Myeloid p38 MAPK signaling in intestinal homeostasis, inflammation and tumorigenesis

Señalización por la MAPK p38 de células mieloides en la homeostasis, inflamación y tumorogénesis intestinal

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Dedicated to my family and my wonderful daughter Angelina, my angel.

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ABBREVIATIONS

53BP1	p53-binding protein 1
A.U.	Arbitrary units
ACF	Aberrant crypt foci
ALP1	Alkaline phosphatase 1
AOM	Azoxymethane
APC	Antigen presenting cell
APS	Ammonium persulfate
Arg1	Arginase 1
Ascl2	Achaete scute-like 2
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CAC	Colitis-associated cancer
CAF	Cancer-associated fibroblast
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
cDNA	Complementary DNA
CDX-2	Caudal type homeobox 2
CgA	Chromogranin A
CLP	Common lymphoid progenitor
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CSF1R	Colony stimulating factor 1 receptor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CX_3CR1	CX_3C chemokine receptor 1
CXCL	C-X-C motif chemokine
DAB	3,3'-Diaminobenzidine

DAI	Disease activity index
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethylsulfoxide
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotides triphosphate
DSS	Dextran sodium sulfate
DTT	DL-dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EPC	Endothelial progenitor cell
Epo	Erythropoietin
ER	Endoplasmatic reticulum
ERK	Extracellular signal–regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc-γ binding protein	Fcgbp
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
g	Gravity (centrifugal force)
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GH	Growth hormone
GI	Gastrointestinal
GLP-2	Glucagon-like peptide 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor

H&E	Hematoxylin & eosin
HB-EGF	Heparin-binding epidermal-like growth factor
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
Hsp70	Heat-shock protein 70
IBD	Inflammatory bowel disease
IBDU	Inflammatory bowel disease unclassified
IC	Indeterminate colitis
ID	Integrated density
IEC	Intestinal epithelial cells
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IGF	Insulin-like growth factor
IGF1R	IGF-1 Receptor
IGF2R	IGF-2 Receptor
IGFBP	IGF-binding protein
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-	Interleukin-
iNOS	Inducible nitric oxide synthase
IR	Insulin Receptor
IRS-1	Insulin receptor substrate 1
JNK	Jun N-terminal kinase
KGF	Keratinocyte growth factor
KO	Knockout
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
Lin	Lineage
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6 complex, locus
MAdCAM-1	Mucosal addressin cell adhesion molecule-1

MALT	Mucosa-associated lymphoid tissue
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
M-CSF	Macrophage-colony stimulating factor
MDC	Macrophage derived chemokine
MDP	Macrophage and dendritic cell precursor
MDSC	Myeloid-derived suppressor cell
MEF	Mouse embryo fibroblast
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α
MK2	Mitogen-activated protein kinase 2
MKP	MAPK phosphatase
MMC	Mitomycin C
MPP	Multipotential progenitor
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MSK1	Mitogen- and stress-activated protein kinase 1
NF-κb	Nuclear factor-κB
NK cell	Natural killer cell
NO	Nitric oxide
NP-40	Nonyl phenoxypolyethoxylethanol
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGE2	Prostaglandin E2
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor

qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RCL	Red Cell Lysis
RELM	Resistin-like molecule
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute 1640
RT	Room temperature
SCA1	Stem-cell antigen 1
SDS	Sodium dodecyl sulfate
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
sTNFRII	Soluble tumor necrosis factor Receptor II
TAM	Tumor-associated macrophage
TCK-1	Thymus chemokine-1
TEMED	N,N,N´,N´-tetramethylethylenediamine
TFF	Trefoil factor
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TREM-1	Triggering receptor expressed on myeloid cells-1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WT	Wild type
YAP1	Yes-associated protein 1
γ-H2AX	Phosphorylated histone H2AX

INTRODUCTION

1. INTRODUCTION

The mammalian gastrointestinal (GI) tract consists of the mouth, pharynx, esophagus stomach, small intestine, caecum and large intestine. Its main function is to process food and allow the efficient reabsorption of nutrients and water. The small intestine, which includes the duodenum, jejunum, and ileum, is the major organ for the absorption of nutrients. The large intestine or colon consists of the ascending, transcending, and descending colon. It reabsorbs water and salts from food and excretes waste material from the body (Atuma et al., 2001; Mowat, 2003). The GI tract is the largest surface of the body in contact with the external environment, approximately 400 m^2. This barrier between the host and the external environment has to be accessible to dietary products, therefore creating vulnerability to be attacked from pathogenic organisms (Mowat, 2003).

1.1 Homeostasis in the colon

The amount of bacteria in the intestinal lumen of the colon represents one of the most densely and complex colonized environments (Kurashima et al., 2013; Pott and Hornef, 2012). In humans, the gut commensal resident bacteria (also called microbiota) increases in number and complexity from the stomach to the colon, and is estimated to be made up by 10^{14} bacteria pertaining as many as 1000 species, most of which are poorly identified and functionally uncharacterized (O'Hara and Shanahan, 2006; Wang et al., 2015b). However, the host and the gut microbiota peacefully coexist in a mutualistic relationship under homeostatic conditions. The host provides a safe niche, rich in nutrients to its gut microbiota. In return, intestinal microbes provide important functions and profits to the host (O'Hara and Shanahan, 2006), which are related to pathogen protection, nutrition, host metabolism and immune modulation (Guinane and Cotter, 2013). Apart from that, the intestinal lumen is also continuously exposed to a huge number of foreign antigens, and although most of them are innocuous and include food antigens as well as commensal bacteria and their microbial products, they represent a challenge for the immune system (Baumgart and Dignass, 2002; McCole and Barrett, 2007). This co-evolution of mammals with their intestinal flora has led to a situation of tolerance in the gut (Mowat and Bain, 2011).

Based on the classical interpretation of the immune system, this huge variety of antigens should be recognized as foreign material and induce an immune response. However, the intestinal immune response against common food antigens would lead to a

massive inflammatory response of the gut, compromising the host viability (Baumgart and Dignass, 2002; McCole and Barrett, 2007). A constant molecular interaction between the microbiota and the host is required for the establishment and maintenance of intestinal homeostasis and tolerance. This requires finely tuned pro-and anti-inflammatory immune responses to antigens within the GI tract (Kaser et al., 2010; Maloy and Powrie, 2011). The maintenance of this thin equilibrium between immune activation and suppression is marked by a situation of controlled 'physiological inflammation', where distinct populations of intestinal epithelial cells (IECs) and immune cells, amongst them resident and inflammatory macrophages in the gut maintain a balance and ensure protective immunity when required (Mowat and Bain, 2011) (section 1.2.3).

1.1.1 The mucosal epithelial barrier

The intestinal epithelial barrier is a highly organized mucosal surface that consists of a monolayer of IECs covered by a stratified mucus layer (Muniz et al., 2012). There are four major IEC lineages that arise from pluripotent stem cell progenitors (Barker et al., 2008). These include absorptive enterocytes, the mucus-producing goblet cells, enteroendocrine cells, which are specialized hormone-producing endocrine cells in the GI tract, and Paneth cells, which are the main producers of antimicrobial peptides (Kim and Ho, 2010). Besides other critical functions, the mucosal epithelial barrier also prevents the entry of microbes into the lamina propria and as explained above, it is in permanent cross-talk with a huge load of microorganisms (Zheng, 2012).

To provide a balanced response (homeostasis) to these massive antigenic challenges without impeding nutrient absorption and the protection of the body against potentially harmful pathogenic bacteria and toxic substances, the intestine has developed powerful defense mechanisms that comprise physical, biochemical and cellular controls (Baumgart and Dignass, 2002; McCole and Barrett, 2007). Physical controls include peristalsis, mucus secretion by goblet cells, and an uninterrupted monolayer of epithelial cells regulated by tight junctions. Moreover, it has become clear that the epithelium is more than an inert anatomical barrier and contributes to intestinal health in a much more complex and multi-layered fashion than simply by regulating intestinal permeability (Tomasello and Bedoui, 2013). For instance, it is able to initiate inflammatory responses by the expression of Pattern Recognition Receptors (PRRs) by enterocytes that act as dynamic sensors of the microbial environment and as active participants in directing mucosal immune cell responses (Peterson and Artis, 2014; Rakoff-Nahoum et al., 2004;

Vaishnava et al., 2008). Biochemical controls include secretion of antibacterial molecules such as defensins, cathelicidins, cryptidins, lactoferrin or lysozyme mainly by Paneth cells in the base of the crypts of Lieberkuhn, which together with the mucus layer form the glycocalix that traps and aids expulsion of invading microorganisms, a process aided by peristaltic movement (Mowat, 2003). Moreover, globet cells synthesize and secrete bioactive molecules such as secretory and membrane-bound mucins, trefoil peptides, resistin-like molecule (RELM)β, and Fc-γ binding protein (Fcgbp), which are components of mucus and contribute to both barrier function and mucosal repair (Kim and Ho, 2010; Taupin and Podolsky, 2003). Finally, an intense trafficking of immune cells in and out of the gut, and a highly specialized immunological system warrant the integrity of the mucosa and the constant surveillance of the full system (Guarner and Malagelada, 2003). It is therefore not surprising, that to deal with its massive antigenic load, the intestine exhibits one of the largest immunologic compartments in the body (Fiocchi, 2003). In summary, the mucus layer, epithelial cells and immune cells together constitute the intestinal mucosal barrier and prevent the trillions of microorganisms living in the intestine from reaching systemic sites (Perez-Lopez et al., 2016).

1.2 Mucosal immunity in homeostasis

By definition, the immune system is the system that protects the body from foreign antigens, such as microbes, viruses, cancer cells, and toxins by producing the immune response (McGhee and Fujihashi, 2012; Warrington et al., 2011). The mucosal immune system, consists of an organized mucosa-associated lymphoid tissue (MALT) and is the part of the immune system juxtaposed to the mucosal surfaces and in direct contact with the external antigens (McGhee and Fujihashi, 2012). It comprises the largest mass of lymphoid tissue, containing up to 70% of the body's immune cells, since the mucosa is the main place of pathogen recognition, tolerance induction and generation of Immunoglobulin A (IgA) antibody response to luminal environmental antigens (Jung et al., 2010). The gut-associated lymphoid tissue (GALT), either as organized lymphoid structures or diffusely distributed, plays a central role in the induction of immune tolerance against harmless materials, but also in the induction of the protective immunity to harmful antigens (Chistiakov et al., 2014).

1.2.1 Intestinal tolerance

The involvement of intestinal microbiota in the generation, regulation, differentiation and maturation of the immune system has become one of the hottest topics in the field (Agace and McCoy, 2017; Ericsson and Franklin, 2015; Thaiss et al., 2016). The immune system is highly complex, but can be simplistically viewed as having two “lines of defense”, namely the innate immunity and adaptive immunity (Warrington et al., 2011). However, the two systems are not mutually exclusive mechanisms, they overlap and interrelate extensively (Figure 1).

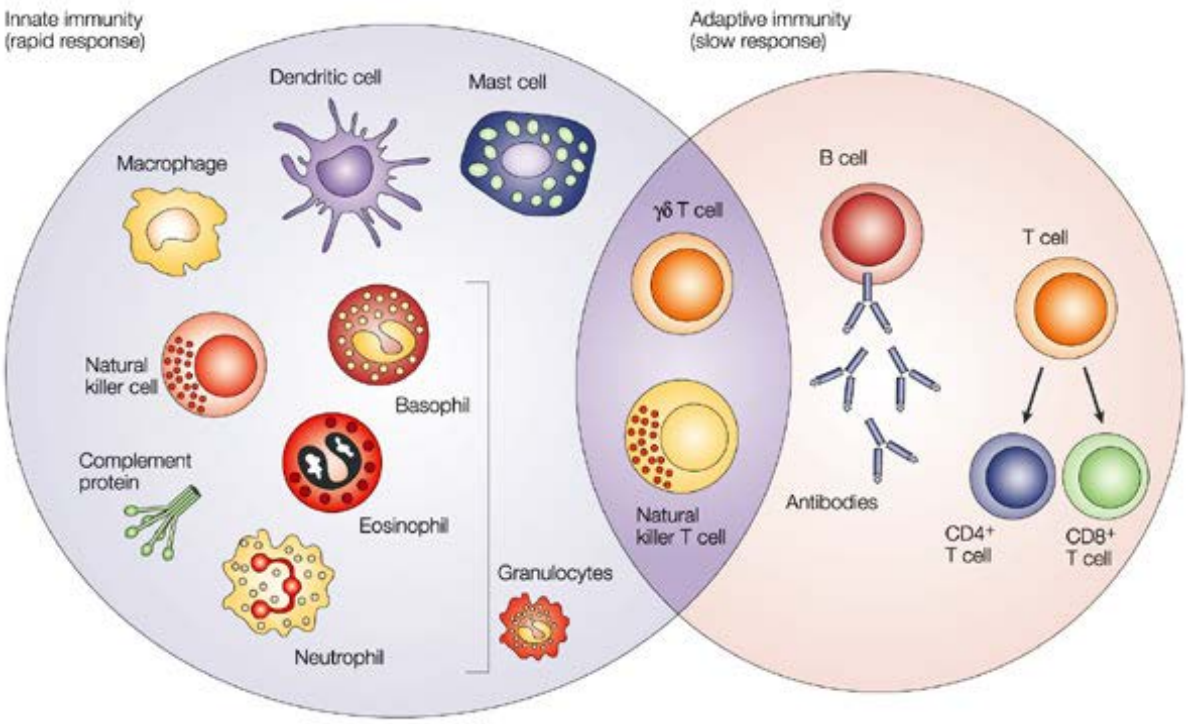


Figure 1. The innate and adaptive immune response.

In addition to soluble factors and tissue-specific cells the innate immune response consists of hematopoietic immune cells including granulocytes (neutrophils, eosinophils and basophils), mast cells, macrophages, dendritic cells, and natural killer cells. The adaptive immune response is slower to develop, but is antigen specific and has memory. It consists of B cells and T lymphocytes. NK T cells and T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity (obtained from Nat Rev Cancer (Dranoff, 2004)).

The innate immunity represents the first line of defense against a foreign threat. It is an antigen-independent (non-specific) defense mechanism and involves mechanical and chemical barriers (Dranoff, 2004; McGhee and Fujihashi, 2012). However, resident commensal bacteria occupying the ecological niche in the gut may also be regarded as part of the host’s innate defense system as they provide a colonization resistance to

pathogenic microbes (Stecher and Hardt, 2008). The cellular components of the innate immune system include neutrophils, eosinophils, basophils, mastocytes, natural killer (NK)-cells, γδT cells, dendritic cells (DCs), monocytes and macrophages (Dranoff, 2004). Leukocytes of hematopoietic origin, in particular monocytes and macrophages, are considered one of the central cell types of the intestinal innate immunity (Dranoff, 2004; Parihar et al., 2010). However, the innate immune system has no immunologic memory. The adaptive immunity in contrast is antigen-dependent and specific and is therefore able to recognize or "memorize" the same antigen in case of re-exposure. This immunologic memory is the hallmark of the adaptive immunity and allows the host to mount a more rapid and efficient immune response upon subsequent exposure to the same antigen than in the first encounter with the antigen (Peterson and Artis, 2014; Warrington et al., 2011).

The switch between the induction of active immunity or tolerance depends on the local microenvironment, which makes a significant contribution to determining the initiation of the response (Faria and Weiner, 2005). In this sense, the cross-talk between innate and adaptive immunity in the gut maintains and exports the tolerogenic response to the systemic immune system, a process regulated by a variety of cells, their secretion products and their interactions with endogenous mediators (Abraham and Medzhitov, 2011; Mowat, 2003). Briefly, intestinal microorganisms are recognized by innate immune cells such as macrophages and DCs through their PRRs, which detect a broad spectrum of microbial molecules. In response to invading bacteria, the signals from PRRs converge to transcription factors, which start the transcription of genes responsible for the synthesis of pro-inflammatory proteins mounting a defensive inflammatory reaction (Belkaid and Hand, 2014; Shalapour and Karin, 2015) (see section 1.3). However, non-pathogenic bacteria, which might present a different pattern of surface markers than pathogenic ones, may also elicit regulatory cytokines such as transforming growth factor β (TGFβ) or Interleukin-(IL-)10 leading to the induction of tolerance. The way in which macrophages discriminate between pathogens and commensal bacteria in the gut remains one of the major studied questions of mucosal immunology (Bermudez-Brito et al., 2012; Shanahan, 2001b).

Resident intestinal macrophages reside in the lamina propria in close association with the intestinal epithelial layer and are the largest reservoirs of macrophages in the body (Mowat and Bain, 2011). The immunobiology of intestinal macrophages remains poorly understood, partly due to the difficulty in isolating these cells. It is assumed that

resident intestinal macrophages perform crucial homeostatic functions, such as capture of any luminal bacteria breaching the epithelial monolayer without provoking overt inflammation, but also contribute to the induction of T cell anergy, deletion of reactive lymphocytes and the generation and expansion of cluster of differentiation (CD) 4^+ T regulatory cells (Treg) in the lamina propria (Bain and Mowat, 2014; Mowat, 2003; Mowat and Bain, 2011) (see section 1.2.3). Treg are responsible for secreting suppressive mediators, including inhibitory molecules such as cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) as well as the cytokines IL-10, TGFβ and IL-35 (Faria and Weiner, 2005; Tsuji and Kosaka, 2008). TGFβ is mainly produced by both, stromal and Treg in the gut and, in addition to its immunosuppressive properties, is also a critical factor for IgA class switching, which helps to generate the IgA-predominant milieu characteristic of the intestine (Stavnezer and Kang, 2009). In addition, TGFβ plays a key role in the induction of IL-10-secreting regulatory cells (Apetoh et al., 2010).

Thus, the innate and adaptive intestinal immune system has the constant task to survey the intestinal milieu and respond robustly to pathogenic insult, while repressing such responses towards innocuous antigens, e.g. dietary proteins or commensal microorganisms, since they would be dangerous for the body integrity. Indeed breakdown in the discrimination between harmless and harmful antigens results in the development of chronic inflammatory conditions such as inflammatory bowel disease (IBD) (Round and Mazmanian, 2009) (see section 1.3.1).

1.2.2 The mononuclear phagocyte system (MPS)

The mononuclear phagocyte system (MPS) is a family of functionally related innate immune cells including bone marrow precursors, blood monocytes tissue macrophages and DCs. They play crucial roles in the maintenance of homeostatic surveillance, reaction to infection and injury, as well as in the regenerative response after injury (Chow et al., 2011; Yona et al., 2013). MPS cells share many features, importantly their phagocytic activity, but are also extremely plastic in their patterns of gene expression, defying identification based upon surface markers. The proliferation and differentiation of MPS cells is controlled by macrophage colony-stimulating factor (M-CSF) and IL-34, acting through a common receptor, colony stimulating factor 1 receptor (CSF1R) (Jenkins and Hume, 2014). Within the mammalian blood system, only hematopoietic stem cells (HSCs) possess the ability of multi-potency and self-renewal over the life span of an

organism (Figure 2) (Gabrilovich et al., 2012; King and Goodell, 2011; Seita and Weissman, 2010).

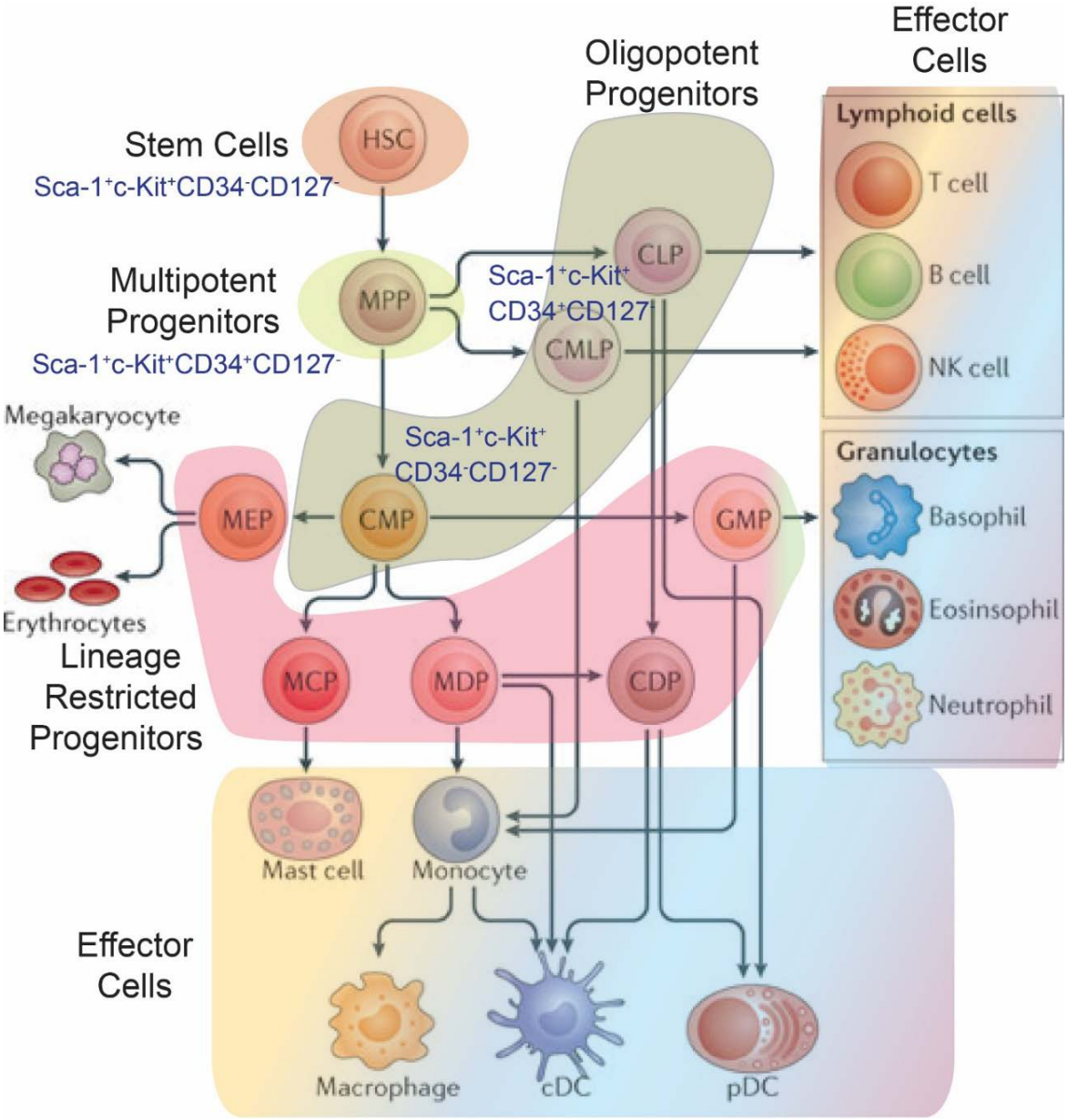


Figure 2. The haematopoietic tree.

Haematopoietic stem cells (HSCs) have the capacity to self-renew over the lifespan and give rise to all the cell types of the bone marrow and peripheral blood. They differentiate in a stepwise manner. Other pluripotent progenitors and multipotent progenitors (MPPs) have less self-renewal capacity. Over the course of the differentiation process, progenitor cells choose a specific branch of the haematopoietic system and loose the potential to differentiate into other lineages until the cells reach the stage of committed progenitors, which can give rise to only one lineage. The figure illustrates the network of progenitor cells that gives rise to the various haematopoietic cell lineages. This process is more complex and simplified in this illustration. CMP, common myeloid progenitor; CLP, common lymphoid progenitor; CMLP, common myelolymphoid progenitor; GMP, granulocyte and macrophage progenitor; MCP, mast cell progenitor; MDP, macrophage and DC progenitor; CDP, common DC progenitor; MEP, megakaryocyte and erythrocyte progenitor; cDC, conventional DC; pDC, plasmacytoid DC (modified from Nat Rev Immunol (Gabrilovich et al., 2012)).

Van Furth and colleagues initially proposed the concept of MPS in the 1970's with a linear model as a family of cells that are derived from a common progenitor and differentiate into blood monocytes, which then give rise to macrophages and DCs throughout the body (van Furth and Cohn, 1968). However, although this simplistic model still remains, it has become clear that the model is much more complex. It has now been expanded to include subpopulations regulated by specific growth and transcription factors as well as epigenetic modifications that result in subset-specific gene expression signatures, and distinct ontogenies (Alvarez-Errico et al., 2015; Davies and Taylor, 2015). Functional HSCs are found in the population of bone-marrow cells that does not express the cell-surface markers normally present on lineage (Lin)-committed differentiated haematopoietic cells. However, they express high levels of stem-cell antigen 1 (SCA1) and c-Kit (Chow et al., 2011). HSCs initially give rise to the multipotent progenitors (MPPs) which no longer possess self-renewal ability. However they keep their full lineage differentiation potential (Seita and Weissman, 2010). Further downstream, MPPs advance to oligopotent progenitors, which then give rise to lineage restricted progenitor cells and finally to effector cells, such as monocytes, macrophages and DCs (Gabrilovich et al., 2012) (Figure 2). Nevertheless, the MPS system is complex and still currently under revision (Geissmann et al., 2010; Seita and Weissman, 2010; Woolthuis and Park, 2016).

1.2.3 Monocytes and macrophages in the colon

Macrophages are found in all tissues and play important roles in development, homeostasis, tissue repair and immunity. They are remarkably plastic cells, present in different tissues throughout the body, which allows them to assume a plethora of phenotypes and functions (Murray and Wynn, 2011). In mice, especially in the intestinal mucosa, the best and most commonly used marker to identify macrophages is F4/80 (Austyn and Gordon, 1981; Gordon et al., 2011; Platt and Mowat, 2008). In addition to F4/80, most murine macrophage populations also express the pan-myeloid markers CD11b and CD68, as well as CD115, consistent with their derivation from monocytes (Sasmono et al., 2003).

Resident intestinal macrophages, exhibit a limited capacity to respond to bacterial adjuvants due to downregulation of their bacterial recognition receptors, such as toll-like receptors (TLRs). Therefore, in conventional conditions, they do not respond to bacterial antigen stimuli through upregulation of pro-inflammatory cytokines, reactive oxygen

species (ROS) or reactive nitrogen species (RNS), but they produce anti-inflammatory cytokine IL-10 (Mahida et al., 1989; Murai et al., 2009; Smith et al., 2011). These features enable them not to react with commensal bacteria that have crossed the epithelial barrier with inflammatory response. In addition, IL-10 produced by macrophages is crucial for regulatory T cell differentiation in the intestine (Murai et al., 2009). However they contribute to the local clearance of bacteria from the tissue, translate alert signals to other immune cells, secrete cytokines to establish the local homeostatic immune cell network, and participate in the stimulation of the adaptive immune system and maintenance within the lamina propia (Zigmond and Jung, 2013). Alternatively, activated macrophages represent a wide array of phenotypes. This spectrum includes macrophages with wound-healing and regulatory properties, which arise during innate or adaptive immune responses, under the influence of IL-10 and TGFβ to dampen the immune response and limit inflammation (Mosser and Edwards, 2008) (see section 1.3.1.3).

The modern view of macrophage population's origin is that monocytes do not always substantially contribute to all tissue macrophage compartments in the body and therefore do not depend entirely on the recruitment of monocytes derived from HSCs in the bone marrow (Epelman et al., 2014a; Ginhoux and Jung, 2014; van Furth and Cohn, 1968). In many tissues, they develop from primitive macrophages existing in the yolk sac or fetal liver and are maintained independently from HSCs in the bone marrow in steady-state conditions, as it is the case for instance for Kupffer cells in the liver, Langerhans cells in the skin, alveolar macrophages in the lung, microglia in the central nervous system, peritoneal macrophages in serosal tissues and red pulp macrophages in the spleen. These macrophages are maintained in adults independently of the bone marrow in homeostatic conditions through a process of self-renewal (Davies and Taylor, 2015; Epelman et al., 2014a; Ginhoux and Jung, 2014; Hashimoto et al., 2013). However, in some tissues, especially in the intestinal mucosa, the vast majority of resident macrophages are continually replenished from circulating monocytes originating from the bone marrow (see section 1.2.2). It takes around four to five days for those monocytes to acquire the phenotype of resident mucosal and lamina propria macrophages, characterized by the expression of F4/80, CD64, major histocompatibility complex (MHC) class II, CD11c, and CX3C chemokine receptor 1 (CX_3CR_1) (Bain et al., 2013). Moreover, by tracking macrophage development from birth to adulthood in the intestinal mucosa, it was observed that although primitive macrophages are present after birth in neonatals, they are not maintained in adulthood (Bain et al., 2014; Bain et al., 2013). This further

confirms that in contrast to other tissues, macrophages that reside in the GI mucosa are replaced continuously by blood monocytes and that yolk sac–derived macrophages are short-lived or rare (Cipriani et al., 2016; Zigmond and Jung, 2013).

Nevertheless, in the context of inflammation, the recruitment and differentiation of blood monocytes from bone marrow stem cells is involved in the maintenance of the tissue-resident macrophage population through highly regulated differentiation events, also in tissues that do not rely on this source of macrophages under homeostatic conditions, e.g. skin, liver, brain or lung (Bain et al., 2014; Hoeffel et al., 2015; Lavin et al., 2015). The ontogeny and classification of different tissue macrophages are summarized in Figure 3.

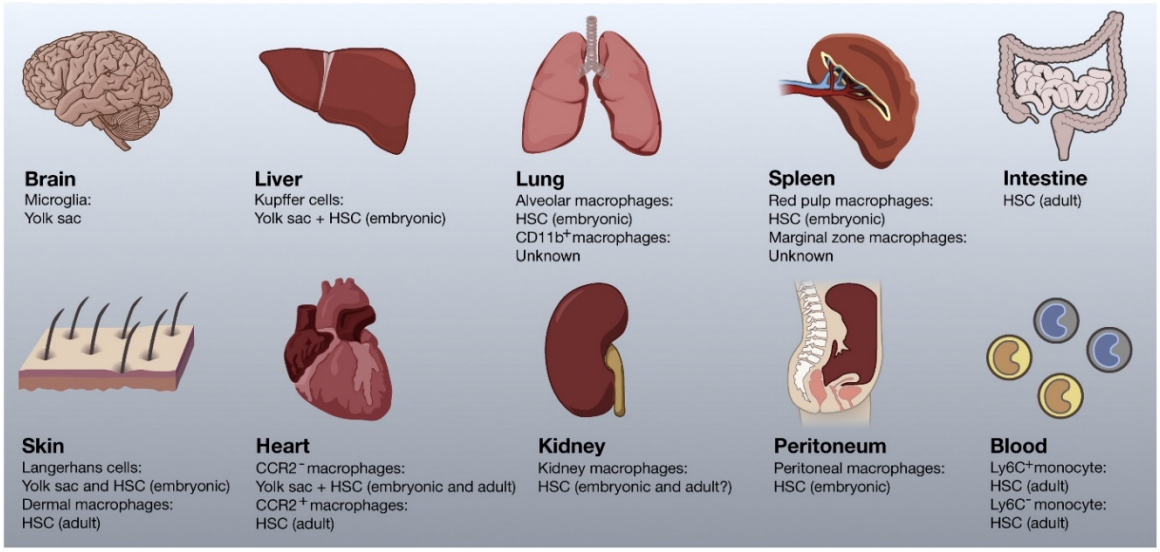


Figure 3. Macrophage ontogeny and contribution to populations of resident tissue macrophages.

Contribution of macrophage lineages to populations of adult resident macrophages. HSC-derived populations include embryonic populations and no definitive evidence of yolk sac origin (embryonic), whereas HSCs (adult) are continually replaced by circulating adult monocytes from the bone marrow (modified from Immunity (Epelman et al., 2014b)).

Circulating monocytes in mice constitute around 1.5 to 4 % of the total peripheral leukocyte blood pool during the steady state (Robbins and Swirski, 2010). Monocytes are defined as cells that express CD11b and CD115, and may also express the F4/80 antigen at intermediate or low levels, particularly if they are in the process of differentiating into macrophages (Sunderkotter et al., 2004; Youn and Gabrilovich, 2010). As described above (see section 1.2.2), they develop from myeloid precursors in the bone marrow, prior to intravasation into the bloodstream (Epelman et al., 2014a). In the differentiation

process, monocyte commitment is influenced primarily by the presence of the growth factor M-CSF (Gow et al., 2010; Sunderkotter et al., 2004). Heterogeneous monocyte subsets have been identified across several mammalian species. In mice in particular, monocytes can be subdivided based on the expression of the lymphocyte antigen 6 complex, locus (Ly6) C into $Ly6C^{lo}$ and $Ly6C^{hi}$ populations (Bain et al., 2014; Epelman et al., 2014a; Epelman et al., 2014b). Mouse $LY6C^{hi}$ and $LY6C^{lo}$ cells are the equivalent of human $CD14^{+}$ and $CD14^{lo}CD16^{+}$ monocyte subsets, respectively (Ginhoux and Jung, 2014). Morphologically, the $Ly6C^{hi}$ population is larger and more granulocytic than its $Ly6C^{lo}$ counterpart and expresses lower levels of chemokine surface receptor CX_3CR1 (Randolph et al., 1998; Shi and Pamer, 2011).

The C-C chemokine receptor type (CCR) 2 is very important for $Ly6C^{hi}$ monocyte trafficking, and it is well accepted that $Ly6C^{hi}$ monocytes rely on CCR2 to mediate egress from the bone marrow (Bain et al., 2014; Nakanishi et al., 2015) (see section 1.3.1.2). Mice lacking this receptor have severely reduced circulating levels of all monocytes, and in particular $Ly6C^{hi}$ monocytes, while mice lacking CX_3CR1, which is highly expressed in $Ly6C^{lo}$ but not $Ly6C^{hi}$ monocytes, show little modification of circulating monocyte numbers. As such, deployment of monocytes into the circulation from the bone marrow relies on CCR2 but is independent of CX_3CR1 (Fairbairn et al., 2011; Shi and Pamer, 2011). Macrophage-like $Ly6C^{lo}$ cells in the blood patrol the endothelial surface of the vessel and coordinate its repair by recruiting neutrophils as required, whereas $Ly6C^{hi}$ monocytes are poised to traffic to sites of infection and inflammation and differentiate by default into $CX_3CR1^{hi}Ly6C^{lo}$ cells (Ginhoux and Jung, 2014; Zigmond et al., 2012). As stressed above, the intestinal macrophage compartment is unique in that it continuously recruits monocytes in homeostasis for its replenishment, most probably because of tonic low-grade inflammation (as described above as “physiological inflammatory state”), caused by the microflora exposure of this tissue (Zigmond et al., 2012).

Given the plasticity of monocytes to give rise to various macrophage and DC populations, once at tissues, they can acquire a wide range of functions and phenotypes, which are modulated by signals from the environment (Figure 4) (Davies and Taylor, 2015; Mosser and Edwards, 2008).

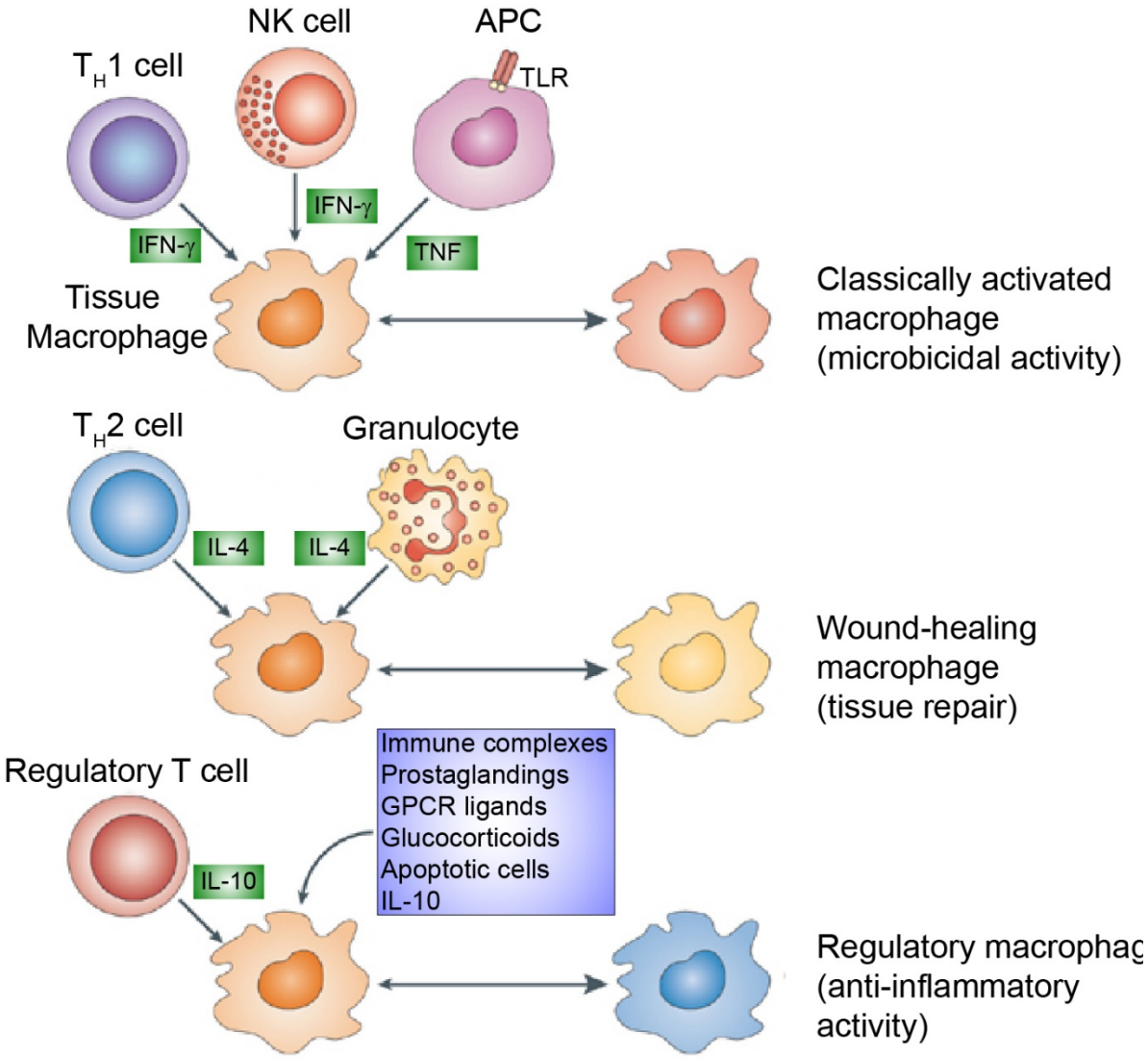


Figure 4. Macrophage plasticity is orchestrated by cytokines.

Classically activated macrophages arise in response to interferon-γ, which can be produced during an adaptive or innate immune response and TNF, which is produced by APCs. Wound-healing (alternatively activated) macrophages arise in response to IL-4, which can be produced during an adaptive or innate immune response. Regulatory macrophages are generated in response to various stimuli, including immune complexes, prostaglandins, G-protein coupled receptor ligands, glucocorticoids, apoptotic cells or IL-10. Each of these three populations has a distinct physiology. Classically activated macrophages have microbicidal activity, whereas regulatory macrophages produce high levels of IL-10 to suppress immune responses. Wound-healing macrophages have a role in tissue repair (modified from Nat Rev Immunol (Mosser and Edwards, 2008)).

1.3 Mucosal immunity in inflammation

The intestinal immune system has the constant task to survey the intestinal milieu and respond robustly to pathogenic insult, while repressing such responses towards innocuous antigens. However, deregulation in this equilibrium as occurs in IBD, implicates an

imbalance between pro- and anti-inflammatory cytokines, impeding the resolution of inflammation and instead leading to disease perpetuation and tissue destruction. Although cytokines participate in the repair of the intestinal mucosal epithelial barrier, they have been also implicated in mucosal injury and tissue damage as well as in tumor angiogenesis, progression and metastasis (Bouma and Strober, 2003; Fukata and Abreu, 2009; Sartor, 2006).

1.3.1 Inflammatory bowel disease

IBD is a chronically relapsing inflammatory disorder (Triantafillidis et al., 2009) characterized by an overactive immune system and an excessive production of cytokines (Sartor, 2006). The two major types of IBD are ulcerative colitis (UC), which is limited to the colon, and Crohn's disease, which can affect any part of the gastrointestinal tract (Bouma and Strober, 2003; Li et al., 2013). Histologically, UC is characterized by non-transmural inflammation that is limited to the mucosa and submucosa with cryptitis and crypt abscesses (Baumgart and Sandborn, 2007; Khor et al., 2011). On the other hand, in Crohn's disease the inflammation of the gastrointestinal mucosa is transmural and typically implicate the development of complications including strictures, abscesses or fistulas. It is characterized by thickened submucosa, transmural inflammation, fissuring ulceration and granulomas (Abraham and Cho, 2009c; Baumgart and Sandborn, 2007; Khor et al., 2011). Differential diagnosis in patients with IBD is nowadays of pivotal importance for a personalized clinical management, as each entity involves specific therapeutic strategies (Dignass et al., 2012; Dignass et al., 2010; Kornbluth et al., 2004; Lichtenstein et al., 2009). Nevertheless, non-classical forms of either UC or Crohn's disease still represent a remarkable clinical matter, as due to the heterogeneity of these diseases no single diagnostic core standard could be established yet (Dignass et al., 2012; Dignass et al., 2010; Kornbluth et al., 2004; Lichtenstein et al., 2009; Magro et al., 2013). Therefore, 5% to 15% of cases do not meet strict criteria for either UC or Crohn's disease and in up to 14% of patients classified as UC and Crohn's disease the diagnosis changes over time (D'Arcangelo and Aloi, 2017; Louis, 2015; Tontini et al., 2015). For these reasons, the terms indeterminate colitis (IC) or inflammatory bowel disease unclassified (IBDU) were introduced by pathologists for the diagnosis of surgical colectomy specimens showing an overlap between the features of UC and Crohn's disease (Tremaine, 2012).

Both forms significantly impair quality of life and require prolonged health care interventions. Their unknown cause, unpredictable presentations and symptoms, the less than optimal treatments and a continuous rise in their incidence and prevalence worldwide makes the treatment of these diseases particularly challenging (Lamhonwah et al., 2003). Current IBD therapies are rather broad in action and not particularly focused on patient characteristics. Understanding the contribution of host and environmental factors will promote individualized management of this heterogeneous patient population (Abreu, 2002; Baumgart and Sandborn, 2007; Michielan and D'Inca, 2015).

IBD is associated with a range of extra-intestinal manifestations (EIMs). Most IBD patients have colonic inflammation, although some patients develop EIMs prior to the onset of colonic symptoms and might be the initial presenting symptoms of the IBD. Up to 36% of patients with IBD have at least one EIM (Bernstein, 2001; Levine and Burakoff, 2011). These manifestations can involve nearly any organ system and some are related to active inflammation (i.e. joint, skin, ocular and oral manifestations) (Langholz, 2010). In addition, the risk of cancer was found to be higher in patients suffering from UC than from Crohn's disease, predisposing factors being the duration of the disease, EIMs, and the onset of the disease at early age (Pohl et al., 2000) (see section 1.3.2).

Although the exact etiologies of IBD are unknown, it is considered as multifactorial. It has been proposed that, in genetically susceptible individuals it is due to disruption of gut homeostasis leading to altered and chronic activation of the immune system and inflammatory cascades against the microorganisms of the intestinal flora, which triggers the disease (Kole and Maloy, 2014; Michielan and D'Inca, 2015; Muniz et al., 2012; Podolsky, 2002a; Podolsky, 2002b; Rubin et al., 2012). However, how and why individuals generate inappropriate host immune responses and initiate the development of IBD is not completely understood (Gulbake et al., 2016). Despite tight and highly evolved regulation mechanisms of the immune system, dysfunctions and disturbances can arise, leading to hyper-inflammatory conditions and autoimmune disease, which has been suggested to be a result of several factors (Abraham and Medzhitov, 2011; Autschbach et al., 2002; Kaser et al., 2010; McGhee and Fujihashi, 2012). In particular, the altered immune response taking place in IBD may be caused by a loss of barrier function, which overwhelms normal immune regulation, as well as dysfunction of regulatory mediators of the immune system. In this sense the intestinal mucosal epithelial barrier is thought to be the effector arm that mediates intestinal inflammation (McGhee and Fujihashi, 2012; Neurath, 2014; Podolsky, 2002a).

The well-known familial occurrence of IBD has suggested for a long time that this condition could have a genetic basis (Orholm et al., 1991). Over the years, high number of susceptibility loci has been associated with IBD (Machado et al., 2006; McGovern et al., 2010). Although the genetic component is stronger in Crohn's disease than in UC, and despite their distinct clinical and histological features, approximately 30% of these IBD-related genetic loci are shared between both conditions, indicating that these diseases engage common pathways (Andersen et al., 2011; Franke et al., 2010). Candidate genes include those that regulate innate immunity and intestinal homeostasis, including epithelial barrier function, epithelial restitution, microbial defence, innate immune regulation, ROS generation, autophagy, regulation of adaptive immunity, endoplasmatic reticulum (ER) stress and metabolic pathways associated with cellular homeostasis (Imielinski and Hakonarson, 2010; Lees et al., 2011; Machado et al., 2006; McGovern et al., 2010; Thompson and Lees, 2011). Additional genetic studies have revealed that gene products involved in the elimination of endogenous small organic cations, drugs and environmental toxins are linked to Crohn's disease and UC etiology (Bilsborough and Viney, 2004; Cucchiara et al., 2007; Waller et al., 2006).

Over the years a plethora of theories have been proposed to explain the pathology of IBD, ranging from infectious to psychosomatic, social, metabolic, vascular, genetic, allergic, autoimmune and immune-mediated (Fiocchi, 1998; Fiocchi, 2009). However, as illustrated in Figure 5, the general consensus nowadays is that this disease is the result of combined effects of four basic components: global changes in the environment, the input of multiple genetic variations, alterations in the intestinal microbiota and aberrations of innate and adaptive immune responses (Sartor, 2006). Of note, it has also been concluded that none of these four components can by itself trigger or maintain intestinal inflammation (Triantafillidis et al., 2011).

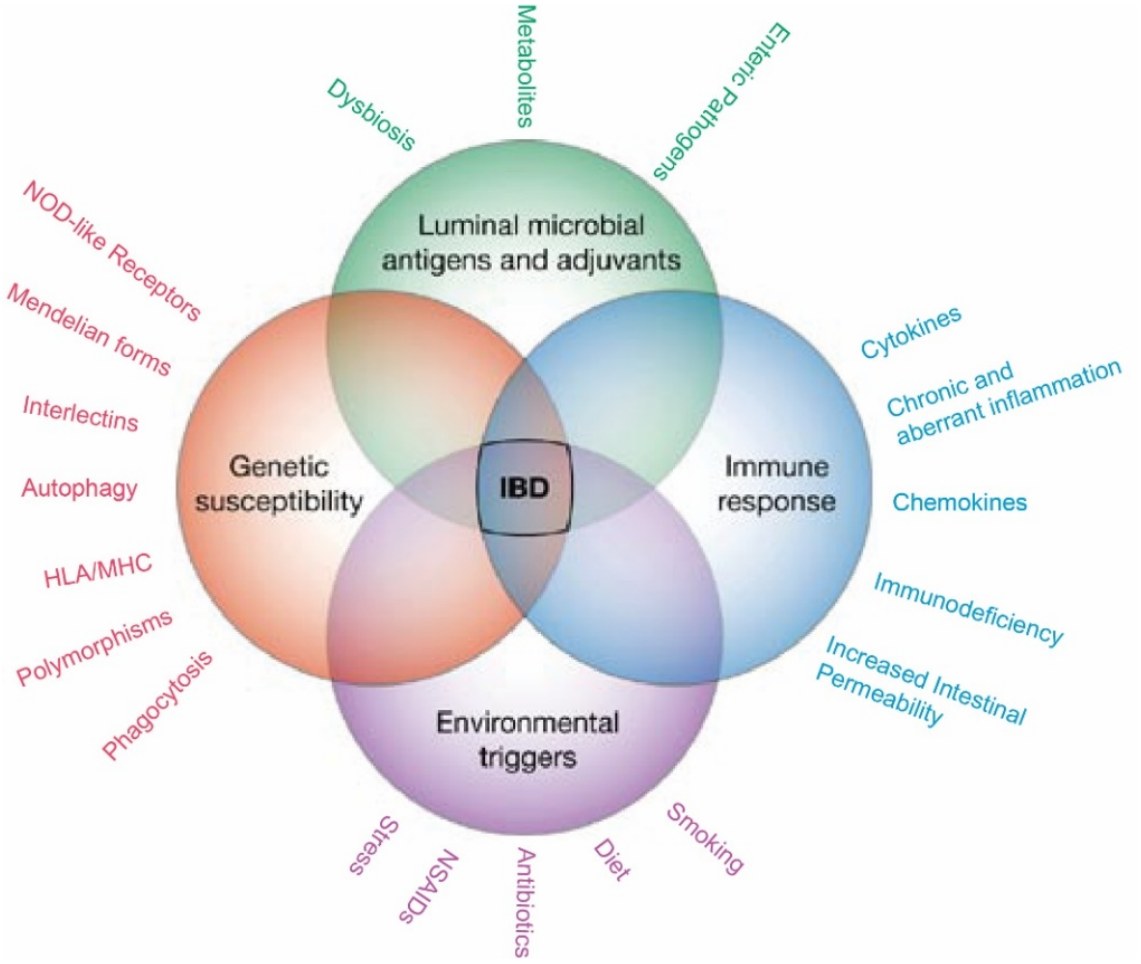


Figure 5. The four basic components of IBD etiology.

Interaction of various factors contributing to chronic intestinal inflammation in a genetically susceptible host. Genetic susceptibility is influenced by the intestinal microbiota, which provide antigens and adjuvants that stimulate either pathogenic or protective immune responses. Environmental triggers are necessary to initiate or reactivate the disease (modified from Nat Clin Pract Gastroenterol Hepatol (Sartor, 2006)).

Lifestyle choices such as diet are thought to contribute to IBD by altering the commensal flora or promoting obesity related inflammatory responses (Ferguson et al., 2007). Thus, the interactions between genetic and environmental factors will shape the gut epithelial-innate immune interface and lead to unique phenotypes in patients with IBDs. Moreover, experimental and clinical evidence suggests that uncontrolled activation of macrophages and T cells is a central mechanism in the pathophysiology of chronic intestinal inflammation (Fiocchi, 1998; Rubin et al., 2012; Shanahan, 2001a). Furthermore, luminal bacteria also play a role in the initiation and progression of the disease. It has been demonstrated that in inflamed mucosa or even during inactive IBD, the composition of the fecal microbiota changes (dysbiosis), and these bacteria can potentially translocate into the intestinal barrier. This is mediated by a reduction of the

interlayer biofilm and the mucus layer, which results in the loss of the epithelial integrity leading to the translocation of harmful bacteria and the consequent chronic activation of the inflammatory immune response. Finally, cell-mediated immunity against the luminal microbiota generates intestinal lesions and impairs resolution of the lesions leading to chronic intestinal disease (Pirzer et al., 1991). In this sense, IBD patients exhibit increased intestinal permeability, however, it is not clear whether this is a cause or consequence of the disease (Uluckan and Wagner, 2017).

1.3.1.1 Disturbances in innate and adaptive immunity

IBD was traditionally viewed as predominantly T-cell-driven (Podolsky, 2002a) and the adaptive immune system was regarded to be the key contributor to the pathogenesis of IBD (Huang and Chen, 2016; Xavier and Podolsky, 2007). However, it is now clear that the innate immune response is equally as important in inducing gut inflammation in these patients, implicating altered innate microbial sensing and autophagy, mucosal barrier functions, innate immune responses, cytokine, chemokine and microbial peptide production (Huang and Chen, 2016; McGhee and Fujihashi, 2012; Muniz et al., 2012; Neurath, 2014). Supporting this conclusion, patients with innate immunodeficiencies tend to develop IBD, and similarly patients with Crohn's disease have defective innate immune responses (Smith et al., 2009). This has refocused the attention to this arm of the immune system.

Belonging to the innate immune system, macrophages, along with DCs, are the main antigen presenting cells (APCs) in the intestinal mucosa and present foreign antigens to cells of the adaptive immune system, thereby integrating the innate and adaptive systems for quick, yet broad, responses to microorganisms and antigens (Abraham and Medzhitov, 2011; Joeris et al., 2017; Sanders et al., 2017; Sansonetti, 2004). The specific role of monocytes and macrophages in the context of intestinal inflammation will be further discussed in detail below (see section 1.3.1.2). Similarly to macrophages, intestinal DCs, can function to provide protection and defense, induce tolerance or mediate inflammation (Bilsborough and Viney, 2004; Mowat and Bain, 2011). These cells are able to acquire inflammatory properties during intestinal inflammation, such as the ability to produce IL-6 and the stimulation of Th1 responses (Interferon-γ (IFN-γ), IL-2, IL-10, IL-12, Tumor necrosis factor- (TNF-) α, TNF-β) (Laffont et al., 2010) and are able to promote colonic Th17 responses (IL-17A, IL-17F, Th17, IL-6, TNF-α, IL-21 and IL-22, IL-23, TGF-β) to commensal-derived ATP. Of note,

acute and chronic mouse models of colitis, were also associated with a significant increase in recruited monocyte-derived DCs, besides monocyte-derived macrophages, that produced high levels of pro-inflammatory cytokines and showed enhanced TLR responsiveness (Damsker et al., 2010; Platt et al., 2010; Platt and Mowat, 2008; Varol et al., 2009). Moreover, in colonic mucosa of IBD patients, interactions between DCs and T cells promote production of inflammatory cytokines and chemokines as well as an increase of expression of chemokines receptors (Drakes et al., 2005).

A cell type belonging to the innate immune system and that accumulates in the lamina propria and within epithelial crypts are the neutrophils. They similarly to macrophages and DCs, correlate with clinical disease activity and epithelial injury in IBD. Moreover, the activation of neutrophils, as for macrophages, implicates the production of ROS and nitrogen species as well as myeloperoxidase (MPO) within intestinal mucosa, which induces oxidative stress that participates in the intestinal damage associated to these conditions (Chin and Parkos, 2006; Smith et al., 2009). However, neutrophils also contribute to the resolution of inflammation, by synthesizing anti-inflammatory mediators such as lipoxin A4 (Witte et al., 2010).

The NK cells also belong to the innate leukocyte population and contribute to intestinal inflammation by secreting Th1 and Th17 cytokines (Colonna, 2009; Cua and Tato, 2010; Maloy and Kullberg, 2008; Wolk et al., 2010). Another important NK cell feature is that they contribute to the control of viral and bacterial infections, kill tumor cells and establish a relationship between innate immunity and adaptive immunity (Colucci et al., 2003). Other leukocyte populations, such as NK T cells and γδ T cells that straddle the interface of innate and adaptive immunity (Figure 1) can similarly to NK cells secrete Th1 and Th17 cytokines that contribute to the pathogenesis of intestinal inflammation (Colonna, 2009; Cua and Tato, 2010; Maloy and Kullberg, 2008; Rescigno et al., 2008).

Of note, the recruitment of innate immune cells to sites of tissue injury and inflammation is choreographed by integrins, chemokines, chemokine receptors and microbial signals. Therefore, its modulation may be useful for disease intervention (Xavier and Podolsky, 2007). Upregulation of chemokines and chemokine receptors has been described in studies examining clinical and experimental colitis (Charo and Ransohoff, 2006). The major chemokine and chemokine receptors associated with the onset of IBD include C-X-C motif chemokine (CXCL)8/IL-8, CCL5/regulated on

activation, normal T cell expressed and secreted (RANTES), CCL2/monocyte chemotactic protein-(MCP-) 1, CCL6/ MCP-3, CCR5, CCR2, CCR1 and Chemerin, which regulate the infiltration of inflammatory cells such as neutrophils, macrophages, and T cells (Ajuebor and Swain, 2002; Buechler, 2014; Lin et al., 2014).

It has been reported that chemokine or chemokine receptor antagonists as well as monoclonal antibodies specific for adhesion molecules can be used to treat IBD patients (Bizzarri et al., 2006; Danese et al., 2005). Accordingly, the influx of inflammatory cells into the mucosa increases the release of many metabolic and inflammatory mediators including cytokines, chemokines, nitric oxide (NO), oxygen radicals, prostaglandins, leukotrienes, histamine, proteases, and matrix metalloproteinases, contributing to further tissue damage and the onset of tumorigenesis (Abraham and Cho, 2009a; Abraham and Cho, 2009b; Abreu, 2002; Capaldo and Nusrat, 2009; Dranoff, 2004; Fukata and Abreu, 2009; Neurath, 2014).

Abnormal innate immune responses result in the imbalance of the adaptive immunity, which leads to its excessive activation and therefore a vicious cycle forms. Therefore, the comprehensive analyses of innate and adaptive immunity and exploration of how they are connected is important for understanding the pathology of IBD and developing more effective treatments (Khor et al., 2011; Shanahan, 2001a; Xavier and Podolsky, 2007).

Basically, the adaptive immune responses are effected by a combination of resident and recruited cell populations, such as mucosal B cells producing secretory IgA and of IgG as well as a complex mixture of T cells (mainly Th1, Th17 or Th2) and regulatory T and B cells (Xavier and Podolsky, 2007). In this sense, antibody production in active IBD has been found to be increased in the blood circulation, but also in the intestinal mucosa (MacDermott et al., 1981). Furthermore B cells are able to synthesize the immunomodulatory cytokines IL-10 and TGF-β, although their role in IBD has not been extensively studied yet (Salinas et al., 2013). The role of B lymphocytes has been studied in animal models of experimental colitis and it seems that, at least in some of these models, B cells might contribute to an inflammatory response (Olson et al., 2004). However, at the moment limited attention is being given to B cells in IBD but renewed interest might occur if B cell depletion for instance by using rituximab, which is targeting CD20, a cell surface molecule expressed mainly on mature B lymphocytes, turns out to be effective in IBD management (Bauer et al., 2012; Furtado and Isenberg, 2013; Perosa et

al., 2010). Nevertheless, a clinical trial in patients with UC showed that this is not a promising treatment strategy, which does not mean that it will not be effective in other clinical manifestations of IBD, neither it rules out that B cells are involved in the pathophysiology of IBD (Leiper et al., 2011).

However, T cells are well established key players of intestinal inflammation in IBD and are the main cells involved in the adaptive immune response. It has been confirmed that a dysregulated T cell response with abnormal development of activated T cell subsets may lead to the onset of inflammation by an excessive release of cytokines and chemokines which have multiple pathogenic effects on both, the adaptive and the innate arms of the immune system (Huang and Chen, 2016; Neurath, 2014; Xavier and Podolsky, 2007). In particular, while Crohn's disease has long been considered to be driven by a Th1 response, UC has been rather associated with a non-conventional Th2 response (Fuss et al., 1996). However, beside the well-known classical Th1 and Th2 responses, a role for Th17 cells has also evolved in the context of IBD pathogenesis (Geremia and Jewell, 2012). These cells are activated through several cytokines, but the main cytokine mediating Th17 responses is IL-17, and the production of this cytokine is stimulated by the production of IL-6, TGFβ and IL-23 by innate immune cells and APCs, and have been found to be increased in IBD and most forms of experimental colitis (see also Table 1) (Abraham and Cho, 2009b; Lawrance et al., 2001; Sartor, 2006).

Altered patterns of cytokine production by immune cells in patients with IBD were initially described in the mid to late 1980s. Over the years, they were found to play a major role in driving intestinal inflammation and leading to local complications, such as neoplasms and EIMs in patients with IBD (Neurath, 2014). The cytokine responses characterizing these diseases are the key pathophysiologic elements that govern the initiation, development and the resolution of the inflammation. Therefore, some cytokines are strongly associated to the severity of the disease and could be future candidate biomarkers of the clinical course and therapeutical decision making (Florholmen and Fries, 2011; Kochhar and Lashner, 2017; Ministro and Martins, 2017). Selected cytokines implicated in the pathogenesis of IBD are summarized in Table 1 (Neurath, 2014).

Cytokine	Source in the mucosa	Potential function in the pathogenesis of chronic intestinal inflammation
IFNα and IFNβ	DCs	Promote epithelial regeneration and induce IL-10-producing cells
IFNγ	T cells and ILCs	Activates macrophages, augments antigen processing and induces death of epithelial cells
TNF	Macrophages, DCs and T cells	Activates fibroblasts, stimulates pro-inflammatory cytokine production and angiogenesis, induces death of epithelial cells, mediates T cell resistance against apoptosis and induces cachexia
IL-1	Neutrophils and macrophages	Augments recruitment of neutrophils, stimulates IL-6 production by macrophages, activates ILCs and promotes tumour development
IL-6	Macrophages, fibroblasts and T cells	Activates T cells and prevents apoptosis, induces macrophage activation, recruits immune cells, activates acute-phase proteins, induces proliferation of epithelial cells and favours tumour growth
IL-10	T cells	Suppresses pro-inflammatory cytokine production by antigen-presenting cells and T cells and induces STAT3 signalling in regulatory T cells
IL-12	Macrophages and DCs	Induces T_H1 cell differentiation via STAT4 activation in T cells, stimulates T_H1-type cytokine production and activates ILCs
IL-13	T cells and iNKT cells	Induces alterations of intestinal epithelial cells and barrier function
IL-17A and IL-17F	T cells and ILCs	Induce pro-inflammatory and anti-inflammatory effects in the mucosa and IL-17A exerts pro-fibrotic functions
IL-18	Macrophages, DC and epithelial cells	Augments production of pro-inflammatory cytokines
IL-21	T_H1 cells	Induces production of TNF, IL-1, IL-6 and IL-8 in the mucosa, recruits neutrophils, induces secretion of matrix metalloproteinases by fibroblasts and favours tumour development
IL-22	γδ and αβ T cells, ILCs, neutrophils and DCs	Activates production of antimicrobial peptides by epithelial cells, induces proliferation of epithelial cells and favours tumour development via STAT3 activation
IL-23	Macrophages and DCs	Activates mucosal immune cells such as T cells and macrophages, augments TNF production and stabilizes the phenotype of effector T_H17 cells
IL-27	Macrophages	Exerts pro-inflammatory effects by inducing T cell activation and T_H1-type cytokine production and exerts anti-inflammatory effects by blocking T cell expansion and inhibiting cytokine production by neutrophils
IL-33	Epithelial cells and myofibroblasts	Suppresses T_H1-type cytokine production and induces neutrophil influx
IL-35	DCs	Blocks the production of pro-inflammatory cytokines by mucosal immune cells
IL-37	Epithelial cells	Suppresses innate mucosal immune responses and reduces IL-1β and TNF production

DC, dendritic cell; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; iNKT cell, invariant natural killer T cell; STAT, signal transducer and activator of transcription; T_H cell, T helper cell; TNF, tumour necrosis factor.

Table 1. Key cytokines implicated in the pathogenesis of IBD

Cytokines play a fundamental role in controlling mucosal inflammation in IBD and in the pathogenesis of progressive and destructive forms of IBD that are associated with complications such as intestinal stenosis, rectal bleeding, abscess and fistula formation, and the development of colitis-associated neoplasias (obtained from Nat Rev Immunol (Neurath, 2014)).

1.3.1.2 Monocytes and macrophages in the intestine

Macrophages in the intestinal mucosa represent the largest pool of tissue macrophages in the body under healthy homeostatic conditions (Smith et al., 2011). Moreover, their numbers dramatically increase in the intestinal mucosa during the acute phase of IBD, wound-healing and malignancy, mainly due to recruitment as monocytes from HSC in the bone marrow (Bain et al., 2014; Bain and Mowat, 2014; Hume, 2006; Mowat and Bain, 2011). The largest amount is found in the lamina propria but they can be found in all layers of the intestine and their number closely correlates with the amount of bacteria in the intestine, being very reduced in germ free mice (Lee et al., 1985). Macrophages in the intestine express a large number of T cell co-stimulating molecules and triggering receptor expressed on myeloid cells-1 (TREM-1), which promotes secretion of macrophage pro-inflammatory factors (Schenk et al., 2007). Moreover, its expression is low in normal intestinal tissue, but higher in experimental colitis models and IBD patients

(Baj-Krzyworzeka et al., 2006), indicating that abnormal innate immunity dominated by macrophages plays a vital role in pathogenesis of IBD. Of note, pro-inflammatory cytokines are normally produced by monocytes and polymorphonuclear cells that have migrated to the inflammatory focus, rather than by resident intestinal macrophages (Smith et al., 2011).

Besides their crucial role in immune tolerance, monocytes and macrophages represent essential players during inflammation and immune assaults in a plethora of tissues, including the intestine (Bain et al., 2014; Bain and Mowat, 2014). Inflammation mediated by these cells is basically composed of tissue recruitment, differentiation and activation in situ, conversion to anti-inflammatory phenotype and restauration of tissue homeostasis (Murray and Wynn, 2011). Once activated, pro-inflammatory macrophages (M1 or classically activated phenotype) produce a large number of mediators and cytokines including IL-1, IL-6, IL-12, TNFα and inducible nitric oxide synthase (iNOS) amongst others (Barrientos et al., 2008; Granata et al., 2006; Sanchez-Munoz et al., 2008), as well as chemoattractants resulting in the recruitment of additional leukocytes (Ajuebor and Swain, 2002; Barrientos et al., 2008; Charo and Ransohoff, 2006; DiPietro, 1995). In order to control inflammation, it has been suggested, that these plastic cells switch from being pro-inflammatory to a broad spectrum of alternatively activated macrophages, exhibiting anti-inflammatory and pro-tissue repair functions (Daley et al., 2010; Mosser and Edwards, 2008; Murray and Wynn, 2011; Stein et al., 1992) (Brancato and Albina, 2011) (see section 1.3.1.3).

Monocytes and macrophages are recruited by chemokines in a process known as chemotaxis, and the most important chemokines involved in inflammatory cell recruitment were mentioned above (see section 1.3.1.1). $Ly6C^{hi}CCR2^{+}$ pro-inflammatory monocytes, are recruited over CCR2 and its ligands CCL2, but also CCL7, which are induced by infections and result in high circulating levels in the serum as well as in the inflamed tissues. This is the most important mechanism for the recruitment of these cells (Bain et al., 2014; Platt et al., 2010; Shi and Pamer, 2011). However, monocytes also express the receptors CCR1 and CCR5, which bind to a variety of chemokines, including the shared ligands CCL3, also known as macrophage inflammatory protein-1α (MIP-1α), and CCL5. The receptor CX_3CR1 is particularly important for $Ly6C^{lo}$ monocyte recruitment. However, also other chemokine receptors and adhesion molecules have been implicated in monocyte recruitment (Imhof and Aurrand-Lions, 2004; Shi and Pamer,

2011). Recruited to the healthy intestinal lamina propria, they give rise to resident CX_3CR1^+ macrophages that contribute to the maintenance of gut homeostasis (Zigmond et al., 2012). Although migration is critical in immune response and homeostasis maintenance, it was shown to have a negative role in some diseases. In this line, several studies revealed that reduced macrophage motility is associated with a decreased capacity to enhance tumor cell invasion or inflammatory disorders including IBD (Chanmee et al., 2014; Mowat and Bain, 2011; Zigmond et al., 2012). Consistently, ablation of either CCR2 or CCL2 has been demonstrated to ameliorate acute gut inflammation (Bain and Mowat, 2014; Ginhoux and Jung, 2014; Mowat and Bain, 2011)

As illustrated in Figure 6, $Ly6C^{hi}CCR2^+$ monocytes are continuously generated in the bone marrow from macrophage and DC precursor (MDP) and common monocyte progenitor (cMoP) intermediates. They are recruited in a CCR2 dependent manner, to give rise to a number of macrophage subsets in both healthy and inflamed colon, which in turn trigger the recruitment of other immune cells and initiate the adaptive immune response (Bain et al., 2014; Ginhoux and Jung, 2014; Nakanishi et al., 2015) .

Infiltrated monocytes are responsible for the amplification of the local immune response through the synthesis of pro-inflammatory soluble molecules and the secretion of vascular and extracellular matrix remodeling factors such as vascular endothelial growth factors (VEGFs) and proteolytic enzymes like metalloproteases (Mosser and Edwards, 2008; Yona and Jung, 2010; Yona et al., 2013). Once infiltrated, they give rise to macrophages and inflammatory DCs that amplify the inflammatory response by secreting further pro-inflammatory mediators. Additionally, recruited macrophages and inflammatory DCs amplify the chemoattractant signal for successive waves of other monocyte-derived myeloid cells or neutrophils, and also for other immune cell lineages such as the T and B lymphocytes (Koh and DiPietro, 2011; McGhee and Fujihashi, 2012; Shi and Pamer, 2011). This monocyte-derived cell infiltration is essential to establish an initial pro-inflammatory and chemoattractant microenvironment and allows the adaptive immune system to trigger further immune responses when inflammation cannot be resolved by the innate immune system. In this sense, unrestrained pro-inflammatory responses of lamina propria macrophages and DCs participate in the induction of chronic inflammation by continued recruitment of inflammatory cells (Cekici et al., 2014; Koh and DiPietro, 2011; Merle et al., 2015; Ortega-Gomez et al., 2013).

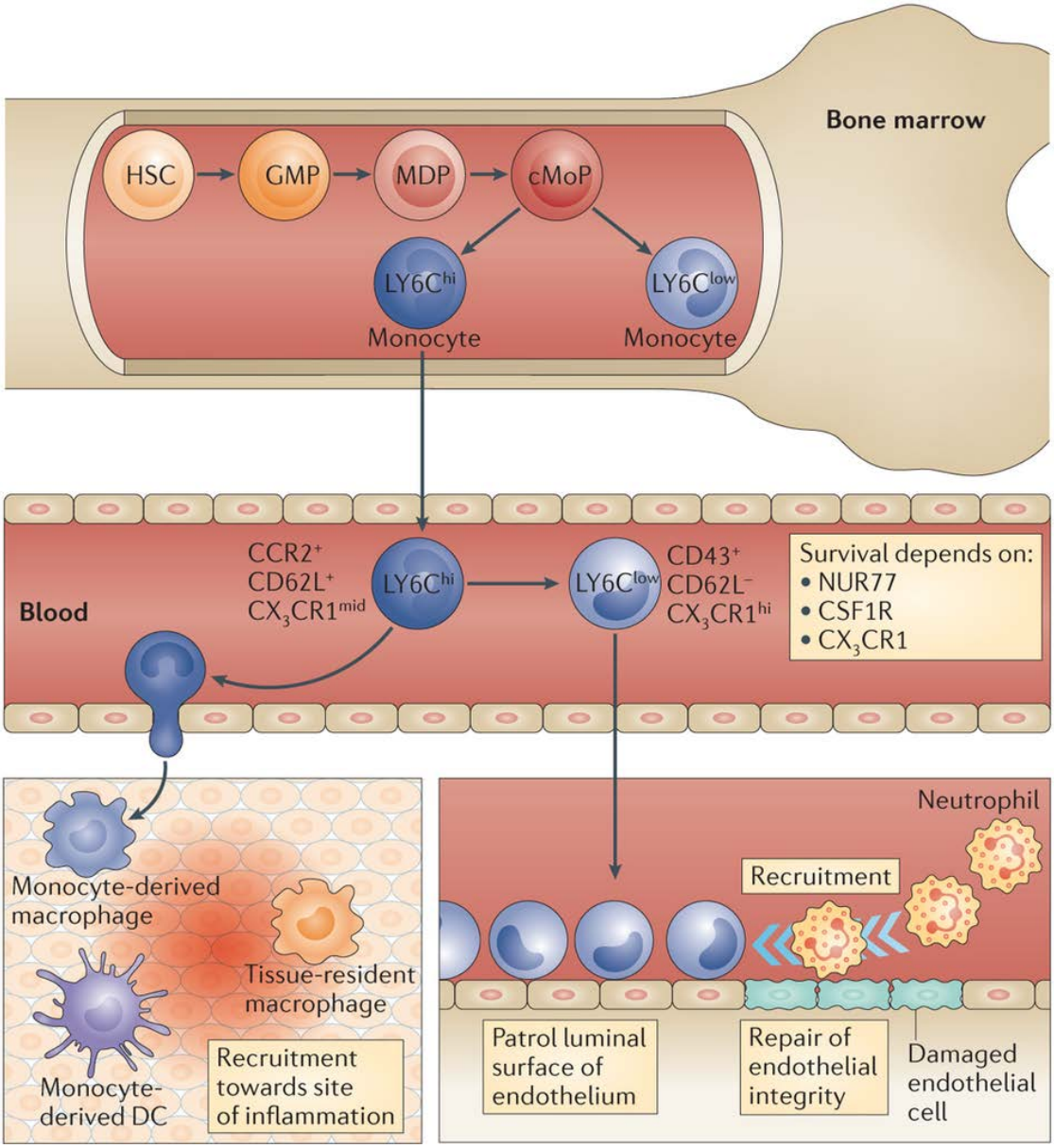


Figure 6. Monocyte recruitment from haematopoietic stem cells in the bone marrow.

Monocytes are continuously generated in the bone marrow from HSCs via macrophage and MDP and cMoP intermediates. In the steady state, Ly6C^{hi} and Ly6C^{lo} monocyte subsets in the circulation form a developmental continuum but are functionally distinct. GMP, granulocyte–macrophage progenitor (obtained from Nat Rev Immunol (Ginhoux and Jung, 2014)).

It is therefore not surprising, that increased leukocyte infiltration into the lamina propria is a hallmark of active IBD and experimental colitis, with several immune cells, such as T cells, monocytes, DCs and neutrophils contributing to disease initiation, subsequent tissue damage and disease perpetuation (Abraham and Cho, 2009a; Abraham and Cho, 2009b; Abreu, 2002; Fukata and Abreu, 2009). The critical role of innate immune responses in IBD is further underscored by the observation that infliximab, a monoclonal antibody against TNF (see Table 1), has emerged as an efficient treatment

with quick disease activity reduction and IBD patient life quality improvement (Hindryckx et al., 2017; Nanda et al., 2013; Radin et al., 2017).

1.3.1.3 Wound-healing

IBD is characterized by chronic inflammation due to an imbalance between pro- and anti-inflammatory cytokines at the submucosal level leading to disruption of epithelial barrier function and tissue destruction. The complete repair of the epithelial layer is a key goal of current IBD treatment, as it has been related to long-term remission of this pathology (Bernstein, 2015; Henderson et al., 2011; Maloy and Powrie, 2011; Neurath and Travis, 2012). However, epithelial cell migration, apart from being important for wound-healing processes, is also a pro-tumorigenic property of cancer cells and cancer has been proposed to be an over healing wound. This suggests a potential problem in targeting epithelial repair mechanisms for IBD treatment (Schafer and Werner, 2008).

Regardless of the type of wound, the same basic physiological principles apply to how wounds heal (Johnstone and Farley, 2005). Due to its exposure to external environmental factors, the intestinal epithelium can be injured by various factors, such as toxic luminal substances, normal digestion, inflammation, interactions with microbes, oxidative stress, and pharmaceuticals (Banan et al., 2000a; Banan et al., 2000b; Vongsa et al., 2009). Rapid resealing of the epithelial surface barrier following injuries or physiological damage is essential to preserve the normal homeostasis and depends on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area (Danese and Peyrin-Biroulet, 2012; Feil et al., 1987; Moyer et al., 2007; Okamoto and Watanabe, 2005; Papi and Aratari, 2014). First, epithelial cells surrounding the wound lose their columnar polarity and take on a flattened morphology, dedifferentiate, form pseudopodia-like structures and reorganize their cytoskeleton to rapidly migrate into the wound area to restore barrier integrity. After migration, they re-differentiate and adopt their initial polarity and phenotype. This process has been termed “epithelial restitution”, it is independent of cell proliferation and occurs within minutes to hours both, *in vitro* and *in vivo* (Dignass and Podolsky, 1993; Dise et al., 2008; Sturm and Dignass, 2008; Taupin and Podolsky, 2003). Then, epithelial cell proliferation is necessary to replenish the decreased cell pool. This generally begins hours or days after the injury (Dignass and Podolsky, 1993; Taupin and Podolsky, 2003). Finally, maturation and differentiation of undifferentiated epithelial cells is needed to maintain the mucosal barrier function (Sturm and Dignass, 2008). However, the separation of intestinal

epithelial wound-healing in these described three distinct processes is rather artificial and simplified and may overlap particularly *in vivo* due to its regulation by a broad spectrum of structurally distinct regulatory factors, including cytokines, growth factors, adhesion molecules, neuropeptides and phospholipids (Fiocchi, 1998; Okamoto and Watanabe, 2004; Okamoto and Watanabe, 2005; Sturm et al., 1999).

As stressed above, macrophages constitute an essential element of inflamed tissues, where they contribute to a variety of functions, due to their property to adopt different functional phenotypes that differ in the expression of surface proteins and the production of cytokines (Murray and Wynn, 2011; Pull et al., 2005; Sica and Mantovani, 2012). Two of the best characterized *in vitro* phenotypes are the pro-inflammatory M1 phenotype that mediates the defense of the host from microorganisms and the wound-healing M2 phenotype. *In vivo*, hyper-activation of the M1 phenotype leads to massive tissue destruction and further recruitment of inflammatory cells, leading to additional disease perpetuation. Wound-healing macrophages however, exhibit potent anti-inflammatory activity through expression of high levels of anti-inflammatory cytokines and scavenger molecules. They are mainly activated in response to IL-4 and IL-13 and express factors that are important for tissue repair and fibrosis (Daley et al., 2010; Gensel and Zhang, 2015; Novak and Koh, 2013; Xiao et al., 2008). They also antagonize M1 macrophage responses, which may be crucial for the activation of the wound-healing response and for tissue homeostasis to be restored (Sindrilaru et al., 2011). Recent studies have shown that M1 macrophages can themselves ‘convert’ into anti-inflammatory macrophages with an M2 wound-healing phenotype (Arnold et al., 2007; Biswas and Mantovani, 2010). A third type of macrophages are the regulatory macrophages that can arise following innate or adaptive immune responses. Regulatory macrophages do not contribute to the production of the extracellular matrix, and may express high levels of co-stimulatory molecules and present antigens to T cells. The main characteristics of these three macrophage populations are summarized in Table 2 (Mosser and Edwards, 2008).

Marker	Function	Expression
Classically activated macrophages		
IL-12	Induces T_H1-cell development	Induced by IFNγ
iNOS	Produces NO and citrulline from arginine to kill microorganisms	Depends on IFNγ
CCL15	Attracts monocytes, lymphocytes and eosinophils	Upregulated by IFNγ
CCL20	Chemoattractant for DC and T cells	Upregulated by IFNγ
CXCL9	Involved in T-cell trafficking	Induced by IFNγ
CXCL10	Attracts NK and T cells; signals through CXCR3	Induced by IFNγ
CXCL11	Attracts NK and T cells; signals through CXCR3	Induced by IFNγ
Wound-healing macrophages		
CCL18	Attracts lymphocytes, immature DCs and monocytes	Induced by IL-4
YM1	Chitinase-like protein that can bind to extracellular matrix	Strongly induced by IL-4
RELMα	Can promote deposition of extracellular matrix	Strongly induced by IL-4
CCL17	Attracts T cells and macrophages	Induced by IL-4 and suppressed by IFNγ
IL-27Rα	Inhibits pro-inflammatory cytokine production	Upregulated by IL-4
IGF1	Stimulates fibroblast proliferation and survival	Induced by IL-4
CCL22	Attracts T_H2 cells and other CCR4-expressing cells	Induced by IL-4
DCIR	C-type lectin containing an ITIM motif	Induced by IL-4
Stabilin 1	Endocytic receptor that may be involved in lysosomal sorting	Induced by IL-4
Factor XIII-A	Can bind to extracellular matrix proteins and contribute to wound healing	Induced by IL-4 and suppressed by IFNγ
Regulatory macrophages		
IL-10	Potent anti-inflammatory cytokine	Induced by TLRs in combination with other stimuli
SPHK1	Catalyses the conversion of sphingosine to sphingosine-1 phosphate	Induced by TLRs and immune complexes
LIGHT	Provides co-stimulatory signals for T cells through HVEM	Induced by TLRs and immune complexes
CCL1	Attracts eosinophils and T_H2 cells; binds CCR8	Induced by TLRs in combination with several other stimuli

Table 2. Characteristics and potential biomarkers of classically activated, wound-healing and regulatory macrophages.

DCIR, DC immunoreceptor; HVEM, herpesvirus entry mediator; IL-27Rα, IL-27 receptor α-chain; ITIM, immunoreceptor tyrosine-based inhibiting motif; SPHK1, sphingosine kinase 1 (obtained from Nat Rev Immunol (Mosser and Edwards, 2008)).

Several growth factors play important roles in intestinal growth as well as aiding intestinal repair after injury or inflammation, and many of them are known to be produced by macrophages, but also by mesenchymal cells. An increasing body of evidence shows that a number of peptides are important in the regulation of gastrointestinal mucosal growth (Akhurst et al., 1988; Duncan and Grant, 2003; McCole and Barrett, 2007; Sturm and Dignass, 2008; Xian et al., 1999). These peptides include members of the epidermal growth factor (EGF) family, TGF-β family, fibroblast growth factor (FGF) family, insulin-like growth factor (IGF) family, trefoil factor family (TFF), heparin-binding epidermal-like growth factor (HB-EGF), growth hormone (GH), erythropoietin (Epo), granulocyte colony stimulating factor (G-CSF), glucagon-like peptide 2 (GLP-2), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), amongst others (Chen et al., 1999; Chen et al., 2013; Dignass and Podolsky, 1993; Lawrance et al., 2001;

Leppkes et al., 2014; Li et al., 2016a; Scharl et al., 2016; Shen et al., 2015; Smith et al., 2011). Several of these growth factors have been shown to be altered and suggested to play a role in IBD pathogenesis. They can regulate cell proliferation but also mediate processes such as extracellular matrix formation, cell migration and differentiation, immune regulation, and tissue remodeling.

1.3.1.3.1 Insulin-like growth factor-1 (IGF-1)

IGF-1 is a 70 amino acid polypeptide growth hormone synthesized primarily in the liver but also in the GI. It is found in the fetal intestine and in human milk, suggesting a role in intestinal development (Han et al., 1988; Hormi and Lehy, 1994; Menard and Pothier, 1991) and its production can be regulated by GH, insulin, and caloric intake (Bortvedt and Lund, 2012). Nevertheless, IGF-1 can be locally produced through autocrine or paracrine mechanisms apart from its endocrine source in the liver, and macrophages have been proposed as a major source of extra-hepatic IGF-1 (Gow et al., 2010).

As illustrated in Table 2, IGF-1 is a marker of wound-healing macrophages, mainly induced by IL-4, contributing to wound-healing through the induction of migration and helping tissue regeneration (Chen et al., 1999; Leoni et al., 2015; Mosser and Edwards, 2008; Roszer, 2015; Sierra-Filardi et al., 2011). IL-4 is a cytokine produced by various immune cells, which is considered a major regulator of wound-healing and tumor-associated macrophage (TAM) phenotypes and determines the balance of pro- and anti-inflammatory cytokines produced (Mosser and Edwards, 2008; Paul and Zhu, 2010).

Although IGF-1 has been implicated in a number of anti-inflammatory effects, in particular in the context of artherosclerosis, muscle and skin repair (Higashi et al., 2010; Spies et al., 2001; Sukhanov et al., 2007; Tidball and Welc, 2015), other reports suggest that IGF-1 has pro-inflammatory functions. For instance, IGF-1 has been reported to enhance chemotactic migration and to stimulate TNF-α expression in monocytes/macrophages (Hochberg et al., 1992; Renier et al., 1996). Thus, additional studies are required to clarify the action of IGF-1 on intestinal inflammatory processes.

The IGF-1 pathway has been linked to cancer and IBD by modulating the innate and adaptive immune systems as well as through its multi-functional involvement in the tumor microenvironment (TME) (Sanchez-Lopez et al., 2015; Smith, 2010). IGF-1 has been implicated in inflammation, inflammatory cell recruitment, proliferation, cell growth and sustaining macrophage alternative activation, collectively a number of features that promote tumorigenesis upon sustained and high exposure to this growth factor (Knuever

et al., 2015; Lawrance et al., 2001; Li et al., 2015; Mourkioti and Rosenthal, 2005; Pelosi et al., 2007).

Moreover, besides its role as a growth hormone, IGF-1 can function as chemoattractant (Roussos et al., 2011) and experimental evidence suggests that IGF-1 production by myeloid cells regulates inflammatory cell recruitment (Knuever et al., 2015; Mourkioti and Rosenthal, 2005; Pelosi et al., 2007). In fact, IGF-1 can induce macrophage migration in transwell chambers, which is impaired by chemical inhibitors of integrin and p38 signaling (Furundzija et al., 2010). However, migration and wound-healing are not always beneficial processes, as they can also promote tumorigenesis. In this sense, increasing evidence has shown the importance of IGF-1 receptor signaling in colorectal cancer (Brahmkhatri et al., 2015; Dowling et al., 2016; Sanchez-Lopez et al., 2015; Schafer and Werner, 2008; Vigneri et al., 2015; Wang et al., 2015a; Yao et al., 2016; Zhuang et al., 2016). The early evidence linking the IGF-1 receptor to cancer was the finding that the transformation of mouse embryo fibroblasts (MEFs) requires an intact *Igf1r* gene (Salisbury and Tomblin, 2015). Nowadays, the IGF-1 pathway is gaining tremendous interest due to its important role in cancer as well as IBD (Brahmkhatri et al., 2015; El Yafi et al., 2005; Lawrance et al., 2001; Sanchez-Lopez et al., 2015; Sipos et al., 2008). Numerous cancer types have been shown to be associated with aberrant IGF-1 signaling, moreover a large amount of work performed *in vitro*, *in vivo*, and in clinical studies have shown that increased IGF-1 and IGF1R activity contributes to the pathogenesis of colorectal cancer. Thus, IGF-1 has been linked to wound-healing, inflammatory cell recruitment, alternative macrophage activation and cell proliferation as well as activation of the phosphoinositide 3-kinase (PI3K) and β-catenin pathways implicated in resistance to therapeutic agents (Clayton et al., 2011; Mourkioti and Rosenthal, 2005; Spadaro et al., 2017; Vigneri et al., 2015)..

IGF-1 expression is also increased in the intestine of animal models of IBD and in patient's with Crohn’s disease (Theiss et al., 2004). In fact, evidence suggests that IGF-1 may increase the risk of intestinal cancer and fibrosis, and high levels of IGF-1 in the circulation may increase the risk of colon cancer (Shim and Cohen, 1999; Wang et al., 2015a). IGF-1 is one of the most potent natural stimulators of growth and proliferation in multiple cell types in the body, and despite its possible benefits, GH in IBD therapy may introduce some risks and complications, such as colon cancer, due to its effector hormone IGF-1. Therefore, further investigation is required on the role of IGF-1 in IBD (Eivindson

et al., 2005; Lawrance et al., 2001; Lund and Zimmermann, 1996; Pucilowska et al., 2000; Simmons et al., 1995; Simmons et al., 2002; Theiss et al., 2004; Xin et al., 2004; Zatorski et al., 2016).

Binding of IGF-1 to IGF1R, a transmembrane tyrosine kinase receptor with structural homology to insulin receptors (IRs), induces its autophosphorylation, which can lead to the activation of insulin receptor substrate 1(IRS-1)/PI3K/AKT and extracellular signal–regulated kinase (ERK pathways) (Foulstone et al., 2005; Furundzija et al., 2010; Kuemmerle and Zhou, 2002). However, the IGF-1 system involves complex regulatory networks (Figure 7). Key molecules include the ligands IGF-1 and IGF-2, the type 1 and type 2 IGF receptors (IGF1R and IGF2R, respectively), IGF-1R and IR hybrids, the IGF-binding proteins (IGFBPs), and the proteins involved in intracellular signaling distal to IGF-1 receptor (Pollak, 2004). Importantly, IGFs and IGFBPs can be also locally produced through autocrine or paracrine mechanisms apart from its endocrine source from the liver. These mechanisms often involve interactions between stromal- and epithelial-cell subpopulations (Denduluri et al., 2015).

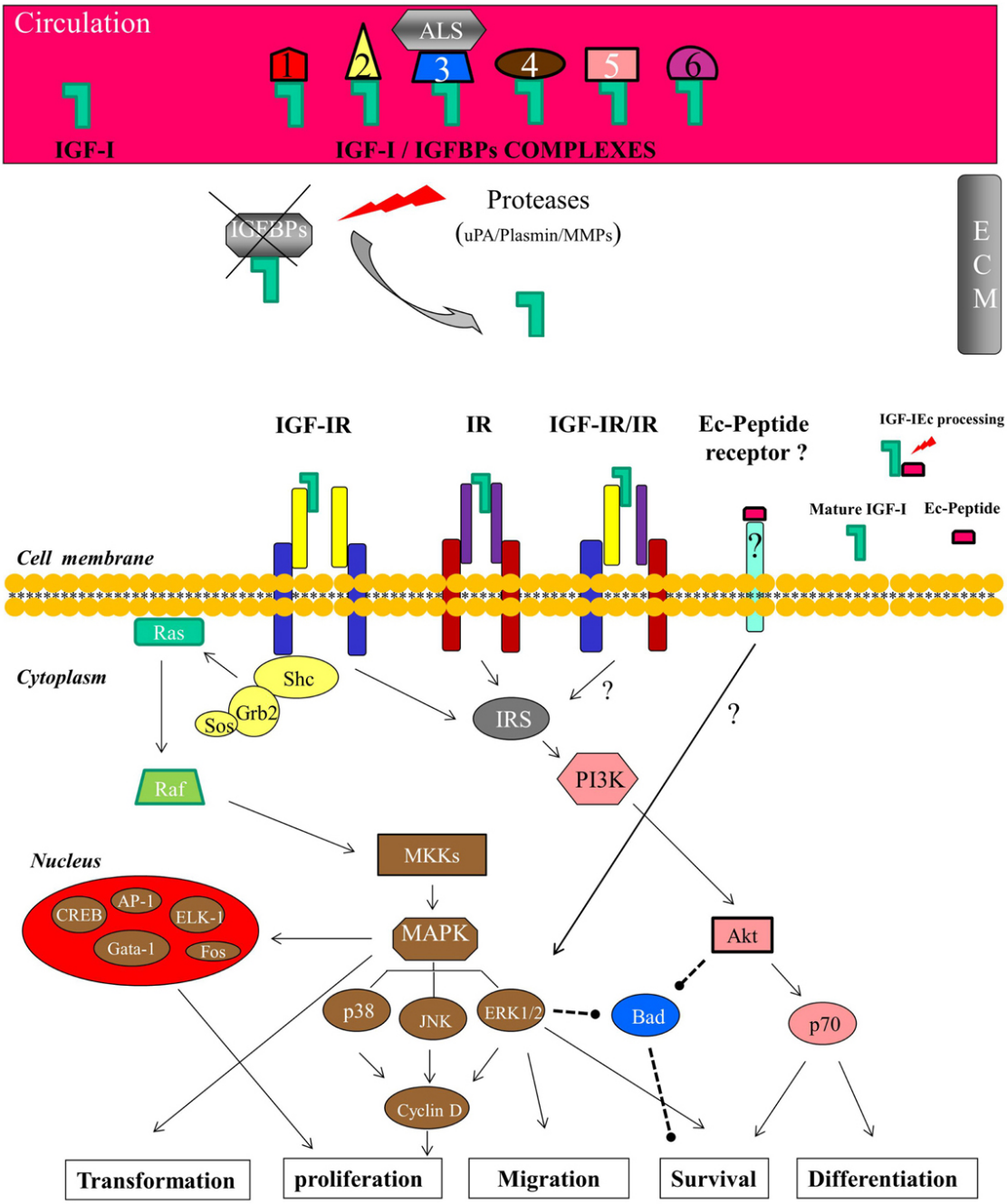


Figure 7. Schematic representation of the IGF-1 bioregulation system, signaling pathways and the cellular processes downstream of IGF-I.

Apart from IGF-I, the system consists of the receptors IGF1R, IGF2R, IR, and IGF-IR, IR hybrids, and at least six high affinity IGFBPs. IGF-I circulates mainly in an IGF/IGFBP-3/ALS complex. Binding of IGFBPs to IGF-I prevents the ligand to interact with the receptor(s) and IGFBPs can modulate, the extent of IGF-1-dependent cellular effects. Proteolysis of IGFBPs by proteases, such as urokinase-type plasminogen activator (uPA), plasmin and MMPs, results in an increase of bioavailability of IGF-1 for interaction with the IGF-1R. Some IGFBPs can exert also an IGF-IR-independent bioactivity. ALS, acid-labile subunit; ECM, extracellular matrix.
The arrows indicate an activating effect and the dashed lines represent an inhibitory effect.
(Modified from Front. Endocrinol. (Philippou et al., 2013))

1.3.2 Colitis-associated cancer (CAC)

Colorectal cancer (CRC) defines the presence of a malignant tumor in the colon or rectum. It is estimated that each year more than one million people will develop CRC worldwide and the mortality of this disease is approximately 35% in the developing world. CRC is the third most common malignancy and fourth most common cause of cancer mortality worldwide (Huxley et al., 2009). The risk of developing CRC throughout a lifetime is about 5%. The statistical lifetime risk is similar in men and women despite higher incidence rates in men because women have longer life expectancy (Siegel et al., 2016). The causes of CRC and its mechanism of pathogenesis are complex and heterogeneous. Diet and lifestyle factors, somatic and hereditary mutations contribute to the development of CRC. Risk factors include diets rich in unsaturated fats, processed and red meat, total energy intake and reduced physical activity as well as excessive alcohol consumption. Most cases of CRC occur sporadically and contributing factors include age, male sex, previous chromosome polymorphisms and family history of CRC (Huxley et al., 2009).

Nevertheless, chronic inflammation is one of the main reasons for development of neoplasia in the colon. Colitis-associated cancer (CAC) is a type of CRC induced by inflammatory disorders in the colon, such as IBD. Although inflammation induced cancer is considered less frequent than those caused by hereditary mutations or sporadic causes, it is a serious complication in IBD (Rogler, 2014; Sussman et al., 2012; Yang and Jobin, 2014). The link between inflammation and carcinogenesis is not a recent one, more than 150 years ago, Rudolf Virchow hypothesized that inflammation could be linked to subsequent carcinogenesis (Balkwill and Mantovani, 2001). Considerable evidence suggests that chronic inflammation plays an important role in the development of CAC. Individuals with longstanding colonic inflammation due to IBD have an increased susceptibility to CAC (Bernstein et al., 2001). The risk of CRC increases with the duration of the disease. Thus, there is an incidence of 2% after 10 years of illness, 9% after 20 years and more than 20% of IBD patients develop CAC within 30 years of disease onset with more than 50% of these patients will dying from this type of cancer (Goel et al., 2011; Lakatos and Lakatos, 2008). Moreover, the risk increases with the duration of illness, severity and extent of inflammation (Itzkowitz and Harpaz, 2004; Lanas, 2009; Rigas and Tsioulias, 2015). Additionally, recent data suggests that chronic

inflammation may partially mediate the link between obesity and CAC (Belkina and Denis, 2012).

Even though immune mediated mechanisms are responsible for CAC, there are similarities between inflammation induced carcinogenesis and other types of CRC. The important stages of colon cancer development such as formation of aberrant crypt foci (ACF), polyps, adenomas and carcinomas are similar between CAC and CRC, even though different pathogenic sequences have been proposed for colitis associated tumorigenesis, such as chronic inflammation and injury-dysplasia carcinoma (Barker et al., 2009; Bernstein et al., 2001; Goldstone et al., 2011). They also exhibit comparable genetic similarities such as chromosomal and microsatellite instabilities. Nevertheless, despite these similarities there are important differences between inflammation and sporadic-induced CRC. The frequency and timing of genetic alterations, as the activation of proto-oncogenes such as Ras and the inactivation of tumor suppressor genes such as adenomatous polyposis coli and p53 that are important for the development of the majority of CRCs, differ remarkably in sporadic CRC and CAC (Eshghifar et al., 2017; Fodde, 2002; Hadziavdic et al., 2008; Hisamuddin and Yang, 2004; Hisamuddin and Yang, 2006).

During colorectal carcinogenesis, colonic epithelial cells accumulate genetic mutations, which are mostly induced by environmental factors that confer a selective growth advantage (Erreni et al., 2011). It has been shown that CRC expands in a bottom-up fashion (Barker et al., 2009; Preston et al., 2003). This model assumes the origin of the cancer cells at the bottom of the crypts, where intestinal stem cells are located, and their expansion towards colon lumen (Preston et al., 2003). At the onset, chronic inflammation causes injuries, which leads to development of dysplasia proceeding to colorectal carcinoma (Radtke and Clevers, 2005; Terzic et al., 2010). Aberrant crypts are the first notable changes that can develop further to tumorigenic tissue. Furthermore, through promotion and progression, adenocarcinoma develops with the possibility of invasion, resulting in metastasis (Goldstone et al., 2011; Radtke and Clevers, 2005; Ullman and Itzkowitz, 2011). It has become clear that besides the occurrence of genetic or epigenetic abnormalities, an inflammatory microenvironment involving growth factors and cytokines secreted by activated monocytes and macrophages also plays a pivotal role in the formation of benign polyps and can lead to the development of invasive colorectal tumors (Erreni et al., 2011). Over the past years the link between inflammation and cancer is starting to have implications for prevention and treatment. However, although

considerable progress has been made in IBD research, a complete understanding of the mechanisms and the pathogenesis of CAC is not yet fully achieved, which makes it particularly challenging (Gulbake et al., 2016).

1.3.2.1 Immunological tumor microenvironment (TME)

Tumors are complex tissues comprising not only the tumor cells, but also the TME, which provide the growing tumor with nutrients and growth factors, and also contribute space for the tumor to expand and to escape the host immune response. The tumor-associated environment is composed of the extracellular matrix (ECM) and normal cells e.g. fibroblasts, vascular and lymphatic endothelial cells, pericytes and smooth muscle cells, as well as cells of innate and adaptive immunity, all of which interact with each other and with the malignant cells (Fridman et al., 2013; Hanahan and Weinberg, 2011; Pietras and Ostman, 2010). Tumor progression depends on the interaction of tumor cells with components of the TME. Moreover, tumor cells are able to remodel the stroma and to establish a permissive microenvironment for their progression (Pietras and Ostman, 2010; Romero-Lopez et al., 2017; Roussos et al., 2011). The capacity of carcinomas to recruit and activate immune cells, including macrophages – referred to as TAMs when recruited to the tumors - largely depends on malignant cells having acquired an undifferentiated phenotype with loss of epithelial markers and expression of mesenchymal markers as part of the so-called epithelial-to-mesenchymal transition (EMT). In turn, tumor cells, particularly those that have undergone an EMT secrete factors that modulate and activate cells in the TME (Su et al., 2014).

A major contributor to the TME is inflammation and inflammatory mediators. The recognition of chronic inflammation as a trait acquired by tumor cells necessary for survival, growth, and metastasis has intensified studies on the role of intratumoral inflammatory cells and pro-inflammatory cytokines in cancer initiation and progression (Colotta et al., 2009; Hanahan and Weinberg, 2011; Mantovani et al., 2008). Similar to most other solid tumors, almost all immune cell types but also non-immune cell types may be found in colon carcinomas, including monocytes, macrophages, neutrophils, myeloid-derived suppressor cells (MDSCs), DCs, mast cells, NK-cells, naive and memory lymphocytes, B cells and various subsets of T cells, cancer-associated fibroblasts (CAFs), endothelial cells, endothelial progenitor cells (EPCs), platelets, and mesenchymal stem cells (MSCs) (Fridman et al., 2012; Lee et al., 2010; Murdoch et al., 2008). Nevertheless, myeloid cells (mainly monocytes and macrophages) are the

predominant leukocytes that infiltrate tumors and are known to support primary tumor growth and progression (Gabrilovich et al., 2012; Murdoch et al., 2008; Parihar et al., 2010; Sanchez-Lopez et al., 2015). Interestingly, targeting the recruitment of immune cells to the site of inflammation has evolved as a strategy to impede tumorigenesis (Grivennikov et al., 2010).

The two main factors contributing to this infiltration are inflammation and chemokines secreted by both tumor cells and stromal cells (Coussens and Werb, 2002; Erreni et al., 2011; Green et al., 2009; Jedinak et al., 2010). Although infiltrates of inflammatory cells can vary in size and composition from tumor to tumor, their presence demonstrates that the host is not ignorant of the developing tumor, but rather attempts to interfere with tumor progression, a process referred to as immune surveillance (Zitvogel et al., 2006). In this sense, the initial role of infiltrating cells is not necessarily tumor promotion, and they may constitute an attempt of the host to eliminate detected tumor cells (de Visser et al., 2006; Zitvogel et al., 2006). However, during tumorigenesis, the dynamic interaction between stromal and tumor cells changes in favor of tumor progression as it influences and exploits stromal cells to promote tumor cell proliferation, survival, and metastasis (de Visser et al., 2006; Murray and Wynn, 2011; Zitvogel et al., 2006).

It has been established that IBD promotes CAC development mainly through activation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Greten et al., 2004; Grivennikov et al., 2009), and possibly yes-associated protein 1 (YAP1) and Notch (Taniguchi et al., 2015). These transcription factors can activate genes that promote the survival of epithelial cells and expose them to growth-promoting inflammatory cytokines (Terzic et al., 2010). More recently, it became clear that even without pre-existing IBD, inflammation occupies a key position in the development of sporadic CRC. As soon as the adenomatous polyposis coli locus is lost in mice and ACF lesions - pre-neoplastic lesions on the mucosa - appear, there is an accompanying loss of mucin-2 production and junctional adhesion molecules, resulting in barrier defects and invasion of ACF lesions and early adenomas with commensal enteric bacteria or their products (Grivennikov et al., 2012). Similarly, humans with germline *adenomatous polyposis coli* mutation develop hundreds of colon tumors in their first few decades of life, a condition referred to as familial adenomatous polyposis (FAP) (Zeineldin and Neufeld, 2013).

Besides different stimuli like growth factors and immunosuppressive cytokines supplied by the TME, one of the factors that determine the nature of inflammatory infiltrates found in the TME is the hypoxic environment. It is created early in the tumor development through activation of hypoxia-responsive genes in tumor cells and favors the influx of inflammatory cells, in particular macrophages and granulocytes (Aller et al., 2004; Denko et al., 2003). These cells not only survive in the hypoxic environment but contribute to it by hyper-production of ROS upon local activation (Lluis et al., 2007). Nevertheless, factors released by the TME can either promote or inhibit tumor cell survival and metastatic dissemination, as well as access and responsiveness to therapy. Therefore, understanding the cellular and molecular interactions operative in the tumor microenvironment is of crucial importance, and changing the chronic to acute inflammation at the tumor site might be therapeutically beneficial (Whiteside, 2008).

1.3.2.2 Tumor-associated macrophages (TAMs)

Macrophages comprise a large amount of the immune cell infiltrate in solid tumors (Lewis and Pollard, 2006). They are derived from blood monocytes that are recruited to the tumor by growth factors, chemokines, and angiogenic factors such as CSF-1, CCL2, CCL3, CCL4, CCL5, VEGF, and angiopoietin-2 and are thought to be involved in every aspect of tumor development and progression (Lewis and Pollard, 2006; Sica et al., 2008; Sica and Bronte, 2007; Tariq et al., 2017).

Given the heterogeneity of macrophages, it is possible to find different TAM populations within the same tumor with a combination of both pro-inflammatory and anti-inflammatory gene expression. Especially at early stages of tumor development macrophages involved in the cancer-initiating inflammatory process may begin acting as pro-inflammatory cells and undergo classical activation (also referred to as TAM1) (Franklin et al., 2014; Noy and Pollard, 2014). However, once tumors are established, macrophages are educated to become anti-inflammatory (also referred to as TAM2) (Colegio et al., 2014; Franklin et al., 2014; Noy and Pollard, 2014). Nevertheless, the mechanisms of this switch are not fully understood, although it has been suggested that environmental signals such as secreted tumor factors, accumulation of lactic acid or hypoxia may mediate this transition (Wen et al., 2015; Wenes et al., 2016; Zhang et al., 2014a). Micro-environmental signals contributing to the switch and differentiation into mature macrophages include M-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), prostaglandin E2 (PGE2), TGF-β, IL-6, IL-4 and IL-10. These factors have

the potential to modulate and polarize monocytes mainly into TAMs (Chanmee et al., 2014; Goerdt and Orfanos, 1999; Mantovani et al., 2002). Several studies identified IL-4 as a major regulator of the phenotypes of TAMs (Wang and Joyce, 2010). IL-4 is produced by Th2 lymphocytes, eosinophils, basophils or macrophages, as well as tumor cells and is an important cytokine in determining the balance of pro-inflammatory and anti-inflammatory cytokines, which in turn impinges on the type of immune reaction generated (Coussens et al., 2013; Huang and Chen, 2016; Martinez and Gordon, 2014).

It is clear that TAMs promote tumor angiogenesis and tissue remodeling with a potent ability to facilitate tumor initiation, progression, suppression of cytotoxic T lymphocyte (CTL) responses and metastasis through the secretion of several cytokines and growth factors (Grivennikov et al., 2010; Mantovani et al., 2002; Ruffell et al., 2014). It remains to be determined which TAM type is more important in tumor promotion, but it should be noted that TAM1 cells secrete classical pro-inflammatory cytokines, chemokines, and effector molecules, including IL-1, IL-6, TNF, IL-23, and iNOS, which are known to contribute to tumor initiation and early promotion (Grivennikov et al., 2010). On the other hand, TAM2 cells produce growth factors and anti-inflammatory molecules, such as IL-10, TGF-β, and arginase 1 (ARG1) (Gabrilovich and Nagaraj, 2009; Ruffell et al., 2014) (57, 60). Moreover, they promote angiogenesis and cancer cell migration and intravasation (Lin and Pollard, 2007). A phenotypic characterization that encompasses all TAMs has not emerged yet, but they seem to exhibit several characteristics of regulatory macrophages and also wound-healing macrophages. It is therefore hypothesized that the original classically activated macrophages that might have participated in tumor formation can progressively differentiate to a regulatory phenotype and eventually become cells that share the characteristics of both regulatory and wound-healing macrophages (Mosser and Edwards, 2008).

1.3.2.3 Wound-healing in CAC

Severe mucosal tissue damage requiring efficient wound-healing is a main feature of IBD (Rieder et al., 2007). As described above (see section 1.3.1.3), proper wound-healing and tissue repair response requires precise coordination of overlapping but distinct phases to achieve resolution of inflammation and restore tissue integrity and homeostasis (Serhan and Savill, 2005). However, in severe injury or chronic inflammation, multiple aspects of wound-healing may be deregulated and can lead to the development of cancer (Balkwill and Mantovani, 2001; Chanmee et al., 2014; Sussman et al., 2012). In this sense, although

wound-healing programs and intestinal barrier integrity generally protect against injury and colitis, dysregulated wound-healing and epithelial regeneration is considered one of the requirements of CAC development, resulting in uncontrolled proliferation (Francescone et al., 2015; Low et al., 2014). Therefore, because of the overlap between mechanisms involved in wound-healing and tumorigenesis, tumors have been described as “wounds that do not heal” (Dvorak, 1986), and it is well accepted that chronic inflammation can result in excessive tissue regeneration and thereby enhance the progression of initiated tumor cells (Kuraishy et al., 2011). In fact, in mice, using the azoxymethane (AOM)/dextran sodium sulfate (DSS) experimental model of CAC, wound-healing pathways has been demonstrated to be required for the development and perpetuation of coltis-associated tumorigenesis (Sussman et al., 2012). This is in accordance with the idea that inflammation caused by tissue injury orchestrates wound-healing and tissue regeneration, but when it cannot be properly resolved or it is + propagated chronically by repetitive injury, may result in tumor promotion by uncontrolled wound-healing processes (Kuraishy et al., 2011).

Injury and ulceration induce wound-healing-regeneration responses, which includes migration of stem cells and their enhanced proliferation and expansion to fill in for damaged mucosa (Kuraishy et al., 2011). However, migration and proliferation, apart from being important for wound-healing processes, are also pro-tumorigenic properties of cancer cells (Schafer and Werner, 2008). If these cells harbor oncogenic mutations, which might be acquired from the pro-inflammatory environment (see section 1.3.1.1), local repetitive injury and regeneration will instigate their proliferation and tumor formation (Kuraishy et al., 2011). Additionally, immune cells secrete various cytokines and chemokines into the TME, some of which, e.g. EGF, IGF-1, IGF-2 or VEGF, actively induce proliferation. This is especially true for cells of the innate immune system, which is important for wound-healing and tissue remodeling (Beck and Podolsky, 1999; Dvorak, 1986; Lund and Zimmermann, 1996; Rieder et al., 2007; Theiss et al., 2004).

In the colon, intestinal stem cells reside at the origin of the migration, which is found just above the crypt base. Hence, the differentiated, functional cells are found toward the top of the colonic crypt. These cells become senescent in the latest stages of the differentiation process and are shed into the lumen. There is a finely tuned steady supply of cells generated in the low-to-mid-crypt region (Kaur and Potten, 1986; Loeffler et al., 1986). Similarly to homeostatic proliferation in the physiological steady-state, crypt regeneration after damage induction also appears to be initiated in the crypts. In fact, it

has been reported that epithelial cell proliferation is increased in association with the inflammatory process in several experimental models (Ioachim et al., 2004; Weiss et al., 2014; Weiss et al., 2004). However, it is unclear whether this proliferative activity is due to the direct stimulation of proliferation by cytokines, growth factors, and other mediators or whether it is attributed to the response of epithelial cell loss and subsequent altered cell-cell contacts. In consequence, enhanced proliferation may be a compensatory response to replace the loss of cells due to necrosis or apoptosis. Although this process is thought to contribute to tissue regeneration and recovery of homeostasis, the increase of cellular turnover in these stem cells enhances the risk of mutations leading to a carcinogenic phenotype (Negroni et al., 2015; Tanaka, 2009; Vogelstein et al., 1988)

1.4 The p38 MAPK signaling pathway

MAPKs are serine/threonine protein kinases that convert a variety of extracellular stimuli into a wide range of cellular responses (Kyriakis and Avruch, 2012). MAPK pathways are ubiquitously expressed and activated by a variety of extracellular stressors including pro-inflammatory cytokines, growth factors, hormones, osmotic shock, UV irradiation, bacterial lipopolysaccharide (LPS) and oxidative stress (Cuenda and Rousseau, 2007; Hazzalin et al., 1996). MAPKs are evolutionarily conserved among eukaryotes and control fundamental cellular processes such as gene regulation, growth, differentiation, migration, apoptosis, and proliferation (Cuadrado and Nebreda, 2010; Keyse, 2000). In mammals five MAPK groups have been characterized, the extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), ERK 3/4, ERK5 and p38 (Roux and Blenis, 2004). There are four isoforms of p38 MAPK, encoded by different genes, namely p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12) and p38δ (MAPK13), each with differential tissue expression and overlapping but also distinct physiological roles (Cuadrado and Nebreda, 2010; Enslen et al., 2000; Keyse, 2000).

BMDMs and peritoneal macrophages express all p38 MAPK isoforms, but p38α is the most abundantly expressed (Hale et al., 1999; Kitatani et al., 2009; Korb et al., 2006; Risco et al., 2012). Coordinated activation of the four p38 MAPKs has been suggested in macrophages and it is considered that all may play a role in inflammation (Fearns et al., 2000). Moreover, analysis of monocytes, macrophages, neutrophils and T cells has shown that expression of p38 family members varies with the differentiation state of the cells (Hale et al., 1999). However, the expression of p38 MAPK isoforms in isolated intestinal macrophages has not been explored yet.

Several signaling events are involved in macrophage activation, phenotype plasticity, regulation of cell proliferation and survival. One of the pathways implicated in these processes includes the protein kinase p38α (Cuenda and Rousseau, 2007), which regulates the production of key inflammatory mediators, including TNFα, IL-1β, and cyclooxygenase-2 (COX-2) (Balkwill and Mantovani, 2001; Kim and Choi, 2015). The p38 MAPK pathway may regulate cytokines by a general effect on transcription, the activation of specific transcription factors, but also by post-transcriptional regulation (mRNA processing, nuclear export, mRNA stability and translation) (Cuadrado and Nebreda, 2010; Cuenda and Rousseau, 2007; Kaminska, 2005; Rani and Ransohoff, 2005; Ronkina et al., 2010). Genetic inactivation of p38α in myeloid cells has provided evidence for the importance of this signaling pathway in cytokine production and inflammatory responses *in vivo* in models of colitis and arthritis (Guma et al., 2012; Otsuka et al., 2010) and *in vitro* in macrophages derived from mice (Bachstetter et al., 2014; Kang et al., 2008).

The MAPK signaling pathway functions as a cascade that is composed of a canonical three tier hierarchy of kinases (Cuenda and Rousseau, 2007). At the very top, the MAPK kinase kinase (MAP3K) is activated by cellular signaling components in response to a stimulus. Once active, the MAP3K phosphorylates an associated MAPK kinase (MAP2K), inducing conformational changes that mediate binding to its target MAPK, the terminal component of the cascade (Roux and Blenis, 2004). Once the MAPK is phosphorylated, and therefore activated, it can phosphorylate a plethora of cellular factors to control gene expression and various other cellular processes (Figure 8) (Kyriakis and Avruch, 2012; Roux and Blenis, 2004; Trempolec et al., 2013).

Multiple MAPK pathways belonging to distinct sub-families are present in nearly all eukaryotic organisms and exist in parallel (Johnson and Lapadat, 2002; Kyriakis and Avruch, 2012). While one might think of each MAPK pathway as being separated and linear, evidence reflects that a stimulus can activate different MAPKs as well as different isoforms within each MAPK pathway. Therefore, it is likely that the final cellular outcome depends on the contribution of different MAPKs. Some components of signaling pathways are shared between families and this seems to increase in components that lie upstream in the cascade (Johnson and Lapadat, 2002).

The canonical activation of p38 MAPKs is achieved through the dual-specificity MAP2Ks MKK3, MKK4 and MKK6, which phosphorylates p38 in the activation loop

sequence Threonine-Glycine-Tyrosine (Cuenda and Rousseau, 2007; Kyriakis and Avruch, 2012). The MKK responsible for the activation of a specific cell type is also dependent on the stimuli and the cell type (Remy et al., 2010). MKK3 and MKK6 are the dominant MKKs in the activation of the p38 MAPK pathway (Alonso et al., 2000; Cuadrado and Nebreda, 2010; Zarubin and Han, 2005). Both are highly selective for p38 MAPKs but do not activate JNKs and ERK1/2 (Brancho et al., 2003; Cuenda and Rousseau, 2007). However, MKK4, an upstream kinase of JNK, has also been shown to be capable of activating p38α and p38δ in specific cell types (Zarubin and Han, 2005).

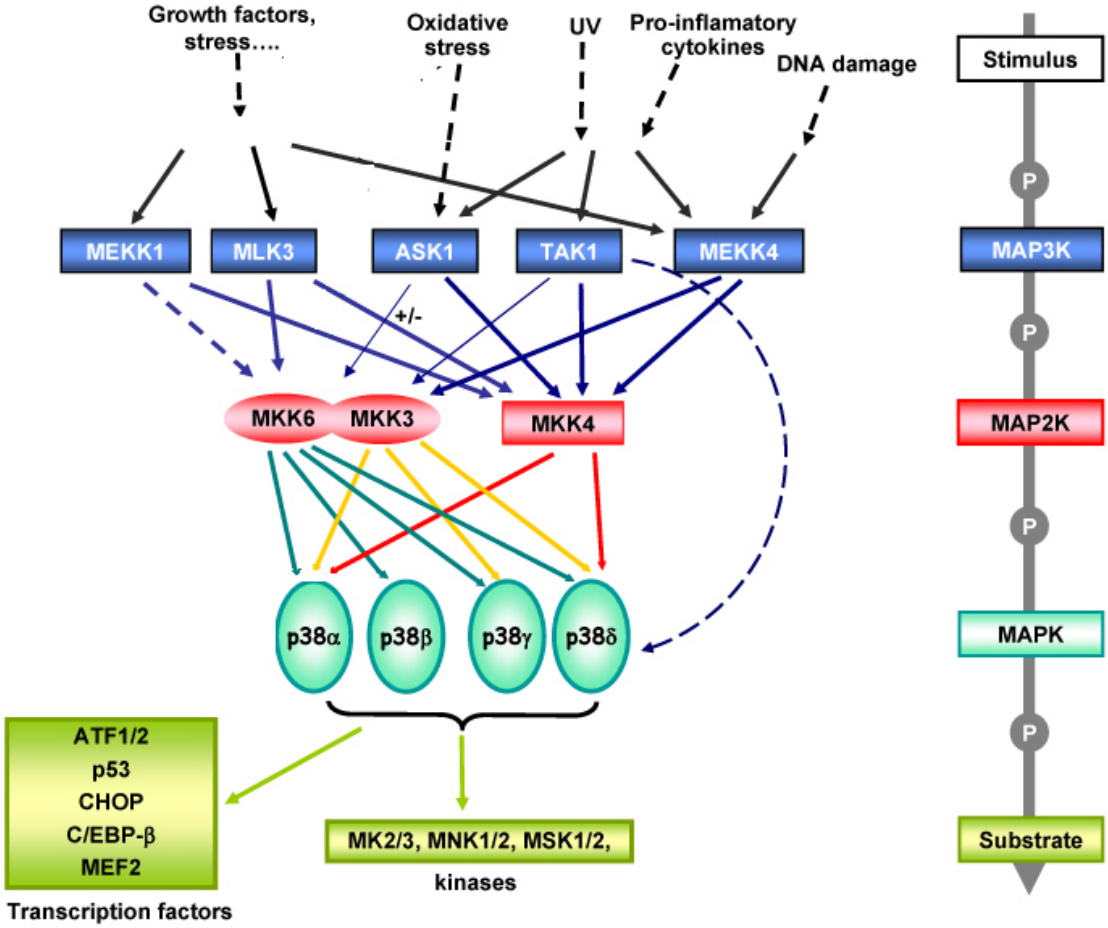


Figure 8. p38 MAPK signaling pathway.

A variety of stimuli, activate p38 MAPK through complex kinase cascades that phosphorylate p38 MAPKs. Once activated, the different p38 MAPKs either phosphorylate cytoplasmic targets or translocate into the nucleus leading to the regulation of transcription factors involved in cellular responses. MKK6 is the major activator of all p38 MAPK isoforms, while MKK3 and MKK4 are more specific and can only activate some isoforms (modified from Atlas of Genetics and Cytogenetics in Oncology and Haematology; 2011, Vol. 15, N° 3 ; p. 316-326)

p38α is ubiquitously expressed at significant levels in most cell types, and has therefore been considered the dominant form within this family, whereas the others seem to be expressed in a more tissue-specific manner, e.g. p38β in brain p38γ in skeletal muscle and p38δ in endocrine glands (Cuadrado and Nebreda, 2010). p38α activation can lead to cellular outcomes in a cell- and context- specific manner. In consistence, the relative contribution of upstream kinases also depends on the stimuli and the cell type. Nevertheless, little is known about how these interconnected components relays and integrate specific information (Brancho et al., 2003; Otto et al., 2012). In contrast to p38α, p38β appears to be required neither for acute nor chronic inflammatory responses (Igea and Nebreda, 2015). The mechanism that accounts for the specificity of MAP2Ks to activate MAPK isoforms is mediated partially by an interaction between an N-terminal region located on the MAP2K and different docking sites present on the MAPK, as well as by the structure of the MAPK activation loop (Bardwell and Thorner, 1996; Biondi and Nebreda, 2003; Enslen et al., 2000). MAPK pathways also use molecular scaffolds to achieve signaling specificity. These can physically co-localize proteins in a cascade, while excluding components of other parallel MAPK pathways, in order to mediate a specific signal. However, although a number of scaffold proteins have been implicated in the regulation of different MAPK signaling modules, not many have been found to participate in the p38 MAPK cascade (Kolch, 2005; Morrison and Davis, 2003).

Feedback inhibition of MAPK signaling is achieved in most cases through MAPK phosphatases (MKPs) that reverse the phosphorylation of its own components or components of a neighboring pathway and return the MAPK to its inactive state. This mechanisms further contributes to signaling fidelity (O'Rourke and Herskowitz, 1998; Owens and Keyse, 2007). Given the strong evidence implicating p38 MAPK in a variety of pathological conditions, it is of interest to interfere therapeutically with the activity of p38 MAPKs. Therefore, several chemical inhibitors have been developed. However, despite the high sequence homology of p38 MAPK isoforms, they differ in their sensitivity to these compounds (Coulthard et al., 2009; Cuenda and Rousseau, 2007). p38 MAPK chemical inhibitors include SB-203580, VX-745, VX-702, RO-4402257, SCIO-469, BIRB-796, SD-0006, PH-797804, AMG-548, LY-2228820, SB-681323 and GW-856553, amongst others (Buhler and Laufer, 2014; Genovese, 2009). Several of the compounds have proven efficacy in preclinical models and have good pharmacological properties and therefore have reached clinical trials, as for instance PH-797804, BIRB-

796, VX-702, SB-203580 and LY-2228820 (Buhler and Laufer, 2014; Cohen, 2009; Cohen et al., 2009; Goldstein et al., 2010; Tate et al., 2013).

The p38 MAPK pathway has been implicated in different pathological conditions. The effect of p38 MAPK inhibition has been studied in a number of chronic inflammatory diseases and cancer (Cai et al., 2017; Del Reino et al., 2014; Lin et al., 2016; Rajashekhar et al., 2011; Sakkinen et al., 2016; Yang et al., 2014; Yong et al., 2009; Yu et al., 2016). Table 3 shows a list of p38 MAPK inhibitors that have been tested in disease models.

Inhibitor	Indication	Model	Outcome	Reference
SB-203580	Asthma	*In vivo*: in BAL fluid of rat	Inhibition of TNF-α and IL-1β production	(Escott et al., 2000; Haddad et al., 2001)
	RA	*In vivo*: Murine model of collagen-induced arthritis	Prevention of RA progression	(Badger et al., 1996)
	IBD	*In vivo*: DSS-induced ulcerative colitis mice model	Reduction of mRNA levels of pro-inflammatory cytokines RICK inhibition	(Hollenbach et al., 2004)
	Cancer-related diseases	*In vitro*: PC12 cancer cells	Block of neuronal outgrowth	(Morooka and Nishida, 1998)
		In vitro: Colon cancer cells	Growth inhibition and apoptosis induction	(Lim et al., 2006)
		In vitro: Human breast epithelial cells	Inhibition of H-Ras-induced invasion/migration	(Kim et al., 2003; Song et al., 2006)
		In vitro: Human breast epithelial cells	Inhibition of TGF-β-induced invasion/migration	(Kim et al., 2004; Kim et al., 2005; Kim et al., 2007)
		In vitro: Human glioma cells	Inhibition of GDNF-induced migration	(Song and Moon, 2006)
		In vivo: MMTV-neu model	Inhibition of tumor suppressor pathways	(Bulavin et al., 2004)
SB-239063	Asthma	*In vivo*: in BAL fluid of rat	Reduction of neutrophil infiltration, IL-8, IL-6 and MMP-9	(Underwood et al., 2000)
	Brain inflammation and stroke	*In vivo*: in rat	Reduction of infarct volume	(Barone et al., 2001a; Barone et al., 2001b; Legos et al., 2001)
SB-220025	RA	*In vivo*: Murine model of collagen-induced arthritis	Prevention of RA progression	(Jackson et al., 1998)
SB-202190	Cancer	*In vitro*: Human gastric cancer cells	Reduction of AP-1 activity and increase of the sensitivity to chemotherapy	(Guo et al., 2008)
	Atherosclerosis	*In vivo*: in rabbit	Prevented macrophage (J774A.1) content of rabbit atherosclerotic plaques through apoptosis	(Croons et al., 2009)

	CRC	*In vivo:* Xenografts of human colon cancer cell lines	Induction of apoptosis and inhibition of proliferation	(Chiacchiera et al., 2009)
		In vivo: APC^{min} model	Induction of apoptosis and inhibition of proliferation	(Chiacchiera et al., 2009)
SB-242235	RA	*In vivo*: Murine model of adjuvant-induced arthritis	Inhibition of TNF-α	(Badger et al., 2000)
RW-J67657	Systemic inflammation	*In vivo*: LPS-injected mice and rats	Reduction of TNF-α release	(Wadsworth et al., 1999)
SD-282	Brain inflammation and stroke	*In vivo*: APP571 mice (have higher activity of p38 MAPK)	Protection of brain against ischemic injury	(Maroney et al., 2001)
BIRB-796	Cancer	*In vitro*: Paracrine tumor cell	Inhibition of IL-6 secretion and proliferation	(Yasui et al., 2007)
R-130823	RA	*In vivo:* Murine model of adjuvant-induced arthritis	Reduction of hind paw swelling	(Wada et al., 2005)
Talmapimod (SCIO-469)	MM	*In vivo*: Mouse plasmacytoma model of MM	Inhibition of MM tumor weight	(Navas et al., 2006)
LY-2228820	Breast Cancer	*In vivo:* Xenografts of human breast cancer cell lines	Reduction of tumor growth	(Campbell et al., 2014)
PH-797804	Breast Cancer	*In vivo:* PyMT model in mice	Sensitization of tumor cells to cisplatin induced apoptosis	(Pereira et al., 2013)
	CAC	*In vivo:* AOM/DSS induced colon tumor	Induction of apoptosis and inhibition of proliferation	(Gupta et al., 2014)

Table 3. p38 MAPK inhibitors used in *in vitro* and *in vivo* studies.

BAL: Bronchoalveolar lavage; GDNF: Glial cell-derived neurotrophic factor; MM: Multiple myeloma; PyMT: polyoma middle T; RA: Rheumatoid arthritis; RICK: Rip-like interacting caspase-like apoptosis-regulatory protein kinase

1.4.1.1 p38α MAPK in IBD

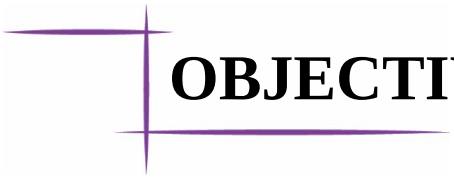
Given the role of p38 MAPK signaling in the regulation of inflammatory pathways, the implication of this pathway in IBD has been extensively investigated. Solid evidence has been found showing that p38 MAPK signaling plays an important role in bowel inflammation and may be responsible for the immune-pathogenesis of the overactive immune system. Moreover, p38 MAPK activity was found increased in patients suffering from IBD (Hommes et al., 2002; ten Hove et al., 2002; van Montfrans et al., 2002). After the activation of p38 MAPK, pro-inflammatory cytokines, such as IL-1β and TNF-α, are increasingly produced and secreted. Inversely, IL-1β and TNF-α can activate p38 MAPK as well, establishing a positive feed-back loop (Li et al., 2006). Moreover, p38α in lymphatic endothelial cells has been involved in the TNFα-dependend up-regulation of mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which regulates lymphocyte homing to Peyer's patches and lymph nodes in the gut and plays

an important role in chronic intestinal inflammation (Oshima et al., 2001). Consistent with this result, in an animal model for mucosal infection based on *Citrobacter rodentium* infection, p38α has been shown to be critical for chemokine expression, subsequent immune cell recruitment into the intestinal mucosa, and clearance of the pathogen (Kang et al., 2010).

On the other hand, p38α has been found to have distinct functions in mouse myeloid cells versus the colonic epithelium in the context of experimental models, such as the DSS-induced acute colitis model (Gupta et al., 2014; Otsuka et al., 2010; Wakeman et al., 2012). When p38α is downregulated in IECs, intestinal barrier function is lost, leading to more epithelial damage and more inflammation. However, p38α downregulation in myeloid cells was found to have a protective function, probably due to well-known involvement in the regulation of cytokines (Cuadrado and Nebreda, 2010; Gupta et al., 2014; Gupta et al., 2015; Otsuka et al., 2010). Of note, the different functions of this kinase in IECs or in myeloid cells in the context of inflammation-associated colon tumorigenesis highlights the importance of selectively targeting specific cell types and should be taken into consideration for more successful therapeutic interventions in this pathway. Perhaps this is why the p38α inhibitors have not yielded clear results in clinical trials so far (Gupta et al., 2015; Patterson et al., 2014).

1.4.1.2 p38α MAPK in CAC

Using several animal models, p38α signaling has been reported to be involved in tumorigenesis and the pathogenesis of a broad range of human cancers, such as breast, colon, lung and liver (Wagner and Nebreda, 2009; Xu et al., 2014; Yong et al., 2009) (Cuadrado and Nebreda, 2010; Igea and Nebreda, 2015). In a mouse model of AOM/DSS induced colitis-associated tumorigenesis, it has been shown that p38α in IECs has a dual function in colon cancer development. On the one hand, p38α in IECs is involved in the maintenance of mucosal homeostasis by regulating intestinal epithelial barrier function and therefore protecting mice from DSS-induced intestinal inflammation and AOM/DSS-induced tumorigenesis. However, p38α inhibition in transformed colon epithelial cells reduces the tumor burden through inhibition of proliferation and induction of apoptosis. Thus, p38α suppresses DSS-induced epithelial damage and inflammation-associated tumorigenesis, but contributes to the proliferation and survival of tumor cells.(Gupta et al., 2014).



OBJECTIVES

2. OBJECTIVES

p38α MAPK plays crucial roles in inflammation and tissue homeostasis. For example, p38α controls the production of leukocyte chemoattractants and other pro-inflammatory mediators, and is therefore a potential target for therapeutic intervention in inflammatory diseases (Corvinus et al., 2005; Cuadrado and Nebreda, 2010; Grivennikov et al., 2010). There is also evidence implicating this kinase in macrophage activation as well as in the regulation of phenotype plasticity, cell proliferation and survival (Cuenda and Rousseau, 2007). Genetic inactivation of p38α in myeloid cells has provided evidence for the importance of this signaling pathway in cytokine production and inflammatory responses in models of colitis, skin inflammation and sepsis (Kim et al., 2008; Otsuka et al., 2010; Wagner and Nebreda, 2009). Although p38α is thought to contribute to the pathogenesis of IBD, clinical trials and animal studies have not yielded promising results so far (Gupta et al., 2015; Patterson et al., 2014). There is evidence that p38α in IECs suppresses inflammation-associated tumorigenesis, but contributes to the survival of colon tumor cells (Gupta et al., 2014), However, whether myeloid p38α signaling can affect intestinal homeostasis, repair mechanisms and tumorigenesis remains unclear. Thus, a better understanding of how macrophage functions are controlled during CAC would provide important insights into their impact on systemic and local inflammation.
Considering the above information, we propose four main objectives:

1. To characterize the role of myeloid p38α signaling in the regulation of intestinal mucosal homeostasis and inflammation using the DSS-induced colitis mouse model.

2. To evaluate the impact of myeloid p38α downregulation on CAC using the AOM/DSS-induced model of colitis-associated tumorigenesis.

3. To characterize novel downstream targets of p38α signaling and how these impinge on the proliferation, migration or survival of IECs.

4. To decipher the molecular and cellular mechanisms regulated by myeloid p38α signaling in IBD and CAC using *in vitro* and in *vivo* models.

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 Commercial reagents and kits

3.1.1 Reagents

Reagent	Reagent Abbreviation	Commercial House	Reference
30% Acrylamide/Bis (37.5:19)		Biorad	1610158
4',6-diamidino-2-phenylindole	DAPI	Invitrogen	MP-36930
Actinomycin D	ActD	Sigma-Aldrich	A1410
Agarose D1 medium EEO		Conda Pronadisa	8021
Ammonium chloride		Sigma-Aldrich	A9434
Ammonium persulfate	APS	Sigma-Aldrich	A3678
Azoxymethane	AOM	Sigma-Aldrich	A2853
Bovine serum albumin	BSA	Sigma-Aldrich	A7906
Bradford Reagent		Sigma-Aldrich	B6916
Bromophenol blue		Sigma-Aldrich	B8026
β-mercaptoethanol		Sigma-Aldrich	M7154
Calcium chloride	$CaCl_2$	Sigma-Aldrich	449709
CD16/32 Blocking Antibody	Cluster of Differentiation 16/32 Blocking Antibody	eBioscience	16-0161-85
Chloroform		Merck	1024451000
Collagenase D		Roche	11088866001
Collagenase V		Sigma-Aldrich	C9263
Deoxynucleotide triphosphate mix (10 mM)	dNTPs mix	Fermentas	R0192
Dextran sodium sulfate (molecular weight 36-50 kDa)	DSS	MP Biomedicals	160110
Dimethylsulfoxide	DMSO	Sigma-Aldrich	D8418
Dispase		Gibco	17105-041

Reagent	Reagent Abbreviation	Commercial House	Reference
DNA Gel Loading Dye (6x)		Thermo Scientific	R0611
DNA ladder		Niborlab	LR100-5
DNase	Deoxyribonuclease	Roche	10104159001
DPX Mounting medium		Leica	3808600E
Dulbecco's modified eagle medium	DMEM	Sigma-Aldrich	D5796
Ethylene glycol tetraacetic acid	EGTA	Sigma-Aldrich	E3889
Ethylenediamine tetraacetic acid	EDTA	Sigma-Aldrich	E5134
Fetal bovine serum	FBS	Thermo Scientific	E6541L
Fluorescein isothiocyanate-dextran	FITC-dextran	Sigma-Aldrich	FD4
Formalin (10%, buffered)		Sigma-Aldrich	HT501128
Glycerol		Sigma-Aldrich	49782
Glycine		Sigma-Aldrich	G7126
Goat Serum		Dako	X090710
Hank's balanced salt solution	HBSS	Gibco	14175-137
Heparin		Sigma-Aldrich	H4784
Hepes		Sigma-Aldrich	90909C
Hoechst		Sigma-Aldrich	861405
Interleukin-4	IL-4	BD pharmingen	550067
Isopropanol		Sigma-Aldrich	I9516
L-glutamine		LabClinics	x0550
Lipopolysaccharide	LPS	Sigma-Aldrich	L4005
Liquid DAB	3,3′-Diaminobenzidine	Dako	K346811

Reagent	Reagent Abbreviation	Commercial House	Reference
Mayer's Hematoxylin Solution		PanReac AppliChem	CA254766.1611
Methanol		PanReac AppliChem	131091-1214
Mitomycin C	MMC	Sigma-Aldrich	M0503
Nitrocellulose membrane 0.2 μm		WhatmanTM	10401396
Nonyl phenoxypolyethoxylethanol	NP-40	AppliChem	A1694,0250
Pellet Paint Co-Precipitant		Millipore	69049
Penicillin/Streptomycin (100x)	P/S	LabClinics	P11-010
Peroxidase-Blocking Solution		Dako	STB14 - S202386
Phenol: chloroform		Sigma-Aldrich	P2069
Phenylmethylsulfonyl fluoride	PMSF	Sigma-Aldrich	P7626
Phosphate buffered saline 10x	PBS 10x	Sigma-Aldrich	D1408
Polyethylene glycol 400	PEG 400	Sigma-Aldrich	81170
Ponceau red		Sigma-Aldrich	P3504
Potassium bicarbonate		Sigma-Aldrich	P9144
Primers for PCR and qRT-PCR		Sigma-Aldrich	-
Prolong Gold Antifade Mountant with DAPI	4’,6-diamidino-2-phenylindole	Invitrogen	P36935
Propidium iodide	PI	Sigma-Aldrich	P4864
Propylene glycol		Sigma-Aldrich	W294004
Protease inhibitor cocktail		Sigma-Aldrich	P8340
Proteinase K solution		Dako	S3020

Reagent	Reagent Abbreviation	Commercial House	Reference
Random primers		Invitrogen	48190-011
Recombinant mouse insulin-like growth factor-1	Rm IGF-1	Abcam	ab9861
RNAsin		Promega	N2111
RNAsin 2500U		Promega	N2111
Roswell Park Memorial Institute 1640	RPMI 1640	Sigma-Aldrich	R8758
Sodium azide		Sigma-Aldrich	438456
Sodium chloride	NaCl	Sigma-Aldrich	433209
Sodium citrate		Sigma-Aldrich	71497
Sodium dodecyl sulfate	SDS	Sigma-Aldrich	71725
Sodium fluoride	SF	Sigma-Aldrich	S7920
Sodium orthovanadate		Sigma-Aldrich	S6508
Sodium Pyruvate		Sigma-Aldrich	P2256
Superscript IV reverse transcriptase		Invitrogen	18090050
SYBR Green		Life technologies	4472954
TEMED	N,N,N´,N´-tetramethylethylenediamine	Sigma-Aldrich	T9281
Triton X-100		Sigma-Aldrich	T9284
TRIZMA-base		Sigma-Aldrich	T6066
TRIZMA-HCl		Sigma-Aldrich	T3253
Trizol		Ambion	15596018
Trypsin-EDTA		Sigma-Aldrich	T3924
Trypsin-EDTA		Sigma-Aldrich	T3924
Tween-20		Sigma-Aldrich	P7949
Tween-80		Sigma-Aldrich	P4780

Table 4. Commercial Reagents.

3.1.2 Inhibitors

Inhibitor	Commercial House	Reference	Target
LY-2228820	Axon Medchem	1895	p38α and p38β
MK2 inhibitor III	Calbiochem	475864	MK2
PF-3644022	Sigma	PZ0188	MK2
PH-797804	Sellech Chemicals	S2726	p38α and p38β
PQ401	MedChem Express	HY-13686	IGF1R
SB-203580	Axon MedChem	1363	p38α and p38β
SB-747651A	Axon Medchem	Axon1897	MSK1

Table 5. Commercial Inhibitors.

3.1.3 Kits

Kit	Commercial House	Reference
Annexin V kit	BD Transduction	556547
BIOTAQ™ PCR Kit	Bioline	BIO-21071
Deoxyribonuclease I	Roche	04716728001
DNase I treatment	Roche	04716728001
FITC BrdU Flow Kit	BD Transduction	559619
IL-1β/IL-1F2 quantikine ELISA kit	R&D Systems	MLB00C
LIVE/DEAD Fixable Yellow Dead Cell Stain Kit	Life Technologies	L-34959
Mouse chemokine array	R&D systems	ARY020
Mouse Cytokine Antibody Array C1000	Raybiotech; RayBio C-Series	AAM-CYT-1000-2
Mouse/Rat IGF-I Quantikine ELISA Kit	R&D Systems	MG100
On-column Purelink Dnase kit	Invitrogen	12185-010
Pure Link RNA mini Kit	Ambion	12183-018A
RNeasy mini kit	Quiagen	74104
Senescence β Galactosidase staining kit	Cell Signaling	9860

Kit	Commercial House	Reference
Super Script II Reverse Transcriptase	Invitrogen	18064-014
TNF-α platinum ELISA kit	eBioscience	BMS607
TUNEL	Roche	11684795910

Table 6. Commercial Kits.

3.2 Buffers and Solutions

Laboratory stock solutions and common buffers were prepared as described in weblink: http://onlinelibrary.wiley.com/doi/10.1002/0471143030.cba02as00/abstract, following Current Protocols in Cell Biology, 2001. Buffers and used in this thesis are specified below, or in the corresponding sections.

Buffer/Solution	Composition	Preparation	Methodology
Cell/Tissue Lysis Buffer	1% NP40, 150 mM NaCl, 50 mM Trizma HCl (pH 7.5), 2mM EDTA, 2 mM EGTA, 20mM SF, 2 mM PMSF, 2 mM Sodium orthovanadate, 1 mM DTT	Add up with distilled water to final volume, store at 4ºC; Protease inhibitor cocktail (Sigma-Aldrich; 1:100) is added just before use.	Western Blotting, ELISA
Citrate Buffer, pH 6	10 mM Sodium citrate	Add up to final volume in distilled water and adjust pH to 6 with 1 M HCl.	IHC, Antigen Retrieval
Electrophoresis Buffer (10x)	250 mM Trizma base, 1.92 M Glycine, 1 % SDS	Dissolved in distilled water and bring volume up to final volume.	Western Blotting

Buffer/Solution	Composition	Preparation	Methodology
Fluorescence-activated cell sorting (FACS) buffer	1mM EDTA, 4% FBS	Add up with PBS to final volume.	Flow Cytometry
Hotshot reagent	25 mM NaOH, 0.2 mM disodium EDTA (pH 12)	Add up to final volume in distilled water.	DNA Extraction
IF Blocking Buffer	3 % BSA, 0.1 % Triton X-100, 0.02 % Sodium Azide	Add up to final volume in PBS. CD16/32 blocking antibody was added in a 1:50 dilution just prior to use.	IF
IHC Blocking Buffer	10% goat serum, 0.3% Triton X-100	Add up to final volume in PBS.	IHC
Neutralizing reagent	40 mM Trizma-HCL (pH 5)	Add up to final volume in distilled water.	DNA Extraction
PBSBT	3 % BSA, 0.1 % Triton X-100, 0.02 % Sodium Azide	Add up to final volume in PBS.	IF
P-buffer	10 mM Hepes, 1 mM Sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, 145 mM NaCl		*In vitro* permeability assay
PI solution	25 μg/ml PI, 0.1 mg/ml RNase	Add up with PBS to final volume.	BrdU labeling
Ponceau Red Staining Solution	0.1 % Ponceau red, 5 % Acetic acid	Dissolved in distilled water, and bring up to final volume.	Western Blotting

Buffer/Solution	Composition	Preparation	Methodology
PQ401 oral gavage vehicle	30% PEG 400, 0.5% Tween-80, 0.5% Propylene Glycol	Add up with distilled water to final volume.	Oral Gavage
Red Cell Lysis (RCL) buffer	150 mM ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA	Dissolved in distilled water and bring volume up to final volume and adjust pH to 7.2-7.4	Flow Cytometry
Sample Loading Buffer (5x)	312.5 mM Trizma-HCl (pH 6.8), 50% Glycerol, 5 % SDS, 0.05 % Bromophenol blue, 10 mM DTT	Add up with distilled water to final volume and rotate on a wheel for 30 min. Store in aliquots at -20ºC.	Western Blotting
TAE Buffer (1x)	40 mM Tris-acetate (pH 7.6), 1 mM EDTA	Add up to final volume in distilled water.	Agarose Gel Electrophoresis
TBS (10x)	0.1 M Tris base 1.5 M NaCl	Add up to final volume in distilled water and adjust pH to 6 with 7.4 M HCl.	Western Blotting
TBST Buffer	50 mM Trizma/HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20	Add up to final volume in distilled water.	Western Blotting
Transfer Buffer (10x)	250 mM Trizma base, 1.92 M Glycine	Dissolved in distilled water and bring volume up to final volume. 20% methanol was added to 1X transfer buffer before use.	Western Blotting

Buffer/Solution	Composition	Preparation	Methodology
Tris-EDTA Buffer, pH 9	10 mM Trizma base, 1 mM EDTA	Add up to final volume in distilled water and adjust pH to 9.	IHC, Antigen Retrieval

Table 7. Preparation of Buffers and Solutions.

3.3 *In Vivo* models

3.3.1 Mice

All mice were healthy and had access to regular chow diet and autoclaved sterile water *ad libitum*. The animals were housed in a barrier system (temperature: 20-26ºC; relative humidity: 40-70%) and were provided with a controlled 12 hour light-dark cycle. For setting-up of breedings, male and female mice were put together at a minimum age of 6 weeks. Litters were weaned at 4 weeks of age and marked with an ear-tag. Genotyping was performed from the obtained ear tissues. Littermate controls were used in all experiments. Experimental groups were age- and sex-matched and mice were randomly allocated to the treatment groups and killed by cervical dislocation. The mice used in this thesis were obtained in C57BL/6 background and housed according to national and European Union regulations in conventional housing conditions. Animal experiments were approved by the Animal Research Committee of the University of Barcelona.

3.3.2 Mice genotypes

p38α-Δ^{MC} mice to downregulate of p38α in myeloid cells were generated by crossing p38$\alpha^{lox/lox}$ mice (Ventura et al., 2007) with LysM-Cre mice (Clausen et al., 1999). p38$\beta^{-/-}$ mice were previously described (Beardmore et al., 2005). To check efficiency of p38α deletion, peritoneal macrophages or bone marrow-derived macrophages (BMDM) were obtained and p38α protein was evaluated by western blotting.

IGF-1Δ^{MC} mice, expressing IGF-1$^{lox/lox}$ and LysM-Cre, were generously provided by Dr. Nadia Rosenthal and Dr. Lina Wang (Monash University, Melbourne, Australia) (Tonkin et al., 2015). To check efficiency of IGF-1 deletion, peritoneal macrophages were analyzed for IGF-1 messenger RNA (mRNA) expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

3.3.3 DSS-induced acute colitis

For induction of acute colitis (resembling colonic inflammation in IBD patients), mice received 1.5% DSS (w/v) *ad libitum* in the drinking water (Wirtz et al., 2007) for 6 days. DSS is toxic for the colonic mucosa and causes colitis (Okayasu et al., 1990). At day 6, DSS was removed and mice were provided with regular drinking water. Body weight and disease activity index (DAI) are commonly used indicators for the severity of DSS-induced colitis (Cooper et al., 1993) and were recorded daily. DAI was determined by combining scores of weight loss, stool consistency and rectal bleeding (divided by 3) as previously described (Cooper et al., 1993) (see Table 8).

Disease Activity Index (based on Cooper et al, 1993)			
Score	**Weight loss**	**Stool consistency**	**Visible blood in stools or rectal bleeding**
0	< 1%	Formed and	absence
1	1-5%	Formed but	--
2	5-10%	Loose stools	presence
3	10-15%	Mid diarrhea	---
4	>15%	Diarrhea	gross bleeding
DAI (0-4) = Σ score of each parameter / n parameters evaluated			

Table 8. Disease activity index (DAI).

During DSS-induced colitis DAI was recorded daily as an indicator for intestinal inflammation susceptibility.

After DSS treatment, mice were sacrificed at the indicated time points and samples were collected for histological, biochemical or cytometric analysis. Colons were collected as described in section 3.3.5.

3.3.3.1 PQ401 treatment

The IGF1R inhibitor PQ401 was administered by oral gavage at a dose of 30 mg/kg in PQ401 oral gavage vehicle. Control mice were treated in the same manner with the vehicle. The first dose was given one day prior to the start of DSS administration and was continued daily until mice were sacrificed. The dosing protocols were well tolerated by the animals.

3.3.4 AOM/DSS-induced tumorigenesis

To induce colon tumors, we applied a combination of the carcinogen AOM with repeated administration of DSS in the drinking water as previously described (Neufert et al., 2007). Mice received a single injection of AOM (10 mg/kg) diluted in PBS, and 5 days later, 1.5% DSS was administered for 5 days, followed by 14 days of regular drinking water. The DSS treatment was repeated for 2 additional cycles. Animals were sacrificed 100 days after the AOM injection, except when indicated otherwise. Colon and tumor samples were collected as described in section 3.3.5.

3.3.4.1 PQ401 treatment

Mice treated with AOM/DSS to induce tumorigenesis, were administered with PQ401 beginning from day 55 after AOM injection daily for 20 consecutive days. The dosing protocols were well tolerated by the animals.

3.3.5 Colon and tumor sample collection

Colons were removed, separated from the cecum at the ileocecal junction and flushed with cold PBS to remove feces and blood. After removing excess fat, the colons were opened longitudinally and samples for RNA and protein extracts were obtained by cutting a tiny piece horizontally beginning from the proximal up to the distal colon with a thickness of around 3 mm using sterile surgical blades (Quirumed #153-HB22). These samples were immediately shock-frozen in liquid nitrogen upon collection and stored at -80ºC until use. Before starting RNA or protein extraction of frozen samples, they were defrosted at 4ºC. The remaining colon (still the mayor part of the colon) was fixed as “swiss-rolls” in 10% formalin solution at RT overnight prior to paraffin embedding using the RM2255 Rotary Microtome (Leica). In the case of mice killed after application of the tumorigenesis protocol, tumor sizes were measured using a digital caliper in a blinded manner, prior to sample collection (from colon and tumors) and fixation.

3.4 Cellular models

3.4.1 Isolation of macrophages

3.4.1.1 Bone marrow-derived macrophages (BMDMs)

Bone marrow precursor cells were isolated from femurs and tibias of mice and cultured in complete DMEM (30% L-cell, 20% heat-inactivated FBS, 1% P/S) as previously described (Bailon et al., 2010) in an incubator at 37°C and 5% carbon dioxide (CO_2)

3.4.1.1.1 Treatment

If not otherwise indicated, cells were deprived from M-CSF for 18 h in DMEM (10% heat-inactivated FBS, 1% P/S) at day 6 of differentiation to synchronize the culture and render the cells quiescent. The inhibitors PH-797804 (2 μM), SB-203580 (10 μM) and LY-2228820 (0.1 μM) were added 1 h before stimulation with LPS (10 ng/ml) or IL-4 (10 ng/ml; R&D Systems).

3.4.1.2 Peritoneal macrophages

Mice were sacrificed and peritoneal cells were collected to assess the efficiency of p38α deletion. Prior to colon dissection, a small incision was made in the abdominal skin of the mice using surgical scissors. The skin was carefully retracted manually to expose the intact peritoneal wall and 5 ml of cold PBS were injected through the peritoneal wall using 5 ml syringes (Sudelab #15922500) and 21G needles (BD Microlance #304432). After a gentle peritoneal massage to wash the peritoneal cavity, the PBS containing peritoneal cells were recovered with the syringe and cells were pelleted at 1200 rpm for 5 min. For isolation and amplification of peritoneal macrophages, the cells were resuspended in 3 ml of DMEM containing 1% P/S and seeded onto non-treated tissue culture plates (Nunc #150239). After 1 h in an incubator at 37°C and 5% CO_2, mainly macrophages and monocytes adhere to the plates, which were then washed twice with ice-cold PBS to remove all dead and non-adherent cells and complete DMEM was added containing 20% heat-inactivated FBS, 30% L-cell conditioned medium and 1% P/S. Depending on the number of macrophages on the plates, cells were left to proliferate in complete DMEM medium for one or two days, until enough cells were obtained and collected for protein extraction (see section 3.6.3.1).

3.4.1.3 Colonic lamina propria macrophages

To obtain colon macrophages, the large intestine was excised, washed and opened longitudinally. They were then washed in HBSS containing 2% FBS, and cut into approximately 0.5 cm sections using sterile surgical blades. These pieces were then shaken vigorously in 10 ml HBSS with 2% FBS and the supernatant was discarded. HBSS containing 2 mM EDTA was then added and the tube was placed in a shaking incubator for 15 min at 37ºC, before being shaken vigorously and the supernatant discarded. Prior to a second incubation with HBSS/EDTA at 37ºC for 30 min, the tissue was washed once with HBSS, shaking vigorously and the supernatant discarded. After this second incubation, the step was repeated and the remaining tissue was digested in pre-warmed RPMI (Roswell Park Memorial Institute) 1640 containing 2 mM L-glutamine, 1% P/S and 10% FBS (complete RPMI 1640 medium) containing collagenase D (1.25 mg/ml), collagenase V (0.85 mg/ml), dispase (1 mg/ml) and DNase (30 μg/ml) for 30-45 min in a shaking incubator at 37° C, shaking vigorously every 7 min until no intact tissue was left, but without exceeding 45 min of total incubation time in the presence of the enzyme mix. The resulting cell suspension was passed through a 40 μm cell strainer (BD Falcon # 352340) and then washed twice in complete RPMI 1640 (centrifuged 5 min at 200 g and resuspended in complete RPMI 1640). This cell suspension was used to seed the cells onto non-treated plastic tissue culture plates in order to select and enrich monocyte/macrophage population from the whole lamina propria digestion in an incubator at 37°C and 5% CO_2. The cells were resuspended in complete RPMI 1640 medium and were pelleted again by centrifugation and recovered in DMEM containing 1% P/S. After 30 min, the cells were collected and used for purification of total RNA.

3.4.2 CMT-93 cell culture

The mouse colon cancer cell line CMT-93 was a kindly gift from Dr. Christine Loscher (Immunomodulation Group, Dublin City University). This cell line was derived from an induced carcinoma of mouse rectum and was used at passages 14-34 (Franks and Hemmings, 1978). Cells were cultured in DMEM (10 % heat-inactivated FBS, 1 % P/S, 2 mM L-glutamine) in an incubator at 37°C and 5% CO_2.

3.4.2.1.1 Sub-culturing

For cell line maintenance and sub-culturing, cells were usually passed at 80% of cellular confluence and never reached confluence. For sub-culturing, cells were washed with PBS and incubated with trypsin solution for 5-10 min. Trypsin was inactivated with serum containing medium and centrifuged (200 g, 5 min) and then in fresh media and passed to a new culture dish.

3.4.2.1.2 Treatment

CMT-93 cells were treated with the inhibitors PH-797804 (2 μM), SB-203580 (10 μM), MK2 inhibitor III (10 μM), PF-3644022 (20 μM) and SB-747651A (10 μM), for the times indicated in the corresponding sections. Mitomycin C (MMC) treatment was performed for the specified times at the concentration of 5 μg/ml if not otherwise indicated in the corresponding section

3.4.3 Generation of L-cell conditioned medium

Conditioned medium (L-cell) to generate macrophages from bone marrow precursor cells was obtained from the mouse fibroblast cell line L929 (ATCC CCL 1, NCTC clone 929), which produces large quantities of M-CSF during proliferation. This is the only growth factor produced by these fibroblasts affecting macrophages. The addition of monoclonal antibodies against M-CSF to the medium blocks production of macrophages in culture (Lokeshwar and Lin, 1988). $7x10^5$ L929 cells were cultured in high glucose DMEM supplemented with 10% heat-inactivated FBS at 37°C and 5% CO_2. Cells were grown in 150 mm flasks up to confluence and after 7 days the supernatant was removed, centrifuged to remove the cells in suspension and kept in aliquots at -80 ºC until the moment of use. Once thawed, the aliquots were stored at 4 ºC to prevent degradation of M-CSF resulting from freezing and thawing cycles. The content of M-CSF was determined by a test of proliferation (BrdU incorporation) in BMDM. In our studies, we used the concentration of 30% of supernatant which is equivalent to 1200 U ml^{-1} of recombinant M-CSF (eBioscience, San Diego, CA), since this dose is able to saturate the M-CSF receptors on the surface of macrophages (Stanley, 1985).

3.5 *In Vivo* studies

3.5.1 Intestinal permeability in mice

For determination of *in vivo* intestinal permeability, the mice were starved from food for 6 h and from water for 2 h, prior to administration of FITC-dextran (MW 3000-5000), prepared in PBS at a concentration of 100 mg/ml and administered to each mouse by oral gavage (0.44 mg/g body weight). After 4 h, mice were anesthetized using isoflurane (induction dose: 5%, maintenance dose: 2% using a precision vaporizer; Esteve #13400264), blood was collected by cardiac puncture using 1 ml syringes (ENFA #JS1) and 25G needles (Sudelab #1780242) and mice were sacrificed by cervical dislocation. The blood was immediately transferred to BD Microtainer SST blood collection tubes (BD #365968) and stored at 4ºC in the dark until samples from all the experimental mice were collected and SST tubes were processed following the manufacturer's instructions for serum separation. The serum was diluted with an equal volume of PBS and 100 μl of the diluted serum were added in triplicates to a 96-well microplate (Corning #3650). The FITC concentration in the serum of the mice was determined by spectrofluorometric measurement (BioTek #FLx800), with an excitation of 485 nm (20 nm band width) and an emission wavelength of 528 nm (20 nm band width), using serially diluted FITC-dextran as standard.

3.6 *In Vitro* studies

3.6.1 Mouse genotyping

3.6.1.1 DNA extraction

Hotshot reagent (80μl) was added to the tube containing the ear tissue, and was heated at 95ºC for 30 min. Samples were left to cool down for 5 min on RT and short spinned. Then, 80μl of neutralizing agent was added to the samples, mixed and centrifuged for 1 min at maximum speed. Samples were cooled down to 4ºC and stored at the same temperature until use. Some undissolved tissue usually remains that does not interfere with the PCR reaction (Zheng, 2012). For the PCR reactions, 0.5μl of this extraction was used.

The combination of alkaline lysis reagent and neutralizing yields a buffer consisting of 20mM Trizma-HCl (pH 8.1) and 0.1 mM EDTA, which is similar to a common DNA storage buffer.

3.6.1.2 Genotyping PCR

3.6.1.2.1 Primers

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size
CRE	ACGAGTGATGAGGTTC GCAAG	CCCACCGTCAGTACGT GAGAT	520 bp
flox-p38α	ATGCTACTGTCTGCGCC TCTCT	CAGCTTCTTAACTGCC ACACGA	WT: 121 bp; flox-p38α: 188 bp
p38β	TGACCGCTTCCTCGTGC TT	GGAGACTATCAGTCT GCCAACCC	WT: 220 bp; KO: 450 bp
flox-IGF-1	CACACATATACGCGCA CACA	CCTGAGCCCATCTTGA CA	WT: 170 bp; Flox-IGF-1: 270 bp

Table 9. Primers used for genotyping PCRs.

3.6.1.2.2 PCR conditions

PCRs were performed using the BIOTAQ™ PCR Kit (2x), in a final volume of 20 µl. To each reaction, 0.2 µl of the required primer were added together with 0.5 µl of DNA from the extracted ear tissues (described above).

3.6.1.2.3 Thermal cycling programs

3.6.1.2.3.1 p38α fox

PCR Program	Temperature (ºC)	Time (', minutes; '', seconds)
denaturation	94	5'
denaturation	94	30"
annealing	60	30"
extend time	72	30"
# cycles	35	
extend time	72	10'
end	4	∞

Table 10. Thermal cycling program for p38α floxed allele PCR.

3.6.1.2.3.2 CRE

PCR Program	Temperature (ºC)	Time (', minutes; '', seconds)
denaturation	94	5'
denaturation	94	30"
annealing	60	30"
extend time	72	30"
# cycles	35	
extend time	72	10'
end	4	∞

Table 11. Thermal cycling program for CRE recombinase PCR.

3.6.1.2.3.3 p38β

PCR Program	Temperature (ºC)	Time (', minutes; '', seconds)
denaturation	94	5'
denaturation	94	30"
annealing	64	30"
extend time	72	30"
# cycles	35	
extend time	72	10'
end	4	∞

Table 12. Thermal cycling program for p38β floxed allele PCR.

3.6.1.2.3.4 IGF-1 flox

PCR Program	Temperature (ºC)	Time (', minutes; '', seconds)
denaturation	94	4'
denaturation	94	30"
annealing	64	1'
extend time	72	1'
# cycles	34	
extend time	72	20'
end	4	∞

Table 13. Thermal cycling program for IGF-1 floxed allele.

The PCR products were separated on a 2% agarose gel by gel electrophoresis.

3.6.2 RNA extraction and gene expression analysis

3.6.2.1 RNA extraction

3.6.2.1.1 Colon tissues

Colon samples were collected as described above (see section 3.3.5). Tissue samples were homogenized in Trizol (500 µl) using the Precellys instrument (Bertin technologies #03119.200.RD000) and incubated for 5 min at RT. Insoluble material was removed from the homogenate by centrifugation (12 000 g, 10 min, 4ºC) and supernatant was transferred to a new tube. Samples were either further processed as described below, or stored at this point at -80ºC until use.

When the samples were previously frozen at -80ºC, they were thawed on ice and then incubated at RT for 5 min. Then, Chloroform (100 µl) was added and samples were vortexed for 10 min. After centrifugation (14 000 g, 15 min, 4ºC), the aqueous phase was transferred to a new tube and placed on ice, then the extraction was continued following the manufacturer's instructions using the RNeasy mini kit. Subsequently, DNase I treatment was performed following user's manual instructions.

3.6.2.1.2 BMDM and CMT-93 cells

Total RNA from quiescent BMDM or CMT-93 cells (DMEM and 10% FBS for 17 h) unstimulated, or stimulated with the indicated treatments was extracted using PureLink™ RNA miniKit following manufacturer's instructions. DNAse treatment was performed using on-column DNase Treatment following manufacturer's instructions.

3.6.2.1.3 Intestinal macrophages

Intestinal macrophages were isolated from the colonic lamina propia as described above (see section 3.4.1.3), total RNA was extracted using Trizol and Pellet Paint Co-Precipitant. Trizol (300 µl) was added to each well containing intestinal macrophages, incubated for 5 min at RT and chloroform (60 µl) was added. The samples were shaken vigorously for 15 sec and allowed to stand for 5 min at RT prior to centrifugation for 15 min at 12000 g (4°C). The colorless aqueous upper phase containing the RNA was then transferred to a new tube. Pellet Paint (2 µl) and 1 volume of isopropanol were added to the samples and incubated on ice for 5 min. Samples were then centrifuged for 30 min at 12000 g (4°C). Supernatants were removed and the pellets were washed with 300 µl of ethanol by vortexing. The tube was put 180° relative to the previous step and centrifuged

for 10 min at 12000 g. The supernatant was removed and 15 µl of RNase free water was added to the pellet and left for 5 min at RT. Samples were vortexed for 1 min and spun down briefly. Samples were pipetted up and down to further homogenize and spun down again. DNase I treatment was performed following user's manual instructions.

3.6.2.2 cDNA synthesis

3.6.2.2.1 Colon tissues, BMDM and CMT-93 cells

Samples were quantified by Nano Drop and reverse transcription was performed using 1 µg of total RNA using Superscript II reverse transcriptase, RNAsin and random primers following Invitrogene user's instructions for complementary DNA (cDNA) synthesis using SuperScript II RT.

3.6.2.2.2 Intestinal macrophages

Samples were quantified by Nano Drop and reverse transcribed using 1 µg of total RNA, RNAsin, random primers and Superscript IV reverse transcriptase as recommended by Invitrogen.

3.6.2.3 qRT-PCR analysis

3.6.2.3.1 Colon tissues, BMDM and CMT-93 cells

qRT-PCR was performed in triplicates using 4 µl of 1/16 diluted cDNA and SYBR green in 10 µl final volume. Data were obtained as relative mRNA levels normalized in each sample to the GAPDH expression level.

3.6.2.3.2 Intestinal macrophages

qRT-PCR was performed in triplicates using 4 µl of 1/32 diluted cDNA and SYBR green in 10 µl final volume. Data were obtained as relative mRNA levels normalized in each sample to the GAPDH expression level.

3.6.2.4 Primer sequences

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Alp1	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Ascl2	CCGTGAAGGTGCAAACGTC	CCCTGCTACGAGTTCTGGTG
CCL12	ATTTCCACACTTCTATGCCTCCT	ATCCAGTATGGTCCTGAAGATCA
CCL2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
CCL21	GTGATGGAGGGGGTCAGGA	GGGATGGGACAGCCTAAACT
CCL6	TATCCTTGTGGCTGTCCTTGG	TTACATGGGATCTGTGTGGCA
CCL9	CCCTCTCCTTCCTCATTCTTACA	AGTCTTGAAAGCCCATGTGAAA
Cdx2	CAAGGACGTGAGCATGTATCC	GTAACCACCGTAGTCCGGGTA
CgA	CCAAGGTGATGAAGTGCGTC	GGTGTCGCAGGATAGAGAGGA
Chemerin	GCTGATCTCCCTAGCCCTATG	CCAATCACACCACTAACCACTTC
CXCL12	TGCATCAGTGACGGTAAACCA	TTCTTCAGCCGTGCAACAATC
GAPDH	CTTCACCACCATGGAGGAGGC	GGCATGGACTGTGGTCATGAG
IGF-1	CTGGACCAGAGACCCTTTGC	GGACGGGGACTTCTGAGTCTT
IGF1R	GTGGGGGCTCGTGTTTCTC	GATCACCGTGCAGTTTTCCA
IL-16	AAGAGCCGGAAATCCACGAAA	GTCTCAAAAGGGTCAGGGTACT
Lgr5	GGACCAGATGCGATACCGC	CAGAGGCGATGTAGGAGACTG
Lysozyme	GGGGCTTGAATTATTTTGAGGGC	GAGCCATCTTGTGGGTCCT
Mucin-2	GCCCGTGGAGTCGTACGTGC	TTGGGGCAGAGTGAGGCGGT
Neurogenin-3	CCAAGAGCGAGTTGGCACT	CGGGCCATAGAAGCTGTGG
p38α (exon 12)	GCCCTCCCTCACTTCAGGAG	TGTGCTCGGCACTGGAGACC
p38α (exon 2)	GCATCGTGTGGCAGTTAAGA	GTCCTTTTGGCGTGAATGAT
STAT3	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACTGCCCT
Tff3	TGCTCTGGTAGAGGGCGAG	CGACGCTAGAGTCAAAGCAG

Table 14. Primers used for quantification of mRNA expression by qRT-PCR.

3.6.2.4.1 PCR conditions

PCRs were performed in 384-well reaction plates (Applied Biosystem; #4309849) with SYBR green in 10 µl total volume using the QuantStudio™ 6 Flex Instrument (Life Technologies). Each primer was used at a final concentration of 0.2 µM and cDNA used in the reactions are added and prepared as described above (see section 3.6.2.3).

PCR thermal cycling program was set as follows: 50ºC for 2 min, 95ºC for 10 min, 40 cycles of denaturation at 95ºC for 15 s, annealing at 56ºC for 15 s, elongation at 72ºC for 60 s, and three final steps of 95ºC for 15 s, 60ºC for 2 min and 95ºC for 15 s.

3.6.3 Protein extraction

3.6.3.1 BMDMs, peritoneal macrophages and CMT-93 cells

Cells were cultured and treated as described in section 3.4. For protein extraction, the plates were washed using ice-cold PBS and collected with protein lysis buffer on ice using a cell scraper (Costar #3008). Cell lysates were left for 15 min on a rotatory mixer at 4ºC, prior to centrifugation (20 000 g, 15 min, 4ºC). Supernatants were transferred to a new tube, quantified by Bradford assay and either directly used for Western Blotting or ELISA analysis or stored at -80ºC until use. Frozen samples were always defrosted on ice.

3.6.3.2 Colon and tumor tissues

Frozen colon samples were collected as described in section 3.3.5. The samples were defrosted at 4ºC and lysed in the protein lysis buffer using the Precellys instrument (Bertin technologies #03119.200.RD000). Cell and tissue lysates were left for 15 min on a rotatory mixer at 4ºC, prior to centrifugation (20 000 g, 15 min, 4ºC). Supernatants were transferred to a new tube, quantified by Bradford assay and either directly used for Western Blotting or ELISA analysis or stored at -80ºC until use. Frozen samples were always defrosted on ice.

3.6.4 Protein quantification

Total protein was quantified using Bradford assay according to manufacturer instructions. Protein concentrations were measured with a spectrophotometer (BioTek #FLx800) at an absorbance of 570 nm. For the calculation of protein concentrations, a BSA-standard curve was used.

3.6.5 Western blotting

For Western blotting, 40 µg of lysates were boiled for 5 min at 95ªC and separated by SDS-PAGE (see section 3.6.5.1) prior to transfer onto a nitrocellulose membrane using a semi-dry transfer system (GE Healthcare) (75 mA, 90 min). After transfer, membranes were stained with Ponceau red to confirm the efficiency of transfer. Ponceau red was washed with tap water and the nitrocellulose membrane was blocked with 5% non-fat

milk in TBST buffer. After 3 washes with TBST, membranes were incubated with the primary antibodies overnight at 4ºC in TBST containing 5 % of BSA and 0.02 % Sodium Azide at 4ºC. The primary antibodies used are listed in Table 15. GAPDH and α-Tubulin were used as loading control.

Antibody	Commercial House	Reference	Source	Dilution
Claudin-1	Invitrogen	374900	Mouse	1:500
Claudin-2	Thermo Scientific	PA5-13334	Rabbit	1:1000
GAPDH	Sigma-Aldrich	SAB2100894	Rabbit	1:5000
p38α	Cell Signaling	9218	Rabbit	1:1000
p38α	Santa Cruz	sc5356	Goat	1:600
Phospho-p38	Cell Signaling	9211	Rabbit	1:1000
ZO-1	Invitrogen	40-2200	Rabbit	1:600
α-Tubulin	Sigma-Aldrich	T9026	Mouse	1:5000

Table 15. Primary antibodies used for Western Blotting.

After 3 washes with PBST, membranes were incubated with Alexa Fluor 680 or 800-conjugated antibodies (Table 16) for 1 h at RT prior to visualization using Odyssey Infrared Imaging System (Li-Cor, Biosciences).

Antibody	Commercial House	Reference	Dilution
Goat IgG (Alexa Fluor 680)	Invitrogen	A21084	1:5000
Mouse IgG (Alexa Fluor 680)	Invitrogen	A21057	1:5000
Mouse IgG (Alexa Fluor 800)	Rockland	610-731-124	1:5000
Rabbit IgG (Alexa Fluor 680)	Invitrogen	A21076	1:5000
Rabbit IgG (Alexa Fluor 800)	Rockland	611-131-122	1:5000

Table 16. Secondary antibodies used for Western Blotting.

3.6.5.1 SDS-PAGE gels

3.6.5.1.1 Resolving gels (for 20 ml)

V (ml)	8%	12%	15%
H_2O	9.3	6.6	4.6
30% Acrylamide solution	5.3	8	10
1.5 M Trizma (pH 8.8)	5	5	5
10% SDS	0.2	0.2	0.2
10% APS	0.2	0.2	0.2
TEMED	0.008	0.008	0.008
Proteins resolved	ZO-1	p38α, phospho-p38	Claudin-1, Claudin-2

Table 17. Composition of resolving gels.

3.6.5.1.2 Stacking gels (for 5 ml)

V (ml)	4%
H_2O	3.07
30% Acrylamide solution	0.67
1.5 M Trizma (pH 8.8)	1.25
10% SDS	0.05
10% APS	0.05
TEMED	0.005

Table 18. Composition of stacking gels.

3.6.6 ELISA (Enzyme-linked immunosorbent assay) and protein arrays

3.6.6.1 ELISA

For analysis of IGF-1 protein at basal conditions, BMDMs were generated (see section 3.4.1.1) and supernatants and cells were collected at day 7 after bone marrow extraction. Supernatants were collected and centrifuged at 7500 g for 5 min at 4ºC to remove dead cells and cellular debris, and diluted 1:5 for analysis using the mouse/rat IGF-1 quantikine

ELISA kit following the manufacturer's instructions. The adhered BMDMs were washed once with ice-cold PBS and directly lysed on the plates using a cell scraper on ice. Protein extracts from BMDMs and colon lysates were analyzed by ELISA using 100 μg per sample and well (see section 3.1.3).

IL-1β and TNFα were analyzed by using 100 μg per sample from whole colon protein extracts and the mouse IL-1β/IL-1F2 quantikine Enzyme-linked immunosorbent assay (ELISA) kit or mouse TNF-α platinum ELISA kit following manufacturer's instructions (see section 3.1.3).

3.6.6.2 Cytokine array

BMDMs were generated and cultured as described in (section 3.4.1.1). Medium was changed at day 6 and macrophages were deprived from M-CSF. Cytokine array was performed by pooling supernatants at day 7 derived from BMDM of three animals per group (WT and p38α-Δ^{MC}) using RayBio C-Series mouse cytokine antibody array C1000 (see section 3.1.3).

3.6.6.3 Chemokine array

For the chemokine array, whole colon extracts were obtained from mice either WT or deficient for p38α in myeloid cells (five different mice each), and a total of 400 μg of protein was pooled (80 μg protein per mouse) and analyzed following manufacturer's instructions (see section 3.1.3).

3.6.7 Hematoxylin and eosin staining

For histological analysis of colon and tumor sections, formalin-fixed and paraffin-embedded sections of 5 μm thickness were de-waxed in xylol for 10-15 min and then rehydrated in descending series of ethanol solutions (100%, 95%, 75%, 50% and then in water). The de-wax, rehydrated colon sections were then stained with H&E using the CoverStainer (Dako).

3.6.7.1 Epithelial damage scoring

H&E stained slides were scanned using the digital scanner Nanozoomer 2.0HT (Hamamatsu) with a 40x objective. Epithelial damage and inflammation was determined using a scoring system previously described (Gupta et al., 2014). Briefly, first a score (1-4) was assigned for observed epithelial damage in the colon using following criteria: 1-intact crypts, 2- basal one-third damaged, 3-basal two-thirds damaged, 4- damaged

surface epithelium. The colon sample was given the score of the highest epithelial damage observed in the entire intestinal mucosa. Then, another score (1-4) was assigned assessing the extent of involvement in percentage of given epithelial damage: 1- for 1-25%, 2- for 26-50%, 3- for 51-75%, and 4- for 76-100%. The scores from the two values were added up and divided by two, resulting in the final epithelial damage score.

3.6.8 Immunohistochemistry

For IHC staining, tissue sections on slides were de-waxed and re-hydrated as described above (see section 3.6.7). After washing with tap water for 5 min, endogenous peroxidase activity was blocked for 15 min at RT in peroxidase blocking buffer to reduce background and unspecific staining. Then, slides were washed in tap water and antigen unmasking was performed as indicated in Table 19, depending on the primary antibody that will be used for staining (Table 20).

Antigen Retrieval Buffer	Incubation Time (min)	Incubation Temperature (ºC)
Citrate Buffer, pH 6	20	97
Proteinase K	5	RT
Tris-EDTA Buffer, pH 9	20	97

Table 19. Antigen retrieval buffers used for paraffin-embedded colon and tumor sections.

The preparation of the indicated buffers is described above in Table 7, except of Proteinase K solution, which was commercially purchased (Table 4).

After antigen retrieval, slides were washed with PBS and unspecific staining was blocked with IHC blocking buffer. Diluted primary antibodies were then incubated as indicated in Table 20.

Antigen	Commercial House	Reference	Antigen Retrieval	Dilution	Incubation Conditions	
					Time	Temper-ature
CD45	BD Biosciences	550539	Citrate Buffer	1:100	overnight	4 ºC
CD3	Dako	A0452	A0452	1:10	2 h	RT
MPO	Dako	A0398	Tris/EDTA Buffer	1:1000	30 min	RT
STAT3 (p-Tyr705)	Cell Signaling	9145	Tris/EDTA Buffer	1:200	90 min	RT
IGF1R (p-Y1161)	Abcam	ab39398	Citrate Buffer	1:500	1 h	RT
Ki67	Novacastra	NCL-Ki67	Citrate Buffer	1:500	1 h	RT
CD31	abcam	ab28364	Tris/EDTA Buffer	1:500	2 h	RT
F4/80	eBioscience	14-4801	Proteinase-K	1:50	2 h	RT

Table 20. Primary antibodies used for IHC.

After washing the primary antibody with tap water, slides were incubated in HRP-conjugated secondary antibodies (Table 21).

Antigen	Commercial House	Reference	Dilution	Incubation Conditions	
				Time	Temperature
anti-goat	Dako	P0449	1:80	30 min	RT
anti-mouse	Dako	P0447	1:100	30 min	RT
anti-rabbit	ImmunoLogic	DPVR110HRP	1:100	45 min	RT
anti-rat	Dako	P0450	1:75	30 min	RT

Table 21. Secondary Antibodies used for IHC (Horseradish Peroxidase (HRP)-conjugated).

Signals were visualized with DAB, using hematoxylin (Mayer's hematoxylin solution) as a counterstaining. Tissue was mounted with DPX mounting medium after washing with PBS.

3.6.8.1 Colon and tumor IHC quantification

IHC slides were scanned using the digital scanner Nanozoomer 2.0HT (Hamamatsu) with a 40x objective. The number of positively stained cells was calculated from several high magnification fields per animal. For the analysis of the epithelium of untreated or DSS treated mice, almost the whole distal part of the epithelium in the slide of each animal was analyzed. For the analysis of the non-tumoral epithelium in AOM/DSS-treated mice, almost the whole distal part of the epithelium was analyzed, excluding the tumors. In order to compare tumors of the same size, several tumors of 3 mm of size were cut out of the distal part of the colons after sacrifice of the animals and embedded in paraffin separately. High magnification images were taken for the whole tumor tissue stained per slide and animal analyzed. Detection of positive staining and cell number was performed with ImageJ software using the colour-deconvolution plug-in that has a built-in vector for separating hematoxylin and diaminobenzidine (DAB) staining. After color deconvolution DAB images are processed separately. Suitable threshold levels of DAB were determined for each staining and kept constant for all analysis. The results were expressed as percentage of positively stained cells.

Described analysis was performed for all IHC stainings, except for the quantification of different staining intensities for phospho-IGF1R positive cells. This was performed with Tmarker software (Schuffler et al., 2013) using the plugin for "color deconvolution" (H&E DAB staining protocol) and "cancer nucleus classification". Several training images were used in order to train the Tmarker classification using its active learning algorithm prior to performing the analysis of the samples from several high magnification fields. The results were expressed as percentage of positively stained cells of the corresponding staining intensities.

3.6.9 TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a commonly used method to detect DNA fragmentation resulting from apoptosis by labeling the terminal end of nucleic acids. This method was applied to detect apoptosis in paraffin-embedded colon samples using the Fluorescein In Situ Cell Death Detection Kit according to the manufacturer's instructions.

IHC slides were scanned using the digital scanner Nanozoomer 2.0HT (Hamamatsu) with a 40x objective. Quantification was performed with Tmarker software (Schuffler et al., 2013) using the plugin for "color deconvolution" (CMY staining protocol) and "cancer nucleus classification". Several training images were used in order to train the Tmarker classification using its active learning algorithm prior to performing the analysis of the samples from several high magnification fields. The results were expressed as percentage of positively stained cells.

3.6.10 Immunofluorescence

To confirm the purity of macrophages isolated from lamina propria (see section 3.4.1.3), cells were left to adhere to the plastic petri-dishes for 30 min, then were recollected and seeded onto 8-well glass chamber slides (Millipore #PEZGS0816), for immunofluorescence staining. Cells were left to adhere for further 30 min, the culture media was removed and cells were immediately fixed with ice-cold Methanol (100%) for 5 min at RT. Methanol was removed and cells were washed 3 times with PBS and incubated with 100 μl of IF Blocking Buffer with shaking for 30 min. The CD16/32-containing blocking buffer was removed and replaced with 100 μl of PBSBT containing the primary antibody for 1h at RT or overnight at 4ºC with shaking (Table 22). In the case of double-staining (γ-H2AX and 53BP1), the slides were washed 3 times with PBSBT prior to addition of the other primary antibody.

Antigen	Commercial House	Reference	Source	Dilution
53BP1	Novus Biologicals	NB 100-904	Rabbit	1:200
CD115	BioLegend	135520	Rat (conjugated)	1:100
E-Cadherin	BD Biosciences	610181	Mouse	1:200
F4/80	eBioscience	14-4801	Mouse	1:100
Phospho-p38	Cell Signaling	4631	Rabbit	1:100
γ-H2AX	Millipore	05-636	Mouse	1:200

Table 22. Primary antibodies used for IF.

After incubation with the primary antibodies, the slides were washed three times with PBSBT. Then secondary antibodies diluted in PBSBT were added for 1 h with shaking (Table 23), except in the case of the conjugated antibody (CD115).

Antibody	Commercial House	Reference	Dilution
Mouse IgG (Alexa Fluor 555)	Invitrogen	A-21422	1:400
Rabbit IgG (Alexa Fluor 488)	Invitrogen	A21441	1:400

Table 23. Secondary antibodies used for IF.

Slides were then washed again in three times in PBSBT prior to mounting them on coverslips using ProLong Gold antifade reagent with DAPI.

3.6.11 Fluorescence-activated cell sorting (FACS)

3.6.11.1 Tissue preparation

3.6.11.1.1 Bone marrow

Bone marrow cells were isolated as previously described (Bailon et al., 2010). Cells were collected out of the bones, transferred to 15 ml Falcon tubes, centrifuged (200 g, 5 min), resuspended in 5 ml RCL buffer and incubated for 5 min at RT. Ice-cold DMEM containing 10% FBS was added and cells were centrifuged once more (200 g, 5 min) and resuspended in ice-cold FACS buffer prior to staining and analysis (see section 3.6.11.2).

3.6.11.1.2 Blood

For analysis of blood leukocytes, blood was collected using heparinized needles by cardiac puncture into prepared 15 ml Falcon tubes containing 50 µl of heparin (50 mg/ml). Tubes were filled up using 5 ml RCL buffer and incubated for 5 min at RT. After centrifugation (200 g, 5 min), cells were lysed once more for further 3 min at RT. Ice-cold DMEM containing 10% FBS was and centrifuged (200 g, 5 min). Cells were resuspended in ice-cold FACS buffer prior to staining and analysis (see section 3.6.11.2).

3.6.11.1.3 Spleen

For collection of splenic cells, the spleen was removed, excess fat was eliminated and the soft mouse spleen was forced through a 40 µm cell strainer. Ice-cold DMEM containing 10% FBS was added, cells transferred to a 15 ml tube and centrifuged (200 g, 5 min). The pellet was resuspended in 5 ml RCL buffer, incubated for 5 min and centrifuged (200 g, 5

min). Ice-cold DMEM containing 10% FBS was added prior to centrifugation (200 g, 5 min) and resuspension in ice-cold FACS buffer for staining and analysis (see section 3.6.11.2).

3.6.11.2 Cell staining and flow cytometry

Cells obtained from bone marrow, blood and spleens from different experiments were washed in FACS buffer and counted to treat $4x10^6$ cells per tube. Fc Receptors were blocked in FACS buffer containing CD16/CD32 antibody (1:100) for 20 min at 4ºC. Cells were washed again in FACS buffer and stained with combinations of fluorescence-labeled antibodies to cell surface markers (Table 24).

Antigen	Channel	Commercial House	Reference	Dilution
CCR2	FITC	R&D Systems	FAB5538F-025	1:100
CD11b	Brilliant Violet 711	BioLegend	101241	1:200
CD127	PE	Biolegend	12-1271-81	1:100
CD34	FITC	Biolegend	11-0341-81	1:100
CD4	PerCP-Cy5.5	BioLegend	100433	1:200
CD45	APC-eFluor 780	ebioscience	47-0451-80	1:400
CD45	PE-Cy7	BD Pharmingen	552848	1:300
CD8	APC	Miltenyi biotec	130-091-606	1:200
c-Kit	APC	Biolegend	17-1171-81	1:100
F4/80	PE	Biolegend	123109	1:100
Lineage Cocktail	e-Fluor 450	Biolegend	88-77772-72	1:100
Ly6C	APC	BD Pharmingen	560595	1:400
Ly6G	PerCP-Cy5.5	BioLegend	127615	1:100
Sca-1	PE-Cy7	BioLegend	108114	1:100

Table 24. Antibodies used for FACS analysis.

Cells were stained for viability using the LIVE/DEAD Fixable Yellow Dead Cell Stain Kit following the manufacturer's instructions and analyzed using Gallios (Beckman Coulter) or Cytoflex (Beckman coulter), flow cytometers. All experiments were analyzed

using FlowJo software (Phoenix Flow Systems, Inc., San Diego) and data were represented as % indicated positive cells.

3.6.12 AnnexinV and propidium iodide (PI) staining

Apoptosis was measured using a fluorescent AnnexinV kit. As positive control, CMT-93 cells were stimulated with ActD (5 μg/ml) for 12 h. For sample analysis, the medium was collected and CMT-93 cells were washed with PBS, which was then collected as well. CMT-93 cells were incubated with trypsin-EDTA solution for 5-10 min at 37ºC, and were collected washing the wells once again with PBS to collect the remaining cells. The tubes containing medium, cells and PBS were centrifuged (200 g, 5 min), washed with PBS and $1x10^5$ cells/sample were resuspended in 500 μl annexin-V binding buffer. Annexin-V (25 μM) was incubated for 20 min at RT (in the dark) and Propidium Iodide (PI) (0.2 μg/ml) was added just before measurement. Samples were analyzed by flow cytometry using FC500 cytometer (Beckman Coulter, Inc., Pasadena, CA). FlowJo software was used to analyze data, which were represented as % of Annexin-V and PI positive cells.

3.6.13 BrdU labelling

Analysis of proliferation was performed in treated or untreated sub-confluent CMT-93 cell cultures, unless otherwise indicated. 10μl of BrdU (10mM) was added 2 h prior to sample collection.

For sample collection, cells were incubated with trypsin-EDTA solution for 5-10 min at 37ºC and trypsin was inactivated with serum containing medium (DMEM, 10% FBS, 1% P/S). CMT-93 cells were collected and the wells washed once again with PBS to collect remaining cells. $1x10^6$ cells/sample cells were fixed with 5 ml EtOH (70%) and stored at –20°C for at least 2 h, up to 2 weeks.

For BrdU staining, tubes were centrifuged (150 g, 5 min), resuspended in 1 ml of cold PBS and centrifuged again (220 g, 7 min), before carefully removing the supernatant and adding 0.5 ml of HCl (0.1M) and Tween 20 (0.5%) in water. Cells were then incubated on ice for 10 min, 1 ml of water was added and tubes centrifuged (220 g, 7 min). The supernatant was discarded and 1 ml of distilled water was added prior to incubation of the samples at 95°C for 5 min and transferring them on ice. Then, 500 μl of 0.5%Tween/1%BSA in PBS was added, samples centrifuged (220 g, 7 min) and supernatant was discarded. Afterwards, 750 μl of 0.5%Tween/1%BSA in PBS was added, centrifuged (220 g, 7 min) and supernatant was discarded. Finally, cells were resuspended

in 90 µl of 0.5%Tween/1%BSA in PBS with 10 µl of anti-BrdU-FITC or anti-isotype-FITC antibody contained in the kit (see section 3.1.3). Samples were then incubated at RT in the dark for 90 min. Then, 750 µl of 0.5%Tween/1%BSA in PBS was added, samples were centrifuged (220 g, 7 min) and supernatant was discarded. Finally, the cells were resuspended in 300 µl of PI solution and analyzed after 20 minutes by flow cytometry using FC500 cytometer (Beckman Coulter, Inc., Pasadena, CA). FlowJo software was used to analyze data, which were represented as % of BrdU positive cells.

3.6.14 β-Galactosidase staining

To evaluate cellular senescence, CMT-93 cells were seeded on 6-well plates at the indicated confluences in the presence of absence of p38 MAPK inhibitors. The Senescence β-Galactosidase staining kit was used and all the steps were performed following the manufacturer's indications. After staining, the wells were scanned using the CellR/ScanR (Olympus) microscope and the integrated density (ID)/μm^2 was quantified using ImageJ software.

3.6.15 *In-Vitro* paracellular permeability assay

The paracellular flux in CMT-93 cells was measured as previously described (Inai et al., 1999). Briefly, CMT-93 cells were seeded at sub-confluence and analyzed at 2 days post-confluence if not otherwise indicated onto 0.4 µm pore size cell inserts (Corning; #353095). Treatment of cells was performed as described in the corresponding sections. For analysis, the culture medium was replaced with P-buffer for 30 min for equilibration (350 µl in the apical, and 900 µl in the basolateral chamber). Then, the P-buffer was removed from both sides, 900 µl of fresh P-buffer was put in the basolateral chamber and in the apical chamber 1 mg/ml of 4 kDa FITC-Dextran was added (Figure 9).

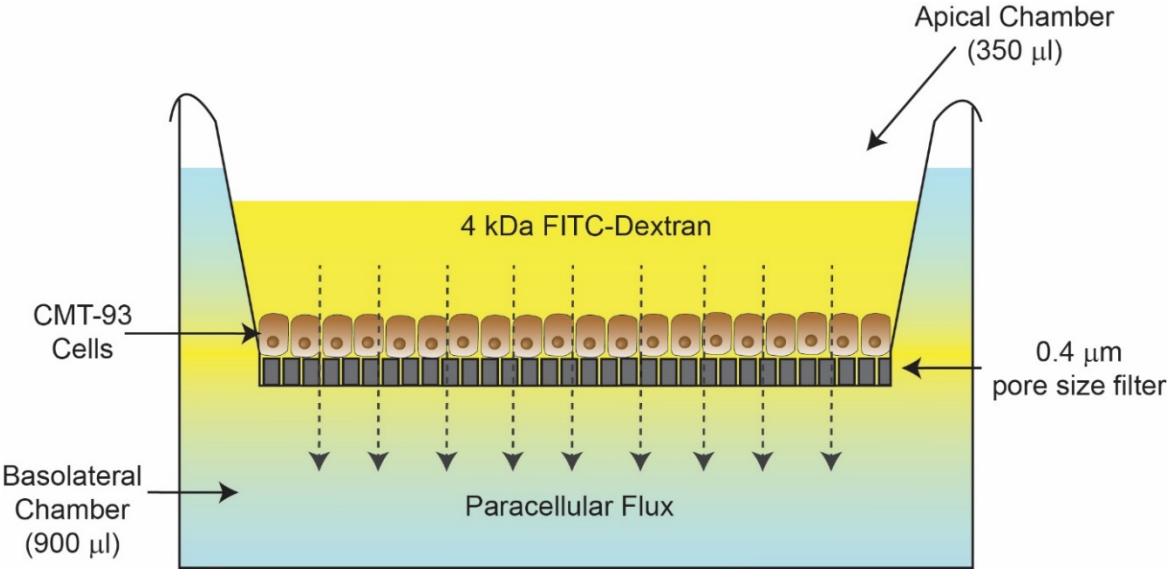


Figure 9. Schematic representation of the paracellular permeability assay.

The paracellular flux of 4 kDa FITC-Dextran using 0.4 µm pore size filter from the apical to the basolateral chamber was measured using a spectrofluorometer.

The media from the lower compartment were collected each h after the addition of the tracer and the cell inserts were moved to fresh 900 µl P-buffer. This was repeated 4 times (a total of 4 h). The paracellular flux was determined by spectrofluorometric measurement (BioTek #FLx800), with an excitation of 485 nm (20 nm band width) and an emission wavelength of 528 nm (20 nm band width), calculating an average of flux per hour from technical duplicates in each experiment performed, using serially diluted FITC-Dextran as standard.

3.6.16 Wound-healing assay

To measure wound-healing, CMT-93 cells were seeded in sub-confluence onto 24-well tissue culture plates, treated as indicated in the corresponding sections and analyzed at 2 days post-confluence. The cell monolayer was scraped in a straight line to create a scratch using a p10 pipet tip. Cellular debris was removed by carefully washing the cells twice with PBS, prior to the addition of the recovery media indicated in the corresponding sections. To keep the humidity of the cells, additional PBS was added in the empty space between the wells of the plate. The wound-closure was followed up using the CellR/ScanR (Olympus) live cell imaging microscope on a 10x objective (phase contrast). Sensors located throughout the instrument were set to keep the temperature inside the microscope chamber at 37° C and CO_2 at 5 %. The software integrated in the microscope (Excellence; Olympus), was programmed using the experiment manager to acquire

images of the same field every 10 min during the whole acquisition period. Per well, 4 acquisition points were selected (Figure 10).

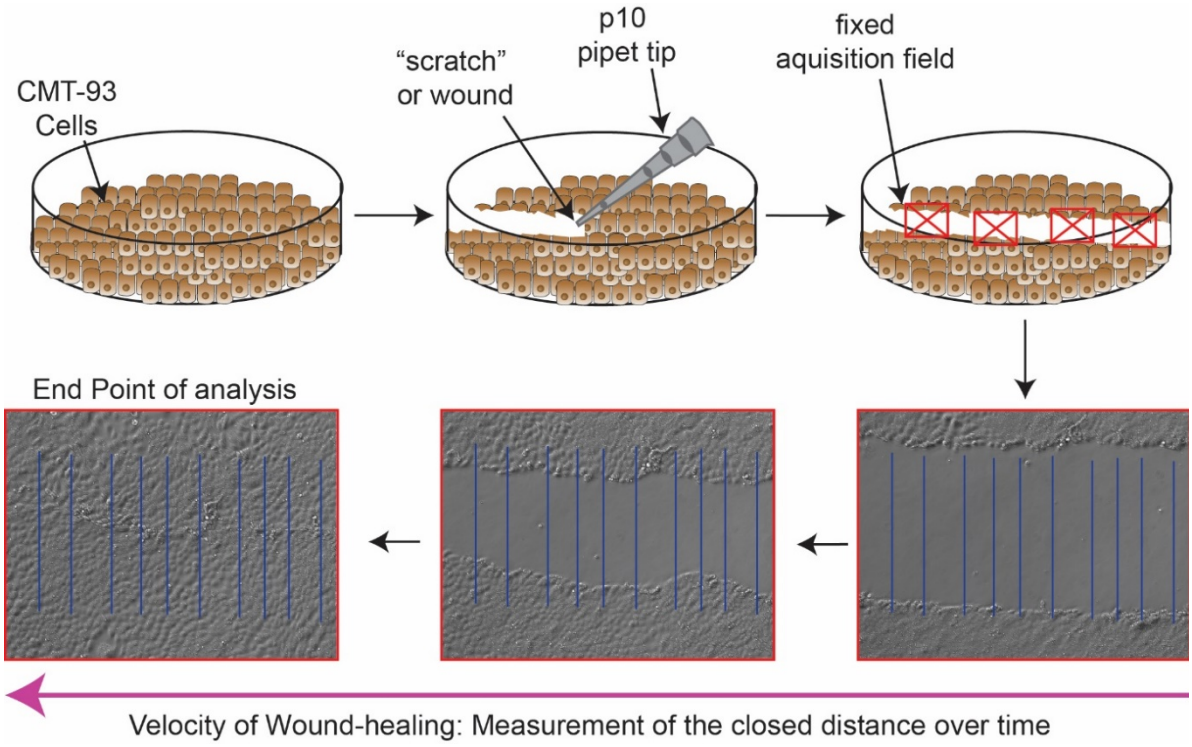


Figure 10. Schematic representation of the scratch assay.

CMT-93 cell monolayers were wounded and 4 fixed acquisition fields per well were randomly chosen. Images were taken every ten minutes and wound-closure was calculated from 10 positions within each acquisition field (image) chosen.

The analysis of wound closure velocity was performed with ImageJ using the plugin "ImageJ Kymograph", following the plugin description. For each acquisition point, 10 positions within the image were analyzed for the migration velocity of CMT-93 cells and results were expressed as the velocity of wound closure (μm/min).

3.7 Statistical analysis

Data are expressed as average ± SD. Statistical analysis was performed by using a two-tailed Student's t-test (Mann-Whitney) for the comparison of two groups or ANOVA using Bonferroni post-hoc correction for multiple groups using GraphPad Prism Software 6 (GraphPad Software, Inc., La Jolla CA). p-values are expressed as *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ and ****, $p \leq 0.0001$.

RESULTS

4. RESULTS

4.1 Regulation of IGF-1 production by p38α in myeloid cells promotes inflammation-associated colon cancer

4.1.1 Mice with p38α-deficient myeloid cells show decreased susceptibility to colon tumorigenesis

Myeloid cells are the predominant leukocytes found in solid tumors and are known to support primary tumor growth and progression (Schouppe et al., 2012). Since p38α has been implicated in several cancers (see section 1.4) and is known to control the production of leukocyte chemoattractants and other pro-inflammatory mediators (Cuadrado and Nebreda, 2010; Kim et al., 2008), we first investigated the contribution of myeloid p38α signaling to CAC.

The AOM/DSS model in C57/BL6 mice is a well-established model for CAC. A single intraperitoneal administration of AOM is followed by three cycles of five-days DSS treatment separated by 14-day intervals of regular water (see section 3.3.4). This treatment is based on the induction of mutations by AOM and inflammation by DSS, resulting in multiple neoplasms in the distal colon that can be visible within a period of 20 weeks. Mice treated with this protocol are usually sacrificed 100 days after AOM injection (Figure 11) and exhibit tumors that resemble human colon tumors both, in distribution, and at the molecular level (Kohno et al., 2005; Neufert et al., 2007; Suzuki et al., 2004; Tanaka et al., 2003).

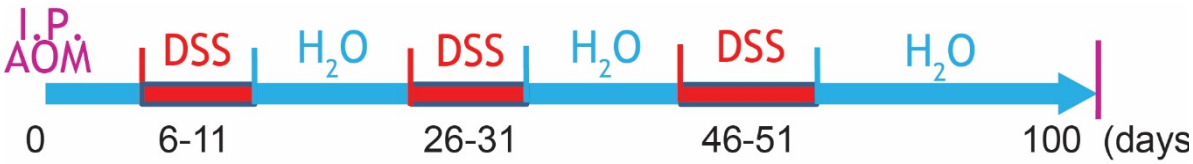


Figure 11. AOM/DSS-induced tumorigenesis protocol.

Schematic representation of the AOM/DSS protocol to induce colitis-associated colon cancer. Animals were sacrificed at day 100, unless otherwise indicated

To evaluate the role of myeloid p38α in inflammation-associated colon tumorigenesis, our group generated mice expressing LysM-Cre and p38α-lox alleles as previously described (Clausen et al., 1999; Kang et al., 2008; Otsuka et al., 2010; Ventura et al., 2007). Mice deficient in p38α in myeloid cells (p38α-Δ^{MC}) have been described to

contain monocytes and macrophages, but also neutrophils with significant p38α deletion (Abram et al., 2014). The *LysM* gene is a marker of myeloid differentiation and is progressively turned on during differentiation from myeloid precursor cells to mature macrophages, in which it is fully active (Cross et al., 1988; Mollers et al., 1992). p38α-Δ^{MC} mice appeared to be healthy and had no obvious physiological phenotype compared to their WT littermates (p38$\alpha^{fl/fl}$).

The efficiency of p38α downregulation in myeloid cells was confirmed using peritoneal macrophages. A representative western blot is shown in Figure 12.

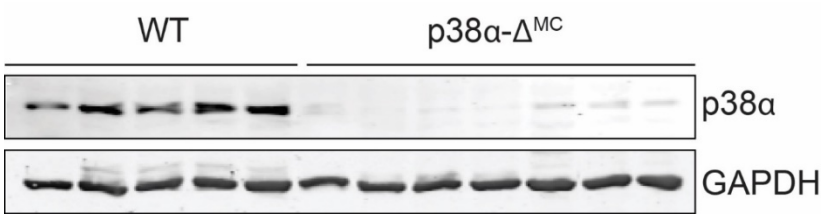


Figure 12. p38α downregulation of p38α-Δ^{MC} mice.

Representative western blot of p38α in cell lysates obtained from peritoneal macrophages to confirm p38α downregulation in myeloid cells of p38α-Δ^{MC} mice used in the experiments.

Six days after the AOM injection, once the DSS treatment was started, and throughout the experiment, several parameters were evaluated to estimate disease susceptibility of the mice. We monitored the animal body weight, occurrence of diarrhea, and the presence of gross blood in the faeces for each mouse. These factors were compiled to calculate the DAI (Table 8). Our results indicated that p38α-Δ^{MC} mice treated with AOM and DSS lost less body weight (Figure 13A) and exhibited a lower DAI compared to WT mice (Figure 13B).

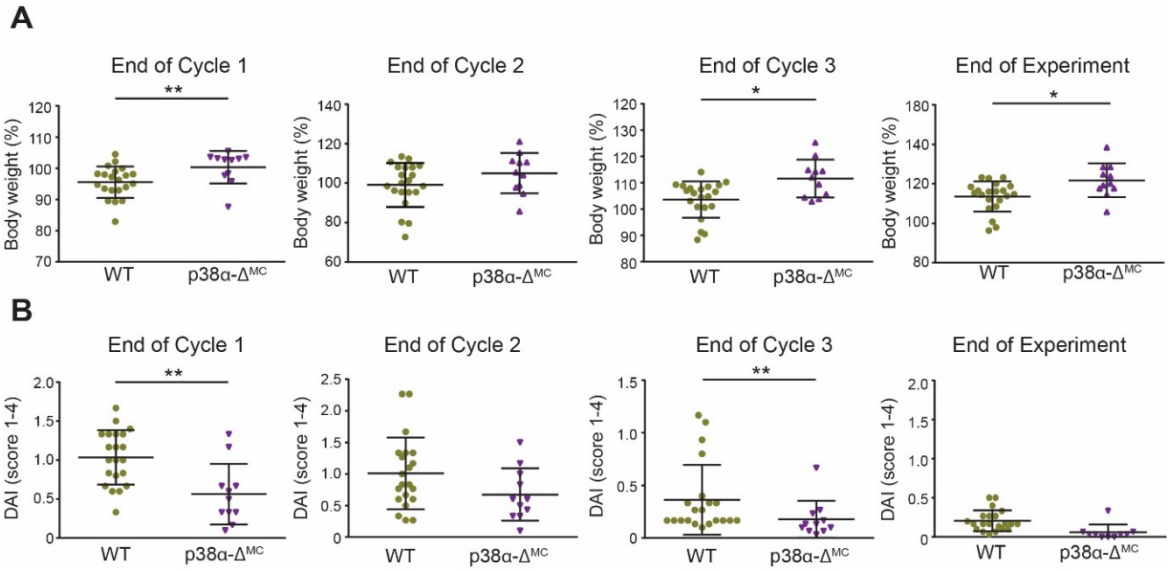


Figure 13. Deficiency of p38α in myeloid cells decreases susceptibility to CAC.

(A and B) Body weight (A) and DAI (B) were recorded at the indicated days during AOM/DSS induced tumorigenesis (n ≥ 11).
Data are expressed as the average±SD. *, p≤0.05, **; p ≤ 0.01.

Moreover, WT mice often developed rectal prolapse, which was rarely observed in the p38α-Δ^{MC} mice (Figure 14A), further indicating reduced disease susceptibility in p38α-Δ^{MC} mice. Analysis of animals sacrificed 100 days after the treatment started showed that macroscopic tumors were mainly located in the distal to middle colon of both WT and p38α-Δ^{MC} mice (Figure 14B).

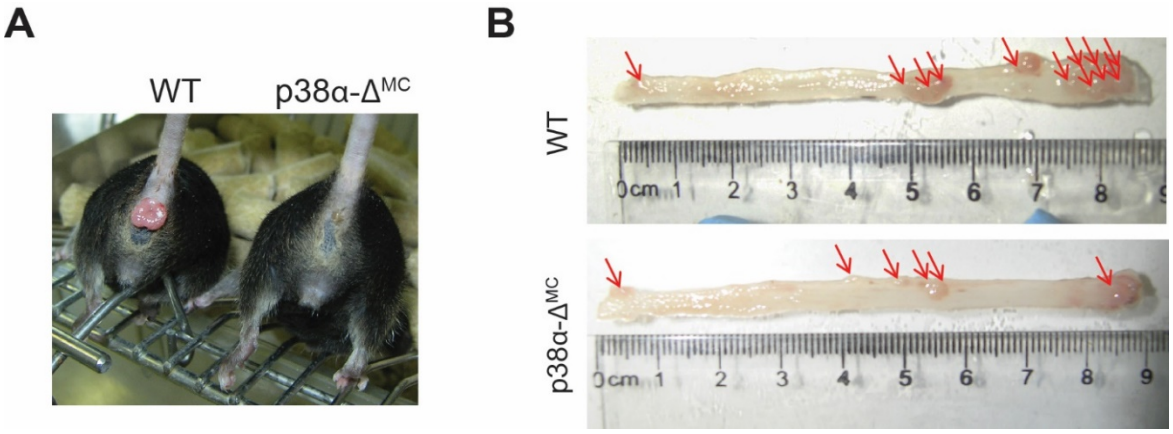


Figure 14. AOM/DSS treated p38α-Δ^{MC} mice develop less tumors and anal prolapses.

(A) Representative anorectal prolapse occurring in WT but very rarely in p38α-Δ^{MC} mice.
(B) Representative images of colon tumors. Red arrows indicate macroscopically visible and measured tumors.

The average tumor size was similar, but p38α-Δ^{MC} mice had significantly less tumors compared to WT mice (Figure 15A). In particular, p38α-Δ^{MC} mice had fewer tumors larger than 4 mm, and no tumors larger than 6 mm (Figure 15B). These results demonstrate that deficiency of p38α in myeloid cells reduces the susceptibility and severity of clinical and pathological parameters in the AOM/DSS-induced colon carcinogenesis model.

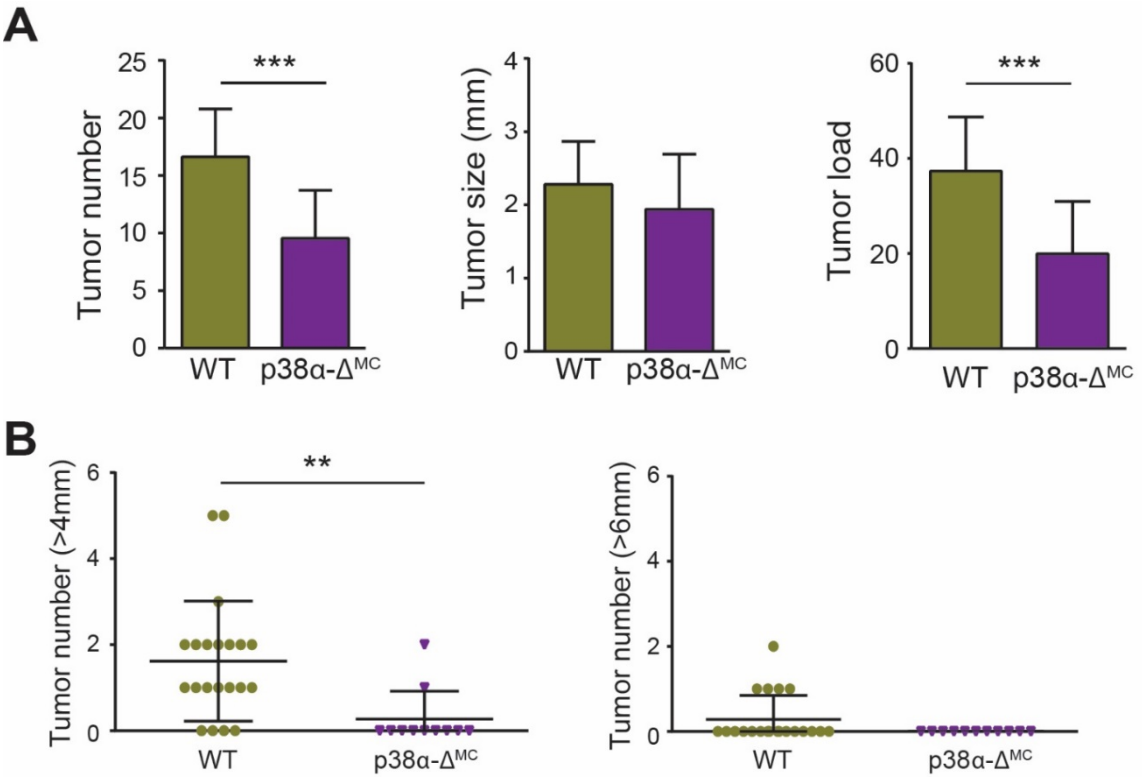


Figure 15. p38α deficiency in myeloid cells reduces colitis-associated tumorigenesis.

(C) Average tumor number, size and load (n ≥ 11).
(D) Number of tumors >4 mm and >6 mm in AOM/DSS-treated mice (n ≥ 11).
Data are expressed as the average±SD. **, p≤0.01, ***; p ≤ 0.001.

4.1.2 p38α deficiency in myeloid cells reduces the egression of Ly6C^{hi} monocytes from the bone marrow during AOM/DSS treatment

Monocytes are found in the blood and spleen, however, their largest reservoir is the bone marrow. The recruitment of these cells to sites of inflammation and their contribution to pathogenesis has been demonstrated in various conditions, including cancer (Biswas and Mantovani, 2010; Pittet and Swirski, 2011; Richards et al., 2013). Therefore, we analyzed if there were differences at the immunological level between p38α-Δ^{MC} and WT mice.

The bone marrow is considered a primary hematopoietic organ and besides of giving rise to cells of the MPS system (see section 1.2.2), it has also been implicated in function and trafficking of several other immune cells, such as T cells (Zhao et al., 2012). Interestingly, we found significantly less Ly6C^{hi}CCR2^{+} inflammatory monocytes in the bone marrow of p38α-Δ^{MC} mice compared to WT mice, indicating a weaker inflammatory response in tumor-bearing p38α-Δ^{MC} mice (Figure 16A and Figure 16B).

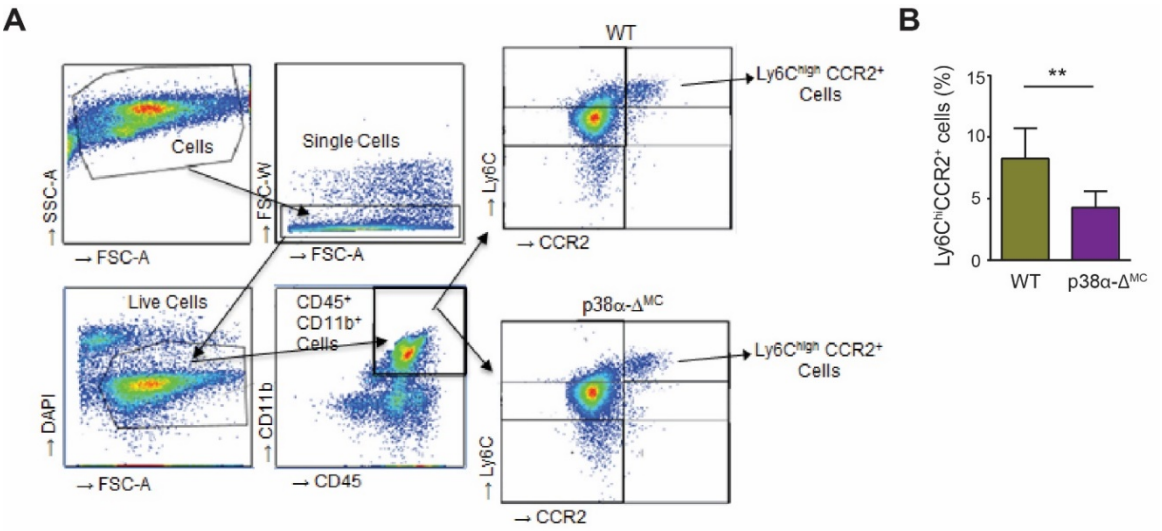


Figure 16. Downregulation of p38α in myeloid cells decreases pro-inflammatory monocyte recruitment during colitis-associated tumorigenesis.

(A) Representative FACS analysis of bone marrow cells from untreated mice. Frequency of each gated population as a percentage of the displayed cells is shown.
(B) Percentage of CD45^{+} CD11b^{+} cells that were Ly6C^{hi} and CCR2^{+} in the bone marrow ($n \geq 3$).
Data are expressed as the average±SD. **, $p \leq 0.01$.

However, other immune cell populations of the bone marrow, such as general leukocytes (CD45^{+}), myeloid cells (CD45^{+}CD11b^{+}), CD8^{+} T-cells, CD4^{+} T-cells or CD4^{+}CD8^{+}T-cells were not significantly different between WT and p38α-Δ^{MC} mice (Figure 17C).

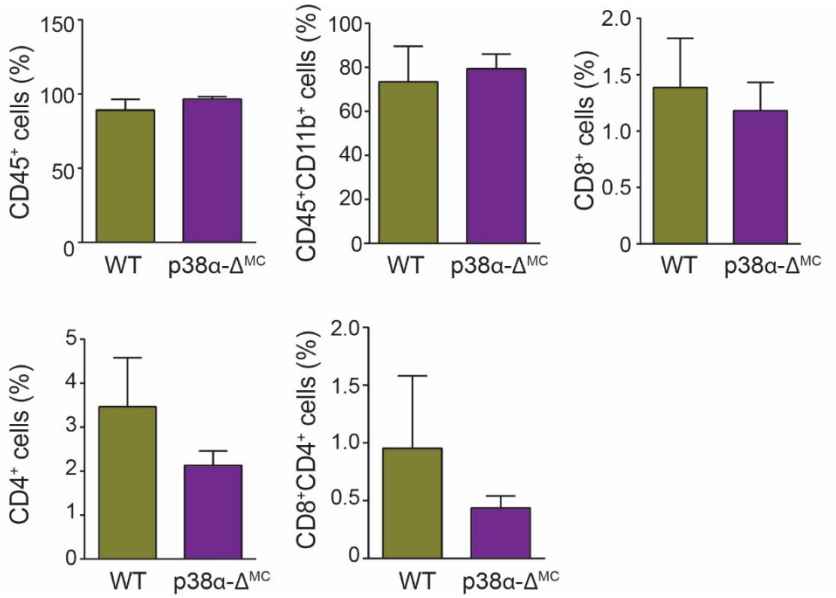


Figure 17. Downregulation of p38α in myeloid cells does not affect the general myeloid cell population or T-cells in the bone marrow.

Percentage of $CD45^+$, $CD45^+$ $CD11b^+$, $CD45^+CD4^+$, $CD45^+CD8^+$ and $CD45^+$ cells that were $CD4^+$ $CD8^+$ in bone marrow ($n \geq 3$).
Data are expressed as the average±SD.

Nevertheless, some WT animals showed spleen enlargement (splenomegaly), an indicator of systemic inflammation that has been correlated to tumor burden (Baltgalvis et al., 2009), which was rarely observed in p38α-Δ^{MC} mice (Figure 18A). However, we did not find significant differences in various immune cell populations, such as general leukocytes ($CD45^+$), myeloid cells ($CD45^+CD11b^+$), neutrophils ($CD11b^+Ly6G^+$), inflammatory monocytes ($Ly6C^{hi}CCR2^+$), $CD8^+$ T-cells, $CD4^+$ T-cells or $CD4^+CD8^+$T-cells of the spleen between WT and p38α-Δ^{MC} mice (Figure 18B). The observed splenomegaly could be due to increased hematopoiesis to replenish blood lost through stools due to tumors or ulcerations in WT mice. Splenic hematopoiesis has been reported in several animal models of disease including cancer and colitis, suggesting that spleens of sick animals are more skewed toward erythropoiesis and lymphopoiesis (Bronte and Pittet, 2013).

However, although we could not observe changes in the percentages of the analyzed immune cells in the spleen, given the observed enlargement of this organ we think it is likely that the total immune cell number between WT and p38α-Δ^{MC} mice could be affected, although this has not been evaluated so far. Thus, our results suggest that p38α-Δ^{MC} mice exhibit a reduced population of $Ly6C^{hi}CCR2^+$ monocytes in the bone marrow, which might reflect suppression of systemic and local inflammation and tumorigenic processes compared to WT mice.

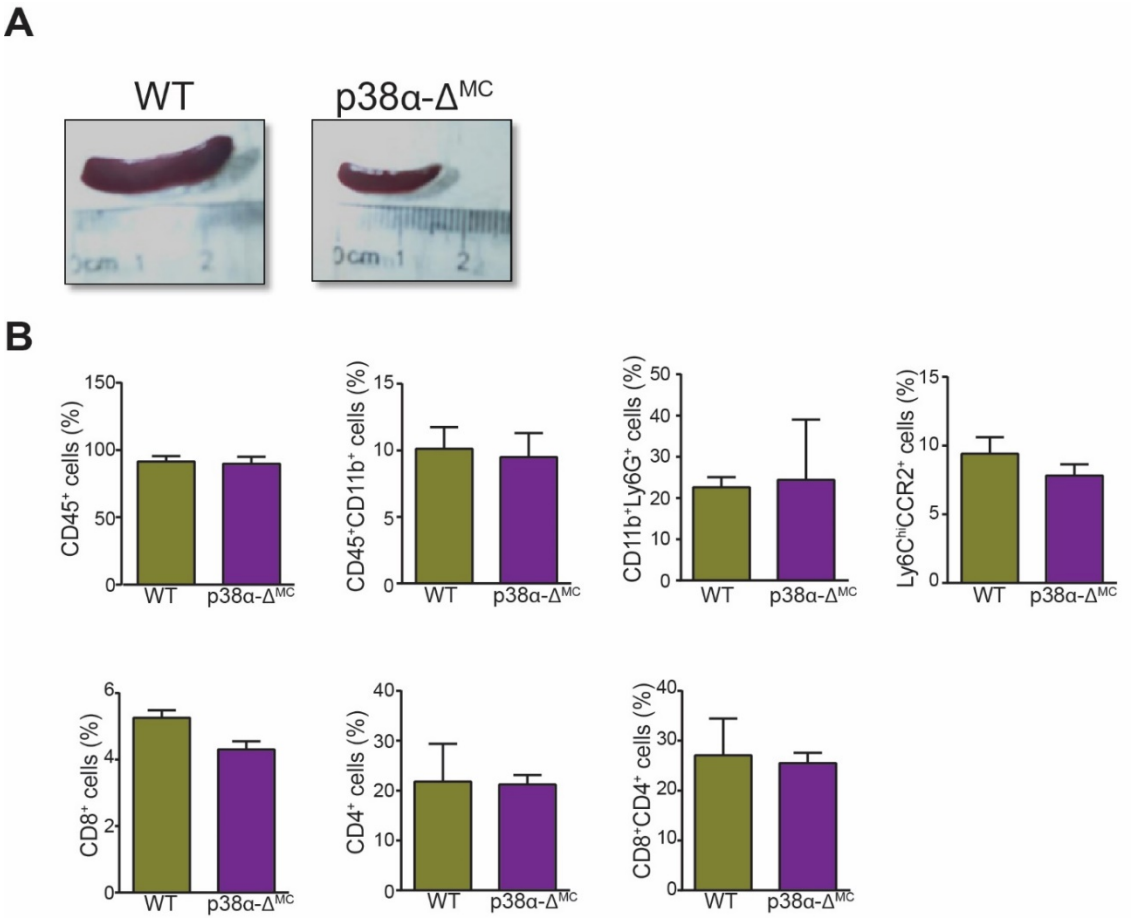


Figure 18. Analysis of spleens from AOM/DSS treated animals.

(A) Representative images of spleens in AOM/DSS-treated mice at day 100.
(B) Percentage of $CD45^+$, $CD45^+$ $CD11b^+$, $CD45^+$ $CD11b^+$ cells that were $Ly6G^+$, $CD45^+$ cells that were $CD4^+$ $CD8^+$ and $CD45^+CD11b^+$ cells that were $Ly6C^{hi}$ $CCR2^+$ in the spleens ($n \geq 3$).
Data are expressed as the average±SD.

4.1.3 Myeloid p38α controls the tumor promoting inflammatory microenvironment

Inflammation affects various aspects of tumorigenesis and immune cells infiltrated in tumors are engaged in an extensive and dynamic crosstalk with cancer cells (Grivennikov et al., 2010). Therefore, given that immune cells are major contributors to the inflammatory microenvironment, we evaluated the immune cell infiltrate in the tumors. In agreement with the reduced levels of $Ly6C^{hi}CCR2^+$ inflammatory monocytes detected in the bone marrow of p38α-Δ^{MC} mice compared to WT mice, we observed in the tumors of mice harboring p38α deficient myeloid cells a reduced macrophage infiltration ($F4/80^+$) measured by IHC (Figure 19).

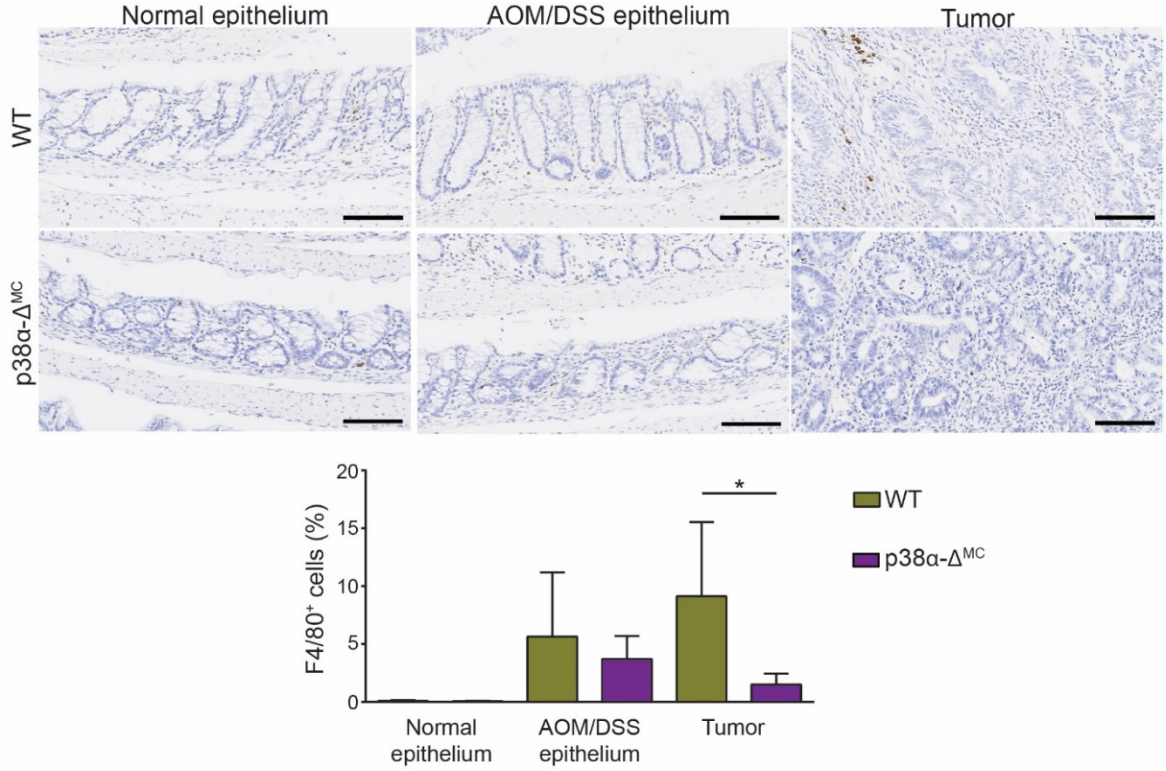


Figure 19. p38α deficiency in myeloid cells inhibits macrophage recruitment to tumors.

Representative sections from normal colon epithelia, epithelia after AOM/DSS treatment and colon tumors stained for F4/80. Quantifications are shown in the histogram below ($n \geq 3$). Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$.

We further measured the abundance of general leukocytes ($CD45^+$) and T-cells ($CD3^+$) in tumor and epithelial sections of mice treated with AOM/DSS, and observed a reduced infiltration in tumors of p38α-Δ^{MC} mice compared to WT mice (Figure 20), although differences were not as striking as for macrophages.

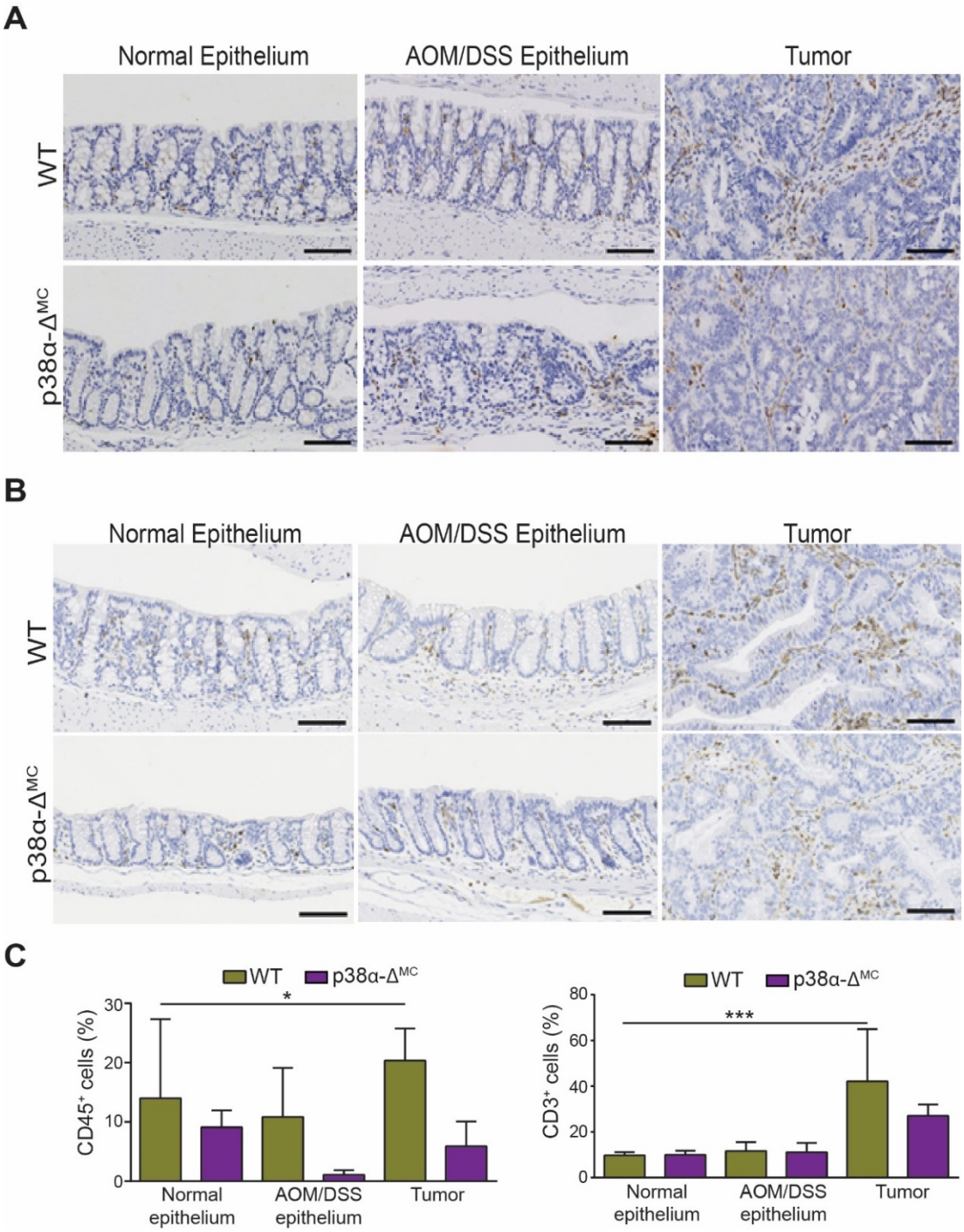


Figure 20. Leukocyte recruitment is suppressed in mice with myeloid p38α deficiency.

(A and B) Representative sections of normal colons, AOM/DSS treated epithelia and tumors stained for CD45 (A) and CD3 (B).
(C) Quantifications of normal colons, AOM/DSS treated epithelia and tumors stained for CD45 and CD3 (n ≥ 3).
Scale bars, 100 μm. Data are expressed as the average±SD. *, p≤0.05; ***, p ≤0.001.

Moreover, no significant changes in activated neutrophils (MPO^+) were observed between colon tumors of WT and p38α-Δ^{MC} mice (Figure 21). Taken together we

conclude that infiltration of immune cells is suppressed in tumors from p38α-Δ^{MC} compared to WT mice.

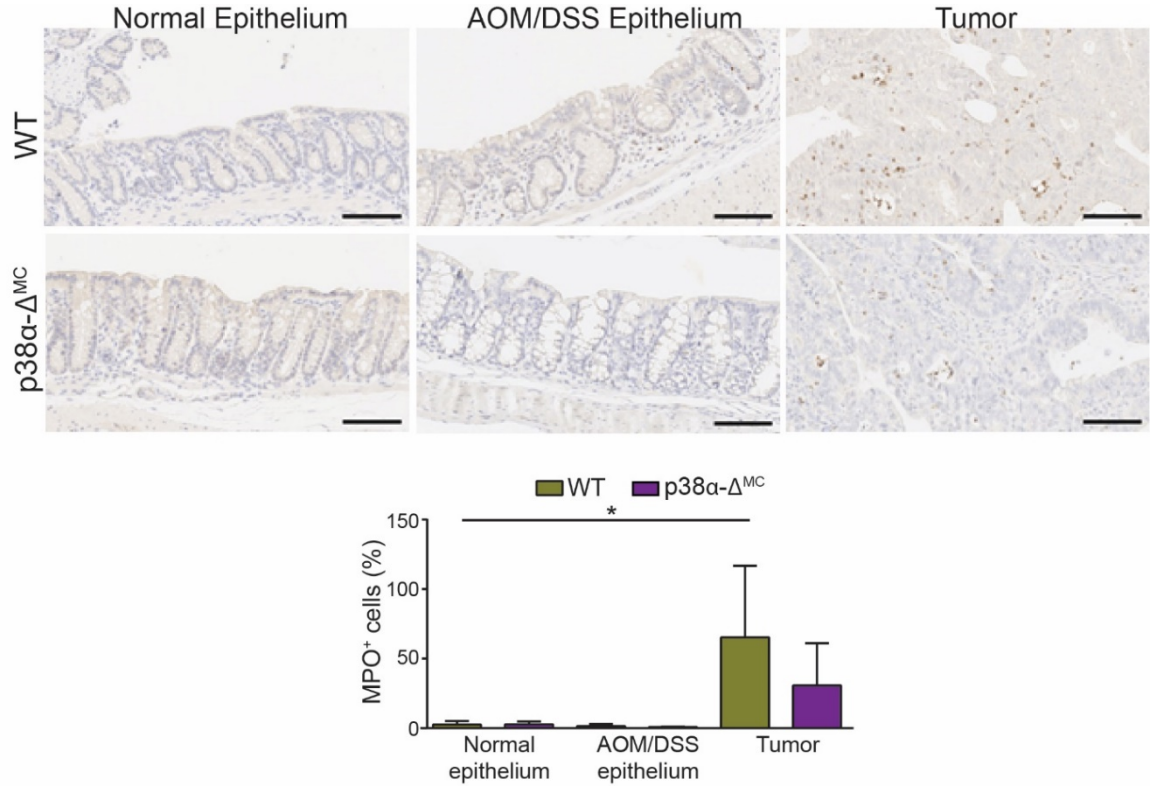


Figure 21. Activated neutrophils in AOM/DSS treated mice.

Representative sections of normal colons and AOM/DSS treated epithelia and tumors stained for MPO. Quantifications are shown in the histograms ($n \geq 3$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$.

The inflammatory TME is also characterized by augmented cellular proliferation (Colotta et al., 2009; Whiteside, 2008). We evaluated the phosphorylation status of STAT3, a potent activator of inflammatory pathways that contributes to oncogenic signaling leading to enhanced cell proliferation and tumor growth (Corvinus et al., 2005; Kim and Bae, 2016). As expected, tumors showed enhanced STAT3 phosphorylation compared to normal epithelium and, interestingly, STAT3 phosphorylation was reduced in tumors from p38α-Δ^{MC} mice compared to tumors from WT mice (Figure 22A and Figure 22C). Cell proliferation, measured as $Ki67^{+}$ cells, was upregulated in the tumors from WT mice compared to normal epithelium, whereas differences observed in tumors from p38α-Δ^{MC} mice were not significant (Figure 22B and Figure 22C).

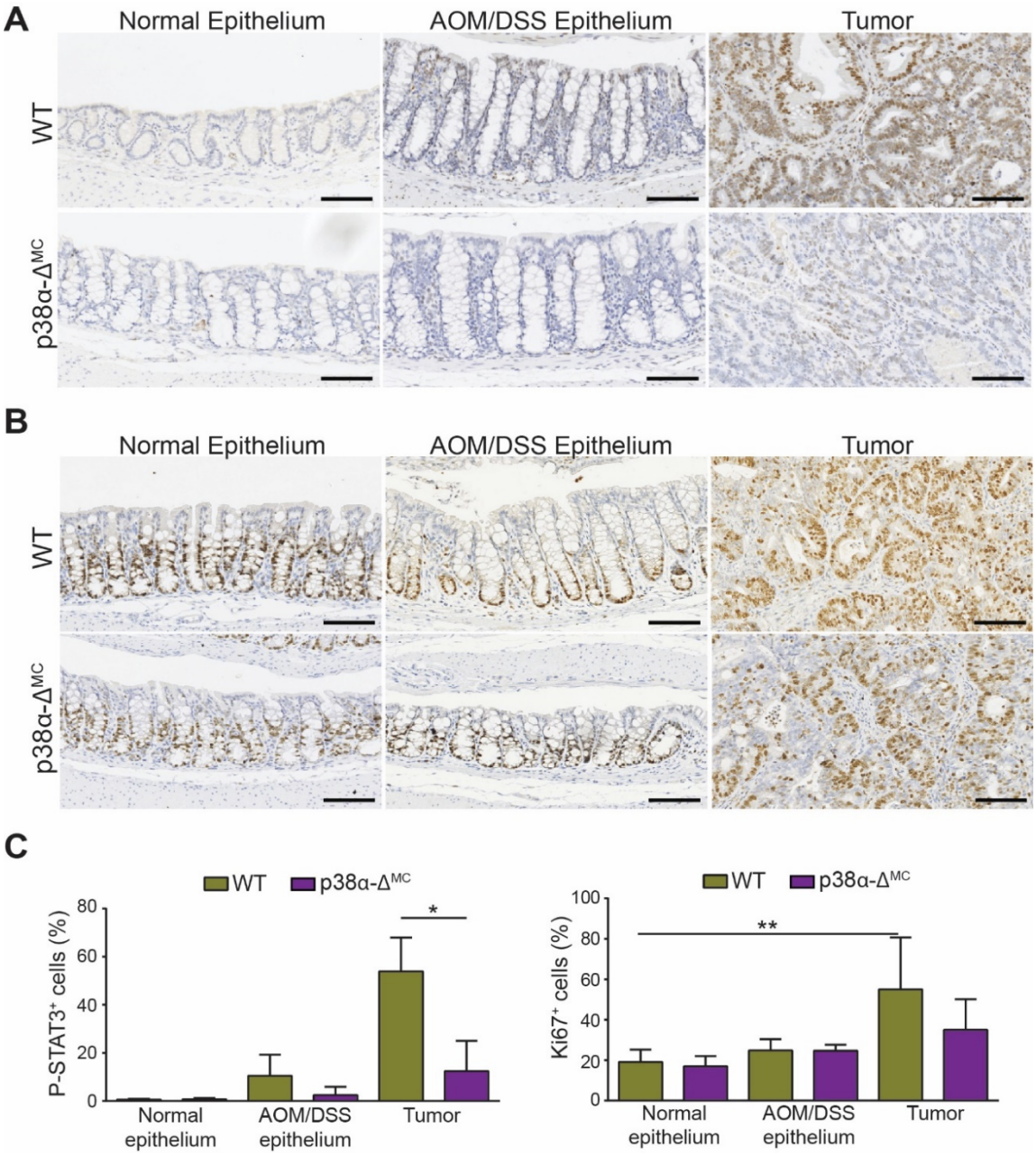


Figure 22. Deficiency of p38α in myeloid cells reduces inflammation in the tumor.

(A and B) Representative sections of normal colons, AOM/DSS treated epithelia and tumors stained for phospho-STAT3 (A) and Ki67 (B).
(C) Quantifications of normal colons, AOM/DSS treated epithelia and tumors stained for phospho-STAT3 and Ki67 ($n \geq 3$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$, **; $p \leq 0.01$.

However, although proliferation was higher in the tumors of WT mice compared to p38α-Δ^{MC} mice, we could not observe significant differences in apoptosis measured by TUNEL in the tumors (Figure 23). These results suggest that p38α in myeloid cells affects STAT3 phosphorylation without significantly affecting the proliferation and apoptosis ratios in colon tumors.

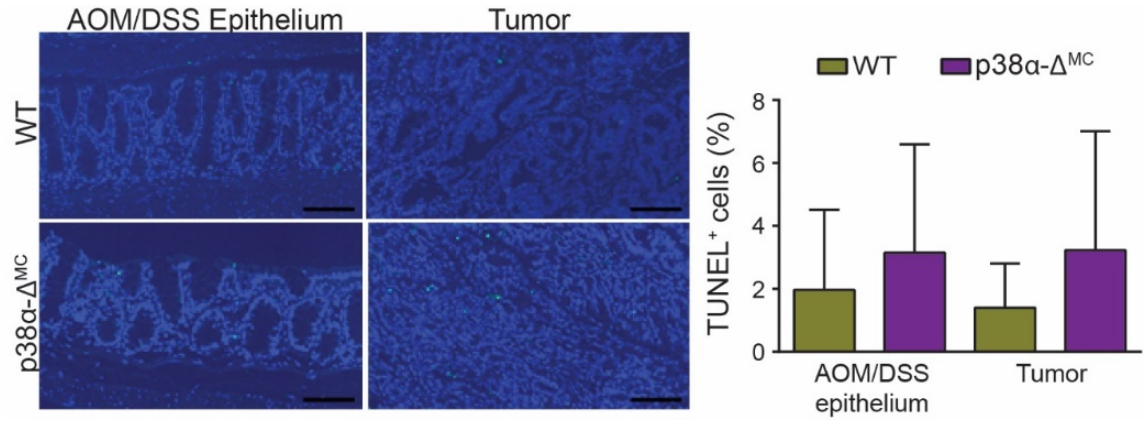


Figure 23. Apoptosis in AOM/DSS treated mice.

Representative sections of AOM/DSS treated epithelia and tumors stained for TUNEL. Quantifications are shown in the histogram ($n \geq 3$).
Scale bars, 100 μm. Data are expressed as the average±SD.

Since macrophages can promote angiogenesis by expressing extracellular factors and recruiting other haematopoietic cells (Pollard, 2004), we hypothesized that angiogenesis could be affecting the tumor burden in our mouse model. Therefore we measured endothelial $CD31^+$ cells as a marker for angiogenesis (Basilio-de-Oliveira and Pannain, 2015; Sharma et al., 2013), but could not observe any differences between tumor derived from WT and p38α-Δ^{MC} mice (Figure 24).

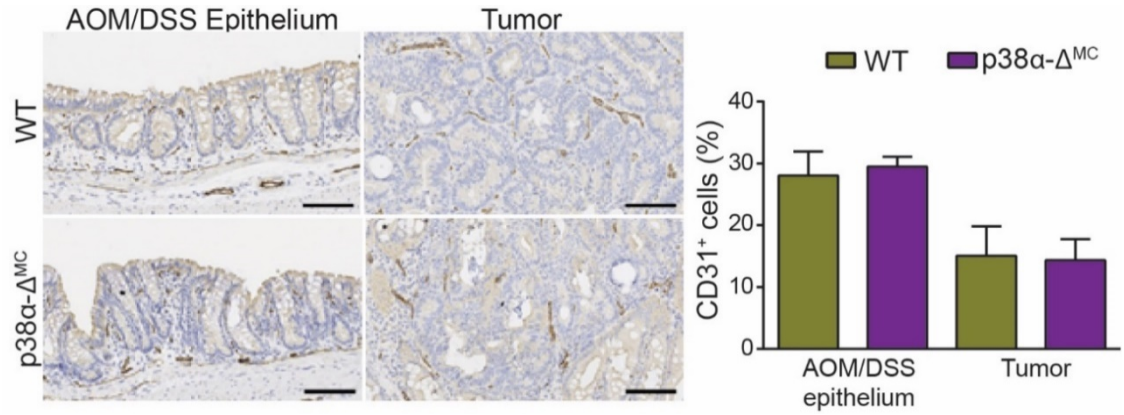


Figure 24. Angiogenesis is not affected by downregulation of p38α in myeloid cells.

Representative sections of AOM/DSS treated epithelia and tumors stained for CD31. Quantifications are shown in the histogram ($n \geq 3$).
Scale bars, 100 μm. Data are expressed as the average±SD.

4.1.4 Deficiency of p38α in myeloid cells reduces DSS-induced inflammation

Given that inflammation is an essential component of AOM/DSS-induced tumorigenesis, we investigated the contribution of myeloid p38α to the DSS response. The model of DSS-induced acute colitis is a widely used experimental model in which DSS acts as an irritant to the epithelial mucosa of the colon, resulting in diarrhea and initiation of inflammatory processes (Okayasu et al., 1990; Perse and Cerar, 2012; Seril et al., 2003).

Colon inflammation in this model is induced by providing mice *ad libitum* with DSS, typically in the drinking water. Depending on various factors, such as mouse strain and housing conditions, the concentration and duration of the DSS treatment needs to be adjusted to the mice used in the experiments (Chassaing et al., 2014; Perse and Cerar, 2012; Stevceva et al., 1999). In our case, we treated mice for 6 days with DSS (1.5 %) and replaced DSS by regular drinking water for 7 more days, until day 13 (Figure 25).

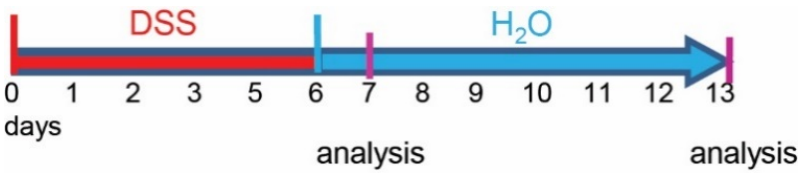


Figure 25. Schematic representation of the experimental design for DSS-induced acute colitis.

Pink lines indicate days 7 and 13, the time points at which animals were sacrificed.

The administration of DSS induced the expected colonic inflammatory status, exhibiting several characteristics similar to human UC, such as diarrhea, shortening of colon, multiple erosions and inflammatory mucosal changes, loss of crypts and ulcerations (Clapper et al., 2007; Randhawa et al., 2014). As previously described, the colonic inflammation process induced by DSS was associated with a decrease in mouse body weight, which was less severe in p38α-Δ^{MC} mice compared to WT mice (Figure 26A). In concordance with the body weight loss, the DAI score was also increased to a higher extent in WT than in p38α-Δ^{MC} mice (Figure 26B).

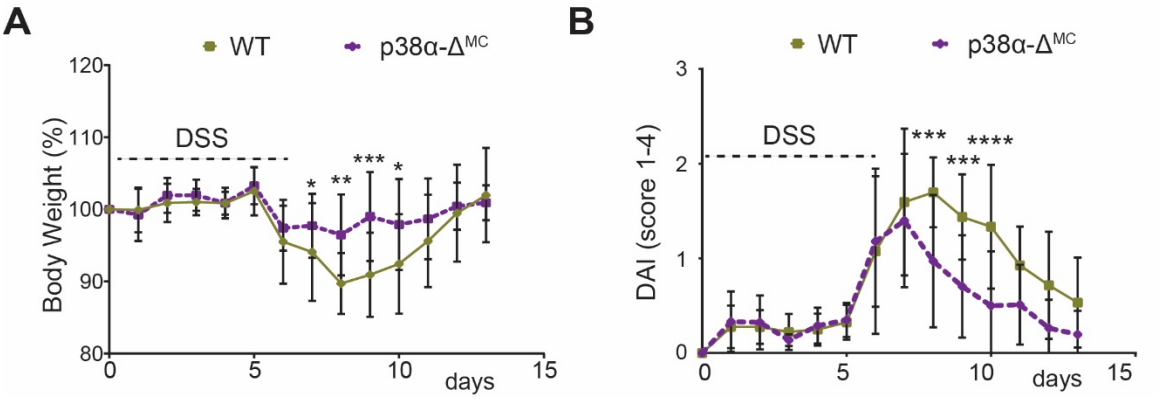


Figure 26. Myeloid deletion of p38α decreases DSS-induced body weight loss and DAI.

(A and B) Body weight (A) and DAI (B) were recorded daily in DSS-treated mice (n ≥ 9).
Scale bars, 100 μm.
Data are expressed as the average±SD. *, p≤0.05; **, p ≤0.01; ***, p≤0.001; ****, p≤0.0001.

Another inflammatory indicator of colitis is the colon length (Diaz-Granados et al., 2000), which was slightly shorter in DSS-treated WT mice compared to p38α-Δ^{MC} mice (Figure 27).

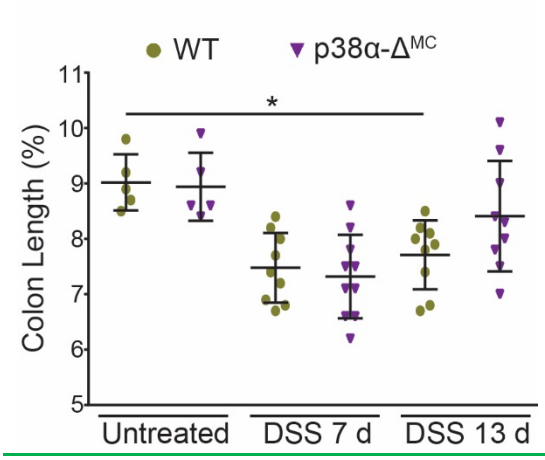


Figure 27. Myeloid deletion of p38α alleviates DSS-induced colon shorting.

The total length of each colon dissected from untreated or DSS-treated mice was measured under a constant weight (2 g; n ≥ 5).
Data are expressed as the average±SD. *, p≤0.05.

The epithelial damage was also assessed microscopically in H&E stained colon sections using a semi-quantitative scoring system, by evaluating the extent (1-4) and percentage (1-4) of epithelial damage observed in the intestinal mucosa (divided by 2), as previously described (Gupta et al., 2014). In line with the decreased severity of colitis indicators observed, the large areas of complete crypt loss and erosions detected in colons from DSS-treated WT mice were strongly reduced in p38α-Δ^{MC} mice. Of note, no differences in colon histology were observed between WT and p38α-Δ^{MC} mice prior to DSS administration (Figure 28). Therefore, our results indicate that p38α-Δ^{MC} mice are

less susceptible to DSS-induced colitis, in agreement with a previous report (Otsuka et al., 2010).

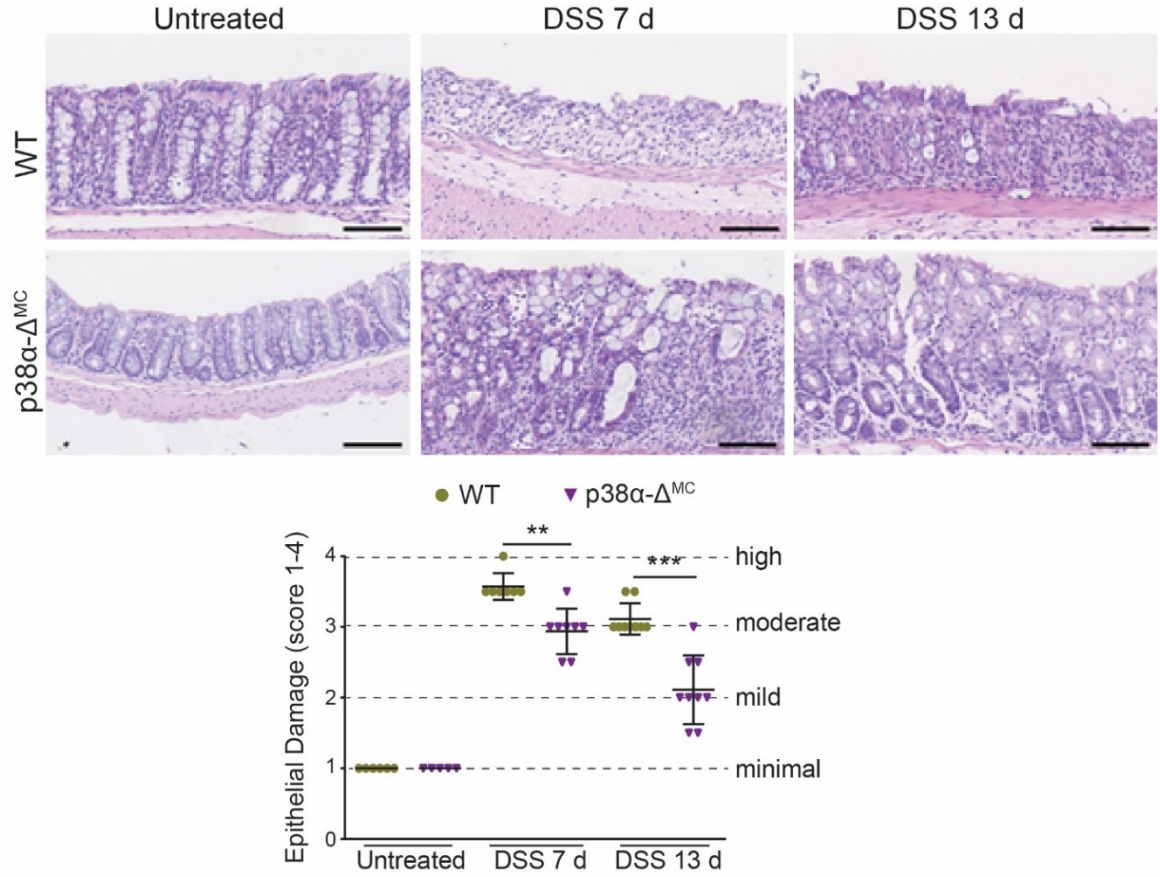


Figure 28. Myeloid deletion of p38α decreases DSS-induced colitis susceptibility.

Representative images of H&E-stained colon sections from animals either untreated or treated with DSS for 6 days and analyzed at days 7 and 13. The histogram below illustrates the quantification of epithelial damage in untreated mice or mice treated with DSS for 6 days and analyzed at days 7 and 13 using H&E-stained colon sections ($n \geq 5$).
Scale bars, 100 μm. Data are expressed as the average±SD. **, $p \leq 0.01$; ***, $p \leq 0.001$.

The analysis of tumors indicated that p38α downregulation in myeloid cells affected inflammatory cell recruitment (see section 4.1.3). We therefore extended this analysis in the epithelia of DSS treated mice to induce acute colitis. Indeed, IHC analysis of different immune cell markers in untreated and DSS treated mice sacrificed at day 7 and day 13 revealed a prominent inhibition of inflammatory cell recruitment in p38α-Δ^{MC} mice during acute colitis, in particular during the pro-inflammatory phase at day 7 of treatment. Both, macrophages ($F4/80^{+}$) and general leukocytes ($CD45^{+}$) were reduced in the immune cell infiltrates of DSS-treated p38α-Δ^{MC} mice compared to WT mice (Figure 29A and Figure 29B).

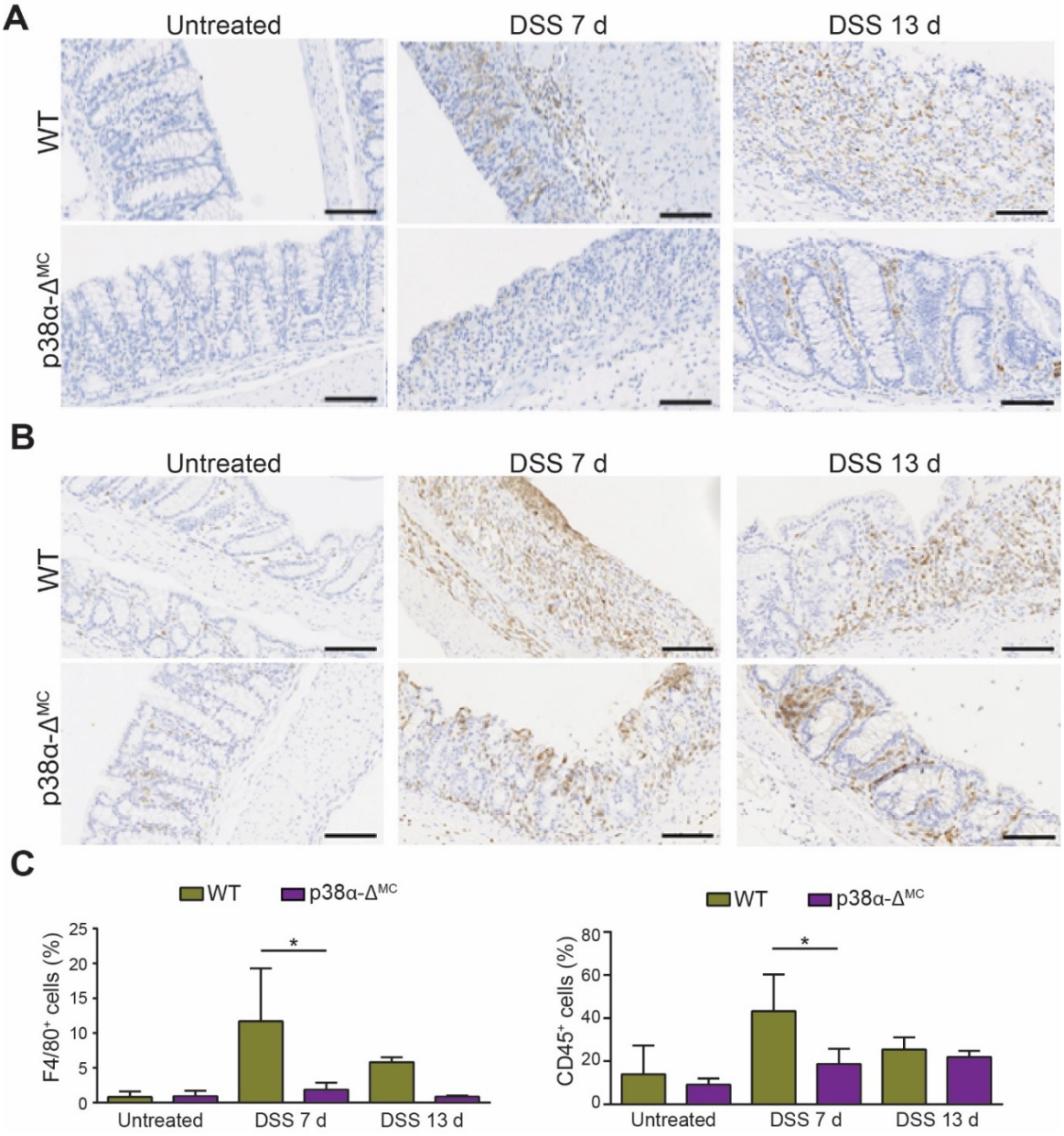


Figure 29. Myeloid deletion of p38α decreases leukocyte recruitment to the inflamed intestine.

(A and B) Representative colon sections stained for F4/80 (A) and CD45 from untreated mice or mice treated with DSS for 6 days and analyzed at days 7 and 13.
(C) Quantifications of F4/80 and CD45 in the epithelia from the indicated mice ($n \geq 4$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$.

Similarly, activated neutrophils (MPO^+) and T-cells ($CD3^+$) were less recruited to the epithelia of p38α-Δ^{MC} mice compared to WT mice, 7 days after starting the DSS administration (Figure 30A and Figure 30B).

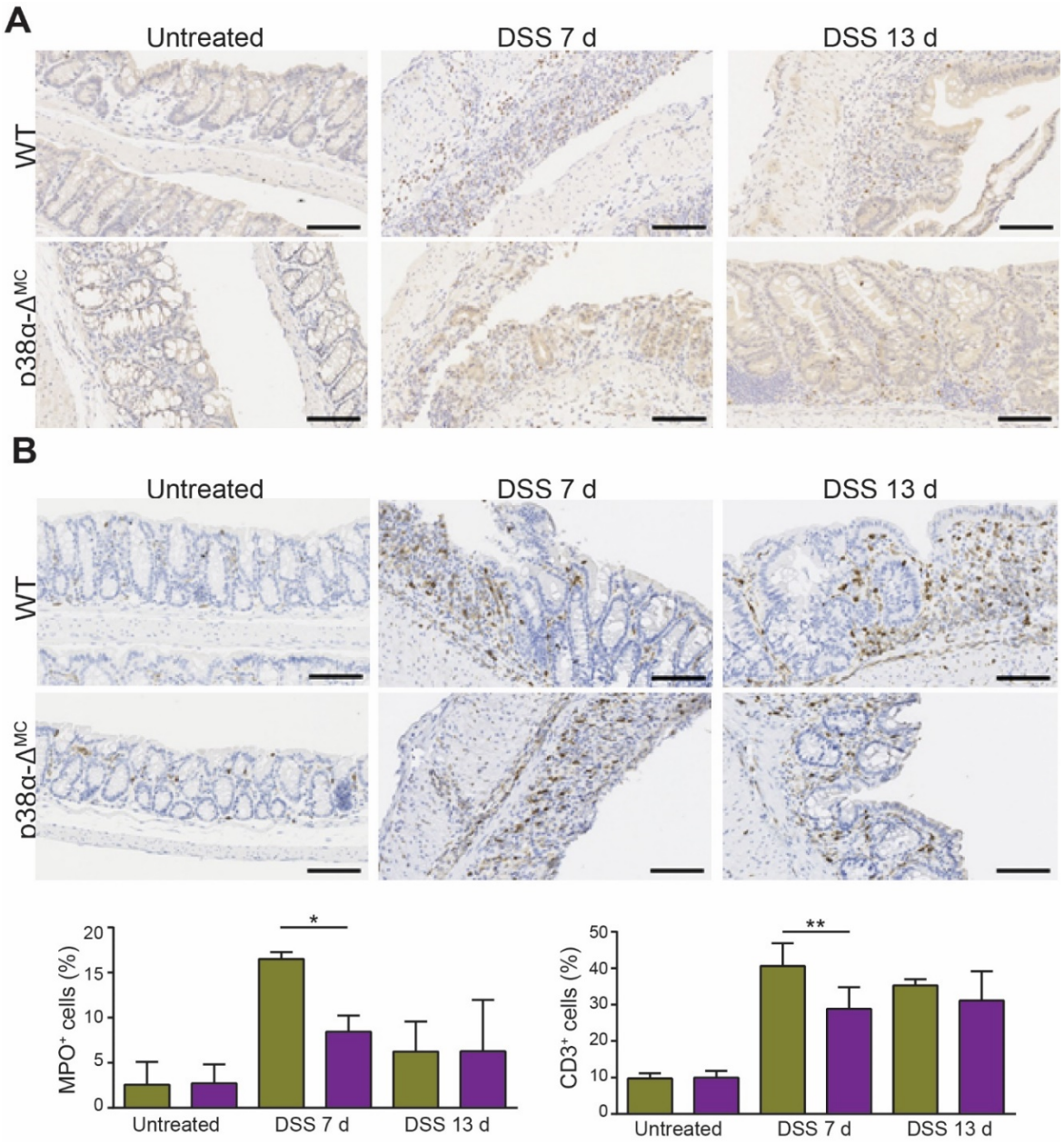


Figure 30. Myeloid deletion of p38α decreases neutrophil and T-cell recruitment to the inflamed intestine.

(A and B) Representative colon sections stained for MPO (A) and CD3 (B) from untreated mice or mice treated with DSS for 6 days and analyzed at days 7 and 13.
(C) Quantifications of MPO and CD3 in the epithelia from the indicated mice ($n \geq 4$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$.

Moreover, infiltrating immune cells produce cytokines that activate STAT3 and its target genes contributing to tumor-promoting inflammation (Jarnicki et al., 2010; Yu et al., 2009). Accordingly, STAT3 phosphorylation was significantly reduced in the colon epithelia of DSS-treated p38α-Δ^{MC} mice compared to WT mice at day 7 after initiation of DSS administration (Figure 31A and Figure 31B). However, STAT3 mRNA levels were

similar in the colons from WT and p38α-Δ^{MC} mice (Figure 31C). These results indicated that STAT3 phosphorylation is induced during active inflammation and this signal transduction pathway is dependent on the activation of p38α in myeloid cells.

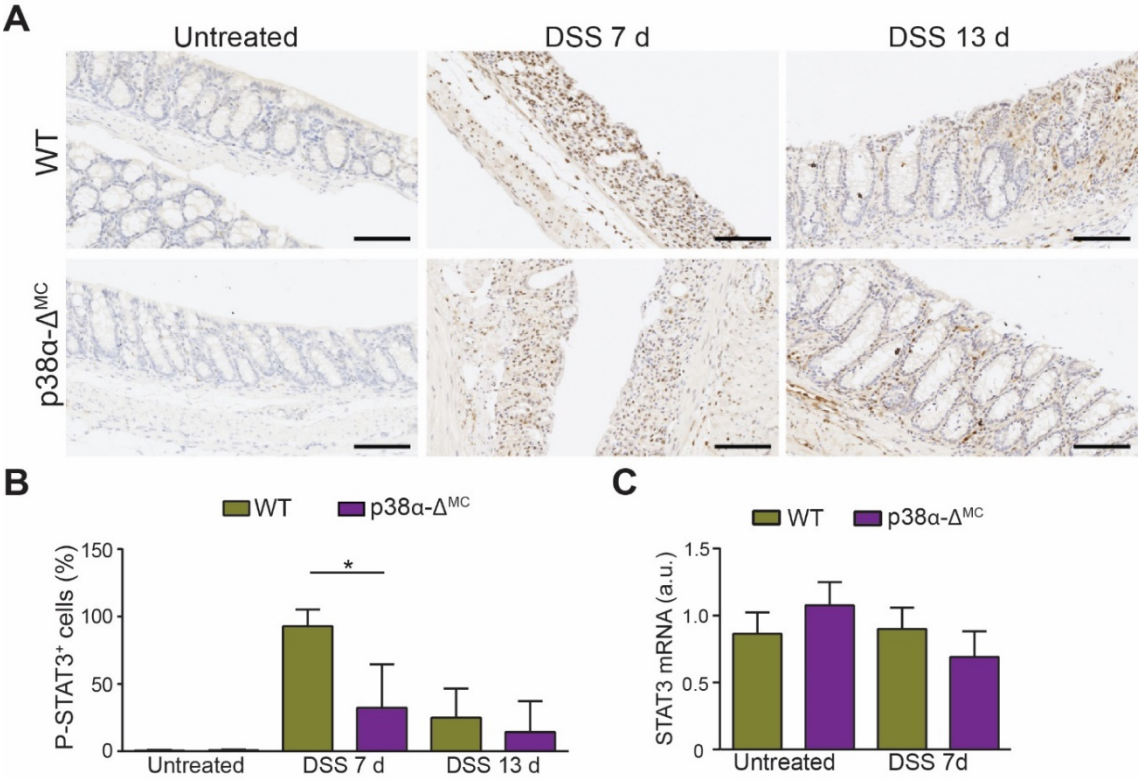


Figure 31. Downregulation of p38α in myeloid cells suppresses expression of inflammatory mediators in colons from DSS treated mice.

(A and B) Representative colon sections from untreated mice and mice treated with DSS for 6 days and analyzed at day 7 and day 13 were stained for phospho-STAT3. Quantifications are shown in the histogram ($n \geq 4$) (B).
(C) STAT3 mRNA levels in mouse colons from DSS-treated mice analyzed at day 7 and 13 were determined by qRT-PCR ($n \geq 5$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$.

Colon inflammation in DSS-treated mice also induces the production of pro-inflammatory cytokines (e.g. IL-1β and TNFα) and their intestinal expression has been correlated to the severity of colitis (Bertevello et al., 2005; Egger et al., 2000; Ghia et al., 2007). ELISA analysis revealed that inflammatory molecules such as IL-1β were downregulated in DSS-treated p38α-Δ^{MC} mice compared to WT mice (Figure 32A), and TNF-α was also induced at lower levels upon DSS treatment in p38α-Δ^{MC} mice (Figure 32B). Taken together, the results indicate that p38α signaling in myeloid cells contributes to the colitis severity induced by DSS.

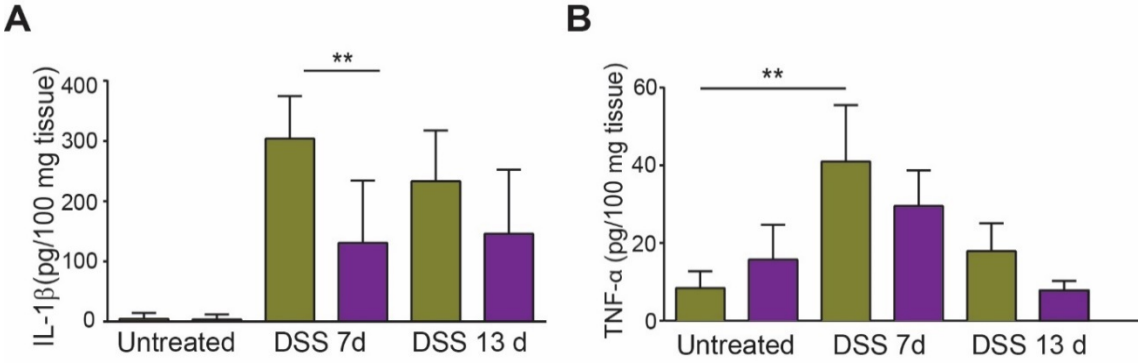


Figure 32. Downregulation of p38α in myeloid cells suppresses expression of inflammatory mediators in colons from DSS treated mice.

(A and B) Colon lysates from mice untreated or treated with DSS for 6 days were analyzed at day 7 by ELISA for IL-1β (A) and TNF-α (B) expression levels ($n \geq 5$).
Scale bars, 100 μm. Data are expressed as the average±SD. **; $p \leq 0.01$.

4.1.5 p38α regulates IGF-1 expression in macrophages

Our results clearly implicate p38α signaling in myeloid cells in intestinal inflammation and CAC. Therefore, we aimed to identify downstream mediators of p38 MAPK signaling in macrophages that might impinge on the disease by affecting the inflammatory microenvironment. Supernatants from BMDMs obtained from mice WT or with myeloid cells deficient in both p38α and p38β were used to analyze a cytokine array. Indeed, we found several cytokines and chemokines, potentially involved in intestinal inflammatory pathogenesis, which were differentially expressed in supernatants derived from p38α-Δ^{MC} BMDM compared to WT supernatants. Among the downregulated ones we found IGF-1, IGFBP-2, Resistin, Lungkine (CXCL15), macrophage derived chemokine (MDC) and thymus chemokine-2 (TCK-1; CXCL7). Upregulated molecules included IGFBP-5, IGFBP-6, vascular cell adhesion molecule-1 (VCAM-1), CX3CL1, IL-13 and soluble tumor necrosis factor Receptor II (sTNFRII) (Figure 33).

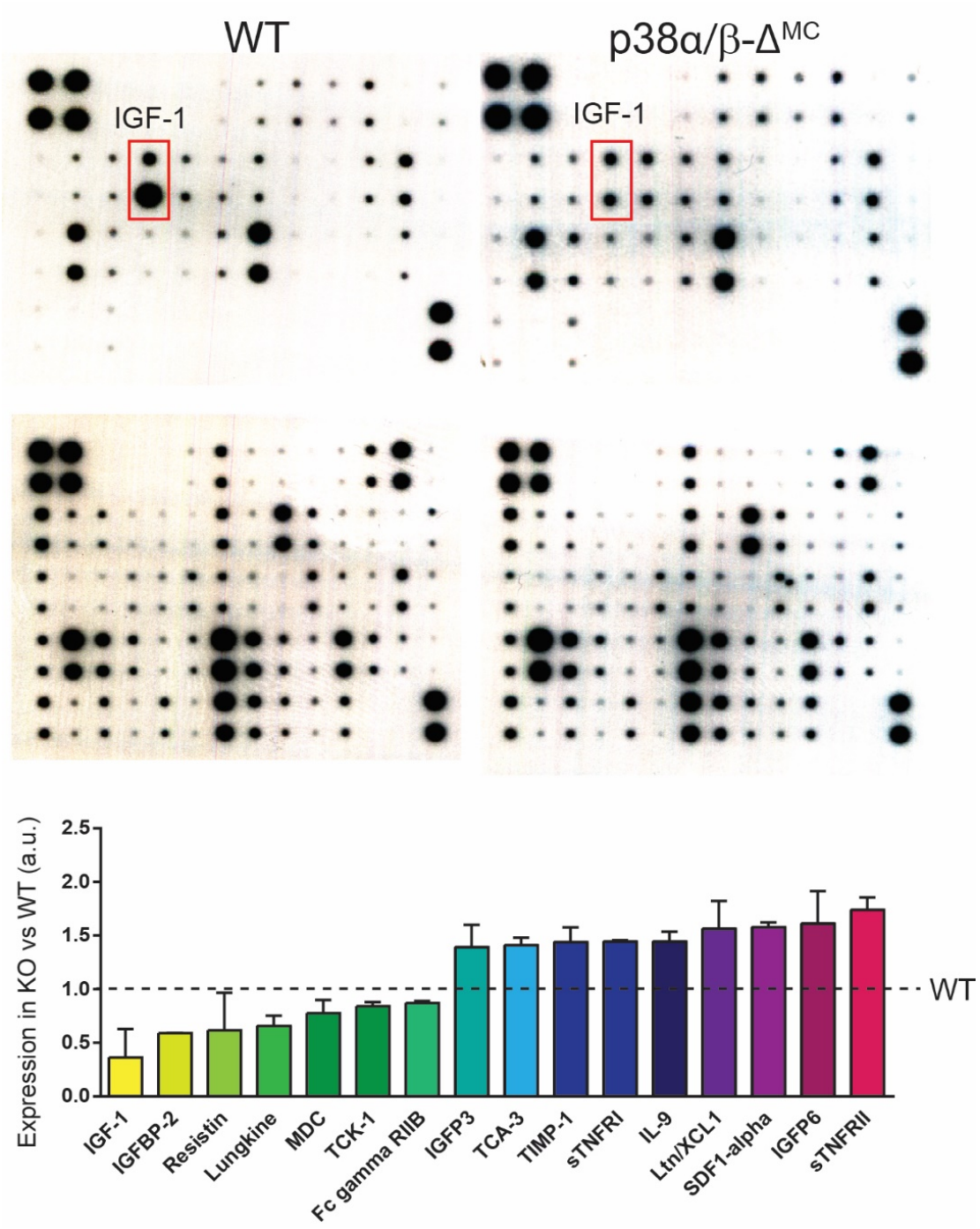


Figure 33. Cytokine arrays analyzed with supernatants derived from BMDMs.

A mouse cytokine antibody array was interrogated using a pool of supernatants derived from non-stimulated mice BMDM (n=3/genotype). Quantifications of the most prominent changes are shown in the lower panel. Arbitrary units are referred to the expression level of each chemokine in WT mice, which was given the value of 1.

We decided to further evaluate the link with IGF-1, which was the most obvious change, and because it has not been previously reported to be regulated by p38α in

macrophages. Moreover IGF-1 has been implicated in the regulation of inflammatory processes, inflammatory cell recruitment and tumorigenesis (see section 1.3.1.3.1).

First, we confirmed the results from the cytokine array analyzing IGF-1 protein and mRNA expression in BMDMs derived from WT and p38α-Δ^{MC} mice. Indeed, we found reduced intracellular and extracellular IGF-1 protein levels in p38α-deficient BMDMs compared with WT BMDMs (Figure 34A).

The IGF-1 mRNA expression level detected under basal conditions in WT macrophages was induced by different stimuli. Thus, LPS from bacteria, related to the classical macrophage activation phenotype (M1) and inflammation (Ying et al., 2013), as well as IL-4, which is related to tissue healing processes (Ferrante and Leibovich, 2012) were both able to upregulate IGF-1 mRNA expression. However, our results showed that IGF-1 mRNA was more potently induced by IL-4 than by LPS (Figure 34B), in agreement with its proposed role as marker for wound-healing macrophages (Roszer, 2015; Tonkin et al., 2015), which contribute to tumor progression (Murray and Wynn, 2011). Finally, the implication of p38α signaling in IGF-1 production by macrophages was confirmed by using the chemical inhibitors SB-203580, PH-797804 and LY-2228820 (Figure 34B and Figure 34C).

The *in vitro* studies demonstrated that IGF-1 expression is controlled by p38α activation in BMDMs. Moreover, IGF-1 expression is potently induced in BMDM activated by IL-4, a wound-healing cytokine also involved in tumor progression and a major regulator of TAM phenotypes (Novak and Koh, 2013; Paul and Zhu, 2010; Varin and Gordon, 2009).

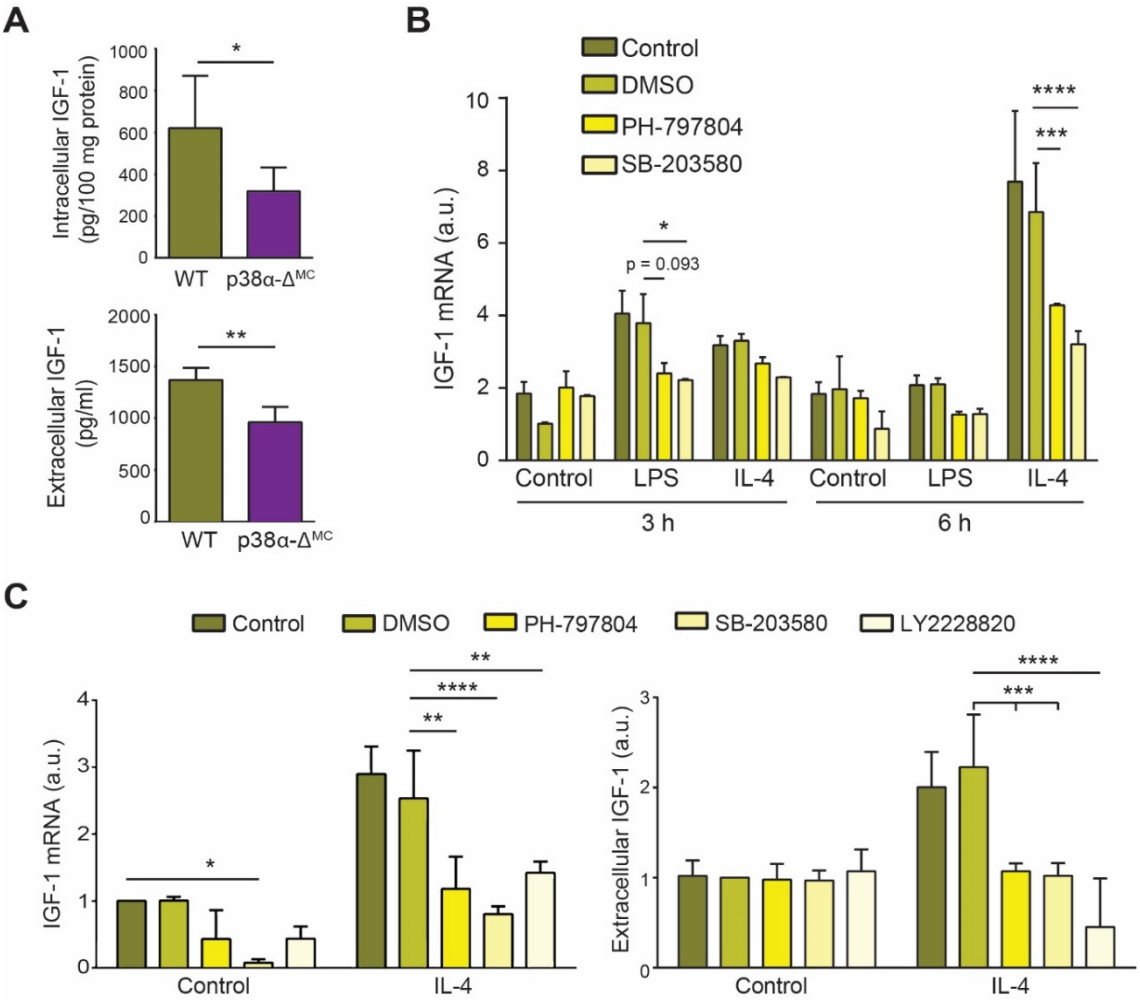


Figure 34. p38α regulates IGF-1 production by BMDMs.

(A) Whole protein lysates (upper panel) or supernatants (lower panel) of BMDMs were used to analyze IGF-1 protein levels by ELISA (n = 5).
(B) BMDMs obtained from WT mice were starved for 17 h prior to stimulation with either LPS (10 ng/ml) or IL-4 (10 ng/ml) for the indicated times in the presence or absence of the indicated p38α inhibitors or vehicle (DMSO). The inhibitors were added to the medium 1 h prior to stimulation with LPS or IL-4 and IGF-1 mRNA expression levels were measured by qRT-PCR (n = 3). Arbitrary units are referred to the expression level in DMSO-treated Control at 3 h, which was given the value of 1.
(C) WT BMDMs were starved for 17 h, then the indicated p38α inhibitors or DMSO were added to the medium 1 h prior to stimulation with IL-4 for 6 h. Supernatants were collected and used to measure IGF-1 mRNA expression levels (left panel) and IGF-1 protein levels by ELISA (right panel) (n = 3). Arbitrary units are referred to the expression level in the DMSO-treated Control (about 700 pg/ml), which was given the value of 1.
Data are expressed as the average±SD. *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001

To corroborate the *in vitro* results in BMDMs, we isolated intestinal macrophages and confirmed by F4/80, and by CD115 (CSFR-1), another widely used marker for macrophages (Sasmono et al., 2003), the identity of the isolated cells (Figure 35).

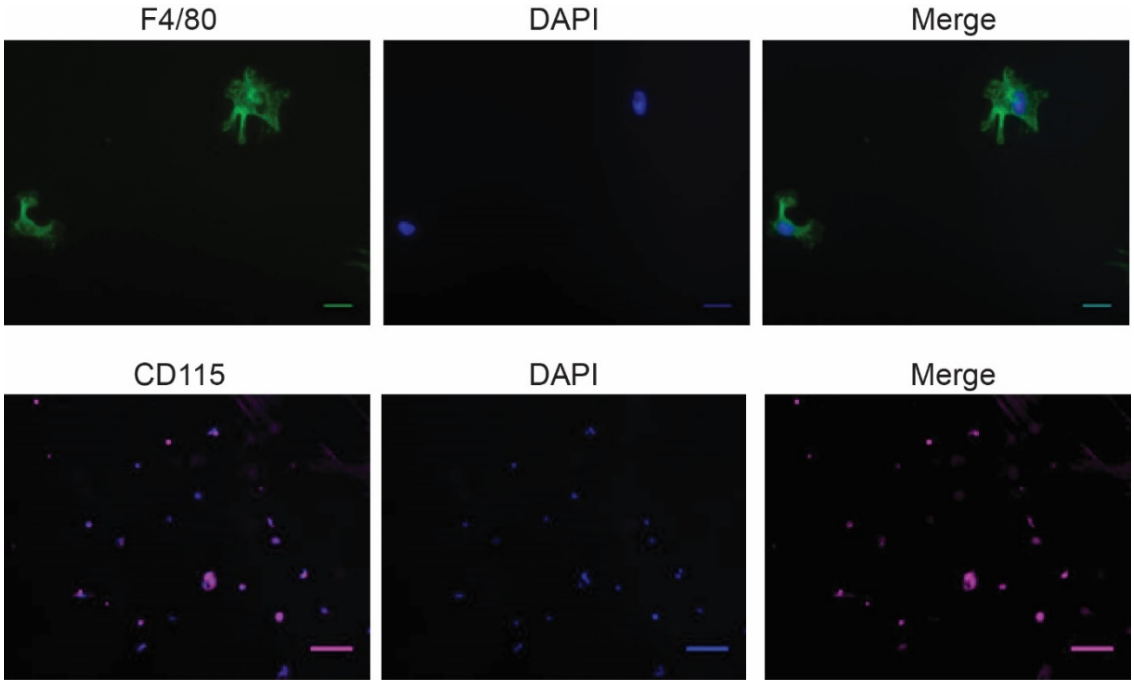


Figure 35. Isolation of intestinal macrophages.

Representative images of intestinal macrophages isolated from the colonic lamina propria and stained for F4/80 and CD115.
Scale bars, 10 μm (F4/80) and 100 μm (CD115).

This analysis showed decreased IGF-1 mRNA levels in intestinal macrophages from p38α-Δ^{MC} mice compared to WT mice (Figure 36A). In response to DSS, intestinal macrophages switch from the initial classical activation phenotype (day 7) to a wound-healing phenotype in the repair phase (day 13) (Enderlin Vaz da Silva et al., 2014; Ortega-Gomez et al., 2013; Serhan and Savill, 2005; Sugimoto et al., 2016). Accordingly, intestinal macrophages isolated from WT and p38α-Δ^{MC} mice 7 days after the DSS treatment, showed stronger differences in IGF-1 expression levels compared to the untreated animals (Figure 36A, Day 13). The downregulation of p38α in intestinal macrophages from p38α-Δ^{MC} mice was verified by quantifying the deletion of the floxed exon 2 of p38α (Figure 36B). Thus, these results suggest that p38α MAPK signaling in myeloid cells contributes to IGF-1 expression in macrophages.

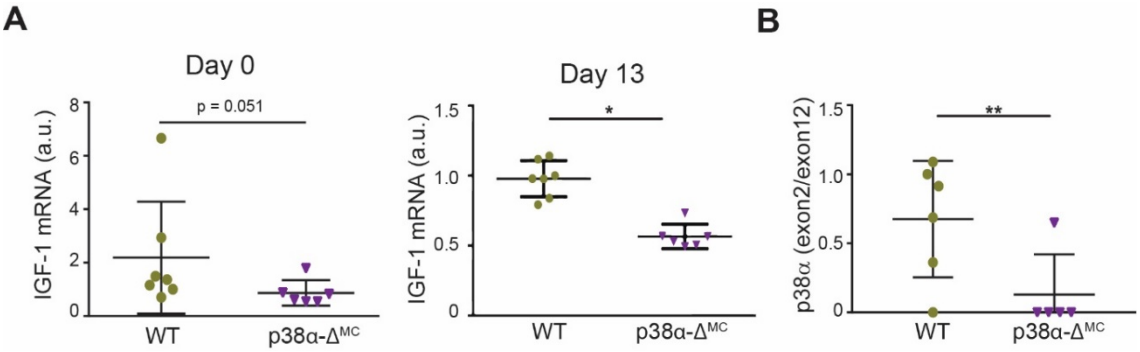


Figure 36. p38α regulates IGF-1 production in intestinal macrophages.

(A) Intestinal macrophages were isolated from mice at the indicated days. IGF-1 mRNA levels were quantified by qRT-PCR ($n \geq 5$).
(B) Analysis by qRT-PCR of the levels of floxed exon 2 versus exon 12 (as a control) of the p38α gene in intestinal macrophages ($n \geq 6$).
Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$.

4.1.6 Downregulation of p38α in myeloid cells reduces IGF-1 signaling during intestinal inflammation and tumorigenesis

To confirm that p38α in myeloid cells regulates IGF-1 signaling during intestinal inflammation and tumorigenesis, we evaluated IGF-1 levels and IGF-1 signaling in mice treated with DSS to induce colitis or with AOM/DSS to induce CAC.

In agreement with our findings in intestinal macrophages (see Figure 36), IGF-1 protein levels were significantly reduced in whole colon extracts from DSS-treated p38α-Δ^{MC} mice compared to WT mice during the repair phase at day 13 (Figure 37A), when macrophages adopt the alternative activation phenotype. Moreover, qRT-PCR analysis indicated lower levels of IGF-1 mRNA at day 13 in colon extracts from p38α-Δ^{MC} mice compared to WT mice (Figure 37B). However, the differences using whole colon extracts were not as clear as in the case of isolated intestinal macrophages, supporting the importance of myeloid cells as a source of IGF-1 in the colon.

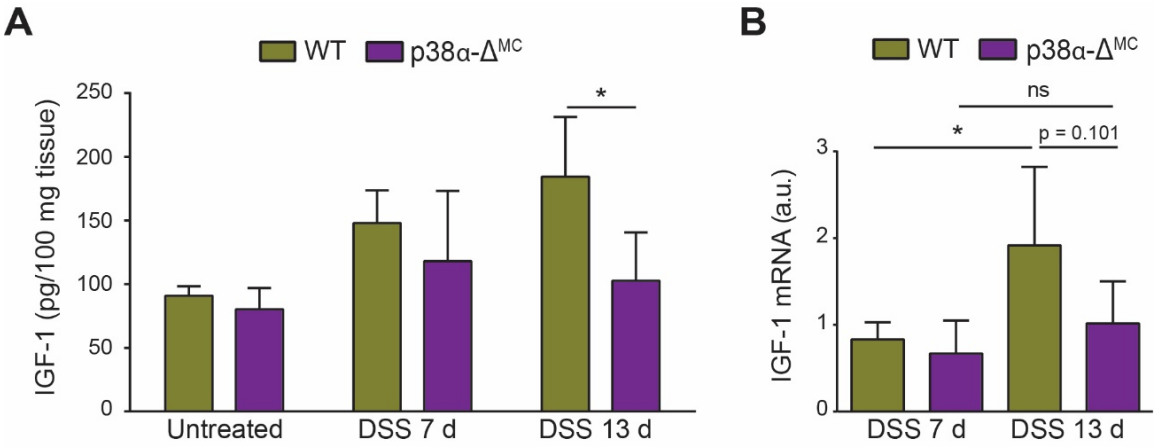


Figure 37. Downregulation of myeloid p38α reduces IGF-1 production during intestinal inflammation.

(A and B) Colon protein lysates obtained from mice either untreated or treated with DSS for 6 days, were analyzed at days 7 and 13 to measure IGF-1 levels by ELISA ($n \geq 3$) (A) or for IGF-1 mRNA levels by qRT-PCR ($n \geq 4$) (B).
Data are expressed as the average±SD. *, $p \leq 0.05$.

It is well established that IGF-1 binds to and induces auto-phosphorylation of the IGF1R (Laviola et al., 2007; Pessin and Frattali, 1993). Therefore, we performed IHC stainings to assess IGF-1 signaling activity during intestinal inflammation and tumorigenesis. Since phospho-IGF1R staining was very heterogeneous in the intestines, ranging from unstained cells to very strongly stained cells, we decided to analyze this staining using the software "TMarker" (Schuffler et al., 2013) to distinguish between the different staining intensities observed (Figure 38).

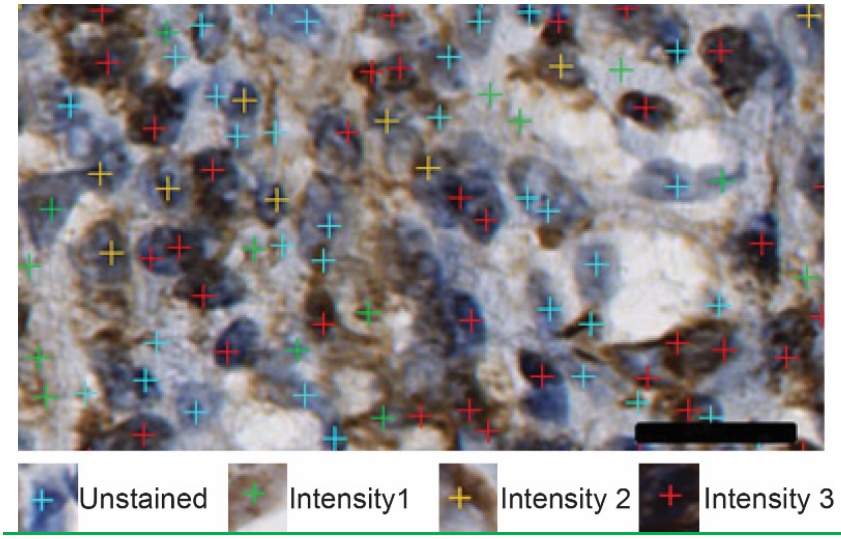


Figure 38. Differentiation of phospho-IGF1R staining intensities using TMarker.

Representative examples of cells classified as unstained, or stained with low (intensity 1), moderate (intensity 2) and high intensities (intensity 3) for phospho-IGF1R using the software Tmarker. Scale bar, 20 µm.

Analysis of IGF1R phosphorylation confirmed that the IGF-1 pathway was less active in the colon of DSS-treated p38α-Δ^{MC} mice compared to WT mice during DSS-induced acute colitis (Figure 39).

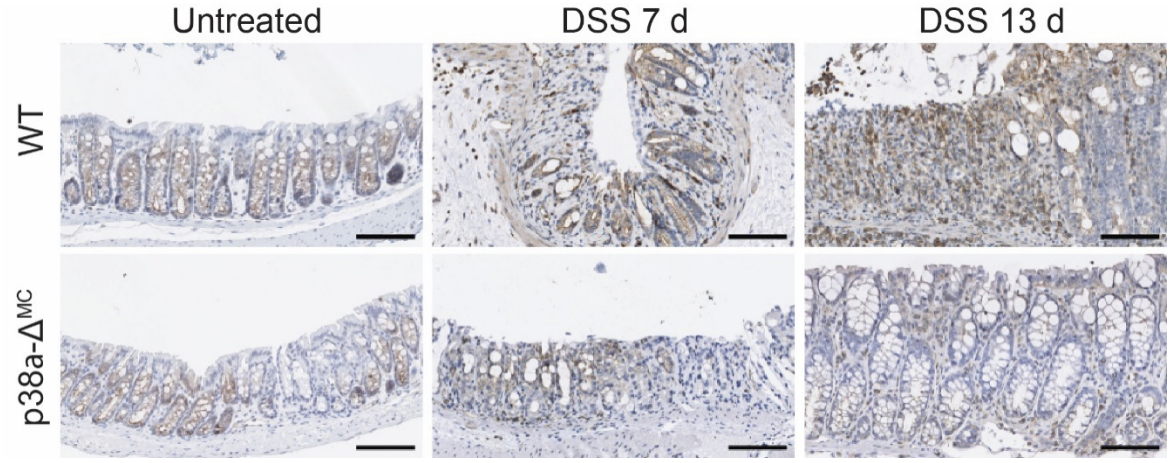


Figure 39. p38α in myeloid cells promotes IGF1R phosphorylation in DSS-treated colons.

Representative colon sections from mice either untreated or treated with DSS for 6 days were analyzed at days 7 and 13 for phospho-IGF1R staining.
Scale bars, 100 μm.

Quantification of the phospho-IGF1R stainings confirmed that the IGF-1 pathway was less active in the colon of DSS-treated p38α-Δ^{MC} mice compared to WT mice. However, the differences were mainly observed in the number of colon cells with stronger staining, indicating high levels of phospho-IGF1R and pathway activity, rather than in the total number of stained cells, although the changes were not statistically significant between WT and p38α-Δ^{MC} mice at days 7 and 13 (Figure 40A). Of note, IGF1R mRNA was also reduced in DSS-treated p38α-Δ^{MC} mice compared to WT mice (Figure 40B). We hypothesized that this could be a consequence of the elevated IGF-1 levels, as in patients with adenomatous polyps a significant positive correlation between serum IGF-1 and mucosal IGF1R mRNA has been suggested to play important roles in the development of both adenomatous polyps and colorectal carcinoma (Zhang et al., 2013b).

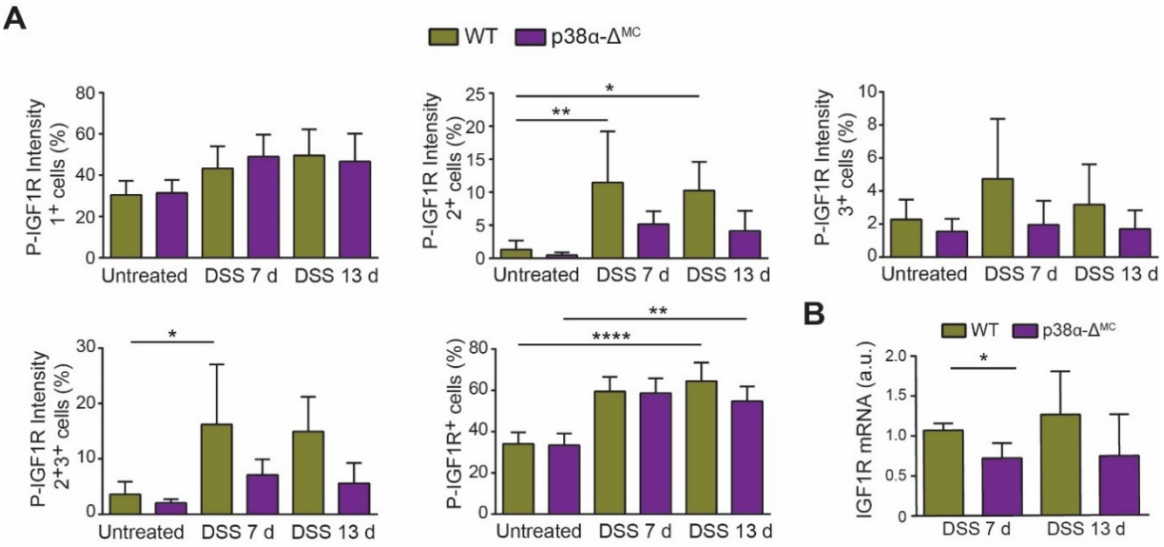


Figure 40. Downregulation of myeloid p38α reduces IGF-1 signaling during colitis.

(A) Colon sections from mice either untreated or treated with DSS for 6 days were analyzed at days 7 and 13 by phospho-IGF1R staining and the number of cells with low (1+), moderate (2+) and high (3+) intensity was identified and calculated using the software Tmarker as indicated in Figure 38 from several high magnification fields per animal ($n \geq 5$).
(B) Relative IGF1R mRNA levels in colons from DSS-treated mice were analyzed at day 7 and day 13 by qRT-PCR and are referred to the expression level in the WT, which was given the value of 1 ($n \geq 5$).
Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

Consistent with the known mitogenic properties of IGF-1, we found significant differences in cell proliferation, as determined by Ki67 staining between the colons of DSS-treated WT and p38α-Δ^{MC} in the repair phase at day 13 (Figure 41), when IGF-1 protein levels were significantly different in colon extracts.

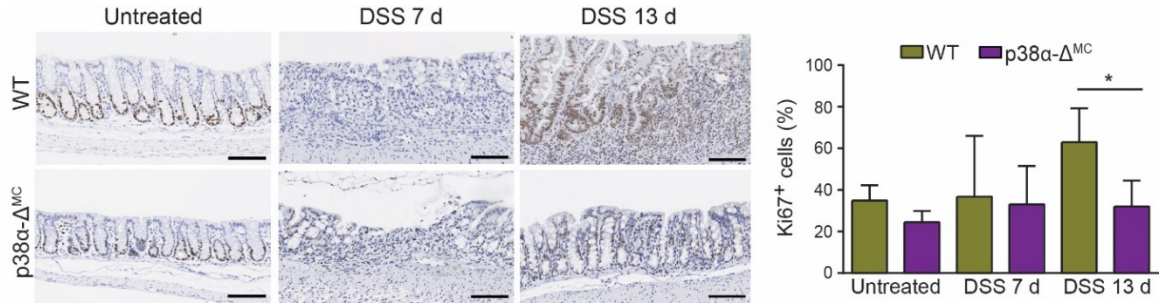


Figure 41. Deficiency of p38α in myeloid cells reduces DSS-induced proliferation.

Representative colon sections from mice either untreated or treated with DSS for 6 days were analyzed at days 7 and 13 for Ki67 staining (left panel). Quantifications are shown in the histogram ($n \geq 4$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$.

We also stained phospho-IGF1R in the colon epithelium of mice to which we applied the AOM/DSS-tumorigenesis model (Figure 42).

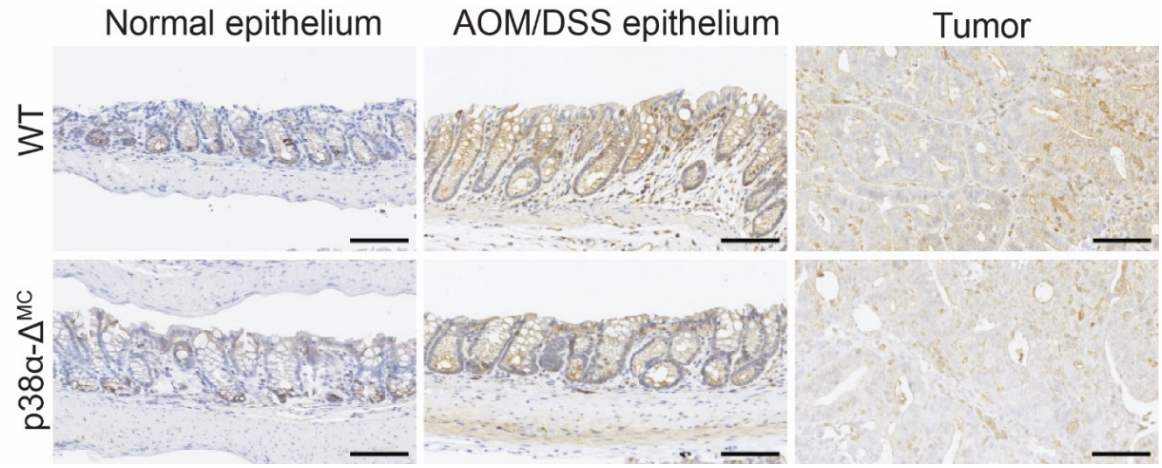


Figure 42. Myeloid p38α promotes AOM/DSS induced IGF1R phosphorylation in the tumors.

Representative colon sections from mice either untreated or treated with AOM/DSS were stained for phospho-IGF1R.
Scale bars, 100 μm.

We observed enhanced IGF1R phosphorylation in the colon epithelium and tumors of AOM/DSS-treated mice (100 days after AOM injection) compared to normal epithelium (Figure 43A). Quantification of IGF1R phosphorylation revealed no significant differences in the epithelium of AOM/DSS-treated WT compared to p38α-Δ^{MC} mice. However, IGF1R phosphorylation was reduced in colon tumors from p38α-Δ^{MC} mice compared to WT mice (Figure 43B).

Finally, ELISA analysis showed increased IGF-1 protein levels in tumors compared to non-tumoral colon tissue obtained from AOM/DSS treated animals, and this increase tent to be higher in WT mice compared to p38α-Δ^{MC} mice (Figure 43C). Taken together these results suggest that p38α signaling in macrophages is involved in the production of IGF-1 and this results in enhanced activation of IGF1R.

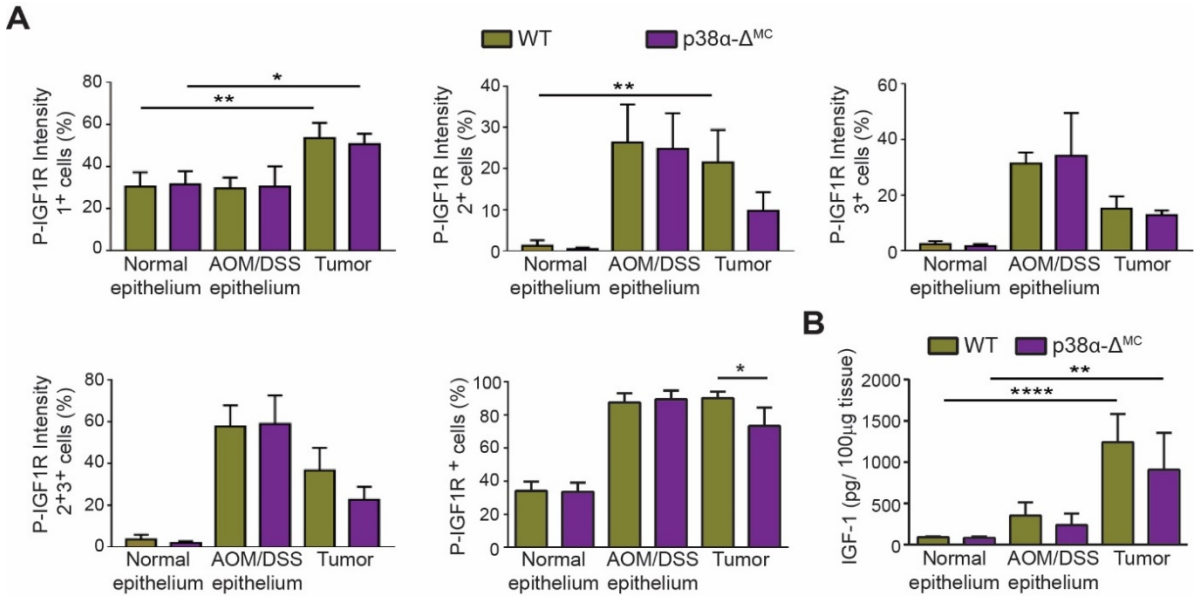


Figure 43. Downregulation of myeloid p38α reduces IGF-1 signaling during intestinal tumorigenesis.

(A) Normal colon epithelium from untreated mice and epithelium or tumor sections from AOM/DSS-treated mice were stained for phospho-IGF1R and the number of cells stained with low (1+), moderate (2+) and high (3+) intensity was calculated using the software Tmarker from several high magnification fields per animal (n = 4).

(B) Total protein lysates from whole colons of untreated or AOM/DSS-treated mice and from isolated tumors, were used to measure IGF-1 protein levels by ELISA ($n \geq 5$).

Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

4.1.7 IGF-1 promotes intestinal inflammation

Since we confirmed that IGF-1 signaling was enhanced in WT mice compared to p38α-Δ^{MC} mice during intestinal inflammation and tumorigenesis, we next questioned the implication of this observation. Given that IGF-1 has been implicated in inflammatory processes and immune cell recruitment (Mourkioti and Rosenthal, 2005), we evaluated the role of myeloid IGF-1 in intestinal inflammation. For this purpose, we used mice expressing LysM-Cre and IGF-1-lox alleles (IGF-1-Δ^{MC}). The efficiency of IGF-1 downregulation was confirmed by qRT-PCR in isolated peritoneal macrophages (Figure 44A). Moreover, we observed reduced IGF1R mRNA levels in colons from IGF-1-Δ^{MC} mice compared to WT mice (Figure 44B), in agreement with the DSS-treated p38α-Δ^{MC} mice (Figure 40B above).

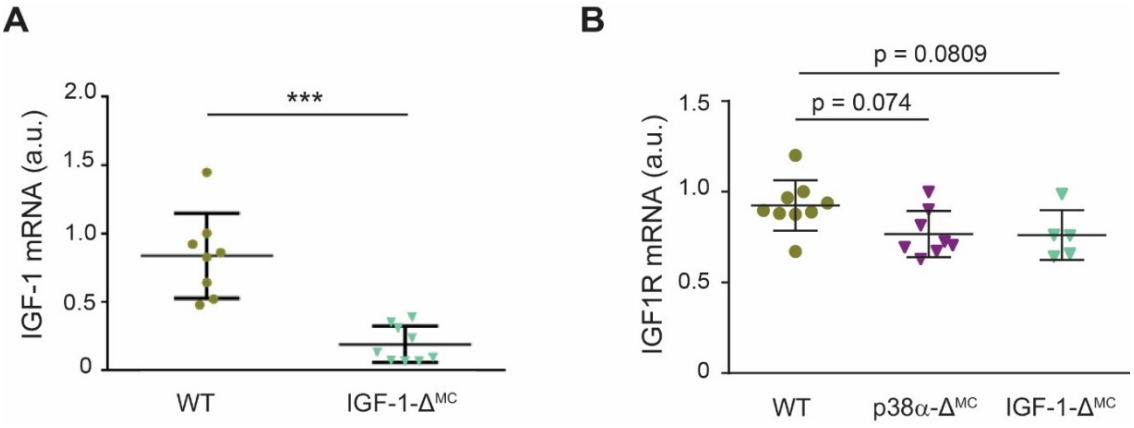


Figure 44. Downregulation of IGF-1 signaling in IGF-1-Δ^{MC} mice.

(A) Relative IGF-1 mRNA levels were quantified in peritoneal macrophages to confirm IGF-1 downregulation in myeloid cells of IGF-1-Δ^{MC} mice used in the experiments ($n \geq 8$).
(B) IGF-1R mRNA levels in mouse colons from untreated mice were determined by qRT-PCR ($n \geq 5$).
Data are expressed as the average±SD. ***, $p \leq 0.001$.

Colitis was then induced in these mice to evaluate the role of IGF-1 produced by myeloid cells in the context of intestinal inflammation. We observed that DSS treatment did not result in significant differences in body weight loss between WT and IGF-1-Δ^{MC} mice (Figure 45A). However, we observed that DSS-treated IGF-1-Δ^{MC} mice had a lower DAI compared to WT mice (Figure 45B).

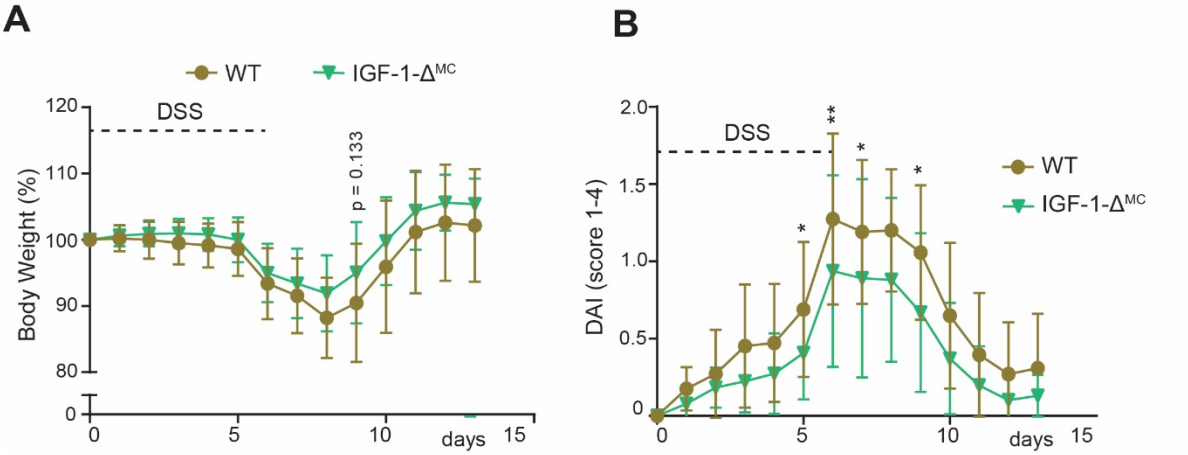


Figure 45. IGF-1 deficiency in myeloid cells slightly reduces DSS susceptibility.

(A and B) Body weight (A) and DAI (B) were recorded daily during DSS treatment ($n \geq 31$).
Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$.

As expected, the colons of WT and IGF-1-Δ^{MC} mice showed no histological differences prior to DSS administration. Moreover, we observed less epithelial damage (Figure 46A and Figure 46B) and longer colons (Figure 46C) at day 7 of the DSS-induced

colitis protocol in IGF-1-Δ^{MC} mice compared to WT mice. In general, the differences between WT and IGF-1-Δ^{MC} mice were not as strong as between WT and p38α-Δ^{MC} mice (Figure 26 and Figure 28), suggesting that p38α-regulated inflammatory mediators other than IGF-1 contribute to the process (Wagner and Nebreda, 2009).

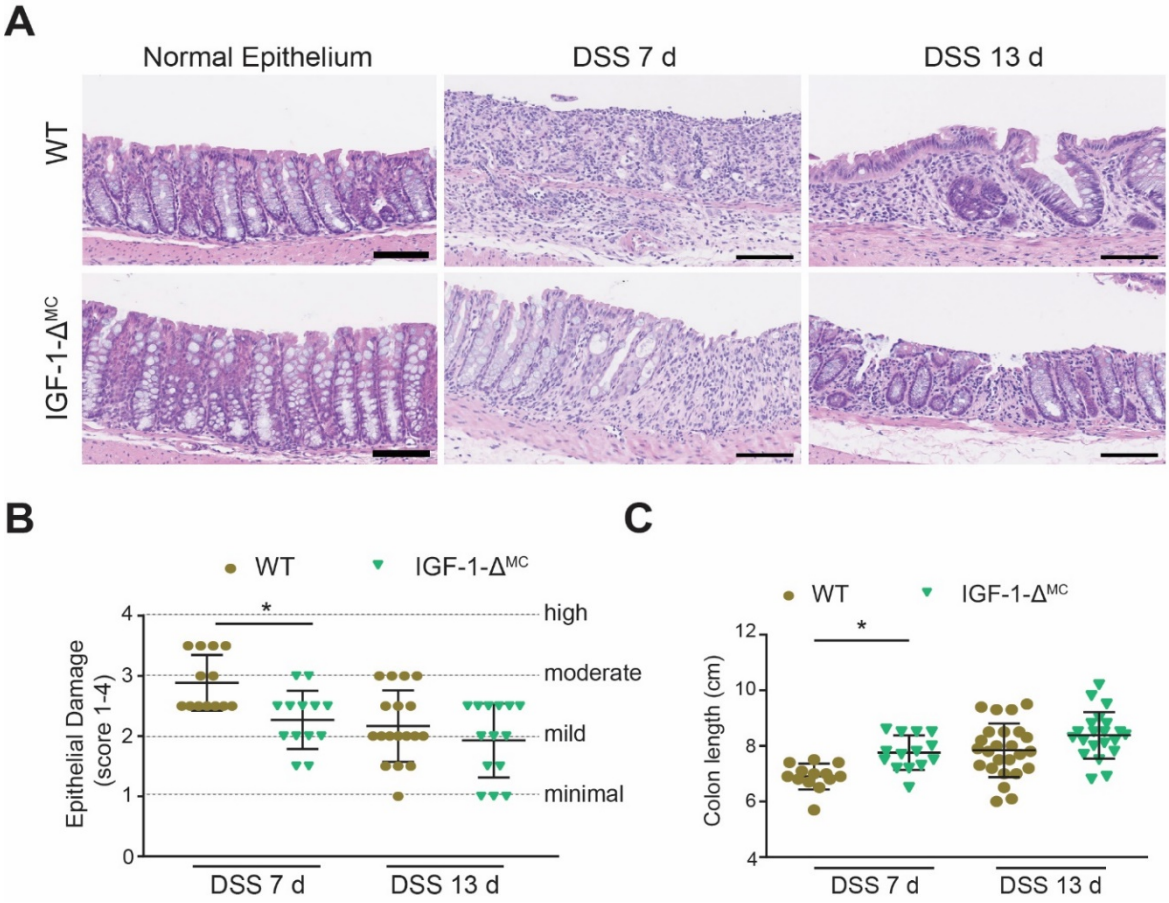


Figure 46. Downregulation of myeloid IGF-1 decreases immune cell mobilization from the bone marrow.

(A and B) Representative H&E-stained colon sections from animals untreated or treated with DSS for 6 days and analyzed at days 7 and 13 (A). Epithelial damage was evaluated in the H&E-stained colon sections ($n \geq 13$) (B).
(C) The total length of each colon dissected from DSS-treated mice was measured under a constant weight (2 g; $n \geq 13$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$.

Next, we investigated the role of IGF-1 in inflammatory cell recruitment, and found significantly more Ly6C^{hi}CCR2^{+} inflammatory monocytes in the bone marrow of DSS-treated WT mice compared to IGF-1-Δ^{MC} mice (Figure 47A, left panel), whereas no significant differences were observed in the blood (Figure 47B). However, we confirmed

less circulating monocytes (F4/80^{+}) in the blood of IGF-1-Δ^{MC} mice (Figure 47A, right panel).

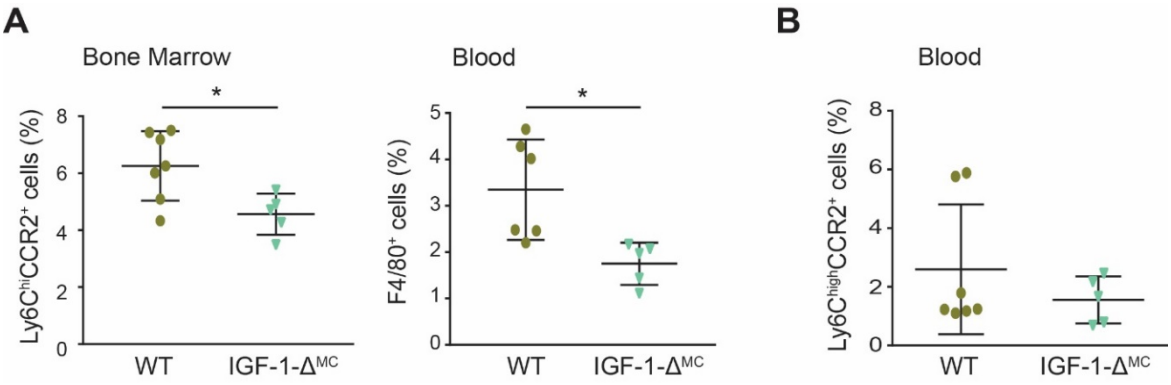


Figure 47. Downregulation of myeloid IGF-1 decreases immune cell recruitment.

(A) WT and IGF-1-Δ^{MC} mice were treated for 6 days with DSS and analyzed at day 7 for bone marrow CD45^{+} CD11b^{+} cells that were Ly6C^{hi} and CCR2^{+} (left panel; n ≥ 5), and for blood CD45^{+} CD11b^{+} cells that were F4/80^{+} (right panel; n ≥ 5).
(B) WT and IGF-1-Δ^{MC} mice were treated for 6 days with DSS and analyzed at day 7 for blood CD45+ CD11b^{+} cells that were Ly6C^{hi} CCR2^{+} (n ≥ 5).
Data are expressed as the average±SD. *, p≤0.05.

In agreement with this, we observed a reduced number of macrophages (F4/80^{+}) in the intestines of DSS-treated IGF-1-Δ^{MC} mice compared to WT mice (Figure 48).

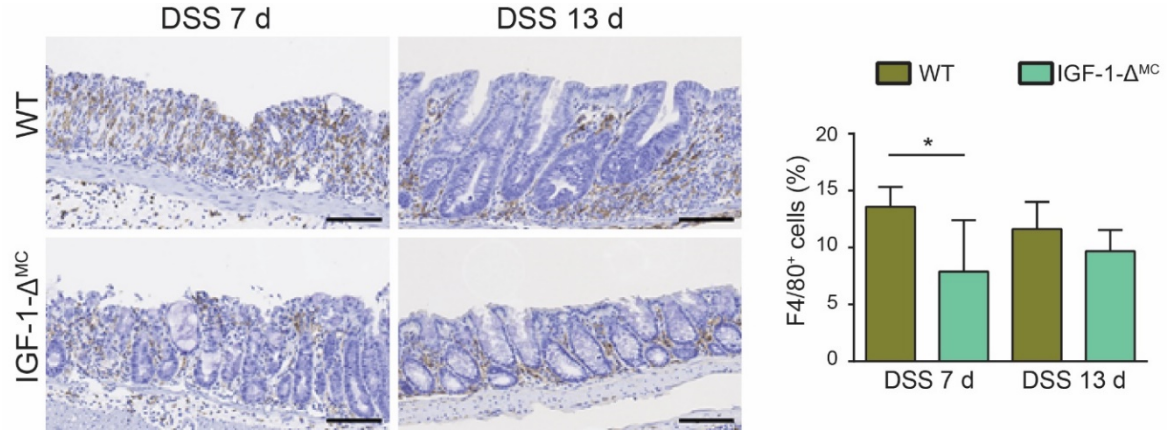


Figure 48. IGF-1-Δ^{MC} mice recruit less macrophages to the colon upon DSS treatment.

Representative colon sections from mice either untreated or treated with DSS for 6 days were analyzed at days 7 and 13 for F4/80 staining. Quantifications are shown in the histogram (n ≥ 5).
Scale bars, 100 μm. Data are expressed as the average±SD. *, p≤0.05.

In addition, infiltration of activated neutrophils (MPO^+) and T-cells ($CD3^+$ cells), as well as the STAT3 phosphorylation levels tent to be all reduced in colons from IGF-1-Δ^{MC} mice without reaching statistical significance (Figure 49A - Figure 49C).

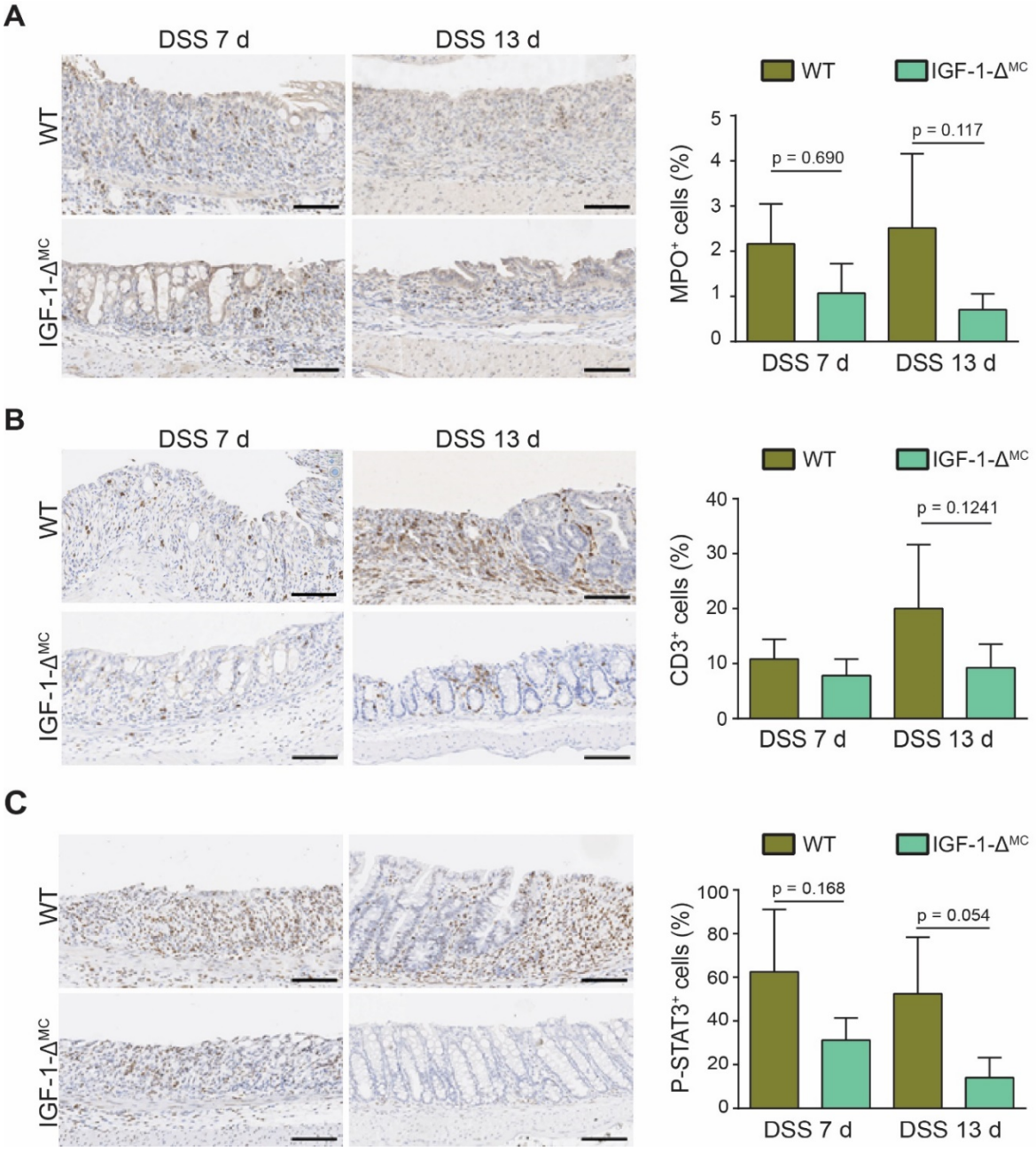


Figure 49. IGF-1-Δ^{MC} mice show decreased immune cell recruitment to the inflamed colon.

(A - C) Representative colon sections from mice either untreated or treated with DSS for 6 days were analyzed at days 7 and 13 for MPO (A), CD3 (B) and phospho-STAT3 (C). Quantifications are shown in the histograms ($n \geq 4$).
Scale bars, 100 μm. Data are expressed as the average±SD.

Importantly, quantification of IGF1R phosphorylation revealed a strong reduction in IGF-1 signaling in colons from DSS-treated IGF-1-Δ^{MC} mice compared to WT mice (Figure 50A). Of note, IGF-1 mRNA levels were also reduced in colons from untreated IGF-1-Δ^{MC} mice compared to WT mice, suggesting an important contribution of IGF-1 produced by myeloid cells to the activation of this pathway in the intestine (Figure 50B).

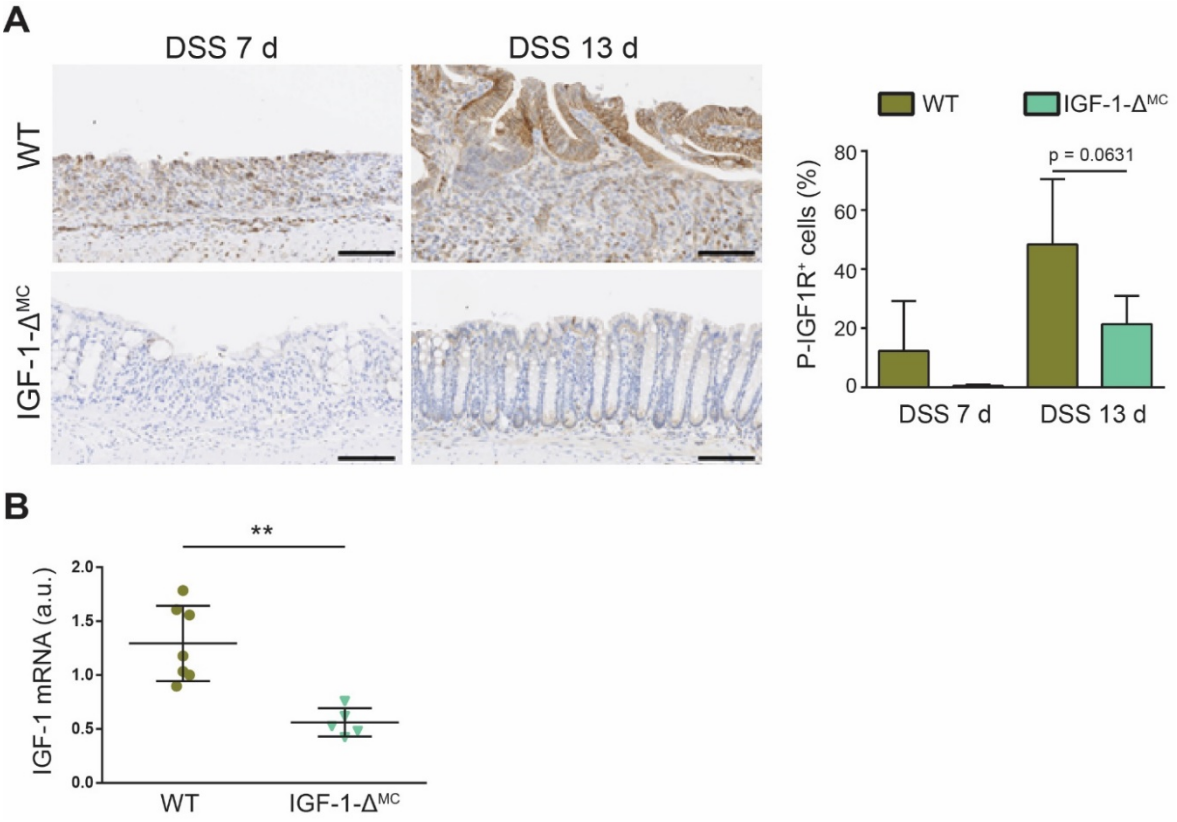


Figure 50. Downregulation of IGF-1 in myeloid cells reduces IGF-1 signaling in the colon.

(A) Representative colon sections from mice either untreated or treated with DSS for 6 days were analyzed at days 7 and 13 for phospho-IGF-1R. Quantifications are shown in the histogram ($n \geq 4$).
(D) IGF1R mRNA levels in mouse colons from untreated mice were determined by qRT-PCR ($n \geq 5$).
Scale bars, 100 µm. Data are expressed as the average±SD. **, $p \leq 0.01$.

To confirm the implication of IGF-1 signaling in DSS-induced intestinal epithelial damage and inflammation, we used PQ401, a chemical inhibitor of IGF1R autophosphorylation (Gable et al., 2006; Troib et al., 2011; Zhou et al., 2016). WT mice were treated daily with PQ401, starting 1 day before the DSS treatment, and were compared with WT and p38α-Δ^{MC} mice treated with vehicle and DSS (Figure 51).

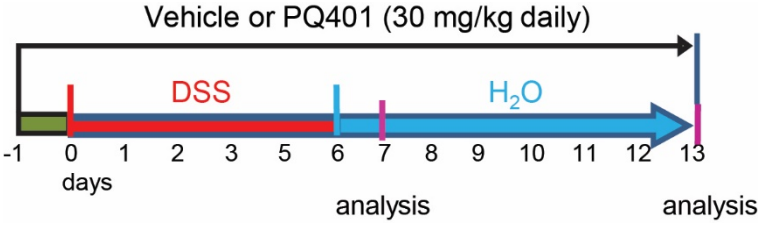


Figure 51. Schematic representation of the protocol used to test the effect of the phospho-IGF1R inhibitor PQ401 in DSS-induced acute colitis.

Animals were treated one day prior to the start of DSS-induced colitis treatment by either vehicle or PQ401 (30mg/kg). Pink lines indicate the time points when animals were sacrificed at days 7 and 13.

In agreement with our results using mice with IGF-1-deficient myeloid cells, treatment of WT mice with PQ401 reduced DSS-induced body weight loss (Figure 52A) and DAI (Figure 52B).

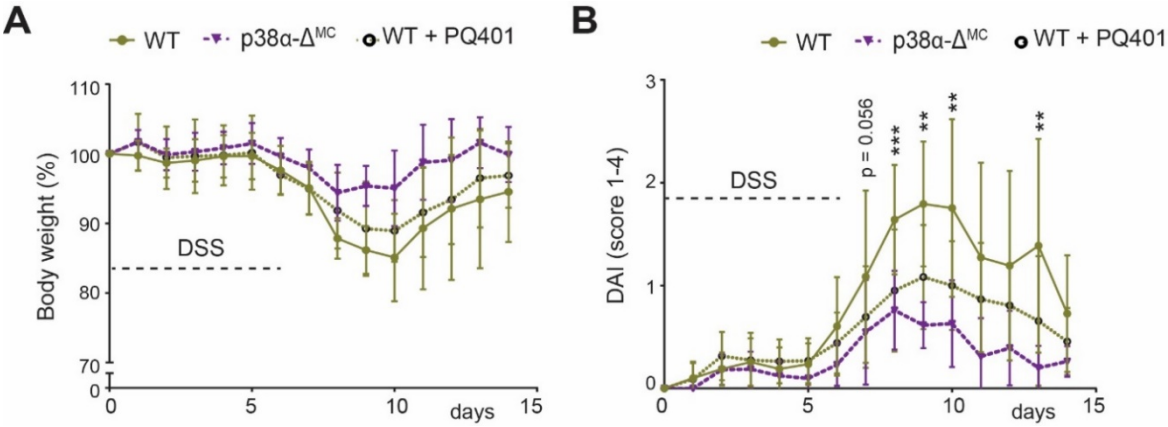


Figure 52. Inhibition of IGF-1 signaling ameliorates DSS-induced colitis.

(A and B) WT mice treated with the IGF1R inhibitor PQ401 or vehicle, and p38α-Δ^{MC} mice treated with vehicle were analyzed daily for body weight (A) and DAI (B) ($n \geq 7$).
Data are expressed as the average±SD. **, $p \leq 0.01$; ***, $p \leq 0.001$.

Epithelial damage values in PQ401-treated mice were also closer to those observed in p38α-Δ^{MC} mice (Figure 53).

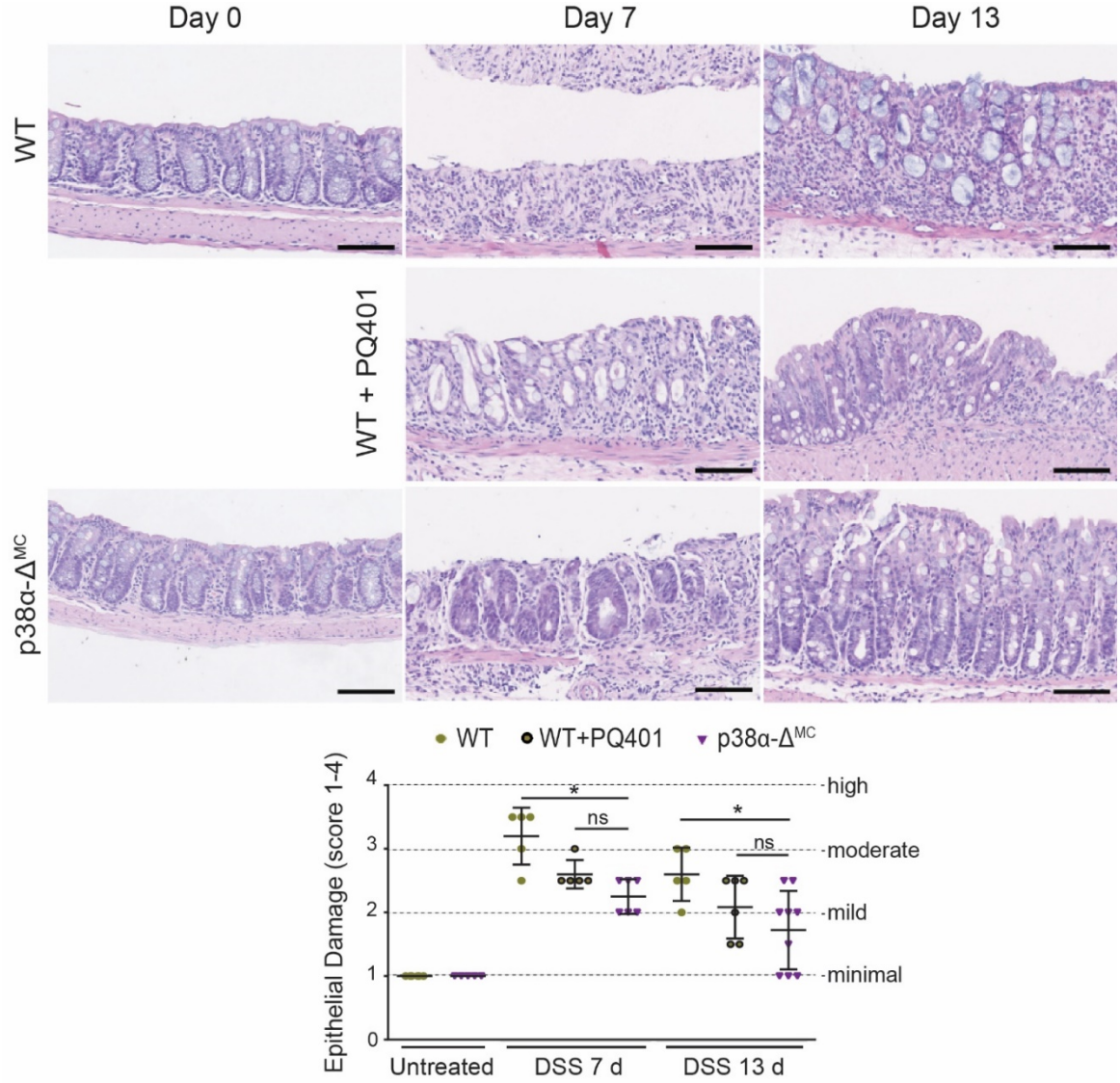


Figure 53. Pharmacological inhibition of IGF-1 receptor signaling ameliorates DSS induced epithelial damage.

Representative H&E-stained colon sections from mice untreated or treated with either PQ401 or vehicle, and with DSS for 6 days were analyzed at days 7 and 13. Epithelial damage was evaluated and quantifications are shown in the histogram ($n \geq 7$).
Scale bars, 100 μm. Data are expressed as the average±SD . *, $p \leq 0.05$.

In order to verify that PQ401 treatment inhibited IGF-1 signaling in the intestines of DSS-treated animals, we performed IHC staining of phospho-IGF1R at the end of the experiment (day 13). As expected, the levels of IGF1R phosphorylation were reduced in the colon of mice treated with PQ401 (Figure 54).

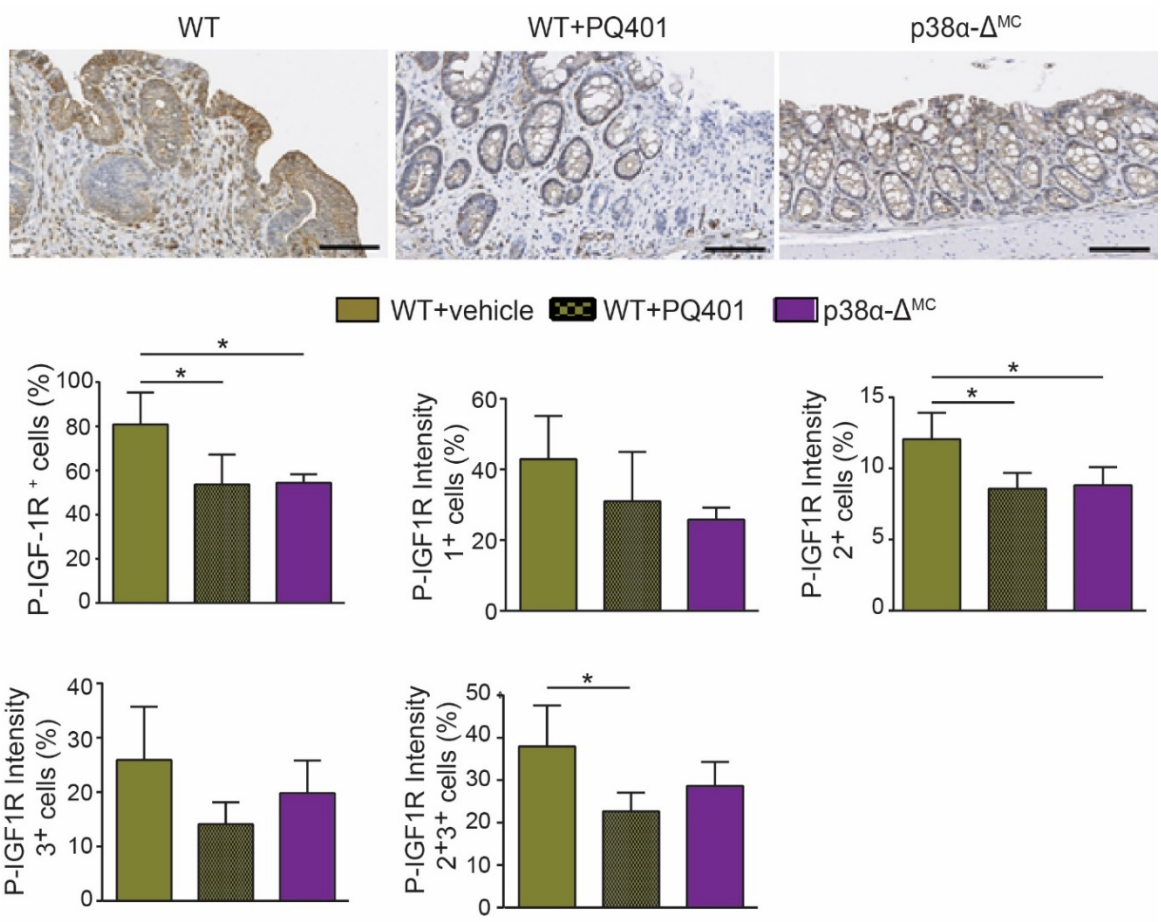


Figure 54. PQ401 treatment during DSS-induced colitis inhibits phosphorylation of IGF-1R.

Colon sections from mice untreated or treated with DSS for 6 days were analyzed at day 13 by phospho-IGF1R staining. Cells stained with low (1+) moderate (2+) or high (3+) intensity, as indicated in Figure 38, were identified using the software Tmarker and quantified from several high magnification fields per animal. Quantifications are shown in the histogram (n = 4).
Scale bars, 100 μm. Data are expressed as the average±SD . *, p≤0.05.

Interestingly, and in consistence the ameliorated epithelial damage, PQ401 treatment impaired macrophage recruitment to the colon of DSS-treated WT mice (Figure 55), indicating a reduction of inflammatory processes resulting from inhibition of IGF1R signaling.

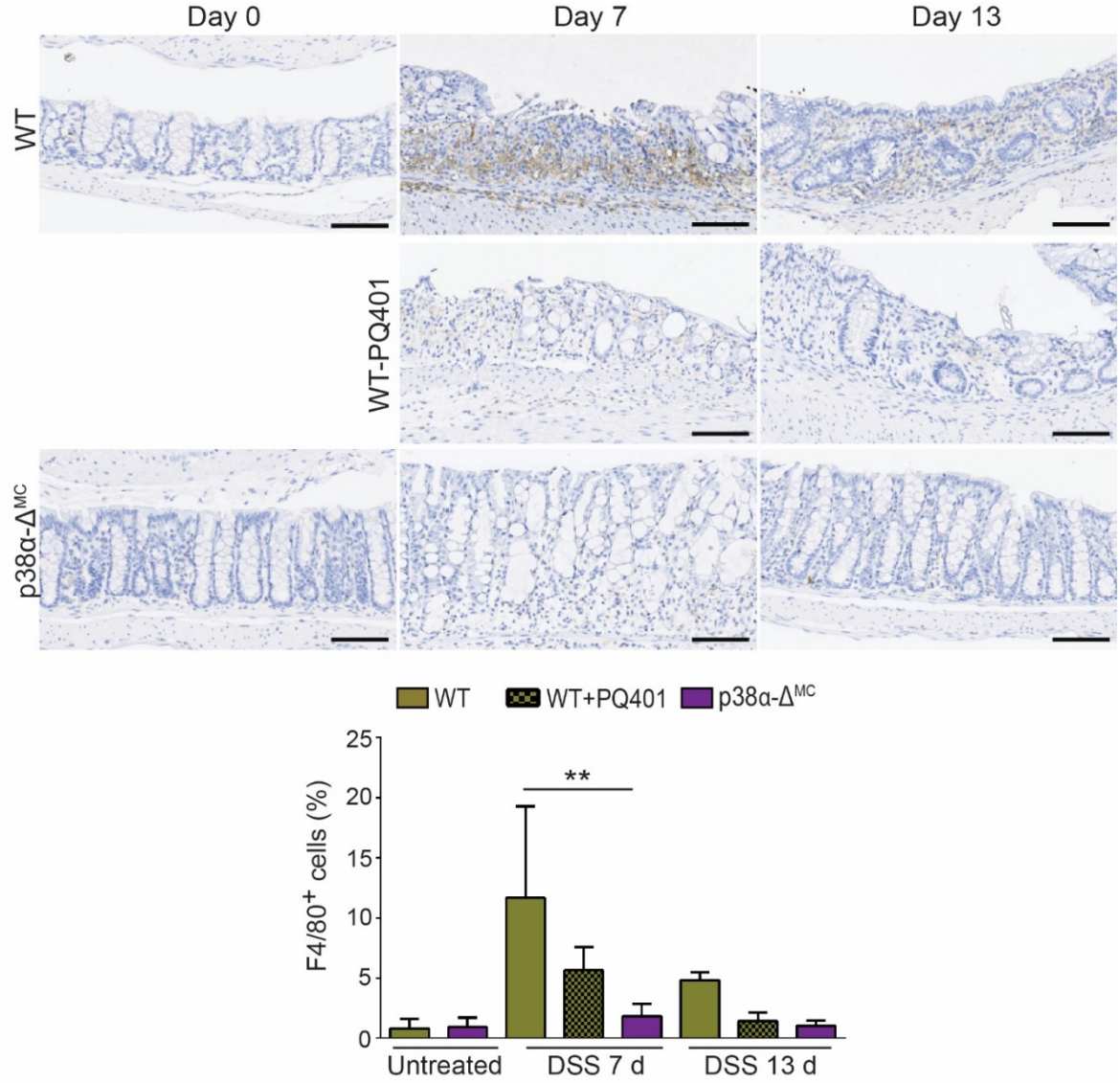


Figure 55. Pharmacological inhibition of IGF1R signaling reduces DSS-induced macrophage recruitment.

Representative colon sections from mice untreated or treated with DSS for 6 days and with either PQ401 or vehicle were analyzed at day 7 and day 13 by staining for F4/80. Quantifications are shown in the histogram ($n \geq 3$).
Scale bars, 100 μm. Data are expressed as the average±SD. **, $p \leq 0.01$.

Similarly, the inhibition of IGF-1 signaling by PQ401 treatment during DSS-induced colitis also slightly reduced the STAT3 phosphorylation levels (Figure 56). These results support that inhibition of IGF1R signaling suppresses DSS-induced inflammation.

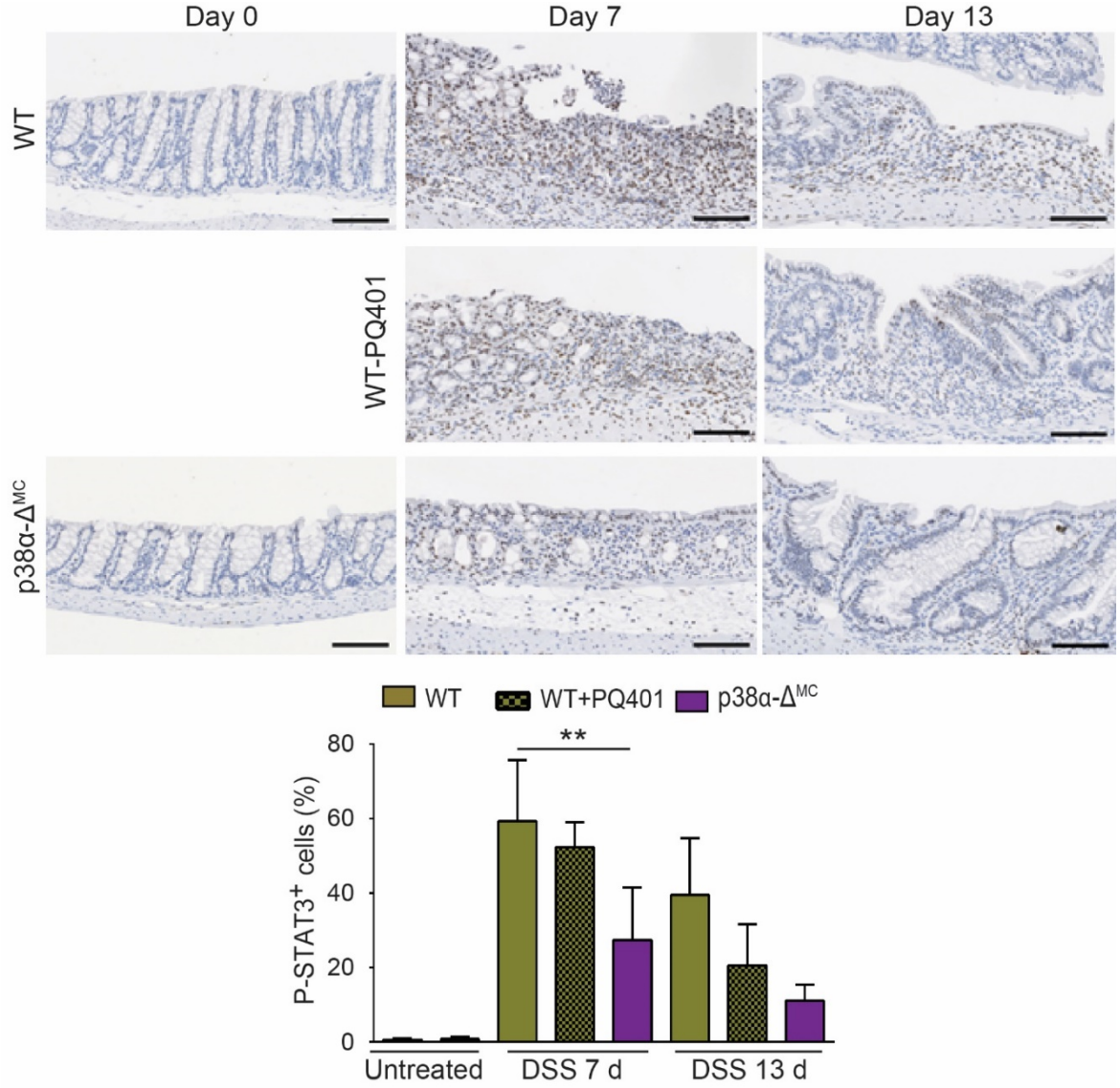


Figure 56. Pharmacological inhibition of IGF1R signaling reduces DSS-induced STAT3 phosphorylation.

Representative colon sections from mice untreated or treated with DSS for 6 days and with either PQ401 or vehicle were analyzed at day 7 and day 13 by staining for phospho-STAT3. Quantifications are shown in the histogram ($n \geq 3$).
Scale bars, 100 μm. Data are expressed as the average±SD. **, $p \leq 0.01$.

Consistent with this idea, the numbers of leukocytes ($CD45^+$), myeloid cells ($CD45^+CD11b^+$), macrophages ($F4/80^+$) and inflammatory monocytes ($Ly6C^{hi}CCR2^+$) were significantly reduced in the bone marrow of PQ401 treated WT mice, as for p38α-Δ^{MC} mice (Figure 57A). These cell populations were also reduced in the blood of WT mice treated with PQ401 but to a lesser extent than in the bone marrow (Figure 57B).

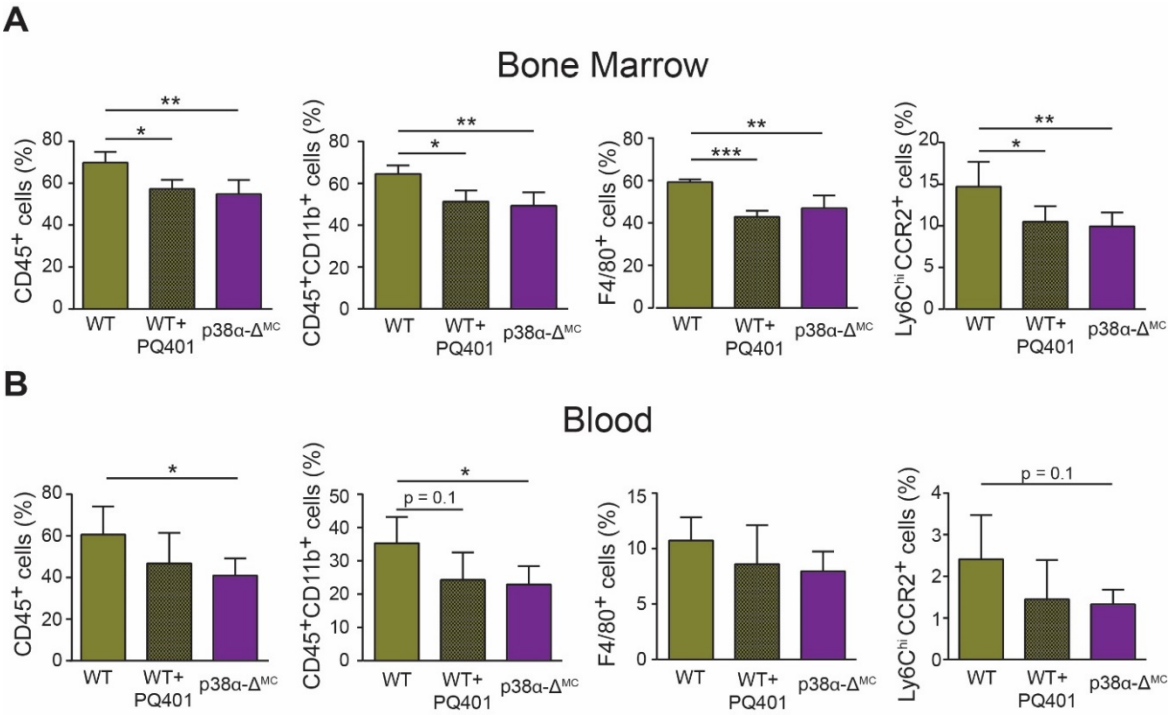


Figure 57. Pharmacological inhibition of IGF1R signaling suppresses DSS-induced inflammatory cell recruitment.

(A and B) Percentage of CD45+, CD45+CD11b+, CD45+CD11b+ F4/80+ and CD45+CD11b+ Ly6C^{hi} CCR2^{+} cells in the bone marrow (A) and blood (B) from mice treated with DSS for 6 days in the presence of either PQ401 or vehicle and sacrificed at day 7 ($n \geq 3$).
Data are expressed as the average±SD . *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

4.1.8 IGF-1 signaling stimulates inflammation-associated carcinogenesis

The above results implicate IGF-1 signaling in DSS-induced inflammation. To address the importance of IGF1 signaling in inflammation-associated tumorigenesis, we treated WT and IGF-1-Δ^{MC} mice with the AOM/DSS protocol. We found that IGF-1-Δ^{MC} mice exhibited a decreased colon tumor number compared to WT mice, with fewer tumors larger than 4 mm and no tumors larger than 6 mm (Figure 58A and 35B). Of note, analysis of colon tumors of IGF-1Δ^{MC} mice revealed reduced macrophage infiltration (F4/80^{+}) compared to those of WT mice (Figure 58C), further suggesting the implication of IGF-1 in immune cell recruitment.

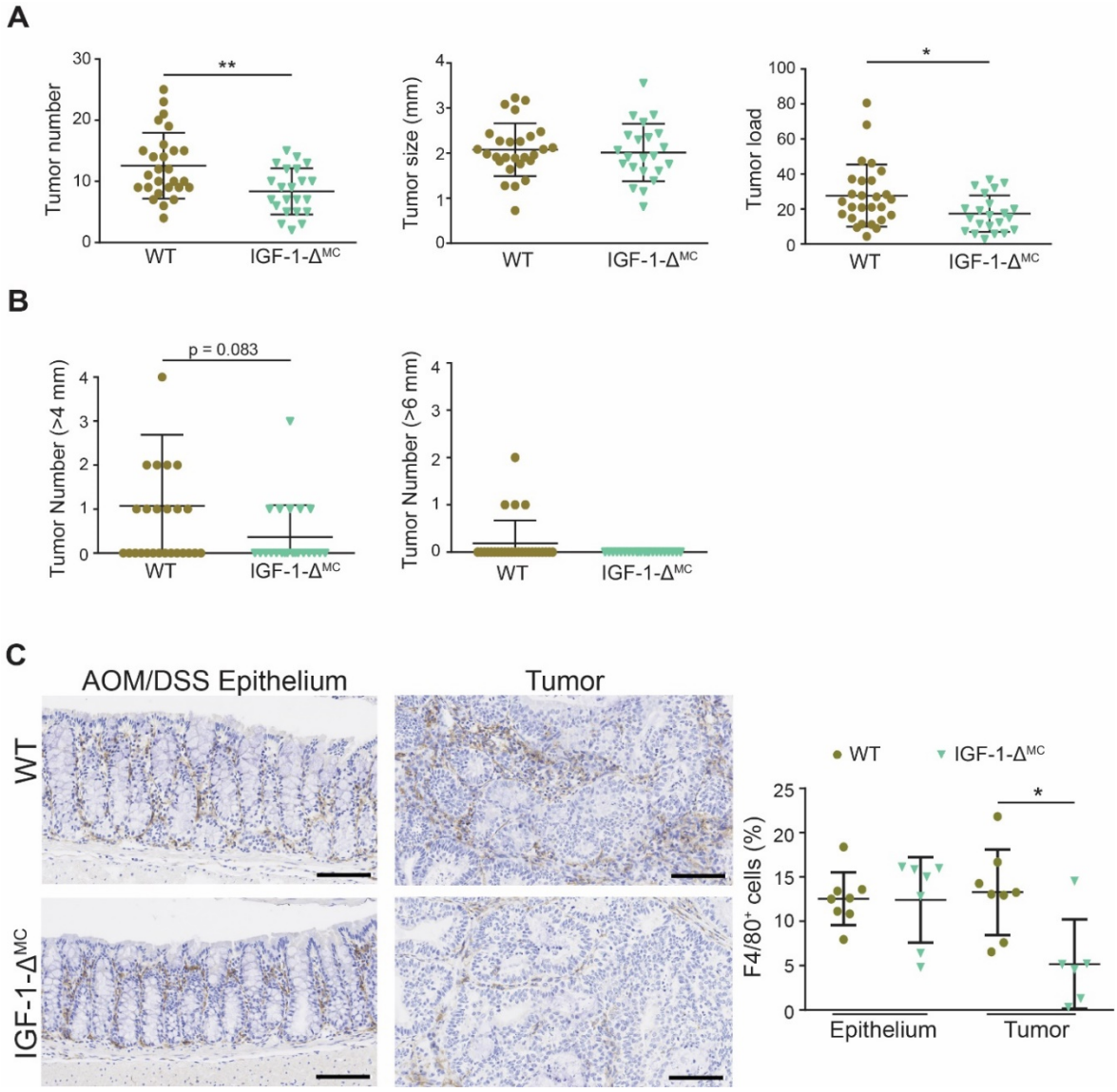


Figure 58. Downregulation of IGF-1 in myeloid cells reduces colitis-associated tumorigenesis induced by AOM/DSS and macrophage recruitment to the tumors.

(A and B) Average tumor number, size and load (A) and number of tumors with size >4 mm or >6 mm (B) in AOM/DSS-treated mice at day 100 ($n \geq 22$).
(C) Representative sections from AOM/DSS treated epithelia and colon tumors stained for F4/80. Quantifications are shown in the histogram ($n \geq 6$).
Scale bars, 100 μm. Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$.

To evaluate the potential therapeutic interest of targeting IGF-1 signaling in colorectal tumorigenesis, WT mice were treated with the AOM/DSS protocol and then were split in two groups, one received PQ401 and the other one vehicle for 20 days (Figure 59A). The results indicated that inhibition of IGF1R significantly reduced the colon tumor number in WT mice to similar levels as in vehicle treated p38α-Δ^{MC} mice. Moreover, the number of tumors larger than 4 and 6 mm was significantly reduced in the

PQ401-treated animals compared to WT mice (Figure 59B). To sum up, our results indicate that IGF-1 produced by myeloid cells promotes inflammatory cell recruitment and inflammation-associated intestinal tumorigenesis.

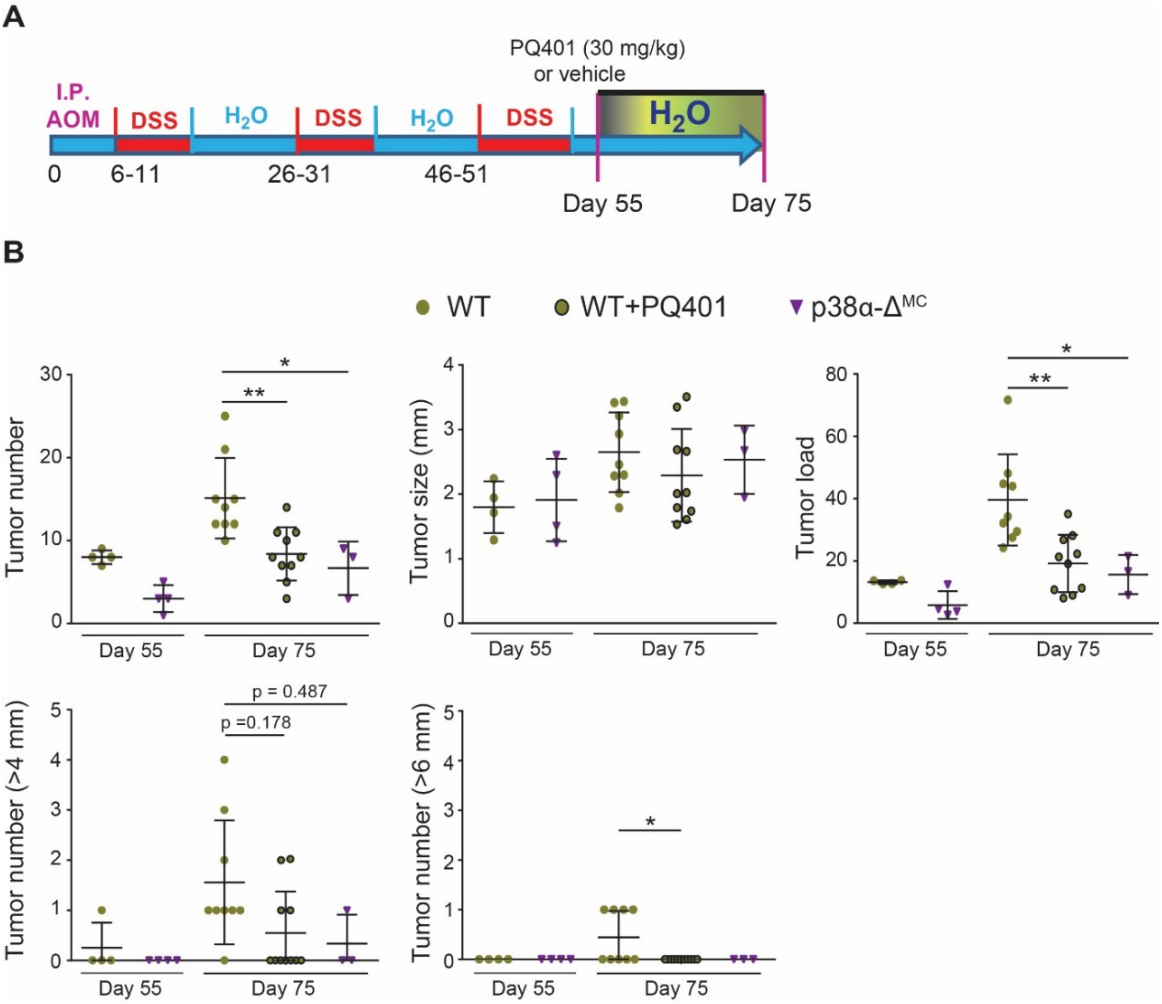


Figure 59. Pharmacological inhibition of IGF-1 signaling reduces colitis-associated tumorigenesis induced by AOM/DSS.

(A) Schematic representation of the protocol used for the combined treatment of mice with AOM/DSS and the IGF1R inhibitor PQ401 (30mg/kg) or vehicle. Pink lines indicate when animals were sacrificed at day 55 and 75.

(B) Average tumor number, size and load (upper panel) and number of tumors with size >4 mm or >6 mm (lower panel) in WT and p38α-Δ^{MC} mice treated with PQ401 or vehicle ($n \geq 3$).

Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$.

4.1.9 Myeloid p38α signaling controls immune cell recruitment to the colon through the regulation of chemokines

Chemokines are important to modulate inflammatory processes, and inappropriate chemokine expression can cause a massive and destructive leukocyte infiltration (Balkwill and Mantovani, 2001). Mice used in this study were maintained under conventional housing conditions resembling a more natural situation than specific pathogen-free (SPF) housing. Under natural conditions, the gut is marked by a situation of controlled "physiological inflammation", due to the constant exposure to environmental microbiota (Mowat and Bain, 2011). Therefore we next evaluated if downregulation of p38α and IGF-1 in myeloid cells affects monocyte recruitment in untreated mice. Indeed, resembling a physiological inflammatory state, p38α-Δ^{MC} mice showed a decreased amount of Ly6C^{hi}CCR2^{+} pro-inflammatory monocytes in the bone marrow and accordingly fewer circulating monocytes in the blood, compared to WT mice under homeostatic conditions (Figure 60A). Besides its role as a growth hormone, IGF-1 can function as chemoattractant (Roussos et al., 2011). In accordance, monocyte recruitment was reduced in the bone marrow of untreated IGF-1-Δ^{MC} mice compared to WT mice (Figure 60B), as reported above also for IGF-1-Δ^{MC} mice and PQ401-treated WT mice upon DSS induced colitis (Figure 47 and Figure 57).

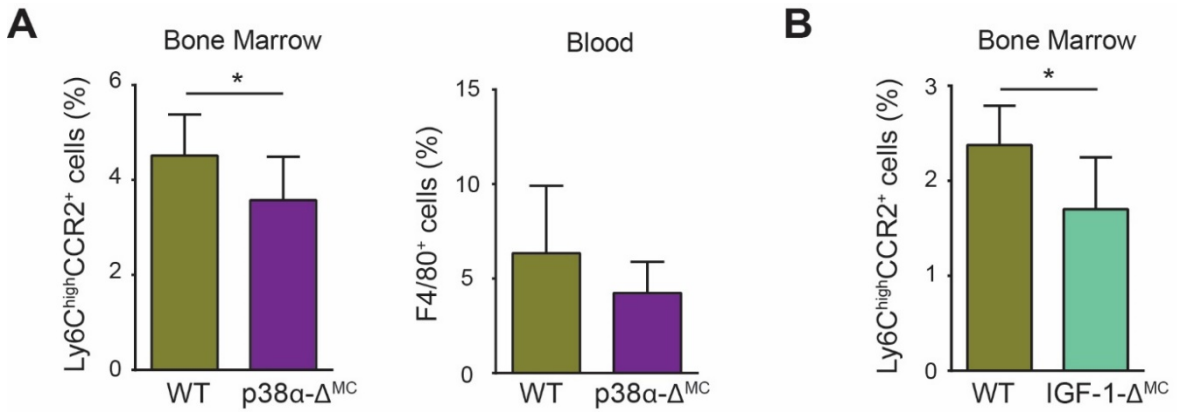


Figure 60. Deficiency of p38α in myeloid cells suppresses inflammatory cell recruitment under homeostatic conditions.

(A) DSS-treated mice were analyzed for bone marrow CD45^{+} CD11b^{+} cells that were Ly6C^{hi} and CCR2^{+} (left panel; n=11), and for blood CD45^{+} CD11b^{+} cells that were F4/80^{+} (right panel; n = 11).
(B) DSS-treated mice were analyzed for bone marrow CD45^{+} CD11b^{+} cells that were Ly6C^{hi} and CCR2^{+} (n ≥ 5).
Data are expressed as the average±SD. *, p≤0.05.

Next, we evaluated whether changes in intestinal chemokine expression between colons from untreated WT and p38α-Δ^{MC} mice accounted for the differences in inflammatory cell recruitment observed. Interestingly, analyzing a mouse chemokine array, we observed an overall downregulation of chemokines potentially important for myeloid cell recruitment in p38α-Δ^{MC} mice compared to WT mice, consistent with the reduced recruitment of monocytes/macrophages. The downregulated chemokines included Chemerin, CCL6, CCL2, CXCL12, IL-16, CCL21, CCL12 and CCL9 (Figure 61), which have been all reported to play crucial roles in leukocyte trafficking (Buechler, 2014; Cao et al., 2016; Shi and Pamer, 2011).

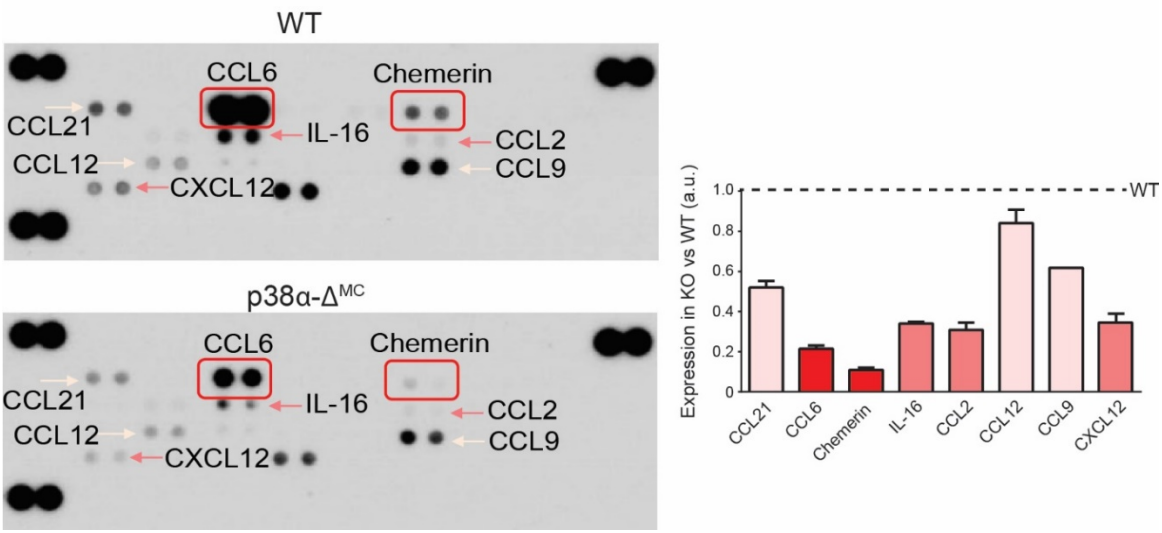


Figure 61. Myeloid p38α downregulation reduces chemokine expression in the colon.

A mouse chemokine antibody array was interrogated using a pool of whole colon extracts derived from non-stimulated mice (n=5/genotype). Quantifications are shown in the right panel. Arbitrary units are referred to the expression level of each chemokine in WT mice, which was given the value of 1.

We confirmed the downregulation of Chemerin, CCL2 and CCL12 mRNAs in intestinal macrophages isolated from p38α-Δ^{MC} mice compared to WT mice, whereas only CCL2 was downregulated in IGF-1Δ^{MC} mice (Figure 62A), however without reaching statistical significance. In addition, we analyzed the expression of these chemokines in whole colons and observed significant downregulation of IL-16 mRNA in p38α-Δ^{MC} mice and of CCL-9 in IGF-1Δ^{MC} mice compared to WT mice (Figure 62B). These observations suggest that myeloid p38α could engage paracrine mechanisms that inhibit chemokine expression by other cell types. We have also identified several chemokines that might be regulated by myeloid p38α at the translation or post-

translational levels, since they are downregulated at the protein level in colons from p38α-Δ^{MC} mice (Figure 61) without noticeable changes in mRNA expression (Figure 62A and Figure 62B).

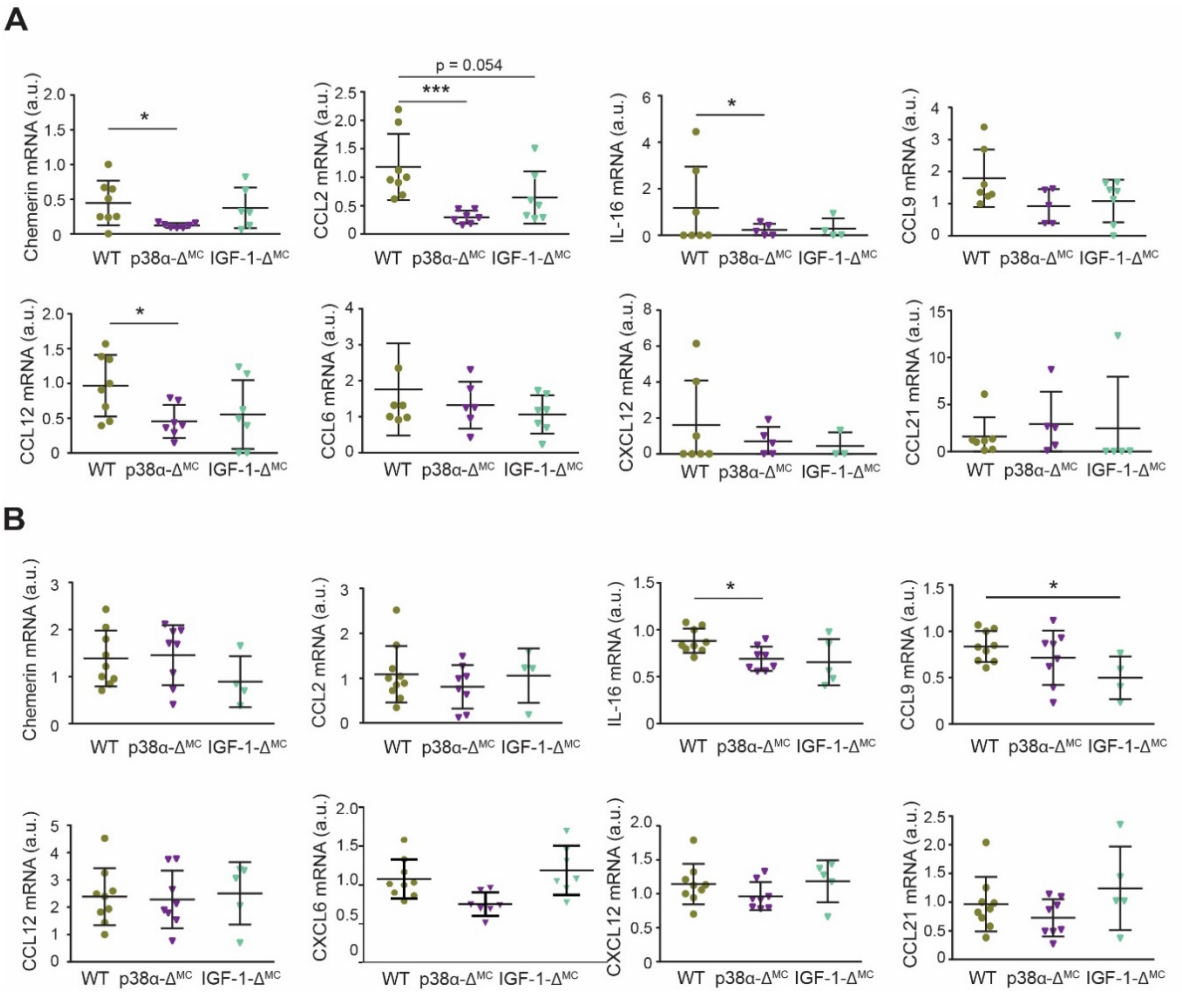


Figure 62. Chemokine expression in isolated intestinal macrophages and whole colons.

(A and B) Relative mRNA levels for the indicated genes in isolated intestinal macrophages (A) and whole colons (B) from untreated mice were determined by qRT-PCR ($n \geq 4$).
Data are expressed as the average±SD. *, $p \leq 0.05$; ***, $p \leq 0.001$.

p38 MAPK signaling has been linked to the differentiation of several cell types, including osteoclasts and lung progenitor cells (Cuadrado and Nebreda, 2010), but we are not aware of reports implicating p38α in monocyte/macrophage differentiation. To evaluate whether impaired generation or differentiation of monocytes/macrophages in p38α-Δ^{MC} mice could contribute to the reduced monocyte recruitment observed, we analyzed different stem cell populations in the bone marrow. Our results indicated that hematopoietic stem cells (HSCs; Sca1^{+}c-kit^{+}CD34^{-}CD127^{-}), multipotent progenitors

(MMPs; Sca1^{+}c-kit^{+}CD34^{+}CD127^{-}), common myeloid progenitors (CMPs; Sca1^{+}c-kit^{+}CD34^{+}CD127^{-}) or common lymphoid progenitors (CLPs; Sca1^{-}c-kit^{-}CD34^{-}CD127^{+}) were not significantly different between p38α-Δ^{MC} and WT mice (Figure 63A). We therefore hypothesized that the reduced recruitment of inflammatory cells to the intestine is likely due to reduced chemokine expression, directly or indirectly regulated by myeloid p38α in the colon due to a "physiological inflammatory state" and potentiated by DSS induced inflammation.

Alterations in the intestinal epithelium barrier function have been associated with IBD and colon cancer (Michielan and D'Inca, 2015; Westbrook et al., 2010). Therefore, we investigated whether the decreased susceptibility to DSS induced acute colitis observed in mice with myeloid p38α downregulation could be due to altered epithelial barrier function. This would be consistent with the "physiological inflammatory state" observed in these mice under conventional housing conditions without any treatment. However, analysis of intestinal permeability *in vivo* using FITC dextran did not reveal significant differences in epithelial barrier function between untreated WT and p38α-Δ^{MC} mice (Figure 63B).

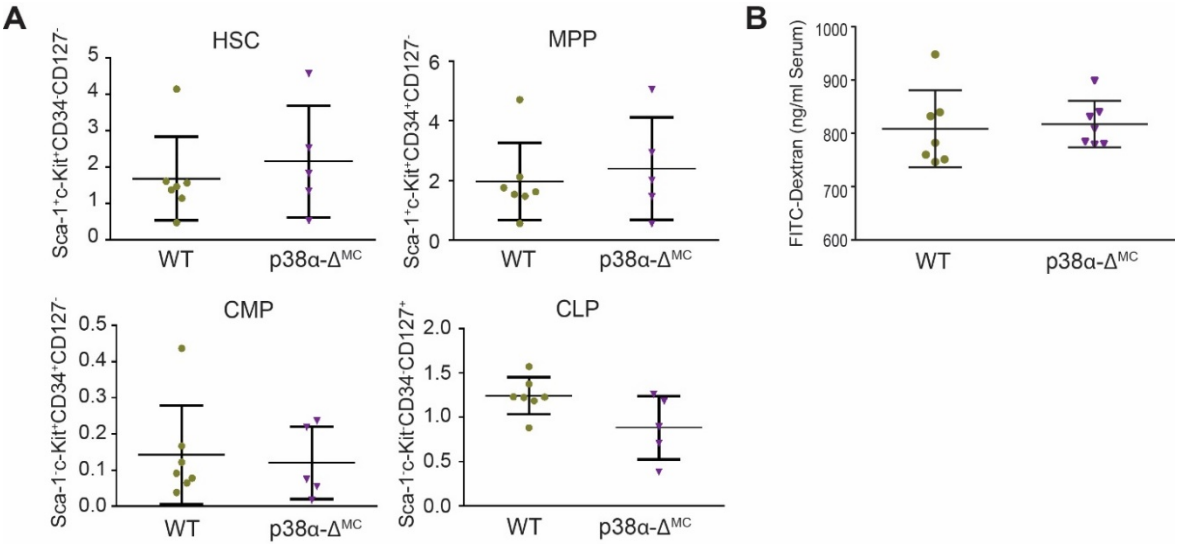


Figure 63. Myeloid downregulation of p38α affects neither precursor cells in the bone marrow nor intestinal permeability.

(A) The bone marrow of untreated mice were analyzed by first removing differentiated blood cells with the so-called lineage cocktail, which contains antibodies against differentiation markers (Lin- selection). Percentages of cells positive or negative for the indicated markers are illustrated from the total Lin- cell population ($n \geq 5$). Data are expressed as the average±SD.
(B) Intestinal permeability was measured by determining the concentration of FITC-dextran in blood serum (n=7).
Data are expressed as the average±SD. *, $p \leq 0.05$.

In summary, our results identify IGF-1 as a novel target of p38α signaling in macrophages, and provide evidence that IGF-1 facilitates inflammatory cell recruitment upon DSS induced colitis and contributes to CAC.

4.2 Study of p38α and IGF-1 signaling in intestinal epithelial cells

To complement the results obtained in mouse models, we performed studies *in vitro* to characterize how the p38α-IGF-1 axis regulates colon homeostasis and pathological disorders such as IBD and CAC. Since the occurrence of colitis-associated tumorigenesis is commonly ascribed to the transformation of normal colonic epithelium to adenomatous polyps and ultimately invasive tumors (Gryfe et al., 1997; Kriegl, 2013), we first focused our efforts in charactering the p38α-IGF-1 axis in the intestinal epithelium.

We addressed whether p38α and IGF-1 signaling affect crucial functions of IECs, such as proliferation, differentiation, apoptosis and migration. The required criteria for studying the role of p38 MAPK in cells that resemble as much as possible normal IECs and in murine derived macrophages, as well as the interactions amongst them, left us with only a few commercially available cell lines to choose from. Finally, we selected the murine colon epithelial cell line CMT-93. Since this cell line was poorly described when we started our experiments a few years ago, we decided to characterize it.

4.2.1 Characterization of the intestinal epithelial cell line CMT-93

The CMT-93 cell line is a transformed epithelial cell line isolated from a mouse rectal carcinoma (Franks and Hemmings, 1978). Firstly, we evaluated E-cadherin by immunofluorescence, which is a widely used marker to identify epithelial cells (Agiostratidou et al., 2007; Nachtigal et al., 2001). Figure 64 confirms the epithelial origin of CMT-93 cells in agreement with other studies (Assi et al., 2013; Bocuk et al., 2017).

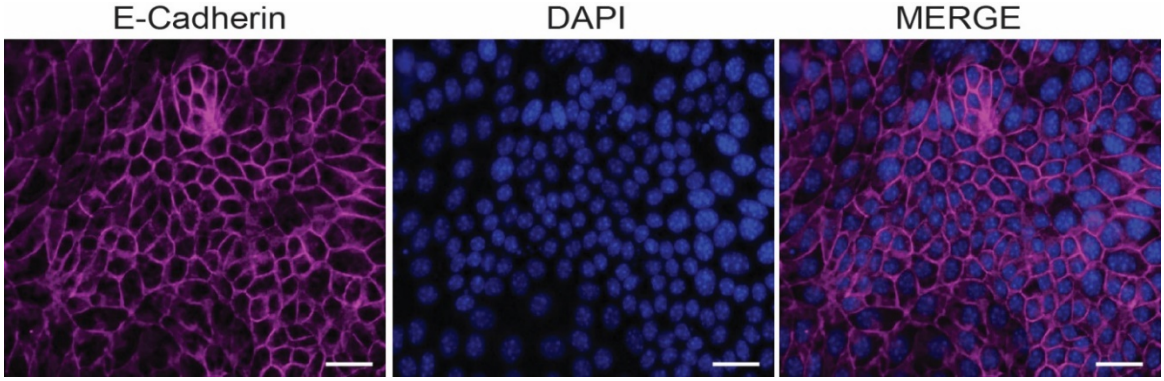


Figure 64. CMT-93 cells express E-Cadherin.

CMT-93 cells were stained by immunofluorescence for E-Cadherin. Scale bars, 20 μm

Next, we evaluated whether CMT-93 cells exhibit characteristics of normal IECs in terms of proliferation, differentiation and response to survival signals, and if they can mimic a normal epithelial barrier, reducing proliferation when confluence increases, or if they exhibit a more tumoral phenotype with loss of contact-inhibition (Puliafito et al., 2012). As previously described, when human cell lines such as Caco-2 and HT-29 are maintained for a long period of time under normal culture conditions in a confluent manner, they display a differentiated phenotype and a reduction of the proliferation rate, similar to the normal epithelial phenotype in the colon (Comalada et al., 2006). Therefore, we analyzed whether the maturation and differentiation of CMT-93 cells depended on the cellular confluence. As expected for normal IECs, the proliferative status of CMT-93 cells decreased significantly once they became confluent and was maintained low in post-confluent cultures of 5 and 10 days (Figure 65A and Figure 65B).

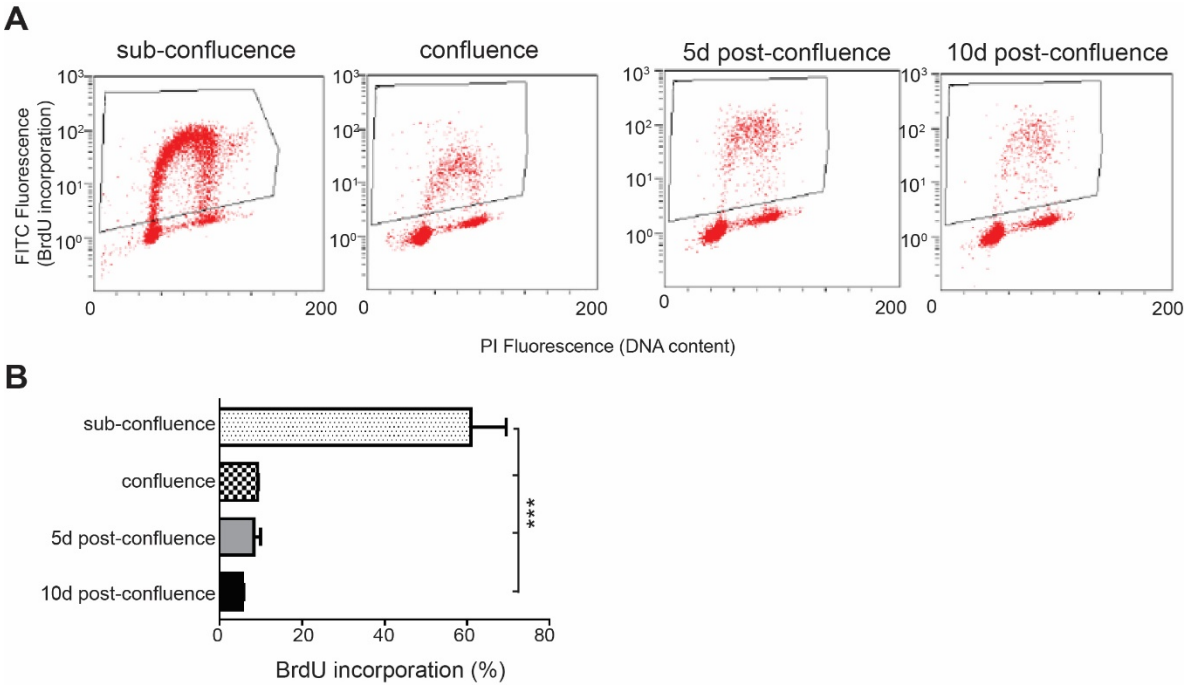


Figure 65. CMT-93 cells exhibit contact inhibition.

(A and B) CMT-93 cells were maintained in complete medium and analyzed for proliferation by BrdU at the indicated confluences. Representative FACS analysis of BrdU incorporation (A). Quantifications are shown in the histogram (B).
Data are expressed as the average±SD. ***, p ≤0.001.

IECs at the crypt base migrate along the so-called crypt-villus axis toward the intestinal lumen where their life cycle is terminated (Grossmann et al., 2002). In order to analyze if reduced proliferation correlated with apoptosis in CMT-93 cells, we performed AnnexinV/PI and Trypan Blue stainings. Our results showed that with increasing confluence of the cell culture, the number of apoptotic cells increased (Figure 66).

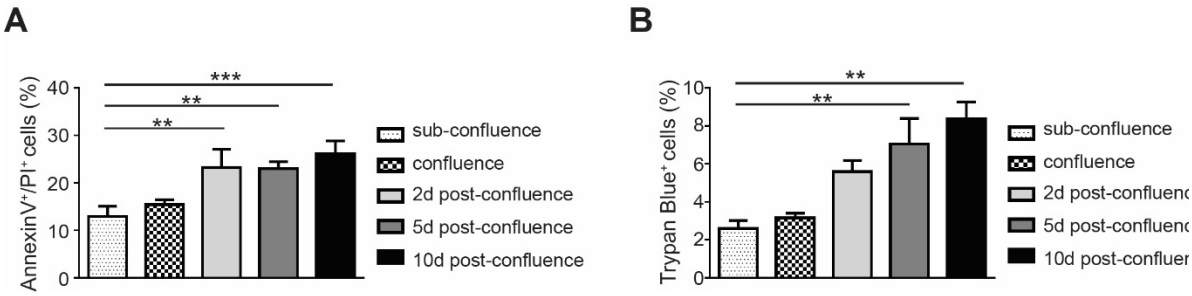


Figure 66. Apoptosis in CMT-93 cells increases with cellular confluence.

(A and B) CMT-93 cells were cultured for the indicated times and analyzed by AnnexinV/PI (A) and trypan blue (B) for cell death.
Data are expressed as the average±SD. **, p ≤0.01; ***, p ≤0.001.

IEC differentiation stages can be measured indirectly by analyzing general intestinal differentiation markers, such as caudal type homeobox 2 (CDX-2) and the intestinal alkaline phosphatase (ALP1) or by analyzing β−galactosidase as a marker for cellular senescence (Debacq-Chainiaux et al., 2009; Severino et al., 2000). Our results showed that confluent CMT-93 cells increased the mRNA expression of CDX-2 and ALP1 compared to sub-confluent cells (Figure 67).

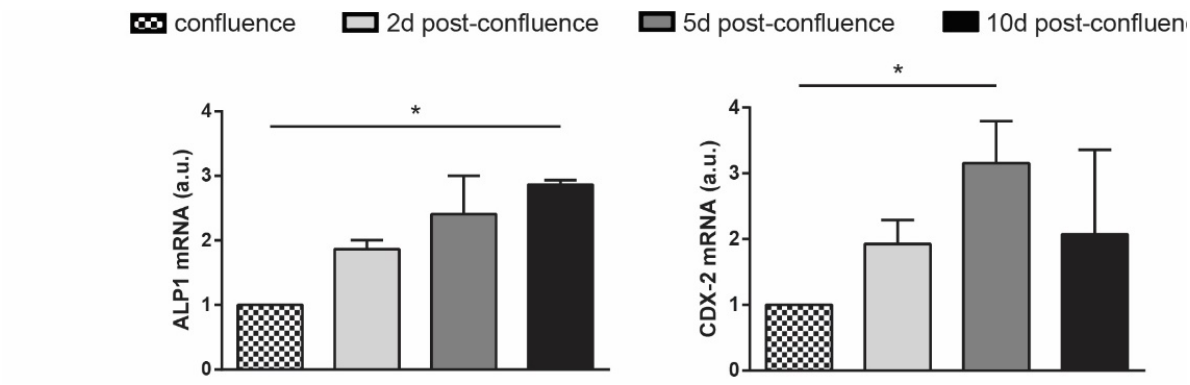


Figure 67. Intestinal differentiation markers increase with CMT-93 cell confluence.

Relative mRNA levels for the indicated genes were quantified by qRT-PCR at the indicated confluences in CMT-93 cells. Arbitrary units are referred to the expression level in the confluent control, which was given the value of 1.
Data are expressed as the average±SD. *, p≤0.05.

Consistently, the number and staining intensity of the senescence marker ß-galactosidase was also increased with confluency in CMT-93 cells (Figure 68). Of note, we observed that the cells in post-confluent cultures were not homogenously stained, and we could detect non-stained, slightly stained and strongly stained cells. This heterogeneity might resemble the behavior of IECs in the intestine, which progressively become senescent until they finally become apoptotic and shed into the lumen (Childs et al., 2014; Williams et al., 2015).

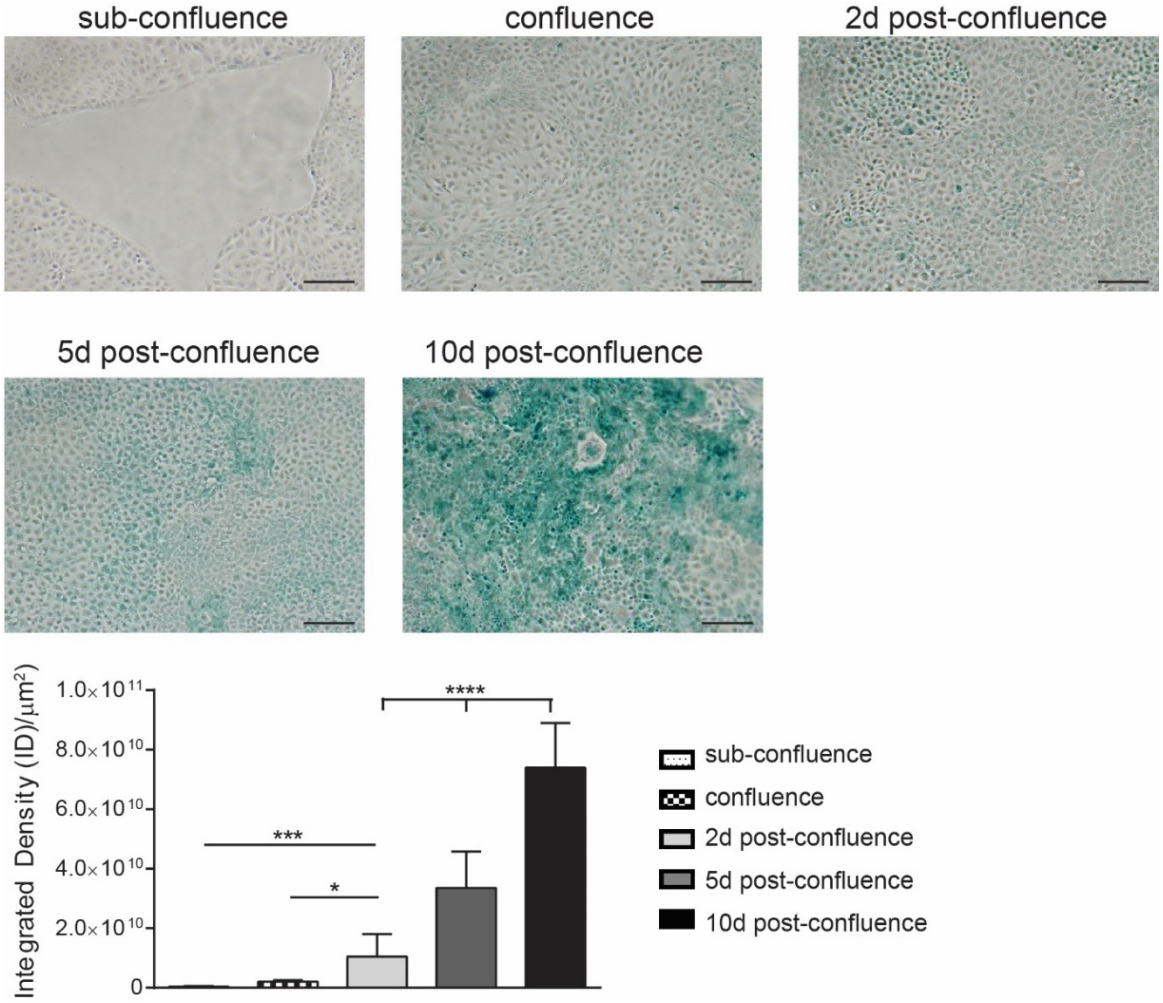


Figure 68. Cellular senescence increases with CMT-93 cell confluence.

CMT-93 cells were stained with β-galactosidase at the indicated confluences. Quantifications are shown in the histogram. Scale bars, 50 µm.
Data are expressed as the average±SD. *, p≤0.05; ***, p ≤0.001; ****, p ≤0.0001.

The above results were confirmed by analyzing stem and progenitor cell markers, such as leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), Achaete scute-like 2 (Ascl2) and Neurogenin-3 (Hardingham et al., 2015; Schonhoff et al., 2004; Stange, 2013; Zhang and Huang, 2013). We observed that LGR5 decreased with increasing time of the culture in confluency, but we did not observe statistically significant changes in the case of Ascl2 and Neurogenin-3 (Figure 69).

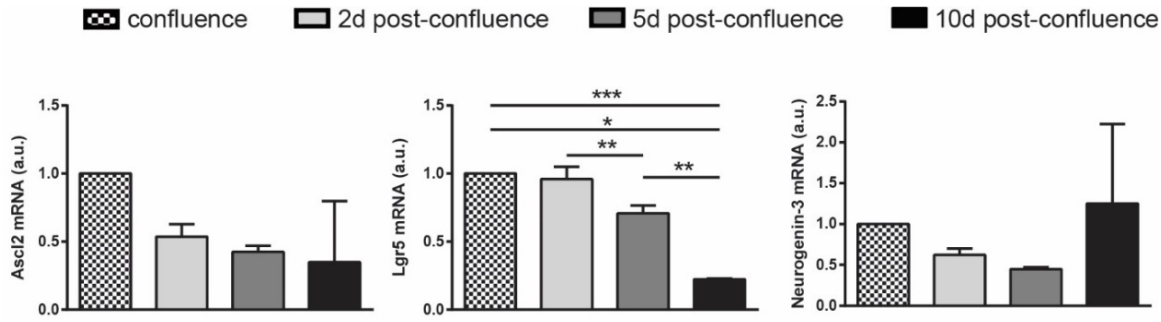


Figure 69. Expression of stem and progenitor cell markers in CMT-93 cells.

Relative mRNA levels for the indicated genes were quantified by qRT-PCR at the indicated confluences in CMT-93 cells. Arbitrary units are referred to the expression level in the confluent control, which was given the value of 1.
Data are expressed as the average±SD. *, p≤0.05; **, p ≤0.01; ***, p ≤0.001.

Since apoptosis and cellular senescence might be triggered by the DNA-damage response (d'Adda di Fagagna, 2008), we evaluated whether the observed increase in apoptosis and senescence, might result from the accumulation of DNA damage in these cells. The co-localization of phosphorylated histone H2AX (γ-H2AX) and p53-binding protein 1 (53BP1) is a key step in the DNA damage repair (Bekker-Jensen et al., 2005; Stewart et al., 2003; van Attikum and Gasser, 2009; Xu and Stern, 2003). By immunofluorescence staining, we could only rarely detect the co-localization of these two proteins in different CMT-93 cell confluences, and we did also not observe DNA damage accumulation with increased confluency (Figure 70).

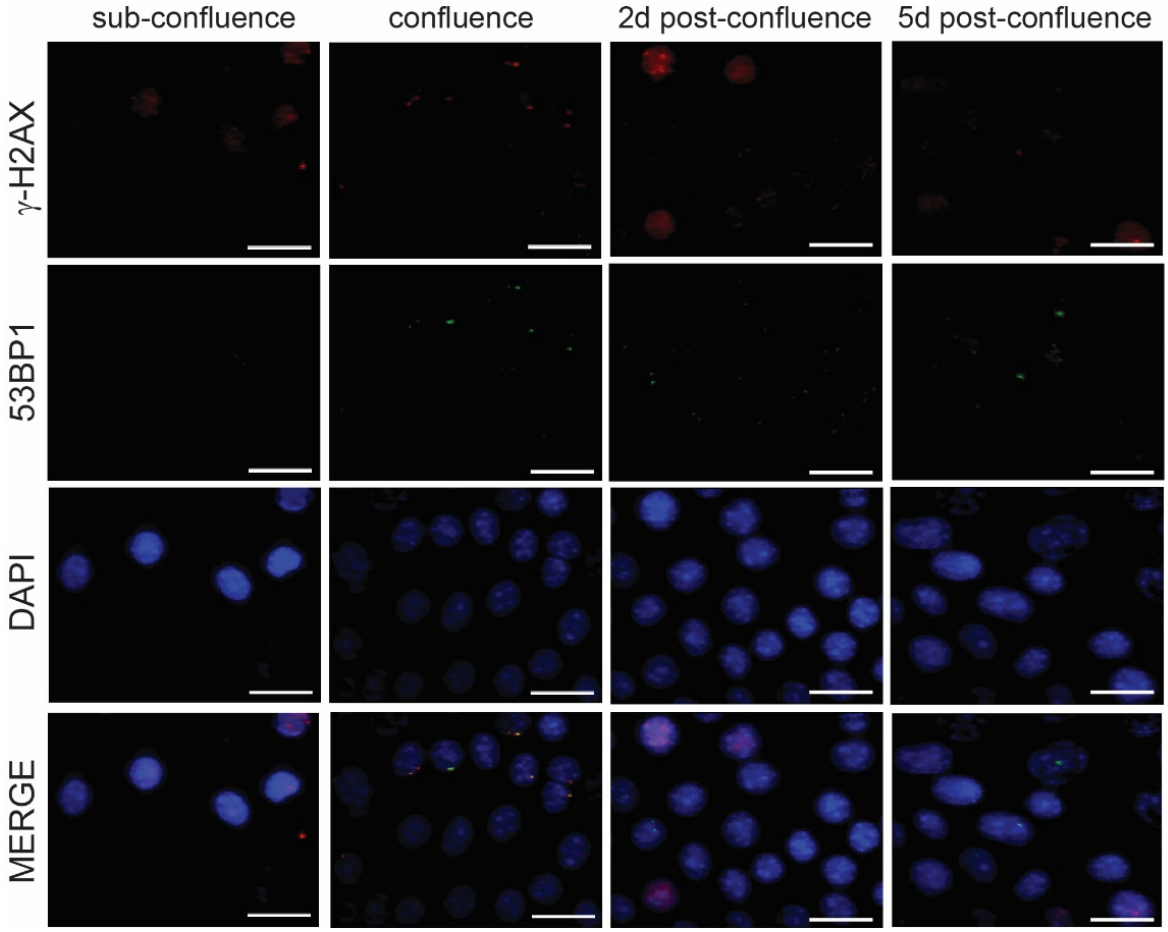


Figure 70. DNA damage foci do not accumulate in post-confluent CMT-93 cell cultures.

CMT-93 cells were stained by immunofluorescence for γ-H2AX and 53BP1 at the indicated confluences to visualize DNA damage foci. Scale bars, 20 μm

These results illustrate that CMT-93 cells in long term cultures (2-, 5- and 10 days post-confluence) showed a reduced proliferative ratio with differentiated and senescent phenotypes, which resemble normal IEC behavior.

The classical physical barrier functions of IECs include the secretion of mucins and trefoil factors (Pitman and Blumberg, 2000; Taupin and Podolsky, 2003). According to the colorectal origin of CMT-93 cells, the mRNA levels for mucin-2 and TFF3, two well-known markers for goblet cells in the intestine (Kim and Ho, 2010; Taupin and Podolsky, 2003), augmented with increased CMT-93 cell confluence (Figure 71).

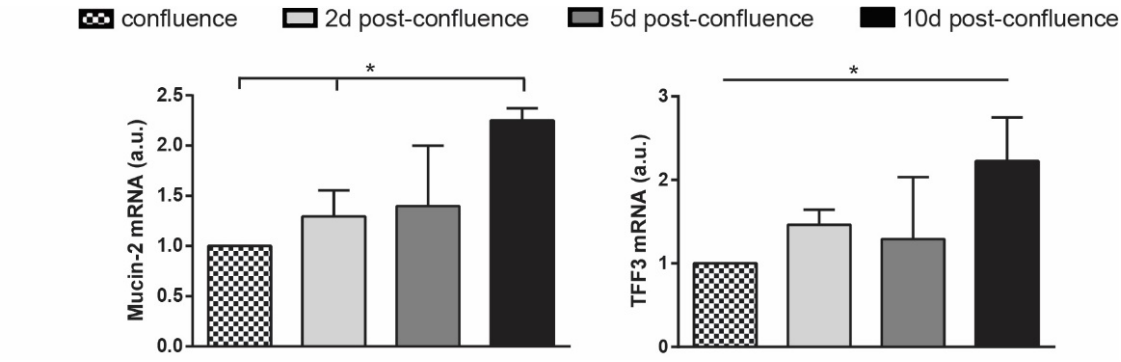


Figure 71. Goblet cell markers increase with CMT-93 cell confluence.

Relative mRNA levels for the indicated genes were quantified by qRT-PCR at the indicated confluences in CMT-93 cells. Arbitrary units are referred to the expression level in the confluent control, which was given the value of 1.
Data are expressed as the average±SD. *, p≤0.05.

In addition, we analyzed the expression of the enteroendocrine cell marker chromogranin A, as well as the paneth cell marker lysozyme (Ho et al., 1989; Quinlan et al., 2006; Szumilo et al., 2005). Our results showed that mRNA expression of chromogranin A and lysozyme tent to increase with confluence, but we could not observe statistical significant differences between late confluent and sub-confluent CMT-93 cell cultures (Figure 72).

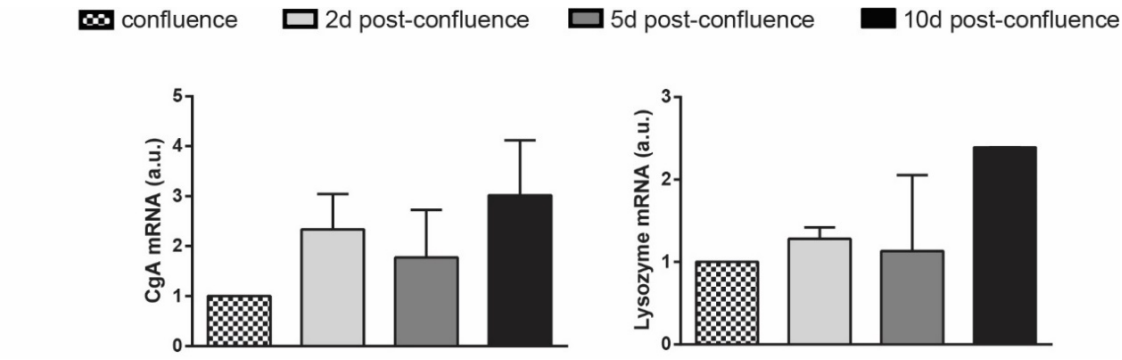


Figure 72. Chromogranin A and Lysozme mRNA are not significantly induced upon CMT-93 cell confluence.

Relative mRNA levels for Chromogranin A (CgA) and Lysozyme genes were quantified by qRT-PCR at the indicated confluences in CMT-93 cells. Arbitrary units are referred to the expression level in the confluence control, which was given the value of 1.
Data are expressed as the average±SD. *, p≤0.05.

Since tight junctional proteins are involved in the maintenance of the intestinal epithelial barrier (Turner, 2006), we analyzed the expression of key junctional proteins at different cellular confluences. Western blot analysis showed that zona occludens-1 (ZO-1), claudin-1 and claudin-2 were mainly detected 2 days post-confluence (Figure 73).

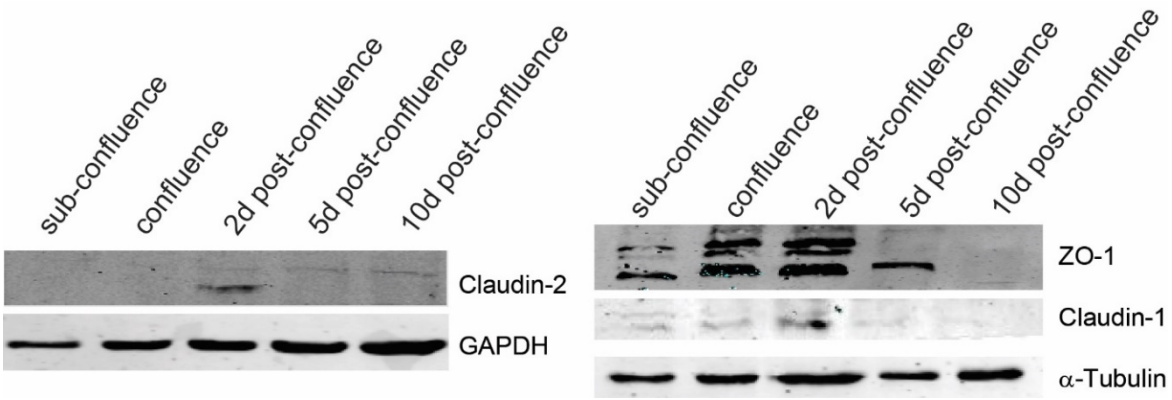


Figure 73. Expression of junctional proteins in CMT-93 cells.

Western blot of the indicated proteins in CMT-93 cells collected at the indicated confluences.

Given that tight junctional proteins modulate paracellular permeability through the formation of a selectively permeable seal between adjacent epithelial cells (Suzuki, 2013), we next questioned whether the changes observed in the expression of key junctional proteins in different cell confluences affect the paracellular permeability. Therefore, we measured paracellular permeability using FITC–dextran (Cong et al., 2017; Maher et al., 2007; Markov et al., 2016). Interestingly, CMT-93 cells exhibited the lowest paracellular permeability at 2 days post-confluence, correlating with the increased expression of ZO-1, claudin-1 and claudin-2 (Figure 74).

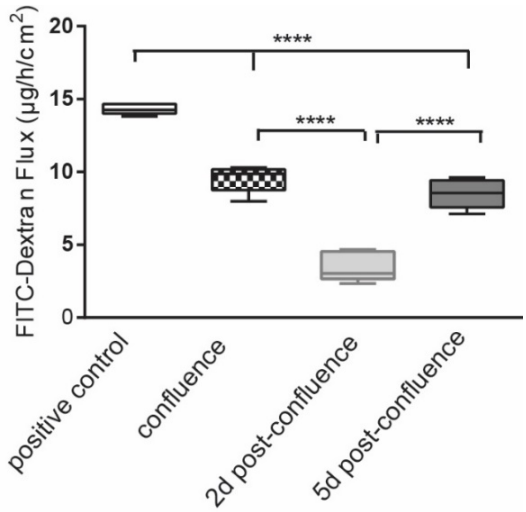


Figure 74. Paracellular permeability of CMT-93 cells in different confluences.

Paracellular Flux of 4 kDa FITC-dextran from the upper to the lower compartment of transwell chambers containing CMT-93 cells at the indicated confluences was measured by spectrophotofluorometry. IL-1β (15 ng/ml) as positive control was incubated for 3 days. Data are expressed as the average±SD. ***, p ≤0.001; ****, p ≤0.0001.

In summary, we concluded that CMT-93 cells could serve as an adequate model for the colon intestinal epithelial barrier due to their ability to form monolayers. Moreover, we decided to perform experiments at 2 days post-confluency, since these cultures express higher levels of junctional proteins and seem to form a more "intact" monolayer at this time point.

4.2.2 p38 MAPK signaling in CMT-93 cells

It has been previously shown that intestinal permeability of mice lacking p38α in IECs was notably increased (Gupta et al., 2014). Indeed, we could corroborate this result in CMT-93 cells *in vitro*. For this purpose, CMT-93 cells were incubated with two p38α inhibitors (PH-797804 and SB-203580) and then paracellular permeability was measured using FITC-dextran. IL-1β stimulation was used as a positive control for the permeability assay (Al-Sadi and Ma, 2007). Our results showed a significant increase of paracellular permeability in CMT-93 cells incubated with p38α inhibitors compared to vehicle (DMSO) incubated control cells (Figure 75).

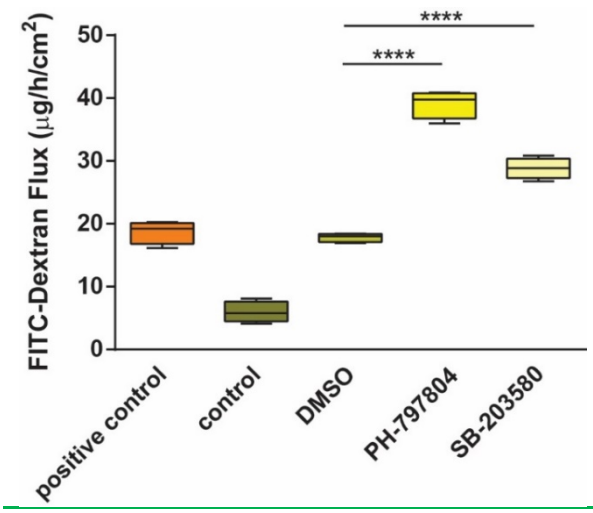


Figure 75. p38α MAPK signaling maintains epithelial integrity of CMT-93 cells.

Paracellular flux of 4 kDa FITC dextran from upper to lower compartment of transwell chambers containing CMT-93 cells was measured by spectrophotofluorometry. IL-1β as positive control and the indicated p38α inhibitors or DMSO vehicle were incubated for 3 days. Data are expressed as the average±SD. ****, $p \leq 0.0001$.

Cellular senescence is considered an important regulator of tumor suppression (Ishikawa, 2003). Previous studies have suggested that p38 MAPK signaling plays a central role in cellular senescence in various cell types, including epithelial cells and fibroblasts (Debacq-Chainiaux et al., 2010; Koul et al., 2013; Xu et al., 2014). It has been demonstrated that p38 MAPK is activated during cellular senescence, and expression of its upstream kinase MKK6 suffices to induce cellular senescence (Cargnello and Roux, 2011; Wang et al., 2002). Moreover, pharmacological or genetic inactivation of p38α

inhibited the onset of cellular senescence (Iwasa et al., 2003; Kuilman et al., 2010; Zhou et al., 2011). Consistent with these results, we observed that p38α inhibitors significantly inhibited the onset of confluence-induced cellular senescence in CMT-93 cells (Figure 76A and Figure 76B). Of note, we also observed increased p38 MAPK phosphorylation in CMT-93 cells with increasing cellular confluence (Figure 76C).

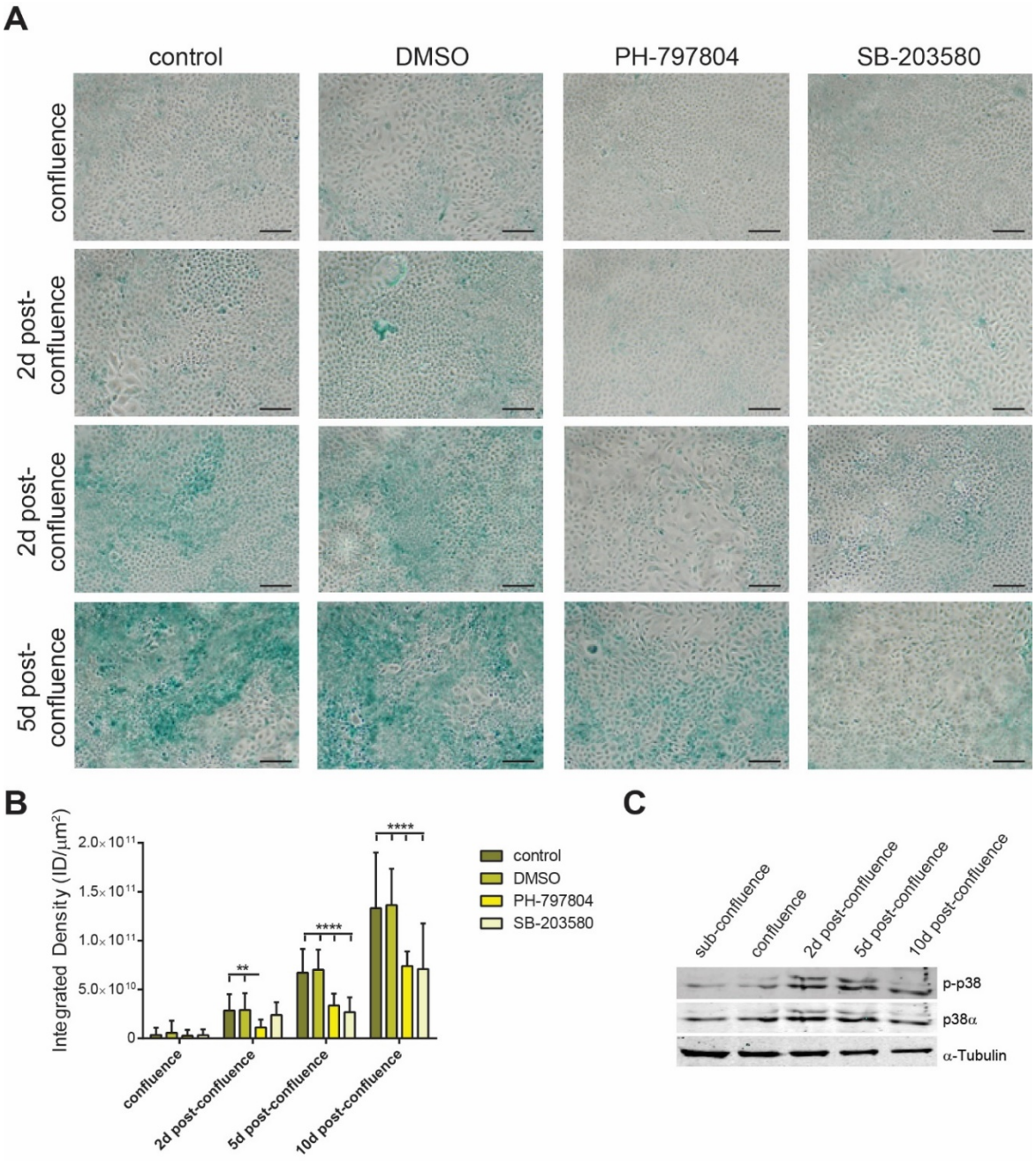


Figure 76. p38α mediates senescence induced upon CMT-93 cellular confluence.

(A and B) Representative images of CMT-93 cells grown in the presence or absence of the indicated p38 MAPK inhibitors or DMSO for 3 days at the indicated confluences and stained with β-galactosidase. Scale bars, 50 µm. (A) Quantifications are shown in the histogram (B).
(C) Western blots of the indicated proteins in CMT-93 cells collected at the indicated confluences.
Data are expressed as the average±SD. **, p ≤0.01; ****, p ≤0.0001.

4.2.3 p38α promotes cell migration in CMT-93 cells

Inflammation and inflammation-associated cancer are linked to excessive wound-healing processes, which strongly predispose to the development of neoplasms and tumor progression, although these mechanisms are also important for mucosal integrity (Balkwill and Mantovani, 2001; Schafer and Werner, 2008; Sussman et al., 2012) (see 1.3.2.3 Wound-healing in CAC).

In order to evaluate the role of p38α in this process, we opted for the scratch assay, which is a simple and sensitive method to define the wound-healing capacity of cells. This assay is one of the earliest developed methods to study directional cell migration *in vitro*, and is used to mimic cell migration during wound-healing *in vivo* (Liang et al., 2007a; Rodriguez et al., 2005). The method is based on the observation that, upon induction of a physical wound, by scratching a confluent epithelial cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the scratch until new cell–cell contacts are established (Liang et al., 2007b; Rodriguez et al., 2005; Ueck et al., 2017).

CMT-93 cells were seeded in clear-bottom well plates at low density and allowed to form 2 days-post confluent monolayers. We observed that the wound-healing process initiated just shortly after wounding with a pipette tip. CMT-93 cells were also incubated in the presence of the p38α inhibitors, PH-797804 and SB-203580, which are added to the culture just after the scratch. A significant cell reduction in migration could be observed with both p38α inhibitors. Therefore, we concluded that p38α positively regulates migration in a scratched monolayer of CMT-93 cells (Figure 77). Indeed, p38α activity has been shown to increase cell migration *in vivo* and *in vitro* in a variety of cell types, including cancer cells, endothelial cells and leukocytes (Chung et al., 2012; Ghosh et al., 2014; Lam and Huttenlocher, 2013; Loesch and Chen, 2008; Wagner and Nebreda, 2009; Yoshizuka et al., 2012).

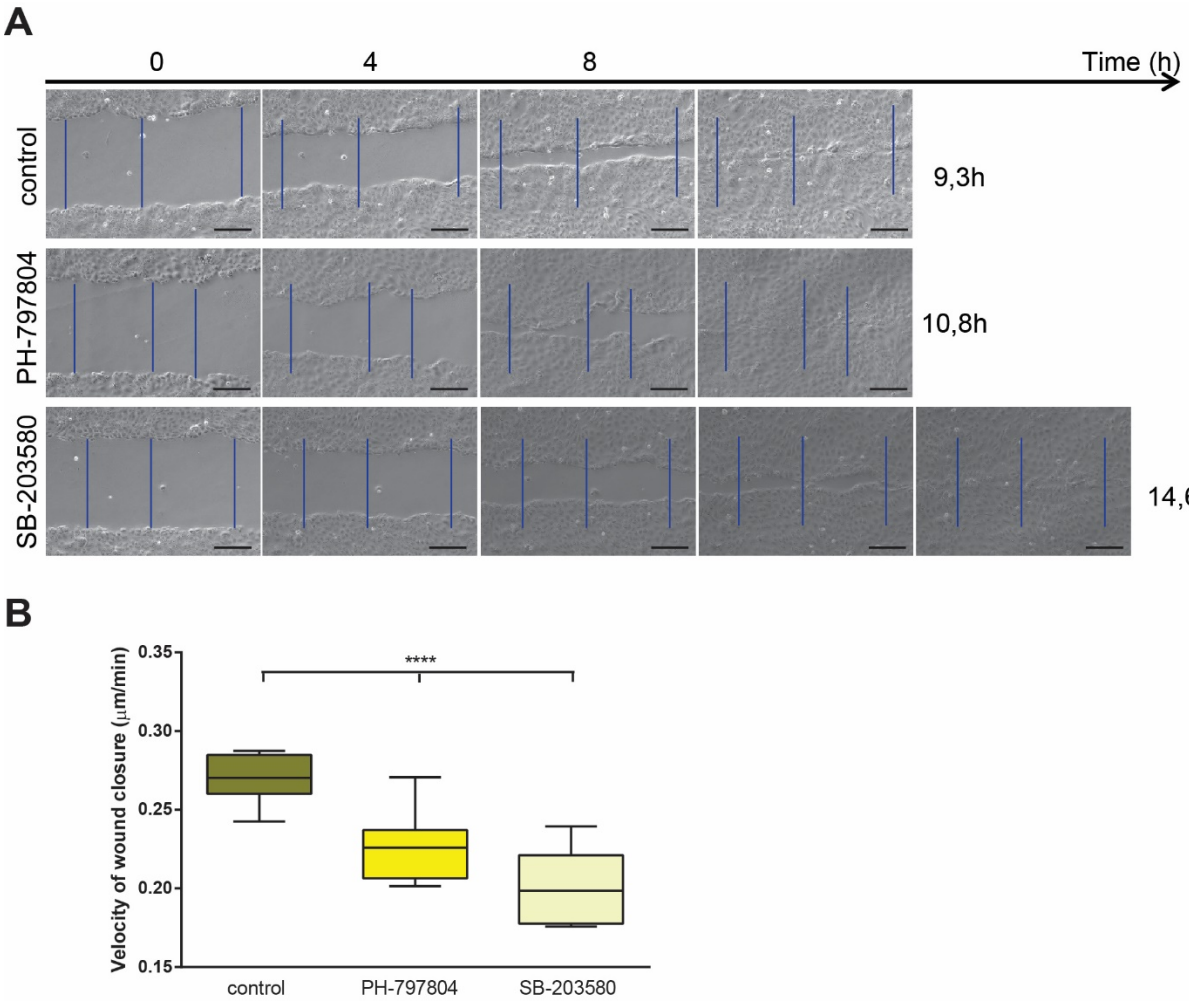


Figure 77. p38α inhibition impairs CMT-93 cell migration.

(A and B) Representative images of CMT-93 cells that were physically wounded by a scratch in the presence of the indicated inhibitors or DMSO (control) (A). Quantification is shown in the histogram (B). Scale bars, 50 µm.
Data are expressed as the average±SD. ****, p ≤0.0001

In order to validate the implication of p38α in this process, we decided to analyze its phosphorylation by immunofluorescence at different time points after scratching. Interestingly, we detected p38 MAPK phosphorylation shortly after scratching the CMT-93 cell monolayer. Of note, this phosphorylation was strongest and preferentially detected at the edges of the scratch, suggesting the involvement of p38 MAPK signaling at the migratory leading front, and further confirming the implication of this signaling pathway in the wound-healing process (Figure 78).

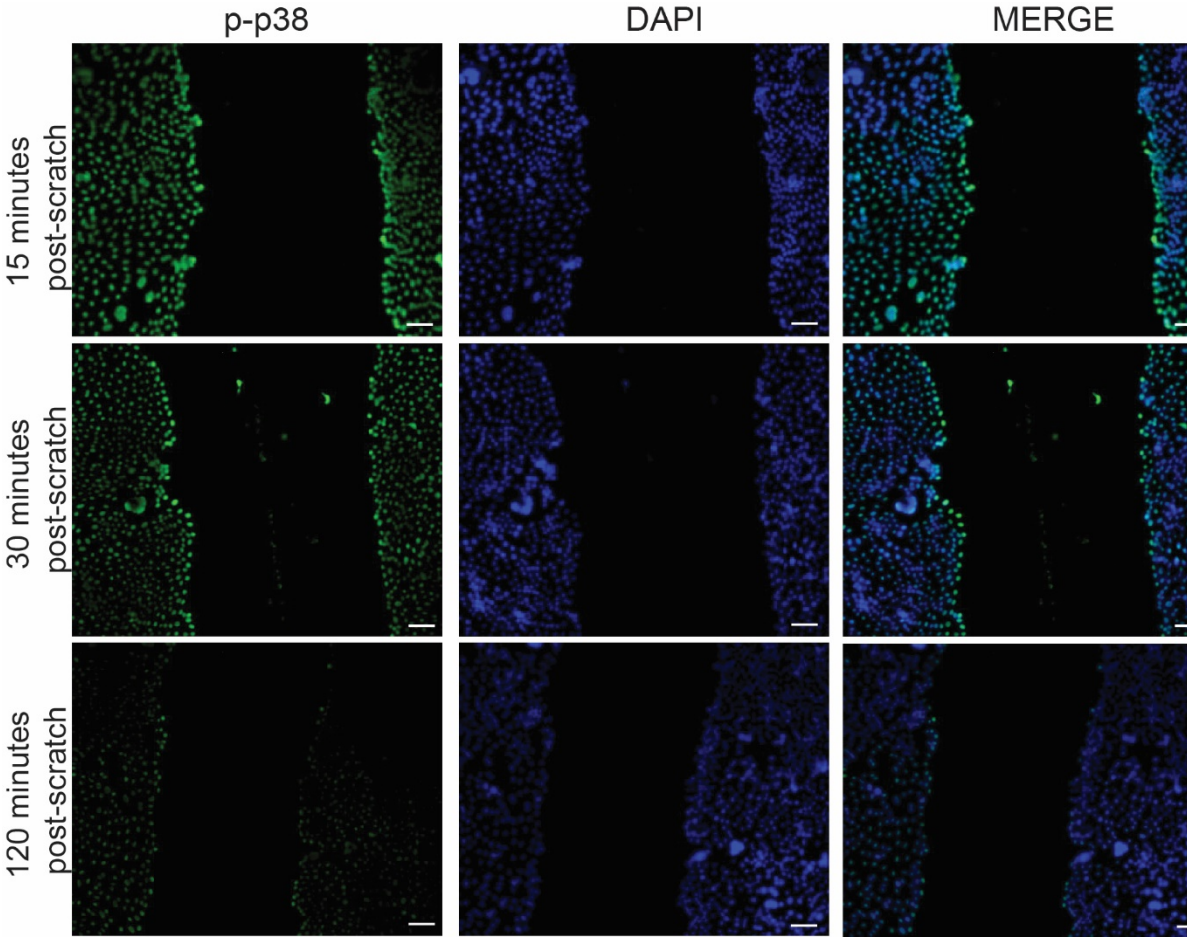


Figure 78. p38 MAPK is activated upon induction of physically induced damage in CMT-93 cells.

CMT-93 cells were scratched and stained for phospho-p38 MAPK at the indicated times. Scale bars, 50 μm

We next aimed to define the mechanism by which CMT-93 cells close the physically induced wound. Mainly, we evaluated three possible mechanisms by which this wound closing could occur. One possible mechanism is that the cells close the wound by proliferating into the wound bed. However, the gap induced by scratching the CMT-93 cell monolayer usually closes within 9-10 hours (Figure 77 above), therefore we concluded that this mechanism is unlikely to explain the wound-healing. Moreover, the experiments were performed at 2 days post-confluence and, at this stage, prior to inducing the scratch, proliferation is strongly impaired due to contact inhibition (Figure 65 above). Nevertheless, to confirm that proliferation does not contribute to this process, we performed the wound-healing assay in the presence of Mitomycin C (MMC), an antibiotic, that causes cross-linking of double-stranded DNA and inhibits cell proliferation (Glenn et al., 2016; Shikatani et al., 2012). We first evaluated the concentration of MMC that would efficiently inhibit proliferation of CMT-93 cells. Since

CMT-93 cells stop proliferation upon confluence due to contact inhibition, this analysis was performed in sub-confluent cell cultures. We also analyzed apoptosis at 2 days post-confluency, the time we performed most experiments. We found that MMC inhibited BrdU incorporation starting at a concentration of 5 μg/ml in sub-confluent CMT-93 cell cultures (Figure 79A), whereas apoptosis measured by AnnexinV/PI staining was not significantly affected at this concentration in 2 days post-confluent CMT-93 cell cultures Figure 79B). Therefore, we decided that 5μg/mL of MMC was an appropriate concentration for our experiments.

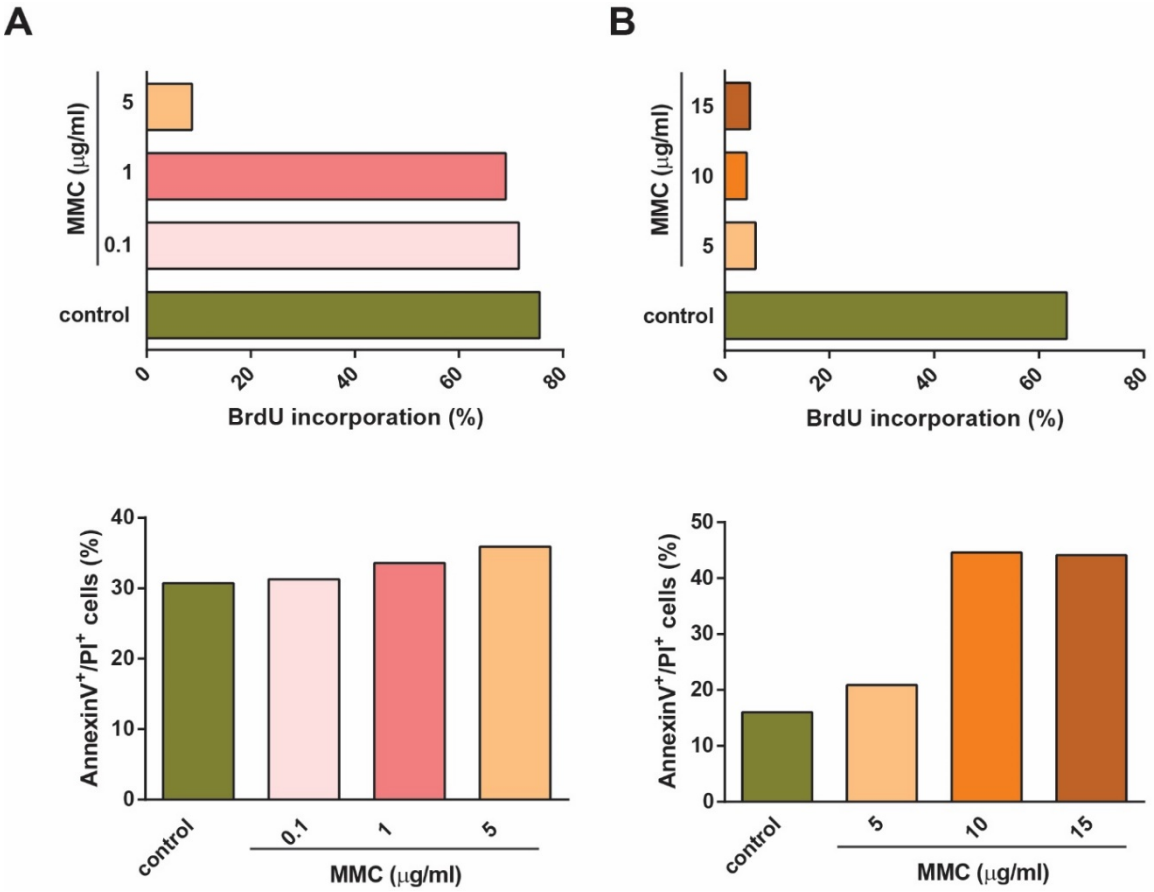


Figure 79. Effect of Mitomycin C in CMT-93 cell proliferations.

(A) Sub-confluent proliferating CMT-93 cells were incubated for 24 hours with or without the indicated concentrations of MMC and proliferation was analyzed by BrdU incorporation.
(B) Two days post-confluent CMT-93 cells were incubated for 24 hours in the presence or absence of the indicated concentrations of MMC and apoptosis was analyzed by AnnexinV/PI.

When the wound-healing assay was performed in 2 days post-confluent CMT-93 cell monolayers in the presence of MMC (5 μg/ml) added immediately after induction of

the scratch, we did not observe any delay in the velocity of wound closure (Figure 81). This confirms that the cell proliferation is not required for the process.

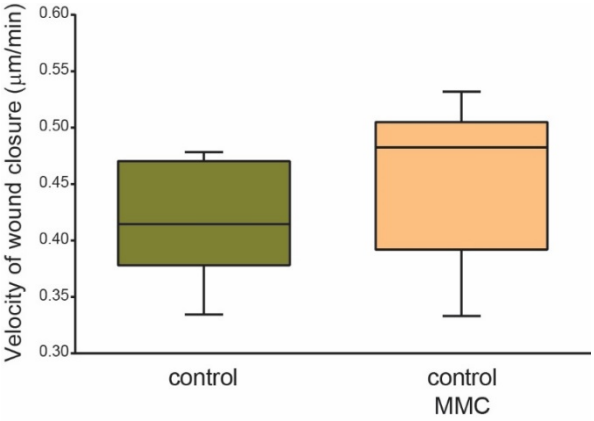


Figure 80. Proliferation is not involved in closure of physically induced wounds in CMT-93 cells.

CMT-93 cells were physically wounded by a scratch in the presence or absence of MMC. Data are expressed as the average±SD.

As previously mentioned, experiments were performed in 2 days post-confluent CMT-93 cell cultures. At this time point, in contrast to sub-confluent cell cultures, the cells in the monolayer were squeezed together, due to space limits and exhibited a "smaller appearance" (Figure 81). Therefore, another factor that might contribute to wound-closure in CMT-93 cell monolayers is the physical pressure exerted by cells lying at both sides of the scratch.

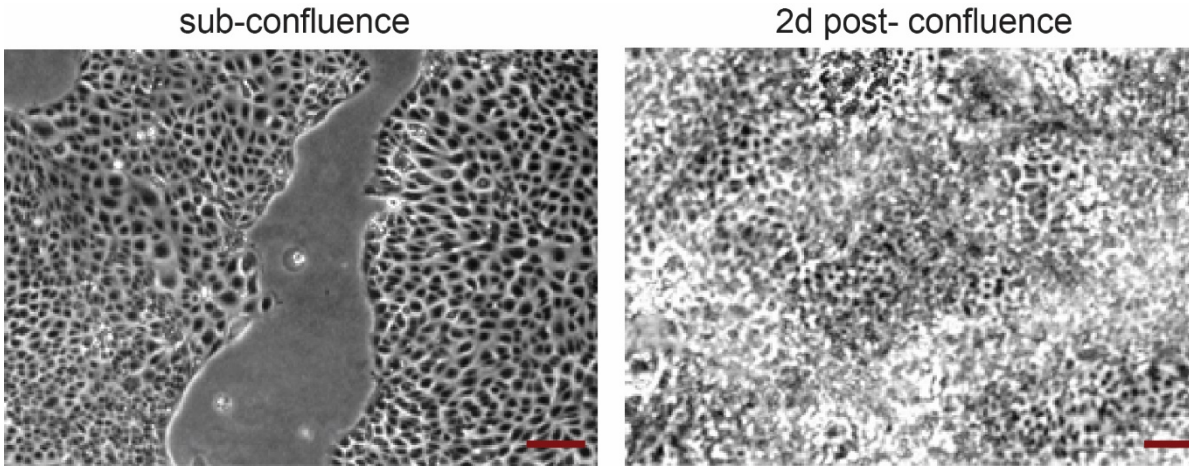


Figure 81. Increased cellular density in post-confluent CMT-93 cell cultures.

Images were taken from sub-confluent and 2d post-confluent CMT-93 cell cultures. Scale bars, 25 µm.

Our results indicated that the wound-closure observed in CMT-93 cell cultures probably occur due to active migration into the wound bed. Consistent with this idea, we

found that the wound-healing process occurred similarly in sub-confluent cell cultures, supporting that the process does not occur due to cellular expansion (Figure 82). We therefore conclude that CMT-93 cells promote wound closure mainly by migration.

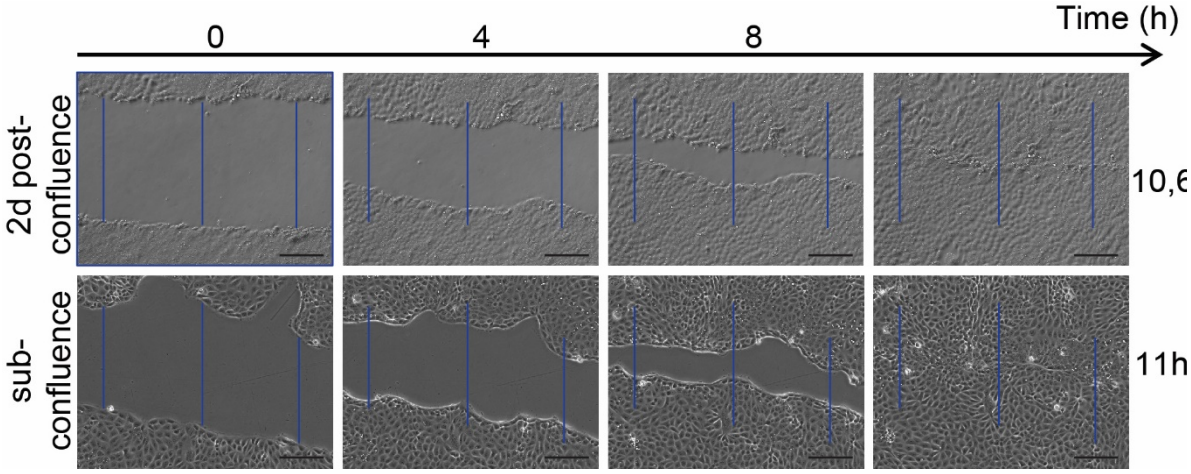


Figure 82. Wound-healing in CMT-93 cells is not mediated by cellular expansion.

Representative images of CMT-93 cells growing as indicated at 2d post-confluence or in sub-confluence (80-90%), which were physically wounded by a scratch. Scale bars, 50 μm.

Next, we aimed to identify the mechanism by which p38α regulated migration of CMT-93 cells upon scratch of the monolayer. The downstream p38α substrate MAPK-activated protein kinase 2 (MAPKAPK-2 or MK2), plays an important role in cell migration by remodeling the actin cytoskeleton (Cargnello and Roux, 2011; Koul et al., 2013). This cytoskeletal remodeling may be mediated by phosphorylation of the 27 kDa heat shock protein (Hsp27), inducing its release from F-actin caps (Kobayashi et al., 2006). To check if MK2 was implicated in the wound-healing process in CMT-93 cells, we used the chemical inhibitors, MK2 inhibitor III and PF-3644022. We also used SB-747651A, an inhibitor of stress-activated protein kinase 1 (MSK1) inhibitor, which is another downstream p38α MAPK target (Figure 68). Our results showed a significant reduction of migration in CMT-93 cells treated with the p38α or MK2 inhibitors, whereas no significant changes could be observed with the MSK1 inhibitor (Figure 83).

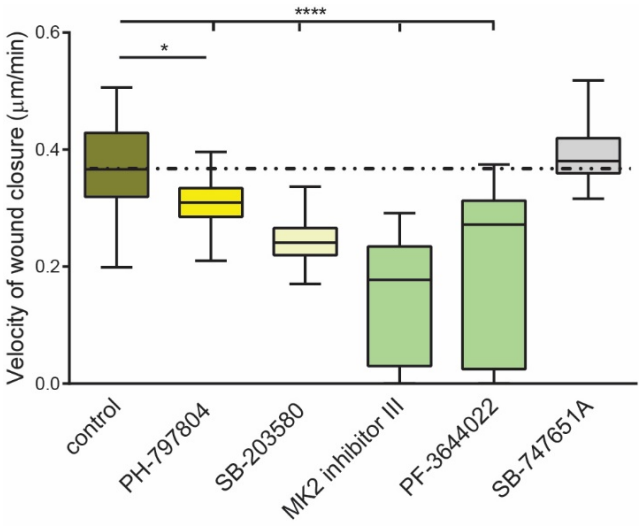


Figure 83. MK2 activation is involved in CMT-93 cell migration.

CMT-93 cells were physically wounded by a scratch and incubated in the presence or absence of the indicated chemical inhibitors or DMSO vehicle. Data are expressed as the average±SD. *, p ≤0.05; ****, p ≤0.0001.

4.2.4 Regulation of IGF-1 by p38α in macrophages stimulates the migration of CMT-93 cells

We next investigated, whether p38α signaling in macrophages regulates cytokines and growth factors that affect the wound-healing process in CMT-93 cells. We observed that conditioned media derived from BMDMs significantly increased the migration velocity of CMT-93 cells upon scratch. Interestingly, pre-incubation of BMDMs with p38α inhibitors for 24 hours reduced the ability of conditioned media to induce CMT-93 cell migration compared with untreated BMDMs (Figure 84A). These results indicated that p38α in BMDMs regulates the expression and/or secretion of extracellular factors that are able to stimulate migration of CMT-93 cells. This result was confirmed by using supernatants derived from BMDMs obtained from p38α, p38β and p38α/p38β *knock-out* (KO) mice (Figure 84B). Of note, the L-cell media used for macrophage differentiation was not able by itself to increase the migration velocity of CMT-93 cells (Figure 84B).

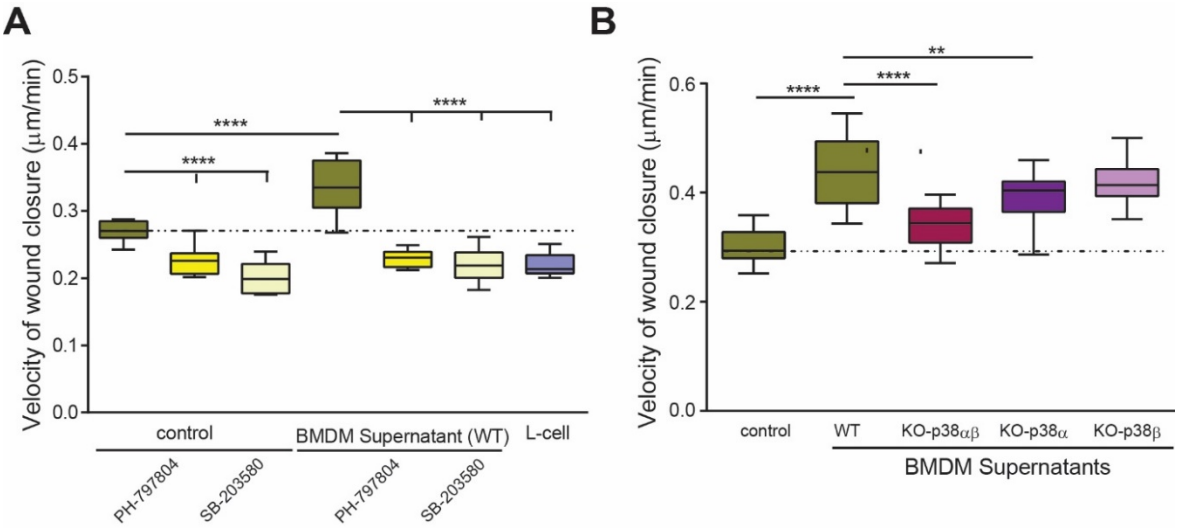


Figure 84. Extracellular factors regulated by p38α in BMDMs increase the migration velocity of CMT-93 cells.

(A) CMT-93 cells were physically wounded by a scratch and incubated in the presence or absence of supernatants derived from WT BMDMs that were previously treated for 24 h with the indicated p38α inhibitors or DMSO control. The inhibitors or DMSO control as well as L-cell conditioned medium were also added to the assay without BMDM supernatants.
(B) CMT-93 cells were physically wounded by a scratch and incubated in the presence or absence of supernatants derived from the indicated BMDMs.
Data are expressed as the average±SD. **, p ≤0.01; ****, p ≤0.0001.

Experiments using MMC confirmed that proliferation was not involved in the increased migration velocity of CMT-93 cells induced by BMDM supernatants (Figure 85).

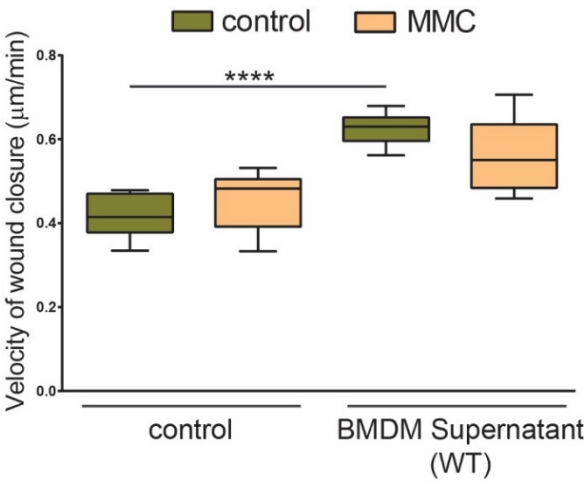


Figure 85. Proliferation is not involved in the increased of migration velocity of CMT-93 cells cultured in the presence of BMDM supernatants.

CMT-93 cells were physically wounded by a scratch in the presence or absence of MMC and supernatants derived from BMDM. Data are expressed as the average±SD. ****, p ≤0.0001.

We had observed that p38α regulates IGF-1 in macrophages (Figure 34 above), and this growth factor is known to stimulate migration in several cell types, including

IECs (Chen et al., 1999; Furundzija et al., 2010; Guvakova, 2007; Jeong et al., 2014; Nadzir et al., 2013; Pelosi et al., 2007). Therefore, we evaluated whether IGF-1 was able to induce migration in CMT-93 cells. We found that, similarly to WT BMDM supernatants, incubation of CMT-93 cells in the presence of IGF-1 significantly increased their wound-healing velocity compared to both the control and to supernatants derived from p38α-Δ^{MC} BMDMs (Figure 86).

In summary, our results strongly suggest the involvement of p38α signaling in wound-healing of IECs at two levels. On one hand, p38α regulates migration through MK2 in IECs. In addition, p38α regulates the production of IGF-1 and probably other cytokines and growth factors by macrophages, which lead to migration of IECs and wound closure.

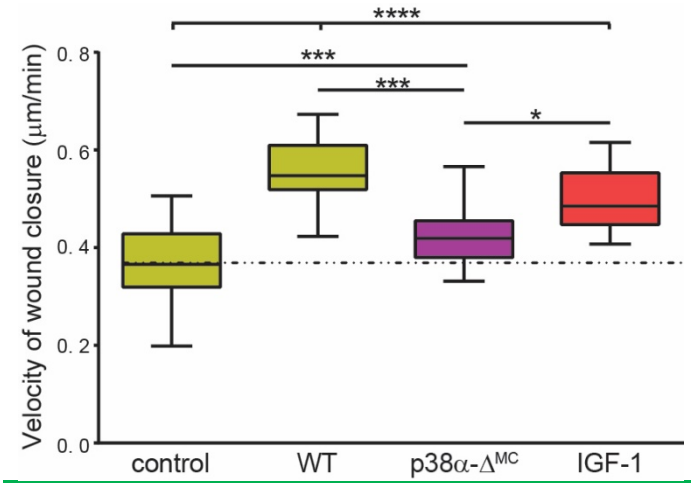


Figure 86. IGF-1 induces migration in CMT-93 cells.

CMT-93 cells were physically wounded by a scratch and incubated in the presence or absence of IGF-1 (100 ng/ml) or supernatants derived from WT or p38α-Δ^{MC} BMDM.
Data are expressed as the average±SD. *, p ≤0.05; ***, p ≤0.001; ****, p ≤0.0001.

4.2.5 IGF-1 induces CMT-93 cell proliferation and protects from DSS induced apoptosis

We were interested in confirming the role of IGF-1 in IECs subjected to inflammatory conditions. IGF-1 is known to promote cell proliferation and survival (Clayton et al., 2011; Shanmugalingam et al., 2016; Wagner et al., 2007; Yu and Rohan, 2000). The effect of DSS in IECs has been reported in several studies, including *in vitro* models (Ahmad et al., 2014; Chang and Kuo, 2015; Han et al., 2015; Viennois et al., 2013). We observed that IGF-1 induced, in a dose dependent manner, the proliferation of sub-confluent CMT-93 cells (Figure 87A). DSS treatment also enhanced CMT-93 cell proliferation but did not significantly modify proliferation induced by IGF-1 (Figure 87A). On the other hand, DSS treatment induced the expected increased apoptosis in

CMT-93 cells, and treatment with IGF-1 significantly reduced both the DSS-induced apoptosis and the basal apoptosis observed at 2 days post-confluence in CMT-93 cells (Figure 87B). These results support a pro-survival role of IGF-1 in the IEC cell line CMT-93.

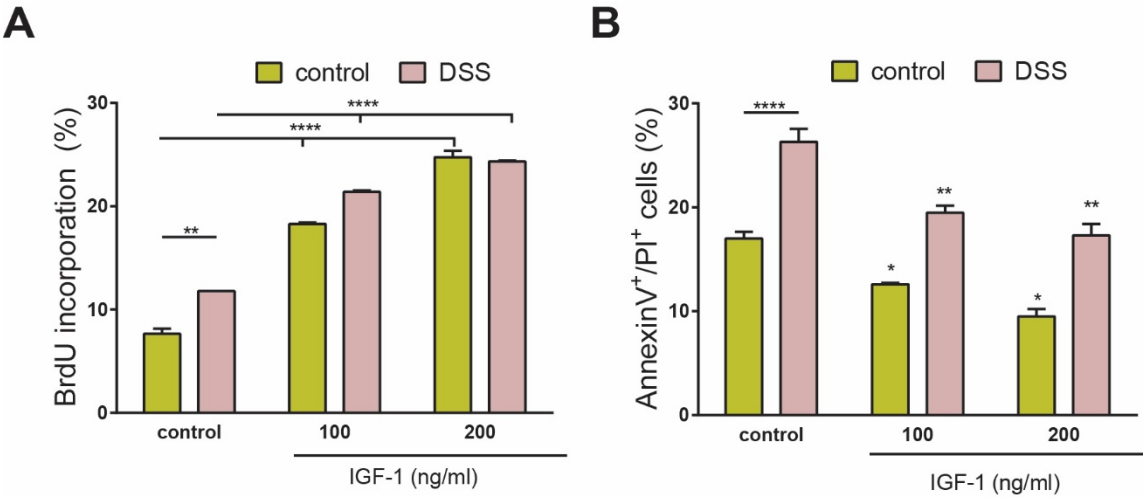


Figure 87. IGF-1 induces proliferation and inhibits apoptosis in CMT-93 cells.

(A) Sub-confluent proliferating CMT-93 cells were incubated for 24 hours in the presence or absence of DSS (2%) and with or without IGF-1 at the indicated concentrations. Proliferation was analyzed by BrdU incorporation.
(B) Two days post-confluent CMT-93 cells were incubated for 24 hours in the presence or absence of DSS and with or without IGF-1 at the indicated concentrations. Apoptosis was analyzed by AnnexinV/PI.
Data are expressed as the average±SD. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$

To sum up, our *in vitro* studies demonstrate the involvement of the IGF-1-p38α-MK2 axis in migration of CMT-93 cells. We show that p38α signaling regulates the secretion of factors that increase the migration velocity of CMT-93 cells, and identify IGF-1 as a novel p38α effector that induces CMT-93 cell migration. These results are in concordance with our *in vivo* results, since migration is not only important for wound-healing processes but is also a pro-tumorigenic property of cancer cells, and cancer has been proposed to be an overt healing wound (Schafer and Werner, 2008)

DISCUSSION

5. DISCUSSION

Chronic inflammation is a hallmark of colon cancer and can predispose to carcinogenesis as observed in patients with IBD and other inflammatory disorders that are prone to developing tumors (Coussens and Werb, 2002; Thorsteinsdottir et al., 2011). However, the molecular and cellular events involved in the pathogenesis of CAC are not fully understood (Kaplan and Ng, 2017). Myeloid cells play a key role in the TME, regulating tumor growth and therapeutic responses. (Grivennikov et al., 2010; Mantovani et al., 2017; Tariq et al., 2017; Waldner and Neurath, 2015). One of the pathways that has been implicated in a plethora of inflammatory diseases and cancers is controlled by the protein kinase p38α (Gupta and Nebreda, 2015; Kim and Choi, 2015; McKinnon et al., 2016; Qian et al., 2016). Specifically, p38α is a key regulator of IEC homeostasis protecting against inflammation-associated colon tumorigenesis in mice (Gupta et al., 2014; Otsuka et al., 2010; Wakeman et al., 2012). However, the contribution of myeloid p38α to colitis-associated tumorigenesis has been largely neglected, and this was the main objective of this doctoral thesis.

Our results demonstrate the implication of p38α in myeloid cells in inflammation-associated colon cancer using well established experimental models in mice, and identify IGF-1 as a novel effector downstream of p38α signaling in macrophages. While p38α is known to regulate cytokine production, to our knowledge, this is the first report describing the regulation of IGF-1 by p38α in macrophages. Previous studies have shown that chemical inhibition of p38 MAPK signaling reduces IGF-1 production by TNF-treated MSCs and adipose progenitor cells in culture (Wang et al., 2006). Our results suggest, that myeloid cells are a major source of IGF-1 in the large intestine: Consistent with this idea, genetic or pharmacological inhibition of IGF-1 suppresses inflammatory cell recruitment and reduces colitis-associated colon tumor burden.

This work suggests that targeted inhibition of the p38α pathway in myeloid cells could be therapeutically useful especially in tumors associated with chronic inflammation. Of note, IGF-1 is known to have mitogenic and anti-apoptotic functions, in addition to its role in inflammatory cell recruitment (Clayton et al., 2011; Furundzija et al., 2010; Musaro et al., 2004; Sanchez-Lopez et al., 2015; Smith, 2010).

5.1 p38α signaling in myeloid cells promotes intestinal inflammation and tumorigenesis

We show that suppression of p38α in myeloid cells ameliorates intestinal inflammation mainly through repression of inflammatory cell recruitment, resulting in reduced AOM/DSS-induced tumor burden in mice.

In agreement with a previous report (Otsuka et al., 2010), we have found that p38α-Δ^{MC} mice are less susceptible to colitis and epithelial damage caused by DSS administration. Of note, tumors induced by AOM/DSS are highly dependent on the inflammation and linked epithelial damage caused by DSS (Chassaing et al., 2014; Neufert et al., 2007; Perse and Cerar, 2012). Moreover, we have observed an increased number of Ly6C^{hi}CCR2^{+} monocytes in the bone marrow and, in accordance, higher numbers of infiltrated innate and adaptive immune cells in the intestines of DSS-treated animals. It is well known, that persistent activation and recruitment of leukocytes, especially innate immune cells, including macrophages and DCs, can cause continuous tissue destruction and atrophy through the release of numerous factors, increasing the risk of malignant transformation (see section 1.3.1.1 and section 1.3.2) (Balkwill and Mantovani, 2001; Colotta et al., 2009; Coussens and Werb, 2002; Macarthur et al., 2004).

We have demonstrated a stronger induction of two key pro-inflammatory cytokines, TNF-α and IL-1β in WT mice compared to p38α-Δ^{MC} mice upon DSS treatment. Several human and mouse studies support that these cytokines might play important roles in the pathogenesis of IBD due to their immunological up-regulatory and pro-inflammatory activities (Dionne et al., 1999; Muzes et al., 2012; Sanchez-Munoz et al., 2008). IL-1β belongs to the IL-1 family can be produced by various cell types, including monocytes, macrophages, neutrophils, DCs, endothelial cells, and some epithelial cells (Fonseca-Camarillo and Yamamoto-Furusho, 2015; Guan and Zhang, 2017; Hendrikx et al., 2013; Hogmalm et al., 2014; Mladenovic et al., 2014). TNF-α is a cytokine mainly produced by macrophages and monocytes, but also adipocytes, fibroblasts and differentiated T cells (Atreya et al., 2011; Kamada et al., 2008; Strober, 2006; Strober et al., 2002), which exerts pleiotropic effects through increased production of IL-1β and IL-6, expression of adhesion molecules, proliferation of fibroblasts and pro-coagulant factors, as well as initiation of cytotoxic, apoptotic and acute-phase responses, inhibition of apoptosis and activation of macrophages and effector T cells (Baumann and Gauldie, 1994; Begue et al., 2006; Di Sabatino et al., 2007; Gunther et al., 2011; Su et al.,

2013). It has been suggested that IL-1 plays a role in the initiation rather than in the perpetuation of colonic inflammation (Neurath, 2014). On the other hand IL-1β can also activate the release of other pro-inflammatory cytokines such as TNFα, IL-23 and IL-6 (Sahoo et al., 2011; Tsianos and Katsanos, 2009). Indeed, p38α is known to control the production of TNF-α and IL-1β (see section 1.4.1.1) (Cuadrado and Nebreda, 2010; Kim et al., 2008; Lee et al., 1994; Lee et al., 2000; Zarubin and Han, 2005). However, our results indicate that the decrease of these inflammatory mediators observed in DSS-treated p38α-Δ^{MC} mice correlates with a reduced number of infiltrated immune cells.

Similarly, mice with p38α deletion in myeloid cells show reduced STAT3 phosphorylation in the context of both intestinal inflammation and tumorigenesis compared to WT mice. Several lines of evidence associate STAT3 with inflammation and cancer in humans and mice (Aggarwal and Gehlot, 2009; Aggarwal et al., 2009a; Aggarwal et al., 2009b; Corvinus et al., 2005; Grivennikov et al., 2009; Jarnicki et al., 2010; Sanchez-Lopez et al., 2015). STAT3 plays a crucial role in mediating the biological functions of cytokines belonging to the IL-6 family, amongst others (Hirano et al., 2000; Yu et al., 2009). Also the other way around, the expression of pro-inflammatory genes like IL-6, IL-8 and VEGF, was shown to be dependent on STAT3 activity (Carey et al., 2008; Fritzenwanger et al., 2006a; Fritzenwanger et al., 2006b; Fritzenwanger et al., 2007; Sumimoto et al., 2006). Additionally, a number of studies demonstrated the activation of STAT3 signaling in IBD pathogenesis, as well as in experimental mouse models. Accordingly, the expression of several STAT3 dependent genes, including various chemokines, cytokines and growth factors was found to be increased in IBD (Atreya et al., 2000; Carey et al., 2008; Mudter et al., 2005; Musso et al., 2005; Nguyen et al., 2013; Siegmund et al., 2002; Suzuki et al., 2001). Nevertheless, it is unclear why in other studies STAT3 signaling in IECs increased the sensitivity of mice to DSS-induced colitis (Bollrath et al., 2009; Grivennikov et al., 2009; Pickert et al., 2009; Tebbutt et al., 2002). These discrepancies in the cellular outcomes of STAT3 signaling could be linked to the experimental settings, such as molecular weight of DSS, DSS dose and length of treatment, mouse strains and background, housing conditions and food, microbiota (Bramhall et al., 2015; Chassaing et al., 2014; Laukens et al., 2016; Mahler et al., 1998; Perse and Cerar, 2012). It should be considered that some properties of STAT3, such as suppression of apoptosis, induction of proliferation and induction of cyto-protective factors, such as intestinal TFF3 or the chaperone heat-shock protein 70 (Hsp70), might be

beneficial in the context of acute colitis, where epithelial damage depends on repair and protective mechanisms (Becker et al., 2005; Klampfer, 2008; Madamanchi et al., 2001).

Nevertheless, although some discrepancies have been reported in the context of acute colitis, there is little doubt that STAT3 exhibits a potent tumor promoting role in intestinal carcinogenesis, which is probably linked to its crosstalk with NFκB activity whose sustained activation promotes tumorigenesis by inducing the expression of inflammatory mediators and growth factors (Greten et al., 2004; Greten and Karin, 2004; Grivennikov et al., 2009; Grivennikov and Karin, 2010; Karin, 2006; Li et al., 2011; Pacifico and Leonardi, 2006; Rakoff-Nahoum et al., 2004; Tebbutt et al., 2002; Yu and Kone, 2004).

We detected a higher tumor burden in WT mice compared to p38α-Δ^{MC} mice, but the tumors were morphologically and histologically indistinguishable and no significant differences in tumor size could be observed between both genotypes. This is in line with the fact that apoptosis and proliferation were very similar in tumors of WT and p38α-Δ^{MC} mice, suggesting that p38α signaling in myeloid cells mainly participates in the tumor promotion upon AOM/DSS treatment of mice. The detection of larger tumors in WT mice compared to p38α-Δ^{MC} mice, could be explained by the earlier initiation of WT tumors, which therefore have more time to grow.

Taken together, our data suggest that p38α downregulation in myeloid cells suppresses the production of key inflammatory mediators and cytokines, ameliorating the susceptibility to DSS-induced colitis and resulting in reduced inflammation-associated tumor formation, in line with other reports linking inflammation and cancer (Aggarwal and Gehlot, 2009; Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Del Reino et al., 2014; Macarthur et al., 2004; Ullman and Itzkowitz, 2011).

5.2 IGF-1 as a novel downstream effector of p38α signaling

Since mice with p38α downregulation in myeloid cells showed reduced susceptibility to intestinal inflammation and tumorigenesis, we investigated macrophage-produced cytokines or chemokines regulated by p38 MAPK signaling, which could be involved in inflammatory processes and tumor promotion. We identified several cytokines and chemokines that seemed to be regulated by p38 MAPK signaling in BMDMs, with IGF-1 showing the most obvious differential expression. A recent study proposed a role for p38 MAPK signaling in the IL-4-induced activation of peritoneal macrophages, but IGF-1

was not analyzed (Jimenez-Garcia et al., 2015). IGF-1 is a growth factor that has been implicated in tumor promotion, as well as in the inflammatory disorders, playing a prominent role in the regulation of immunity and inflammation (Baserga, 2009; Heemskerk et al., 1999; Sanchez-Lopez et al., 2016).

To the best of our knowledge, there are no studies reporting the regulation of IGF-1 by p38 MAPK in macrophages. Our results using genetic models and chemical inhibitors confirmed the implication of p38α signaling in IGF-1 expression in both BMDMs and intestinal macrophages. In agreement with the proposed role of IGF-1 as a marker for wound-healing macrophages (Roszer, 2015; Tonkin et al., 2015), we found that it was more potently induced by IL-4 (induction of alternative activation) than by LPS (induction of classical activation)(Ying et al., 2013).

Similarly, it has been established that intestinal macrophages switch in response to DSS from the initial classical activation phenotype to a wound-healing phenotype in the repair phase (Enderlin Vaz da Silva et al., 2014; Ortega-Gomez et al., 2013; Serhan and Savill, 2005; Sugimoto et al., 2016). Accordingly, intestinal macrophages isolated from WT and p38α-Δ^{MC} mice 7 days after the end of the DSS treatment (day 13, in the anti-inflammatory repair phase), showed stronger differences in IGF-1 expression levels compared to the untreated animals. Moreover, the differences of IGF-1 protein levels in whole colon extracts were also stronger at this time point, than in the acute inflammatory phase.

A point to keep in mind is the induction of IGF-1 expression by LPS, although IGF-1 is a marker for alternatively activated macrophages. This apparent controversy might be explained by the fact that both IL-4 and LPS can activate p38α in macrophages (Jimenez-Garcia et al., 2015; Kang et al., 2008; Meng et al., 2014; Xagorari et al., 2002), and is consistent with the current idea of a spectrum of macrophage activation stages, which cannot be easily binned into defined groups (Biswas and Mantovani, 2010; Edwards et al., 2006; Mantovani et al., 2005; Sica and Mantovani, 2012; Stout et al., 2005; Stout and Suttles, 2004; Stout and Suttles, 2005). Nevertheless, in accordance to our findings, other studies have reported IGF-1 induction by LPS in macrophages and in acute models of inflammation (Chand et al., 2012; Pang et al., 2010; Suh et al., 2013).

Another example of macrophage phenotype marker potentially controversial is Arg1, which is considered a marker for alternatively activated macrophages but is also expressed in classically activated macrophages of some resident macrophage populations

and is highly induced in mycobacteria-infected macrophages (El Kasmi et al., 2008; Raes et al., 2002; Wagener et al., 2017). The conclusion is that relying on a single marker can be problematic, and a combination of markers should be applied to define the phenotype of macrophages (Edwards et al., 2006; Mosser and Edwards, 2008).

Tissue resident and inflammatory macrophages are actively recruited from circulating bone marrow-derived monocytic precursors, which therefore contribute to modify the TME (Chanmee et al., 2014; Murray and Wynn, 2011). TAMs facilitate tumor initiation, progression and metastasis at various levels (Grivennikov et al., 2010; Mantovani et al., 2017). IL-4 is a cytokine produced by various immune cells, which is considered a major regulator of TAM phenotypes and determines the balance of pro- and anti-inflammatory cytokines produced (Paul and Zhu, 2010). Although we could not observe significant differences in IGF-1 protein expression in the colon, quantification of IGF1R phosphorylation indicated that the IGF-1 pathway is more active in tumors obtained from WT mice than in those from p38α-Δ^{MC} mice. This discrepancy is probably a result of the complexity of the IGF signaling system. Although further experiments will be needed to define the factor(s) responsible for this inconsistency, we can postulate several possibilities. One is that tumors produce IGF-2, which is known to bind to the IGF1R independently of IGF-1 (Baserga, 2009; Lawrence et al., 2007). However, ligand-binding affinities vary depending on cell type and experimental conditions, and it is not clear whether both ligands bind with identical affinities or IGF-1 exhibits a higher affinity for IGF1R than IGF-2 (Danielsen et al., 1990; Forbes et al., 2002). Nevertheless, preliminary analysis in colon and tumor samples indicates very low expression levels of IGF-2 mRNA, making unlikely the possibility that IGF-2 expression accounts for the discrepancy between IGF-1 protein levels and phospho-IGF1R staining in our model. Moreover, although IGF-1 and IGF-2 elicit similar biological responses, their pattern of expression *in vivo* is significantly different. IGF-1 is preferentially expressed after birth and is produced mostly in the liver through autocrine or paracrine mechanisms, with macrophages being its largest extra-hepatic source. On the other hand, IGF-2 is preferentially expressed in a variety of somatic tissues during early embryonic and fetal development. The adult expression of IGF-2 takes place in the liver and in the epithelial cells lining the brain surface (Engstrom et al., 1998; Gow et al., 2010; Pan and Anthony, 2000; Underwood et al., 1986). Nonetheless, there are studies reporting an implication of IGF-2 in various cancers, including colon, which has been proposed to derive from

cancer-associated fibroblasts (CAFs) (Brouwer-Visser and Huang, 2015; Liu et al., 2013; Livingstone, 2013; Tian et al., 2017; Unger et al., 2017; Xu et al., 2017).

Another factor to consider concerning the above discrepancy is the complex regulation of IGF1 activity and sensitivity by six IGFBPs, which have been shown to regulate IGF1-mediated IGF1R activation (Brahmkhatri et al., 2015; Yu and Rohan, 2000; Zhang et al., 2013a). In addition, several IGFBP proteases hydrolyzes IGFBPs, resulting in the release of bound IGFs, which then can interact with IGF1R. Thus, IGFBP proteases may indirectly regulate the action of IGFs (Ferry et al., 1999; Helle, 2004; Moschos and Mantzoros, 2002; Rodriguez et al., 2007; Shim and Cohen, 1999). Indeed, our preliminary data suggests that p38α can regulate the expression of IGFBP-5 and IGFBP-6 in BMDMs, but these results need to be validated. There is also some evidence in the literature suggesting that p38α can regulate IGFBPs (Frost et al., 2000; Kuemmerle and Zhou, 2002; Zhang et al., 2014b). An additional potential regulatory mechanism of IGF-1 signaling is the modulation of IGF2R expression, which has been postulated to act as a tumor suppressor by binding and clearing IGF2, thereby preventing activation of IGF1R (Yu and Rohan, 2000). Future studies should analyze the potential implication of IGFBPs and/or IGF2R in colitis and CAC, and whether they account for the disagreement between the levels of IGF-1 protein and IGF1R phosphorylation observed in AOM/DSS-induced tumors.

Our cytokine array analysis comparing WT and p38α KO BMDMs (see section 4.1.5) identified a number of other cytokines and chemokines potentially involved in intestinal inflammatory pathogenesis, which should be further evaluated in the future. Nonetheless, we conclude that p38α regulates IGF-1 expression in macrophages *in vitro* and *in vivo*, and this correlates with enhanced IGF-1 signaling during DSS induced colitis and AOM/DSS induced tumorigenesis.

5.3 IGF-1 promotes intestinal inflammation and tumorigenesis

The IGF-1 pathway has been linked to cancer and IBD by modulating the innate and adaptive immune systems as well as through its multi-functional involvement in the TME (Sanchez-Lopez et al., 2015; Smith, 2010). Aberrant IGF-1 signaling has been associated with numerous human cancer types, and a large amount of work has implicated IGF-1 receptor activity in cancer cell proliferation, migration, and invasion (Clayton et al., 2011). Consistently, our results show significant downregulation of IGF-1 signaling in the colons of IGF-1-Δ^{MC} mice during inflammation and colitis-associated tumorigenesis.

Downregulation of IGF-1 in myeloid cells or treatment of WT mice with the IGF1R inhibitor PQ401 partially improves disease condition and epithelial damage induced by DSS, which correlates with a strong reduction of $Ly6C^{hi}CCR2^{+}$ monocytes in the bone marrow and decreased macrophages in the colon of these mice. However, DSS-induced colitis susceptibility was not reduced to the same levels as in p38α-Δ^{MC} mice, perhaps due to the implication of IGF-1 signaling in mucosal repair mechanisms (Werner and Grose, 2003). Nonetheless, although these mechanisms are important for mucosal integrity, excessive wound-healing activity strongly predispose to the development of neoplasms and tumor progression (see 1.3.1.3.1 section and section 1.3.2.3). In fact, in our experimental model, the IGF-1 increase in the intestinal repair phase also associates with increased IEC proliferation. This might be explained by a compensatory proliferation as previously suggested (Gupta et al., 2014), or be the result of increased macrophage-produced IGF-1 in the colonic microenvironment.

In addition, p38α is known to control the production by myeloid cells of other cytokines involved in acute intestinal inflammation, which are not affected by the inhibition of IGF-1 signaling (see section 1.4.1.1). There is evidence indicating that IGF-1 signaling may favor mitogenesis and neoplastic transformation of normal IECs (Vigneri et al., 2015; Yao et al., 2016). This is consistent with our observation that differences in IGF-1 signaling between WT and p38α-Δ^{MC} mice are stronger during acute colitis than at the end of the tumorigenesis experiment.

The reduced colon tumorigenesis observed in IGF-1-Δ^{MC} mice or in PQ401-treated WT mice is consistent with the importance of this factor in tumor initiation and progression (Clayton et al., 2011; Yu and Rohan, 2000). In agreement with our results, a chemical compound that inhibits both IGF-1 and STAT3 signaling has been reported to reduce intestinal tumorigenesis induced by Apc deletion by affecting several cell types in the TME (Sanchez-Lopez et al., 2015). In accordance, mice with p38α deletion in myeloid cells show reduced STAT3 phosphorylation in the context of both, intestinal inflammation and tumorigenesis (see section 5.1). There is also evidence suggesting that IGF-1 is able to induce STAT3 activation (Zong et al., 2000)

In conclusion, we propose that p38α signaling in myeloid cells supports colon inflammation and tumorigenesis through the production of IGF-1. It seems likely that IGF-1 acts on various cell types, given that it is secreted to the microenvironment and that

the IGF-1 receptor is ubiquitously expressed in normal tissues (Sanchez-Lopez et al., 2015).

5.4 p38α signaling in myeloid cells regulates leukocyte recruitment to the intestine

Increased leukocyte infiltration is a hallmark of IBD and experimental colitis, contributing to disease initiation and tissue damage (Abraham and Cho, 2009c). Accordingly, the decreased inflammation observed in DSS-treated p38α-Δ^{MC} mice correlates with reduced colon infiltration of leukocytes. Importantly, Ly6C^{hi}CCR2^{+} monocytes are continuously generated in the bone marrow from HSCs and recruited to healthy and injured tissues, where they give rise to intestinal effector cells (Bain and Mowat, 2014). Our studies demonstrated that this cell population is reduced in p38α-Δ^{MC} mice compared to WT mice, even without any treatment. We believe this is important for the observed phenotype, given the key role of inflammatory monocytes in triggering the recruitment of other immune cells as well as in the initiation of the adaptive immune response.

The mobilization of Ly6C^{hi}CCR2^{+} monocytes from the bone marrow depends on the CCL2-CCR2 chemokine axis and, as a result, deletion of either of these molecules markedly reduces circulating monocytes and ameliorates acute intestinal inflammation (Bain and Mowat, 2014; Ginhoux and Jung, 2014; Mowat and Bain, 2011). Although this increase of inflammatory monocytes in homeostatic conditions was not observed to affect intestinal permeability, we hypothesize that the differences in Ly6C^{hi}CCR2^{+} monocytes observed in untreated p38α-Δ^{MC} mice are probably due to environmental factors exposure, such as microbiota (Bain et al., 2014), leading to a state of controlled "physiological inflammation", which is boosted upon DSS induced inflammation and is linked to differences in expression of chemokines crucial for immune cell recruitment.

The intestinal microbiota is well known to play an important role in health maintenance. In the last years, it became evident that the results of experimental studies may vary from animal facility to animal facility or even over time within the same facility, depending on the local microbiota, housing conditions, and diet (Abraham and Medzhitov, 2011; Belkaid and Hand, 2014; Chassaing et al., 2014; Ericsson and Franklin, 2015). The experiments described in this Thesis were performed with mice housed in conventional conditions, but could not be reproduced with the same mice in SPF housing

conditions, indicating an important role of myeloid p38α in the response to microbiota and thereby the development of inflammatory diseases. Preliminary data from our laboratory also suggests that there are no changes between WT and Ly6C^{hi}CCR2^{+} monocytes in the bone marrow of mice under homeostatic conditions when housed in SPF conditions. This is striking and, in the future, it should be of great interest to further dissect the role of myeloid p38α in the response to changes in the gut microbiome. We believe that the experiments performed in conventional housing conditions resemble a more natural situation than the animals in SPF housing conditions, given the fact that in real life, people and animals are exposed to a myriad of microbiota and external factors.

It should be noted that especially for intestinal pathologies, the gut microbiota plays a crucial role in modulation of the immune system and disease perpetuation. In accordance, a number of studies have reported similar differences in their models when changing the housing conditions and therefore the microbiota to which the animals are exposed. Considering the role of immune cells in the response to microbiota, this seems to be of particular importance in studies of the immune system (Frosali et al., 2015; Gronbach et al., 2010; Kuhn and Stappenbeck, 2013; Landman and Quevrain, 2016; Nunez, 2017; O'Rourke et al., 1988; Reinisch, 2017; Round and Mazmanian, 2009; Shono et al., 2015; Stecher, 2015; Yamada et al., 2016; Zhan et al., 2013). Of note, there is evidence showing the importance of gram-positive commensal bacteria in inducing colitis and recruiting colitogenic monocytes and macrophages, particularly bacteria of the Lachnospiraceae family has been shown to specifically downregulate the expression of CCR2 ligands in colonic epithelial cells (Nakanishi et al., 2015).

Besides its function as a growth hormone, IGF-1 can mediate chemotaxis of several cell types including tumor cells (Roussos et al., 2011). In fact, IGF-1 can induce macrophage migration in transwell chambers, which is impaired by chemical inhibitors of integrin and p38 MAPK signaling (Furundzija et al., 2010), and has been reported to mediate the recruitment of bone marrow cells to damaged muscle sites (Musaro et al., 2004). Indeed, intestinal macrophages isolated from untreated p38α-Δ^{MC} mice showed reduced IGF-1 expression, which might account for the differences in bone marrow inflammatory cells.

We have observed a general pattern of chemokine downregulation in colons from untreated p38α-Δ^{MC} mice. Previous work has implicated p38 MAPK signaling in the regulation of chemokines by T helper lymphocytes and myeloid cells (Granata et al.,

2006; Kim et al., 2008; Sun et al., 2008; Wong et al., 2007). In this study, we show that p38α regulates Chemerin, CCL2 (MCP-1) and CCL12 (MCP-5) mRNAs in intestinal macrophages. Chemerin is a potent macrophage chemoattractant that potentiates intestinal inflammation in DSS-induced colitis (Lin et al., 2014). Interestingly, CCL2 is regulated by both p38α and IGF-1 in intestinal macrophages, and p38 MAPK signaling has been previously linked to CCL2 regulation in murine macrophages (Sun et al., 2008). How IGF-1 might regulate CCL2 production by macrophages is unknown but, consistent with our results, CCL2 was also downregulated in tumors from mice treated with an inhibitor of IGF-1 and STAT3 signaling (Sanchez-Lopez et al., 2015).

In summary, taken all our findings into consideration, we propose the following model (Figure 88). Commensal microbiota lead to a situation of tightly controlled inflammation, implicating constant recruitment of monocytes from HSCs in the bone marrow to the intestine. In intestinal macrophages, p38α controls the expression of chemokines and IGF-1 important for this process. This is pathologically boosted through inflammation and a vicious cycle starts if inflammation gets out of control leading to the development of chronic inflammation. In this process, IECs accumulate genetic mutations eventually leading to the formation of tumors.

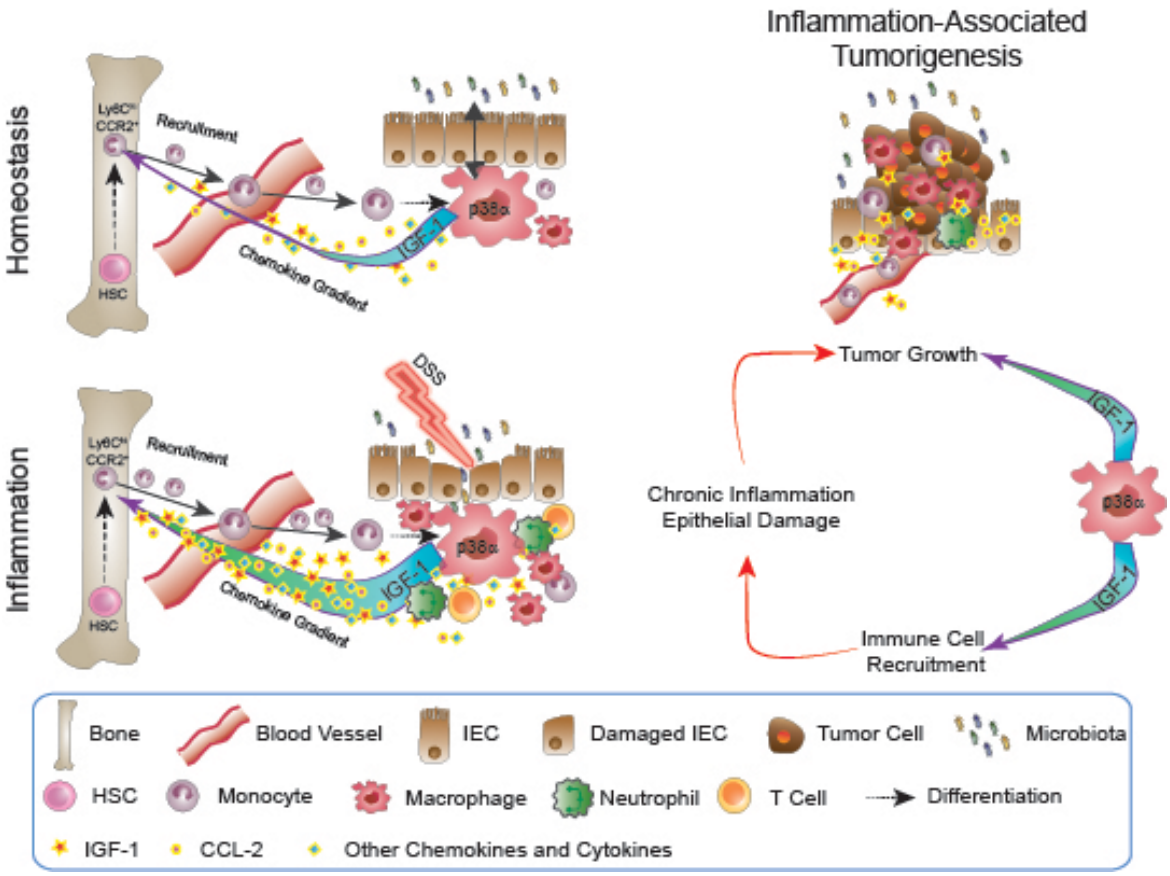


Figure 88. p38α-IGF-1 axis in intestinal homeostasis, inflammation and tumorigenesis.

Schematic model representing a summary for the implication of p38a in myeloid cells in the context of intestinal inflammation and CAC.

5.5 p38α and IGF-1 as regulators of wound-healing

In parallel to the *in vivo* studies, in which we show that p38α in myeloid cells exerts a pro-inflammatory role in the epithelial mucosa, and taking into account that p38α in IECs plays an important role in the maintenance of intestinal homeostasis (Gupta et al., 2014), we have evaluated the role of p38α in both macrophages and IECs using an *in vitro* model of wound-healing. This model could be relevant to understand effects occurring *in vivo* within the intestinal epithelium injured by colitis. Additionally, we have evaluated IGF-1 in the *in vitro* model of wound-healing, as a downstream target of p38α signaling in macrophages.

The use of macrophages derived from WT and p38α-Δ^{MC} mice implicated that the interactions with IECs should be better studied using a murine model of IECs. The CMT-93 cell line was almost unknown to the scientific community four years ago, when this

project started. Therefore, due to the limited information available about these cells, we performed a series of characterization experiments. We concluded that the CMT-93 cell line was a good *in vitro* model for IECs performing experiments at 2 days post-confluence, when they show a more intact monolayer.

We show that the wound-healing process initiated through a physically induced scratch in CMT-93 cell monolayers is due to the induction of active migration, rather than cell proliferation or expansion. To our knowledge, neither the implication of p38α in wound-healing nor the contribution of macrophage-secreted factors to this process has been studied in CMT-93 cells. In line with previous reports, we observed that p38α regulates migration via its downstream substrate MK2 (Hedges et al., 1999; Kobayashi et al., 2006; Rousseau et al., 1997). MK2 is known to phosphorylate Hsp27, which plays a pivotal role in cell migration by promoting actin polymerization and remodeling (Manetti, 2012). Moreover, paxillin, a focal adhesion-associated phospho-tyrosine protein, and caldesmon, an important regulator of cell morphology and motility that associates with actin filaments, are two other p38α substrates that have been implicated in cell migration (Goncharova et al., 2002; Huang et al., 2004). Our results using chemical inhibitors indicate that CMT-93 cell migration is mainly regulated via MK2, although it remains to be evaluated whether this is mediated by Hsp27 phosphorylation.

Wound-healing experiments performed with BMDM supernatants demonstrated that p38α is also important for the production of macrophage cytokines that induce migration of CMT-93 cells. We observed that treating BMDMs with p38α inhibitors had a stronger inhibitory effect than using p38α-Δ^{MC} BMDM. This could be explained because we used inhibitors that target both p38α and p38β (Cuenda and Rousseau, 2007; Selness et al., 2011), and is consistent with the observation that supernatants derived from p38α/p38β KO BMDMs induced less migration in CMT-93 cells than supernatants from p38α KO, indicating a synergistic effect of p38α and p38β in the production of wound-healing regulatory molecules. It remains to be analyzed whether p38β cooperates with p38α in the regulation of IGF-1 in macrophages. It should be also noted that, since p38α inhibitors used to treat the macrophages are not removed from the collected supernatants, they are also potentially transferred to the CMT-93 cell scratch assays, where the inhibitors can block migration therefore producing an additive effect.

In conclusion, IGF-1 is downregulated in supernatants derived from p38α-deficient macrophages, and consistent with previous *in vitro* and *in vivo* studies, we

confirmed that IGF-1 contributes to the increased migration observed in CMT-93 cells (Chen et al., 1999; Furundzija et al., 2010; Jeong et al., 2014; Nadzir et al., 2013). To further verify that macrophage derived IGF-1 contributes to migration of CMT-93 cells, we plan to use in the future macrophage supernatants derived from IGF-1-Δ^{MC} macrophages. Nevertheless, our results do not exclude the possibility that p38α regulates other cytokines contributing to the induction of migration observed in CMT-93 cells. In addition, supernatants derived from p38α-Δ^{MC} BMDM can still induce some migration of CMT-93 cells, suggesting that p38α is not the only regulator of factors involved in the induction of migration by macrophage supernatants.

Treatment of CMT-93 cells with DSS produces a cytotoxic effect and increased apoptosis, in line with other *in vitro* studies (Araki et al., 2006) and with what occurs in DSS-treated mice *in vivo* (Siegmund et al., 2002; Zhan et al., 2013). Curiously, DSS treatment slightly induces CMT-93 cell proliferation *in vitro*, as it has been described to occur *in vivo* upon DSS treatment, but DSS treatment in cell lines other than CMT-93 has been described to inhibit proliferation (Araki et al., 2010; Araki et al., 2006). Moreover, according to previous studies (Badr et al., 2012; Li et al., 2016b; Yan et al., 2016), we show that IGF-1 protects from apoptosis and induces proliferation in CMT-93 cells, and also helps the cells to survive in response to DSS (Figure 89).

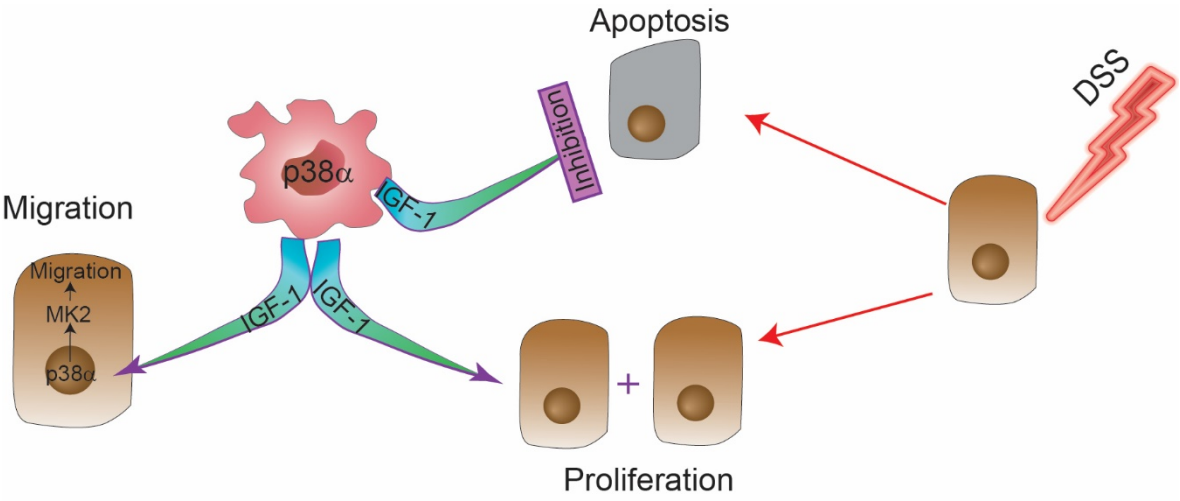


Figure 89. IGF-1 is regulated by p38α in macrophages inducing proliferation, survival and migration in CMT-93 cells.

The IGF-1-p38α-MK2 axis was identified to regulate migration in CMT-93 cells. Moreover, IGF-1 induces CMT-93 cell proliferation and inhibits cell death, contributing to cell survival upon DSS treatment.

5.6 Is this novel p38α-IGF-1 axis a potential therapeutic target for intestinal inflammatory disorders and cancer?

Our results strongly suggest that the p38α-IGF-1 axis promotes inflammatory cell recruitment, inflammation and subsequent tumorigenesis in the colon. This depicts myeloid p38α as a tumor promoter and is in line with the detection of increased phosphorylation of p38α in human tumors (Wagner and Nebreda, 2009). Although it has not been analyzed whether p38α in macrophages is hyperactivated in human cancer, we think this is very likely, since the TME exhibits a milieu with a myriad of cytokines, chemokines, hormones and growth factors secreted by macrophages and CAFs and p38α is known to be activated in macrophages and other cells by a variety of these molecules (Alonso et al., 2000; Brancho et al., 2003; Buhler and Laufer, 2014; Cuadrado and Nebreda, 2010; Cuenda and Rousseau, 2007; Docena et al., 2010; Jimenez-Garcia et al., 2015; Risco et al., 2012; Ying et al., 2013). Strengthening a potential clinical importance is the fact that macrophages are prominently found and recruited to tumors (see section 1.3.2.1) (Gabrilovich et al., 2012; Murdoch et al., 2008; Parihar et al., 2010; Sanchez-Lopez et al., 2015)

Of note, the different functions of p38α in IECs or in myeloid cells in the context of inflammation-associated colon tumorigenesis highlights the importance of selectively targeting specific cell types and should be taken into consideration for more successful therapeutic interventions in this pathway. Perhaps this is why the p38α inhibitors have not yielded clear results in clinical trials so far (Gupta et al., 2015; Patterson et al., 2014). Given the difficulty inherent to targeting a specific p38 MAPK family member in a specific cell type, the alternative of targeting a combination of effector pathways with compounds that are already in clinical trials, often as separate medications, should be considered. It has been proposed that some IBD patients could benefit from IGF-1 treatment due to its involvement in growth and repair processes, as well as anti-inflammatory effects (Zatorski et al., 2016). However, our results suggest that the inflammatory features and local IGF-1 levels assessed from colonic biopsies should be taken into account.

Moreover, circulating IGF-1 levels rise during juvenile life and then decline after puberty (Sara et al., 1983), which might be linked to the high incidences of pediatric IBD (Benchimol et al., 2017). In addition, and further emphasizing a potential clinical importance of our findings is the fact that IGF-1 expression is known to positively

correlate with caloric intake and the risk of developing colon cancer, also a number of other cancers also increase with obesity and several studies in humans indicate that this can be reversed by caloric restriction (Baserga, 2009; Bowers et al., 2015; Chen and Sharon, 2013; Cohen and LeRoith, 2012; D'Addio et al., 2015; Denduluri et al., 2015; Gallagher and LeRoith, 2011; Moschos and Mantzoros, 2002; Renehan et al., 2006).

Finally, it is generally accepted that IGF-1 contributes to the pathogenesis of CRC through a number of features that collectively promote tumorigenesis, such as wound-healing, inflammatory cell recruitment, sustained alternative macrophage activation, cell proliferation and activation of the PI3 kinase and β-catenin pathways and its implication in several resistance mechanisms to therapeutic agents (Clayton et al., 2011; Mourkioti and Rosenthal, 2005; Spadaro et al., 2017; Vigneri et al., 2015). In summary, we propose that IGF-1 signaling might be an attractive target in the context of intestinal diseases with prominent inflammatory cell recruitment.

Taken together the results of this Thesis obtained from *in vivo* and *in vitro* models, we conclude that the myeloid p38α-IGF-1-axis contributes to tumorigenesis mainly by inducing excessive wound-healing Figure 90. In accordance, it has been postulated decades ago that tumors are over healing wounds (Dvorak, 1986; Haddow, 1972). We hypothesize that this occurs *in vivo* due to a combination of various factors contributing to carcinogenesis, such as cell survival and immune cell recruitment, resulting in further release of pro-tumorigenic mediators including IGF-1. IEC cell migration might also contribute to the pro-tumorigenic properties of cancer cells in our model, however this has not been analyzed *in vivo* so far.

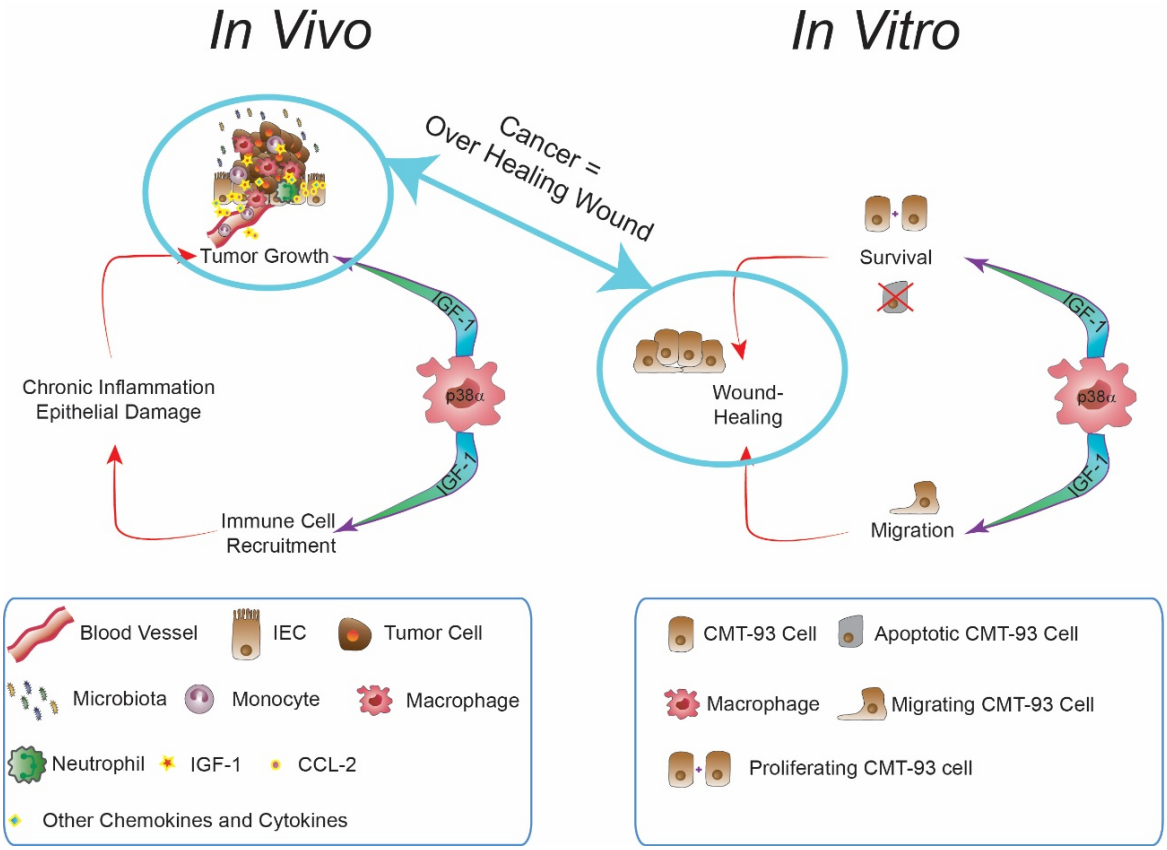


Figure 90. Schematic summary of myeloid p38α signaling in intestinal carcinogenesis.

CONCLUSIONS

6. CONCLUSIONS

1. Myeloid p38α suppresses DSS-induced colitis and inflammation-associated colon tumorigenesis.

2. IGF-1 is a novel target of p38α signaling in macrophages

3. IGF-1 regulated by p38α in myeloid cells contributes to colitis and colon tumorigenesis

4. Inhibition of p38α or IGF-1 signaling in myeloid cells suppresses inflammatory cell recruitment

5. Myeloid p38α regulates the expression of several chemotactic factors in the intestine

6. The myeloid p38α-IGF-1 axis should be considered as a potential therapeutic target in inflammation associated intestinal diseases and cancers.

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7. BIBLIOGRAPHY

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RESUMEN EN CASTELLANO

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Introducción y objetivos

El cáncer colorectal es el tercer tipo de cáncer más común y el cuarto en mortalidad (Pan et al., 2016). El riesgo de desarrollar este tipo de cáncer se encuentra significativamente incrementado en individuos diagnosticados con Enfermedad inflamatoria intestinal o IBD (*inflammatory bowel disease*), una enfermedad inflamatoria crónica recurrente (Coussens and Werb, 2002; Dyson and Rutter, 2012; Thorsteinsdottir et al., 2011). El termino IBD comprende dos patologías muy relacionadas, la colitis ulcerosa y la enfermedad de Crohn, que se caracterizan por una inflamación crónica del intestino y por presentar períodos de exacerbación de los síntomas, seguidos de intervalos más o menos prolongados de remisión de los mismos. La colitis ulcerosa se presenta solamente en el intestino grueso y la enfermedad de Crohn puede manifestarse en cualquier parte del tracto digestivo, desde la boca hasta el ano (Bouma and Strober, 2003). Estas enfermedades se caracterizan por un sistema inmunitario excesivamente activo y asociado a una exagerada producción de citoquinas (Sartor, 2006). La relación entre inflamación y carcinogénesis ya fue descrita hace más de 150 años, cuando Rudolf Virchow hipotetizó que un estado inflamatorio crónico podría llevar a un proceso tumoral (Balkwill and Mantovani, 2001). Aunque se ha logrado un progreso considerable en la investigación de la IBD, aún no se ha logrado una comprensión completa de los mecanismos y la patogénesis en el cáncer asociado a la colitis (CAC: colitis-associated cancer) (Kaplan and Ng, 2017).

La co-evolución de los mamíferos con su flora intestinal o microbiota permite una situación de tolerancia intestinal. El mantenimiento de este fino equilibrio o homeostasis se encuentra marcado por una situación controlada de "inflamación fisiológica" basada en mecanismos de activación y supresión por parte del sistema inmunitario, dónde distintas poblaciones celulares, entre ellas macrófagos residentes e inflamatorios, participan en el mantenimiento de este balance y aseguran una inmunidad protectora (Mowat and Bain, 2011). Un desequilibrio en esta homeostasis, como ocurre con la IBD, implica una desregulación de citoquinas pro- y anti-inflamatorias, que impiden la resolución de la inflamación conduciendo a su perpetuación y a la destrucción tisular. Por tanto, la producción de citoquinas juega un papel clave, tanto en los mecanismos implicados en el daño de la mucosa y tejido intestinal, como en las funciones de reparación de la mucosa epitelial intestinal, así como en los procesos de angiogénesis, progresión y metástasis tumoral (Mattner and Wirtz, 2017; Rakoff-Nahoum and Medzhitov, 2009).

Durante la carcinogénesis colorectal, las células epiteliales colónicas acumulan mutaciones genéticas, muchas de las cuales son inducidas por factores ambientales que les confieren ventajas como un crecimiento selectivo. Los factores de crecimiento secretados por monocitos activados y macrófagos ayudan a la formación de pólipos benignos y pueden conducir al desarrollo de tumores colorrectales invasivos (Baylin and Ohm, 2006; Schwitalla et al., 2013). Por lo tanto, la búsqueda de nuevas estrategias que impidan el reclutamiento de células del sistema inmunitario en los sitios de inflamación para impedir la tumorogénesis es un tema de gran interés en la actualidad (Grivennikov et al., 2010). Las características del estroma tumoral reactivo incluyen la presencia de infiltrado de leucocitos, y las células mieloides (monocitos y macrófagos) son las predominantes dentro del microambiente tumoral (Mantovani and Sica, 2010). Actualmente está establecido los monocitos con el fenotipo $Ly6C^{hi}CCR2^{+}$ (también llamados monocitos inflamatorios) de la médula ósea entran en la circulación estimulados por la quimiocina CCL2 y su receptor CCR2 y, como resultado, la deleción de cualquiera de estas moléculas reduce notablemente los monocitos circulantes. Además, se ha demostrado que la menor infiltración de monocitos al torrente circulatorio reduce la inflamación intestinal aguda (Bain and Mowat, 2014; Ginhoux and Jung, 2014; Mowat and Bain, 2011).

p38α es miembro de una superfamilia de serina/treonina proteína-quinasas llamadas MAPK (*mitogen-activated protein kinase*), que pueden ser activadas por una gran variedad de estímulos. p38α regula principalmente la respuesta celular a estímulos de estrés, pero también está involucrada en otros procesos como la inflamación y homeostasis tisular. Por ejemplo, p38α controla la producción de quimioatrayentes y otros mediadores inflamatorios en los leucocitos, y por tanto se ha considerado una potencial diana para la intervención terapéutica de patologías inflamatorias (Corvinus et al., 2005; Cuadrado and Nebreda, 2010; Grivennikov et al., 2010). Por otro lado, en macrófagos, la vía de señalización de p38α se ha implicado en distintas funciones celulares como la activación, la diferenciación a distintos fenotipos (plasticidad fenotípica), proliferación y supervivencia celular (Cuenda and Rousseau, 2007).

La inactivación genética de p38α en células mieloides ha demostrado la importancia de esta vía de señalización en la producción de citocinas y respuestas inflamatorias nivel *in vitro* como *in vivo* (Kim et al., 2008; Otsuka et al., 2010; Wagner and Nebreda, 2009). Por ejemplo, en un modelo *in vivo* de CAC, p38α en las células

epiteliales intestinales suprime la iniciación de la tumorogénesis asociada a inflamación, pero contribuye a la supervivencia de las células tumorales de colon (Gupta et al., 2015; Patterson et al., 2014). También se ha demostrado que existe menos inflamación intestinal en un modelo de colitis experimental con ratones en los que p38α se ha eliminado específicamente en las células mieloides (Otsuka et al., 2010). Sin embargo, aún no existen estudios de cómo la p38α presente en las células mieloides puede afectar a la homeostasis intestinal, o a los mecanismos de reparación tisular y tumorogénesis intestinal. Por tanto, una mejor comprensión de cómo se regulan las funciones de los macrófagos en el cáncer asociado a colitis proporcionaría importantes conocimientos sobre su impacto dentro de la inflamación local y sistémica.

El principal objetivo de esta tesis consistió en investigar cómo la p38α presente en las células mieloides regula funciones tan importantes como son la homeostasis e inflamación intestinal, y cómo afecta esta proteína al proceso de tumorogénesis colorectal asociado a colitis. Dada la importancia demostrada de las citoquinas secretadas por los macrófagos durante la IBD, nos propusimos identificar qué citoquinas podían estar reguladas por p38α y por tanto, podrían afectar a funciones como la proliferación, migración o supervivencia de las células epiteliales intestinales. Los resultados que obtuvimos al estudiar distintos modelos *in vivo,* fueron ampliados en estudios usando células en cultivo para intentar descifrar el mecanismo celular y molecular involucrado.

Resultados

Para evaluar el papel de p38α de las células mieloides en el proceso tumorogénico asociado a la inflamación intestinal, utilizamos ratones modificados genéticamente que expresan los alelos LysM-Cre y p38α-lox (p38α-Δ^{MC}). Estos ratones presentan una deleción especifica del gen que codifica para p38α sólo en las células mieloides (monocitos y macrófagos). Para los primeros estudios *in vivo,* utilizamos un modelo experimental de cáncer de colon asociado a inflamación que combina el carcinógeno azoximetano (AOM) con el compuesto sulfato sódico de dextrano (DSS) utilizado para inducir colitis. Nuestros resultados indicaron que a diferencia de los ratones control o wild-type (WT), los ratones p38α-Δ^{MC} tratados con AOM/DSS tenían menor pérdida de peso y un menor índice de actividad de la enfermedad o DAI (*disease activity index*) (Cooper et al., 1993). Este índice mide diferentes parámetros entre ellos, pérdida de peso, consistencia de las heces y presencia de sangre en heces, y proporciona información del

estado de la inflamación intestinal del animal. Además, la mayoría de los ratones WT desarrollaban prolapso rectal, el cual era raramente observado en los ratones p38α-Δ^{MC}. Por tanto, los ratones p38α-Δ^{MC} presentaban una susceptibilidad reducida a la colitis en comparación con los WT. Por otra parte, observamos que los tumores se localizaban principalmente en el colon distal y medio de ambos ratones WT y p38α-Δ^{MC}. Sin embargo, los ratones p38α-Δ^{MC} mostraron una carga tumoral significativamente menor en comparación con los ratones WT, correlacionándose con el estado de la enfermedad de los animales durante el tratamiento. El tamaño medio del tumor fue similar, pero los ratones p38α-Δ^{MC} tenían significativamente menos tumores en comparación con los WT.

Curiosamente, observamos que había menos monocitos inflamatorios ($Ly6C^{hi}CCR2^{+}$) en la medula ósea de los ratones p38α-Δ^{MC} en comparación con los WT, lo cual indicaba que los ratones p38α-Δ^{MC} que presentaban tumores tenían una respuesta inflamatoria más débil. Cabe mencionar que no se observaron diferencias en otras poblaciones celulares del sistema inmunitario en la medula ósea. En concordancia con estos resultados, se detectó menos infiltración de macrófagos en los tumores de los ratones p38α-Δ^{MC}. Por otro lado, los tumores de colon de los ratones p38α-Δ^{MC} también mostraron menos infiltración de leucocitos ($CD45^{+}$) y células T ($CD3^{+}$). Por tanto, estos resultados se correlacionan con la menor inflamación detectada en los ratones p38α-Δ^{MC}.

Ampliamos nuestros estudios evaluando la fosforilación de STAT3, un activador de la vía de la inflamación que contribuye a la oncogénesis potenciando las funciones de proliferación y crecimiento tumoral (Corvinus et al., 2005; Kim and Bae, 2016). Como era de esperar, los tumores presentaban un incremento de la fosforilación de STAT3 comparado con el epitelio normal y, curiosamente, la fosforilación de STAT3 estaba reducida en los tumores de los ratones p38α-Δ^{MC} cuando se comparaban con los tumores del mismo tamaño de ratones WT. Mediante la técnica de inmunohistoquímica (IHC) en el colon se observó que había un incremento de proliferación celular ($Ki67^{+}$) en los tumores de colon en comparación con el epitelio normal de los ratones WT, sin embargo, no se detectaron diferencias significativas en los ratones p38α-Δ^{MC}. Finalmente, se analizaron la apoptosis celular en el colon mediante la técnica TUNEL y el proceso de angiogénesis mediante marcaje de células $CD31^{+}$, pero no se observaron diferencias en ambos casos entre los ratones WT y p38α-Δ^{MC}.

Dado que la inflamación es un componente esencial del modelo de AOM/DSS, el siguiente paso fue investigar la contribución de p38α de las células mieloides a la

inflamación intestinal inducida mediante la administración de DSS en el agua de bebida. De acuerdo con otros autores (Otsuka et al., 2010), observamos que los ratones p38α-Δ^{MC} eran menos susceptible a la colitis inducida por DSS que los ratones WT, ya que presentaban menos pérdida de peso y valores de DAI más bajos. Además, la longitud del colon, otro parámetro utilizado para medir inflamación intestinal (Diaz-Granados et al., 2000), era menor en los ratones WT que en los ratones p38α-Δ^{MC}, indicándo que p38α en las células mieloides juega un papel clave en el proceso inflamatorio intestinal. A nivel microscópico, observamos una mayor pérdida de criptas y más erosiones tisulares en los tejidos colónicos de los ratones WT tratados con DSS en comparación con los ratones p38α-Δ^{MC}. La infiltración en el tejido dañado de macrófagos ($F4/80^{+}$), leucocitos ($CD45^{+}$), neutrófilos activados (MPO^{+}) y células T ($CD3^{+}$) también estaba reducida en los ratones p38α-Δ^{MC} tratados con DSS en comparación con los ratones WT.

Puesto que las células inmunitarias infiltradas en el foco de inflamación producen citoquinas que pueden activar la vía de STAT3, la cual está implicada en la progresión tumoral dependiente de inflamación (Jarnicki et al., 2010; Yu et al., 2009), analizamos la fosforilación de esta proteína en los ratones tratados con DSS. De acuerdo con los resultados anteriores, los niveles de fosforilación de STAT3 inducidos por la administración del DSS eran más bajos en las células epiteliales intestinales de los ratones p38α-Δ^{MC} en comparación con los WT. Además, observamos que los niveles de citoquinas pro-inflamatorias como IL-1β y TNFα secretadas en el colon durante el proceso inflamatorio intestinal producido por el DSS se encontraban disminuidas en los ratones p38α-Δ^{MC} en comparación con los ratones WT.

Dada la importancia de las citoquinas secretadas por los macrófagos durante la inflamación y los procesos tumorogénicos, analizamos la expresión de varias citoquinas en sobrenadantes de macrófagos procedentes de medula ósea de ratones WT y p38α-Δ^{MC}. Estos experimentos nos permitieron identificar el IGF-1 (*insulin-like growth factor-1*) como un factor extracelular regulado por la señalización de la vía de p38 MAPK. Estudios posteriores permitieron confirmar una menor producción de IGF-1 tanto a nivel intracelular como extracelular en distintas preparaciones de macrófagos de medula ósea procedentes de ratones p38α-Δ^{MC}. Además, observamos que la expresión de mRNA de IGF-1 se inducía por IL-4 (citoquina tipo Th2 que participa en reparación tisular) más que por LPS (lipopolisacarido procedente de las bacterias y activador de inflamación), corroborando el papel de IGF-1 como marcador del proceso cicatrización de herida

(Roszer, 2015), el cual también contribuye a la progresión del tumor (Murray and Wynn, 2011). La implicación de p38 MAPK en la expresión de IGF-1 por parte de los macrófagos también fue confirmada utilizando distintos inhibidores químicos de ésta ruta de señalización. Además, observamos que el mRNA de IGF-1 se encontraba disminuido en macrófagos intestinales de ratones p38α-Δ^{MC} comparado con los ratones WT, sugiriéndo que el eje p38α-IGF-1 podría jugar un papel importante en la mucosa intestinal. Por tanto, el siguiente paso consistió en analizar la implicación de IGF-1 en la inflamación intestinal y la tumorogénesis asociada.

El IGF-1 puede promover la tumorogénesis regulando distintos procesos incluidos la proliferación y supervivencia celular (Clayton et al., 2011; Yu and Rohan, 2000). Por tanto, nos planteamos investigar como la falta de p38α en las células mieloides podría afectar los niveles de IGF-1 *in vivo* en respuesta tanto a la inflamación intestinal inducida por DSS como al proceso carcinogénico asociado a la inflamación inducido por AOM/DSS. Primero observamos que existe menos proteína IGF-1 en los extractos colónicos de ratones p38α-Δ^{MC} tratados con DSS en comparación con los ratones WT durante la fase de reparación tisular a día 13. A continuación analizamos la fosforilación del receptor tipo I de IGF (IGF1R), cuya autofosforilación es inducida por IGF-1 (Foulstone et al., 2005; Furundzija et al., 2010; Kuemmerle and Zhou, 2002). Observamos que 7 días después de finalizar el tratamiento con DSS (día 13), la vía de señalización de IGF-1 estaba menos activa en el colon de los ratones p38α-Δ^{MC} que en los WT. Esta menor actividad de IGF-1 en los extractos colónicos se correlacionaba con diferencias significativas en la proliferación celular ($Ki67^{+}$) a día 13 (fase de reparación tisular) en el colon de los ratones tratados p38α-Δ^{MC} con DSS, de acuerdo con las propiedades mitogénicas atribuidas al IGF-1. Además, observamos diferencias en la fosforilación de IGF1R en el epitelio colónico de ratones tratados con AOM/DSS en comparación con los no tratados, aunque no había diferencias entre ratones WT y p38α-Δ^{MC}. Sin embargo, la fosforilación de IGF1R estaba reducida en los tumores de los ratones p38α-Δ^{MC} comparados con los tumores de ratones WT.

El IGF-1 se ha implicado en los procesos de inflamación y reclutamiento de células del sistema inmunitario (Mourkioti and Rosenthal, 2005). Para evaluar el papel del IGF-1 producido por células mieloides en la inflamación intestinal, utilizamos ratones que expresan los alelos LyM-Cre e IGF-1-lox (IGF-1-Δ^{MC}). Nuestros resultados indicaron que en respuesta al DSS, los ratones IGF-1-Δ^{MC} presentaban un menor DAI, menos daño

epitelial a día 7 y menor longitud de colon en comparación con los ratones WT, mientras que no había diferencias significativas en la perdida de peso. El siguiente paso fue investigar el papel de IGF-1 en el reclutamiento celular y encontramos que el DSS inducía en ratones WT una producción más alta de monocitos inflamatorios ($Ly6C^{hi}CCR2^{+}$) en la medula ósea que en los ratones IGF-1-Δ^{MC}. De acuerdo con estos resultados, también observamos una disminución de macrófagos ($F4/80^{+}$) en el intestino de ratones IGF-1-Δ^{MC} tratados con DSS en comparación con los ratones WT. La infiltración de neutrófilos activados y células T, así como la fosforilación de STAT3 estaban reducidas en los ratones IGF-1-Δ^{MC}. Es importante resaltar que la cuantificación de la fosforilación del IGF1R en los colones tratados con DSS reveló una reducción muy drástica de la señalización en los ratones IGF-1-Δ^{MC} comparado con los WT. La expresión de mRNA de IGF-1 también se vio disminuida en los macrófagos intestinales de ratones IGF-1-Δ^{MC} no tratados en comparación con los ratones WT, sugiriendo la importancia del IGF-1 producido por las células mieloides a la activación de esta vía en el intestino.

Para abordar el efecto de la inhibición de la señalización por IGF-1 en la inflamación intestinal, realizamos un tratamiento con el compuesto PQ401 que inhibe la autofosforilación de IGFR1 (Gable et al., 2006; Troib et al., 2011; Zhou et al., 2016). De acuerdo con los resultados obtenidos en ratones deficientes para IGF-1 en las células mieloides, el tratamiento con PQ401 redujo la colitis inducida por el DSS en ratones WT. Estos ratones tratados con PQ401 presentaban una pérdida de peso, un DAI y un daño epitelial muy similares a los ratones p38α-Δ^{MC}. Como era de esperar, los niveles de fosforilación de IGF1R estaban disminuidos en el colon de los ratones tratados con PQ401. Una observación interesante es que el tratamiento con PQ401 redujo el reclutamiento de macrófagos en el colon de los ratones WT tratados con DSS. Estos resultados apoyan que la inhibición de la señalización por la vía IGF1R puede reducir la inflamación intestinal inducida por DSS. Consistente con esta idea, observamos una reducción significativa del número de leucocitos ($CD45^{+}$), células mieloides ($CD45^{+}CD11b^{+}$), macrófagos ($F4/80^{+}$) y monocitos inflamatorios ($Ly6C^{hi}CCR2^{+}$) en la medula ósea de ratones WT tratados con DSS y PQ401, con valores similares a los obtenidos en los ratones p38α-Δ^{MC} tratados con DSS.

Para confirmar la importancia de la señalización por IGF-1 en el desarrollo del CAC, tratamos los ratones WT y IGF-1-Δ^{MC} con el protocolo AOM/DSS. Nuestros

resultados indicaron que los ratones IGF-1-Δ^{MC} mostraban una menor carga de tumores de colon asociados a colitis en comparación con los ratones WT. Además, los tumores de los ratones IGF-1-Δ^{MC} presentaban menos infiltración de macrófagos (F4/80^{+}) comparados con los tumores de ratones WT, sugiriéndo la implicación de IGF-1 en el reclutamiento de células inmunitarias. Para evaluar el potencial efecto terapéutico que podría suponer inhibir la vía de IGF-1 en cáncer colorectal, los ratones WT tratados con AOM/DSS se trataron con PQ401 durante 20 días. La evaluación del proceso tumorogénico indicó que la inhibición de la señalización por IGFR1 al tratar con PQ401 redujo significativamente el número de tumores en ratones WT de forma muy similar a lo que observado en los ratones p38α-Δ^{MC}.

Es importante mencionar que los ratones utilizados en este estudio se mantuvieron en un estabulario convencional, el cual se asemeja más a una situación normal que en los estabularios libres de patógenos o SPF (*specific pathogen-free).* En condiciones normales, el intestino se encuentra en una situación de "inflamación fisiológica" debido a la exposición constante a la microbiota del individuo (Mowat and Bain, 2011). Dada la importancia del continuo reemplazamiento de los macrófagos intestinales por monocitos circulantes que se produce en la medula ósea (Bain and Mowat, 2014), decidimos analizar las diferencias que podría haber en esta población entre ratones WT y p38α-Δ^{MC} en condiciones de homeostasis (o no patogénicas). Observamos que los ratones p38α-Δ^{MC} presentaban menos cantidad de monocitos inflamatorios (Ly6C^{hi}CCR2^{+}) en la médula ósea correlacionándose con una menor circulación de estas células en sangre cuando se comparaba con los ratones WT.

Como el reclutamiento de células inmunitarias en ratones a los que se les indujo colitis por DSS era más reducido en los ratones WT tratados con PQ401 y en ratones IGF-1-Δ^{MC}, nos planteamos analizar el reclutamiento de monocitos inflamatorios en condiciones basales de homeostasis en estos ratones. Efectivamente, observamos una reducción similar de monocitos Ly6C^{hi}CCR2^{+} en la medula ósea de ratones IGF-1-Δ^{MC} sin tratar en comparación con los ratones WT. A parte de su papel como hormona inductora de crecimiento, el IGF-1 puede funcionar como agente quimiotáctico (Tsai et al., 2014). Por este motivo, analizamos si la p38α en las células mieloides podría afectar a la producción de quimioquinas, comparando los extractos colónicos procedentes de ratones WT y p38α-Δ^{MC}. Interesantemente, observamos una reducción generalizada de quimioquinas importantes para el reclutamiento de células mieloides en ratones p38α-

Δ^{MC}, que correlacionaba con la menor infiltración de monocitos/macrófagos en los tejidos inflamados de estos ratones. Las quimioquinas que se encontraban expresadas a menor nivel incluyen Chemerin, CCL6, CCL2, CXCL12, IL-16, CCL21, CCL12 y CCL9, todas ellas con un importante papel en el reclutamiento de leucocitos (Buechler, 2014; Cao et al., 2016; Shi and Pamer, 2011). Mediante RT-PCR, confirmamos la reducción de la expresión del mRNA de chemerin, CCL2 y CCL12 en macrófagos intestinales aislados del colon de ratones p38α-Δ^{MC} comparados con los WT. Además, observamos una disminución significativa de IL-16 en los extractos colónicos de los ratones p38α-Δ^{MC} y de CCL9 en los ratones IGF-1-Δ^{MC} comparados con los ratones WT. Todos estos resultados sugieren que p38α regula la expresión de quimioquinas en las células mieloides tanto a nivel transcripcional como post-transcripcional.

La señalización por p38α ha sido implicada en la diferenciación de distintos tipos celulares (Cuadrado and Nebreda, 2010) pero hasta la fecha no se ha relacionado p38α con la diferenciación de monocitos/macrófagos. Para evaluar si la generación o diferenciación de monocitos/macrófagos podría encontrarse alterada en los ratones p38α-Δ^{MC}, analizamos diferentes poblaciones de células madre hematopoyéticas en la médula ósea. Sin embargo, no observamos diferencias entre los ratones p38α-Δ^{MC} y WT. Por lo tanto, hipotetizamos que la reducción del reclutamiento de células inflamatorias en el intestino podría ser debida a una expresión reducida de quimioquinas, directa o indirectamente reguladas por la p38α presente en las células mieloides del colon debido a la exposición de la microbiota intestinal conduciendo a un estado de “inflamación fisiológica”, la cual se encontraría potenciada por la inflamación inducida por el DSS.

Para complementar los estudios en modelos animales, utilizamos la línea celular de epitelio intestinal de ratón CMT-93. Esta línea fue caracterizada exhaustivamente antes de seleccionarla, puesto que cuando se empezó a trabajar con ella no había mucha información en la literatura. Esta caracterización indicó que las células CMT-93 exhiben inhibición por contacto al alcanzar la confluencia celular, y que el aumento de la confluencia celular conlleva un aumento en la expresión de marcadores típicos de diferenciación celular, además de la aparición de células apoptóticas y senescentes. El primer objetivo fue realizar ensayos utilizando las células CMT-93 y los macrófagos derivados de médula ósea para evaluar el papel de p38α en la interacción entre ambos tipos de células. Nuestros resultados demostraron que la inhibición de p38α mediante incubación con distintos inhibidores, aumentó la permeabilidad de monocapas de células

CMT-93, confirmando resultados previos del grupo que demostraron una mayor permeabilidad intestinal en los ratones deficientes en p38α en las células del epitelio intestinal (Gupta et al., 2014).

Por otra parte, realizamos estudios para evaluar el mecanismo de reparación tisular o cicatrización implicado en la inflamación intestinal y en los procesos de tumorogénesis. Los ensayos de cierre de herida (*wound-healing*) nos permitieron concluir que el proceso de migración celular es el principal mecanismo utilizado por las células CMT-93 para cerrar físicamente la herida. Además, observamos que la migración se encontraba reducida cuando se utilizaban inhibidores de p38α, y que la activación de p38 MAPK principalmente se localizaba en los bordes de la herida que se cerraba. La migración, a parte de ser muy importante en la reparación tisular, también es una propiedad pro-tumorogénica de las células cancerosas (Schafer and Werner, 2008). Las citoquinas secretadas por las células tumorales frecuentemente inducen la diferenciación de macrófagos a un fenotipo denominado macrófago asociado al tumor o TAM (*tumor-associated macrophage*), cuyas características son más parecidas a los macrófagos alternativos o M2 que participan en la reparación de tejido dañado (Mosser and Edwards, 2008; Paul and Zhu, 2010)

Interesantemente, los sobrenadantes procedentes de macrófagos contienen muchas citoquinas, las cuales pueden ser parcialmente reguladas por la vía de p38 MAPK, como se ha demostrado ampliamente utilizando tanto modelos genéticos como inhibidores químicos. De acuerdo con estos resultados, los sobrenadantes de macrófagos activan p38 MAPK en las células CMT-93. Ya que identificamos el IGF-1 como una nueva diana regulada por p38α en macrófagos, y se ha demostrado que IGF-1 activa p38 MAPK (Kuemmerle and Zhou, 2002), decidimos investigar la posible implicación de IGF-1 en este proceso. El IGF-1 se ha implicado en la migración de células epiteliales *in vitro* y es considerado un marcador para macrófagos M2 activados por la vía alternativa, por tanto se encuentra involucrado en el proceso de reparación tisular y tumorogénesis (Chen et al., 1999; Wang et al., 2015a). De acuerdo con esta información, observamos que el IGF-1 era capaz de inducir la migración de las células CMT-93 en los ensayos de cierre de herida.

En resumen, nuestros resultados identifican a IGF-1 como una nueva diana de la vía de señalización de p38α en macrófagos, y además, aportan evidencias de que el IGF-1 facilita el reclutamiento de células inflamatorias en los modelos de colitis inducida por

DSS y contribuye al proceso de tumorogénesis asociado a colitis inducido por AOM/DSS.

Discusión

Las respuestas inflamatorias crónicas pueden predisponer a la carcinogénesis tal como se observa en la IBD y otras patologías inflamatorias (Coussens and Werb, 2002; Thorsteinsdottir et al., 2011). Nuestros resultados amplían los hallazgos anteriores que indicaban que la p38α en las células mieloides es un mediador clave de las respuestas inflamatorias, e identificamos el IGF-1 como un nuevo efector de la señalización por p38α en los macrófagos. Aunque se conoce el papel de la señalización por p38α en la regulación de citoquinas proinflamatorias, no se ha descrito previamente la regulación de IGF-1 por parte de p38α en macrófagos.

Nuestros resultados muestran que la supresión de p38α en las células mieloides mejora la inflamación intestinal principalmente a través de la inhibición del reclutamiento de células inflamatorias, que a su vez reduce la carga tumoral. Existe una relación establecida entre p38α e inflamación. Los macrófagos asociados a tumores o TAM promueven el proceso tumorogénico en numerosos aspectos (Grivennikov et al., 2010). Varios estudios han identificado la IL-4 como una de las principales citoquinas reguladoras del fenotipo de los TAM. La IL-4 se produce en varios tipos celulares del sistema inmunitario y es una citoquina clave para determinar el balance entre citoquinas pro- y anti-inflamatorias (Paul and Zhu, 2010). Un estudio reciente propone el papel de p38 MAPK en la señalización inducida por IL-4 en macrófagos peritoneales, sin embargo este estudio no analiza el IGF-1 (Jimenez-Garcia et al., 2015). En esta tesis, demostramos que p38α regula la expresión de IGF-1 en macrófagos. Estudios anteriores han implicado p38 MAPK en la producción de IGF-1 por parte de otros tipos celulares como células mesenquimales, células progenitoras adiposas o células de músculo liso intestinal (Wang et al., 2006). Los macrófagos activados por la vía alternativa pueden producir IGF-1, el cual puede favorecer la reparación tisular induciendo la migración (Roszer, 2015; Wang et al., 2015a). De acuerdo con estos resultados, hemos demostrado que IL-4 es un mejor inductor de IGF-1 en macrófagos que LPS, y en ambos casos esta expresión se encuentra regulada por p38 MAPK. Además de la implicación atribuida a IGF-1 en el proceso de reparación tisular, el IGF-1 también se ha relacionado con procesos de reclutamiento de células inflamatorias, proliferación celular y crecimiento así como con el mantenimiento de la activación alternativa en macrófagos, funciones que en conjunto llevarían a

promover el proceso de carcinogénesis (Coussens and Werb, 2002; Mourkioti and Rosenthal, 2005; Tonkin et al., 2015). En este sentido, una falta de señalización correcta de IGF-1 se ha asociado a distintos cánceres, y existen numerosos estudios que implican la actividad del receptor IGF-1 en la proliferación, migración e invasión de las células cancerosas (Clayton et al., 2011). Los macrófagos han sido propuestos como los principales productores de IGF-1 después del hígado (Gow et al., 2010), lo cual es coherente con nuestros resultados que muestran una baja señalización por IGF-1 en los intestinos de los ratones IGF-1-Δ^{MC}.

La inhibición de la vía de señalización de IGF-1 en las células mieloides o el tratamiento de ratones WT con PQ401 parcialmente mejora las condiciones de la inflamación y el daño epitelial inducido por el DSS. Además observamos una fuerte reducción de los monocitos Ly6C^{hi}CCR2^{+} en la medula ósea de estos ratones, lo cual se correlaciona con menos macrófagos en el colon de los ratones IGF-1-Δ^{MC} y WT tratados con PQ401. Sin embargo, esto no fue suficiente para reducir la colitis inducida por el DSS al mismo nivel que en los ratones p38α-Δ^{MC}, probablemente debido a la implicación de la señalización por IGF-1 en los mecanismos de reparación de la mucosa. Además, p38α en las células mieloides puede regular otras citoquinas proinflamatorias involucradas en la inflamación intestinal aguda que pueden no ser afectadas por la inhibición de la vía IGF-1. La reducción en la carga tumoral detectada en los ratones IGF-1-Δ^{MC} o en los ratones WT tratados con PQ401 confirman la importancia de este factor en el proceso de tumorogénesis (Clayton et al., 2011).

Hemos observado una reducción en el número de los monocitos Ly6C^{hi}CCR2^{+} en los ratones p38α-Δ^{MC}, incluso en condiciones basales o de homeostasis. Creemos que es importante analizar exhaustivamente el fenotipo de estas células, puesto que estos monocitos inflamatorios participan en el reclutamiento de otras células del sistema inmunitario así como participan en la activación de la inmunidad adaptativa (Bain and Mowat, 2014). Nuestra hipótesis es que las diferencias observadas en los monocitos Ly6C^{hi}CCR2^{+} en ratones no tratados probablemente sean debidas a un estado controlado de "inflamación fisiológica" que se incrementa en condiciones inflamatorias producidas por ejemplo por el DSS y se encuentra relacionada con la diferente expresión de quimioquinas cruciales para el reclutamiento de las células mieloides. Además, a parte de su función como hormona que estimula el crecimiento, el IGF-1 es también conocido como una quimioatrayente que media la quimiotaxis en distintos tipos celulares (Roussos

et al., 2011). Los macrófagos intestinales aislados de ratones p38α-Δ^{MC} no tratados muestran menos mRNA de IGF-1 que los ratones WT, sugiriendo que podría estar involucrado en las diferencias en las células inflamatorias observadas en la médula ósea.

El análisis de quimioquinas reveló la reducción de varias quimioquinas en los colones de ratones con células mieloides deficientes en p38α, en ausencia de ningún tratamiento. La ruta de p38 MAPK se ha involucrado en la regulación de quimioquinas en linfocitos T *helper*, macrófagos de pulmón humano y macrófagos peritoneales así como en modelos experimentales de inflamación de la piel en queratinocitos y en células mieloides (Granata et al., 2006; Kim et al., 2008; Sun et al., 2008; Wong et al., 2007). En esta tesis demostramos que p38α regula los niveles de mRNA de Chemerin, CCL2 (MCP-1) y CCL12(MCP-5) en macrófagos intestinales, que no se había descrito previamente. Es interesante mencionar que CCL2 parece estar regulada tanto por p38α como por IGF-1 en macrófagos intestinales, de hecho se ha propuesto que la ruta de p38 MAPK regula CCL2 en macrófagos murinos (Sun et al., 2008).

o se sabe cómo IGF-1 puede regular la producción de CCL2 en macrófagos, pero, de acuerdo con nuestros resultados, se ha demostrado que CCL2 se encuentra reducida en los tumores de colon de ratones tratados con NT157, un inhibidor de IGF-1 y STAT3 (Sanchez-Lopez et al., 2015). Parece probable que el IGF-1 pueda actuar sobre diversos tipos de células, dado que se secreta en el microambiente y que el receptor de IGF-1 se expresa de forma ubicua en los tejidos normales. Sobre la base de los resultados obtenidos en nuestro estudio, proponemos que la señalización de IGF-1 podría ser un blanco potencial en el contexto de la inflamación intestinal y la tumorogénesis.

Conclusiones

1. p38α en las células mieloides suprime la colitis inducida por DSS y la tumorogénesis de colon asociada a la inflamación.
2. IGF-1 es una nueva diana de p38α en macrófagos.
3. IGF-1 regulado por p38α en las células mieloides contribuye a la colitis y a la tumorogénesis de colon asociada a la inflamación.
4. La inhibición de la señalización por p38α o IGF-1 en células mieloides suprime el reclutamiento de células inflamatorias al intestino.
5. p38α en células mieloides regula la expresión de distintos factores quimiotácticos en el intestino.

6. El eje p38α-IGF-1 en células mieloides debe ser considerado como un potencial objetivo terapéutico en la inflamación asociada a enfermedades intestinales y cáncer.