THE ROLES OF SECRETED PROTEIN ACIDIC AND RICH IN CYSTIENE (SPARC) IN INTESTINAL INFLAMMATION, HEALING AND FIBROSIS.

Yoke Leng NG

BSc(Hons.)

This thesis is presented for the degree of Doctor of Philosophy of Murdoch University,

School of Veterinary and Biomedical Sciences,

2012

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

.....
(Yoke-Leng NG)

ABSTRACT

Secreted Protein Acidic and Rich in Cysteine (SPARC) is a matricellular protein expressed during tissue repair and regulates cell proliferation and migration. It binds to, and interacts with collagen and regulates matrix metalloproteinase (MMP) expression. The aim was to determine if SPARC modifies intestinal inflammation, healing and fibrogenesis.

Intestinal disease was investigated using SPARC null (KO) and wild-type (WT) mice in which inflammation was induced by 3% dextran sodium sulphate (DSS) in the drinking water for 7 days. Inflammation was assessed endoscopically, clinically and histologically on days 7, 14, 21 and 35 after initiation of DSS treatment. Systemic and colonic cytokines and chemokines were quantitated by ELISA and CBA. Colon, mesenteric lymph node and spleen were analysed by flow cytometry and immunofluorescence for inflammatory cell infiltrates. To determine the effect of SPARC on the extracellular matrix (ECM) genes regulation, RNA from colonic tissue, and colonic myofibroblasts from WT and KO mice, were analysed by real time PCR for expression of ECM related genes.

KO animals had significantly lower endoscopic scores of inflammation, suffered less weight loss, diarrhoea, faecal blood and had lower spleen/body weight ratios compared to WT animals consistent with less colonic and systemic inflammation. WT mice had higher levels of histological inflammation and damage when compared to KO animals and in the majority of KO animals the colonic mucosa had completely regenerated by day 35 in contrast to the WT mice. Compared to WT mice, in KO animal colons at day 7 there was significantly less IL1β and MIG expression while TGFβ1 levels were higher. Flow cytometric analysis identified a significantly greater percentage of FoxP3+ regulatory T cells in the spleen and draining lymph nodes of KO animals. KO mice also

had fewer of cells, such as CD68+ macrophages and Ly6G+ neutrophils, of the innate immune e system infiltrating the inflamed colon.

Collagen (Col) 1α1, Col3α1 MMP13 and MMP3 expression levels were reduced in DSS-treated WT colons at day 7 and these were significantly lower than those observed in the KO colons. TIMP1 expression was significantly lower in KO mice at day 35 when healing was complete in this group. TIMP2 and TGFβ1, TGFβ3 were not different between the groups at any time point. The observation by others that collagen fiber diameters in KO colons were noted to be significant smaller than in WT animals suggesting that SPARC modifies the collagen bundling. Compared to unstimulated WT fibroblasts, KO cells had lower Col1α1 and Col3α1 expression. Stimulation with PMA reduced Col1α1 and Col3α1 and increased MMP13 and TIMP1 expression in all the isolated cells, but PMA had no effect on MMP3, TIMP2, TGFβ1 and TGFβ3 expression.

DSS induced less colonic and systemic inflammation in KO compared to WT mice and the inflammation appeared to resolve faster. This may be secondary to increased numbers of regulatory T cells and increased colonic TGF-β levels which may inhibit effector cell activity, including cytokine and chemokine expression and aid in the more rapid resolution of inflammation and restoration of the intestinal mucosa. SPARC is able to modify tissue healing potentially through the regulation of collagen expression, bundling and its degradation by MMPs, which impacts on tissue turnover rate and thus delays healing. Hence, SPARC might represent a promising therapeutic target in clinical management of inflammatory bowel diseases (IBD).

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First of all, I wish to express my deepest gratitude to my supervisor Prof. Ian Lawrance for giving me the opportunity to extend my study in his lab, as well as his continuous guidance and support throughout the course of my PhD. My sincere appreciations also go to my co-supervisors: To Dr Borut Klopcic for his encouragement, expertise and critical yet helpful comments on my research project and thesis; and to Dr. Wayne Greene for his valuable comments and suggestions regarding my project and administrative matters.

I'd especially like to thank A/Prof Jane Allan for proofreading my thesis and being patient with me on lab related issues. A great "Terima Kasih" to Dr. Anita Chua for her expert guidance and friendship throughout. You two was like my extra "uncredited" supervisors who always willing to help and support me academically and emotionally.

I was really lucky to work in Freo's IBD research group because of the following great people. Mrs. Frances Lloyd, the super RA, who provided technical brilliance to me right from my honours till now and all those delicious sweet treats. Constant moral support and guidance was also given to me by Dr. Angela Chew, who always broadened my thinking and boast up my caffeine levels. My fellow PhD student, Kristine Fu, thanks for cutting my cyrosections and for your histo knowledge, not to mention that all the fun and jokes you brought to me (your mum's home cooking too). Thanks girls.

Sincere thanks must go to those that helped in term of technical support throughout my PhD. Alison and Carla from the animal facility at Fremantle Hospital for their invaluable assistance. Staff from the Pathwest Histopathology Department at Fremantle Hospital, in particular; Dr Cynthia Forrest for her knowledge and precious time on the histological scoring. Staff from the Centre for Microscopy, Characterisation and Analysis, UWA, helped me regarding flow cytometry, immunofluorescence and TEM, especially Lyn Kirilak; Dr Leanne Scott from the statistics department, Murdoch

University, for her statistical advice, HDR scholarships officer, Julie Blake from the Murdoch University for her assistance with my scholarship extension. Many thanks to Irma Larma for her tireless technical support with the flow cytometry; both present and past lab members at MSL, Fremantle Hospital: Carly, Amber, Gwen, Ross, Ro, Desiree, Brenda, Kerry, Janina and many more for their continual assistance throughout my work.

To family and friends, thanks for providing me with unconditional love and support in every way. Last but very essential, I truly appreciated the Murdoch University Research Scholarship that providing me the financial support.

I thank you all again (those that have been mentioned and sorry for those that I missed) from the bottom of my heart. I couldn't have done it without your support.

SPECIAL DEDICATION

To my parent, thanks for all the hard work and sacrifices you have made to allow me to pursue my dream and choose the path I want; forgive me for being "irresponsible" child sometimes. Nothing can express my thankfulness and how grateful I am besides dedicated this to you both.

&

The "MouthSky Gang" and Aunty K. Lan, thanks for putting up with me all the time, providing me with support, encouragement and most importantly "babies's stories" to brighten these tough years.

CONFERENCE ABSTRACTS

Ng Y.L., Klopcic B., Lloyd F, Greene W, Lawrance IC "Combined Transforming Growth Factor-β1 and -3 Fibroblast stimulation modifies the Extracellular Matrix gene expression through Smad independant signalling"
Poster presented at Digestive Diseases Week – May 2007; Washington, DC
Gastroenterology Volume 132, Issue 4, Supplement 1 A568 (April 2007) Annual Abstract Supplement

Ng Y.L., Klopcic B., Greene W, Lawrance IC "Combined Transforming Growth Factor-β1 and -3 Fibroblast stimulation modifies the Extracellular Matrix gene expression through Smad independant signalling"
Oral presented at Australian Society for Medical Research (ASMR)- June 2007; Perth, Western Australia

Ng Y.L., Klopcic B., Lloyd F, Greene W, Lawrance IC "SPARC attenuated Intestinal Inflammation in the DSS-treated Mouse?"
Poster presented at 9th Euroconference on Clinical Cell Analysis- September 2009; St. Etienne, France

Ng Y.L., Klopcic B., Lloyd F, Greene W, Lawrance IC "A role for SPARC in modifying inflammation and healing in the DSS murine model of colitis"
Poster presented at 18th united European Gastroenterology Week- October 2010; Barcelona, Spain

Ng Y.L., Klopcic B., Lloyd F, Fu S.K., Lawrance I.C. "SPARC modifies colonic tissue healing and inflammation by regulating collagen and MMP expression"
Oral presented at 7th Congress of European Crohn's and Colitis Organisation – February 2012; Barcelona, Spain

Ng Y.L., Klopcic B., Lloyd F, Greene W., Lawrance I.C. "SPARC enhances intestinal inflammation in dextran sodium sulphate-induced murine colitis"
Poster presented at 7th Congress of European Crohn's and Colitis Organisation – February 2012; Barcelona, Spain

LIST OF FIGURES

LIST OF TABLES

ABBREVATIONS

The following abbreviations are used throughout this thesis:

α-SMA	α-smooth muscle actin
AF	Alexa fluor®
APC	Antigen presenting cells
BCP	Bromochloropropane
BSA	Bovine serum albumin
CCL28	Mucosal epithelial chemokine
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
COX	Cyclooxygenase
CrD	Crohn's disease
CTGF	Connective tissue growth factor
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
FBS	Foetal bovine serum
FSC	Forward scatter
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
H&E	Haemotoxylin and eosin
HBSS	Hank's balanced salt solution
HCl	Hydrogen chloride
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBD	Inflammatory bowel diseases
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILF	Isolated lymphoid follicles
KCl	Potassium chloride

$KHCO_3$	Potassium hydrogen carbonate
KO	Knockout
LAP	latency-associated peptide
LP	Lamina propria
LPMC	Lamina popria mononuclear cell
LPS	lipopolysaccharide
LTBP	Latent TGF-β binding protein
MAdCAM	Mucosal vasacular addressin cellular adhesion molecules
MgCl	Magnesium chloride
M cell	Specialised epithelial cells in GLT
MCP-1	monocyte chemotactic protein-1, CCL2
MEICS	Murine endoscopic score of colitis severity
MHC	Major histocompatibility complex
MIG	Monokines induced by IFN-γ, CXCL9
MIP	Macrophage inflammatory proteins
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NF	Nuclear factor
NH_4Cl	Ammonium Chloride
NOD	Nucleotide oligomerisation domain
OD	Optical density
Oligo	Oligonucleotide
PAI-1	Type-1 plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PRR	Pattern recognition receptors
PMA	Phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leucocytes
PDGF	platelet derived growth factor
PG	Prostaglandin
PP	Peyer's Patches
PSF	Penicillin /Streptomycin/Fungizone

RANTES	Regulated on activation normal T cell expressed and excreted, CCL5
RNAse	Ribonuclease
RNAsin	Ribonuclease inhibitor
ROR	Retinoic-acid-receptor-related orphan receptors
RT	Room temperature
SEM	Standard error of mean
SPARC	Secreted Protein Acidic and Rich in Cystiene
SSC	Side scatter
STAT	Signal transducer and activators of transcription
TBS	Tris-buffered saline
TBST	TBS-Tween
TGF	Transforming growth factor
Th cell	T helper cell
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptors
TNBS	trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
tPA	tissue type plasminogen activator
Treg cell	Regulatory T cell
TREM-1	Triggering Receptor Expressed on Myeloid cells-1
UC	Ulcerative colitis
uPA	Urokinase type plasminogen activator
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter ***1***

General introduction

1.1 INFLAMMATORY BOWEL DISEASES

The inflammatory bowel diseases (IBD), Crohn's disease (CrD) and ulcerative colitis (UC), are life-long, chronic, inflammatory disorders of complex, and aetiology still largely unknown, that primarily present in the gastrointestinal tract of young adults. Their development, however, is believed to result from defects in the interaction between the host and their intestinal microflora, in combination with intestinal epithelial cell dysfunction resulting in an abnormal mucosal immune response (Kumar 2010; Podolsky 2002a). Morbidity is high (costing $2.7 billion annually in Australia), and despite advances in both the understanding of the genetic aspects behind the diseases and improvements in the medical therapies available, both UC and CrD are increasing in incidence and prevalence worldwide and surgical intervention is still required in the majority of CrD patients (Harper *et al.* 1987; Podolsky 2002b).

The IBD, especially CrD, have many characteristics of an autoimmune disease. CrD is characterised by a transmural granulomatous inflammation that may occur anywhere throughout the gastrointestinal tract (GIT), but it most frequently affects the terminal ileum and colon. UC, in contrast, is characterised by superficial inflammation resulting in epithelial cell destruction that only involves the colon and rectum (Fichtner-Feigl *et al.* 2005; Podolsky 2002b; Finkelstein *et al.* 2002; Bouma and Strober 2003). Inflammatory cells within these regions release factors that are chemotactic for fibroblasts. These factors can stimulate both fibroblast activation and proliferation leading to the synthesis and deposition of extracellular matrix (ECM) components (Kumagai *et al.* 2001). Intestinal fibrosis can then result from the excessive deposition of these fibrous ECM components and is a frequent occurrence as a consequence of the chronic inflammation that is the hallmark of IBD. In the intestine, the development of

fibrosis may result in the serious complication of stenosis, or stricture, of the intestinal lumen, an adverse outcome particularly associated with CrD (Abreu *et al.* 2002; Harper *et al.* 1987). To date, there is a marked lack of understanding of the process of intestinal fibrosis and in the availability of effective therapies for the treatment or prevention of this complication. The data on current therapies such as, corticosteroids, immune modulators, antibiotics and surgery, primarily focus on the short term control of clinical disease activity, but these therapies may not alter the natural history of the diseases or the long term development of intestinal fibrosis in CrD (Harrison and Hanauer 2002; Rutgeerts *et al.* 2005). These anti-inflammatory treatments target specific components of the immune response and while colectomy (partial or total surgical removal of the large bowel) is occasionally necessary, it is not a cure for CrD. Hence, there is still a great need for alternative and more specific therapeutic strategies for IBD.

To understand the pathogenesis behind IBD, one first needs to understand the normal function of the GIT mucosal immune system. The upper and lower respiratory tract, urogenital tract and the GIT have a combined surface of greater than 400 m^2 that is primarily covered by a single layer of epithelial cells. This single cell layer has many important physiological functions, including as a barrier in face of an external environment rich in pathogens. The gut is exposed to an estimated 10-15 kg of food protein per year and is colonised by at least 1000 different species of commensal microorganisms (Murphy 2008; Savage 1977). As a defense against the multitude of microorganisms, the mucosal tissue is populated with many immune cells. The normal healthy intestinal mucosa is thus often described as experiencing a “physiological inflammation”, as it contains large numbers of effector lymphocytes and other leucocytes that are normally associated with chronic inflammation, and are generally

not present in other organs in the absence of tissue injury or disease. In order to monitor, and control, the "physiological inflammation", a tight regulatory system is required. (Neurath *et al.* 2002; Neutra *et al.* 1996; Murphy 2008).

The immune response seen in the intestine is also different from the systemic inflammation that is controlled by the spleen and other peripheral immune organs. This difference is mainly due to the antigenic environment of the intestine (intestinal microflora and dietary antigen) which is in contrast to the generally sterile environment that surrounds systemic immune system (Mayer 2005). The fact that the mesenteric lymph node (MLN) are the first, and largest, lymph nodes to be formed during embryogenesis further highlights the importance of intestinal immune system (Newberry and Lorenz 2005).

1.1.1 Intestinal immune system in colon

In order for the intestinal immune system to function optimally it requires a mechanism that is finely balanced between mounting an effective immune response to pathogenic antigens while suppressing any potentially destructive responses against the commensal microflora or food antigens. The intestinal immune system thus has certain distinctive features that allow it to maintain this balance, such as the presence of an intestinal barrier, gut-associated lymphoid tissue (GALT) as well as both the innate and adaptive immune systems that are phenotypically and functionally specifically adapted to the intestinal microenvironment.

The intestinal epithelium is composed primarily of intestinal epithelial cells and is the first line of defense. Under normal conditions, the intestinal epithelium, with the

overlaying mucus layer, serves as a barrier between the luminal contents and the GI mucosa. A defect in this barrier, however, is frequently observed in both CrD and UC and is central to the development of the colitis in animal models of these diseases (Su *et al*. 2009). The layer of epithelial cells, however, not only forms a barrier between the body and the luminal contents but is also responsible for importing nutrients into the body, the release of antibodies into the lumen and the production of antimicrobial peptides as well as pro-inflammatory cytokines in response to activation of the immune defense system (Artis 2008). The epithelial layer also contains other specialised cell populations that confer further protection to the host. These include the goblet cells that secrete a protective mucus layer that covers the epithelium and paneth cells that secrete antimicrobial peptides such as the defensins and lysozymes which help to prevent direct access of microorganism to the intestinal mucosa (Cash *et al*. 2006; Schenk and Mueller 2007).

The intestine is associated with several types of lymphoid organs collectively known as GALT. As the intestine is a primary site for encounters with foreign antigens, the presence of GALT, not only provides a rapid immunological response, but also provides an immune response that is specific to the gut. GALT includes the Peyer's Patches (PP) found within the small intestine and isolated lymphoid follicles (ILF), which are distributed throughout the large intestine and are sites where the immune response is frequently initiated. PP and ILF are connected by a lymphatic network to the draining MLNs that play a crucial role in initiating and shaping the immune responses to the intestinal foreign antigens. (Lorenz *et al*. 2003; Murphy 2008). The PP and ILF have a similar structure with each containing specialised M cells, antigen presenting dendritic

cells (DC)s, and T and B lymphocytes which play important roles in generating the mucosal immune response.

The innate immune responses occur rapidly in order to provide immediate protection to the host. Overlapping with this system, there is the adaptive immune system that takes a longer time to respond (>96 hours), but is more efficient in the elimination of specific pathogens (Murphy 2008). The innate immune system eliminates harmful pathogens by phagocytic mechanisms and production of cytokines, it also promotes the release of co-stimulatory signals that can then activate an adaptive immune response.

Immune responses in the colon take place in three distinct locations: the lamina propria (LP), the ILF scattered throughout the colon, and the MLN as shown in **Figure 1.1**. DCs are present in large numbers within the LP and ILF, where they are able to capture and sample bacterial antigen from the gut lumen by penetrating their finger-liked processes across the epithelium without disrupting its integrity (A and B in ***Figure 1.1***). Antigens from pathogens that penetrate the mucosal barrier are taken up by local DCs and carried via the lymphatics to the draining MLN. DC can also signal to the innate and adaptive immune cells that are present within the LP to eliminate the harmful pathogens (C in ***Figure 1.1***). In addition, DCs situated in GALT such as ILF can sample luminal antigens via specialised epithelial cells (M cells). The M cells continually monitor the luminal contents, transport molecules and particles into the mucosa via phagocytosis and endocytosis from the gut lumen and releasing them into the extracellular space by transcytosis. The transported antigen is then taken up and sampled by the underlying antigen presenting DCs. Antigen containing DCs then meet and activate the naïve, antigen–specific T lymphocytes located within in the ILF in order to generate an

immune response (D in ***Figure 1.1***). These DCs also migrate to the draining MLN in order to present the antigen to other naïve lymphocytes (E in ***Figure 1.1***) (Murphy 2008; Izcue *et al.* 2006, 2009; Banchereau *et al.* 2000). Following antigen stimulation, the activated lymphocytes migrate into the draining MLNs where further maturation occurs. Following this, the mature cells migrate through the lymphatic circulation, though the thoraric duct and via the bloodstream in order to return to the LP layer of the colon as mature lymphocytes (or effector cells) (F in ***Figure 1.1***). This occurs with the help from the gut-specific homing integrins and chemokines, mucosal vasacular addressin cellular adhesion molecules (MAdCAM)-1 and CCL28 (mucosal epithelial chemokine) (Izcue *et al.* 2009; Iwata *et al.* 2004). Once the mature lymphocytes have returned to the gut, they are able to clear the intestinal mucosa of the foreign antigens (Eksteen *et al*. 2006; Kato *et al*. 2000) and once these foreign antigens are cleared, most of the effector lymphocytes will die by apoptosis, leaving a small population of memory cells with a longer lifespan that can rapidly mount another immune response for any future encounter with the same antigen (Eksteen *et al.* 2005; Murphy 2008).

A second immune response that can also be generated through the activation of cells located in the GALT is driven through B lymphocytes that produce antigen specific immunoglobulins (Ig). These Igs within the mucosal tissues or MLN can promote the differentiation of the B cells into plasma cells that are able to secrete higher amounts of IgA and other Ig isoforms into the LP. Secreted IgA is then able to inhibit mucosal adherence by the bacteria, neutralise viruses and enhance clearance of the pathogenic microorganism (Murphy 2008; Neurath *et al.* 2002).

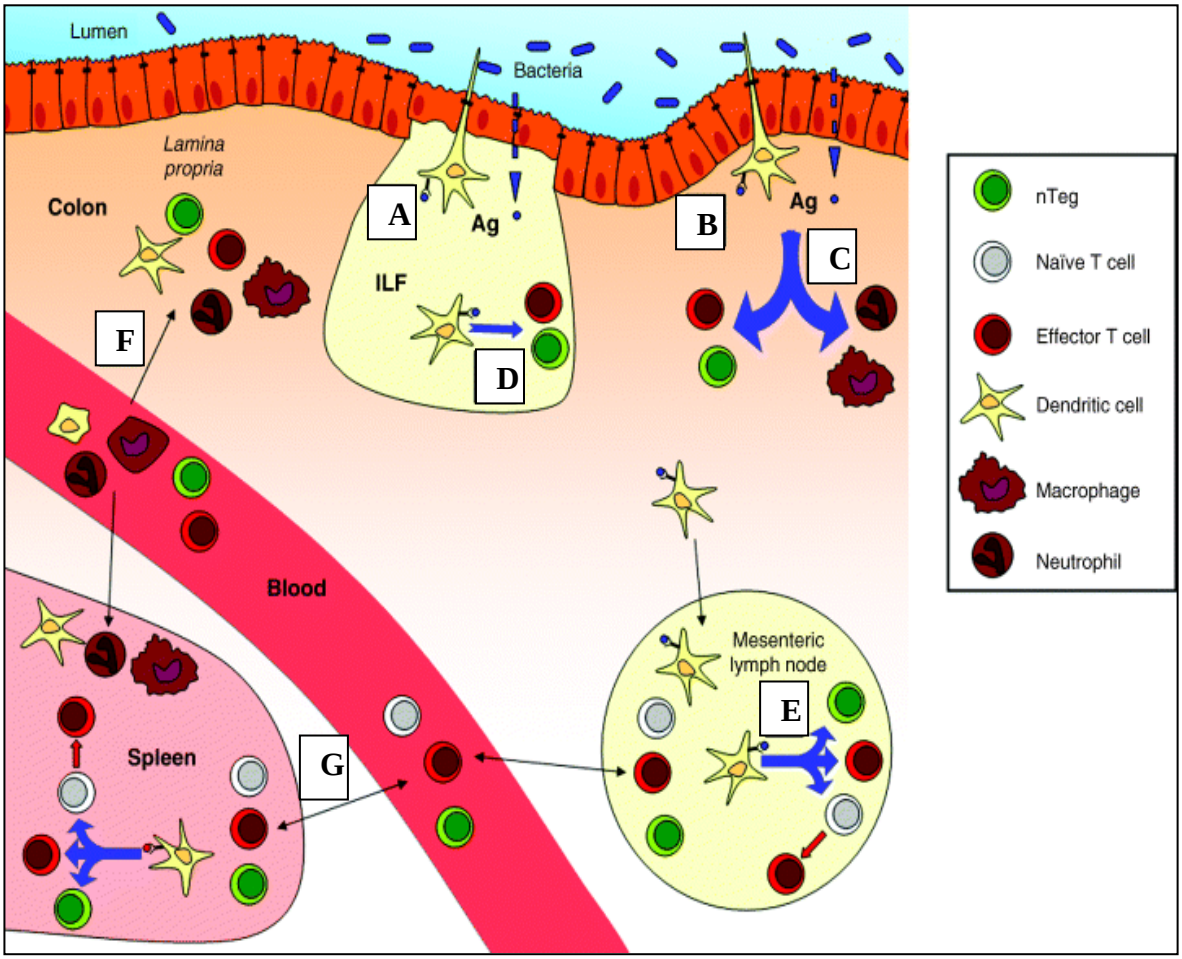


Image adapted and modified from (Izcue *et al.* 2006)

Figure 1.1 The intestinal immune responses occur within the epithelium, LP, and GALT and MLN. **A, B**) DCs can sample bacterial antigen by penetrating the epithelium with their dendrites or by capturing antigen carried across the epithelium. **C**) DCs can then signal to effector T cells, Tregs and also other components of the innate immune system. **D**) M cells that are present in the ILF can transport antigen from the lumen and are then taken up by local DCs in the ILF, antigen containing DCs then meet and activate the na ïve, antigen–specific T lymphocytes located within the ILF in order to generate an immune response. **E)** In addition to their local role, intestinal DCs also migrate into the draining MLN where they present foreign antigen to T cells. **F**) Na ïve T cells activated this way return to the blood and preferentially migrate back into the colon, where they will again encounter their cognate antigen. **G**) T cells, including effector T cells, also migrate to the spleen; in many disease models, local intestinal inflammation induces the accumulation of activated T and innate immune cells in the spleen.

1.1.2 Innate immunity in the colon

The innate immune response is composed of mechanical, chemical and cellular elements. The intestinal epithelial cells form the cellular barrier between the host and the environment as well as providing a mucus secretion that covers and protects the monolayer of cells. This constitutes the mechanical aspect. The chemical elements include the involvement of pattern recognition receptors (PRR) like the Toll-like receptors (TLR) and nucleotide oligomerisation domain (NOD) receptors that are able to recognise and bind "highly conserved pathogen-associated molecular patterns" that are located on the cell surface of most of the noneukaryotic organisms. These PRR are subsequently able to activate the nuclear factor (NF)-κB signaling pathway that controls expression of many inflammatory cytokines and chemokines. The release of these factors then can promote the attraction of effector immune cells to the site of the insult. Each class of the innate immune cells (e.g. DC, eosinophil, neutrophils, mast cells etc) then secrete a characteristic profile of cytokines and pro-inflammatory mediators that have overlapping functions in order to progress the immune response (Kumar 2010; Takeda and Akira 2005). This cellular innate immune response also protects the tissue through the processes of phagocytosis by macrophages and the killing of the invading microbial agent by natural killer cells (Biron *et al.* 1999; Arseneau *et al.* 2007).

1.1.2.1 Macrophages

Intestinal macrophages (especially in the colon) represent the largest population of mononuclear phagocytes in the body (Lee *et al.* 1985), and are capable of phagocytosing (engulfing and killing) pathogens as a non-specific defense against any invading antigen to maintain tissue homoestasis. It has been shown that depletion of macrophages results in more severe intestinal inflammation in the dextran sodium

sulphate (DSS)-induced colitis model in the mouse (Qualls *et al*. 2006). Macrophage depletion, however, has also been shown to reduce the development of the spontaneous colitis observed in interleukin (IL)-10 deficient mice (Wirtz and Neurath 2007) suggesting that the intestinal macrophages could have a dual function within the gut.

Blood monocytes originate in the bone morrow from a common myeloid progenitor and are the progenitors for all resident macrophages subsets, e.g. Kupffer cells in the liver, osteoclasts in the bone and intestinal macrophages in the gut. From the circulation, the blood monocytes migrate into the tissue where they mature into fully differentiated resident macrophages. The intestinal resident macrophage, unlike other resident macrophages subsets (e.g. Kupffer cells), has not been shown to proliferate suggesting that continuous replenishing of the intestinal population of macrophages must occur solely by the migration of blood monocytes to the intestine in response to the potent chemokine activity of transforming growth factor (TGF)-β and IL-8 that are constitutively produced by mucosal epithelial cells and mast cells under homeostasic conditions (Smythies *et al.* 2006; Smith *et al.* 2010). When this homeostasis is disturbed, such as in the presence of inflammation, there will be an increase in recruitment of blood-borne monocyte precursors to boost the mononuclear phagocyte numbers in inflamed tissue. This macrophage type is considered to be the inflammatory macrophage (Schenk and Mueller 2007; Heinsbroek and Gordon 2009). Depending on the extracellular microenvironment, murine studies suggest that these inflammatory macrophages may further polarise into macrophages with either M1 and/or M2 characteristics (Smith *et al.* 2010).

Intestinal resident macrophages are phenotypically and functionally different from the inflammatory macrophages and their precursor blood monocytes as shown in **Table 1.1**. The intestinal macrophages do not express the lipopolysaccharide (LPS) receptor (CD14) and several innate response receptors (e.g. TLR4), make it unresponsive to LPS and other microbe associated molecular patterns that present abundantly in the intestinal microflora. Intestinal macrophages also lack the Fc receptors for Igs (CD16, CD32 and CD640), the complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), and the integrin α2β1. These Fc and complement receptors on phagocytes usually mediate cellular activation, secretion of pro-inflammatory cytokines and induction of potent adaptive immune responses. Low, or absent, cell surface expression of CD40, CD80 and CD86 give intestinal macrophages a reduced ability to respond to certain immune stimuli and also make then unable to activate a T cell-mediated immune response, hence they do not act as antigen presenting cells (APCs). The intestinal macrophages, however, retain and even have enhanced phagocytic and bactericidal capabilities. In combination this allows them to maintain the homeostasis in the gut without an over-response to the 'healthy microflora' found within the intestinal lumen (Smythies *et al*. 2005; Smith *et al*. 2001; Heinsbroek and Gordon 2009; Sartor 2003; Smith *et al*. 2010).

By contrast, the inflammatory macrophages recruited to the mucosa during intestinal inflammation produce pro-inflammatory cytokines (Burgio *et al*. 1995; Rugtveit *et al*. 1994) that result in epithelial cell apoptosis, epithelial barrier dysfunction, tissue necrosis, development of granulomas and the formation of fibrosis (Heinsbroek and Gordon 2009). The LP macrophages isolated from the inflamed intestine of IBD patients also demonstrate high expression of CD14, CD80, CD86 and increased activation of NF-κB (Rugtveit *et al*. 1997; Schenk *et al*. 2007; Neurath *et al*. 1998). In

patients with CrD, the number of CD14 expressing macrophages was significantly increased in the intestine compared with normal control subjects, these cells also produce higher levels of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-23 (Kamada *et al.* 2008; Reimund *et al.* 1996). In addition, and in marked contrast to the resident intestinal macrophage, the inflammatory macrophage also expresses Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) which is an efficient amplifier of both the acute and chronic inflammatory reaction and is generally expressed on peripheral blood neutrophils and the monocytes and macrophages located within the secondary lymphoid organs. Engagement of TREM-1 to its receptor on TREM-1-positve myeloid cells results in enhanced secretion of the pro-inflammatory cytokines (e.g. TNF, IL-1β, -6, -8, MCP-1) (Bouchon *et al.* 2000; Schenk *et al.* 2007).

To date, there is still a lack of a definite, unique marker that can fully distinguish the resident intestinal macrophage from the recruited inflammatory macrophages. This is particularly true in the murine immune system. The human intestinal macrophages generally express the myeloid markers CD13, CD33, CD68 and HLA-DR (major histocompatibility complex (MHC) class II molecule) at levels comparable to blood monocytes and most resident macrophages in other organs (Smith *et al.* 2001) and in an elegant study by Schenk *et al*., CD68 (a cytoplasmic marker) was used as a marker to identified intestinal macrophage from both human and murine model (Schenk *et al.* 2007). CD11b was used in this study to identify the infiltrating inflammatory macrophages as there is a lack of CD11b expression by the intestinal resident macrophages. There was high expression of CD11b on colonic macrophages isolated from IBD patients (Rogler *et al*. 1997) and inflammatory peritoneal macrophages in

mice (Rosas *et al.* 2010). This suggests that CD68 and CD11b can be used to identify the resident intestinal macrophages and inflammatory macrophages respectively.

Table 1.1 Phenotypic and functional differences between monocytes, infiltrated and intestinal macrophages. (Adapted from (Heinsbroek and Gordon 2009; Smith *et al.* 2010))

Phenotype	Monocytes	Inflammatory macrophage	Intestinal macrophage
Toll-like receptors- TLR-2	+	+	-
TLR-4	+	+	+
CD4	+	+	Low
CD11a/LFA-1	+	+	-
CD11b/MAC-1	+	+	-
CD11c/integrin α	+	+	-
CD14	+	Low	-
Fc receptors (CD16, CD32, CD64)	+	+	-
Complement receptors (CR3, CR4)		+	-
Chemotaxis (CCR5, CXCR4)	+	+	-
Costimulatory molecules- CD40, CD86	+	+	-
CD80	-	+	-
MHC molecules HLA-DR	+	+	+
Function			
Phagocytosis	+	+	+
Killing	+	+	+
Chemotaxis	+	+/-	-
Antigen presentation	+	+	**?**
Cytokines production (TNF-α, IL-1,-6,-8)	+	+	-
Activation inflammatory responses (TREM-1)	+	+	-

1.1.2.2 Dendritic Cells

Dendritic Cells (DC) are professional APCs that are involved in most of the antigen presentation to T cells located within mucosal tissue and the draining MLN. As the DC are part of both the innate and adaptive immune system, they enable immune system to determine whether there should be a response, or not, to a potential invading antigen. The DCs are central to determining whether a regulatory or effector cell response predominates, and whether tissue specific homing of antigen specific effector cells takes place. The microbial products play a central role in modulating DC function and influencing these different immune outcomes (Zhang *et al.* 2006). The LP and MLN

contain unusual subsets of DCs, which have unique functional properties that are different from those DCs found in the spleen. For example $CD103^{+}CD11c^{+}CD11b^{+}$ DCs promote T cell expression of the gut homing receptors, CCR9 and α4β7, by producing retinoic acid while another subset of DCs ($CX_3CR1^{+}CD11c^{low}CD11b^{+}$) were shown to promote the generation of regulatory T (Treg) cells through the production of retinoic acid and TGF-β in the colon (Kelsall and Strober 1997; Williamson *et al.* 2002; Svensson *et al.* 2008). Besides the antigen presenting functions and the maintenance of the local Treg cell pool, LP DCs also are able to promote colonic Th17 cell generation following activation by commensal microorganisms (Atarashi *et al.* 2008), can mediate IL-23-dependent granuloma formation as well as the chronic intestinal inflammation observed in CrD patients (Mizoguchi *et al*. 2007; Krajina *et al*. 2003; Mowat 2003).

1.1.3 Adaptive Immunity in the Colon

Adaptive immunity is the immune response of antigen–specific lymphocytes to an antigen and the development of an immunological memory, which can increase the immune response with a subsequent exposure to the same pathogen and act as the second wave of mucosal immunity. Lymphocytes are the primary cell involved in the adaptive immune response and can be subcategorised into T lymphocytes (T-cells) and B lymphocytes (B cells). In the intestine, T lymphocytes are located within the LP and the epithelial monolayer. T lymphocytes consist of two major subclass based on their expression of the CD markers. The CD4 cells are commonly known as T helper (Th) cells owing to the "help" they provide for B cells to trigger antibody production. The CD8 cells are known as cytotoxic T cells due to the high expression of macrophages–activating IFN-γ and granzymes. Both groups of cells are only able to recognise antigen bound to self- MHC proteins. The CD4 cells recognise peptides bound on to MHC class

II molecules and the CD8 cytotoxic T cells recognise peptides bound to the MHC class I molecules (Murphy 2008).

1.1.3.1 CD4 T cells Subclasses

The development and differentiation of naïve CD4 T cells or Th cells is under the control of transcription factors such as T box that is expressed in T cells (T-bet) and the signal transducer and activators of transcription (STAT)-4, -6, GATA-binding protein-3 and retinoic-acid-receptor-related orphan receptor (ROR)-γt as well as a range of cytokines. The pattern of cytokines that are secreted have been used to define the different types of CD4+ Th cells found both in the mouse and human (Brand 2009; Mosmann *et al*. 1986; Rotteveel *et al.* 1988) (***Figure 1.2***). Depending on which cytokines are produced, different immune responses can be generated (Agnello *et al.* 2003; Brand 2009).

IL-12 activates the STAT4 signaling pathway, which is necessary for optimal differentiation of naïve T cells into IFN-γ producing Th1 cells. IFN-γ then activates the STAT1 pathway and subsequently T-bet expression (Kaplan *et al.* 1996). Th1 cells cannot be generated *in vitro* or *in vivo* without expression of T-bet, and over expression of T-bet is able to override and suppress Th2 signal and drive T cells to become Th1 cells (Szabo *et al*. 2002). T-bet activation is also required for optimal IL-12 receptor expression and IL-12 responsiveness in Th1 cells (Lugo-Villarino *et al*. 2003). Th1 cells primarily produce IFN-γ, which is important for macrophages activation, and IL-12 that is crucial in directing the cytotoxic CD8 T cells responses. In combined these provide protection against the presence of intracellular pathogens such as viruses and

intracellular bacteria. Aberrant Th1 cell responses, however, are thought to be involved in autoimmune diseases and chronic inflammation as in IBD.

IL-4 stimulation leads to activation of the STAT-6 pathways that are required for the expression of the Th2 regulator transcription factor, GATA-3. GATA-3, together with other proteins, drives T cells towards a Th-2 phenotype. GATA-3 further drives the expression of IL-4, creating a positive feedback loop and inducing expression of further cytokines such as IL-4, IL-5 and IL-13. These cytokines are primarily involved in allergic type reactions and the elimination of extracellular pathogens (Zhu *et al*. 2002; Shimoda *et al*. 1996; Heller *et al*. 2005). IL-13 produced by T cells, and natural killer cells, increases gut epithelial cell turnover during infection in order to enhance repair of any damage (Murphy 2008).

It has been postulated that the inflammation that occurs in CrD is primarily mediated through Th1 cells, while the inflammation of UC is driven by Th2 cells. In support of this paradigm, active intestinal inflammation in CrD is associated with high levels of the Th1-polarising cytokine IL-12 when compared to the healthy controls and patients with UC (Monteleone *et al.* 1997). This concept, however, has been challenged. Both human and animals studies show that both the Th1 and Th2 adaptive immune responses are involved in CrD pathogenesis, and at distinct stages of the disease. The initiation of the CrD-like intestinal inflammation in the SMAP1/YitFc mouse model of colitis is indeed Th1 mediated and requires the expression of IFN-γ and TNF-α. The chronic stage of the inflammation in these mice, however, is driven by IL-5 and IL-13, the Th2-polarising cytokines (Bamias *et al*. 2005). In addition, in children with CrD, intestinal mucosal T cells initially demonstrate a strong Th1 response in the acute stages of the disease,

which is characterized by the high levels of IFN-γ. This response, however, is noted to diminish over time with more prolonged inflammation (Kugathasan *et al.* 2007). The expression of IL-4 and IL-5 has also been shown to be involved in human CrD pathogenesis (Desreumaux *et al.* 1997) and this is further complicated by the recent discovery of the Th17 cell that appears to play a major role in both autoimmunity and the pathogenesis of inflammation in CrD. Collectively, the strict Th1/Th2 paradigm no longer appears to adequately explain the IBD pathogenesis.

Development of the Th17 cell can be divided into three stages, differentiation, amplification and stabilisation. Naïve T cells can differentiate into Th17 cells in the presence of TGF-β, which is able to drive the Smad signaling pathway, and IL-6 that activates the STAT3 pathway. STAT3 transcription factor is then involved in the expression of the ROR-γt and ROR-α, which induce IL-17A expression in T cells. IL-21 is essential for the amplification of Th17 cell numbers. IL-21, which is induced by IL-6 through STAT3 activation independently of ROR-γt, leads to activation of ROR-γt that induces further expression of IL-17A (Nurieva *et al*. 2007).

The naive T cell is not responsive to IL-23 demonstrating that IL-23 is not required for the differentiation of naïve T cells into Th17 cells. IL-23 is, however, important for the expansion of these cells (Veldhoen *et al*. 2008). TGF-β upregulates IL-23R expression and promotes T cell responsiveness to IL-23 (Zhou *et al*. 2008; Mangan *et al.* 2006). Th17 T cells are then able to produce IL-17A, IL-17F, IL-21, and IL-22, which enhance neutrophil trafficking to the site of infection and expression of colony stimulating factors that link the innate with the adaptive immunity (Stockinger and Veldhoen 2007). Th17 cells are associated with the inflammation in IBD where the Th17 cytokines listed

above are upregulated in IBD patients and are present at higher levels in the inflamed colon tissue and serum when compared to healthy controls (Fujino *et al.* 2003; Nielsen *et al.* 2003; Seiderer *et al.* 2008). IL-17 also stimulates IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) secretion via NF-κB and MAP kinase activation in the intestinal subepithelial myofibroblasts where these cytokines are shown to contribute to intestinal inflammation (Hata *et al.* 2002).

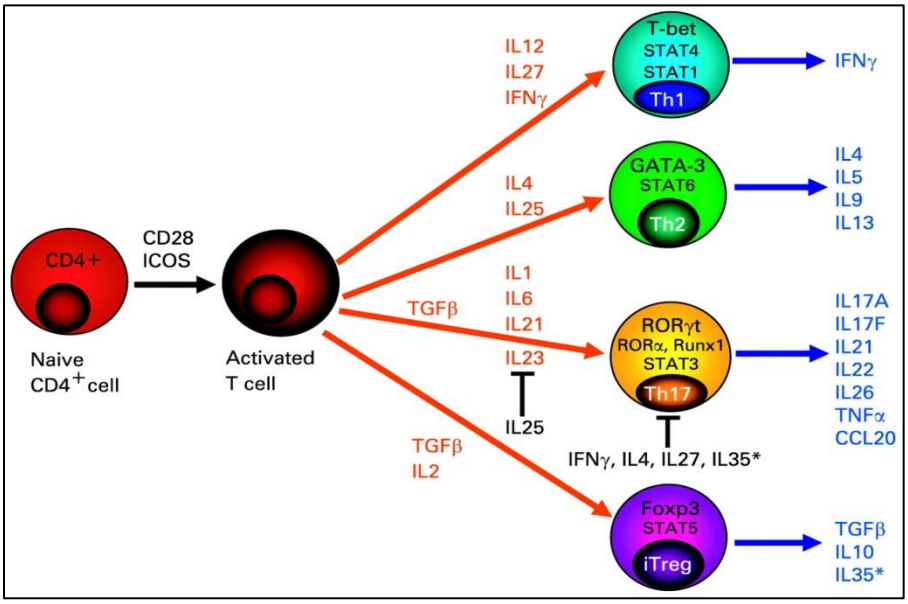


Image adapted from (Brand 2009)

Figure 1.2 Development of Th1, Th2, Th17 and iTreg cells from naïve CD4+ T cells. An APC (e.g. DC) provides factors derived from the innate immune response to naive T cells together with antigenic peptides in the context of MHC class II molecules to the TCR, which is associated with the CD3 protein and the costimulatory signals (CD28 and ICOS), that ultimately lead to polarized T cells responses. (Cytokines inducing the development of Th1, Th2, Th17 and iTreg cells are marked in red. Main effector cytokines of these four cell lines are marked in blue. Cytokines which inhibit the development of Th17 cells are marked in black.)

1.1.3.2 Regulatory T cells

Regulatory T (Treg) cells are vital for normal mucosal immune homeostasis. The gastrointestinal tract is frequently the first organ to suffer inflammation when the Treg

cells are dysregulated, or deficient, due to the constant antigenic stimulation that bathes the intestinal mucosa (Wan and Flavell 2008). They also appear to play a role in the inflammatory processes involved in IBD as reduced numbers of Treg cells have been observed in the blood and sera in IBD patients when compared to controls (Boden and Snapper 2008).

Treg cells are able to be defined by to the presence of a marker for the transcription factor forkhead box P3 (FoxP3). These cells also express other markers that include IL-2 receptor α chain (CD25) and cytotoxic T lymphocytes associated protein 4 (CTLA-4), which have been used in the past to identify the Treg cells. These markers, however, are not exclusive to the Treg cell. The differentiation of naïve T cells into Treg cells and the triggering of FoxP3 expression appears to be dependent on the TGF-β-Smad3 signaling pathway that is enhanced by the vitamin A metabolite retinoic acid (Mucida *et al*. 2007; Coombes *et al*. 2007). Treg cells secrete the two anti-inflammatory cytokines, IL-10 and TGF-β, which are important in both the control, and downregulation, of the effector T cells and innate immune cellular responses. IL-10 not only acts on innate immune cells in the intestine and also reduces Th cell proliferation. TGF-β has a similar effect on the differentiation of Th cells, but also inhibits the differentiation of naïve T cells into effector Th cells (Zheng *et al.* 2007; Zenewicz *et al.* 2009; Wan and Flavell 2008; Maloy *et al.* 2003).

Tregs also promote the differentiation of naïve T cells into the Th17 phenotype in a TGF-β-dependent manner. Naïve T cells can express both FoxP3 and ROR-γt when stimulated with TGF-β and depending on what other signals are received these cells can become either Treg or Th17 cells. High levels of TGF-β promote the expression of

FoxP3 and permit the generation of Treg cells, while low levels of TGF-β, or TGF-β in the presence of IL-6, induces IL-23R expression and mediates Th17 cell differentiation. Retinoic acid, which enhances Treg cell differentiation, however, can also inhibit the development of the Th17 cell (Mucida *et al*. 2007; Veldhoen *et al*. 2006; Zhou *et al*. 2008). IL-2, which promotes the development of Treg cells, can also inhibit the generation of Th17 cells, further supporting the reciprocal relationship between Treg and Th17 cells (Laurence *et al*. 2007).

1.1.4 Summary

In summary, if potential harmful pathogens breach the epithelial barrier of the gut, APCs such as DC, macrophages, and potentially fibroblasts (Parsonage *et al*. 2005), phagocytose or process the pathogens before presenting the antigen components to naïve CD4 T cells. The activated T cells then undergo differentiation into either effector (Th1, Th2 or Th17) or regulatory cells. The effector T cells can then mount a specific immune response through the expression of unique cytokines and aid in B cell activation. Once the pathogen has been cleared, the Treg cells help in the suppression of inflammation in order to restore the homeostasis of the GIT. Together, both innate and adaptive immune systems work synergetically to maintain homeostasis within the vulnerable gut microenvironment.

1.2 WOUND HEALING AND TISSUE FIBROSIS

As mentioned earlier, fibrosis resulting in stricture formation that leads to intestinal obstruction and surgery is not an uncommon occurrence in IBD, especially in CrD. Fibrosis is the undesired end result of the wound repair process resulting from chronic

injury. When tissue injury occurs a signal from the damaged tissue i.e. epithelial cells, triggers the release of inflammatory mediators that initiate haemostasis (***Figure 1.3***). Thereafter, epithelial cells secrete growth factors and chemokines that stimulate the proliferation and recruitment of leukocytes that produce pro-fibrotic cytokines and include TGF-β, IL-13 and TNF-α (Wynn 2004; Theiss *et al*. 2005; Fiocchi *et al*. 2006; Fichtner-Feigl *et al*. 2006). Matrix metalloproteinases (MMPs) are produced by stimulated myofibroblasts, and epithelial cells, and are able to disrupt the ECM to allow efficient cellular recruitment to the site of injury. Neutrophils and macrophages remove the tissue debris and dead cells, and secrete the chemical signals that promote the recruitment and activation of T cells. The T cells have an important role in the production of IL-13, which is involved in the formation of granulation tissue (Seno *et al*. 2009).

Fibroblasts and subepithelial myofibroblasts are subsequently recruited and activated within the granulation tissue and these cells are the primary source of collagen synthesis and deposition. In the normal intestine, both fibroblasts and subepithelial myofibroblasts in the submucosa, intermuscle connective tissue of the muscularis propria, and serosa are the primary sources of collagen mRNA and protein expression (Pucilowska, McNaughton, *et al*. 2000). Subepithelial myofibroblasts are later involved in wound contraction that aids in the closure of the wound site following angiogenesis. Lastly, the epithelial cells divide and migrate over the basal membrane in order to regenerate the epithelium to complete the wound repair process without the formation of scar tissue.

When injury continues, however, as in the case of the chronic inflammation associated with CrD, fibrosis can develop. Fibrosis is characterised by excessive ECM deposition without complete regeneration of the injured tissue and results in the formation of a permanent fibrotic scar with disruption to the normal tissue architecture (Lund and Rigby 2008; Wynn 2004; Diegelmann and Evans 2004).

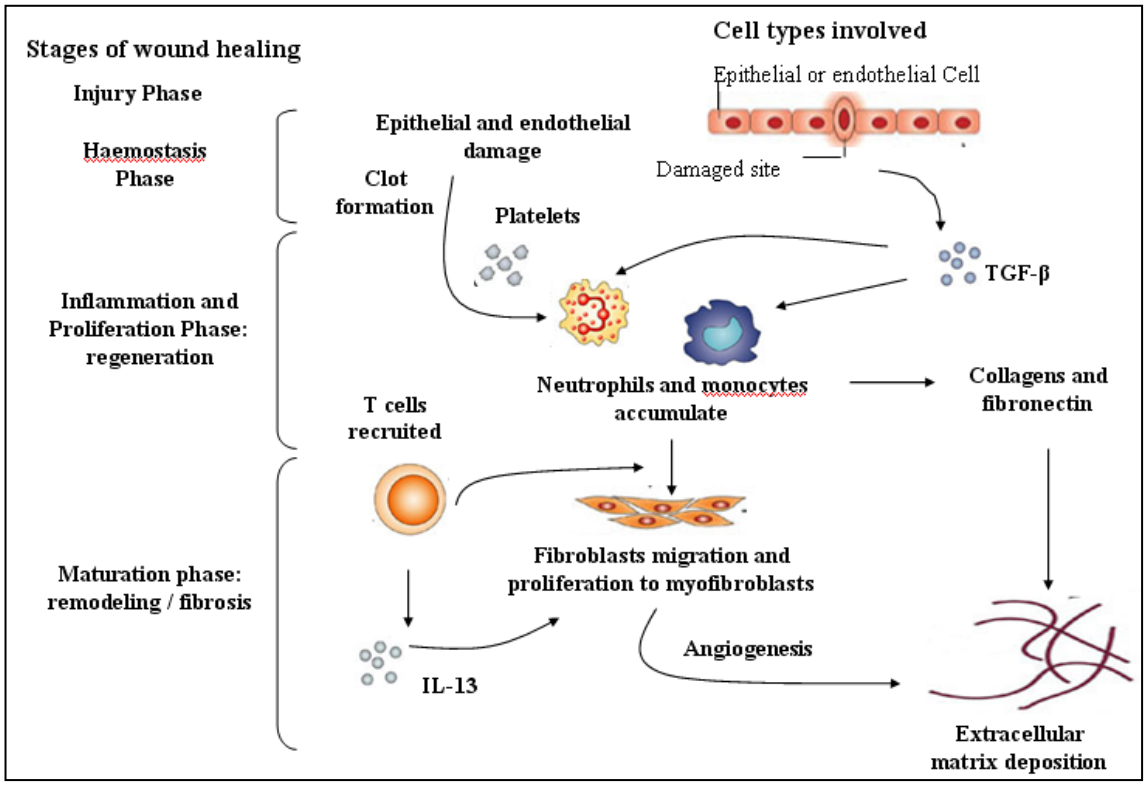


Image adapted from (Wynn 2004)

Figure1.3 The pathogenesis of fibrotic disease

Inflammation normally occurs prior to fibrosis formation, but several experimental models have demonstrated that the level of fibrosis is not necessary linked with the severity of inflammation. This suggests that the mechanisms of fibrogenesis may be different from those that regulate inflammation. The schistosomiasis–induced liver

fibrosis model has demonstrated that fibrosis can occur in the liver prior to the induction of chronic inflammation (Wynn *et al*. 1995) and mediated by a Th2 response that involves the secretion of IL-3, IL-4 and IL-13 (Kaviratne *et al*. 2004). This showed that CD4 T cells and the developing Th cellular responses are involved and play important roles in both the development of inflammation and fibrosis (Wynn *et al*. 1995; Hoffmann *et al*. 2000; Fichtner-Feigl *et al*. 2005).

1.3 SECRETED PROTEIN ACIDIC AND RICH IN CYSTIENE

Secreted Protein Acidic and Rich in Cystiene (SPARC), is also known as basement membrane 40 and osteonectin, and was first described as a major component in human and bovine bones. It is, however, expressed by a wide variety of cell types in adult vertebrates and is located primarily within the tissues that undergo consistent cellular turnover i.e. the bone and gut. It is also strongly expressed in fibroblasts, endothelial cells, epithelial cells and macrophages located within sites of injury (Reed *et al*. 1993; Puolakkainen *et al*. 1999; Latvala *et al*. 1996). SPARC has demonstrated numerous functions in the modulation of cell-matrix interactions and cell function without actually participating in the structural scaffold of ECM (Brekken and Sage 2000; Tai and Tang 2008). Due to many of its novel roles, SPARC has been directly, and indirectly, showed to be involved in the processes of both inflammation and fibrosis in many organs including the lung, liver, pancreas heart and adipose tissue. Its function within the colon is less well understood and is the focus of this thesis (Wang *et al*. 2010; Klopcic *et al*. 2008; Chang *et al*. 2010; Kos *et al*. 2009; Pershouse *et al*. 2009; Reding *et al*. 2009; Savani *et al*. 2000).

1.3.1 Structure and general functions

SPARC is a 32 kD protein after the cleavage of the signal sequence, and is secreted in a form that migrates at a molecular weight of 43 kD on a SDS-PAGE gel. The sequence of the SPARC gene, and the protein it encodes, are highly conserved among species (Yan and Sage 1999) with the structure of the human protein consisting of 286 residues organized in three domains (***Figure 1.4***). *In vitro* studies have shown that SPARC can be proteolytically cleaved into peptides with functions that are different from the native protein (Phan *et al.* 2007; Tai and Tang 2008; Kos and Wilding 2010; Bradshaw and Sage 2001; Brekken and Sage 2000). The NH_2 terminal domain is an acidic region that contains the major immunological epitopes of the protein. Peptide 1.1, a sequence of this domain, has been shown to promote cellular rounding, induces de-adhesion in endothelial cells and fibroblasts, a process that involves loosening of the structural links between the cytoskeleton and the ECM, and can inhibit the migration of cells (Lane and Sage 1990; Hasselaar and Sage 1992).

The second domain is a Cys-rich, follistain-like domain which binds activin, inhibin and proteoglycans. It contains a sequence named peptide 2.1 located at the NH_2- terminal end of the domain that is involved in, de-adhesion and inhibition of endothelial cell proliferation (Murphy-Ullrich *et al*. 1995; Funk and Sage 1993, 1991). It also contains two copper binding sites one of which, with the sequence KGHK, stimulates cell proliferation of endothelial cells and fibroblasts, and angiogenesis (Funk and Sage 1993).

The third domain, the C-terminal extracellular domain, contains two EF-hand motifs that bind calcium with high affinity. It contains a sequence known as peptide 4.2 that interacts with endothelial cells and binds fibril-forming collagens (Tremble *et al*. 1993; Sasaki *et al*. 1998; Lane and Sage 1990).

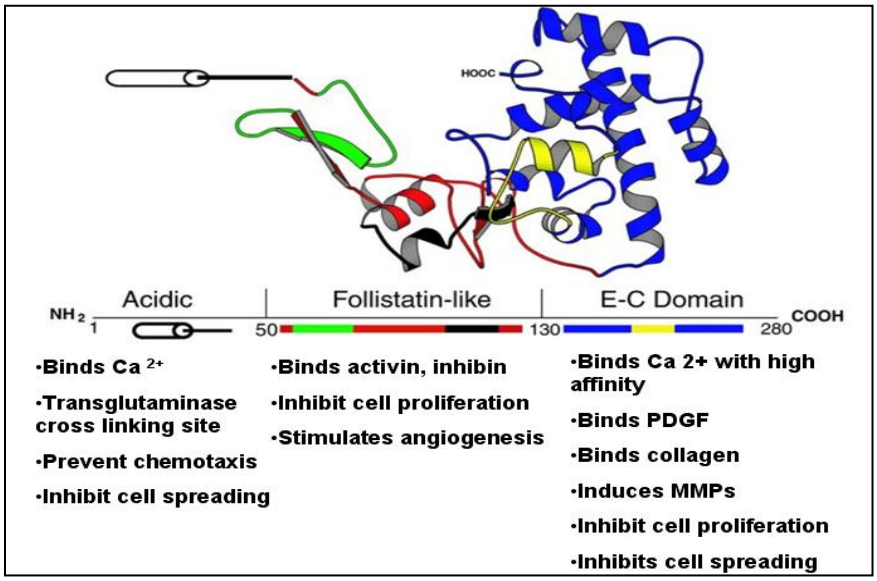


Image adapted and modified from (Bradshaw and Sage 2001; Kos *et al*. 2009) .

Figure 1.4 Structure of the SPARC protein. A ribbon diagram derived from crystallographic data shows the three modular domains of SPARC. Representative activities attributed to each domain are shown beneath the designated amino acids. (The follistatin-like domain contains the peptide 2.1 shown in green; The (K)GHK angiogenic peptide (amino acids 114–130) shown in black; The E-C domain contains the amino acids 255–274 (peptide 4.2) shown in yellow.)

From its structure, SPARC appears to be an inhibitor of the cell cycle and is an anti-adhesion molecule, which causes cellular rounding. *In vitro* studies have also demonstrated that SPARC is able to regulate cellular differentiation, inactivate cellular response to certain growth factors and is also able to modulate cellular proliferation and migration. SPARC interacts with the ECM components and to induce MMP expression and has a crucial role in cell-matrix/cell-cell involved events like tissue renewal, remodeling, and embryonic development. Owing to these properties, SPARC is

considered a regulatory protein of ECM reorganisation, wound healing-fibrosis, angiogenesis, inflammation and even tumorigenesis (Raines *et al.* 1992; Bassuk *et al.* 1999; Hasselaar *et al.* 1991; Kupprion *et al.* 1998; Tai and Tang 2008; Brekken and Sage 2000; Bradshaw and Sage 2001).

1.3.2 SPARC and ECM

The ECM not only provides a structural framework for the support of cells, but also acts as a reservoir for embedded cytokines and growth factors. The ECM harbours cryptic information within the molecules that make up the ECM network. The ECM is composed of a complex mixture of insoluble structural macromolecules, including collagen, laminin, fibronectin, and proteoglycans that are produced locally by fibroblasts, epithelial, smooth muscle and endothelial cells. Besides that, matricellular proteins that consist of thrombospondin 1, 2, tenascin and SPARC, are also transiently secreted into the ECM but do not become part of the ECM mesh. They are not detected within the established normal ECM but are abundantly expressed at sites of ECM turnover and can act to modulate cell-cell/cell-matrix interactions (Podhajcer *et al.* 2008; Simon-Assmann *et al.* 1995; Aumailley and Gayraud 1998; Phan *et al.* 2007). SPARC knockout (KO) mice are characterised with decreased bone turnover, early cataract development, altered collagen content in the lens and collagen fibril diameter in the dermis and greater size of subcutaneous adipose tissue pads when compared to control animals. This highlights the essential interaction of SPARC with the ECM in multiple tissues (Kos and Wilding 2010; Chlenski and Cohn 2010).

ECM components such as the fibril-forming collagen I, III, V, and basement membrane collagen IV, vitronectin, laminin are able to bind to SPARC (Brekken and Sage 2000). Collagen may serve as a storage site for SPARC in the ECM or may directly modulate the de-adhesive and anti-proliferative activity of SPARC (Sasaki *et al*. 1998). SPARC has been shown to increase MMP production and activity (Tremble *et al*. 1993), which play important roles in healing and fibrosis and will be discussed further. The affinity of collagen binding to SPARC is increased when a single peptide bond located in the SPARC, within the third C-terminus extracellular domain, is cleaved by several different MMPs including MMP-2, -3, -7, and -13. Collectively, these observations suggest that SPARC acts in both an autocrine, or paracrine, positive feedback loop such that SPARC stimulates the expression of MMP-2, which is then able to proteolytically cleave SPARC, that in turn increases the affinity of SPARC for collagen (Brekken and Sage 2000). This interaction between collagen and SPARC may also result in further remodeling of the ECM. This was further demonstrated in the *Mov*-13 mice, which carry a mutation in the collagen 1α1 gene, and do not express collagen I in most tissues. These mice have impaired deposition of SPARC within the ECM and die *in utero* (Raines *et al*. 1992).

Besides collagen, vitronectin in the ECM interacts and binds to SPARC *in vivo*, which is also demonstrated by the colocalisation of both proteins in blood vessel walls of a variety of tissues. This binding, however, can be inhibited by type-1 plasminogen activator inhibitor (PAI-1), inhibitor of both urokinase type plasminogen activator (uPA) and tissue type plasminogen activator (tPA). Plasma vitronectin normally will not bind to SPARC, but it can be converted to a multimeric SPARC binding form by PAI-1 (Rosenblatt *et al.* 1997). Thus, PAI-1 can affect and interfere with the binding of

vitronectin to SPARC, due to the SPARC stimulated production of PAI-1 by endothelial cells. This can act as another potential feedback loop for the regulation of SPARC function and its association with the ECM (Lane and Sage 1994).

Finally, SPARC has been shown to enhance fibronectin–induced assembly of ECM via the intergrin-linked kinase (Barker *et al*. 2005). All these interactions suggest that SPARC participates in the regulation of tissue remodeling and repair where it is necessary to facilitate production of an ECM permissive for cell migration, proliferation, and differentiation (Bradshaw and Sage 2001).

1.3.3 SPARC and other proteins

Binding and interacting with other proteins and cytokines is the other major characteristic of SPARC. SPARC was shown to bind the platelet derived growth factor (PDGF) dimers AB and BB and vascular endothelial growth factor (VEGF), which has 20% homology to PDGF. Both PDGF and VEGF lose their mitogenic potency after binding to SPARC as SPARC prevents the binding of PDGF to its receptors on fibroblasts thus affecting the biological activity of PDGF. SPARC also decreases the binding of VEGF to the endothelial cells and reduces the association of VEGF with its cell surface receptor Flt-1, which in turn inhibits endothelial cell proliferation induced by VEGF. In addition, SPARC has been shown to interact with and inhibit the proliferative effect of basic fibroblast growth factor on smooth muscle cells (Bradshaw and Sage 2001; Yan and Sage 1999).

1.3.3.1 SPARC and TGF-β

TGF-β is a homodimeric 25kDa protein. It is secreted and maintained in a latent complex where the cytokine non-covalently interacts with a latency-associated peptide (LAP). The latent TGF-β complex is sequestered within the ECM by a covalent attachment between LAP and a latent TGF-β binding protein (LTBP). Both the LAP and LTBP need to be proteolytically removed before the mature TGF-β protein can function. Latent TGF-β can be activated by a number of physical processes including heat, acid, reactive oxygen species, and biological processes such as proteolysis or integrin mediated activation. A number of proteases including plasmin, thrombin, MMP-2 and MMP-9 have been shown to be capable of directly activating latent TGF-β *in vitro* (Annes *et al.* 2003; Border and Noble 1994; Jenkins 2008).

SPARC can induce MMP-2 and MMP-9 (Tremble *et al.* 1993; Kato *et al.* 2001), which can cleave the LAP releasing active TGF-β (Ge and Greenspan 2006; Yu and Stamenkovic 2000). Both SPARC and TGF-β increases the production of PAI-1, which inhibits the formation of plasmin that is also known to be an activator of latent TGF-β1. Recently, SPARC has been showed to regulate the basal expression of PAI-1 in lung fibroblasts through the β catenin pathway (Chang *et al*. 2010). Reduction of plasmin not only down regulates the expression of TGF-β and also reduces the protease activity against SPARC, thus prolonging its activity in tissue (Hasselaar *et al*. 1991). SPARC has been shown to inhibit the production of thrombospondin-1 which is a major activator of TGF-β1 in the endothelial cells (Murphy-Ullrich and Poczatek 2000; Lane *et al.* 1992). SPARC is also able to interact with TGF-β through the TGF-β/TGF-βRII complex and increase the downstream signaling of the SMAD-2 pathway. Incubation of

epithelial and endothelial cells with SPARC induces SMAD-2 phosphorylation and increased expression of the TGF-β resposive genes (Francki *et al*. 2004).

SPARC has the ability to modulate the activity of TGF-β and vice versa with TGF-β1 reported to regulate the expression of SPARC in fibroblasts (Lane and Sage 1994). Mesangial cells isolated from SPARC null mice demonstrate a diminished expression of TGF-β1 that can be restored to WT levels by the exogenous application of SPARC (Francki *et al.* 1999). From the above, SPARC appears to have a positive correlation with TGF-β but the exact mechanism underlies the interaction of SPARC and TGF-β remain unknown to date, but modulation of SPARC/TGF-β pathway might represent a potential target to restore the mucosal immunity as TGF-β is a potent anti-inflammatory cytokine.

1.3.4 SPARC in wound healing and fibrosis

Wound repair is a complex, dynamic process in which various cell populations interact in different ways. Immunohistochemical and *in situ* hybridization techniques have demonstrated that SPARC is expressed spatially and temporally in injured tissue, implying that SPARC is involved in repairing dermal wounds (Reed *et al*. 1993), the healing of intestinal anastomosis and in short bowel syndrome (Puolakkainen *et al*. 1999), wounded cornea (Seet *et al.* 2010; Latvala *et al*. 1996) and ischemic myocardium (Porter *et al.* 1995). Fibroblasts and macrophages start expressing SPARC in the healing wounds where it is also released by platelet degranulation (Reed *et al*. 1993). SPARC in turn induces MMP-1, MMP-3, MMP-9 expression by fibroblasts which are required in the wound healing processes (Tremble *et al*. 1993).

Besides the capacity of SPARC to interact with the ECM, and various growth factors (e.g. PDGF, TGF-β and etc), SPARC also plays a vital role in angiogenesis that is important in wound healing process. SPARC is expressed in the newly synthesised blood vessels from a variety of tissues (Reed *et al*. 1993). SPARC is also able to interact with vascular ECM and modulate the proliferation and migration of the endothelial cells. *In vitro,* intact SPARC protein has an anti-angiogenic activity and inhibits endothelial cell proliferation, however, the KGHK motif on the second domain is a strong inducer of angiogenesis and stimulates endothelial cell proliferation both *in vitro* and *in vivo* (Lane *et al*. 1994).

SPARC KO mice have an enhanced rate in wound closure and this might be linked with the withdrawal of the inhibitory effect of SPARC on cellular proliferation or with the increase in subdermal fat in the KO animals. As many cells that infiltrate into the wound bed to repair the injured tissue have been shown to originate from the subdermal fat and muscle layer (Kurman and Argyris 1975), it been speculated that if there is an increase in fat there may also be an increase in the availability of cells that contributed to wound closure thus accelerating wound healing (Bradshaw and Sage 2001).

There is a strong association between SPARC and tissue scarring and fibrosis. There are, however, contradictory reports on its role in the development of fibrosis as demonstrated in two bleomycin-induced pulmonary fibrosis murine models. In one SPARC null mice were seen to develop a more prominent fibrotic response than the wild type controls with enhanced collagen deposition and greater alveolar architectural destruction (Savani *et al*. 2000). This suggested that SPARC was anti-fibrogenic. In the second "bleomycin-induced pulmonary fibrosis" model, Strandjord *et al*. however,

showed that SPARC KO mice's lung had decreased collagen accumulation (Strandjord *et al*. 1999) and this finding was supported by recent work that demonstrated that the inhibition of SPARC with siRNA decreased the expression of type I collagen and attenuated the pro-fibrotic effect of TGF-β1 in cultured normal human fibroblasts (Zhou *et al.* 2005) and murine skin fibroblasts (Wang *et al*. 2010).

It is thus unclear whether SPARC plays an anti- or pro-fibrogenic role in intestinal inflammation. As mentioned previously, SPARC can down-regulate the expression of ECM components such as fibronectin and laminin (Raines *et al.* 1992; Lane and Sage 1994) and increases ECM degradation by MMPs. It has been showed that SPARC can induce cyclooxygenase (COX)-2 mediated prostaglandin (PG)-E_2 synthesis, which then increases MMP-1 and -9 expression by macrophages and fibroblasts (Shankavaram *et al*. 1997; Tremble *et al.* 1993). On the other hand, SPARC also can reduce PGE_2 levels which are linked with the development of intestinal fibrosis in the rat (Elson *et al.* 1995; Shankavaram *et al.* 1997). Furthermore, SPARC interacts with other growth factors in ECM which may enhance its anti-fibrogenic role as PDGF-BB and AB bind to SPARC and are prevented from binding to their fibroblast receptors when they bind to SPARC (Brekken and Sage 2000), thereby potentially inhibiting the induction of interstitial collagen secretion and the mitogenic effect of fibroblasts, smooth muscle cells, and myofibroblasts resulting in diminished ECM deposition (Bradshaw and Sage 2001).

The complex interaction of SPARC with the pro-fibrogenic TGF-β further complicates our understanding of SPARC's role in fibrosis. As TGF-β is a pleiotropic cytokine that is ubiquitously expressed by all cells and tissues within the body and besides its crucial

role in maintaining homeostasis in the gut as mentioned earlier, it is also implicated in the pathogenesis of fibrosis (Border *et al.* 1992; Sato *et al.* 2003). Alveolar macrophages in the bleomycin model of pulmonary fibrosis are thought to produce the active TGF-β that is involved in the pathological matrix remodelling process (Khalil *et al*. 1996). TGF-β is also able to pair with IL-13 to causes fibrosis, IL-13 can activate TGF-β by upregulating the expression of MMPs that can cleave the active TGF-β from its inactive, latent form and stimulate macrophages production of TGF-β1 that in turn activates the fibroblasts and enhances collagen deposition (Lee *et al*. 2001). Hence, the roles that SPARC plays in intestinal wound healing and fibrosis require further investigation.

1.3.5 SPARC in inflammation

As in fibrosis, SPARC's role in inflammation and the recruitment of inflammatory cells can vary in different organs. In the bleomycin-induced pulmonary fibrosis model, SPARC KO mice exhibited increased leucocytes infiltration and a greater fibrotic response (Savani *et al*. 2000), by contrast, decreased recruitment of inflammatory cells and reduced liver fibrosis was observed in the thioacetamide-liver fibrosis model with enforced down regulation of SPARC (Camino *et al*. 2008). It has been suggested that SPARC KO animals have an impaired immune response with decreased numbers of neutrophils and B cells but increased numbers of T cells, and are unable to mount an innate immune response to the bacterial product LPS in the mouse footpad swelling model (Rempel *et al*. 2007). These differences in the recruitment of inflammatory cells may also be a result of anomalies in the collagen content and the collagen bundle size in SPARC KO mice that make the ECM more "permeable" than their WT counterparts. This may allow the infiltrating inflammatory cells to migrate more freely (Llera *et al*.

2010), an explanation that has been shown to be valid in the contact hypersensitivity model in the SPARC KO mice where DC migration was increased *in vivo*. This accelerated the onset of T cell priming with greater numbers of epidermal Langerhans cells migrating to draining lymph nodes (Sangaletti *et al*. 2005). SPARC was shown to be pro-inflammatory in the kidney as significantly less recruitment of macrophages in response to angiotensin II was found in the tubulointestinal space of SPARC KO mice when compared to the WT (Socha *et al.* 2007). SPARC, therefore, is possibly pro-inflammatory in the GIT as increased SPARC expression levels were found in inflamed, non fibrosed colonic surgical UC specimens (Lawrance, Fiocchi, *et al.* 2001).

The pro-inflammatory cytokines TNF-α, IL-1β and IL-6, are crucial in the amplification of the mucosal inflammation observed in IBD. TNF-α and IL-1β are primarily secreted by monocytes, macrophages, activated intestinal macrophages, neutrophils, fibroblasts, and SMC, inducing the production of prostaglandins, proteases and other mediators of inflammation and tissue injury (Bosani *et al.* 2009). The mRNA expression of these cytokines is increased in the inflamed intestinal mucosa of IBD patients, with IL-6 noted to be particularly high (Raddatz *et al.* 2005). SPARC has demonstrated an inhibitory effect on lysophosphatidic acid-induced proliferation and survival in mesothelial-ovarian cancer cells by the downregulation of IL-6 production (Said, Najwer, *et al.* 2007). SPARC might play a regulatory role on the expression of these pro-inflammatory cytokines due to its capacity to modulate the activities of the cells that produce the cytokines.

1.3.6 SPARC in tumorigenesis

There is clear evidence that chronic inflammation is linked the development of cancer as it has been observed that the inflammatory cells recruited to an area of tissue remodeling will secrete proteases and angiogenic mediators that favour tumour development (Coussens and Werb 1996). Inflammatory cells, however, can also attack tumour cells (Podhajcer *et al.* 2008). Reduced tumour growth has been observed in SPARC KO mice that have been injected with mammary tumour cells compared to the wild type controls, where there were also greater numbers of infiltrating inflammatory cells which were primarily polymorphonuclear leucocytes (PMN)s and macrophages (Sangaletti *et al*. 2003). Melanoma cells lacking SPARC expression can also induce PMN recruitment and tumour cell rejection via a mechanism that involves the increased release of chemotactic factors for inflammatory cells such as IL-8 (Alvarez *et al*. 2005). In a recent murine model of pancreatic carcinoma, however, macrophage recruitment and polarisation to the M2 phenotype was increased and this resulted in accelerated tumour progression and enhanced metastasis in the SPARC KO mice (Arnold *et al*. 2010).

Genome expression analysis of human colorectal cancer cell lines resistant to chemotherapeutic agent showed deceased levels of SPARC (Tai *et al.* 2005). The frequent inactivation of SPARC expression by promoter hypermethylation is related to rapid progression of colon cancers, immunohistochemicaal analysis of 292 primary colorectal cancers showed that SPARC expression in neoplastic cells improves 5 year survival rate (Yang *et al.* 2007). These studies suggested that SPARC has anti-tumorigenic role in colon cancer. So, a better understanding on the role of SPARC in intestinal inflammation could be helpful in our understanding of colon carcinogenesis.

1.4 ANIMAL MODELS OF INTESTINAL INFLAMMATION

Animal models of intestinal inflammation have been proven to be extremely useful tools in the study of the mechanisms involved in IBD and can be divided into 3 categories depending on the cellular defect involved. 1) Models that depend on a defect in the epithelial barrier function (e.g. DSS, TNBS/oxazalone induced colitis); 2) Models with a defect in the innate immune system (e.g. mice with STAT3 deficiency in myeloid cells); and 3) Models with a defect in the adaptive immune system (e.g. $CD45RB^{Hi}$ transfer model) where mutant mice which lack certain genes, are required (Heinsbroek and Gordon 2009; Wirtz and Neurath 2007). The disadvantage of using animal models is that none of the models are able to represent all aspect of the human disease. Choosing an appropriate animal model, however, can still be of use in the investigation of specific pathophysiological mechanisms and the testing of new or emerging therapeutic agents. Of the models, the barrier disruption colitis models with chemical such as DSS and trinitrobenzenesulfonic acid (TNBS) are the most widely used due to the low cost and ease to perform. They also exhibited many of the immunological and histopathological features seen in human IBD (Wirtz *et al*. 2007).

Feeding mice for several days with DSS polymers in the drinking water induces a very reproducible acute colitis characterised by bloody diarrhea, ulcerations and infiltrations with granulocytes (Wirtz *et al*. 2007). Most *in vivo* and *in vitro* studies on the DSS-induced colitis model suggest that DSS interferes directly with the intestinal epithelial cell barrier as the primary event that then leads to a mucosal inflammation (Wirtz and Neurath 2007; Yan *et al*. 2009). The acute DSS colitis model is very useful in the investigation of the contribution of the innate immune mechanisms of colitis. The

adaptive immune system does not appear to be involved in the acute phase of the inflammation in this model as both T and B cell deficient mice are also able to develop severe colitis (Dieleman *et al*. 1998). Feeding susceptible mouse strains (e.g. C57BL/6 and Swiss Webster mice) with several cycles of DSS, however, can result in chronic inflammation that is characterised by marked infiltration of mononuclear cells (Melgar *et al.* 2005; Mahler *et al.* 1998; Wirtz and Neurath 2007). In both the acute and chronic DSS-induced colitis models, DSS is able to induce the secretion of large amount of pro-inflammatory cytokines like IL-6 and TNF-α which caused tissue damage (Strober *et al.* 2002). The DSS-induced model of colitis was used in our studies to investigate the effect that SPARC can play on both the innate and adaptive immunity associated with intestinal inflammation.

1.5 AIMS AND OBJECTIVES OF THE PROJECT

The IBD are chronic incurable conditions and not all patients respond to current therapies. The development of alternate therapeutic strategies is thus a priority. SPARC is involved in many of the cellular processes associated with inflammation, healing and fibrosis and may be a possible therapeutic target in the management of IBD. A greater understanding of the mechanisms through which SPARC is involved in inflammation is required.

SPARC is expressed at a low level in uninflamed adult colon, but it is expressed at the mesenchymal-epithelial border and in the smooth muscle layer in normal foetal intestine and also at the sites of inflammation and fibrosis in the adult GIT. SPARC can influence the infiltration of innate immune cells such as neutrophils and macrophages, into sites of inflammation but its overall role is unclear. SPARC might also be able to affect the adaptive immunity by influencing the production of various cytokines that promote the differentiation of T cells either into effector T cells such as Th17 T cells, or into Treg cells that suppress the immunological responses. Both SPARC and TGF-β have regulatory roles on cell proliferation, differentiation, inflammation and wound healing and they also modulate the activities of each other. Lastly, the capacity of SPARC to modify the activities of fibroblasts/myofibroblasts in ECM turnover also makes SPARC a potential target to improve healing and in the management of fibrotic diseases. Both human and animal studies, however, suggest that the roles of SPARC in inflammation, fibrosis as well as on tumorigenesis, appear to depend on diverse functions that may be specific to a given microenvironment. The studies described in this thesis, therefore, were undertaken to investigate possible effects of SPARC on the *in vivo* and in *vitro* development of intestinal inflammation, healing and fibrosis.

The specific hypothesis:

SPARC induces intestinal inflammation due to its regulatory role on the immune system and ECM modulation with consequences on both tissue damage and healing.

General aim:

To analyse the role of SPARC on intestinal inflammation in the murine model of DSS-induced colitis.

The specific research aims:

1) **To characterise and compare the effect of SPARC on intestinal inflammation in the murine model of DDS-induced colitis.**
    - **a.** To characterise the inflammatory symptoms and involvement of SPARC in DSS induced colitis.
2) **To determine differences in the immune cellular infiltrates during intestinal inflammation in the presence and absence of SPARC.**
    - **a.** To establish which innate immune cells infiltrate to colon during intestinal inflammation.
    - **b.** To establish which adaptive T cells infiltrate to colon during intestinal inflammation.
3) **To determine the roles of SPARC in ECM expression *in vivo* and *in vitro***
    - **a.** To determine the collagen content *in vivo* in the DSS induced colitis.
    - **b.** To investigate which the expression ECM genes are modulated by the presence or absence of SPARC *in vitro*.

Chapter **2**

Materials and Methods

2.1 MATERIALS

All chemical reagents used in the experiments outlined in this thesis were of analytical or biochemical grade, unless otherwise indicated and were purchased from the following manufacturers:

2.1.1 General reagents and biochemicals

Ammonium chloride (NH_4Cl)	BDH Biochemicals (UK)
Anaesthetics (Ketamine; Xylazine)	Troy Laboratories (AUS)
Agarose	Sigma Aldrich (USA)
Bovine serum albumin (BSA)	Sigma Aldrich (USA)
Dextran sodium sulphate (DSS)	MP Biomedicals (AUS)
DePeX mounting medium, Gurr®	Merck Pty Ltd (AUS)
Dimethyl sulphoxide (DMSO)	Sigma Aldrich (USA)
Dithiothreitol (DTT)	Sigma Aldrich (USA)
EDTA	Merck Pty Ltd (AUS)
Ethanol (100%)	Sigma Aldrich (USA)
Ethiduim bromide	Merck Pty Ltd (AUS)
Goat serum	DAKO (Denmark)
Hydrogen chloride (HCl)	Sigma Aldrich (USA)
Hydrogen peroxidase 30%	BDH Biochemicals (UK)
Ionomycin calcium salt	Sigma Aldrich (USA)
Isopropanol	Sigma Aldrich (USA)
Magnesium chloride (MgCl)	Merck Pty Ltd (AUS)
PBS tablet	Amersham Biosci (UK)
Percoll ™	GE Healthcares (USA)
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich (USA)
Picric acid	Sigma Aldrich (USA)
Potassium chloride (KCl)	Merck Pty Ltd (AUS)
Potassium hydrogen carbonate ($KHCO_3$)	BDH Biochemicals (UK)
Sirius red	Merck Pty Ltd (AUS)
Sodium azide (NaN_3)	Sigma Aldrich (USA)
Sodium carbonate ($Na_2CO_{3)}$	Merck Pty Ltd (AUS)

Sodium chloride (NaCl)	Merck Pty Ltd (AUS)
Sodium hydroxide (NaOH)	Sigma Aldrich (USA)
Tris (Hydroxymethyl Methane)	BDH Biochemicals (UK)
Trypan blue	Sigma Aldrich (USA)
Tween-20	Sigma Aldrich (USA)
Water,"Ultrapure", DNase/RNase-free	Gibco® Invitrogen (USA)
Xylene	Sigma Aldrich (USA)

2.1.2 Tissue culture reagents and cell line

Dulbecco's modified eagle medium (DMEM)	Gibco® Invitrogen Co. (USA)
Foetal bovine serum (FBS)	Gibco® Invitrogen Co. (USA)
L-Glutamine	Gibco® Invitrogen Co. (USA)
Hank's balanced salt solution (HBSS)	Gibco® Invitrogen Co. (USA)
HEPES	Gibco® Invitrogen Co. (USA)
Penicillin /Streptomycin/Fungizone (PSF)	Gibco® Invitrogen Co. (USA)
RPMI 1640 Medium	Gibco® Invitrogen Co. (USA)
TrypLE Express™.	Gibco® Invitrogen Co.(USA)
A549 human epithelial cells	Gift from A/P. J.Allan, UWA, AUS

2.1.3 Enzymes

Collagenase IV	Worthington Biochemical (USA)
Dispase II	Roche diagnostics (Germany)
DNase I	Invitrogen Co. (USA)
Trypsin 250	Difco™, BD Biosciences (USA)

2.1.4 RNA studies and reverse transcription reagents

Bromochloropropane (BCP)	Sigma Aldrich (USA)
dNTP	Invitrogen Co. (USA)
GlycoBlue™ Co-precipitant	Ambion (USA)
OligodT_{15}	Promega (USA)
PCR reaction buffer, 10x	Invitrogen Co. (USA)
Magnesium chloride, 25mM	Invitrogen Co. (USA)
RNAsin ®	Promega (USA)
SuperScript™III First-stand synthesis SuperMix for qRT-PCR, 50 reactions	Invitrogen Co. (USA)
SYBR green I	Molecular Probes, Invitrogen Co. (USA)
Platinum Taq DNA polymerase	Invitrogen Co. (USA)
TRI Reagent®	Ambion (USA)

2.1.5 Antibodies

Immunofluorescence

Rat anti mouse CD11b	BD Pharmingen (USA)
Rat anti mouse CD68	abcam® (USA)
Rat anti mouse F4/80	AbD serotec (UK)
Rat anti mouse Ly6G	Biolegend® (USA)
Goat anti rat Alexa Fluor® 594 IgG	Molecular Probes, Invitrogen (USA)

Flow cytometry

Hamster anti mouse FITC CD3e	BD Pharmingen (USA)
Rat anti mouse FITC CD4	eBioscience (USA)
Rat anti mouse PERcp-Cy5.5CD4	BD Pharmingen (USA)
Rat anti mouse Allophycocyanin CD25	eBioscience (USA)
Rat anti mouse PE IFN γ	BD Pharmingen (USA)
Rat anti mouse Alexa Fluor® 488 IL-4	Biolegend® (USA)
Rat anti mouse Alexa Fluor® 647 IL-17A	Biolegend® (USA)
Rat anti mouse PE FoxP3	eBioscience (USA)

Immunohistochemistry

Monoclonal mouse anti human α-SMA	Chemicon, Millipore Corp (USA)
Monoclonal mouse anti desmin	Novacastra, Leica (Germany)
Polyclonal goat anti-human vimentin	Chemicon, Millipore Corp (USA)
Polyclonal rabbit anti-cow keratin	Dako (Germany)

2.1.6 Commercial kits

BD™ CBA Mouse Flex set (100 tests): IL-1β IL-4 IL-5 IL-6 IL-10 IL-13 IL-17A IFN-γ TNF-α MCP-1 MIG MIP-1α MIP-1β RANTES Mouse/Rat soluble protein master buffer kit (500 tests)	BD Biosciences (USA)
Biotin blocking system	DAKO (Denmark)
Cytofix/cytoperm™ fixation/permeabilisation kit with Golgistop™ (monensin)	BD Biosciences (USA)
DNase removal kit	Ambion (USA)
Liquid DAB substrate chromogen system	DAKO (Denmark)
LSAB® Streptavidin HRP	DAKO (Denmark)
Mouse regulatory T cell staining kit	eBioscience (USA)
ProLong® Gold antifade reagent with DAPI	Invitrogen Co. (USA)
Quantikine® mouse/rat/porcine/canine immunoassy for mouse TGF-β kit	R&D System (Aus)

2.1.7 Laboratory instruments, equipment and software

25 cm^2 vented tissue culture flasks	BD Biosciences (USA)
175 cm^2 vented tissue culture flasks	BD Biosciences (USA)
6 cm culture dishes	BD Biosciences (USA)
24 well microtitre plates	BD Biosciences (USA)
96 well microtitre plates	BD Biosciences (USA)
0.6, 1.5 & 2 ml MAXYMum recovery™ microfuge tubes	Axygen Scientific (USA)
15 & 50 ml Falcon tubes	BD Biosciences (USA)
19 G needles	Terumo Medical Corp (USA)
23 G needle	Terumo Medical Corp (USA)
10, 20, 50ml syringes	BD Biosciences (USA)
Aperio Scanscope Digital Slide Scanner	Aperio (USA)
Aperio's ImageScope Viewer-Spectrum positive pixel count algorithm software	Aperio (USA)
BD CBA array software	BD Biosciences (USA)
Benchtop centrifuge 5414 D (refrigerated)	Eppendorf (Germany)
Bio-Rad power packs	Bio-Rad Laboratories (USA
Cell scraper	Sarstedt AG & Co (Germany)
Cell strainer (40, 70, 100μm nylon)	BD Biosciences (USA)
CO2 incubator	Forma Scientific Inc (USA)
Disposable 0.2 μM bottle top filter	Nalgene Nunc International (USA)
Double deionised water system	Millipore Pty, Ltd (AUS)
DU 640 Spectrophotometer	Beckman Coulter (USA)
FACSCanto™ Flow Cytometer	BD Biosciences (USA)
FACSDIVA™ software	BD Biosciences (USA)
FlowJO 7.6 software	Treestar Inc (USA)
Fluostar Optima microtitre plate reader	BMG Laboratories (Aus-Pacific)
Freezing microtome cryostat: Leica Cryocut 1800	Leica Microsystems (Germany)
GraphPad Prism 5.0	GraphPad Software (USA)
J2-MI Centrifuge	Beckman Coulter (USA)
Megafuge 1.0R centrifuge	Heraeus Sepatech (Germany)
McIlwain tissue chopper	Mickle Laboratory Engineering (UK)
Microtome: Microm HM 325	GMI Inc (USA)

Millipore filter 0.22 µM	Millipore Corp (USA)
MultiScreen$_{HTS}$ vacuum manifold	Millipore Corp (USA)
MultiScreen$_{HTS}$ 96-well filter plates	Millipore Corp (USA)
Nanodrop 1000 spectrophotometer	Thermo Fisher Scientific (USA)
Nikon TE2000-E Microscope/camera	Coherent Life Science (AUS)
Rotor-Gene 3000 System	Corbett Research (USA)
Olympus IX-81 inverted fluorescence microscope	Olympus (Japan)
PTC-100 thermocycler	MJ research Inc (USA)
Rotor-Gene 6 software	Corbett Research (USA)
Sakura Tissue Tek VIP-E150 tissue processor	GMI Inc (USA)
Shandon Linistain™ GLX Linear Stainer	Thermo Scientific (AUS)
Superfrost ®plus slides	Menzel-Gläser (Germany)
VersaDOC 3000 Imaging System	Bio-Rad Laboratories (USA

2.1.8 Common solution and buffers

ACK lysis buffer	8.9g NH_4Cl, 1g KHCO3, 37.2mg EDTA in 1lt of double deionised water , and stored at 4 ℃.
Acetone/methanol fixative	Acetone-methanol mixture 1:1(v/v), and stored at -20°C.
BSA/PBS:	1mg/ml stock solution prepared in 1 x PBS, and stored at -20°C.
Digestion solution	Dissolve 0.05 g of collagenase IV, 0.05 g of DNase I and 0.3 g of dispase II in 100 ml of 1 × PBS.
FBS	Heat-inactivated by heating for 30 minutes at 56 ℃.
Flow buffer	PBS supplemented with 5% FBS, 0.01% NaN_3, filtered sterilised and stored at 4°C.
High serum Dulbecco's Modified Eagle Medium (H-DMEM):	10% foetal bovine serum (FBS), 0.025M HEPES buffer. 1X PSF, 2% L-glutamine in DMEM.
Low serum Dulbecco's Modified Eagle Medium (L-DMEM):	2% FBS, 0.025M HEPES buffer. 1X PSF, 2% L-glutamine in DMEM.
Percoll solution	9 parts percoll mixed with 1 part 10x PBS, sterile solution and stored at 4°C.
Phosphate buffered saline (PBS)	50mM KPO_4, 150mM NaCl, in deionised water, pH 7.2 (without adjustment). Alternatively, 1 PBS tablet (Amersham Biosci) to 100ml of deionised water (1x).
PMA	1mg/ml stock solution prepared in DMSO, and stored at -20°C.
Predigestion solution	1 × HBSS containing 5 mM EDTA and 1 mM DTT.
10% SDS:	10% (w/v) in double deionised water, stored at RT.
Tris-buffered saline (TBS):	10mM Tris, 150mM NaCl in double deionised water. pH adjusted to 7.5 with 1M HCl
TBS-Tween (0.2%) (TBST):	0.2% Tween-20 detergent in TBS

2.2 METHODS

2.2.1. Animal care and housing

129/SvJ × C57/BL6 wild-type (WT) and SPARC null (KO) mice were originally obtained from Dr E. Helene. Sage, Hope Heart Institute, Washington State, USA on a mixed background (Norose *et al*. 1998). Animals were maintained under specific pathogen free (except for norovirus) conditions at Fremantle Hospital. The use of mice, the colitis model and interventional procedures were approved by the Animal Ethics Committee (AEC) of the University of Western Australia (UWA). Unless specifically stated, female mice aged 10-12 weeks were used in all experiments and only animals weighing >19g at time of commencing the experiments were used. Mice were allowed food and water *al libitum*. Wet food was provided to particular mice, if required, during the experiments. Confirmation of complete ablation of SPARC gene was demonstrated in all SPARC null mice by genotyping using PCR (by Mrs. Frances Lloyd).

2.2.2 Induction of colitis by Dextran Sodium Sulphate (DSS)

Colitis was induced by the addition of DSS [3% (wt/vol) (MP Biomedicals LLC, Aus)] to the drinking tap water for 7 days. Control mice received plain tap water without DSS. DSS at a concentration of 3% has been shown previously in our lab to induce colitis with low animal mortality. As the efficacy of DSS varies between batches, the majority of the experiments were undertaken using the same batch number. Each new DSS batch was tested for efficacy.

Mice were given 7 days of DSS (induction), followed by a recovery period of 1-7 weeks without DSS (***Figure 2.1***). The mean daily DSS-water consumption was recorded for each group. Body weights were assessed and stool samples were examined for blood daily during the treatment period and weekly during the recovery period.

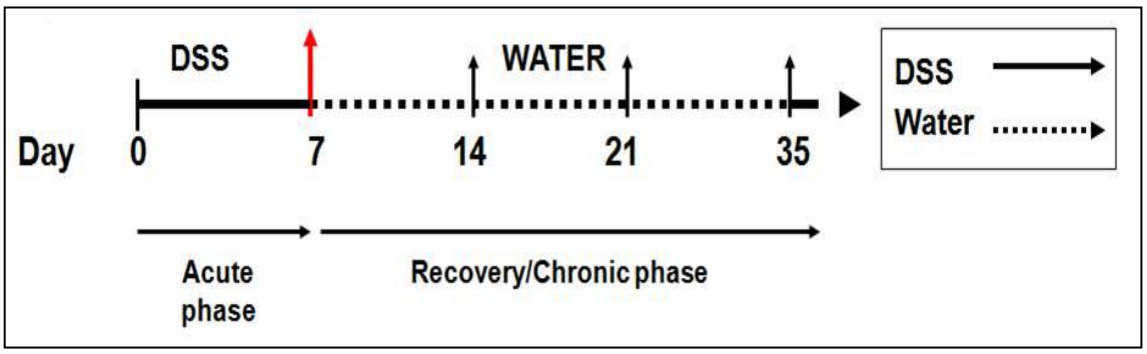


Figure 2.1: Overview of the experimental design to investigate acute, recovery and chronic phases. Mice receiving DSS for 7 days were used to examine the acute inflammatory response. Mice that received 3% DSS for 7 days followed by plain tap water for 1, 2 and 4 weeks (D14, D21 and D35 respectively) were used to investigate the progression to chronic inflammation or recovery. Age matched control groups (non DSS treated) were present for each time point. (↑ and ↑ represents points of analysis)

2.2.3 Endoscopic monitoring of colitis

A high resolution mouse video endoscopic system was used to monitor the colitis and the mice were routinely scoped 1 day prior to sacrifice. Mice were anaesthetised using an intraperitoneal ketamine/xylazil injection of a 0.01ml/g body weight. The experimental endoscopy setup, denoted "*Coloview system*", consisted of a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (Karl Storz, Germany) to achieve regulated inflation of the mouse colon. The endoscopic procedure was viewed on a colour monitor and digitally recorded. All endoscopic procedures were viewed by Dr. Borut Klopcic.

Colitis was staged using the modified murine endoscopic score of colitis severity (MEICS, **Table 2.1**)(Becker *et al*. 2005). The MEICS system consists of 5 parameters: Thickening of the colon wall, changes of the normal vascular pattern, presence of fibrin, mucosal granularity and stool consistency. Endoscopic grading was performed for each parameter (scored between 0 and 3) leading to a cumulative score of between 0 (no signs of inflammation) and 15 (endoscopic signs of very severe inflammation). Healthy mice usually have a score of 0-3.

Table 2.1 The MEICS system.

					Total
Colonic Thickening	0 Transparent	1 Moderate	2 Marked	3 Non- transparent	0-3
Vascular Pattern	0 Normal	1 Moderate	2 Marked	3 Bleeding	0-3
Visible Fibrin	0 None	1 Little	2 Marked	3 Extreme	0-3
Mucosal Granularity	0 None	1 Moderate	2 Marked	3 Extreme	0-3
Stool Consistency	0 Normal+solid	1 Still shaped	2 Unshaped	3 Spread	0-3
				Overall	0-15

2.2.4 Tissue dissection

2.2.4.1 Colon

All mice were scarified by lethal injection followed by cervical dislocation unless stated elsewhere. The colon was removed from each animal and the colon length was measured from the end of anus to caecum (***Figure 2.2*** #). Approximately 1mm from the anus was discarded to remove the rectum as it has a different tissue fibro-structure to the colon. Tissue was then harvested for the different analyses as shown in figure 2.2: i) cryosection, ii) paraffin section, iii) tissue explant for cytokine analysis, iv) flow cytometric analysis v) RNA expression if not performing flow cytometry.

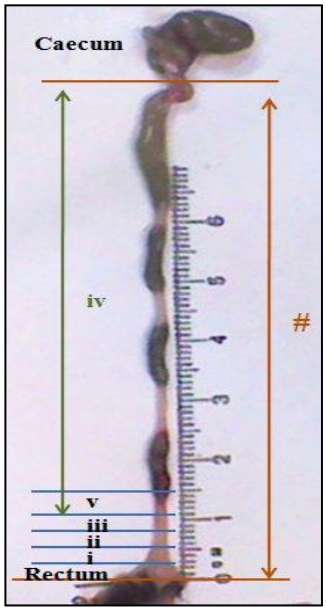


Figure 2.2: Picture of the colon showing regions of tissue collected for different experimental purposes: i) cryosection, ii) paraffin section, iii) tissue explant for cytokine analysis, iv) flow cytometric analysis v) RNA expression if not performing flow cytometry.

2.2.4.2 Lymphoid organs-spleen and mesenteric lymph nodes

The spleen and mesenteric lymph nodes were removed from each WT and SPARC KO mouse and collected into H-DMEM (Gibco® Invitrogen Co., Aus) and kept on ice until use.

2.2.4.3 Plasma and explant cultured supernatant

Blood was drawn by cardiac puncture immediately prior to sacrifice. Blood was collected in microtubes containing heparin and spun at 8400 g for 20 minutes at 4℃. The plasma was transferred to fresh tubes and snap frozen and kept at -80℃.

The colonic tissues for cytokine detection were washed in RPMI-1640 (Gibco® Invitrogen Co., Aus) supplemented with antibiotics. The tissues were then placed in a 96 well tray that contained serum free RPMI-1640 supplemented with antibiotics for ~22-24 hour. The culture supernatants were collected and spun at 12000 g at 4℃ to remove any tissue debris then snap frozen and stored at -80℃ until use.

2.2.5 Preparation of paraffin sections

Colonic tissue was cleaned and then fixed by immersion in neutral-buffered formalin and processed in the Sakura Tissue Tek VIP-E150 tissue processor (GMI Inc, USA) at the Pathwest Histopathology Department at Fremantle Hospital. It was then embedded in paraffin wax and stored at RT. Sections, 4.5μm thick, were cut using a Microm HM 325 Microtome (GMI Inc, USA), floated on water of a 45℃ water bath prior to mounting on Superfrost™ slides. Paraffin sections were dried at 55°C overnight and stored at RT until H&E stained.

2.2.6 Haemotoxylin and eosin (H&E) staining

Paraffin sections were stained with H&E using the Shandon Linistain™ GLX Linear Stainer (Thermo Scientific, AUS).

All H&E stained paraffin sections were assessed blindly by Dr Cynthia Forrest (specialist gastroenterological histopathologist) according to the scoring system by Dieleman (Dieleman *et al*. 1998). In this scoring system, 4 features are graded as outlined in **Table 2.2:** The severity of inflammation (acute and chronic) and depth of inflammation were scored from 0-3, while the level of crypt damage and regeneration were scored between 0 and 4. These changes were also quantified as to the percentage of the total colonic circumference that was involvement by the disease process: i) 1-25%; ii) 26-50%; iii) 51-75%; iv) 76-100%. Total inflammatory score (a score of 0-56) then was given to each sample based on multiplying the score of each feature by the percentage of the tissue involved and adding the totals together. The regeneration feature was not graded in the day 7 sections, the peak of acute inflammation.

Table 2.2 Histological grading of colitis.

Feature	Grade	Description
Inflammation	0	None
	1	Slight
	2	Moderate
	3	Severe
Depth	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Precent involvement	1	1-25%
	2	26-50%
	3	51-75%
	4	76-100%

2.2.7 Preparation of cryosections

Colon and spleen were embedded in Tissue-Tek® O.C.T (optimum cutting temperature) freezing medium (ProSciTech, Aus), snap frozen in liquid nitrogen and stored at -80 ℃. Briefly, cryoembedded tissues were cut by Miss Kristine Fu into 7μm thick sections on a freezing microtome cryostat chamber at a temperature of between -15 and -20 ℃. Sections were mounted onto Superfrost™ slides, dried at RT for approximately 1 hour and then stored at -80 ℃.

2.2.8 Immunofluorescent staining of cryosections

Slides were fixed in ice cold acetone for 2 minutes and then rehydrated in PBS for 10-15 minutes and blocked with 5% goat serum for 30 minutes. The sections were incubated in the optimal concentration (**Table 2.3**) of primary antibody prepared in commercial antibody diluent (DAKO, Denmark) overnight at 4℃ in a humidified chamber and then washed 3 times for 10 minutes in PBS. The sections were then incubated in goat anti-rat IgG secondary antibody conjugated to AF 594 (Invitrogen Co., USA) for 1 hour at RT in a dark, humidified chamber. After washing a further 3 times in PBS, slides were mounted with Prolong Gold Antifade reagent with DAPI (Invitrogen Co., Aus). Slides were viewed and photographed with Olympus IX-81 inverted fluorescence microscope. The DAPI stained nuclei blue when viewed under the ultra violet (UV) filter while the proteins of interest stained red when view with green filter. Each section was viewed at x200 magnification and photographed. The numbers of positive cells were counted in 5-15 fields.

Table 2.3 Rat monoclonal antibodies directed against cell surface markers used in fluorescence staining.

Antibody clone	Dilution	Source	Isotype
CD11b (M1/70)	1:400	Pharmingen	IgG2b,κ
CD68 (Kp1)	1:400	abcam	IgG1, κ
F4/80 (CI:A3-1)	1:200	AbD serotec	IgG2b
Ly6G (1A8)	1:400	Biolegend	IgG2a,κ

2.2.9 Sirius red staining and analysis.

Sirius red staining, described previously in the literature (Lopez-De Leon and Rojkind 1985), was used in order to quantify the collagen content in the colon. Briefly, colonic paraffin sections were dewaxed by immersion for 2 minutes each in 3 changes of xylene, followed by ethanol with of 2 changes of 100% and 1 change of 70% ethanol. The dewaxed tissue sections were then rehydrated in tap water followed by double deionised water. Sections were stained by immersion in a saturated solution of picric acid in double deionised water that contained 0.1% Sirius red (Merck Pty Ltd, AUS) for 30 minutes at RT. Thereafter, excess Sirius red stain was washed off by briefly immersing the sections in 3 changes of double deionised water. Slides were then blotted dry and transferred to xylene before mounting with DePeX.

Stained slides were scanned and digitally captured by an Aperio Scanscope Digital Slide Scanner. The images were analysed with Aperio's ImageScope Viewer-Spectrum positive pixel count algorithm software, which converts the images according to investigator defined parameters (based on a series of positive and negative control of collagens and picric acid) into markup images and quantifies the area and intensities of the positive and negative stains. The software quantified the pixels and colour-coded them to strong, medium, and weak positive and negative staining as shown below. Only tissue sections of the full colon quantified and ignored those with artifact area caused by tissue overlapping. Below shown a markup image generated by the software (***Figure 2.3***).

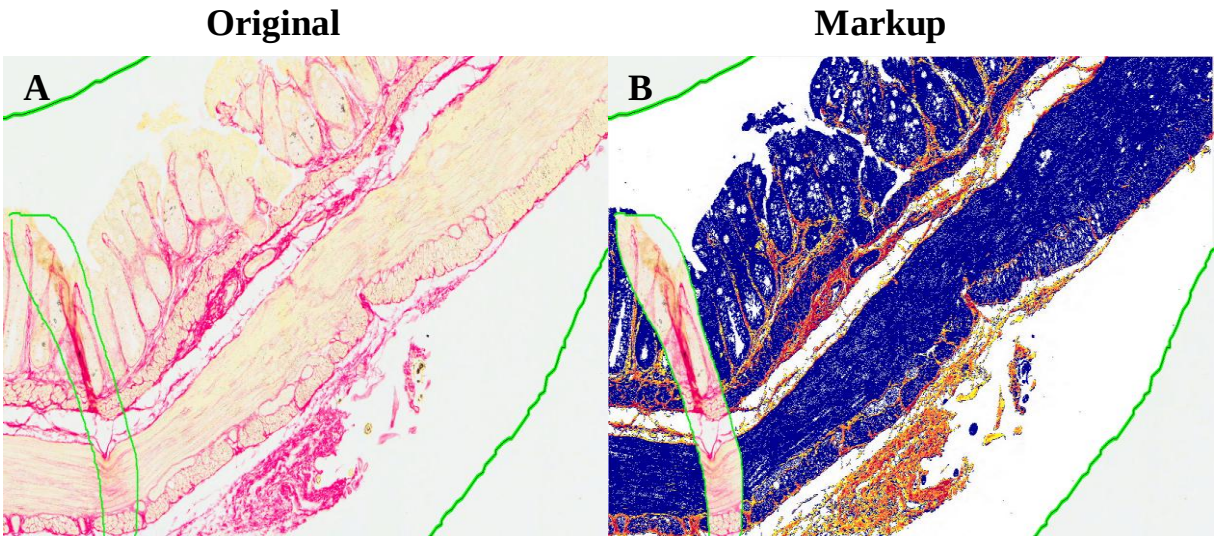


Figure 2.3 Original and markup images generated by Aperio's ImageScope Viewer software. A) Collagen stained red while picric acid stained non-collagenous components to pale yellow. B) The software quantified the pixels and colour-coded them to strong, medium, and weak positive and negative staining. Areas with tissue overlapping were not included.

2.2.10 Detection of cytokines

2.2.10.1 Cytokine bead assay

Beads with different fluorescent intensities were coated with analyte-specific capture antibodies. This enabled the simultaneous detection of multiple analytes from a single sample. The capture beads were incubated with recombinant standard or test samples and phycoerythrin (PE)-conjugated detection antibodies that were added to form sandwich complexes.

A customized 15plex BD™ Cytometric bead array (CBA) Flex set was used to detect the following cytokines and chemokines simultaneously in the tissue explants and serum samples from individual mice: IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-

12/IL23p40, TNF-α, IFN-γ; RANTES, MCP-1, MIP-1α, MIP-1β, MIG. Serum samples were diluted 1:4 in assay diluent and supernatants from tissue explants were used undiluted (except 1:60 for IL-6 analysis). The assay was set up in multiscreen HTS filter plates (Milipore) and performed according to the manufacturer's instructions. A typical standard obtained by serial dilution of recombinant protein. The detection limit for most cytokines was ≤ 2pg/ml (**Table 2.4**). Samples were analysed on a BD FACSCanto™ instrument using BD FACSDIVA™ software and BD FCAP Array™ software. All cytokines were measured at the protein level without *ex vivo* stimulation of the cells/tissues (serum free) as this may reflect the *in vivo* conditions better (Melgar *et al.* 2005).

Table 2.4 Detection limit of cytokines according to the manufacturer (BD, USA).

Cytokine	Detection limit (pg/ml)
Proinflammatory :	
Mouse IL-6 (Bead B4)	1.4
Mouse TNF-α (Bead C8)	2.8
Mouse IL-1ß (Bead E5)	1.9
Th 1 involved	
Mouse IFN-γ (Bead A4)	0.5
Th 2 involved	
Mouse IL-5 (Bead A6)	0.9
Mouse IL-4 (Bead A7)	0.3
Mouse IL-13 (Bead B8)	2.4
Th 17 involved	
Mouse IL-17A flex set (Bead C5)	0.95
Chemokines/Chronic inflammation involved	
Mouse MCP (Bead B7)	2.7
Mouse MIP-Iα (Bead C7)	2.3
Mouse MIP-Iβ (Bead C9)	0.6
Mouse RANTES (Bead D8)	3.3
Mouse MIG (Bead D9)	3
Anti inflammatory	
Mouse IL-10 Flex Set (Bead C4)	9.6

2.2.10.2 Enzyme-linked immunosorbent assay (ELISA)

To measure TGF-β protein levels the Quantikine® mouse/rat/porcine/canine immunoassy for mouse TGF-β (R&D Systems, Aus) was used according to the manufacturer's instructions modified to include neutralisation of the sample with 2.4 N NaOH instead of 1.2N NaOH following activation with acid. The explant culture supernatants and serum samples were diluted 1.3 and 60- fold respectively following sample activation. Recombinant protein from 31.2 to 2000 pg/ml served as a standard curve.

2.2.11 Preparation of single cell suspensions for flow cytometry

Single cell suspensions from the spleen and mesenteric lymph nodes were obtained by gentle dispersion using frosted glass slides. For flow cytometry, the cells were filtered through a 40μm wire mesh and the red blood cells were lysed with ACK lysis buffer. The samples were kept on ice until stained.

To obtain sufficient cells for flow cytometry, cells from 4 murine colons were pooled. Single cell suspensions from the colon were prepared as described (Weigmann *et al.* 2007) with slight modifications. Briefly, the colons were washed in PBS and incubated with predigestion solution for 20 minutes at 37℃ to release the intraepithelial lymphocytes (IEL). The colons were then denuded of the epithelial layer and washed twice with PBS to remove the EDTA and DTT from the predigestion solution as these affect the collagenase activity. They were then finely cut with a McIlwain tissue chopper followed by 2 washes in PBS. The minced tissue was then digested with Digestion solution in order to obtain the LP mononuclear cells (LPMC). The cells were washed twice with H-DMEM and purified with percoll (GE Healthcare, Aus). The

leucocytes from the IEL and LPMC fractions were then recovered from the interphase of a 70/40% percoll gradient for 20 minutes at 1000g at RT.

2.2.12 Flow cytometry

2.2.12.1 Surface and Intracellular Staining

The single leucocyte cell suspensions were seeded into U bottom plates, washed with flow buffer and then analysed by FACS. The cells were then incubated in 50µl stimulation solution (DMEM that contained 750ng/ml of ionomycin (Sigma), 50ng/ml PMA, 10ug/ml monensin (BD)) for 12 hours at 37℃ to enhance cytokine production and prevent secretion out of the cell. The cells were then washed twice with flow buffer, and stained with flurochrome conjugated antibodies solution (50µl per well, in optimised dilution, ***Table 2.5***) at 4℃ in the dark for 30 minutes. Cells were then washed twice with flow buffer, fixed and permeabelised with the BD Cytofix/Cytoperm kit according to the manufacturer's instructions.

The intracellular stain (50µl per well, in optimised dilution, ***Table 2.5***) was applied and incubated for 30 minutes in the dark at 4℃. Cells were then washed twice with Perm buffer and resuspended in flow buffer and analysed with BD FACSCanto™ instrument using BD FACSDIVA™ software.

All staining for Treg cells was performed using the mouse regulatory T cell staining kit (eBioscience, USA) according to the manufacturer's instructions. The single cells were suspended in the H-DMEM and keep at 4℃ until ready for staining.

Table 2.5 Primary antibodies directed against cell surface markers and intracellular secretory molecules in flow cytometry.

Host species	Antibody clone	Dilution	Conjugate	Isotype
Surface				
Hamster	CD3e (145-2C11)	1:250	FITC	IgG1,κ
Rat	CD4 (RM4-5)	1:250	FITC	IgG2a
		1:250	PerCP-Cy™5.5	IgG2a,κ
Rat	CD25 (PC61.5)	1:250	Allophycocyanin	IgG1
Intracellular				
Rat	IFN γ	1:250	PE	
Rat	IL-4(11B11)	1:250	AF488	IgG2b,κ
Rat	IL-17A	1:250	AF647	IgG1, κ
Rat	FoxP3(FJK-16s)	1:100	PE	IgG2a

2.2.12.2 Analysis by flow cytometry

All data were collected with BD FACSCanto™ instrument using BD FACSDIVA™ software with analysis by FlowJo software (version 7.6, Tree Star Inc, USA). The lymphocyte population was identified on the basis of size (forward scatter [FSC]) and granularity (side scatter [SSC]). The lymphocytes were further characterized by their cell surface markers. All gating was set based on the appropriate isotype control as shown in ***Figure 3.1***.

In order to quantify the regulatory T cells, the CD4+ CD25+ population within the lymphocyte population was selected and the Treg marker, FoxP3+ cells analysed. For each sample 100 000 gated lymphocyte events were recorded. The results represent the pooled data of mean percentage of cells of interest out of the total gated cells from 1-3 independent experiments.

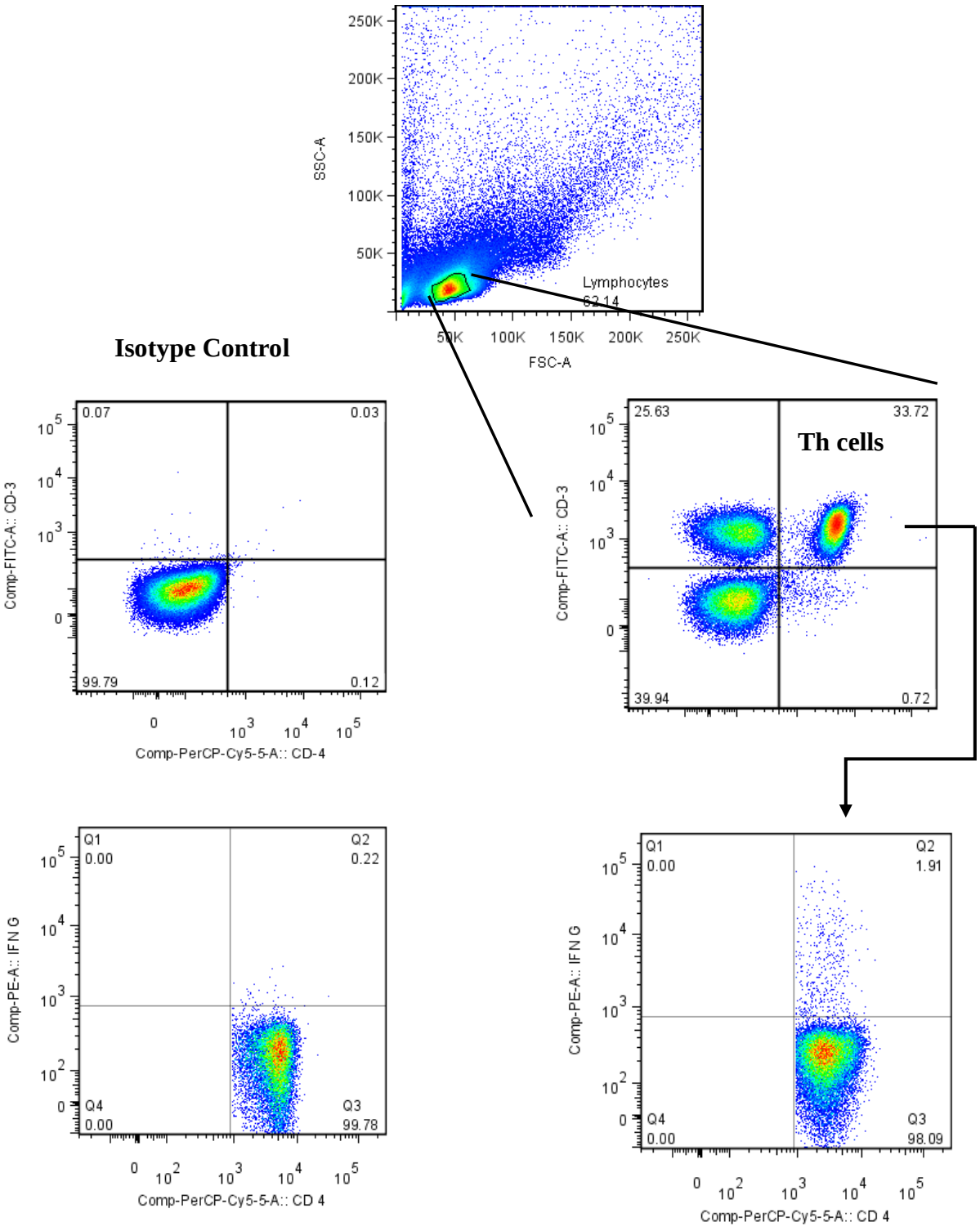


Figure 2.4: The lymphocyte population was gated on the basis of the forward scatter (FSC) and side scatter (SSC). T helper cells (CD3+ CD4+) were further analysed based on intracellular marker (e.g. IFN-γ). All gating was determined by the appropriate isotype control.

2.2.13 RNA Analysis

All buffers, distilled water, microfuge tubes, micropipette tips, and any implements that were used in RNA work were RNase free.

2.2.13.1 Tissue homogenisation for RNA isolation from colon tissue

Colonic tissue was harvested and snapped frozen in liquid nitrogen and kept at -80℃. The colonic pieces were transferred to a pre-chilled 2ml microtube that contained stainless steel beads. Sufficient amount of TRI Reagent® (~1ml; Ambion Inc., USA) was added to each tube and homogenised with a tissue lyser (QIAGEN Pty Ltd, USA) at 30Hz, 10 minutes RT.

2.2.13.2 Cell lysis for RNA isolation from fibroblasts cells

Fibroblasts were lysed directly in the culture wells with 1ml TRI Reagent® (Ambion Inc., USA) per $10cm^2$ of culture dish area. Fibroblasts were incubated at RT for 5 minutes then passed through a pipette several times to ensure complete lysis. The cell lysate was transferred to a labelled 1.5ml MAXYMum Recovery™ microfuge tube (Axygen Scientific) and stored at 20℃ prior to RNA extraction.

2.2.13.3 RNA Extraction

Briefly, cell lysates in TRI Reagent® were thawed at RT, and 100µl of BCP (Ambion Inc., USA) per 1ml of Tri Reagent® added prior to vigorous vortexing for 10 seconds. The tubes were then incubated for 10 minutes at RT and centrifuged at 12000 g for 15 minutes at 4℃. The upper, aqueous phase, containing RNA was transferred to fresh 1.5ml microfuge tubes, then 500µl of isopropanol per 1ml of Tri Reagent® and 4µl of

GlycoBlue CoPrecipitant (diluted 1:20 in RNA storage buffer) (Ambion Inc., USA) were added. Tubes were vortexed and incubated for 15 minutes at RT to allow for RNA precipitation. RNA was pelleted by centrifugation at 12000g for 10 minutes at 4℃, and the supernatants removed. RNA pellets were washed with 1ml of 75% ethanol per 1ml of Tri Reagent® then re-centrifuged at 12000g for 5 minutes at RT. The supernatants were decanted and excess ethanol removed using a pipette. RNA pellets were air dried for 5-10 minutes then dissolved in 15µl of Ultrapure DNAse RNAse distilled water (Gibco®, Invitrogen Co., USA). RNA was allowed to fully dissolve by incubating at 4°C overnight prior to DNase treatment and assessment of the yield and quality.

2.2.13.4 DNase treatment

Genomic DNA was removed using a DNase removal kit (Ambion Inc., USA). The RNA was incubated with DNase I (2 Unit/µl) for 30 minutes at 37℃ and subsequent steps were carried out according to manufacturer's instructions.

2.2.13.5 Quantification of RNA

The concentration and purity of RNA was determined using a Beckman DU640 spectrophotometer. RNA was diluted at 1:50 in RNAase free distilled water and the OD_{260} and OD_{280} measured against a RNase free distilled water blank. To determine the RNA concentration in µg/ml, the A_{260} was multiplied by the dilution factor and the extinction coefficient (1 A_{260} = 40µg RNA/ml). Alternatively, RNA samples were measured undiluted using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific. The A_{260}/A_{280} ratio of the RNA is an indication of its purity. The RNA isolated with this protocol typically exhibited an A_{260}/A_{280} ratio of 1.8–2.1.

2.2.13.6 Reverse transcription of RNA

Reverse transcription of RNA to cDNA was performed using SuperScript™ III Reverse Transcriptase (Invitrogen Co., USA) according to manufacturer's protocol with a slight modification to use half reaction volume. Briefly, 0.75µL of oligo dT_{15} (Promega, USA) and 0.5µl of 10mM of dNTPs were added to 1µg of RNA, and made up to 6.75µL with RNAase free distilled water in RNAase free PCR tubes. The RNA mixture was incubated for 5 minutes at 65℃ to disrupt the secondary structures, then rapidly chilled to 4^{o}C for at least 1 minute. To each tube, a master mix that consisted of 2µl of 5x first strand buffer, 0.5µl of 0.1M of DTT, 0.25µl of recombinant RNasin® ribonuclease inhibitor (2500 Units) (Promega, USA) and 0.5µl of SuperScript™ III reverse transcriptase (200 Units) (Invitrogen Co., USA) was added to yield final reaction volume of 11.25µl. Samples were mixed and centrifuged before being incubated for 1 hour at 50^{o}C for DNA extension. Finally, tubes were heated to 70°C for 10 minutes to inactivate the reverse transcriptase. All cDNA samples were stored at 4°C, and analysed within 3 days, otherwise stored at -20°C.

2.2.13.7 Real-time Quantitative Polymerase Chain Reaction

Real-time quantitative PCR was performed using continuous monitoring of the amplification process with SYBR green, a high-affinity double-stranded DNA-binding fluorescent dye. Each PCR reaction consisted of 1µg cDNA, 75 pmoles $MgCl_2$, 20mmoles each dNTP, 10µmol each forward and reverse primer, 0.5 Units of Taq polymerase (Invitrogen Co., USA), and 1× PCR buffer (Invitrogen Co., USA), 4x SYBR to give a final volume of 20µl. Samples were run alongside a set of serially

diluted plasmid DNA standards (10^2 - 10^8 copies) specific for each gene examined in order to allow copy number quantitation, as well as a no template negative control.

PCR was performed using a Rotor Gene 3000^{TM} Real Time thermal cycle and analysed using the commercial software (Corbett Life Science). During the amplification process, SYBR green dye intercalates with double stranded cDNA, and is excited at 470nm to emit a fluorescence signal detected by the Rotor Gene at 510nm. The level of fluorescence was measured after the 72℃ elongation step of each cycle. The fluorescence increased proportionally to the amount of DNA present, theoretically doubling every cycle (which gives an efficiency of 1 on the standard curve).

The PCR cycling profiles comprised an initial enzyme activation step of 95°C for 5 minutes, then 35-45 cycles consisting of denaturation at 95°C for 15 seconds, a variable annealing temperature for 20 seconds (**Table 2.6**) and primer extension of 72°C for 20 seconds. Data were acquired after each cycle, after the 35-40 cycles, the reaction was held for 15 seconds at 72°C followed by a melting curve program (72–99℃ with a rising heating rate by 1°C on each step and a continuous fluorescence measurement) and finally a cooling step to 40℃ for 3 minutes. At the completion of the PCR, the melt curve was generated. PCR products were analysed and quantified using the Rotor Gene 6 software (Corbett Life Science).

First, all genes were normalised to a housekeeping gene (β-actin or HPRT) and then expressed as a relative ratio with its non-treated control.

Table 2.6: The sequences and PCR conditions of different primers.

Gene of interest	Primer sequences (5'-3')	Annealing temp (℃)	Cycles
β-actin	**Fwd**: CTGGCACCACACCTTCTA	68	35
	Rev: GGTGGTGAAGCTGTAGCC		
HPRT	**Fwd**: GTAATGATCAGTCAACGGGGGAC	60	40
	Rev: CCAGCAAGCTTGCAACCTTAACCA		
TGF-β1	**Fwd**: GCCCTGGACACCAACTATTGC	58	40
	Rev: AGCTGCACTTGCAGGAGCGCAC		
TGF-β3	**Fwd**: GCTCTTCCAGATACTTCGAC	60	40
	Rev: AGCAGTTCTCCTCCAGGTTG		
Collagen Iα1	**Fwd**: GGCGGCCAGGGCTCCGACCC	66	35
	Rev: AATTCCTGGTCTGGGGCACC		
Collagen IIIα1	**Fwd**: CCCAGAACATYACATAYCAC	56	40
	Rev: GATTARAACAAGAKGAACACA		
MMP-13	**Fwd**: TGAACATCCATCCCGTGACC	58	45
	Rev: GGCATGACTCTCACAATGCG		
MMP-3	**Fwd**: GTGGTTGTGTGCTCATCCTACC	60	45
	Rev: TAGTGTTGGAGTCCAGCTTC		
TIMP-1	**Fwd**:ARTCAACSAGACCACCTTATACCA	54	40
	Rev: ASCTGRTCCGTCCACAARCA		
TIMP-2	**Fwd**: CTGGACGTTGGAGGAAAGAA	55	40
	Rev: GTCRAGAAACTCYTGCTTGG		
SPARC	**Fwd**: ACTGAGAAGCAGAAGCTGCG	58	40
	Rev: CAGGCGGAACAGCCAACCAT		
COX-2	**Fwd**: AAAACCGTGGGGAATGTATGAGCAC	62	40
	Rev: AAACTTCGCAGGAGGGGGATGTTG		

2.2.14 *Primary colonic fibroblasts cultures*

All cell culture media and reagents were obtained from Gibco® Invitrogen Co., AUS unless otherwise stated.

2.2.14.1 Tissue Collection and Fibroblast Isolation

The procedure used to culture primary mouse colon fibroblasts was modified from the original method (Strong *et al.* 1998; Freshney 2005) where the colon was enzymatically disaggregated. First, the colon was opened up longitudinally and cut horizontal into 2-3mm strips. These strips were then washed in warm Ca^{2+} Mg^{2+} free HBSS® solution buffered with 20mM HEPES, 2mM L-glutamine, 100 IU/ml penicillin/streptomycin/fungizone, and 10% FBS for 15 minutes, subsequently washed in RMPI solution as supplemented as above for 15 minutes. Thereafter, the strips were washed with PBS twice and minced with a McIlwain tissue chopper. The minced tissue was incubated with Difco trypsin 1:250 (2.5mg/ml)/collagenase IV (400U/ml)/DNase I (350U/ml) enzyme cocktail at 37℃. The cells were collected every 30 minutes until complete disaggregation. Trypsin was removed by centrifugation and neutralized by with H-DMEM to stop the trypsin reaction. The pellets were resuspended in H-DMEM and plated in culture flasks. The non-attached cells were carefully removed wash with PBS 24 hours later.

2.2.14.2 Maintenance and subculturing of cell cultures

Culture media were replaced every 3 to 4 days with pre-warmed H-DMEM. Cells were passaged upon reaching 80-95% confluency. To passage cells, media were aspirated from the flask and cells rinsed with PBS. An appropriate volume of prewarmed TrypLE Express™, diluted 1:5 in PBS, was added and the flask rocked to coat the cell sheet.

Flasks were then incubated at 37℃ for approximately 5 minutes, or until cells were completely dislodged. Flasks were checked at regular intervals using a light microscope to ensure the cells were completely detached from the surface of the flask and, if necessary, gently tapped to dislodge the remaining cells. Once the cells were completely detached from the surface of the flask, an equal volume of H-DMEM was added to inactivate the TrypLE Express™. Equal volumes of the cell suspension were seeded into 2 or more new flasks and topped up with an appropriate volume of H-DMEM.

2.2.14.3 Immunohistochemistry

The established fibroblast cultures were stained for α-smooth muscle actin (SMA), desmin, vimentin and keratin to confirm that the cells were mesenchymal fibroblasts/myofibroblasts and not epithelial cells. Cells were grown as monolayers on glass coverslips, or directly in 24 wells tissue culture plates. The cells were washed twice with 1x PBS, fixed with ice cold methanol and acetone in a 1:1 ratio for 3 minutes, air dried and washed twice with 1x PBS at RT. Subsequently, cells were stained with the avidin-biotin-peroxidase complex system (streptABcomplex /HRP; Dako, Denmark) as described by the suppliers. Briefly, the cells were incubated in TBST for 5 minutes and in ARK diluent containing 1% BSA for 3 minutes to permeablise the cells. Following permeablisation, cells were incubated in DAKO^{TM} peroxidase block solution for 5 minutes to block endogenous peroxidase activity and rinsed with double deionised water. The cells were then incubated with DAKO^{TM} serum free protein biotin blocking solution to block non-specific staining and washed 3x with TBS. Primary antibodies, at various dilutions (**Table 2.7**), were applied to the cells and incubated in a humidified environment overnight at 4℃. A no-primary control was undertaken in each run.

Table 2.7: Primary antibodies and their corresponding dilutions and diluent used for immunohistochemistry.

Primary Antibody	Dilution	Diluent
Monoclonal mouse anti human α-SMA (Chemicon Inc)	1:500	ARK diluent prepared by 1% of BSA in TBS supplemented with biotinylatation and blocking reagent from DAKO™
Monoclonal mouse anti desmin (Novacastra)	1:50	ARK diluent prepared by 1% of BSA in TBS supplemented with biotinylatation and blocking reagent from DAKO™
Polyclonal Goat anti-human vimentin (Chemicon Inc)	1:20	TBS
Polyclonal rabbit anti-cow keratin (DAKO)	1:200	TBS

Following incubation and extensive washing (3x in TBS, 3x in double deionised water after 2 minutes of TBS bath), the cells were incubated with biotinylated primary antibody (mouse monoclonal α-SMA and desmin) and treated with DAKO LSAB$^+$ yellow linking solution diluted 1:2 in TBS. Those cells incubated in non mouse origin antibody were further incubated with IgG (with respective origin) for 20 minutes at RT and washed 3x in a TBS bath. All cells were then incubated with DAKO LSAB$^+$ red label streptavidin-HRP for 30 minutes at RT. Cells were washed as above with TBS and double deionised water 3 times. The cells were then stained with DAB staining reagent for 5 minutes and washed with double deionised water.

2.2.14.3.1 Visualisation of cells after staining

The cells were visualised using the Nikon TE2000-E microscope at the magnification of 200-300x. The actin filaments on nucleus in α-SMA positive cells shown brown, while

desmin and vimentin had brown cytoplasmic stain with DAB. Fibroblasts cells stained negative with keratin, which is an epithelial cell marker.

2.2.14.4 Stimulation of fibroblasts cell cultures

Cells were seeded in T-25 culture flasks, and grown to 80-90% confluence to avoid contact inhibition. Upon reaching the desired confluency, fibroblast growth was arrested by replacing the H-DMEM with L-DMEM (DMEM with 2% FCS, 0.025M HEPES buffer. 1X PSF, 2% L-glutamine), for 24 hours prior to stimulation.

All fibroblasts were stimulated with PMA (Sigma Aldrich, USA), which mimics the state of active inflammation that occurs during tissue injury (Lyass *et al*. 1988; Sturges 2004). The PMA was prepared by diluting a stock solution to the working concentration of 10ng/ml in L-DMEM. Fibroblast-like cells seeded in T25 culture flasks were incubated with PMA treated L-DMEM for 24 hours. Following the 24 hours stimulation, cells were harvested for RNA analysis.

2.2.15 Statistical Analysis

Statistical significance was calculated using GraphPad Prism (Version 5.0) (GraphPad software, Inc, USA). All graphs and comparison of differences between groups were assessed using Student's unpaired t-test.

Chapter 3

Contribution of SPARC to the inflammatory response in DSS colitis

3.1. INTRODUCTION

IBD are chronic inflammatory disorders which affect the human gastrointestinal tract. CrD and UC are categorised as typical IBD. There are numerous murine models that have been developed to investigate the pathology that lies behind IBD. The DSS-induced model of colitis is one of the most commonly used. DSS is directly toxic to the gut epithelial cells located within the basal crypts. It reduces the integrity of the mucosal barrier allowing the translocation of proteins across the epithelial surface that then promote acute inflammation with recruitment and activation of immune cells (Okayasu *et al.* 1990; Wirtz *et al.* 2007). The destructive effects of DSS are generally reversible with the regeneration of colon mucosal tissue upon withdrawal of the DSS (Da Silva *et al.* 2006). Administration of DSS to mice over a long period of time however has been shown to induce chronic intestinal inflammation with symptoms and histological features similar to those observed in human IBD (Mahler *et al.* 1998; Melgar *et al.* 2005; Okayasu *et al.* 1990).

SPARC is a matricellular glycoprotein that is involved in diverse biological processes, including tissue remodeling, wound repair, morphogenesis, cell differentiation, adhesion, proliferation, migration, and angiogenesis (Brekken and Sage 2000; Jendraschak and Sage 1996; Norose *et al.* 2000). A deficiency in SPARC has been reported to reduce both renal inflammation and fibrosis (Socha *et al.* 2007). These effects, however, may not be universal across different tissues as the bleomycin-induced lung injury in SPARC KO mice resulted in increased recruitment of neutrophils to the site of acute inflammation (Savani *et al*. 2000) and enhanced inflammation, though not always with evidence of fibrosis.

Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, enhance the inflammatory process, which is crucial to the recruitment of leucocytes and their subsequent activation to secrete various growth factors including PDGF and TGF-β. TNF-α is a chemotactic agent for both macrophages and neutrophils, and it also modulates the MMPs and tissue inhibitors of metalloproteinase (TIMP)s levels within the ECM (Dayer *et al.* 1985; Gharaee-Kermani and Phan 2001; Lee *et al.* 1995). It is not surprising, therefore, that high levels of the pro-inflammatory protein TNF-α are present within the serum and mucosal samples taken from patients with IBD (Murch *et al.* 1991; Braegger *et al.* 1992; Reimund *et al.* 1996) . IL-1β is also an important mediator of inflammation in multiple models of autoimmunity, including collagen-induced arthritis, experimental autoimmune encephalomyelitis, as well as IBD. Serum levels of IL-6 are increased in patients with active IBD and have been shown to decrease following successful treatment of the inflammation (Suzuki *et al.* 2001; Mahida *et al.* 1991). SPARC has been suggested to modulate inflammation through affecting the bioavailability of cytokines and chemokines via MMPs (Soehnlein and Lindbom 2010), e.g. MMP-2, and MMP-3 which can cleave pro-IL1β to form IL-1β (Schonbeck *et al.* 1998). Thus, SPARC might represent a potential therapeutic target that worked as Infliximab, a monoclonal antibody against TNF-α, which is widely used to treat CrD.

The contribution of SPARC on the inflammation in the intestine will be investigated in this chapter through examination of several aspects of the disease including endoscopic, clinical, histological, and assessment of the pro-inflammatory cytokines levels within tissues. This investigation will be performed on SPARC KO mice with DSS-induced colitis. Tissues will be analysed at early and late time points to elucidate any potential

role for SPARC in the development of acute inflammation, recovery, or in the development of chronic inflammation.

3.2 RESULTS

3.2.1 Clinical symptoms and histopathological changes during the periods of acute inflammation and recovery

3.2.1.1 Weight loss

Clinical symptoms appeared in both the WT and SPARC KO mice between 2-7 days after DSS exposure included loss of body weight, diarrhoea and faecal blood, which providing a gross clinical indication of disease. Loss of body weight was observed from day 2 in all DSS-treated mice (***Figure 3.1***). The WT mice generally underwent a greater weight loss than the SPARC KO mice when treated with DSS although that was not statistically significant. Both groups of mice, however, regained the weight once DSS treatment was stopped. Both WT and SPARC KO mice had bloody liquid stools during the DSS treated period.

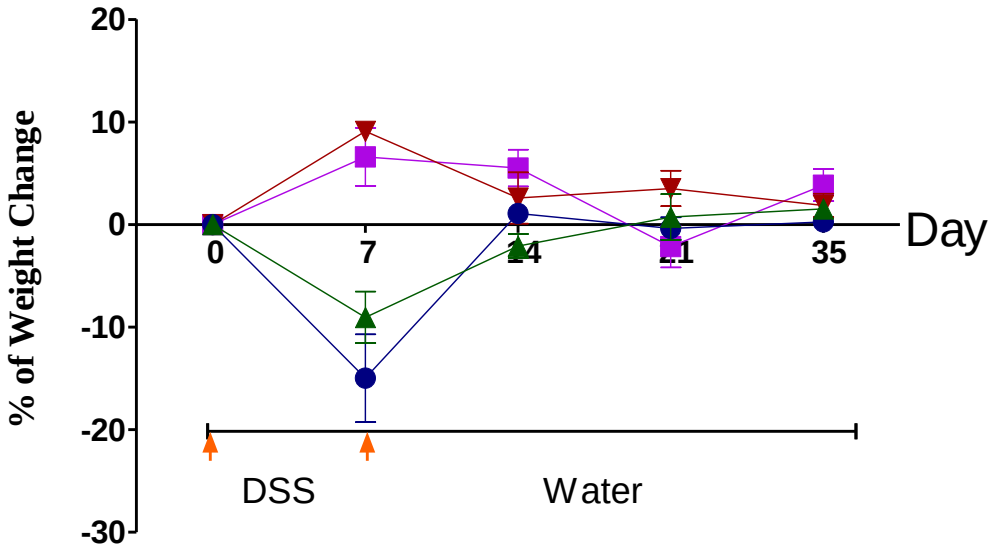


Figure 3.1: Changes in the body weight of WT and SPARC KO treated with DSS and water control. The loss of body weight was calculated by dividing the difference in weight on the specified day by the body weight of day 0 and expressed as percentage. Values are mean ± SEM *n*=15) ■ WT untreated; ▼ SPARC KO untreated; ● WT DSS-treated; ▲ SPARC KO DSS-treated.

3.2.1.2 Colon length

The colon length is often used as morphological parameter for the degree of inflammation in DSS colitis. During the peak of inflammation, it was observed that the colon shortened. At day 7, both DSS-treated WT and SPARC KO mouse colons had an average colon length of 6.5cm while those of the control mice averaged 8.6cm although this was not statistically significant. During the recovery stage (Day 14, 21 and 35), the length of the colon from both groups of mice, treated with DSS, gradually returned to normal length consistent with resolution of the inflammation (***Figure 3.2***). The WT mice, however, had a shorter average colon length than the SPARC KO mice consistent with continuing inflammation and a slower healing rate.

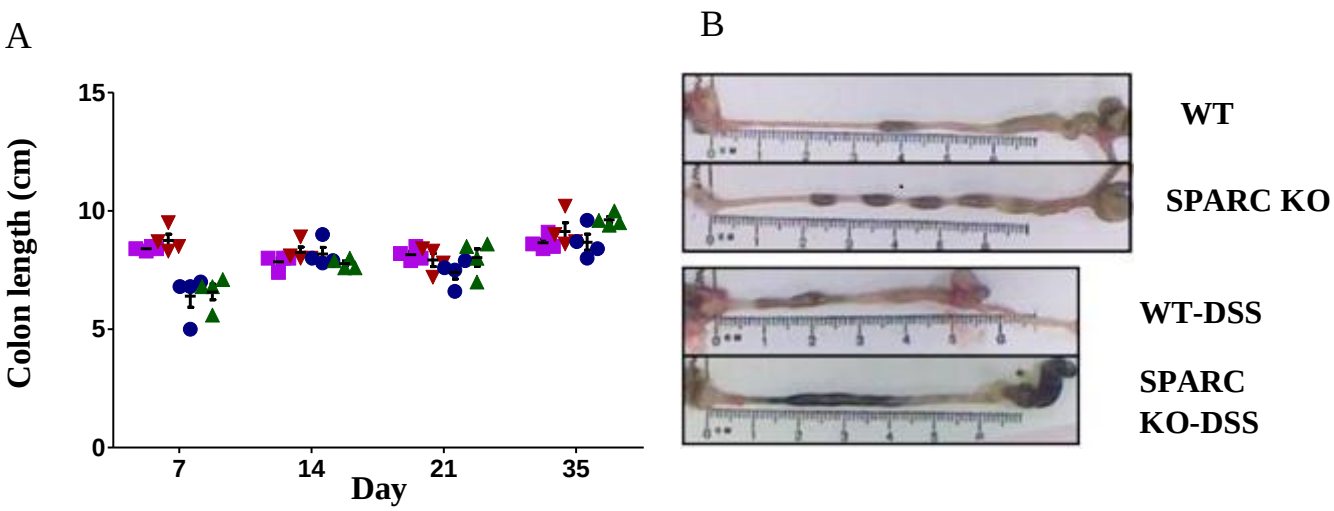


Figure 3.2: **A)** Colon length in WT and SPARC KO mice during acute, chronic/recovery phases of colitis. **B)** Representative image of colon immediately after removal from the mice at day 7. (*n*=4) ■ WT untreated; ▼ SPARC KO untreated; ● WT DSS-treated; ▲ SPARC KO DSS-treated.

During acute inflammation, all the WT mice treated with DSS had an increase in spleen weight (Chapter 4) and enlargement of the MLNs (data not shown), where such enlargement were observed less frequently in the SPARC KO mice treated with DSS.

3.2.1.3 Endoscopic Scoring

Endoscopically, the inflammation was primarily located in the distal colon (2-3cm from the anus) in both WT and SPARC KO animals. At day 6, both WT and SPARC KO showed marked signs of mucosal inflammation as described in Table 2.1: soft and bloody stool, thickening of the mucosa, , bleeding, loss of visible blood vessel structures and the presence of fibrin (***Figure 3.4 a)***. After DSS removal, the stool almost back to normal solid shaped and translucency and vascular pattern gradually restored as seen in day 13 (***Figure 3.4 b)***, 20 (***Figure 3.4 c)*** and 34(***Figure 3.4 d)***. Granular mucosa surface formed on the colon tissue closest to rectum.

WT mice had significantly higher MEICS scores than SPARC KO especially at day 6 ($p=0.039$), 20 ($p=0.019$) and 34 ($p<0.0001$) (***Figure 3.3 A***). This suggests that inflammation is worse and that the speed of recovery from the inflammation is slower in the presence of SPARC.

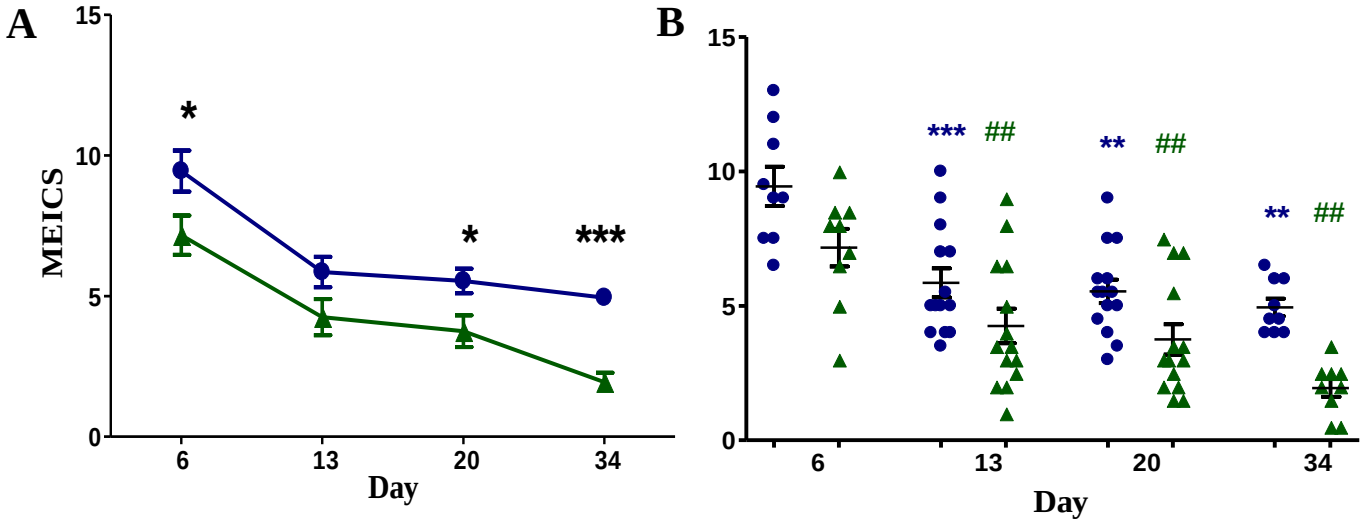


Figure 3.3: **MEICS of WT and SPARC KO mice**. **A**) Line graph (*****p<0.05, *******p<0.001) comparing SPARC KO-DSS to WT-DSS mice at difference time points. **B**) Scatter plot to show the variation in each group and compared with day 6: WT ******p<0.01;******* p<0.001; SPARC KO- ## p<0.01. Values are mean ± SEM based on records of 8-15 mice per time point from 3 different experiments. Statistical values using Student's unpaired t-test. The untreated controls (WT, KO) are not shown as they had a score of "0" for MEICS. ● WT DSS-treated; ▲ SPARC KO DSS-treated.

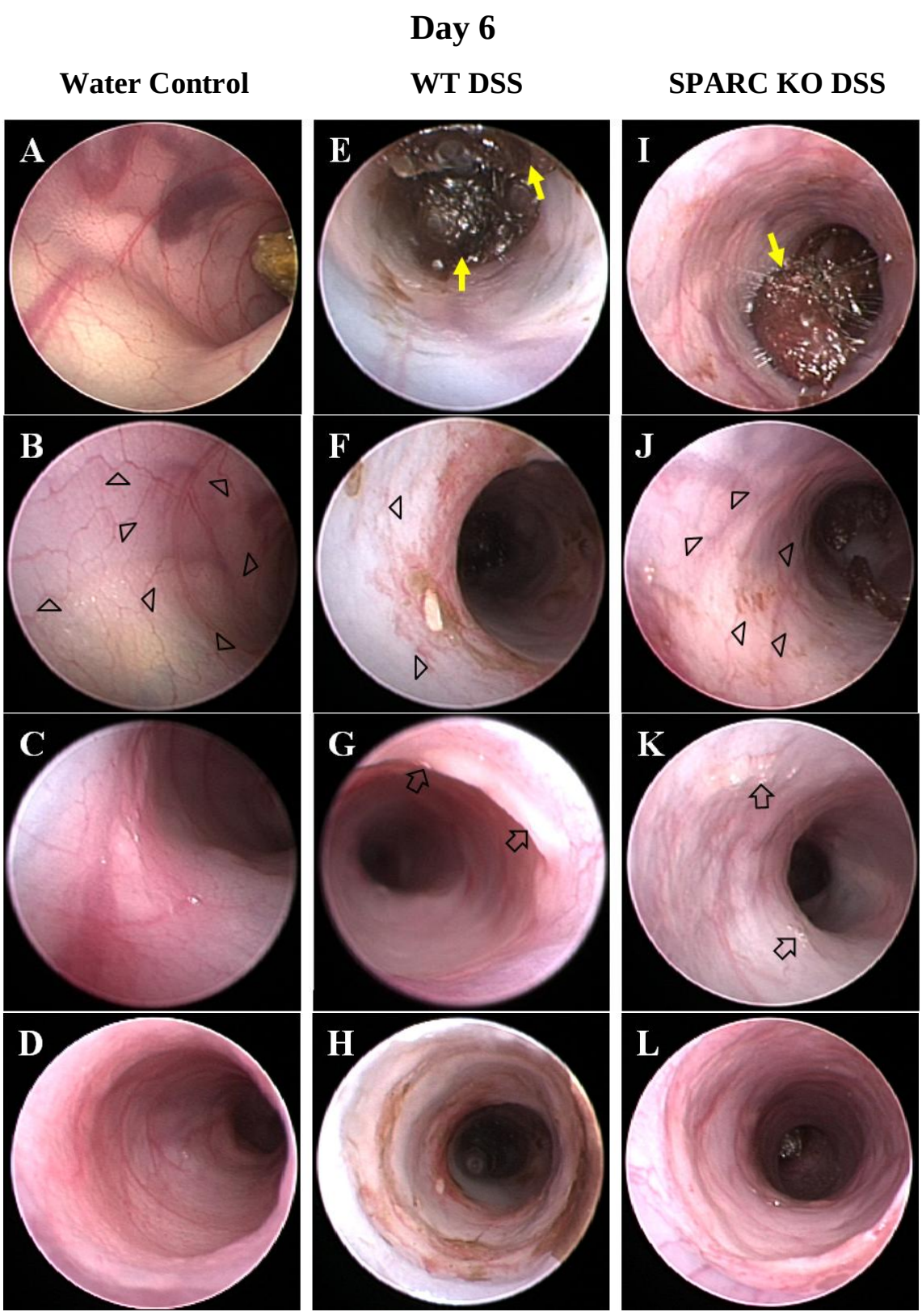


Figure 3.4 a: **Representative endoscopic images at day 6 showing the colon of healthy control (A, B, C, D), DSS-treated WT (E, F,G, H) and DSS-treated SPARC KO (I, J, K, L) mice.** When treated with DSS for 7 days, both WT and KO mice showed signs of inflammation: loose and bloody stool (E, I), thickening of colonic wall with reduction in translucency (F, J), altered vascular pattern (F, J) and present of fibrin (G, K). The lowest panel (D, H, L) showed the colon closest to rectum, which has a different fibrosturcture to the distal and proximal colon.

Day 13

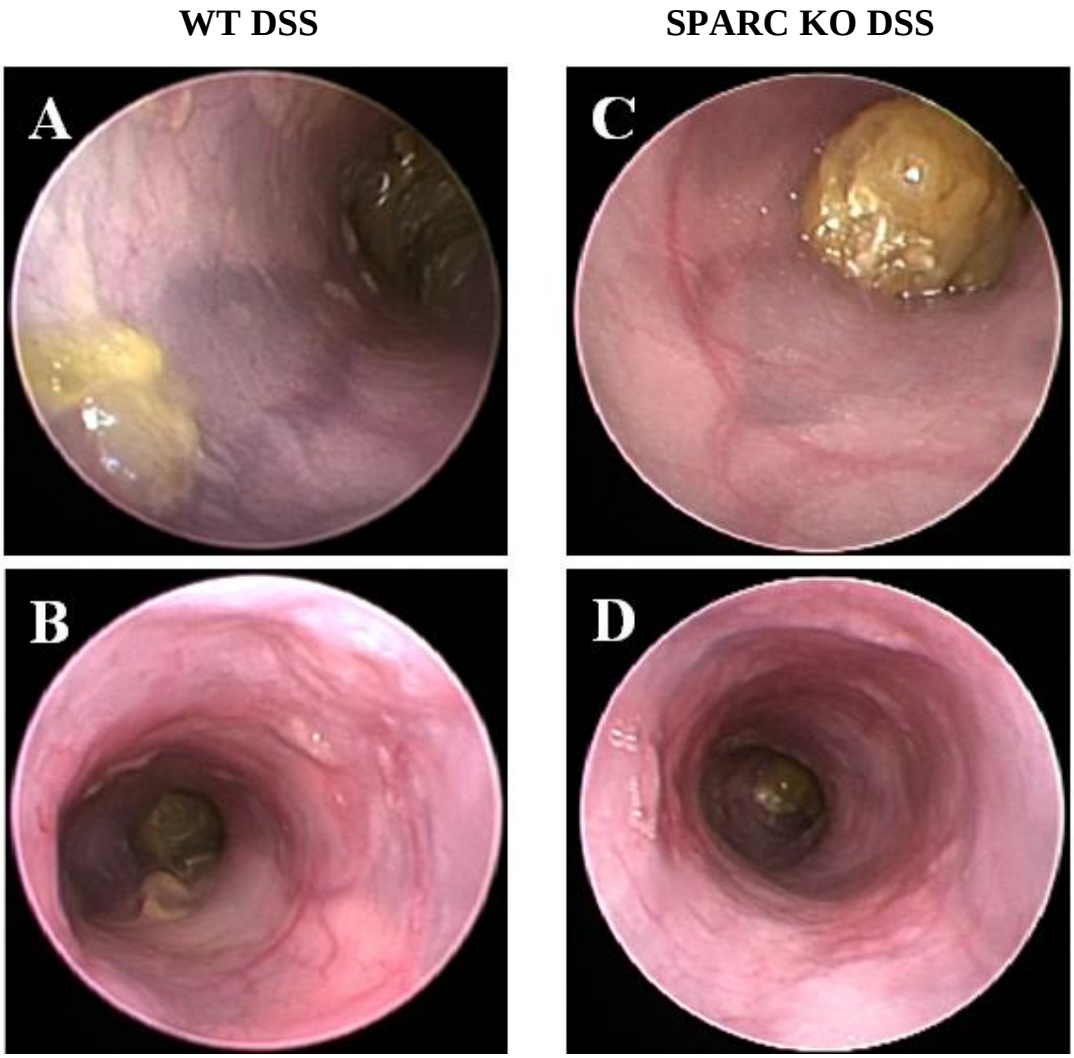


Figure 3.4 b: Representative endoscopic images at day 13 showing the colons of mice recovering from DSS treatment. The stool is an almost normal solid shape but thickening of the colonic wall and altered vascular patterns are still present in both WT and SPARC KO mice (A, C). The lowest panel (B, D) shows the colonic closest to rectum where there was the presence of granular mucosa.

Day 20

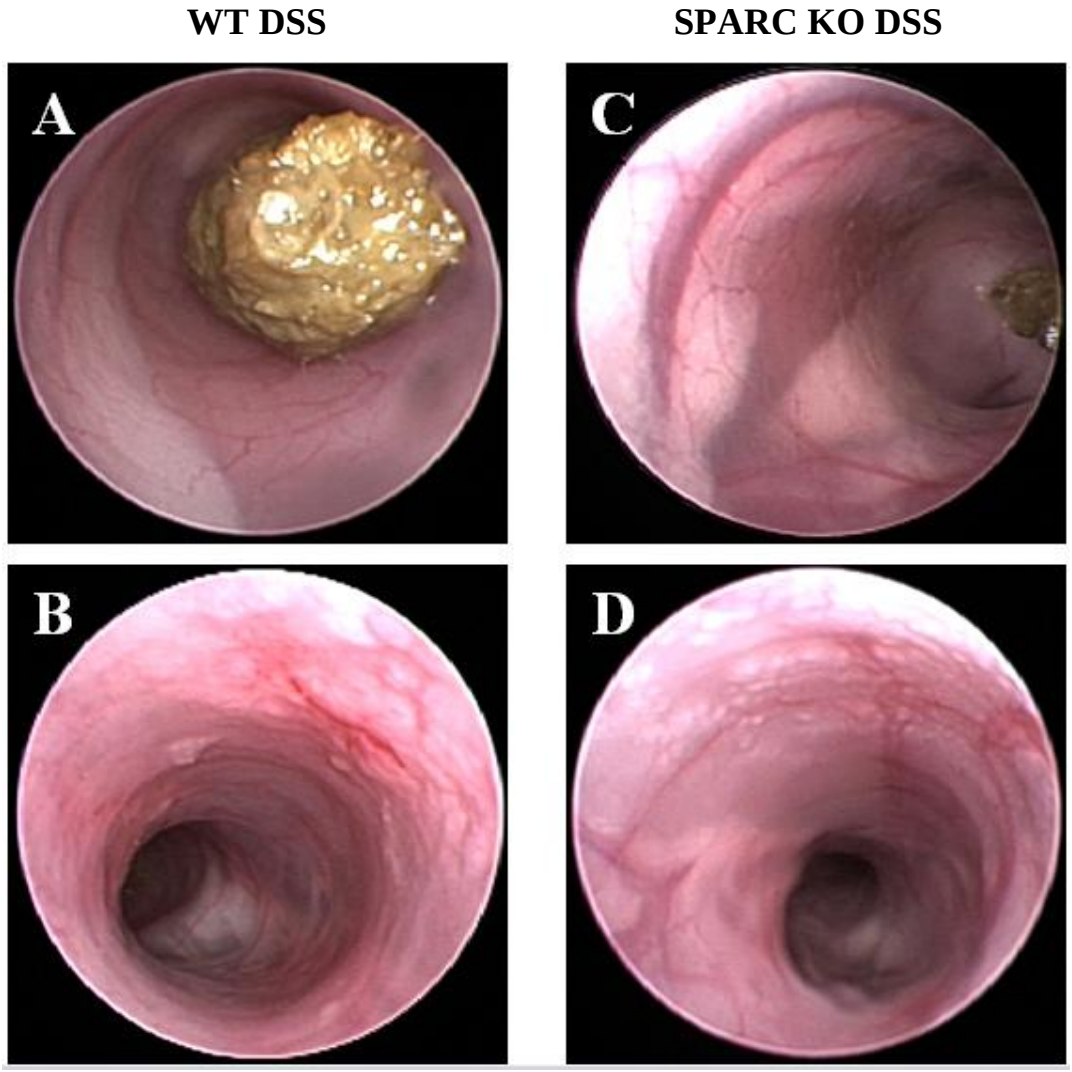


Figure 3.4 c: **Representative endoscopic images showing the colon of DSS-treated WT and SPARC KO mice at day 20.** The translucency has returned to the SPARC KO colons (C) while some WT mice still demonstrate mucosal thickening. The mucosal surface was still "granulated" and this persisted until day 34 (B and D).

Day 34

WT DSS **SPARC KO DSS**

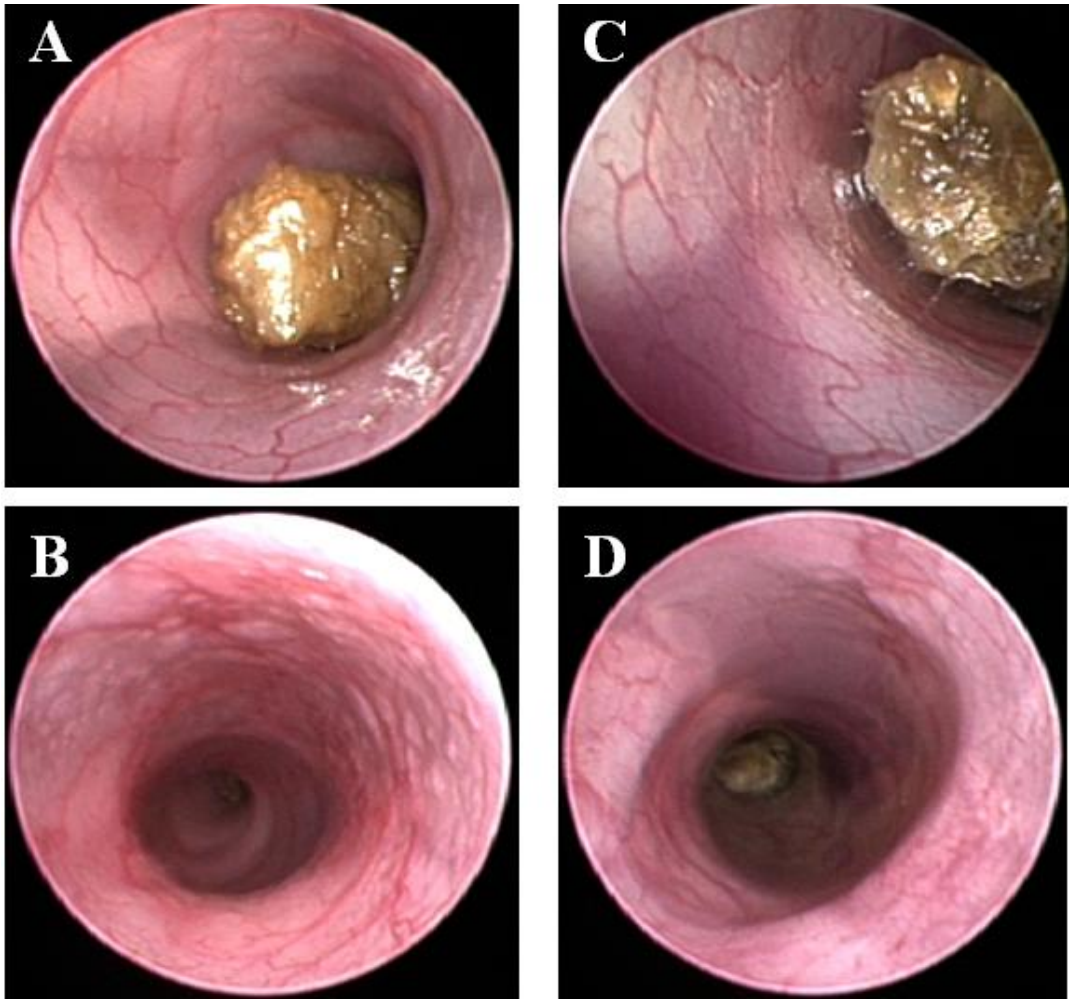


Figure 3.4 d: Representative endoscopic images showing the colon of DSS-treated WT and SPARC KO mice at day 34. The translucency and vascular pattern had returned to normal in majority of the animals, but some of the WT mice still demonstrate thickening of the mucosa and the mucosal closet to the rectum was still "granulated" (B).

3.2.1.4 Histological score

Histopathological analysis was undertaken following H&E staining. SPARC KO animals that received DSS treatment appear to have a trend towards a lower total inflammatory score (***Figure 3.5 A***) and in all the histological features (***Figure 3.5 B***) although not statistically significant. Tissues at day 7 demonstrated moderate to severe inflammation in both WT and SPARC KO mice, with transmural inflammation detected in some animals, particularly the WT animals. The crypts were lost from the colon with a reduction in the goblet cell number, focal ulceration, infiltration of inflammatory cells, including neutrophils, into the mucosa and oedema of the submucosa (***Figure 3.6 A***). Regeneration, indicated by reepithelisation and proliferation of new crypts, began following the removal of the DSS as seen at days 14 to 35. Overall, the WT DSS-treated mice still demonstrated crypt damage and ulceration at day 21 in contrast to the SPARC KO mice (***Figure 3.6 C***). At day 35, majority of SPARC KO DSS-treated mice had completely regenerated the colonic mucosa (***Figure 3.6 D***), while the WT DSS-treated animals had still not fully resolved the inflammation. A small number of treated WT mice also still demonstrated moderate inflammation, crypt damage with ulceration.

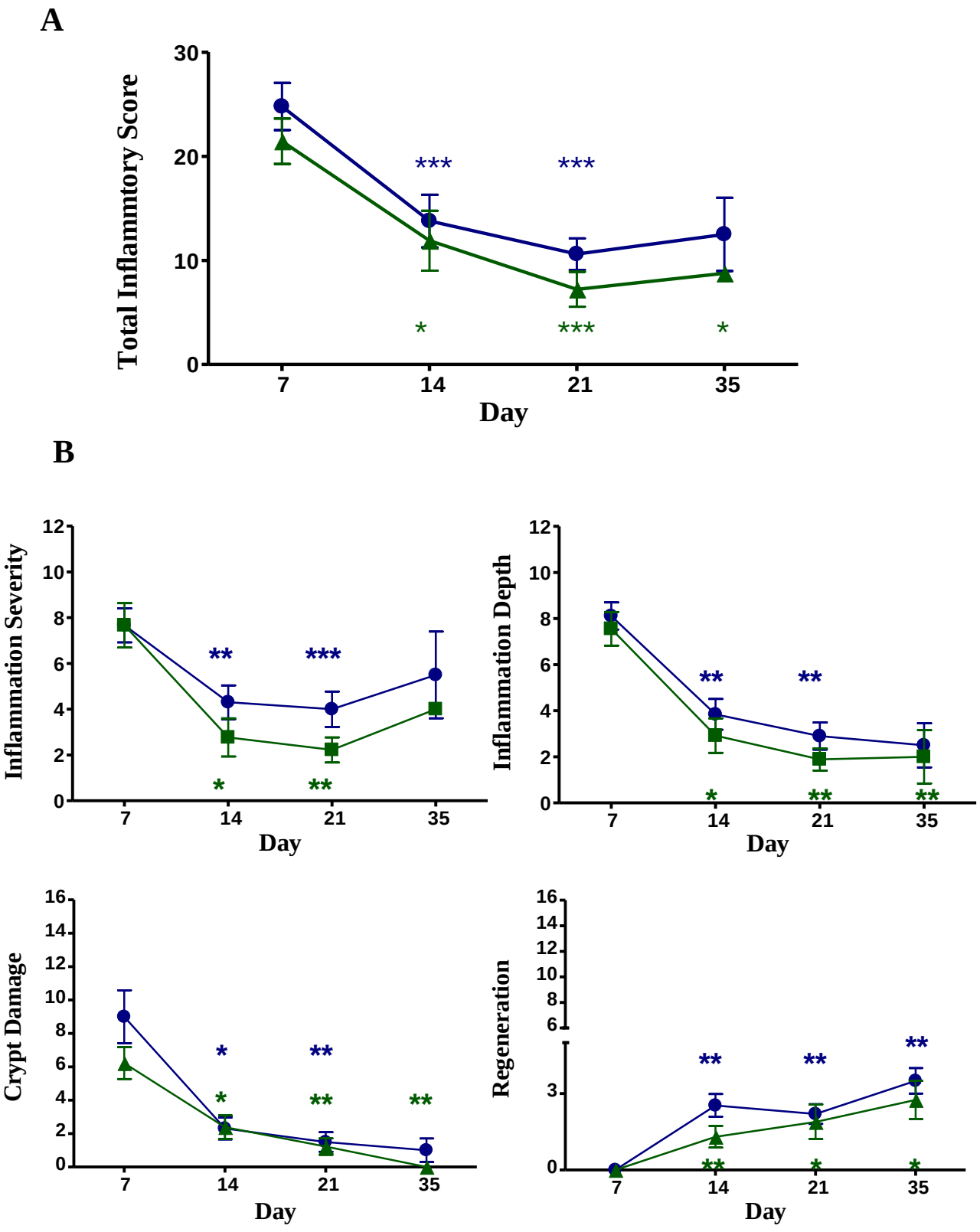


Figure 3.5: A) Total inflammatory score. Extent of inflammation quantified and expressed as an inflammation score as shown in table 2.3. **B) The score for 4 different features of histology**. The untreated controls are not shown as they had a score of "0" to all assessed features. Each time point consisted of 10-15 mice from 3 different experiments. Value is mean ± SEM. Statistical values using Student's unpaired t-test comparing mice at difference time points with mice at peak of inflammation (day 7): *****p<0.05; ******p<0.01; *******p<0.0001. ● WT DSS-treated; ▲ SPARC KO DSS-treated.

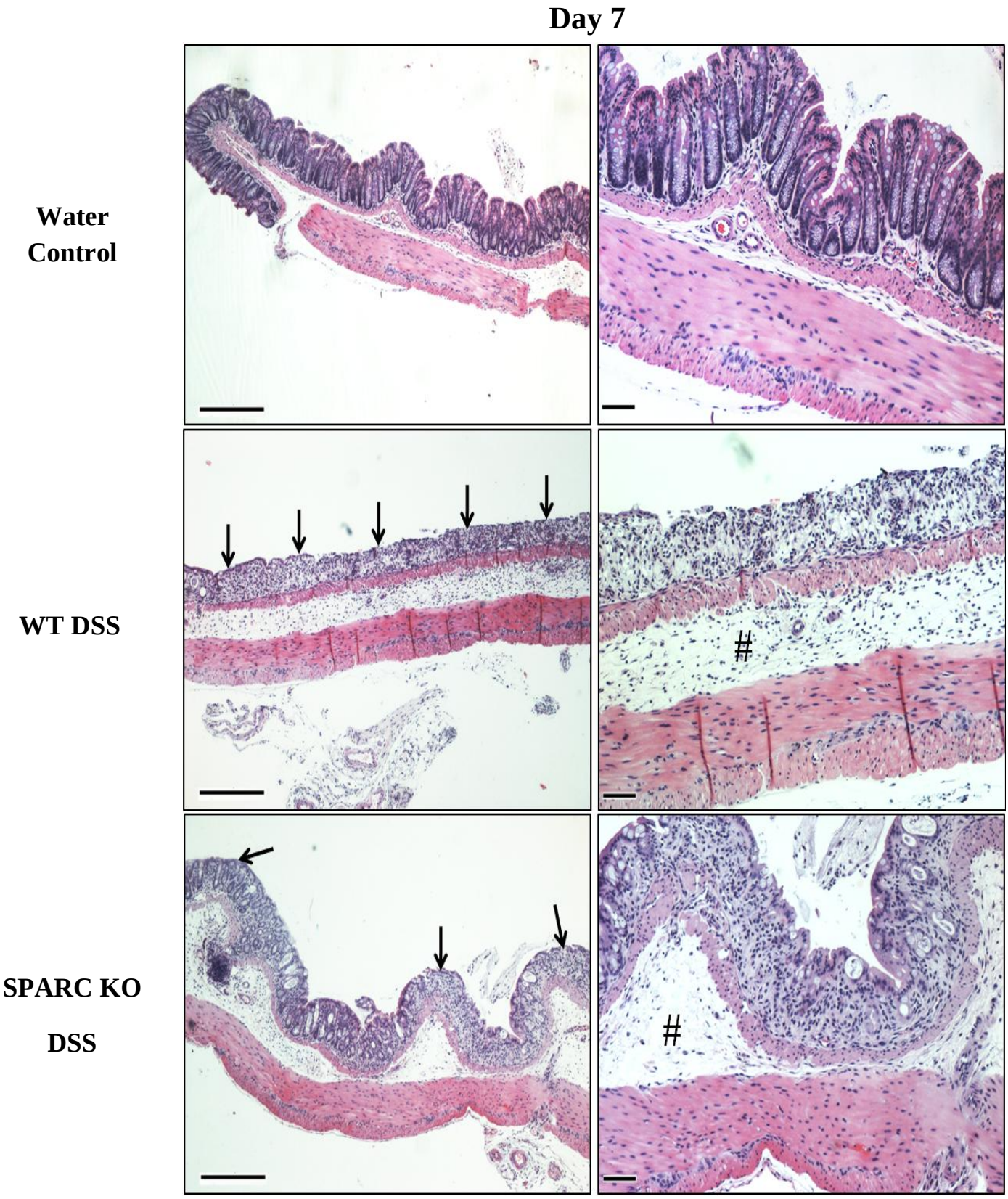


Figure 3.6 A: Representative photographs of H&E-stained sections of the distal colon at day 7 demonstrating ulceration, mononuclear cell infiltrate involving part to all of the colonic wall with disruption of the normal crypt architecture and loss of goblet cells (↑) and submucoa oedema (#). Left panel-40x, Bar: 500 µm; Right panel-100x, Bar: 100 µm)

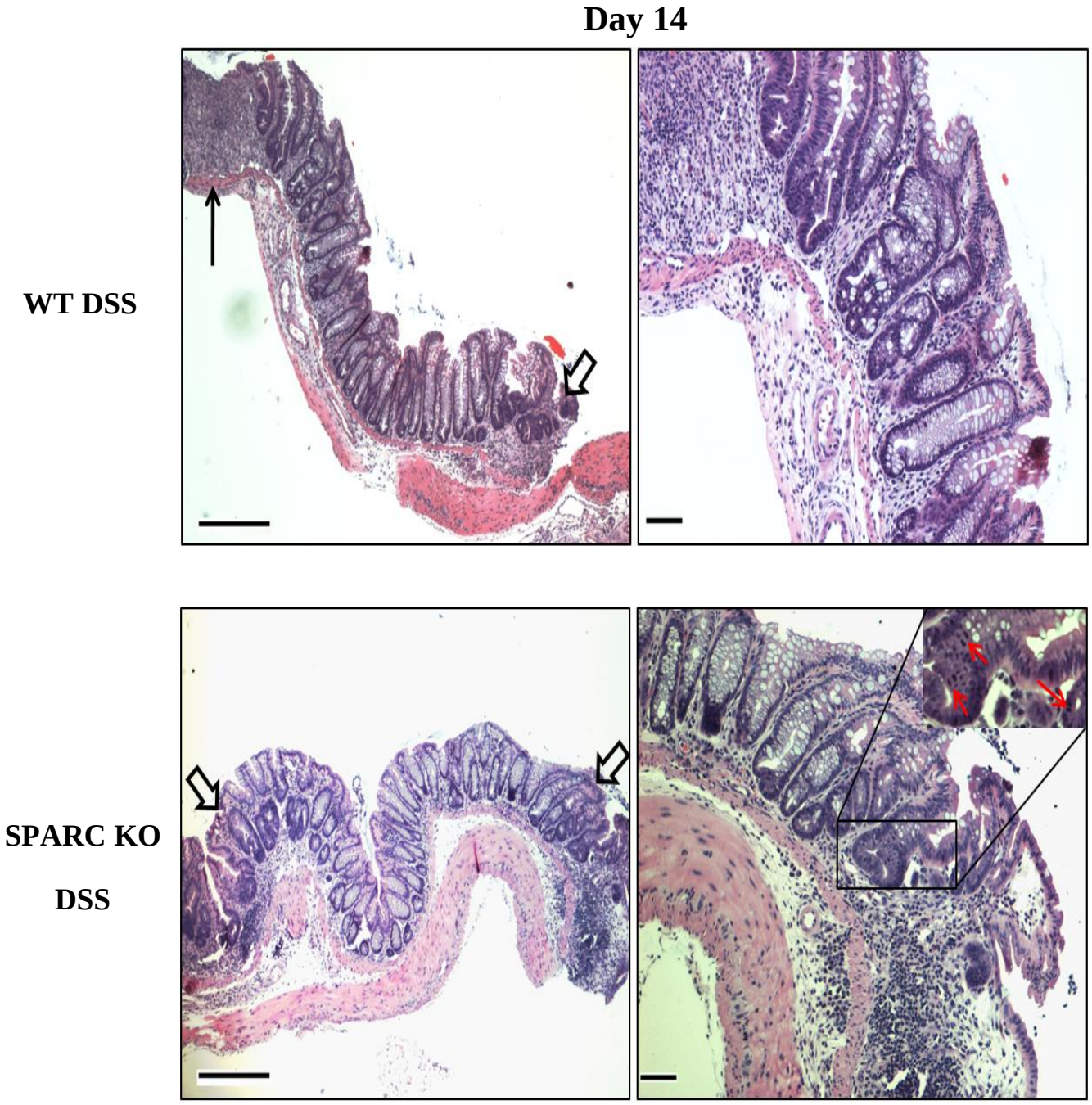


Figure 3.6 B: Representative photographs of H&E-stained sections of the distal colon at day 14. Ulceration (↑); Regeneration of crypts (⇧) and reepithelisation that occurred from the edge of the ulcerations was seen in both WT and KO-treated mice. Inset showing mitotic figures (↑) 400x. Left panel-40x, Bar: 500 µm; Right panel-100x, Bar: 100 µm.

Day 21

WT DSS

SPARC KO DSS

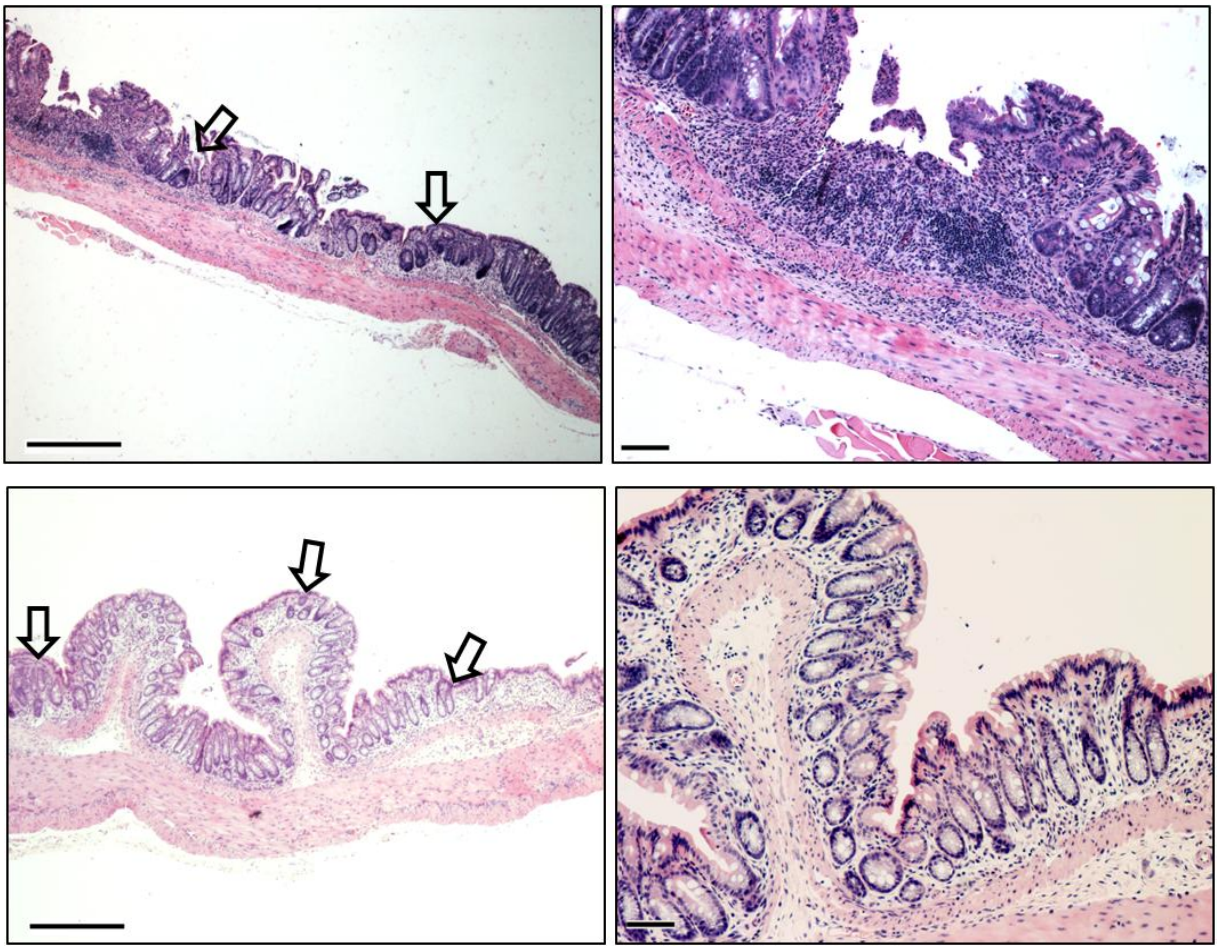


Figure 3.6 C: Representative photographs of H&E-stained sections of the distal colon at days 21. Both WT and SPARC KO mice colons continued to heal. Left panel-40x, Bar: 500µm; Right panel-100x, Bar:100 µm

Day 35

SPARC KO DSS

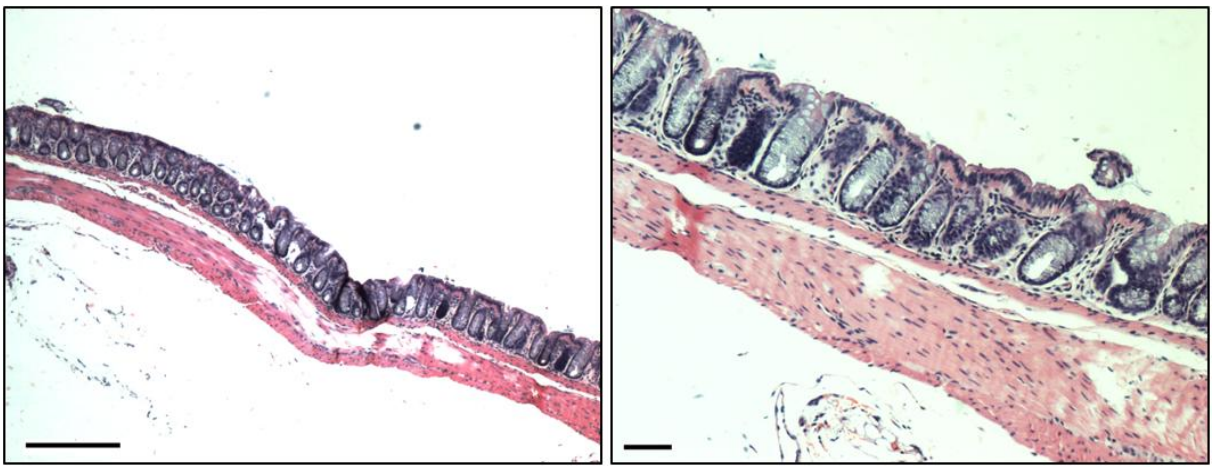


Figure 3.6 D: Representative photographs of H&E-stained sections of the distal colon of SPARC KO at day 35 where complete regeneration occurred Left panel-40x, Bar: 500µm; Right panel-100x, Bar:100 µm

3.2.2 Cytokine production by DSS treated mice during the periods of acute inflammation and recovery

To further investigate the impact of SPARC on the infiltration of inflammatory cells during the development of colitis, the production of selected cytokines within the colonic tissue was determined. IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines that are not only present during the early stage of inflammation but are also implicated in pathogenesis of IBD. The DSS-treated animals demonstrated significantly elevated levels of all 3 of these cytokines at day 7 when compared to the untreated animals. The production of IL-1β was also noted to be significantly higher ($p=0.025$) in the WT DSS-treated animals when compared to the DSS-treated SPARC KO mice at day 7 (***Figure 3.7 C***). The levels of all 3 cytokines fell rapidly following withdrawal of the DSS in both the WT and SPARC KO animals. WT DSS-treated animals, however, maintained a slightly higher mean concentration of these cytokines when compared to the SPARC KO mice. This trend is consistent with the MEICS (***Figure 3.3***) and histological scores (***Figure 3.5***) detailed above.

In an attempt to maintain homeostasis, regulatory or anti-inflammatory cytokines, such as IL-10 and TGF-β, are produced by the body to counteract the inflammatory reaction triggered by pro-inflammatory cytokines. The change in production of IL-10 in DSS-treated colons was similar to that observed for the pro-inflammatory cytokines and peaked at day 7 with a rapid decrease following withdrawal of the DSS. DSS-treated WT animals also demonstrated significantly higher ($p=0.034$) levels of IL-10 production than the untreated WT animals (***Figure 3.10 A***). No statistically significant trend was found between the DSS-treated WT and SPARC KO mice (***Figure 3.10 C***).

Despite the variation between animals, TGF-β1 was produced at significantly higher concentrations ($p=0.017$) by the DSS-treated SPARC KO animals when compared to the DSS-treated WT mice (***Figure 3.11 C***). This is in contrast to the trend observed for the other cytokines as detailed above.

The levels of IL-1β, IL-6 and TNF-α in the plasma of the DSS-treated mice were low (<10 pg/ml; data not shown), suggesting that DSS-induced colitis is a more localized inflammation (colonic) rather than a systemic inflammation. In contrast, the level of TGF-β1, and to lesser extend of IL-10, were abundantly present in the plasma, but no statistically significant differences were detected between the DSS-treated WT and SPARC KO animals.

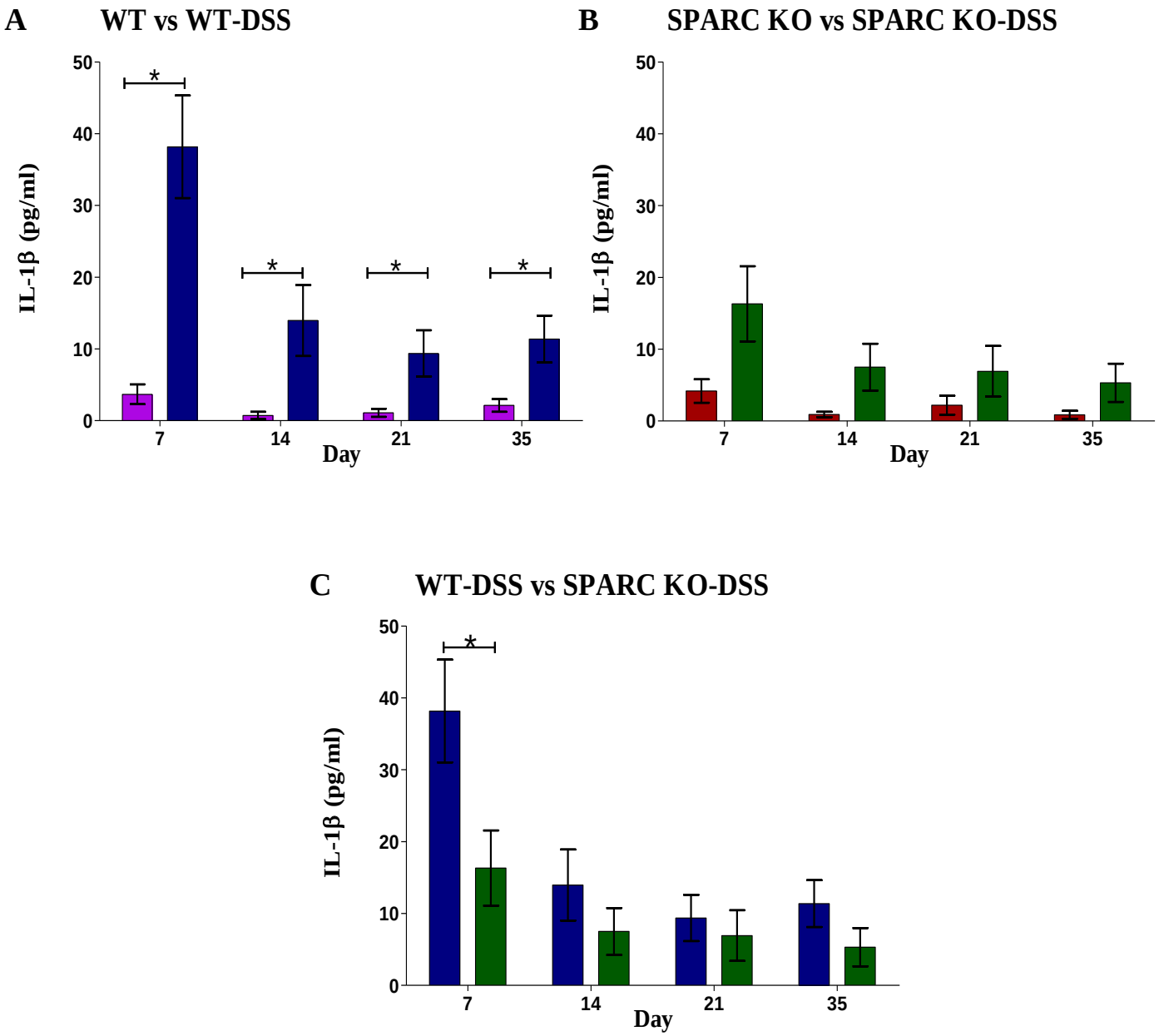


Figure 3.7: **IL-1β production in the colon of WT and SPARC KO mice measured with CBA.** (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. IL-1β production in the acute, chronic/recovery phases. Mean ± SEM (*n* =10-20 mice per group from 3 different experiments per time point) (*****p=0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

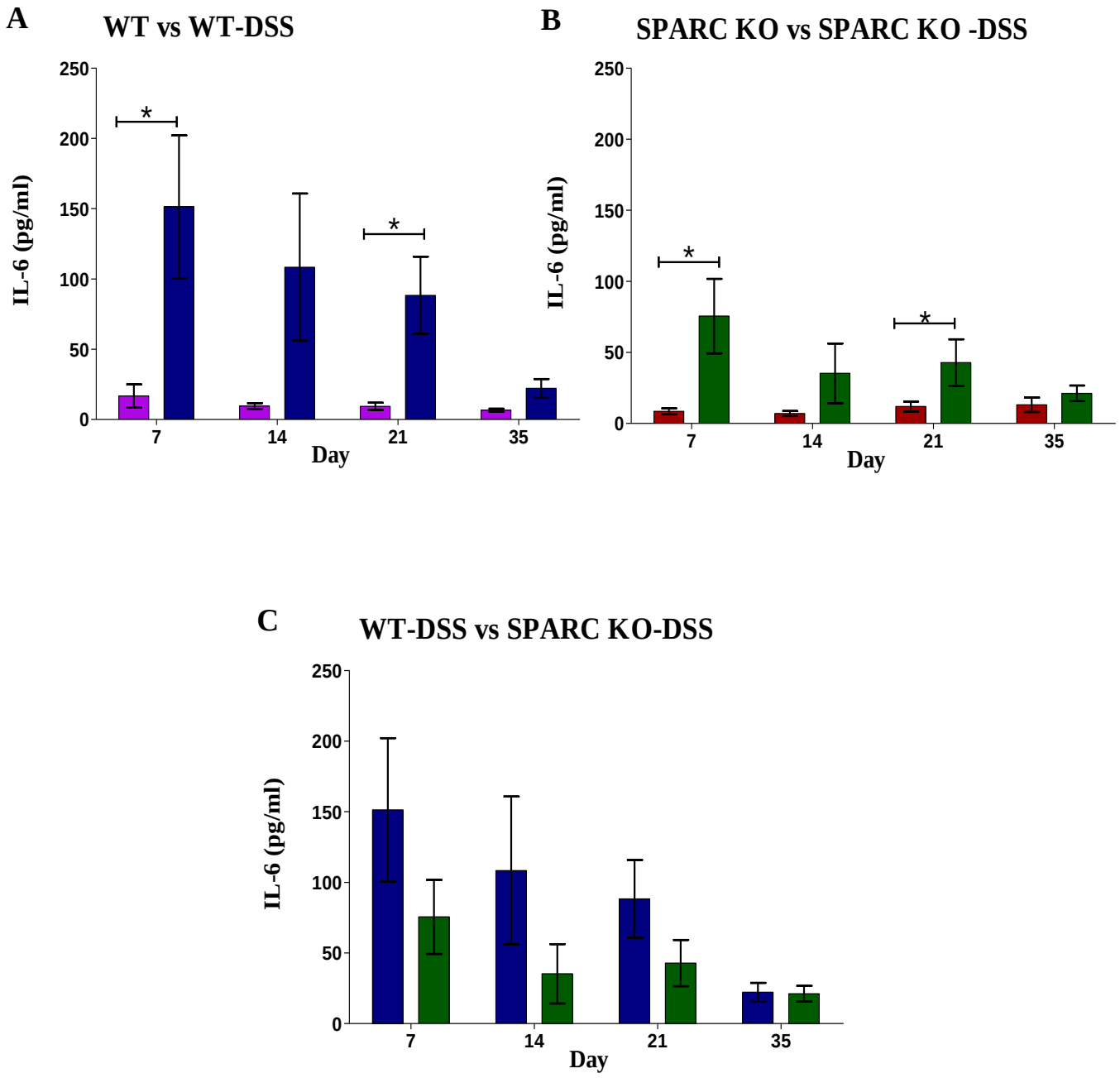


Figure 3.8: **IL-6 production in the colon of WT and SPARC KO mice measured with CBA.** (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. IL-6 production in the acute, chronic/recovery phases. Mean ± SEM (*n* =10-20 mice per group from 3 different experiments per time point) (*****p=0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

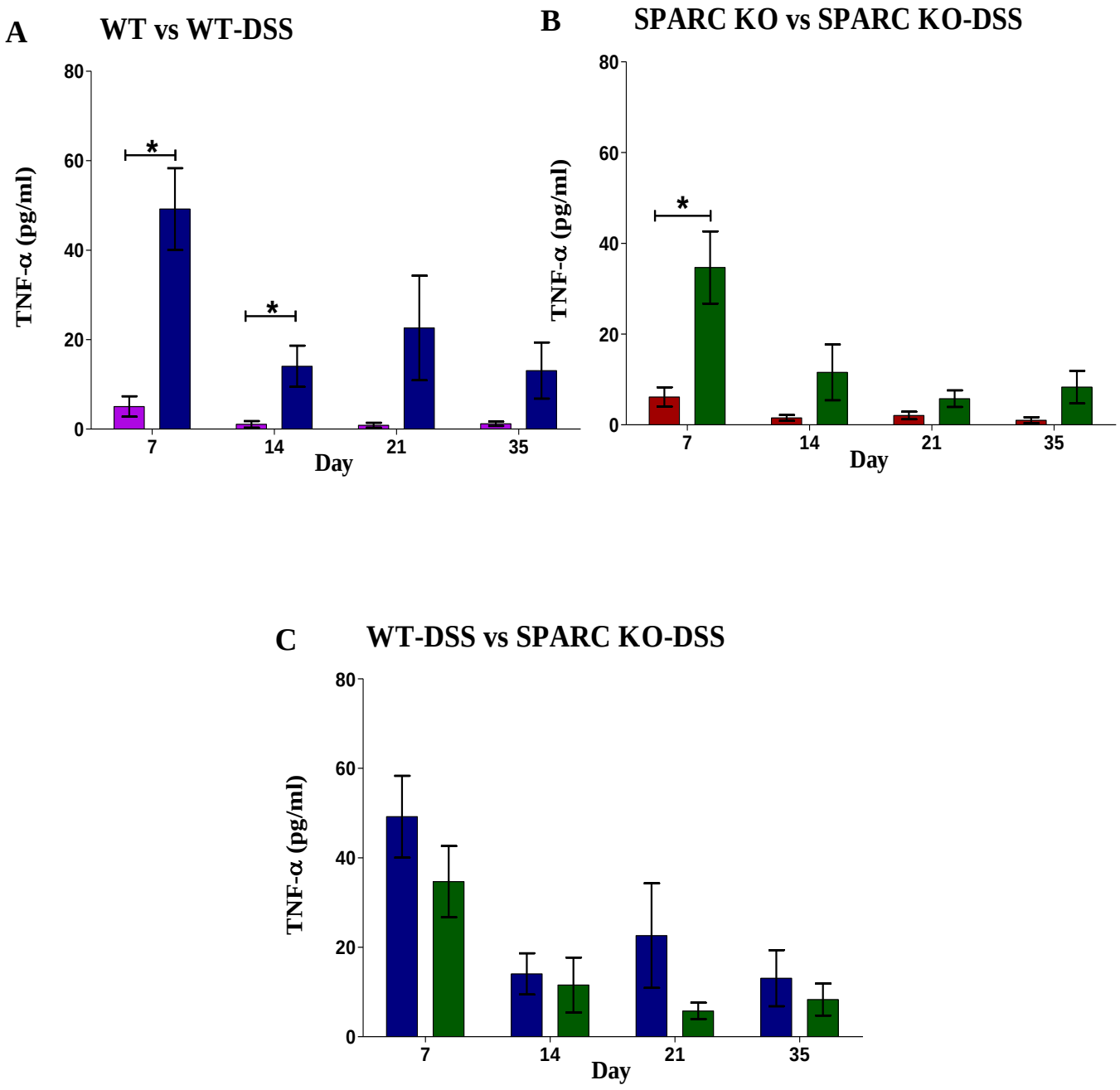


Figure 3.9: **TNF-α production in the colon of WT and SPARC KO mice measured with CBA.** (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. TNF-α production in the acute, chronic/recovery phases. Mean ± SEM (*n* =10-20 mice per group from 3 different experiments per time point) (*****p=0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

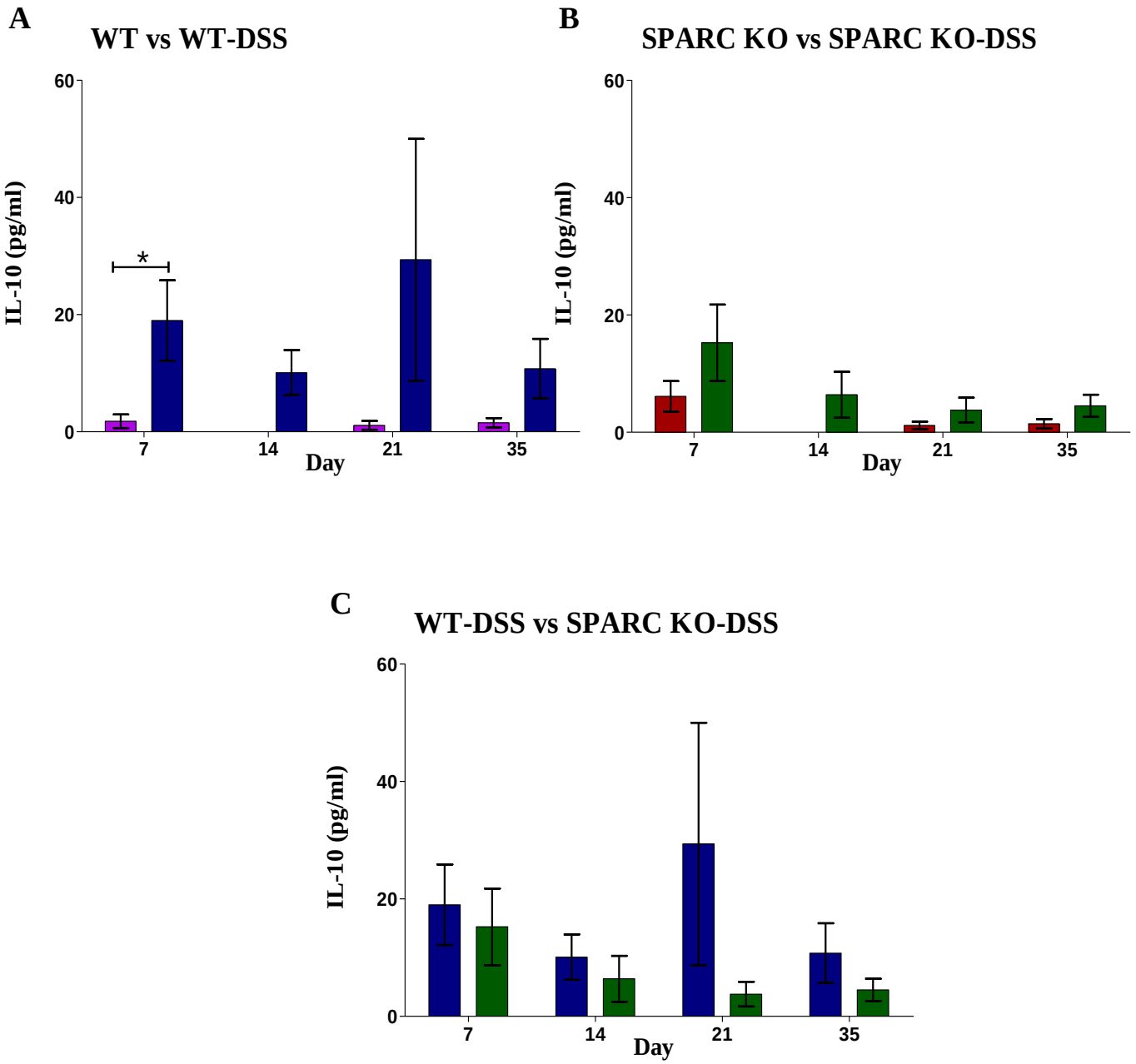


Figure 3.10: **IL-10 production in the colon of WT and SPARC KO mice measured with CBA.** (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. IL-10 production in the acute, chronic/recovery phases. Mean ± SEM (*n* =10-20 mice per group from 3 different experiments per time point) (***** p=0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

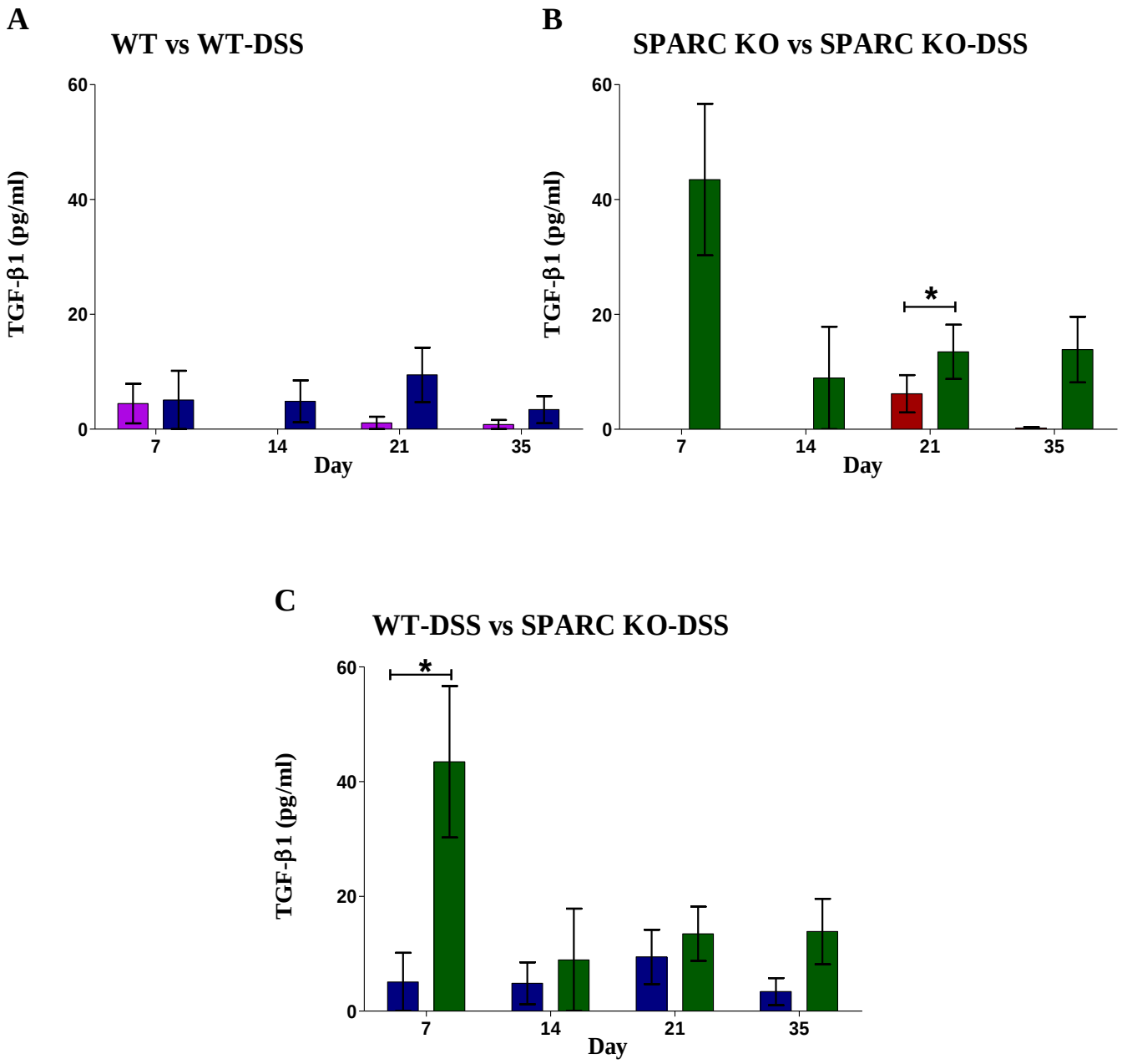


Figure 3.11: **TGF-β1production in the colon of WT and SPARC KO mice measured with ELISA.** (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. TGF-β1 production in the acute, chronic/recovery phases. Mean ± SEM (*n* =10-20 mice per group from 3 different experiments per time point) (***** p=0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

3.3 DISCUSSION

SPARC has been shown to play an active role in cell migration, ECM regulation and wound healing (Bradshaw and Sage 2001; Brekken and Sage 2000; Lane and Sage 1994; Latvala *et al.* 1996). A role for SPARC in colonic inflammation and pathologies, however, has not been studied extensively. There are conflicting conclusions in the literature regarding the role of SPARC in inflammation, suggesting that its function could be organ and model specific (Savani *et al.* 2000; Rempel *et al.* 2007; Socha *et al.* 2007). The widely used DSS-induced colitis model not only exhibits inflammatory and histopathological features that are similar to the human colitis, but it also can be used to analyse the patency and repair of the epithelial barrier, leucocytes behavior, lymphocyte responses and the production of both pro and anti-inflammatory cytokines as described in chapter 1(Dieleman *et al.* 1998; Dieleman *et al.* 1994; Hall *et al.* 2011; Wirtz *et al.* 2007). Thus, this model was chosen to investigate the role of SPARC in intestinal inflammation.

Ingestion of 3-10% DSS for 7-10 days has been shown to induce acute inflammation that depends on the susceptibility of the mice strain and the molecular weight of DSS (Okayasu *et al.* 1990; Melgar *et al.* 2005; Mahler *et al.* 1998; Wirtz and Neurath 2007). Chronic inflammation can also be achieved by the administered of 3-5 cycles of DSS in the drinking water with a period of 2 weeks without DSS between each DSS cycle (Okayasu *et al*. 1990). A single DSS cycle, however, is sufficient to induce chronicity in the C57BL/6 mice strain (Melgar *et al.* 2005) and the WT mice used in this study. The data presented in this chapter demonstrated that there is a potential pro-inflammatory role of SPARC in the colon in the DSS-induced model of acute colitis in mice. The clinical parameters of weight loss, colonic shortening and splenic enlargement were less

in the SPARC KO mice when compared to the WT mice and this was accompanied with significantly lower micro-endoscopic score ($p<0.001$). Several WT mice also developed a chronic, slow healing inflammation while majority of SPARC KO mice resolved the intestinal inflammation by day 35 as determined by endoscopy and lesser extend on histology. In a pilot study using a small number of mice (n=4) which received 2-3 cycles of DSS in order to induce chronic inflammation, SPARC KO mice also had less inflammation and lower MEICS than the WT mice (9 vs 5.5; $p=0.029$, data not shown).

In humans, endoscopy of the colon is an important diagnostic method for the investigation of colonic diseases. The use of the mouse micro-endoscopic system provides valuable and accurate information in the assessment and monitoring of intestinal inflammation and its resolution (Becker *et al.* 2005). The micro-endoscopic assessment of SPARC KO mice clearly demonstrated that they develop less inflammation when treated with DSS than the WT controls. The endoscopic scoring was more sensitive at detecting differences between the animals and was a better way to evaluate inflammation levels than the histology or the use of surrogate markers to evaluation disease activity such as weight loss and stool consistency.

Histopathologically, DSS-treated SPARC KO mice had lower histological score (total inflammatory score) and less crypt damage than WT mice. At day 7, both, WT and SPARC KO treated animals, showed similar levels of inflammation, but the SPARC KO mice had less severe inflammation at each subsequent time point. This suggests that the SPARC KO animals were able to recover more rapidly with a potentially faster healing rate although the regeneration sub-score did not detect a difference again suggesting that the histology scoring of colonic regeneration is difficult, complex and

not particularly sensitive. Regeneration could not be assessed during the peak of inflammation (day 7) but only from day 14 so it is possible that the peak time point of regeneration (perhaps day 9-11) may have been missed in the SPARC KO animals. The published effects of SPARC on wound healing appear to be contradictory, but this study is similar that of Bradshaw and colleagues where SPARC null mice demonstrated accelerated healing of 5 mm induced cutaneous wounds (Bradshaw *et al.* 2002). The anti-proliferative properties of SPARC (Brekken and Sage 2000) may cause a slower healing in the WT animals as re-epithelialisation usually started from the neighbouring epithelium as revealed by focal mitoses in neighboring epithelial cells bridging the lesions (Dieleman *et al.* 1998). SPARC KO animals healed faster than the WT and this could also be related to SPARC inhibiting the function of PDGF, VEGF, and basic fibroblast growth factor which are important mediators in healing. It has been showed that the dermal structure in SPARC KO mice is difference to WT animals with smaller collagen fibrils suggesting a defect in collagen bundling in the absence of SPARC. Similar findings were also observed in the colon (Chapter 5) again suggesting that the less structured ECM allows for a faster cellular infiltrate and improved healing (Bradshaw, Puolakkainen, *et al.* 2003; Bradshaw and Sage 2001).

Cytokines are important immunoregulatory modulators of IBD pathology. The colonic explants used in this study allowed evaluation of a wide array of cytokines that were produced by the whole intestine under inflammatory conditions (Siegmund *et al.* 2002; Suzuki *et al.* 2001). The WT mice had greater tissue damage than SPARC KO and this might due to the relatively greater local production of the pro-inflammatory cytokines. One of roles of SPARC is to promote cell migration (Basu *et al.* 2001; Yan and Sage 1999). SPARC allows for enhanced migration of macrophages, neutrophils and

epithelial cells and it is primarily from these cells that pro-inflammatory cytokines IL-6, IL-1β and TNF-α derived (Melgar *et al.* 2005). The DSS-treated SPARC KO animals had significant lower levels of IL-1β ($p=0.025$), which has been suggested to be involved in the initiation and perpetuation of colitis (Youngman *et al.* 1993). The inflammation in IBD patients and the experimental models of colitis are associated with increased levels of IL-1β which is thought to be secreted by the macrophages activated within the mucosa and sub-mucosa (Kwon *et al.* 2005; Guimbaud *et al.* 1998).

IL-6 works synergistically with IFN-γ to induce B cell differentiation (Jego *et al*. 2003), and IL-6 has been showed to mediate the resistance of T cells to apoptosis in CrD and is thus able to contribute to the perpetuation of chronic intestinal inflammation (Atreya *et al.* 2000). In contrast, IL-6 deficient mice developed less severe colitis when submitted to the DSS model of colitis (Suzuki *et al.* 2001). The level of IL-6 in SPARC KO was lower than the WT animals and although the differences were not statistically significant. This may be a reason behind the differences observed in the levels of inflammation between the animal groups and remains to be confirmed statically in more detailed experiments. It might also indicate that specific targeting of the SPARC-IL-6/IL-1β pathway may be a promising new approach for the treatment of CrD.

As mentioned earlier, DSS disrupts the epithelial cell barrier and increases exposure to luminal antigens which results in inflammation that involves PMN, macrophages, B and T cells as major sources of the pro-inflammatory cytokines. These cytokines then cause more tissue damage later, hence, recruitment of leucocytes to the intestine seems to be the key event in the DSS induced colitis (Abdelbaqi *et al.* 2006; Chidlow *et al.* 2009; Stevens *et al.* 1992) and this will be investigated in Chapter 4.

The SPARC KO animals were found to have higher TGF-β1 levels which contrasted with the report by Socha and colleagues in which, had less expression of TGF-β in the kidney in the absence of SPARC (Socha *et al*. 2007). It is unclear why more TGF-β is observed in the SPARC KO colon than the WT, but it may be due to the unique immune adaptations of the colon which is colonised with great number of Treg cells that able to modulate activity of the immunosuppressive TGF-β. In addition, it has been shown that the intestinal mucosa promotes epithelial restitution after injury through the increased production of bioactive TGF-β1 by the epithelial cells (Dignass and Podolsky 1993) and subepithelial myofibroblasts (McKaig *et al*. 1999). Hence, the increase in TGF-β1 may have contributed to the maintenance of intestinal barrier integrity and intestinal healing in the SPARC KO mice that experienced less tissue destruction.

3.4 CONCLUSION

The investigations here have shows that SPARC appears to have pro-inflammatory properties in the DSS-induced model of colonic inflammation thereby delaying healing. This is most likely due to roles in cell migration and regulation of the ECM, which subsequently modulate cellular infiltration and the secretion of the pro-inflammatory cytokines. Therefore, the cellular components which include cells of the innate and adaptive immune responses will be investigated in the next chapter.

Chapter ***4***

The roles of SPARC in the innate and adaptive immune system in DSS colitis

4.1 INTRODUCTION

Both the innate and adaptive immune systems in the gut have developed unique characteristics in order to maintain homeostasis in this antigen-rich environment (Murphy 2008; Schenk and Mueller 2007). The intestinal wall is populated with immune cells (e.g. intestinal macrophages) that eliminate invading pathogens without inducing an over response to the commensal microorganisms located in the lumen (Qualls *et al.* 2006). There is also an enhanced frequency of the presence of regulatory T cell (Treg) subsets that are characterised by the production of the potent anti-inflammatory cytokines, TGF-β and IL-10 (Izcue *et al.* 2009; Groux *et al.* 1997). IEL that may potentially exert immunoregulatory functions are found abundantly, and strategically positioned, in the LP (Newberry and Lorenz 2005). There is ample evidence to suggest that disruption of the immune regulatory networks within the intestine may result in IBD. These include abnormalities in the function of the intestinal epithelial cell barrier, excessive production of Th1, Th2 or Th17 cytokines, and dysregulated activation of the intestinal mucosal inflammatory cells by normal luminal bacteria (Fiocchi 1998; Strober *et al.* 2002; Fujino *et al.* 2003; Su *et al.* 2009; Izcue *et al.* 2009; Uguccioni *et al.* 1999; Sanchez-Munoz *et al.* 2008).

So far, no studies have been done to investigate the role of SPARC on immunity in the GIT despite its properties as a matricellular protein, which have been shown to modulate numerous cell functions such as cell proliferation and migration as well as ECM regulation (Yan and Sage 1999; Tremble *et al.* 1993; Tai and Tang 2008). As SPARC may play an important role in regulating the immune balance within the GIT it could also play a role in the pathogenesis of IBD.

The effects of SPARC could result in differences in the recruitment and activation of inflammatory cells across different systems. Savani and colleagues showed that SPARC KO mice had increased neutrophil and leucocyte infiltration in an intratracheal bleomycin-induced lung injury model (Savani *et al*. 2000). In a mouse footpad swelling model, SPARC null mice also exhibited an altered immune system with decreased numbers of neutrophils and B cells but increased numbers of T cells, and these animals were unable to mount an innate immune response to the bacterial product LPS (Rempel *et al*. 2007). In the absence of SPARC, however, T cell priming was enhanced in a murine model of contact hypersensitivity due to the accelerated migration of DCs to the draining lymph nodes (Sangaletti *et al*. 2005).

As discussed in the previous chapter, the increase in production of pro-inflammatory cytokines by infiltrating inflammatory cells in WT mice may be the main driver of the tissue damage observed in DSS-induced colitis. Cytokines are important mediators of both the innate and adaptive arms of the immune responses in mucosal inflammation (Dignass and Podolsky 1993). Cytokines also play a key role in IBD as they determine T cell differentiation of Th1, Th2, T regulatory and Th17 cells (Sanchez-Munoz *et al.* 2008). In addition to cytokines, chemokines and their receptors contribute to the regulation of intestinal immune responses and mucosal inflammation (Tokuyama *et al*. 2005) and monokines induced by IFN-γ (MIG) and RANTES (regulated on activation normal T cell expressed and excreted) are elevated in the intestine of IBD patients (Danese and Gasbarrini 2005).

Identification of the different subsets of immune cells such as the Th lymphocytes, neutrophils and macrophages within the intestinal mucosa is vital and will be investigated in this chapter as will the expression of key cytokines and chemokines.

These findings will allow us to further determine the role that SPARC may play in cell recruitment in the DSS-induced model of colitis.

4.2 RESULTS

4.2.1 Spleen to body weight ratios in WT and SPARC KO mice

Splenic enlargement was used as one marker for the severity of DSS-induced colitis. The spleen wet weight was recorded and normalised to the body weight to determine the systemic effect of DSS treatment.

In control animals given plain tap water to drink, there was no statistical difference in the ratio of the spleen wet weight to the body weight between WT and SPARC KO mice at all time points except at day 35 (0.49 vs 0.37, p=0.017; data not shown). Following treatment with DSS, both WT and SPARC KO mice had an increased spleen/body weight ratio (***Figure 4.1; Figure 4.2 A and B***). The WT mice, however, had an approximately 2 to 4 fold greater increase in this ratio than KO mice. This was particularly noted at the time of maximal inflammation on days 7 (1.01 vs 0.61; $p<0.01$) and 21 (0.92 vs 0.60; p=0.037), where DSS-treated WT mice had a statistically larger spleen to body weight ratio (***Figure 4.2 C***). A similar trend was observed throughout the experiment, from the acute through to the recovery, or chronic, phase of disease.

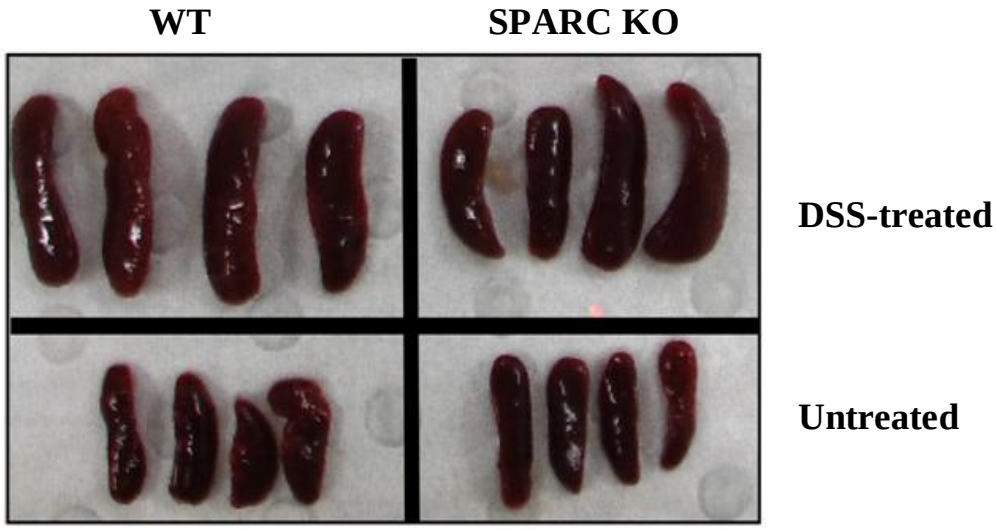


Figure 4.1: Spleen size in WT and SPARC KO mice after 7 days of DSS treatment

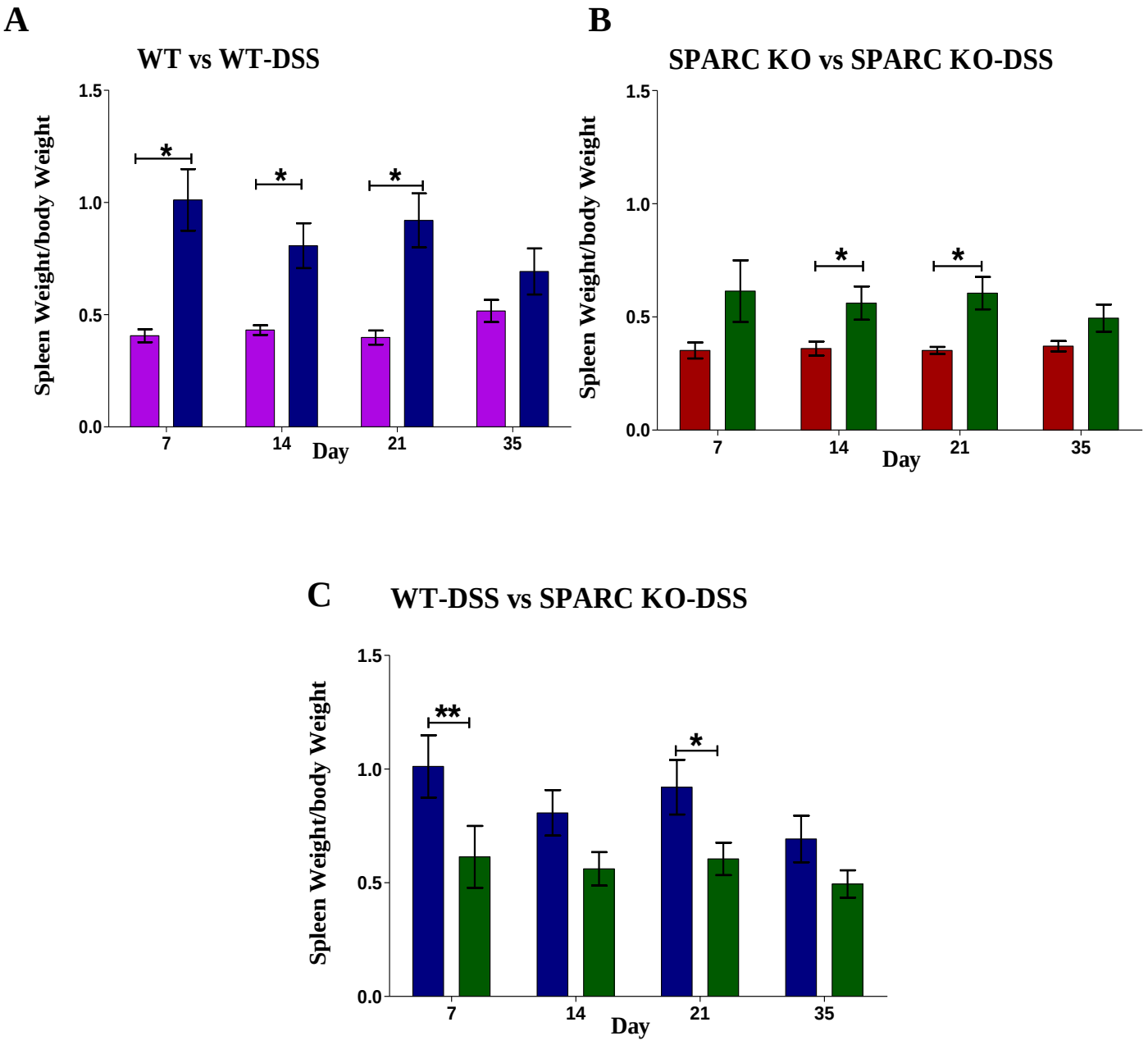


Figure 4.2: Wet spleen weight was measured at sacrifice and expressed as ratio to body weight after DSS treatment. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Ratio was expressed as a mean ± SEM based on 10-15 mice per time point from 3 different experiments. Statistical values using Student's unpaired t-test (*$p<0.05$; **$p<0.01$). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

4.2.2 CD4+ T cell changes in WT and SPARC KO mice

It has been suggested that an alteration in the CD4+ T cell response is central to the immunological pathogenesis of IBD. In order to determine if the CD4+ T cell subset differs between WT and SPARC KO following DSS administration, T cells in the MLN and the spleen were analysed by flow cytometry. Attempts were made to analyse the T cell subtypes in the colon, however, the yield of cells varied between the treatment groups and despite pooling cells from several animals, insufficient numbers were obtained for reliable analysis.

4.2.2.1 Total Lymphocytes

When compared to the control animals, the percentage of lymphocytes in the spleen was reduced at day 14 and 21 in DSS-treated WT mice (***Figure 4.3 A***). This was in contrast to SPARC KO animals, where the percentage lymphocytes did not change with DSS treatment (***Figure 4.3 B***). It was observed that the SPARC KO animals had a significantly higher percentage of lymphocytes than WT mice in the spleen at days 7 ($p=0.022$) and 14 ($p<0.01$) following DSS treatment (***Figure 4.3 C***).

In the local draining lymph nodes, there were no differences observed at any time point between the WT controls and the DSS-treated WT animals. There were also no differences in the percentage of lymphocytes following DSS treatment in the SPARC KO mice compared to their untreated controls except on day 14 where a significant increase was noted ($p=0.028$) (***Figure 4.4 B***).

Spleen

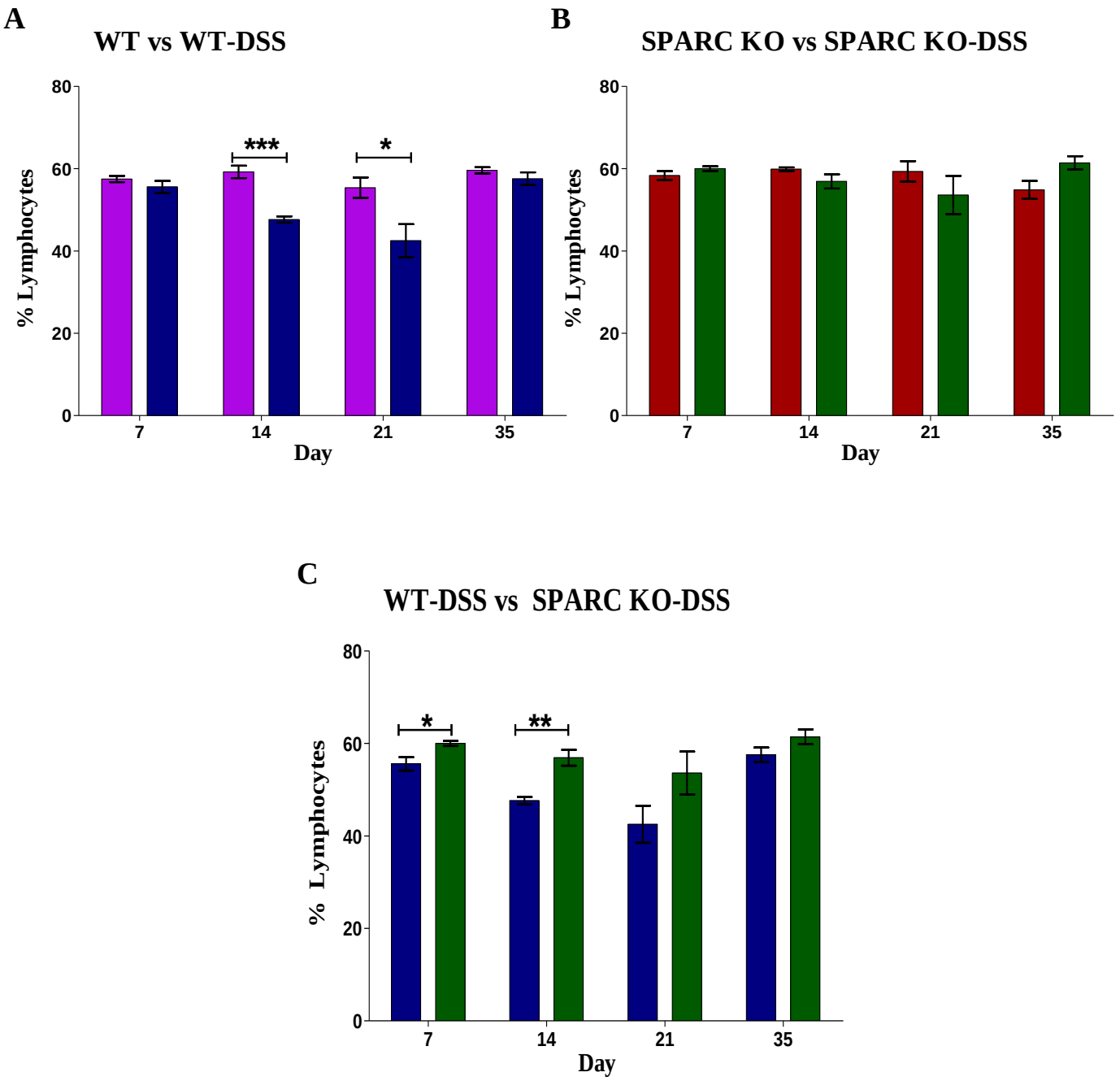


Figure 4.3: Percentage of total lymphocytes in spleen following DSS treatment. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 and determined by flow cytometry. Data represent the mean percentage of lymphocytes. ± SEM from 3 independent experiments each consisting of 4-5 animals; * $p<0.05$; **$p<0.01$; *** $p<0.001$). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

MLN

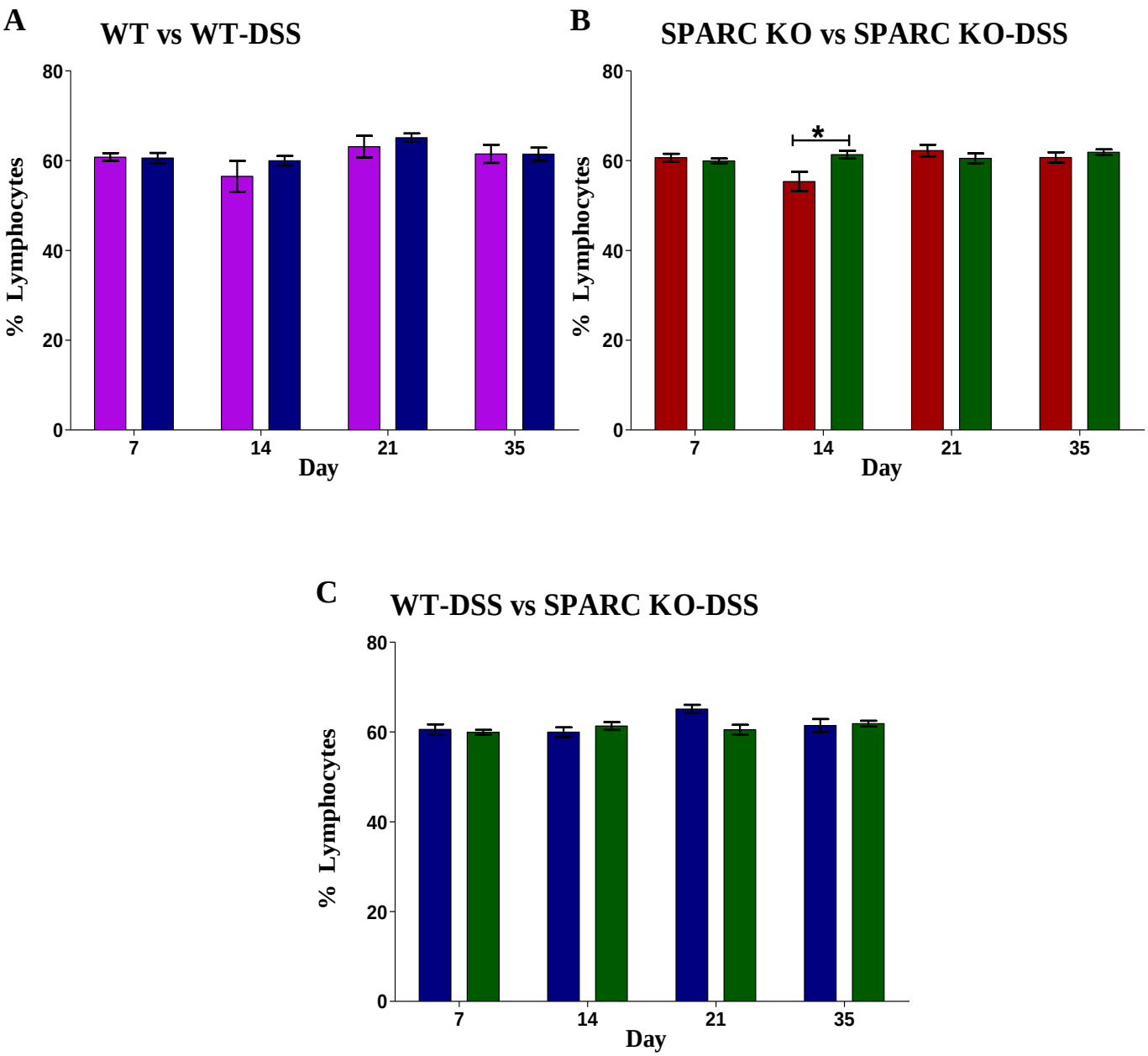


Figure 4.4: Percentage of total lymphocytes in MLN following DSS treatment. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 and determined by flow cytometry. Data represent the mean percentage of lymphocytes. ± SEM from 3 independent experiments each consisting of 4-5 animals; WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

4.2.2.2 CD4+ T lymphocytes

The lymphocytes were stained for surface markers, CD3 and CD4, to define the CD4+ T lymphocytes or Th cells. The cells were analysed in order to determine if SPARC impacts on this cell population. There was a lower percentage of CD4+ lymphocytes in the DSS-treated groups compared to the untreated groups in both tissues (***Figure 4.5 and 4.6 A and B***). The SPARC KO DSS-treated mice, however, had a higher percentage of CD4+ T lymphocytes in the spleen relative to the WT DSS-treated mice, which was significantly different at day 7 ($p=0.017$) (***Figure 4.5 C***). There were no statistical differences in the percentage of Th lymphocytes in the MLN (***Figure 4.6 B***).

4.2.2.3 CD4+ T lymphocyte subsets

As differences were found in the percentage of CD4+ T lymphocytes further investigations were performed to determine which subset of CD4+ T lymphocytes were associated with the DSS-induced colitis. The isolated leucocytes were stimulated *in vitro* and stained to identify intracellular IFN-γ, IL-4 and IL-17A by flow cytometry.

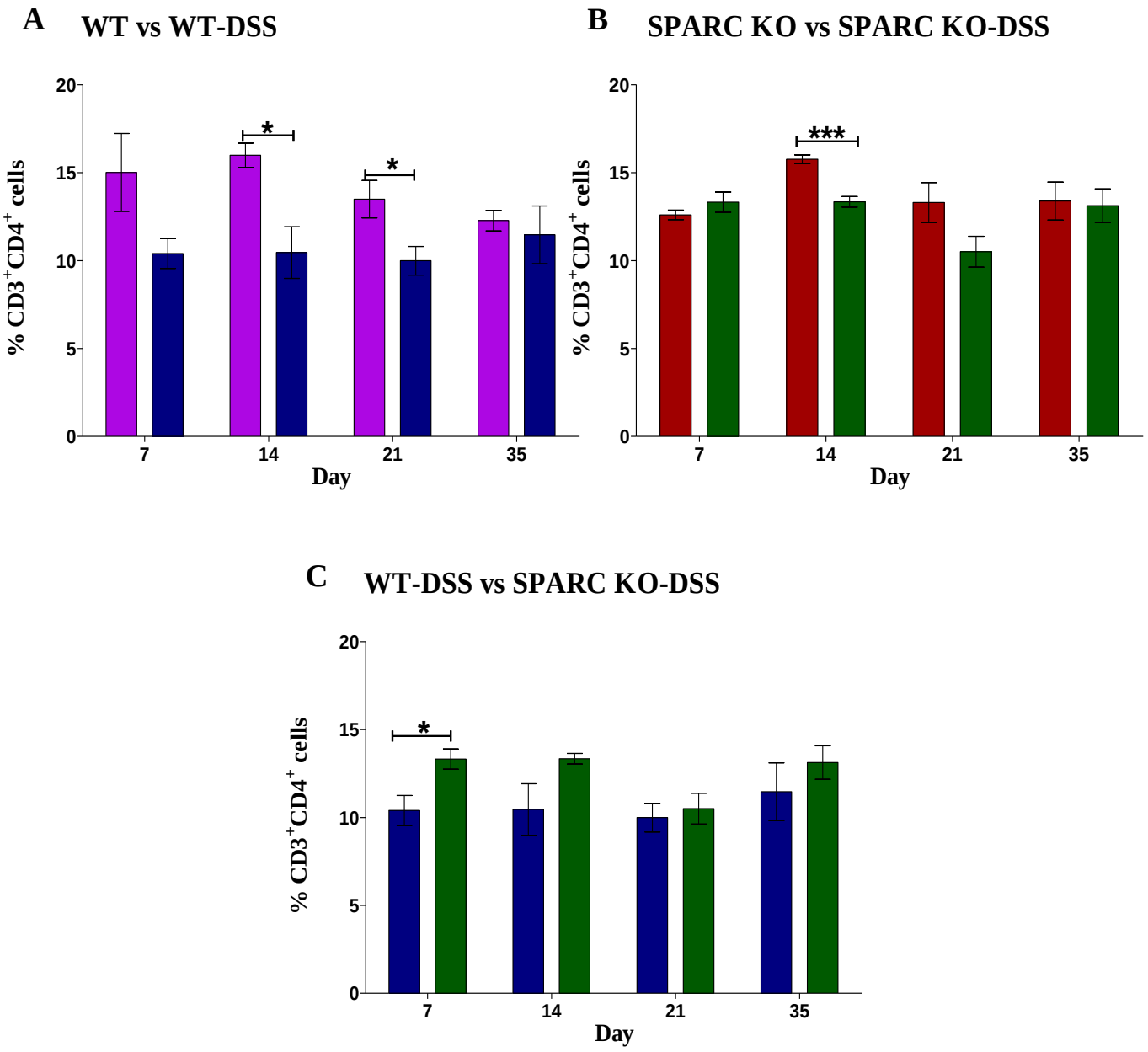
DSS treatment reduced the number of IFN-γ+ expressing CD4+ T lymphocytes in both WT and SPARC KO mice when compared to the untreated animals in the spleen ***(Figure 4.7 A and B***). The SPARC KO DSS-treated animals had a higher percentage of IFN-γ+ producing CD4+ T lymphocytes in the spleen than the WT-DSS treated mice at most time points and this difference was statistically significant ($p=0.035$) at day 14 ***(Figure 4.7 C***). The percentage of IFN-γ producing cells located in the MLNs did not statistically differ between the DSS-treated and untreated groups or between WT and SPARC KO mice ***(Figure 4.8)***.

The IL-4+ and IL-17A+ expressing cells were present in low levels in all animals but with no differences detected between the groups (data not shown). These findings, however, suggest that both WT and SPARC KO mice mounted a Th1 inflammatory response when treated with DSS as none of the other Th subset specific cytokines were able to be detected.

Treg cells are important in maintaining gut immunity (Izcue *et al.* 2009) and high levels of TGF-β1 were found following DSS treatment of the SPARC KO animals. TGF-β plays a role in the Treg-mediated inhibition of systemic and mucosal immune responses. The presence of these cells was measured by flow cytometry for intracellular FoxP3 following DSS-induced colitis.

The percentage of Treg cells (CD4+CD25+FoxP3) was higher in the healthy SPARC KO mice compared to the WT animals in both the spleen (13.2 vs 16.8; $p<0.01$) and MLNs (8.55 vs 11.14; $p=0.013$) (data not shown). Following the induction of colitis, this increase was still present and continued to be observed at day 14 in both the spleen ($p<0.01$) (***Figure 4.9 C)*** and MLN ($p<0.01$) ***(Figure 4.10 C***); and day 21 in the spleen ($p<0.01$) (***Figure 4.9 C***).

Spleen



.

Figure 4.5: Percentage of CD4+ T lymphocytes in the spleen. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 following DSS treatment and determined by flow cytometry. Data represent the mean percentage of CD3+CD4+ (Th lymphocytes) ± SEM from 3 independent experiments each consisting of 4-5 animals; *p<0.05; ***p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

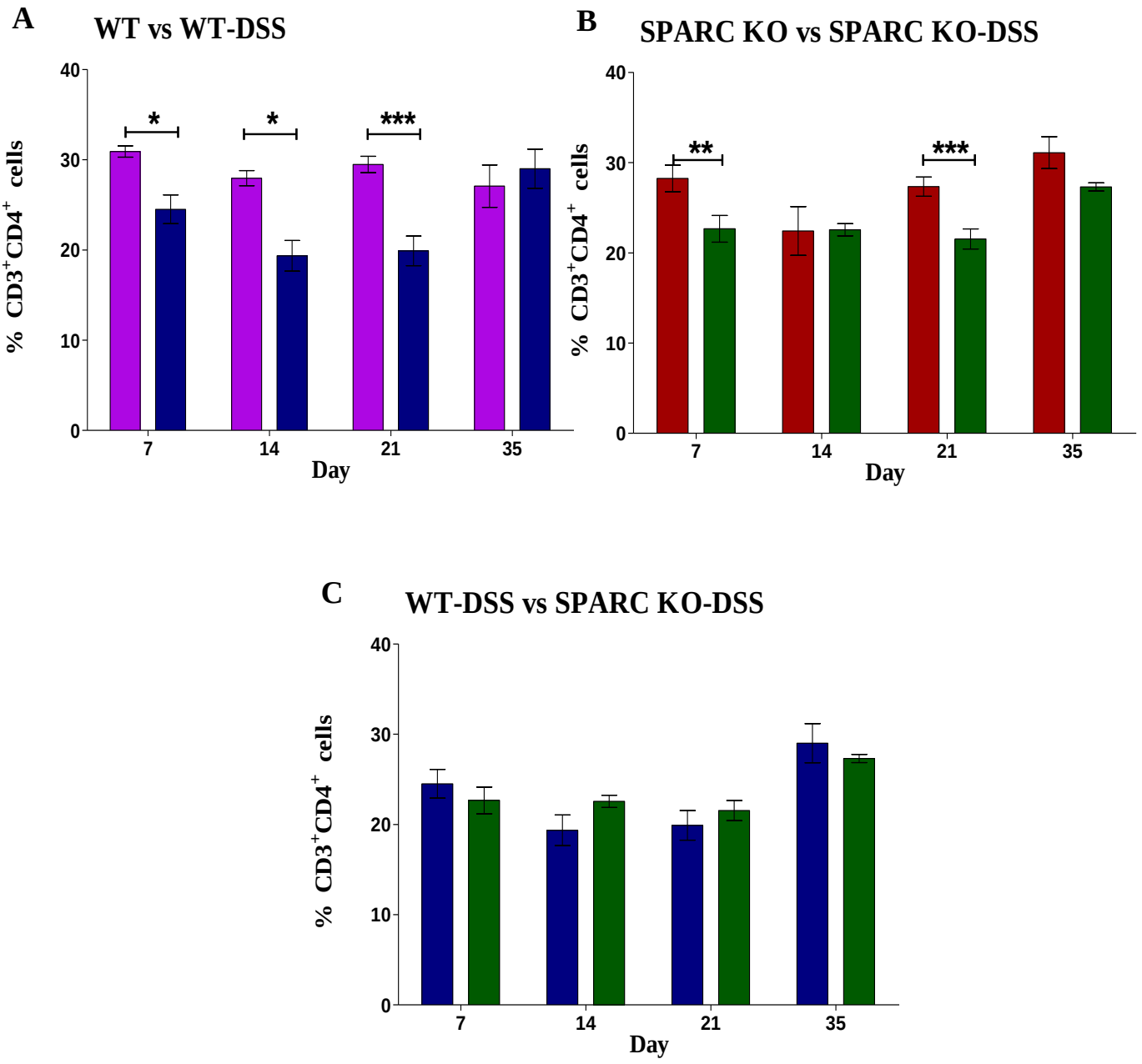


Figure 4.6: Percentage of CD4+ T lymphocytes in the MLN. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 following DSS treatment and determined by flow cytometry. Data represent the mean percentage of CD3+CD4+ (Th lymphocytes) ± SEM from 3 independent experiments each consisting of 4-5 animals; *p<0.05; **p<0.01; ***p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

Spleen

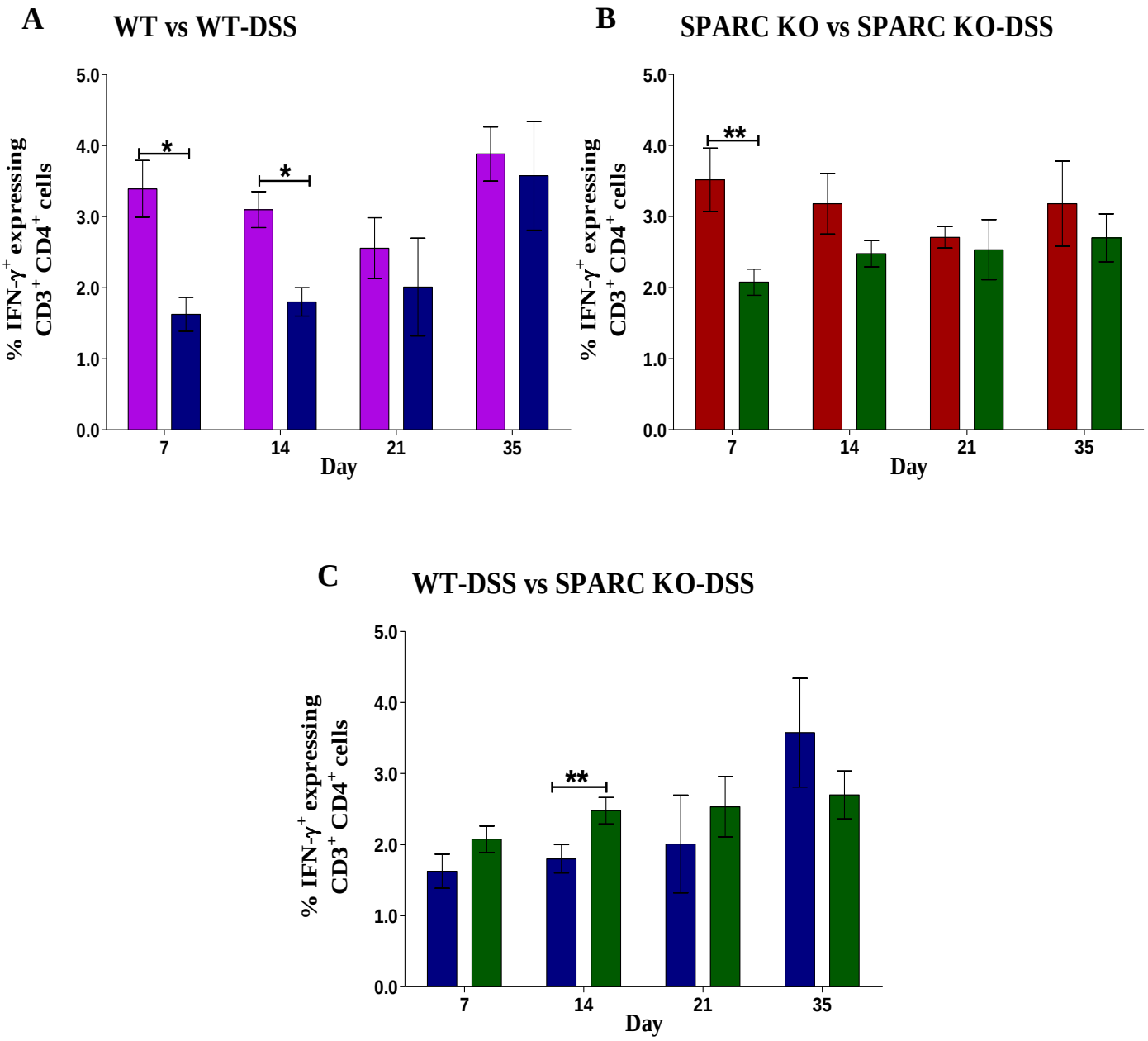


Figure 4.7: Percentage of CD3+CD4+ cells expressing IFN-γ in the spleens. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 following DSS treatment and determined by flow cytometry. Data represent the mean percentage of CD3+CD4+ (Th lymphocytes) ± SEM from 3 independent experiments each consisting of 4-5 animals; *p<0.05; **p<0.01). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

MLN

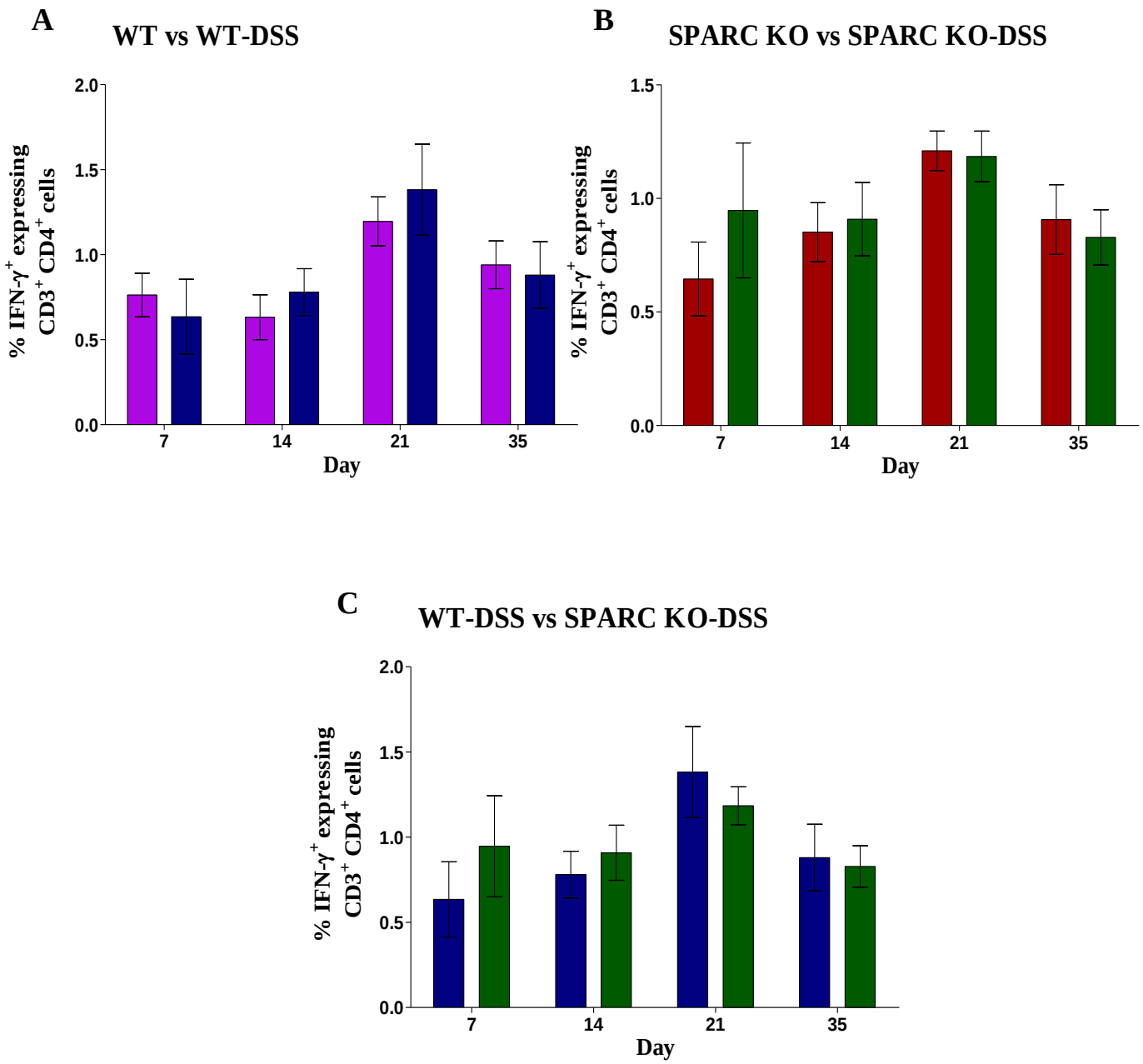


Figure 4.8: Percentage of CD3+CD4+ cells expressing IFN-γ in the MLN. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 following DSS treatment and determined by flow cytometry. Data represent the mean percentage of CD3+CD4+ (Th lymphocytes) ± SEM from 3 independent experiments each consisting of 4-5 animals; WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

Spleen

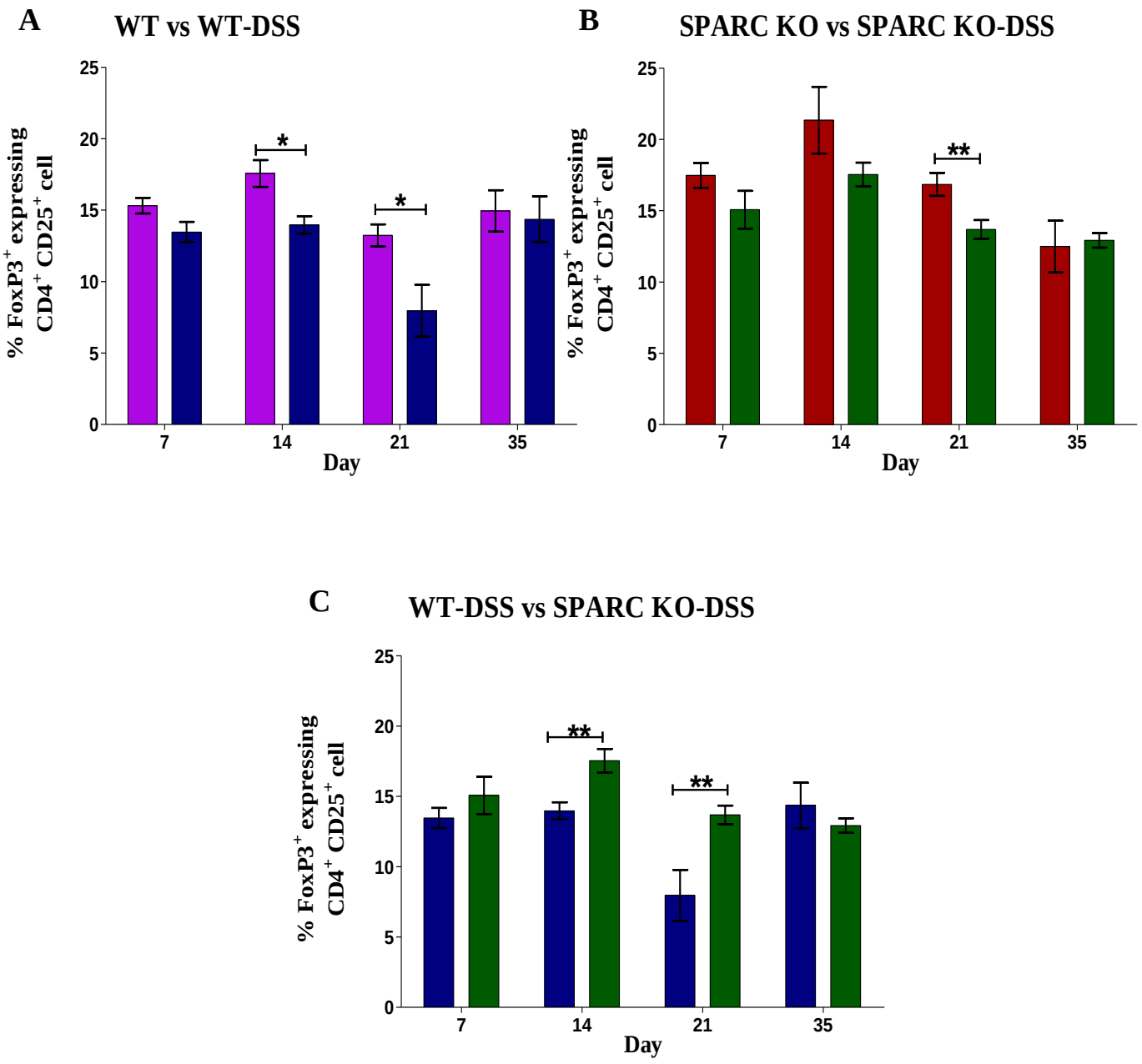


Figure 4.9: Percentage of Treg cells (CD4+CD25+FoxP3+) in the spleen. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 following DSS treatment and determined by flow cytometry. Data represent the mean percentage of CD4+CD25+FoxP3+ ± SEM from 3 independent experiments each consisting of 4-5 animals; *p<0.05; **p<0.01). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

MLN

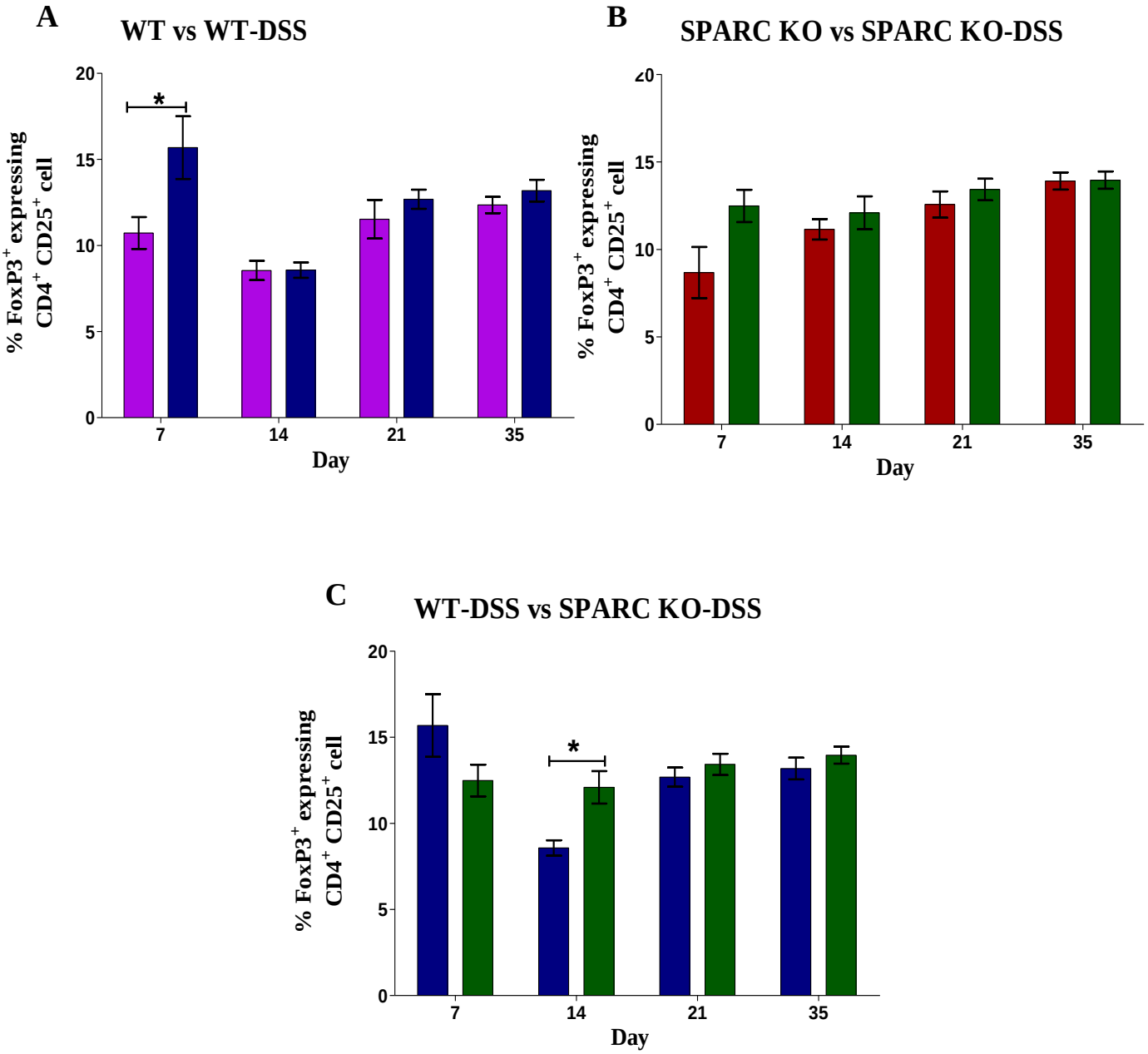


Figure 4.10: Percentage of Treg cells (CD4+CD25+FoxP3+) in the MLN. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Cells were isolated from spleen and MLN on day 7, 14, 21, and 35 following DSS treatment and determined by flow cytometry. Data represent the mean percentage of CD4+CD25+FoxP3+ ± SEM from 3 independent experiments each consisting of 4-5 animals; *p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

4.2.3 The cytokine profile in the colon of WT and SPARC KO mice

As each subset of CD4+ T cells produce a specific set of cytokines. CD4+ T cells have been classified into distinct subsets namely Th1, Th2, and Th17 and depending on which cytokines are produced, different immune responses can be generated (Agnello *et al.* 2003; Brand 2009; Rotteveel *et al.* 1988). The panels of cytokines to be measured were chosen as they have been implicated in the pathogenesis of IBD, and these were also used to subcharacterise the CD4+ T cells located within the colon after unsuccessful attempts to isolate enough cells from the colons for flow cytometric analysis. The cytokines secreted from the colon were measured without *ex vivo* stimulation with a CBA kit.

DSS treatment increased the production of IFN-γ in the colon in both WT and SPARC KO mice when compared to untreated animals (***Figure 4.11 A***). DSS-treated SPARC KO animals had a higher mean level of IFN-γ secretion compared to WT DSS-treated mice. This was noted during both the acute phase of inflammation and following the withdrawal of DSS (day 7-14) with a gradually decrease over time (***Figure 4.11 A***), but it was not statistically higher due to the high variability between the samples.

Consistent with the flow cytometric analyses, no IL-4 producing lymphocytes were detected either in the spleen or MLNs. This was also the case in the colon where no IL-4 production was detected (***Figure 4.11 B***). There were, however, detectable levels of the other Th-2 related cytokines, IL-5 (***Figure 4.11 C***) and IL-13 (***Figure 4.11 D***). Due to the high inter-individual variation, however, these did not reach statistical significance between the untreated and DSS-treated groups. IL-17A production was elevated following the administration of DSS in the WT animals (day 21 $p=0.035$; day 35

p=0.049; ***Figure 4.12 A)***. No significant differences were observed in between the DSS-treated WT and SPARC KO animals (***Figure 4.12 C)***.

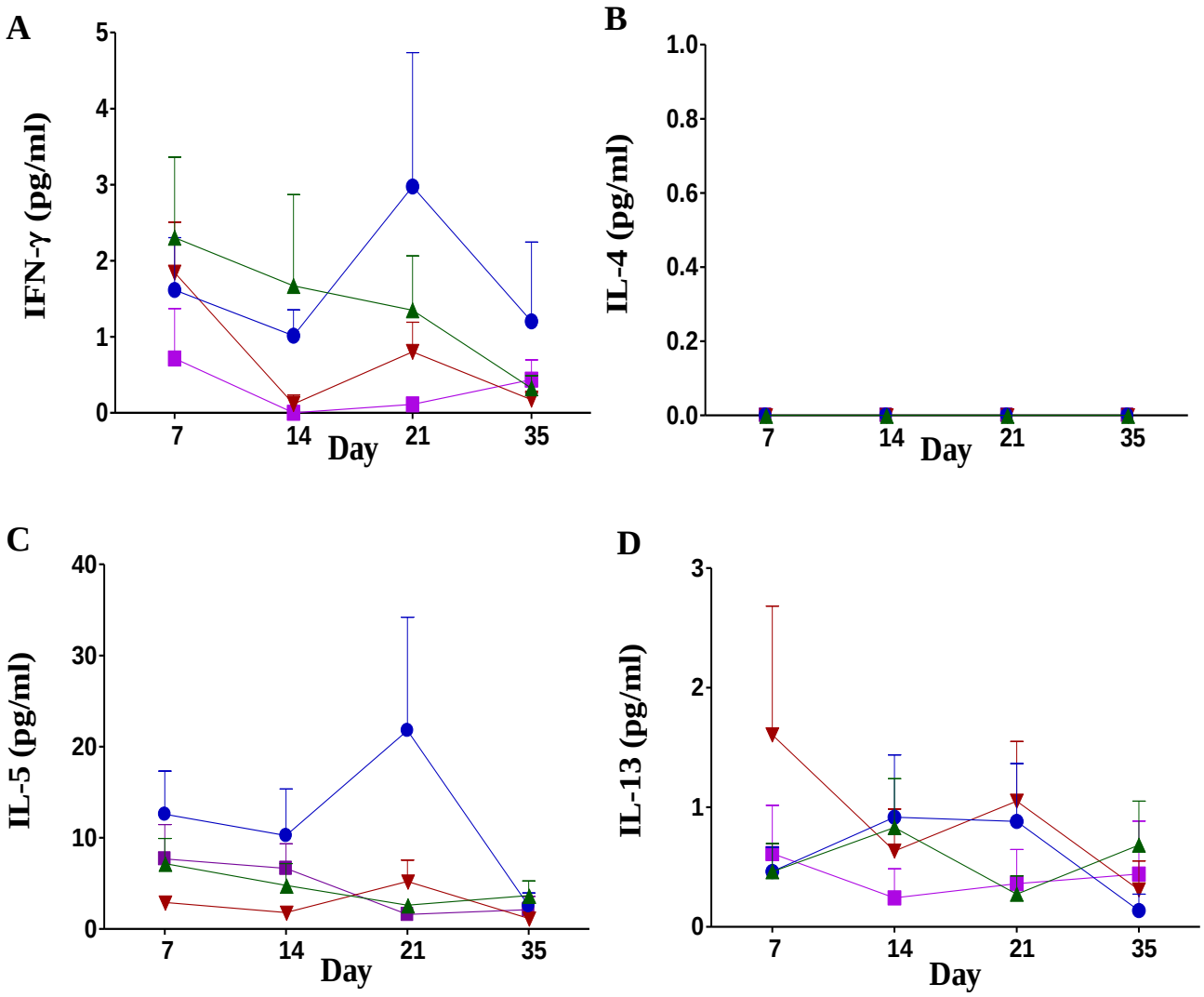


Figure 4.11: Th1 (IFN-γ) and Th2 related cytokines secretion of the colon during the acute and chronic/recovery phases in WT and SPARC KO mice measured with CBA. A) IFN-γ B) IL-4, C) IL-5, D) IL-13 production in the acute, chronic/recovery phases in SPARC WT and KO mice. Mean ± SEM (*n* =12 to mice per group from 3 different experiments per time point). ■ WT untreated; ▼ SPARC KO untreated; ● WT DSS-treated; ▲ SPARC KO DSS-treated.

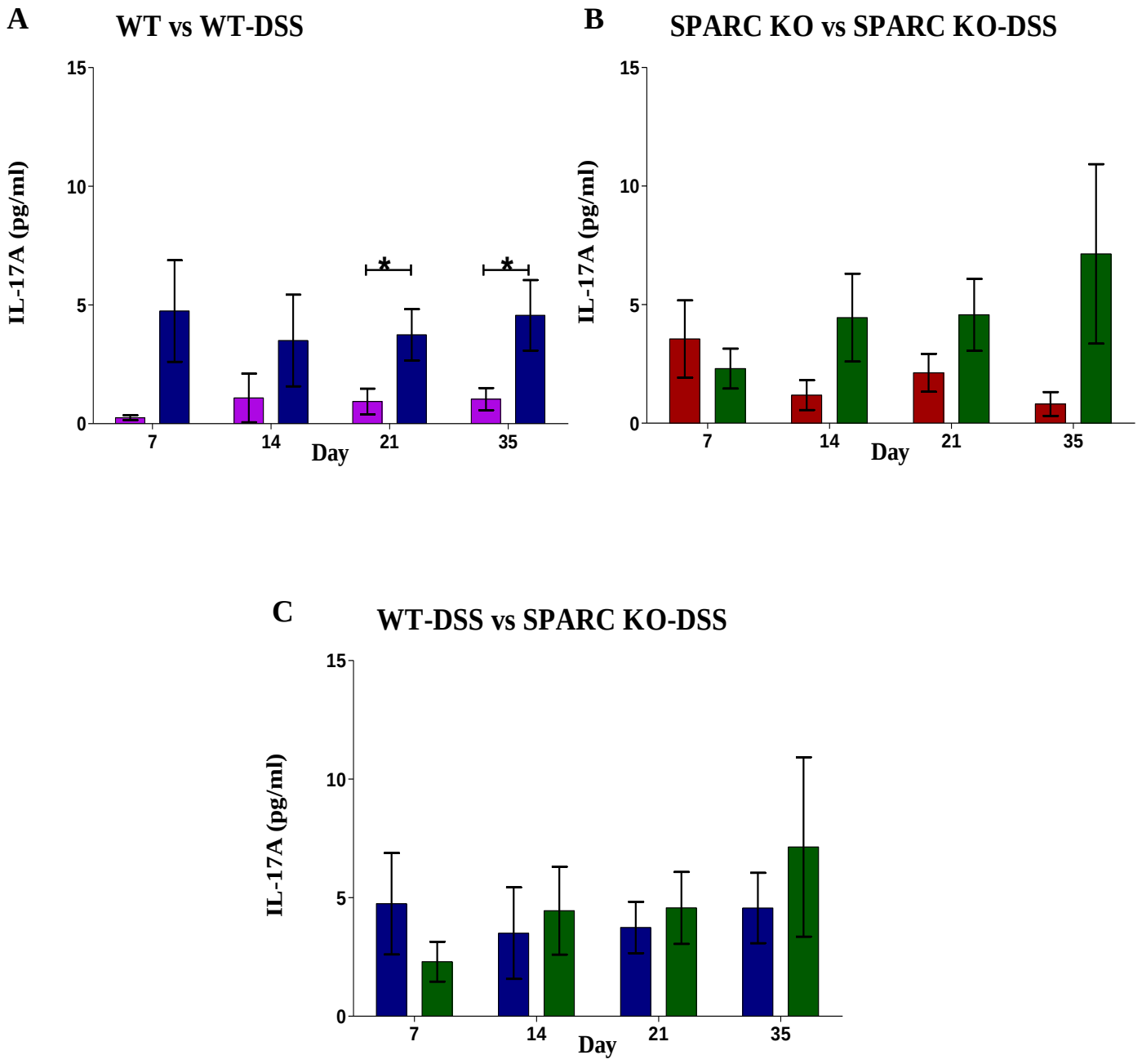


Figure 4.12: Th 17 related cytokine, IL-17A, secretion by the colon in WT and SPARC KO mice measured with CBA. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Mean ± SEM (*n* =12 to mice per group from 3 different experiments per time point) (*****p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

4.2.4 The chemokines expression in WT and SPARC KO mice

Chemokines contribute to the regulation of the intestinal immune response and mucosal inflammation, as they are critical elements in regulating the trafficking of cells to inflammatory sites (Tokuyama *et al*. 2005). Chemokine production was thus measured. Both WT and SPARC KO untreated animals had similar levels of MCP-1. MCP-1 production rose when the animals received DSS (***Figure 4.13 A, B***). WT mice generally had higher levels than SPARC KO animals after DSS treatment. Coinciding with the maximal colonic inflammation at day 7 WT animals demonstrated the highest expression of MCP-1. This fell by day 14, but rose at day 21 before decreasing again by day 35. Due to high sample variability no significant differences were detected between the disease groups (***Figure 4.13 C***).

MIG and RANTES were detected in the untreated animals and both were significantly induced following DSS treatment for 7 days in both the WT and SPARC KO animals although to a lesser extend in the SPARC KO animals (***Figure 4.14 and 4.15 A and B***). The level of MIG was significantly higher in the WT DSS-treated group when compared to the SPARC KO at day 7 ($p=0.031$) and 14 ($p=0.014$) (***Figure 4.14 C***).

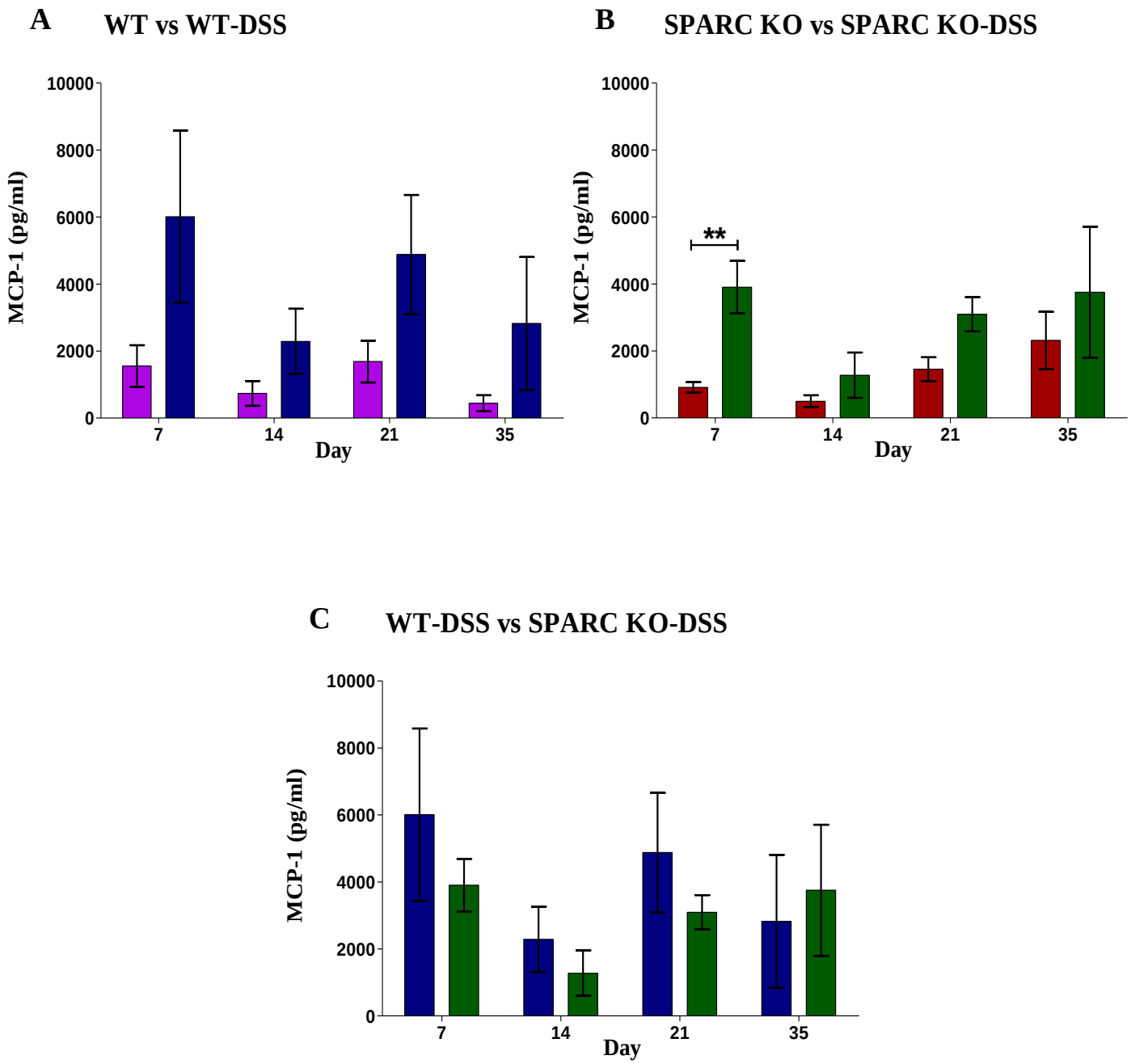


Figure 4.13: Colonic chemokines, MCP-1 production of the colon by SPARC WT and KO mice measured with CBA. MCP-1 production in the acute, chronic/recovery phases. Mean ± SEM (*n* =10 mice per group from 3 different experiments per time point) (**p<0.01). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

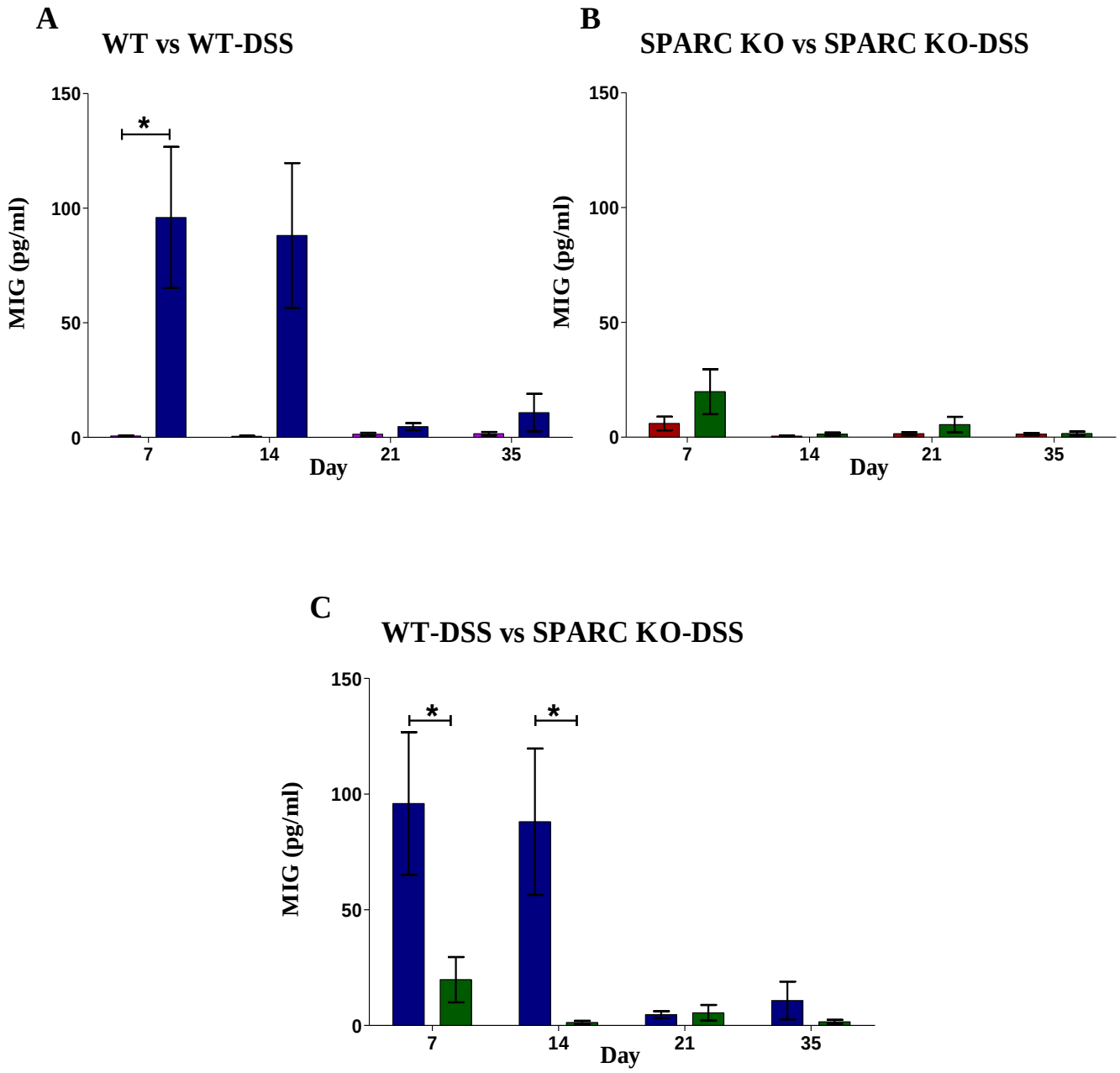


Figure 4.14: Colonic chemokines, MIG production of the colon by SPARC WT and KO mice measured with CBA. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Mean ± SEM (*n* =10 mice per group from 3 different experiments per time point) (*****p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

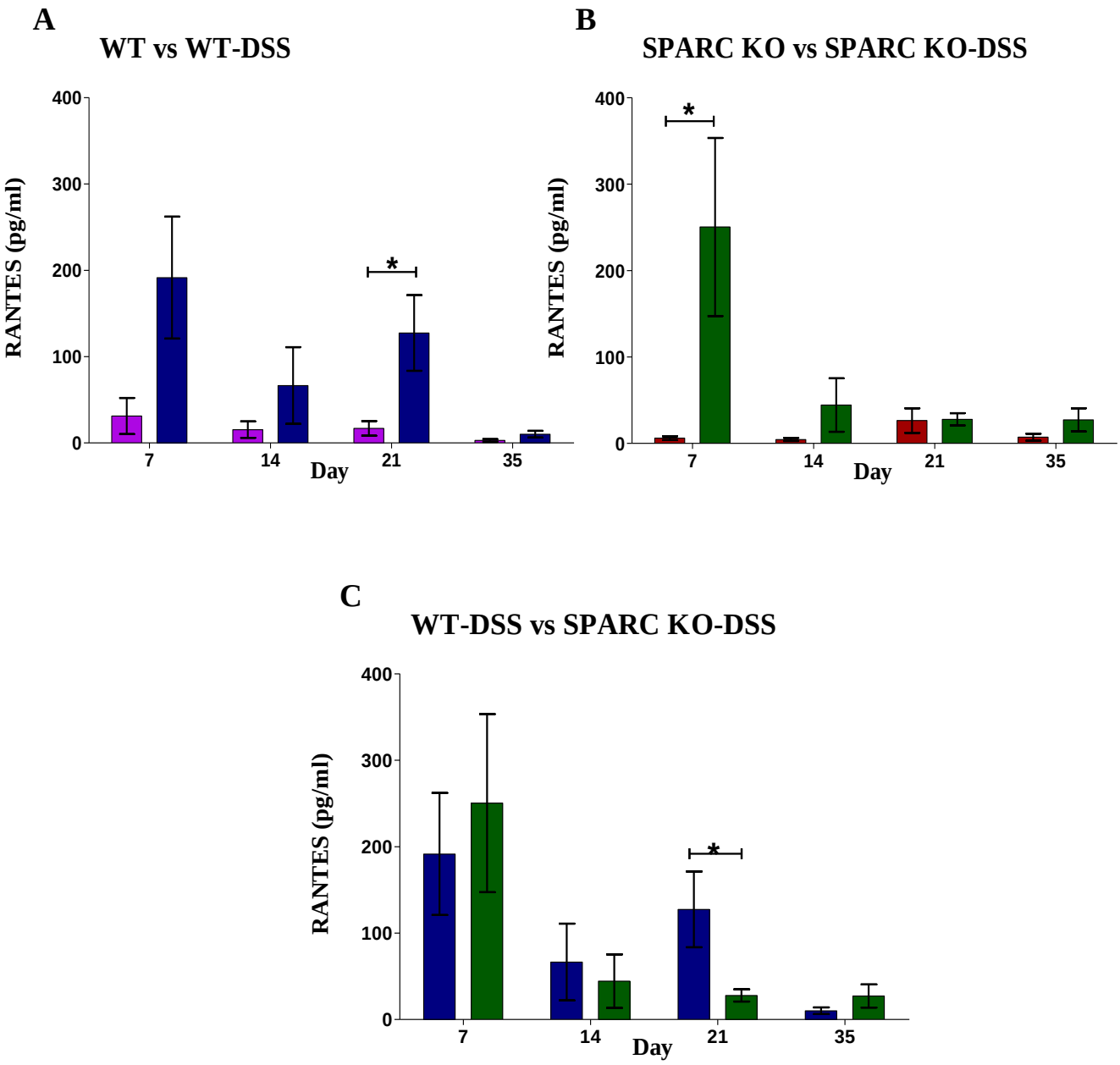


Figure 4.15: Colonic chemokine, RANTES production of the colon by SPARC WT and KO mice measured with CBA. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Mean ± SEM (*n* =10 mice per group from 3 different experiments per time point) (*****p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

4.2.5 Innate cell pattern in WT and SPARC KO Mice

The colon is populated with large numbers of resident intestinal macrophages that are able to phagocytoses harmful pathogen but lack certain receptors that allow them to generate an inflammatory stimuli. Inflammatory macrophages, however, are recruited from the systemic circulation and can secrete pro-inflammatory cytokines thus promoting inflammation (Smith *et al.* 2010; Heinsbroek and Gordon 2009). Lower levels of chemokines and IL-1β were detected in SPARC KO animals in this study and these changes possibly contributed to the less colonic inflammation that was seen as those mediators are associated with innate cell recruitment. To determine any differences in the inflammatory cell infiltration, serial cryosections of untreated and DSS-treated mice were stained for the distribution and number of the macrophage subsets and neutrophils, to determine if this could account for the differences observed in the level of cytokines, chemokines and inflammation. Immunofluorescence was chosen instead of flow cytometry analysis due to difficulties in cell isolation from the colon and as it allows localisation of the cells of interest. Unfortunately, there is a lack of markers that accurately differentiate the intestinal macrophage from the inflammatory macrophage, however, CD68 and CD11b can be used to characterise some features of these cells and were used in this study.

CD68+ cells were primarily located in the LP within the colonic mucosa, and low numbers were also detected in the submucosa of control mice. Following DSS treatment, the CD68+ cells, in both locations, increased in number and more cells infiltrated the submucosa (***Figure 4.16 D***). WT animals had statistically greater levels of CD68+ cells than SPARC KO mice following DSS removal at day 21 ($p=0.021$) and 35 ($p<0.01$) (***Figure 4.16 C***).

The number of CD11b+ cells was heterogeneous throughout the experiments, which might be explained by the fact that CD11b+ stains both monocytes and neutrophils. Double staining with another macrophage marker and/or a neutrophil specific marker will be required to further distinguish CD11b+ macrophages from other leucocytes. Different numbers of CD11b+ cells, however, were still observed between the WT and KO mice. DSS treatment significantly increased (WT: $p=0.016$; SPARC KO: $p<0.01$) the number of CD11b+ cells in both sets of mice. CD11b+ cells were also found around the muscle and serosa layers instead of just in the LP and submucosa of the colon (***Figure 4.17 D***). SPARC KO had greater numbers of CD11b+ cells than the WT at day 7 but this decreased rapidly with a significantly lower number of CD11b+ cells in the SPARC KO animals by day 35 ($p=0.025$) (***Figure 4.17 C***).

Staining was also undertaken for Ly6G, a maker for neutrophils, as these are the first innate immune cells to infiltrate sites of inflammation. In water control animals, almost no neutrophils were present and those that were observed were located within the LP. Following DSS treatment, more neutrophils were observed in the LP, particularly at sites of ulceration, as well as in the submucosa (***Figure 4.18 D***). WT animals had significantly higher Ly6G+ neutrophils numbers than the SPARC KO mice throughout the 4 assessment time points consistent with the greater levels of inflammation (***Figure 4.18 C***).

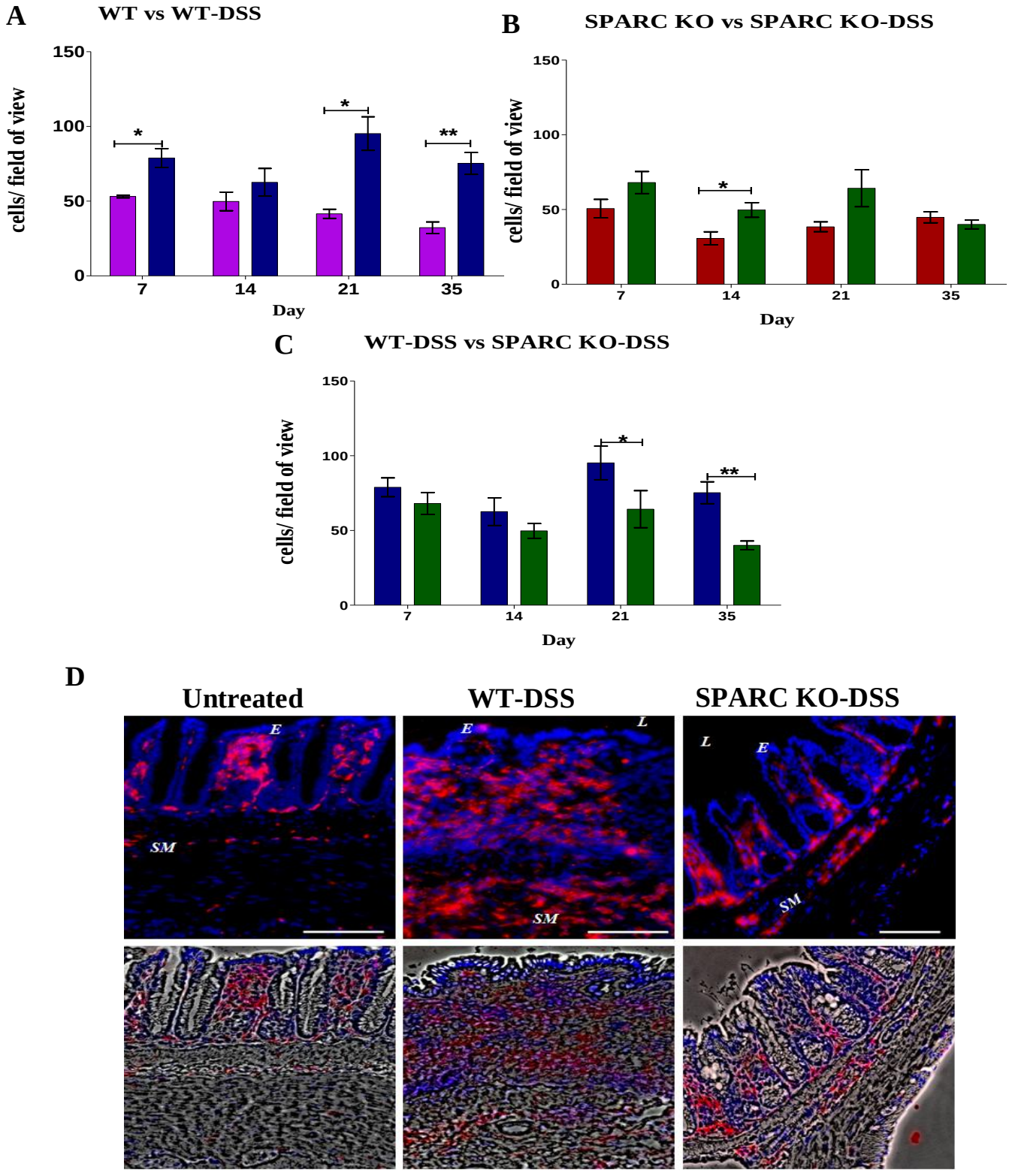


Figure 4.16: CD68+ cells in WT and SPARC KO colon. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Total numbers of CD68+ cells per field of view. Mean ± SEM (n=4 mice per time point; *****p<0.05; ******p<0.01). (D) *In situ* visualisation of colonic macrophages at day 21. CD68 staining (red) and co-stained nuclei (blue). E - epithelium layer, L - lumen and SM – submucosa. Lower panels are the merged phase contrast-florescence images. WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated. Bar: 100 µm

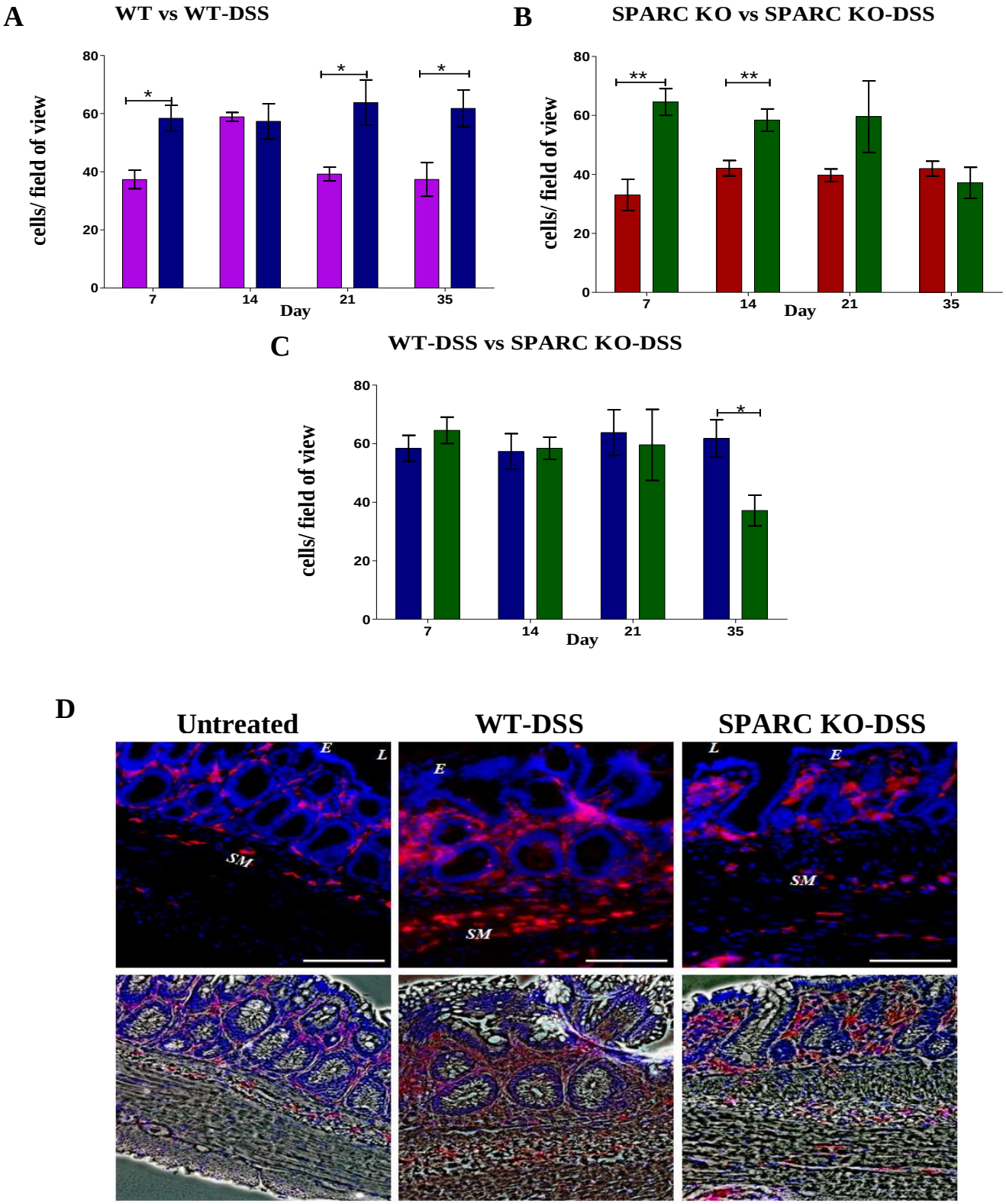


Figure 4.17: **CD11b+ cells in WT and SPARC KO colon.** (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Total numbers of CD11b+ cells per field of view. Mean ± SEM (n=4 mice per time point; ***** $p<0.05$; ******$p<0.01$). (D) *In situ* visualisation of colonic CD11b+ cells. CD11b staining (red) and co-stained nuclei (blue). E - epithelium layer, L - lumen and SM – submucosa. Lower panels are the merged phase contrast-florescence images. WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated. Bar: 100 µm

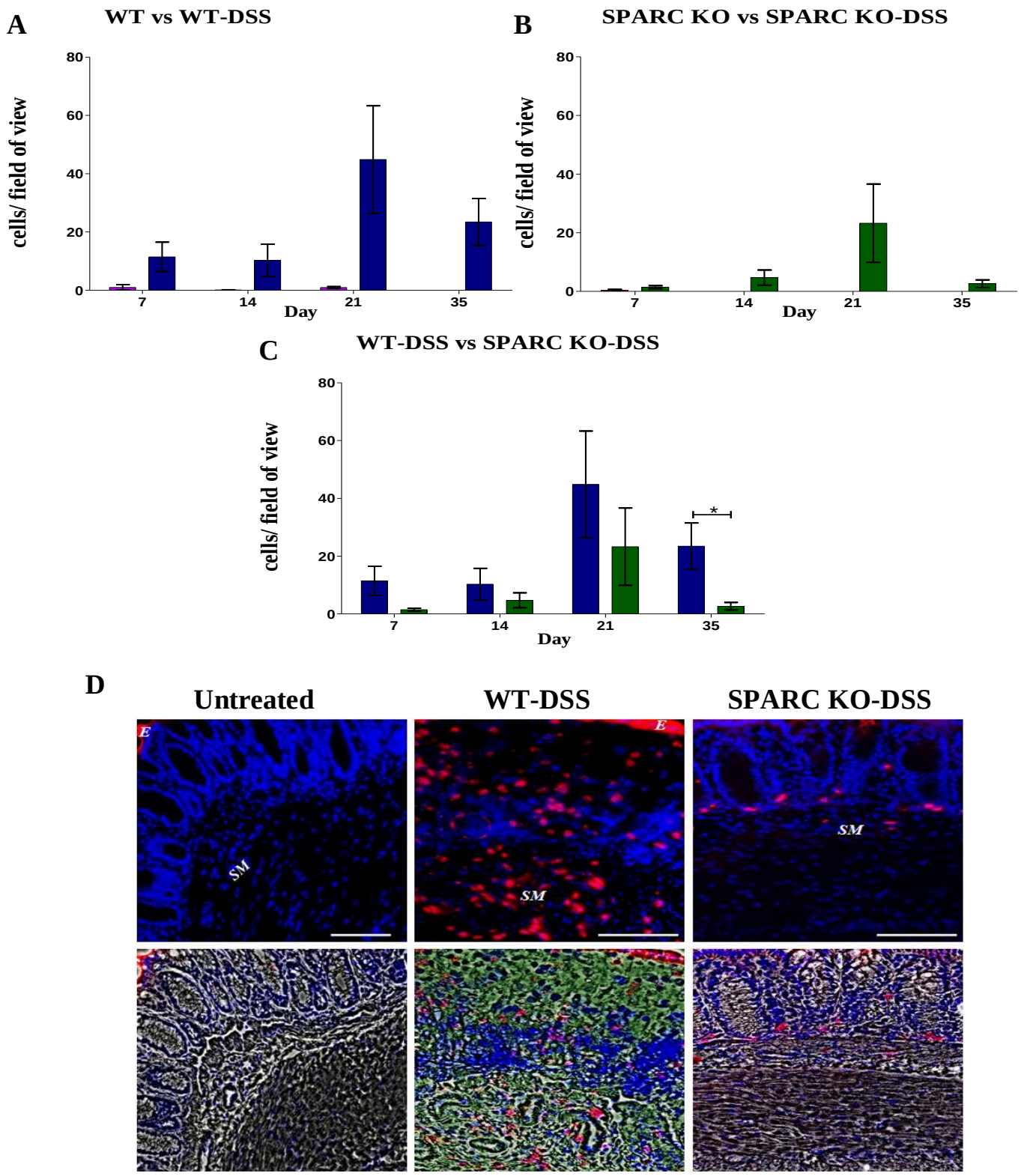


Figure 4.18: Ly6G+ cells in SPARC WT and KO colon. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. Total numbers of Ly6G+ cells per field of view. Mean ± SEM (n=4 mice per time point; *****p<0.05). (D) *In situ* visualisation of colonic Ly6G staining (red) and co-stained nuclei (blue). E - epithelium layer, L - lumen and SM – submucosa. Lower panels are the merged phase contrast-florescence images. WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated. Bar: 100 µm

4.3 DISCUSSION

The activation of the adaptive immune system is important in protecting the intestinal mucosa that is continuously in contact with numerous harmless antigens as well as micropathogens and other potentially harmful agents. Numerous studies, however, also point out that an uncontrolled adaptive immune response could play a role in the pathogenesis of IBD (Fujino *et al.* 2003; Izcue *et al.* 2009; Monteleone *et al.* 1997). For many years, the Th1 and Th2 paradigm has been used to define the inflammation observed in CrD and UC respectively. Recently, however, emerging evidence has demonstrated that Th17 cells are also involved in IBD pathogenesis and the mucosal Th1 and Th2 responses of CrD and UC also suggest that these may be a secondary effect to defects in the innate immune response. Thus characterisation of the levels of local and systemic inflammation, the pattern of the innate and adaptive CD4+ T cellular responses in the DSS model of colitis were conducted. These allowed us to investigate whether SPARC affects the immune response as SPARC appears able to modify cytokine production thus influencing cell recruitment and polarisation of the T cell response.

The level of colonic inflammation was different between SPARC KO and WT animals with SPARC KO colons being less inflamed than WT (Chapter 3). The splenic weights and the spleen/body weight ratio, which act as markers of systemic inflammation, also demonstrated significant differences between the SPARC KO and the WT animals in both the water controls ($p<0.05$) and following DSS-induced colitis ($p<0.01$). Rempel and colleagues reported that SPARC KO animals had a greater splenic wet weight when in the healthy state (Rempel *et al*. 2007). The present study, however, found that SPARC KO mice had smaller spleens and a lower spleen/body ratio than WT. This might be due to the different mouse strain or the age of mice used experiments.

Younger (8 to 12 weeks old) mice were used in the Remple study whereas the animals in this study were slightly older with the first assessment occurring after 13 weeks of age. Embryonic and developmental mouse studies have previously demonstrated subtle phenotypic differences between SPARC KO and WT mice but these phenotypic characteristics become more apparent with age (Norose *et al.* 2000; Bradshaw *et al.* 2002). This effect of aging might thus explain the observed differences between these studies.

With larger and heavier spleens, the DSS-treated WT animals had higher numbers of cells isolated from these organs. The total number of leucocytes isolated from the WT spleens was higher, but these had a lower percentage of lymphocytes than the SPARC KO mice. This suggests that other cell populations may be contributing more than lymphocytes to the splenic enlargement observed in the DSS-treated WT animals and also agrees with a role for SPARC through affecting lymphocyte trafficking (Rempel *et al.* 2007) as SPARC is a ligand of VCAM-1 that mediates leucocyte transmigration (Kelly *et al.* 2007). IFN-γ producing Th lymphocytes (Th1) were present in a higher percentage in the SPARC KO DSS-treated mice than WT (***Figure 4.7 and 4.8***). This might be due to accelerated DC migration and subsequent increased T cell priming, i.e DC can move faster to the draining lymph nodes and activate a stronger adaptive immune response because of a less dense collagen network present in the SPARC KO mice (Sangaletti *et al.* 2005). A similar event may also possibly account for the higher percentage of Treg cells that seen in the SPARC KO mice.

Significantly higher TGF-β levels were detected in the colonic tissue of the KO DSS-treated animals at day 7 compared to the WT mice (Section 3.2.2). This might allow for greater differentiation of CD4+ T lymphocytes into Treg cells and possibly explain why

the KO DSS-treated animals had significantly higher levels of Treg cells in the isolated tissues compared to the WT DSS-group at the time points after day 7. With more Treg cells present in the KO-DSS treated animals, more TGF-β may be released, reducing the level of inflammation by inhibiting the effector cells, and also aiding in the restoration of the integrity of the intestinal mucosal barrier (Izcue *et al.* 2009).

SPARC has been shown to have a negative regulatory role on levels of IL-6sR, which is an IL-6 agonist implicated in IL-6 trans-signalling (Kallen 2002) , and SPARC is also able to modulate the expression of TGF-β (Lane and Sage 1994; Francki *et al.* 2004). Both IL-6 and TGF-β are essential in Th17 differentiation. Unfortunately, neither flow cytometric or cytokines analyses were able to clearly distinguish any differences in CD4+ T cells subsets between WT and SPARC KO. It may be that the antibodies used to characterise the cells were not sensitive enough to detect subtle differences if they were present or due to the use of the C57/BL6 strain which is known to be prone to mount a Th1 response (Sacks and Noben-Trauth 2002). Besides TGF-β, IFN-γ has been shown to be capable of inducing the Treg cell phenotype as IFN-γ KO mice have a more severe form of experimental autoimmune encephalomyelitis and impaired Treg function (Wang *et al*. 2006). Although not statically significant, SPARC KO animals in this study had numerically higher levels of IFN-γ and IFN-γ-expressing CD4+ T lymphocytes (at least during the early phase) that could possibly contribute to the greater number of Treg cells that were found.

The greater levels of inflammation observed in the WT mice could be secondary to the role that SPARC plays in promoting cell migration, with less neutrophils, monocytes and possibly inflammatory macrophages seen in the SPARC KO colons following DSS administration. Cell migration is a complex process, which requires many different, and

precise, signals to be present and chemokines are part of this signalling process. Production of the chemokines, MCP-1, MIG, macrophage inflammatory protein (MIP)-1α and -1β (data not shown) were lower in the colons of the DSS-treated SPARC KO mice when compared to the WT animals. This may explain the reduced levels of infiltrating leucocytes observed in the colonic cyrosections of the SPARC KO mice. With less production of chemokines, leucocytes, especially neutrophils, may have reduced migrating into the colon. A similar effect can also be observed in chemokine receptor-deficient mice, which are protected from the DSS-induced inflammation due to lesser immune cellular infiltration (Siegmund *et al.* 2002; Andres *et al.* 2000).

4.4 CONCLUSION

Differences were observed in the level of inflammation in the SPARC KO animals compared to WT. Although the only genetic difference between the animals was the absence of SPARC, the exact mechanism that caused all the observed differences remains unknown since SPARC plays important roles in numerous cell functions. Differences in the neutrophil, macrophage and lymphocyte populations; in chemokine expression, as well as cytokine levels, suggest that numerous cellular activities are closely associated with regulation of the ECM and that SPARC is responsible for some of these functions. The ECM of SPARC KO mice has been shown to be different to WT animals and this may be important to the observed differences (Bradshaw 2009). The higher TGF-β levels seen in the KO animals at day 7 also support the observation that the colonic ECM of SPARC KO animals differs from WT since ECM is one of the major reservoirs of TGF-β. The aim of the next chapter is, therefore, to examine the ECM of the colon in both WT and SPARC KO mice to determine what the differences that impact on the observed cellular events described above.

Chapter 5

The roles of SPARC in ECM changes

5.1 INTRODUCTION

From the data presented in chapter 4, it appears that SPARC affects the recruitment of immune cells through the modulation of chemokine expression, and also through an effect on the ECM composition and architecture as proposed form the literature. SPARC is involved in many processes that regulate proliferation, migration, and growth factor activities, but some of its major functions involve binding to, and interacting with, collagen (Brekken and Sage 2000). SPARC can alter the interaction between collagen and the cell surface possibly through the modulation of receptor binding and the regulation of collagen incorporation into fibrils that subsequently affecting collagen fibril maturation (Bradshaw 2009). SPARC also regulates the expression of several MMPs in certain cell types (Shankavaram *et al.* 1997). Thus, SPARC seems to possess the necessary biological properties that are required to regulate the balance between ECM deposition and degradation. Fibrosis is over deposition of ECM and also is a nonspecific feature of chronic inflammation and is observed in CrD and UC (Harper *et al.* 1987; Pucilowska, Williams, *et al.* 2000). Many mouse models investigating fibrosis in numerous organs such as the skin, lung, heart and kidney have demonstrated that SPARC KO mice have reduced collagen deposition when compared to their WT counterparts (Bradshaw *et al.* 2009; Bradshaw *et al.* 2002; Socha *et al.* 2007; Strandjord *et al.* 1999).

Mesenchymal cells such as fibroblasts and myofibroblasts are the major effector cells of wound repair and regenerative healing. They coordinate the deposition and further organisation of the collagenous ECM in the wound bed, and they determine the final outcome of the scarring process (Powell *et al.* 1999a; Tomasek *et al.* 2002). Colonic subepithelial myofibroblasts are located at the interface between the epithelium and LP,

immediately adjacent to the basement membrane in the normal intestinal mucosa. These cells are specialised mesenchymal cells and have ultrastructural features that resemble both fibroblasts and smooth muscle cells. It is these cells that play a vital role in the regulation of epithelial cell function and ECM metabolism as well as the development of fibrosis in the gut (Brenmoehl *et al.* 2009; Powell *et al.* 1999a, 1999b; Andoh *et al.* 2005). Recently, the pro-fibrogenic response of hepatic stellate cells, myofibroblast-like cells that reside in the liver and are involved in hepatic fibrogenesis, has been attenuated by knock down of SPARC expression with siRNA (Atorrasagasti *et al*. 2011). Therefore, an investigation of the precise mechanisms by which these mesenchymal cells are involved in the ECM modulation and development of fibrosis in colitis is vital and will be examined in this chapter.

5.2 RESULTS

5.2.1 Collagen content of colonic section in WT and SPARC KO mice

Colons commonly become rigid in the presence of acute and/or chronic inflammation (Melgar *et al.* 2005). In order to investigate whether this was due to the presence of inflammatory cells or the resulting fibrosis, distal colonic tissues were stained with Sirius red to analyse the production of collagen as a marker of fibrosis and ECM changes.

The collagen content was colour-coded by the Aperio's ImageScope Viewer-Spectrum positive pixel count algorithm software. The results are presented as the amalgamation of all 3 groups of positive staining pixels (weak, medium and strong) and normalised to the area selected. SPARC KO animals had less colonic collagen on average when compared to the WT mice in both untreated (0.428 vs 0.238; p=0.015; data not shown) and following DSS-treatment (***Figure 5.1***). At day 7 following the commencement of DSS treatment, both WT and SPARC KO DSS-treated animals had more collagen than their water treated group. The difference in collagen content diminished over the course of the experiment and the collagen content in the treated animals dropped in comparison to the water control mice following removal of the DSS at day7.

Collagen deposition in the mucosa and submucosa of both the SPARC KO and WT mice increased with the DSS treatment and was most marked at day 7 (***Figure 5.2 A***). Following removal of the DSS, the level of colonic collagen in SPARC KO animals rapidly returned to the pretreatment levels while the collagen levels in the WT remained elevated until days 21 and 35 (***Figure 5.2 B***). These findings suggest that the WT animals are be more prone to the development of intestinal fibrosis than SPARC KO mice.

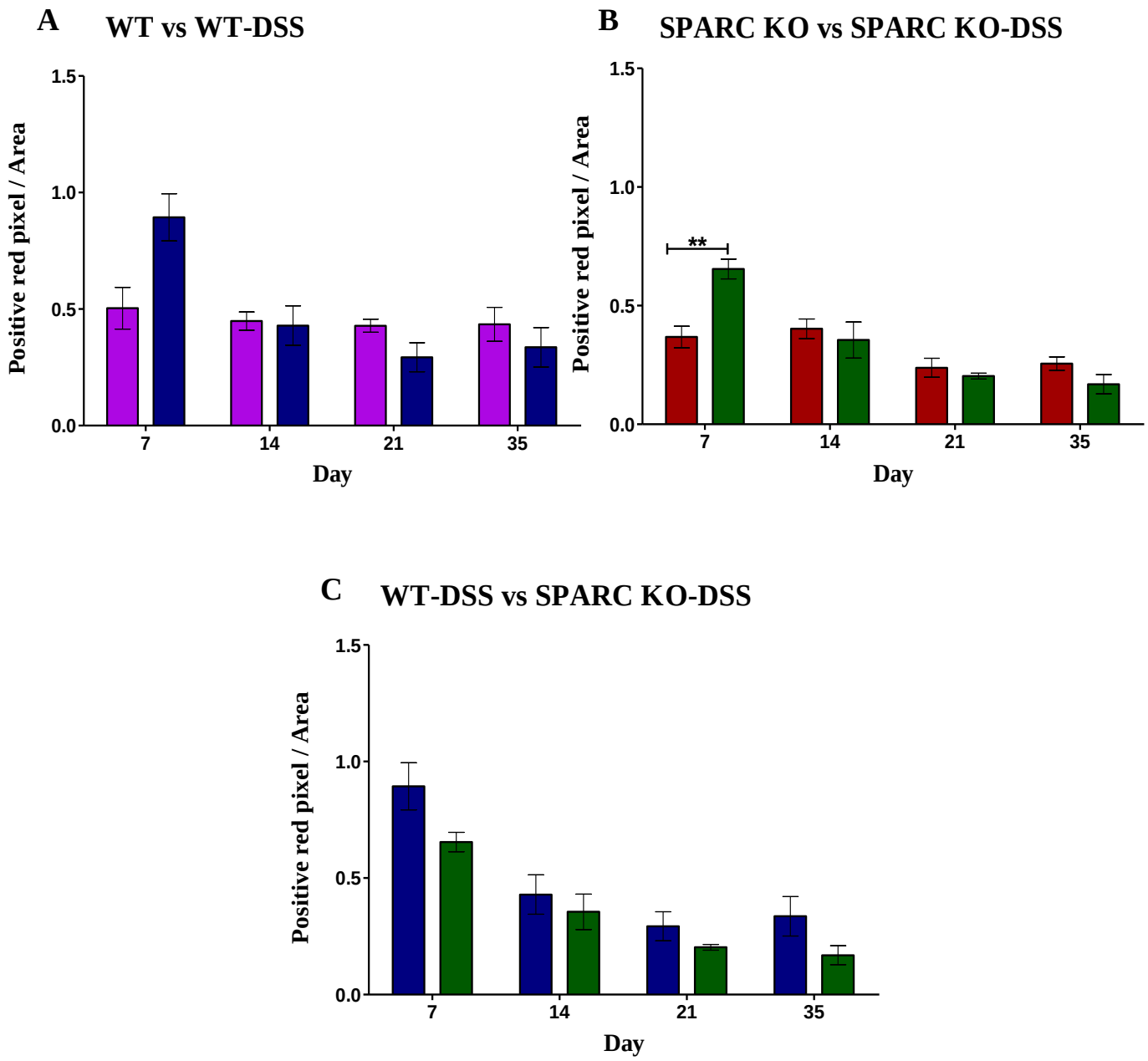


Figure 5.1: Positive Sirius red stain pixel to area ratio. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS.Values are mean ± SEM based on records of 5 mice at each time point. **(****p<0.01). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

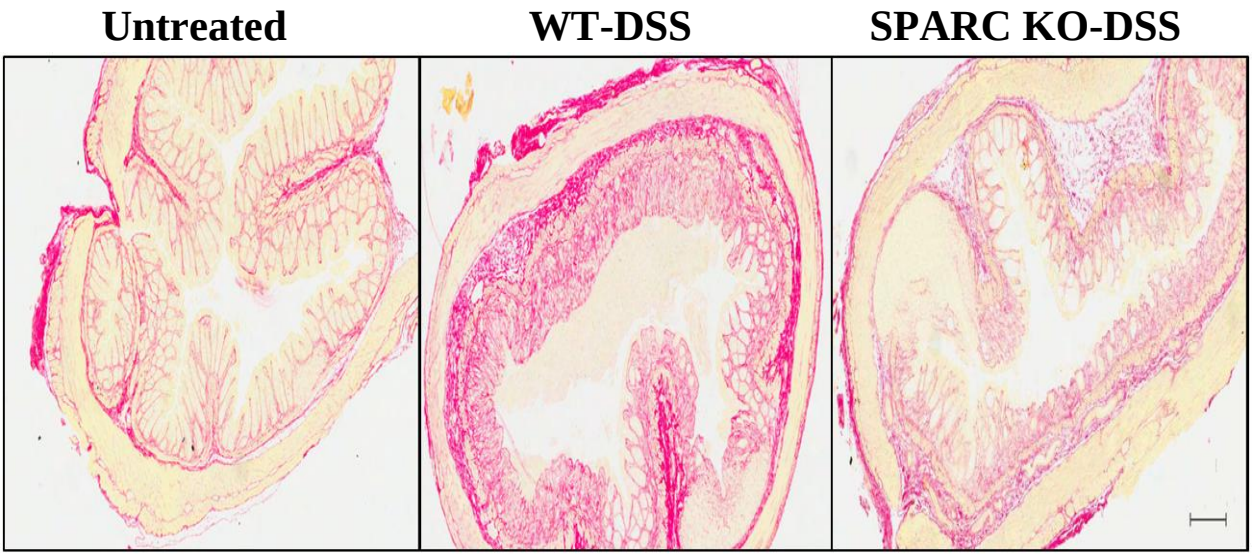


Figure 5.2 A: Increased levels of collagen in the DSS-induced inflamed colons at day 7: Representative tissue sections of SPARC WT and KO stained with Sirius red. Following 7 days exposure to DSS, both SPARC KO and WT mice demonstrated extensive deposition of collagen within the LP, submucosa and the serosa layer. WT-DSS - WT DSS-treated; SPARC KO-DSS –SPARC KO-DSS treated. Bar:200μm

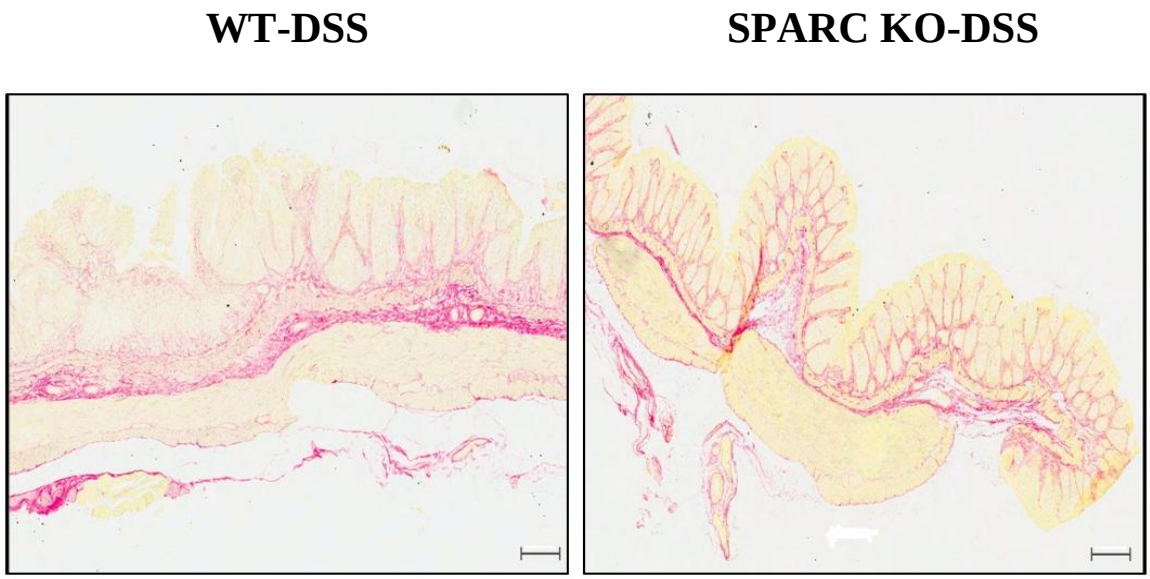


Figure 5.2 B: Colonic collagen at day 21 in SPARC KO and WT mice following DSS treatment: Representative tissue sections of SPARC WT and KO stained with Sirius red. At day 21, WT-T animals still had great amounts of collagen compared to KO mice which appeared to have returned to pre-DSS treatment levels. WT-DSS - WT DSS-treated; SPARC KO-DSS –SPARC KO DSS-treated. Bar: 200μm

5.2.2. Colonic ECM gene expression in DSS-treated WT and SPARC KO mice

5.2.2.1 Collagen expression

ECM-related gene expression levels were measured in the colonic tissue to determine if there were any differences between SPARC KO and WT animals. Collagen type 1 (collagen 1α1) mRNA expression in WT DSS-treated group was significantly lower ($p<0.001$) than in the WT untreated group (***Figure 5.3 A***). No statistical differences observed between the water and DSS-treated SPARC KO animals (***Figure 5.3 B***). The SPARC KO animals had significantly higher collagen type 1 expression ($p<0.01$) than the WT animals at day 7 after DSS treatment (***Figure 5.3 C***). There were, however, no statistically significant differences detected between the WT and SPARC KO DSS-treated mice at the other time points (***Figure 5.3 C***).

The collagen type 3 (collagen 3α1) gene expression in WT DSS-treated group was significantly lower ($p<0.001$) than in the WT untreated group at day 7 and no differences observed in other time points (***Figure 5.4 A***). SPARC KO mice receiving DSS treatment had significantly greater collagen 3α1 expression ($p<0.01$) than the SPARC KO untreated group only at day 21 (***Figure 5.4 B***). Similar to collagen 1α1 expression, the collagen 3α1 expression in the SPARC KO animal was significantly higher than WT in the DSS treated group($p<0.001$) (***Figure 5.4 C***).

5.2.2.2 MMP expression

MMPs play a crucial role in ECM degradation. MMP-13 (homologous to human MMP-1) cleaves the triple helixes of native fibrillar collagen that includes collagens I, II, III and VII. Stromelysins (MMP-3) degrade the noncollagen components of ECM, such as fibronectin, laminin and proteoglycans. These two MMPs are postulated as being the

most important of the proteolytic enzymes responsible for the breakdown of ECM in IBD (von Lampe *et al.* 2000; McKaig *et al.* 2003).

The transcription levels of MMP-13 in the WT DSS-treated animals were significantly lower ($p<0.01$) than in water control WT animals at day 7 (***Figure 5.5 A***). By contrast, the water control SPARC KO animals had significantly higher ($p=0.015$) MMP-13 mRNA expression at day 14 compared to the DSS-treated KO group (***Figure 5.5 B***). The WT-DSS treated animals at day 7 also demonstrated significantly less ($p=0.013$) MMP-13 expression compared to the SPARC KO DSS-treated animals (***Figure 5.5 C***), which also reflected the change in the collagen expression levels.

MMP-3 was also significantly increased ($p=0.031$) in the DSS-treated SPARC KO mice compared to the DSS-treated WT group at day 7, although there was some variability in the sample levels (***Figure 5.6 C***). No statistical differences in the MMP3 expression levels were demonstrated at any of the other time points.

5.2.2.3 TIMP expression

The TIMPs also are regulators involved in the ECM balance as they regulate the actions of the MMPs. The SPARC KO animals demonstrated less TIMP-1 expression when compared to the WT mice and this difference was significantly different at day 35 between the DSS-treated groups (***Figure 5.7 C***). No other differences between the WT and SPARC KO animals were observed, but DSS was noted to reduce colonic TIMPs expression, especially TIMP-2 expression, which was significantly reduced in both WT

and SPARC KO groups when compared to the untreated control animals at day 7 ($p<0.001$) and 35 (WT: $p= 0.026$; SPARC KO: $p<0.01$) (***Figure 5.8 A and B***).

5.2.2.4 TGF-β expression

Colonic TGF-β1 and β3 expression levels were low with a great deal of variation between the different groups when compared to each other across the time points and no trend in expression was observed (***Figure 5.9 and 5.10***). Untreated SPARC KO animals, however, had significantly higher TGF-β1 expression than the WT untreated controls at day 21 (data not shown).

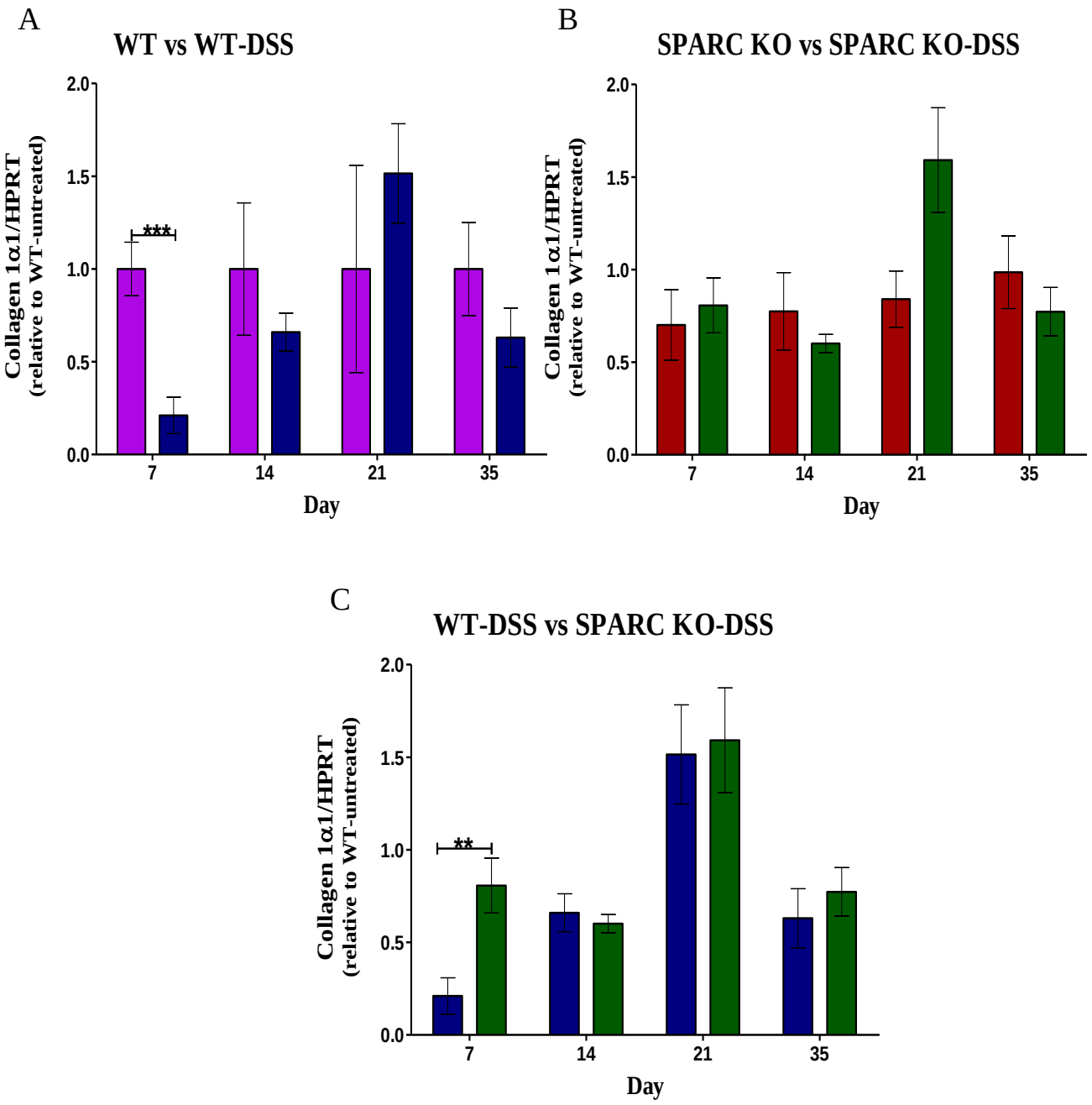


Figure 5.3: Collagen 1α1 mRNA expression in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. (**p<0.01; ***p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

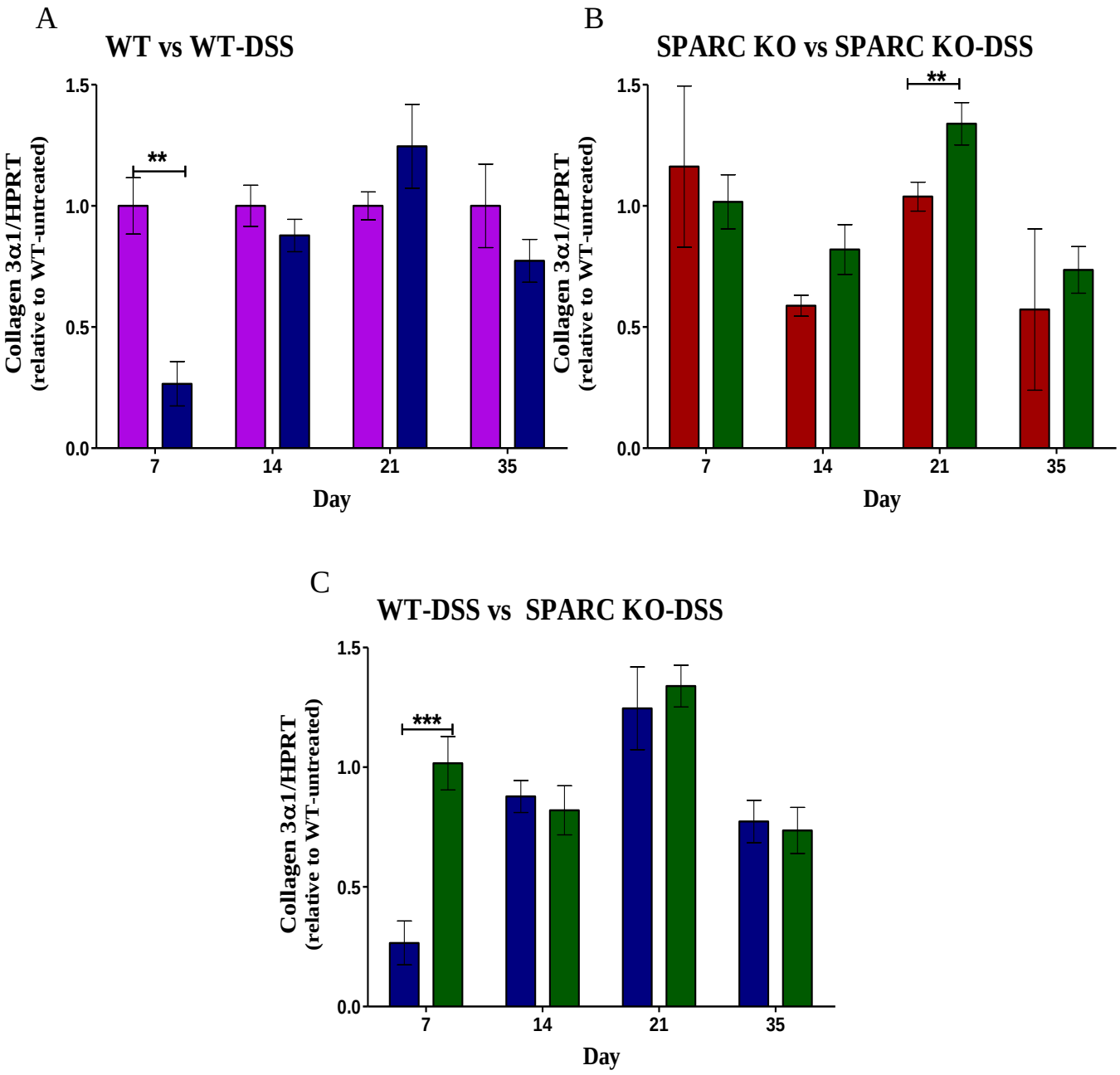


Figure 5.4: Collagen 3α1 mRNA expression in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. (**p<0.01; ***p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

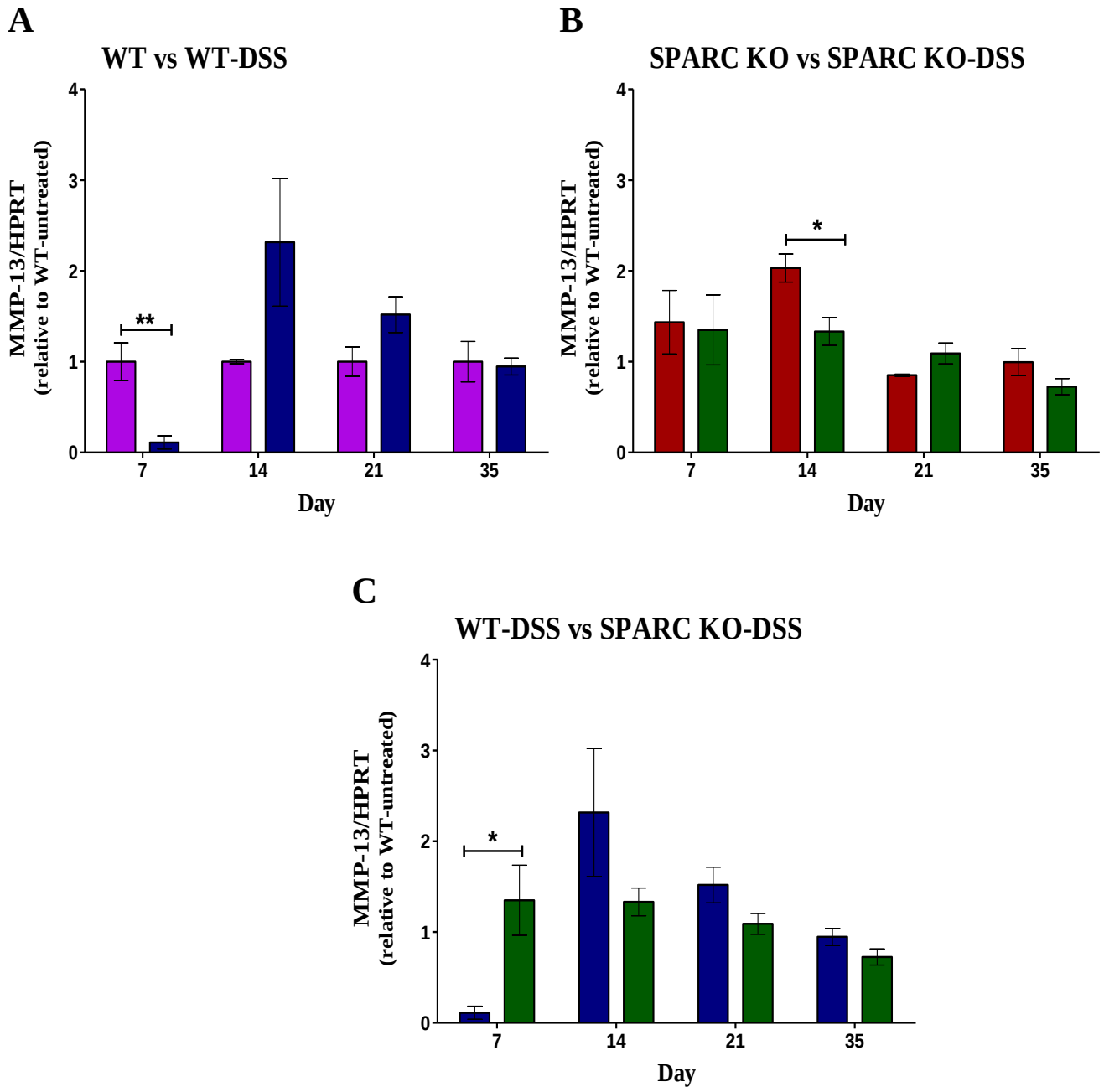


Figure 5.5: MMP-13 mRNA expression in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. (*****p<0.05; ******p<0.01). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

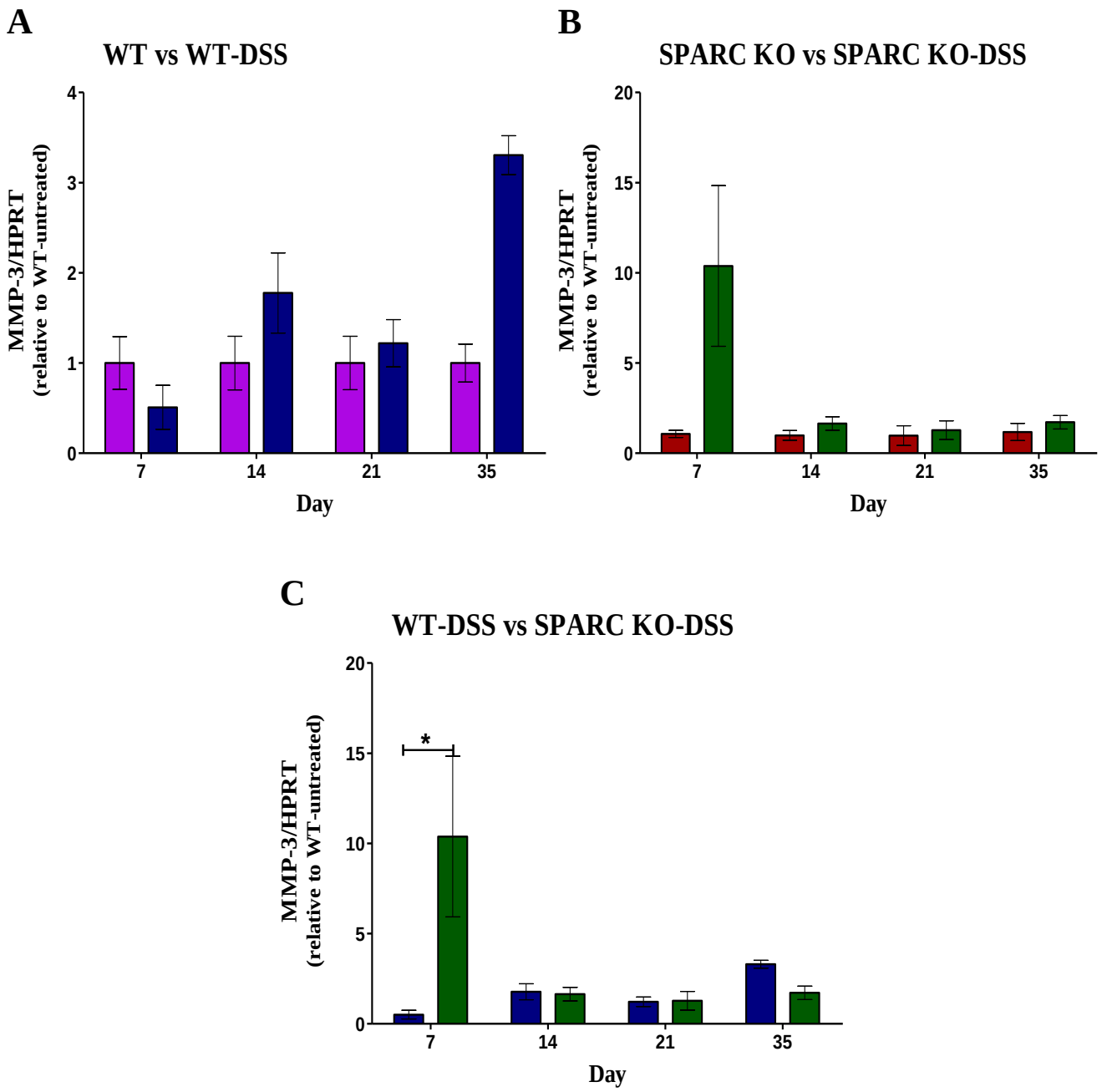


Figure 5.6: MMP-3 mRNA expression in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. (*****p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

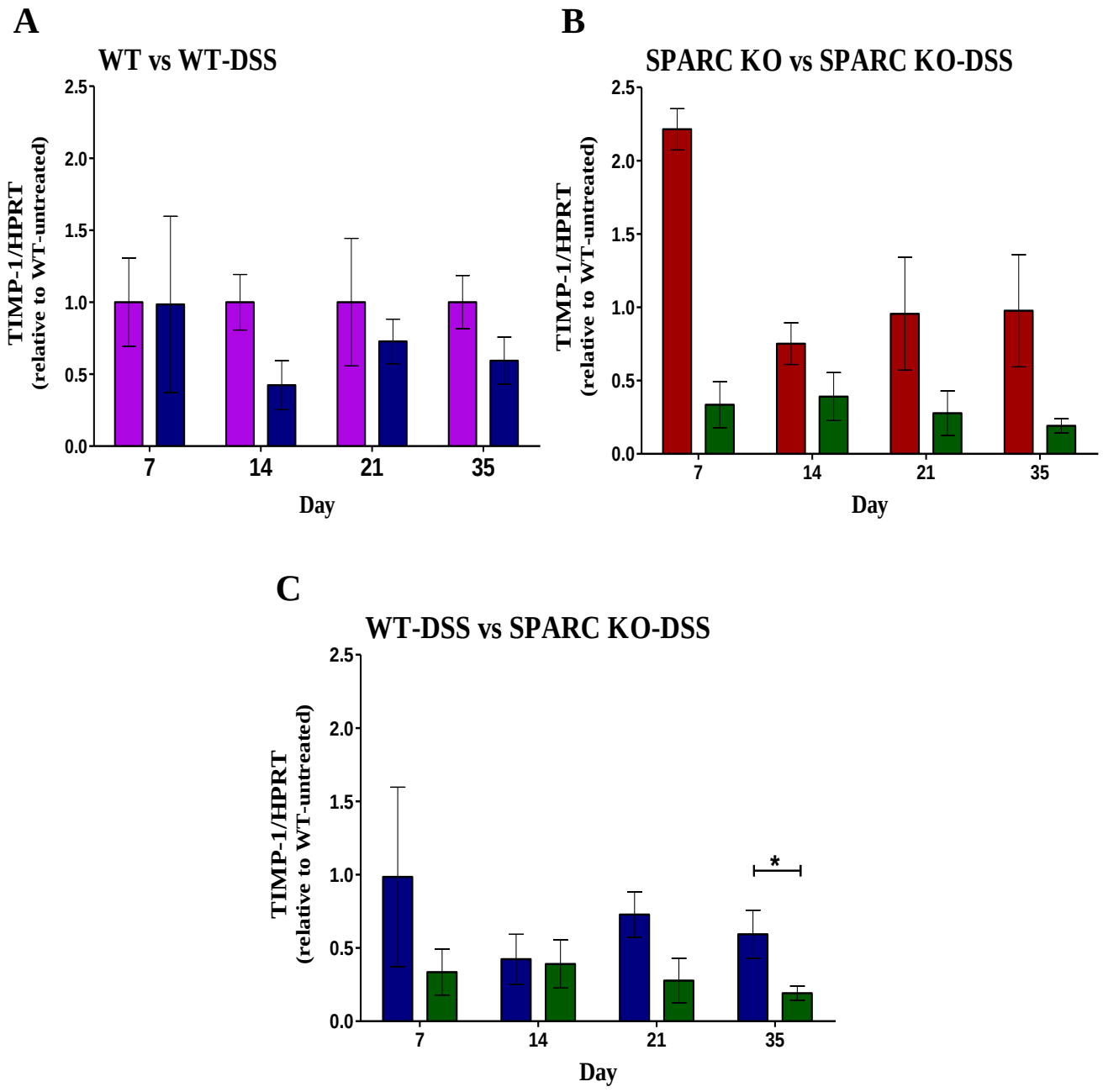


Figure 5.7: TIMP-1 mRNA expressions in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. (*****p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

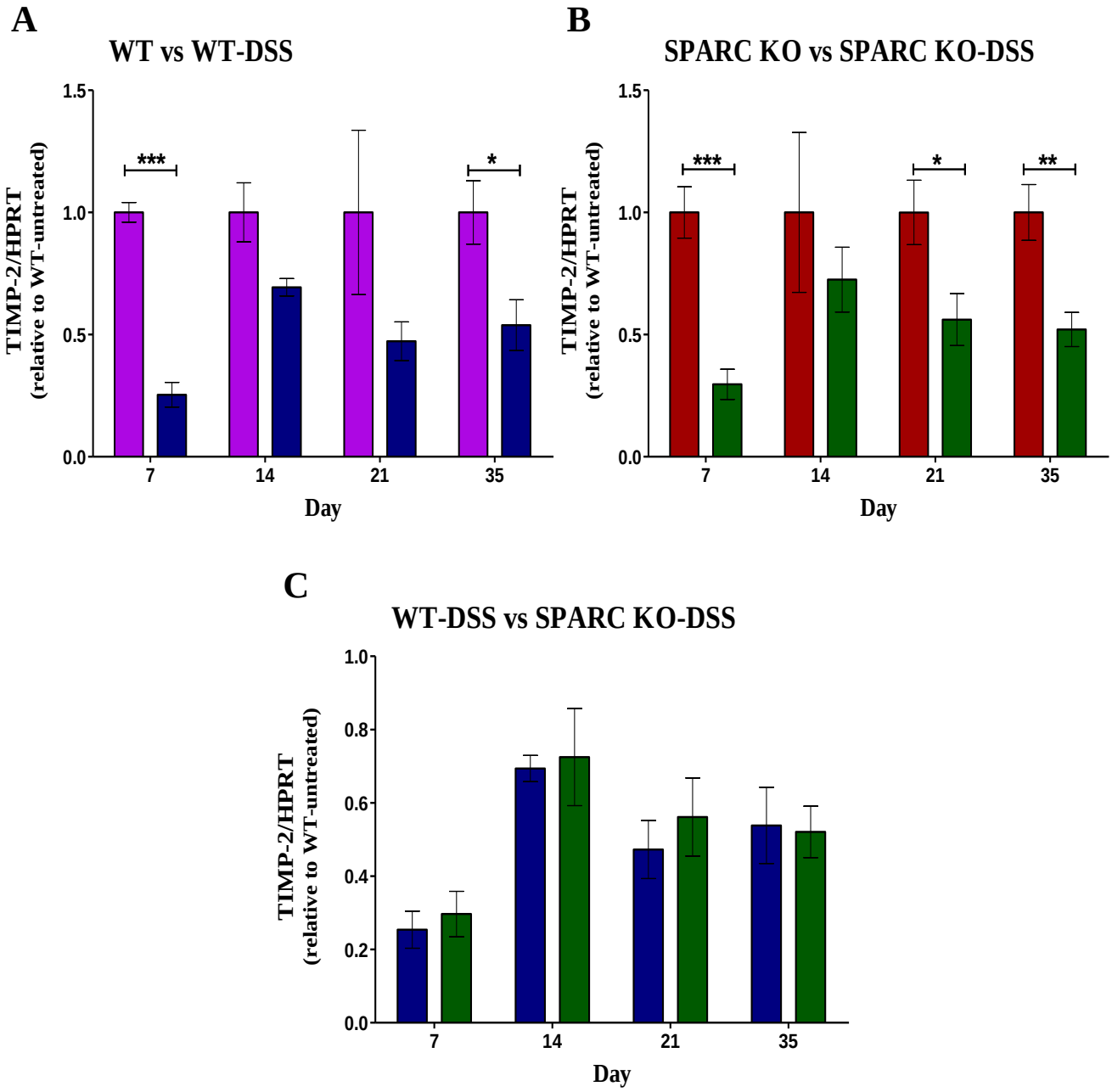


Figure 5.8: TIMP-2 mRNA expressions in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. (*****p<0.05; ******p<0.01; *******p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

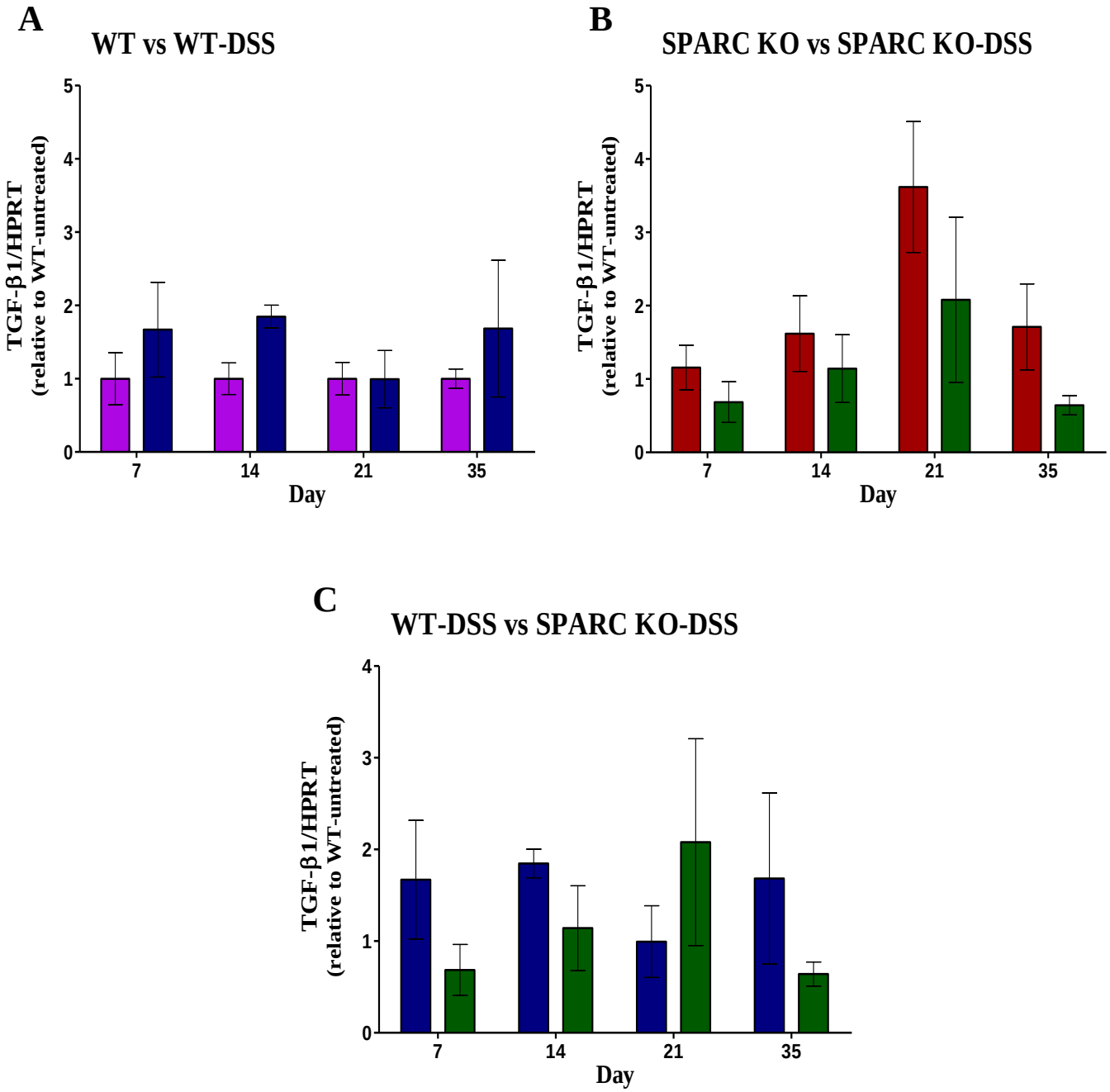


Figure 5.9: TGF-β1 mRNA expression in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 mice per time point. WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

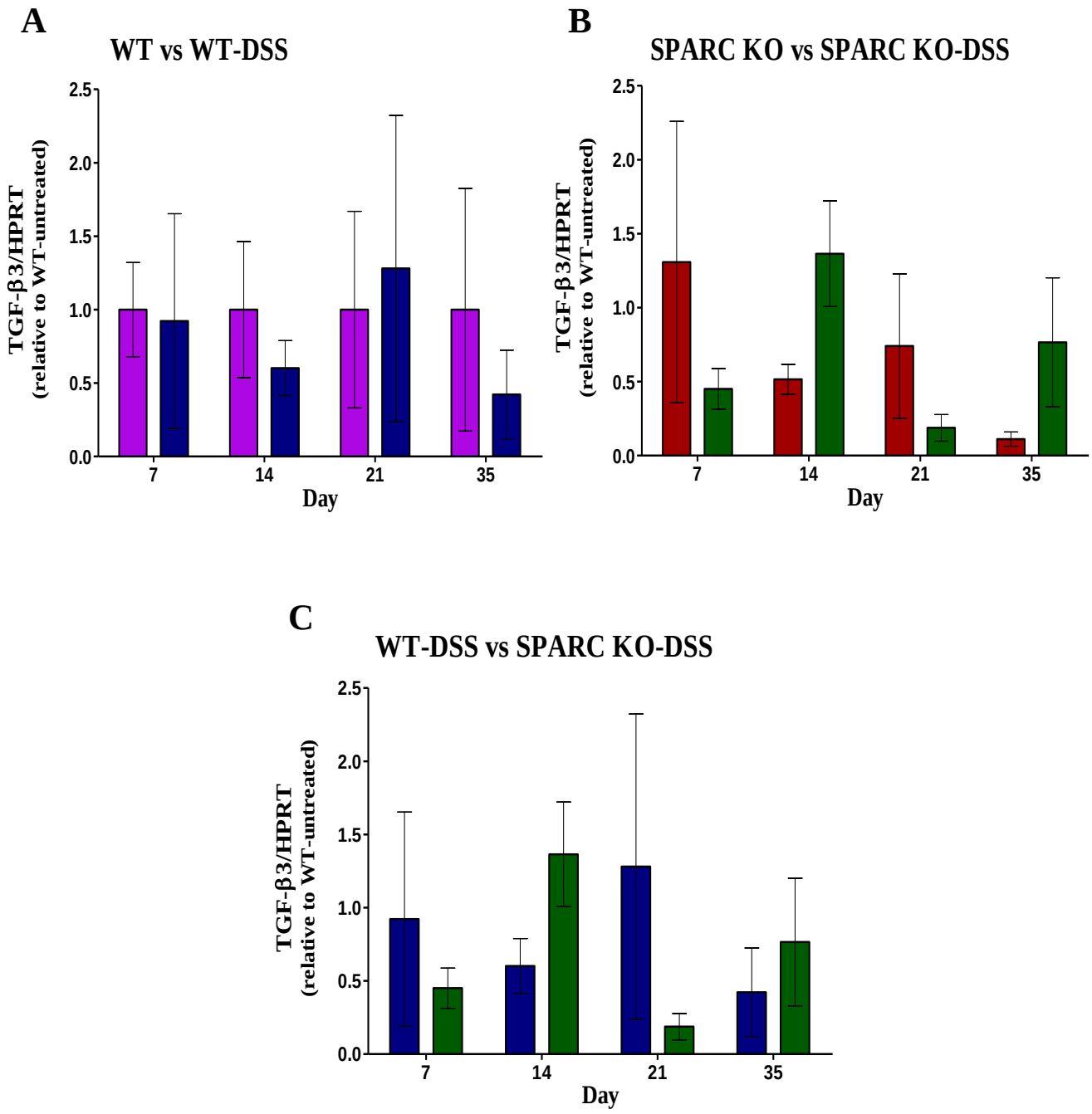


Figure 5.10: TGF-β3 mRNA expression in colonic tissue. (A) WT VS WT-DSS (B) SPARC KO VS SPARC KO-DSS (C) WT-DSS VS SPARC KO-DSS. After normalising with the house keeping gene and expressed relative to untreated WT controls.. Values are mean ± SEM based on records of 5 mice per time point. WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

5.2.3 The ECM gene expression of primary subepithelial myofibroblasts cell cultures

The colonic ECM gene expression levels in KO mice demonstrated that at some time points the collagens were expressed differently compared to WT animals. At day 7, the collagen 1α1 and 3α1 expressions were downregulated in WT but not SPARC KO. WT animals, however, have more inflammation than SPARC KO animals at this time point. Since gene expression measurements were normalised against a house keeping gene (HPRT) which is presumably expressed by inflammatory cells as well, hence, it is not clear whether collagen expression by fibroblasts is actually decreased on a cellular level or whether the observed differences are an indirect effect due to the abundant presence of inflammatory cells in the WT animals.

The cells of mesenchymal orgin, fibroblast/myofibroblast cells, appear to be implicated in ECM modulation and intestinal fibrosis. The colonic fibroblasts were, therefore, isolated from both WT and SPARC KO mice, in order to determine the effect of SPARC on fibroblasts gene expression on a cellular level.

5.2.3.1 Characterisation of primary myofibroblasts cells

The isolated colonic fibroblast-like cell cultures demonstrated the normal anchorage dependent fibroblast characteristics. The fibroblasts grew as flat, elongated cells with spindle shaped cytoplasm and cytoplasmic extensions of variable length when examined under light microscope at confluency. The vast majority of isolated cells were shown to be of mesenchymal origin and were myofibroblasts, and not epithelial cells, as they stained positive for vimentin (***Figure 5.11 B***), α-smooth muscles actin (***Figure 5.11 A***) and negative for keratin (***Figure 5.11 E***). A small percentage of the isolated cells, however, did stain positive for desmin (***Figure 5.11 C***) suggesting that they had the contractile nature that is part of the smooth muscle/myofibroblast cell. There were no

detectable differences in light microscopy morphology or immunohistochemistry between the cell cultures from WT and SPARC KO mice.

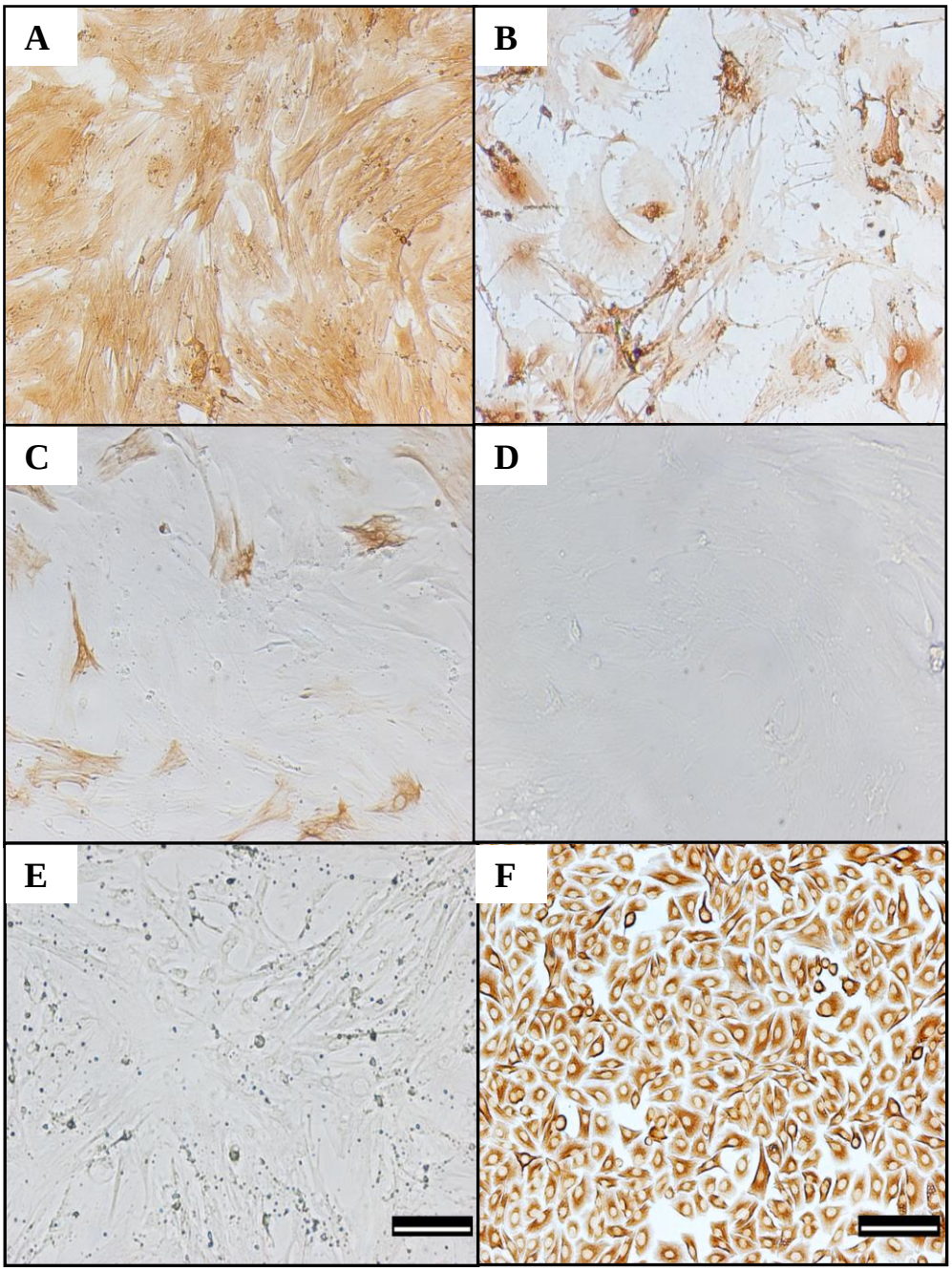


Figure 5.11: Characterisation of the isolated colonic myofibroblasts. Once confluent, cellular morphology was examined and immunohistochemical staining undertaken **A)** α-smooth muscle actin, **B)** Vimentin **C)** Desmin **D)** No primary antibody control. **E)** The isolated cells stained negative for the epithelial cell marker: keratin **F) A549** epithelial cell line stained positive for the keratin as a positive control to **E**. A representative photomicrograph was shown for each antibody. Bar:100 µm

5.2.3.2 ECM genes expression in the colonic myofibroblast culture

The *in vitro* colonic myofibroblast cultures were stimulated with PMA, a mammalian tumour promoter, to mimic the *in vivo* inflammatory conditions in the *in vitro* experiments.

5.2.3.2.1 Collagen expression

Collagen 1α1 and 3α1 mRNA expressions were significantly lower in the unstimulated SPARC KO compared to the control WT myofibroblasts ($p<0.001$). PMA treatment was also noted to reduce collagen expression in both sets of myofibroblast cultures. No statistical difference, however, was found between the PMA-treated WT and SPARC KO myofibroblast cultures (***Figure 5.12 A and B***).

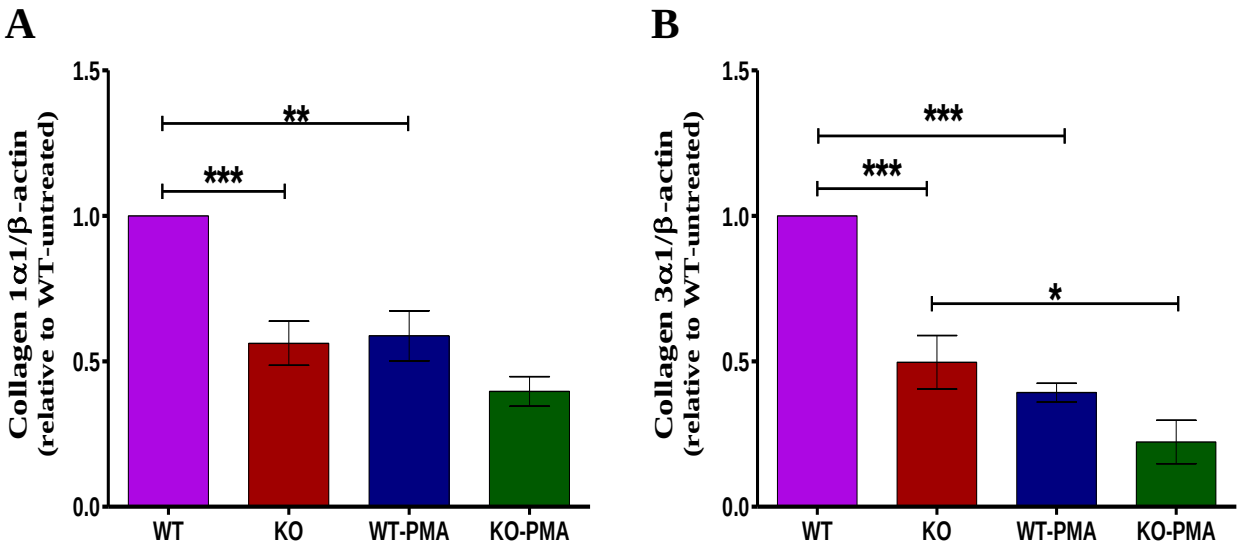


Figure 5.12: A) Collagen 1α1 B) Collagen 3α1 mRNA expression in myofibroblast cultures with and without PMA stimulation. After normalising with the house keeping gene and expressed relative to untreated WT controls. Values are mean ± SEM based on records of 5 independent experiments, performed in triplicate. (*****$p<0.05$; ******$p<0.01$; ******* $p<0.001$). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

5.2.3.2.2 MMP expression

PMA treatment significantly increased MMP-13 expression in both the WT ($p<0.001$) and SPARC KO ($p<0.01$) myofibroblasts, and the SPARC KO cells were noted to have a trend of greater expression of MMP-13 than the WT cells (***Figure 5.13 A***). The expression of MMP-3 did not differ between the stimulated and unstimulated isolated cells or between the SPARC KO and WT groups (***Figure 5.13 B***).

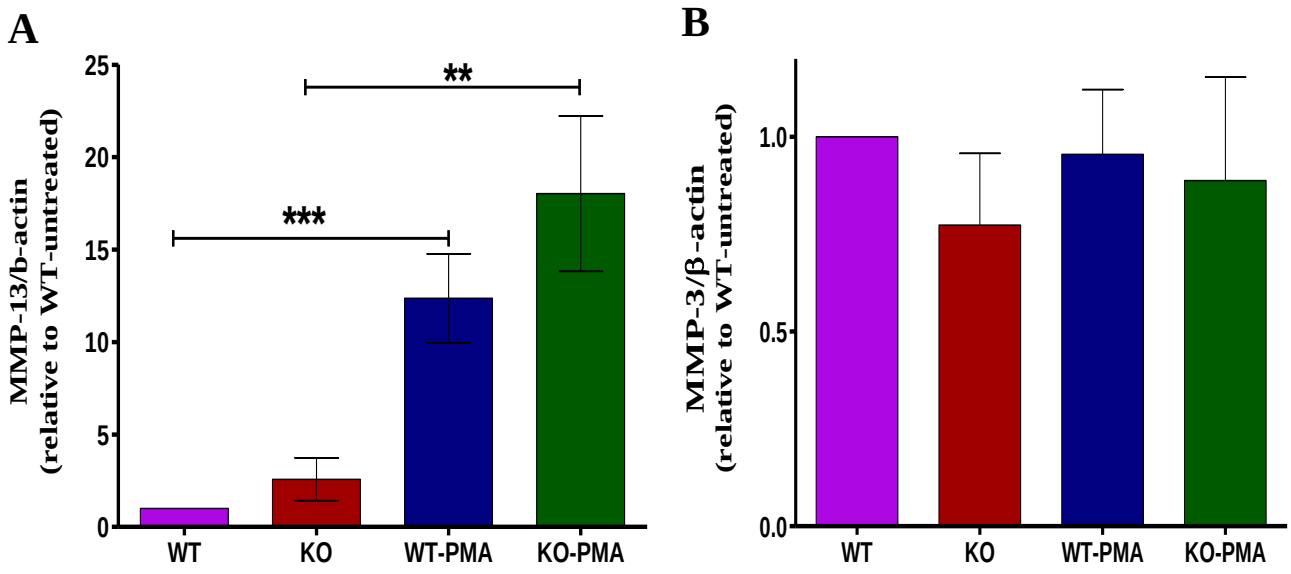


Figure 5.13: A) MMP-13 B) MMP-3 mRNA expression in the myofibroblast cultures with and without PMA. After normalised with the house keeping gene and expressed relative to wild type controls. Values are mean ± SEM based on 5 independent experiments, performed in triplicate. (**p<0.01; ***p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

5.2.3.2.3 TIMP expression

In the non PMA treated cells, the SPARC KO expressed more TIMP-2 and significantly higher (p=0.045) TIMP-1 expression compared to the WT controls (***Figure 5.14***). PMA stimulation significantly increased the expression of TIMP-1 by myofibroblasts in both the WT (p=0.044) and SPARC KO (p=0.041) cells (***Figure 5.14 A***). This was in contrast to TIMP-2 mRNA expression, which, although there was a trend to a reduction in expression following PMA stimulation, was not statistically significant (***Figure 5.14 B***).

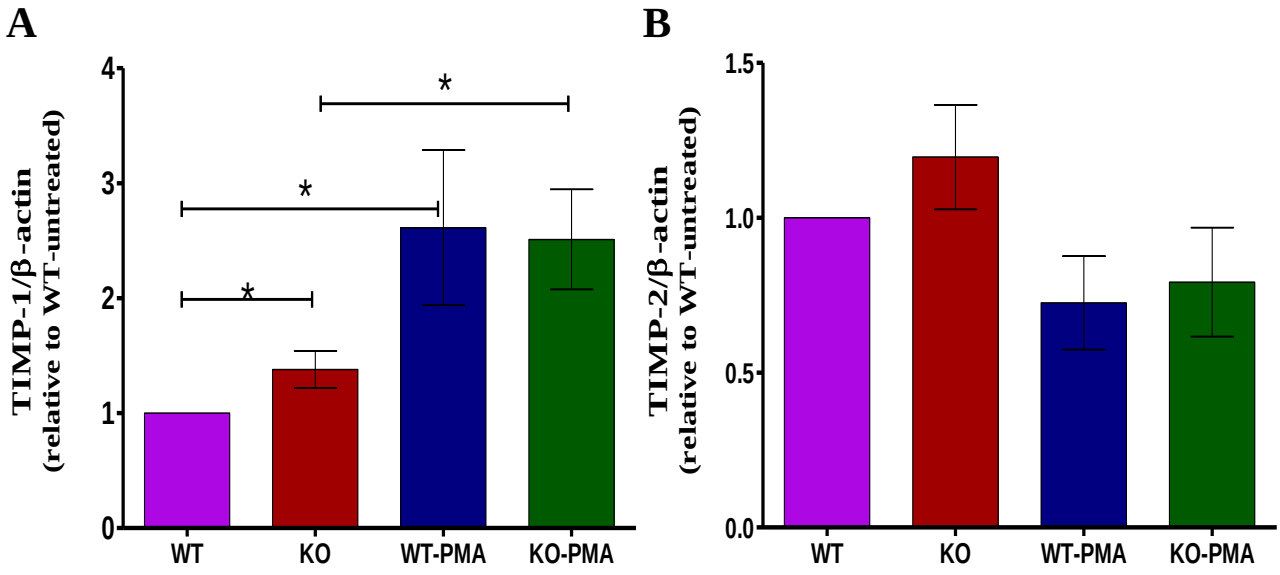


Figure 5.14: A) TIMP-1 B) TIMP-2 mRNA expression in the myofibroblast cultures with and without PMA. After normalised with the house keeping gene and expressed relative to wild type controls. Values are mean ± SEM based on 5 independent experiments, performed in triplicate. (*****p<0.05). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

5.2.3.2.4 TGF-β expression

No differences were observed between the expression of TGF-β1 in the unstimulated myofibroblast cultures (***Figure 5.15 A***). PMA, however, significantly reduced TGF-β3 RNA expression in the WT myofibroblasts, but this was not significantly different to the SPARC KO cells (***Figure 5.15 B***).

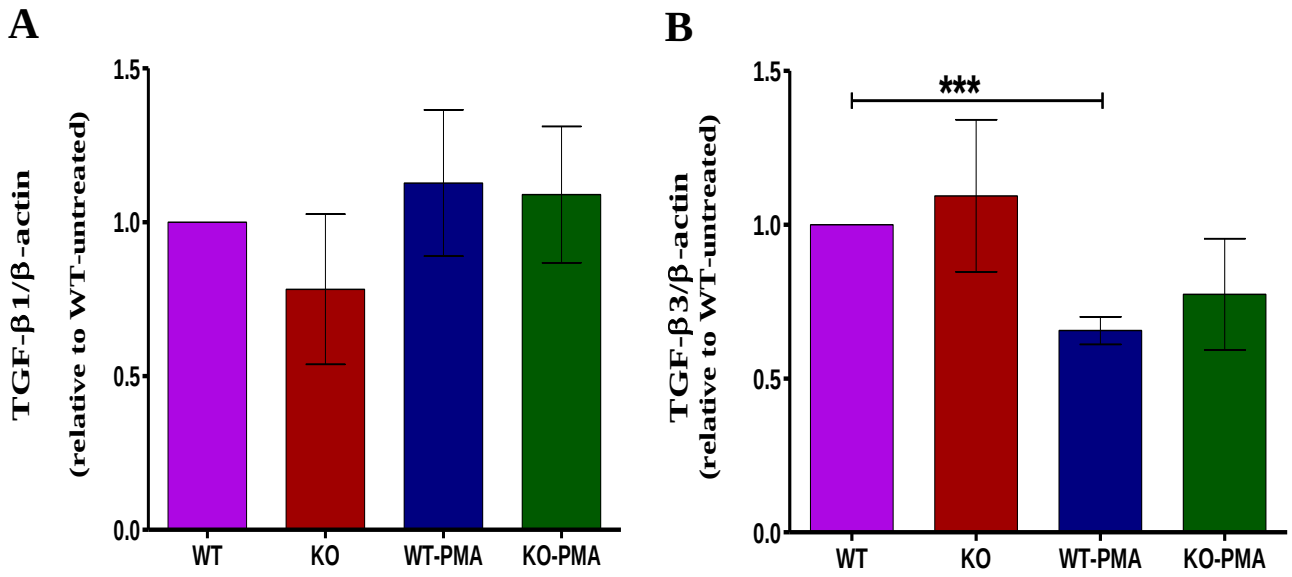


Figure 5.15: A) TGF-β1 B) TGF-β3 mRNA expression in the myofibroblast cultures with and without PMA. After normalised with the house keeping gene and expressed relative to wild type controls. Values are mean ± SEM based on 5 independent experiments, performed in triplicate. (***p<0.001). WT untreated; SPARC KO untreated; WT DSS-treated; SPARC KO DSS-treated

5.3 DISCUSSION

Intestinal wound healing is a tightly regulated process of tissue repair and regeneration following tissue injury. Inappropriate tissue repair, resulting in intestinal fibrosis and stricture, as seen in CrD patients, can greatly impair function of the GIT and may also result in the persistence of mucosal ulceration and the formation of fistulous tracts (Suzuki *et al.* 2011; Abreu *et al.* 2002). SPARC has been shown to play an important role in both wound healing and the regulation of the ECM and potentially the development of fibrosis and may thus represent a potential target in the management of intestinal wound healing. Therefore, the gene expression of key regulatory genes of ECM formation was analysed and compared between WT and SPARC KO mice in this chapter.

Collagen content was evaluated in colonic tissue taken from WT and SPARC KO mice in order to determine if there were any differences between the ECM, including the presence of excessive collagen deposition. Sirius red staining clearly reveals the thin collagen fibres and enables quantitative morphometric assessment of the collagen amounts (van den Borne *et al.* 2008). Comparisons between Sirius red and Masson's trichrome staining, another collagen stain, did not demonstrate any significant differences (data not shown), but due to ease of use and the potential of the examination of the collagen morphology under polarised light (Trombetta and Bradshaw 2010; Junqueira *et al.* 1979), Sirius red staining was chosen for use in this study. The lower amount of collagen in the SPARC KO colons demonstrated by this method not only indicated that the ECM in the KO animals is different to that of WT mice, but also supports the observation of more rapid colonic healing in the KO DSS-treated animals. SPARC KO animals treated with DSS also demonstrated lower levels of collagen

deposition with histological signs of healing from day 21 onwards. These findings are consistent with the published literature which states that in the kidney, heart, lung and skin fibrosis models SPARC KO mice also have lower collagen deposition (Strandjord *et al.* 1999; Socha *et al.* 2007; Bradshaw *et al.* 2009).

It has been reported that collagen type III is increased in CrD strictures (Graham *et al.* 1988; Stallmach *et al.* 1992), such that the ratio of type III:I is >1, while in normal tissue the ratio is <0.3 (Lawrance, Maxwell, *et al.* 2001). Gut fibrosis has also been observed in adenovirus expressing murine MCP-1 treated mice, where the ratio of collagen type III and I demonstrated a significant increase from <0.3 at day 3 to >0.8 at day 21(Motomura *et al*. 2006). This suggests that there is a need for future work to be done to further investigate the collagen type III and I ratio.

Intracellular accumulation of procollagen has been noted to be less in SPARC KO dermal fibroblasts compared to WT cells (Rentz *et al*. 2007), and the proposed rationale for this observation is that without SPARC the precise regulation of procollagen maturation into collagen fibres is altered and the efficiency of collagen to form the cross-linked helical structure that forms the insoluble ECM is compromised (Bradshaw 2009). The data presented in this chapter comparing unstimulated colonic myofibroblasts demonstrated a similar effect in the colon with significantly less collagen expression (***Figure 5.12***; $p<0.001$) and higher levels of MMP-13 gene expression (***Figure 5.13 A***) in the SPARC KO animals. This suggests that in combination with the lower collagen production there is also enhanced collagen degradation further reducing the net deposition of collagen. In addition, when the tissues were examined under the transmission electron microscope , it was noted that

the collagen bundles in the SPARC KO colon had significantly smaller diameters (WT: 40nm vs SPARC KO: 30nm; $p<0.001$) than the WT controls (Lawrance and colleagues unpublished observation) similar to what has been observed by others in the skin (Rentz *et al.* 2007). Again this supports the concept that SPARC is implicated in collagen fibril deposition and organisation.

ECM deposition and the degradation need to be in balance in order to maintain intestinal homeostasis. Fibrosis is a major, and often a frequent, complication in CrD and may lead to intestinal obstruction and the need for surgical intervention. Fibrosis also may complicate UC but due to the superficial nature of the colonic inflammation in UC, it is less likely to result in clinically significant changes. It has been shown that increased procollagen RNA transcription levels but differs in collagen deposition in CrD and UC patients and is has thus been proposed that there are different posttranscriptional, or posttranslational, regulatory mechanisms that affect collagen deposition, such as collagen degradation, that may account for any observed differences (Matthes *et al.* 1992). In the work presented here, there was enhanced expression of the colonic procollagens but there was less collagen protein deposition in SPARC KO DSS-treated animals. This could be related to the level of MMP activity as increased MMP-13 and -3 mRNA expression levels were observed in the DSS-treated SPARC KO animals and degrade collagen thereby reducing the net deposition. MMP-2 and -9 are the two MMPs referred to as gelatinases that are also highly expressed in inflamed colonic mucosa of IBD patient and are associated with the level of disease activity (Stallmach *et al*. 2000; von Lampe *et al*. 2000). These MMPs have also been suggested to contribute to reduced levels of intestinal fibrosis in the DSS-induced colitis model (Suzuki *et al.* 2011). While this thesis focused in MMP-3 and -13, it appears more work, however, will need to be done in order to investigate the impact of these MMPs in

SPARC KO animals. TIMPs differ in their expression patterns and affinity for the various MMPs but overall all MMPs are inhibited by one of the TIMPs once they are activated (Goldberg *et al.* 1992). SPARC KO had less TIMP-1 expression than WT may contribute to the high MMP expression that present. Hence, MMPs/TIMP-1that act as another point of regulates ECM balance shall further be investigated.

The TGF-β family of growth factors is important for many different cell functions, including cellular differentiation, epithelial-mesenchymal cell transformation, and these growth factors are also regarded as central in the process of tissue fibrosis (Schiller *et al.* 2004; Martens *et al.* 1992). TGF-β1 in involved in foetal development from early gestation through to the postnatal period with high expression in fetal dermis and is known to promote fibrosis, while TGF-β3 has, lower expression levels and is postulated to be antifibrogenic (Chen *et al.* 2005). The 'scar-reducing' effect of TGF-β3 has been observed in the incised and sutured mouse lip model, where elevated levels of TGF-β3 reduce type I collagen deposition by restricting myofibroblasts differentiation and thereby collagen synthesis. It can also promote collagen degradation by enhancing MMP expression (Hosokawa *et al*. 2003). Although no statistical differences were observed in the work presented in this chapter, the DSS-induce colitis model and the stimulated myofibroblasts demonstrated numerically higher expression of TGF-β3 in the SPARC KO animals with less collagen deposition and higher in MMP expression. It has also been suggested that TGF-β3 does not work alone in reducing scar formation but it requires TGF-β1 in the appropriate ratio to promote scar-free regeneration as observed in the healing of fetal wounds (Shah *et al.* 1995; Hsu *et al.* 2001; Herdrich *et al.* 2010). Further investigation into to better defining the actions of TGF-β1 and TGF-β3 and the way that these ratio link to wound healing phenotype are, however, still required since SPARC showed to closely interact with TGF-β1.

5.4 CONCLUSION

When the results are taken together, SPARC would appear to be involved in the matrix remodeling of the colon by several interacting mechanisms at the same time, by affecting the expression and processing of procollagen, as well as collagen fibrillogenesis, and by modulating MMP/TIMP-1 expression levels that further altering the net deposition of ECM. Together with the synergistic interaction with TGF-βs suggested by others makes SPARC a promising target for development of therapeutic approaches to improve healing and avoid intestinal fibrosis.

Chapter **6**

General discussion and Future directions

6.1 SUMMARY OF THE FINDINGS FROM THIS THESIS

The addition of DSS to the drinking water of mice promotes the disruption of the intestinal mucosal epithelial integrity, which then allows for the influx of luminal bacterial components into the gut mucosa that were able to promote the recruitment and activation of cells of the innate immune system, such as neutrophils, monocytes and macrophages. These cells then further contributed to colonic mucosal damage. This appears to constitute a self-amplifying process where colonic bacteria activate immune cells and these activated innate cells secrete pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) that recruit additional inflammatory cells into the intestinal mucosa, which, in turn, stimulate metalloproteinases and other factors that result in further tissue damage.

In the studies presented in this thesis, SPARC KO animals demonstrated less mucosal damage, more rapid resolution of the mucosal inflammation and tissue damage, and lower levels of inflammatory cell infiltrates when compared to the WT animals. It was also demonstrated that SPARC KO animals had higher expression levels of the anti-inflammatory cytokine, TGF-β1. The lower numbers of innate immune cells seen in the SPARC KO animals may also be due to the lower levels of chemokine expression (MCP-1, MIG) compared to the WT animals. It appears, therefore, that SPARC is able to affect the inflammatory response by modulating inflammatory cell recruitment via chemokine and cytokine expression levels,

SPARC has previously been noted to alter tissue ECM and this was also observed for the colon in this study. The level of collagen deposition in any tissue is a balance between its production and degradation. SPARC KO mice were noted to have lower

colonic collagen deposition than WT animals. The expression of the procollagens, however, in the colonic tissue was noted to be higher in KO animals compared to WT mice at day 7, but the expression of the MMPs was noted to be higher as well. The combination of these findings suggests that the net deposition of collagens in the SPARC KO animals would be reduced. Colonic subepithelial myofibroblasts are the cells primarily responsible for ECM deposition and were isolated from the SPARC KO and WT mice. SPARC KO myofibroblasts had higher MMP-3 expression than the WT cells in line with the findings within the colon. SPARC KO myofibroblasts, however, had less expression of the procollagens again supporting the concept that the colonic ECM is different without SPARC. A higher frequency of Treg cells was also observed in the SPARC KO mice and this might be secondary to the less dense ECM that has been previously noted SPARC KO animals. This differentially structured ECM could potentially allow for more TGF-β to be released and could also be associated with more rapid DC migration and subsequent T cells priming.

6.2 HOW THESE FINDINGS ADD TO THE PUBLISHED LITERATURE

SPARC is a matricellular protein expressed in variety of tissues during embryogenesis. In adult vertebrates, its expression is restricted to those tissues undergoing remodeling such as gut, bone, or following tissue injury. It also has been suggested that SPARC has an important modulatory role in healing and inflammation as well as fibrosis. (Bradshaw and Sage 2001; Brekken and Sage 2000). Increased SPARC expression levels have already been demonstrated in inflamed, non fibrosed colonic surgical UC specimens compared to normal colonic tissue (Lawrance, Fiocchi, *et al.* 2001) and the DSS-induced model of colitis is considered to be a model that mimics UC. The finding

that SPARC KO animals exposed to DSS exhibited less inflammation than control animals is consistent with a previous report that SPARC null mice also exhibit attenuated inflammation and fibrosis of the kidneys (Socha *et al*. 2007). Recent reports have also shown that levels of the inflammatory markers also correlate with increased SPARC expression (Reding *et al.* 2009; Kos *et al.* 2009). In contrast, however, SPARC null mice were noted to have increased levels of neutrophil and leucocyte infiltration in bleomycin-induced lung injury and thioglycolate-induced peritonitis (Savani *et al*. 2000). It seems that SPARC inhibition is thus able to alter the composition of the tissue microenvironment, which may either promote, or restrain, the inflammatory response depending of the tissue specific context. This may be due to the degradation products of SPARC as these smaller peptide fragments that represent different domains of SPARC also have biological activity. These fragments can oppose the activity of other SPARC fragments or the native SPARC protein itself. (Yin *et al.* 2010). These findings are also not surprising since the protease profile of the microenvironment may differ between different tissue types and SPARC is known to undergo rapid proteolysis by MMPs (Iruela-Arispe *et al*. 1995), not to mention the effect that the local composition of matrix molecules and cytokines play in contributing to the complex behaviour of SPARC in different types of inflammation.

Permeabilisation of the microvascular endothelial-cell barrier permits leucocytes, immunoglobulins, and other immunogenic agents to translocate into the site of tissue injury and is a necessary condition for an inflammatory response to occur. While its role in inflammation is controversial, and may vary between different systems, SPARC has been proposed as a promoter of inflammation by a process of enhancing leucocyte passage into the extravascular tissues (Workman and Sage 2011). Recently, a binding

site for the receptor, stabilin-1, has been identified on SPARC and the monoclonal antibody that recognises the binding site has also been characterised. SPARC has been shown to interact with stabilin-1 (Kzhyshkowska *et al.* 2006) and stabilin-1 promotes inflammation by arresting leucocyte flow in the blood and aiding their positioning for extravasation (Workman and Sage 2011). These observations suggest a possible therapeutic target to modify inflammation through the inhibition of the SPARC/stabilin-1interaction thereby reducing leucocyte recruitment to inflamed tissue (Workman and Sage 2011). In addition, there are already molecules being investigated in the management of both UC and CrD like MLN0002 that reduces recruitment of leucocytes through blocking the integrin α4β7 (Feagan *et al.* 2008).

TGF-β is secreted in a latent, inactive form and is stored at the cell surface or covalently linked to the ECM where it is proteolytically cleaved to its active form (Annes *et al.* 2003; Border and Noble 1994). SPARC is known to positively regulate TGF-β1 activity and vice versa although the precise mechanism is yet unknown. In the SPARC KO DSS-treated animals, low TGF-β1 expression levels were predicted but the TGF-β1 levels were instead noted to be lower in the WT than SPARC KO mice. Chlenski and colleagues, however, also demonstrated that SPARC could have a negative effect on TGF-β1 activity both *in vitro* and *in vivo* (Chlenski *et al.* 2007) as SPARC null pericytes displayed increased basal TGF-β1 activity (Rivera and Brekken 2011). Without SPARC being present, the ECM in the gut was observed to be different to the WT mice and this could potentially allowed easier cleavage of TGF-β from its ECM storage. As TGF-β1 is very important for multiple different cellular activities (Kisseleva and Brenner 2008), SPARC KO mice may generate an "adaptive" mechanism that allows for an increase in the availability of TGF-β1 in order to maintain homeostasis.

SPARC deficient HSCs have also been shown to have increased levels of Adamst8, thrombospondin-1 and other MMPs, that are known to be involved in the activation of TGF-β1, to avoid cell death (Atorrasagasti *et al.* 2011). In addition, SPARC null mice have increased accumulation of white adipose tissue (Bradshaw *et al.* 2002), which is associated with increased TGF-β (Keophiphath *et al.* 2009). As TGF-β1 is a potent anti-inflammatory cytokine, the high levels of TGF-β1 may account for the lower intestinal inflammation that was observed in the DSS-treated SPARC KO mice. TGF-β1 is also vital in the differentiation of Treg cells that also regulate inflammation, while, the secretion of TGF-β1 by macrophages is known to suppress the pro-inflammatory signalling from Toll-like receptors further enhancing the speed of tissue repair (Barker *et al.* 1999).

SPARC is linked with leptin. Leptin is a key secretory product of adipose tissue (Kos and Wilding 2010) and is also a mediator of intestinal inflammation. Leptin deficient mice have reduced colitis severity and lower secretion levels of pro-inflammatory cytokines in both the DSS- and TNBS-induced models of colitis (Siegmund *et al.* 2002). This suggests that a lack of SPARC will promote intestinal inflammation as the SPARC KO mice have increased fat accumulation and leptin levels (Bradshaw, Graves, *et al.* 2003). This trend, however, was not observed in this study and might be due to the strain of mice used, despite the fact that the SPARC KO animals used in the experiment presented having increased of leptin mRNA levels (data not show). It also suggests that leptin might require an intact SPARC pathway in order to induce intestinal inflammation. Conversely, the increased level of leptin may speed up the healing process in the SPARC KO animals as leptin has previously been shown to improve dermal healing (Frank *et al*. 2000).

Fibrosis occurs when the wound healing response is out of control and causes excessive deposition of ECM. Although DSS-induced colitis is not an ideal fibrosis model, the findings presented suggest that SPARC might impair healing and be pro-fibrogenic. TGF-β1 has anti-inflammatory properties but it is also a key pro-fibrogenic factor. A low fibrogenic response was seen in SPARC KO mice following DSS-treatment despite high TGF-β1 levels being present in the colon. The lack of fibrosis might be due to the fact that the fibrogenic properties of TGF-β are not mediated directly but occur through its induction of connective tissue growth factor (CTGF), which acts in an autocrine loop to increase collagen synthesis (Duncan *et al.* 1999) and SPARC is known as an upstream regulator of the CTGF (Zhou *et al.* 2006) and may impact on this interaction. Further research is, therefore, required in order to decipher any interaction between TGF-β/CTGF/SPARC and their effects on inflammation.

In conclusion, the work presented in this thesis indicates that SPARC is pro-inflammatory in the DSS-induced colitis model. SPARC also remodeled ECM to prolong the healing process and potentially has a pro-fibrogenic activity. It also raises several important avenues of future research that should be undertaken in order to clarify the precise functional involvement of SPARC in intestinal inflammation.

6.3 FUTURE DIRECTIONS

In addition to those areas of future work mentioned in previous chapters, more analysis is required in order to improve the experimental protocol. In chapter 4, the numbers of Ly6G+ neutrophils at day 7 and 14 were numerical lower than those at day 21 and 35 but this was most likely due to the poor tissue structure of the severely inflamed tissue

with less area for viewing and subsequent counting, and thus acted as secondary evidence that neutrophils were causing severe colonic tissue destruction possibly through reactive oxygen species despite lower numbers numerically. That SPARC KO mice had fewer neutrophils also supports the proposal that SPARC KO mice heal faster than the WT animals as effective elimination of neutrophils is a prerequisite for resolution of the inflammatory response. Further biochemical tests could also be performed to measure the enzyme myeloperoxidase within the colonic tissue and this may be able to better determine the level of neutrophils present in the colonic tissue particularly during the early acute phase of disease. TREM-1 could also be stained in colonic cryosections in order to distinguish the inflammatory macrophage from the resident macrophage as TREM-1 enhances the secretion of the pro-inflammatory cytokines by the inflammatory macrophages.

The adaptive immune system does not appear to be primarily involved in the acute stage of the DSS-induced colitis model as severe combined immunodeficient mice that lack both T and B cells are still able to develop the DSS-induced acute colitis (Dieleman *et al*. 1994). The adaptive immune system, however, plays an important role in the development of chronic inflammation as observed in certain mice strains such as C57BL/6, while chronic inflammation is not seen in BALB/c mice (Melgar *et al.* 2005). The progression of the acute colitis towards that of chronicity observed in C57BL/6 mice suggests that this is a potential animal model for the pathological inflammatory changes seen in patients with UC, while the acute model of colitis in the BALB/c may be useful in the study of normal mucosal healing (Melgar *et al.* 2005). The chronicity seen in C57BL/6 mice after a single dose of DSS might also only reflect prolonged repair of acute colitis and it is not defined simply as chronic colitis (Suzuki *et al.* 2011).

As mentioned above, DSS-induced colitis is not a fibrosis model although intestinal fibrosis was observed in C57BL/6 mice (Melgar *et al.* 2005). TNBS with concomitant administration of ethanol, which disrupts the colonic epithelial barrier, induces colitis and is considered as more of a CrD model where chronic, transmural inflammation and fibrosis as well as a skewed immune reaction towards the Th1 profile is observed in mice (Lawrance *et al.* 2003). Thus, the use of the DSS experimental model in different mice strain could be repeated in the future, as well as using the TNBS-induced colitis model to allow for further investigates of the mechanism that SPARC may have on both the acute and chronic changes in gut immunity.

The role for SPARC in tumorigenesis is also unclear, as in inflammation, and it appears to depend on its functions within a given microenvironment. SPARC has been found to be increased in melonama cell lines and other neoplastic cells within tumours (Ledda, Bravo, *et al.* 1997). When the expression of SPARC was suppressed by antisense RNA, this resulted in a decrease in both the adhesive and invasive characteristics of the cells *in vitro* and resulted in a complete loss of invasive-metastatic phenotype development in human melonoma cells (Alvarez *et al.* 2005; Ledda, Adris, *et al.* 1997). In contrasted, to the observed effects of SPARC in melanomas, it has been shown to anti-tumorigenic in ovarian cancers (Chlenski and Cohn 2010) where overexpression of SPARC is able to attenuate mitogenic, chemotactic, and pro-invasive effects that induced by infiltrating macrophages in ovarian cancer cells (Said, Socha, *et al.* 2007; Said and Motamed 2005). In colorectal tumours, low or no SPARC is expressed by the neoplastic cells, but it is present within the stromal cells that surround the glandular tissue (Tai *et al.* 2005; Lussier *et al.* 2001). Recently, our group identified that SPARC expression levels in the primary tumour of patients with colorectal cancer correlated with their long-term

cancer-specific-survival (Chew *et al.* 2011). Chronic inflammation has been linked with the developed of neoplasia and cancer in numerous tissue types and this has particularly been noted in patients with chronic inflammatory bowel disease that may lead to colorectal cancer (Seril *et al.* 2003; Katsanos *et al.* 2011; Neumann *et al.* 2011). Our studies in the animal colitis models demonstrated that the presence of SPARC is associated with greater levels of inflammation, but how this may impact on carcinogenesis in human colorectal cancer require further research. SPARC, however, may represent a potentially clinically important therapeutic target in this matter. Currently, this is being further investigated in our lab by using the murine tumouroigenesis models of azoxymethane–alone and azoxymethane in combination with DSS.

There is evidence to suggest that ECM fragments or ECM molecules can influence the inflammatory response. SPARC has been shown here to exacerbate intestinal inflammation. Inflammation is also closely associated with cancer where tumour development might arise from sites of unresolved chronic inflammation as seen in IBD patients. It is, therefore, possible that SPARC might be a potential therapeutic protein for the modification of inflammation levels and reduction of tumour development in the colon.

Chapter 7

Bibliography

Abdelbaqi, M., J. H. Chidlow, K. M. Matthews, K. P. Pavlick, S. C. Barlow, A. J. Linscott, M. B. Grisham, M. R. Fowler, and C. G. Kevil. 2006. Regulation of dextran sodium sulfate induced colitis by leukocyte beta 2 integrins. *Lab Invest* 86 (4):380-90.

Abreu, M. T., K. D. Taylor, Y. C. Lin, T. Hang, J. Gaiennie, C. J. Landers, E. A. Vasiliauskas, L. Y. Kam, M. Rojany, K. A. Papadakis, J. I. Rotter, S. R. Targan, and H. Yang. 2002. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* 123 (3):679-88.

Agnello, D., C. S. Lankford, J. Bream, A. Morinobu, M. Gadina, J. J. O'Shea, and D. M. Frucht. 2003. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol* 23 (3):147-61.

Alvarez, M. J., F. Prada, E. Salvatierra, A. I. Bravo, V. P. Lutzky, C. Carbone, F. J. Pitossi, H. E. Chuluyan, and O. L. Podhajcer. 2005. Secreted protein acidic and rich in cysteine produced by human melanoma cells modulates polymorphonuclear leukocyte recruitment and antitumor cytotoxic capacity. *Cancer Res* 65 (12):5123-32.

Andoh, A., S. Bamba, Y. Fujiyama, M. Brittan, and N. A. Wright. 2005. Colonic subepithelial myofibroblasts in mucosal inflammation and repair: contribution of bone marrow-derived stem cells to the gut regenerative response. *J Gastroenterol* 40 (12):1089-99.

Andres, P. G., P. L. Beck, E. Mizoguchi, A. Mizoguchi, A. K. Bhan, T. Dawson, W. A. Kuziel, N. Maeda, R. P. MacDermott, D. K. Podolsky, and H. C. Reinecker. 2000. Mice with a selective deletion of the CC chemokine receptors 5 or 2 are protected from dextran sodium sulfate-mediated colitis: lack of CC chemokine receptor 5 expression results in a NK1.1+ lymphocyte-associated Th2-type immune response in the intestine. *J Immunol* 164 (12):6303-12.

Annes, J. P., J. S. Munger, and D. B. Rifkin. 2003. Making sense of latent TGFbeta activation. *J Cell Sci* 116 (Pt 2):217-24.

Arnold, S. A., L. B. Rivera, A. F. Miller, J. G. Carbon, S. P. Dineen, Y. Xie, D. H. Castrillon, E. H. Sage, P. Puolakkainen, A. D. Bradshaw, and R. A. Brekken. 2010. Lack of host SPARC enhances vascular function and tumor spread in an orthotopic murine model of pancreatic carcinoma. *Dis Model Mech* 3 (1-2):57-72.

Arseneau, K. O., H. Tamagawa, T. T. Pizarro, and F. Cominelli. 2007. Innate and adaptive immune responses related to IBD pathogenesis. *Curr Gastroenterol Rep* 9 (6):508-12.

Artis, D. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* 8 (6):411-20.

Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455 (7214):808-12.

Atorrasagasti, C., J. B. Aquino, L. Hofman, L. Alaniz, M. Malvicini, M. Garcia, L. Benedetti, S. L. Friedman, O. Podhajcer, and G. Mazzolini. 2011. SPARC downregulation attenuates the profibrogenic response of hepatic stellate cells induced by TGF-beta1 and PDGF. *Am J Physiol Gastrointest Liver Physiol* 300 (5):G739-48.

Atreya, R., J. Mudter, S. Finotto, J. Mullberg, T. Jostock, S. Wirtz, M. Schutz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J. F. Schlaak, H. A. Lehr, F. Autschbach, G. Schurmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P. R. Galle, S. Rose-John, and M. F. Neurath. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 6 (5):583-8.

Aumailley, M., and B. Gayraud. 1998. Structure and biological activity of the extracellular matrix. *J Mol Med* 76 (3-4):253-65.

Bamias, G., C. Martin, M. Mishina, W. G. Ross, J. Rivera-Nieves, M. Marini, and F. Cominelli. 2005. Proinflammatory effects of TH2 cytokines in a murine model of chronic small intestinal inflammation. *Gastroenterology* 128 (3):654-66.

Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767-811.

Barker, R. N., L. Erwig, W. P. Pearce, A. Devine, and A. J. Rees. 1999. Differential effects of necrotic or apoptotic cell uptake on antigen presentation by macrophages. *Pathobiology* 67 (5-6):302-5.

Barker, T. H., G. Baneyx, M. Cardo-Vila, G. A. Workman, M. Weaver, P. M. Menon, S. Dedhar, S. A. Rempel, W. Arap, R. Pasqualini, V. Vogel, and E. H. Sage. 2005. SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity. *J Biol Chem* 280 (43):36483-93.

Bassuk, J. A., T. Birkebak, J. D. Rothmier, J. M. Clark, A. Bradshaw, P. J. Muchowski, C. C. Howe, J. I. Clark, and E. H. Sage. 1999. Disruption of the Sparc locus in mice alters the differentiation of lenticular epithelial cells and leads to cataract formation. *Exp Eye Res* 68 (3):321-31.

Basu, A., L. H. Kligman, S. J. Samulewicz, and C. C. Howe. 2001. Impaired wound healing in mice deficient in a matricellular protein SPARC (osteonectin, BM-40). *BMC Cell Biol* 2:15.

Becker, C., M. C. Fantini, S. Wirtz, A. Nikolaev, R. Kiesslich, H. A. Lehr, P. R. Galle, and M. F. Neurath. 2005. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut* 54 (7):950-4.

Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 17:189-220.

Boden, E. K., and S. B. Snapper. 2008. Regulatory T cells in inflammatory bowel disease. *Curr Opin Gastroenterol* 24 (6):733-41.

Border, W. A., and N. A. Noble. 1994. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331 (19):1286-92.

Border, W. A., N. A. Noble, T. Yamamoto, J. R. Harper, Y. Yamaguchi, M. D. Pierschbacher, and E. Ruoslahti. 1992. Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 360 (6402):361-4.

Bosani, M., S. Ardizzone, and G. B. Porro. 2009. Biologic targeting in the treatment of inflammatory bowel diseases. *Biologics* 3:77-97.

Bouchon, A., J. Dietrich, and M. Colonna. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol* 164 (10):4991-5.

Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 3 (7):521-33.

Bradshaw, A. D. 2009. The role of SPARC in extracellular matrix assembly. *J Cell Commun Signal* 3 (3-4):239-46.

Bradshaw, A. D., C. F. Baicu, T. J. Rentz, A. O. Van Laer, J. Boggs, J. M. Lacy, and M. R. Zile. 2009. Pressure overload-induced alterations in fibrillar collagen content and myocardial diastolic function: role of secreted protein acidic and rich in cysteine (SPARC) in post-synthetic procollagen processing. *Circulation* 119 (2):269-80.

Bradshaw, A. D., D. C. Graves, K. Motamed, and E. H. Sage. 2003. SPARC-null mice exhibit increased adiposity without significant differences in overall body weight. *Proc Natl Acad Sci U S A* 100 (10):6045-50.

Bradshaw, A. D., P. Puolakkainen, J. Dasgupta, J. M. Davidson, T. N. Wight, and E. Helene Sage. 2003. SPARC-null mice display abnormalities in the dermis characterized by decreased collagen fibril diameter and reduced tensile strength. *J Invest Dermatol* 120 (6):949-55.

Bradshaw, A. D., M. J. Reed, and E. H. Sage. 2002. SPARC-null mice exhibit accelerated cutaneous wound closure. *J Histochem Cytochem* 50 (1):1-10.

Bradshaw, A. D., and E. H. Sage. 2001. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. *J Clin Invest* 107 (9):1049-54.

Braegger, C. P., S. Nicholls, S. H. Murch, S. Stephens, and T. T. MacDonald. 1992. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet* 339 (8785):89-91.

Brand, S. 2009. Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut* 58 (8):1152-67.

Brekken, R. A., and E. H. Sage. 2000. SPARC, a matricellular protein: at the crossroads of cell-matrix. *Matrix Biol* 19 (7):569-80.

Brenmoehl, J., S. N. Miller, C. Hofmann, D. Vogl, W. Falk, J. Scholmerich, and G. Rogler. 2009. Transforming growth factor-beta 1 induces intestinal myofibroblast differentiation and modulates their migration. *World J Gastroenterol* 15 (12):1431-42.

Burgio, V. L., S. Fais, M. Boirivant, A. Perrone, and F. Pallone. 1995. Peripheral monocyte and naive T-cell recruitment and activation in Crohn's disease. *Gastroenterology* 109 (4):1029-38.

Camino, A. M., C. Atorrasagasti, D. Maccio, F. Prada, E. Salvatierra, M. Rizzo, L. Alaniz, J. B. Aquino, O. L. Podhajcer, M. Silva, and G. Mazzolini. 2008. Adenovirus-mediated inhibition of SPARC attenuates liver fibrosis in rats. *J Gene Med* 10 (9):993-1004.

Cash, H. L., C. V. Whitham, C. L. Behrendt, and L. V. Hooper. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313 (5790):1126-30.

Chang, W., K. Wei, S. S. Jacobs, D. Upadhyay, D. Weill, and G. D. Rosen. 2010. SPARC suppresses apoptosis of idiopathic pulmonary fibrosis fibroblasts through constitutive activation of beta-catenin. *J Biol Chem* 285 (11):8196-206.

Chen, W., X. Fu, S. Ge, T. Sun, G. Zhou, D. Jiang, and Z. Sheng. 2005. Ontogeny of expression of transforming growth factor-beta and its receptors and their possible relationship with scarless healing in human fetal skin. *Wound Repair Regen* 13 (1):68-75.

Chew, A., P. Salama, A. Robbshaw, B. Klopcic, N. Zeps, C. Platell, and I. C. Lawrance. 2011. SPARC, FOXP3, CD8 and CD45 correlation with disease recurrence and long-term disease-free survival in colorectal cancer. *PLoS One* 6 (7):e22047.

Chidlow, J. H., Jr., J. J. Greer, C. Anthoni, P. Bernatchez, C. Fernandez-Hernando, M. Bruce, M. Abdelbaqi, D. Shukla, D. N. Granger, W. C. Sessa, and C. G. Kevil. 2009. Endothelial caveolin-1 regulates pathologic angiogenesis in a mouse model of colitis. *Gastroenterology* 136 (2):575-84 e2.

Chlenski, A., and S. L. Cohn. 2010. Modulation of matrix remodeling by SPARC in neoplastic progression. *Semin Cell Dev Biol* 21 (1):55-65.

Chlenski, A., L. J. Guerrero, Q. Yang, Y. Tian, R. Peddinti, H. R. Salwen, and S. L. Cohn. 2007. SPARC enhances tumor stroma formation and prevents fibroblast activation. *Oncogene* 26 (31):4513-22.

Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Carcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 204 (8):1757-64.

Coussens, L. M., and Z. Werb. 1996. Matrix metalloproteinases and the development of cancer. *Chem Biol* 3 (11):895-904.

Da Silva, A. P., A. Pollett, S. R. Rittling, D. T. Denhardt, J. Sodek, and R. Zohar. 2006. Exacerbated tissue destruction in DSS-induced acute colitis of OPN-null mice is associated with downregulation of TNF-alpha expression and non-programmed cell death. *J Cell Physiol* 208 (3):629-39.

Danese, S., and A. Gasbarrini. 2005. Chemokines in inflammatory bowel disease. *J Clin Pathol* 58 (10):1025-7.

Dayer, J. M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. *J Exp Med* 162 (6):2163-8.

Desreumaux, P., E. Brandt, L. Gambiez, D. Emilie, K. Geboes, O. Klein, N. Ectors, A. Cortot, M. Capron, and J. F. Colombel. 1997. Distinct cytokine patterns in early and chronic ileal lesions of Crohn's disease. *Gastroenterology* 113 (1):118-26.

Diegelmann, R. F., and M. C. Evans. 2004. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 9:283-9.

Dieleman, L. A., M. J. Palmen, H. Akol, E. Bloemena, A. S. Pena, S. G. Meuwissen, and E. P. Van Rees. 1998. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 114 (3):385-91.

Dieleman, L. A., B. U. Ridwan, G. S. Tennyson, K. W. Beagley, R. P. Bucy, and C. O. Elson. 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 107 (6):1643-52.

Dignass, A. U., and D. K. Podolsky. 1993. Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor beta. *Gastroenterology* 105 (5):1323-32.
Duncan, M. R., K. S. Frazier, S. Abramson, S. Williams, H. Klapper, X. Huang, and G. R. Grotendorst. 1999. Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *FASEB J* 13 (13):1774-86.
Eksteen, B., A. Miles, S. M. Curbishley, C. Tselepis, A. J. Grant, L. S. Walker, and D. H. Adams. 2006. Epithelial inflammation is associated with CCL28 production and the recruitment of regulatory T cells expressing CCR10. *J Immunol* 177 (1):593-603.
Eksteen, B., L. S. Walker, and D. H. Adams. 2005. Immune regulation and colitis: suppression of acute inflammation allows the development of chronic inflammatory bowel disease. *Gut* 54 (1):4-6.
Elson, C. O., R. B. Sartor, G. S. Tennyson, and R. H. Riddell. 1995. Experimental models of inflammatory bowel disease. *Gastroenterology* 109 (4):1344-67.
Feagan, B. G., G. R. Greenberg, G. Wild, R. N. Fedorak, P. Pare, J. W. McDonald, A. Cohen, A. Bitton, J. Baker, R. Dube, S. B. Landau, M. K. Vandervoort, and A. Parikh. 2008. Treatment of active Crohn's disease with MLN0002, a humanized antibody to the alpha4beta7 integrin. *Clin Gastroenterol Hepatol* 6 (12):1370-7.
Fichtner-Feigl, S., I. J. Fuss, J. C. Preiss, W. Strober, and A. Kitani. 2005. Treatment of murine Th1- and Th2-mediated inflammatory bowel disease with NF-kappa B decoy oligonucleotides. *J Clin Invest* 115 (11):3057-71.
Fichtner-Feigl, S., W. Strober, K. Kawakami, R. K. Puri, and A. Kitani. 2006. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* 12 (1):99-106.
Finkelstein, S. D., E. Sasatomi, and M. Regueiro. 2002. Pathologic features of early inflammatory bowel disease. *Gastroenterol Clin North Am* 31 (1):133-45.
Fiocchi, C. 1998. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 115 (1):182-205.
Fiocchi, C., K. Ina, S. Danese, A. Z. Leite, and J. D. Vogel. 2006. Alterations of mesenchymal and endothelial cells in inflammatory bowel diseases. *Adv Exp Med Biol* 579:168-76.
Francki, A., A. D. Bradshaw, J. A. Bassuk, C. C. Howe, W. G. Couser, and E. H. Sage. 1999. SPARC regulates the expression of collagen type I and transforming growth factor-beta1 in mesangial cells. *J Biol Chem* 274 (45):32145-52.
Francki, A., T. D. McClure, R. A. Brekken, K. Motamed, C. Murri, T. Wang, and E. H. Sage. 2004. SPARC regulates TGF-beta1-dependent signaling in primary glomerular mesangial cells. *J Cell Biochem* 91 (5):915-25.
Frank, S., B. Stallmeyer, H. Kampfer, N. Kolb, and J. Pfeilschifter. 2000. Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair. *J Clin Invest* 106 (4):501-9.
Freshney, R.I. 2005. Primary culture. In *Culture of Animal Cells: A manual of basic technique*

Canada: John Wiley & Sons, Inc.
Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52 (1):65-70.
Funk, S. E., and E. H. Sage. 1991. The Ca2(+)-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* 88 (7):2648-52.
Funk, S. E., and E. H. Sage. 1993. Differential effects of SPARC and cationic SPARC peptides on DNA synthesis by endothelial cells and fibroblasts. *J Cell Physiol* 154 (1):53-63.
Ge, G., and D. S. Greenspan. 2006. BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. *J Cell Biol* 175 (1):111-20.
Gharaee-Kermani, M., and S. H. Phan. 2001. Role of cytokines and cytokine therapy in wound healing and fibrotic diseases. *Curr Pharm Des* 7 (11):1083-103.
Goldberg, G. I., A. Strongin, I. E. Collier, L. T. Genrich, and B. L. Marmer. 1992. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents

dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J Biol Chem* 267 (7):4583-91.
Graham, M. F., R. F. Diegelmann, C. O. Elson, W. J. Lindblad, N. Gotschalk, S. Gay, and R. Gay. 1988. Collagen content and types in the intestinal strictures of Crohn's disease. *Gastroenterology* 94 (2):257-65.
Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389 (6652):737-42.
Guimbaud, R., V. Bertrand, L. Chauvelot-Moachon, G. Quartier, N. Vidon, J. P. Giroud, D. Couturier, and S. Chaussade. 1998. Network of inflammatory cytokines and correlation with disease activity in ulcerative colitis. *Am J Gastroenterol* 93 (12):2397-404.
Hall, L. J., E. Faivre, A. Quinlan, F. Shanahan, K. Nally, and S. Melgar. 2011. Induction and activation of adaptive immune populations during acute and chronic phases of a murine model of experimental colitis. *Dig Dis Sci* 56 (1):79-89.
Harper, P. H., V. W. Fazio, I. C. Lavery, D. G. Jagelman, F. L. Weakley, R. G. Farmer, and K. A. Easley. 1987. The long-term outcome in Crohn's disease. *Dis Colon Rectum* 30 (3):174-9.
Harrison, J., and S. B. Hanauer. 2002. Medical treatment of Crohn's disease. *Gastroenterol Clin North Am* 31 (1):167-84, x.
Hasselaar, P., D. J. Loskutoff, M. Sawdey, and E. H. Sage. 1991. SPARC induces the expression of type 1 plasminogen activator inhibitor in cultured bovine aortic endothelial cells. *J Biol Chem* 266 (20):13178-84.
Hasselaar, P., and E. H. Sage. 1992. SPARC antagonizes the effect of basic fibroblast growth factor on the migration of bovine aortic endothelial cells. *J Cell Biochem* 49 (3):272-83.
Hata, K., A. Andoh, M. Shimada, S. Fujino, S. Bamba, Y. Araki, T. Okuno, Y. Fujiyama, and T. Bamba. 2002. IL-17 stimulates inflammatory responses via NF-kappaB and MAP kinase pathways in human colonic myofibroblasts. *Am J Physiol Gastrointest Liver Physiol* 282 (6):G1035-44.
Heinsbroek, S. E., and S. Gordon. 2009. The role of macrophages in inflammatory bowel diseases. *Expert Rev Mol Med* 11:e14.
Heller, F., P. Florian, C. Bojarski, J. Richter, M. Christ, B. Hillenbrand, J. Mankertz, A. H. Gitter, N. Burgel, M. Fromm, M. Zeitz, I. Fuss, W. Strober, and J. D. Schulzke. 2005. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 129 (2):550-64.
Herdrich, B. J., E. Danzer, M. G. Davey, D. M. Bermudez, A. Radu, L. Zhang, Z. Zhang, L. J. Soslowsky, and K. W. Liechty. 2010. Fetal tendon wound size modulates wound gene expression and subsequent wound phenotype. *Wound Repair Regen* 18 (5):543-9.
Hoffmann, K. F., A. W. Cheever, and T. A. Wynn. 2000. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol* 164 (12):6406-16.
Hosokawa, R., K. Nonaka, M. Morifuji, L. Shum, and M. Ohishi. 2003. TGF-beta 3 decreases type I collagen and scarring after labioplasty. *J Dent Res* 82 (7):558-64.
Hsu, M., Z. M. Peled, G. S. Chin, W. Liu, and M. T. Longaker. 2001. Ontogeny of expression of transforming growth factor-beta 1 (TGF-beta 1), TGF-beta 3, and TGF-beta receptors I and II in fetal rat fibroblasts and skin. *Plast Reconstr Surg* 107 (7):1787-94; discussion 1795-6.
Iruela-Arispe, M. L., T. F. Lane, D. Redmond, M. Reilly, R. P. Bolender, T. J. Kavanagh, and E. H. Sage. 1995. Expression of SPARC during development of the chicken chorioallantoic membrane: evidence for regulated proteolysis in vivo. *Mol Biol Cell* 6 (3):327-43.
Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S. Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21 (4):527-38.
Izcue, A., J. L. Coombes, and F. Powrie. 2006. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 212:256-71.
Izcue, A., J. L. Coombes, and F. Powrie. 2009. Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol* 27:313-38.

Jego, G., A. K. Palucka, J. P. Blanck, C. Chalouni, V. Pascual, and J. Banchereau. 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 19 (2):225-34.

Jendraschak, E., and E. H. Sage. 1996. Regulation of angiogenesis by SPARC and angiostatin: implications for tumor cell biology. *Semin Cancer Biol* 7 (3):139-46.

Jenkins, G. 2008. The role of proteases in transforming growth factor-beta activation. *Int J Biochem Cell Biol* 40 (6-7):1068-78.

Junqueira, L. C., G. Bignolas, and R. R. Brentani. 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* 11 (4):447-55.

Kallen, K. J. 2002. The role of transsignalling via the agonistic soluble IL-6 receptor in human diseases. *Biochim Biophys Acta* 1592 (3):323-43.

Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M. T. Kitazume, A. Sugita, K. Koganei, K. S. Akagawa, and T. Hibi. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* 118 (6):2269-80.

Kaplan, M. H., Y. L. Sun, T. Hoey, and M. J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382 (6587):174-7.

Kato, S., R. Hokari, K. Matsuzaki, A. Iwai, A. Kawaguchi, S. Nagao, T. Miyahara, K. Itoh, H. Ishii, and S. Miura. 2000. Amelioration of murine experimental colitis by inhibition of mucosal addressin cell adhesion molecule-1. *J Pharmacol Exp Ther* 295 (1):183-9.

Kato, Y., J. M. Lewalle, Y. Baba, M. Tsukuda, N. Sakai, M. Baba, K. Kobayashi, S. Koshika, Y. Nagashima, F. Frankenne, A. Noel, J. M. Foidart, and R. I. Hata. 2001. Induction of SPARC by VEGF in human vascular endothelial cells. *Biochem Biophys Res Commun* 287 (2):422-6.

Katsanos, K. H., A. Tatsioni, N. Pedersen, M. Shuhaibar, V. H. Ramirez, P. Politi, E. Rombrechts, M. Pierik, J. Clofent, M. Beltrami, P. Bodini, J. Freitas, I. Mouzas, G. Fornaciari, B. Moum, P. L. Lakatos, S. Vermeire, E. Langholz, S. Odes, C. O. Morain, R. Stockbrugger, P. Munkholm, and E. V. Tsianos. 2011. Cancer in inflammatory bowel disease 15years after diagnosis in a population-based European Collaborative follow-up study. *J Crohns Colitis* 5 (5):430-42.

Kaviratne, M., M. Hesse, M. Leusink, A. W. Cheever, S. J. Davies, J. H. McKerrow, L. M. Wakefield, J. J. Letterio, and T. A. Wynn. 2004. IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. *J Immunol* 173 (6):4020-9.

Kelly, K. A., J. R. Allport, A. M. Yu, S. Sinh, E. H. Sage, R. E. Gerszten, and R. Weissleder. 2007. SPARC is a VCAM-1 counter-ligand that mediates leukocyte transmigration. *J Leukoc Biol* 81 (3):748-56.

Kelsall, B. L., and W. Strober. 1997. Peyer's patch dendritic cells and the induction of mucosal immune responses. *Res Immunol* 148 (8-9):490-8.

Keophiphath, M., V. Achard, C. Henegar, C. Rouault, K. Clement, and D. Lacasa. 2009. Macrophage-secreted factors promote a profibrotic phenotype in human preadipocytes. *Mol Endocrinol* 23 (1):11-24.

Khalil, N., S. Corne, C. Whitman, and H. Yacyshyn. 1996. Plasmin regulates the activation of cell-associated latent TGF-beta 1 secreted by rat alveolar macrophages after in vivo bleomycin injury. *Am J Respir Cell Mol Biol* 15 (2):252-9.

Kisseleva, T., and D. A. Brenner. 2008. Mechanisms of fibrogenesis. *Exp Biol Med (Maywood)* 233 (2):109-22.

Klopcic, B., A. Appelbee, W. Raye, F. Lloyd, J. C. Jooste, C. H. Forrest, and I. C. Lawrance. 2008. Indomethacin and retinoic acid modify mouse intestinal inflammation and fibrosis: a role for SPARC. *Dig Dis Sci* 53 (6):1553-63.

Kos, K., and J. P. Wilding. 2010. SPARC: a key player in the pathologies associated with obesity and diabetes. *Nat Rev Endocrinol* 6 (4):225-35.

Kos, K., S. Wong, B. Tan, A. Gummesson, M. Jernas, N. Franck, D. Kerrigan, F. H. Nystrom, L. M. Carlsson, H. S. Randeva, J. H. Pinkney, and J. P. Wilding. 2009. Regulation of the fibrosis and angiogenesis promoter SPARC/osteonectin in human adipose tissue by weight change, leptin, insulin, and glucose. *Diabetes* 58 (8):1780-8.

Krajina, T., F. Leithauser, P. Moller, Z. Trobonjaca, and J. Reimann. 2003. Colonic lamina propria dendritic cells in mice with CD4+ T cell-induced colitis. *Eur J Immunol* 33 (4):1073-83.

Kugathasan, S., L. J. Saubermann, L. Smith, D. Kou, J. Itoh, D. G. Binion, A. D. Levine, R. S. Blumberg, and C. Fiocchi. 2007. Mucosal T-cell immunoregulation varies in early and late inflammatory bowel disease. *Gut* 56 (12):1696-705.

Kumagai, S., H. Ohtani, T. Nagai, K. Funa, N. O. Hiwatashi, Shimosegawa, and H. Nagura. 2001. Platelet-derived growth factor and its receptors are expressed in areas of both active inflammation and active fibrosis in inflammatory bowel disease. *Tohoku J Exp Med* 195 (1):21-33.

Kumar, V., Abbas, A.K, Fausto,N., Aster, J.C. 2010. *Pathologic Basic Disease*. 8th ed. Philadelphia: Saunders Elsevier

Kupprion, C., K. Motamed, and E. H. Sage. 1998. SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. *J Biol Chem* 273 (45):29635-40.

Kurman, M., and T. S. Argyris. 1975. The proliferative response to epidermis of hairless mice to full thickness wounds. *Am J Pathol* 79 (2):301-10.

Kwon, K. H., A. Murakami, R. Hayashi, and H. Ohigashi. 2005. Interleukin-1beta targets interleukin-6 in progressing dextran sulfate sodium-induced experimental colitis. *Biochem Biophys Res Commun* 337 (2):647-54.

Kzhyshkowska, J., A. Gratchev, and S. Goerdt. 2006. Stabilin-1, a homeostatic scavenger receptor with multiple functions. *J Cell Mol Med* 10 (3):635-49.

Lane, T. F., M. L. Iruela-Arispe, R. S. Johnson, and E. H. Sage. 1994. SPARC is a source of copper-binding peptides that stimulate angiogenesis. *J Cell Biol* 125 (4):929-43.

Lane, T. F., M. L. Iruela-Arispe, and E. H. Sage. 1992. Regulation of gene expression by SPARC during angiogenesis in vitro. Changes in fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1. *J Biol Chem* 267 (23):16736-45.

Lane, T. F., and E. H. Sage. 1990. Functional mapping of SPARC: peptides from two distinct Ca+(+)-binding sites modulate cell shape. *J Cell Biol* 111 (6 Pt 2):3065-76.

Lane, T. F., and E. H. Sage. 1994. The biology of SPARC, a protein that modulates cell-matrix interactions. *FASEB J* 8 (2):163-73.

Latvala, T., P. Puolakkainen, M. Vesaluoma, and T. Tervo. 1996. Distribution of SPARC protein (osteonectin) in normal and wounded feline cornea. *Exp Eye Res* 63 (5):579-84.

Laurence, A., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E. M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26 (3):371-81.

Lawrance, I. C., C. Fiocchi, and S. Chakravarti. 2001. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Hum Mol Genet* 10 (5):445-56.

Lawrance, I. C., L. Maxwell, and W. Doe. 2001. Inflammation location, but not type, determines the increase in TGF-beta1 and IGF-1 expression and collagen deposition in IBD intestine. *Inflamm Bowel Dis* 7 (1):16-26.

Lawrance, I. C., F. Wu, A. Z. Leite, J. Willis, G. A. West, C. Fiocchi, and S. Chakravarti. 2003. A murine model of chronic inflammation-induced intestinal fibrosis down-regulated by antisense NF-kappa B. *Gastroenterology* 125 (6):1750-61.

Ledda, F., A. I. Bravo, S. Adris, L. Bover, J. Mordoh, and O. L. Podhajcer. 1997. The expression of the secreted protein acidic and rich in cysteine (SPARC) is associated with the neoplastic progression of human melanoma. *J Invest Dermatol* 108 (2):210-4.

Ledda, M. F., S. Adris, A. I. Bravo, C. Kairiyama, L. Bover, Y. Chernajovsky, J. Mordoh, and O. L. Podhajcer. 1997. Suppression of SPARC expression by antisense RNA abrogates the tumorigenicity of human melanoma cells. *Nat Med* 3 (2):171-6.

Lee, C. G., R. J. Homer, Z. Zhu, S. Lanone, X. Wang, V. Koteliansky, J. M. Shipley, P. Gotwals, P. Noble, Q. Chen, R. M. Senior, and J. A. Elias. 2001. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 194 (6):809-21.

Lee, H. G., I. Choi, K. H. Pyun, and K. W. Park. 1995. Peritoneal lavage fluids stimulate NIH3T3 fibroblast proliferation and contain increased tumour necrosis factor and IL-6 in experimental silica-induced rat peritonitis. *Clin Exp Immunol* 100 (1):139-44.

Lee, S. H., P. M. Starkey, and S. Gordon. 1985. Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. *J Exp Med* 161 (3):475-89.

Llera, A. S., M. R. Girotti, L. G. Benedetti, and O. L. Podhajcer. 2010. Matricellular proteins and inflammatory cells: a task force to promote or defeat cancer? *Cytokine Growth Factor Rev* 21 (1):67-76.

Lopez-De Leon, A., and M. Rojkind. 1985. A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J Histochem Cytochem* 33 (8):737-43.

Lorenz, R. G., D. D. Chaplin, K. G. McDonald, J. S. McDonough, and R. D. Newberry. 2003. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. *J Immunol* 170 (11):5475-82.

Lugo-Villarino, G., R. Maldonado-Lopez, R. Possemato, C. Penaranda, and L. H. Glimcher. 2003. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proc Natl Acad Sci U S A* 100 (13):7749-54.

Lund, P. K., and R. J. Rigby. 2008. What are the mechanisms of fibrosis in IBD? *Inflamm Bowel Dis* 14 Suppl 2:S127-8.

Lussier, C., J. Sodek, and J. F. Beaulieu. 2001. Expression of SPARC/osteonectin/BM4O in the human gut: predominance in the stroma of the remodeling distal intestine. *J Cell Biochem* 81 (3):463-76.

Lyass, L. A., A. D. Bershadsky, J. M. Vasiliev, and I. M. Gelfand. 1988. Microtubule-dependent effect of phorbol ester on the contractility of cytoskeleton of cultured fibroblasts. *Proc Natl Acad Sci U S A* 85 (24):9538-41.

Mahida, Y. R., L. Kurlac, A. Gallagher, and C. J. Hawkey. 1991. High circulating concentrations of interleukin-6 in active Crohn's disease but not ulcerative colitis. *Gut* 32 (12):1531-4.

Mahler, M., I. J. Bristol, E. H. Leiter, A. E. Workman, E. H. Birkenmeier, C. O. Elson, and J. P. Sundberg. 1998. Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. *Am J Physiol* 274 (3 Pt 1):G544-51.

Maloy, K. J., L. Salaun, R. Cahill, G. Dougan, N. J. Saunders, and F. Powrie. 2003. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 197 (1):111-9.

Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441 (7090):231-4.

Martens, M. F., C. M. Huyben, and T. Hendriks. 1992. Collagen synthesis in fibroblasts from human colon: regulatory aspects and differences with skin fibroblasts. *Gut* 33 (12):1664-70.

Matthes, H., H. Herbst, D. Schuppan, A. Stallmach, S. Milani, H. Stein, and E. O. Riecken. 1992. Cellular localization of procollagen gene transcripts in inflammatory bowel diseases. *Gastroenterology* 102 (2):431-42.

Mayer, L. 2005. Mucosal immunity. *Immunol Rev* 206:5.

McKaig, B. C., S. S. Makh, C. J. Hawkey, D. K. Podolsky, and Y. R. Mahida. 1999. Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-beta3. *Am J Physiol* 276 (5 Pt 1):G1087-93.

McKaig, B. C., D. McWilliams, S. A. Watson, and Y. R. Mahida. 2003. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol* 162 (4):1355-60.

Melgar, S., A. Karlsson, and E. Michaelsson. 2005. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am J Physiol Gastrointest Liver Physiol* 288 (6):G1328-38.

Mizoguchi, A., A. Ogawa, H. Takedatsu, K. Sugimoto, Y. Shimomura, K. Shirane, K. Nagahama, T. Nagaishi, E. Mizoguchi, R. S. Blumberg, and A. K. Bhan. 2007. Dependence of intestinal granuloma formation on unique myeloid DC-like cells. *J Clin Invest* 117 (3):605-15.

Monteleone, G., L. Biancone, R. Marasco, G. Morrone, O. Marasco, F. Luzza, and F. Pallone. 1997. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 112 (4):1169-78.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136 (7):2348-57.

Motomura, Y., W. I. Khan, R. T. El-Sharkawy, M. Verma-Gandhu, E. F. Verdu, J. Gauldie, and S. M. Collins. 2006. Induction of a fibrogenic response in mouse colon by overexpression of monocyte chemoattractant protein 1. *Gut* 55 (5):662-70.

Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 3 (4):331-41.

Mucida, D., Y. Park, G. Kim, O. Turovskaya, I. Scott, M. Kronenberg, and H. Cheroutre. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317 (5835):256-60.

Murch, S. H., V. A. Lamkin, M. O. Savage, J. A. Walker-Smith, and T. T. MacDonald. 1991. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut* 32 (8):913-7.

Murphy-Ullrich, J. E., T. F. Lane, M. A. Pallero, and E. H. Sage. 1995. SPARC mediates focal adhesion disassembly in endothelial cells through a follistatin-like region and the Ca(2+)-binding EF-hand. *J Cell Biochem* 57 (2):341-50.

Murphy-Ullrich, J. E., and M. Poczatek. 2000. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 11 (1-2):59-69.

Murphy, K. Travers P. Walport, M. 2008. *Janeway's Immunobiology*. 7th ed: Garland Science , Taylor & Francis Group.

Neumann, H., M. Vieth, C. Langner, M. F. Neurath, and J. Mudter. 2011. Cancer risk in IBD: How to diagnose and how to manage DALM and ALM. *World J Gastroenterol* 17 (27):3184-91.

Neurath, M. F., S. Finotto, and L. H. Glimcher. 2002. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* 8 (6):567-73.

Neurath, M. F., I. Fuss, G. Schurmann, S. Pettersson, K. Arnold, H. Muller-Lobeck, W. Strober, C. Herfarth, and K. H. Buschenfelde. 1998. Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease. *Ann N Y Acad Sci* 859:149-59.

Neutra, M. R., E. Pringault, and J. P. Kraehenbuhl. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu Rev Immunol* 14:275-300.

Newberry, R. D., and R. G. Lorenz. 2005. Organizing a mucosal defense. *Immunol Rev* 206:6-21.

Nielsen, O. H., I. Kirman, N. Rudiger, J. Hendel, and B. Vainer. 2003. Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. *Scand J Gastroenterol* 38 (2):180-5.

Norose, K., J. I. Clark, N. A. Syed, A. Basu, E. Heber-Katz, E. H. Sage, and C. C. Howe. 1998. SPARC deficiency leads to early-onset cataractogenesis. *Invest Ophthalmol Vis Sci* 39 (13):2674-80.

Norose, K., W. K. Lo, J. I. Clark, E. H. Sage, and C. C. Howe. 2000. Lenses of SPARC-null mice exhibit an abnormal cell surface-basement membrane interface. *Exp Eye Res* 71 (3):295-307.

Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448 (7152):480-3.

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

Parsonage, G., A. D. Filer, O. Haworth, G. B. Nash, G. E. Rainger, M. Salmon, and C. D. Buckley. 2005. A stromal address code defined by fibroblasts. *Trends Immunol* 26 (3):150-6.

Pershouse, M. A., A. M. Smartt, C. Schwanke, and E. A. Putnam. 2009. Differences in gene expression profiles from asbestos-treated SPARC-null and wild-type mouse lungs. *Genomics* 94 (2):101-9.

Phan, E., A. Ahluwalia, and A. S. Tarnawski. 2007. Role of SPARC--matricellular protein in pathophysiology and tissue injury healing. Implications for gastritis and gastric ulcers. *Med Sci Monit* 13 (2):RA25-30.

Podhajcer, O. L., L. G. Benedetti, M. R. Girotti, F. Prada, E. Salvatierra, and A. S. Llera. 2008. The role of the matricellular protein SPARC in the dynamic interaction between the tumor and the host. *Cancer Metastasis Rev* 27 (4):691-705.

Podolsky, D. K. 2002a. The current future understanding of inflammatory bowel disease. *Best Pract Res Clin Gastroenterol* 16 (6):933-43.

Podolsky, D. K. 2002b. Inflammatory bowel disease. *N Engl J Med* 347 (6):417-29.

Porter, P. L., E. H. Sage, T. F. Lane, S. E. Funk, and A. M. Gown. 1995. Distribution of SPARC in normal and neoplastic human tissue. *J Histochem Cytochem* 43 (8):791-800.

Powell, D. W., R. C. Mifflin, J. D. Valentich, S. E. Crowe, J. I. Saada, and A. B. West. 1999a. Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 277 (1 Pt 1):C1-9.

Powell, D. W., R. C. Mifflin, J. D. Valentich, S. E. Crowe, J. I. Saada, and A. B. West. 1999b. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol* 277 (2 Pt 1):C183-201.

Pucilowska, J. B., K. K. McNaughton, N. K. Mohapatra, E. C. Hoyt, E. M. Zimmermann, R. B. Sartor, and P. K. Lund. 2000. IGF-I and procollagen alpha1(I) are coexpressed in a subset of mesenchymal cells in active Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 279 (6):G1307-22.

Pucilowska, J. B., K. L. Williams, and P. K. Lund. 2000. Fibrogenesis. IV. Fibrosis and inflammatory bowel disease: cellular mediators and animal models. *Am J Physiol Gastrointest Liver Physiol* 279 (4):G653-9.

Puolakkainen, P., M. Reed, P. Vento, E. H. Sage, T. Kiviluoto, and E. Kivilaakso. 1999. Expression of SPARC (secreted protein, acidic and rich in cysteine) in healing intestinal anastomoses and short bowel syndrome in rats. *Dig Dis Sci* 44 (8):1554-64.

Qualls, J. E., A. M. Kaplan, N. van Rooijen, and D. A. Cohen. 2006. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol* 80 (4):802-15.

Raddatz, D., M. Bockemuhl, and G. Ramadori. 2005. Quantitative measurement of cytokine mRNA in inflammatory bowel disease: relation to clinical and endoscopic activity and outcome. *Eur J Gastroenterol Hepatol* 17 (5):547-57.

Raines, E. W., T. F. Lane, M. L. Iruela-Arispe, R. Ross, and E. H. Sage. 1992. The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. *Proc Natl Acad Sci U S A* 89 (4):1281-5.

Reding, T., U. Wagner, A. B. Silva, L. K. Sun, M. Bain, S. Y. Kim, D. Bimmler, and R. Graf. 2009. Inflammation-dependent expression of SPARC during development of chronic pancreatitis in WBN/Kob rats and a microarray gene expression analysis. *Physiol Genomics* 38 (2):196-204.

Reed, M. J., P. Puolakkainen, T. F. Lane, D. Dickerson, P. Bornstein, and E. H. Sage. 1993. Differential expression of SPARC and thrombospondin 1 in wound repair: immunolocalization and in situ hybridization. *J Histochem Cytochem* 41 (10):1467-77.

Reimund, J. M., C. Wittersheim, S. Dumont, C. D. Muller, J. S. Kenney, R. Baumann, P. Poindron, and B. Duclos. 1996. Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut* 39 (5):684-9.

Rempel, S. A., R. C. Hawley, J. A. Gutierrez, E. Mouzon, K. R. Bobbitt, N. Lemke, C. R. Schultz, L. R. Schultz, W. Golembieski, J. Koblinski, S. VanOsdol, and C. G. Miller. 2007. Splenic and immune alterations of the Sparc-null mouse accompany a lack of immune response. *Genes Immun* 8 (3):262-74.

Rentz, T. J., F. Poobalarahi, P. Bornstein, E. H. Sage, and A. D. Bradshaw. 2007. SPARC regulates processing of procollagen I and collagen fibrillogenesis in dermal fibroblasts. *J Biol Chem* 282 (30):22062-71.

Rivera, L. B., and R. A. Brekken. 2011. SPARC promotes pericyte recruitment via inhibition of endoglin-dependent TGF-beta1 activity. *J Cell Biol* 193 (7):1305-19.

Rogler, G., T. Andus, E. Aschenbrenner, D. Vogl, W. Falk, J. Scholmerich, and V. Gross. 1997. Alterations of the phenotype of colonic macrophages in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 9 (9):893-9.

Rosas, M., B. Thomas, M. Stacey, S. Gordon, and P. R. Taylor. 2010. The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B. *J Leukoc Biol* 88 (1):169-80.

Rosenblatt, S., J. A. Bassuk, C. E. Alpers, E. H. Sage, R. Timpl, and K. T. Preissner. 1997. Differential modulation of cell adhesion by interaction between adhesive and counter-adhesive proteins: characterization of the binding of vitronectin to osteonectin (BM40, SPARC). *Biochem J* 324 (Pt 1):311-9.

Rotteveel, F. T., I. Kokkelink, R. A. van Lier, B. Kuenen, A. Meager, F. Miedema, and C. J. Lucas. 1988. Clonal analysis of functionally distinct human CD4+ T cell subsets. *J Exp Med* 168 (5):1659-73.

Rugtveit, J., P. Brandtzaeg, T. S. Halstensen, O. Fausa, and H. Scott. 1994. Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. *Gut* 35 (5):669-74.

Rugtveit, J., E. M. Nilsen, A. Bakka, H. Carlsen, P. Brandtzaeg, and H. Scott. 1997. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* 112 (5):1493-505.

Rutgeerts, P., W. J. Sandborn, B. G. Feagan, W. Reinisch, A. Olson, J. Johanns, S. Travers, D. Rachmilewitz, S. B. Hanauer, G. R. Lichtenstein, W. J. de Villiers, D. Present, B. E. Sands, and J. F. Colombel. 2005. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 353 (23):2462-76.

Sacks, D., and N. Noben-Trauth. 2002. The immunology of susceptibility and resistance to Leishmania major in mice. *Nat Rev Immunol* 2 (11):845-58.

Said, N. A., I. Najwer, M. J. Socha, D. J. Fulton, S. C. Mok, and K. Motamed. 2007. SPARC inhibits LPA-mediated mesothelial-ovarian cancer cell crosstalk. *Neoplasia* 9 (1):23-35.

Said, N., and K. Motamed. 2005. Absence of host-secreted protein acidic and rich in cysteine (SPARC) augments peritoneal ovarian carcinomatosis. *Am J Pathol* 167 (6):1739-52.

Said, N., M. J. Socha, J. J. Olearczyk, A. A. Elmarakby, J. D. Imig, and K. Motamed. 2007. Normalization of the ovarian cancer microenvironment by SPARC. *Mol Cancer Res* 5 (10):1015-30.

Sanchez-Munoz, F., A. Dominguez-Lopez, and J. K. Yamamoto-Furusho. 2008. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* 14 (27):4280-8.

Sangaletti, S., L. Gioiosa, C. Guiducci, G. Rotta, M. Rescigno, A. Stoppacciaro, C. Chiodoni, and M. P. Colombo. 2005. Accelerated dendritic-cell migration and T-cell priming in SPARC-deficient mice. *J Cell Sci* 118 (Pt 16):3685-94.

Sangaletti, S., A. Stoppacciaro, C. Guiducci, M. R. Torrisi, and M. P. Colombo. 2003. Leukocyte, rather than tumor-produced SPARC, determines stroma and collagen type IV deposition in mammary carcinoma. *J Exp Med* 198 (10):1475-85.

Sartor, R. B. 2003. Innate immunity in the pathogenesis and therapy of IBD. *J Gastroenterol* 38 Suppl 15:43-7.

Sasaki, T., E. Hohenester, W. Gohring, and R. Timpl. 1998. Crystal structure and mapping by site-directed mutagenesis of the collagen-binding epitope of an activated form of BM-40/SPARC/osteonectin. *Embo J* 17 (6):1625-34.

Sato, M., Y. Muragaki, S. Saika, A. B. Roberts, and A. Ooshima. 2003. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 112 (10):1486-94.

Savage, D. C. 1977. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* 31:107-33.

Savani, R. C., Z. Zhou, E. Arguiri, S. Wang, D. Vu, C. C. Howe, and H. M. DeLisser. 2000. Bleomycin-induced pulmonary injury in mice deficient in SPARC. *Am J Physiol Lung Cell Mol Physiol* 279 (4):L743-50.

Schenk, M., A. Bouchon, F. Seibold, and C. Mueller. 2007. TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J Clin Invest* 117 (10):3097-106.

Schenk, M., and C. Mueller. 2007. Adaptations of intestinal macrophages to an antigen-rich environment. *Semin Immunol* 19 (2):84-93.

Schiller, M., D. Javelaud, and A. Mauviel. 2004. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 35 (2):83-92.

Schonbeck, U., F. Mach, and P. Libby. 1998. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 161 (7):3340-6.

Seet, L. F., R. Su, V. A. Barathi, W. S. Lee, R. Poh, Y. M. Heng, E. Manser, E. N. Vithana, T. Aung, M. Weaver, E. H. Sage, and T. T. Wong. 2010. SPARC deficiency results in improved surgical survival in a novel mouse model of glaucoma filtration surgery. *PLoS One* 5 (2):e9415.

Seiderer, J., I. Elben, J. Diegelmann, J. Glas, J. Stallhofer, C. Tillack, S. Pfennig, M. Jurgens, S. Schmechel, A. Konrad, B. Goke, T. Ochsenkuhn, B. Muller-Myhsok, P. Lohse, and S. Brand. 2008. Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD. *Inflamm Bowel Dis* 14 (4):437-45.

Seno, H., H. Miyoshi, S. L. Brown, M. J. Geske, M. Colonna, and T. S. Stappenbeck. 2009. Efficient colonic mucosal wound repair requires Trem2 signaling. *Proc Natl Acad Sci U S A* 106 (1):256-61.

Seril, D. N., J. Liao, G. Y. Yang, and C. S. Yang. 2003. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. *Carcinogenesis* 24 (3):353-62.

Shah, M., D. M. Foreman, and M. W. Ferguson. 1995. Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 108 (Pt 3):985-1002.

Shankavaram, U. T., D. L. DeWitt, S. E. Funk, E. H. Sage, and L. M. Wahl. 1997. Regulation of human monocyte matrix metalloproteinases by SPARC. *J Cell Physiol* 173 (3):327-34.

Shimoda, K., J. van Deursen, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. Vignali, P. C. Doherty, G. Grosveld, W. E. Paul, and J. N. Ihle. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380 (6575):630-3.

Siegmund, B., H. A. Lehr, and G. Fantuzzi. 2002. Leptin: a pivotal mediator of intestinal inflammation in mice. *Gastroenterology* 122 (7):2011-25.

Simon-Assmann, P., M. Kedinger, A. De Arcangelis, V. Rousseau, and P. Simo. 1995. Extracellular matrix components in intestinal development. *Experientia* 51 (9-10):883-900.

Smith, P. D., L. E. Smythies, M. Mosteller-Barnum, D. A. Sibley, M. W. Russell, M. Merger, M. T. Sellers, J. M. Orenstein, T. Shimada, M. F. Graham, and H. Kubagawa. 2001. Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. *J Immunol* 167 (5):2651-6.

Smith, P. D., L. E. Smythies, R. Shen, T. Greenwell-Wild, M. Gliozzi, and S. M. Wahl. 2010. Intestinal macrophages and response to microbial encroachment. *Mucosal Immunol*.

Smythies, L. E., A. Maheshwari, R. Clements, D. Eckhoff, L. Novak, H. L. Vu, L. M. Mosteller-Barnum, M. Sellers, and P. D. Smith. 2006. Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. *J Leukoc Biol* 80 (3):492-9.

Smythies, L. E., M. Sellers, R. H. Clements, M. Mosteller-Barnum, G. Meng, W. H. Benjamin, J. M. Orenstein, and P. D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 115 (1):66-75.

Socha, M. J., M. Manhiani, N. Said, J. D. Imig, and K. Motamed. 2007. Secreted protein acidic and rich in cysteine deficiency ameliorates renal inflammation and fibrosis in angiotensin hypertension. *Am J Pathol* 171 (4):1104-12.

Soehnlein, O., and L. Lindbom. 2010. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* 10 (6):427-39.

Stallmach, A., C. C. Chan, K. W. Ecker, G. Feifel, H. Herbst, D. Schuppan, and M. Zeitz. 2000. Comparable expression of matrix metalloproteinases 1 and 2 in pouchitis and ulcerative colitis. *Gut* 47 (3):415-22.

Stallmach, A., D. Schuppan, H. H. Riese, H. Matthes, and E. O. Riecken. 1992. Increased collagen type III synthesis by fibroblasts isolated from strictures of patients with Crohn's disease. *Gastroenterology* 102 (6):1920-9.

Stevens, C., G. Walz, C. Singaram, M. L. Lipman, B. Zanker, A. Muggia, D. Antonioli, M. A. Peppercorn, and T. B. Strom. 1992. Tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 expression in inflammatory bowel disease. *Dig Dis Sci* 37 (6):818-26.

Stockinger, B., and M. Veldhoen. 2007. Differentiation and function of Th17 T cells. *Curr Opin Immunol* 19 (3):281-6.

Strandjord, T. P., D. K. Madtes, D. J. Weiss, and E. H. Sage. 1999. Collagen accumulation is decreased in SPARC-null mice with bleomycin-induced pulmonary fibrosis. *Am J Physiol* 277 (3 Pt 1):L628-35.

Strober, W., I. J. Fuss, and R. S. Blumberg. 2002. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 20:495-549.

Strong, S. A., T. T. Pizarro, J. S. Klein, F. Cominelli, and C. Fiocchi. 1998. Proinflammatory cytokines differentially modulate their own expression in human intestinal mucosal mesenchymal cells. *Gastroenterology* 114 (6):1244-56.

Sturges, N. 2004. The Role that Transforming Growth Factor beta isoforms play in fibrosis formation in intestinal fibroblasts. (Honours thesis), School of Biological Science and Biotechnology, Murdoch University, Perth.

Su, L., L. Shen, D. R. Clayburgh, S. C. Nalle, E. A. Sullivan, J. B. Meddings, C. Abraham, and J. R. Turner. 2009. Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis. *Gastroenterology* 136 (2):551-63.

Suzuki, A., T. Hanada, K. Mitsuyama, T. Yoshida, S. Kamizono, T. Hoshino, M. Kubo, A. Yamashita, M. Okabe, K. Takeda, S. Akira, S. Matsumoto, A. Toyonaga, M. Sata, and A. Yoshimura. 2001. CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. *J Exp Med* 193 (4):471-81.

Suzuki, K., X. Sun, M. Nagata, T. Kawase, H. Yamaguchi, V. Sukumaran, Y. Kawauchi, H. Kawachi, T. Nishino, K. Watanabe, H. Yoneyama, and H. Asakura. 2011. Analysis of intestinal fibrosis in chronic colitis in mice induced by dextran sulfate sodium. *Pathol Int* 61 (4):228-38.

Svensson, M., B. Johansson-Lindbom, F. Zapata, E. Jaensson, L. M. Austenaa, R. Blomhoff, and W. W. Agace. 2008. Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells. *Mucosal Immunol* 1 (1):38-48.

Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295 (5553):338-42.

Tai, I. T., M. Dai, D. A. Owen, and L. B. Chen. 2005. Genome-wide expression analysis of therapy-resistant tumors reveals SPARC as a novel target for cancer therapy. *J Clin Invest* 115 (6):1492-502.

Tai, I. T., and M. J. Tang. 2008. SPARC in cancer biology: its role in cancer progression and potential for therapy. *Drug Resist Updat* 11 (6):231-46.

Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int Immunol* 17 (1):1-14.

Theiss, A. L., J. G. Simmons, C. Jobin, and P. K. Lund. 2005. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. *J Biol Chem* 280 (43):36099-109.

Tokuyama, H., S. Ueha, M. Kurachi, K. Matsushima, F. Moriyasu, R. S. Blumberg, and K. Kakimi. 2005. The simultaneous blockade of chemokine receptors CCR2, CCR5 and

CXCR3 by a non-peptide chemokine receptor antagonist protects mice from dextran sodium sulfate-mediated colitis. *Int Immunol* 17 (8):1023-34.

Tomasek, J. J., G. Gabbiani, B. Hinz, C. Chaponnier, and R. A. Brown. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3 (5):349-63.

Tremble, P. M., T. F. Lane, E. H. Sage, and Z. Werb. 1993. SPARC, a secreted protein associated with morphogenesis and tissue remodeling, induces expression of metalloproteinases in fibroblasts through a novel extracellular matrix-dependent pathway. *J Cell Biol* 121 (6):1433-44.

Trombetta, J. M., and A. D. Bradshaw. 2010. SPARC/osteonectin Functions to Maintain Homeostasis of the Collagenous Extracellular Matrix in the Periodontal Ligament. *J Histochem Cytochem*.

Uguccioni, M., P. Gionchetti, D. F. Robbiani, F. Rizzello, S. Peruzzo, M. Campieri, and M. Baggiolini. 1999. Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. *Am J Pathol* 155 (2):331-6.

van den Borne, S. W., S. Isobe, J. W. Verjans, A. Petrov, D. Lovhaug, P. Li, H. R. Zandbergen, Y. Ni, P. Frederik, J. Zhou, B. Arbo, A. Rogstad, A. Cuthbertson, S. Chettibi, C. Reutelingsperger, W. M. Blankesteijn, J. F. Smits, M. J. Daemen, F. Zannad, M. A. Vannan, N. Narula, B. Pitt, L. Hofstra, and J. Narula. 2008. Molecular imaging of interstitial alterations in remodeling myocardium after myocardial infarction. *J Am Coll Cardiol* 52 (24):2017-28.

Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24 (2):179-89.

Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9 (12):1341-6.

von Lampe, B., B. Barthel, S. E. Coupland, E. O. Riecken, and S. Rosewicz. 2000. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut* 47 (1):63-73.

Wan, Y. Y., and R. A. Flavell. 2008. TGF-beta and regulatory T cell in immunity and autoimmunity. *J Clin Immunol* 28 (6):647-59.

Wang, J. C., S. Lai, X. Guo, X. Zhang, B. de Crombrugghe, S. Sonnylal, F. C. Arnett, and X. Zhou. 2010. Attenuation of fibrosis in vitro and in vivo with SPARC siRNA. *Arthritis Res Ther* 12 (2):R60.

Wang, Z., J. Hong, W. Sun, G. Xu, N. Li, X. Chen, A. Liu, L. Xu, B. Sun, and J. Z. Zhang. 2006. Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest* 116 (9):2434-41.

Weigmann, B., I. Tubbe, D. Seidel, A. Nicolaev, C. Becker, and M. F. Neurath. 2007. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nat Protoc* 2 (10):2307-11.

Williamson, E., J. M. Bilsborough, and J. L. Viney. 2002. Regulation of mucosal dendritic cell function by receptor activator of NF-kappa B (RANK)/RANK ligand interactions: impact on tolerance induction. *J Immunol* 169 (7):3606-12.

Wirtz, S., C. Neufert, B. Weigmann, and M. F. Neurath. 2007. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2 (3):541-6.

Wirtz, S., and M. F. Neurath. 2007. Mouse models of inflammatory bowel disease. *Adv Drug Deliv Rev* 59 (11):1073-83.

Workman, G., and E. H. Sage. 2011. Identification of a sequence in the matricellular protein SPARC that interacts with the scavenger receptor stabilin-1. *J Cell Biochem* 112 (4):1003-8.

Wynn, T. A. 2004. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* 4 (8):583-94.

Wynn, T. A., A. W. Cheever, D. Jankovic, R. W. Poindexter, P. Caspar, F. A. Lewis, and A. Sher. 1995. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. *Nature* 376 (6541):594-6.

Yan, Q., and E. H. Sage. 1999. SPARC, a matricellular glycoprotein with important biological functions. *J Histochem Cytochem* 47 (12):1495-506.

Yan, Y., V. Kolachala, G. Dalmasso, H. Nguyen, H. Laroui, S. V. Sitaraman, and D. Merlin. 2009. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. *PLoS One* 4 (6):e6073.

Yang, E., H. J. Kang, K. H. Koh, H. Rhee, N. K. Kim, and H. Kim. 2007. Frequent inactivation of SPARC by promoter hypermethylation in colon cancers. *Int J Cancer* 121 (3):567-75.

Yin, J., G. Chen, Y. Liu, S. Liu, P. Wang, Y. Wan, X. Wang, J. Zhu, and H. Gao. 2010. Downregulation of SPARC expression decreases gastric cancer cellular invasion and survival. *J Exp Clin Cancer Res* 29:59.

Youngman, K. R., P. L. Simon, G. A. West, F. Cominelli, D. Rachmilewitz, J. S. Klein, and C. Fiocchi. 1993. Localization of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells. *Gastroenterology* 104 (3):749-58.

Yu, Q., and I. Stamenkovic. 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14 (2):163-76.

Zenewicz, L. A., A. Antov, and R. A. Flavell. 2009. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol Med* 15 (5):199-207.

Zhang, S. Z., X. H. Zhao, and D. C. Zhang. 2006. Cellular and molecular immunopathogenesis of ulcerative colitis. *Cell Mol Immunol* 3 (1):35-40.

Zheng, Y., S. Z. Josefowicz, A. Kas, T. T. Chu, M. A. Gavin, and A. Y. Rudensky. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445 (7130):936-40.

Zhou, L., J. E. Lopes, M. M. Chong, Ivanov, II, R. Min, G. D. Victora, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, S. F. Ziegler, and D. R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453 (7192):236-40.

Zhou, X. D., M. M. Xiong, F. K. Tan, X. J. Guo, and F. C. Arnett. 2006. SPARC, an upstream regulator of connective tissue growth factor in response to transforming growth factor beta stimulation. *Arthritis Rheum* 54 (12):3885-9.

Zhou, X., F. K. Tan, X. Guo, D. Wallis, D. M. Milewicz, S. Xue, and F. C. Arnett. 2005. Small interfering RNA inhibition of SPARC attenuates the profibrotic effect of transforming growth factor beta1 in cultured normal human fibroblasts. *Arthritis Rheum* 52 (1):257-61.

Zhu, J., L. Guo, B. Min, C. J. Watson, J. Hu-Li, H. A. Young, P. N. Tsichlis, and W. E. Paul. 2002. Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. *Immunity* 16 (5):733-44.