Interplay between Pathogenic and Immune Regulatory Mechanisms in Gαi2 Deficient Colitis

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Cover illustration: Possible immune pathogenesis of Gαi2−/− colitis, by the author

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To my husband, Torsten

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ABSTRACT

The two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are gastrointestinal disorders characterized by chronic and relapsing inflammation. Mice deficient in the inhibitory G protein subunit Gαi2 spontaneously develop chronic colitis and have been used as a model for IBD, with particular similarities to UC. They have been used in this thesis to study the changes in immune pathology during disease progression; to investigate the role of regulatory T cells (Tregs) in chronic intestinal inflammation; and as a model for testing anti-inflammatory agents.

During the progression of the colitis, continuing increases in colon weight/cm and spleen weight, and a gradual decrease in thymus weight were observed. The protein levels of proinflammatory cytokines/chemokines IL-1β, IL-6, IL-12p40, IL-17, TNF-α, CCL2 and CXCL1 increased during colitis progression and were all significantly increased in mice with moderate and severe colitis in colonic, but not small intestinal tissues. In colon, IFN-γ mRNA and IL-27 mRNA were gradually elevated during the course of the colitis, whereas IL-21 mRNA expression was significantly enhanced in mice with severe colitis. Thus, the lack of Gαi2 elicits an expansion of gut Th17 responses, possibly as a result of a $G\alpha i2^{-/-}$-driven epithelial barrier defect; this drives the production of neutrophil-attractant chemokines, resulting in the influx of neutrophils; in turn promoting an adaptive Th1 response.

Numbers of lamina propria $CD4^{+}FoxP3^{+}$ cells were significantly increased in colitis and were dispersed in the tissue, in contrast to non-inflamed colon in which they concentrated within organized lymphoid structures. *In vitro* data showed that both $G\alpha i2^{-/-}$ and wild-type (WT) splenic Tregs were able to suppress wild type (WT) effector T cells (Teffs), but that pathogenetic $G\alpha i2^{-/-}$ Teffs could not be suppressed by either type of Tregs. Adoptive transfer

experiments in the colitis model showed that neither type of Tregs could prevent disease induced by co-transfer of Gαi2$^{-/-}$ Teffs. It is possible that Treg regulatory function is suppressed in the inflamed colonic milieu, and/or that they are unable to overcome the heightened activity of Gαi2$^{-/-}$ Teffs.

Acute colitis induced by dextran sodium sulphate (DSS) and spontaneous Gαi2$^{-/-}$ chronic colitis were used to assess the efficacy of *ex vivo* anti-inflammatory treatment. The gene profiles reflected the different mechanisms underpinning these two types of colitis. Thus, genes related to pro-inflammatory innate cytokines, chemokines and chemokine receptors were up-regulated in DSS-induced colitis, whereas genes related to T cell markers were preferentially elevated in Gαi2$^{-/-}$ colitic tissues. In general, the same panel of genes displayed increased transcription in the *in vivo* and *ex vivo* cultured tissues in DSS model. The well-characterised corticosteroid methyl-prednisolone and the proteasome inhibitor MG132, were used to compare the efficacy between *in vivo* treatment and *ex vivo* cultures of colon obtained from DSS-induced and Gαi2$^{-/-}$ colitic mice. After steroid treatment, IL-1β, IL-6 mRNA were suppressed in both models, both *in vivo* and *ex vivo,* wheras iNOS was not suppressed *in vivo* in Gαi2$^{-/-}$ mice. The anti-inflammatory function of methyl-prednisolone was mostly at the innate level, as shown in DSS-colitis, but MG132 acted effectively on the chronically activated adaptive immune response in the Gαi2$^{-/-}$ colitis model. Thus the changes in inflammatory gene expression in the murine *ex vivo* culture system reflected the *in vivo* response in the inflamed colonic tissue, suggesting that the murine culture system can be used for validation of future IBD therapies.

We conclude that the genetic Gαi2 deficiency leads to amplification of gut Th17 responses, possibly mediated by a gut barrier defect, and this leads to a mixed Th17/Th1 effector phenotype in late-stage colitis. The hyper-reactive Gαi2$^{-/-}$ effector T cells further amplify the inflammation and increased numbers of memory effector T cells are generated as disease progresses. The lack of appropriate regulation by Tregs further exacerbates the Gαi2$^{-/-}$ colitis.

Keywords: IBD, Gαi2$^{-/-}$colitis, Tregs, Teffs, Th17/Th1

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Interplay between Th1 and Th17 effector T cell pathways in the pathogenesis of spontaneous colitis in the Gαi2-deficient mouse.**
Yu-Yuan Götlind*, Maria Fritsch Fredin*, Ashok Kumar Kumawat, Roger Willén, Hilja Strid, Ignacio Rangel, Paul W. Bland, Elisabeth Hultgren Hörnquist *Submitted manuscript, * both authors contributed equally*

II. **$CD4^+FoxP3^+$ regulatory T Cells from $G\alpha i2^{-/-}$ mice are functionally active in vitro, but do not prevent colitis.**
Yu-Yuan C. Götlind, Sukanya Raghavan, Paul W. Bland, Elisabeth Hultgren Hörnquist *PLoS One*. 2011, *6:*e25073.

III. **The application and relevance of ex vivo culture systems for assessment of IBD treatment in murine models of colitis**.
Maria Fritsch Fredin, Alexander Vidal, Helena Utkovic, Yu-Yuan Götlind, Roger Willén, Liselotte Jansson, Elisabeth Hultgren Hörnquist, Silvia Melgar S. *Pharmacol Res*. 2008, *58:*222-231.

CONTENT

ABBREVIATIONS

5-ASA	5-aminosalicylate
APC	Antigen Presenting Cell
ATP	adenosine 59-triphosphate
CD	Crohn's disease
CLP	colonic lymphoid patch
CP	cryptopatch
DC	dendritic cell
DSS	dextran sodium sulphate
EBI3	Epstein-Barr virus–induced gene 3
GPCR	G protein-coupled receptor
GWAS	Genome-wide association study
Gαi2	G alpha i 2
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFN-γ	interferon-gamma
ILF	isolated lymphoid follicle
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, and X-l inked inheritance
KO	gene knockout

LTi	lymphoid tissue–inducer
MDP	muramyl dipeptide
MMP	matrix metalloproteinase
NKT	natural killer T cell
NOD2	nucleotide-binding oligomerization domain-containing protein 2
nTreg	natural regulatory T cell
PAMP	pathogen-associated molecular pattern
PRR	Pattern Recognition Receptor
SCID	severe combined immunodeficiency
SFB	segmented filamentous bacterium
SNP	single-nucleotide polymorphism
TCR	T cell receptor
Teff	effector T cell
TGFβ	transforming growth factor beta
TLR	Toll-like receptors
TNBS	2,4,6-trinitrobenzenesulphonic acid
TNF-α	tumour necrosis factor-alpha
Treg	$FoxP3^+$ regulatory T cell
UC	ulcerative colitis
WT	wild-type

1 INTRODUCTION

1.1 Inflammatory Bowel Disease (IBD)

The two major clinically defined forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory conditions. Ulcerative colitis is confined to the colon and rectum, the mucosa of which becomes inflamed with superficial ulcers, macroscopically evident as redness and swelling of the intestinal lining. The clinical presentation includes abdominal pain, diarrhoea and gastrointestinal bleeding. In contrast, CD most commonly affects the ileum and proximal large intestine but can affect any part of the digestive tract from mouth to anus. In CD the inflammation extends transmurally across all layers of the intestinal wall. Unlike UC, in which the inflammation is continuous, the lesions in CD can be discontinuous, with so-called skip lesions occurring simultaneously in different regions of the intestine. The main histopathological features of UC comprise depletion of goblet cells, distortion of crypt architecture, crypt abscesses, haemorrhage and a significant infiltrate of neutrophils and mononuclear cells within the lamina propria. Crohn's disease is characterized by marked infiltration of lymphoid cells, plasma cells, other inflammatory cells and macrophages. Non-caseating granulomas, fissures, fibrotic stenoses and fistulae are also features of CD (1). The aetiopathogenic mechanisms of IBD are multifactorial, and include both genetic and environmental influences.

1.1.1 Experimental Models of IBD

Experimental models continue to be important tools for investigating the mechanisms of pathogenesis and for detecting potential therapeutic agents. An appropriate animal model should exhibit morphological alterations, inflammatory characteristics, course of disease and symptoms, as well as a suggested pathophysiology, similar or identical to human IBD.

IBD models can be divided into four categories: (i) gene knockout (KO) models where key molecules are deleted; (ii) transgenic mice; (iii) inducible colitis models where administration of different chemical compounds induce colitis; and (iv) adoptive transfer models where different cell populations are transferred into immunodeficient recipient mice. The animal models used in this thesis are one gene KO model, the $G\alpha i2^{-/-}$ mouse, and one inducible colitis model, dextran sodium sulphate (DSS)-induced colitis.

Gαi2-deficient Mice

Heterotrimeric guanine nucleotide-binding proteins (G proteins), a complex of three subunits termed α, β, and γ, are classified into four families: Gi, Gs, Gq, and $G_{12/13}$. G protein-coupled receptors (GPCRs) transduce diverse extracellular signals, such as a large variety of chemical stimuli (hormones, neurotransmitters, chemoattractants, calcium ions) and sensory stimuli, into intracellular responses through activation of the G proteins. Following ligand binding, the receptor undergoes conformational changes, which promote the coupling with G proteins (Gαβγ), and catalyze the exchange of GDP for GTP on the α-subunit. GPCRs are expressed in virtually every cell and consist of seven membrane-spanning α-helical structures with the N- and C-termini exposed to the extracellular and intracellular environment, respectively (2).

$G\alpha i2^{-/-}$ mice on a 129SvEv background spontaneously develop colitis (3-5). The inflammation is restricted to the large intestine, resembling the pathology seen in human UC with crypt distortion, loss of mucin-producing goblet cells, and abscess formation together with erosion and ulcerations of the mucosa (3-5).

Gαi2 deficiency leads to immune alterations characterized by activation of pro-inflammatory T helper 1 (Th1) cells in late colitis (3), enhanced spontaneous production of proinflammatory cytokines in the small and large intestine, increased frequencies of activated T and B lymphocytes expressing mucosal homing receptors, and antibodies specific for normal intestinal flora and autoantigens present in intestinal lavage fluid (5, 6). The precolitic changes also include regression of Peyer´s patches (4), as well as a switch from an IL-10 dominated dietary antigen-specific T cell response in wild-type mice to a Th1 cytokine profile in $G\alpha i2^{-/-}$ mice (7).

$G\alpha i2^{-/-}$ mice have a higher frequency of mature thymocytes, compared to control mice (8). This disturbed T cell development with increased production of IFN-γ, together with high levels of IL-1, IL-6 and TNF-α in inflamed tissue (9) drives a typical Th1-skewed $CD4^+$ immune response. Increased serum concentrations of IL-18 and IL-1 receptor antagonist (IL-1Ra) in $G\alpha i2^{-/-}$ mice with established disease, and increased serum IL-1Ra levels already in the precolitic stage, suggests IL-1Ra to be an early serum marker of disease progression. In contrast, serum concentrations of IL-1β were not different between $G\alpha i2^{-/-}$ and wild type controls, irrespective of colitis stage (10).

The lack of the Gαi2 subunit has also been associated with functional disorders in epithelial cells (11), professional antigen-presenting cells (9) and B lymphocytes (4, 9, 12). Thus, this mouse model reveals multiple requirements for Gαi2 in the development of mucosal immunoregulation. However, the cellular and molecular mechanisms, which require the participation of Gαi2 for regulating immune responses, still remain unclear.

Dextran Sodium Sulphate (DSS) Induced Colitis

The administration of DSS dissolved in water to mice or rats causes rectal bleeding, weight loss, shortening of the intestine, mucosal ulcers, and mucosal infiltration of neutrophils. Both acute colitis, which occurs during the administration of DSS, and chronic colitis, which occurs approximately five days after the administration of DSS, can be examined in this model. The acute phase is thought to be induced by innate immunity, as it also occurs in DSS-treated severe combined immunodeficiency (SCID) mice (13).

Studies of DSS-induced colitis have shown that DSS alters mucosal barrier function prior to the onset of colitis (14). One possible consequence of this barrier destruction is that mucosal phagocytes are activated by components of the mucosal microbiota, leading to release of pro-inflammatory cytokines. Although preventive antibiotics attenuate DSS-induced colitis, they have no therapeutic benefit once colitis establishes (15).

There is also clear evidence for the involvement of T cells in the development of DSS-induced colitis. Shintani et al. demonstrated in adoptive transfer experiments that T cells from mice with DSS-induced colitis, primed with DSS-pulsed macrophages in vitro, and then transferred into mice treated with DSS, showed more severe colitis than mice treated with oral DSS only (16).

Similar to Gαi2-deficient mice, colitis induced by DSS is generally considered as a model of UC, based on clinical symptoms and histopathological features.

1.1.2 The Intestinal Microbiota in IBD

The intestinal microbiota profoundly affects host immune composition under physiologic conditions and is likely to be the most important environmental factor in IBD as the target of the inflammatory response (17). Numerous genetic mouse models of intestinal inflammation do not develop disease after germ-free rederivation (18), and antibiotic treatment may induce remission in IBD patients (19). Also, T cell lines specific for bacterial antigens can induce intestinal inflammation, but not when activated with polyclonal activators

(20, 21). This has led to an intense interest in the composition of the intestinal microbiota, its regulation by the host and by other environmental factors, and the interactions between the microbiota and host.

Changes in the composition of the intestinal microbiota in IBD patients are characterized by a markedly reduced diversity in the *Clostridium* cluster IV and XIVa (belonging to the *Firmicutes* phylum) and in the *Bacteroidetes* phylum, with a concurrent increase in the *Proteobacteria* phylum and the *Bacillus* genus within the *Firmicutes* phylum (22, 23). In addition, intestinal inflammation has been related to a marked increase in *Enterobacteriaceae* (24, 25).

With regard to a possible infectious aetiology, some studies have suggested that *Mycobacterium avium paratuberculosis* plays a role in CD (26), but there are conflicting data (27). Mannose-bearing antigens (mannans) from yeast elicit antibody responses in some CD patients (28) and an invasive *Escherichia coli* of novel phylogeny is involved in depletion of *Clostridia* strains in CD (29). An increased amount of colonic sulphate-reducing bacteria has been observed in some patients with UC, resulting in higher concentrations of hydrogen sulphide, although the exact role of hydrogen sulphide in UC pathogenesis is still unclear (30).

Regarding possible autoimmune mechanisms, autoantibodies are detectable in IBD, but there is no evidence that autoimmunity plays a direct pathogenic role in either UC or CD. It is more likely that these autoantibodies result from cross-reactivity with bacterial antigens (31).

1.1.3 Role of Genetic and Environmental Factors in IBD

Genome-wide association studies (GWASs) have significantly increased our understanding of the importance of genetic susceptibility loci in IBD (32). The GWASs have identified more than 30 risk-conferring loci in CD (32-34), and have revealed a considerable overlap in genetic risk factors between CD and UC (35, 36). These studies highlight pathways examined in this thesis, including IL-23 and T helper 17 (Th17) cells (37), but have also pointed to pathways such as autophagy (38). GWASs have identified several single-nucleotide polymorphisms (SNPs) in the IL-23R locus that are associated with either increased susceptibility or resistance to IBD (37, 39). Mutations in the IL-10 receptor subunits *IL10RA* or *IL10RB* have also been associated with early-onset IBD (40).

The strongest genetic risk factor for CD identified so far is the mutation in Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (41), an intracellular receptor sensing muramyl dipeptide (MDP) in peptidoglycans from gram-negative and gram-positive bacteria, leading to activation of nuclear factor-κB (NF-κB) (42-44). Ironically, *in vitro* evidence indicates that the most common variants of NOD2 appear to represent loss-of-function mutations that actually decrease NF-κB activation (44, 45), and its correlation to the up-regulation of NF-κB observed in patients with IBD remains unclear. It is possible that NOD2 variants fail to trigger protective response pathways to bacterial products such as muramyl dipeptide, which may be mediated by NF-κB, resulting in a secondary diffuse activation of NF-κB in intestinal tissue by NOD2-independent mechanisms (46). An alternative explanation is that defective eradication of intracellular pathogens, as demonstrated with *Salmonella typhimurium* in NOD2-negative intraepithelial cell lines transfected with the CD-associated frameshift mutated form of NOD2, leads to persistent innate activation of inflammatory pathways (47). In support of this interpretation, NOD2-deficient mice have been shown to harbour increased loads of commensal resident bacteria, and also a reduced ability to prevent colonization with pathogenic bacteria (48). Interestingly, the regulation seems to be reciprocal, as NOD2 expression was reduced in germ-free mice (48). NOD2 is expressed in myelomonocytic and dendritic cells (DC), intestinal epithelial cells (IEC) and Paneth cells (49). The discovery of NOD2 expression in Paneth cells is particularly interesting as these cells have an antibacterial function in the gut, secreting potent antimicrobial substances such as lysozyme, phospholipase A2, and α- and β-defensins (50). Paneth cells are most highly concentrated in the terminal ileum, the most common site of inflammation in CD.

Several additional genetic loci associated with IBD, e.g. Autophagy related 16-Like 1 (*ATG16L1*) and X-box binding protein-1 (*XBP1*) likewise affect the ability of Paneth cells to secrete antimicrobial factors (51-54). Hence, the function of a number of genes could affect microbial community structure and promote inflammation.

Nevertheless, the rapid increase in IBD incidence in certain developing countries undergoing a rapid westernization, the development of IBD in immigrants to high-prevalence countries (55), and the discordance of IBD among monozygotic twins (56, 57) all underpin the importance of environmental factors as essential components of the pathogenesis of IBD (58).

1.1.4 The Intestinal Epithelium as an Immunophysiological and Structural Barrier

The intestinal mucosal barrier includes mucus secreted on the apical surfaces of IECs, IECs themselves, and the stromal compartment below. However, the epithelium is also a crucial mediator of communication between the lumen and the mucosal immune system via the release of cytokines and chemokines, signalling the intrusion of pathogens (59). The sensing of, and response to, luminal antigen involves absorptive IECs, M cells in the follicle-associated epithelium of Peyer's patches, and subepithelial DCs.

The single layer of IECs is sealed by tight junctions (60). A leaky intestinal epithelium is likely one of the key factors in the pathophysiology of IBD, and defects in epithelial barrier function have been observed in a number of intestinal disorders, including IBD (61).

The immune system plays an important role in modulating intestinal permeability. The cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) are found in high levels in the intestinal mucosa in IBD patients (62), and have been found to decrease barrier function of cultured intestinal epithelial monolayers (63). Likewise, incubation of intestinal epithelial cell monolayers with IFN-γ and TNF-α leads to reorganization of tight junction proteins and, thereby, disruption of the barrier function (64). TNF-α and IFN-γ have also been shown to upregulate transcellular transport of macromolecules (65), and elevated transcellular transport of macromolecules across epithelial cells in biopsies from CD patients correlates with expression levels of TNF-α (66). Thus, regulation of epithelial barrier function by proinflammatory factors can involve both tight junctions and transcellular pathways.

1.1.5 Dendritic Cells as the Interface between the Innate and Adaptive Immune Systems

The intestinal inflammation in IBD is likely due to dysregulation both in innate and adaptive immune mechanisms. In the innate immune response, activated dendritic cells (DC) and macrophages may secrete cytokines that promote the inflammatory response. Subsequently, these cytokines trigger and determine T cell differentiation in the adaptive immune response.

Sensing of pathogen-associated molecular patterns (PAMPs) by the innate immune system is mediated by a heterogenous set of receptors, including Toll-like receptors (TLR), NOD-like receptors, and various C-type lectins. Upon TLR engagement, DCs undergo a maturation process required for

further induction of adaptive immune responses (67, 68). Full DC activation also involves endogenous signals generated upon interaction between CD40 on the DCs and CD40L expressed by activated T cells. Additional tissue-derived signals, e.g., pro- or anti-inflammatory cytokines, affect the maturation of DCs, and thereby the type of T cell response they will induce (67, 69).

Dysfunctional dendritic cells (DCs) may act by priming abnormal T cell responses to the enteric flora in organized lymphoid tissues; sustain T cell reactivity within the inflamed mucosa through the interaction with T cells, and function as effector cells via the release of proinflammatory cytokines. Furthermore, there may be an imbalance between Th17 cell– versus Treg–inducing DC subsets, favouring Th17 cell differentiation and thus driving inflammation.

The involvement of DCs during the late phases of colitis development may be caused by aberrant activation of resident DCs, recruitment of DCs that were not exposed to the local tolerogenic microenvironment and hence are immunogenic, and an imbalance between tolerogenic and immunogenic DCs. Analysis of the DC phenotype in murine colitis has shown an expansion of mature DCs expressing higher levels of costimulatory molecules (CD40, CD80, and CD86) and increased amounts of IL-12p40 and IL-23p19 upon CD40 ligation (70).

There is evidence that TLR-mediated induction of IL-23 is enhanced by NOD2, resulting in the generation of DCs promoting the release of IL-17 by T cells.(71) Nonetheless, a recent study has shown that MDP activation of NOD2 may have inhibitory effects by inhibiting cytokine responses of mouse DCs to various TLR ligands (72).

Resident DCs could protect the colon during the initiation of colitis, in which their ability to induce Treg development may play a primary role. For example, clusters of DCs and Tregs are described in the colonic mucosa of mice during amelioration of colitis, where Tregs were observed located between clusters of $CD11c^+$ cells and pathogenic T effector cells and found to be in contact with both cell types (73). During colitis induced by adoptive transfer of $CD45RB^{hi}$ $CD4^+$ T cells into immunocompromised $RAG^{-/-}$ mice, $FoxP3^+$ regulatory T cells accumulate in colonic DC aggregates producing TGF-β, suggesting that DC aggregates are important structures to regulate inflammation (74).

1.1.6 Role of Cytokines from the Innate Immune System in IBD Pathogenesis

Cytokines are involved in cell activation, growth, and differentiation, and they are central to the development of inflammation and immunity (75, 76). Cells of the innate immune system, such as macrophages and monocytes, are able to mount a rapid response to a danger signal, e.g. an infectious agent, by secreting several proinflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, and TNF-α. The cytokine milieu subsequently directs the development of adaptive immunity mediated by T and B cells (77).

There is an increased production of a variety of cytokines from the innate immune system in IBD, e.g. TNF-α, IL-1, IL-6, IL-12, IL-13, IL-18 and IL-23.

Secretion of IL-6 is increased in both CD and UC mucosa (78), and plays a key role in many cellular processes such as cell growth and apoptosis. IL-6 *trans*-signalling (in which IL-6 signalling is mediated by soluble IL-6R, thereby activating cells that do not express membrane bound IL-6R) is important for survival of $CD4^+$ T cells, and possibly macrophages, and for their production of inflammatory cytokines such as TNFα, IFN-γ, and IL-1β (78, 79). IL-6 is also pivotal for differentiation of T helper cells into a more inflammatory Th17 phenotype (described below) (80-82). In accordance, blockade of IL-6 *trans*-signalling via a gp130-Fc decoy receptor, or *cis*-IL-6R signalling via an anti-IL-6R antibody, ameliorates colitis in several experimental mouse models (78, 79).

IL-1β expression is increased in IBD intestinal tissues relative to expression of IL-1Ra (83). Several α-defensins decrease IL-1β secretion, raising the possibility that elevated IL-1β is a result of Paneth cell dysfunction (84). Furthermore, IL-1β, together with IL-6, increases the frequency of Th17 cells (85), where IL-6 participates in the generation of Th17 cells from naïve T cells and IL-1β expands the Th17 cell pool in combination with TGF-β (86).

In addition, IL-18 expression is increased in IBD, particularly in IECs (87, 88). Increased IL-18 expression in DSS-induced colitis localizes to IECs, and neutralization of IL-18, either by antibody or by the natural IL-18 antagonist, IL-18 binding protein (IL-18BP), ameliorates DSS-induced colitis (89, 90). A major proinflammatory role of IL-18 in experimental intestinal inflammation was also deduced from experiments in the 2,4,6-trinitrobenzenesulfonic acid induced (TNBS) colitis and the SCID transfer models of colitis (91, 92).

Key components in the IL-12-related cytokine family are also involved in IBD development. Interleukin-12 and IL-23, secreted by DCs in response to Pattern Recognition Receptor (PRR)-derived signals, are closely related heterodimeric cytokines (34). IL-12, consisting of a p40/p35 heterodimer promotes the differentiation of Th1 cells, promoting macrophage activation and cell mediated immunity, and was originally linked with intestinal inflammation in the TNBS colitis model, in which anti-IL-12p40 treatment resulted in decreased IFN-γ production from lamina propria $CD4^+$ T cells (93). However, the p40 subunit is shared with IL-23, forming a heterodimer with a unique p19 chain. Interleukin-23 promotes the differentiation of inflammation-promoting Th17 cells (94, 95). Hence, whereas IL-12 (p40/p35) drives Th1 cell differentiatoin, IL-23 (p40/p19) supports the Th17 cell pathway (96).

Thus, enhanced production of IL-1 and IL-6 in the mucosa during innate surveillance of the luminal interface may promote IBD through an enhanced differentiation of naive T cells into inflammation-endorsing Th17 cells, and maintenance of their phenotype, while IL-18 acts as a costimulatory molecule with IL-12 in the development of the Th1 phenotype (97, 98).

1.1.7 The T helper 17 cell subset

Effector T cells play a central role in cell-mediated immunity. There are two main subsets of T cells; $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells. $CD8^+$ T cells detect and destroy virally infected cells and tumour cells, while $CD4^+$ T helper cells assist immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages.

$CD4^+$ T effector cells can be divided into different subsets, such as Th1, Th2 and Th17 cells, whose development is dictated by the transcription factors T-bet, GATA3 and RORγt, respectively (99-101). Th1 cells are inflammatory cells that release IFN-γ and are involved in immunity against intracellular pathogens, whereas Th2 cells are primarily involved in B cell help as they release B cell growth factors, for example, IL-4 and IL-5. Th17 cells, characterized by IL-17 (also known as IL-17A and IL-17F), IL-21 and IL-22 production, promote an acute inflammatory response, but can have anti-inflammatory roles, for example, promoting the intestinal epithelial barrier (102, 103).

Th17 cells mediate host defence against a variety of extracellular bacteria and fungi, by inducing production of neutrophil-recruiting chemokines and G-

CSF and GM-CSF from subepithelial myofibroblasts, promoting granulopoiesis. In addition, IL-22 from Th17 cells stimulates epithelial cell proliferation and restores mucus production by goblet cells (104).

In mice, TCR ligation in the presence of IL-6 and TGF-β is required for Th17 lineage commitment, inducing the specific transcription factor retinoic acid-related orphan receptor (ROR)γt (80, 98). Interleukin-6 induces expression of IL-23R on RORγt^{+} Th17 cells, which makes them responsive to IL-23, thereby expanding this lineage further (105). In addition, the Th17-derived cytokine IL-21 functions as an autocrine growth factor for Th17 cells in the presence of TGF-β in mice (106, 107). Interleukin-6 also induces the expression of IL-21R. In the presence of IL-21, IL-23 and TGF-β, IL-17 expression is induced independently from IL-6, whereas IL-6 orchestrates the downstream IL-21R and IL-23R signalling pathways which amplify Th17 differentiation in concert with TGF-β (106).

Apart from promoting Th17 cell differentiation, IL-21 has been reported to suppress IFN-γ expression in Th1-polarized cells without affecting the Th1-associated transcription factor T-bet or IL-12R expression (108). Furthermore, IL-21-knockout mice showed reduced pulmonary and hepatic granuloma formation with a general reduction of both Th1 and Th2 cytokines, suggesting that IL-21 might be involved in the amplifications of T-cell activation in both responses rather than promoting either one of the two pathways (109).

Th17 associated cytokines in IBD

Th17 cells and IL-23 are constitutively present in the intestinal lamina propria, and their presence is dependent on the microbial flora (105), as in the germ-free state or after treatment with broad-spectrum antibiotics the number of lamina propria Th17 cells decreases (105, 110, 111). In addition, adenosine 59-triphosphate (ATP) derived from commensal bacteria has been shown to activate a unique subset of myeloid CD70^{high}CD11c^{low} cells in the lamina propria, which then enhance differentiation of Th17 cells (111). Germ-free mice have much lower ATP levels in their intestinal lumen compared to specific pathogen-free (SPF) mice (111) and instead exhibit an increase in FoxP3^{+} regulatory T cells (Tregs) (described below) (111, 112).

The Th17 cell lineage is unique in that one single commensal microbe, segmented filamentous bacterium (SFB), which adheres tightly to the surface of IECs in the terminal ileum, has been demonstrated to induce Th17 cells upon colonization of germ-free mice (113, 114).

In normal human colonic mucosa, IL-17-producing $CD4^+$ T cells are markedly few compared to IFN-γ-producing cells, but their frequency is substantially increased in both CD and UC mucosa (115). In experimental models of colitis, the absence of IL-17 can lead to both ameliorated and more aggressive colitis. A recent study demonstrated that transfer of IL-17 deficient $CD45RB^{high}$ T cells into $RAG^{-/-}$ hosts resulted in increased severity of colitis, accompanied by increased production of IFN-γ in the colon, compared to transfer of wild type (WT) $CD45RB^{high}$ T cells (116). It was demonstrated that IL-17A suppressed Th1 polarization (116). Similarly, transfer of IL-17 receptor (IL-17R) deficient T cells to $RAG^{-/-}$ mice also elicited an accelerated colitis, compared to wild-type T cells (116). Neutralization of IL-17 with monoclonals (117) or genetic deletion of IL-17 (118) is also associated with exacerbation of DSS-induced colitis. However, in the TNBS colitis model IL-17R signalling is critical for colitis development (119).

Lately a subset of Th17 cells has been identified that co-produce IFN-γ (115, 120), possibly representing an intermediate cell, and TH17 cells have been shown to be able to differentiate into Th1 cells (121).

IL-21 also contributes to gut damage by enhancing the secretion of matrix metalloproteinases (MMPs), a family of neutral endopeptidases that can cleave multiple components of the extracellular matrix, resulting in abnormal deposition of collagen, from lamina propria myofibroblasts and fibroblasts during inflammation (122). Neutralization of IL-21 markedly reduces the amounts of MMPs released when fibroblasts are cultured with CD lamina propria mononuclear cell supernatants (122).

In addition, it seems clear that IL-23 promotes chronic mucosal inflammation in animal models (123-125) and likely human disease (37). In addition to promoting the expansion and/or maintenance of Th17 cells and induce Th17-type cytokine production from non-T cells IL-23 has also been shown to block Treg function (126, 127) (128).

Whereas patients with established CD display a Th1/Th17 immune response with high production of IFN-γ, TNF-α, IL-12, IL-17, IL-22 and IL-23, an atypical cytokine profile with high production of IL-5 and IL-13, possibly from NKT cells dominate in patients with UC (129).

1.1.8 T Lymphocytes with Regulatory Properties

Peripheral immune tolerance is mediated by several mechanisms, including anergy; deletion; ignorance; or suppression of effector T cell function by different populations of regulatory T cells (130). Regulatory lymphocytes prevent immune pathological responses induced by the enteric flora (131), or following microbial infection (127, 132); mediate transplantation tolerance (133); and have been linked to maternal tolerance to the foetus (134). However, activation of these cells is not always beneficial to the host, as they can obstruct anti-tumour immunity (135) and protective immunity to pathogens (136, 137).

In 1970, Gershon and Kondo discovered that T cells not only elicited, but also dampened, immune responses and that this suppression was mediated by T cells that were different from effector T cells (138). Although this discovery was pursued by intensive studies over the following years, research of these suppressor T cells collapsed in the mid-1980s, when molecular biology techniques showed no existence of the proposed I-J region of the mouse MHC gene, a region which had been assumed to encode a putative molecule associated with the suppressive function (139, 140). In 1990, Powrie and Mason reconstituted athymic nude rats with splenic T-cell suspensions depleted of $CD45RB^{low}CD4^{+}$ T cells, showing that transfer of this T cell population elicited a systemic disease resembling graft-versus-host disease and autoimmune tissue damage in multiple organs (141). Powrie et al. later showed that transfer of $CD45RB^{high}CD4^{+}$ T cells to T/B-cell-deficient SCID mice induced an IBD-like disease (142). In 1995, Sakaguchi et al. identified CD25, the IL-2 receptor α-chain, as a candidate marker for these regulatory T lymphocytes, because $CD4^{+}$ cells depleted of $CD25^{+}$ cells induced autoimmune disease when transferred into athymic nude (nu/nu) mice, whereas reconstitution with $CD4^{+}CD25^{+}$ cells within a limited period after transfer of $CD4^{+}CD25^{-}$ cells prevented autoimmunity in a dose-dependent fashion (143). Thus the discoveries of the ability to experimentally switch on autoimmune disease by depletion of a regulatory T cell population from normal rodents, and the ability to turn off autoimmune disease by reconstitution of the same population (9-15, 141, 144-146) highlighted the importance of these regulatory T cell populations.

Subsequently, several different lymphocyte populations have been shown to exert regulatory activities, including naturally occurring $TCR\gamma\delta^{+}$cells; NKT cells; $CD8^{+}CD25^{+}$ T cells; conditionally-induced Th3 and Tr1 (T regulatory type 1) cells; and the natural and induced $FoxP3^{+}$ regulatory T cells. The latter are described in detail below. However, the former cell types are

distinct from $CD4^+FoxP3^+$ regulatory T cells with respect to their mode of development, mechanism(s) of suppression and sites of actions (147).

T cell receptor $\gamma\delta^+$cells are present at high numbers in the epithelium lining the gut mucosa and are important in maintaining homeostasis of intestinal epithelia (148). It has been shown that aerosol nasal delivery of insulin results in the expansion of TCR$\gamma\delta^+$cells that express CD8 which have an immunosuppressive effect probably mediated by IL-10 (148). Furthermore, in low-dose oral tolerance induction, TCR$\gamma\delta^+$cells have been shown to increase the production of IL-10 by $CD4^+$ T cells (149).

Tr1 cells can be generated from peripheral T cells by *ex vivo* stimulation with antigens in the presence of IL-10, or by *in vitro* propagation after *in vivo* induction of oral tolerance (150), and once mature, secrete large amounts of IL-10.

In addition, oral co-administration of IL-4 and myelin basic protein resulted in increased differentiation of $CD4^+$ T cells with high production of TGF-β. This cell population is termed Th3 cells and they have the ability to reduce the severity of experimental autoimmune encephalitis (151).

Both TGF-β and IL-10 have immunosuppressive effects on both antigen presenting cells (APC) and T cells, but Tr1 and Th3 cells differ from the $CD4^+FoxP3^+$ regulatory T cell in their dependency on cytokines for their maintenance and suppressive function (152).

A $CD8^+CD25^+$ T cell population with regulatory properties was induced through T cell receptor stimulation with a genetically modified α-CD3 monoclonal antibody in patients with type I diabetes. This population required cell-cell contact for inhibition of antigen-specific $CD4^+$ effector T cells *in vitro*, and they expressed CTLA4 and FoxP3, similar to the $CD4^+$ $FoxP3^+$ regulatory T cells (153).

Experiments using adoptive transfer of effector and regulatory T cell populations into immunodeficient mice indicate a crucial role for regulatory T cells in maintaining mucosal immune homeostasis (131, 154), suggesting that dysfunction of regulatory T cells may be a contributory factor in the pathogenesis of human IBD.

FoxP3$^+$ regulatory T cells – Treg

In 1982, the autoimmune disease IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance), affecting multiple endocrine organs including symptoms of IBD and atopic dermatitis, was described as an X-linked immunodeficiency syndrome (155). IPEX shares many phenotypic features with the natural mutant mouse "Scurfy" described in the late 1950´s (156). These mice have an X-linked recessive inheritance of scaly skin; small, thickened ears; diarrhoea and malabsorption causing runting, and death in homozygous males within a month after birth, with hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines (156). The gene responsible for this syndrome was subsequently identified as FoxP3, encoding a transcription factor belonging to the forkhead/winged-helix family (157) and subsequently FoxP3 was found to be the causative gene for IPEX (158, 159). In the following years it was demonstrated that the development, function, and homeostasis of a subset of regulatory T cells are controlled by FoxP3 (160-162). This $CD4^+FoxP3^+$ T cell subset is hereafter referred to as Tregs.

There are two subsets of Treg: natural Tregs (n Tregs), which develop in the thymus; and inducible Tregs (iTregs), which are derived from naïve $CD4^+$ T cells in the periphery. The term iTregs is now used for the type 1 regulatory T cell (Tr1), induced by IL-10 (163) and the T helper 3 (Th3) cells induced by TGF-β (164), as described above, as well as the induced $FoxP3^+$ Tregs.

Before the identification of FoxP3 as a functional marker for Tregs, CD25 was considered a useful marker for distinguishing Tregs from effector T cells (143). However, as the CD25 molecule is the high affinity α-chain of the IL-2 receptor, it is not restricted to Treg, but is also expressed on activated T cells. However, at least in humans, a very high density of expression of CD25 distinguishes Tregs from other activated T cells with a lower expression of CD25. IL-2-deficient mice contain few $CD4^+CD25^+$ T cells and spontaneously develop severe autoimmunity (165). CD25-deficient or CD122 (IL-2Rβ-chain)-deficient mice also develop autoimmunity, which can be prevented by the transfer of $CD4^+CD25^+$ T cells from normal mice (166).

However, it has now been demonstrated that naturally arising $CD4^+$ Tregs are not limited to the $CD4^+CD25^+$ population, as $CD4^+CD25^-$ T cells with suppressive function *in vivo* and *in vitro* have been described in normal naive rodents: $CD4^+CD25^-CD45RB^{low}$ T cells from normal mice inhibited the development of IBD in SCID mice transferred with $CD4^+CD45RB^{high}$ T cells (167). In addition, most of the $CD4^+CD25^+$ T cells from normal mice lose

their expression of CD25 when transferred to SCID mice, but they still exhibit their suppressive activity both *in vivo* and *in vitro* (168).

Both the $CD25^+$ and $CD25^-$ Tregs express Cytotoxic T-Lymphocyte Antigen 4 (CD152, CTLA-4) which transmits an inhibitory signal to T cells when binding to CD80 and CD86 (also called B7) on APC. Both subsets also express Glucocorticoid-induced TNF Receptor-related protein (GITR), but neither GITR nor CTLA-4 are specific for Tregs (169). Since FoxP3, as an intracellular protein, cannot be used as a marker for sorting of live $CD4^+$ Tregs, CD25 has remained the most commonly used marker to obtain a relatively pure population of $CD4^+FoxP3^+$ Tregs enabling functional studies.

$CD4^+FoxP3^+$Tregs are anergic, but absolutely dependent on exogenous IL-2 for their generation and survival *in vitro* and *in vivo*. The anergic state is closely linked to suppression. Thus, if the anergic state is broken through *in vitro* TCR stimulation in the presence of high doses of IL-2 or anti-CD28 antibody, the suppressive activity is also lost (161, 162). They can then regain the suppressive and anergic characteristics once IL-2 or anti-CD28 is removed (170, 171).

The suppressive activities of anergic Treg are dependent on antigen dose. Thus, whereas incubation with a suboptimal antigen dose induces hyporesponsiveness, a higher (optimal) antigen dose induces an anergic state capable of exerting immunoregulatory effects. A supraoptimal antigen dose leads to an anergic and suppressive phenotype that is not reversed by APC, antigen, or IL-2 (171, 172).

Many studies have specified the role of FoxP3 in the development and function of Tregs (161, 162, 173). Retroviral transduction of FoxP3 can convert murine naive T cells into Tregs that phenotypically and functionally resemble naturally arising Tregs (161, 162), and FoxP3-transduced $CD4^+CD25^-$ T cells are able to suppress proliferation of other T cells *in vitro* and the development of autoimmune disease and IBD *in vivo* (173). FoxP3 transduction induced expression of CD25, CTLA-4, CD103, and GITR, all closely associated with the function of Tregs (173). It has also been shown that inoculation of Tregs prepared from normal mice can prevent autoimmune disease in FoxP3-defective mice (162). Finally, targeted disruption of the *Foxp3* gene prevents development of Tregs, and these mice develop a lethal inflammatory disease (162).

In contrast to observations *in vitro*, Tregs are capable of extensive proliferation *in vivo* (174). Proliferation is highly dependent on the presence of specific antigen. Antigen-specific $CD4^+CD25^+$ Tregs were found not to suppress the initial priming but to prevent subsequent clonal expansion of co-transferred $CD4^+$ effector cells (175).

Analysis of the half-life of polyclonal Tregs transferred into normal recipients suggests that the Treg population is heterogeneous; consisting of two populations: One is long-lived, persisting without dividing, while the other proliferates as a result of continuous stimulation, possibly in response to self-antigens in the periphery (176). The rapid turnover of a large proportion of Treg fits with the finding that both their development and maintenance is highly dependent on CD28 (177) and IL-2 (178).

Tregs utilize a variety of mechanisms to suppress the immune response. Generally, nTregs are believed to suppress by a cell–cell contact-dependent mechanism *in vitro* (possibly by granzyme or perforin-mediated cytotoxicity, or by cAMP), while their regulatory effects *in vivo* are assumed to be mainly cytokine-mediated (179, 180).

Tregs in the intestinal mucosa

$FoxP3^+$ Tregs are particularly abundant in the small intestine and colon, where they control potentially harmful responses to dietary and microbial stimuli (154). In addition to thymus-derived Tregs, the intestine is also a preferential site for TGF-β-dependent induction of $FoxP3^+$ Treg lymphocytes from naive $CD4^+$ T-cell precursors (154). These antigen-induced Tregs are expanded in models of oral tolerance and can control local and systemic antigen-induced hypersensitivity responses. Interestingly, Treg numbers in the colon are reduced in germ-free mice and can be increased by particular indigenous bacteria, suggesting a role for the microbiota in promoting intestinal Treg responses (181).

CD103 has now been demonstrated to identify the most potent subpopulation of mucosal Treg lymphocytes (182-185). The integrin $\alpha_E\beta_7$ (in which CD103 defines the integrin α_E chain) is expressed by more than 90% of intestinal intraepithelial lymphocytes and 40% of lamina propria T cells in the intestine. By binding to E-cadherin on epithelial cells, it contributes to mucosa-specific retention of T cells within epithelia. It has also been proposed that $\alpha_E\beta_7$ has an accessory function for activation of intraepithelial lymphocytes (186). Both the $CD25^+$ and $CD25^-CD103^+$ Treg subsets express CTLA-4, suppress T cell proliferation *in vitro*, and protect mice from colitis

in the SCID transfer model (153). However, they exert profound differences in their migratory behaviour, which could possibly explain their differences in regulatory capacity. Thus, whereas $CD25^+CD103^-$ Treg express the lymph node homing receptors CD62L and CCR7, their $CD103^+$ counterparts express E/P-selectin-binding ligands, and receptors for inflammatory chemokines, e.g. CCR5, allowing efficient migration into inflamed sites (149, 151). Also, the CD25 marker distinguishes two types of Treg populations. In general, $CD4^+CD25^+$ Tregs produce lower amounts of proinflammatory cytokines compared with non-regulatory $CD4^+CD25^-$ T cells. Although both populations expressed CD103, only the $CD25^-$ and not the $CD25^+$ subset, produced high amounts of IL-2, IFN-γ and Th2-cytokines such as IL-4, IL-5, and IL-13, and this production was linked with suppressive function (153). Thus, CD103 can be regarded as a useful marker for subsets of highly potent regulatory T cells, possibly specialized for crosstalk within the gut mucosa and especially the epithelium.

1.1.9 Th17–Treg Balance in IBD

An important role of Treg-mediated control of intestinal homeostasis is their ability to survive and compete with effector T cells in the intestinal niche (187). Differentiation of both Treg and Th17 requires TGF-β, which induces FoxP3 and RORγ, and their differentiation is reciprocally regulated in a highly dynamic manner depending on further signals (82,188). This mechanism has recently been shown to involve the expression of co-stimulatory pathways and transcription factors (189, 190): Mice with a *Stat3* deletion in $FoxP3^+$ Tregs develop aggressive colitis owing to uncontrolled Th17 responses (189). In addition to STAT3, Tregs can express several transcription factors associated with particular effector responses, including T-bet, IRF4 and GATA3 (187). A certain degree of plasticity does, however, exist between induced Tregs (iTregs) and Th17 cells. Thus, DCs conditioned in TGF-β-rich environments, as the gut mucosa, produce retinoic acid (RA), derived from dietary vitamin A, and present antigens and induce differentiation of naive $CD4^+$ T cells to $FoxP3^+$-induced Tregs (191-193). However, in the presence of pro-inflammatory stimuli, e.g. pathogenic microbes, DCs downregulate RA synthesis and instead start to produce IL-6, thereby inducing Th17 cell differentiation. Interleukin-6, together with IL-21 suppresses FoxP3 expression and up-regulates expression of the inducible component of the IL-23R and further Th17 cell development (66, 176-177, 194). In contrast, high concentrations of TGF-β in the absence of pro-inflammatory signals repress IL-23R expression (188). In addition, FoxP3 directly interacts with RORγt to inhibit its function, resulting in decreased

Il17 gene transcription, whereas IL-6, IL-21, and IL-23 decrease the FoxP3-mediated inhibition of RORγt (188).

Further with regard to plasticity, high-level T-bet (the transcription factor for Th1) expression in the presence of infection in the mucosa drives Treg into an inflammatory IFN-γ-secreting phenotype (195).

Altogether, it is possible that abnormalities in the pathways that determine the balance between induced Th17 or Treg development due to e.g., polymorphism in the *IL23R,* or the composition of the microbiota, may be involved in the pathogenesis of IBD. In another proposed model, $FoxP3^+$ Tregs are normal in IBD (196, 197), but the effector T cells are resistant to the effects of inhibitory cytokines such as TGF-β in the context of inflammation (198).

1.2 Treatment of IBD

A variety of drugs that target the inflammatory processes of IBD are usually effective in controlling active disease in most patients and in sustaining remission for prolonged periods. In general, a "step-up" therapy approach is used, in which more potent agents are added if less active drugs fail to achieve an adequate response.

The 5-aminosalicylate (5-ASA)–based compounds have remained as the main treatment for patients with mild to moderate UC and CD. 5-ASAs mechanisms of action include blocking of the production of prostaglandins and leukotrienes, inhibition of bacterial peptide–induced neutrophil chemotaxis, and the scavengeing of reactive oxygen metabolites (199).

Anti-TNF blockers or immunosuppressive agents such as Azathioprine and its active metabolite mercaptopurine or prednisone are used to relieve the symptoms of the disease and to prolong remission (199), despite an increased risk of lymphoma and other side effects (200). In general, side effects correlate with the dose and duration of treatment (201). Corticosteroids are commonly used when 5-ASA–based compounds are inadequate. They are mainly used to control acute inflammatory activity, as they have no proven maintenance benefit in the treatment of either UC or CD.

Clinical experience has led to the recognition that antibiotics are useful in the treatment of subgroups of patients with CD (202). Similarly probiotics, which manipulate the intestinal flora by administration of "healthy" bacteria, appear to be promising in some cases, but their mechanisms are far from clear. Thus,

patients with active CD, treated with a mixture of commensal bacteria, have been shown to respond positively (203).

However, so far no drugs which permanently lower the inflammatory pathology and keep the patient in remission have been identified. Severe cases may require surgery, including bowel resection, a temporary or permanent colostomy or ileostomy.

2 AIMS OF THE PRESENT STUDY:

The overall goal of this thesis was to elucidate the role of dysregulated adaptive immune responses in the intestinal mucosa in the etiology and pathogenesis of IBD, using the Gαi2$^{-/-}$ mouse spontaneous colitis model, and the DSS-induced colitis model. The specific aims were to:

- Explore the transcription and production of a selected panel of proinflammatory cytokines and chemokines involved in the Th1 and Th17 pathways at different time points before and after the onset of colitis in the Gαi2$^{-/-}$ model.

- Test the hypothesis that colitis in Gαi2$^{-/-}$ mice is a consequence of a qualitative or quantitative defect in CD4^{+}FoxP3^{+} regulatory T cells.

- Investigate new ways of assessing colitis and effects of treatment strategies, as well as comparing gene expression in the Gαi2$^{-/-}$ and DSS-induced models of colitis by analyzing the *in vivo* and *ex vivo* cultured colonic tissue gene expression profiles.

3 MATERIALS AND METHODS

A general description of Materials and Methods used in this thesis is given in this section. More detailed descriptions of protocols can be found in the Papers I-III.

3.1 Mice

3.1.1 Gαi2 deficient mice (Paper I-III)

Gαi2 deficient ($G\alpha i2^{-/-}$) mice on a pure 129SvEv background were bred as heterozygotes and maintained under specific pathogen-free conditions in micro-isolator cages with filtered air at the Department of Experimental Biomedicine, University of Gothenburg. Offspring were genotyped by Polymerase Chain Reaction (PCR) amplification of specific DNA sequences containing either the gene coding for Gαi2, or the gene for the neomycin cassette, inserted into both alleles to disrupt the Gαi2 gene, on purified tail genomic DNA. Homozygous $G\alpha i2^{-/-}$ mice on this background develop a lethal colitis between the ages of five and seven weeks.

Mice deficient in T and B cells (recombination-activating-gene 2, $RAG2^{-/-}$) on the 129SvEv background were used as homozygous recipients for adoptive T cell transfer studies. The phenotype of $RAG2^{-/-}$ mice was screened regularly for the presence of $CD3^+$ or $CD19^+$ lymphocytes in peripheral blood by flow cytometry.

All animal procedures were carried out under local and national ethical guidelines (Swedish Board of Agriculture) and were approved by the regional ethical committee, Gothenburg Administrative Court of Appeal.

3.1.2 Mice with DSS-induced colitis (Paper III)

Specific pathogen free female C57BL/6JOlaHsD mice (Harlan, the Netherlands), seven to nine weeks old, weighing 20–24 g, were housed in groups of six mice per cage and acclimatised for at least two weeks prior the study. Animals were kept in the animal house facilities at AstraZeneca R&D Mölndal under standard conditions of temperature and light, and were fed with standard laboratory chow and water *ad libitum*. In the *in vivo* study, a chronic coltic inflammation was induced by adding 3% dextran sodium sulphate (DSS) in the drinking water for five days, followed by one day of

tap water (204), while 5% DSS was applied for the same number of days in the *ex vivo* study.

3.1.3 Assessment of Gαi2$^{-/-}$ and DSS-induced colitis

Macroscopic scoring (Papers I-III)

The colon wall was macroscopically scored for the following parameters: thickness (0–2), stiffness (0–3), oedema (0–3) and visible ulcerations (0–1), with a total maximum score of 10. The same parameters were applied to both models.

Histological scoring (Papers I & III)

Because of the resemblance of the histopathology between Gαi2$^{-/-}$ colitis and the colitis seen in UC patients, a scale of 1-5, originally developed for grading the inflammation in UC patients (205), was used for the histological scoring in Gαi2$^{-/-}$ mice, where 1 = normal mucosa, 2 = mild inflammation (enhanced glands with intraepithelial granulocytes, influx of cells and/or eosinophis in the stroma), 3 = intermediate inflammation (goblet cell depletion, loss of tubular parallelism and reduced mucin seretion in some glands, marked influx of inflammatory cells in the stroma), 4 = severe inflammation (marked gland and mucosal atrophy, evident crypt abscesses and pus on the surface, massive influx of acute inflammatory cells and follicle formation in deeper cell layers), and 5 = fulminate inflammation (ulcerations with pus, gland and mucosal atrophy, crypt abscesses, extensive stromal inflammation and deep follicles).

The histopathology in the DSS model is characterized by loss of epithelial cells and infiltration of immune cells in the mucosa and submucosa (204), where 0 = no obvious pathology, 1 = few inflammatory cells (no signs of epithelial degeneration), 2 = mild inflammation (some signs of epithelial degeneration), 3 = moderate inflammation (some epithelial ulcerations), 4 = moderate to severe inflammation, 5 = moderate to severe inflammation (large ulceration of more than 50% of the tissue section plus extensive ulceration), 6 = severe inflammation (ulceration of more than 75% of the tissue section)

3.2 Preparation of cell suspensions (Paper II)

Single cell suspensions were prepared from whole thymus, spleen or mesenteric lymph nodes (MLN) by forcing the tissues through a nylon net using a syringe plunger. Splenic erythrocytes were lysed with ammonium chloride and cells were then washed in Phosphate buffered saline (PBS) containing 1% heat inactivated fetal calf serum (FCS). For isolation of colonic lamina propria lymphocytes (LPL), dissected colons were washed free of fecal contents by flushing with saline. They were then opened longitudinally, washed extensively in Hank's Balanced Salt Solution without Calcium and Magnesium (CMF-HBSS) supplemented with 15 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and incubated for 5 x15 min at 37º C, in CMF-HBSS containing 5 mM EDTA (Ethylenediaminetetraacetic acid) to remove epithelial cells and intraepithelial lymphocytes. The remaining tissue was incubated for 15 min in RPMI 1640 (bicarbonate buffering system) containing 15 mM HEPES and 10% heat inactivated FCS, followed by three successive 60 min incubations at 37º C in Liberase/blendzyme 3 in RPMI 1640 medium + GlutaMAX I containing 15 mM HEPES and 20% heat inactivated horse serum, yielding LPLs.

3.3 Live cell fractionation (Paper II)

In order to study cell function *in vivo* and *in vitro*, certain cell subpopulations were partially purified by negative selection on Auto Magnetic Activated Cell Sorter (AutoMACS). The pre- sorted cell populations were then labeled with fluorochrome-conjugated antibodies and sorted using a FACSAria® (BD Biosciences) flow cytometer.

3.4 Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) and gene arrays (Papers I & III)

In paper I, the thermal cycler TaqMan 7500 Fast Real-Time PCR System with 7500 Fast Sequence Detection and Relative Quantification software packages were employed to detect and quantify a chosen panel of genes. PCR cycling conditions were: Step one; 95º C for 2 min, step two; 95º C for 3 s and 60º C for 30 s. The second step was repeated 40 times. Combined primers and probes were purchased from Applied Biosystems (Sweden):

Il12a p35 (Mm01208555), Gapdh (Mm99999915), Actb(Mm01205647_g1), Gusb (Mm01197698_m1), Foxp3 (Mm01351178_g1), Rorc (Mm01261022_m1), Aire (Mm00477461_m1), Il23a (Mm00518984), Il21 (Mm00517640), Il27 (Mm00461164), Ifng (Mm01168134) and Il17a (Mm00439618).

In paper III, TaqMan™ Low-Density mouse immune-arrays were used to assess the expression of immune-related genes in tissues and organ cultures. The gene arrays are micro fluidic cards designed for analyzing gene expression patterns in many samples across a defined set of gene targets. Samples were assayed in triplicates or quadruplicates and the expression levels were determined with an ABI Prism 7900HT Sequence Detector (Applied Biosystems). Quantitative RT-PCR (Taqman 7500) was employed to detect further altered expression of chosen genes.

3.5 Protein extraction and analysis (Papers I & II)

In paper I, the protein expression of IL-1β, TNF-α, IL-6, IL-12p40, IL-17, CXCL1 (KC/GROα), CCL2 (MCP-1) and CCL5 (RANTES) was analyzed using the xMAP technology developed by Luminex (Austin, TX, USA). The technology is built on color-coded microspheresin 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. In this way, xMAP technology allows multiplexing of chosen assays within a single sample. The concentrations were determined using Bioplex Multiplex Suspension Array System kits (Bio-Rad Laboratories, Hercules, CA).

In paper II, cytokine concentrations of IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A and IL-10 in supernatants from the suppression assays were measured using a cytometric bead array (CBA) kit, mouse Th1/Th2/Th17 cytokine kit (BD Pharmingen). The CBA assay provides a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytomery in a single sample. Each capture bead in the kit has been conjugated with a specific antibody. The detection reagent comprised a mixture of phycoerythrin (PE)-conjugated antibodies, which provide a fluorescent signal in proportion to the amount of

bound analyte. Cytokine levels were quantified using calibrated standards of each cytokine and analyzed by the CBA software.

3.6 Statistical analysis

As most of the data obtained from our analyses were not normally distributed, data are shown from individual mice or as median values, and the unpaired two-way *t*-test or Mann-Whitney non-parametrical test for unmatched data were used for comparison of data. One-way ANOVA (Tukey's Multiple Comparison Test) was used for comparison between groups. Differences were considered statistically significant at $p < 0.05$.

In paper I, correlation patterns for the relative values of cytokines and chemokines and the histological grading of colitis were carried out by constrained or "canonical" correspondence analysis (CCA) and are presented as ordination plots using R package vegan. CCA can be used as a multivariate method to elucidate the relationships between biological elements. The method is designed to extract synthetic gradients from the data-sets for relating two set of variables.

4 RESULTS AND DISCUSSION

4.1 Longitudinal analysis of Gαi2$^{-/-}$ colitis (Paper I)

Colonic inflammation in the Gαi2$^{-/-}$ model arises spontaneously, probably as a result of multiple immunologic and physiologic changes resulting from the G protein deletion. Although it has been noted previously that colitis in this model increases in severity with age, immune parameters which may be associated with disease induction and progression have never been followed through longitudinal analysis. Such an analysis may provide important information regarding immune aetiology, chronicity and targets for therapy

Previous studies in the group have demonstrated that well-established, severe Gαi2$^{-/-}$ colitis is characterized by a colonic pro-inflammatory Th1 cytokine profile (3, 7) with accompanying high levels of IL-17 transcription (206). However, as the colitis arises from a genetic defect, it is likely that the inflammatory pathology in the colon is progressive and that the mixed Th1/Th17 effector T cell response observed in late-stage disease is not informative of pathogenesis. Accordingly, this study was designed to examine proinflammatory cytokine and chemokine transcription and translation in colonic and other tissues at all stages during the progression of colitis.

Onset of colitis was defined as the time of first appearance of diarrhoea. Mice without diarrhoea were categorised as having no colitis, or mild colitis; mice with diarrhoea but without any other clinical signs of colitis were categorised as moderately colitic; and mice with body weight loss, diarrhoea, piloerection and lethargy were categorised as severely colitic.

In Paper I, Fig 1, colitis was graded histopathologically, blind, using a scale of 1-5 originally developed for grading of inflammation in UC patients (207), using the following grading: 1 = normal mucosa; 2 = mild inflammation (enhanced glands with intraepithelial granulocytes, influx of cells and/or eosinophils in the stroma); 3 = intermediate inflammation (goblet cell depletion, loss of tubular parallelism and reduced mucin seretion in some glands, marked influx of inflammatory cells in the stroma); 4 = severe inflammation (marked gland and mucosal atrophy, evident crypt abscesses and pus on the surface, massive influx of acute inflammatory cells and follicle formation in deeper cell layers); and 5 = fulminate inflammation

(ulcerations with pus, gland and mucosal atrophy, crypt abscesses, extensive stromal inflammation and deep follicles). Mice were further separated into three groups based on these gradings: 1-2, with no apparent disease, or very mild disease; 3, in which the entire lamina propria was infiltrated with large numbers of lymphocytes, expanding this compartment; and 4-5, severe disease, which was typified by a massive increase also in granulocytes, not seen in score 3.

The data showed that macroscopic observations correlated with histological score, during the development of colitis (Fig 1A, Paper I). Colon weight per cm, spleen weight and thymus weight were also recorded (Fig 1, Paper I). The inflamed colon of colitic Gαi2$^{-/-}$ mice becomes progressively enlarged due to increasing oedema and infiltration by inflammatory cells (Fig 1A, Paper I). The increased colon weight is presumably associated with oedema, alongside disease progression. At the onset of infection, or barrier destruction, inflammatory cell infiltration, their activation and secretion of mediators will result in vasodilatation with increased blood flow and increased permeability of the blood vessels resulting in an exudation (leakage) of plasma proteins and fluid into the tissue. These vascular changes, together with release of mediator molecules, proinflammatory cytokines and chemokines, permit the migration of leukocytes - initially neutrophils, followed by monocytes and lymphocytes - into the tissue (extravasation). This process of acute inflammation is initiated by resident cells, mainly resident macrophages, dendritic cells, mast cells and activated epithelial cells. This acute response will ultimately drive the adaptive immune system, priming effector T and B cells, which will proliferate at the site of the inflammation.

In agreement with the histological scores (Fig 1A, Paper I), distorted crypt architecture and the presence of crypt abscesses increased significantly during disease progression, as a result of attempted repair of the epithelial barrier and neutrophil influx. Colonic dysplasia or colon cancer was only observed in mice with severe colitis.

Colon weight in mice with severe colitis was more than 4 x that of WT controls and 60% greater than that of mice with no colitis or mild colitis. Interestingly, already in the mice with no colitis or mild colitis, there was a small but detectable increase in colon weight compared to WT littermates. Thymic involution was likewise evident already in mice with no colitis or mild colitis compared to WT mice and became more pronounced as the colitis developed further (Fig 1B, Paper I). In mice with severe colitis, thymi were extremely small and sometimes difficult to detect, in line with our

previous findings (15). The spleen weight was not significantly altered in mice with no colitis or mild colitis compared to WT mice, but was increased in mice with moderate and severe colitis (Fig 1C, Paper I). These results indicate that some, but not all, tissue indicators of inflammation were raised in Gαi2$^{-/-}$ mice, compared to WT mice, even before clinical signs of inflammation.

As a result of the observations in clinical, macroscopic and histological parameters, the levels of transcription and translation of key proinflammatory cytokines were assessed to provide mechanistic links to the clinical and macroscopic data. Importantly, levels of innate and adaptive inflammatory cytokines were elevated even in Gαi2$^{-/-}$ mice with no clinical signs of disease.

In Fig 2, Paper I, colonic protein levels of IL-1β, IL-6, IL-17, IL-12p40, TNF-α, CXCL1 and CCL2, and CCL5, were measured. For all cytokines/chemokines, except CCL5, there was an increasing trend with disease progression. In all cases, significantly more cytokine was present in moderately and severely diseased tissue than in WT tissue. In the case of IL-12p40 and TNF-α, higher levels of significance were achieved in severely diseased tissue over moderately diseased tissue, suggesting a progression with disease severity. In the case of TNF-α, Gαi2$^{-/-}$ mice with no clinical disease showed higher levels in colonic tissue than WT mice, but this was not apparent for the other cytokines. However, these data may be skewed by a single mouse (of four) which, although categorised as having no colitis or low level colitis, consistently showed the highest levels of all cytokines in this group. Subsequent back-analysis of the tissue from this mouse confirmed minimal histopathology. It is not clear from these data, therefore, whether proinflammatory cytokines/chemokines are already raised as a consequence of the Gαi2$^{-/-}$ genotype, prior to the onset of disease, and therefore factors involved in the *initiation* of disease, or whether all of these mediators gradually increase as a *consequence* of disease. Greater numbers of mice within this group with no pathology, or minimal pathology, would be required to resolve this.

With regard to function, elevated levels of IL-1β and TNF-α elicit the onset of the inflammation. Both are involved in regulating barrier permeability through their effects on tight junctions (6,7). CXCL1 is a known chemoattractant for neutrophils to the site of inflammation and, not surprisingly, increased gradually with the progression of colitis. In fact, the neutrophil granulocyte is one of the most prominent infiltrating leukocytes in

this model and has been suggested to contribute significantly to the development of tissue injury and inflammation in human IBD (208).

Levels of IL-6 in Gαi2$^{-/-}$ colon displayed a progressively high level from the moderate colitis stage to the severe colitis stage, indicating an influx and activation of monocytes. Transition from innate to adaptive immunity, critical for host defense, requires functional immune regulation. This transition is characterized by an initial influx of neutrophils, which is subsequently replaced by inflammatory monocytes and T cells (209). Studies have shown that IL-6 is a crucial regulator of neutrophil trafficking during the inflammatory response by orchestrating chemokine production and leukocyte apoptosis (210, 211). IL-6 has also been reported to protect neutrophils from apoptosis in osteomyelitis (212). During the transition, IL-6 and its soluble receptor (sIL-6R) contribute to the suppression of neutrophil recruitment and the concurrent attraction of mononuclear leukocytes (213). Thus, inappropriate regulation of this neutrophil trafficking can lead to impaired neutrophil clearance and increased tissue damage from the accumulation of neutrophil-secreted proteases and reactive oxygen species at the site of inflammation (214). Increases in IL-6 correlated with high levels of CCL2, a monocyte chemotactic protein, indicating concurrent neutrophil recruitment and promotion of monocytes to the site of inflammation (210). These cytokine levels correspond to the histology at the severe stage of colitis in Gαi2$^{-/-}$ mice, showing crypt abscesses and massive influx of acute inflammatory cells.

In contrast, CCL5 levels in colonic tissue from Gαi2$^{-/-}$ mice showed no significant difference from WT mice. As CCL5 is a chemoattractant for memory and activated T cells, the lack of upregulation might suggest a clonal expansion of T cells locally at the site of inflammations rather than recruitment of peripheral T cells to the inflamed mucosa. Such an interpretation is consistent with the reported high expression of regulator of G protein signaling-1 (RGS1) proteins in the gut mucosa, regulating T cell chemotaxis, which is correlated with reduced T cell migration to mucosal-homing chemokines (215). In accordance, it has been reported that effector T cells with the Gαi2$^{-/-}$ defect display a hyperproliferative response to the TCR/CD3 stimulation (216). Thus, the histological findings in Gαi2$^{-/-}$ mice with moderate colitis, in which high level lymphocyte infiltration into the lamina propria was seen compared to mice with no colitis or mild colitis, may result from in situ proliferation of effector T cells.

Furthermore, IL-1β together with IL-6 and TGF-β increase the frequency of IL-17-producing effector cells. (85), which could explain the high IL-17 protein levels at all stages of the Gαi2$^{-/-}$ colitis. As our group has demonstrated that aberrant T cell responses of Gαi2$^{-/-}$ lead to a typical Th1-skewed CD4^{+} immune response (9), this new discovery of high IL-17 protein secretion throughout disease progression may shed a new light on the established Th1 response in the Gαi2$^{-/-}$ colitis model. As discussed in the Introduction, increased production of Th1 cytokines has been a feature of active IBD (217, 218), but the discovery of the Th17 lineage has led to a re-evaluation of IBD pathogenesis. A number of inflammatory conditions in which Th1 was previously considered essential may actually be Th17-dependent. Th17 cells are driven by IL-23 (as well as IL-6 and TGF-β) and are characterized by IL-17 production (96). IL-12 (consisting of a p35 and p40 heterodimer) drives Th1, while IL-23 (consisting of a p19 and p40 heterodimer) supports Th17 pathways (96). As a member of both the IL-23 and IL-12 families, p40 has a reciprocal position in both the Th1 and Th17 pathways. The protein results show that p40 significantly increased in mice with moderate colitis and that correlated with a significant rise in IL-17 protein. However, the contribution of this p40 protein to Th1 or Th17 effector cells is somewhat clarified by consideration of the mRNA data in Fig 5, Paper I. Thus, IL-17 transcripts were significantly greater in Gαi2$^{-/-}$ mice than WT mice, regardless of colon pathology, whereas IL-12p35 transcript levels did not reach significance over WT mice until late-stage disease and IFN-γ transcripts showed a gradual increase with pathology. IL-23 transcript levels were greater in Gαi2$^{-/-}$ mice than WT, significantly so at the medium pathology level. It may be proposed from these combined data that IL-17 is elevated *a priori* as a result of the Gαi2 deletion, whereas the Th1-driven IFN-γ, which has been noted in our previous studies, arises as a consequence of the colonic tissues being primed for inflammation, perhaps in part mediated by IL-17. Although the IL-23 mRNA data support its involvement in the IL-17 response, confirmation of this requires measurement of IL-23 protein.

Interleukin-21, also a Th17 pathway cytokine – although with many other functions (219) - was found to be significantly increased in the colon of Gαi2$^{-/-}$ mice with severe colitis (Fig 5, Paper I). IL-21 has been reported to contribute to gut damage by enhancing the secretion of matrix metalloproteinases (MMPs), a family of neutral endopeptidases that can cleave multiple components of the extracellular matrix secreted by lamina propria myofibroblasts and fibroblasts during homeostasis, inflammation, and wound healing (122). With regard to effector T cell biasing, IL-21 is expressed by Th17 cells and serves as an autocrine factor, sustaining Th17

lineage commitment (220). Thus, in this study, the increased levels of IL-21 - and IL-23p19 - transcription in Gαi2$^{-/-}$ mice, compared to WT mice, both support a possible role in Gαi2 modulation of colonic IL-17 levels as a possible precursor to colitis (see above). Additionally, IL-21 has been shown to oppose the differentiation of Treg and enhance the resistance of target effector T cells to Treg suppression (221) , relevant to the studies described in Paper 2 (below).

It has been shown in human tissue that a significant proportion of Th17 cells also secrete IFN-γ, thus displaying a mixed Th17/Th1 cytokine phenotype, particularly in the gut (115). Previous data from the group, together with the data in this thesis, support the hypothesis that, as in human IBD, late-stage colitis in Gαi2$^{-/-}$ is of a mixed Th1/Th17 phenotype and, further, that this is preceded by a dysregulated Gαi2$^{-/-}$-driven Th17 effector T cell response.

IL-27, a member of the IL-12 family, also plays an important role as a negative regulator of Th17 cell development. As shown in Fig 5, Paper I, we found a progressive up regulation of IL-27 in the colon of Gαi2$^{-/-}$ mice with increasing severity of colitis. IL-27 is a heterodimeric cytokine composed of an EBI3 (Epstein-Barr virus–induced gene 3) and a p28 chain. Several studies have shown that activation of T cells in the presence of IL-27 induces T-bet, a transcription factor critical for the differentiation of naive CD4^{+} cells into Th1 cells (222). Thus, enhanced transcription of IL-27 is also consistent with our hypothesis of initial Th17 inflammation driving an eventual Th17/Th1 colitis in the model.

In summary, the data in Paper I are consistent with the deletion in Gαi2 causing an expansion of Th17 responses – perhaps resulting from a Gαi2$^{-/-}$-driven barrier defect – followed in turn by expansion of Th1 effector T cells, perhaps mediated by attempted restitution of the barrier defect by IL-27 (223).

4.2 Immune regulation in the Gαi2$^{-/-}$ colon (Paper II)

In an immunologically intact individual, inflammation is normally tightly regulated such that, even if the adaptive immune response becomes involved, a return to tissue homeostasis is achieved. However, in this model – as in human IBD – the gut inflammation is chronic, progressive, and does not return to normal. The possibility of dysregulation of regulatory T cell (Tregs) function prompted the analysis of these mechanisms in the model.

Inflammation is regulated at many checkpoints. However, because the colitis seen in the Gαi2$^{-/-}$ model mimics human CD by presenting a mixed Th17/Th1 effector T cell pathology, an obvious candidate for defective regulation is the Tregs which would normally modulate these effector T cell functions. This leads to the next hypothesis which was addressed in this work: That regulatory T cell function is defective in the Gαi2$^{-/-}$ mouse model.

The aim of this part of the study, therefore, was to determine whether the presence of colitis in the Gαi2 deficient mouse model correlated with changes in frequency, distribution, and/or function of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells.

Firstly, a possible numerical defect in regulatory T cells was analyzed. As a measure of natural regulatory T cells (nTregs) production, levels of FoxP3$^+$ cells in the thymus were measured, and frequencies of CD4$^+$ FoxP3$^+$ cells in peripheral tissues, including the colon, were also measured.

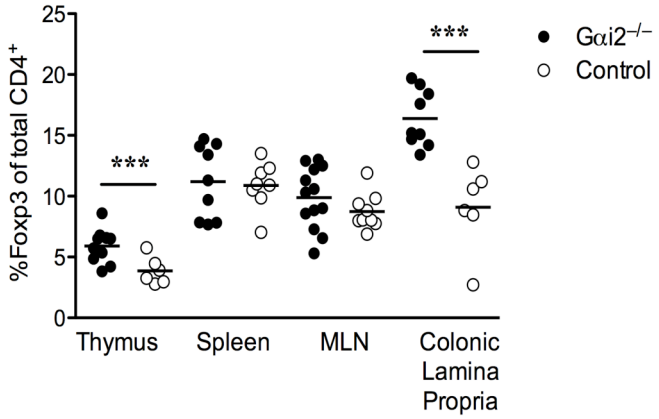


Figure 1. *Frequencies of regulatory T cells in the thymus and peripheral lymphoid tissues in the Gαi2$^{-/-}$ model of colitis. doi:10.1371/journal.pone.0025073.g001*

Frequencies of CD4$^+$FoxP3$^+$ T cells in the thymus and colonic lamina propria of the Gαi2$^{-/-}$ model were significantly enhanced, but were not different from WT mice in spleen and mesenteric lymph nodes (MLN) (Fig 1). The increased frequency of FoxP3$^+$ cells in the inflamed colon correlates with studies in human UC patients, in which Tregs are increased in the lamina propria of the inflamed mucosa (224).

While FoxP3$^+$ frequency in the colon is a useful indicator, suggesting that simple, numerical loss of regulators does not explain the lack of regulation, it does not provide information regarding the co-localization of these regulators with effector cells in the colonic inflammation. The data were derived from total cells isolated from colonic tissue. However, T cell distribution in the colon is not uniform, but partitioned between the lamina propria and organised lymphoid structures. These include: colonic lymphoid patches (CLPs), clusters of lymphoid follicles in the colon containing germinal centers and defined B cell and T cell/DC areas (225); isolated lymphoid follicles (ILFs), single B-cell follicles with associated small numbers of T lymphocytes (226); and cryptopatches (CP), small follicular structures comprising lymphoid tissue–inducer cells (LTi cells) surrounded by DC (74). In contrast to mice, CPs are not found in humans, whereas ILFs and lymphocyte aggregates within the muscularis mucosa have been described (227).

Distribution of T cells between these distinct colonic tissues is not equal and in the Rag$^{-/-}$ transfer model of colitis (74) , transferred T cells were found to accumulate and proliferate in subepithelial dendritic cell (DC) aggregates located in regions of the colonic lamina propria, where inflammation later develops (228). Further, FoxP3-expressing cells were found in DC aggregates in the colon of Rag$^{-/-}$ recipients, whereas few were seen in the lamina propria, two weeks post-transfer. At four weeks post-transfer, FoxP3$^+$ cells were present at low density in DC aggregates in the severely inflamed gut.

However, the distribution of T cells in the colon and the site frequency of Tregs have not been analysed in the Gαi2$^{-/-}$ mouse model, nor have changes within these lymphoid compartments during colonic inflammation.

Thus, an immunohistochemical study of CD4$^+$FoxP3$^+$ expressing cells in colon was performed.

Few CD4$^+$FoxP3$^+$ cells in WT controls were found in the lamina propria, whereas the majority of CD4$^+$FoxP3$^+$ T cells were localized in the T cell zone of CLP. In Gαi2$^{-/-}$ mice with colitis, CLPs and ILFs were not present, consistent with earlier findings from our group of regression of Peyer´s patches in Gαi2$^{-/-}$ mice (229). We observed that CD4$^+$ T cells were scarce in the lamina propria of WT mice, mostly restricted in the crypt bases, in which few FoxP3$^+$ cells were found (1-4%, Fig 2A-B, K-L). In CLPs of WT mice, large numbers of CD4$^+$FoxP3$^+$ Tregs were observed within the interfollicular T cell areas (Fig 6E-F), comprising 10-15 % of the total CD4$^+$ T cells (Fig

2L). The distribution was quite different in colitic Gαi2$^{-/-}$ mice. No discrete CLP with associated clustered CD4^{+}FoxP3^{+} Treg were observed in the inflamed colon. Moreover, a heavy infiltration of CD4^{+} cells was observed in the colonic lamina propria, particularly above the level of crypt bases – a 10x increase compared to the equivalent WT tissues (Fig 2A-J, K). High FoxP3 expression was found among these CD4^{+} infiltrating cells (Fig 2K), with a 10-20x increase in the number of CD4^{+}FoxP3^{+} cells in the basal and upper mucosa, respectively. These data correlated well with the flow-cytometric analysis, as the CD4^{+}FoxP3^{+} frequency in the upper lamina propria was increased 2-3x compared to CD4^{+} cells in WT colon (Fig 2L). Thus, the hyperplastic mucosa in established colitis was heavily infiltrated by a ten-fold increase in CD4^{+} T cells, and the proportion of FoxP3^{+} cells rose by two- to three-fold. This may result from the loss of CLP and ILF, which in the WT colon contain the major proportion of Tregs.

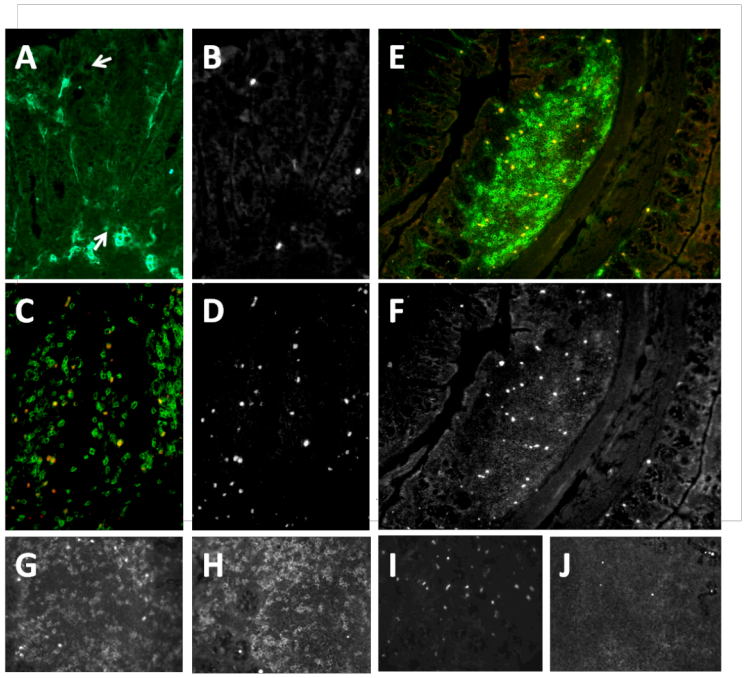


Figure 2. *Regulatory T cells in the normal mouse colon and in colitis. A, B, E, F = WT mice; C, D = Gαi2$^{-/-}$ mice with colitis. Tissues were double stained for CD4 (green) and FoxP3 (red). In WT colonic mucosa CD4^{+} T cells were few and mostly confined to the basal lamina propria. In colitis CD4^{+} T cells infiltrated the entire lamina propria and included many double positive (DP) cells. Within CLP of WT mice (E, F), interfollicular T cell areas contained many DP CD4^{+}FoxP3^{+} cells. G-J, controls. doi:10.1371/journal.pone.0025073.g002*

These data show that the overwhelming majority of colonic FoxP3$^+$ Tregs in WT mice are associated with organized CLPs and ILFs structures, possible sites for the accumulation and/or regeneration of Tregs. With the loss of CLPs and ILFs in inflamed Gαi2$^{-/-}$ colons, the numbers of lamina propria CD4$^+$FoxP3$^+$ cells were significantly increased in inflammation and dispersed in the tissue. It is possible that the higher frequency of FoxP3$^+$ cells in the colonic lamina propria of colitic Gαi2$^{-/-}$ mice is a consequence of local ongoing inflammation, as there was no significant difference in the frequency of CD4$^+$FoxP3$^+$ T cells at peripheral sites, such as spleen and MLN, compared to WT mice.

These findings are supported by the reports from human disease - in patients with active IBD, the number of FoxP3$^+$ cells in the affected intestinal mucosa correlates with disease activity (196, 230).

The data indicate that whereas colitis occurs despite apparently normal frequencies of Treg, organised lymphoid tissue in the colon disappears in colitis, so sampling was not biased by selective isolation. Thus, the next hypothesis was that there are normal frequencies of Treg, but that their functional capacity is diminished.

To understand the role of CD4$^+$ Treg in the Gαi2$^{-/-}$ mouse model of colitis, freshly isolated and sorted splenic CD4$^+$CD25$^+$ cells regulatory T cell (Treg) and CD4$^+$CD25$^-$ effector T cell (Teff) subpopulations, at the ratio of 4:1 or Teff alone, from colitic Gαi2$^{-/-}$ and control (Gαi2$^{+/-}$) mice were adoptively transferred into RAG2$^{-/-}$ mice by i.p. injection. Five to seven weeks after T cell transfer, mice receiving WT-Teff cells did not develop disease, regardless of the presence or absence of Tregs from either donor.

However, mice receiving effector cells from colitic Gαi2$^{-/-}$ (KO-eff) developed progressive diarrhoea, rectal bleeding and other clinical signs of disease, irrespective of either co-transfer of Tregs from Gαi2$^{-/-}$ mice (KO-Treg), or Tregs from WT control mice (WT-Treg). Thus, Tregs from Gαi2$^{-/-}$, but also WT mice, are apparently unable to exert sufficient regulation of the expanding pathogenic effector T cells *in vivo*. Apparently, the KO-Teff cells were highly pathogenic and the pathology induced was unaffected by co-transfer of Treg from either WT or Gαi2$^{-/-}$ mice.

The hypothesis that Treg from Gαi2$^{-/-}$ mice are functional, but that effector T cells from these mice are less easily regulated by Treg was further addressed using an *in vitro* co-culture system and flow cytometric analysis of cell division.

A model suppression assay was constructed with mixed combinations of Tregs and Teff from Gαi2$^{-/-}$ mice and WT (Gαi2$^{+/-}$) control mice at different ratios. Regulation of these effector responses were analyzed in Teff:Treg co-cultures by cell cycle analysis, using CFSE labeling (Fig 3).

It is apparent from the cell division data (Fig 3A, B, D) that both sets of regulators were able to suppress proliferation of both sets of effectors, with WT-Teff being more readily suppressed (even at 8:1) than KO-Teff (Fig 3A-B) (suppressed less readily with regulator dilution, at 2:1).

Furthermore, there was a clear difference in the rate of cell division in KO-Teff versus WT-Teff, with significantly more Teff from Gαi2$^{-/-}$ mice entering cell cycle in response to a-CD3 stimulation, compared to Teff from WT mice (Fig 3C). Therefore, KO-Teff displayed a hyper-responsiveness to TCR stimulation compared to WT-Teff and were apparently less easily regulated compared to WT-Teff. Thus, the data showed that the *in vitro* suppressive function of WT and Gαi2$^{-/-}$ Tregs was indistinguishable.

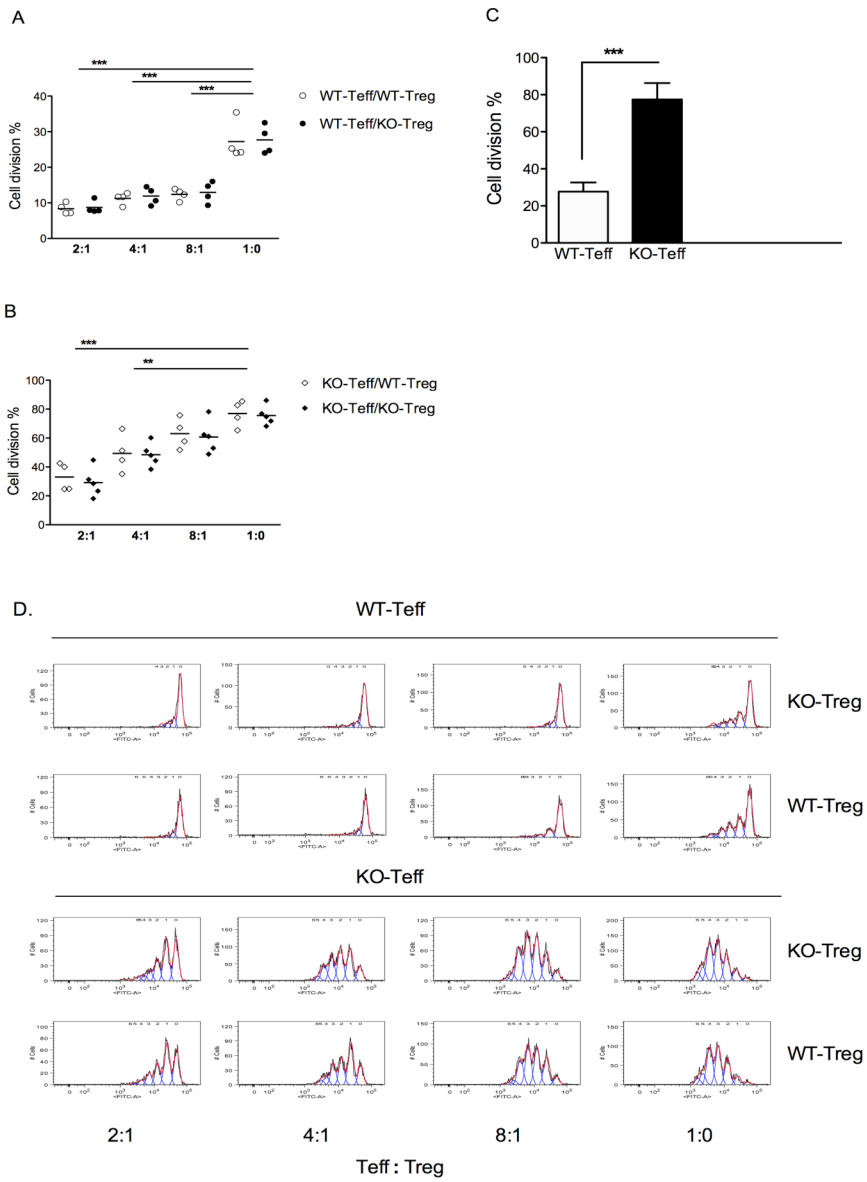


Figure 3. *Sorted splenic CD4^{+}CD25^{-} Teff pre-stained with CFSE and CD4^{+}CD25^{+} Treg from colitic Gαi2$^{-/-}$ mice and WT littermates were mixed at various ratios and in different combinations. A-B. The mixtures of cell populations were co-cultured with irradiated T cell depleted splenocytes from WT mice as accessory cells and soluble α-CD3 mAb for 72 hours. C. Means ± SEM of cell divisions of CFSE WT-Teff and KO-Teff from the wells with Teff only. D. Representative histograms showing the division of CFSE labeled cells in the absence or presence of KO- or WT-Treg. doi:10.1371/journal.pone.0025073.g006*

In conclusion, $CD4^+$ T cells were distributed throughout the inflammatory infiltrate with no evidence of organised, follicular structures in the colon of $G\alpha i2^{-/-}$ colitic mice, and $CD4^+Foxp3^+$ cells in these mice were distributed randomly among the $CD4^+$ population in the crypts and lamina propria. However, neither WT nor $G\alpha i2^{-/-}$ $FoxP3^+$ Treg can downregulate chronic $G\alpha i2^{-/-}$ T cell-driven inflammation. This is likely due to $G\alpha i2^{-/-}$ T effector cells being dramatically less susceptible to suppression, with Treg demonstrating no endogenous functional defect.

4.3 Ex vivo cultures for assessment of treatment strategies of IBD – comparisons between $G\alpha i2^{-/-}$ and DSS-induced colitis (paper III)

Although the regulatory system of $G\alpha i2^{-/-}$ colitis is functionally intact, it is unable to overcome the pathogenic mixed Th17/Th1effector T cell response in the colon. The next questions raised were therefore: Can the $G\alpha i2^{-/-}$ colitis be affected by anti-inflammatory treatment *in vivo,* and if so, how does this compare to the well characterized DSS-induced colitis model, and can these treatment effects be mirrored in *ex vivo* cultures?

Ulcerative colitis (UC) is characterized by chronic uncontrolled inflammation of colonic mucosa, with relapsing and remitting phases. As an agent of induced colitis in mice, dextran sulphate sodium (DSS) has been widely used to study the mechanisms of colonic inflammation and to evaluate the effect of any candidate drug. As corticosteroids are widely used as a primary treatment for acute flares in IBD, the well-characterised corticosteroid methyl-prednisolone was used. In addition, a member of a novel group of anti-inflammatory agents functioning by blocking proteasomes assessed in a number of colitis models (231-234), the proteasome inhibitor MG132, were used to compare the efficacy between *in vivo* treatment and *ex vivo* cultures of colon obtained from DSS-induced and $G\alpha i2^{-/-}$ colitic mice.

Firstly, the *in vivo* dose-dependent effect of methyl-prednisolone treatment in the DSS model was investigated based on clinical symptoms and on local inflammatory markers, whereas the highest dose only was used in $G\alpha i2^{-/-}$ colitic mice, due to lower availability of these mice.

Not surprisingly, the weight of the spleen and thymus, organs known to be affected by steroids, was significantly reduced in DSS-induced colitis. In

contrast to the findings in the DSS model, body weight loss and iNOS mRNA expression in Gαi2$^{-/-}$ mice were not significantly altered by the treatment. However, the colitis–associated increased weight of the colons, as well as colonic mRNA expression of IL-1β and IL-6 were significantly reduced with methyl-prednisolone treatment in both DSS-induced and Gαi2$^{-/-}$ colitis (Fig 4). In contrast, colon length, colonic inflammatory score, nor histopathological changes were significantly affected by methyl-prednisolone in either colitis model.

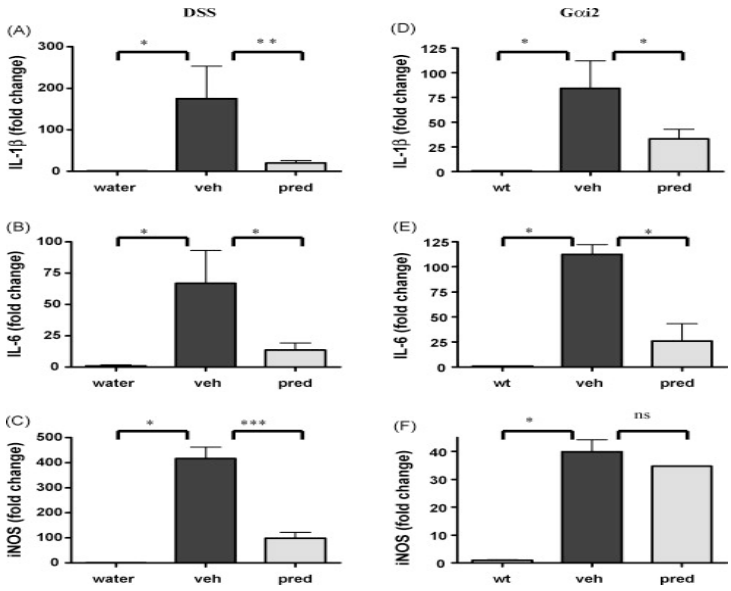


Figure 4. *mRNA expression of IL-1β (A, D), IL-6 (B, E) and iNOS (C, F) in inflamed colon obtained from mice treated per-orally with methyl-prednisolone. (A–C) DSS-induced colitic mice, (D–F) Gαi2$^{-/-}$ colitic mice. Pharmacol Res. 2008;58:222–231.*

Whereas only female mice were used in the DSS-induced colitis, Gαi2$^{-/-}$ mice of both sexes were used. To our surprise, only female mice responded to methyl-prednisolone treatment. The reason for this is still unresolved, but highlights the importance of taking gender differences into account when evaluating new therapies.

We next compared the *in vivo* colonic transcriptional profile to that obtained in *ex vivo* cultured tissue of mice with DSS-induced colitis. In general, the same sets of genes displayed increased transcription in the *in vivo* and *ex vivo* cultured tissues, although the magnitude of the enhancement was higher in *in vivo*. The mRNAs most highly up-regulated in *in vivo* were those encoding

IFN-γ, IL-1β, IL-17, CXCL10, CCL2 and iNOS, and in *ex vivo* cultured tissue IL-17 and CCL19 (Fig 5).

This also allowed us to compare the inflammatory gene profile of DSS-induced and Gαi2$^{-/-}$ cultured colitic tissue: Both models demonstrated enhanced expression of cytokines such as IL-1α, IL-6, IL-10, p35 and IL-17, chemokines attracting T cells and monocytes/macrophages (CCL3 and CCL5) and chemokine receptors (CCR7 and CXCR3), B and T lymphocyte surface markers and co-stimulatory molecules (CD19, CD28, CD80, CD86, CTLA-4, GITR, ICOS and Fas) as well as mediators of oxidative stress (iNOS and COX-2).

Nevertheless, profound differences were also recorded, where genes related to pro-inflammatory innate cytokines, chemokines and chemokine receptors were preferentially up-regulated in DSS-induced colitis (e.g. G-CSF, GM-CSF, CD62E, CCL2, CCL19, CCR2, CXCL10 and CXCL11), whereas genes related to T cell markers were preferentially elevated in Gαi2$^{-/-}$ colitic tissues (e.g. CD3, CD4, CD8α, CD40L, CD45, T-bet, MHC class II, IL-1β, IFN-γ, TGF-β, TNF-α, granzyme B and perforin). Among the genes up-regulated >100-fold, IL-17 was up-regulated in both models, while IFN-γ, CCL3, IL-2Rα, CD3 and CD4 were only up-regulated in the Gαi2$^{-/-}$ mice (Fig 5, Table 3, paper III). These differences likely reflect the different stages of colitis in the two models: The inflammatory process in the Gαi2$^{-/-}$ mice is likely to have started several weeks before symptoms of colitis are evident, as immune activation has been observed in precolitic Gαi2$^{-/-}$ mice (5). In comparison, the inflammatory response evaluated at day 6 in mice with DSS-induced colitis rather reflects an acute inflammation (204).

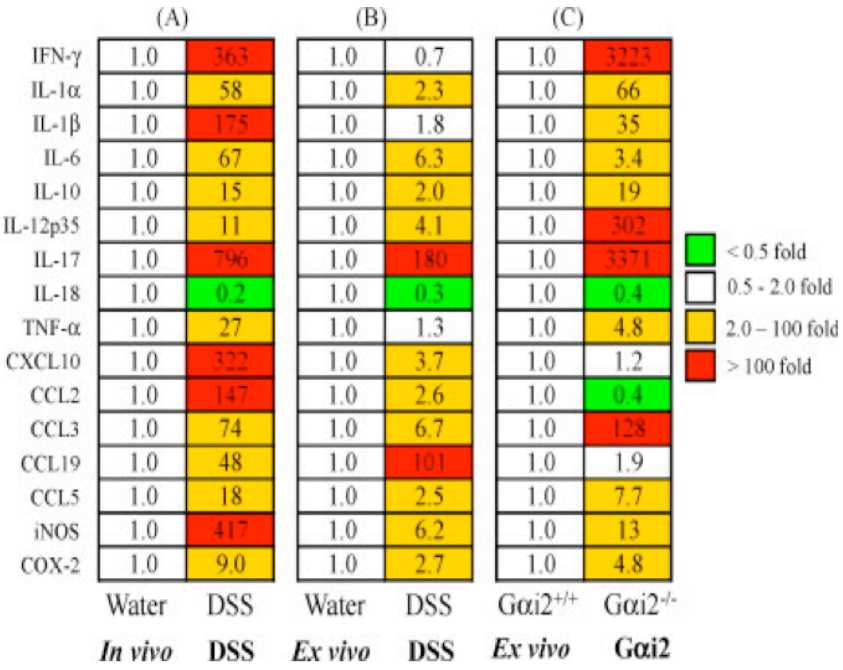


Figure 5. *Heat maps showing the regulation of inflammatory genes in colonic tissue directly excised from healthy mice and DSS-induced colitic mice (in vivo) (A) and in ex vivo cultures from healthy and inflamed colon from DSS-induced colitic mice (DSS/VEH) (B) and Gαi2$^{-/-}$ colitic mice (Gαi2$^{-/-}$/VEH) (C). The expression in the healthy tissue was normalised to 1.0 (left panels), and the fold induction of the genes in the inflamed tissues (right panel) is shown compared to healthy C57BL/6 mice or Gαi2$^{+/+}$ WT mice, respectively. Pharmacol Res. 2008;58:222–231.*

To further confirm the relevance of the *ex vivo* culture system, the gene expression profile was compared between *ex vivo* cultures of inflamed colons from the DSS-induced colitis and those of Gαi2$^{-/-}$colitic mice treated with methyl-prednisolone or MG132. In general, down-regulation of immune gene expression was more pronounced in DSS-induced colitic tissue compared to tissue from Gαi2$^{-/-}$mice with colitis, and some of the genes were down-regulated in DSS-induced colitis only. Confirming the *in vivo* findings, iNOS was not reduced in methyl-prednisolone treated Gαi2$^{-/-}$colonic tissue, whereas this expression was inhibited by MG132. Similarly CCL19, mediating recruitment of naive T cells and antigen-matured tissue DCs to lymph nodes; the regulatory cytokine IL-10; the cytokines p35 and IL-18; and CCL5, recruiting T cells, eosinophils and basophils to inflamed tissue, were not, or only marginally, down-regulated by methyl-prednisolone in Gαi2$^{-/-}$colonic tissue, but more-so by MG132 (Fig 6, Table 3, paper III). These data can be interpreted as that methyl-prednisolone acts mainly by

inhibiting innate immune responses, as shown in DSS-colitis, whereas MG132 can act effectively on both the innate arm of the immune response, as well as on the adaptive immune response, seen in the Gαi2$^{-/-}$ colitis model.

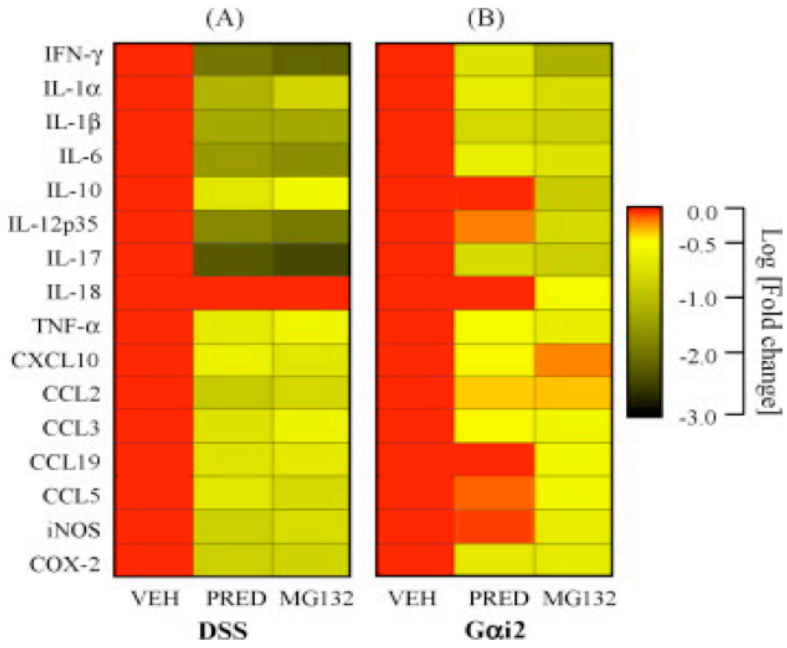


***Figure** 6. Heat maps showing inflammatory markers from ex vivo cultures of inflamed colons from DSS-induced (5%) colitic mice (A) and Gαi2$^{-/-}$ mice (B). The expression of each gene from inflamed colons were normalised to the logarithm of 1.0 (red colour, left panels). The right panels show the relative expression after treatment with methyl-prednisolone or MG132 in relation to untreated inflamed colon according to the scale on the right. VEH denotes inflamed vehicle-treated colon from DSS-induced and Gai2$^{-/-}$ colitic mice, respectively. Pharmacol Res. 2008;58:222–231.*

Somewhat surprisingly, although IL-12p35 was up-regulated in both models, IL-12p40 was not detected before or after treatment. This raises the possibility that the enhanced p35 transcription and EBI3 formed a heterodimeric cytokine, IL-35 (235). Interestingly, IL-35 is important in regulating mucosal immune responses by inhibiting Th1 and Th17 cells (236), and thus further studies are needed to shed light on this issue.

Finally, expression of IL-1, IL-6, and iNOS mRNA was investigated by quantitative RT-PCR and IL-6 protein by ELISA in *ex vivo* cultures from both mouse models to confirm the findings from the mouse arrays. mRNA levels of IL-1, IL-6 and iNOS were significantly up-regulated in both models,

and the expression of these markers were significantly down-regulated in the steroid treated groups. Similar results were obtained for IL-6 levels in colons of DSS-induced colitic mice, but not in Gαi2$^{-/-}$ mice with colitis.

Several of the genes assayed in this study (e.g. CCL2, CCL3, CCL5, IL-1β and IL-6) have been reported to be up-regulated in UC patients and suppressed after steroid treatment (237). This finding was partly confirmed by our analysis in the murine *in vivo* studies and in the *ex vivo* cultures, further supporting the relevance of both animal models for the evaluation of future therapies.

In summary, an innate cytokine/chemokine profile dominated in the DSS model, whereas a T cell related gene profile dominated in the Gαi2$^{-/-}$ model. After steroid treatment, IL-1β, IL-6 and iNOS were suppressed in both models both in *in vivo* and *ex vivo* analyses. Thus the changes in inflammatory gene expression in the murine *ex vivo* culture system reflected the *in vivo* response in the inflamed colonic tissue, suggesting that the murine culture system can be used for validation of future IBD therapies. In conclusion, detailed analysis of immune parameters, and their regulation, in the Gαi2$^{-/-}$ mouse model of IBD benefits the understanding of the complexity of inflammatory pathogenesis in IBD and potential treatments.

5 GENERAL DISCUSSION

Animal models have improved our understanding of the pathogenesis of human IBD. It is becoming evident that in genetically susceptible hosts aberrant immune responses and loss of tolerance to environmental parameters are major factors contributing to the mucosal inflammation.

In the Gαi2$^{-/-}$ model, the Gαi2 deletion has been associated with many functional defects: The Gαi2 subunit is critical to tight junction formation (11), and functional defects in the epithelial component of the barrier thereby allows inappropriate access of microbial antigens into the lamina propria in Gαi2$^{-/-}$ mice. The microbial signals, sensed by lamina propria macrophages and DC will drive an acute inflammatory response and direct the adaptive immune response. Gαi2 has also been shown to be essential in the regulation of proinflammatory cytokine production. For example, in CD8α^{+} DCs in mice, Gαi2 is involved in constitutive suppression of proinflammatory cytokine production *in vivo*, mediated by 7TDR ligands that activate Gi proteins (9). Further studies have demonstrated disturbed T cell development, with increased frequencies of mature thymocytes, in the absence of Gαi2 (8). Increased IFN-γ, together with high levels of IL-1, IL-6 and TNF-α in inflamed tissue (9) drives a typical Th1-skewed CD4^{+} immune response in Gαi2$^{-/-}$ mice.

The major gaps in our knowledge addressed by this thesis concern a) the details of how this inflammatory response progresses and b) why it is not appropriately regulated and progresses to a chronic colitis.

The chronic inflammation in Gαi2$^{-/-}$ colitis is maintained by effector T cells and previous work in the group had defined the effector T cell response in late-stage colitis as a mixed Th1/Th17 character. However, in the work described here, it was possible to, at least to some extent, map the pathogenesis of this, and the preceding innate response, in a longitudinal study.

Neutrophil granulocytes are prominent both in human UC (208) and Gαi2$^{-/-}$ mice with colitis. Linked lines of evidence emerge from this thesis to support a defect in the regulation of this induced innate response, driven by the Gαi2 deletion. Firstly, the colonic IL-17 response, which is known to drive neutrophilia (238, 239), was shown to be upregulated in Gαi2$^{-/-}$ mice, even in the absence of disease and, secondly, CXCL1, a known chemoattractant for neutrophils, increased with the progression of Gαi2$^{-/-}$ colitis. Although IL-17

does not directly affect transcription of CXCL1, recent work shows that the IL-17-driven enhancement of neutrophil infiltration by CXCL1 is mediated by stabilization of CXCL1 mRNA, in TNFa-stimulated myeloid cells (240). The enhanced IL-17 levels in Gαi2$^{-/-}$ mice may be driven by the epithelial barrier defect, present from birth (11), in turn causing prolonged proinflammatory cytokine secretion, concurring with our findings of increased levels of IL-1β, IL-6 and TNF-α in Gαi2$^{-/-}$ mice with colitis.

The data show that, as with human CD (241), late-stage disease in Gαi2$^{-/-}$ colitis has a mixed Th17/Th1 character. Interleukin-12/23p40 is the common subunit in both IL-12 and IL-23 which are key cytokines in Th1 and Th17 pathways, respectively (242). Its increase during disease pathogenesis in the model suggests that IL-12/23p40 promotes the Th1 pathway during the development of disease. This is also evidenced by progressive increases in IFN-g. As it has been observed recently that Th17 cells induce colitis and promote Th1 responses through IL-17 induction of innate IL-12 and IL-23 production (243), it is likely that in Gαi2$^{-/-}$ colitis the Th17 response promoted the development of colitis before the Th1 pathway was triggered.

Interferon-γ has been shown to have an inhibitory effect on the production of IL-17 (244), and evidence has emerged recently in CD, of a subset of intestinal Th17 cells co-producing IFN-γ (115, 120). This phenomenon is particularly prominent at sites of inflammation in CD (115). Thus, IFN-γ may not always downregulate IL-17A production and may contribute to the pathogenetic and proinflammatory functions of Th17 cells. Human CCR6^{+}CXCR3^{+}-expressing Th1 cells also include a subset that produces both IL-17A and IFN-γ (120). The human data are in line with experimental colitis. Thus, purified Th17 cells also upregulate the Th1-associated transcription factor Tbet and secrete IFN-γ upon exposure to IL-12 *in vitro* and *in vivo* (245). IL-23 promoted intestinal Th17 cell accumulation, and enhanced the emergence of an IL-17A^{+}IFN-γ^{+} population of T cells (246). These studies challenge the traditional concept in the recent decade that IFN-γ suppresses Th17 and enhances Th1 development. Therefore, the interactions between Th1 and Th17 cells and the role of IFN-γ on Th17 cells may be more complex than previously assumed.

An important finding of this thesis is that Gαi2$^{-/-}$ Tregs were functional *in vitro* but did not prevent colitis in Gαi2$^{-/-}$ mice. The observation that CCL5 levels in Gαi2$^{-/-}$ mice were not different from WT, indicates a local clonal expansion of effector T cells within the colonic mucosa, rather than immigration. Gαi2$^{-/-}$ effector T cells have been demonstrated to be resistant to the inhibitory effects of TGF-β (247) and display a hyperproliferative

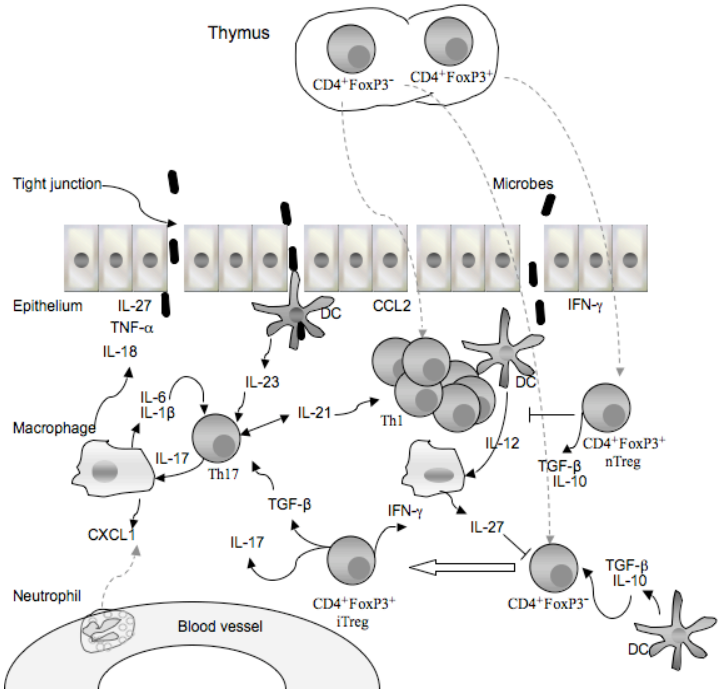
response to TCR/CD3 stimulation (216). In line with these data, our study showed that effector T cells from Gαi2$^{-/-}$ colitic mice cannot be suppressed *in vitro* by either WT or Gαi2$^{-/-}$ Tregs.

The Tregs used for *in vitro* assays and for transfer to Gαi2$^{-/-}$ recipients were sorted from splenic lymphocytes and, although they were clearly functional in *in vitro* suppression assays, their activity *in situ* may be compromised by the inflammatory milieu. Within an inflammatory site, effector T cell and Treg phenotype and function is somewhat plastic. Data has shown that FoxP3^{+} Tregs suppression is lost upon iTreg-specific ablation of Stat3, a transcription factor critical for Th17 differentiation, and results in the development of a fatal intestinal inflammation (189). Ironically, Stat3 deficiency in FoxP3^{+} Tregs resulted in increased expression of *Il6* and *Tgfb1*. Although FoxP3^{+} Treg-derived TGF-β1 can mediate suppression (81), TGF-β1 in combination with IL-6 also facilitates the generation of Th17 cells. In addition to STAT3, iTregs can express several transcription factors associated with effector T cell responses, including T-bet (Th1), IRF4 (Th2, Th17) and GATA3 (Th2)(187). In healthy individuals, this mechanism would allow Treg-mediated control of distinct effector polarity under homeostatic conditions. However, the system is delicately sensitive and can sometimes lead to Tregs instability. For example, high-level T-bet expression in the presence of acute intestinal infection drives Tregs into an inflammatory IFN-γ-secreting phenotype (195). Furthermore, a subset of IL-17 producing FoxP3^{+} regulatory cells in human has been identified. These CD4^{+}FoxP3^{+}CCR6^{-} Tregs are able to differentiate into IL-17-producing cells upon T-cell receptor (TCR) stimulation in the presence of IL-1β, IL-2, IL-21, and IL-23 (248).

These phenomena might explain why Tregs from neither Gαi2$^{-/-}$ colitic mice nor WT mice were able to suppress the Gαi2$^{-/-}$ pathogenic effector T cell responses due to the strong pro inflammatory cytokines/chemokines milieu in the mixed Th17/Th1 pathway. Thus, there might be several decisive factors which could determine the fate of Gαi2$^{-/-}$ Tregs in the Th17/Th1 inflamed colon. Firstly, although high numbers of FoxP3^{+} Tregs were detected in the inflamed colitic tissue, in the colonic pro-inflammatory milieu, Gαi2$^{-/-}$ FoxP3^{+} Tregs might be suppressed by IL-21; co-secrete IL-17 and/or IFN-γ, which in turn may transform them into effector T cells; or their suppressive function may be insufficient to overcome the enhanced effector cell activity.

The altered efficacy of the anti-inflammatory agents between the acute DSS-induced colitis and the chronic inflammation of the Gαi2$^{-/-}$ colitis model clearly reflects the different mechanisms behind these two types of colitis. The anti-inflammatory function of methyl-prednisolone acted mostly at the level of innate immunity, as shown in DSS-colitis, whereas MG132 can act effectively on the chronically activated adaptive immune response in the Gαi2$^{-/-}$ colitis model. It has been shown that methyl-prednisolone induces Annexin-1 (249), which in turn modulates T-cell activation and differentiation (250). This may then allow Tregs to suppress the dampened effector T cells. However, the pathogenic Gαi2$^{-/-}$ effector T cells, which comprise a high frequency of differentiated memory T cells (paper II), clearly cannot be switched off or influenced by the anti-inflammatory effect of methyl-prednisolone. On the other hand, the proteasome inhibitor MG132 efficiently suppressed the proinflammatory gene expression in both Gαi2$^{-/-}$ and DSS-induced colitic mice. Studies have shown that MG132 induces apoptosis in human Jurkat T cells (251) and in mice through formation of reactive oxygen species (ROS) (252). Thus, the most effective way to counter the established inflammation in this model appears to be to induce apoptosis in differentiated effector T cells.

Altogether, we conclude that the Gαi2 deficiency leads to the dysregulation in the intestinal barrier, which amplifies the gut Th17 response. As the Th17 reponse proceeds, the tissue destruction initiates the Th1 pathway and leads to a mixed Th17/Th1 response. The hyper-reactive Gαi2$^{-/-}$ effector T cells amplify the inflammation and more memory effector T cells are generated during the disease progression. Clearance of overreactive effector T cells may be disturbed, adding to the established intestinal inflammation. The lack of appropriate regulation from Tregs, will then exacerbate the Gαi2$^{-/-}$ colitis.



Possible immune pathogenesis of Gαi2$^{-/-}$ colitis

- Gαi2 deletion leads to defective epithelial tight junctions.
- Access of commensals to innate receptors on IEC basolateral membrane and on macrophages, DC.
- Secretion of proinflammatory cytokines from IEC, macrophages and DC signalling microbe intrusion.
- The attempt of IL-27 to heal the barrier defect.
- Th17 cell expansion, driven by IL-6, IL-23 and TGFβ.
- IL-17 feeds back on macrophage, stabilizing CXCL1 mRNA and up regulating CXCL1 secretion - neutrophil ingress.
- Th-17/iTreg plasticity - Th17 cells generation rather than iTregs in the local inflammatory milieu – transformed character of existing iTregs – reduced regulation – amplification of Th1.

6 FUTURE PERSPECTIVES

The future studies of Gαi2$^{-/-}$ colitis should focus on:

A. Characterization of Tregs under the influence of proinflammatory cytokines, mimicking the inflamed colon in Gαi2$^{-/-}$ colitic mice.

- the gene expression of Gαi2$^{-/-}$ Tregs, such as transcription factors associated with particular effector responses, Tbet (Th1) and Stat3 (Th17)

- the suppressive function of splenic Gαi2$^{-/-}$ Tregs *in vitro* in different cytokine milieux.

- examine Gαi2$^{-/-}$FoxP3^{+}Tregs for possible IL-17 and/or IFN-γ-secreting phenotype

B. Dissection of the early events in *in-situ* Tregs responses in the colon of RAG2$^{-/-}$ recipients, where different combinations of Gαi2$^{-/-}$ (KO)/WT-Tregs are co transferred with KO/WT-Teffs. To investigate the role of Gαi2$^{-/-}$ FoxP3^{+} Tregs, while cross-talking with DCs, in T cell priming during the development of the colitis.

- By comparing the quantity of the DC aggregates of isolated lymphoid aggregates (ILAs) between groups of mice co-transferred with different combinations of Treg/Teff.

- By comparing (quantifying) FoxP3 expression in DC aggregates of ILAs between groups of mice co-transferred with different combinations of Treg/Teff.

- Cytokine profiles from spleens and MLN from the transferred models, as above.

Hopefully, these future studies will further advance our insight of the disease development in Gαi2$^{-/-}$ colitis.

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