The role of SOCS3 signaling in ulcerative colitis and ulcerative colitis-related carcinogenesis

Yi Li

Colophon

ISBN: 978-90-8570-766-0

The work presented in this dissertation was conducted at the Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, the Netherlands.

Cover design: http: //pdldj.taobao.com
Layout: Bo Han & Yi Li
Printing: Wöhrmann Print Service B.V., Zutphen

Financial support for printing this thesis was kindly given by: Dr. Falk Pharma Benelux B.V., Abbott B.V., Ferring B.V., UCB Pharma B.V., MSD Nederland B.V., Greiner bio-one, Pentax Nederland B.V., Dynavin GmbH, the Nederlandse Vereniging voor Gastroenterologie and the Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam.

The role of SOCS3 signaling in ulcerative colitis and ulcerative colitis-related carcinogenesis

De rol van SOCS3 signalering in colitis ulcerosa en colitis ulcerosa-gerelateerde carcinogenese

Thesis

to obtain the degree of Doctor
from the Erasmus University Rotterdam
by command of the rector magnificus
Prof. dr. H.G. Schmidt
and accordance to the decision of the
Doctorate Board

The public defense shall be held on
Wednesday 11 of January 2012 at 15: 30 hrs
By

Yi Li

Born in Hubei, China

PROMOTION COMMITTEE

Promotor:	Prof. dr. E.J.Kuipers
Small committee:	Prof. dr. M.P. Peppelenbosch
	Prof. dr. P.M. van Hagen
	Prof. dr. G. d'Haens
Co-promotor:	Dr. C.J. van der Woude
	Dr. C. De Haar

My heart will go on

ABBREVIATIONS

5-ASA	5-aminosalicylic acid
AOM	azoxymethan
AP1	activator protein 1
CAMP	cyclic AMP or 3'-5'-cyclic adenosine monophosphate
CD	Crohn’s disease
CRC	colorectal cancer
CRE	cAMP-response element
CREB	cAMP response element-binding
DAC	5-aza-2 –deoxycytidine
DMBT1	deleted in malignant brain tumor 1
DNA	deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DSS	Dextran sulfate sodium
H&E	hematoxylin and eosin
HGD	high-grade dysplasia
IBD	inflammatory bowel disease
IEC	intestine epithelial cells
IHC	immunohistochemistry
IL	interleukin
LGD	low-grade dysplasia
LPS	lipopolysaccharide
MSP	methyaltion specific PCR
MTT	3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide
MUC	mucin
NSAID	Nonsteroidal anti-inflammatory drugs
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PKA	protein kinase A
REG-I	the growth factor regenerate gene I
RNA	ribonucleic acid
SOCS	suppressor of cytokine signaling
SRE	STAT-response element
STAT	the Signal Transducers and Activators of Transcription protein
SNP	single-nucleotide polymorphism
TGF	Transforming growth factor
Th	T helper
TIFIID	Transcription factor II D
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	T regular
UC	ulcerative colitis
WB	western blot

CONTENTS

Chapter I

General introduction and thesis outline

GENERAL INTRODUCTION

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterized by a chronic recurrent inflammation of the gastrointestinal tract and is subdivided into two major phenotypes: Crohn's disease (CD) and ulcerative colitis (UC). CD could potentially affect any location of the gastrointestinal tract, but inflammation of the terminal ileum and colon (ileocolic) dominates this disease. The pathology of CD is characterized by its patchy distribution and transmural inflammation and the presence of granuloma in the lamina propria is an important characteristic for the diagnosis of CD. UC is a chronic gastrointestinal disorder that is limited to the colon. The pathology of the UC is characterized by an even and continuous distribution of the inflammatory infiltrate that only affects the lamina propria. Furthermore, the disruption of normal crypt architecture and the presence of crypt abscesses are important histological characteristics of UC. The peak age of onset for IBD between 15 and 30 years, although it may occur at any age. About 10% of the cases occur in individuals under the age of 18. UC is slightly more common in males, whereas CD is marginally more frequent in women. Although it was thought that IBD occurred less frequently in ethnic or racial minority groups compared with Caucasians, the differences seem to be narrowing [1]. The existing epidemiology data suggest that the worldwide incidence rate of UC varies between 0.5-24.5/100,000 persons, while that of CD varies between 0.1-16/100,000 persons worldwide, with the prevalence rate of IBD reaching up to 396/100,000 persons [2].

The mucosal immune system enables us to fight pathogens while preventing responses against 'harmless' substances using ignorance or the induction of tolerance. As such, dysregulation of this immune system could encompass either the inability to fight pathogens or lead to autoimmunity. IBD is an example of this latter immune dysregulation, where a loss of tolerance or ignorance towards the intestinal microbiota leads to chronic inflammation in the intestine [3].

IBD is a multifactorial disease. This means that, in addition to the immune response, other factors like the luminal microbiota, environmental triggers and genetic susceptibility are also contributing to the disease (Figure 1). Our understanding of the other factors like microbiota and genetics has been greatly enhanced by the development of new high-

throughput sequencing methods [4-7]. Microbiota and some of their products affect epithelial cell function as well as the development of our immune system. As such, specific differences in composition may be linked to dysregulated barrier and/or immune function in IBD [8]. Although microbiota are generally beneficial, they can only be considered harmless because of the strict barrier between them and the host. This barrier consists of the intestinal epithelial cells (IEC) with their produced mucus, antimicrobial peptides and tight-junctions, preventing bacterial translocation that could lead to inflammation. Phagocytes are localized underneath this epithelial layer as a backup to capture bacteria or other substances that are able to make their way through the barrier [9]. In addition to their beneficial effects, evidence show that commensal bacteria are also critically involved in driving the pathogenesis IBD [10].

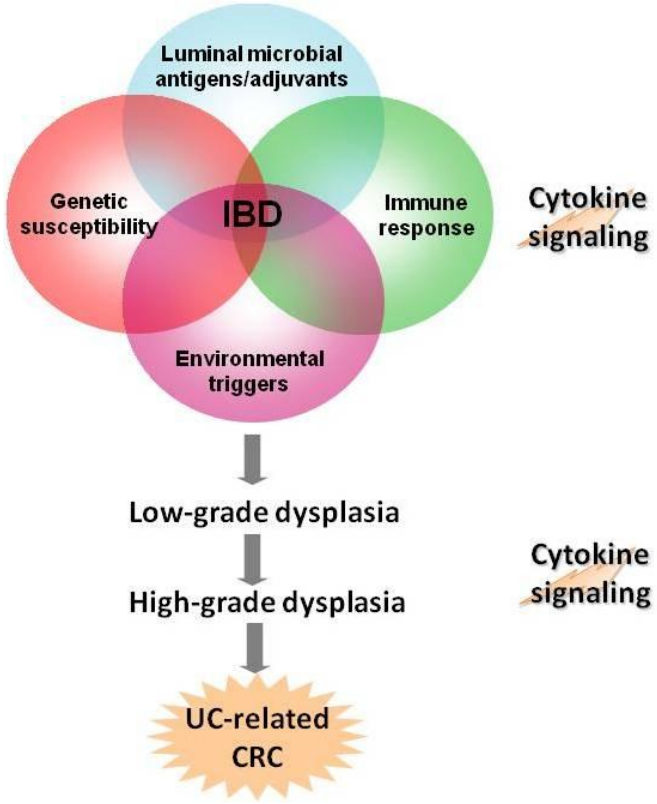


Figure 1. Interactions of various factors contributing to chronic intestinal inflammation in IBD patietns. Genetic susceptibility is influenced by the luminal microbiota, which provides antigens and adjuvants that stimulate immune responses in either pathogenic or protective way. Environmental triggers are necessary to initiate or reactivate disease expression. The long-lasting intestinal inflammation stimulates cell proliferation in the mucosal and could eventually lead to the low-grade dysplasia, high-grade dysplasia and ultimately carcinoma.

Several genes linked to the various IBD associated SNPs regulate important biologic functions, many of which are affecting the immune system or mucosal barrier integrity via the intestinal epithelial cell function [11-12]. The environmental factors affecting IBD include smoking which is protective in UC but detrimental in CD, diet, the use of antibiotics and

nonsteroidal anti-inflammatory drugs (NSAIDs) and stress [1]. All of which can directly or indirectly affect the epithelial barrier, the immune system as well as the microbiota.

IBD-related colorectal cancer

Chronic inflammation has been associated with an increased risk for developing cancer [13-14]. The risk for IBD-related colorectal cancer (CRC) increases by 0.5-1% yearly at 8-10 year after diagnoses. Although IBD contributes to only 1-2% to all cases of colorectal cancer (CRC), the mortality rate in patients with IBD-related CRC is higher than those in sporadic CRC case. CRC accounts for approximately 15% of all deaths in patients with IBD [13-14]. Risk factors for colorectal cancer in patients with IBD include disease duration, early onset, extensive disease, primary sclerosing cholangitis and a family history of sporadic colorectal cancer.

The etiology is clearly different between IBD-CRC and sporadic CRC. Whereas IBD-CRC typically arises from flat dysplastic mucosa, sporadic CRC often arises from polyps. Various studies have started to unravel some of the underlying mechanisms that may drive the development of IBD-CRC [15]. The effects of the immune system on IEC seem to play a crucial role in the development of colitis-associated cancer. The activation of the immune system in IBD leads to the production of mediators that cause further damage of IEC enhancing the inflammation. Some of these mediators may also have mutagenic potential enhancing the risk for CRC development. Other inflammatory mediators produced by immune cells or the IEC themselves are enhancing tissue-repair stimulating IEC proliferation. This combination of events forms the basis for IBD-CRC development [14, 16-18].

Cytokine signaling in IEC

As mentioned above, IEC play an important role in both IBD and IBD-CRC. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) 3 pathway is critically involved in IEC proliferation during homeostasis as well as the enhance proliferation needed for tissue repair in active IBD. Furthermore, STAT3 signaling is associated with enhanced survival and proliferation of CRC-tumor cells [19].

When cytokines bind to the receptor and trigger the activation of JAKs, the activated JAK phosphorylates the tyrosine residues of the cytoplasmic tail of the cytokine receptor. This provides a docking site for proteins that contain a phosphotyrosine-binding Src homology 2 (SH2) domain like STAT proteins [20]. STATs are recruited to the receptors, and tyrosine-phosphorylated by JAKs, enabling their homo- or heterodimerization, and subsequent translocation to the nucleus where they initiate transcription of target genes [21]. A very important target gene of STAT signaling is the suppressor of cytokine signaling (SOCS) protein, that functions as a feedback inhibitor [22] (Figure 2).

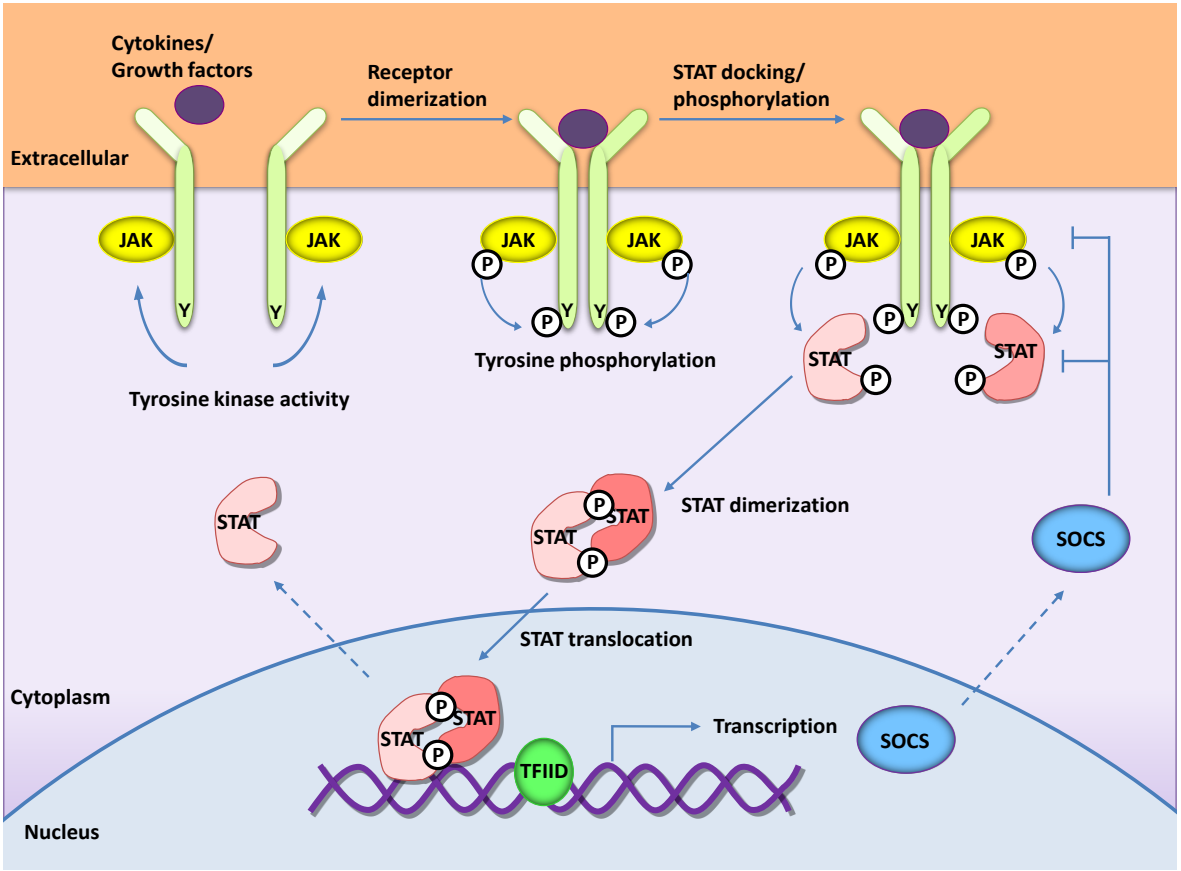


Figure 2. Schematic representation of the STAT-signaling pathway from cytokine stimulation of the receptor till gene expression. Cytokine binding to the receptor induces its dimerization and phosphorylation of Janus kinases (JAK) by the intrinsic tyrosine kinase activity. The activated JAK phosphorylates tyrosine residues that are then able to bind STAT proteins. Next, JAK phosphorylates STAT enabling its dimerization and translocation to the nucleus. In the nucleus the STAT-dimers bind to the DNA and recruit cofactors like the transcription Factor II D (TFIID) to start transcription of STAT-target genes. One of these target genes, the suppressor of cytokine signaling (SOCS) is able to negatively regulate STAT activation by binding to the phophorylted tyrosine residues on the receptor.

Considering the importance of STAT signaling in IBD and IBD-CRC, SOCS protein will have an important regulatory function in these diseases.

Suppressor of cytokine signaling (SOCS)

The mammalian SOCS family contains eight members, which include CIS (cytokine-induced SH2 containing protein) and SOCS1-SOCS7. Each SOCS member has a central SH2 domain, a mino-terminal domain of variable length and sequence, and a carboxy-terminal 40-amino-acid module known as the SOCS box. The SH2 domain of SOCS3 contains a 35-residue unstructured PEST (proline-, glutamicacid-, serine- and threonine-rich)-motif insertion to increase its protein stability [23]. The SOCS box has also been found in various other proteins like ASBs (ankyrin repeatcontaining proteins with a SOCS box), SSBs (SPRY domaincontaining proteins with a SOCS box) and WSBs (WD40 repeat-containing proteins with a SOCS box) [24].

As mentioned above, SOCS proteins inhibit JAK/STAT signal transduction, forming a negative feedback loop that modulates cytokine signaling by different mechanisms. i) Direct inhibition of all four JAK kinases. i.e. SOCS1 [25-26]. ii) Indirect inhibition of JAKs by binding of SOCS to the membrane proximal regions of the receptor chains, resulting in steric hindrance of constitutive JAK binding to the receptor chains. i.e. SOCS3 [27-28]. iii) Inhibition of JAK tyrosine kinase activity through their kinase inhibitory region (KIR), which is proposed to function as a pseudo substrate. i.e. SOCS1 and SOCS3 [29]. iv) Competitive inhibition of STAT binding to receptor chains by SOCS proteins. i.e. CIS and SOCS2 [28]. v) Inhibition of downstream signaling pathways. i.e. SOCS3 mediate inhibition of signaling via erythropoietin receptor (EpoR), gp130 and leptin receptor (LeptinR), resulting in the blockade of SHP-2-mediated ERK activation [30-32]. SOCS1 and SOCS6 inhibit Kit signaling similarly [33-34].

SOCS3 in IBD

Increased SOCS3 expression has been detected in mouse models of IBD where it seems to limit the extent of inflammation [35]. In addition, increased SOCS3 mRNA expression has been detected in inflamed biopsies of UC patients compared with non-inflamed biopsies [36]. Recently Sewel et al, proposed a three-stage model for IBD [37]. In this model, enhanced translocation of bacteria by impaired barrier function represents the first stage of disease

development. Next, the innate immune system seems to fall short leading to impaired clearance of these translocated bacteria. In the third stage, the adaptive immune system is getting involved and will drive the chronic inflammation. Although this represents an attractive model it will probably only explain the development of disease in a subset of IBD patients as overactive responses at various stages of this model could also lead to disease [38]. To illustrate the importance of SOCS3 in IBD we will address its function at the different stages of disease development.

IEC barrier function

Only a few studies have provided direct insight into the role of SOCS3 in epithelial cells. In general on could argue that SOCS3 will inhibit various STAT3 dependent processes in IEC. The major function of STAT3 in IEC during inflammation would be the induction of proliferation during wound healing. Both IL-6 and IL-22 have been shown to enhance proliferation and reconstitution of IEC using STAT3 signaling [39-42]. This is in accordance with the enhanced susceptibility of mice with IEC-specific ablation of STAT3 expression [43]. A direct effect of SOCS3 on STAT3 signaling was shown in skin epithelial cells where overexpression of SOCS3 hampered wound healing, further enhancing inflammation [44-46]. These data also suggest that SOCS3 expression may play an important role in inhibiting the inflammation associated hyperproliferation that may lead to CRC [47].

Innate immunity

Both LPS and TNF-α have been shown to induce the expression SOCS3 in macrophages, inhibiting IL-6 but not IL-10-induced gene expression via STAT3 [48-49].

In addition to IL-10-induced expression of SOCS3, constitutive expression of SOCS3 has been shown to inhibit LPS-induced expression of NO, TNF, IL-6 and GM-CSF all of which are important mediators involved in the pathogenesis of IBD [50]. Furthermore IL-6 induced an IL-10-like anti-inflammatory response in macrophages by inhibiting LPS-induced expression of TNF-α and IL-12 in the absence of SOCS3 expression [51].

G-CSF induces SOCS3 expression in granulocytes, which has been shown to inhibit G-CSFR-mediated STAT activation in a negative feedback loop [52]. The enhanced anti-apoptotic effects of G-CSF-induced STAT3 in SOCS3 deficient neutrophils, support the idea that normal induction of SOCS3 expression is needed to induce apoptosis as part of

resolution of inflammation [53]. Dysregulation of the proper SOCS3 expression may therefore enhance neutrophil survival contributing to chronic inflammation, or enhance apoptosis inducing impaired clearance of translocated bacteria.

Adaptive immunity

Cytokines play a major role in T helper cell (Th) proliferation and differentiation. With regard to proliferation, SOCS3 limits the CD28-induced production of IL-2 during early T-cell activation [54]. Polarization of Th0 cells towards Th1 or Th2 is primarily driven by IL-12 and IL-4, which respectively signal via STAT4 and STAT6 [55]. SOCS3 is predominantly expressed in committed Th2 cells [56]. Furthermore, SOCS3 inhibits IL-12-induced STAT4 activation and Th1 differentiation [57-58]. This latter effect of SOCS3 seems to be mediated via STAT5α activation of SOCS3 expression [59]. Besides effecting Th1/Th2 differentiation, SOCS3 is a major regulator of Th17 generation by inhibiting both IL-6 and IL-23-mediated STAT3 signaling [60-61]. Consistently, T cells from SOCS3-deficient mice differentiate towards the Th17 type due to the hyperactivation of STAT3 signaling [62]. TGF-β has been shown to be one of the substances that induce enhanced SOCS3 expression thereby enabling enhanced IL-6 and or IL-23-induced Th17 differentiation [63]. In conclusion SOCS3 expression in T-cells could influence the development of either CD (Th1/Th17) or UC (Th2).

SOCS3 in IBD-CRC

As mentioned earlier, STAT3 signaling has a stimulating effect on cell survival and proliferation. As such, reduced SOCS3 might also be involved in the development and progression of cancer. Reduced SOCS3 expression has been observed in a variety of inflammation-related human cancers and cancer cell lines and is correlated with strong STAT3 activity in these cells [64-70].

The existence of a similar mechanism in IBD-CRC is based on the tumor promoting potential of IL-6/p-STAT3 signaling in azoxymethan (AOM)/dextran sulfate sodium (DSS) induced colitis-associated CRC mouse models [43, 71]. These studies showed that during colitis-associated CRC development, IL-6 produced by cells in the lamina propria enhances STAT3-dependent proliferation of tumor-initiating cells and protection of premalignant IECs from apoptosis [43, 71].

CONCLUSION

SOCS3 protein is an important regulator of cytokine signaling that could affect all stages involved in IBD as well as IBD-CRC development. Since many of the current data are based on indirect evidence and/or are solely obtained in either in vitro or mouse models, the role of SOCS3 in IBD will be further investigated in this thesis.

AIMS AND OUTLINE OF THIS THESIS

Cytokine-signaling plays an important role in IBD. Proinflammatory cytokines like IL-6 contribute to the protection against pathogens, whereas they are also involved in chronic inflammation and are able to drive the survival and proliferation of cancer cells. Anti-inflammatory cytokines like IL-10 could help-out with the resolution of inflammation or prevent autoimmunity. On the other hand these anti-inflammatory cytokines can contribute to cancer development by inhibiting proper immune surveillance. All these effect warrant proper regulation of cytokine-signaling especially in a complex environment like the intestinal mucosa. In this thesis we focus on the role of SOCS3 the inhibitor of STAT3-signaling and its role in UC and UC-related CRC.

Chapter II provides a kaleidoscopic view on the role of STAT3 in IBD. We describe its role in IEC function and contribution to the balancing between Th17 cells and Tregs in IBD, and also point out new therapeutic strategies that directly or indirectly affect STAT3 signaling. This information puts our work on the STAT3-regulator SOCS3 in a wider perspective.

In **chapter III** we studied the disease-related IL-6/STAT3/SOCS3 expression in intestine epithelial compartment using immunohistochemistry. Our data showed a strong expression of IL-6 and STAT3, without the expression of SOCS3 in UC-CRC. The latter could be the effect of promoter methylation of the SOCS3 gene which is further addressed in **chapter IV**. Another remarkable observation was the high expression of SOCS3 in inactive UC something we studied in more detail in **chapters V and VI**.

In **chapter IV**, using patient material and the CRC cell lines we provide further evidence that hypermethylation of the SOCS3 gene is involved in the lack of SOCS3 expression in UC-CRC. We also provide some evidence that IL-6-induced DNMT1 activity might be involved in this link between inflammation and carcinogenesis. Our data suggest that methylation of SOCS3 may be a critical factor in the progression towards CRC development.

In **chapter V** we further addressed the clinical significance of the increased SOCS3 expression IEC of UC patients with inactive disease. Since this expression can make the IEC more vulnerable to various insults, we investigated whether it correlated with a shorter

time to mucosal relapse. Furthermore we provide some initial data that show a positive correlation between p-STAT1 and SOCS3 suggesting that p-STAT1 could be involved in the SOCS3 expression.

This latter observation was extended in **chapter VI**, where we assessed the involvement of other STAT3-independent pathways, which could induce high SOCS3 expression in UC patients with inactive disease.

REFERENCES

1. Loftus EV, Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology 2004;126:1504-17.
2. Lakatos PL. Recent trends in the epidemiology of inflammatory bowel diseases: up or down? World J Gastroenterol 2006;12:6102-8.
3. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995;102:448-55.
4. Garrett WS, Gordon JI, Glimcher LH. Homeostasis and inflammation in the intestine. Cell 2010;140:859-70.
5. Latiano A, Palmieri O, Latiano T, Corritore G, Bossa F, Martino G, Biscaglia G, Scimeca D, Valvano MR, Pastore M, Marseglia A, D'Inca R, Andriulli A, Annese V. Investigation of multiple susceptibility loci for inflammatory bowel disease in an Italian cohort of patients. PLoS One 2011;6:e22688.
6. Newman B, Siminovitch KA. Recent advances in the genetics of inflammatory bowel disease. Curr Opin Gastroenterol 2005;21:401-7.
7. Schreiber S, Rosenstiel P, Albrecht M, Hampe J, Krawczak M. Genetics of Crohn disease, an archetypal inflammatory barrier disease. Nat Rev Genet 2005;6:376-88.
8. Gordon JN, Di Sabatino A, Macdonald TT. The pathophysiologic rationale for biological therapies in inflammatory bowel disease. Curr Opin Gastroenterol 2005;21:431-7.
9. Shi J. Defensins and Paneth cells in inflammatory bowel disease. Inflamm Bowel Dis 2007;13:1284-92.
10. Crump BC, Hopkinson CS, Sogin ML, Hobbie JE. Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. Appl Environ Microbiol 2004;70:1494-505.
11. Henderson P, van Limbergen JE, Wilson DC, Satsangi J, Russell RK. Genetics of childhood-onset inflammatory bowel disease. Inflamm Bowel Dis 2011;17:346-61.
12. Momozawa Y, Mni M, Nakamura K, Coppieters W, Almer S, Amininejad L, Cleynen I, Colombel JF, de Rijk P, Dewit O, Finkel Y, Gassull MA, Goossens D, Laukens D, Lemann M, Libioulle C, O'Morain C, Reenaers C, Rutgeerts P, Tysk C, Zelenika D, Lathrop M, Del-Favero J, Hugot JP, de Vos M, Franchimont D, Vermeire S, Louis E, Georges M. Resequencing of positional candidates identifies low frequency IL23R coding variants protecting against inflammatory bowel disease. Nat Genet 2011;43:43-7.
13. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001;48:526-35.
14. Eaden J. Review article: colorectal carcinoma and inflammatory bowel disease. Aliment Pharmacol Ther 2004;20 Suppl 4:24-30.

15. Saleh M, Trinchieri G. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. Nat Rev Immunol 2011;11:9-20.
16. Bamias G, Nyce MR, De La Rue SA, Cominelli F. New concepts in the pathophysiology of inflammatory bowel disease. Ann Intern Med 2005;143:895-904.
17. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 2006;3:390-407.
18. Rhodes JM, Campbell BJ. Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. Trends Mol Med 2002;8:10-6.
19. Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. Cancer Lett 2004;206:193-9.
20. Hebenstreit D, Horejs-Hoeck J, Duschl A. JAK/STAT-dependent gene regulation by cytokines. Drug News Perspect 2005;18:243-9.
21. Li Y, de Haar C, Peppelenbosch MP, van der Woude CJ. New insights into the role of STAT3 in IBD. Inflamm Bowel Dis 2011.
22. Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol 2002;2:410-6.
23. Babon JJ, McManus EJ, Yao S, DeSouza DP, Mielke LA, Sprigg NS, Willson TA, Hilton DJ, Nicola NA, Baca M, Nicholson SE, Norton RS. The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability. Mol Cell 2006;22:205-16.
24. Yoshimura A, Nishinakamura H, Matsumura Y, Hanada T. Negative regulation of cytokine signaling and immune responses by SOCS proteins. Arthritis Res Ther 2005;7:100-10.
25. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, Tanimura S, Ohtsubo M, Misawa H, Miyazaki T, Leonor N, Taniguchi T, Fujita T, Kanakura Y, Komiya S, Yoshimura A. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997;387:921-4.
26. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, Nishimoto N, Kajita T, Taga T, Yoshizaki K, Akira S, Kishimoto T. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997;387:924-9.
27. Aman MJ, Migone TS, Sasaki A, Ascherman DP, Zhu M, Soldaini E, Imada K, Miyajima A, Yoshimura A, Leonard WJ. CIS associates with the interleukin-2 receptor beta chain and inhibits interleukin-2-dependent signaling. J Biol Chem 1999;274:30266-72.
28. Ram PA, Waxman DJ. SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. J Biol Chem 1999;274:35553-61.
29. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol 2003;4:1169-76.

30. Sasaki A, Yasukawa H, Shouda T, Kitamura T, Dikic I, Yoshimura A. CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2. J Biol Chem 2000;275:29338-47.
31. Schmitz J, Weissenbach M, Haan S, Heinrich PC, Schaper F. SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. J Biol Chem 2000;275:12848-56.
32. Bjorbaek C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers MG, Jr., Flier JS. Divergent roles of SHP-2 in ERK activation by leptin receptors. J Biol Chem 2001;276:4747-55.
33. De Sepulveda P, Okkenhaug K, Rose JL, Hawley RG, Dubreuil P, Rottapel R. Socs1 binds to multiple signalling proteins and suppresses steel factor-dependent proliferation. Embo J 1999;18:904-15.
34. Bayle J, Letard S, Frank R, Dubreuil P, De Sepulveda P. Suppressor of cytokine signaling 6 associates with KIT and regulates KIT receptor signaling. J Biol Chem 2004;279:12249-59.
35. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. J Exp Med 2001;193:471-81.
36. Miyanaka Y, Ueno Y, Tanaka S, Yoshioka K, Hatakeyama T, Shimamoto M, Sumii M, Chayama K. Clinical significance of mucosal suppressors of cytokine signaling 3 expression in ulcerative colitis. World J Gastroenterol 2007;13:2939-44.
37. Sewell GW, Marks DJ, Segal AW. The immunopathogenesis of Crohn's disease: a three-stage model. Curr Opin Immunol 2009;21:506-13.
38. van Lierop PP, Damen GM, Escher JC, Samsom JN, Nieuwenhuis EE. Defective acute inflammation in Crohn's disease. Lancet 2006;368:578.
39. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkuhn T, Goke B, Auernhammer CJ, Dambacher J. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 2006;290:G827-38.
40. Nagalakshmi ML, Rascle A, Zurawski S, Menon S, de Waal Malefyt R. Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells. Int Immunopharmacol 2004;4:679-91.
41. Ziesche E, Bachmann M, Kleinert H, Pfeilschifter J, Muhl H. The interleukin-22/STAT3 pathway potentiates expression of inducible nitric-oxide synthase in human colon carcinoma cells. J Biol Chem 2007;282:16006-15.

42. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest 2008;118:534-44.
43. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91-102.
44. Ivory CP, Wallace LE, McCafferty DM, Sigalet DL. Interleukin-10-independent anti-inflammatory actions of glucagon-like peptide 2. Am J Physiol Gastrointest Liver Physiol 2008;295:G1202-10.
45. Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S. The suppressor of cytokine signaling (SOCS)-3 determines keratinocyte proliferative and migratory potential during skin repair. J Invest Dermatol;130:876-85.
46. Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S. Epithelial overexpression of SOCS-3 in transgenic mice exacerbates wound inflammation in the presence of elevated TGF-beta1. J Invest Dermatol;130:866-75.
47. Rigby RJ, Simmons JG, Greenhalgh CJ, Alexander WS, Lund PK. Suppressor of cytokine signaling 3 (SOCS3) limits damage-induced crypt hyper-proliferation and inflammation-associated tumorigenesis in the colon. Oncogene 2007;26:4833-41.
48. Bode JG, Nimmesgern A, Schmitz J, Schaper F, Schmitt M, Frisch W, Haussinger D, Heinrich PC, Graeve L. LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. FEBS Lett 1999;463:365-70.
49. Niemand C, Nimmesgern A, Haan S, Fischer P, Schaper F, Rossaint R, Heinrich PC, Muller-Newen G. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. J Immunol 2003;170:3263-72.
50. Berlato C, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J Immunol 2002;168:6404-11.
51. Yoshimura A, Ohishi HM, Aki D, Hanada T. Regulation of TLR signaling and inflammation by SOCS family proteins. J Leukoc Biol 2004;75:422-7.
52. Hortner M, Nielsch U, Mayr LM, Johnston JA, Heinrich PC, Haan S. Suppressor of cytokine signaling-3 is recruited to the activated granulocyte-colony stimulating factor receptor and modulates its signal transduction. J Immunol 2002;169:1219-27.

53. Kimura A, Kinjyo I, Matsumura Y, Mori H, Mashima R, Harada M, Chien KR, Yasukawa H, Yoshimura A. SOCS3 is a physiological negative regulator for granulopoiesis and granulocyte colony-stimulating factor receptor signaling. J Biol Chem 2004;279:6905-10.
54. Matsumoto A, Seki Y, Watanabe R, Hayashi K, Johnston JA, Harada Y, Abe R, Yoshimura A, Kubo M. A role of suppressor of cytokine signaling 3 (SOCS3/CIS3/SSI3) in CD28-mediated interleukin 2 production. J Exp Med 2003;197:425-36.
55. Egwuagu CE, Yu CR, Zhang M, Mahdi RM, Kim SJ, Gery I. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. J Immunol 2002;168:3181-7.
56. Seki Y, Inoue H, Nagata N, Hayashi K, Fukuyama S, Matsumoto K, Komine O, Hamano S, Himeno K, Inagaki-Ohara K, Cacalano N, O'Garra A, Oshida T, Saito H, Johnston JA, Yoshimura A, Kubo M. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. Nat Med 2003;9:1047-54.
57. Dimitriou ID, Clemenza L, Scotter AJ, Chen G, Guerra FM, Rottapel R. Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins. Immunol Rev 2008;224:265-83.
58. Yamamoto K, Yamaguchi M, Miyasaka N, Miura O. SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor beta2 subunit. Biochem Biophys Res Commun 2003;310:1188-93.
59. Takatori H, Nakajima H, Kagami S, Hirose K, Suto A, Suzuki K, Kubo M, Yoshimura A, Saito Y, Iwamoto I. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of suppressor of cytokine signaling 3 expression. J Immunol 2005;174:4105-12.
60. Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, Yoshimura A, Hennighausen L, O'Shea JJ. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. Proc Natl Acad Sci U S A 2006;103:8137-42.
61. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, Takahashi H, Sun HW, Kanno Y, Powrie F, O'Shea JJ. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. Immunity;32:605-15.
62. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006;24:677-88.
63. Qin H, Wang L, Feng T, Elson CO, Niyongere SA, Lee SJ, Reynolds SL, Weaver CT, Roarty K, Serra R, Benveniste EN, Cong Y. TGF-beta promotes Th17 cell development through inhibition of SOCS3. J Immunol 2009;183:97-105.
64. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, Yoshikawa H. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene 2005;24:6406-17.

65. Tokita T, Maesawa C, Kimura T, Kotani K, Takahashi K, Akasaka T, Masuda T. Methylation status of the SOCS3 gene in human malignant melanomas. Int J Oncol 2007;30:689-94.
66. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A 2003;100:14133-8.
67. Isomoto H. Epigenetic alterations in cholangiocarcinoma-sustained IL-6/STAT3 signaling in cholangio- carcinoma due to SOCS3 epigenetic silencing. Digestion 2009;79 Suppl 1:2-8.
68. Ogata H, Chinen T, Yoshida T, Kinjyo I, Takaesu G, Shiraishi H, Iida M, Kobayashi T, Yoshimura A. Loss of SOCS3 in the liver promotes fibrosis by enhancing STAT3-mediated TGF-beta1 production. Oncogene 2006;25:2520-30.
69. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. Gastroenterology 2007;132:384-96.
70. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.

Chapter II

New insights into the role of STAT3 in inflammatory bowel disease

Yi Li[1], Colin de Haar[1], Maikel P. Peppelenbosch[1], C. Janneke van der Woude[1].

1. Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, Netherlands.

Inflamm Bowel Dis 2011;Oct 12 [Epub head of print].

ABSTRACT

Although it is clear that IBD involves an inappropriate immune response to floral components, the molecular determinants that mediate the gene transcription underlying and aggravating disease remain poorly understood. There is building momentum, however, that implicates differential regulation of the signal transducer and activator of transcription (STAT) 3 as an important factor in mediating pathogenic gene transcription in IBD, and this notion was reinforced by studies presented at the recent 2011 DDW in Chicago. In the present report we shall integrate the existing body of literature with the novel data presented at this meeting to present a kaleidoscopic scheme as to provide further insight into the role of STAT3 in IBD. A genetic propensity to its overactivation in the monocyte and epithelial compartment compromises the innate defense to allow low-level bacterial infection to fester and eventually set-off disease. The subsequent STAT3 activation in various relevant mucosal immune compartments, in particular epithelial cells proliferation and survival, and the function in the regulatory T cells (Tregs) and Th17 cell allow the mucosal immune system to fight the infection to return to steady state. As such, the action of STAT3 in IBD is highly context dependent but always important.

KEY WORDS: STAT3; IEC; Th17; IBD

STAT signaling and gene expression in the inflammatory reaction

Like almost all biological processes, the inflammatory reaction is critically guided by the activation of gene expression programs. Some of these programs are associated with activation of host defenses in general, in particular the gene expression program dependent on the activation and nuclear translocation of NF- B, others confer specificity of the immune response. The family of Signal Transducers and Activators of Transcription (STATs) is a group of transcription factors especially important for such specificity as the activation of specific STATs is associated with distinct modalities in host defense [1, 2]. The STAT family constitutes 6 members, numbered 1 through 6, which share important structural and functional characteristics. All family members contain a so-called SRC-homology 2 (SH2) domain, a motif that allows specific association to phosphorylated tyrosine residues. These tyrosine residues are present in the cytoplasmic tail of tyrosine kinase receptors (*e.g.* the Colony Stimulating Factor receptor *c-fms* or the receptors for platelet-derived growth factor) and cytokine receptors (*e.g.* the receptors for interleukins and interferons). Activation of these receptors by inflammatory mediators is associated with rapid activation of associated Janus kinases (JAK) that causes phosphorylation of the intracellular tyrosine residues inducing an early recruitment of STAT factors to the plasma membrane and their SH2 domain mediated docking to the phosphorylated residues of the receptor. After docking, JAK activity provokes tyrosine phosphorylation of the STAT proteins themselves, enabling their homodimerisation which is followed by their translocation to the nucleus, DNA binding to specific motifs by virtue of a DNA binding domain in the STAT proteins, and finally resulting in specific transcriptional responses through the recruitment of the transcription Factor II D (TFIID) to the transactivation domain of the STAT protein [3]. Figure 1A provides an overview of STAT structure.

Because each different STAT proteins binds to its own specific palindromic response element in the DNA, transcriptional responses to activation are highly specific, and the body exploits this property to provide direction to the immune response (Figure 1B). It is not the intention of this review to give a full account of the molecular biology of the role of different STATs in the control of immunological transcription, but in general it can be stated that interleukin (IL)-12-induced activation of STAT4 in combination with T-bet signals are important for Th1 differentiation; IL-4-induced STAT6 provokes Th2 responses in combination with GATA3 signals; STAT3 activation by IL-6 and IL-23, in combination

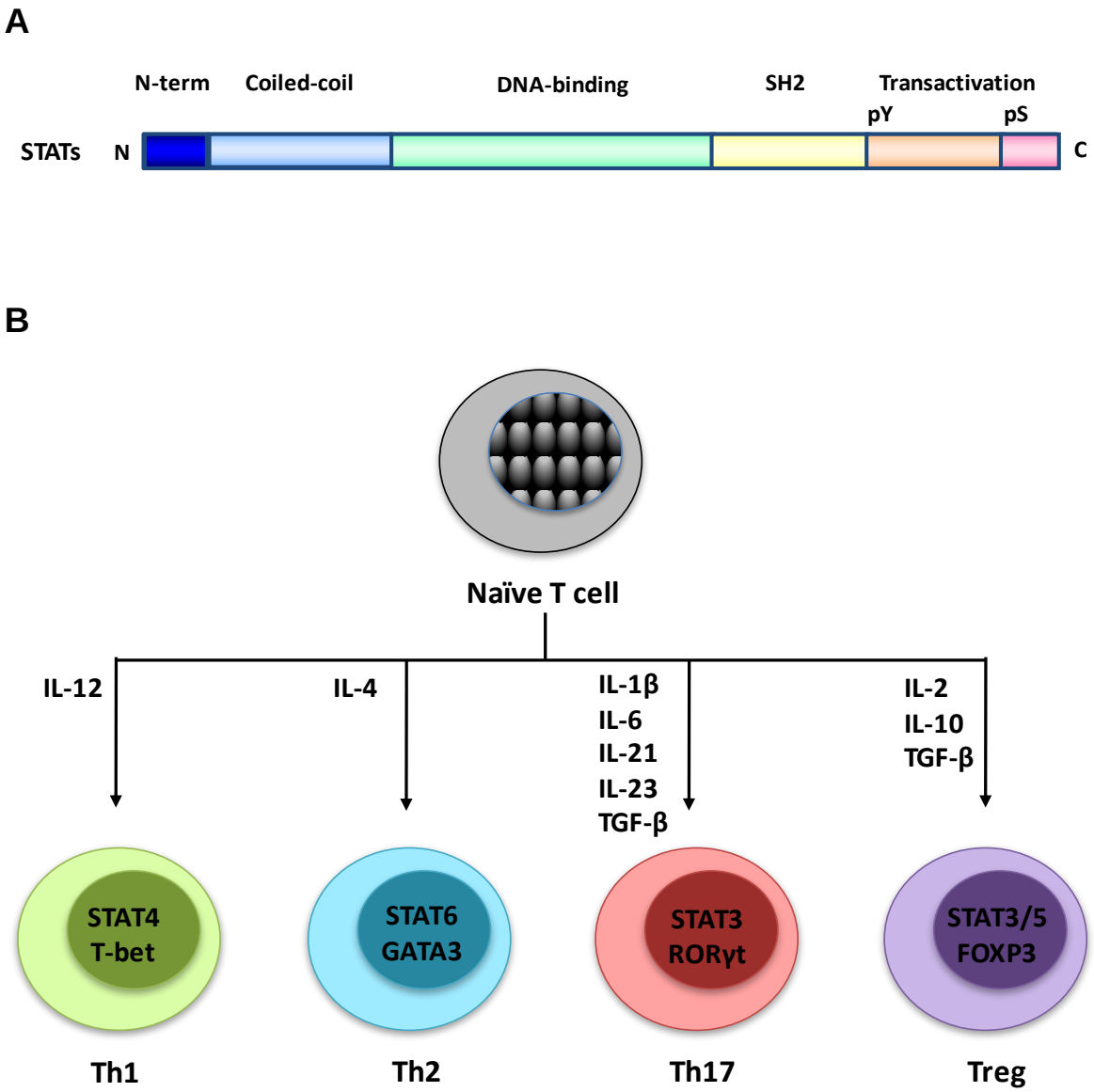


Figure 1. The structural domains, activation pathways and impact on T-cell differentiation of Signal Transducers and Activators of Transcription (STATs) proteins

A) The STAT protein contains 5 structural domains. The amino-terminal domain (N-term) of STATs mediates the formation of higher order structures when two STAT dimers bound neighboring STAT binding sites on the DNA. The coiled-coil domain is involved in the receptor binding but also the nuclear translocation of STATs. The DNA-binding domain with the consensus core sequence TT($N_{4–6}$)AA enable binding to STAT response elements promoter region of STAT target genes. The SRC-homology 2 (SH2) domain enables docking of STATs to the receptor or the formation of dimers via binding to phosphorylated tyrosine residues (pY). The transactivation domain is involved in recruitment and binding of transcription coregulators, such as histone acetyltransferases. Some

STATs contain serine phosphorylation (pS) residue in the carboxy-terminal domain (C-term) that enhances transcriptional activity.

B) Contribution of different STATs in helper T (Th) cells differentiation. Naïve CD4+ T cells can differentiate to Th1 cells in the presence of interleukin (IL)-12, and require activation of the master regulator transcription factor, T-bet, through STAT1. IL-4-induced STAT6 mediates Th2 differentiation in combination with GATA3 signals. Th17 cells require a combination of TGF-β and proinflammatory cytokines (IL-1β, IL-6, IL-21, IL-23) with the activation of STAT3 and RoRγt. IL-2-induced activation of STAT5 is needed for the induction of $FOXP3^+$ regulatory T cells (Tregs), but in these cells STAT3 is also needed for their regulatory function.

with factors like IL-1β, TGF-β, and RoRγt, is associated with Th17 formation 4. IL-2-induced STAT5 activation is required for the development of $FOXP3^+$ Tregs whereas STAT3 activation in these cells by IL-10, is important for the function of Tregs, especially their ability to suppress coinciding pathogenic Th17 responses [3, 5].

This STAT3 activation by both the tolerogenic cytokine IL-10 as well as the proinflammatory cytokines IL-6 is a good representation of the complex and fascinating function of this transcription factor. As IBD is associated with reduced tolerance of the mucosal flora, there has always been a fair amount of interest in the protein with respect to the pathogenesis of IBD, but recent data in conjunction with those presented at this year DDW 2011 in Chicago now highlight its pivotal importance in this disease, but also the complexity of its action.

STAT3 and the genetic basis of IBD

Studies comparing monozygotic and dizygotic twins have long suggested that IBD is a disease in which environmental factors trigger pathology in a genetically susceptible host. The large-scale GWAS studies have now identified the genetic location of the risk factors involve and demonstrated the existence of DNA polymorphisms that confer increased risk to contracting IBD, providing strong support for the notion that aberrant STAT3 signaling is important in IBD [3]. In apparent agreement, variants of JAK2, which is associated with STAT3 activation, also coincide with increased susceptibility to contracting IBD. Nevertheless, the increase in relative risk conferred by these risk alleles is moderate, already suggesting that the role of STAT3 in pathogenesis is not simple. Studies in patients carrying risk alleles and comparing patients and healthy individuals with or

without risk alleles have previously provided important insight into their functional effects on disease development [6]. This approach was employed by Kuhn et al. who investigated the functional effects of the STAT3 rs744166 risk allele variants [7]. This is especially important, because although the association of this allele to disease is evident, the intronic location makes functional effects difficult to predict. Data was presented showing that EBV-transformed B-cells from genotyped IBD patients display increased STAT3 activation and nuclear translocation in patients homozygous for the risk allele (possibly an effect the absolute amount of STAT3 mRNA produced), suggesting that IBD patients have too much rather as too little activation of the transcription factor. As STAT3 activation is associated with immunoactivation and Th17 driven inflammation but also the immunosuppression associated with signaling downstream of IL-10, the interpretation this observation more complex. Considering that the primary defect in IBD, or at least Crohn's disease, is an inability to control low-level infections because of enhanced immunosuppressive IL-10 signaling, this will subsequently fester and cause chronic inflammation. On the other hand, overactive IL-17 activation may also contribute to chronic inflammation when the adaptive immune system is recruited to help out the IL-10 hampered innate immune response. New developments presented at this year DDW have again strengthened the importance of STAT3 signaling in the mucosal immune regulation between intestine epithelial cells and T cells of IBD.

New insights into the role of STAT3 function in IEC related to IBD

Intestinal epithelial cells (IEC) form the physical barrier in our intestine to protect us from harmful luminal contents and form the first line of defense. The epithelial cells uses a variety of mechanism to protect the body from bacterial invasion, important are for instance the maintenance of the mucus layer lining the gut by the goblet, the antimicrobial peptides by Paneth cells, but also the formation of tight-junction to prevent luminal bacteria to cross the barrier in a non-controlled fashion. As such, defects in barrier function will lead to a commensal driven chronic inflammatory response like IBD. Importantly, more and more data suggest that the immunomodulatory function of STAT3 is not limited to cells from the hematopoietic lineage but present in other cell types as well, where it may modulate immune functions of non-professional host defense cells.

Ali et al. demonstrated higher levels of phosphorylated STAT3 (p-STAT3) in IEC of patients with active IBD as compared to patients with inactive IBD [8], which fits the

previous observation that the p-STAT3 positive intestine epithelial cells increased in active UC compared with inactive UC patients [9]. The finding that mice with an IEC-specific ablation of STAT3 signaling are more susceptible to develop dextran sodium sulphate (DSS)-induced colitis [10, 11] and the data presented by Wittkopf et al, that these animals show decreased secretion of antimicrobial peptides (Reg3g, Reg3b, Cryptdin, Lysozyme, Pla2g5 and Pla2g2a) encompass the defective epithelial barrier function and enhanced susceptibility to Citrobacter rodentium [12]. These data suggest that the increased levels of STAT3 signaling in IEC may limit the extent of mucosal inflammation. Unfortunately, this activity is far from sufficient to induce mucosal healing in the patients suffering from IBD.

One of the physiological functions of STAT3 activation in the IEC of the intestine may be to allow regenerative responses following inflammatory insult. It has been suggested that especially IL-22 would employ signaling through STAT3 to allow reconstitution of intestinal architecture [13, 14]. Indeed, a microarray study presented at the DDW 2011 showed that colon cell lines stimulated with IL-22 upregulate expression of anti-microbial proteins like deleted in malignant brain tumor 1 (DMBT1) and the growth factor regenerate gene I (REG-I), which is involved in the generation and maintenance of the mucosal villus structure among other functions [15], and thus would support such a notion. In addition to the induction of various antibacterial mediators, IL-22 is also reported to induce MUC1 expression in IEC through the activation of STAT3. The effects of MUC1 expression were studied using Th1- and Th2-mediated colitis models and MUC1 deficient mice. It was shown that the absence of MUC1 exacerbated colitis in both models coincided with a shift in immune response from Th1 or Th2 to Th17. Together these data suggest that IL-22 may function as a regulator of Th17 responses via the induction of MUC1 from IEC [16].

Hence, STAT3 activation in IEC functions in terminating inflammatory reaction and allowing the mucosa to return to steady state, making activation of STAT3 in IEC would be an attractive possible therapeutic target. The observation presented by Goldsmith et al, in which they showed that using the specific mu-opioid receptor agonist DALDA induces STAT3 activation dependent proliferation and migration of IEC is very interesting in this regard [17]. Since activation of STAT3 seems so crucial for proper induction of healing and the maintenance of homeostasis, the expression and activity of its downstream negative regulator suppressor of cytokine signaling 3 (SOCS3) could play an important regulatory function in these processes. High expression of SOCS3 could conceivably interfere with regenerative responses and thus epithelial cell homoeostasis and enhance the sensitivity

of these epithelial cells to inflammatory damage. In support of this idea the high SOCS3 expression in IEC at time of UC remission is associated with a shorter time till relapse and the increased severity of inflammation during relapse [18]. The major drawback of the STAT3 activation mediated proliferation and survival of IEC is that it provides a rational mechanism for cancers to develop and thrive [1].

Taken together, the role of STAT3 in the epithelial compartment is complex, important for inducing return to homeostasis following acute infection, but at the same time its chronic activation induces a susceptibility to low-level bacterial infection in turn producing ulceration.

Below the surface: the STAT3/Th17 axis in IBD

In contrast to its general protective effects in IEC, activation of STAT3 downstream IL-6, IL-23 and IL-21 receptors is required for efficient generation of highly inflammatory Th17 cells. Th17 cells possibly specific for commensal microbiota have been implicated in IBD [19, 20]. The IL-17 produced by these cells has a wide variety of effects mainly involving the induction of cytokines and chemokines expression by a variety of cells [21]. In this way T-cells are able to enhance recruitment of innate and adaptive immune cells all contributing intestinal inflammation in the case of IBD [22]. Conversely, the IL-10-induced STAT3 activation, necessary for Treg-mediated control of Th17 responses, is in accordance with the immunosuppressive function of this transcription factor [23]. Thus STAT3 function in the T cell compartment is truly kaleidoscopic.

Metwali et al. reported that infection with the Helminth Heligmosoides bakeri reduced disease severity in a variety of IBD mouse models. The infected mice have fewer Th17 cells in the mesenteric lymph nodes and reduced STAT3 signaling, probably in the Th17 compartment [24]. These data suggest that infection with specific nematodes or the use of nematode excreted substances could provide a future therapy by suppressing Th17 cells response. A novel drug named Vidofludimus (which inhibits pyrimidine biosynthesis through blocking DHODH) is beneficial in preclinical models of IBD and the presented data suggests that part of the effect may be through the inhibition of STAT3-mediated Th17 generation [25]. Likewise it is possible that the effects seen with the oral JAK inhibitor, CP-690,550 also involve effects on STAT3 signaling in the T cell compartment. CP-690,550 (CP) was used to treat 194 patients with moderate-to-severe active UC.

Treatment with CP was associated with dose-dependent improvement in clinical response and remission rates (Clinical response was 5 mg BID: 27% and 15mg BID: 38%. Clinical remission was 5 mg BID: 33% and 15 mg: 35%) [26, 27]. Although the drug was much effective in UC than in CD, the response and remission rate in fact didn't reach much significance. Because CP-690,550 targets more than one JAK, its exact mode of action in the setting of IBD has not been resolved, but because Th17 cytokines decrease at least an effect on Th17 generation is likely. In mouse arthritis model demonstrated CP-690,550 not only interferes with Th17 cell differentiation, but also prevents the activation of STAT1, induction of T-bet, and subsequent generation of Th1 cells. Thus, CP-690,550 may improve autoimmune diseases by suppressing the differentiation of pathogenic Th1 and Th17 cells as well as innate immune cell signaling [28]. The possible mechanisms are needed to be proved in IBD models.

The cytokine IL-23 is one of the key components involved in the induction and maintenance of Th17 cells through STAT3 activation, but the importance of this cytokine for maintaining mucosal inflammation remains unclear, maybe also because of the pleiotropic functioning of this cytokine in the T cell compartment. Blocking IL-23 in murine models of IBD has not yielded consistent results, and thus genetic ablation of the receptor for IL-23 was used as a novel approach by Meelu et al. to study its impact on mucosal inflammation. DSS colitis in IL-23R-deficient mice had a less severe phenotype and these mice also had a better recovery compared to the control IL-23R GFP reporter mice [29]. Interestingly, the IBD GWAS studies revealed that mutations in the IL-23R gene were associated with an increased risk for developing IBD. Clark et al. studied the functional effects of the R381Q IL-23R variant that is associated with a protection from developing IBD. Using T-cells from IL-23R R381 and IL-23R Q381 positive donors they show that the reduced function of the individuals carrying the IL-23R Q381 allele (rs11209026) may contribute to their protective role for developing CD, probably by limiting STAT3-dependent generation of Th17 cells [30].

The recent advances in our knowledge on the biological contribution of Th17 cells to IBD and various other diseases makes it an interesting target for the development of new biologicals [31], with i.e. the trials on anti-IL-6, anti-IL-23R and more recently anti-IL-17R and anti-IL-17. The dependence on Th17 cells for gut inflammation seems certainly not

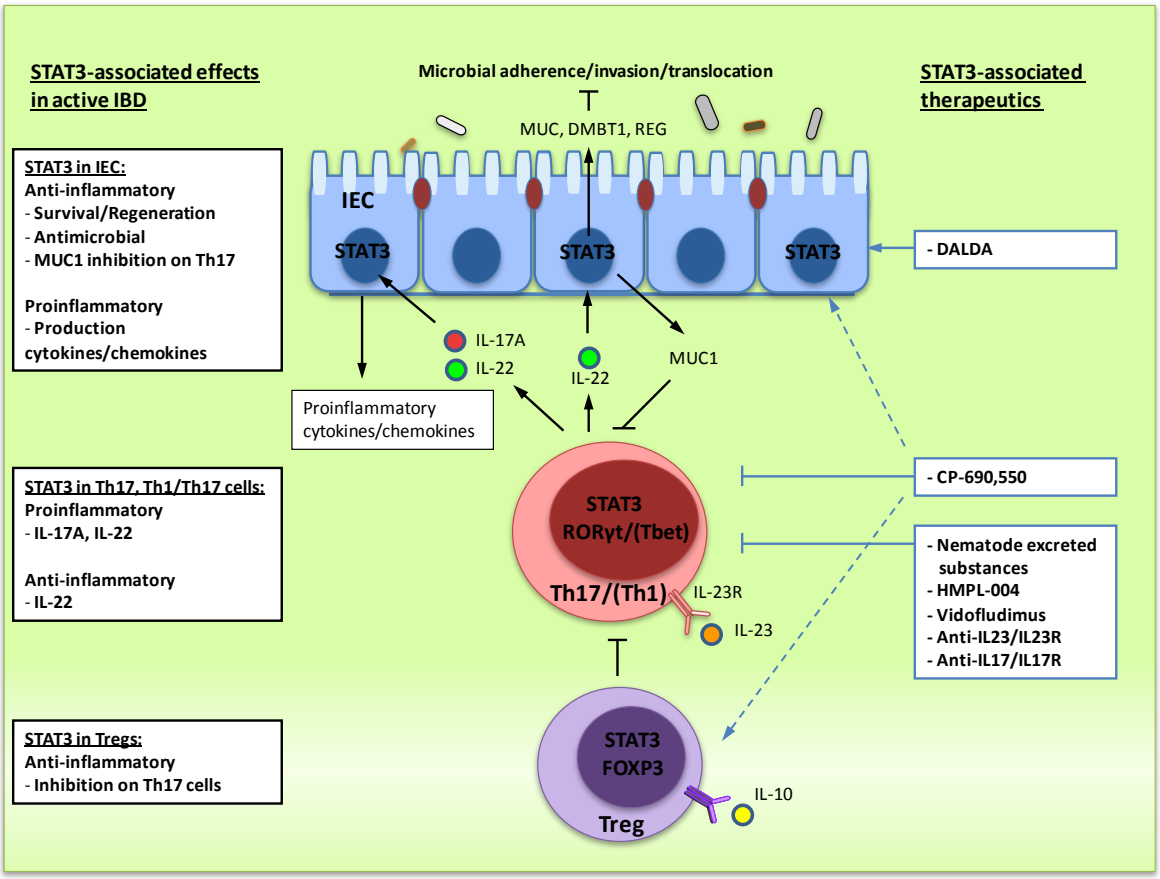


Figure 2. Kaleidoscopic view on the central and bivalent role of STAT3 in IEC and T-cells during mucosal inflammation in IBD.

In intestine epithelial cells (IEC), the activation of STAT3 can have both pro- and anti-inflammatory effects. IL-22 induces STAT3-dependent IEC survival, regeneration and the production of antimicrobial compounds that directly limit the bacterial component or the inflammatory T-cell component of the inflammatory response. IL-22 induced STAT3 activation can also induce the production and release of a variety of proinflammatory cytokines and chemokines further enhancing IL-17A driven inflammation. DALDA is shown to enhance IEC survival en regeneration in a STAT3-dependent fashion and has therapeutic effects in IBD mouse models. STAT3 is activated in Th17 and Th1/Th17 cells by a variety of cytokines (ie IL-23, IL-6 and IL-21) driving the differentiation and survival of these cells. Although these cells are known for their proinflammatory function, the bivalent function of their produced cytokines, like IL-22, also suggest some inflammation limiting mechanisms to be in place. A variety of new drugs is shown to inhibit mucosal inflammation which was associated with effects on this inflammatory subset of T-cells. IL-10-induced STAT3 is important for the regulatory function of Tregs on Th17 cells. The wide expression, bivalent role and its downstream effects make STAT3 an interesting but difficult therapeutic target. CP-690,550 is a JAK-inhibitor that could affect both the proinflammatory Th17 and Th1/Th17 cells but also likely to have the general protective function of STAT3 in IEC and the inhibitory effects of Tregs, which needs to be further proved.

absolute, as blocking the Th17 cytokine does not prevent colitis in spontaneous mouse colitis model with lacking STAT3 in macrophages and granulocytes. In this model, whose existence dramatically demonstrates the pleiotropic nature of STAT3 action in mucosal immunology, a lack of regulatory signaling through STAT3 in the myeloid compartment drives virulent phagocyte overactivation. In this colitis model, IFN-γ/IL-17 double positive T-cells that express both T-bet and ROR are present, whose generation seems to depend on IL-23 [32]. It further supports the increasing body of evidence that this cytokine might constitute an interesting therapeutic target. Another interesting therapeutic possibility is HMPL-004, an extract of the herbaceous plant Andrographis Paniculata, which was tested at 300 mg/kg, p.o. in a T cell-dependent murine colitis model and demonstrated to inhibit the development of colitis by affecting T cell proliferation as well as Th1/Th17 differentiation [33]. Nevertheless, as STAT3 action in the mucosal compartment contains a plethora of both pro- as well as anti-inflammatory actions, testing of medication will always contain a large trial and error component and does not always lend itself easily to rational prediction.

Conclusion

Both the recent increases to the compendium of biomedical literature as well as the novel data presented at the 2011 DDW display an increasing momentum towards a central organizing role for STAT3 in the molecular coordination of mucosal immune responses (Figure 2). The transcription factor is important in almost all cell types in the intestine, the epithelial compartment (where it suppresses and terminates immune responses and allows regeneration), the innate immune system (where it provides essential feedback to prevent fulminant infection), the Th17 cell (a highly aggressive pro-inflammatory cell type whose generation requires STAT3) and the regulatory T cell (which require STAT3 for efficient regulation of other cell types) compartment, amongst others. In addition, the role of inflammation per se in relation to pathology is not clear-cut. Small time infections require efficient immune responses to prevent bacteria from fester, but later during infection down modulation of inflammation is necessary for regenerative responses and return to steady state, whereas resolution of chronic infection requires strong activation of immune responses yet again. Together this provides a kaleidoscopic picture as to the role of STAT3 activation in the intestine and strongly suggests that STAT3-based therapy will need to be highly targeted for optimal results.

References

1. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009;9:798-809.
2. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol 2007;7:41-51.
3. Ferguson LR, Han DY, Fraser AG, Huebner C, Lam WJ, Morgan AR, Duan H, Karunasinghe N. Genetic factors in chronic inflammation: single nucleotide polymorphisms in the STAT-JAK pathway, susceptibility to DNA damage and Crohn's disease in a New Zealand population. Mutat Res;690:108-15.
4. Hundorfean G, Neurath MF, Mudter J. Functional relevance of T helper 17 (Th17) cells and the IL-17 cytokine family in inflammatory bowel disease. Inflamm Bowel Dis.
5. Burchill MA, Yang J, Vang KB, Farrar MA. Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. Immunol Lett 2007;114:1-8.
6. Braat H, Stokkers P, Hommes T, Cohn D, Vogels E, Pronk I, Spek A, van Kampen A, van Deventer S, Peppelenbosch M, Hommes D. Consequence of functional Nod2 and Tlr4 mutations on gene transcription in Crohn's disease patients. J Mol Med (Berl) 2005;83:601-9.
7. Kuhn B, Jurickova I, Guthery SL, Denson L. The Inflammatory Bowel Disease (IBD) Signal Transducer and Activator of Transcription 3(STAT3) Risk Allele is Associated With Increased Interleukin-6(IL-6)/STAT3 Activation in Epstein-Barr Virus(EBV) Transformed B Lymphocytes From Pediatric IBD Patients. Gastroenterology;140:S-1-S-2.
8. Raja Ali RA, Hartnett L, Lee JM, Egan LJ. The Level of Serum Cytokines, Chemokines and the Colonic STAT3 Activation Pattern in IBD Patients Are Influenced by Disease Activity and Duration. Gastroenterology;140:S-842.
9. Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, Kuipers EJ, van der Woude CJ. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut;59:227-35.
10. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.
11. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91-102.

12. Wittkopf N, Billmeier U, G 眉 nther C, Wirtz SJ, Neurath MF, Becker C. STAT3-Mediated Production of Antimicrobial Peptides by Intestinal Epithelial Cells Helps Controlling C. rodentium Induced Infection. Gastroenterology;140:S-325.
13. Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, Lehr HA, Hirth S, Weigmann B, Wirtz S, Ouyang W, Neurath MF, Becker C. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med 2009;206:1465-72.
14. Neufert C, Pickert G, Zheng Y, Wittkopf N, Warntjen M, Nikolaev A, Ouyang W, Neurath MF, Becker C. Activation of epithelial STAT3 regulates intestinal homeostasis. Cell Cycle;9:652-5.
15. Ose T, Kadowaki Y, Fukuhara H, Kazumori H, Ishihara S, Udagawa J, Otani H, Takasawa S, Okamoto H, Kinoshita Y. Reg I-knockout mice reveal its role in regulation of cell growth that is required in generation and maintenance of the villous structure of small intestine. Oncogene 2007;26:349-59.
16. Nishida A, Lau CW, Mizoguchi E, Mizoguchi A. Contribution of a Membrane-Bound Mucin for Controlling TH17 Responses. Gastroenterology;140:S-149-S-150.
17. Goldsmith JR, Uronis JM, Gipson G, Jobin C. Mu Opioid Signaling and STAT3 Activation Protects Against Acute Murine Intestinal Injury in Mice. Gastroenterology;140:S-654.
18. Li Y, de Haar C, Nuij VJ, Baars JE, Kuipers EJ, van der Woude CJ. SOCS3 Expression is a Predictive Factor of Relapse of Mucosal Inflammation in Chronic UC. Gastroenterology;140:S-1.
19. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, Takahashi H, Sun HW, Kanno Y, Powrie F, O'Shea JJ. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. Immunity;32:605-15.
20. Liu X, Lee YS, Yu CR, Egwuagu CE. Loss of STAT3 in CD4+ T cells prevents development of experimental autoimmune diseases. J Immunol 2008;180:6070-6.
21. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity 2004;21:467-76.
22. van Lierop PP, de Haar C, Lindenbergh-Kortleve DJ, Simons-Oosterhuis Y, van Rijt LS, Lambrecht BN, Escher JC, Samsom JN, Nieuwenhuis EE. T-cell regulation of neutrophil infiltrate at the early stages of a murine colitis model. Inflamm Bowel Dis;16:442-51.
23. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, Jack RS, Wunderlich FT, Bruning JC, Muller W, Rudensky AY. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity;34:566-78.
24. Metwali A, Winckler S, Vennalaganti RP, Ince MN, Urban JF, Elliott DE. Helminth-Mediated Suppression of TH17 Cell Function is Associated With Decreased IL6 Signaling. Gastroenterology;140:S-177.

25. Fitzpatrick LR, Small JS, Ammendola A. Inhibition of IL-17 Release by the Novel Anti-Inflammatory Drug Vidofludimus Involves Attenuation of STAT3 and NF-kappa B Signaling Pathways in Murine Splenocytes and Hapten-Induced Colitis. Gastroenterology;140:S-837.
26. Sandborn WJ, Ghosh S, Panes J, Vranic I, Su C, Spanton J, Niezychowski W. Phase 2 Study of CP-690,550, an Oral Janus Kinase Inhibitor, in Active Ulcerative Colitis. Gastroenterology;140:S-110.
27. Sandborn WJ, Ghosh S, Panes J, Vranic I, Spanton J, Niezychowski W. Phase 2 Randomized Study of CP-690,550, an Oral Janus Kinase Inhibitor, in Active Crohn's Disease. Gastroenterology;140:S-124.
28. Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsup JW, Warner JD, Tanaka M, Steward-Tharp SM, Gadina M, Thomas CJ, Minnerly JC, Storer CE, LaBranche TP, Radi ZA, Dowty ME, Head RD, Meyer DM, Kishore N, O'Shea JJ. Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). J Immunol;186:4234-43.
29. Meelu P, Awasthi A, Kuchroo VK, Yajnik V. Deficiency in IL23R Accelerates Wound Healing in Experimental Colitis. Gastroenterology;140:S-142-S-143.
30. Clark H, Pidasheva S, Trifari S, Hackney J, Ma Y, Smith A, Sohn S, Spits H, Little R, Behrens T, Honigberg L, Ghilardi N. Human IL23R R381Q Genetic Variant Implicated in Crohn's Disease Pathogenesis is Hypomorphic Resulting in Reduced Function of the Receptor. Gastroenterology;140:S-273.
31. van der Woude CJ, Hommes DW. Biologics in Crohn's disease: searching indicators for outcome. Expert Opin Biol Ther 2007;7:1233-43.
32. Billmeier U, Neurath MF, Wirtz SJ. IL-23 is a Key Pathogenic Inducer of Chronic Intestinal Inflammation. Gastroenterology;140:S-836.
33. Michelsen KS, Wong MH, Ko B, Thomas LS, Dhall D, Targan SR. Mode of Action of HMPL-004 (Andrographis Paniculata Extract) in the Prevention of Murine Colitis: Blockage of T Cell Proliferation and TH1/ TH17 Responses. Gastroenterology;140:S-177.

Chapter III

Disease-related expression of the IL-6/STAT3/SOCS3 signaling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis

Yi Li[1], Colin de Haar[1], Min Chen[2], Jasper Deuring[1], Monique M. Gerrits[1], Ron Smits[1], Bing Xia[3], Ernst J. Kuipers[1], C. Janneke van der Woude[1].

1. Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, Netherlands.
2. Department of Geriatric, Zhongnan Hospital, Wuhan University, China.
3. Department of Gastroenterology, Internal Medicine, Zhongnan Hospital, Wuhan University, China.

Gut 2011;59:277-35.

ABSTRACT

Background and Aims: Mouse models showed that IL-6 stimulates survival, proliferation and progression to cancer of intestinal epithelial cells via the activation of STAT3. To apply in human data, we investigated the expression of IL-6/p-STAT3/SOCS3 in biopsies from patients with ulcerative colitis (UC) and UC related-colorectal cancer (CRC) progression.

Methods: Biopsies from patients with inactive UC (n=18), active UC (n=28), UC with low-grade dysplasia (LGD) (n=9), UC with high-grade dysplasia (HGD) (n=7), UC-CRC (n=11) and sporadic CRC (n=14) were included. Biopsies (n=9) from patients without colonic abnormalities served as control. The protein expression of IL-6, p-STAT3 and SOCS3 was determined immunohistochemically.

Results: Patients with active UC had significantly more IL-6 and p-STAT3 positive epithelial cells than both inactive UC patients and controls (strong positive IL-6: 53.6%, 11.1% and 0% respectively; p-STAT3: 64.3%, 22.2% and 11.1% respectively; all $p \leq 0.012$). SOCS3-positive cells were significantly increased in colonic epithelium of both inactive and active UC compared with controls (strong positive: 94.4%, 96.4% and 11.1% respectively; both $p<0.001$). In subsequent stages of dysplasia and cancer, significant more epithelial cells expressed IL-6 and p-STAT3 compared with controls (strong positive IL-6: 72.7% and 0% respectively; p-STAT3: 54.5% and 11.1% respectively; both $p<0.05$), whereas the proportion of strongly SOCS3-positive cells in this progression reduced (LGD 33.3%; HGD 14.3%; UC-CRC 9.1%). In addition, methylation of the SOCS3 gene was detected in epithelial cells from UC-CRC biopsy specimens.

Conclusion: The importance of IL-6/p-STAT3 in patients with inflammation-induced CRC was demonstrated. Moreover, SOCS3 may be involved in UC pathogenesis and the absence of SOCS3 seems critical for CRC progression.

KEYWORDS: UC; CRC; IL-6; STAT3; SOCS3

INTRODUCTION

Ulcerative colitis (UC) is a phenotype of inflammatory bowel disease (IBD) characterized by a chronic recurrent colonic inflammation, which is associated with an increased risk of developing colorectal cancer (CRC) [1, 2]. Chronic inflammation in the intestine leads to damage of the epithelium. Locally produced cytokines cause inflammation and stimulate the proliferation of crypt cells to compensate for the loss of epithelial cells. This chronically stimulated state of the epithelium may eventually lead to the development of UC-CRC [3-5]. Interleukin (IL)-6 is a proinflammatory cytokine that has been detected in both colon biopsies and serum samples of UC patients, and the levels of IL-6 correlated with the disease severity [6, 7].

Interestingly, unlike epithelial cells, most lamina propria T-cells of IBD patients do not express the membrane bound IL-6R. In addition to the membrane bound IL-6R, IL-6 can act via soluble IL-6 receptor (sIL-6R) in cells expressing gp130 in a process known as trans-signaling. Several observations support an important role of IL-6 trans-signaling in the development and maintenance of IBD [8, 9], and the specific inhibitors of sIL-6R-signaling pathway are utilized to ameliorate IBD [10]. The IL-6 (trans)-signaling initiates the dimerization and activation of receptor associated Janus kinases (JAK). Subsequently, the activated JAK phosphorylates the tyrosine residues on the cytoplasmic tail of the receptor, providing a docking site for proteins with Src homology 2 (SH2) domains like the signal transducers and activators of transcription (STAT)3 protein [8]. Next, STAT3 binds to the receptor and is activated by phosphorylation through JAK, leading to homo- and heterodimerization of phosphorylated STAT3 (p-STAT3) with translocation to the nucleus, where they interact with specific DNA sequences and induce transcription of target genes.

One of the targets genes of the IL-6/p-STAT3 pathway is the suppressor of cytokine signaling 3 (SOCS3). SOCS are a family of proteins that regulate negative feedback to the JAK/STAT cytokine signaling cascade [11, 12]. There are eight members of the SOCS family: the cytokine inducible SH2 domain-containing protein (CIS) and SOCS1 to SOCS7 [13]. SOCS3 protein is expressed in intestinal epithelial cells and lamina propria in mouse colitis models and in UC patients [14, 15], suggesting a role in the pathogenesis of colitis [16].

In addition to some human observations [17, 18], there is strong experimental evidence from mouse studies that suggest a key role for IL-6/STAT3/SOCS3 in regulating intestinal

epithelial cell homeostasis. These studies also imply that an imbalance between SOCS3 expression and IL-6/p-STAT3 signaling will lead to inflammation [10] and eventually to inflammation-induced carcinogenesis [19]. The latter observations support the idea that IL-6 signaling is a potential therapeutic target in IBD and IBD-related CRC [9, 20]. There is however a general lack of clinical data from UC and UC-CRC patients to support this. We studied the expression of IL-6/p-STAT3 and SOCS3 in colon biopsies from patients with UC in different phases of the disease. Our study provides clinical data supporting animal model-derived mechanisms on IL-6/STAT3, which suggests the involvement of SOCS3 in UC-related cancer, and supports the IL-6 signaling pathway as a therapeutic target in UC and UC-related CRC.

MATERIALS AND METHODS

Patients and biopsies

To investigate the involvement of IL-6/STAT3/SOCS3 pathway in UC and UC related carcinogenesis, we collected biopsies from 9 healthy controls, 18 patients with inactive UC, 28 patients with 28 active UC, 9 UC patients with low-grade dysplasia (LGD), 7 UC patients with high-grade dysplasia (HGD) (n=7), 11 patients with UC-CRC and 14 patients with sporadic CRC. Colon biopsies were obtained during colonoscopy performed for clinical care and surveillance studies after informed consent. The diagnosis of UC was based on conventional clinical, endoscopic and pathohistological criteria as described by Lennard-Jones [21]. Colonic biopsies from non-UC patients without abnormalities at colonoscopy and histories of gastrointestinal disease served as controls. An independent pathologist reconfirmed colitis, dysplasia (low grade or high grade) and colorectal cancer in the biopsies of UC patients based on histological scoring. In addition, the colitis severity was graded from 0-5 according to Geboes-criteria [22].

Immunohistochemistry

All the biopsies were fixed in 10% formalin and embedded in paraffin. Hematoxylin eosin (HE) staining on paraffin embedded biopsies was performed to evaluate the severity of colitis. Four micrometer sections of paraffin embedded tissues were mounted onto slides of Superfrost Plus (Thermo Scientific), these sections were deparaffinized in xylene, and rehydrated. The endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 15 min at room temperature. Antigen retrieval was performed by boiling in preheated

buffer (Tris/EDTA pH 9.0 for SOCS3 or IL-6 and EDTA pH 8.0 for p-STAT3) for 15 min at 200W in a microwave. Next, slides were transferred to Shandon chambers and blocked by 10% normal human serum, 10% goat serum in phosphate-buffered saline (PBS) pH 7.4 for SOCS3 and IL-6, 5% goat serum in Tris-buffered saline (TBS) and 0.1% Tween20 for p-STAT3 for 1 hour at room temperature. Monoclonal rabbit anti-p-STAT3 tyrosine 705 (cell signaling, #9145), polyclonal rabbit anti-SOCS3, and polyclonal rabbit anti-IL-6 (both abcam ab53984, ab6672) were diluted in 10% blocking buffer at 1:50; 1:200; 1: 200 respectively and incubated at 4 °C overnight. Envision goat anti-rabbit-HRP (DAKO) was used as secondary antibody. Immunoreactions were detected using 3-3-diaminobenzidine (10 mg/ml) in Tris-HCl 0.05 M pH 7.6 containing imidazol stock (0.068g/10ml) and 0.03% H_2O_2, followed by counterstaining with hematoxylin. Negative controls were performed by staining with the secondary antibody only.

Immunohistochemical scoring

Two experienced scientists scored the staining of epithelial cells in a blinded manner. The percentage of cells that stained positive (immunoreactivity above background) in the area consistent with that used for diagnoses was quantified. The immunoreactivity was defined in epithelial cells for SOCS3 as: strong positive (>60% of the cell population in the biopsy stains positive), positive (30%-60%) or mild (<30%) cytoplasm staining; in epithelial cells and non-epithelial cells for p-STAT3 as: strong positive (>20%), positive (10%-20%) or negative (<10%) nuclear staining. The expression of IL-6 cytoplasm staining was evaluated in both epithelial and non-epithelial cells, and defined as strong positive (>60%), positive (30%-60%) or mild (<30%) in epithelial cells and strong positive (>70%), positive (40%-70%) or mild (<40%) in non-epithelial cells.

Macro-dissection and DNA processing

Intestinal epithelial cells and/or adenomatous tissue were isolated from paraffin-embedded intestinal biopsy specimens (three controls, three inactive UC, three active UC and four UC-CRC) using macro-dissection.23 DNA was isolated using a Fixed-Tissue Genomic DNA Purification kit (Cat. #MD1180, Promega, USA) and bisulphate-modified using the EZ DNA methylation kit (ZYMO Research, USA). Universal methylated and unmethylated DNA (Millipore, USA) were used as controls.

Methylation-specific PCR (MSP)

MSP was performed on bisulphite-treated genomic DNA using specific primers as previously described.24 Sequences of methylation-specific SOCS3 primers were 59-GGAGATTTTAGGTTTTCGGAATATTTC-39 (forward) and 59-CCCCCGAAACTACCTAAACGCCG-39 (reverse), corresponding to -525 through -499 and -384 through -406, respectively. Sequences of the unmethylation-specific primers were 59-GTTGGAGATTTTAGGTTTTTGGAATATTTT-39 (forward) and 59-AAACCCCCAAAACTACCTAAACACCA-39 (reverse). A PCR was performed in a 25 ml volume containing 40 ng bisulphate-modified DNA, 53 Gold Taq buffer, 0.2 mMdNTPs, 10 pmol specific primer mix (forward and reverse primers) and one unit Gold Taq enzyme (Promega). The PCR condition was set up as 958C for 5 min, followed by 40 cycles of 958C for 30 s, 578C for 30 s and 728C for 30 s, and ending with a final extension of 728C for 5 min. The PCR products were visualised on a 2% agarose gel using ethidium bromide and UV illumination.

Statistical analysis

Statistic analysis was performed using SPSS software version 15.0. Data were presented as mean±SD. The relationship between each group for SOCS3, p-STAT3 and IL-6 was determined by chi-square. The association between IL-6/p-STAT3 positive cells and severity of disease was demonstrated by Spearman correlation. P value<0.05 was considered statistically significant.

RESULTS

Patients' characteristics

To investigate the possible involvement of the IL-6/STAT3/SOCS3 signaling pathway in UC and UC-related carcinogenesis we studied the expression of these proteins in biopsies from UC patients in different stages. As shown in Table 1, both the mean age and duration of disease were significantly higher in the UC-CRC group compared with the other disease status groups ($p<0.01$), which is in accordance increasing risk of developing UC-CRC overtime. In addition, the mean age of sporadic CRC patients was also significantly higher than those of patients in other groups ($p<0.01$). There were no other significantly differences among the other groups including therapy. Inactive UC defines patients in long-term remission with no endoscopically signs of colitis and a Geboes

histology score of 0 or 1. Active UC defines patients in acute phase of the disease with endoscopically visible signs of colitis and a Geboes histology score of 2 or higher.

Table 1. Patients' characteristics

	Healthy control (N=9)	**Inactive UC (N=18)**	**Active UC (N=28)**	**LGD (N=9)**	**HGD (N=7)**	**UC-CRC (N=11)**	**Sporadic CRC (N=14)**
Gender							
Male	6	8	15	8	5	7	8
Female	3	10	13	1	2	4	6
Mean age, yr±SD	39.6±17.8	43.3±13.5	41.2±15.6	41.0±10.5	39.7±10.7	(56.1±16.5)[a]	(68.3±12.2)[a]
Mean duration of disease, yr±SD	-	16.8±10.2	11.0±9.5	14.8±5.5	14.6±9.4	(22.3±5.8)[b]	NA
Extent of disease							
Extensive colitis	-	0	4	6	4	5	7
Left side colitis	-	11	18	1	1	2	2
Proctitis	-	7	5	2	2	4	5
Therapy							
Amino-salicylates	-	7	14	3	1	0	-
Corticosteroids	-	6	13	4	4	3	-
Immune-suppressives	-	6	11	4	4	7	-
Biologicals	-	3	1	1	1	0	-
None	-	3	6	0	2	4	-
Pathological severity							
Grade 0-1	-	18	0	6	1	2	
Grade 2-5	-	0	25	0	3	6	

a. The mean age of patients in UC-CRC group (56.1±16.5) and sporadic CRC group (68.3±12.2) were significantly larger than those of other UC-CRC precursor groups ($p<0.01$).

b. The mean duration of disease of patients in UC-CRC patients (22.3±5.8) was significantly longer than that of patients in other groups ($p<0.01$).

IL-6 expression in active UC and inactive UC patients

Immunohistochemistry (IHC) was performed to determine the protein expression of IL-6 in different cells and in different phases of disease (Figure 1A). The colonic epithelial cells revealed significantly more IL-6 positive cells in the biopsies from patients with active UC compared with inactive UC ($p=0.011$) or controls ($p<0.001$) (Figure 2A). In addition, there were significantly more IL-6 positive cells in the non-epithelial cells in biopsies from patients with active UC compared with those with inactive UC ($p<0.001$) or controls ($p<0.001$) (Figure 2B). Figures 2c and d show a positive correlation between IL-6 expression and the severity of colitis in both epithelial ($r=0.56$, $p=0.0001$) and non-epithelial cells ($r=0.62$, $p<0.0001$) when biopsies with inactive and active UC are combined.

P-STAT3 and SOCS3 expression in active UC and inactive UC patients

To see whether IL-6 expression correlates with the activation of STAT3/SOCS3 signaling pathway in the different biopsies, we investigated p-STAT3 and SOCS3 expression (Figure 1B and C). As shown in figure 3A, significantly more p-STAT3 positive epithelial cells could be observed in biopsies with active UC compared with both inactive UC ($p=0.007$) and controls ($p=0.012$). The number of p-STAT3 positive epithelial cells and non-epithelial cells both correlated positively with the severity of colitis (Figure 3B. $r=0.44$, $p=0.001$; Figure 3C. $r=0.51$, $p<0.001$).

Since non-epithelial cells (eg: leukocytes) have been suggested to be the major source of IL-6 in the inflamed intestine, we correlated the expression of IL-6 in non-epithelial cells to the p-STAT3 expression in epithelial cells. As shown in Figure 3D, IL-6 positive non-epithelial cells correlate with p-STAT3 positive epithelial cells ($r=0.35$, $p=0.016$).

SOCS3 positive epithelial cells were uniformly increased in both inactive and active UC biopsies compared with those of the control (inactive UC vs. normal control: $p<0.001$; active UC vs. normal control: $p<0.001$) (Figure 3A). So, whereas no positive correlation between SOCS3 expression and severity of colitis was found in the epithelial cells, a positive correlation was found between SOCS3 positive non-epithelial cells and the severity of the colitis (Figure. 3F, $r=0.42$, $p=0.005$).

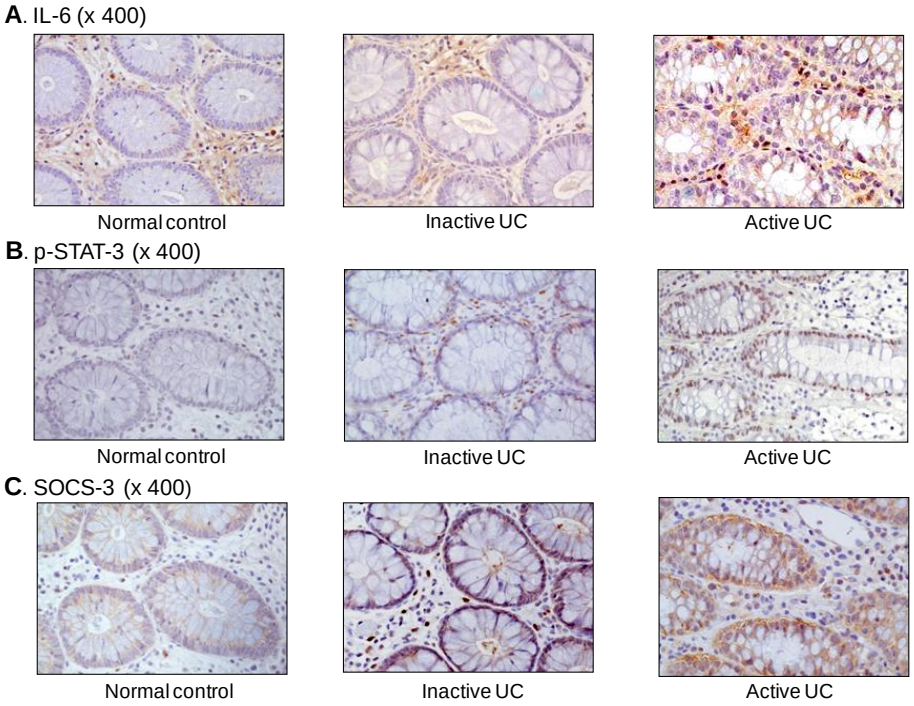


Figure 1. Immunohistochemical staining of IL-6 (cytoplasm staining), p-STAT3 (nucleus staining) and SOCS3 (cytoplasm staining) in colonic bi0psies of control, inactive UC and active UC patients.

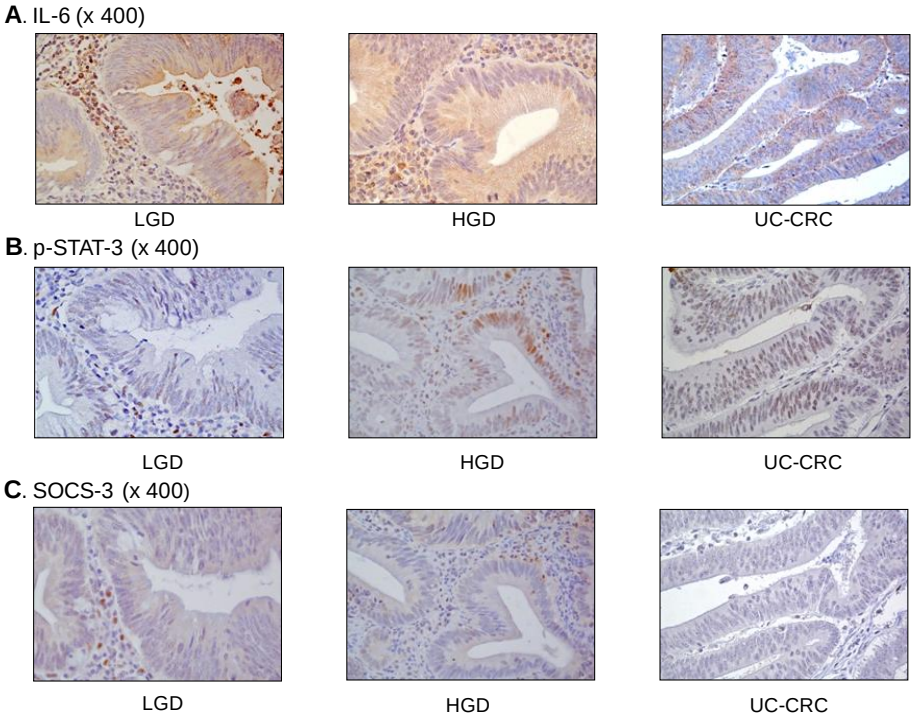


Figure 4. Immunohistochemical staining of IL-6, p-STAT3 and SOCS3 in colonic biopsies of UC-patients with LGD, HGD and CRC.

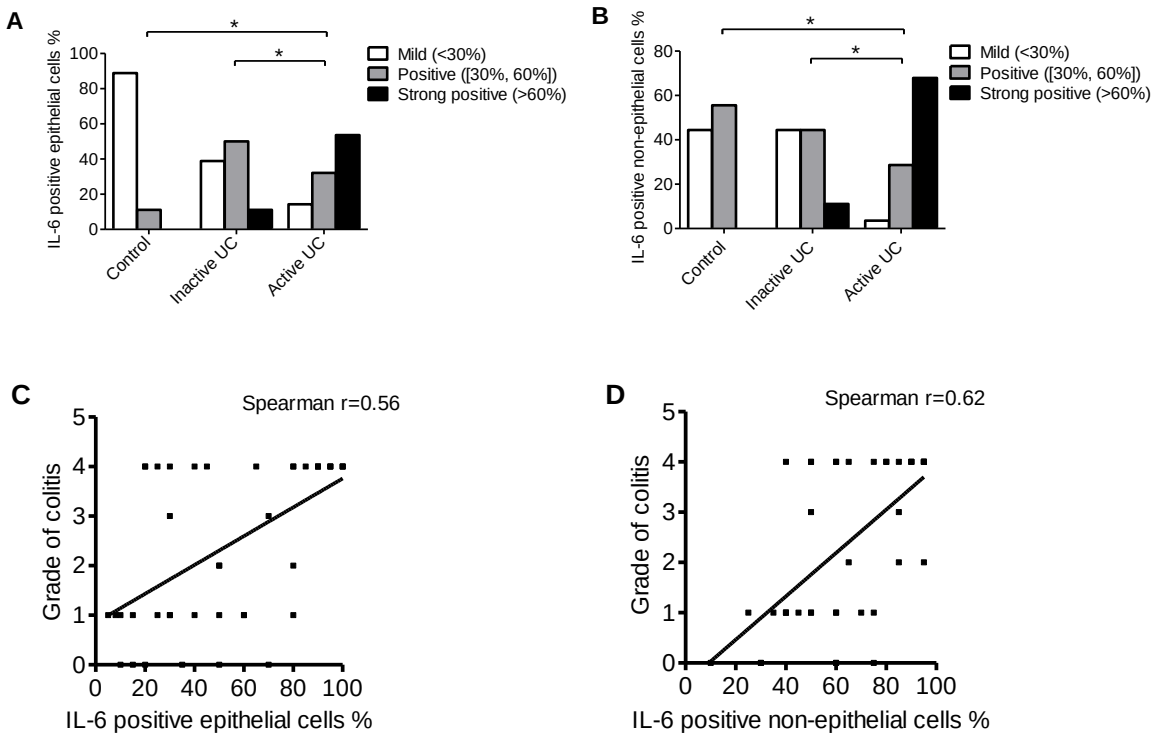


Figure 2. Analysis of the immunohistochemical IL-6 staining in (A) colonic epithelial cells and (B) non-epithelial cells from control, inactive UC and active UC biopsies. * IL-6 expressed on significantly more positive cells in active UC than in both inactive UC and control groups. In epithelial cells: active UC vs. inactive UC: p=0.011; active UC vs. normal control: p<0.001. In non-epithelial cells: active UC vs. inactive UC: p<0.001; active UC vs. normal control: p<0.001. Correlations between IL-6 positive (C) epithelial/(D) non-epithelial cells and the severity of colitis in UC patients (r=0.56, p=0.0001/r=0.62, p<0.0001).

IL-6 expression in colonic epithelial cells of patients during UC-CRC progression

Since IL-6 has both proinflammatory as well as carcinogenic potential, we investigate IL-6 expression in biopsies from patients in different stages of progression to UC related CRC (LGD, HGD and UC-CRC) (Figure 4A), and analyzed IL-6 levels in epithelial and non-epithelial cells. Significantly more IL-6 positive epithelial cells were observed in all stages of progression to UC-related CRC compared with IL-6 in the controls (p<0.001) (Figure 5A). Although a trend was observed for the non-epithelial cells, there was no significant difference in IL-6 expression between biopsies from patients with UC-CRC progression and controls (data not shown).

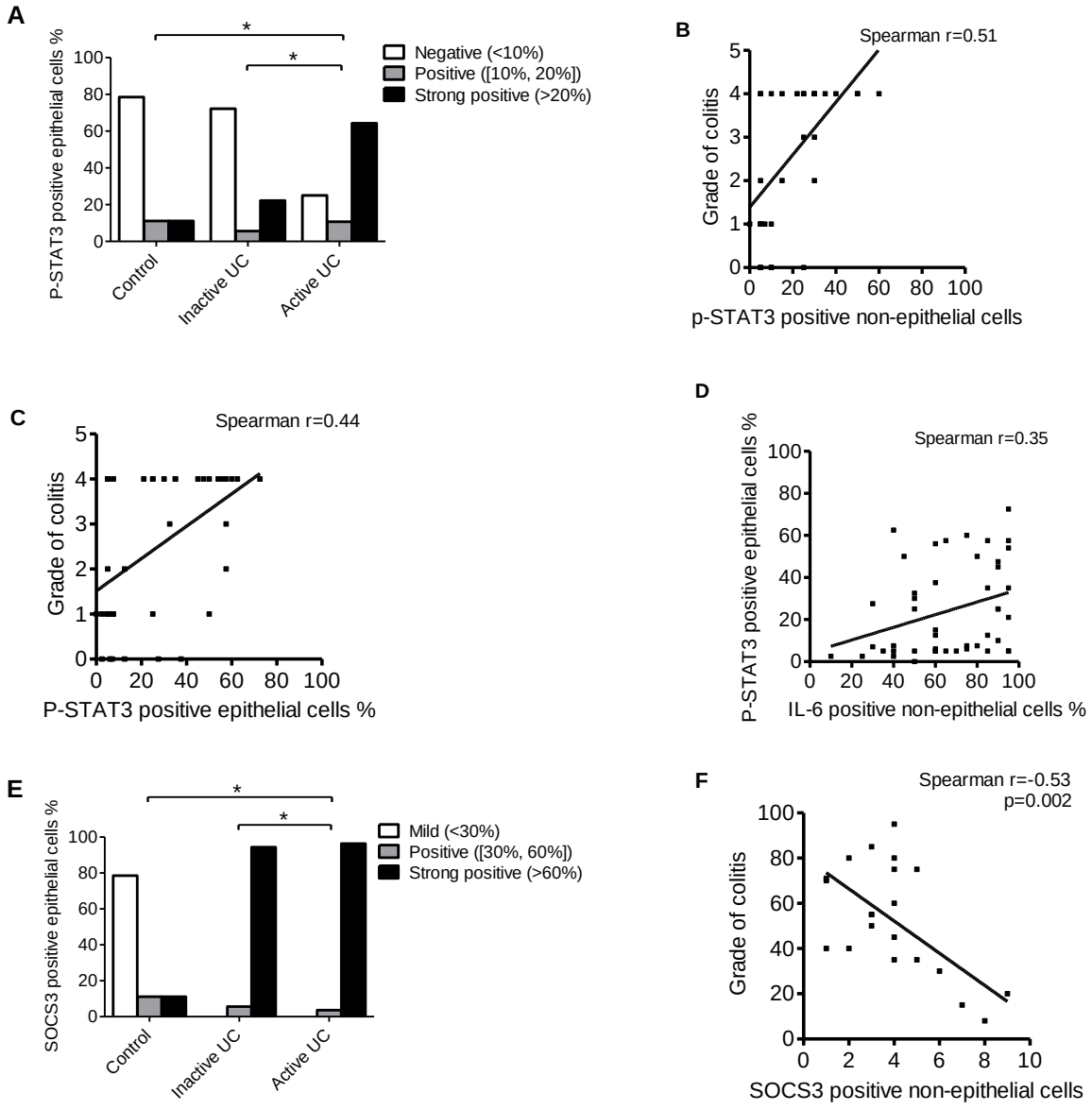


Figure 3. (A) The differences in p-STAT3 protein expression in IEC from control, inactive UC and active UC biopsies. * P-STAT3 expressed on significantly more cells in active UC than in inactive UC and control groups (active UC vs. inactive UC: p=0.007; active UC vs. normal control: p=0.012). (B) The correlation between p-STAT3 positive epithelial cells and severity of colitis of UC patients (r=0.44, p=0.0009). (C) The correlation between p-STAT3 positive non-epithelial cells and severity of colitis of UC patients (r=0.51, p<0.001). (D) The correlation between IL-6 positive non-epithelial cells and p-STAT3 positive epithelial cells (r=0.35, p=0.016). (E) The differences in SOCS3 protein expression in IEC from control, inactive UC and active UC biopsies. * SOCS3 expressed on significantly more cells in inactive and active UC than those of controls (inactive UC vs. normal control: p<0.001; active UC vs. normal control: p<0.001). (F) The correlation between SOCS3 positive non-epithelial cells and severity of colitis of UC patients (r=0.42, p=0.005).

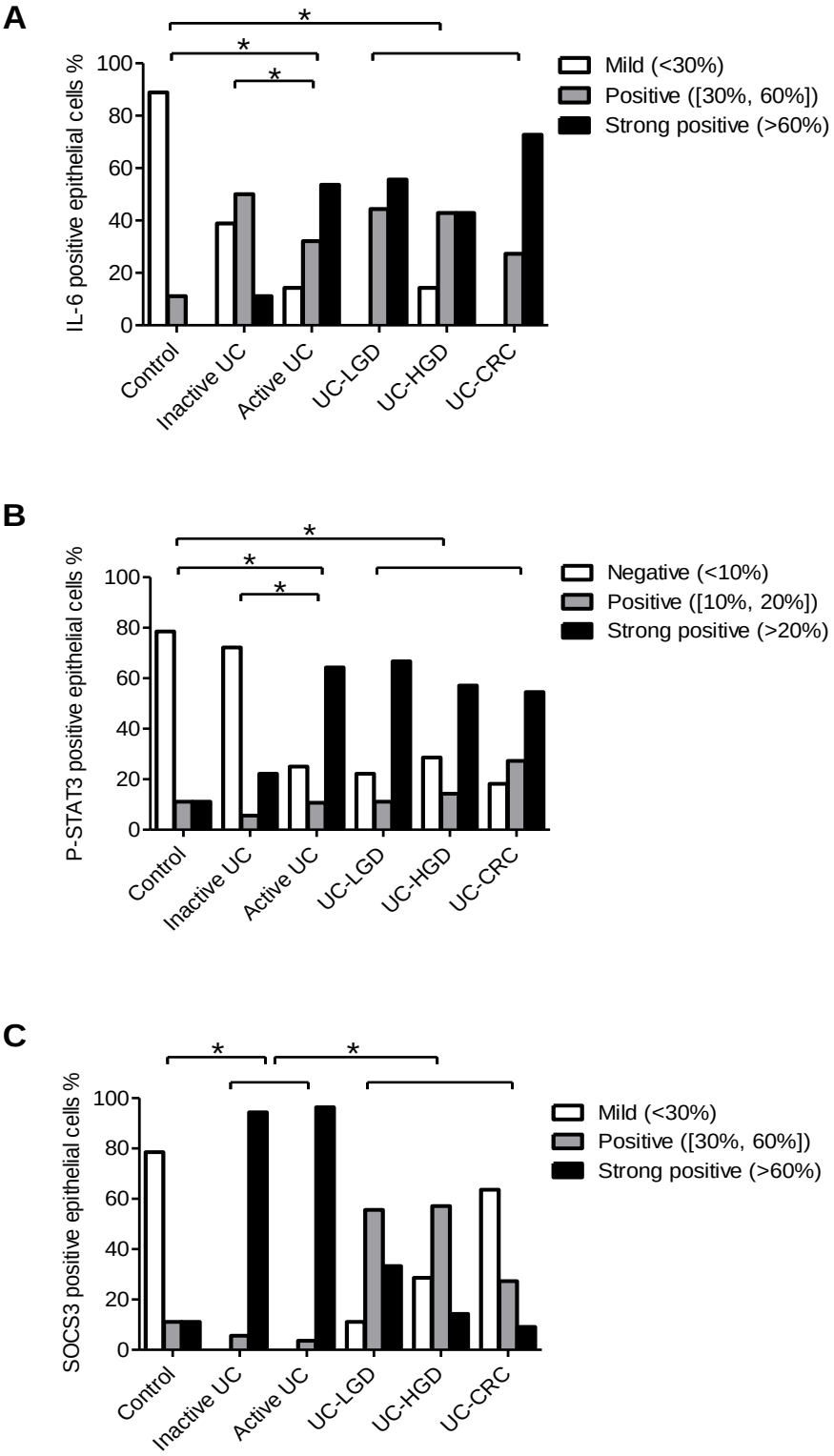


Figure 5. (A) Analysis of the IL-6 protein expression in epithelial cells in the progression to UC-CRC. Significantly more IL-6 positive cells were observed in UC-CRC progression than in controls

(p<0.001). (B) P-STAT3 protein expression in epithelial cells in the progression to UC-CRC. Significantly more p-STAT3 positive cells were observed in UC-CRC progression than in controls (p=0.010). (C) SOCS3 protein expression in epithelial cells in the progression to UC-CRC. SOCS3 expression was significantly reduced in the progression to UC-CRC (LGD, HGD and UC-CRC) compared with UC (inactive and active UC) patients (p<0.001).

P-STAT3 and SOCS3 in colonic epithelial cells of patients during the UC-CRC progression

Since chronic inflammation in UC, partially mediated by IL-6, can progress to CRC, we studied the downstream p-STAT3 and SOCS3 expression in the progression to UC-CRC (LGD, HGD and UC-CRC) and compared those data with the UC and normal control biopsies (Figure 4B and C). The number of p-STAT3 positive cells was, like in active UC, significantly increased in colonic epithelial cells during progression (LGD, HGD and UC-CRC) when compared with the control group (p=0.010) (Figure 5B). In contrast, the number of SOCS3 positive epithelial cells was significantly reduced during UC-CRC progression compared with UC (inactive and active UC) patients (p<0.001) (Figure 5C). SOCS3 expression was reduced during UC-CRC progression from LGD to CRC, and the reduced expression was strictly found at the dysplastic and cancer areas of the patients.

P-STAT3 and SOCS3 expression in UC-CRC and sporadic CRC

Although p-STAT3 expression was lower and SOCS3 expression was higher in sporadic CRC compared with UC-CRC group, no significant difference was detected for SOCS3 and p-STAT3 expression in these two groups (data not shown).

SOCS3 gene methylation in UC-CRC

To assess whether SOCS3 methylation is involved in the different phases of disease, we performed MSP on epithelial cellderived and bisulphate-treated genomic DNA in a subset of our samples. As shown in figure 6, SOCS3 methylation was detected in all the investigated samples of patients with UC-CRC. SOCS3 methylation was not detected in the normal control biopsy specimens and in biopsy specimens from patients with inactive UC. In active UC, SOCS3 methylation was detected in only one of the biopsy specimens.

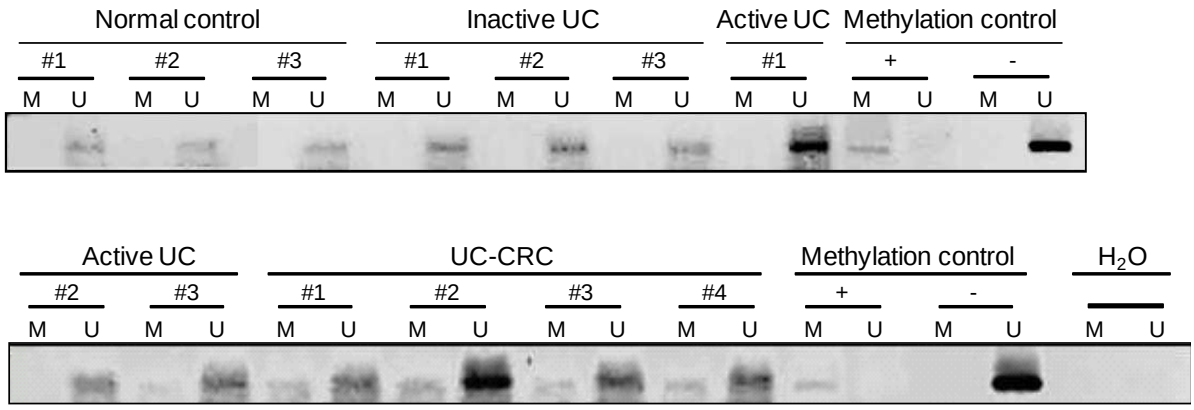


Figure 6. Methylation-specific PCR analysis of the suppressor of cytokine signalling 3 (SOCS3) gene in epithelial cells from healthy controls and from patients with inactive ulcerative colitis (UC), active UC and UC-colorectal cancer (CRC). PCR products in lanes marked "M" and "U" indicate the use of methylation and unmethylation-specific primers, respectively. Universal methylated and unmethylated DNA were used as a positive and negative controls. All the samples of normal control, inactive UC and active UC showed unmethylated alleles. One sample of active UC and all the samples of patients with UC-CRC showed alleles on both methylated and unmethylated lanes.

DISCUSSION

The IL-6/p-STAT3 pathway plays a crucial role in maintaining gastrointestinal homeostasis by regulating epithelial turnover and mucosal healing [23]. As such, inappropriate stimulation and (or) inhibition of this pathway may lead to dysregulation of gastrointestinal homeostasis causing colitis or even colorectal cancer.

We demonstrated significantly more IL-6/p-STAT3 expression in epithelial cells of biopsies from active UC patients compared with inactive UC patients and controls, which is in accordance with earlier studies [7, 14, 24]. We further show that the IL-6/p-STAT3 positive staining on both epithelial and non-epithelial cells correlates with the severity of the colitis. As demonstrated earlier, non-epithelial cells play an important role in the pathogenesis of chronic inflammation, as IL-6 signaling via p-STAT3 can affect potentially pathogenic laminia propria in IBD by inducing T-cell apoptosis resistance [25]. This increased IL-6 signaling in active UC supports the potential of IL-6 signaling blockade as a therapeutic strategy for IBD [7, 9, 26]. When compared with healthy controls, SOCS3 levels in biopsies from active UC patients were higher. Hypothetically, the increased SOCS3 expression in

these biopsies may be the downstream effect of increased IL-6/p-STAT3 signaling. In this phase, SOCS3 activity may not be sufficient to completely inhibit IL-6/STAT3 activation. This phase with overactive IL-6 signaling can be an ideal time point for therapeutic blockade of the IL-6 signaling pathway.

In mouse models, it was shown that the absence of gp130/IL-6/p-STAT3 signaling in intestinal epithelial cells does increase the sensitivity to dextran sodium sulfate (DSS) induced colitis, possibly due to the reduced epithelial cell survival and proliferation [27, 28]. As such, the high constitutive expression of SOCS3 in inactive UC epithelial cells we found could prevent normal IL-6/p-STAT3-mediated epithelial cell homeostasis and lead to enhanced sensitivity of these epithelial cells to inflammatory damage. Since IL-6/p-STAT3 are hardly detected in inactive UC, they cannot explain the high SOCS3 expression in the epithelial cells of patients with inactive UC, however, SOCS3 has been shown inducible in various tissues by a range of stimuli besides IL-6, including LPS, TNF-α, IFN and IL-10 [29-33]. We are currently conducting experiments to determine whether stimulators like LPS and IL-1β are involved in the increased SOCS3 expression in inactive UC. The lack of IL-6 signaling in inactive UC suggest that targeting IL-6 signaling pathway may not be effective as a remission or maintenance therapy in UC patients. In addition, the possible increase of the vulnerability of intestinal epithelial cells in the absence of IL-6 signaling further support this idea.

Studies of UC-related carcinogenesis have shown the involvement of IL-6 in this cancer development [28, 34, 35]. In the epithelial cells of UC-CRC biopsies, we found IL-6/p-STAT3 expression, this implicates that the epithelial cells are an important source of IL-6. In vitro data showed that IL-6 stimulates colonic epithelial cell proliferation and up-regulated anti-apoptotic molecules [35]. The IL-6 expression in epithelial cells, that we observed, may stimulate survival and tumor progression in an autocrine way. This idea is further supported by recent evidence that IL-6/p-STAT3 has a key role in the DSS-induced tumor formation in mice models [34], and IL-6/p-STAT3 are involved in epithelial cell survival and tumor progression using conditional deletion of p-STAT3 in epithelial cells in DSS treated mice [28]. Additionally, it was demonstrated that the absence of the negative regulator of IL-6/p-STAT3 signaling, SOCS3, enhanced the sensitivity of mice to DSS induced colon carcinoma [36]. All these data makes intervention in the IL-6 signaling a feasible therapeutic target for preventing cancer in UC patients.

The major difference between patients with UC and patients with UC-CRC in our study was the expression of SOCS3 in intestinal epithelial cells. Whereas there was a high expression of SOCS3 in UC, this expression gradually declined during the progression to UC-CRC. Several data showed SOCS3 is silenced in other non-colorectal cancers by promoter hypermethylation [37] [38] [39]. Therefore, the methylation silencing of SOCS3 can be an obvious explanation for the observed lack of SOCS3 expression in our UC-CRC biopsies. The absence of this negative regulator of IL-6/pSTAT3 signaling, SOCS3, can enhance the sensitivity of mice to DSS induced colon carcinoma [36], and the restoration of SOCS3 reduces STAT3 activation, induces apoptosis, and decreases tumor growth.

Although p-STAT3 and SOCS3 were expressed uniformly between UC-CRC and sporadic CRC, other factors, which we did not investigate, can affect the pathogenesis of both disorders in different ways.

In conclusion, our study provides clinical support for the importance of IL-6/p-STAT3 and SOCS3 expression in UC and UC-induced carcinogenesis. As such, our data support the IL-6 signaling pathway as an interesting therapeutic target in active UC and UC-CRC. Moreover, the high expression of SOCS3 in inactive UC compared with non-disease controls suggests the involvement of this protein in UC development, while the absence of SOCS3 expression in UC-CRC may be a critical factor in the progression towards CRC development.

ACKNOWLEDGEMENTS

The authors thank Prof. Gregory J. Gores (Mayo Clinic, Rochester, Department of Gastroenterology and Hepatology) for advice and discussion.

REFERENCES

1. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001;48:526-35.
2. Eaden J. Review article: colorectal carcinoma and inflammatory bowel disease. Aliment Pharmacol Ther 2004;20 Suppl 4:24-30.
3. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 2006;3:390-407.
4. Schottelius AJ, Dinter H. Cytokines, NF-kappaB, microenvironment, intestinal inflammation and cancer. Cancer Treat Res 2006;130:67-87.
5. Rubie C, Frick VO, Pfeil S, Wagner M, Kollmar O, Kopp B, Graber S, Rau BM, Schilling MK. Correlation of IL-8 with induction, progression and metastatic potential of colorectal cancer. World J Gastroenterol 2007;13:4996-5002.
6. Mitsuyama K, Sasaki E, Toyonaga A, Ikeda H, Tsuruta O, Irie A, Arima N, Oriishi T, Harada K, Fujisaki K, et al. Colonic mucosal interleukin-6 in inflammatory bowel disease. Digestion 1991;50:104-11.
7. Carey R, Jurickova I, Ballard E, Bonkowski E, Han X, Xu H, Denson LA. Activation of an IL-6:STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease. Inflamm Bowel Dis 2007.
8. Mitsuyama K, Sata M, Rose-John S. Interleukin-6 trans-signaling in inflammatory bowel disease. Cytokine Growth Factor Rev 2006;17:451-61.
9. Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol 2006;80:227-36.
10. Mitsuyama K, Matsumoto S, Rose-John S, Suzuki A, Hara T, Tomiyasu N, Handa K, Tsuruta O, Funabashi H, Scheller J, Toyonaga A, Sata M. STAT3 activation via interleukin 6 trans-signalling contributes to ileitis in SAMP1/Yit mice. Gut 2006;55:1263-9.
11. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol 2003;4:1169-76.
12. Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu Rev Immunol 2004;22:503-29.
13. Yoshimura A, Nishinakamura H, Matsumura Y, Hanada T. Negative regulation of cytokine signaling and immune responses by SOCS proteins. Arthritis Res Ther 2005;7:100-10.
14. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. J Exp Med 2001;193:471-81.

15. Mizoguchi E, Xavier RJ, Reinecker HC, Uchino H, Bhan AK, Podolsky DK, Mizoguchi A. Colonic epithelial functional phenotype varies with type and phase of experimental colitis. Gastroenterology 2003;125:148-61.
16. Greenhalgh CJ, Miller ME, Hilton DJ, Lund PK. Suppressors of cytokine signaling: Relevance to gastrointestinal function and disease. Gastroenterology 2002;123:2064-81.
17. Grutkoski PS, Chen Y, Chung CS, Ayala A. Sepsis-induced SOCS-3 expression is immunologically restricted to phagocytes. J Leukoc Biol 2003;74:916-22.
18. Corvinus FM, Orth C, Moriggl R, Tsareva SA, Wagner S, Pfitzner EB, Baus D, Kaufmann R, Huber LA, Zatloukal K, Beug H, Ohlschlager P, Schutz A, Halbhuber KJ, Friedrich K. Persistent STAT3 activation in colon cancer is associated with enhanced cell proliferation and tumor growth. Neoplasia 2005;7:545-55.
19. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. Gastroenterology 2007;132:384-96.
20. Atreya R, Neurath MF. New therapeutic strategies for treatment of inflammatory bowel disease. Mucosal Immunol 2008;1:175-82.
21. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl 1989;170:2-6; discussion 16-9.
22. Geboes K, Riddell R, Ost A, Jensfelt B, Persson T, Lofberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. Gut 2000;47:404-9.
23. Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. Nat Med 2002;8:1089-97.
24. Kusugami K, Fukatsu A, Tanimoto M, Shinoda M, Haruta J, Kuroiwa A, Ina K, Kanayama K, Ando T, Matsuura T, et al. Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent. Dig Dis Sci 1995;40:949-59.
25. Mudter J, Neurath MF. Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. Inflamm Bowel Dis 2007;13:1016-23.
26. Mitsuyama K, Matsumoto S, Masuda J, Yamasakii H, Kuwaki K, Takedatsu H, Sata M. Therapeutic strategies for targeting the IL-6/STAT3 cytokine signaling pathway in inflammatory bowel disease. Anticancer Res 2007;27:3749-56.
27. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91-102.

28. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.
29. Albanesi C, Fairchild HR, Madonna S, Scarponi C, De Pita O, Leung DY, Howell MD. IL-4 and IL-13 negatively regulate TNF-alpha- and IFN-gamma-induced beta-defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. J Immunol 2007;179:984-92.
30. Bode JG, Nimmesgern A, Schmitz J, Schaper F, Schmitt M, Frisch W, Haussinger D, Heinrich PC, Graeve L. LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. FEBS Lett 1999;463:365-70.
31. Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D, Hanada T, Takeda K, Akira S, Hoshijima M, Hirano T, Chien KR, Yoshimura A. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nat Immunol 2003;4:551-6.
32. Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO, Benveniste EN. Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. J Immunol 2007;179:5966-76.
33. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 2007;7:454-65.
34. Becker C, Fantini MC, Schramm C, Lehr HA, Wirtz S, Nikolaev A, Burg J, Strand S, Kiesslich R, Huber S, Ito H, Nishimoto N, Yoshizaki K, Kishimoto T, Galle PR, Blessing M, Rose-John S, Neurath MF. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. Immunity 2004;21:491-501.
35. Schneider MR, Hoeflich A, Fischer JR, Wolf E, Sordat B, Lahm H. Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells. Cancer Lett 2000;151:31-8.
36. Rigby RJ, Simmons JG, Greenhalgh CJ, Alexander WS, Lund PK. Suppressor of cytokine signaling 3 (SOCS3) limits damage-induced crypt hyper-proliferation and inflammation-associated tumorigenesis in the colon. Oncogene 2007;26:4833-41.
37. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, Yoshikawa H. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene 2005;24:6406-17.
38. Evans MK, Yu CR, Lohani A, Mahdi RM, Liu X, Trzeciak AR, Egwuagu CE. Expression of SOCS1 and SOCS3 genes is differentially regulated in breast cancer cells in response to proinflammatory cytokine and growth factor signals. Oncogene 2007;26:1941-8.

39. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A 2003;100:14133-8.

Chapter IV

SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer

Yi Li[1], Maikel P. Peppelenbosch[1], Ernst J. Kuipers[1, 2], Colin de Haar[1#], C. Janneke van der Woude[1#].

1. Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, Netherlands.
2. Department of Internal Medicine, Erasmus Medical Center, Rotterdam, Netherlands.
Authors share senior authorship.

Submitted for publication

ABSTRACT

Background: Ulcerative colitis is associated with a high incidence of colorectal cancer and the mechanisms that allow inflammatory responses to develop in full-blown cancer remain only partially understood, but may involve reduced control of IL-6 signaling towards STAT3 through the loss of SOCS3 expression.

Materials and methods: Downregulation of SOCS3 was analyzed at non-dysplasia and dysplasia areas of intestinal epithelial cells (IEC). Methylation of the SOCS3 promoter region was examined in CRC cell lines by methylation specific PCR (MSP) and pyrosequencing. Methylation effects were confirmed by studying the IL-6/p-STAT3 induced expression of SOCS3 after demethylation using 5-aza-2'-deoxycytidine (DAC). IL-6 and DNMT1 expression were immunohistochemically assessed in the biopsies from patients with UC, UC-CRC, sporadic CRC and controls.

Results: We observe that loss of epithelial SOCS3 expression delimits the areas subject to dysplasia in UC, suggesting an important tumor suppressive role of SOCS3 in UC. The SOCS3 methylation status in CRC cell lines were correlated with the disability to upregulate SOCS3 upon IL-6 stimulation. DNMT1-inhibition restores SOCS3 expression and restricts the induction of IL-6 induced p-STAT3 activation and proliferation in DLD1 cells. IL-6 is correlated with overexpression of DNMT1 in CRC patients and cell lines.

Conclusion: We establish that DNMT1-mediated methylation in the SOCS3 promoter may underlie the phenomenon of the loss of SOCS3 expression. In apparent agreement methylation inhibitors restore the capacity of IL-6 to induce SOCS3 expression. We propose that when IL-6 signaling results in DNMT1 expression, SOCS3 induction and consequently inhibition of IL-6 signaling through STAT3 is lost. Thus DNMT1 emerges as a rational target in preventive strategies aimed at counteracting UC-CRC.

KEYWORDS: SOCS3; IL-6; DNMT1; methylation; UC-CRC

INTRODUCTION

Ulcerative colitis (UC) is a major manifestation of inflammatory bowel disease (IBD) and is clinically characterized by chronic recurrent colitis and histopathologically by the presence of a continuous inflammatory infiltrate in the intestinal mucosa ascending from the rectum into the colon [1]. Chronic UC is associated with an increased risk to develop colorectal cancer (CRC) and UC-CRC underlies approximately 10-15% of all mortality in IBD patients and risk for contracting CRC is approximately 20 to 100 times higher as the population at large, depending on the absence or presence of PSC as co-morbidity [2, 3]. As thus UC-CRC represents a special challenge in the prevention and treatment of gastrointestinal cancer.

The pathophysiological mechanisms explaining the unusually high risk for contracting colon cancer in UC remain only very partially understood, but may involve the chronic proliferation necessary to repair the damage inflicted on the monolayer by the constant inflammation [4, 5]. In this context, the high interleukin (IL)-6 detected in colonic biopsies and serum samples of UC patients are considered an especially deleterious factor with respect to the development of malignant disease. IL-6 is a proinflammatory cytokine that is associated with carcinogenesis at multiple places in the human body. Signaling through the canonical cytokine receptor pathway, IL-6 evokes activation of Janus kinase 2 (JAK), in turn activating the signal transducer and activator of transcription 3 (STAT3), which can mediate immunosurveillance evasion, promote cell growth and increase survival signaling [6, 7]. Full manifestation of these pro-oncogenic effects of STAT3, however, requires inactivation of the negative regulators of IL-6 signaling. The most important of the latter is the suppressor of cytokine signaling 3 (SOCS3), which is a direct target gene of STAT3 and mediates suppression of IL6 signaling through the ubiquitination and degradation of signaling intermediates. The question how during UC-CRC carcinogenesis the negative influence of STAT3-dependent induction of SOCS3 is overcome, remains one of the most important questions in the field.

We have previously reported on the silencing of SOCS3 expression as a potential mechanism explaining sustained IL-6-dependent STAT3 phosphorylation and activation in the inflammation-dysplasia-carcinoma sequence in UC [8], but left the underlying molecular mechanism unexplored. Obviously, delineation of these mechanisms might be

exceedingly useful for devising rational strategies for the prevention and maybe also treatment of UC-associated CRC. Interestingly, in cholangiocarcinoma, a disease that shares many characteristics with UC-CRC, IL-6 signallng has been associated with increased the expression of DNA methyltransferase-1 (DNMT1) [9], a CpG island methylator, which has thus been proposed to provide a link between IL-6 levels and inflammation-associated cholangiocarcinoma [10] and UC-related carcinoma [11] through DNMT1-mediated tumor suppressor gene silencing. Causative relationships, however, remain to be established and especially identification of the tumor suppressor genes involved remains a very open issue.

Here we explore the possibility that DNMT1 induction by IL-6 in UC mediates silencing of SOCS3 expression and consequently CRC. We observe that spatially UC-CRC is restricted to areas with loss of SOCS3 expression, that SOCS3 promoter methylation makes the gene resistant to IL-6-dependent induction of its expression in a DNMT1-dependent fashion and that both in vivo as well as in patients IL-6 dependent activation of STAT3 signaling is strictly dependent on DNMT1 enzymatic activity. The dependence of carcinogenic progression of UC-CRC on DNMT1 and the subsequent inhibition of SOCS3 transcription make this methyltransferase a prime candidate target for chemopreventive strategies in this disease.

MATERIALS AND METHODS

Patient biopsies and cell lines

Paraffin embedded biopsies from 9 normal controls, 27 active UC, 11 UC-CRC and 14 sporadic CRC were included. Colon biopsy specimens were obtained during colonoscopy performed for clinical care and surveillance studies after informed consent. The diagnosis of UC was based on conventional clinical, endoscopic and pathohistological criteria as described by Lennard-Jones [12]. An independent pathologist reconfirmed carcinoma by hematoxylin and eosin (H&E) staining and histological scoring. Colonic biopsies from patients without abnormalities at colonoscopy and histories of gastrointestinal disease served as controls. Five CRC cell lines were included, which are caco-2, HT29, SW480, SW48 and DLD1. They are cultured in medium DMEM, supplemented with streptomycin 10% (v/v) fetal calf serum, 1% (v/v) penicillin and streptomycin, at 37°C in a humidified

atmosphere containing 5% CO2. All the cell experiments were repeated in three times independently.

Immunohistochemistry staining and scoring

All biopsies were fixed in 10% formalin and embedded in paraffin. The deparaffinization, endogenous peroxidise blocking and staining processes were perform as previously described [8]. Antigen retrieval was performed by boiling in preheated buffer Tris/EDTA pH 9.0 for 15 min at 200 W in a microwave. Next, slides were transferred to Shandon chambers and blocked by 10% normal human serum, 10% goat serum in phosphate-buffered saline (PBS) pH 7.4 for 1 h at room temperature. Primary antibodies rabbit polyclonal anti-DNMT1 (Abcam, ab19905) and rabbit polyclonal anti-IL-6 (Abcam, ab6672) were added and incubated at 4 degrees overnight. Envision goat anti-rabbit-HRP (DAKO, Denmark) was used as secondary antibody. Two experienced people scored the staining of epithelial cells in a blinded manner. The percentage of epithelial cells that stained positive (immunoreactivity above background) in the area consistent with that used for diagnoses was quantified.

DNA isolation and bisulfate modification

Different cell line DNA was isolated using trizol reagent. Cell line DNA samples were bisulfate modified by EZ DNA methylation ^{TM} kit (ZYMO Research). Methylated DNA and unmethylated DNA (Millipore) were purchased and used as a positive and negative control, which have been treated by the methylation kit as well.

Methylation-specific PCR (MSP)

MSP specific primers were designed by Methprimer or previous described by Weber Hengge [13] to amplify methylated and unmethylated sequence respectively, shown in supplementary figure 2. MSP was performed on bisulfite-treated genomic DNA. PCR reaction was performed in a 25 ml volume containing 40 ng bisulfite-modified DNA, 5x Gold Taq buffer, 0.2 mM dNTPs, 10 pmol specific primer mix (forward and reverse primers) and one unit Gold Taq enzyme (Promega). Three region of SOCS3 promoter were targeted, the PCR conditions and sequences of each primer mix were shown in table 1. The PCR products were visualized on a 2% agarose gel using ethidium bromide and UV illumination.

Normal PCR and Pyrosequencing

Bisulfite genomic DNA was amplified region exon 1 to intron before exon 2, on SOCS3 promoter. Product was purified and sequenced by input 15ng per 100bp purified PCR product per 20ul reaction. SOCS3 promoter region 5'-UTR before exon 1 was amplified and prepared using PyroMark™ Vacuum Prep Workstation. The methylation status was quantified by pyrosequencing machine PyroMark™MD, Biotage. The PCR conditions and sequences of each primer mix were shown in table 1.

Demethylation treatment

All the culture cells were seeded 1: 10 ratio in 12-wells plate. Twenty-four hours later, 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma) was added to a final concentration of 10 μM. Three days (72 h) after 5-Aza-dC treatment, the cells were stimulated by IL-6 (100ng/ml) and harvested for Q-PCR and Western blot. Independent cells (control and demethylated cells) cultured after three days and six days were viewed by microscope.

RNA isolation and Real time PCR

Total RNA was isolated from cell lines using TRIzol (Sigma). 1μg of RNA was reversely transcribed using iScript^{TM} cDNA synthesis kit (Bio-rad). QPCR reactions were performed by using real-time PCR kit sensimix^{TM} SYBR&Flurescence (Quntace) in a total volume of 25μl containing 10pmol primers in IQ5 (Bio-rad). The working conditions and primers are shown in table 1. Calculated data was the ratio between SOCS3 and GAPDH mRNA delta-Ct presented as the fold induction in mRNA expression relative to each control samples.

Western blot

The total cell lysates were prepared and a Western blot was performed as described earlier [14]. Immobilon IF transfer membranes (Millipore) were probed with primary antibodies specific for SOCS3 (rabbit polyclonal, abcam, ab16030), p-STAT3 (rabbit monoclonal, #9145, cellsignal), DNMT1 (rabbit polyclonal, Abcam, ab19905) and Cyclin D1 (rabbit monoclonal, #2922, cellsignal). α-tublin (Abcam ab6046) and β-actin (Santa Cruz, E0610) was used as a reference protein. HRP labelled goat anti-rabbit and goat anti-mouse was used as secondary antibody (LI-COR Biosciences). Transfer membranes were incubated with antibodies and washed in 50ml sterile centrifuge tubes and light

	Primer sequence	Annealing tempera-ture (cycle)	SOCS3 Gene amplified	Pro-duct size (bp)
SOCS3 MSP				
M1F	GTTTCGGTTTCGTACGTAGTTAGTC	55 (x 45, 20s)	Exon 1	M=180
M1R	TAAAATCCACAAAAAAACCTTCG			
U1F	TTTTGGTTTTGTATGTAGTTAGTTG	56.5 (x 40, 30s)		U=179
U1R	TAAAATCCACAAAAAAACCTTCAC			
M2F	GGAGATTTTAGGTTTTCGGAATATTTC	56.5 (x 40, 30s)	Intron before exon 2, stat3 binding site	M=142
M2R	CCCCCGAAACTACCTAAACGCCG			
U2F	GTTGGAGATTTTAGGTTTTTGGAATATTTT	56.5 (x 40, 30s)		U=151
U2R	AAACCCCCAAAACTACCTAAACACCA			
M3F	GTTTGATTCGTAGTTGGGTTTTC	55 (x 40, 25s)	5'-UTR before exon 1	M=104
M3R	GACCTAAAAAACCTCCCGAT			
U3F	GTTTGATTTGTAGTTGGGTTTTTG	55 (x 40, 25s)		U=105
U3R	CAACCTAAAAAACCTCCCAAT			
SOCS3 pyrosequencing				
F	GTTTGGTTGTGGGGTAGTTTTATT	63 (x45, 45s)	5'-UTR before exon 1	259
R	AAGTGTGAATGAGAAGTTGGGG			
Q-PCR				
GAPDH				
F	GCATTGCCCTCAACGACCAC	60 (x40, 15s)		
R	CCACCACCCTGTTGCTGTAG			
SOCS3				
F	TCTGTCGGAAGACCGTCAAC	60 (x 40, 15s)		
R	GGTCCAGGAACTCCCGAATG			
IL-6R				
F	TGTGCGTCGCCAGTAGTGTC	60 (x40, 15s)		
R	CGGCAGTGACTGTGATGTTG			

	Primer sequnece	Annealing temperature (cycle)	SOCS3 Gene amplified	Product size (bp)
DNMT1				
F	AGCCCGTAGAGTGGGAATGG	60 (x40, 15s)		
R	TGAGGCAGGAGGGTCTCTTG			

protection centrifuge tubes (greiner bio-one). Protein expression was visualized using fluorescence Odyssey system (LI-COR Biosciences, Lincoln, NE, USA). Quantitive expression data were determined by Odyssey 3.0 software and normalized using β-actin or α-tublin for reference gene expression.

Thiazolyl Blue Tetrazolium Bromide (MTT) assay

Control cells and demethylated cells were cultured in 96-well plates for in total four days. Final concentration of 0.5mg/ml MTT was added to the cell medium. As described previously [15], the absorbance was measured using a microplate reader Model 680XR (BIO-RAD) at 490 nm. All experiments were repeated three times. Percentage of cell viability after demethylation was calculated as % Viability = mean OD in sample well/mean OD in control well x 100, where OD is optical density.

Statistical analysis

Statistical analysis was performed using SPSS software version 15.0. The difference between each two groups was performed by Mann Whitney test. The difference between more groups was performed by Kruskal-Wallis test. The correlation between two variables was performed by spearman correlation test. P value <0.05 was considered statistically significant.

RESULTS

Loss of epithelial SOCS3 expression delimits the neoplastic domain in UC-CRC

We previously proposed that silencing of SOCS3 expression explains sustained IL-6-

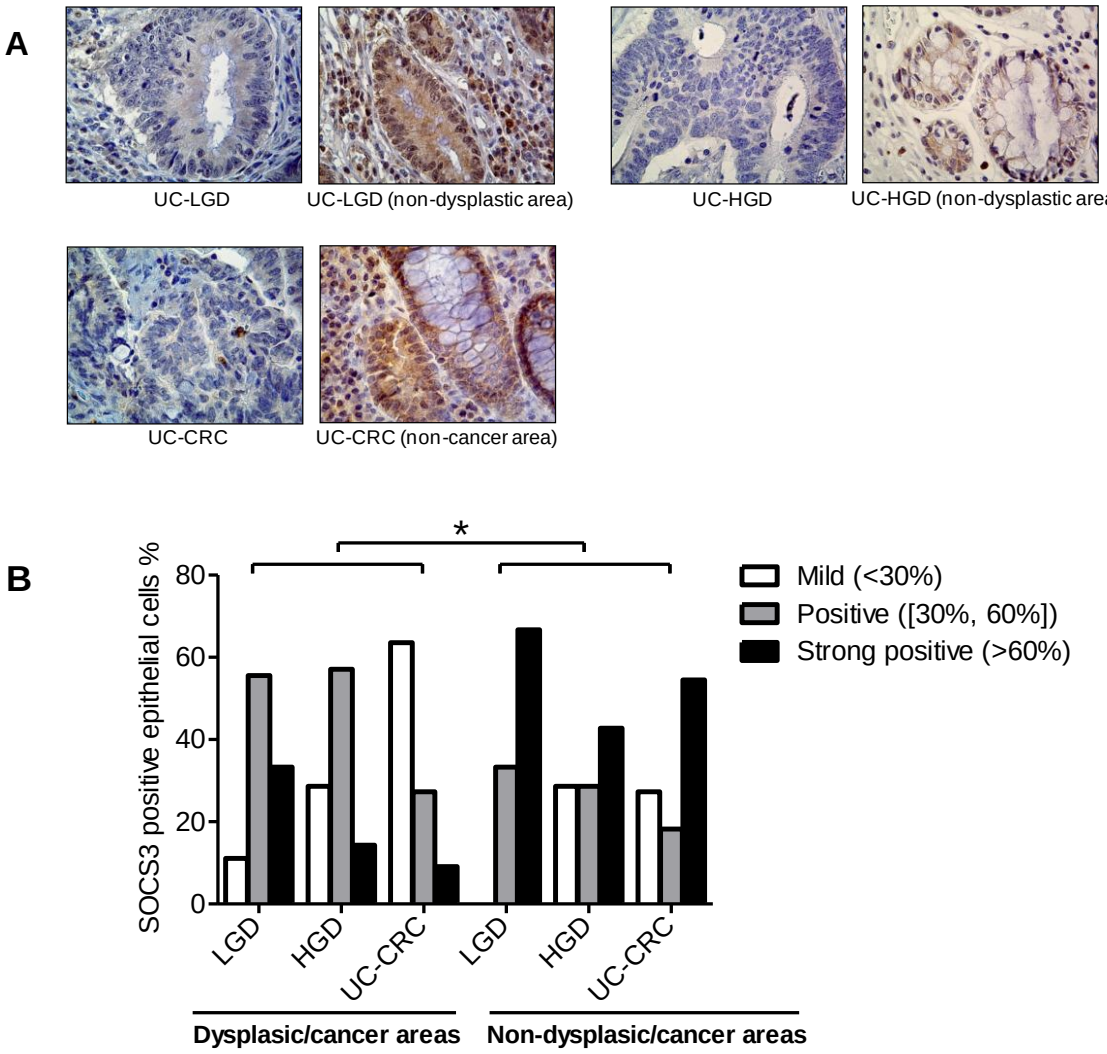


Figure 1. Specific SOCS3 down regulation in epithelial cells during progression from LGD to UC-CRC in the dysplastic and/or cancer region. (A). IHC staining showed SOCS3 expression in dysplastic/cancer and adjacent non-dysplastic/cancer area in the same biopsies. (B). SOCS3 positive epithelial cells were significantly more in the non-dysplastic/cancer area than in the dysplastic/cancer from the same biopsies ($p<0.05$)

dependent STAT3 phosphorylation and activation in the inflammation-dysplasia-carcinoma sequence in UC [8]. The same situation was not found in CD-dysplasia and CD-CRC patients (Supplementary Figure 1), which points out the specificity of SOCS3 downregulation in UC-carcinogenesis. A prediction from the hypothesis is that the cancerous process in UC-CRC should be limited to areas with loss of SOCS3 expression. Hence we investigated SOCS3 expression in the dysplastic as well as the adjacent non-

dysplastic tissue (Figure 1A). Non-dysplastic regions were characterized by relatively high levels of SOCS3 expression, but both the dysplasia itself as well as the degree of transformation was strongly correlated shows loss of epithelial SOCS3 expression. This was not a field effect, as in areas directly adjacent to the dysplastic regions such a correlation was absent (Figure 1A-B). It thus appears that dysplasia per se only occurs in context of loss of SOCS3 expression and subsequent experiments were initiated to identify the mechanisms underlying the loss of SOCS3 in the inflammation-dysplasia-carcinoma sequence in UC.

SOCS3 promoter methylation is a major regulator of SOCS3 expression in the colonic epithelial compartment

We reasoned that the mechanisms mediating inhibition of SOCS3 expression in UC-associated carcinogenesis might be reflected in vitro models for colon cancer. As such a panel of colon cancer cell line cultures was checked for both unstimulated SOCS3 levels as well as for the potential of IL-6 to provoke SOCS3 gene expression. It appeared that SOCS3 mRNA levels already varied substantially between different cell lines (Figure 2B), demonstrating that regulation is different in established colon cancer cell cultures, but that especially the capacity of IL-6 to stimulate transcription of SOCS3 was widely divergent, such induction being very strong in caco-2 cultures, but absent in DLD1. A preliminary analysis of IL-6 receptor levels revealed that alternative expression of IL-6 signal transduction elements was unlikely to account for these differences (Supplementary Figure 3). As identification of the mechanisms underlying differences in expression of SOCS3 may hold important clues as to the regulation of the protein in vivo, we checked promoter methylation in this panel as well. The methylation status was determined in five CRC cell lines by MSP at three different regions of SOCS3 gene promoter. It revealed the high negative correlations between promoter methylation with basal SOCS3 levels, but especially with the sensitivity to SOCS3 induction with IL-6 (Figure 2A-B). Consistent with MSP results, pyrosequencing confirmed increased CpG methylation in SOCS3 promoter from colon cell cultures: HT29 17.12%, sw480 18.06%, sw48 18.46%, DLD1 19.33% and caco-2 cells only 10.86%. It thus seems that methylation is an important mechanism controlling IL-6-dependent SOCS3 expression in colon cancer cultures and subsequent experiments were initiated to investigate the importance of methylation in UC-CRC proper.

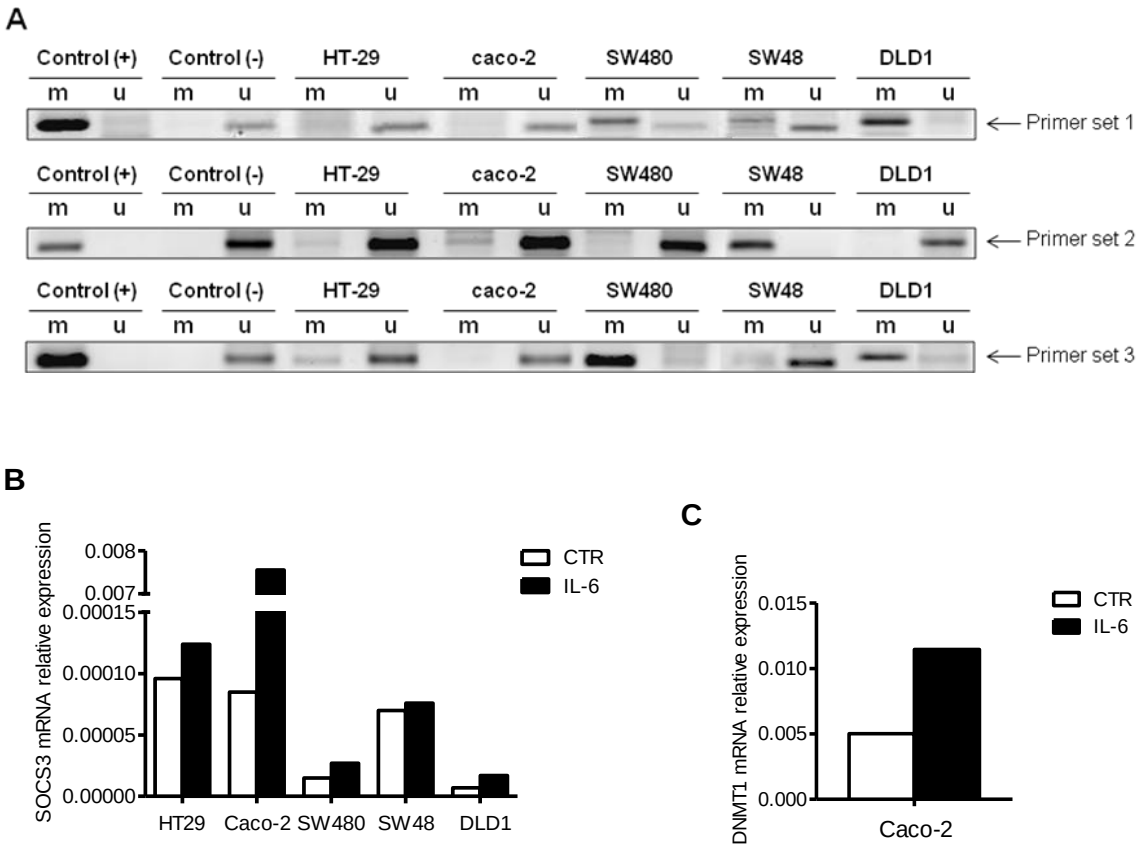


Figure 2. (A). The methylation status was determined in five CRC cell lines by MSP at three different regions of SOCS3 gene promoter. MSP showed cells of HT29, sw480, sw48 and DLD1 had denser SOCS3 methylation status than caco-2 cells. (B). SOCS3 induction upon IL-6 stimulation in CRC cell lines. IL-6 stimulation does not increase SOCS3 expression in DLD1 cells because of high SOCS3 gene methylation. (C). DNMT1 induction upon IL-6 stimulation in caco-2 cells.

Since DNMT1 is expressed in CRC [16] and IL-6 is highly expressed in UC-CRC able to upregulate the expression of DNMT1 [10], we checked the expression levels of DNMT1 in caco-2 cell lines which had the most abilities of upregulating SOCS3 upon IL-6 stimulation (Figure 2C).

DNMT1 can regulate the SOCS3 promoter in colon cancer cell cultures

A prediction from the concept that IL-6-dependent DNMT1 induction mediates SOCS3 downregulation, thus releasing IL-6 signaling towards STAT3 from its negative regulation

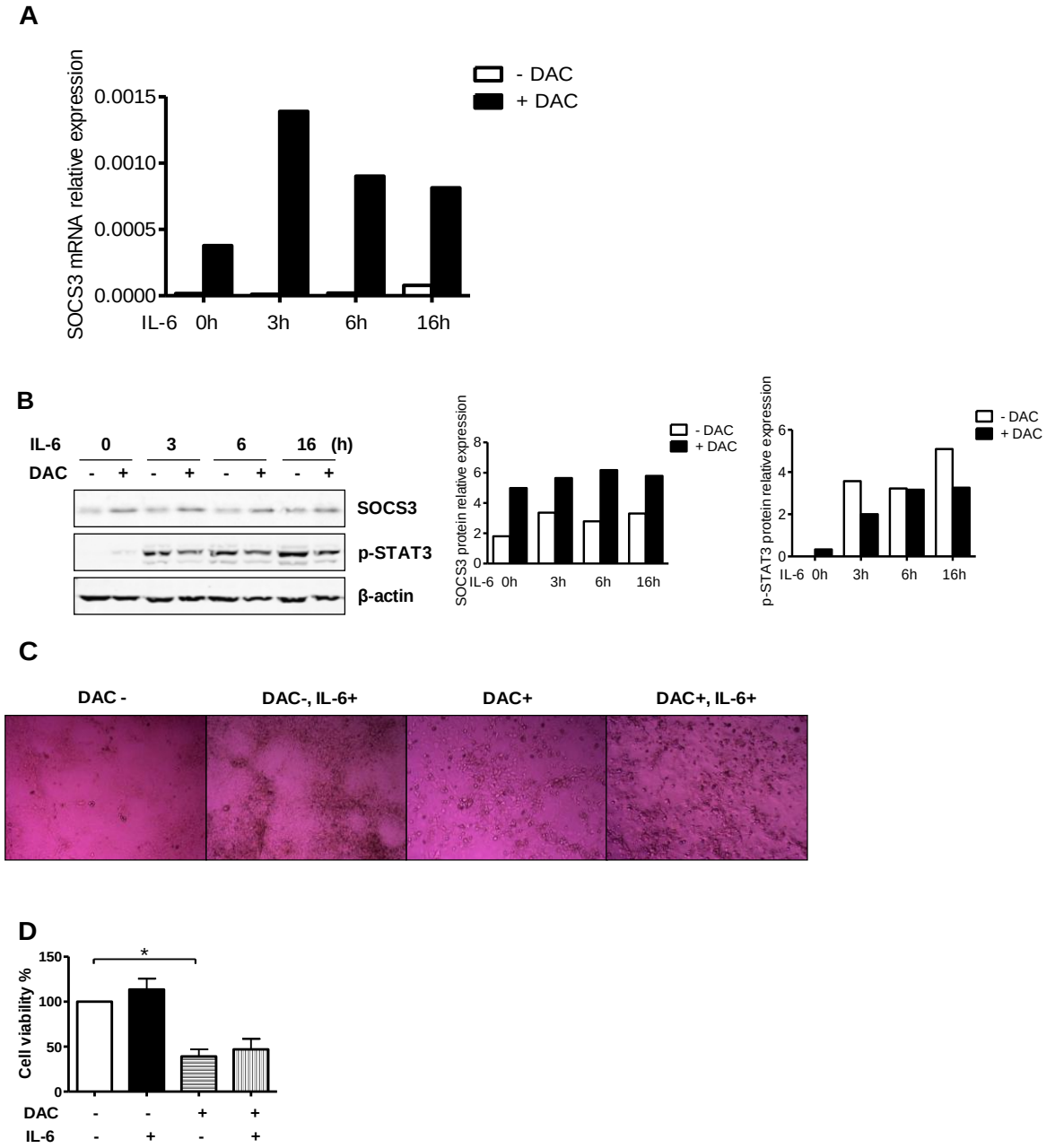


Figure 3. In DLD1 cells, DAC treatment increased the basal levels of SOCS3 expression both at mRNA (A) and protein level (B), and this expression was further increased after IL-6 stimulation (B). DAC restoration of SOCS3 expression at basal levels reduced the STAT3 phosphorylation induced by IL-6 in DLD1 cells (B). (C-D). The restoration of SOCS3 expression by DNMT1 inhibition affects cell growth. DLD1 cells were cultured in medium with or without DAC for 3 days, then for an additional 3 days, with or without IL-6. Cells treated with DAC for the entire 6 days shown a significant reduction in cell growth compared to those cultured without DAC ($p<0.05$). Whereas IL-6 stimulation further increased the growth of cells in the absence of DAC, DAC treatment prevented enhanced cell growth by IL-6.

through SOCS3 is that inhibition of DNMT1 should restore IL-6-dependent induction of SOCS3. Since SOCS3 methylation data suggest that the DLD1 cells provide a good model for further elucidating the mechanisms behind the SOCS3 hypermethylation observed in UC-CRC, the proof of this hypothesis was attempted through the treatment of DLD1 cells with the methylation inhibitor DAC. As expected from the observed regulation of SOCS3 in colonic cell cultures, DAC treatment increased SOCS mRNA and protein levels in otherwise unchallenged cells (Figure 3A-B). Importantly, following treatment with the methylation inhibitor, cells became susceptible to IL-6-dependent SOCS3 induction. Furthermore, concomitantly IL-6-dependent induction of STAT3 was impaired (Figure 3B) as well as IL-6-dependent cell proliferation was impaired (Figure 3C-D). To illustrate the effect of methylation inhibitor on cell growth, DLD1 cells were cultured in medium with or without DAC for 3 days, then for an additional 3 days, with or without IL-6. Cells treated with DAC for the entire 6 days shown a significant reduction in cell growth compared to those cultured without DAC ($p<0.05$). Whereas IL-6 stimulation further increased the growth of cells in the absence of DAC, DAC treatment prevented enhanced proliferation by IL-6.

Thus these in vitro experiments provide support for the notion that an IL-6-dependent downmodudulation of methyltransferases reduces regulation of IL-6 signaling towards STAT3 and is permissive for uncontrolled colonic cell proliferation.

IL-6 is associated with DNMT1 overexpression in CRC

Earlier data obtained in cholangiocarcinoma indicated that DNMT1 is a methyltransferase induced by IL-6 and capable of epigenetic reprogramming gene expression. As such it is a prime candidate to mediate SOCS3 methylation and hence we investigated biopsies from controls, UC, UC-CRC and sporadic CRC that were stained for IL-6 (showed previously [8]) and DNMT1 (Figure 4A). As expected, IL-6 expression was significantly higher in UC-CRC ($p=0.0002$), sporadic CRC ($p=0.0017$) and active UC ($p<0.0001$) than in normal controls, in agreement with a role of IL-6 in tumor progression in the colon. Furthermore, biopsies from UC-CRC have even higher IL-6 expression than sporadic CRC ($p=0.006$) (Figure 4B). Interestingly, also DNMT1 expression was significantly higher in UC-CRC ($p=0.020$) and active UC ($p=0.004$) compared with controls (Figure 4C). IL-6 and DNMT1 showed a good positive correlation with each other (spearman $r=0.371$, $p=0.002$).

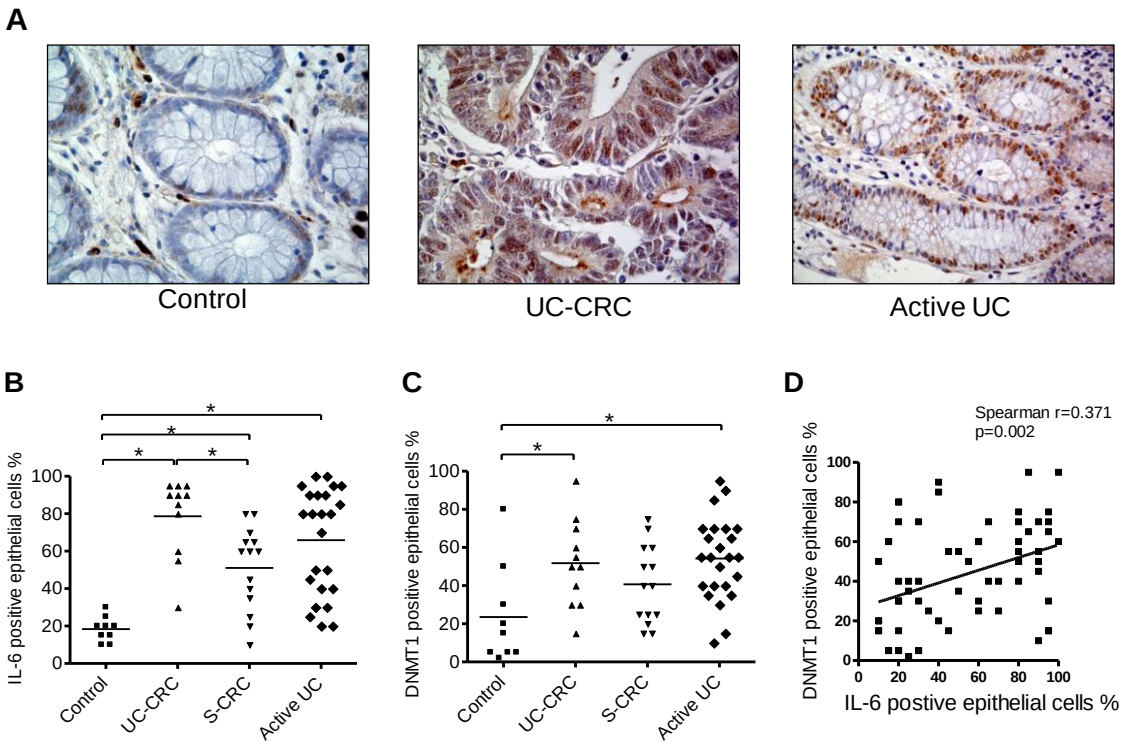


Figure 4. IL-6, DNMT1 expression and their correlation in the biopsies from patients. (A). IHC staining showed DNMT1 expression in the biopsies from controls, UC-CRC and active UC. (B). Analysis of IL-6 positive epithelial cells. IL-6 expression was significantly higher in UC-CRC (p=0.0002), sporadic CRC (p=0.0017) and active UC (p<0.0001) than in normal controls. Biopsies from UC-CRC had even higher IL-6 expression than sporadic CRC (p=0.006). (C). Analysis of DNMT1 positive epithelial cells. DNMT1 expression was significantly higher in UC-CRC (p=0.020) and active UC (p=0.004) compared with controls. (D). Overall the expression levels of IL-6 and DNMT1 in all the biopsies were positively correlated (spearman r=0.371, p=0.002).

DISCUSSION

The highly increased incidence of CRC associated with chronic inflammatory disorders lumped together under the common denominator IBD is of complex and poorly understood etiology, but a large body of evidence in the corpus of biomedical literature implicates increased IL6 signaling through STAT3 in this phenomenon [17-19]. We previously presented the hypothesis that SOCS3 methylation in UC-CRC patients would be an important factor in allowing IL-6 to exert its deleterious consequences in colonic carcinogenesis [8]. In the present study, we demonstrate that SOCS3 downregulation

delimits the extent of the dysplastic domain in UC-CRC and that methylation controls the activity of the SOCS3 promoter in the colonic compartment. Furthermore, we show that IL-6 signaling can provoke an increase in methylation of SOCS3, releasing its own signaling towards STAT3 from inhibition. Finally, we provide evidence that DNMT1 is upregulated in vivo in UC and that is expression correlates to both the areas with high IL-6 signaling and those which develop cancer. In total, our data strongly support the concept that IL-6-dependent DNMT1-mediated methylation of the SOCS3 promoter is an important event in progression towards CRC in UC patients. Thus the lack of SOCS3 expression is like to be the consequence of IL-6 driven methylation, and meanwhile the cause UC carcinogenesis. DNMT1 emerges as an attractive target for rational strategies aimed at preventing CRC in IBD patients.

From our data emerges that SOCS3 methylation involves large areas of its promoter (see also Supplementary Figure 2), as MSP showed strong methylation in three different regions of the promoter. Importantly, one of areas positively identified as being methylated includes the STAT3 binding element, which is considered essential for induction of SOCS3 in response to IL-6, and seen as the main element responsible for limiting the extent of SOCS3 induction in vivo. The relevance of this finding for the cancerous process is illustrated by our observation that absence of SOCS3 expression delimits the dysplastic domain in UC. Thus it seems that methylation of this particular element might constitute a rate limiting step in the generation UC-CRC.

IL-6/p-STAT3 signaling in vitro has been shown to both stimulate colonic epithelial cell proliferation and induce resistance to apoptosis [20]. In apparent agreement, in animals genetically devoid of SOCS3 in the colonic mucosa, enhanced sensitivity to repeated DSS challenge is noted with respect to the occurrence of colon carcinoma [21]. The current data add to this concept as they provide a mechanistic link to the previous murine data to actual patients and also establish that through methylation a phenocopy of the murine genetically induced situation is established in the human colon. Importantly, however, the role of STAT3 in colonic physiology is not clear cut. Recent data indicate that STAT3 expression can also inhibit inflammation in the colon through multiple but mainly tolerogenic mechanisms [22]. Hence strategies at increasing SOCS3 expression may be accompanied exacerbation of disease. Disregarding complication in the clinical management of UC per se, however, our data do show that SOCS3 inhibition eg: by inducing methyltransferase is an important component of the sequence of events leading

to the colorectal carcinogenesis associated with IBD and thus opens the door to novel avenues for the rational management of this complication of UC.

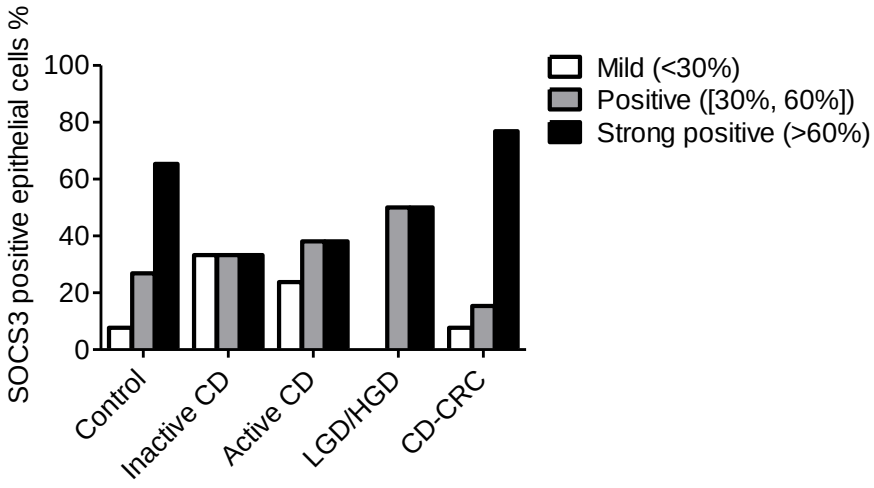
REFERENCES

1. Cho, J.H. (2008) The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol*, **8**, 458-66.
2. Eaden, J.A., Abrams, K.R. and Mayberry, J.F. (2001) The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut*, **48**, 526-35.
3. Eaden, J. (2004) Review article: colorectal carcinoma and inflammatory bowel disease. *Aliment Pharmacol Ther*, **20 Suppl 4**, 24-30.
4. Sartor, R.B. (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol*, **3**, 390-407.
5. Schottelius, A.J. and Dinter, H. (2006) Cytokines, NF-kappaB, microenvironment, intestinal inflammation and cancer. *Cancer Treat Res*, **130**, 67-87.
6. Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L. and Karin, M. (2009) IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell*, **15**, 103-13.
7. Bollrath, J., Phesse, T.J., von Burstin, V.A., Putoczki, T., Bennecke, M., Bateman, T., Nebelsiek, T., Lundgren-May, T., Canli, O., Schwitalla, S., Matthews, V., Schmid, R.M., Kirchner, T., Arkan, M.C., Ernst, M. and Greten, F.R. (2009) gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell*, **15**, 91-102.
8. Li, Y., de Haar, C., Chen, M., Deuring, J., Gerrits, M.M., Smits, R., Xia, B., Kuipers, E.J. and van der Woude, C.J. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. *Gut*, **59**, 227-35.
9. Wehbe, H., Henson, R., Meng, F., Mize-Berge, J. and Patel, T. (2006) Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. *Cancer Res*, **66**, 10517-24.
10. Braconi, C., Huang, N. and Patel, T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. *Hepatology*, **51**, 881-90.
11. Foran, E., Garrity-Park, M.M., Mureau, C., Newell, J., Smyrk, T.C., Limburg, P.J. and Egan, L.J. Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6. *Mol Cancer Res*, **8**, 471-81.
12. Lennard-Jones, J.E. (1989) Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl*, **170**, 2-6; discussion 16-9.
13. Weber, A., Hengge, U.R., Bardenheuer, W., Tischoff, I., Sommerer, F., Markwarth, A., Dietz, A., Wittekind, C. and Tannapfel, A. (2005) SOCS-3 is frequently methylated in head

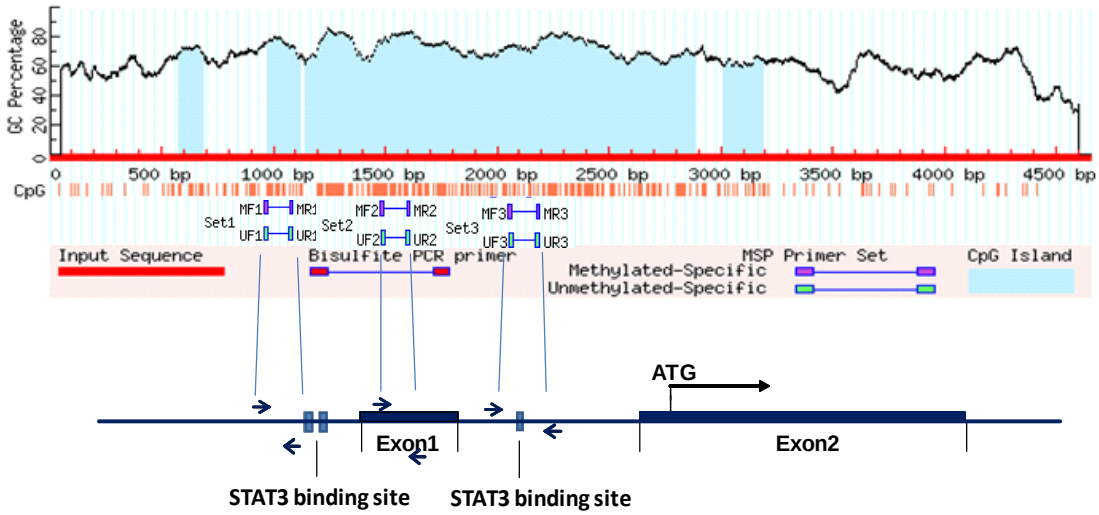
and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition. *Oncogene*, **24**, 6699-708.

14. Wang, M., Markel, T., Crisostomo, P., Herring, C., Meldrum, K.K., Lillemoe, K.D. and Meldrum, D.R. (2007) Deficiency of TNFR1 protects myocardium through SOCS3 and IL-6 but not p38 MAPK or IL-1beta. *Am J Physiol Heart Circ Physiol*, **292**, H1694-9.
15. Yin, Y., Cao, L.Y., Wu, W.Q., Li, H., Jiang, Y. and Zhang, H.F. Blocking effects of siRNA on VEGF expression in human colorectal cancer cells. *World J Gastroenterol*, **16**, 1086-92.
16. Zhu, Y.M., Huang, Q., Lin, J., Hu, Y., Chen, J. and Lai, M.D. (2007) Expression of human DNA methyltransferase 1 in colorectal cancer tissues and their corresponding distant normal tissues. *Int J Colorectal Dis*, **22**, 661-6.
17. Kim, S., Takahashi, H., Lin, W.W., Descargues, P., Grivennikov, S., Kim, Y., Luo, J.L. and Karin, M. (2009) Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature*, **457**, 102-6.
18. Neurath, M.F. and Finotto, S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev*, **22**, 83-9.
19. Fantini, M.C. and Pallone, F. (2008) Cytokines: from gut inflammation to colorectal cancer. *Curr Drug Targets*, **9**, 375-80.
20. Schneider, M.R., Hoeflich, A., Fischer, J.R., Wolf, E., Sordat, B. and Lahm, H. (2000) Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells. *Cancer Lett*, **151**, 31-8.
21. Rigby, R.J., Simmons, J.G., Greenhalgh, C.J., Alexander, W.S. and Lund, P.K. (2007) Suppressor of cytokine signaling 3 (SOCS3) limits damage-induced crypt hyper-proliferation and inflammation-associated tumorigenesis in the colon. *Oncogene*, **26**, 4833-41.
22. Li, Y., de Haar, C., Peppelenbosch, M.P. and van der Woude, C.J. (2011) New insights into the role of STAT3 in IBD. *Inflamm Bowel Dis*.

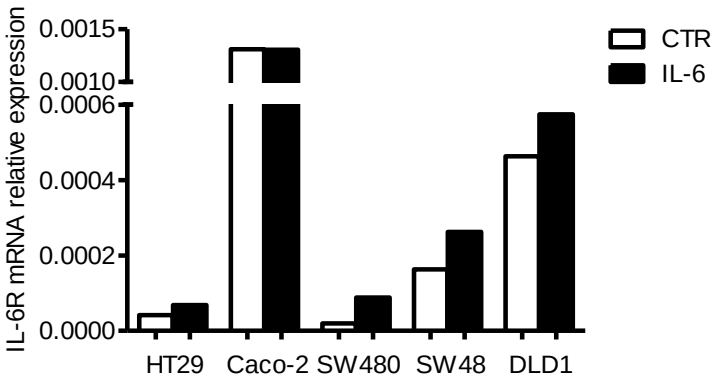
SUPPLEMENTARY FIGURES



Supplementary figure 1. SOCS3 protein expression in epithelial cells in the progression to CD-CRC. SOCS3 expression was investigated in the biopsies of controls (26), inactive CD (9), active CD (21), CD dysplasia (2) and CD-CRC (12). No significant difference of SOCS3 postive epithelial cells were found out between each groups (all p>0.05).



Supplementary figure 2. The design of MSP primers on SOCS3 gene promoter in CpG islands-rich fraction. Three pairs of MSP primers were design targeting on the elements before exon1, on the exon1 and the intron after exon1, respectively.



Supplementary figure 3. IL-6R expression upon IL-6 stimulation in CRC cell lines. Caco-2 Cells which without clear signs of SOCS3 methylation was induced with highest level of SOCS3 upon IL-6 stimulation. In all the other cell lines IL-6 was not able to induce strong SOCS3 expression.

Chapter V

Increased SOCS3 expression predicts mucosal relapse in ulcerative colitis

Yi Li[1], Veerle J. A. A. Nuij[1], Judith E. Baars[1], Katharina Biermann[2], Ernst J. Kuipers[1, 3], Maikel P. Peppelenbosch[1], Colin de Haar[1#], C. Janneke van der Woude[1#].

1. Departments of Gastroenterology and Hepatology
2. Department of Pathology
3. Department of Internal Medicine

Erasmus Medical Center, Rotterdam, Netherlands.

[#] Authors share senior authorship.

Submitted for publication

ABSTRACT

Background: Most biomarkers predicting mucosal relapse of ulcerative colitis (UC) patients in clinical remission represent low levels of mucosal inflammation. Since SOCS3 expression may increase the vulnerability of intestinal epithelial cells (IEC) to various insults, we investigated whether its expression predict mucosal relapse in UC patients in clinical remission without any signs of mucosal inflammation.

Design: UC patients (n=32) in clinical, endoscopic and histological remission who underwent a baseline surveillance colonoscopy between 2001 and 2003 were included. Follow-up ended on December 31st, 2010. IEC expression of SOCS3, p-STAT3 and p-STAT1 was assessed in biopsies from the baseline colonoscopy, last colonoscopy before relapse and colonoscopy at relapse. Clinical data, endoscopy and histology reports were collected from patient charts.

Results: Twenty-six (81%) patients had histological relapse, 19 (59%) developed an endoscopic relapse, and 17 (53%) had a clinical relapse during follow-up. SOCS3 expression in biopsies from 1st colonoscopy during remission correlated with a shorter time to histological, endoscopic and clinical relapse. SOCS3 expression was increased at the last colonoscopy before relapse and approached relapse levels, whereas p-STAT3 expression was low during the entire remission. A positive correlation showed between IEC SOCS3 and one of its inducers p-STAT1.

Conclusion: SOCS3 IEC expression during remission is a predictor for mucosal relapse in patients without any signs of mucosal inflammation. These data strengthen our hypothesis that SOCS3 contributes to enhanced vulnerability of IEC during remission. Thus SOCS3 levels during remission may function as a therapeutic target for clinical monitoring and early induction of mucosal healing.

KEY WORDS: SOCS3, p-STAT1, UC, prediction

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD). Most patients suffer from an intermittent disease course with periods of remission and relapses. Relapse requires adjustment of medication and could probably be preventable if immunosuppressive therapy is intensified before on-set. A relapse period is however difficult to predict [1]. Markers which are proposed to predict systematic clinical relapse include the C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), prostaglandin E2 and fecal calprotectin [2-6]. Unfortunately, these markers either lack specificity or are not sensitive and specific enough to predict relapse in long-term remission. This also pertains to others factors like interleukin-6 (IL-6) and soluble tumor necrosis factor-α (TNF-α) [7, 8]. Most studies that attempt finding predictors of relapse in IBD link the involvement of biological parameters to clinical symptoms. Although these clinical symptoms to some extent correlated with the macroscopic disease activity as defined by endoscopy [9], they poorly correlated with histological signs of mucosal inflammation. The mucosal compartment is mediating the inflammatory response during relapse. It is likely that functional information from this compartment could provide important clues as to an impending relapse of disease. The epithelial compartment might be especially important here because of its major role in UC.

Interesting candidate markers for such a study are the suppressor of cytokine signaling (SOCS) proteins from an important family of negative regulators of cytokine-induced signal transducers and activators of transcription (STAT) protein signaling [10]. SOCS proteins are expressed following cytokine-induced STAT phosphorylation, functioning as a classical negative feedback loop by inhibiting STAT signaling [11] (Supplementary figure 1). SOCS3 is one of the SOCS family members controlling STAT3 signaling downstream of a variety of cytokines and growth factors [12]. SOCS3 protein has been detected in IEC and other cells of the lamina propria in both mouse models of colitis and IBD patients, suggesting a role in the pathogenesis of IBD [13]. Whether the expression of SOCS3 is related to an upcoming relapse, however remains unknown.

Preclinical data, however prompt investigation into the relation between epithelial SOCS3 expression and UC relapse. Mice with an IEC-specific ablation in STAT3 signaling are more vulnerable to dextran sodium sulphate (DSS)-induced colitis, suggesting that IL-6 driven STAT3 signaling in epithelial cell enhances survival and proliferation, contributing

to the protection against colitis [14, 15]. We thus hypothesized that a high constitutive expression of SOCS3 in epithelial cells of UC patients in remission could interfere with normal IL-6/p-STAT3-mediated epithelial cell homoeostasis, enhancing the sensitivity of these cells to inflammatory damage [16]. This would suggest that high expression of SOCS3 may correlate with a shorter time-to-relapse. If so, SOCS3 would provide as a very valuable biomarker for adjusting immunosuppressive therapy in UC to prevent relapse of the disease.

In the present study, we therefore investigated whether SOCS3 expression in IEC predicted the time-to-relapse of UC patients in histological, endoscopic and clinical remission. Since no active STAT3 signaling was detected in IEC during remission, we also checked the expression of another possible inducer of SOCS3 expression, p-STAT1 [17]. The results demonstrate a strict correlation between epithelial SOCS3 levels and upcoming relapse of the disease.

MATERIALS AND METHODS

Patients

This study was conducted with the approval of the Netherlands ethics committee. UC patients were selected from a colorectal cancer surveillance colonoscopy program in the Erasmus University Medical Center, Rotterdam, the Netherlands between January 2001 and December 2003. Thirty-two patients in entire remission (clinical, endoscopic and histological) and with the follow-up data will the end of December 31st 2010 were included in this study. During follow-up, clinical, endoscopic and histological relapses were assessed based on the following criteria: Clinical activity was defined as having one of the following symptoms: abdominal pain, diarrhea, blood loss, fever, weight loss and/or a physician global assessment of clinical disease activity. Endoscopic severity of disease was scored according to the four-point Mayo score as suggested by the most recent guidelines for the surveillance colonoscopies [18]: 0, normal; 1, mild disease (erythema, decreased vascular pattern, mild friability, no contact bleeding); 2, moderate disease (marked erythema absent vascular pattern, friability, erosion, contact bleeding); 3, severe diseases (spontaneous bleeding, ulceration). Histological disease activity was scored according to the Geboes criteria [19] on the scales of 0-3 respectively: 0, no active histological disease activity; 1, mild active inflammation (cryptitis, but no crypt abscesses);

2, moderate active inflammation (few crypt abscesses); 3, severe active inflammation (numerous crypt abscesses). The histological disease activity was confirmed by two independent pathologists and the endoscopic disease activity was evaluated by two physicians together. Clinical data as well as endoscopy and histology reports were collected from the patients charts. Extent of disease was subdivided into pancolitis, left-sided colitis and proctitis. Pancolitis was defined as colitis beyond the splenic flexure, left-sided colitis as disease located distal to the splenic flexure. Proctitis was defined as an inflammation limited to the last 6 inches of the rectum.

Immunohistochemistry staining and scoring

Biopsies from the descending colon and rectum where the most UC injury occurs and easy to obtain are used for this study. Biopsies were taken from the baseline colonoscopy during remission, the last follow-up colonoscopy before histological relapse and is applicable the colonoscopy at histological relapse after informed consent (Figure 1A). The similarity of SOCS3, p-STAT3 and p-STAT1 expression between descending colon and rectum were compared. Biopsies were fixed in 10% formalin, embedded in paraffin and stained for SOCS3, p-STAT3 and p-STAT1 protein expression. Sections of paraffin-embedded tissues (4 μm) were mounted onto slides of Superfrost Plus (THERMO SCIENTIFIC, UK), deparaffinised in xylene and rehydrated. The endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 15 min at room temperature. Antigen retrieval was performed by boiling in preheated buffer (Tris/EDTA pH 9.0 for SOCS3 and EDTA pH 8.0 for p-STAT1 and p-STAT3) for 15 min at 200 W in a microwave. Next, slides were transferred to Shandon chambers and blocked by 10% normal human serum, 10% goat serum in phosphate-buffered saline pH 7.4 for SOCS3, 5% goat serum in Tris-buffered saline and 0.1% Tween 20 for p-STAT1 and p-STAT3 for 1 h at room temperature. Polyclonal rabbit anti-SOCS3 (ABCAM ab53984, 1: 200), rabbit polyclonal anti-p-STAT1 (CELL SIGNALING, #9167, 1: 400) and monoclonal rabbit anti-p-STAT3 tyrosine 705 (CELL SIGNALING, #9145, 1: 50) were diluted in 10% blocking buffer and incubated at 4 degrees overnight. Envision goat anti-rabbit-HRP (DAKO, DENMARK) was used as secondary antibody. Immunoreactions were detected using 3-3-diaminobenzidine (10 mg/ml) in Tris-HCl 0.05 M pH 7.6 containing imidazole stock (0.068 g/10 ml) and 0.03% H2O2, followed by counterstaining with haematoxylin. Negative controls were performed using only the secondary antibody. The percentage of IEC in the whole

biopsies that stained positive (immunoreactivity above background) was scored. A trained pathologist scored basal plasmacytosis after haematoxylin-eosin (H&E) staining.

Statistical analysis

Statistical analysis was performed using SPSS software version 15.0 and the analysis tool embedded in the GraphPad Prism 5.0 software. The descriptive data were presented as mean and individual data points. Differences between subgroups were analyzed using Kruskal-Wallis test, which was followed by Dunn's Multiple Comparison Test. The relationship between SOCS3 and p-STAT1 positive epithelial cells was determined by χ^2. Two-sides p values <0.05 were considered statistically significant.

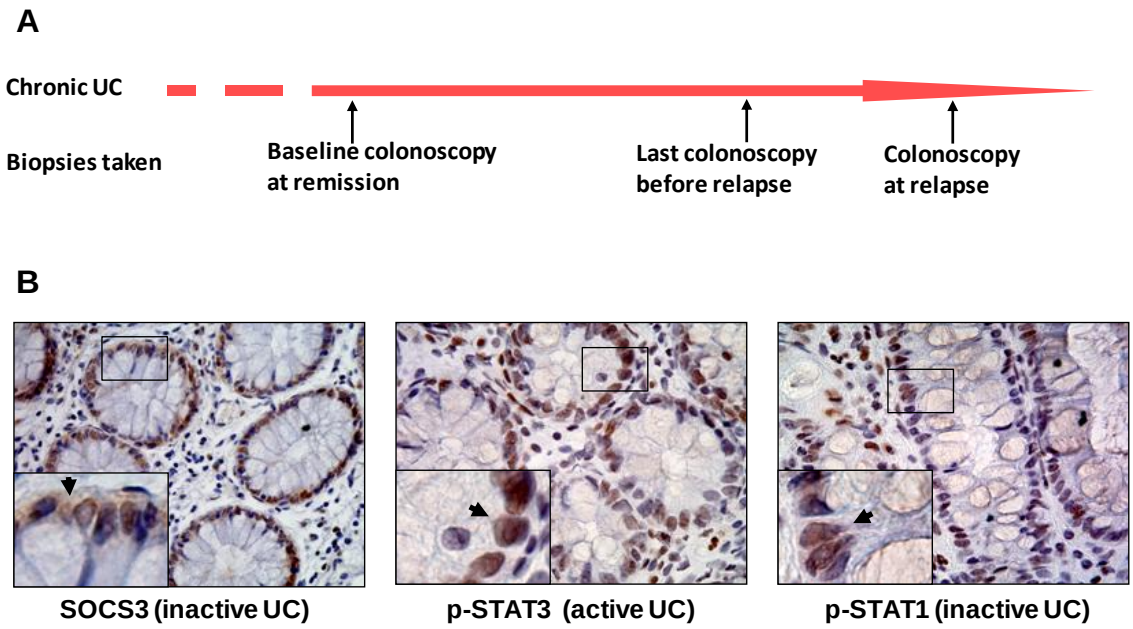


Figure 1. Colonoscopy time-line and biopsy staining

1A. Biopsies from UC patients were used, that were taken during the baseline colonoscopy, the last colonoscopy before relapse and the colonoscopy where histological colitis relapse was detected. 1B. Biopsies of both the descending colon and the rectum were stained for the presence of SOCSS, p-STAT3 and p-STAT1 (x 400 and x 2400 inserts).

RESULTS

Patient characteristics

Table 1. Patients' characteristics

Characteristics	Ulcerative colitis (n=32)
Gender	
Male	16 (50%)
Female	16 (50%)
Mean age, years ± SD	52.7 ± 16.5
Mean age of diagnose UC, years ± SD	24.6 ± 12.9
Mean duration of disease, years ± SD	26.1 ± 8.5
Mean duration to histological relapse, years ± SD	4.19 ± 2.38
Histological relapse during follow-up	26 (81.2%)
Relapse in 1-3 (years)	11
Relapse in 4-6 (years)	10
Relapse in 7-9 (years)	5
Mean duration to endoscopic relapse, years ± SD	3.79 ± 2.30
Endoscopic relapse during follow-up	19 (59.4%)
Relapse in 1-4 (years)	13
Relapse in 5-9 (years)	6
Mean duration to clinical relapse, years ± SD	4.24 ± 3.05
Clinical relapse during follow-up	17 (53.1%)
Relapse in 1-4 (years)	10
Relapse in 5-9 (years)	7

Thirty-two UC patients in remission (both clinically, endoscopic and histological), undergoing surveillance colonoscopy were selected. Of these patients, 16 were males (50%), 16 were females (50%), with a mean age of 52.7 years (± 16.5 years). The mean age at diagnosis of UC was 24.6 years (± 12.9 years). The mean duration of disease was 26.1 years (± 8.5 years). Patients were under endoscopic surveillance, which implied colonoscopy at 6-24 months intervals depending on histology following standard protocol

in the Erasmus MC and at the discretion of the treating physician. Among the 32 patients, 26 (81.2%) developed histological relapse, 19 (59.4%) had endoscopic relapse and 17 (53.1%) had clinical evidence of relapse within 9 years follow-up (Table 1). The population of histological, endoscopic and clinical relapse was presented in different intervals depends on the different distributions during follow-up.

Table 2. Patients characteristic of patient with different times to relapse

Characteristics	**Remission time before histological relapse (y)**			
	1-3	4-6	7-9	No relapse
No. of subjects	12	9	5	6
Gender (M/F)	6/6	5/4	3/2	2/4
Mean age (y) *	60.9 (11.5)	42.8 (12.6)	47.0 (6.5)	68.2 (13.0) #
Mean age at diagnosis (y) *	31.9 (12.2)	15.1 (8.5)	22.4 (5.3)	34.3 (12.2)
Mean disease duration (y) *	27.1 (10.6)	26.1 (4.4)	23.2 (7.1)	33.8 (10.1)
Maximum disease extent in history				
Pancolitis	4	5	4	4
Left-side colits	5	2	1	1
Proctitis	0	0	0	0
Unknown	3	2	0	1
Severity of disease in history (histological)				
Mild	1	1	0	2
Moderate	4	1	2	2
Severe	4	6	3	2
Unknown	3	1	0	0
Severity of disease in history (endoscopic)				
Mild	0	1	0	3
Moderate	4	4	2	0
Severe	5	2	3	3
Unknown	3	2	0	0
Medication use at 1 year before surveillance				
5-ASA	9	5	3	3

Characteristics	Remission time before histological relapse (y)			
Ursochol	1	0	0	0
†Combination	2	3	2	2
No	0	1	0	1
Medication use at 1 month before surveillance				
5-ASA	8	7	3	3
Imuran	0	0	1	0
Ursochol				
†Combination	2	1	1	2
No	1	1	0	1
Medication use after surveillance				
5-ASA	7	5	3	3
Imuran				
Corticosteroids	0	1	0	0
Ursochol	1	0	0	0
†Combination	4	2	1	2
No	0	1	0	1
Basal plasmacytosis at 1st histological remission				
No	7	6	3	6
Mild	1	3	2	0
Moderate	4	0	0	0

* mean (SD)

The mean age in the group without a relapse during follow-up was significantly higher (p=0.014) than other groups.

† The specific combination of drugs were explained in the last section of results.

SOCS3 expression in IEC during early remission predicts the time-to-relapse

SOCS3 expression in IEC was assessed using immunohistochemical staining of biopsies from the descending colon and rectum (Figure 1B). Patients with a histological relapse within 1-3 years or 4-6 years had a higher percentage of SOCS3-positive IECs in biopsies from descending colon than those with a histological relapse within 7-9 years or no relapse at all during follow-up (Kruskal-Wallis test p=0.0003; 1-3(y) vs. 7-9(y), p=0.0006;

1-3(y) vs. no >9(y), p<0.0001; 4-6(y) vs. no >9(y), p=0.0015) (Figure 2A). A Kaplan-Meier curve was used to illustrate for the probability of persistent histological remission in relation to the baseline SOCS3 expression (p<0.0001) (Figure 2B).

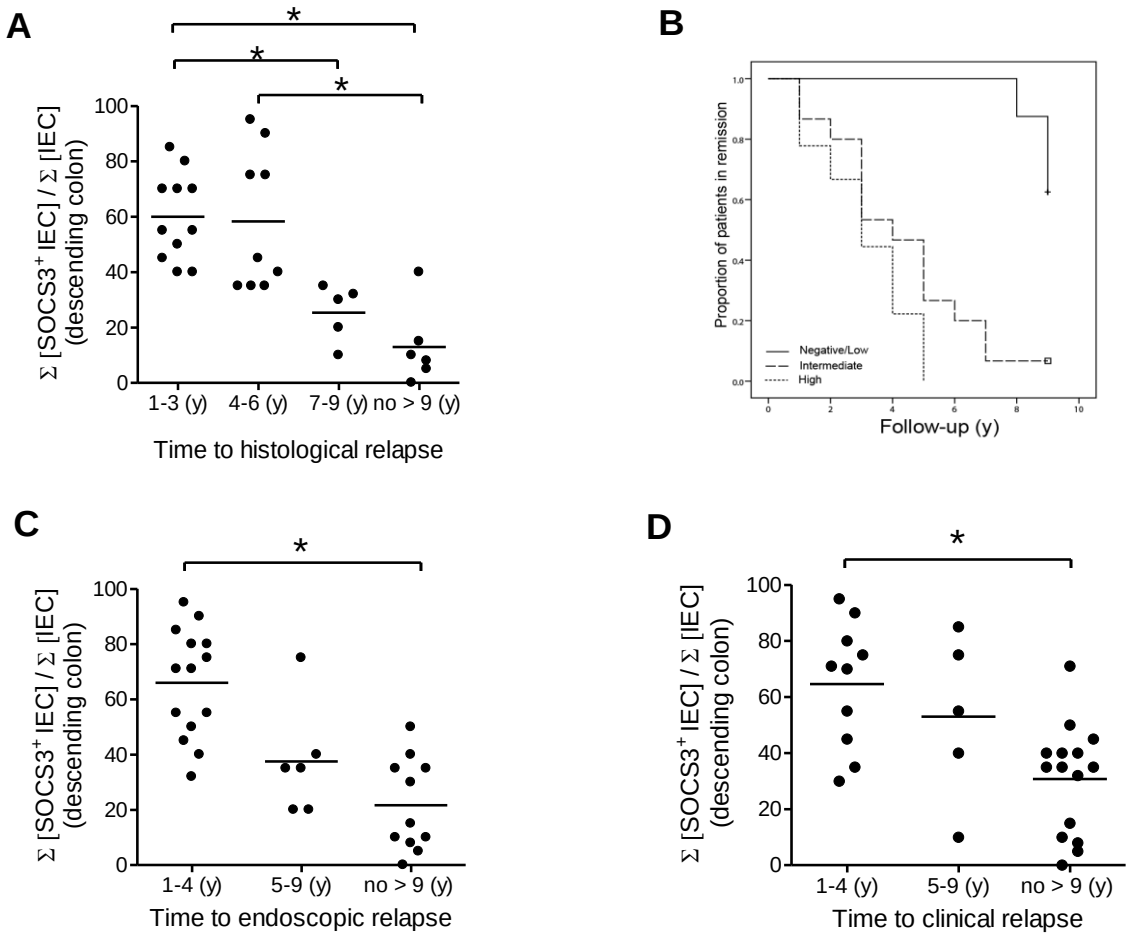


Figure 2. SOCS3 expression in IEC correlates with time-to-relapse

The percentage of SOCS3 positive IEC was assessed in the biopsies from the baseline colonoscopy during remission. The SOCS3 expression in IEC from descending colon was compared between individuals who had a histological relapse (A) within 1-3, 4-6, 7-9 years or who did not relapse during follow up (no > 9). Kaplan-Meier curve showing the proportion of patients remaining in remission with negative/low (0-30 %), intermediate (31-60 %) or high (61-100 %) levels of SOCS3 expression in IEC from descending colon (B). The SOCS3 expression in IEC from descending colon was compared between individuals who had an endoscopic (C) or clinical (D) relapse within 1-4, 5-9 years or who did not relapse during follow up (no > 9). The mean and individual expression is depicted and * means statistic significance $p < 0.05$ (Dunn's Multiple Comparison Test).

When using endoscopic or clinical relapse as endpoints, again a significant correlation between the numbers of SOCS3-positive IECs and the time to relapse was found (endoscopic: Kruskal-Wallis test p=0.0002; 1-4(y) vs. no >9(y), p<0.0001; clinical, Kruskal-Wallis test p=0.0086; 1-4(y) vs. no >9(y), p=0.0005) (Figure 2C-D). This effect was observed both at the level of the descending colon and rectum (Supplementary figure 2A-C).

To determine whether any of the clinical characteristics were linked to the time to histological relapse, patients characteristics were analyzed accordingly (Table 2). The mean age in the group without a relapse during follow-up was significantly older (p=0.014) than other groups. While the basal plasmacytosis was overall low (negative/mild) in biopsies taken at baseline histological remission, there were some patients in the group that relapsed within 1-4 years, who showed moderate basal plasmacytosis (Supplementary figure 3A). Within the group relapsing in 1-4 years, there was no correlation between basal plasmacytosis and time-to-relapse. There was also no significant correlation between basal plasmacytosis and SOCS3 expression (Supplementary figure 3B). Thus, epithelial SOCS3 status is a specific and independent predictor of upcoming relapse in UC.

P-STAT1 correlates with SOCS3 expression in IEC in the baseline biopsies taken during remission

SOCS3 expression is under the control of STAT3, a transcription factor normally residing in cytoplasm but upon stimulation is phosphor-related (p-STAT3), dimmerizes and translocates to nucleus to alert transcription[21]. To investigate which factors are involved in the increased SOCS3 biopsies were stained for STAT3 and STAT1 activity (Figure 1B). The percentage of p-STAT3-positive IECs was low in all biopsies taken at baseline colonoscopy during remission (Figure 4B). As such, there was no correlation between IEC expression of p-STAT3 and SOCS3. The expression of p-STAT1, an alternative SOCS3 inducer, correlated with the SOCS3 expression in the IEC from the biopsies taken at baseline colonoscopy during remission (p<0.0001) (Figure 3A).

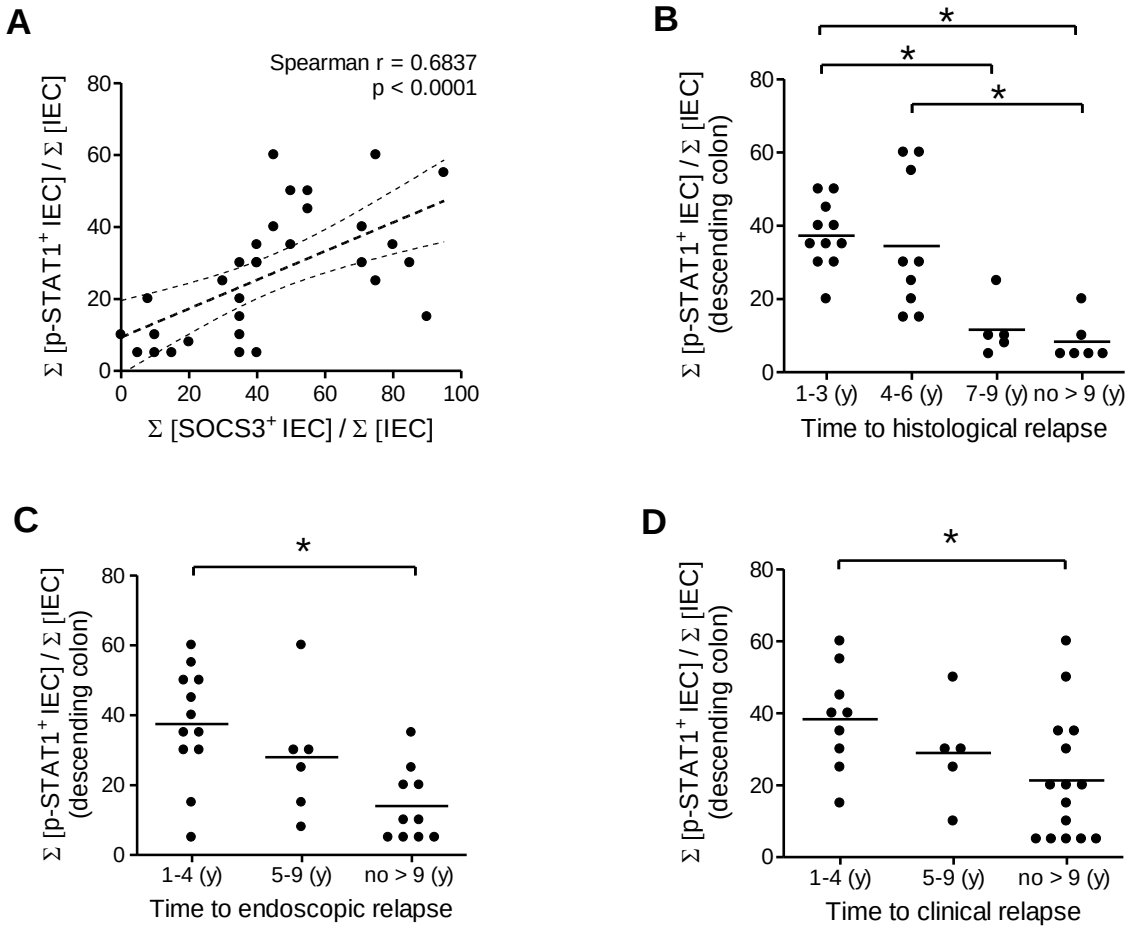


Figure 3. P-STAT1 expression in IEC correlates with SOCS3 expression and time-to-relapse

(A) Correlation between p-STAT1 and SOCS3 positive IEC in biopsies from the baseline colonoscopy during remission. The p-STAT1 expression in IEC from descending colon was compared between individuals who had a histological relapse (B) within 1-3, 4-6, 7-9 years or who did not relapse during follow up (no > 9). The p-STAT1 expression in IEC from descending colon was compared between individuals who had an endoscopic (C) or clinical (D) relapse within 1-4, 5-9 years or who did not relapse during follow up (no > 9). The mean and individual expression is depicted and * means statistic significance $p < 0.05$ (Dunn's Multiple Comparison Test).

The percentage of p-STAT1 positive IEC was significantly higher in remission biopsies from patients with a histological relapse within 1-3 years than in those with histological relapse within 7-9 years or those not relapsing at all during follow-up (Kruskal-Wallis test p=0.0003; 1-3(y) vs. 7-9(y), p<0.0001; 1-3(y) vs. no >9(y), p<0.0001; 4-6(y) vs. no >9(y),

p=0.0062) (Figure 3B). In biopsies from patients with endoscopic or clinical relapse, the percentage of p-STAT1 positive IEC was significantly higher in patients who relapse within 1-4 years compared with those who did not relapse during follow-up relapse (endoscopic: Kruskal-Wallis test p=0.0067; 1-4(y) vs. no >9(y), p=0.0008; clinical, Kruskal-Wallis test p=0.0481; 1-4(y) vs. no >9(y), p=0.0219) (Figure 3C-D). Consistent data were found using biopsies from either descending colon or rectum. Thus although STAT1 activation seems to be involved in driving SOCS3 expression in the upcoming-relapse epithelium, SOCS3 is the more reliable biomarker.

SOCS3, p-STAT3 and p-STAT1 expression increase at time of relapse

The SOCS3-positive IEC in the baseline biopsies taken during remission differs significantly between individuals, and is associated with time-to-relapse. The average SOCS3 expression slightly increased from the baseline remission biopsies to last remission biopsies, and significantly increased at time of relapse (baseline remission vs. relapse, p<0.0001; last remission vs. relapse, p<0.0001) (Figure 4A). Due to the increasing trend of SOCS3 in the whole period of remission, the SOCS3-positive IEC, at the last remission before relapse, did not correlate significantly with the time to relapse that mostly occurred within 3 years (data not shown), indicating the neat correlation of SOCS3 to the time of relapse appears more in long term remission.

In addition to the previously mentioned lack of p-STAT3 expression IEC in biopsies from the baseline colonoscopy during remission, the expression was low in the last biopsies taken before relapse and significantly increased at the time relapse (baseline remission vs. relapse, p<0.0001; last remission vs. relapse, p=0.0002) (Figure 4B). The percentage of p-STAT1 positive IEC was also significantly increased in biopsies taken at time of relapse (baseline remission vs. relapse, p=0.0002; last remission vs. relapse, p=0.0026) (Figure 4C). Thus we view SOCS3 expression as an integrate part of a momentum-building mucosal inflammatory response, whose early appearance provides an opportunity for therapeutic intervention.

SOCS3 expression was not affected by drug use

In the year before surveillance colonoscopy, 20/32 of these patients used 5-ASA, 1/32 used ursochol, 9/32 patients had combination medicine use of 5-ASA, corticosteroids or immunosuppressives, 1/32 patients didn’t use any medicine for UC treatment. In the

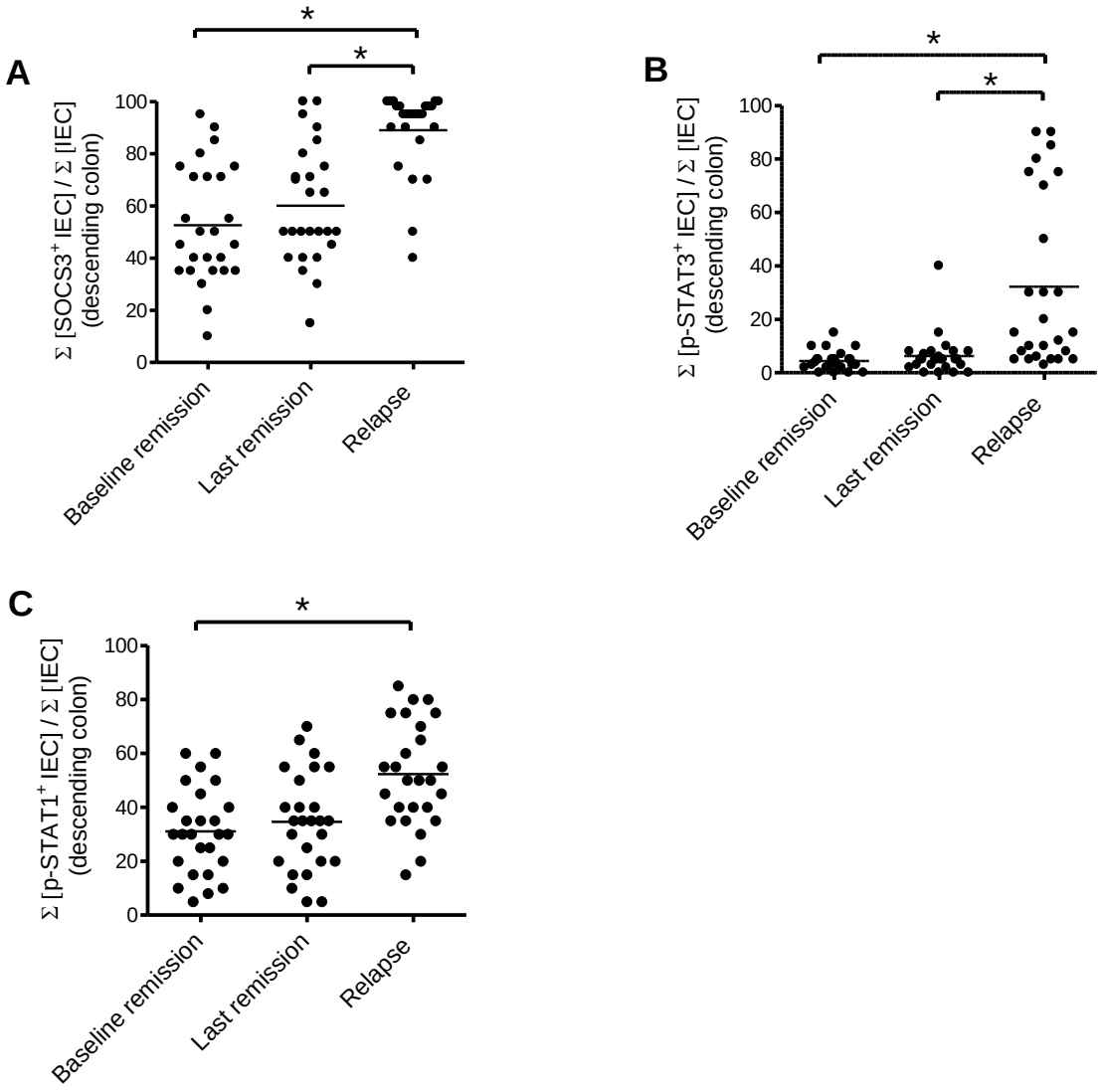


Figure 4. SOCS3, p-STAT3 and p-STAT1 are upregulated in IEC during relapse

The percentage SOCS3 (A), p-STAT3 (B), p-STAT1 (C) positive IEC was compared between biopsies taken at baseline remission, last remission or relapse. The mean and individual expression is depicted and * means statistic significance $p < 0.05$ (Dunn's Multiple Comparison Test).

month before surveillance colonoscopy, 21/32 of these patients used 5-ASA, 1/32 patients used imuran, 1/32 used ursochol, 6/32 patients had combination medicine use of 5-ASA, corticosteroids or immunosuppressives, 3/32 patients didn't use any medicine for UC treatment. No significantly difference was found between SOCS3 expression at first remission and above medicine use ($p>0.05$). In the period from surveillance till the end of

follow-up, 15/26 of these patients used 5-ASA, 1/26 patients used imuran, 1/26 patients used steroids, 1/26 used ursochol, 7/26 patients had combination medicine of 5-ASA, corticosteroids, immunosuppressives, folic acid, or ursodeoxycholic acid. 1/26 patients didn't use any medicine for UC treatment. SOCS3 expression at last remission time was not related to the maintenance usage of above medicine after surveillance (all $p>0.05$). In addition, p-STAT1 expression from first/or last colonoscopy at remission was not related to the drug use of 5-ASA, corticosteroids and immunosuppressives before/after surveillance ($p>0.05$).

DISCUSSION

The identification of a reliable biomarker predicting relapse would contribute to improved care for UC patients. Our aim was to investigate whether the expression of SOCS3 in IEC from UC-patients in complete remission was related to the time to relapse. This was based on the hypothesis that a high levels of SOCS3 expression observed during inactive disease may interfere with IEC homeostasis making epithelial cells more vulnerable to various insults thus leading to earlier relapse [20].

In the present study high SOCS3 expression in colonic IEC of UC patients in complete remission was indeed associated with a shorter time until relapse. Similar to our results, Miyanaka et al. reported that UC patients in clinical remission with a low expression of mucosal SOCS3 mRNA had a lower relapse rate within 12 months [22]. Unfortunately, only mRNA levels were determined in this study, so no insight was provided which cells were expressing SOCS3. In addition, that report showed SOCS3 expression correlated positively with endoscopic and histological disease activity scores, but no information about these scores and their link to SOCS3 expression was provided in the patients in clinical remission per se.

It has been well established that after the induction of clinical remission, a significant proportion of UC patients remain to show endoscopic and histological signs of disease activity, which are related to the time to relapse [23]. Still, most studies on predictive factors for relapse used clinical assessment of remission and relapse [5, 24]. In those studies the factors which in particular predict relapse are early signs of mucosal inflammation predicting clinical relapse within the next few months/year. As we have only used UC patient biopsies without any clinical, endoscopic, or histological signs of disease activity

and a much longer follow-up period of 9 years, it is unlikely that the SOCS3 expression is just another early marker for reactivation of disease. Since the SOCS3 expression increased from the baseline remission to last remission biopsies, approaching the level of high expression at time of relapse, the prediction of SOCS3 in short period behaves as an increasing trend instead of regular correlation.

As with other early signs of reactivation, the enhanced recruitment of basal plasma cells has been suggested to be an important immunologic event in the progression to local inflammation and eventually clinical relapse [24]. The presence of basal plasmacytosis has been independently associated with a shorter time till clinical relapse [24]. In our data the majority of patients displayed no signs of basal plasmacytosis in the baseline biopsies after remission. As such, we did not find a significant association between basal plasmacytosis and time-to-relapse, which may be due to our definition of remission based on histology instead of only endoscopic activity assessment. However, moderate basal plasmacytosis was detected in some patients in the group with a relapse within 1-4 years, partly supporting the previously observed correlation with time-to-relapse [24]. Unfortunately, the previously reported relapse predicting factors calprotectin and lactoferrin are not part of the UC surveillance parameters and therefore not assessed in our studies [5, 26]. However, since it merely represents the influx of neutrophils and the time to relapse in previous studies has always been within a year this is not a likely factor to predict relapse over a longer time.

Although the high expression of SOCS3 correlates with a shorter time till relapse, the question remains whether it actively contributes to remission or merely defines a subset of UC patients prone to have faster and more severe relapses. As a relapse will be the consequence of a variety of disease-affecting factors like genetics, microbiota and other environmental factors, the actual trigger for relapse is not know and may be different for every UC patient. The previously reported defects in IEC homeostasis in mice that lack normal STAT3 signaling and their enhanced vulnerability to experimental colitis support an active contribution of SOCS3 in risk for relapse [14, 15]. It is therefore of interest which factors are driving the expression of SOCS3. Our current data support our previous observation that IEC in biopsies from UC patients with inactive disease did not express p-STAT3 [16]. The expression of another possible SOCS3-inducer, p-STAT1, correlated positively with the expression of SOCS3 and resembled the SOCS3 correlation with time to relapse and severity of relapse. These data suggests that p-STAT1-induced SOCS3

expression interferes with normal STAT3 signaling and it's anti-inflammatory and proliferative effects, as was previously reported for endothelial cells [17]. Further investigations are ongoing to elucidate the mechanisms involved in the increased SOCS3 expression, potentially holding therapeutic targets for the down-regulation of SOCS3 during remission.

In conclusion, we show that high expression of SOCS3 is associated with a shorter time until relapse. We propose that the high expression of SOCS3 represents a subgroup of patients that may be more vulnerable to whatever trigger is involved in the induction of relapse. Having SOCS3 as a predictor, we are able to estimate the risk of relapse and adjust the therapeutic strategies. It would be beneficial if apply more intensive therapy for the patients who have more potential of getting relapse, and avoid unnecessary medical treatment in inactive UC patients who are not at significant risk of relapse.

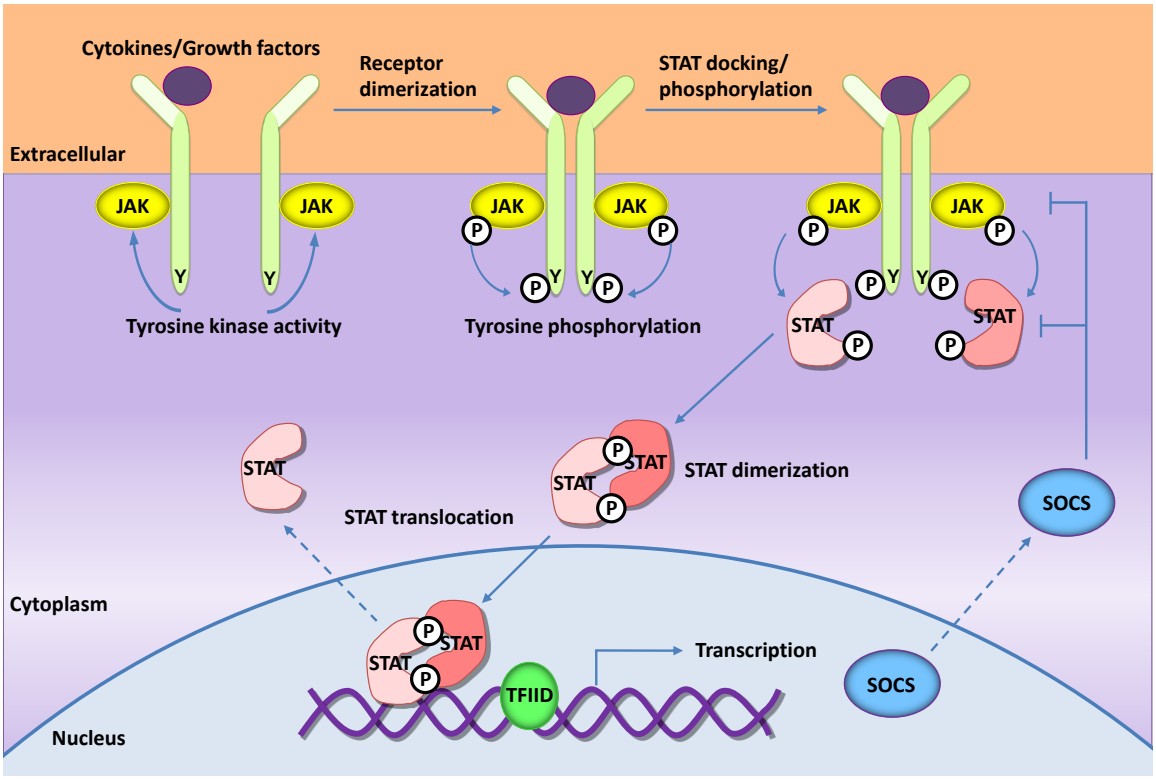
REFERENCES

1. Langholz E, Munkholm P, Davidsen M, Binder V. Course of ulcerative colitis: analysis of changes in disease activity over years. Gastroenterology 1994;107:3-11.
2. Henriksen M, Jahnsen J, Lygren I, Stray N, Sauar J, Vatn MH, Moum B. C-reactive protein: a predictive factor and marker of inflammation in inflammatory bowel disease. Results from a prospective population-based study. Gut 2008;57:1518-23.
3. Consigny Y, Modigliani R, Colombel JF, Dupas JL, Lemann M, Mary JY. A simple biological score for predicting low risk of short-term relapse in Crohn's disease. Inflamm Bowel Dis 2006;12:551-7.
4. Lauritsen K, Laursen LS, Bukhave K, Rask-Madsen J. Use of colonic eicosanoid concentrations as predictors of relapse in ulcerative colitis: double blind placebo controlled study on sulphasalazine maintenance treatment. Gut 1988;29:1316-21.
5. Gisbert JP, Bermejo F, Perez-Calle JL, Taxonera C, Vera I, McNicholl AG, Algaba A, Lopez P, Lopez-Palacios N, Calvo M, Gonzalez-Lama Y, Carneros JA, Velasco M, Mate J. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. Inflamm Bowel Dis 2009;15:1190-8.
6. Ho GT, Lee HM, Brydon G, Ting T, Hare N, Drummond H, Shand AG, Bartolo DC, Wilson RG, Dunlop MG, Arnott ID, Satsangi J. Fecal calprotectin predicts the clinical course of acute severe ulcerative colitis. Am J Gastroenterol 2009;104:673-8.
7. Garcia-Sanchez V, Gonzalez R, Iglesias-Flores E, Gisbert JP, Angel-Rey JM, Soto-Escribano P, Galvez-Calderon C, Reyes-Lopez A, Perez-Jimenez F, de Dios-Vega JF, Muntane J, Gomez-Camacho F. Can systemic cytokines predict relapse of inflammatory bowel disease? Hepatogastroenterology;57:524-30.
8. Louis E, Belaiche J, van Kemseke C, Franchimont D, de Groote D, Gueenen V, Mary JY. A high serum concentration of interleukin-6 is predictive of relapse in quiescent Crohn's disease. Eur J Gastroenterol Hepatol 1997;9:939-44.
9. Higgins PD, Schwartz M, Mapili J, Zimmermann EM. Is endoscopy necessary for the measurement of disease activity in ulcerative colitis? Am J Gastroenterol 2005;100:355-61.
10. Elliott J, Johnston JA. SOCS: role in inflammation, allergy and homeostasis. Trends Immunol 2004;25:434-40.
11. Hebenstreit D, Horejs-Hoeck J, Duschl A. JAK/STAT-dependent gene regulation by cytokines. Drug News Perspect 2005;18:243-9.
12. Dimitriou ID, Clemenza L, Scotter AJ, Chen G, Guerra FM, Rottapel R. Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins. Immunol Rev 2008;224:265-83.
13. Greenhalgh CJ, Miller ME, Hilton DJ, Lund PK. Suppressors of cytokine signaling: Relevance to gastrointestinal function and disease. Gastroenterology 2002;123:2064-81.

14. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.
15. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91-102.
16. Theiss AL, Fuller CR, Simmons JG, Liu B, Sartor RB, Lund PK. Growth hormone reduces the severity of fibrosis associated with chronic intestinal inflammation. Gastroenterology 2005;129:204-19.
17. Bluyssen HA, Rastmanesh MM, Tilburgs C, Jie K, Wesseling S, Goumans MJ, Boer P, Joles JA, Braam B. IFN gamma-dependent SOCS3 expression inhibits IL-6-induced STAT3 phosphorylation and differentially affects IL-6 mediated transcriptional responses in endothelial cells. Am J Physiol Cell Physiol;299:C354-62.
18. Cairns SR, Scholefield JH, Steele RJ, Dunlop MG, Thomas HJ, Evans GD, Eaden JA, Rutter MD, Atkin WP, Saunders BP, Lucassen A, Jenkins P, Fairclough PD, Woodhouse CR. Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). Gut;59:666-89.
19. Geboes K, Riddell R, Ost A, Jensfelt B, Persson T, Lofberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. Gut 2000;47:404-9.
20. Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, Kuipers EJ, van der Woude CJ. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut;59:227-35.
21. Li Y, de Haar C, Peppelenbosch MP, van der Woude CJ. New insights into the role of STAT3 in IBD. Inflamm Bowel Dis;Oct 12 [Epub head of print].
22. Miyanaka Y, Ueno Y, Tanaka S, Yoshioka K, Hatakeyama T, Shimamoto M, Sumii M, Chayama K. Clinical significance of mucosal suppressors of cytokine signaling 3 expression in ulcerative colitis. World J Gastroenterol 2007;13:2939-44.
23. Riley SA, Mani V, Goodman MJ, Dutt S, Herd ME. Microscopic activity in ulcerative colitis: what does it mean? Gut 1991;32:174-8.
24. Roda G, Caponi A, Benevento M, Nanni P, Mezzanotte L, Belluzzi A, Mayer L, Roda A. New proteomic approaches for biomarker discovery in inflammatory bowel disease. Inflamm Bowel Dis;16:1239-46.

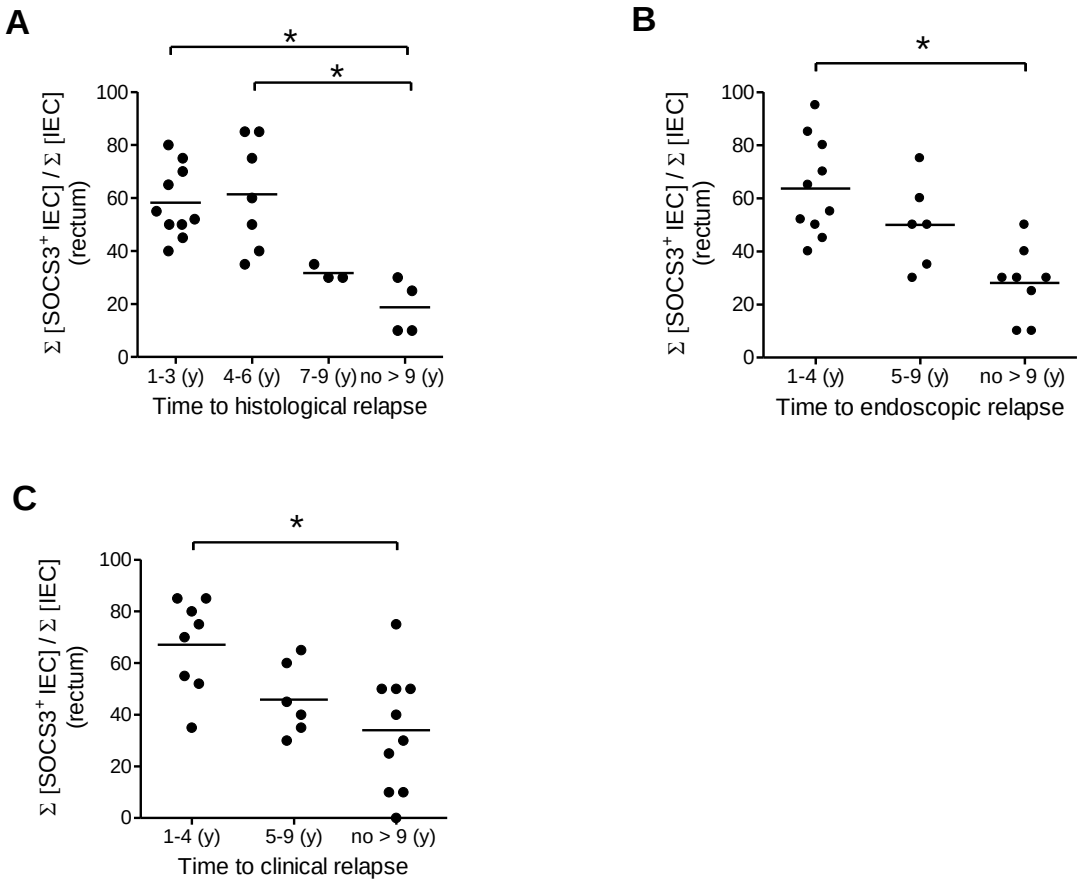
25. Bitton A, Peppercorn MA, Antonioli DA, Niles JL, Shah S, Bousvaros A, Ransil B, Wild G, Cohen A, Edwardes MD, Stevens AC. Clinical, biological, and histologic parameters as predictors of relapse in ulcerative colitis. Gastroenterology 2001;120:13-20.
26. Costa F, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. Gut 2005;54:364-8.

SUPPLEMENTARY FIGURES



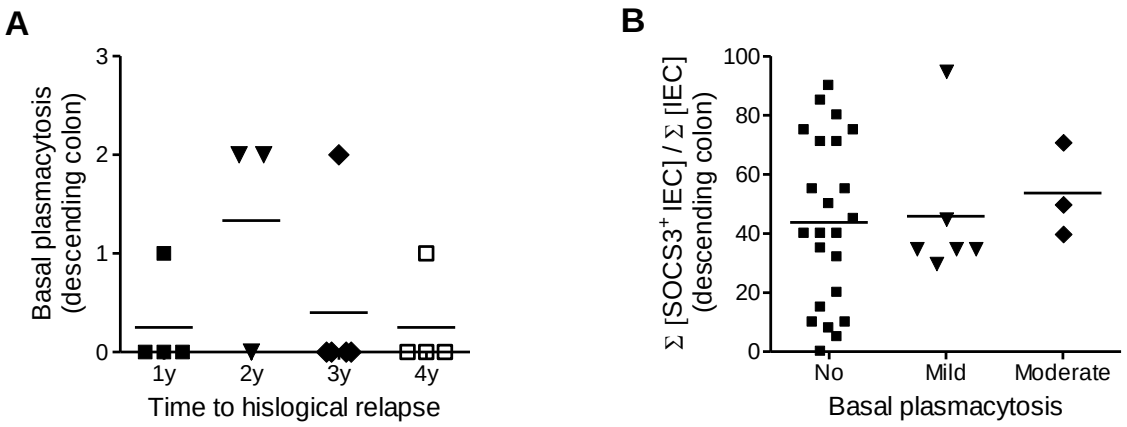
Supplementary Figure 1. Schematic representation of the STAT-signaling pathway from cytokine stimulation of the receptor till gene expression

Cytokine binding to the receptor induces its dimerization and phosphorylation of Janus kinases (JAK) by the intrinsic tyrosine kinase activity. The activated JAK phosphorylates tyrosine residues that are then able to bind STAT proteins. Next, JAK phosphorylates STAT enabling its dimerization and translocation to the nucleus. In the nucleus the STAT-dimers bind to the DNA and recruit cofactors like the transcription Factor II D (TFIID) to start transcription of STAT-target genes. One of these target genes, the suppressor of cytokine signaling (SOCS) is able to negatively regulate STAT activation by binding to the phophorylted tyrosine residues on the receptor.



Supplementary Figure 2. The percentage of SOCS3 positive IEC was assessed in expression biopsies from the baseline colonoscopy during remission

The SOCS3 expression in IEC from rectum biopsies was compared between individuals who had a histological relapse (A) within 1-3, 4-6, 7-9 years or who did not relapse during follow up (no > 9). The SOCS3 expression in IEC from rectum biopsies was compared between individuals who had an endoscopic (B) or clinical (C) relapse within 1-4, 5-9 years or who did not relapse during follow up (no > 9). The mean and individual expression are depicted and * means statistical significance $p < 0.05$ (Dunn's Multiple Comparison Test).



Supplementary Figure 3. No (0), mild (1), moderate (2) or high (3) basal plasmacytosis in descending colon biopsies taken during baseline colonoscopy was expressed for each patient relapsing within 4 years after the biopsy was taken (A). The percentage of SOCS3 positive IEC in these biopsies is expressed for all patients with similar basal plasmacytosis.

Chapter VI

STAT1, STAT6 and cAMP signaling drive SOCS3 expression in inactive ulcerative colitis

Yi Li[1], Maikel P. Peppelenbosch[1], Ernst J. Kuipers[1, 2], Colin de Haar[1#], C. Janneke van der Woude[1#].

1. Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, Netherlands.
2. Department of Internal medicine, Erasmus Medical Center, Rotterdam, Netherlands.
[#] Authors share senior authorship.

Submitted for publication

ABSTRACT

Background: SOCS3 is expressed in absence of STAT3-phosphorylation (p-STAT3) in the intestinal epithelial cells (IEC) of ulcerative colitis (UC) patients in remission. The upcoming relapse in UC is associated with high mucosal expression of SOCS3 and hence knowledge of the mechanisms driving mucosal SOCS3 expression may provide important clues as to rational therapy aimed at preventing exacerbation of disease. Here we aimed to characterize the molecular driving SOCS3 in the mucosal compartment.

Methods: The colon epithelial cell line caco-2 was stimulated with IFN-γ, IL-4 or PGE2 to allow correlations between SOCS3 expression with STAT1, STAT6 and cAMP signalling respectively. The physiological relevance of the findings obtained was assessed by immunohistochemical staining for the activated forms of STAT1, STAT6, PKA-Cγ and CREB in biopsies from inactive UC patients and controls.

Results: Stimulation with IFN-γ, IL-4 or PGE2 induced activation of STAT1, STAT6 and cAMP respectively, without any signs of STAT3 activation. The activation of all these pathways led to increased expression of SOCS3. Biopsies from patients with inactive UC showed significant increase of p-STAT1, p-STAT6, p-PKA-Cγ and p-CREB expression (all $p<0.01$) compared with controls.

Conclusions: STAT3-independent SOCS3 induction in inactive UC involves multiple pro-inflammatory signalling pathways arguing against the usefulness of pathway-specific anti-inflammatory drugs for preventing relapse and suggesting that broad-spectrum anti-inflammatory drugs are essential to counteract increases in SOCS3 expression and exacerbation of disease.

KEY WORDS: SOCS3; STAT1; STAT6; cAMP; UC

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD), characterised by alternating periods of remission and relapse. Although the majority of studies focus on the pathways that drive active inflammation, it can be argued that the development of strategies focussing on remission maintenance is the larger clinical challenge in the management of this disease [1]. Nevertheless, the insights into the molecular mechanisms that cause the colonic mucosa to relapse after prolonged periods of quiescent disease remain largely unknown, urgently prompting further investigation.

We have previously reported that an increase of mucosal SOCS3 protein levels in colonic epithelium in biopsies otherwise devoid from signs of a mounting inflammatory reaction is a highly reliable predictor of upcoming relapse[2], suggesting a causative role of increased SOCS3 expression for disease exacerbation. As SOCS3 is an intracellular inhibitor of the signal transduction of various immunomodulatory cytokines and growth factors that normally signal via the potent immunoregulator STAT3, it is well possible that a SOCS3-dependent decrease in the levels of activated SOCS3 releases pro-inflammatory activities from regulation, driving a mucosal inflammatory response. If true, characterisation of the molecular mechanisms that drive SOCS3 expression in the mucosal epithelium of UC patients would thus be of utmost importance for both our fundamental understanding of processes driving UC and open the possibility to devise rational strategies for preventing relapse.

Analysis of the molecular structure of the genetic elements in the SOCS3 promoter that drive transcription of the gene can already provide important clues as to mechanisms potentially involved here. The SOCS3 promoter contains two putative STAT-response elements (SREs), a GC-rich region and an AP-1/CRE (cAMP-response element) region (Figure 1) [3, 4]. It is known that in response to IFN-γ, a Th1 cytokine expressed during UC remission [5], janus kinases associated with the IFN-γ-receptor complex phoshorylate the latent transcription factor STAT1 at plasma membrane and that the thus generated tyrosine phosphorylated form of STAT1 (p-STAT1) translocates to the nucleus upon homodimerisation. In the nucleus p-STAT1 transactivates a variety of gene products, including SOCS3 expression, through binding to the SREs like those present in the SOCS3 promoter (6-7). The importance of STAT1 activation for epithelial SOCS3 expression, both in colonic epithelium in general and during UC in particular has, however,

not been addressed. The SREs in general are also capable of binding p-STAT6 homodimers, which are generated as a cellular response to Th2 cytokines like IL-4 or IL-13 [6]. Since UC is regarded as a Th2-mediated disease, it is well possible that especially STAT6 activation would be responsible for SOCS3 induction, but again this has not been investigated (9). In addition p-STAT5 homodimers should be able bind and transactivate SREs, but the methylation of STAT5 observed in UC patients suggests that it is an unlikely activator of SOCS3 expression [7]. In total, however, the existing body of biomedical literature suggests different STATs are potential mediators of driving SOCS3 expression in UC to provoke relapse and their function needs urgently to be addressed.

Beside SREs, the transcription factors that bind to either AP-1/CRE binding element of the CG-rich element could also be involved in the increased IEC SOCS3 expression. Recently, Prostaglandin E2 (PGE2) was demonstrated to induce SOCS3 expression in breast cancer cells via the activation of Cyclic-(c)AMP and then the AP-1/CRE region in the SOCS3 promoter [4, 8]. In the latter pathway, the downstream cAMP-dependent protein kinase A (PKA) is activated by the binding of cAMP to the regulatory subunit and releasing of catalytic subunits (PKA-C) [9, 10]. In UC patients and experimental colitis, PGE2 expression was increased and related to the severity of colitis (14-15), which may contribute to the SOCS3 induction.

Our aim was to investigate STAT3-independent pathways involved in the induction of SOCS3 in intestinal epithelial cells to show its contribution of impairing IEC for the UC regeneration and increased risk for relapse.

MATERIALS AND METHODS

Patients and biopsies

This study was approved by the ethical committee of the Erasmus MC Rotterdam and after informed consent biopsies from UC patients were obtained during colonoscopy form March 2009 to June 2009. Only patients with a diagnosis of UC over more than one year and in remission for at least three months were included. The diagnosis of UC was based on conventional clinical, endoscopic and histological criteria as described by Lennard-Jones [11]. Inactive UC was defined as patients in three months clinical remission without endoscopic abnormalities and with a Geboes histology score of 0 or 1 [12]. Demographics and current medical therapy were noted. Colonic biopsies from non-UC patients without

abnormalities at colonoscopy and no history of gastrointestinal disease served as control. Patient characteristic are summarized in table I.

Cells and reagents

Caco-2 cells were grown into a fully differentiated and confluent monolayer in transwells for 18-21 days. They were cultured in DMEM, supplemented with fetal calf serum, 10% (v/v) penicillin and streptomycin, at 37°C in a humidified atmosphere containing 5% CO2. Cells were stimulated with IL-6 (100ng/ml, cell signaling, #8904), IL-4 (10ng/ml, R&D, 204-IL), PGE2 (250ng/ml, Sigma, P5640) or IFN-γ (1000U/ml, R&D). Each condition was repeated in three independent experiments.

RNA isolation and RT-PCR

Total RNA was isolated from cell lines using TRIzol (Sigma). RNA pellets were resuspended in RNAse free water and total RNA concentrations were determined by nanodrop. 1µg of RNA was reversely transcribed using iScript^{TM} cDNA synthesis kit (Bio-rad). QPCR reactions were performed by using real-time PCR kit sensimix^{TM} SYBR&Flurescence (Quntace) in a total volume of 25µl containing 10pmol primers. QPCR was performed in an IQ5 (Bio-rad) with 40 cycles of amplification (15s at 95°C, 15s at 60°C and 15s at 72°C). GAPDH served as a household gene. Primers 5'-TCTGTCGGAAGACCGTCAAC-3' (sense), 5'-GGTCCAGGAACTCCCGAATG-3' (antisense) were used to amplify SOCS3. Primers 5'-GCATTGCCCTCAACGACCAC-3' (sense), 5'-CCACCACCCTGTTGCTGTAG-3' (antisense) were used to amplify GAPDH (both from Isogen Biosolutions). Presented data are the ratio between SOCS3 and GAPDH mRNA delta-Ct.

Western blot

Total cell lysates were prepared and Western blot was performed as described earlier [13]. Transfer membranes (Immobilon-FL, pore size 0.45um) were probed with primary antibodies specific for SOCS3 (rabbit polyclonal, abcam, ab16030), p-STAT3 (cell signaling, #9145), p-STAT1 (Cell signaling, #9167), p-STAT6 (Cell signaling, #9361), p-PKA-Cγ (Abcam, ab75991), p-CREB (Cell signaling, #9198). β-actin (Santa Cruz, E0610) was used as a reference protein. HRP labelled goat anti-rabbit (channel 680 and 800) and goat anti-mouse was used as secondary antibody (LI-COR Biosciences). Transfer

membranes were incubated with antibodies and washed in 50ml sterile centrifuge tubes and light protection centrifuge tubes (greiner bio-one). Protein expression was visualized using fluorescence Odyssey system (LI-COR Biosciences, Lincoln, NE, USA). Quantitative expression was determined by Odyssey 3.0 software and normalized using β-actin and α-tubulin as reference.

Immunohistochemistry staining and scoring

All biopsies were fixed in 10% formalin and embedded in paraffin. Haematoxylin eosin staining on paraffin embedded biopsies was performed to confirm lack of inflammation. Sections (4 μm) were mounted onto Superfrost Plus slides (Thermo Scientific, UK), deparaffinised in xylene and rehydrated. The endogenous peroxidase activity was blocked by 3% H2O2 in methanol for 15 min at room temperature. Antigen retrieval was performed by boiling in preheated buffer (EDTA pH 8.0 for p-STAT1 and p-STAT6; Tris/EDTA pH 9.0 for PKA-Cγ; Citric acid pH 6.0 for p-CREB) for 15 min at 200 W in a microwave. Next, slides were transferred to Shandon chambers and blocked by 5% goat serum in Tris-buffered saline (TBS) and 0.1% Tween 20 for p-STAT1, p-STAT6 and p-CREB; 10% normal human serum, 10% goat serum in phosphate-buffered saline (PBS) pH 7.4 for PKA-Cγ, for 1 h at room temperature. Primary antibodies rabbit polyclonal anti-p-STAT1 (1: 400, cell signalling, #9167), anti-p-STAT6 (1: 100, Abcam, ab28829), rabbit monoclonal anti-PKA-Cγ (1: 250, Abcam, ab75991) and rabbit monoclonal anti-p-CREB (1: 400, Cell signalling, #9198) were added and incubated at 4 degrees overnight. Envision goat anti-rabbit/mouse-HRP (DAKO, Denmark) was used as secondary antibody. Immunoreactions were detected using 3-3-diaminobenzidine (10 mg/ml) in Tris-HCl 0.05 M pH 7.6 containing imidazole stock (0.068 g/10 ml) and 0.03% H2O2, followed by counterstaining with haematoxylin. Negative controls were performed using only the secondary antibody. Two experienced people scored the staining of epithelial cells in a blinded manner. The percentage of cells that stained positive (immunoreactivity above background) in the area consistent with that used for diagnoses was quantified. P-STAT1, p-STAT6, PKA-Cγ and p-CREB were counted for nuclear staining in epithelial cells.

Statistical analysis

Statistical analysis was performed using SPSS software version 15.0. Data were presented as mean±SD. The difference between each two groups was performed by Mann Whitney test. The relationships between p-STAT1, p-STAT6 and PKA-cr positive

IEC was determined by Spearman correlation testing. p values <0.05 were considered statistically significant.

RESULTS

Patients' characteristics

In total, biopsies from 17 patients with inactive UC were included. There were 8 males (47.1 %) and 9 females (52.9%) with the mean age of 45.0 years (± 12.0 years), and a disease duration of 17.7 years (± 9.8 years) .The control group consisted of 6 males and 3 females with a mean age of 39.6 (± 17.8) years. There were no significant differences in patients' gender and mean age between groups of control and inactive UC patients (table 1).

Table 1. Patients' characteristics

	Controls (N=9)	Inactive UC (N=17)	P value
Gender			0.429
Male	6	8	
Female	3	9	
Mean age, yr±SD	39.6±17.8	45.0±12.0	0.366
Mean duration of disease, yr±SD	-	17.7±9.8	-
Extent of disease			-
Extensive colitis	-	0	
Left side colitis	-	11	
Proctitis	-	6	
Therapy			-
Aminosalicylates	-	11	
Corticosteroids	-	7	
Immunosuppressives	-	3	
Biologicals	-	2	
None	-	3	

STAT1, STAT6 and cAMP signaling induces STAT3-independent SOCS3 expression in vitro

To study the possible factors involved in STAT3-independent upregulation of SOCS3 in patients with inactive UC (supplementary Figure 1), we studied several other factors involved in UC and signaling pathways reported to induce SOCS3 expression[3, 4, 6, 14] (Figure 1).

Since, in addition to p-STAT3, the SRE in the SOCS3 promoter can be bound and activated by p-STAT1 as well as p-STAT6, caco-2 cells were treated with IFN-γ and IL-4 respectively (Figure 1). First we confirmed that the different cytokines were able to activate their specific signaling pathway in IEC without activation of STAT3. IFN-γ induced the phosphorylation of STAT1 without affecting STAT6 or STAT3 (Figure 2A-C). IL-4 induced the phosphorylation of STAT6 without affecting STAT1 or STAT3 (Figure 2A-C). IL-6 was used as a positive control and increased STAT3 as well as STAT1 phosphorylation (Figure 2A-C).

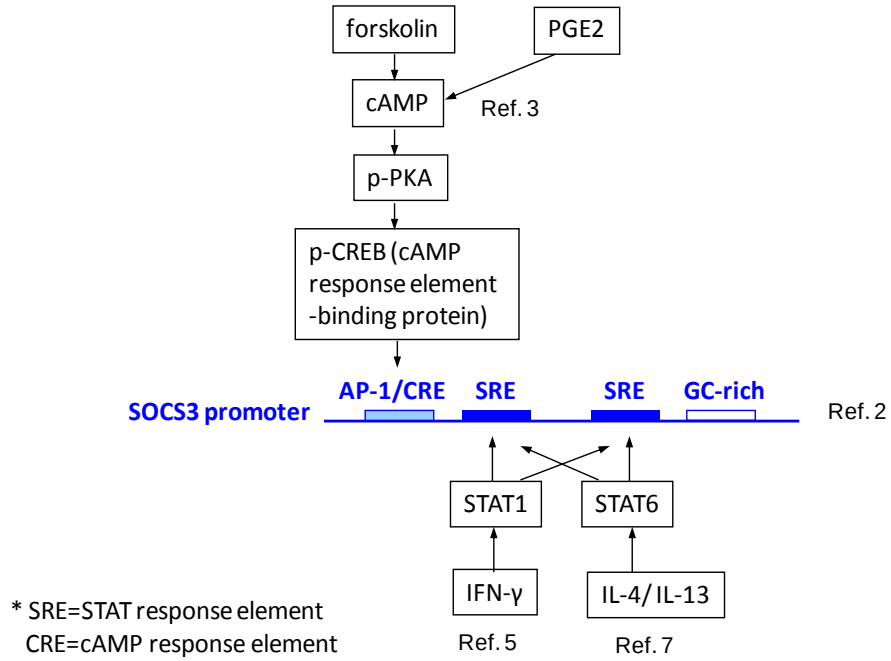


Figure 1. The signaling pathways reported to induce SOCS3 expression [2-3, 5, 7] in STAT3-independent manners. PGE2 or forskolin induced cAMP signaling can activate PKA and CREB binding to AP1/CRE element on SOCS3 promoter. IFN-γ induced STAT1 signaling and IL-4/IL-13induced STAT6 signaling stimulate SOCS3 promoter by binding to SRE elements.

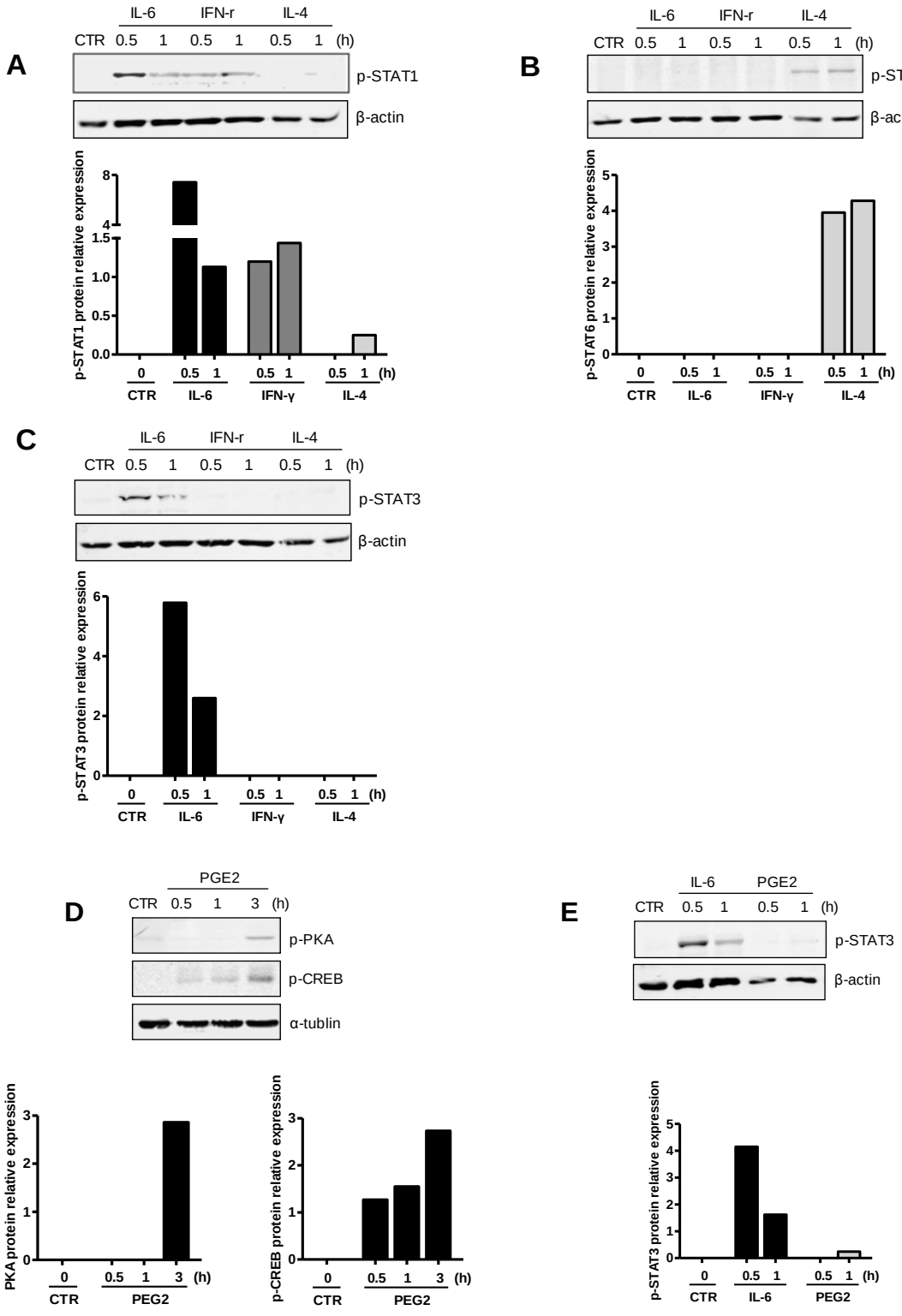


Figure 2. Different cytokines were able to activate their specific signaling pathway in IEC without activation of STAT3 (2A-C). In caco-2 cells, IFN-γ induced the phosphorylation of STAT1 without affecting STAT6 or STAT3. IL-4 induced the phosphorylation of STAT6 without affecting STAT1 or

STAT3. IL-6 was used as a positive control and increased STAT3 as well as STAT1 phosphorylation. (2D-E). Stimulation with PGE-2 induced p-PKA and p-CREB expression in caco-2 cells without any signs of STAT3 phosphorylation.

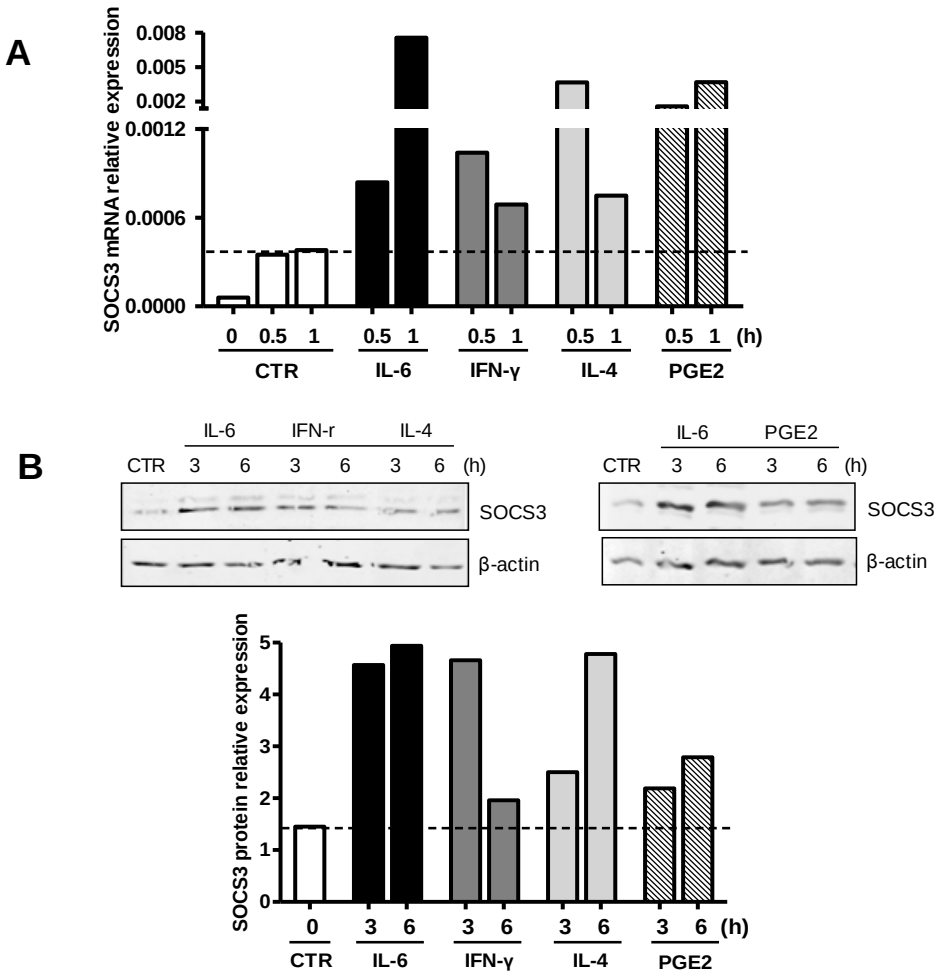


Figure 3. STAT3-independent SOCS3 expression induced by different UC-associated factors and signaling pathways. 3A-B. The cytokines stimulation IFN-γ and IL-4 as well as the PGE2 increased SOCS3 expression at both mRNA and protein levels.

In addition to the SREs, the promoter region of SOCS3 also contains an AP-1/CRE binding site which is a well-known down-stream target of cAMP signalling. To see if cAMP mediated signal transduction could induce SOCS3 expression, caco-2 cells were stimulated with PGE2 (Figure 1). Stimulation with PGE-2 induced p-PKA and p-CREB expression (Figure 2) in IEC without any signs of STAT3 phosphorylation (Figure 2E).

Next we tested whether the different UC-associated factors and signaling pathways were actually able to induce STAT3-independent SOCS3 expression. The cytokines IFN-γ and IL-4 as well as the hormone-like substance PGE2 were able to induce increased SOCS3 expression at both mRNA and protein levels (Figure 3A-B).

These data showed that SOCS3 expression in IEC can be induced by several UC associated factors without affecting STAT3.

Active STAT1 and STAT6 signaling in IEC of patients with inactive UC

The see whether the STAT1 and/or the STAT6 signaling pathway were active in UC biopsies with high SOCS3 but no p-STAT3 expression, we stained the biopsies for p-STAT1 and p-STAT6 expression and analyzed nuclear staining (Figure 4A-B). Quantification of the percentage positive IEC in each biopsy showed a significantly increased expression of both p-STAT1 ($p<0.0001$) and p-STAT6 ($p=0.0001$) in inactive UC patients compared with control biopsies (Figure 4C-D).

These data provide evidence that active STAT1 and STAT6 signaling might be involved in the high SOCS3 expression in IEC of UC patients with inactive disease.

Active cAMP signaling in IEC of patient with inactive UC

The same biopsies were also stained for p-PKA-Cγ and p-CREB as markers of active cAMP signaling (Fig 5A-B). Analysis of these staining showed significantly increased percentages of p-PKA-Cγ ($p=0.0003$) and p-CREB ($p=0.0025$) positive IEC in biopsies from patients with inactive UC compared with control biopsies (Fig 5C-D).

These data show that the cAMP signaling pathway may also be involved in the high SOCS3 expression in IEC of UC patients with inactive disease.

No significant correlation between three signaling pathways and medicine use

To check whether the signaling pathways were mutually exclusive or had corresponding expression we correlated the expression between the different staining within the biopsy from the same patient. In the biopsies of inactive UC no significant correlation were found between the different parameters (all $p>0.05$) (Figure 6).

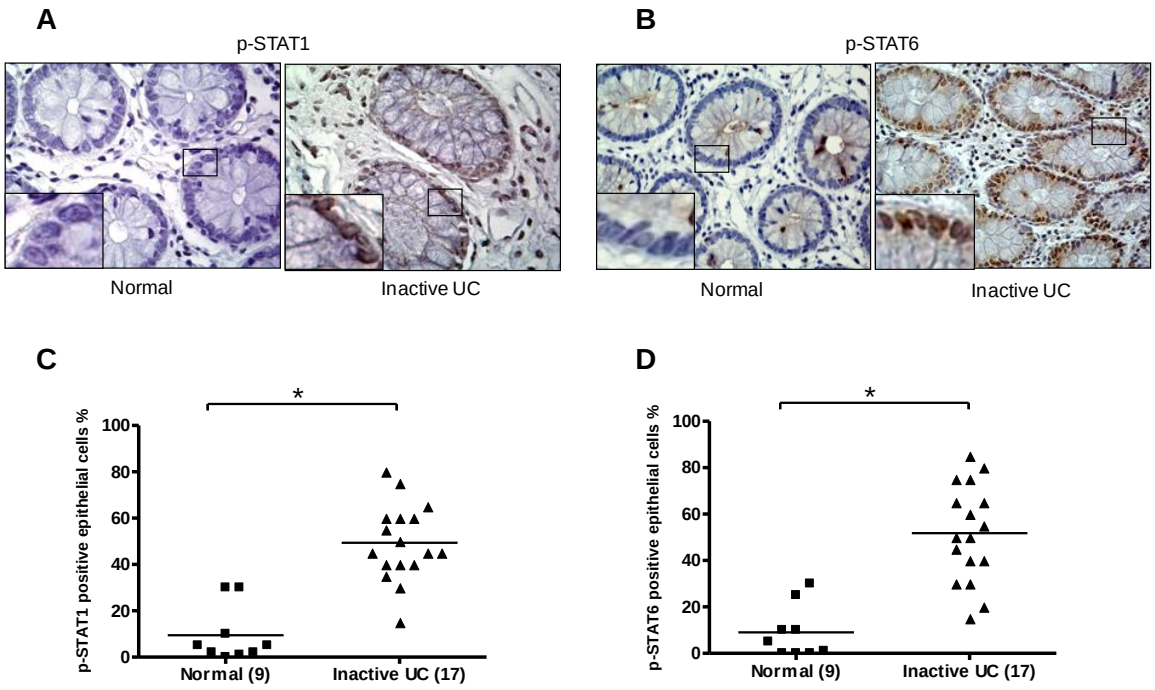


Figure 4. IHC staining of p-STAT1 (A) and p-STAT6 (B) IEC positive cells between patients with inactive UC and controls (nuclear staining). Quantification of the percentage positive IEC in each biopsy showed a significantly increased p-STAT1 ($p<0.0001$) (C) and p-STAT6 ($p=0.0001$) (D) positive IEC in biopsies from patients with inactive UC compared with control biopsies.

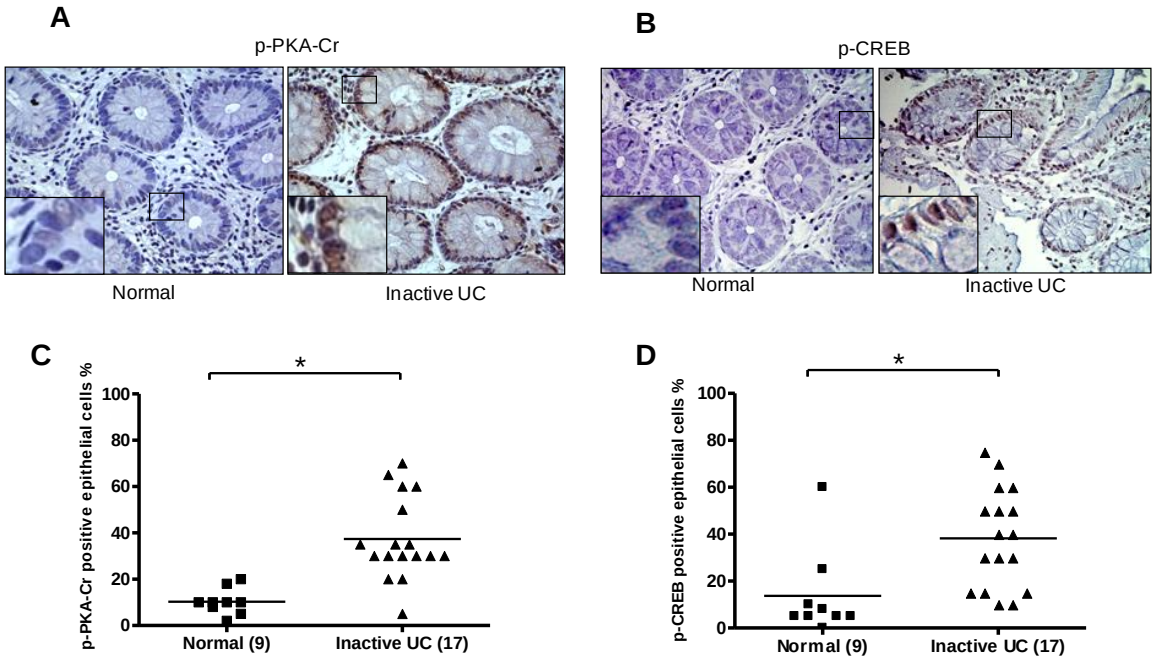


Figure 5. IHC staining of p-PKA-Cγ (A) and p-CREB (B) IEC positive cells between patients with inactive UC and controls (nuclear staining). Quantification of the percentage positive IEC in each

biopsy showed significantly increased percentages of p-PKA-Cγ (p=0.0003) (C) and p-CREB (p=0.0025) (D) positive IEC in biopsies from patients with inactive UC compared with control biopsies.

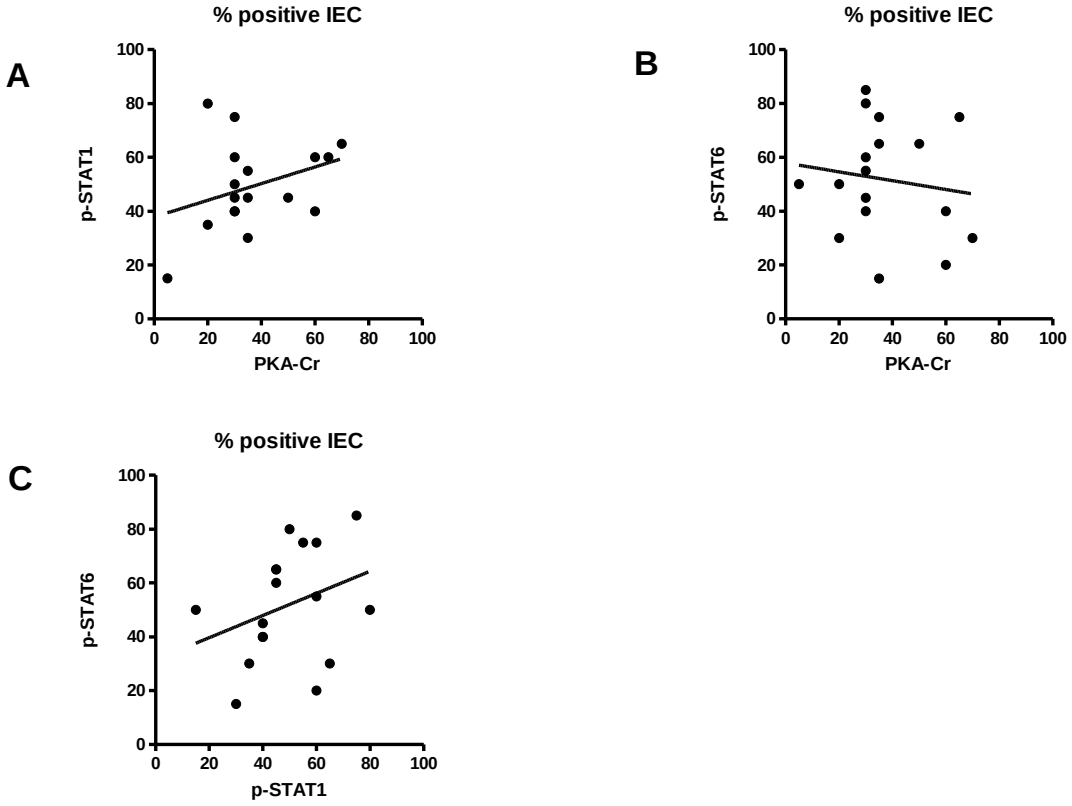


Figure 6. The relation of different signaling pathways between the staining within the biopsy from the same patient with inactive UC. No significant correlations were found between the IEC expressions of p-STAT1 and PKA-Cr (A), p-STAT6 and PKA-Cr (B), p-STAT1 and p-STAT6 (C). All p>0.05.

We further compared the medication use with the signaling pathways that were dominant in each patient. However, when we sub-identified the signaling activities according to different medicine use in these patients, no significant difference was found between these signaling activities and different medicine users (data now shown).

DISCUSSION

In the current study we show that SOCS3 expression can be induced in IEC via the STAT1, STAT6, or cAMP pathway independent of STAT3. We found that the same pathways were active in IEC of colonic biopsies from UC patients with inactive disease and with high SOCS3 expression. These data provide new insight into the mechanism involved in induction of SOCS3 expression.

Mice lacking of IL-6/gp130/p-STAT3 signaling were more sensitive to chemically-induced colitis due to reduced IEC survival and proliferation [15, 16]. In addition, transgenic SOCS3 expression in epithelial cells negatively affected STAT3-dependent wound healing [17, 18]. These data suggested that due to the high SOCS3 expression, normal STAT3 signaling is impaired and could lead to enhanced vulnerability of IEC, which could promote the development of UC or at least to an enhanced risk for UC relapse as we have recently seen [19].

Our observation of enhanced p-STAT1 and SOCS3 expression in biopsies of patients with inactive UC and the IFN-γ-induced SOCS3 expression via p-STAT1 in vitro are consistent with the previous detection of increased level of IFN-γ and STAT1 in UC pouchitis patients without clinical and endoscopic evidence [5]. However IFN-γ has not been shown to be upregulated in any other studies looking at gene or protein expression in biopsies of patients with inactive UC. Other IFN-family members could be involved but also via the induction of p-STAT1. Although not much is known about their expression during remission, it will be interesting to confirm whether peg-IFN could cause UC exacerbation in view of the inconsistent trial data [20], and to see whether the observed exacerbation of UC in HCV patients treated with peg-IFN is mediated by increased SOCS3 expression in their IEC [21]. The observed STAT1 activation could also be a downstream effect of TLR signaling, suggesting that IEC of UC patients are more sensitive to bacterial stimulation [22, 23].

In our study we showed for the first time that IL-4 induced STAT6 phosphorylation and subsequent SOCS3 expression in IEC, and p-STAT6 in combination with high SOCS3 are expressed in the biopsies from UC patients with inactive disease. These data fit with the STAT6-dependent induction of SOCS3 by stimulation keratinocytes by a combination of

IL-4 and IL-13 [24] Various reports have mentioned a balancing effect between STAT1 and STAT6 signaling, where IL-4/IL-13 inhibit IFN-γ via the induction of SOCS1 and SOCS3 and vice versa [24, 25], unfortunately due to the small group of patients we didn't see it in our biopsies.

In addition to STAT signaling we also saw that PGE2-induced cAMP signaling can induce SOCS3 upregulation in IEC. Phosphorylation of PKA-Cγ and CREB was used as a read-out for cAMP signaling as demonstrated previously [4]. Our data also showed for the first time that cAMP signaling is active in IEC of UC patients with inactive inflammation, as detected by enhanced expression of p-PKA-Cγ and p-CREB expression. Although PGE2 worked well as a positive control in our in vitro assays, it may not be the most likely candidate to induce the observed cAMP activity in biopsies. A recent study has shown that PGE2 was highly expressed in actively inflamed tissue [26]. In the same study the pro-resolution mediator prostaglandin D2 (PGD2) and its receptor DP1 were expressed in biopsies of UC patients in remission [26]. This observation combine with the even stronger induce of cAMP by PGD2 compared to PGE2 make the PGD2 a good candidate for the increased SOCS3 expression in some of the UC patients [27].

No significant correlations were found between the different pathways, suggesting that multiple pathways could be active in the same IEC in specific biopsies.

In conclusion, our data provide evidence that STAT1, STAT6 and cAMP signaling activation may be involved in the p-STAT3 independent SOCS3 expression in IEC as detected in biopsies of UC patients with histological inactive disease. As such, these pathways provide interesting therapeutic targets for maintaining UC remission.

REFERENCES

1. Peyrin-Biroulet L, Lemann M. Review article: remission rates achievable by current therapies for inflammatory bowel disease. Aliment Pharmacol Ther 2011;33:870-9.
2. Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, Kuipers EJ, van der Woude CJ. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut;59:227-35.
3. He B, You L, Uematsu K, Matsangou M, Xu Z, He M, McCormick F, Jablons DM. Cloning and characterization of a functional promoter of the human SOCS-3 gene. Biochem Biophys Res Commun 2003;301:386-91.
4. Bousquet C, Chesnokova V, Kariagina A, Ferrand A, Melmed S. cAMP neuropeptide agonists induce pituitary suppressor of cytokine signaling-3: novel negative feedback mechanism for corticotroph cytokine action. Mol Endocrinol 2001;15:1880-90.
5. Leal RF, Ayrizono ML, Milanski M, Coope A, Fagundes JJ, Velloso LA, Coy CS. Activation of signal transducer and activator of transcription-1 (STAT-1) and differential expression of interferon-gamma and anti-inflammatory proteins in pelvic ileal pouches for ulcerative colitis and familial adenomatous polyposis. Clin Exp Immunol;160:380-5.
6. Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P. DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem 2001;276:6675-88.
7. Lin Z, Hegarty J, Cappel J, Yu W, Chen X, Faber P, Wang Y, Kelly A, Poritz L, Peterson B, Schreiber S, Fan JB, Koltun W. Identification of disease-associated DNA methylation in intestinal tissues from patients with inflammatory bowel disease. Clin Genet.
8. Barclay JL, Anderson ST, Waters MJ, Curlewis JD. Characterization of the SOCS3 promoter response to prostaglandin E2 in T47D cells. Mol Endocrinol 2007;21:2516-28.
9. Lohmann SM, Walter U. Regulation of the cellular and subcellular concentrations and distribution of cyclic nucleotide-dependent protein kinases. Adv Cyclic Nucleotide Protein Phosphorylation Res 1984;18:63-117.
10. Kammer GM. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. Immunol Today 1988;9:222-9.
11. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl 1989;170:2-6; discussion 16-9.
12. Geboes K, Riddell R, Ost A, Jensfelt B, Persson T, Lofberg R. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. Gut 2000;47:404-9.
13. Wang M, Markel T, Crisostomo P, Herring C, Meldrum KK, Lillemoe KD, Meldrum DR. Deficiency of TNFR1 protects myocardium through SOCS3 and IL-6 but not p38 MAPK or IL-1beta. Am J Physiol Heart Circ Physiol 2007;292:H1694-9.

14. Gatto L, Berlato C, Poli V, Tininini S, Kinjyo I, Yoshimura A, Cassatella MA, Bazzoni F. Analysis of SOCS-3 promoter responses to interferon gamma. J Biol Chem 2004;279:13746-54.
15. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.
16. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91-102.
17. Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S. Epithelial overexpression of SOCS-3 in transgenic mice exacerbates wound inflammation in the presence of elevated TGF-beta1. J Invest Dermatol;130:866-75.
18. Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S. The suppressor of cytokine signaling (SOCS)-3 determines keratinocyte proliferative and migratory potential during skin repair. J Invest Dermatol;130:876-85.
19. Li Y, de Haar C, Nuij VJ, Baars JE, Kuipers EJ, van der Woude CJ. SOCS3 Expression is a Predictive Factor of Relapse of Mucosal Inflammation in Chronic UC. Gastroenterology;140:S-1.
20. Tilg H, Vogelsang H, Ludwiczek O, Lochs H, Kaser A, Colombel JF, Ulmer H, Rutgeerts P, Kruger S, Cortot A, D'Haens G, Harrer M, Gasche C, Wrba F, Kuhn I, Reinisch W. A randomised placebo controlled trial of pegylated interferon alpha in active ulcerative colitis. Gut 2003;52:1728-33.
21. Mitoro A, Yoshikawa M, Yamamoto K, Mimura M, Yoshikawa Y, Shiroi A, Mochi T, Sakamoto T, Yamao J, Kikuchi E, et al. Exacerbation of ulcerative colitis during alpha-interferon therapy for chronic hepatitis C. Intern Med 1993;32:327-31.
22. Rhee SH, Jones BW, Toshchakov V, Vogel SN, Fenton MJ. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. J Biol Chem 2003;278:22506-12.
23. Wu F, Dassopoulos T, Cope L, Maitra A, Brant SR, Harris ML, Bayless TM, Parmigiani G, Chakravarti S. Genome-wide gene expression differences in Crohn's disease and ulcerative colitis from endoscopic pinch biopsies: insights into distinctive pathogenesis. Inflamm Bowel Dis 2007;13:807-21.
24. Albanesi C, Fairchild HR, Madonna S, Scarponi C, De Pita O, Leung DY, Howell MD. IL-4 and IL-13 negatively regulate TNF-alpha- and IFN-gamma-induced beta-defensin

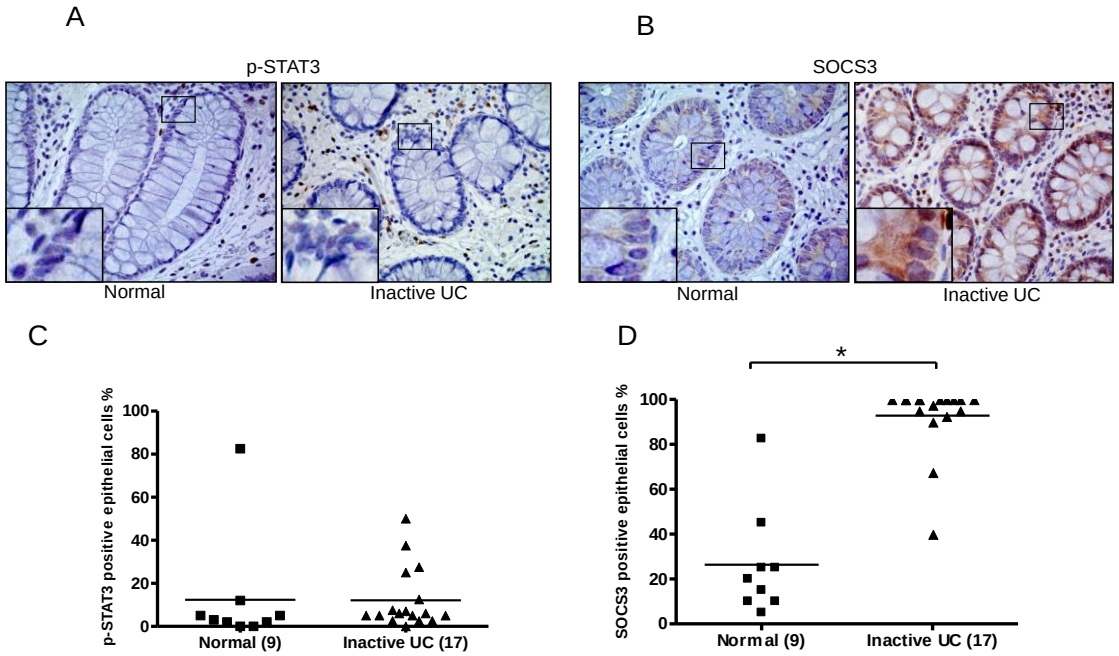
expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. J Immunol 2007;179:984-92.

25. Venkataraman C, Leung S, Salvekar A, Mano H, Schindler U. Repression of IL-4-induced gene expression by IFN-gamma requires Stat1 activation. J Immunol 1999;162:4053-61.

26. Vong L, Ferraz JG, Panaccione R, Beck PL, Wallace JL. A pro-resolution mediator, prostaglandin D(2), is specifically up-regulated in individuals in long-term remission from ulcerative colitis. Proc Natl Acad Sci U S A;107:12023-7.

27. Craven PA, Saito R, DeRubertis FR. Role of local prostaglandin synthesis in the modulation of proliferative activity of rat colonic epithelium. J Clin Invest 1983;72:1365-75.

SUPPLEMENTARY FIGURES



Supplementary figure 1. Difference in IHC staining of p-STAT3 (A) and SOCS3 (B) in IEC of patients with inactive UC and controls. Quantification of the percentage positive IEC in each biopsy showed increased SOCS3 expression ($p<0.0001$) (D) corresponded with low p-STAT3 expression ($p=0.1286$) (C) in patients with inactive UC compared with controls.

Chapter VII

General summary and discussion

Yi Li

Introduction

Inflammatory bowel disease (IBD) is characterized by a chronic recurrent colonic inflammation, which results from the interaction from as yet poorly understood environmental factors and the intestinal microbiome in a genetically predisposed host. Two major manifestations, Crohn's disease and ulcerative colitis, are distinguished on basis of clinical and pathophysiological criteria. IBD is associated with an increased risk of developing colorectal cancer (CRC) [1-2]. The ever increasing incidence of IBD and associated CRC makes it imperative that we establish better understanding of the underlying mechanisms. This thesis aimed to contribute to this quest, focusing on the colonic epithelium, which acts as metaphorical waterbarrier between the body and the mucosal immune system and the luminal constituents. When the epithelium layer barrier function is defective, due to bacterial or microbial invasion, loss of goblet cells and mucus protection especially UC can arise an thus the present work with its focus on the colonic epithelium has more-or-less naturally been drawn to this manifestation of IBD.

In this thesis we explore the role of the SOCS3 pathway in the epithelium. This pathway can be activated by a large amount of stimuli, including bacterial compounds, growth factors, pro-inflammatory/anti-inflammatory cytokines and proteins involved in different pathways. In **Chapter I and II** of this thesis, we discussed the dichotomal role of STAT3-SOCS3 signaling pathway in intestinal epithelial cells (IEC) and other immune cells (i.e., T cells, macrophages, dendrtic cells) of inflammatory disease and malignant disorders, especially inflammatory bowel disease and its related carcinoma [3]. STAT3 is importantly involved in integrating oncogenic (activated by intrinsic genetic or epigenetic alterations in transformed cells) and environmental pathways (extrinsic factors like ultraviolet (UV) radiation, chemical carcinogens, infection and stress stimulate cytokine receptors, growth factor receptors and Toll-like receptors). As discussed in these chapters, SOCS3 is induced by STAT3 but also a negative regulator of STAT3 and acts to limit STAT3 signaling in the body. Thus the dysregulation of SOCS3 mediated negative feedback causes immune imbalance and inflammatory disease due to aberrant STAT3 activation [4-5] and eventually inflammation-induced carcinogenesis [6]. In subsequent chapters we further elucidated this important function of SOCS3 in ulcerative colitis and ulcerative colitis-related carcinoma.

Disease-related expression of IL-6/p-STAT3/SOCS3 in UC/UC-CRC

As mentioned the IL-6/p-STAT3 pathway plays a crucial role in maintaining gastrointestinal homeostasis by regulating epithelial turnover and mucosal healing [7]. As such, inappropriate stimulation and (or) inhibition of this pathway may lead to dysregulation of gastrointestinal homeostasis causing colitis or even colorectal cancer.

In **Chapter III**, we show a significant expression of IL-6/p-STAT3 expression in the IEC compartment in biopsies from active UC patients and document that this represents a substantial increased when compared with inactive UC patients and controls [8-10]. When combining our data of IL-6/p-STAT3 positive epithelial and non-epithelial cells and especially their correlation with the severity of the colitis, we obtain evidence that the IL-6/p-STAT3 is involved in the initiation of UC and thus therapeutic blockade of the IL-6 signaling pathway emerges as a novel avenue for preventing UC initiation or rekindling. The high SOCS3 levels in biopsies from active UC patients compared with non-inflammatory controls may be a downstream effect of increased IL-6/p-STAT3 signaling. Nevertheless, IL-6/p-STAT3 are hardly detected in inactive UC, and this therefore the high SOCS3 expression in the epithelial cells of patients with inactive UC which is observed in a subset of patients must be brought about in a STAT3-independent fashion. The nature of the molecular signals involved would be the subject of experimental work elsewhere in this thesis.

The role of STAT3 signaling in IEC with respect to inflammatory and carcinogenic reactions has been corroborated using mouse models genetically depleted of gp130/IL-6 or STAT3. IEC defective in gp130/IL-6/p-STAT3 signaling are more sensitive to dextran sodium sulfate (DSS) induced colitis, maybe due to the reduced epithelial cell survival and proliferation [11-12]. As such, the high constitutive expression of SOCS3 in inactive UC epithelial cells we observed could prevent normal IL-6/p-STAT3-mediated epithelial cell homeostasis and thus cause enhanced the sensitivity of these epithelial cells to inflammatory damage. STAT3 signaling might well for instance suppress immune responses in IEC and facilitates epithelial regeneration to allow epithelium to be less sensitive to various insults. Since STAT3, as noted, is a double-edged sword, STAT3 may have profound anti-inflammatory effects in macrophages and epithelial cells [13]. Therefore, when one contemplates STAT3 inhibition in humans, careful attention must be directed toward the cell population that is being targeted.

Concerning UC-related carcinogenesis, studies have shown the involvement of IL-6 in this cancer development [12, 14-15]. The IL-6/p-STAT3 expression in the IEC of UC-CRC biopsies we found may stimulate survival and proliferation of these cells and tumor progression [12, 14-15]. The absence of the negative regulator of IL-6/p-STAT3 signaling, SOCS3, therefore enhances the sensitivity of mice to DSS induced colon carcinoma [16]. All these data makes intervention in the IL-6 signaling a feasible therapeutic target for preventing cancer in UC patients. Additionally, we have shown SOSC3 downregulation in UC-CRC patients, consistent with previous data that SOCS3 is silenced in other non-colorectal cancers by promoter hypermethylation [17-19]. Our patient data on SOCS3 gene methylation obviously explain the lack of SOCS3 expression in the UC-CRC biopsies.

Our data support the notion that the IL-6 signaling pathway is an interesting therapeutic target in active UC and UC-CRC. Moreover, the high expression of SOCS3 in inactive UC compared with non-disease controls suggests the involvement of this protein in UC development, while the absence of SOCS3 expression in UC-CRC may be a critical factor in the progression towards CRC development.

SOCS3 gene methylation relates to UC-CRC

Following the findings of SOCS3 methylation in UC-CRC patients in cancer epithelial cells [20] from chapter III, **Chapter IV** further extended these data by demonstrating the existence of multiple methylation sites in the SOCS3 promoter in CRC cells, the induction of SOCS3 gene methylation by the proinflammatory cytokine IL-6 in UC-CRC patients and CRC cell lines and the effects of SOCS3 demethylation on p-STAT3 induced cell growth and proliferation.

IL-6/p-STAT3 signaling in vitro has been shown to stimulate colonic epithelial cell proliferation and up-regulate anti-apoptotic molecules [15]. Our observations show that the lack of SOCS3 expression caused by methylation in IEC may enable the constitutive increased IL-6/p-STAT3 signaling, leading to enhanced cell proliferation the sensitivity these IEC to inflammatory substances induced colon carcinoma [16]. We show that cells with dense SOCS3 methylation have higher IL-6 induced p-STAT3 induction but less SOCS3 expression, while restoration of SOCS3 expression reduces p-STAT3 activation, thus inhibits IL-6/p-STAT3 induced cell proliferation and decreases tumor growth.

In the initiation of inflammation-related carcinogenic events like IBD-related cancer, inflammatory effects from immune cells (eg: macrophages, T cells) in the tumor microenvironment contribute to neoplastic transformation in the epithelium [21-23]. In this chapter we linked chronic inflammation with carcinogenesis. IL-6 signaling induced methyltransferase DNMT1 expression then promotes the neoplastic phenotype of colonic epithelial cells via the CpG island methylation. DNMT1 is inducible by IL-6 in cell lines with mild SOCS3 methylation and the enzyme is highly expressed in UC-CRC compared with sporadic-CRC and controls. DNMT1 in IEC from our patient biopsies correlates with IL-6 expression, which is consistent with the results obtained in several other cancers [13, 24]. The DNMT1 activity induced by increased IL-6 levels might be derived from signals via STAT3 pathway, thus induce cell growth and induce positive feedback on these signalings by epigenetic silencing of tumor-suppressor genes like SOCS3 [25], explaining the lower SOCS3 expression and neoplastic phenotype in colon epithelial cells. These findings provide a potential molecular explanation for how an inflammatory tumor microenvironment induces an neoplastic phenotype in colon epithelial cells [26].

In addition to our findings in patients with UC-CRC, we confirmed in vitro the SOCS3 promoter region methylation, explaining lack of SOCS3 induction by IL-6 stimulation in various CRC cell lines. The methylation of the SOCS3 promoter enables uncontrolled IL-6/STAT3 signaling, leading to the enhanced proliferation and survival associated with carcinogenesis. Restoration of SOCS3 expression or targeting the IL-6-induced DNMT1 overexpression and CpG island methylation may provide interesting therapeutics in colitis-associated carcinogenesis [27-28].

SOCS3, a predictive marker of mucosal relapse in UC remission

Although clinical disease activity of UC is the consequence of mucosal inflammation, quiescent UC patients can have, to some extent, residual chronic inflammation in the mucosa. Several studies have shown that endoscopy and histology are potential tools for the prediction of UC outcome [29-30]. The identification of a reliable biomarker specially predicting mucosal relapse would contribute to improved care for UC patients. In **Chapter V,** we addressed the high SOCS3 expression in colonic IEC of UC patients in complete remission was associated with a shorter time until relapse. It is known that STAT3 signaling is important for mucosal homeostasis by stimulating IEC proliferation and survival. The hypothesis of this chapter was based on the findings and implications from

chapter IV that a high level of negative regulator of STAT3, SOCS3 expression observed during inactive disease may interfere with IEC homeostasis making epithelial cells more vulnerable to various insults thus leading to earlier relapse thus leading to earlier relapse [20].

It has been well established that in a significant proportion of UC patients with clinical remission, the endoscopic and histological signs of disease activity can still remain, the severity of which are related to the time to relapse [30]. Most studies on predictive relapse used only clinical assessments to gauge remission and relapse [31-32], but such clinical parameters are probably just signs of overt mucosal inflammation and thus only useful for predicting very imminent clinical relapse. One study related low SOCS3 mRNA expression from biopsies to a higher rate of remission maintenance over a 12-month period [33]. This study entailed a poor description on the cell types involved and which kinds of remission/relapse, prompting us to perform a more expansive study with a larger group and longer follow-up, with clear criteria to define remission (absence of endoscopic and histological disease activity). Once SOCS3 expression is linked to endoscopic and histological or clinical relapse, the early predictive role of SOCS3 is more convincing. The SOCS3 level in the biopsies from the last colonoscopy before relapse approaches that of relapse supports the active contribution of SOCS3 in risk for relapse other than the induction of remission. The data presented in this chapter also lend further credit to the conclusions in chapter IV that IEC from UC patients with inactive disease do have activated STAT3 [34].

We also demonstrated that p-STAT1 levels in IEC correlate with the SOCS3 expression suggesting a role for this signal transducer in SOCS3 induction. Indeed the correlation of p-STAT1 expression with the time to relapse and severity of relapse further highlights the predictive nature of SOCS3 expression with respect to upcoming relapse. The data suggest, through negative correlation, that p-STAT1-induced SOCS3 expression interferes with normal STAT3 signaling and its anti-inflammatory and proliferative effects, as was also previously reported for endothelial cells [35]. Such a relation between SOCS3 and STAT1 has only been observed before in chronic hepatitis. Higher SOCS3 and p-STAT1 expression in the liver biopsies prior to interferon therapy is associated with poor response to antiviral response and is related to the severity of inflammation [36-38]. Activated STAT1 has been suggested as an important therapeutic target for modification of host immune response to viral infection [39].

High expression of SOCS3 is associated with a shorter time until relapse. Staining of surveillance biopsies for SOCS3 expression might provide a tool for clinical monitoring to step up or drop down in therapy regime. Induction of mucosal healing with low levels of SOCS3 expression in the IEC might be a therapeutic goal to ensure long lasting (deep) remission.

Possible SOCS3 inducers in inactive UC

Chapter VI demonstrated SOCS3 expression can be induced in IEC via the STAT1, STAT6, or cAMP pathway independent of STAT3, as these pathways were active in IEC of colonic biopsies from UC patients with inactive disease and with high SOCS3 expression.

Although we have show IFN-γ-induced SOCS3 expression via p-STAT1 (which consistent with previous findings [40]), STAT1 signaling can also be induced by other IFN-family members or TLR signaling. Further investigations as to whether the exacerbation of UC in patients treated with peg-IFN is mediated via increased IEC SOCS3 expression [41] and whether as a consequence the IEC of UC patients are more sensitive to bacterial stimulation [42-43] should prove interesting. To our knowledge, we are the first to show that STAT6 phosphorylation (probably IL-4 induced) and subsequent SOCS3 expression in IEC from biopsies. These data fit with the STAT6-dependent induction of SOCS3 by stimulation keratinocytes by a combination of IL-4 and IL-13 [44]. A larger group of patients might be more valuable to see the balancing between STAT1 and STAT6 signaling, which has been suggested as IL-4/IL-13 inhibits IFN-γ via the induction of SOCS1 and SOCS3 and vice versa [44-45]. We have also shown that PGE2-induced cAMP signaling can induce SOCS3 in IEC, employing the phosphorylation of PKA-Cγ and CREB as a read-out [46]. We are the first to show that cAMP signaling is active in IEC of UC patients with inactive inflammation. Except for PGE2, the pro-resolution mediator prostaglandin D2 (PGD2) and its receptor DP1 were expressed in biopsies of UC patients in remission [47]. This observation suggests PGD2 may be another good candidate via cAMP signaling to increase SOCS3 expression in some of the UC patients [48].

Since mice with IL-6/gp130/p-STAT3 signaling depletion are more sensitive to chemically-induced colitis due to reduced IEC survival and proliferation [11-12] and transgenic SOCS3 expression in epithelial cells negatively affected STAT3-dependent wound healing [49-50], it

seems that high SOCS3 expression impairs STAT3 signaling and leads to enhanced vulnerability of IEC. This would promote UC development or at least enhance the risk for UC relapse, consistent with the data in chapter VI. Our findings in this chapter suggest at least three different signaling pathways that all may contribute the high SOCS3 expression and consequent inihibition of normal STAT3 signaling. With this study we provided new insight into the mechanism involved in induction of SOCS3 expression, which is that STAT1, STAT6 and cAMP signaling activation may be involved in the p-STAT3 independent SOCS3 expression in IEC as detected in biopsies of UC patients with histological inactive disease. These pathways could be the interesting therapeutic targets for maintaining UC remission.

Conclusive remarks and future studies

In conclusion, through this work we identify SOCS3 regulation of STAT3 signaling as a principal regulator of inflammatory phenotype in UC and its loss as an important driver for inflammation-related carcinogenesis in the colon. Future work should be aimed at exploiting this paradigm for future therapy.

REFERENCES

1. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001;48:526-35.
2. Eaden J. Review article: colorectal carcinoma and inflammatory bowel disease. Aliment Pharmacol Ther 2004;20 Suppl 4:24-30.
3. Li Y, de Haar C, Peppelenbosch MP, van der Woude CJ. New insights into the role of STAT3 in IBD. Inflamm Bowel Dis 2011.
4. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009;9:798-809.
5. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 2007;7:454-65.
6. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. Gastroenterology 2007;132:384-96.
7. Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. Nat Med 2002;8:1089-97.
8. Kusugami K, Fukatsu A, Tanimoto M, Shinoda M, Haruta J, Kuroiwa A, Ina K, Kanayama K, Ando T, Matsuura T, et al. Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent. Dig Dis Sci 1995;40:949-59.
9. Carey R, Jurickova I, Ballard E, Bonkowski E, Han X, Xu H, Denson LA. Activation of an IL-6:STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease. Inflamm Bowel Dis 2007.
10. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, Akira S, Matsumoto S, Toyonaga A, Sata M, Yoshimura A. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. J Exp Med 2001;193:471-81.
11. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell 2009;15:91-102.
12. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009;15:103-13.

13. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol 2003;4:1169-76.
14. Becker C, Fantini MC, Schramm C, Lehr HA, Wirtz S, Nikolaev A, Burg J, Strand S, Kiesslich R, Huber S, Ito H, Nishimoto N, Yoshizaki K, Kishimoto T, Galle PR, Blessing M, Rose-John S, Neurath MF. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. Immunity 2004;21:491-501.
15. Schneider MR, Hoeflich A, Fischer JR, Wolf E, Sordat B, Lahm H. Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells. Cancer Lett 2000;151:31-8.
16. Rigby RJ, Simmons JG, Greenhalgh CJ, Alexander WS, Lund PK. Suppressor of cytokine signaling 3 (SOCS3) limits damage-induced crypt hyper-proliferation and inflammation-associated tumorigenesis in the colon. Oncogene 2007;26:4833-41.
17. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, Yoshikawa H. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene 2005;24:6406-17.
18. Evans MK, Yu CR, Lohani A, Mahdi RM, Liu X, Trzeciak AR, Egwuagu CE. Expression of SOCS1 and SOCS3 genes is differentially regulated in breast cancer cells in response to proinflammatory cytokine and growth factor signals. Oncogene 2007;26:1941-8.
19. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A 2003;100:14133-8.
20. Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, Kuipers EJ, van der Woude CJ. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut;59:227-35.
21. Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, Luo JL, Karin M. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 2009;457:102-6.
22. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. Cytokine Growth Factor Rev;22:83-9.
23. Fantini MC, Pallone F. Cytokines: from gut inflammation to colorectal cancer. Curr Drug Targets 2008;9:375-80.
24. Boisclair YR, Wang J, Shi J, Hurst KR, Ooi GT. Role of the suppressor of cytokine signaling-3 in mediating the inhibitory effects of interleukin-1beta on the growth hormone-dependent transcription of the acid-labile subunit gene in liver cells. J Biol Chem 2000;275:3841-7.

25. Zhang Q, Wang HY, Woetmann A, Raghunath PN, Odum N, Wasik MA. STAT3 induces transcription of the DNA methyltransferase 1 gene (DNMT1) in malignant T lymphocytes. Blood 2006;108:1058-64.
26. Foran E, Garrity-Park MM, Mureau C, Newell J, Smyrk TC, Limburg PJ, Egan LJ. Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6. Mol Cancer Res;8:471-81.
27. Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol 2006;80:227-36.
28. Atreya R, Neurath MF. New therapeutic strategies for treatment of inflammatory bowel disease. Mucosal Immunol 2008;1:175-82.
29. Allez M, Lemann M. Role of endoscopy in predicting the disease course in inflammatory bowel disease. World J Gastroenterol;16:2626-32.
30. Riley SA, Mani V, Goodman MJ, Dutt S, Herd ME. Microscopic activity in ulcerative colitis: what does it mean? Gut 1991;32:174-8.
31. Gisbert JP, Bermejo F, Perez-Calle JL, Taxonera C, Vera I, McNicholl AG, Algaba A, Lopez P, Lopez-Palacios N, Calvo M, Gonzalez-Lama Y, Carneros JA, Velasco M, Mate J. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. Inflamm Bowel Dis 2009;15:1190-8.
32. Roda G, Caponi A, Benevento M, Nanni P, Mezzanotte L, Belluzzi A, Mayer L, Roda A. New proteomic approaches for biomarker discovery in inflammatory bowel disease. Inflamm Bowel Dis;16:1239-46.
33. Miyanaka Y, Ueno Y, Tanaka S, Yoshioka K, Hatakeyama T, Shimamoto M, Sumii M, Chayama K. Clinical significance of mucosal suppressors of cytokine signaling 3 expression in ulcerative colitis. World J Gastroenterol 2007;13:2939-44.
34. Theiss AL, Fuller CR, Simmons JG, Liu B, Sartor RB, Lund PK. Growth hormone reduces the severity of fibrosis associated with chronic intestinal inflammation. Gastroenterology 2005;129:204-19.
35. Bluyssen HA, Rastmanesh MM, Tilburgs C, Jie K, Wesseling S, Goumans MJ, Boer P, Joles JA, Braam B. IFN gamma-dependent SOCS3 expression inhibits IL-6-induced STAT3 phosphorylation and differentially affects IL-6 mediated transcriptional responses in endothelial cells. Am J Physiol Cell Physiol;299:C354-62.
36. Miyaaki H, Ichikawa T, Nakao K, Matsuzaki T, Muraoka T, Honda T, Takeshita S, Shibata H, Ozawa E, Akiyama M, Miuma S, Eguchi K. Predictive value of suppressor of cytokine signal 3 (SOCS3) in the outcome of interferon therapy in chronic hepatitis C. Hepatol Res 2009;39:850-5.

37. Kim KA, Lin W, Tai AW, Shao RX, Weinberg E, De Sa Borges CB, Bhan AK, Zheng H, Kamegaya Y, Chung RT. Hepatic SOCS3 expression is strongly associated with non-response to therapy and race in HCV and HCV/HIV infection. J Hepatol 2009;50:705-11.
38. Miyaaki H, Ichikawa T, Nakao K, Takeshita S, Shibata H, Ozawa E, Akiyama M, Miuma S, Eguchi K. Predictive value of the phosphorylation of signal transducers and activators of transcription in the outcome of interferon therapy for chronic hepatitis C. Intervirology 2008;51:394-9.
39. Ning Q, Berger L, Luo X, Yan W, Gong F, Dennis J, Levy G. STAT1 and STAT3 alpha/beta splice form activation predicts host responses in mouse hepatitis virus type 3 infection. J Med Virol 2003;69:306-12.
40. Leal RF, Ayrizono ML, Milanski M, Coope A, Fagundes JJ, Velloso LA, Coy CS. Activation of signal transducer and activator of transcription-1 (STAT-1) and differential expression of interferon-gamma and anti-inflammatory proteins in pelvic ileal pouches for ulcerative colitis and familial adenomatous polyposis. Clin Exp Immunol;160:380-5.
41. Mitoro A, Yoshikawa M, Yamamoto K, Mimura M, Yoshikawa Y, Shiroi A, Mochi T, Sakamoto T, Yamao J, Kikuchi E, et al. Exacerbation of ulcerative colitis during alpha-interferon therapy for chronic hepatitis C. Intern Med 1993;32:327-31.
42. Rhee SH, Jones BW, Toshchakov V, Vogel SN, Fenton MJ. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. J Biol Chem 2003;278:22506-12.
43. Wu F, Dassopoulos T, Cope L, Maitra A, Brant SR, Harris ML, Bayless TM, Parmigiani G, Chakravarti S. Genome-wide gene expression differences in Crohn's disease and ulcerative colitis from endoscopic pinch biopsies: insights into distinctive pathogenesis. Inflamm Bowel Dis 2007;13:807-21.
44. Albanesi C, Fairchild HR, Madonna S, Scarponi C, De Pita O, Leung DY, Howell MD. IL-4 and IL-13 negatively regulate TNF-alpha- and IFN-gamma-induced beta-defensin expression through STAT-6, suppressor of cytokine signaling (SOCS)-1, and SOCS-3. J Immunol 2007;179:984-92.
45. Venkataraman C, Leung S, Salvekar A, Mano H, Schindler U. Repression of IL-4-induced gene expression by IFN-gamma requires Stat1 activation. J Immunol 1999;162:4053-61.
46. Bousquet C, Chesnokova V, Kariagina A, Ferrand A, Melmed S. cAMP neuropeptide agonists induce pituitary suppressor of cytokine signaling-3: novel negative feedback mechanism for corticotroph cytokine action. Mol Endocrinol 2001;15:1880-90.
47. Vong L, Ferraz JG, Panaccione R, Beck PL, Wallace JL. A pro-resolution mediator, prostaglandin D(2), is specifically up-regulated in individuals in long-term remission from ulcerative colitis. Proc Natl Acad Sci U S A 2010;107:12023-7.

48. Craven PA, Saito R, DeRubertis FR. Role of local prostaglandin synthesis in the modulation of proliferative activity of rat colonic epithelium. J Clin Invest 1983;72:1365-75.
49. Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S. Epithelial overexpression of SOCS-3 in transgenic mice exacerbates wound inflammation in the presence of elevated TGF-beta1. J Invest Dermatol;130:866-75.
50. Linke A, Goren I, Bosl MR, Pfeilschifter J, Frank S. The suppressor of cytokine signaling (SOCS)-3 determines keratinocyte proliferative and migratory potential during skin repair. J Invest Dermatol;130:876-85.

NEDERLANDSE SAMENVATTING

Ons immuunsysteem speelt een belangrijke rol bij het ontstaan van inflammatoire darmziekten (IBD) zoals de ziekte van Crohn en colitis ulcerosa (UC). Pro-inflammatoire cytokines, zoals IL-6, geproduceerd door cellen van dit immuunsysteem helpen ons bij de bescherming tegen ziekteverwekkers. Aan de andere kant zijn deze cytokines ook betrokken bij het ontstaan en in stand houden van chronische ziekten en kunnen ze de overleving en proliferatie van kankercellen stimuleren.

Anti-inflammatoire cytokines, zoals IL-10, helpen daarentegen bij het uitblussen van de ontsteking en het voorkomen van chronische ziekten en auto-immuniteit. Aan de andere kant kunnen anti-inflammatoire cytokines ook weer bijdragen aan de ontwikkeling van kanker door het remmen van immunologische surveillance.

Om al deze processen op een goede manier te laten verlopen, dus zonder het ontwikkelen van een chronische ziekte of kanker, is een goede regulering van de cytokine-signalering nodig. Dit geldt in het bijzonder voor de immuniteit in een complexe omgeving als dat van het darmslijmvlies. Zowel IL-6 als IL-10 activeren het signaleringsmolecuul STAT3 wat vervolgens zorgt voor o.a. de expressie van SOCS3, een remmer van de STAT3-signalering. In dit proefschrift wordt de rol van SOCS3 in UC en UC-gerelateerde ontwikkeling van colorectaal carcinoom (UC-CRC) beschreven.

Hoofdstuk I geeft een overzicht van recente inzichten in de verschillende functies van SOCS3, waarbij de focus ligt op de verschillende facetten betrokken bij de ontwikkeling van IBD en IBD-CRC.

Hoofdstuk II geeft vervolgens een caleidoscopische blik op de rol van STAT3 in IBD. We beschrijven de rol van STAT3 in intestinale epitheel cellen (IEC) en de bijdrage van STAT3-signalering aan het evenwicht tussen Th17 en regulatoire T-cellen in IBD. Bovendien bespreken we nieuwe therapeutische strategieën voor de behandeling van IBD welke een direct of indirect effect hebben we STAT3-signalering. Deze informatie helpt ons werk aan de STAT3-regulator SOCS3 in een breder perspectief te plaatsen.

In **hoofdstuk III** bestudeerden we de ziekte-gerelateerde expressie van IL-6/STAT3/SOCS3 in de IEC met behulp van immuunhistochemie. Onze data toonden een sterke expressie aan van zowel IL-6 als STAT3 maar geen expressie van SOCS3 in UC-

CRC. Verder onderzoek hiernaar laat zien dat het gebrek aan SOCS3 expressie het effect kan zijn van promotor methylatie van het SOCS3 gen, iets wat verder wordt behandeld in hoofdstuk IV. Een andere opmerkelijke observatie was de hoge expressie van SOCS3 in de IEC van UC patiënten met inactieve ziekte (remissie) iets wat we in meer detail bestudeerd hebben in de hoofdstukken V en VI.

In **hoofdstuk IV** leveren we, met behulp van patiënten materiaal en cellijnen, verder bewijs dat hypermethylatie van het SOCS3 gen betrokken is bij de afwezigheid van SOCS3 expressie in UC-CRC en CRC cellijnen. Door middel van in vitro experimenten konden we aantonen dat IL-6 de expressie van DNMT1, een enzym betrokken bij gen methylatie, verhoogt in IEC. Omdat de expressie van DNMT1 ook verhoogd is in IEC van patiënten met actieve UC en UC-CRC, zou de IL-6 geïnduceerde DNMT1 activiteit een belangrijke link kunnen vormen tussen ontsteking en carcinogenese.

In **hoofdstuk V** gaan we verder in op de klinische betekenis van de verhoogde expressie van SOCS3 in de IEC van UC patiënten in remissie. Eerder is aangetoond dat STAT3-signalering belangrijk is voor de normale deling en overleving van IEC. De hoge expressie van SOCS3 zou deze STAT3-signalering dus kunnen belemmeren waardoor de IEC kwetsbaarder kunnen worden voor schade en ook moeilijker zouden kunnen herstellen na schade. In dit hoofdstuk hebben we daarom onderzocht of er een correlatie bestond tussen de expressie van SOCS3 in de IEC van patiënten in remissie met de tijd tot een mucosaal recidief. Onze data tonen hierbij duidelijk aan dat een hoge expressie van SOCS3 correleert met een korter tijd tot recidief. Deze bevinding maakt verdere kennis omtrent de mogelijke factoren die betrokken zijn bij deze SOCS3 expressie van groot belang. De expressie van een van de kandidaat factoren p-STAT1 correleerde positief met de SOCS3 expressie wat suggereert dat p-STAT1 betrokken kan zijn bij de verhoogde SOCS3 expressie.

De zoektocht naar factoren die betrokken zouden kunnen zijn bij de hoge SOCS3 expressie is verder uitgebreid en beschreven in **hoofdstuk VI**. We hebben hierbij verschillende signaleringsroutes bestudeerd waarvan eerder aangetoond was dat moleculen ervan de expressie van SOCS3 kunnen induceren door aan specifieke elementen in de promoter regio van het SOCS3 gen te binden. Onze data tonen aan dat de IFN-gamma gestimuleerde STAT1 signalering, IL-4 of IL-13 gestimuleerde STAT6 signalering en de PGE2 of PGD2 gestimuleerde cAMP-signalering allen in staat zijn de

expressie van SOCS3 te stimuleren in cellijnen. Vervolgens hebben we laten zien dat deze signaleringsroutes actief lijken te zijn in de patiënten met hoge SOCS3 expressie.

Door het werk beschreven in dit proefschrift hebben we meer inzicht gekregen in de rol van SOCS3 als een de belangrijkste regulatoren van het inflammatoire processen en ziekte verloop in patiënten met UC. Bovendien hebben we aangetoond dat de afwezigheid van SOCS3 een belangrijke aanjager voor ontsteking-gerelateerde kanker in de dikke darm kan zijn. Vervolg onderzoek zal er dus op gericht zijn dit paradigma voor de toekomstige therapieën te benutten.

ACKNOWLEDGEMENTS

Time flies. Looking back, I am very glad that I have gained a rich life in the past four years, in varieties, because all of you:

To my promoter, Prof. Ernst Kuipers, I appreciate the opportunity you provide me to start my career as a scientist in your department. Your generosity and kindness have been always supporting me in both research work and daily life. I have been feeling that not only during my four-year stay in Rotterdam, but since we met for the first time in Wuhan. Thank you very much for your help!

To others, but no less important, my co-promoter, Dr. Janneke van der Woude, your enthusiastic dedication in IBD research has influenced me deeply, and your efficient management on my project led me to achieve my goals smoothly. Beside, what's also important is that, you can always understand me by standing in a woman's point of view and help me out of the woods. I've learnt a lot from you. Thank you very much!

To my co-promoter and supervisor, Dr. Colin de Haar, not only a supervisor, you are a really nice person to work together. Since joining IBD group, you brought us so many brilliant ideas and made me never feel alone in my project. With your solid experience, you've guided me into the world of fundamental science, and encouraged me to think about questions carefully again and again. I thank you so much!

To Prof. Maikel Peppelenbosch, your global views and quick thoughts gave me excellent examples. Thank you for all the open-mind comments in the meetings, for making so many critical remarks on my presentations, and for the brilliant revisions in my papers.

To my previous mentor, Prof. Bing Xia, you are the first teacher who opened the door of IBD to me and enlightened my scientific interest. You showed me determination and passion, you taught me to how to think ahead and look further. You have been standing behind me anytime anywhere. For many I thank you deeply.

Ron, you stimulated my curiosity in the knowledge of mouse models and molecular cloning. Thank you to make me capable of continuing the models in future.

Monique, although worked with you for only three months, you are the one getting me to know all the lab stuff and telling me how to get used to Dutch culture as an initiator. Thank you!

Jasper, your smart ideas are always beside me in laboratory for solving my problems. Thank you so much for all the technical supports, suggestions and discussions in my project.

Katharina, thanks for your contribution to my papers, for all the help of samples searching and immunohistochemistry scoring. Thank you for your patient with my endless questions.

All my group members thank you for providing and teaching me different topics in the weekly meetings and all the suggestions on my project.

Vivi (Vivianna), Veda and Laly (Ayala), I cherish so much the time we spent together, with chatting, playing, sharing lives, comforting each other from frustrations. I will remember all the parties we've had, especially the baby parties. How could I have so much fun without you? Our friendship will continue wherever we are, no matter in Netherlands, Italy, Greece, India, Israel, England, or China, in future!

My roommates, Tony (Anthonie), Petra, Paula and my previous roommates, Ozlem, Dowty, Patrick and Antoine, thank you for cheering up our office all the time, so that I've never been feeling boring during working. Thank you for the help with Dutch translation for many times. Of course, you guys made me learn "een beetje Nederlands" as well. The souvenirs collection is such a treasure in our room, I like them so much because it seems we have been to almost twenty countries. Dank wel.

All other colleagues in MDL, 4 years, in what a good time we shared different cultures, enjoyed so many parties and activities plus working together.

All my Chinese friends in Netherlands, all the lovely partners, I won't mention so many names one by one but how could I forget you all? The numerous parties, drinks, dinners, poker games, trips…… all the time I spent with you made my life full of joy.

All my friends in China, I can always feel you although so far away in distance. Thank you for all the contacts, steps on my Q-zone and caring about my life. You reminded me of

where I came from. Especially to Jing and Xufeng, so nice to see you in Rotterdam. You let me felt like 14 years ago. I believe you had an excellent honey-moon and wish you a beautiful life. To Fei and Heng, thank you very much for your visit. Our 18 years friendship! The nice Chinese desert you brought helped me a lot during my second pregnancy. I'm looking forward to seeing your cute baby girls. Wish you a very prospective career but don't work too hard!

To my father Weimin Li and my mother Jinping Zhu, how can I thank your raising me up by words? You have supported me in whatever way and time, especially for coming to the Netherlands to take care of my child. I will remember all your kindness ever, forever!

To my parents in law, your help with my child and my parents is great appreciated. Good to have you in neighbors.

To Bo, my husband, I always feel I should have known you much earlier. Thank you for all the sweet in the past when we were separated by distance. Thank you for coming to me in Netherlands. Thank you for commuting every day just to make me close to my work. Thank you for your unconditional supporting on my PhD during last four years. The encouragement gives me all the enthusiasm and confidence in the past and present. Thank you for all your love!

To my daughter Cherry (Xiao-niu), for without you, I could not have learnt so much! You have brought me into a new world. My life becomes much more colorful by staying with you and being surprised by how fast you start understanding, talking, singing and dancing.

To my unborn baby, you are a new life entering mine. You are my gift. Take care and Mom is waiting for your coming!

The financial support from Chinese Scholarship Council (CSC) and Erasmus Medical Center (EMC) are gratefully appreciated.

CURRICULUM VITAE

Yi Li was born on 1st November, 1981, in Zhong Xiang, Hubei Province, China. Soon afterwards she moved to Wuhan, Hubei Province, China with her family and spent her childhood there.

From 2000 to 2005, she studied clinical medicine in the medical school of Wuhan University, including 1-year internship in the university-affiliated Zhongnan Hospital. During the clinical rotation, she became so much interested in gastroenterology. Therefore when she finished her undergraduate in 2005, she decided to stay in the same department and continue her master study in internal medicine (gastroenterology) under the supervision of Professor Bing Xia. In her 2-year master program, she performed epidemiological survey of the complications and extra-intestinal manifestations in the patients of inflammatory bowel disease, and investigated the gene polymorphism in patients of ulcerative colitis in the laboratory of autoimmune disease. She got a chance to take another 6-month internship at the end of her master program. She graduated as “excellent master graduate student” in 2007.

During the master study, Yi Li met her current promoter, Prof. Ernst.J. Kuipers, during his visit in the seminar of 100-year’s anniversary of Zhongnan Hospital. Awarded by the national grant of “building high level PhD abroad”, she started with her favorite research topic on inflammatory bowel disease in the department of Gastroenterology and Hepatology, Erasmus MC, the Nertherlands, under promotion of Prof. Ernst.J. Kuipers, and co-promotion of Dr. C.Janneke van der Woude and Dr. Colin de Haar. Since December 2007, she has been focusing on PhD research work on “the role of SOCS3 signaling in ulcerative colitis and ulcerative colitis-related carcinogenesis”. Yi Li will defense her thesis at the beginning of 2012.

After finishing her PhD, she is going back to become a medical doctor in China, which is her 20-year’s dream.

PhD PORTFOLIO

Period

December 2007-January 2012

Courses

Course of English presentation

Introduction of data analysis

English writing and communication

Course of immunology

Photoshop and Illustrator

Conferences

2011

Li Y, de Haar C, Nuij VJ, Baars JE, Kuipers EJ, van der Woude CJ. SOCS3 Expression is a Predictive Factor of Relapse of Mucosal Inflammation in Chronic UC. Gastroenterology;140:S-1. **Digestive Disease Week (DDW)**, Chicago, US. Oral presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. SOCS3 promoter hypermethylation status in ulcerative colitis related colorectal cancer. Gastroenterology. 140(5), S-350. **Digestive Disease Week (DDW)**, Chicago, US. Poster presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. Th2 cytokines and cAMP mediated induction of SOCS3 expression in inactive ulcerative colitis. Gastroenterology. 140(5), S-842. **Digestive Disease Week (DDW)**, Chicago, US. Poster presentation.

Li Y, de Haar C, Nuij VJ, Baars JE, Kuipers EJ, van der Woude CJ. SOCS3 is a predictive factor of relapse of mucosal inflammation in chronic UC. **The molecular biology of inflammatory bowel diseases**, Durham, UK. Poster presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. SOCS3 promoter hypermethylation status in ulcerative colitis related colorectal cancer. **The molecular biology of inflammatory bowel diseases**, Durham, UK. Poster presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. P-STAT3 independent induction of SOCS3 expression in inactive ulcerative colitis. **The molecular biology of inflammatory bowel diseases**, Durham, UK. Poster presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. SOCS3 promoter hypermethylation status in ulcerative colitis related colorectal cancer. **Dutch Experimental Gastroenterology and Hepatology Meeting (NVGE)**, Veldhoven, NL. Poster presentation.

Li Y. The role of SOCS3 in ulcerative colitis and ulcerative colitis-related carcinogenesis. **IBD Eve Erasmus MC**, Rotterdam, NL. Oral presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. Th2 cytokines and cAMP mediated induction of SOCS3 expression in inactive ulcerative colitis. **European Crohn's and Colitis (ECCO)**, Dublin, Ireland. Poster presentation.

Li Y, de Haar C, Kuipers EJ, van der Woude CJ. SOCS3 promoter hypermethylation status in ulcerative colitis related colorectal cancer. **European Crohn's and Colitis (ECCO)**, Dublin, Ireland. Distinguished Poster presentation.

2010

Li Y. Disease-related expression of the IL-6/STAT3/SOCS3 signaling pathway in ulcerative colitis-related carcinogenesis. **Young Initiative on Crohn and Colitis (YICC)**, Utrecht, NL. Oral presentation.

Li Y, de Haar C, Deuring J, Kuipers EJ, van der Woude CJ. Methylation of the Suppressor of Cytokine Signaling (SOCS) 3 Promoter Region in Ulcerative Colitis (UC)-Related Carcinogenesis. Gastroenterology. 138(5), S-488. **Digestive Disease Week (DDW)**, New Oreleans, US. Poster presentation.

Li Y, de Haar C, Deuring J, Kuipers EJ, van der Woude CJ. Methylation of the Suppressor of Cytokine Signaling (SOCS) 3 Promoter Region in Ulcerative Colitis (UC)-

Related Carcinogenesis. **Dutch Experimental Gastroenterology and Hepatology Meeting (NVGE)**, Veldhoven, NL. Poster presentation.

2009

Li Y, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, de Haar C Kuipers EJ, van der Woude CJ. The role of the IL-6/STAT3/SOCS3 pathway in ulcerative colitis related carcinogenesis. Gastroenterology. 136(5), S-114. **Digestive Disease Week (DDW)**, Chicago, US. Poster presentation.

Li Y, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, de Haar C Kuipers EJ, van der Woude CJ. The role of the IL-6/STAT3/SOCS3 pathway in ulcerative colitis related carcinogenesis. **Dutch Experimental Gastroenterology and Hepatology Meeting (NVGE)**, Veldhoven, NL. Poster presentation.

2008

Li Y, Chen M, Gerrits MM, Deuring J, Smits R, Kuipers EJ, van der Woude CJ. The expression of suppressor of cytokine signaling (SOCS)-3 and signal transducer and activator of transcription (STAT)-3 in inflammatory bowel disease-related colorectal cancer. Gut II(57), S-338. **United European Gastroenterology Week (UEGW)**, Vienna, Austria. Poster presentation.

Memberships

Dutch Experimental Gastroenterology and Hepatology Meeting (NVGE)

Young Initiative on Crohn and Colitis associate (YICC)

Peer reviews

Broad Medical Research Program Inflammatory Bowel Disease, 2010.

J Microbial Biochem Technol, 2011.

Clin Invest Med, 2011

PUBLICATIONS

Li Y, de Haar C, Peppelenbosch MP, van der Woude CJ. New insights into the role of STAT3 in IBD. *Inflamm Bowel Dis* 2011; Oct 12 [Epub head of print].

Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R, Xia B, Kuipers EJ, van der Woude CJ. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. *Gut* 2010; 59(2):227-235.

Li Y, Xia B, Lü M, Ge L, Zhang XL. MICB0106 gene polymorphism is associated with ulcerative colitis in central China. *Int J Colorectal Dis* 2010; 25(2): 153-159.

Lü M, Xia B, Ge L, **Li Y**, Zhao J, Chen F, Zhou F, Zhang X, Tan J. Role of major histocompatibility complex class I-related molecules A*A5.1 allele in ulcerative colitis in Chinese patients. *Immunology* 2009; 128(1 Suppl):230-236.

Li Y, Xia B. Chapter XXII. Clinical and Endoscopic Assessments of Activity and Severity of crohn's disease. *Inflammatory Conditions of the Colon* 2009; 147-154. Nova Science Publishers Inc. ISBN10: 1606922408. ISBN13: 9781606922408.

Li Y, Xia B. Clinical and Endoscopic Assessments of Activity and Severity of Crohn's Disease. *A handbook of inflammatory conditions of the colon* 2007; 165-174. China Medical-Pharmaceutical Sciences & Technology Publishing House. ISBN: 750673714.

Li Y, Nuij VJ, Baars JE, Biermann K, Kuipers EJ, de Haar C, van der Woude CJ. Increased suppressor of cytokine signaling-3 expression predicts mucosal relapse in ulcerative colitis. Submitted for publication.

Li Y, Peppelenbosch MP, Kuipers EJ, de Haar C, van der Woude CJ. STAT1, STAT6 and cAMP signaling drive SOCS3 expression in inactive ulcerative colitis. Submitted for publication.

Li Y, Peppelenbosch MP, Kuipers EJ, de Haar C, van der Woude CJ. IL-6 induced DNMT1 mediates SOCS3 promoter hypermethylation in ulcerative colitis related colorectal cancer. Submitted for publication.

Li Y, de Haar C, van der Woude CJ. SOCS3 protein in immune regulation of inflammatory bowel disease and inflammatory bowel disease-related cancer. Submitted for publication.