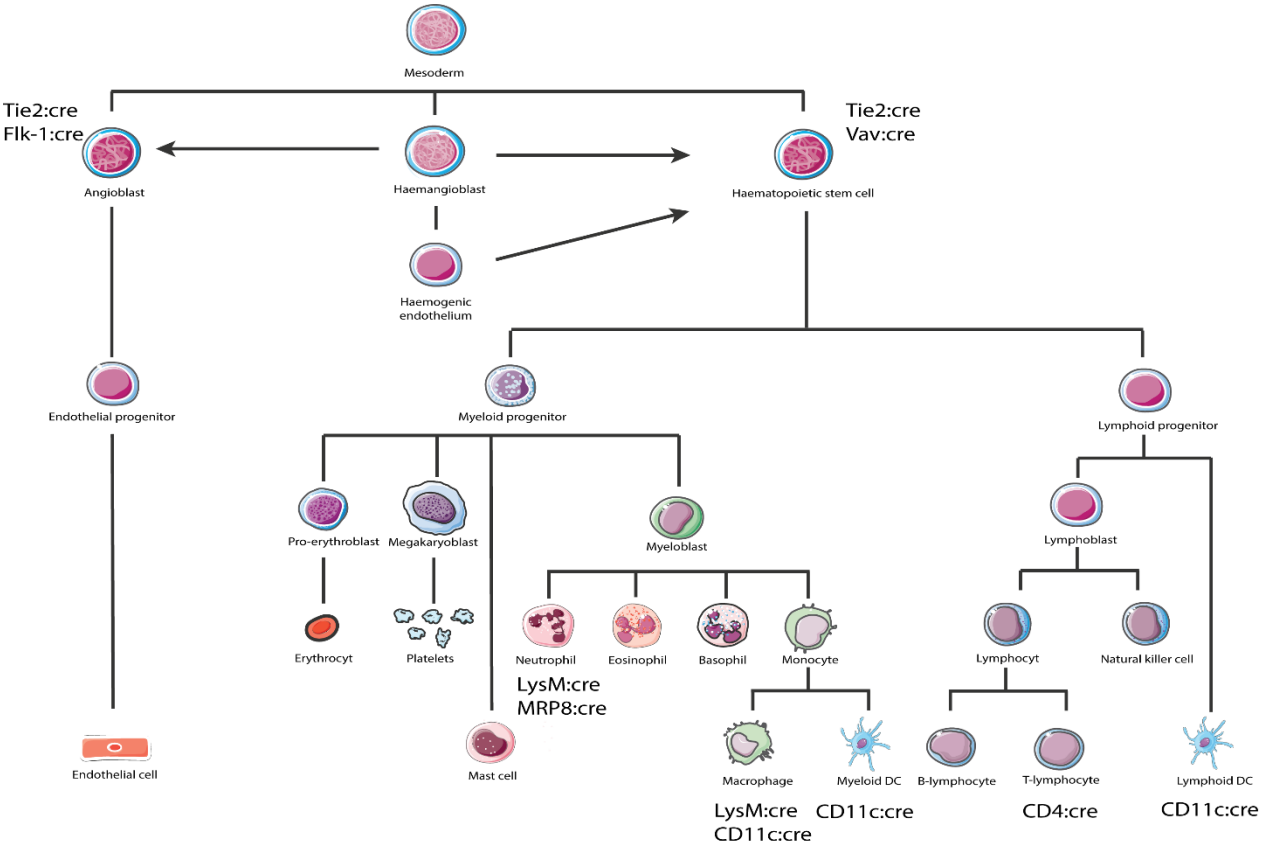

Figure 1: *Schematic overview of the targeted cells using different transgenic cre:mice. Tie2 expression occurs in the angioblast and haematopoietic stem cells which give rise to endothelial and haematopoietic cells respectively. Therefore, Tie2:cre mice will delete a loxP- flanked gene in exactly these cells. To study endothelial and haematopoietic cell-mediated effects separately, Flk-1:cre and Vav:cre mice can be used. When macrophage-specific deletion is required, LysM:cre mice are good model although knockout in neutrophils also occurs. Sole deletion in neutrophils can be achieved by MRP8:cre mice. CD11c:cre mice have been mainly used to investigate DC-mediated effect, but they also generate knockout in macrophages. To explore the impact of T-cell specific deletion, in particular $CD4^+$ and $CD8^+$ T cells, CD4:cre mice are a valid option.*

3.2 Dextran sodium sulphate (DSS)-induced colitis, an experimental IBD model associated with intestinal hypoxia, immune and endothelial cell dysfunction

Although no animal model completely resembles human IBD, they are indispensable to unravel the underlying mechanisms of IBD pathogenesis and to study the therapeutic potential of compounds.

Numerous animal models of IBD exist and can be divided into 1) chemically induced which consists of the administration of agents that promote intestinal inflammation, 2) genetic, where a certain gene is targeted or transgenes are introduced which results in intestinal inflammation and 3) spontaneous mouse models where mice spontaneously develop inflammation without genetic, chemical or immunological manipulation.

Chemically induced models including DSS and 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis are the most commonly used models because of their simplicity, low cost, reproducibility and controllability. Adjusting the concentration, frequency and duration of these compounds can either induce acute or chronic intestinal inflammation and therefore both the innate and adaptive immune response can be studied. In addition, the supplementation of the carcinogen azoxymethane (AOM) prior to DSS administration represents a model for studying colitis-associated carcinogenesis[6]. The disadvantage is that the evoked disease is self-limiting and that mice exhibit differential susceptibilities and responsiveness to both DSS-and TNBS-induced colitis. Important contributing factors to this phenomenon are not only the DSS itself (i.e. concentration, molecular weight, duration of exposure, manufacturer and batch), but also genetic (i.e. strain and gender) and microbiological (microbiological state and intestinal microbiota) factors[7]. More specifically, male mice are more susceptible than females. DBA/2J and C3H/HeJBir mice exhibit the least and highest susceptibility respectively. The susceptibility of C57Bl6 is in between these mice. The presence of the microbiota on the other hand facilitates inflammation during this model. The susceptibility to TNBS is also dependent on the mouse strain used[8]. SJL and BALB/c are susceptible whereas C57BL6 are quite resistant to TNBS-induced colitis. Therefore, every setting requires the optimization of TNBS and DSS to obtain the desired inflammatory response in the chosen mouse strain. Although these chemically-induced models of IBD provide valuable information about mechanism(s) contributing to IBD pathogenesis, human IBD is not caused by a chemical agent, but results from a complex interaction of genetic, immune and environmental factors. Therefore, genetically engineered mice that carry susceptibility genes for IBD such as IL2 and IL10 knockout mice more closely mimic the human context. In the following part, I will further focus and elaborate on the DSS-induced model of colitis which we used throughout our research because it is characterized by hypoxia, immune and endothelial cell dysfunction.

DSS is a water-soluble, negatively charged sulfated polysaccharide. To induce colitis, DSS with a molecular weight of 36-50kDa is dissolved in the drinking water at a concentration ranging from 2-5%. By modifying the concentration and the frequency of administration, acute or chronic and relapsing intestinal inflammation can be achieved. Clinical features include rectal bleeding, diarrhea, weight loss, hunched back and sometimes even death, especially when high dosages are applied. Histologically, mucin and goblet cell depletion, epithelial erosions and infiltration of polymorphonuclear and mononuclear cells in both mucosa and submucosa can be observed [9]. Concomitantly, increased colonic levels of the cytokines IL-1β, IL-6, TNF, IL-12, IFN-γ, IL-18 and G-CSF are seen, while the expression of IL-4 is not significantly altered, indicating a Th1 rather than a Th2 profile[10, 11]. The inflammation is restricted to the colon with the most severe signs in the distal part and inflammation gradually declines more proximally. Considering all described findings, this model mostly resembles human UC. The exact mechanism by which DSS induces inflammation is still not fully unraveled. It has been suggested that DSS complexes with medium-chain-length fatty acids (MCFAs), present at high concentrations in the colonic lumen which could explain its distal preference[12]. These vesicles in turn disrupt intestinal barrier function because DSS appears to be toxic to epithelial cells[13] and hence allows bacterial antigens to easily penetrate the mucosa which causes an inflammatory reaction. In addition, DSS can be found in colonic macrophages already one day after DSS administration and inhibits their phagocytic capacity[14, 15]. This further enhances bacterial invasion and suggests that macrophages play a crucial role in the development of DSS-induced colitis. In addition, there is an M1/M2 imbalance present during this model and transfer of M2 macrophages is able to suppress colitogenesis by inducing IL-10 release and promoting Treg differentiation[16]. Qualls and colleagues were actually the first to investigate the *in vivo* role of macrophages and DCs during the course of this model[17]. They demonstrated that depletion of LP macrophages and DCs using transgenic mice or via the administration of clodronate liposomes before the development of DSS-induced colitis, results in a more severe clinical outcome. Although this suggests that macrophages and DCs play a suppressive role in the development of DSS-induced colitis, two later publications came up with opposite results. In particular, depletion of DCs (and in fact also macrophages since they used CD11c:cre mice) after DSS administration attenuated, while adoptive transfer of BM-DCs exacerbated DSS-induced colitis[18, 19]. However, the latest publication on this issue is in agreement with the first publication and hypothesized that one of the reasons for the conflicting results is the timing of depletion. The main phenotype of DCs before initiation of DSS is probably a regulatory/anti-inflammatory subpopulation, while inflammatory DCs dominate after DSS administration[20]. In accordance, it has been demonstrated that DSS induces the secretion of pro-inflammatory cytokines by DCs such as IL-12 and TNF, but also the chemokines KC, MIP-1α, MIP-2 and MCP-1[19]. Although different DC subtypes have been described

in the gut, the transgenic mice used in the past were not able to reveal their exact functions in gut homeostasis and inflammation. This recently changed and resulted in a publication by Muzaki and colleagues who demonstrated that $CD103^+CD11b^-$ DCs exert a protective role during DSS-induced colitis by inducing the expression of anti-inflammatory proteins in epithelial cells mediated by IFN-γ[21]. Mice lacking this DC subtype exhibit increased susceptibility to intestinal inflammation, while deletion of $CD103^+CD11b^+$ DCs did not alter the disease course[21]. The first studies that investigated a possible role for T cells in the DSS model made use of immunodeficient mice that lack both T-and B-cells. They revealed that the adaptive immunity is not required to induce the acute DSS-induced colitis as the disease pathology could be evoked in these mice[22], but appear to act as an aggravating factor since recombinase-activating gene (RAG)-1 knockout mice are less susceptible to DSS (only when a low dose is administered)[23]. In agreement with a T cell specific aggravating role, mice receiving primed T cells from colitic mice with DSS-pulsed macrophages exhibit exacerbated signs of colitis[24]. The same authors further identified the $CD4^+$ and not $CD8^+$ T cells to be essential to this event. Within these $CD4^+$ T cells, it has been demonstrated that DSS elicits an increase in the percentage colonic Th1 cells, while the percentage Th17 cells remains comparable to non-treated controls[25]. Despite the unaltered frequency of Th17 cells, the acute DSS model induces an increase in colonic IL-17A levels and IL-17A knockout mice are less susceptible to colitogenesis[26]. I mentioned earlier that macrophages also produce this cytokine and may therefore be the main source of this cytokine during the acute phase of DSS. In contrast to Th1 cells, a suppressive role is reserved for Tregs. Boehm and co-workers demonstrated that deletion of Treg worsens the course of the acute DSS model due to elevated levels of IL-17A and IFN-γ[27]. Besides a dysregulated immunologic response, the functioning of the microvasculature and endothelial cells is also compromised during this model. Mori and colleagues reported that DSS-treatment results in a significantly reduced blood flow in the smallest arterioles which may be due to their diminished vasodilatory capacity as observed in response to the vasodilator bradykinin[28]. In addition, the same authors reported that DSS causes an accumulation of platelets and leukocytes adhering in colonic venules starting from day 2 onwards which can be attenuated by blocking endothelial and, to a lesser extent, platelet-associated P-selectin [29]. Furthermore, they demonstrated that DSS administration increases vascular permeability which was confirmed later on in the same setting by Tolstanova *et al.* [30]. Concerning cell adhesion molecules, it has been shown that the expression of ICAM-1, VCAM-1 and MAdCAM-1 is elevated on colonic vessels of DSS-treated mice. Moreover, their selective blockade using antibodies is effective in protecting mice from DSS-induced colitis[31-33]. Taken together, DSS administration provokes a dysfunctional, prothrombogenic endothelial phenotype similar to human IBD. Finally, to determine the presence of hypoxia 2-nitroimidazoles like pimonidazole can be used. This compound leaves the cell when sufficient oxygen is present, but forms

irreversible adducts with thiol groups when the oxygen tension falls below 10mmHg. Using this method, Karhausen and colleagues were the first ones to report that the colonic epithelium is hypoxic and markedly enhanced in colitic lesions of the 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis model[34]. Later on, this physiological hypoxic state of the colonic epithelium was confirmed in the DSS model, but was not evaluated at the peak of inflammation[35].

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CHAPTER II: AIMS

GENERAL HYPOTHESIS

We hypothesize that deletion of *Phd1* suppresses inflammation and microvascular dysfunction during experimental colitis through its role in immune cells and thus represents the primary therapeutic target for IBD.

RATIONALE

Pan-hydroxylase inhibition has been proposed as a novel treatment approach for IBD patients, but this strategy has raised some important concerns about tumor development, cardiovascular events (due to increased erythropoiesis) and fibrogenesis. To minimize the risk of these unwanted side-effects, isoform-specific targeting could be a better approach. Some preliminary work has been done in this field and demonstrated that *Phd1* knockout mice are protected from acute colitis. It was hypothesized that epithelial PHD1 mediated this protection by decreasing epithelial cell apoptosis and barrier dysfunction, both prominent features of active IBD. However, this might be a secondary phenomenon because the epithelial apoptosis rate in UC seems to be driven primarily by the local inflammatory response rather than vice versa. Given the fact that an aberrant immune response and microvascular dysfunction hallmarks IBD and PHDs are involved in multiple aspects of immune cell functioning, we aimed to expand the current knowledge on the involvement of the different PHD isoforms in immune cells and their contribution to IBD pathogenesis.

SPECIFIC RESEARCH AIMS

1) Identifying the key isoform(s) involved in the pathogenesis of IBD. In this study we aimed to:
    - determine the expression of PHD1, PHD2 and PHD3 both at the mRNA and protein level in colonic biopsies from healthy controls, patients with UC, CD and infectious colitis.
    - analyse the cellular distribution of PHD1, PHD2 and PHD3

    The results from this study are described in the **first part of chapter III**.

2) Evaluating if isoform-and cell-specific modulation of PHD expression holds therapeutic potential for the treatment of IBD. In this study we aimed to evaluate the *in vivo* effect of PHD1-3 deletion in endothelial and haematopoietic cells on:
    - blood vessel function,
    - epithelial integrity and
    - the inflammatory burden during acute colitis

    The results from this study are described in the **second part of chapter III**.

CHAPTER III: RESULTS

1 Differential expression of prolyl hydroxylase 1 in patients with ulcerative colitis versus patients with Crohn's disease/infectious colitis and healthy controls

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Abstract

Background: Inhibition of prolyl hydroxylases (PHDs) leads to the induction of a transcriptional program that, in the gut, promotes intestinal epithelial cell survival. PHD inhibitors have recently been suggested as a promising alternative treatment for inflammatory bowel disease (IBD). In this study, we explored the colonic mucosal expression of the different PHD-isoforms (PHD1, 2 and 3) in order to identify the key isoform(s) involved in the pathogenesis of IBD.

Methods: The mRNA expression of inflammatory cytokines (IL-8 and TNF), an apoptosis marker (caspase 3) and PHD1, 2 and 3 was analysed in biopsies of IBD patients (UC and CD), patients with infectious colitis and healthy controls using qRT-PCR. PHD protein levels were evaluated using western blot. Cellular localisation of PHD 1, 2 and 3 was determined by immunohistochemistry.

Results: PHD1 was significantly up-regulated in IBD patients, both at the mRNA (UC: $p<0.0001$ and CD: $p<0.05$) and at the protein level (UC: $p<0.05$ and CD: $p<0.05$), and showed a very good correlation with the expression of the inflammatory cytokines IL-8 and TNF and the apoptosis marker caspase 3. Colonic mucosal PHD2 mRNA and protein expressions were not altered in IBD. PHD3 expression was increased in inflamed biopsies from UC patients ($p<0.0001$), but only at the mRNA level. PHD1 and PHD2 expression was found both in the colonic lamina propria and the epithelium, while PHD3 was mainly located in the endothelium of blood vessels.

Conclusions: In this exploratory expression analysis, PHD1 comes forward as the primary therapeutic target for UC and, to a lesser extent, for (colonic) CD.

Keywords: prolyl hydroxylases, Crohn's disease, ulcerative colitis, infectious colitis

Background

Prolyl hydroxylase domain-containing proteins (PHDs) are oxygen sensing enzymes that, under normoxic conditions, hydroxylate the hypoxia-inducible factor 1 alpha subunit (HIF-1α), leading to its proteasomal degradation. During hypoxia, the PHDs are inhibited, leading to the formation of the active transcription factor HIF-1, which induces the expression of several cell survival genes (i.e. the hypoxic adaptive response)[1]. Several groups have proposed prolyl hydroxylase (PHD) inhibition as a promising novel strategy in the treatment of inflammatory bowel disease (IBD)[2,3,4].

To identify the key PHD isoforms (PHD1-3) involved in the pathogenesis of IBD, we explored their colonic mucosal expressions in endoscopically derived colonic mucosal biopsies from healthy controls and patients with Crohn's disease (CD), ulcerative colitis (UC) and infectious colitis.

Methods

Study populations and samples

Colonic mucosal biopsies were taken from endoscopically inflamed areas of 19 Crohn's disease (CD) patients and 10 ulcerative colitis (UC) patients with active disease, and from completely healed mucosa of 16 CD patients and 5 UC patients in remission. Samples of 20 healthy controls (HC) and inflamed regions of 9 patients with infectious colitis were included as controls. Patients were diagnosed with infectious colitis based on histological findings (5 out of 9) or positive stool sample cultures (4 out of 9). The patients with infectious colitis were not known with IBD. Biopsies were stored immediately after removal in -80°C. IBD patients were either free of medication use or used 5-aminosalicylates in monotherapy. This study was approved by the ethical committee of the University Hospital of Ghent (permit number EC UZG 2004/242) and all participants gave their written informed consent. Patient characteristics are summarized in Table 1.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from the colonic mucosal biopsies using the RNeasy Mini Kit (Qiagen, Westburg BV, Leusden, The Netherlands) and converted to cDNA by reverse transcription (iScript$_{TM}$ cDNA synthese kit, Biorad, CA, USA), according to the manual instructions. Real-time quantification was performed using SensiMix$_{TM}$ SYBR No-ROX kit (Bioline, Gentaur Europe BVBA, Kampenhout, Belgium) and 250 nM forward and reverse primers (BioLegio, Nijmegen, The Netherlands). A twostep

program was run on a LightCycler® 480 II (Roche, Basel, Switzerland). Cycling conditions were 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. All reactions were run in duplicate and normalized to the stably-expressed human succinate dehydrogenase complex subunit (SDHA) levels. The mRNA expression levels of the inflammatory cytokines interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF) were analysed as markers of inflammation. Sequences of the qRT-PCR primers and the PCR efficiencies are given in Table 2.

Immunohistochemistry

Paraffin-embedded colonic sections of 5 controls, 5 active UC, 5 active CD and 5 infectious colitis patients were deparaffinized with xylene, and rehydrated in a graded series of ethanol. Antigen retrieval was performed by boiling the slides in 10mM sodium citrate buffer with 0.05% Tween 20 for 20 minutes. Next, endogenous peroxidase activity was blocked with peroxidase block solution Envision (Dako) for 15 minutes. Sections were subsequently blocked with 10% goat serum for 1,5 hours at room temperature and then incubated overnight with primary antibodies at 4°C. Primairy antibodies used were rabbit monoclonal anti-PHD1 (1/50), anti-PHD2 (1/100) and rabbit polyclonal anti-PHD3 (1/200), obtained from Abcam. The slides were then treated with HRP labeled goat anti-rabbit antibody (Envision+System-HRP kit, Dako) and developed with diaminobenzidine. Counterstaining was performed with hematoxylin.

Western blotting

Human biopsies were lysated, proteins were separated on 4-12% Bis-Tris SDS-polyacrylamide gels and transferred to nitrocellulose membranes using iBlot dry blotting (Invitrogen). Afterwards, membranes were blocked with 5% skimmed milk in TBS-T (50mM Tris (pH 7.6), 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C in 5% skimmed milk in TBS-T with anti-PHD1 (1/5000, Abcam), anti-PHD2 (1/600, Abcam), anti-PHD3 (1/900, Abcam) and anti-GAPDH (1/2500, Abcam). Bound antibodies were visualized using the ECL detection kit BM chemiluminescence Blotting Substrate POD (Roche) according to manufacturer's instructions. Quantitative densitometric analysis using the Image J program was performed to quantify protein expression levels in each sample. Data were normalized to the protein expression of GAPDH.

Statistical analysis

The data were statistically analysed using SPSS Statistics, version 20, for Windows (SPSS, Chicago, IL). Normality of the data was checked using the Kolmogorov-Smirnoff (KS) test. In the case of normally distributed data, the differences between groups were analysed using an unpaired Student's t-test for independent samples. For non-normal or unknown data distribution, groups were compared by using the non-parametric Mann-Whitney U-test. The KS-test also determined the use of either a parametric (Pearson) or a non-parametric (Spearman) correlation test. Two-tailed probabilities were calculated and p-values less than or equal to 0.05 were considered statistically significant.

Results

As a first step, we evaluated the expression of the pro-inflammatory cytokines IL-8 and TNF to confirm and define the degree of inflammation in the inflamed biopsies of IBD patients and patients with infectious colitis. It has been previously reported that they are representative markers of active inflammation [5,6]. Furthermore, we determined the expression of the apoptosis marker caspase 3 to be able to evaluate its correlation with the different PHD isoforms. The inflamed samples were characterized by highly increased IL-8, TNF and caspase 3 mRNA levels compared to biopsies obtained from non-inflamed areas and HC ($p<0.0001$ for all groups). IL-8, TNF and caspase 3 expression levels in UC and CD patients in remission were comparable to those observed in the HC group (data not shown).

Quantitative assessment of PHD1 mRNA levels revealed a significant increase of PHD1 in inflamed colonic biopsies of UC patients ($p<0.0001$). This up-regulation was absent in patients in remission. Expression levels of PHD1 in biopsies from patients with CD and infectious colitis were only slightly elevated ($p<0.05$ and $p=0.063$, respectively) compared to HC, despite similarly elevated IL-8 levels (Figure 1A).
For PHD2, no differences were seen in inflamed biopsies from patients with UC, CD and infectious colitis versus non-inflamed biopsies from IBD patients in remission or healthy controls (Figure 1B).

The expression level of the PHD3 gene was significantly elevated in samples taken from inflamed colonic areas in UC patients compared to samples from HC ($p<0.0001$). Inflamed samples from CD patients or infectious colitis nor non-inflamed biopsies from UC patients in remission showed an up-regulated PHD3 expression (Figure 1C).

A positive correlation was found between IL-8/TNF and PHD1 expression. In contrast, no correlation was found between IL-8/TNF and PHD2, and only a poor correlation was observed between IL-8/TNF

and PHD3. PHD1 and, to a lesser extent, PHD2 correlated positively with caspase 3 ($p<0.0001$ and $p=0.001$, respectively) (Table 3).

All above reported results were confirmed in a second, independent patient cohort (data not shown).

Next, the protein expression levels of the three PHD isoforms were evaluated in biopsies of 5 healthy controls and in inflamed biopsies of 5 UC patients (3 with severe disease (Mayo endoscopic score 3), 2 with mild to moderate disease (Mayo endoscopic score 1-2)) and 5 CD patients (2 with severe disease and 3 with mild to moderate disease). As shown in Figure 2A and Figure 2B, PHD1 protein expression was significantly increased in both UC ($p<0.05$) and CD ($P<0.05$) patients compared to healthy controls. PHD2 protein levels were not altered between all groups. The PHD3 protein expression was not significantly different between inflamed samples of CD patients versus healthy controls (Figure 2B). However, the expression in the inflamed samples from severely diseased UC patients (Figure 2A; lane 6, 9 and 10) was significantly lower compared to healthy controls.

On immunohistochemistry, no disease-dependent localisation of the PHDs was observed. PHD1 was predominantly found in regenerative epithelial cells and in the cytoplasm of mononuclear cells (e.g. dendritic cells, macrophages) in the lamina propria (Figure 3A). Lymphocytes were PHD1 negative. For PHD2, we observed strong nuclear staining in a wider range of cell types than for PHD1. Approximately half of the cells in the epithelium, inflammatory cell infiltrate (mononuclear cells in the lamina propria and lymphocytes) and smooth muscle cells in the muscularis mucosae showed strong PHD2 staining (Figure 3B). Lastly, we found that the PHD3 protein is specifically located in the endothelium of blood vessels (Figure 3C).

Discussion

In this study, we analysed the expression and the localisation of the different PHD isoforms in IBD patients, in order to identify the primary target(s) for the development of specific PHD-inhibitors.
The current treatment strategy for both CD and UC is focused on the suppression of inflammation. Standard therapy includes corticosteroids, 5-ASA preparations, immunomodulating drugs and/or biologicals. Despite these drugs, approximately 70% of the patients with CD and 35% of patients with UC ultimately come to surgery. Therefore, research in IBD is still focused on the identification of novel therapeutics to improve the disease outcome. In this regard, pan-hydroxylase inhibitors have been proposed as promising therapeutic compounds for IBD [1-4], but most of these studies lack human data to support their claim.

We found strongly increased mRNA expression of PHD1 and PHD3 in inflamed biopsies from patients with UC whereas inflamed biopsies from patients with CD and infectious colitis only displayed a slight increase in PHD1 expression. Only PHD1 showed a good correlation with the pro-inflammatory markers IL-8 and TNF. Whether or not inflammatory cytokines directly influence the PHD expression or vice versa is a subject of further research.

In accordance with our mRNA results, a significant elevation of PHD1 protein expression was observed in inflamed biopsies of both UC and CD patients ($p<0.05$), while PHD2 protein levels remained unaltered. PHD3 protein expressions were comparable between all groups except for UC patients. In contrast to the mRNA levels, severely diseased UC patients displayed a significant decrease in PHD3 expression ($p<0.05$). This might, at least in part, be explained by the fact that Siah 2, a E3 ubiquitin ligase, becomes activated as oxygen concentration decreases due to the extensive consumption of oxygen by the inflammatory cells, leading to the proteasomal degradation of PHD3 [7,8]. The same phenomenon was not seen in CD patients, where PHD3 expression did not follow the severity of the disease. This is not unexpected because biopsies from CD patients are always characterized by a discontinuous infiltrate of inflammatory cells so that the fluctuating levels of high and low oxygen give rise to a net hypoxic situation that is less pronounced than in patients with severe UC.

Apart from a role in inflammation, a role of PHDs in apoptosis has also been suggested. It has been shown that inhibition of PHD1 and PHD2 results in activation of HIF-1α and NF-κB [9], both being transcription factors that regulate the expression of several genes involved in apoptosis [10]. Inflammatory bowel disease is hallmarked by an increased rate of intestinal epithelial cell death. In fact, one of the main mechanisms of action by which pan-hydroxylase inhibitors are able to suppress experimental colitis, is probably by reducing colonic epithelial cell apoptosis [11]. Our data also indirectly imply a role of PHD1 and PHD2 in colonic epithelial apoptosis as these isoforms show a positive correlation with caspase 3, a marker of apoptosis. It are exactly these isoforms that can be found in the colonic epithelium.

In conclusion, only PHD1 was up-regulated both at the mRNA and the protein level and showed an excellent correlation with both inflammatory markers and apoptosis in IBD (especially in UC). Although we acknowledge that PHD1 protein expression as such is not directly related to its enzymatic activity, our exploratory expression analysis puts PHD1 forward as the primary therapeutic target for UC and, to a lesser extent, for colonic CD. This is further supported by the observation that PHD1-deficient mice, and not PHD2 and 3, are highly protected against colitis by reducing epithelial cell apoptosis and hence, by maintaining barrier function [12].

List of abbreviations

PHD: prolyl hydroxylase domain-containing protein

IBD: Inflammatory bowel disease

CD: Crohn's disease

UC: ulcerative colitis

Competing interests

None of the authors have competing interest with regard to the manuscript.

Authors' contributions

SVW carried out the studies and data analyses and drafted the manuscript. DL supervised samples analyses and statistics. MDV participated in the design and coordination of the study. PH conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1: mRNA expression of PHD1, PHD2 and PHD3 in human colonic biopsies. mRNA expression levels of PHD1 (A), PHD2 (B) and PHD3 (C) in colonic samples of healthy controls, IBD patients and patients with infectious colitis of the first patient cohort. The data are expressed as medians and presented on a log scale (****P<0.0001, *P<0.05).

Figure 2: Protein expression levels of PHD1, 2 and 3 in human colonic samples. A) Protein expression of PHD1, PHD2 and PHD3 in whole biopsy lysates of 5 healthy controls and 5 UC patients (lane 6, 9 and 10: severe UC and lane 7 and 8:mild to moderate UC). B) Protein expression of PHD1, PHD2 and PHD3 in whole biopsy lysates of 5 healthy controls and 5 CD patients (lane 7 and 8: severe CD and lane 6, 9 and 10: mild to moderate CD). The columns represent the densitometric evaluation of the PHDs, normalized to GAPDH (mean ± SEM)(*P<0.05).

Figure 3: Immunostaining of human biopsies for PHD1, PHD2 and PHD3. A) Immunostaining of PHD1 demonstrates cytoplasmatic staining of mononuclear cells in the lamina propria and of regenerative epithelium. B) Immunostaining of PHD2 shows nuclear staining of epithelium, mononuclear cells in the lamina propria and smooth muscle cells in the muscularis mucosae. C) Immunostaining of PHD3 reveals selective staining of the endothelium of blood vessels. Only the representative images (200x) of UC patients are given as no disease-dependent localisation of the PHDs was observed.

Tables

Table 1: Patient characteristics. CD: Crohn's disease; UC: ulcerative colitis; A1: 0-16yrs, A2: 16-40yrs, A3: >40 yrs. Location of disease in and disease behavior of CD was defined as maximal disease before surgical resection; L1: solely ileal disease, L2: solely colonic disease, L3: ileal and colonic disease, L3 + L4: ileal, colonic and upper gastrointestinal tract disease, L4: upper gastrointestinal tract disease; B1: non-stricturing, non-penetrating, B2: stricturing, B3: penetrating, (X_P): number of patients when concomitant perianal disease was present; E1: ulcerative proctitis, E2: left-sided UC, E3: pancolitis.

Group	*Healthy controls*	*UC inflamed*	*UC remission*	*CD inflamed*	*CD remission*	*Infectious colitis*
N (Biopsies)	20	16	5	19	10	9
Gender (male/female)	5/15	8/8	4/1	8/11	4/6	6/3
Age, years (mean)	49	38	50	32	49	36
Age, years (range)	12-73	14-58	26-70	11-54	28-73	17-58
Age at diagnosis A1/A2/A3		1/9/6	0/4/1	4/11/4	0/6/4	
Max location of disease L1/L2/L3/L3+L4/L4 E1/E2/E3		3/10/3	0/4/1	0/7/9/3/0	3/2/4/0/1	
Max disease behaviour B1/B2/B3				12/3/4 (9^P)	4/3/3 (4^P)	
Medication No 5-aminosalicylates	20	5 11	3 2	13 6	8 2	9

Table 2: Sequences of used qRT-PCR primers and PCR efficiencies. hSDHA, human succinate dehydrogenase complex subunit A; hPHD1, human prolyl hydroxylase domain 1; hPHD2, human prolyl hydroxylase domain 2; hPHD3, human prolyl hydroxylase domain 3; hIL-8, human interleukin 8; hTNF, human tumor necrosis factor alpha; hCasp3, human caspase 3.

Gene Symbol	*Forward Primers (5'-3')*	*Reverse Primers (5'-3')*	*PCR efficiencies (%)*
hSDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	92
hPHD1	CCGGAGGAAAAAGCTCGCCACCC	CCTCTGCGGTCCCTAAGGGCTT	105
hPHD2	CAGCATGGACGACCTGATAC	TACATAACCCGTTCCATTGC	103
hPHD3	AAAGGCGCCCTCCGACTCCT	CGACCCGTTTCCGGACTGGC	103
hIL-8	TGTTCCACTGTGCCTTGGTTTC	TGTGAGGTAAGATGGTGGCTAATAC	102
hTNF	ATGAGCACTGAAAGCATGATCC	GAGGGCTGATTAGAGAGAGGTC	112
hCASP3	GAGTGCTCGCAGCTCATACCT	CCTCACGGCCTGGGATTT	87

Table 3: Correlations. Correlation between the expression of the inflammatory cytokines IL-8 and TNF-α, the apoptosis marker caspase 3 and the expression of the different PHD isoforms in colonic mucosal biopsies (Pearsons r for IL-8 and TNF-α, Spearman's r for caspase 3).

	PHD1	**PHD2**	**PHD3**
IL-8	0.576 (P<0.001)	0.089 (NS)	0.291 (P=0.009)
TNF	0.706 (P<0.001)	0.177 (NS)	0.280 (P=0.012)
Caspase 3	0.594 (P<0.0001)	0.463 (P=0.001)	0.262 (NS)

Figure 1

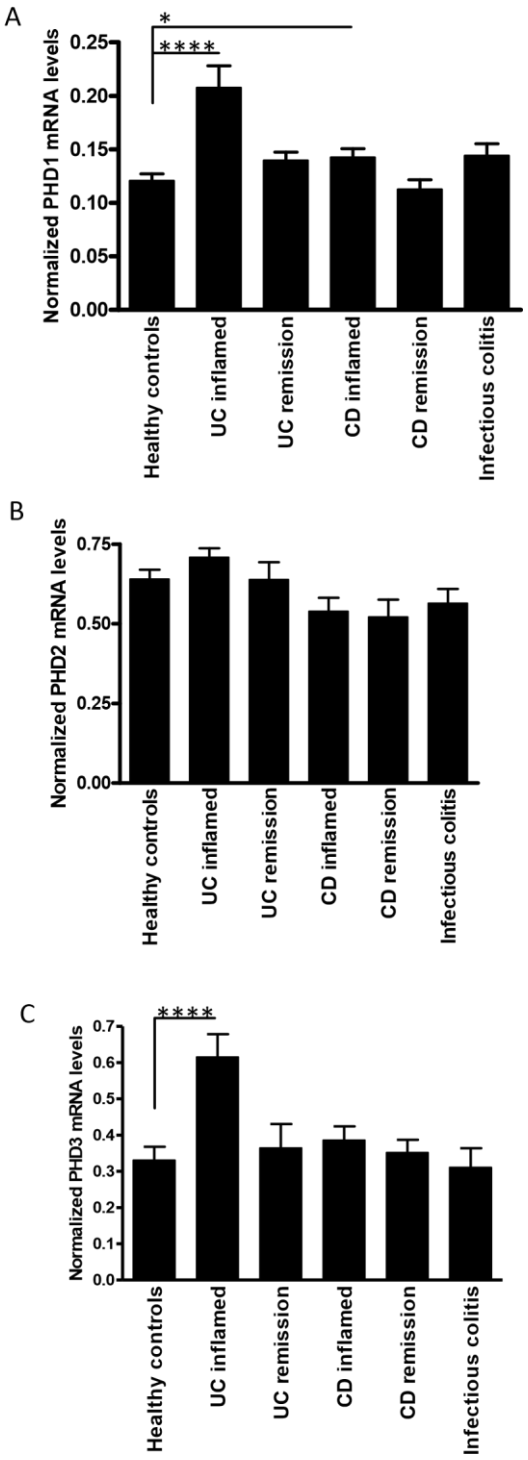
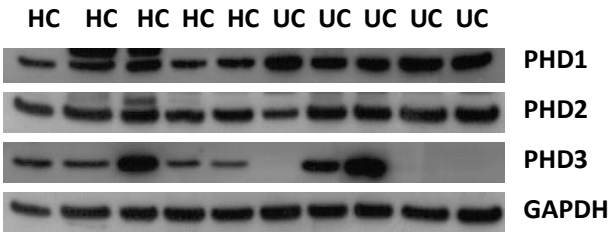
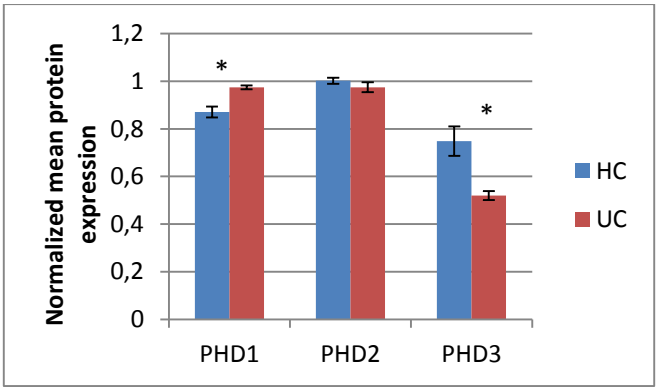

A
*
****
Normalized PHD1 mRNA levels
0.25
0.20
0.15
0.10
0.05
0.00
Healthy controls
UC inflamed
UC remission
CD inflamed
CD remission
Infectious colitis
B
Normalized PHD2 mRNA levels
0.75
0.50
0.25
0.00
Healthy controls
UC inflamed
UC remission
CD inflamed
CD remission
Infectious colitis
C
****
Normalized PHD3 mRNA levels
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Healthy controls
UC inflamed
UC remission
CD inflamed
CD remission
Infectious colitis

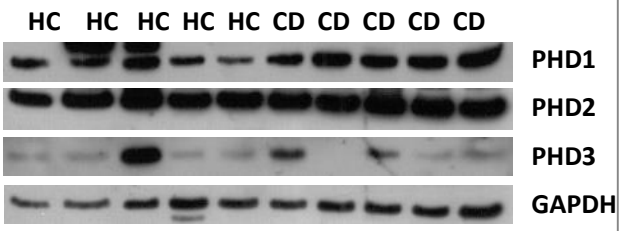
Figure 2

A


HC HC HC HC HC UC UC UC UC UC
PHD1
PHD2
PHD3
GAPDH


Normalized mean protein expression
1,2
1
0,8
0,6
0,4
0,2
0
*
*
PHD1
PHD2
PHD3
HC
UC

B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PHD1
PHD2
PHD3
GAPDH

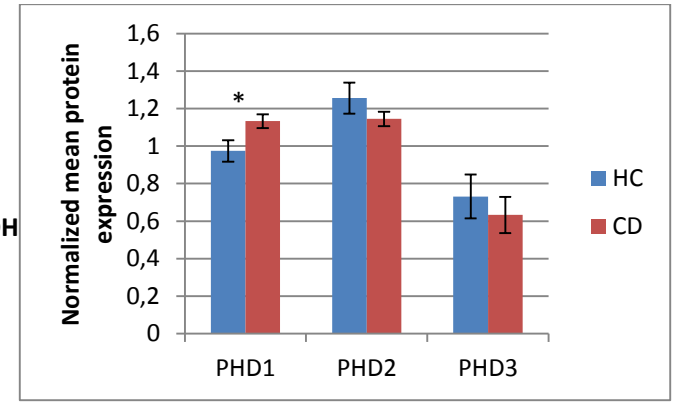
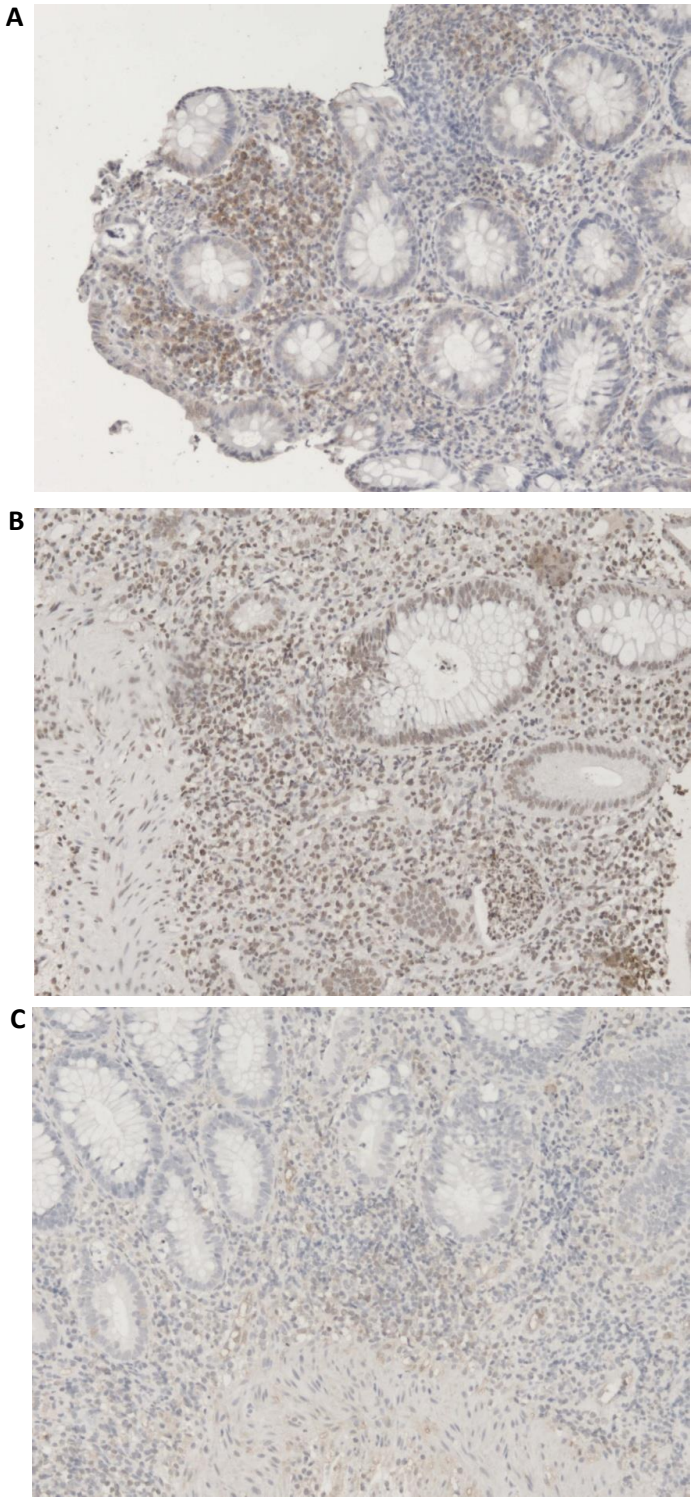

Normalized mean protein expression
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
PHD1
PHD2
PHD3
HC
CD

Figure 3



2 Haematopoietic prolyl hydroxylase-1 deficiency promotes M2 macrophage polarization and is both necessary and sufficient to protect against experimental colitis

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Abstract

Prolyl hydroxylase domain-containing proteins (PHD) regulate the adaptation of cells to hypoxia. Pan-hydroxylase inhibition is protective in experimental colitis in which PHD1 plays a prominent role. However, it is currently unknown how PHD1 targeting regulates this protection and which cell type(s) are involved. Here we demonstrated that *Phd1* deletion in endothelial and haematopoietic cells (*Phd1*$^{f/f}$Tie2:cre) protected mice from dextran sulphate sodium (DSS)-induced colitis with reduced epithelial erosions, immune cell infiltration and colonic microvascular dysfunction, whereas the response of *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice to DSS was similar to their littermate controls. Using bone marrow chimeras and cell-specific cre mice we could demonstrate that ablation of *Phd1* in haematopoietic cells, but not endothelial cells was both necessary and sufficient to inhibit experimental colitis. This effect relied, at least in part, on skewing of *Phd1*-deficient bone marrow-derived macrophages towards an anti-inflammatory M2 phenotype. These cells showed an attenuated NF-κB-dependent response to lipopolysaccharide (LPS) which in turn diminished endothelial chemokine expression. In addition, *Phd1*-deficiency in dendritic cells significantly reduced interleukin (IL)-1β production in response to LPS. Taken together, our results further support the development of selective PHD1-inhibitors for ulcerative colitis and identify haematopoietic cells as their primary target.

Key words:

Prolyl hydroxylase-1, haematopoietic cells, macrophages, dendritic cells, ulcerative colitis

Introduction

Active Inflammatory Bowel Disease (IBD), comprising Crohn's disease and ulcerative colitis (UC), is associated with severe mucosal hypoxia resulting from the increased oxygen consumption of inflammatory cells and decreased oxygen supply by dysfunctional blood vessels. This imbalance, in addition to excessive cytokine levels, leads to increased epithelial cell death and subsequently contributes to impaired mucosal barrier function. The cellular response to low oxygen levels is tightly regulated by oxygen-sensing prolyl hydroxylase domain-containing proteins (PHD). Under normoxic conditions, these enzymes induce the ubiquitination of the hypoxia-inducible factor (HIF) subunits HIF-1α and HIF-2α, which leads to their proteasomal degradation. During hypoxia, the enzymatic activity of PHDs is inhibited, which ensures the stabilization of HIFα subunits and the formation of the active transcription factors HIF-1 and HIF-2. These effects, in turn, transactivate the expression of genes involved in angiogenesis, erythropoiesis, cell migration and metabolism, thereby protecting the host cell against low oxygen conditions [1, 2].

PHDs have been investigated as therapeutic targets in IBD. In particular, pan-hydroxylase inhibitors ameliorate disease in murine IBD models and are set to enter the clinical trial phase [3-5]. However, potential risks including the promotion of tumor growth through increasing angiogenic potential, and cardiovascular events may be associated with systemic pan-hydroxylase inhibition [6-8]. Therefore, current research is focusing on the role of the different PHD isoforms (PHD1-3) in the pathogenesis of IBD. We have recently demonstrated that *PHD1* expression is increased in inflamed IBD biopsies, especially in active UC [9]. Consistent with these findings, Tambuwala and colleagues demonstrated that *Phd1*-deficient mice are protected against colitis [10]. However, it is unknown which cell type(s) need to be targeted to achieve these beneficial effects. It was hypothesized that epithelial PHD1 mediated this protection by decreasing epithelial cell apoptosis and barrier dysfunction, both prominent features of active IBD. However, this might be a secondary phenomenon because the epithelial apoptosis rate seems to be driven by the local inflammatory response rather than vice versa [11]. This is supported by the fact that the currently available effective therapeutic strategies in IBD intervene at the site of the immune cells (corticosteroids, purine-analogues, methotrexate, cyclosporine, anti-tumor necrosis factor alpha (TNF), anti-interleukin 12/23 (IL-12/IL-23)) or at the interaction of these immune cells with the intestinal microvessels (anti-integrins, anti-mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1)) [12, 13]. We therefore investigated the role of PHD1-3 in endothelial and haematopoietic cells on blood vessel function, epithelial integrity and the inflammatory burden in experimental colitis.

Materials and methods

Animals

The mice used in this study were male and female on a C57BL/6 background. The generation of the floxed *Phd1*$^{f/f}$, *Phd2*$^{f/f}$ and *Phd3*$^{f/f}$ mice has been described previously [14-16]. The *Phd1*$^{f/f}$Tie2:cre, *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice were obtained by crossing the *Phd1*$^{f/f}$, *Phd2*$^{f/f}$ and *Phd3*$^{f/f}$ mice with Tie2:cre mice [17]. The heterozygous *Phd2* Tie2:cre mice (*Phd2*$^{f/+}$Tie2:cre) were used because the homozygous mice (*Phd2*$^{f/f}$ Tie2:cre) displayed growth retardation. The *Phd1*$^{f/f}$Flk-1:cre, *Phd1*$^{f/f}$Vav:cre and *Phd1*$^{f/f}$CD11c:cre mice were obtained by breeding Flk-1:cre, Vav:cre and CD11c:cre mice with the *Phd1*$^{f/f}$ mice. The characterization of Flk-1:cre, Vav:cre and CD11c:cre mice has been reported previously [18-20].

Mice from different genotypes were cage-mixed during each experiment to minimize the influence of gut microbiota [21]. The mice were bred and housed in individually ventilated cages in a temperature-controlled room at 22 °C with a 12 h/12 h light-dark cycle. The animals had free access to water and commercial chow (mice maintenance chow, Carfil Labofood, Pavan Service, Belgium). All mice were treated in accordance with the institutional animal health care guidelines, following study approval (ECD2010/40, ECD2013/49, ECD2015/21, ECD2015/58 and ECD2016/29) by the Institutional Review Board at the Faculty of Medicine and Health Sciences of Ghent University.

Induction of colitis and BM transplantation

Eight-to-twelve-week-old *Phd1*$^{f/f}$Tie2:cre, *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice and their corresponding wild-type (WT) littermate control mice *Phd1*$^{f/f}$, *Phd2*$^{f/+}$ and *Phd3*$^{f/f}$, respectively, received 4% dextran sulphate sodium (DSS; MW 36 000–50 000; MP Biomedicals, CA, USA) in their drinking water for seven consecutive days, followed by normal drinking water. The *Phd1*$^{f/f}$Flk-1:cre, *Phd1*$^{f/f}$Vav:cre and their corresponding WT littermate mice were given 3% DSS. Uptake of DSS-containing water was monitored daily and was comparable between cages. Over time, the DSS dosage had to be adjusted to 3% because 4% DSS led to a more severe induction of colitis with a high mortality rate likely because of the environmental and structural changes in our animal facility. The disease activity index (DAI) was determined according to the criteria proposed by Cooper *et al.* [22].

One week prior to irradiation, the mice for bone marrow (BM) transplantation received acidified water (pH between 2.4 and 3.1) that contained 0.1 mg/ml neomycin (Sigma-Aldrich, Diegem, Belgium) and 0.01 mg/ml polymyxin B sulphate (Sigma-Aldrich, Diegem, Belgium). Six-to-eight-week-old mice were subjected to 9.5 Gy lethal total-body irradiation using an X-ray source. Between 4 and 12 hours later, the mice were intravenously reconstituted with BM cells (10^7) prepared from the femur, tibia and humeri of the WT or *Phd1*$^{f/f}$Tie2:cre mice. Following irradiation and BM transplantation, the mice were

maintained on acidified, antibiotic-containing water and were allowed to recover for 8 weeks on a chow diet. Colitis was subsequently induced using 3% DSS in the drinking water. The uptake of donor BM was verified via PCR following sacrifice of the mice.

Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) for vascular permeability measurements during colitis

The MR images were acquired on a 7 Tesla small animal system (Bruker BioSpin® Pharmascan 70/16, Ettlingen, Germany) with a mouse body volume coil. The mice were anesthetized with isoflurane (5% induction, 1.5% maintenance, mixed with medical oxygen) and actively heated with a water-based heating blanket. Anatomical information was obtained with a T1-weighted sequence (RARE) with the following parameters: TR 1453 ms, TE 9.1 ms, 4 averages, echo train length 4, field of view 3 x 2.4 cm, matrix 250 x 200, 120 µm in-plane spatial resolution, 30 contiguous slices with 600 µm thickness, acquisition time 3 min 35 s. Dynamic contrast enhanced MR images were acquired in a single slice using a 2D fast low angle shot (FLASH) sequence with the following parameters: TR 12 ms, TE 3.4 ms, flip angle 25°, field of view 3 x 3 cm, matrix 112 x 112, 268 µm in-plane resolution, 550 repetitions, temporal resolution 1.344 s and acquisition time 12'19". The slice was positioned to contain the colon, based on the anatomical images. The gadolinium-based contrast agent (Dotarem, Guerbet, France, 2 mmol/kg) was injected intravenously 30 seconds after the initiation of the acquisition. The total acquisition time per session was 25'. The DCE data were processed with MiStar® (Apollo, Melbourne, Australia). A two-compartment model (blood and extracellular extravascular space) was used to obtain parametric maps with K^{trans} values on a pixel-by-pixel basis [23]. These K^{trans} values are a measure of the vascular leakage, and the mean K^{trans} values were calculated for each colonic slice of each animal.

Isolation and culture of bone marrow derived macrophages and dendritic cells

Primary BM-derived macrophages (BMDM) and dendritic cells (BMDC) were isolated by flushing the bone marrow out of femur and tibia. The BMDM were subsequently cultured in 10 x 15 mm Petri-dishes in DMEM+glutamax supplemented with 10% foetal calf serum (FCS) (Invitrogen, Ghent, Belgium), penicillin/streptomycin (Invitrogen) and 20 ng/ml recombinant murine macrophage colony stimulating factor (M-CSF, Peprotech, London, UK) with a complete change of medium every 2-3 days. The BMDC cultures were set up according to the protocol originally published in Lutz, et al 1999 [24]. After 5 days, 10^6 BMDM were stimulated for 24 h with 100 ng/ml lipopolysaccharide (Ultrapure LPS from E. coli K, Invivogen, San Diego, California, USA), 20 ng/ml murine recombinant IL-4 (Peprotech), 125 nM ACHP (Tocris, Bristol, UK) and 20 ng/ml IL-4 or an equal volume of placebo. After 10 days in culture, 10^6 BMDC were treated for 16 h with 100 ng/ml LPS (Invitrogen) or an equal volume of placebo. The cells were subsequently lysed for RNA isolation, and the supernatant was collected.

Statistical analysis

Data were analysed using SPSS Statistics, version 22, for Windows (SPSS Inc., Chicago, IL, USA) or GraphPad Prism® software (GraphPad Software Inc., San Diego, CA, USA). In the case of normally distributed data, the differences between the groups were analysed using an unpaired Student's *t*-test for independent samples. For the non-normal or unknown data distributions, the groups were compared using the non-parametric Mann-Whitney U-test. Continuous data (body weight and DAI changes) were analysed using linear mixed models. Two-tailed probabilities were calculated and a probability value of $p < 0.05$ was considered statistically significant.

Results

Characterization of *Phd1*$^{f/f}$Tie2:cre mice

To determine the degree of *Phd1* recombination in cre:mice, qPCR primers were designed to detect the *Phd1* allele as it occurs in the WT mice, whereas the other primers bind the remaining *Phd1* allele as it occurs following recombination in the *Phd1*$^{f/f}$Tie2:cre mice. In the WT mice, the forward primer binds the *Phd1* cDNA sequence at exon 2, before the floxed exons (exon 3 - exon 4), whereas the reverse primer binds exon 3, which will be deleted if recombination occurs (Figure S1F, left). As a positive control for effective recombination, primers were designed in which one binds at the transition between exon 2 and exon 5, which does not occur in WT mice, and the reverse primer binds exon 5 (Figure S1F, right). Thus, the allele detected by the first primer pair will be referred to hereafter as "*Phd1* wt", whereas the "remaining" *Phd1* allele, detected by the second primer pair, will be denoted "*Phd1* ko". The primer sequences are provided in Table S1. It has been reported that when crossed with floxed mice, Tie2:cre mice generate a recombination of the floxed gene in endothelial and haematopoietic cells [17]. We therefore first examined whether efficient recombination occurs in these cellular compartments of our mice. To this end, we isolated bone marrow and colonic endothelial cells from the *Phd1*$^{f/f}$Tie2:cre and WT mice. The bone marrow, bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDC) exhibited a decrease in *Phd1* mRNA of 62%, 85% and 85%, respectively, (Figure S1A-C) and a concomitant increase in the *Phd1* ko expression. Additionally, CD4+ T lymphocytes isolated from the spleen exhibited a 75% reduction in the *Phd1* mRNA level accompanied by increased *Phd1* ko expression (Figure S1D). In addition, we isolated colonic epithelial cells from the *Phd1*$^{f/f}$Tie2:cre and WT mice as a negative control, as well as to confirm potential effects arise exclusively from endothelial or haematopoietic knockout. As expected, the colonic epithelial cells from both *Phd1*$^{f/f}$Tie2:cre and WT mice exhibited comparable *Phd1* mRNA levels. Consistent with this finding, no expression of the *Phd1* ko allele was identified in the groups (Figure

S1E). However, the *Phd1* mRNA expression in the isolated primary colonic endothelial cells was only decreased by 37% with a slight induction of *Phd1* ko expression (Figure S1F left). As a consequence, we concluded that the use of Tie2:cre transgenic mice is not the best model to generate an efficient knockdown in the colonic vasculature. Therefore, the effect of endothelial-specific *Phd1* knockdown was re-evaluated using *Phd1*$^{f/f}$Flk-1:cre mice where the *Phd1* mRNA expression in isolated primary colonic endothelial cells was 75% reduced and a concomitant increase in *Phd1* ko expression was observed (Figure S1F right).

Phd1$^{f/f}$Tie2:cre mice exhibit highly attenuated signs of inflammation and blood vessel dysfunction during DSS-induced colitis

We generated *Phd1*$^{f/f}$Tie2:cre, *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice that are conditionally deficient for *Phd1*, *Phd2* and *Phd3*, respectively, in both endothelial and haematopoietic cells. To investigate the effect of conditional *Phd1-3* deficiency on colitogenesis, we subjected these mice and their respective WT littermate controls to DSS-induced colitis and compared their clinical parameters. The *Phd1*$^{f/f}$Tie2:cre mice, but not the *Phd2*$^{f/+}$Tie2:cre or *Phd3*$^{f/f}$Tie2:cre mice were significantly protected from DSS-induced colitis as determined by weight loss, DAI, colonic shortening and histological inflammation score (Figure 1A-E) compared with the WT mice. In addition, we observed fewer epithelial erosions and less infiltration of inflammatory cells in the *Phd1*$^{f/f}$Tie2:cre mice (Figure 1F-G). In contrast, weight evolution and histological inflammation score were not attenuated in the colitic *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice compared to their respective littermate controls (Figure 1A-C). We subsequently questioned whether *Phd1*-deficiency influenced colonic blood vessel function. We therefore evaluated the *in vivo* colonic microvascular leakage at days 0, 3 and 7 during the course of colitis, using dynamic contrast-enhanced micro magnetic resonance imaging (DCE-MRI) (Figure 2A-B). At day 3, no change in leakage was identified compared with day 0. However, the vascular leakage in the WT mice was significantly increased at day 7, whereas the *Phd1*$^{f/f}$Tie2:cre mice exhibited leakage comparable to the baseline levels. Consistent with these findings, at the end of the DSS experiment (day 11), the mRNA expressions of the known endothelial dysfunction markers *Icam1*, *Vcam1*, *Madcam1*, *Vwf* and *Vegfr2* were strongly induced at the end of the DSS experiment (day 11) in the WT animals, whereas these markers were significantly lower in the *Phd1*$^{f/f}$Tie2:cre littermates (Figure 2C). In addition, the expression of these endothelial dysfunction markers in *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice was comparable with the levels of their corresponding littermate controls (data not shown). Collectively, these findings demonstrate that *Phd1*$^{f/f}$Tie2:cre mice are selectively protected against the development of colitis and its associated endothelial dysfunction.

Endothelial *Phd1*-deficiency alone does not rescue mice from DSS-induced colitis

Next, we questioned whether the strong protective effects identified in the *Phd1*$^{f/f}$Tie2:cre mice resulted exclusively from *Phd1* targeting in endothelial cells. We therefore irradiated *Phd1*$^{f/f}$Tie2:cre mice and their littermate WT controls and intravenously injected them with WT BM to generate WT<-WT BM chimeras and *Phd1*$^{f/f}$Tie2:cre<-WT BM chimeras. Unexpectedly, no amelioration in terms of body weight, colon length or histological inflammation was identified in the *Phd1*$^{f/f}$Tie2:cre<-WT BM chimeras (Figure 3A-C, left). The *Phd1*$^{f/f}$Tie2:cre mice only exhibited a poor reduction in the *Phd1* mRNA expression in colonic endothelial cells as mentioned earlier; thus we re-evaluated the effect of endothelial *Phd1*-deficiency on colitogenesis using Flk-1:cre mice, which generate effective and specific recombination in endothelial cells (Figure S1F right). However, the *Phd1*$^{f/f}$Flk-1:cre mice were also not protected from DSS-induced colitis (Figure 3A-C, right). These findings conclusively demonstrate that endothelial cells are not the primary target for *Phd1*-deletion to protect mice from DSS-induced colitis.

Haematopoietic *Phd1*-deficiency is essential to confer protection from DSS-induced colitis

Since *Phd1*$^{f/f}$Tie2:cre mice also exhibit knockdown of *Phd1* in different cell types from the haematopoietic compartment, we questioned whether selective *Phd1* deletion in these cells may render mice less susceptible to colitogenesis. We therefore irradiated WT mice and half of the mice received WT BM from their WT littermate controls which resulted in WT<-WT BM chimeras. The remaining irradiated WT mice were injected with BM from their littermate *Phd1*$^{f/f}$Tie2:cre mice to generate WT<-*Phd1*$^{f/f}$Tie2:cre BM chimeras which exhibit sole knockout of *Phd1* in all haematopoietic cells. Eight weeks after irradiation, the mice were subjected to 3% DSS. Strikingly, the WT<-*Phd1*$^{f/f}$Tie2:cre BM chimeras were significantly protected from DSS-induced colitis, which was evidenced by reduced weight loss, colon shortening and histological inflammation score compared with the WT<-WT BM chimeras (Figure 4A-D). Also, fewer epithelial erosions and less infiltration of inflammatory cells was observed (Figure 4E-F). In addition, the abundance of *Icam1*, *Vcam1*, *Vwf* and *Vegfr2* mRNAs was significantly reduced in the WT<-*Phd1*$^{f/f}$Tie2:cre BM chimeras (Figure 4G). Successful validation was performed in *Phd1*$^{f/f}$Vav:cre mice, in which *Phd1* is also specifically targeted in all haematopoietic cells. These mice exhibited a significantly improved body weight evolution compared with their WT littermate controls (Figure 4H). Collectively, these findings demonstrate that the loss of *Phd1* in haematopoietic cells is both necessary and sufficient to reduce mucosal inflammation and its associated endothelial dysfunction during colitis.

Loss of *Phd1* in BMDM promotes their skewing towards an M2 phenotype with reduced response to LPS

We subsequently questioned which cell type(s) within this haematopoietic pool is/are the main contributor(s) to these protective effects. We first focused on macrophages because they are key players in both the onset and resolution of inflammation in DSS-induced colitis, depending on their phenotype [25-27]. While M1 macrophages secrete pro-inflammatory cytokines, M2 macrophages secrete anti-inflammatory cytokines, growth and angiogenic factors responsible for vascular remodelling and tissue repair. We found that *Phd1*$^{f/f}$Tie2:cre BMDM showed increased expression of the M2 marker *Arg1* at baseline as compared with WT BMDM. Moreover, when inducing M2 conversion with IL-4, increased levels of the M2 markers *Chi3l3*, *Fizz1* and *Arg1* were seen in the *Phd1*$^{f/f}$Tie2:cre BMDM compared with the WT BMDM (Figure 5A).

To assess the anti-inflammatory nature of these *Phd1*-deficient macrophages, we stimulated BMDM from both WT and *Phd1*$^{f/f}$Tie2:cre mice with LPS and determined the secretion of pro-inflammatory cytokines and chemokines. The supernatant from the *Phd1*$^{f/f}$Tie2:cre BMDM contained significantly lower levels of IL-1β, IL-6, TNF and MCP-1 compared with the protein levels in the supernatant of the WT BMDM (Figure 5B). In addition, when this supernatant was added to MS1 endothelial cells, the induction of the endothelial chemokines *Cxcl2* and *Mcp1* was significantly suppressed when compared with the endothelial cells stimulated with LPS-CM from WT BMDM (Figure S2).

Taken together, these observations indicate that the loss of *Phd1* skews macrophages towards an M2 phenotype with a reduced inflammatory response to LPS.

NF-κB activation state is altered during steady-state and inflammatory conditions upon loss of *Phd1* in BMDM and is partly responsible for the promotion of M2 conversion

Since the expression of TNF, IL-6, IL-1β and MCP-1 can all be regulated by NF-κB, and the latter can also be up-regulated by pan-hydroxylase inhibition [28], we determined the impact of *Phd1*-deletion in macrophages on the expression of signal transducers in the NF-κB pathway in steady-state and inflammatory conditions. At baseline, the *Phd1*$^{f/f}$Tie2:cre BMDM exhibited increased levels of phosphorylated IκBα (P-IκBα) and P-p65 compared with the WT BMDM (Figure 6A-B). Accordingly, the expression of IκBα, a known NF-κB target [29], was significantly increased in the *Phd1*$^{f/f}$Tie2:cre BMDM compared with the WT BMDM (Figure 6B). We next questioned whether the increased NF-κB activity at baseline in the *Phd1*$^{f/f}$Tie2:cre BMDM was responsible for the facilitation towards M2 polarization. We therefore incubated WT and *Phd1*$^{f/f}$Tie2:cre BMDM with IL-4 or with the NF-κB inhibitor ACHP, which is in fact an IκB kinase (IKK) inhibitor, or with ACHP in combination with IL-4. We

observed that inhibition of NF-κB in the *Phd1*$^{f/f}$Tie2:cre BMDM reduced the IL-4-induced increase in *Chi3l3* and *Fizz1* expression to levels comparable with the ACHP and IL-4 treated WT BMDM (Figure 6C). The expression of *Arg1* remained elevated in the ACHP and IL-4 treated *Phd1*$^{f/f}$Tie2:cre BMDM, indicating that NF-κB is not involved in the regulation of this M2 marker (Figure 6C). Taken together, the increased baseline NF-κB activity in the *Phd1*$^{f/f}$Tie2:cre BMDM is partly responsible for their facilitated M2 conversion.

However, after LPS stimulation, levels of P-IκBα were significantly less up-regulated in the *Phd1*$^{f/f}$Tie2:cre BMDM compared with the WT BMDM (Figure 6A) which indicates LPS tolerance in *Phd1*$^{f/f}$Tie2:cre BMDM that could account for the reduced levels of the aforementioned inflammatory cytokines and chemokine.

Loss of *Phd1* in BMDC reduces LPS-induced IL-1β secretion

In addition to macrophages, DCs are also essential in the maintenance of tissue homeostasis in the intestinal microenvironment through the induction of tolerance [30]. Pathological disorders such as IBD alter the DC phenotype, inducing a more pro-inflammatory state and infusion of these inflammatory DCs is sufficient to induce and propagate inflammation [31]. Therefore, we investigated if *Phd1*-deficient DCs could also play a role in the protection seen after haematopoietic *Phd1* knockout. We therefore set up BMDC cultures from WT and *Phd1*$^{f/f}$CD11c:cre mice. The CD11c driven expression of the Cre recombinase transgene efficiently abolished *Phd1* expression in BMDC (data not shown). The resulting BMDCs were stimulated with LPS and the concentration of secreted cytokines was determined. DCs from *Phd1*$^{f/f}$CD11c:cre mice secreted significantly lower amounts of IL-1β and a trend towards lower IL-12 secretion after LPS stimulation compared with the levels from WT DCs (Figure 5C). The expression of IL-6 and TNF was comparable between both groups. This indicates that in addition to macrophages, *Phd1* deletion in DCs renders them partly tolerant to LPS activation.

***Phd1*-deficiency does not influence T-cell cytokine secretion after activation**

In addition to beneficial effects on members of the innate immune system (BMDM and BMDC), we wanted to know if *Phd1* knockout also influenced T-cell functioning because CD4+ T-cells are known to play an important role in the course of DSS-induced colitis [32, 33]. Hence, we stimulated spleen single cell suspensions from WT and *Phd1*$^{f/f}$Vav:cre mice with PMA and ionomycin to specifically accomplish T-cell activation and 4 h later cytokine levels were determined in the supernatant. First, we analysed the *Phd1* knockout degree in CD4+ T-cells. As expected, CD4+ T cells from *Phd1*$^{f/f}$Vav:cre mice displayed

complete deletion of the *Phd1* gene when compared with the expression in WT CD4+ T cells (data not shown). When comparing the concentrations of IFN-γ, TNF and IL-10, no significant differences could be detected between WT and *Phd1*$^{f/f}$Vav:cre mice (Figure S3). These results suggest that *Phd1* deletion does not affect T-cell cytokine release.

Discussion

In the present study, we demonstrated that genetic targeting of *Phd1* in haematopoietic cells prevented colonic inflammation and the associated mucosal microvascular dysfunction in mice. We revealed that these beneficial effects are, at least in part, mediated by a promotion of *Phd1*-deficient macrophages towards M2 polarization with a diminished pro-inflammatory response towards LPS.

In line with the previously reported results of Tambuwala and colleagues [10], we observed that *Phd1*, and not *Phd2* or *Phd3*, needs to be targeted in order to achieve protection from DSS-induced colitis. However, while Tambuwala *et al.* proposed a reduction of colonic epithelial apoptosis with resulting preservation of barrier function as a primary mechanism, we could show that genetic deletion in endothelial and haematopoietic cells was sufficient to elicit a strong protection against DSS-induced colitis with less epithelial erosions, a preservation of mucosal microvascular barrier function and a lower expression of the endothelial dysfunction markers *Icam1*, *Vcam1*, *Madcam1*, *Vwf* and *Vegfr2*.

We endeavoured to identify the key cell types and mechanisms behind this strong protective effect, hereby focusing on the main cell types involved in colitogenesis. Using bone marrow chimeras and cell-specific cre:mice, we were able to show that the deletion of *Phd1* in haematopoietic, but not endothelial cells was critical to confer protection from colitis. Interestingly, whereas *Phd1*-deletion in activated T-cells did not alter their cytokine secretion and PHD1 in neutrophils does not seem to play an apparent role during hypoxic and inflammatory conditions based on previous observations [10, 34], we identified an important role for *Phd1* within the mononuclear phagocyte system. We demonstrated that *Phd1*-deletion in BMDM was accompanied by a facilitation towards M2 polarization. Consistent with this finding, Hams *et al.* showed that the administration of dimethyloxalylglycine (DMOG), a pan-hydroxylase inhibitor, is also associated with enhanced levels of M2 markers [35] which suggests that the inhibition of PHD1 might be involved in this effect. In addition, the M2 phenotype caused by *Phd1*-deletion was accompanied by significantly lower amounts of the pro-inflammatory cytokines IL-1β, IL-6 and TNF and the chemokine MCP-1 following stimulation with LPS. This phenotypic switch could be particularly relevant in IBD in which, on the one hand the intestinal mucosa is characterized by an increased proportion of M1 macrophages [36] that directly contribute to epithelial barrier disruption

mainly through TNF release [37], and on the other hand by a decreased proportion of pro-angiogenic "wound healing" M2 macrophages [38, 39]. This imbalance is also present in DSS-induced colitis and the disease course can be ameliorated by adoptive transfer of M2 macrophages [26]. Also, proteins and metabolites that promote M2 conversion are effective in reducing the severity of experimental colitis [40-42].

To further dissect the molecular mechanism by which *Phd1*-deletion in BMDM altered their phenotype, we focused on NF-κB, since its activation can be regulated by PHDs [43] and it is a key transcriptional regulator of both M1 and M2 polarization [44]. We found increased levels of P-IκBα and P-p65 and elevated expression of the NF-κB target IκBα in the *Phd1*-deficient BMDM compared with the WT BMDM, which is in line with previous observations showing induction of NF-κB activity upon knockdown of *PHD1* in HeLa cells [28]. Moreover, we demonstrated that this elevated baseline NF-κB activity in *Phd1*-deficient BMDM is partly responsible for the facilitation towards M2 polarization. On the other hand, levels of P-IκBα after LPS administration were markedly less pronounced in the *Phd1*$^{f/f}$Tie2:cre BMDM as compared with WT BMDM. This indicates that despite the higher baseline NF-κB activation, *Phd1*$^{f/f}$Tie2:cre BMDM are less responsive to an inflammatory stimulus which explained their reduced LPS-induced secretion of IL-1β, IL-6, TNF and MCP-1 in the *Phd1*$^{f/f}$Tie2:cre BMDM compared with the WT BMDM. A similar apparent paradox has been reported using DMOG. In addition to increasing HIF1-α levels, DMOG moderately increases basal NF-κB activity [28, 35], which renders cells tolerant to subsequent NF-κB activation, induced by LPS, and as a result, limited further NF-κB activation is observed with subsequent diminished NF-κB target gene expression [35]. LPS tolerance is obtained in a similar way [45]. Likewise, DMOG also attenuates the IL-1β-induced NF-kB activity by inhibiting PHD1 and factor inhibiting HIF (FIH)-dependent hydroxylation of proteins associated with the TRAF6 complex [46]. Since the latter is also a major part of the LPS pathway, this could explain how *Phd1*-deletion induces LPS tolerance.

Furthermore, we demonstrated that supernatant from LPS-stimulated *Phd1*-deficient macrophages was able to partly diminish the pro-inflammatory signature of endothelial cells, which suggests that *Phd1*-deficiency in BMDM could be responsible, at least in part, for the attenuation of colitis-associated endothelial dysfunction and leukocyte trafficking. In addition to macrophages, we investigated the effect of genetic *Phd1* deletion in DCs and we showed for the first time that *Phd1*-deficient DCs exhibit a marked decrease in IL-1β release in response to LPS. IL-1β is a pro-inflammatory cytokine that is elevated in active IBD [47] and has been shown to alter tight junction protein expression and distribution which consequently disrupts epithelial barrier integrity [48]. Therefore, *Phd1*-deficient macrophages and DCs may work synergistically in preserving barrier function during colitis through the reduced release

of IL-1β. Thus, *Phd1*-deficiency in mononuclear phagocytes, in addition to epithelial cells, regulates epithelial barrier integrity.

Future studies will have to address the translatability of our findings to human IBD. To date, no PHD1-specific inhibitor exists. However, one company (Aerpio Therapeutics) is currently developing a non-selective PHD inhibitor for clinical use in IBD.

In conclusion, we demonstrated for the first time that PHD1 exerts critical functions in haematopoietic cells, more specific in the mononuclear phagocyte system, that drive experimental colitis. Our results further support the development of PHD1-specific inhibitors for the treatment of UC.

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Author contributions

SVW conducted all animal experiments and the cell culture experiments with the macrophage and endothelial cells, interpreted the data, performed statistical analysis and drafted the manuscript; MDV and DL obtained funding and performed the critical revision of the manuscript for important intellectual content; SJT carried out the experiments with the BMDC, interpreted the data and performed critical revision of the manuscript; BW, MD, SN, BD, SDV, LVD, TH, LD, AB, GB, FDV, CC, SD, CV, GB, BNL, SJ, PC and SD performed the critical revision of the manuscript and provided technical and/or material support; DL conceived, analysed and interpreted the data and supervised the study; PH: study concept and design, analysis and interpretation of data, study supervision

Figure legends

Figure 1. *Phd1*$^{f/f}$Tie2:cre mice are protected against DSS-induced colitis. (A) Weight evolution of WT and *Phd1*$^{f/f}$Tie2:cre, *Phd2*$^{f/+}$Tie2:cre and *Phd3*$^{f/f}$Tie2:cre mice exposed to 4% DSS. (B) Representative 100x H&E-stained colonic sections and (C) total histological inflammation scores of the *Phd1*$^{f/f}$Tie2:cre mice at day 11 (left), the *Phd2*$^{f/+}$Tie2:cre at day 11 (middle) and the *Phd3*$^{f/f}$Tie2:cre at day 10 (right) of DSS-induced colitis. (D) DAI, (E) colon length, (F) epithelial erosion score and (G) inflammatory cell infiltration score of WT and *Phd1*$^{f/f}$Tie2:cre mice on day 11 of DSS-induced colitis (scale bar = 200 μm). n = 8. *p < 0.05, **p < 0.01, ***p < 0.001 and NS: not significant. Data are represented as the mean ± SEM.

Figure 2. *Phd1*$^{f/f}$Tie2:cre mice have less dysfunctional blood vessels. (A) Representative dynamic contrast-enhanced microMRI images of the colon of a WT (left) and a *Phd1*$^{f/f}$Tie2:cre mouse (right) at day 7 of DSS-induced colitis. The color scale in the upper panels represents the K^{trans} values as a measure of vascular leakage that ranges from low vascular leakage (black-purple) to high vascular leakage (red). The lower panel represents the anatomical positions where the K^{trans} values were calculated. (B) Vascular leakage in the WT and *Phd1*$^{f/f}$Tie2:cre mice at days 0, 3 and 7 of DSS-induced colitis. (C) Fold increase in mRNA expression of *Icam1*, *Vcam1*, *Madcam1*, *Vwf* and *Vegfr2* on day 11 of DSS-induced colitis in the WT and *Phd1*$^{f/f}$Tie2:cre mice. n = 8. *p < 0.05, **p < 0.01, ***p < 0.001 and NS: not significant. Data are represented as the mean ± SEM.

Figure 3. *Phd1*-deficiency solely in endothelial cells does not ameliorate DSS-induced colitis. (A) Weight evolution, (B) colon length and (C) representative H&E-stained colonic sections of *Phd1*$^{f/f}$Tie2:cre<-WT BM and WT<-WT BM chimeras (n = 8 per group) (left) and *Phd1*$^{f/f}$Flk-1:cre (n = 5) and WT mice (n = 7) (right) (scale bar = 200 μm). NS: not significant. Data are represented as the mean ± SEM. Original magnification x100.

Figure 4. Haematopoietic *Phd1*-deficiency ameliorates DSS-induced colitis. (A) Weight evolution of the haematopoietic *Phd1*-deficient (WT<- *Phd1*$^{f/f}$Tie2:cre BM) and WT (WT<-WT BM) mice subjected to 3% DSS-induced colitis. (B) Colon length, (C) histological inflammation score, (D) representative H&E-stained colon sections (scale bar = 200 μm), (E) epithelial erosion score, (F) infiltration inflammatory cell score and (G) mRNA expression of *Icam1*, *Vcam1*, *Madcam1*, *Vwf* and *Vegfr2* in the WT<-*Phd1*$^{f/f}$Tie2:cre BM and WT<-WT BM mice on day 9 of DSS-induced colitis. n = 11 per group. (H) Body weight evolution of the WT (n = 2) and *Phd1*$^{f/f}$Vav:cre mice (n = 4) during the course of 3% DSS-induced colitis. *p < 0.05, ***p < 0.0001 and NS: not significant. Data are represented as the mean ± SEM. Original magnification x100.

Figure 5. *Phd1*-deletion in BMDM promotes M2 conversion and impairs the LPS response, while *Phd1*-deficient BMDC release reduced IL-1β levels in response to LPS. (A) mRNA expression levels of *Chi3l3*, *Fizz1* and *Arg1* in the untreated or 20 ng/ml IL-4 treated WT and *Phd1*$^{f/f}$Tie2:cre BMDM. (B) IL-1β, IL-6, TNF and MCP-1 concentrations in the supernatant from the unstimulated and 100 ng/ml LPS-stimulated WT and *Phd1*$^{f/f}$Tie2:cre BMDM. (C) Concentration of IL-1β, IL-12, TNF and IL-6 in the supernatant from LPS-treated BMDC. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and NS: not significant. Results are representative of two independent experiments, each in duplicate. Data are represented as the mean ± SEM.

Figure 6. *Phd1*-deficiency alters the NF-κB activation state in BMDM at baseline and after LPS exposure. (A) Western blot analysis (left) of P-IκBα and GAPDH and densitometric analysis (right) of P-IκBα in the unstimulated and LPS-stimulated WT and *Phd1*$^{f/f}$Tie2:cre BMDM. (B) Western blot analysis of P-p65, IκBα and GAPDH in the untreated WT and *Phd1*$^{f/f}$Tie2:cre BMDM (left) and densitometric analysis of P-p65 and IκBα in the untreated WT and *Phd1*$^{f/f}$Tie2:cre BMDM (right). (C) mRNA expression levels of *Chi3l3*, *Fizz1* and *Arg1* in the untreated, 20 ng/ml IL-4-treated, 125 nM ACHP-treated or 20 ng/ml IL-4 and 125 nM ACHP-treated WT and *Phd1*$^{f/f}$Tie2:cre BMDM. Each condition was performed in duplicate; n=2-3. **p* < 0.05, ****p* < 0.001 and NS: not significant. Data are represented as mean ±SEM.

Supplementary material online

Supplementary materials and methods

Supplementary figure legends

Figure S1. Characterization of the *Phd1*$^{f/f}$Tie2:cre mice

Figure S2. LPS-conditioned medium from *Phd1*-deficient macrophages diminishes endothelial cell activation

Figure S3. *Phd1*-deletion does not alter the cytokine release of activated T-cells

Table S1. Primer sequences

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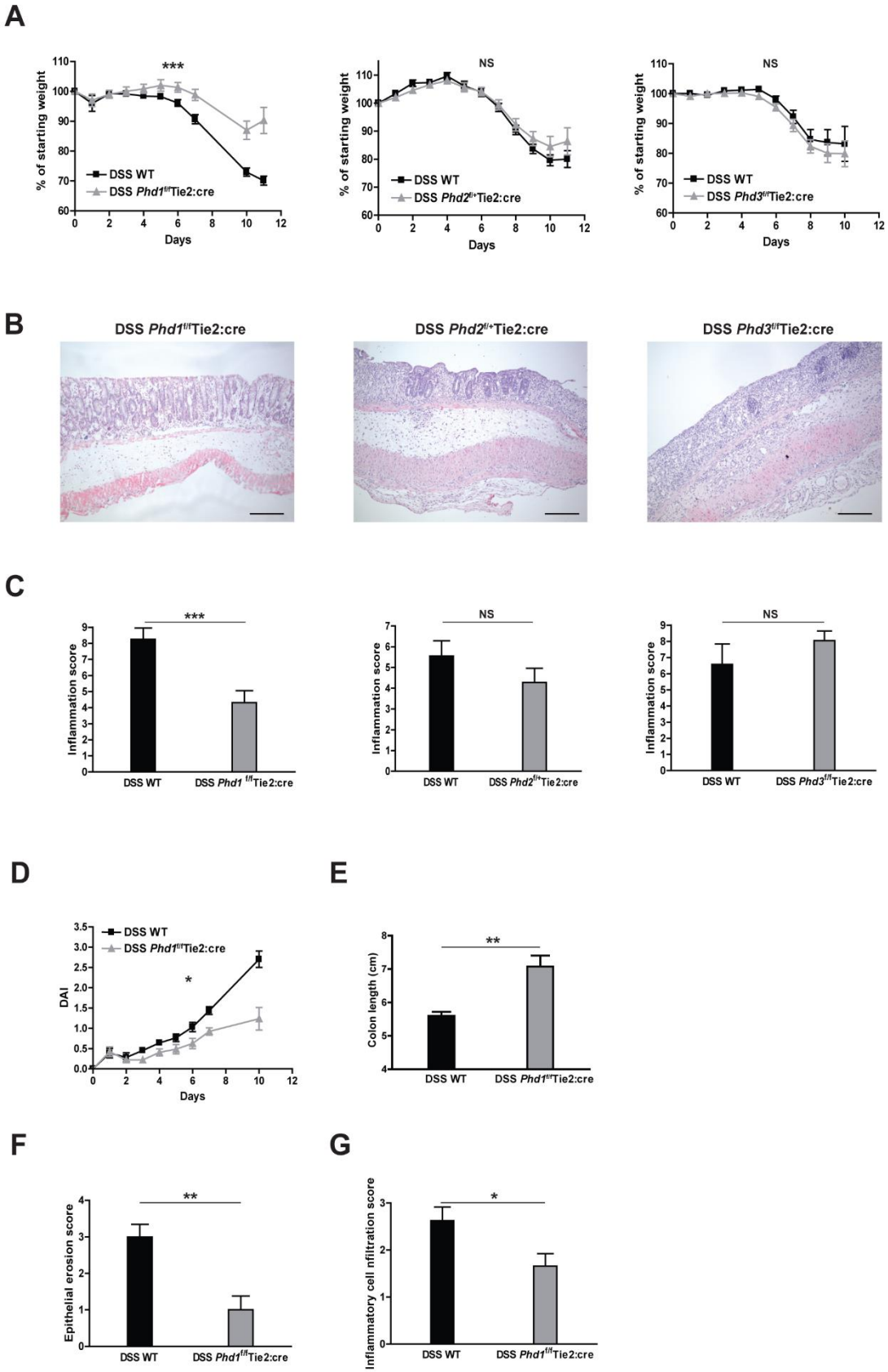
Figure 1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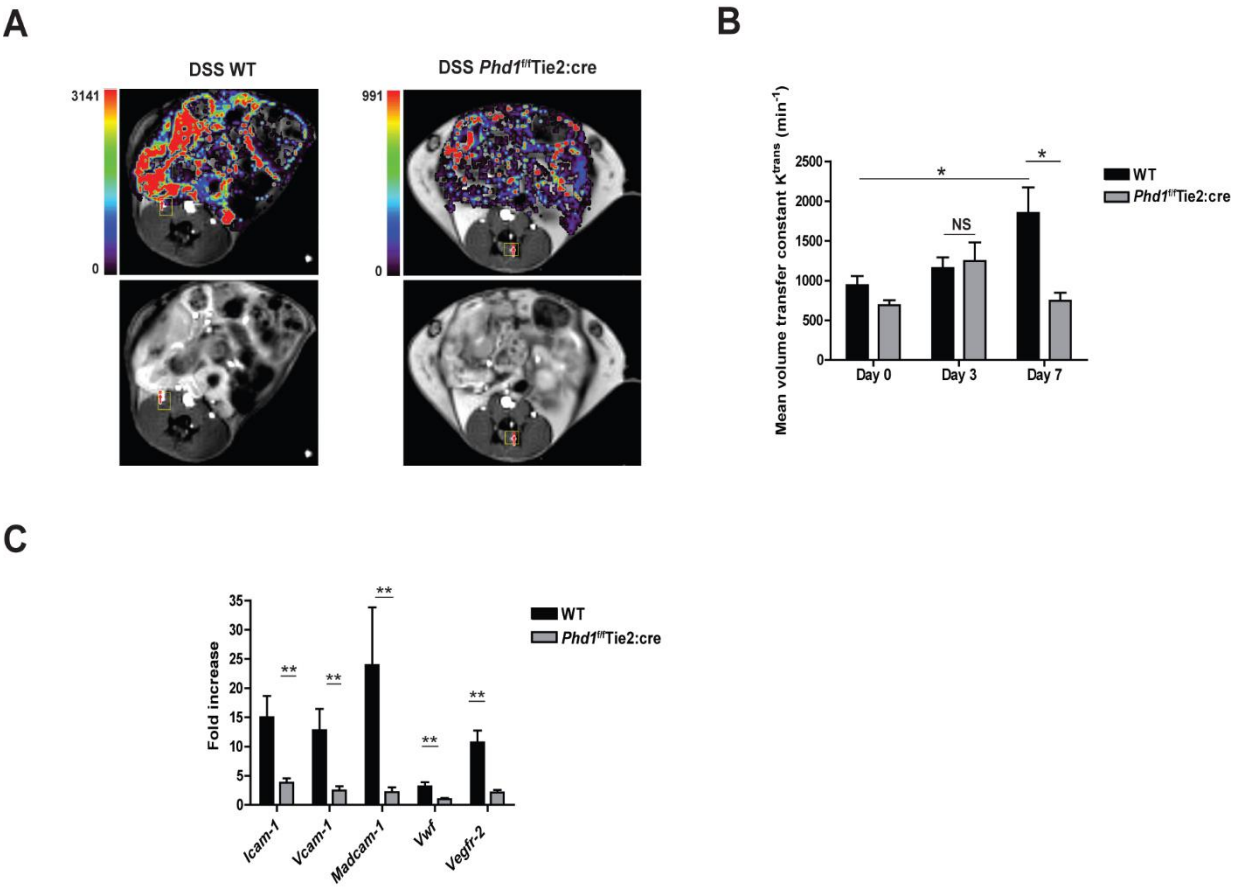
% of starting weight
Days
***
NS
NS
DSS WT
DSS Phd1f/fTie2:cre
DSS Phd2f/+Tie2:cre
DSS Phd3f/fTie2:cre
B
DSS Phd1f/fTie2:cre
DSS Phd2f/+Tie2:cre
DSS Phd3f/fTie2:cre
C
Inflammation score
***
NS
NS
DSS WT
DSS Phd1 f/fTie2:cre
DSS Phd2f/+Tie2:cre
DSS Phd3f/fTie2:cre
D
DAI
DSS WT
DSS Phd1f/fTie2:cre
*
Days
E
Colon length (cm)
**
DSS WT
DSS Phd1f/fTie2:cre
F
Epithelial erosion score
**
DSS WT
DSS Phd1f/fTie2:cre
G
Inflammatory cell infiltration score
*
DSS WT
DSS Phd1f/fTie2:cre

Figure 2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DSS Phd1fl/flTie2:cre
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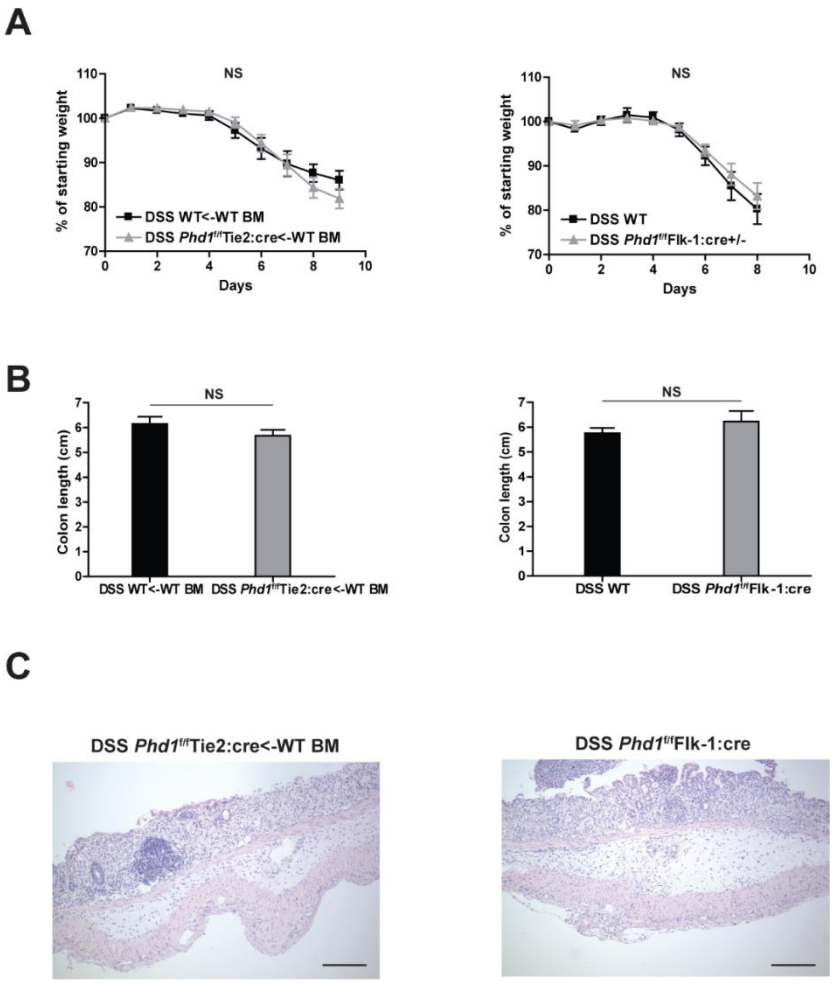
0
991
0
B
Mean volume transfer constant Ktrans (min-1)
2500
2000
1500
1000
500
0
*
*
NS
Day 0
Day 3
Day 7
WT
Phd1fl/flTie2:cre
C
Fold increase
35
30
25
20
15
10
5
0
**
**
**
**
**
Icam-1
Vcam-1
Madcam-1
Vwf
Vegfr-2
WT
Phd1fl/flTie2:cre

Figure 3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NS
% of starting weight
Days
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DSS Phd1f/fTie2:cre<-WT BM
NS
% of starting weight
Days
DSS WT
DSS Phd1f/fFlk-1:cre+/-
B
NS
Colon length (cm)
DSS WT<-WT BM
DSS Phd1f/fTie2:cre<-WT BM
NS
Colon length (cm)
DSS WT
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DSS Phd1f/fFlk-1:cre

Figure 4

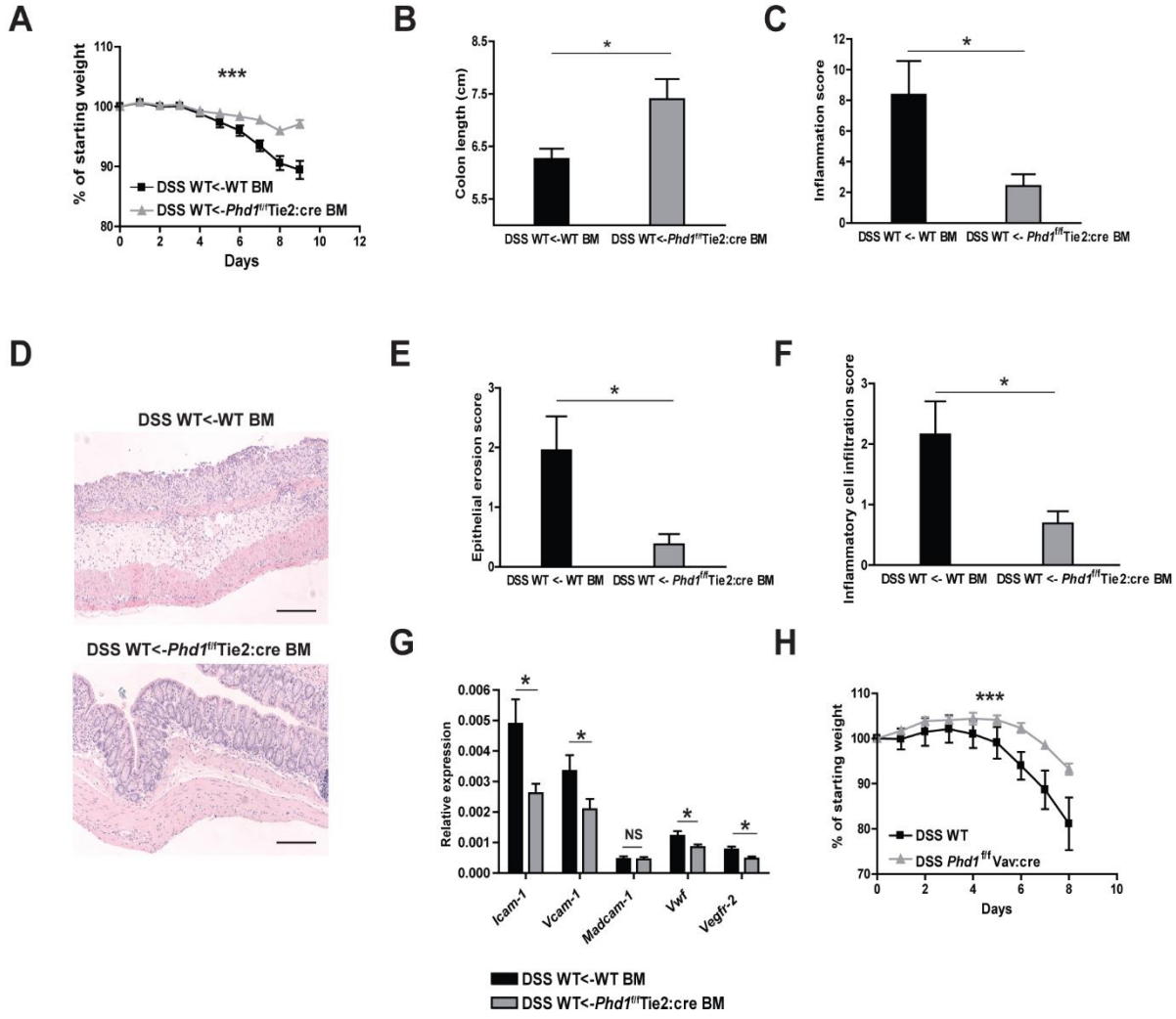
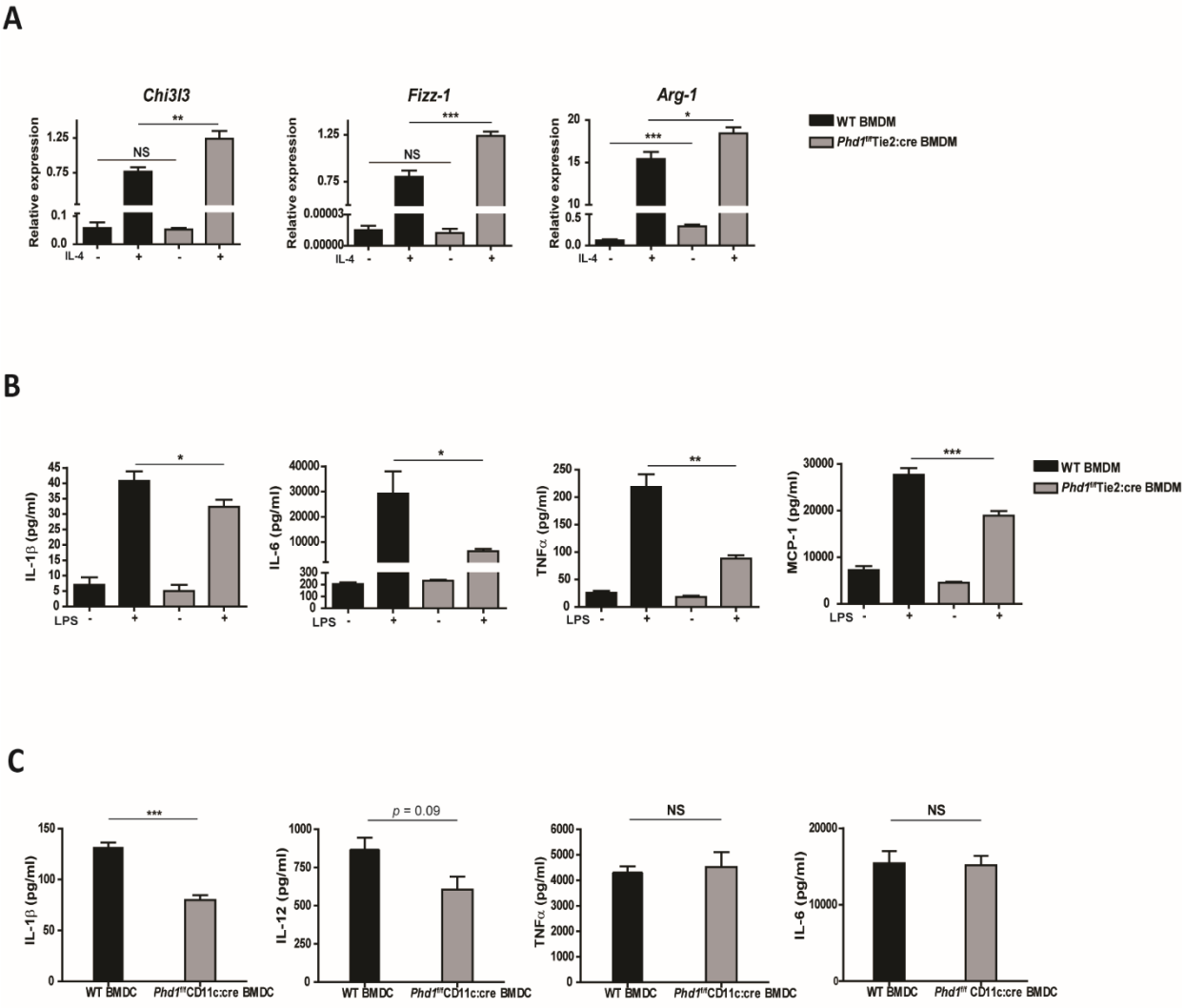

A
% of starting weight
***
DSS WT<-WT BM
DSS WT<-Phd1f/fTie2:cre BM
Days
B
Colon length (cm)
*
DSS WT<-WT BM
DSS WT<-Phd1f/fTie2:cre BM
C
Inflammation score
*
DSS WT <- WT BM
DSS WT <- Phd1f/fTie2:cre BM
D
DSS WT<-WT BM
DSS WT<-Phd1f/fTie2:cre BM
E
Epithelial erosion score
*
DSS WT <- WT BM
DSS WT <- Phd1f/fTie2:cre BM
F
Inflammatory cell infiltration score
*
DSS WT <- WT BM
DSS WT <- Phd1f/fTie2:cre BM
G
Relative expression
*
*
NS
*
*
Icam-1
Vcam-1
Madcam-1
Vwf
Vegfr-2
DSS WT<-WT BM
DSS WT<-Phd1f/fTie2:cre BM
H
% of starting weight
***
DSS WT
DSS Phd1f/f Vav:cre
Days

Figure 5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Chi3l3
Fizz-1
Arg-1
Relative expression
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IL-4
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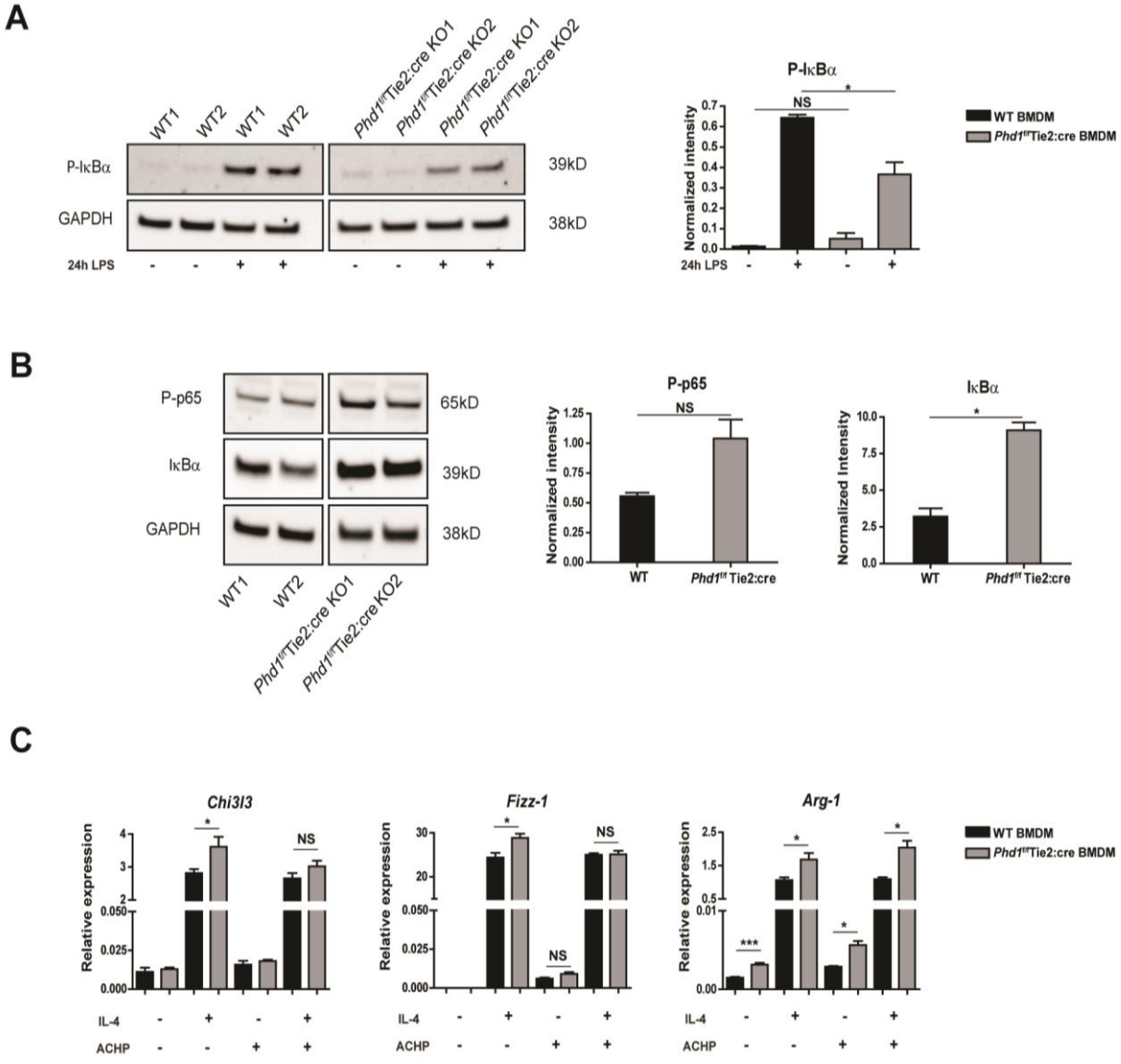
Phd1fl/flTie2:cre BMDM
B
IL-1β (pg/ml)
IL-6 (pg/ml)
TNFα (pg/ml)
MCP-1 (pg/ml)
LPS
C
IL-1β (pg/ml)
IL-12 (pg/ml)
TNFα (pg/ml)
IL-6 (pg/ml)
p = 0.09
NS
WT BMDC
Phd1fl/flCD11c:cre BMDC

Figure 6


A
WT1
WT2
WT1
WT2
Phd1fl/flTie2:cre KO1
Phd1fl/flTie2:cre KO2
Phd1fl/flTie2:cre KO1
Phd1fl/flTie2:cre KO2
P-IκBα
GAPDH
39kD
38kD
24h LPS
P-IκBα
NS
*
Normalized intensity
WT BMDM
Phd1fl/flTie2:cre BMDM
B
P-p65
IκBα
GAPDH
65kD
39kD
38kD
WT1
WT2
Phd1fl/flTie2:cre KO1
Phd1fl/flTie2:cre KO2
P-p65
NS
Normalized intensity
WT
Phd1fl/fl Tie2:cre
IκBα
*
Normalized Intensity
WT
Phd1fl/fl Tie2:cre
C
Chi3l3
Fizz-1
Arg-1
Relative expression
NS
*
***
IL-4
ACHP
WT BMDM
Phd1fl/flTie2:cre BMDM

Supporting information

SUPPLEMENTARY MATERIALS AND METHODS

Histological evaluation of inflammation

Distal colonic sections (5 µm thick) were stained with haematoxylin and eosin (H&E). Colonic epithelial damage and inflammation (mucosal and submucosal inflammatory cell infiltration) were then scored blindly by two independent observers using a validated scoring system [49].

Conditioned media of LPS-activated BMDM

Supernatants from the LPS-stimulated BMDM were obtained following protocols described previously [50]. In brief, the BMDM were stimulated with 100 ng/ml LPS for 20 min. The medium was removed and the adherent cells were washed three times with phosphate-buffered saline $(PBS)^{-/-}$ to eliminate any residual LPS. Fresh DMEM without FCS was added to the cells for 3 h. This conditioned medium (CM) was centrifuged for 15 min at 5 000 X *g*, passed through a 0.2 µm filter and incubated for 2 h at 37 °C with 1 mg/ml polymyxin B sulphate to prevent the remaining LPS residues from binding to their cell receptors.

Cell line culture

Mouse endothelial MS1 cells (ATCC Cell Biology Collection, Manassas, VA, USA) were seeded at a density of 10^5 cells per well of 12-well plates and stimulated the following day for 6 h with LPS-CM or unconditioned medium from WT and *Phd1*$^{f/f}$Tie2:cre BMDM followed by RNA extraction. Each condition was performed in triplicate. The cultured cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2 and the culture supernatant was routinely screened for mycoplasma infections.

RNA collection and extraction

Total RNA was extracted from mouse colonic mucosal samples or cell cultures via the Aurum Total RNA Mini Kit (Biorad, Temse, Belgium), using needle homogenization and on-column DNAse treatment. The concentration and purity of the total RNA was quantified using nanodrop technology (BioPhotometer Plus, Eppendorf, Rotselaar, Belgium). All samples had an $OD_{260/280}$ between 1.8 and 2.1.

cDNA synthesis and quantitative Real-time Polymerase Chain Reaction (qPCR)

Total RNA was converted to single strand complementary DNA (cDNA) by reverse transcription (SensiFAST™ cDNA Synthase Kit, Bioline, London, UK). The cDNA was diluted to a concentration of 5 ng/µl. Real-time quantification was performed using a SensiMix™ SYBR kit (Bioline) and 250 nM of forward and reverse primers (BioLegio, Nijmegen, The Netherlands). A two-step program was run on a LightCycler® 480 II (Roche, Vilvoorde, Belgium). The cycling conditions comprised 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 1 min. A melting curve analysis confirmed the primer specificities. All reactions were conducted in duplicate, and the data were normalized to the expression of the reference genes succinate dehydrogenase complex subunit (*Sdha*), hydroxymethyl-bilane synthase (*Hmbs*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) or a combination based on the stability of the reference genes between the different groups. The efficiency of each primer pair was calculated using a standard curve from reference cDNA. The amplification efficiency was determined using the formula $10^{-1/slope}$. The sequences of the qPCR primers are provided in Supplementary Table S1.

Western blotting

Bone marrow derived macrophages (BMDM) were lysed in RIPA buffer. Proteins were separated on 4-12% Bis-Tris SDS-polyacrylamide gels and transferred to nitrocellulose membranes using iBlot dry blotting (Invitrogen). The membranes were subsequently blocked with 5% skimmed milk in TBS-T (50 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) and incubated overnight at 4 °C in 5% skimmed milk in TBS-T with 1 : 2500 anti-GAPDH (Abcam), 1 : 1000 anti-phosphorylated p65 (Cell Signalling, Leiden, Netherlands), 1 : 1000 anti-phosphorylated IκB-α (Cell Signalling) and 1 : 1000 anti-IκB-α (Cell Signalling). Bound antibodies were visualized using the ECL detection kit BM chemiluminescence Blotting Substrate POD (Roche) on a ChemiDoc™ Touch Imaging System (Biorad).

Chemokine analyses

Chemokines in the supernatant from the BMDM cultures were measured using a multiplex bead-based immunoassay kit for mouse IL-1β, IL-6, MCP-1 and TNF. The samples were processed using the Bio-Plex ProTM Reagent Kit and were analysed with Bio-Plex Manager™ Software (Biorad).

Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-12, IL-6 and TNF concentrations in the supernatant from the BMDC cultures were measured by ELISA (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Isolation of mouse colonic endothelial cells

Eight-to-twelve-week-old mice were euthanized, and the colons were removed. After washing in ice cold $PBS^{-/-}$, the colons were longitudinally cut in 1 cm pieces. The tissues were subsequently incubated for 15 min in 1 mM DTT in $PBS^{-/-}$ at room temperature. Next, the colons were washed twice in ice cold $PBS^{-/-}$, followed by enzymatic digestion for 1 h at 37 °C with gentle shaking. The enzymatic solution included RPMI that contained 5% FCS, 5 mg/ml collagenase D (Roche), 0.5 mg/ml collagenase IV (Sigma), 100 units/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml Fungizone® Antimycotic and 300 µg/ml DNAse I (Roche). The cell suspensions were washed with RPMI, passed through a 40 µm filter, centrifuged and washed again with FACS buffer ($PBS^{-/-}$, 2 mM EDTA and 0.5% BSA). Following centrifugation, the cells were resuspended at 10^8 cells in 900 µL FACS buffer, followed by endothelial cell isolation using CD31+ microbeads (Miltenyi, Leiden, The Netherlands) in accordance with the manufacturer's guidelines. The CD31+ cells were lysed for RNA extraction.

Obtaining spleen suspensions and isolation of CD4+ T cells from the spleen

Eight-to-twelve-week-old WT, *Phd1*$^{f/f}$Tie2:cre and *Phd1*$^{f/f}$ Vav:cre mice were euthanized, and the spleens were removed. The spleens were crushed with the back of a syringe in 5 ml RPMI on a 40 µm cell strainer. After washing with RPMI and centrifugation, the red blood cells were lysed for 5 min at room temperature using RBC lysis buffer (Gentra systems, Minneapolis, USA). The cell suspensions were subsequently washed again with RPMI and centrifuged. Resuspension occurred in RPMI, and the cells were passed again through a 40 µm filter. Spleen suspensions from WT and *Phd1*$^{f/f}$ Vav:cre mice were seeded at a density of 2 x 10^6 cells in a 96-well containing 200 ng/ml PMA (Invitrogen) and 1 µg/ml ionomycin (Sigma Aldrich, Missouri, USA) to accomplish T-cell activation. After 4h, supernatant was collected to determine cytokine concentrations. In addition, CD4+ cells were subsequently acquired from the spleen suspensions from WT and *Phd1*$^{f/f}$Tie2:cre using the Dynabeads® CD4 Positive Isolation Kit (Invitrogen), followed by RNA extraction.

Isolation of colonic epithelial cells (IEC)

Eight-to-twelve-week-old mice were euthanized, and the colons were removed and cut longitudinally. Two 1 cm pieces were incubated overnight on ice in Cell Recovery Solution (BD Bioscience, Belford, MA). The following day, colonic epithelial cells were obtained by vigorous shaking. The remaining colonic tissue was removed, and the cell suspensions were centrifuged. Cell pellets were lysed for RNA isolation.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Characterization of the *Phd1*$^{f/f}$Tie2:cre mice. (A) *Phd1* wt and ko allele expression in BM, (B) BMDM, (C) BMDC, (D) CD4+ T-cells, (E) intestinal epithelial cells (IEC) and (F) CD31+ cells isolated from the colonic tissue of the WT and *Phd1*$^{f/f}$Tie2:cre mice (left) and WT and *Phd1*$^{f/f}$Flk-1:cre mice (right). n=2 mice per group, with the exception of (E) n=1. **p* < 0.05. Values represent mean ± SEM. (G) Schematic overview of the binding sites of the two qPCR primer pairs to distinguish Phd1 expression in the WT and *Phd1*$^{f/f}$Tie2:cre mice. (Left) *Phd1* mRNA sequence with the forward primer binding exon 2 and the reverse primer binding exon 3, thereby amplifying the *Phd1* allele as it occurs in the WT mice and is denoted as the "*Phd1* wt allele". (Right) *Phd1* mRNA sequence as it occurs in the *Phd1*$^{f/f}$Tie2:cre mice with the forward primer binding at the transition of exon 2 and exon 5, whereas the reverse primer binds exon 5. The resulting amplicon is the *Phd1* allele as it occurs in the *Phd1*$^{f/f}$Tie2:cre mice and is denoted as the "*Phd1* ko allele".

Figure S2. LPS-conditioned medium from *Phd1*-deficient macrophages diminishes endothelial cell activation. *Icam1*, *Vcam1*, *Madcam1*, *Il6*, *Cxcl2* and *Mcp1* mRNA levels in MS1 cells stimulated with unconditioned or LPS-conditioned medium (CM) from the WT and *Phd1*$^{f/f}$Tie2:cre BMDM. ***p* < 0.01, ****p* < 0.001 and NS: not significant. Results are representative of two independent experiments, each in duplicate. Data are represented as the mean ± SEM.

Figure S3. *Phd1*-deletion does not alter the cytokine release of activated T-cells. Protein levels of INF-γ, TNF and IL-10 secreted by unstimulated and PMA/ionomycin-stimulated spleen suspensions from WT and *Phd1*$^{f/f}$Vav:cre mice. NS: not significant. Data are represented as the mean ± SEM.

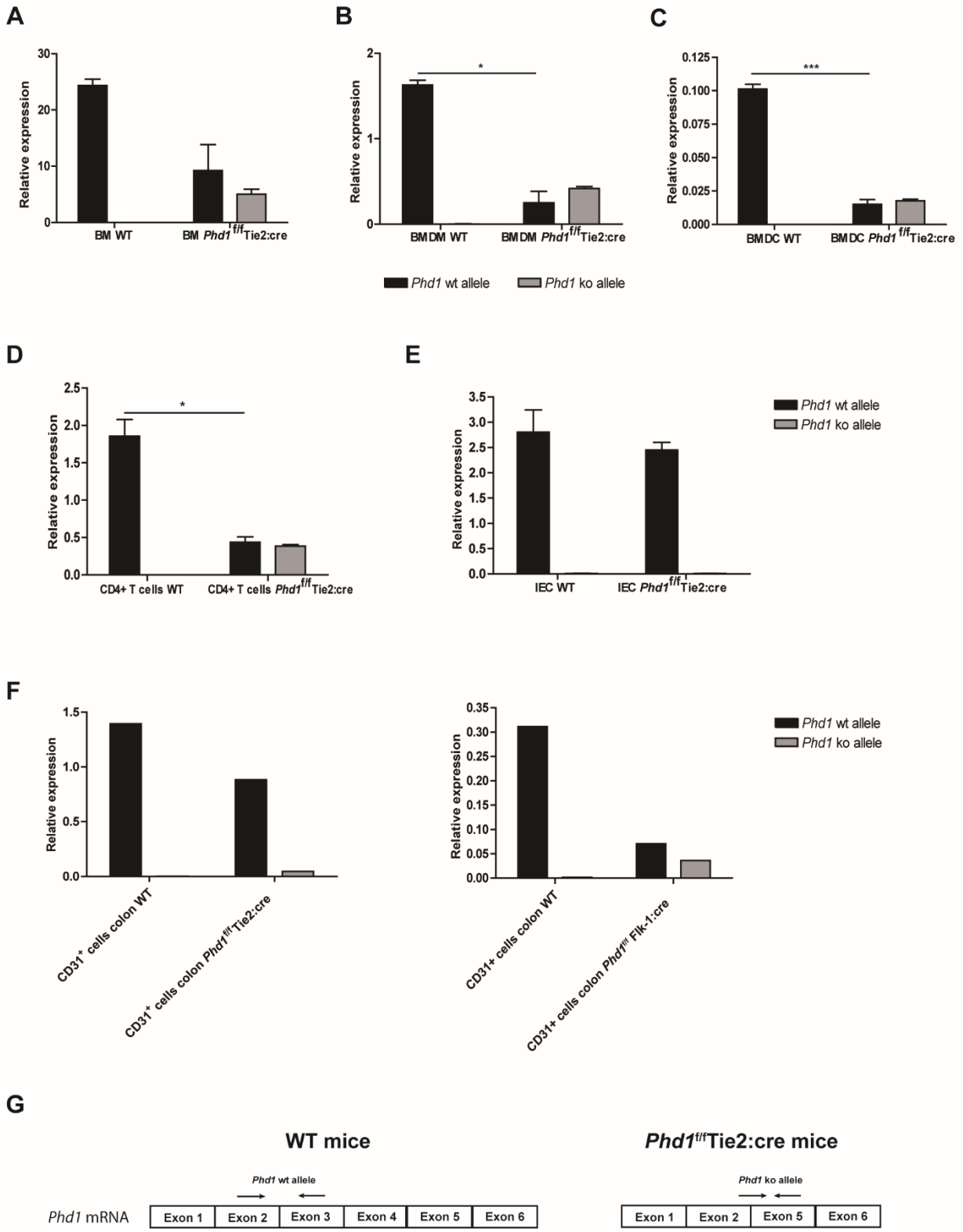
SUPPLEMENTARY TABLES

Table S1: Primer sequences. The primers listed were used for quantitative PCR.

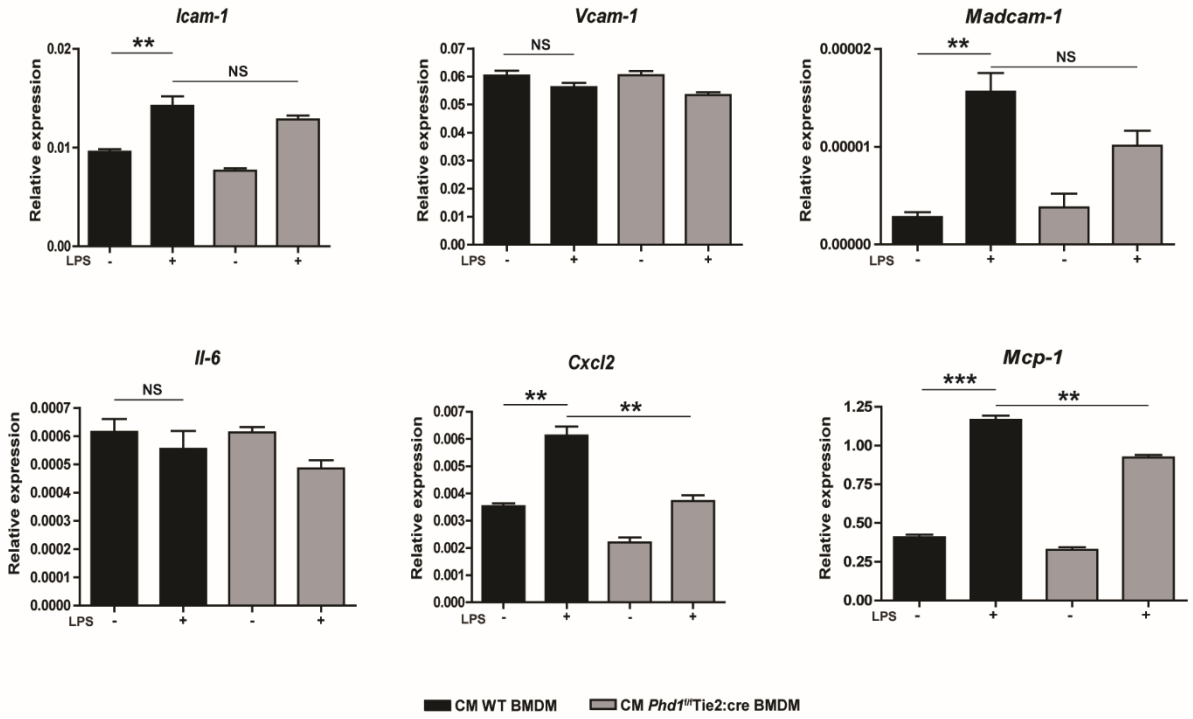
Gene Symbol	*Forward Primers (5'-3')*	*Reverse Primers (5'-3')*
Sdha	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT
Gapdh	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
Hprt	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT
Hmbs	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG
Phd1wt	TCACGTGGACGCAGTAATCC	TAATAGATACAG GTGATGCAGC
Phd1ko	AATGGGCGCACCAAGGTACG	GTGATACTGGTACTTGAACACC
Icam1	GCCTTGGTAGAGGTGACTGAG	GACCGGAGCTGAAAAGTTGTA
Vcam1	TGCCGAGCTAAATTACACATTG	CCTTGTGGAGGGATGTACAGA
Madcam1	CCTGGCCCTAGTACCCTACC	CCGTACAGAGAGGATACTGCTG
Vwf	CCGACAGCCGGGATTGCCTG	CCACCCGATGTGCACGCCTT
Vegfr2	GGGTCGATTTCAAACCTCAATGT	AGAGTAAAGCCTATCTCGCTGT
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Cxcl2	GCGCCCAGACAGAAGTCATAG	AGCCTTGCCTTTGTTCAGTATC
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Chi3l3	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
Fizz1	TCCCAGTGAATACTGATGAGA	CCACTCTGGATCTCCCAAGA
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC

Sdha, mouse succinate dehydrogenase complex A subunit; *Gapdh*, mouse glyceraldehyde-3-phosphate dehydrogenase; *Hprt*, mouse hypoxanthine-guanine phosphoribosyltransferase; *Hmbs*, mouse hydroxymethylbilane synthase; *Phd1wt*, mouse prolyl hydroxylase domain 1; *Phd1ko*, mouse prolyl hydroxylase domain 1 knockout; *Icam1*, mouse intercellular cell adhesion molecule 1; *Vcam1*, mouse vascular cell adhesion molecule 1; *Madcam1*, mouse mucosal addressin cell adhesion molecule 1; *Vwf*, mouse von Willebrand factor; *Vegfr2*, mouse vascular endothelial growth factor receptor 2; *Il6*, interleukin 6; *Cxcl2*, mouse chemokine (C-X-C motif) ligand 2; *Mcp1*, mouse monocyte-chemoattractant protein 1; *Chi3l3*, mouse chitinase-3 like 3; *Fizz1*, mouse resistin-like molecule alpha or found in inflammatory zone protein; *Arg1*, mouse arginase-1.

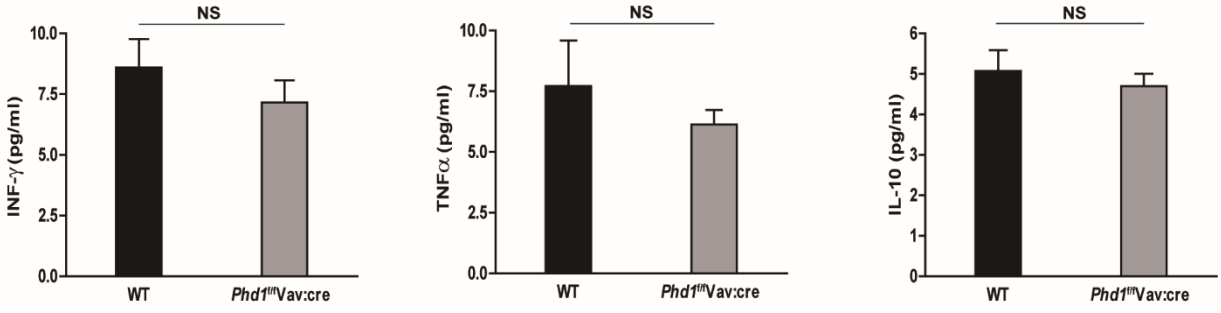
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



CHAPTER IV: DISCUSSION AND FUTURE PERSPECTIVES

1 Discussion

Prolyl hydroxylase domain-containing proteins 1-3 are oxygen-sensing enzymes that orchestrate the response to hypoxia and exert non-immune and immune-modulating functions. They are actively involved in the pathogenesis of intestinal inflammation as inhibition of all three isoforms is protective in animal models of IBD. However, systemically administered pan-hydroxylase inhibitors are associated with some potential risks that discourages their use as therapeutics for IBD. The goal of this project was to identify which isoform(s) play(s) a key role in the pathogenesis of IBD and to establish if and how its targeting provides therapeutic benefit during experimental colitis as an alternative approach to reduce the risk of unwanted side-effects.

1.1 Prolyl hydroxylase expression and cellular distribution in colonic biopsies of IBD patients

The expression pattern of the different PHD isoforms has been studied in healthy tissues. They are widely distributed among different organs; PHD2 is the most abundant isoform except for the testis where PHD1 predominates, while the expression of PHD3 is highest in the heart[1]. However, information about their specific distribution in the healthy and inflamed gut is limited. Tambuwala and colleagues were the first to show that PHD1 expression increases with the severity of IBD, but this was not analysed for UC and CD patients separately. We expanded these initial findings by investigating all three isoforms in colonic inflamed and non-inflamed biopsies from UC and CD patients. In addition, we included inflamed biopsies from patients with infectious colitis as non-IBD inflammatory control group as well as healthy controls. We observed that the PHD1 expression was significantly elevated in inflamed biopsies from both UC and CD patients. Although *PHD1* mRNA levels were higher in samples from inflamed UC patients compared with inflamed CD samples, the protein expression was equally elevated in both groups. Moreover, *PHD1* levels showed a strong positive correlation with the pro-inflammatory markers *IL-8* and *TNF. PHD1* expression was also elevated in samples from patients with infectious colitis, comparable to inflamed CD biopsies, with a tendency towards statistical significance, which indicates that increased PHD1 expression is not IBD specific and associated with inflammation in general. Immunohistochemical staining revealed that PHD1 is strongly expressed by mononuclear cells in the LP. Since these cells infiltrate the mucosa during an acute inflammatory response, it cannot be ruled out at this moment that statistical significance could be reached when the number of infectious colitis patients would be increased to the number of included UC and CD patients. Based on these findings, the elevated PHD1 protein expression is mainly due to the increase in $PHD1^{+}$ inflammatory cells. Furthermore, we demonstrated moderate staining of PHD1 in epithelial cells, while

expression was absent in lymphocytes. This is in agreement with the publication of Soilleux *et al.* who analysed the presence of PHD1 expression in the epithelium and lymphocytes from different healthy tissues, but they did not include the colonic mucosa in their study[2]. In addition, we showed that only UC patients present markedly elevated *PHD3* mRNA levels. This is not unexpected since HIF-1α levels in inflamed colonic biopsies from UC patients are significantly higher compared with healthy controls, but also compared with inflamed colonic CD samples[3], while HIF-1α is known to induce the transcription of *PHD3,* but not *PHD1*[4]. In further support, the expression of other HIF-1α target genes including the glycolytic enzymes aldolase A, phosphoglycerate mutase and pyruvate kinase and antibodies against these enzymes are significantly elevated in active UC patients compared with active CD patients[3]. These elevated PHD3 mRNA levels translate into elevated PHD3 protein levels only in mild-to-moderate UC patients, while PHD3 is no longer detectable in severely diseased UC patients who are classified with Mayo score 3. This observation was confirmed by Chen and co-workers who showed an inverse correlation between PHD3 expression and the severity of UC[5]. We hypothesized that the reason for the disappearance of PHD3 is due to the increased post-translational degradation by Siah2 which expression is already induced under mild hypoxic conditions (10% oxygen concentration)[6]. Recently, our hypothesis was strengthened by Xu and colleagues who demonstrated that HIF-1α expression in the colonic mucosa, and hence hypoxia, is positively correlated with the Mayo scoring in UC patients[7]. The physiological hypoxia present in the gut and the increase in hypoxia during inflammation might therefore contribute, at least in part, to the absence of PHD3 staining in a variety of mucosal cells except for the endothelium which is in contact with generally oxygen-rich blood. Since the intestinal mucosa of active CD patients and especially healthy controls is less hypoxic than severely diseased UC patients, one may expect to observe some PHD3 staining in these samples. We could only detect these expected differences in PHD3 expression between healthy controls and active CD patients versus active UC patients using western blot, but not by immunohistochemical staining. The latter is likely not sensitive enough to detect the differences in PHD3 expression revealed by western blot. Finally, we demonstrated that *PHD1* and *PHD2* are positively correlated with the mRNA levels of *CASP3*. This gene encodes for the protein caspase-3, involved in the regulation of apoptosis. More specifically, the initiator caspases-8, -9 or -10 can all cleave and hereby activate effector caspase-3. The latter in turn cleaves other substrates which results in DNA fragmentation and cytoskeletal reorganization and disintegration of the cell into apoptotic bodies[8]. Whether PHD1 and/or PHD2 take part in this process is currently still unknown. However, other evidence does confirm their involvement in apoptosis. In particular, increased epithelial apoptosis is a prominent hallmark of active IBD patients[9, 10] and we observed that both PHD1 and PHD2 localize in epithelial cells. Moreover, Tambuwala and colleagues demonstrated that deletion of *PHD1* in epithelial cells reduces their

apoptotic rate after DSS exposure[11] hereby demonstrating that PHD1 is a positive regulator of apoptosis. In addition, PHD1 exerts the same function in hepatocytes in a NFκB-dependent manner[12]. Concerning PHD2, silencing this gene attenuates $CoCl_2$-induced apoptosis of renal epithelial cells[13]. However, an involvement in intestinal epithelial apoptosis is yet to be determined. Besides epithelial cells, both isoforms are also present in mononuclear cells in the LP. To date, only an essential role for PHD3 in promoting apoptosis of macrophages under stress conditions like serum deprivation has been demonstrated, while no involvement of PHD1 or PHD2 in mononuclear cell apoptosis has been described thus far.

It was surprising that PHD2, and to a lesser extent PHD1, expression could not be shown by immunohistochemistry in endothelial cells given the fact that others have previously demonstrated PHD2 expression in endothelial cells[14, 15] although not specifically in intestinal endothelial cells. We therefore analysed the expression of both in primary colonic endothelial cells isolated from healthy controls. Western blot analysis revealed expression of all three isoforms with the highest expression seen for PHD3 in accordance with our initial immunohistochemical analysis (data not shown). To obtain additional evidence for a possible role of PHDs in endothelial cells we analysed the gene expression of previously reported endothelial dysfunction markers[16] in biopsies collected from active IBD patients, IBD patients in clinical and endoscopic remission, patients with infectious colitis (as an inflammatory control) and healthy controls. Compared with the healthy tissue, a significant up-regulation of *ICAM-1*, *VCAM-1*, *MAdCAM-1*, *VWF* and *VEGFR-2* was identified in the inflamed biopsies of both patients with IBD and infectious colitis, whereas there were no significant expression changes in the biopsies collected from the IBD patients in remission (Figure 1A). All endothelial dysfunction markers exhibited a strong positive correlation with *TNF*, *IL-8* and *PHD1*, but not with *PHD2* and *PHD3* (data not shown and Figure 1B). Taken together, these findings demonstrate that endothelial dysfunction is a hallmark of active inflammation and is correlated with *PHD1* expression.

2.1 Prolyl hydroxylases and experimental ulcerative colitis

Our research group and others have demonstrated that pan-hydroyxlase inhibition attenuates the inflammatory burden in animal models for IBD. Due to the risk of unwanted side-effects associated with these inhibitors, investigators switched their research to the development and evaluation of alternative approaches that could provide therapeutic benefit without potential side-effects. Today, this way of thinking has resulted in the evaluation of controlled release formulations and isoform-specific targeting. Pioneer in this field was the group by Tambuwala and colleagues who demonstrated the efficacy of DMOG-containing minispheres and *Phd1*-deletion during DSS-induced colitis[11, 17]. The

identified mechanism of action in the latter study was the preservation of epithelial barrier integrity through the reduction of epithelial apoptosis, which is similar to pan-hydroxylase inhibition. However, epithelial barrier disruption in UC is most likely a secondary phenomenon of an unresolved, exaggerated inflammatory response in the intestinal mucosa. Since the different PHD isoforms play an important role in both immune and endothelial cell functioning, PHD inhibition in these cells could therefore represent an additional cause of the preservation of barrier integrity and hence protection against colitis as well as epithelial PHD1 as postulated by the previous study. Therefore, we explored if isoform-specific targeting of PHDs in haematopoietic and endothelial cells exerts therapeutic activity during experimental UC. We demonstrated that deletion of *Phd1*, and not *Phd2* or *Phd3*, in endothelial and haematopoietic cells decreases the susceptibility to experimental colitis. Next, we showed using bone marrow chimeras that haematopoietic *Phd1*-deficiency drives this protection which was in addition validated in cell-specific cre mice. By performing *in vitro* experiments with the main type of immune cells contributing to colitogenesis, we were able to further dissect that deletion of *Phd1* in macrophages and, to a lesser extent, DCs creates an anti-inflammatory phenotype. Therefore, these two cell types are most likely the major contributors to the *in vivo* protection. Importantly, we demonstrated that haematopoietic *Phd1*-ablation also diminished the number of epithelial cell erosions in contrast to what Tambuwala and co-workers hypothesized to be an epithelial PHD1-mediated phenomenon. Nevertheless, their statement may still hold in addition to our observed haematopoietic *Phd1*-mediated effect on the epithelium. Epithelial-specific deletion of *Phd1* using villin:cre mice would provide a decisive answer to this issue. How exactly haematopoietic *Phd1*-deficient macrophages and DCs preserve epithelial barrier integrity needs further investigation. However, their diminished expression of TNF and IL-1β will play a huge role since these cytokines are known to reduce the expression of pore-sealing tight junction proteins and alter their junctional localisation leading to increased epithelial permeability [18, 19]. Besides effects on the epithelial barrier, endothelial chemokine expression was lower after stimulation with medium from LPS-treated *Phd1*-deficient macrophages than from LPS-treated WT macrophages which in turn may be responsible for the reduced infiltration of inflammatory cells in the mucosa, but this hypothesis needs to be confirmed by additional studies. For instance, a co-culture model could be used in which endothelial cells are grown to a confluent monolayer in a transwell permeable support with below the membrane, LPS-CM from *Phd1*-defient macrophages. Next, monocytes are added to the medium at the apical side of the endothelial cells and migration towards the basolateral compartment is evaluated. Finally, we demonstrated that NFκB is partially involved in the promotion of M2 polarization after *Phd1*-deletion. However, it remains speculative how NFκB activation mediates the M2 switch although p50 NFκB may be involved in this effect. Porta and colleagues demonstrated that LPS-tolerant macrophages have

increased levels of p50 which induces the expression of M2-associated genes, while concomitantly repressing M1 markers[20].

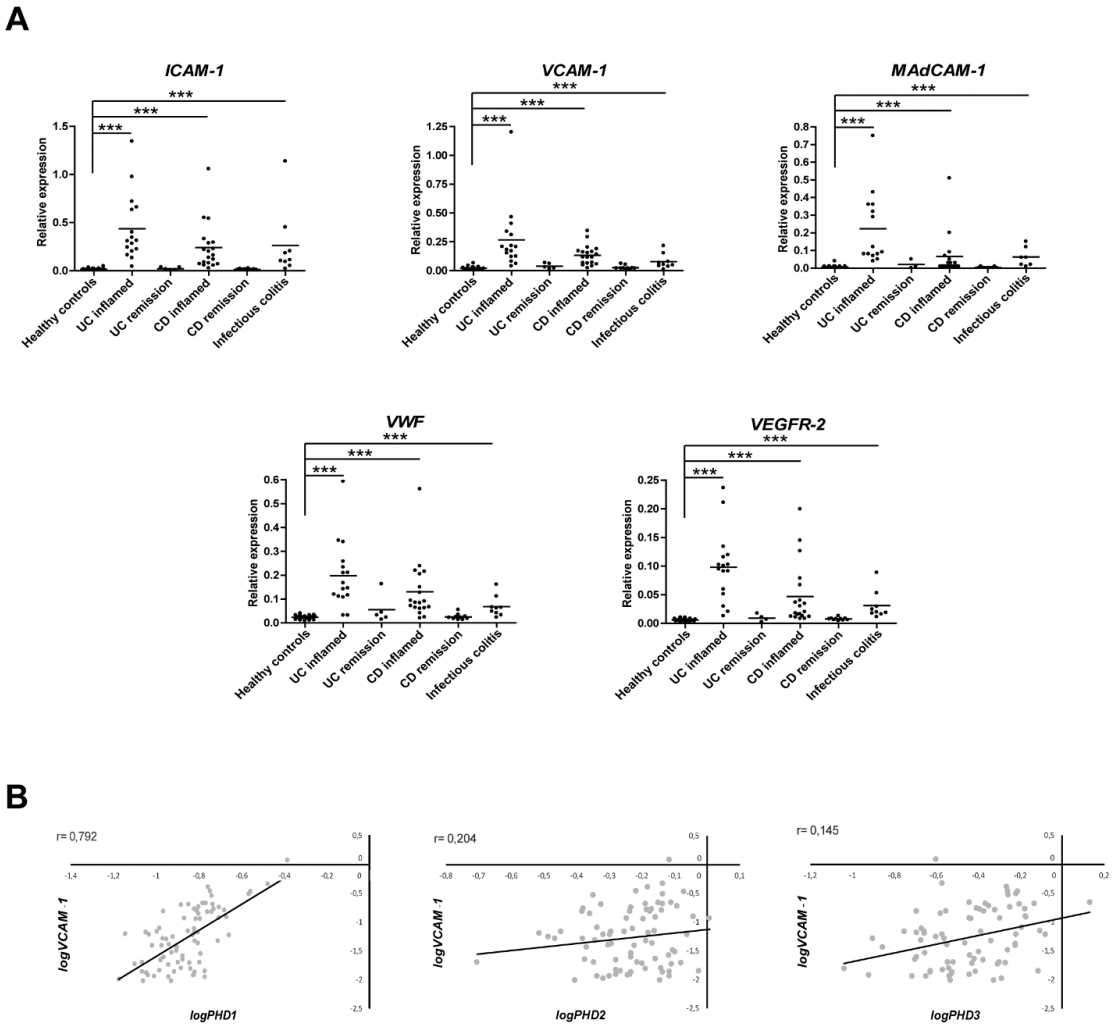


Figure 1. *IBD patients exhibit a dysfunctional endothelial phenotype, which is correlated with the expression of PHD1. A) mRNA expression levels of ICAM-1, VCAM-1, MAdCAM-1, VWF and VEGFR-2 in colonic samples of the healthy controls, UC, CD and infectious colitis patients. ***p < 0.001. B) Illustrative correlation plots between VCAM-1 and PHD1, PHD2 and PHD3 in the colonic biopsies of healthy controls, UC, CD and infectious colitis patients.*

2 Future directions and perspectives

In our initial explorative study, PHD1 came forward as the most promising isoform for therapeutic targeting in IBD due to its elevated expression. Subsequently, we confirmed its therapeutic potential by demonstrating that haematopoietic deletion of *Phd1* protects mice from experimental colitis. Although the work performed is very exciting, a number of questions still remain that need to be addressed.

While we demonstrated *in vitro* that *Phd1*-deletion mainly effects the functioning of macrophages and DCs, confirmation in the *in vivo* setting is missing. Therefore, a logical sequel would be to evaluate the individual involvement of these *Phd1*-deficient immune cells in DSS-induced colitis. We have initiated this quest by investigating the effect of macrophage-specific and T cell specific deletion of *Phd1* on the course of DSS-induced colitis. For that purpose, we crossed *Phd1*$^{f/f}$ mice with CD4:cre and LysM:cre mice to study the T cell and macrophage-specific effects respectively. In line with our *in vitro* experiment with *Phd1*-deficient T cells, we observed equal susceptibility between *Phd1*$^{f/f}$CD4:cre and WT mice to DSS-induced colitis (Figure 2). Despite the facilitation towards M2 conversion and the diminished pro-inflammatory nature of *Phd1*-deficient macrophages, *Phd1*$^{f/f}$LysM:cre mice unexpectedly exhibited no improved clinical parameters including weight loss compared with WT mice (Figure 2). Two main reasons may explain this lack of protection. First, it is possible that the anti-inflammatory phenotype of the *Phd1*-deleted macrophages alone is not sufficient. To elicit an improved clinical outcome, combined targeting of DCs and macrophages might be the requisite which could be performed by using CD11c:cre mice. The concept of combined targeting has proven to be successful in tumorigenesis. In particular, Mamlouk and colleagues demonstrated that *Phd2* needs to be ablated in both myeloid and T cells to impair tumor development, while individual targeting does not affect tumor growth[21]. The second reason might be that not *Phd1*-deficient macrophages, but in fact *Phd1*-deleted DCs drive the beneficial effects during this colitis model. This seems rather unlikely since these DCs only exhibit a diminished IL-1β release, while other pro-inflammatory cytokines remain unaltered in contrast to *Phd1*-deficient macrophages, but the possibility cannot be ruled out at this moment.

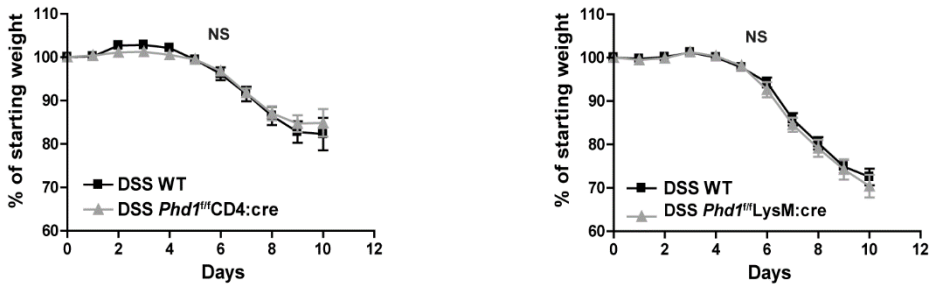


Figure 2. *Weight evolution of Phd1$^{f/f}$CD4:cre, Phd1$^{f/f}$LysM:cre and their corresponding WT littermate controls during DSS-induced colitis. n≥9. NS: not significant.*

Although we have investigated the effect of *Phd1*-deletion in the main immune cells involved during the acute DSS-induced colitis model, we cannot decisively exclude a contribution of *Phd1*-deficient neutrophils. The latter play an important role during both the onset and the resolution of intestinal inflammation,[22] but so far, a role for PHD1 in neutrophils is unknown. Walsmley and colleagues

reported that the protein expression of PHD3 in neutrophils is up-regulated after hypoxic treatment which promotes cell survival, while the expression levels of PHD1 and PHD2 remained unaltered. Nevertheless, this does not implicate that *Phd1*-deletion could not exert an attenuated pro-inflammatory response in these cells similar to our observation in macrophages and DCs. Although we and others have noticed that LysM:cre mice also elicit deletion of a *loxP*-flanked gene in neutrophils, this knockout degree is less efficient than in macrophages and ranges around 50%. Therefore, DSS-induced colitis in MRP-8:cre mice would be a good alternative to study the neutrophil-specific contribution of *Phd1*-deletion as they only show around 10% knockout in other myeloid cells[23-25].

Although our results imply that pan-hydroxylase inhibitors like DMOG most likely exert their therapeutic action through the inhibition of PHD1, it may still be possible that their inhibition of PHD2 also has beneficial effects. Several lines of evidence are suggestive for a role of PHD2 during colitis. In particular, it has been demonstrated that DMOG induces the expression of HIF-1α in epithelial cells[26] and PHD2 is the key isoform that regulates HIF-1α levels[27]. In addition, we demonstrated that PHD2 is positively correlated with an apoptosis-related gene and is located in epithelial cells. Also, one of the protective mechanisms of DMOG is its ability to reduce epithelial cell apoptosis and hence preserve barrier function[26]. Taken together, it would be interesting to investigate the effect of homozygous deletion of *Phd2* in epithelial cells using villin:cre mice during experimental colitis. Besides epithelial cells, we also noticed PHD2 expression in mononuclear cells in the LP of human biopsies. Past publications generally made use of heterozygous PHD2 knockout mice to assess the effect of deletion on immune cell functioning. Although heterozygous PHD2 deletion is sufficient to promote M2 polarization and concomitantly attenuate the M1 phenotype, this knockout degree may not always suffice to elicit an altered immune response or function. Thus far, it has been demonstrated that homozygous *Phd2*-deleted macrophages also exhibit an attenuated pro-inflammatory M1 phenotype and reduced migratory capacity. These data combined with the positive correlation with an apoptosis-related gene might make it worth wild to explore how *Phd2*$^{f/f}$LysM:cre mice respond during DSS-induced colitis.

To make sure the risk of unwanted side-effects is indeed reduced through deletion of PHD1, EPO levels in the serum need to be determined as a marker of erythrocytosis in addition to the potential risk of carcinogenesis and fibrosis. In the context of fibrosis, it has recently been reported that DMOG is able to suppress fibrogenesis in addition to its known anti-inflammatory action which does not require HIF-1α activation in fibroblasts[28]. In this study, the effect of DMOG was studied on fibroblasts which is the most important effector cell type of fibrosis. However, TNF and IL-1β released by M1 macrophages may also promote fibrogenesis by promoting epithelial-mesenchymal transition, myofibroblast

activation through a TGF-β1-mediated mechanism and promote myofibroblast proliferation[29, 30]. Concerning PHDs, Ikeda and colleagues demonstrated that *Phd2*$^{f/f}$LysM:cre mice exhibit less cardiovascular fibrosis compared with WT mice which may be mediated by their attenuated pro-inflammatory M1 phenotype, migration and ability to decrease fibrosis-associated genes such as *Col1a2* and *Tgfb*[31]. In addition, we observed that *Phd1*-deletion also provokes the same anti-inflammatory phenotype. Therefore, it would be interesting to determine if DMOG exerts this anti-fibrotic effect through its inhibition of PHD1 or PHD2. The concern about carcinogenesis through HIF activation may be redundant in the case of *Phd1*-ablation since its silencing is not able to activate HIF levels in various different cells[27]. To confirm that this is also the case in *Phd1*-deleted macrophages and DCs, HIF-1α and HIF-2α levels need to be determined. However, NFκB activation has also been shown to be constitutively expressed in various solid tumors including the colon and plays an active role in the development of colitis-associated cancer[32]. To evaluate the effect of haematopoietic *Phd1*-deletion on tumor development, endoscopic and/or histological examination of the colon in older mice (≥8 months) could be performed. Alternatively, their susceptibility to develop azoxymethane /DSS-induced colitis-associated cancer could be compared with WT mice.

To strengthen our current data about a therapeutic effect of haematopoietic *Phd1*-deletion in IBD in general, confirmation is required in other IBD models including chronic colitis such as chronic DSS-induced colitis and ileitis models. Given the fact that PHD1 expression is increased in inflamed colonic biopsies from CD patients along with its anti-inflammatory effect after deletion in macrophages and DCs, we questioned whether *Phd1*-deletion could also provide therapeutic benefit in a model for CD-like colitis such as TNBS-induced colitis. This model involves the intra-rectal administration of the hapten TNBS in combination with the mucosal barrier breaker ethanol to elicit distal colitis. TNBS is believed to haptenize colonic autologous or microbial proteins which makes them immunogenic to the host. This results in acute transmural inflammation with epithelial erosions, infiltration of neutrophils and macrophages and granuloma formation. However, TNBS-treated *Phd1*$^{f/f}$Tie2:cre mice exhibited similar weight loss, colon shortening and histological inflammation scores compared with TNBS-treated WT mice (Figure 3). The reason for the lack of protection is not immediately clear given the fact that the acute TNBS model evokes a similar cytokine pattern as the acute DSS model[33]. However, despite the marked decrease in body weight and colon length, only 25% of the mice in both groups exhibited clear signs of inflammation after histological examination making it of course very difficult to detect differential effects. This is perhaps not unexpected since our mice are on a C57BL/6 background, known to be quite resistant to TNBS-induced colitis,[34] but which we accounted for by using a higher dose of TNBS. Therefore, optimization of the TNBS concentration or backcrossing onto the sensitive BALB/c mice may resolve this issue.

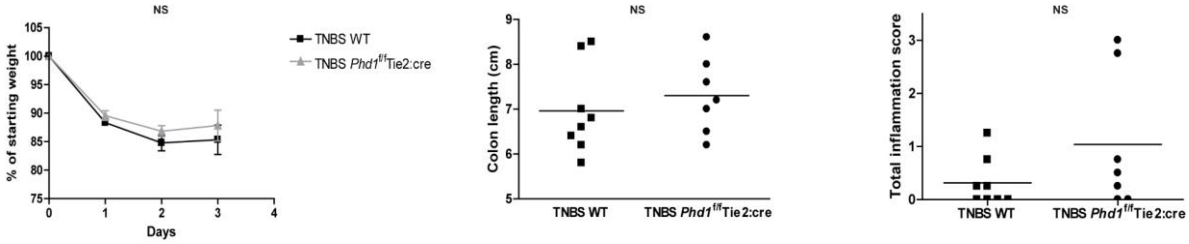


Figure 3. *Haematopoietic and endothelial deletion of Phd1 does not protect against TNBS-induced colitis. Left: Weight evolution of WT and Phd1$^{f/f}$Tie2:cre during the course of TNBS. Middle and right: Colon length and histological inflammation score of WT and Phd1$^{f/f}$Tie2:cre at day 3 of TNBS-induced colitis. n≥7 . NS: not significant.*

Besides a mouse model for CD-like colitis, it would be interesting to know if haematopoietic *Phd1*-deletion exerts a beneficial effect during CD-like ileitis which can be demonstrated by crossing them to TNF$^{\Delta are}$ mice. The latter are genetically modified mice that have a single allele deletion in the AU-rich elements of the *Tnf* gene, stabilizing the *Tnf* transcript, leading to the overproduction of TNF. As such, these mice develop from 8 weeks onward chronic inflammatory arthritis and CD-like ileitis[35]. In a first set-up, we backcrossed *Phd1*$^{f/f}$Vav:cre with TNF$^{\Delta are}$ mice and followed up the weight of their offspring until week 17. No differences in body weight evolution can be observed between all groups (Figure 4). However, further analysis including histological examination, ileal and serum concentrations of pro-inflammatory cytokines still needs to be performed before conclusions about a potential effect can be drawn. In the meanwhile, breeding pairs (*Phd1*$^{f/+}$Vav:cre+/- TNF$^{\Delta are/WT}$ x *Phd1*$^{f/f}$Vav:cre-/-) have been set up to obtain homozygous haematopoietic *Phd1*-deficient TNF$^{\Delta are}$ mice (*Phd1*$^{f/f}$Vav:cre+/- TNF$^{\Delta are/WT}$) which will be compared with their *Phd1*-floxed TNF$^{\Delta are}$ counterparts (*Phd1*$^{f/f}$Vav:cre-/- TNF$^{\Delta are/WT}$).

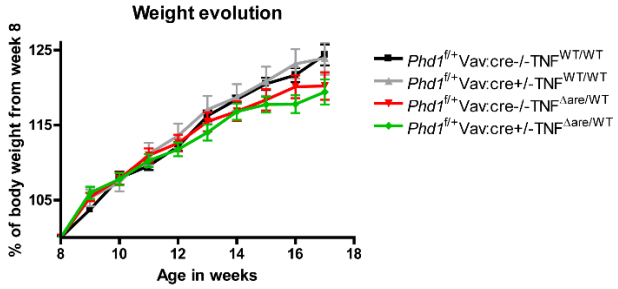


Figure 4. *Weight evolution of the first-generation offspring (n ≥ 10) of the breeding pair Phd1$^{f/f}$Vav:cre+/- x TNF$^{\Delta are/WT}$ starting from the age of 8 till 17 weeks after birth.*

A next important step in this research is the translation of the experimental data to human IBD. In a first attempt to demonstrate that our findings could also apply in the human context, we created *PHD1*-deficient THP-1XB. This is a human monocytic cell line that may be differentiated into macrophages following PMA stimulation. *PHD1*-deficiency was confirmed using qRT-PCR (data not shown). In accordance with the *Phd1*$^{f/f}$Tie2:cre BMDM, the LPS-stimulated sh*PHD1* THP-1XB cells

secreted substantially lower amounts of IL-1β, IL-6, TNF and MCP-1 compared with the shScr control cells (Figure 5).

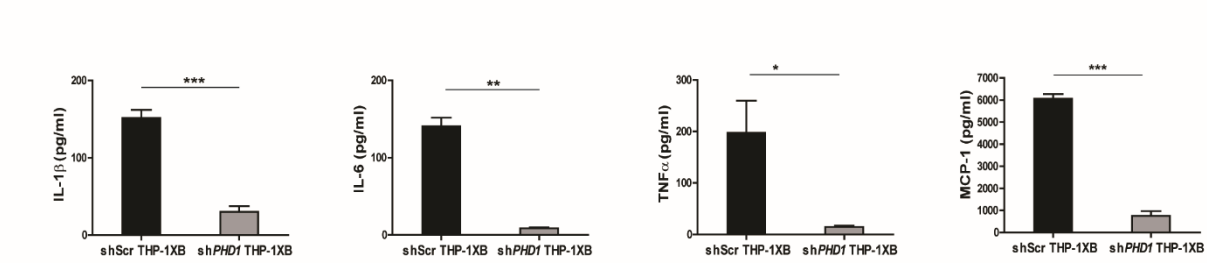


Figure 5. *Deletion of PHD1 in human macrophages impairs the LPS response. Concentrations of IL-1β, IL-6, TNF and MCP-1 in the supernatant from the shScr control and shPHD1 THP1-XB cells following 100 ng/ml LPS-treatment. Data are represented as the means ± SEMs. *p < 0.05, **p < 0.01 and ***p < 0.001*.

Since only pan-hydroxylase inhibitors are available, the development of a specific PHD1 inhibitor is required for application in human IBD. If the development of an inhibitor with high selectivity for PHD1 is not feasible, a PHD1 antisense oligonucleotide may represent a good alternative[36]. In this regard, an oral SMAD7 antisense oligonucleotide has been proven effective in a phase 2 study with active CD patients,[37] while efficacy of ICAM-1 antisense was demonstrated for active UC patients[38].

In summary, this thesis highlights the importance of the oxygen-sensor PHD1 in the pathogenesis of IBD and provides fundamental new insights on how its targeting provides therapeutic benefit during experimental colitis (Figure 6). These findings underscore the potential of PHD1 as novel therapeutic target for IBD patients.

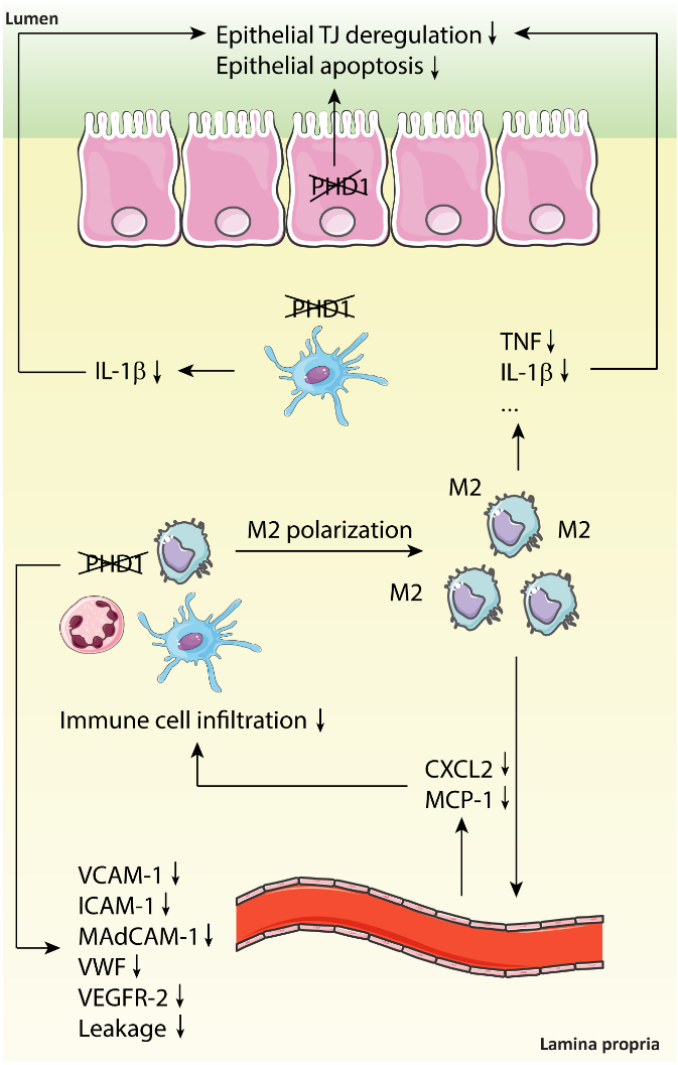


Figure 6. *Overview on how PHD1 targeting confers protection during intestinal inflammation. Initially, it was reported that epithelial deletion of Phd1 decreases apoptosis. We further expanded these findings by showing that haematopoietic Phd1-deficiency is able to reduce endothelial dysfunction, immune cell infiltration in the colonic mucosa and preserve epithelial integrity during experimental colitis. These effects are probably mediated by Phd1-deleted macrophages which polarize towards the M2 phenotype and concomitantly exhibit an attenuated pro-inflammatory response. Their diminished release of pro-inflammatory cytokines reduces 1) endothelial chemokine release which may account for the reduced immune cell infiltration and 2) may be responsible for the preservation of epithelial barrier integrity. In addition, the reduced secretion of IL-1β by Phd1-deficient DCs may also contribute to the latter effect. TJ: tight junction; IL: interleukin; TNF: tumor necrosis factor; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular cell adhesion molecule 1; MAdCAM-1: mucosal addressin cell adhesion molecule 1; VWF: von Willebrand factor; VEGFR-2: vascular endothelial growth factor 2; MCP-1: monocyte chemoattractant protein 1; CXCL2: C-X-C motif ligand 2.*

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Date of Birth:	February 22nd, 1987
Place of Birth:	Zottegem
Nationality:	Belgian

EDUCATION

2012-present: **PhD student** at Ghent University, Faculty of Life sciences and Medicine, Department of Gastroenterology

PhD project: Prolyl hydroxylase domain-containing protein 1 as potential therapeutic target for inflammatory bowel disease.
Promotor: prof. dr. Debby Laukens, PhD; Co-promotor: dr. Pieter Hindryckx, MD

2005- 2011: **Master of Science in bioscience engineering: Cell and Gene Biotechnology** at Ghent University, Faculty of Bioscience Engineering

Master dissertation: Characterization of nanobodies against nucleostemine, a marker of cancer cells
Promotor: prof. dr. Jan Gettemans, Co-promotor: prof. dr. Els Van Damme

SKILLS

- Cell culture: subculture of cell lines, isolation and culture of human and mouse primary cells, transient and stable lentiviral transfections
- RNA extraction, DNA extraction, cDNA synthesis, PCR, qPCR, molecular cloning
- Proteins: SDS-PAGE, Western blotting, ELISA, magnetic bead-based immunoassays (Bio-plex), immunohistochemistry
- Basic knowledge flow cytometry
- Mice: extensive experience with mice breeding, design and set-up of mouse models for colitis and ileitis, mice handling and sampling
- Extensive knowledge of Microsoft Office, SPSS and GraphPad

ADDITIONAL TRAINING

Followed courses organized by the Doctoral Schools of Life sciences and Medicine, Ghent University:

- Applying for a postdoctoral job (2015)
- Personal Effectiveness (2015)
- Speed reading (2014)
- Project management (2014)
- Clinical studies: study design, implementation and reporting (2013)
- Laboratory animal science (FELASA C) (2013)

PUBLICATIONS

Van Welden S., Laukens D., Ferdinande L., De Vos M. and Hindryckx P. Differential expression of prolyl hydroxylase 1 in patients with ulcerative colitis versus patients with Crohn's disease/infectious colitis and healthy controls. Journal of Inflammation (London). 2013 Nov 20;10(1):36. doi: 10.1186/1476-9255-10-36. (IF 2.216)

Van Welden S., De Vos M., Wielockx B., Tavernier SJ., Dullaers M., Neyt S., Descamps B., Devisscher L., Devriese S., Van den Bossche L., Holvoet T., Baeyens A., Correale C., D'Alessio S., Vanhove C., De Vos F., Verhasselt B., Breier B., Lambrecht BN., Janssens S. Carmeliet P., Danese S., Elewaut D., Laukens D. and Hindryckx P. Haematopoietic prolyl hydroxylase-1 deficiency promotes M2 macrophage polarization and is both necessary and sufficient to protect against experimental colitis. The Journal of Pathology. 2017 Mar;241(4):547-558. doi: 10.1002/path.4861. (IF 7.381)

Van Welden S., Selfridge AC, Hindryckx P. Hypoxia and hypoxia-induced signalling as therapeutic targets for inflammatory bowel disease. Nature Reviews Gastroenterology & Hepatology. 2017. Accepted for publication. (IF 14.44)

Van Acker A., Filtjens J., **Van Welden S.**, Taveirne S., Van Ammel E., Vanhees M., Devisscher L., Kerre T., Taghon T., Vandekerckhove B., Plum J., Leclercq G. Ly49E expression on CD8αα-expressing intestinal intraepithelial lymphocytes plays no detectable role in the development and progression of experimentally induced inflammatory bowel diseases. PLoS One. 2014 Oct 13;9(10). doi: 10.1371/journal.pone.0110015. (IF 3.53)

Devriese S., Van den Bossche L., **Van Welden S.**, Holvoet T., Pinheiro I., Hindryckx P., De Vos M., Laukens D. T84 monolayers are superior to Caco-2 as a model system of colonocytes. Histochemistry and Cell Biology. 2017 Mar 6. doi: 10.1007/s00418-017-1539-7. (IF 2.78)

Van den Bossche L., Borsboom D., Devriese S., **Van Welden S.**, Holvoet T., Devisscher L, Hindryckx P., De Vos M., Laukens D. Tauroursodeoxycholic acid protects bile acid homeostasis under inflammatory conditions and dampens Crohn's disease-like ileitis. Laboratory Investigation. 2017 May;97(5):519-529. doi: 10.1038/labinvest.2017.6. (IF 4.202)

Van den Bossche L., Hindryckx P., Devisscher L., Devriese S., **Van Welden S.**, Holvoet T., Vilchez Vargas R., Vanden Bussche J., Vanhaecke L., Van de Wiele T., De Vos M., Laukens D. Ursodeoxycholic acid and its taurine/glycine conjugated species reduce colitogenic dysbiosis and equally suppress experimental colitis in mice. Applied and Environmental Microbiology. 2017 Mar 17;83(7). pii: e02766-16. doi: 10.1128/AEM.02766-16. (IF 3.823)

Holvoet T., Devriese S., Castermans K., Boland S., Leysen D., Vandewynckel Y., Devisscher L., Van den Bossche L., **Van Welden S.**, De Rycke R., Geboes K., Bourin A., Defert O., Hindryckx P., De Vos M.,

Laukens D. Treatment of intestinal fibrosis in experimental inflammatory bowel disease via the pleiotropic actions of a local Rho kinase inhibitor. Under review with Gastroenterology. (IF 18.187)

ABSTRACTS AT NATIONAL AND INTERNATIONAL CONGRESSES

Selected for 3 poster presentations at international congresses. Selected for 5 oral presentations at national congresses and international congresses of which 3 are listed below:

Van Welden S, Laukens D, Devisscher L., Devlies H., K. Olievier, C. Correale, S. D'Alessio, S. Danese, M. De Vos, P. Hindryckx. Silencing endothelial prolyl hydroxylase 1 prevents endothelial dysfunction in intestinal microvascular endothelial cells and dampens murine colitis. **Oral presentation**; UEGW 2014; Vienna.

Van Welden S, Laukens D, Devisscher L., Devlies H., K. Olievier, C. Correale, S. D'Alessio, S. Danese, M. De Vos, P. Hindryckx. Silencing endothelial prolyl hydroxylase 1 prevents endothelial dysfunction in intestinal microvascular endothelial cells and dampens murine colitis. **Oral presentation**; DDW 2014; Chicago.

Van Welden S, Laukens D, Ferdinande L, De Vos M, Hindryckx P. Differential expression of prolyl hydroxylase 1 and 3 in patients with ulcerative colitis versus patients with Crohn's disease/infectious colitis and healthy controls. **Oral presentation**; Belgian Week of Gastroenterology 2013; Antwerp

GRANTS AND AWARDS

- National scholar award by UEGW (United European gastroenterology week) 2014
- Travel grant by UEGW (United European gastroenterology week) 2014
- Travel grant by the VVGE (Flemish association gastroenterology) 2013

“Many of life’s failures are people who did not realize how close they were to success when they gave up.”

Thomas A. Edison

DANKWOORD

Hallelujah, I made it! Na jarenlang experimenteren met muizen en cellen, artikels en revisies schrijven, ben ik eindelijk aan het slot van mijn doctoraat gekomen. Dit was uiteraard niet mogelijk geweest zonder de hulp, steun en het vertrouwen van enkele heel bijzondere mensen:

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