Novel Therapeutic Approaches for Inflammatory Bowel Disease

by

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Abstract

Inflammatory bowel disease (IBD) is chronic relapsing and remitting inflammation of the gastrointestinal tract resulting in abdominal pain, diarrhoea and weight loss. The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Genetic analysis has implicated several genomic regions containing IBD susceptibility genes including genes on 3p21 such as macrophage stimulating1 gene (*MST1*) and Ras-association domain family member 1A gene (*RASSF1A* or *1A*). In this thesis project, we explored the molecular mechanisms by which the loss of RASSF1A can modulate the appearance of IBD in a rodent model. It has been established that RASSF1A is frequently epigenetically silenced by promoter specific methylation in numerous cancers (including colorectal cancer) and in IBD (specifically in ulcerative colitis patients), suggesting its importance for both. The genetic loss of 1A in our *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice resulted in clinical symptoms of colitis including increased intestinal permeability, increased DNA and oxidative damage enhanced cytokine/chemokine production, elevated NF-κB activity, severe colonic epithelial cell injury and poor recovery following dextran sulphate sodium (DSS)-induced inflammation injury, suggesting importance of RASSF1A and haploinsufficiency in the *Rassf1a* locus for DSS inflammation injury. DSS is a chemical inducer of colitis in mice and a potent activator of innate immunity. The absence of *Rassf1a* also resulted in decreased epithelial repair with reduction of several markers of proliferation including Yes-associated protein (YAP)-driven proliferation, an important proliferation regulator within the Hippo pathway. Surprisingly, tyrosine phosphorylation of YAP appeared versus serine phosphorylation in the absence of *Rassf1a* that resulted in enhanced p73 transcriptional upregulation of pro-apoptotoic genes including Bax. These aforementioned events

resulted in increased epithelial cell death and poor survival of DSS-treated mice in the absence of RASSF1A. More importantly to the aims of this thesis, the genetic loss of *Rassf1a* also resulted in increased autophagic signalling linked to the NOD2 pathway. The use of PTK inhibitors and autophagy inhibitors effectively increased recovery from DSS-induced inflammation injury in both the *Rassf1a*$^{-/-}$ mice and in the *IL-10*$^{-/-}$ mice. In this thesis, I have demonstrated that tyrosine kinase inhibitors and anti-autophagy drugs may be novel therapeutic approaches to enhance recovery from inflammation-induced injury and be useful to treat and protect IBD patients from increased risk of developing cancer later in life.

Dedication

To the soul of my father, Dr. Muhammad Fiteih and the soul of my father in law, Dr. Kamal El-Menofy.

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Preface

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List of Abbreviation

ATG	Autophagy related
ATG16L1	Autophagy related 16-like 1
CD	Crohn's Disease
BAX	Bcl2-associated X protein
BSA	Bovine serum albumin
BGS	Bovine growth Serum
CARD15	The other name of NOD2
DSS	Dextran sodium sulphate
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IKK	IκBα kinase
IRGM	Immunity-related GTPase family M protein
LATS1	Large tumour suppressor kinase 1
LATS2	Large tumour suppressor kinase 2
LC3	Microtubule-associated protein 1 light chain 3 alpha
LPS	Lipopolysaccharide
MOAP-1	Modulator of apoptosis
MSP	Macrophage-stimulating protein
MST1	Macrophages stimulating 1 gene
MST1	Mammalian STE20-like kinase 1
MST2	Mammalian STE20-like kinase 2

MyD88	Myeloid differentiation response gene 88
NF-κB	Nuclear factor-κB
NOD2	Nucleotide-binding oligomerization domain containing 2
NOD1	Nucleotide-binding oligomerization domain containing 1
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
RASSFIA	Ras association domain family 1 isoform A
RIP2	Receptor interacting protein 2
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAB	TAK associated binder
TAK	TGFβ activated kinase
TEAD1	Transcriptional enhancer factor TEF-1
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfonic acid
TNFα	Tumour necrosis factor α
TRADD	TRAF2 associated death domain protein
TRAF	TNFα receptor associated factor
TRAIL	TNF-related apoptosis induced ligand
UC	Ulcerative colitis
ULK1	Unc-51 like autophagy activating kinase 1
VDR	Vitamin D receptor
YAP1	Yes associated protein

Chapter 1

Introduction

1.1 Inflammatory bowel disease (IBD)

IBD is chronic relapsing and remitting inflammation of the gastrointestinal tract resulting in abdominal pain, diarrhoea and weight loss [1, 2]. The two major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Crohn's disease can affect any part of gastrointestinal tract from the mouth to the anus with transmural (extending through or affecting the entire thickness of the wall of the colon) inflammation, while ulcerative colitis affects only the colon and the inflammation is limited to the superficial layer (mucosa) [3, 4]. IBD is highly prevalent in Canada [5, 6]. In the fall of 2012, Crohn's and Colitis Canada foundation released its report stating that approximately 233,000 Canadians suffer from Crohn's disease and ulcerative colitis. CD and UC are twice as common as multiple sclerosis or AIDS and about as prevalent as epilepsy and Type 1 diabetes. One of the most important findings of this report is the significantly increasing prevalence of IBD in children less than 10 years, with an estimated 5,900 Canadian children affected. . IBD treatment requires a long term therapy; the economic costs for IBD in Canada were about $2.8 billion in 2012, over than $11.900 for every person with IBD every year. Direct medical costs are about 1.2 billion and are mainly for medication (521 million), hospitalization (395 million) and physician visits (132million), [Crohn's and Colitis foundation of Canada 2012]. 10,200 new cases are being diagnosed every year and incidence rates raising significantly particularly in children under age 10 therefore IBD should be announced as a national and provincial health priority.

1.1.2 Pathogenesis of IBD

The aetiology of IBD has been widely studied during the last several decades; however, contributing factors in disease pathology are not completely understood. IBD is thought to result

from the interaction between genetic and environmental factors that enhance the normal intestinal commensal flora to induce an inappropriate mucosal immune response [7]. Therefore, there are many factors contributing to the pathogenesis of IBD.

Environmental factors

Environmental factors appear to be critical to the pathogenesis of CD and UC, including breastfeeding, smoking, oral contraceptives, diet, hygiene, education, climate, pollution, childhood infections, stress, occupation and miscellaneous components such as toothpaste, appendectomy, tonsillectomy, blood transfusions, contact with animals, and physical activity [8]. For breastfeeding, several studies demonstrated a protective role for breastfeeding in IBD including a meta-analysis of 14 case-control studies [9, 10]. However, some studies have failed to find an association [11, 12]. Appendectomy is negatively associated with UC, especially in children with appendicitis before the age of 10. Meta-analyses studies showed significant reduction in the risk of developing UC after an appendectomy [13, 14]. In contrast, the relationship between appendectomy and CD is still unclear [15] and needs more investigation.

Cigarette smoking is one of the most established associations with IBD. Current smokers have a 2-fold increased risk of CD compared with non-smokers [16, 17]. The relationship between diet and IBD is critical because IBD affects the site of nutrient absorption. Nutritional deficiencies in IBD are well observed especially that of zinc in CD with immunity impairment [8, 18] and vitamin D deficiency [19]. Enteral nutrition (a way to supply food through a tube placed in the nose, the stomach or the small intestine) which is used to induce remission in patients by liquid feeding with polymeric or elemental formulae to reduce the symptoms of IBD, but not widely approved and it is not successful as steroids and aminosalicylates [8, 20-22].

Familial and genetic factors

When epidemiological studies support that genetic factors are important in disease occurrence, then genome-wide association studies (GWAS) and linkage studies are performed to determine genes or genetic variants that are involved. Linkage studies involved in determination of how two genes are linked to a change in a biological process. Linkage is the tendency for genetic markers or genes to be inherited together because of their location near each other on the same chromosome [23, 24]. Genome wide analysis (GWAS) has identified several single nucleotide polymorphism (SNPs) that may be involved in the appearance or progression of IBD. GWAS investigate common genetic variants in different individuals and identify its association with a certain disease. GWAS mainly highlights the associations between single-nucleotide polymorphisms (SNPs) and major diseases. GWAS generally compare the DNA of two groups, people with the disease (cases) and people without the disease (controls). If one type of the variant (one allele) is more constant in people with the disease, then SNP is considered to be associated with the disease [25].

IBD is prevalent among family members, the prevalence of IBD in first-degree family members is about 40%.[26]. A large study in Scandinavians demonstrated that a 10-fold increase in familial risk, strongly suggesting a genetic cause [26]. Furthermore, studies reported that the risk of Crohn's disease in monozygotic twins is 58.3% while in dizygotic twins is 3.9% [27, 28].

With the aforementioned clinical observations, many studies investigating genetic factors in IBD have been performed. A random study in the European population demonstrated increased frequency of HLA-A11 and HLA-A7 in UC and a decreased frequency of HLA-A9 in CD [29]. One of the most reliable and established associations is that with HLA-DR2 in Japanese patients with UC [30]. In contrast, no HLA-DR2 (DR2) association in British and American patients with IBD, although association with DRB1*0103 and DRB1*12 is found in UC [31, 32].

IBD is associated with complex genetic disorders with multiple genes involved in the pathogenesis. Recently, over 160 susceptibility genes have been identified for IBD and they can be classified as microbial sensors, autophagy related regulators and ER stress modulators [33], including cytokine factors (IL-10), cytokine receptors (IL-17R and IL-23R), transcription factors (NFκB and JAK/STAT pathway), kinases (PTPN22, Tyk2), apoptotic elements (CARD9 and 11) and molecules involved in autophagic signaling (ATG16L, IRGM, NOD2) [34]. Autophagy related genes are associated with polymorphism in IBD patients and they are associated with increased susceptibility to Crohn's disease [35]. Genetic linkage studies have demonstrated several genomic regions containing genes involved in IBD. Some genes are specifically related to Crohn's disease (CD) or ulcerative colitis (UC) and other genes are associated with both.

The first and most established replicated linkage region, IBD1 is on chromosome 16q contains the CD susceptibility gene, NOD2/CARD15(Nucleotide-binding oligomerization domain-containing protein 2) [36]. NOD2 is ubiquity expressed in peripheral blood monocytes and has a critical role in innate immunity. Three major coding region polymorphisms are associated with NOD2/CARD15 especially with CD patients of European descent [37, 38]. Having one copy of the risk alleles is associated with a 2–4-fold risk for developing CD, whereas double-dose carriage increases the risk 20–40-fold [39]. Patients with NOD2/CARD15 risk alleles are associated with earlier disease onset, ileal location and stricturing phenotype [40].

Other IBD genomic regions include, IBD2 on chromosome 12q (associated with UC)[41], and IBD3, containing the major histocompatibility complex region [42, 43].A short genomic region has been involved with CD on chromosome 5q and linkages on 3p21, involving macrophages stimulating1 (MST1) (a protein involved in macrophage activation, chemotaxis and inflammatory responses regulation, indicating its role in IBD pathogenesis) [44] [45] and

Ras-association domain family member 1A (RASSF1A), a tumour suppressor protein that is frequently epigenetically silenced by promoter specific methylation in ulcerative colitis and colorectal cancer [46-49]. The discovery of additional IBD susceptibility genes could help in IBD treatment, characterization of the molecular mechanisms behind IBD and aid in understanding the role of environmental factors in intestinal inflammation.

Alteration of the immune system

Recent research suggests that an exaggerated immune response towards antigens in the GIT might be the one of the main causes of IBD [35]. Innate immunity is the first line of defence in our body against invading organisms and tissue destruction. It also activates inflammation and repair to help the body to get rid of invading organisms. The contact between microbial flora and our immune system is restricted by the intestinal barrier that is made of epithelial cells that acts like a physical barrier. This barrier is disrupted in inflammatory diseases specially, IBD [50]. The intestinal epithelium is made up of a single layer of epithelium cells, including M cells, Paneth cells, goblet cells and columnar epithelium cells all of which differentiate from crypt stem cells. These cells cover the mucosa and play a major role in intestinal homeostasis and the balance between intestinal microbiota and immune system. Paneth cells and goblet cells produce mucus layer that has an antimicrobial effect. Immunoglobulin A is produced by transcytosis from columnar epithelium cells [51, 52]. These molecules decrease the bacterial insult to the GIT mucosa and protect the intestinal epithelium from being damaged or disrupted.

When IBD is active, innate immunity cells (macrophages and neutrophils) infiltrate the lamina- propria followed by adaptive immune cells (B and T lymphocytes) where they stimulate T regulatory cells to secrete inflammatory cytokines [50] which will lead to amplification of the

inflammatory response, injury and damage to the intestinal epithelium with loss of the physical barrier. Normally, turning off the active, destructive immunologic and inflammatory events should occur following the resolution of bacterial or viral infection that has been appropriately defended against and controlled by the mucosal immune system. Furthermore, the body tries to protect itself by re-establishing the barrier with a process called epithelial restitution or sealing to prevent direct contact between intestinal bacteria and the active immune system to restrict the exaggerated immune response that will lead to further epithelial damage [53, 54].

In IBD, the down-regulatory events and processes that normally turn off the immunologic and inflammatory protective processes once the pathogenic agent has been removed appear to be working improperly. If inflammation in IBD patients is not shut down and the exaggerated immune response is not properly regulated persistent chronic inflammation ensues to result in a predisposition to colorectal cancer (CRC) later in life.

1.1.3 IBD and cancer

Patients with long standing IBD are at higher risk of developing colorectal cancer. CRC is the most common malignancy associated with IBD patients. The risk of developing CRC after 10,20,30 years of diagnosis of UC are 2,8 and 18% respectively [55]. The overall increase in prevalence of CAC(colitis associated cancer) in IBD patients depends on many factors such as: disease severity and duration, family history of CRC, coexistent of primary sclerosing cholangitis [56, 57], efficacy of anti-inflammatory therapies and IBD management [58, 59]. Utilizing mouse model single injection of AOM, azoxymethane (a potent carcinogen used to induce colon cancer in rats and mice) will give rise to colonic tumors when coupled with repeated inflammation insult. The induction of repeated inflammation will lead to chronic colitis

and eventually to invasive and possibly CRC [60, 61]. These clinical and experimental observations clearly highlight CRC as classical inflammation-driven cancer and make mouse models of CRC extremely valuable for understanding the molecular link between inflammation and cancer [62, 63].

The pathogenesis of CRC in IBD patients is mainly associated with pathological changes associated with IBD development such as: genetic predisposition, increased intestinal epithelium permeability and loss of the intestinal barrier that induces exaggerated and uncontrolled response to bacterial products [64]. Previous studies suggest that the increase in intestinal permeability can be caused by many factors such as oxidative stress [65, 66] and the release of inflammatory mediators like IL-6, IL-1β and TNF-alpha which are prominent in IBD patients [67]. Molecularly, inflammation is characterized by hyperactivation of transcription factors such as NF-κB and uncontrolled increase in inflammatory cytokines production [3, 68] to amplify the inflammatory response [69]. NF-κB is activated by stimulation of surface receptors including TLRs and TNFR that will result in production of pro- inflammatory cytokines such as IL-6 and TNF-alpha [70, 71].

Most of current IBD models outline the major role of NF-κB in linking inflammation in IBD to cancer development [72, 73]. NF-κB has multiple and diverse functions; such as regulation of cytokines production, stimulation of tumour cells proliferation and survival by controlling the anti- apoptotic genes. Furthermore, studies proved that IL-6, one of the main NF-κB-induced pro-inflammatory cytokine, has a critical role in inflammation, immune system regulation and tumour formation in IBD patients. These findings elucidate that NF-κB and pro-inflammatory cytokines especially IL-6 play a major role in tumour development in IBD patients [74], [75]. Recent studies showed that chronic inflammatory changes in IBD patients are

associated with increased levels of pro-inflammatory cytokines secreted by immune cells, such as (IL-6 and TNF-alpha). Also, they found in IBD animal models that the increased levels of NF-κB driven pro-inflammatory cytokines are responsible for exaggerating and sustaining the inflammation and colorectal cancer development [76, 77]. Interestingly, ulcerative colitis and colorectal cancer patients have high levels of circulating pro-inflammatory cytokines (IL-6, IL-8, TNF-alpha and IL-1β) and IL-10, an anti-inflammatory cytokine than healthy individuals [78]. Recently, patients with active UC, dysplasia and cancer were shown to have increased expression of IL-6 and STAT3 (a transcription factor associated with various human cancers and it has anti-apoptotic as well as proliferative effects in epithelial cells). Furthermore, mice studies demonstrated that (IL-6) induces proliferation and progression to cancer via activation of STAT3 [79], indicating that transcription factors and inflammatory cytokines play a critical role in tumourigenesis.

Recently, it was shown that epigenetics plays a role in cancer development. Researchers are focusing on epigenetics changes (such as gene promoter methylation) of tumour suppressor genes in cancers. Hypermethylation of gene promoters can result in their silencing. Silencing of genes responsible for cell cycle and cell death by epigenetic mutations is very dangerous allowing cells to grow and reproduce uncontrollably leading to tumourigenesis. RASSF1A is frequently found to be transcriptionally silenced due to promoter specific methylation in many types of cancers [49]. It has been reported that IL-6 treatment induces DNMT1 (DNA methyltransferase-1) expression in colorectal cancer cells and increases the methylation of promoter regions of genes associated with tumor suppression [80]. Furthermore, a study reported that p53 functions to recruit DAXX and DNMT1 to CpG methylation sites of RASSF1A and epigenetically silence RASSF1A [81]. Interestingly, p53 was found to be accumulated in DSS-

treated mice [82]. In addition, RASSF1A epigenetic silencing can be detected in UC patients [83] and upon IL-6 treatment [84]. These findings suggest that inflammatory mediators will probably result in the epigenetic loss of tumour suppressor genes such as RASSF1A and this may contribute to early changes in the pathogenesis of UC and could be one of the major contributing factors for cancer formation in IBD patients.

1.1.4 Therapeutic control of IBD

Inflammation is a normal physiological response of the immune system as it is tries to protect us from infection and foreign substances such as bacteria, viruses and toxins. In IBD the immune system reacts inappropriately triggering an uncontrolled inflammatory response. As Crohn's disease and UC are diseases that cause inflammation, so anti-inflammatory drugs are the major treatment used to stop or restrict the inflammation.

There are 3 main groups of anti-inflammatory drugs which work in different ways to reduce or control inflammation. Usually, aminosalicylates derivatives are the first medicine used for IBD treatment [85]. Aminosalicylates derivative, 4-Amniosalicylic acid (4-ASA) has been introduced for IBD treatment but recently they have been replaced by 5-Aminosalicylic acid (5-ASA), however their mechanism of action needs more investigations but it is thought that they work by inhibiting cyclo-oxygenase1, IL-1β, TNF-α and ROS (reactive oxygen species) generation. Immunosuppressant such as corticosteroids, azathioprine, 6-mercaptopurine, cyclosporine, tacrolimus, methotrexate and mycophenolate mofetil, comprise the second class of drugs used for IBD treatment. The goal of these drugs is to control the acute inflammation and to

maintain a long remission period by suppression of the immune system. Biologics were proposed for IBD treatment recently. Unlike most medicines that are made from combining chemicals, biologics are made from human or animal proteins. They target specific molecules involved in the body's immune response and inflammatory pathways that work inappropriately in IBD patients. They are only used for severe active disease patients having resistance to traditional treatment. However, the most commonly used drugs are anti-tumour necrosis factor alpha (TNF-α) drugs. These drugs are artificial antibodies against TNF-α and exert there effect by binding to TNF-α and preventing it from activating TNF-receptors.

Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine and one of the main mediators for the abnormal immune response in IBD patients .Targeting TNF-α has significantly improved the management of IBD that is resistant to conventional therapies [86]. Infliximab is the best anti-TNF agent and it was approved by the U.S. Food and Drug Administration (FDA) for the treatment of psoriasis, Crohn's disease [87] and ulcerative colitis [88]. Furthermore, it is currently approved in the European Union for adults and children with Crohn's disease and adults with UC. Adalimumab is indicated for Crohn's disease in adults but not children. In April, 2008 FDA approved certolizumab (Cimzia) to be used in the United States for the treatment of Crohn's disease in people who did not respond sufficiently or adequately to standard therapy but it is still not approved in the European Union for Crohn's disease, treatment. Besides these drugs, antibiotics are sometimes used to treat IBD, particularly Crohn's. Other drugs are also used for IBD to help in reducing symptoms, such as diarrhea or pain, but they do not reduce inflammation.

Most of medications used for adults with IBD are also used for children with similar indications and contraindication. Children and adolescents are in a period of physical and emotional growth and development. Special consideration must be taken for the potential side effects and advantages and disadvantages of the use of the medication. While planning for IBD treatment for children many factors have to be taken into account such as duration and location of inflammation in the intestines, the child's age and size, psychosocial and emotional acceptance of the child and family. In addition, the dose of the drug has to be adjusted according to the child's age and weight (Crohn's and Colitis foundation of America, October 2012). To date, there is no cure for IBD, all of the treatment modalities are depending on reducing inflammation and symptoms associated with IBD.

1.1.5 Animal models of IBD

The list of IBD associated genes is continuously increasing. Some of them are supported by animal genetic knockout models. Several animal models of intestinal inflammation have been generated including chemical induced models, immunological models or genetic models. Animal models help us to understand the pathophysiological mechanism that may be responsible for disease initiation and progression. It has to be mentioned that there is no single animal model that can explain all the pathophysiology behind IBD. Some more commonly and accepted animal models for IBD are described below.

Chemically induced models

This group of animal models requires administration of an exogenous chemical agent for the induction of colitis. Trinitrobenzene sulfonic acid (TNBS), a chemical which induce formation of colonic ulcers for up to 8 weeks with just one dose with resemblance to Crohn's

disease more than UC [89]. Dextran sodium sulfate (DSS), is another chemical inducer of colitis, that irritates the colonic mucosa [90]. Feeding mice for several days with DSS in the drinking water induces a very reproducible acute colitis characterized by bloody diarrhea, ulcerations and infiltrations with granulocytes [90, 91]. It was demonstrated that DSS is very toxic to gut epithelial cells of the basal crypts and therefore it damages the mucosal physical barrier. Acute DSS colitis model is useful in understanding the contribution of innate immune system in colitis and to study the epithelial repair mechanisms [92]. Intra-rectal administration of the hapten oxazolone with ethanol into mice results in acute colitis, characterized by a T helper (Th)2-type immune response with a marked increase in (IL)-5 and IL-4 production, associated with body weight loss, diarrhea, ulcers and loss of epithelial cells in the bowel [93]. It resembles UC more than Crohn's disease, different from (TNBS)-induced colitis (Th1-mediated immune responses) which resemble CD [94].

In conclusion, chemically induced models for IBD are useful for studying biochemical pathways of inflammation and drug study in a simple and relatively inexpensive setting.

Immunological models

Immunologically manipulated models are models of adoptively transferred bone marrow precursors or T cells, which are introduced into immunodeficient mice.

The 2 main examples are the $CD45RB^{high}$ [95] and bone marrow chimera transfer models [96]. Transfer of hsp60-reactive CD8þ T cells promotes intestinal inflammation, mainly in the small intestine [97]. Studies in these models have illustrated the role of pathogenic and regulatory T cells in regulating mucosal immunity and intestinal inflammation and also demonstrated that Th1 has an essential role in IBD pathogenesis [98]. However, the severe immune abnormalities

in recipient mice probably make these models unsuited for studying innate factors causing human IBD.

Genetic models

Transgenic and knockout animal models have been widely used in the field of IBD. Examples include the IL-23, NOD2 and IL-10 knockout mice [46, 94]. Genetic models helps in understanding the role of immune-related molecules and IBD susceptible genes in the pathogenesis of intestinal inflammation.

IL-10 mouse model

IL-10 mouse model is one of the most reliable models for IBD. IL-10 also known as human cytokine synthesis inhibitory factor (CSIF) [99]. IL-10 is an anti-inflammatory cytokine and produced by T cells, monocytes, B cells, macrophages, thymic cells and keratinocytes [100]. IL-10 enhances B cell proliferation and antibody production and negatively regulates the function of T helper-1 cells. IL-10 can block NF-κB activity and it regulates the JAK-STAT signalling pathway [101]. *IL-10*$^{-/-}$ mice spontaneously develop chronic enterocolitis after 12 weeks of age with massive infiltration of lymphocytes, neutrophils and macrophages also they are very sensitive to DSS treatment [102, 103] [104].

Knockout studies in mice suggested the function of this cytokine as an essential immunoregulator in the intestinal tract and fortunately patients with Crohn's disease are ameliorated after treatment with recombinant IL-10-producing bacteria demonstrating the importance of IL-10 for counteracting the hyperactive immune response in IBD patients [105].

IL-23 mouse model

Studies demonstrated that IL-23 is highly expressed in inflamed mucosa of IBD and plays an important role in the induction of IEL (intestinal intraepithelial lymphocytes), NK, T

cell activation, pro-inflammatory cytokine secretion and Th17 cell differentiation [106]. Furthermore, IL-23 knockout mice develop less severe symptoms of colitis, highlighting the role of IL-23 in IBD patients [107, 108]. Furthermore, genetic polymorphisms in the IL-23 receptor are associated with IBD [109]. According to above studies, IL-23 could be one of the novel therapeutic targets for IBD treatment.

NOD2 mouse model

NOD2 (nucleotide-binding oligomerization domain containing 2, also known as CARD15*)* was identified as the first IBD susceptibility gene in 2001 [110, 111]. Polymorphisms in NOD2, is one of the strongest genetic risk for IBD. NOD2 is an intracellular pattern recognition receptor and it recognizes molecules containing specific structure called muramyl dipeptide (MDP) that is found in bacteria.

Two mice models have been established to study the function of NOD2 in IBD [112, 113]. In the first model, *NOD2* has been knocked out and this model is characterized by increased IL-12 production from macrophages upon activation of TLR2 by peptidoglycan, suggesting that the human *NOD2* polymorphisms are loss-of-function mutations [114] and there is coordination between different bacterial sensors. Furthermore, *NOD2* transfection into epithelial cells enhanced their resistance to bacterial infection and its expression in Paneth cells *in vivo* is essential for expression of defensins, suggesting that loss-of-function mutations reduce innate epithelial defence and dysregualtion in mucosal homeostasis [115]. More evidence about the role of NOD2 in CD came from the second animal model (knock-in animals with a mutation similar to most common human CD-associated allele, *3020insC)*. Macrophages from these mice showed increased production of mature IL-1ß upon MDP stimulation suggested to be caused by a gain-of-function mutation in NOD2 that activated caspase-1, an enzyme required for the

activation and maturation step of IL-1ß [116, 117]. At the same time, macrophages from 3020insC knock-in mice have normal TLR signalling.

Surprisingly, neither *Nod2*$^{-/-}$ nor the engineered mice expressing the murine homologue of the human NOD23020insC variant (Nod22939insC) develop spontaneous intestinal inflammation under specific pathogen-free (SPF) conditions. Furthermore, *Nod22939insC* only but not *Nod2*$^{-/-}$ mice display the appearance of experimental colitis using DSS [115]. These findings suggest that dysregulation of the NOD2 pathway alone is insufficient to fully induce intestinal inflammation and additional contributing factors are required for the development of CD in patients carrying a NOD2 mutation, so supporting the multifactorial nature of IBD. Working with genetically manipulated NOD2 mice strains have provided mechanistic understanding of how NOD2 might work, but these studies did not prove the mechanism by which NOD2 variants in human IBD enhance susceptibility to CD.

Unfortunately, in some models such as *IL-10*$^{-/-}$ mice it takes about 3 months for the appearance of colitis, making them unsuitable for large scale drug screening studies. Therefore for the purpose of evaluating novel therapeutic agents, experimentally induced acute colitis models (e.g. DSS- or TNBS colitis) are widely used. Moreover, these easily reproducible models provide the additional advantage of cost-efficiency and they can be used for studying of novel gene targeted mouse strains.

1.2 RASSF1A

1.2.1 Overview

The RASSF (Ras association domain family) family of proteins consists of 10 members, most of them are tumor suppressor proteins that are commonly silenced by promoter specific

methylation in many types of cancers [48, 49].The common pattern among all of RASSF family members is the presence of the Ras association (RA) domain that allows them to potentially associate with the Ras family of GTPases [49]. Ras proteins have direct role in human cancers and about 30% of tumours are associated with Ras mutations. Ras signaling is commonly associated with oncogenic effects such as reduced apoptosis and increased cell division; it is also activates effectors involved in tumor suppression [49].

RASSF1A was the first of the RASSF family member determined to have tumour suppressive activity [49]. The *RASSF1A* gene locus is located on chromosome 3p21.3 and it has 8 exons that undergo alternative splicing to give rise to 8 different isoforms (RASSF1A-RASSF1H) and a 39 kDa protein product for the 1A isoform. Most have not been confirmed to be protein products with the exception of RASSF1C. Interestingly, RASSF1C has been demonstrated to have no tumour suppressor properties and it might be an oncogene. [118-120]. In addition to the Ras binding domain ,RASSF1A also has a SARAH (Salvador-Rassf1A-hippo) domain in the C-terminus ,important for its interaction with the members of Hippo signaling pathway such as mammalian sterile 20-like(MST) kinases and the scaffolding protein Salvador [121]. An ataxia telagectasia mutant (ATM) kinase phosphorylation site on residues 125-138 [122]. A cysteine-rich domain, is present in the N -terminal and it is important for Rassf1a apoptotic activity [123].

The promoter region of *RASSF1A* is hypermethylated and it is one of the most frequently epigenetics inactivation event, in at least 37 types of human cancers [124, 125] [126]. *RASSF1A* is methylated in 20-52% of colorectal cancer [127, 128], 80% of small lung cancer [129], more than 60% of breast tumour (6,7), 90% of liver cancer[130, 131] and 69% of ileal cancers [132]. In addition, epigenetic silencing of *1A* has also been detected in pre-condition diseases to cancer such as IBD [83] and pancreatitis [133].

Rassf1a$^{-/-}$ mice (on the C57BL/6 background) are viable, fertile and retain expression of isoform 1C and other RASSF gene family members. *Rassf1a*$^{-/-}$ mice have an increased tumour incidence by 12–16 months of age (especially in the breast, lung, gastrointestinal (GI) and immune system (B-cell related lymphomas) and develop tumours in response to chemical carcinogens [134, 135], suggesting that Rassf1a has tumour suppressive activity.

1.2.2 The biological functions of RASSF1A

Microtubule organization

Microtubules are highly dynamic polymers of tubulin that form an integral part of the cytoskeletal system. RASSF1A has been found to co-localize with microtubules and deletion analysis studies revealed that both the N-and the C- terminus of RASSF1A are essential for its interaction with microtubules [136, 137]. The loss of RASSF1A microtubule localization results in the inhibition of tumour suppressor properties, loss of tubulin stability, inhibition of death receptor-dependent cell death [138, 139]. *1A* mutant lacking the tubulin association domain was not able to induce cell-cycle arrest in 293-T cells [139]. Furthermore, knockdown of 1A results in loss of cell to cell adhesion and the development of a fibroblast ;like morphology in HeLa cells, this indicates the importance of 1A association with microtubular network to control cell migration [140].

RASSF1A interacts with microtubules through interaction with microtubule associated proteins, such as MAP1B (microtubule-associated protein 1B) and MAP1S (microtubule-associated protein 1S) [138]. Interestingly, RABP1(Rassf1a interacting protein, MAP1S/C190RF5) was recently proved to enhance autophagy by suppressing genomic instability and tumourigenesis [141]. Surprisingly, RABP1 interacts with LC3, one of the main

proteins in the autophagy signaling pathway and is essential for autophagosme formation. These data suggests that RASSF1A stabilization of microtubules may have a role in autophagy regulation [141] and the loss of RASSF1A may promote uncontrolled autophagic response.

Controlling of cell cycle and mitotic progression

Studies showed that RASSF1A overexpression resulted in the accumulation of cells in the G1 phase of the cell cycle and decrease in levels of cyclin D1, an important protein for the progression of the cell cycle to the Rb check point (retinoblastoma protein, a tumour suppressor protein that controls cell division by allowing cell cycle progression only when the cell is ready to divide) to prevent cell cycle progression when DNA is damaged [142] [143]. The mechanism of how RASSF1A can decrease the levels of cyclin D1 needs more investigations but studies suggest that it may be through JNK kinase pathway [144, 145].

Furthermore; studies have shown that interaction of RASSF1A with RABP1 (RASSF1A binding protein 1/C19ORF5/MAP1S) leads to its recruitment to the spindle poles in pro-metaphase and its interaction with Cdc20 [146] [interaction of RASSF1A with Cdc20 is still contradictory and need further investigations, as a study in 2007 reported that there is no interaction between RASSF1A and Cdc20 [147]], inhibition of APC (anaphase promoting complex), accumulation of mitotic cyclins A and B and eventually mitotic arrest [146, 148]. Phosphorylation of RASSF1A by Aurora A kinase restricts its interaction with Cdc20 so relieving APC inhibition, degradation of cyclins and mitotic progression [149]. These studies emphasize the ability of RASSF1A to block the cell cycle in the G1/S phase and control the mitotic progression [144, 145].

Cell death and apoptosis

Apoptosis or programmed cell death can be initiated by intrinsic and extrinsic signals, which eventually will lead to the destruction of the cell. There are 2 types of signaling pathways promote apoptosis utilizing the mitochondria, the main organelle responsible for cell death. The intrinsic pathway is activated by harmful factors, such as DNA damage, un-balanced proliferative stimuli, and nutrient or energy depletion. On the other hand, specific death receptors (such as: tumour necrosis factor α [TNFα] receptor, TNFα apoptosis-inducing related ligand [TRAIL] or Fas [CD95]) stimulation promotes apoptosis via the extrinsic pathway [150, 151]. Mitochondria plays an central role in nearly all apoptotic pathways, supporting upstream pro-apototic signals and promoting the release of small apoptogenic molecules such as cytochrome c [152].

RASSF1A is one of the main effectors in extrinsic cell-death pathway, mediating cell death via direct or indirect interaction with one or more domains in the RASSF1A protein[126]. Rassf1a interacts with the pro-apoptotic, mammalian sterile -20 like kinase 1 and 2 (MST1/2) members of the Hippo pathway (a pathway that controls organ size through regulation of cell proliferation and apoptosis) to modulate their kinase activity and induce cell death. Recently, it has been shown that RASSF1A can modulate the activation of the MST2 pathway by controlling p73 transcriptional activity linked to PUMA (a key pro-apoptotic effector) expression [153, 154] and also it can associate with the pro-apoptotic kinase, MST1 and function with K-Ras to promote apoptosis [155, 156]. MST1/2 are both serine threonine kinases that are activated by auto-phosphorylation in response to several stimuli such as serum starvation and heat shock [157, 158]. Both MST1/2 directly interact with RASSF1A via the SARAH domain in the C-terminal of both MST and 1A [159].

RASSF1A can also induce cell death in a MST1/2 independent manner. Death receptor stimulation such as TNF-R recruits MOAP-1 (modulator of apoptosis a BH3- only pro-apototic protein) to form a complex (TNFR-MOAP-1) and this allows MOAP-1 to interact with RASSF1A to form a trimeric complex and localization of MOAP-1 and RASSF1A to the active death receptor. RASSF1A-MOAP-1 interaction causes conformational changes in MOAP-1 and allowing its BH3 domain to be exposed, facilitating MOAP-1 binding with the pro-apoptotic protein, BAX [123, 160]. RASSF1A indirectly regulates BAX activation by MOAP-1 by inducing conformational changes in BAX that allows it to insert itself in the mitochondrial membrane and activate cell death events. In the absence of RASSF1A, BAX activation via death receptor is inhibited [160], suggesting that RASSF1A is an important mediator of extrinsic apoptotic pathways and highlights the tumour suppressive activity of RASSF1A.

1.2.3 RASSF1A and intestinal inflammation

Recently, it has been shown that both *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice are susceptible to DSS-induced colitis (chemical inducer of colitis) [82]. Under DSS treatment, *Rassf1a* knockout mice displayed clinical symptoms of murine colitis including increased intestinal permeability, enhanced cytokine/chemokine production, elevated NF-κB activity, increased colonic cell death and epithelial cell injury [82] and aged (more than 6 months of age) *Rassf1a*$^{-/-}$ mice have an enhanced susceptibility to spontaneous inflammation (unpublished observation).

Under DSS treatment (condition of acute intestinal inflammation), RASSF1A can restrict NF-κB activation via interfering with membrane proximal complex (TLR4 MYD88/Traf6/Irak2/4) formation required for NF-κB activation [82]. Furthermore, the restriction of NF-κB activity also led to decrease in protein tyrosine kinase activity and thereby, decreasing the tyrosine (Y) 357 phosphorylation of Yes associated protein (YAP), a hippo-

dependent co-transcription factor related to proliferation [82]. Previous studies, have indicated that c-Abl can induce tyrosine 357 phosphorylation of YAP in response to DNA damage, which is prominent during intestinal inflammation injury stimulated by DSS[161-163]. This drives the formation of a pY-YAP/p73 complex to activate pro-apoptotic gene expression, especially Bax [164, 165]. Enhanced Y357 YAP phosphorylation has been detected in *Rassf1a* knockout mice that coincide with increased Bax expression and increased cell death[82]. These data suggest a novel role for 1A in protection from inflammation induced injury and the loss of 1A will not only lead to uncontrolled inflammation but to cancer if the inflammation is not properly regulated.

1.2.4 Other RASSF family members

Other RASSF family members including, RASSF2-RASSF10. Most of them are tumor suppressor proteins that are commonly silenced by promoter specific methylation in many types of cancers [48, 49]. RASSF2 and RASSF6 are involved in NF-κB regulation and RASSF5C is involved in inflammation via regulation of lymphocytes trafficking and adhesion [120].

RASSF1C

In contrast to RASSF1A it has no tumour suppressor properties and studies suggest that it functions as an oncogene in the appearance of breast cancer. It has been demonstrated that RASSF1C stimulates cell growth and migration and promotes metastasis. Furthermore, RASSF1C overexpression stimulates cell growth and migration in breast cancer cells and it has been demonstrated that RASSF1C could enhance proliferation of osteoblasts [120, 166]. All of these findings suggest that RASSF1C has a critical role in tumour formation.

RASSF2

The RASSF2 gene has two major isoforms (A and C) that lack the C1 and ATM domains present in RASSF1. It is primarily a nuclear protein unlike RASSF1A [167]. Promoter methylation and loss of expression of RASSF2 has been identified in different cancers and cancer cell lines [168, 169], suggesting that RASSF2 might have a significant role in tumourigenesis. In addition, transient expression of RASSF2 in 293T embryonic kidney cells showed prominent growth inhibition enhanced by activated K-Ras [170]. Furthermore, studies demonstrated that MST1 regulates RASSF2 protein stability, knocking down MST1 in cancer cells destabilizes RASSF2 and MST1-deficient mice revealed reduced RASSF2 protein levels. Finally, RASSF2-deficient mice have bone remodelling abnormalities and it was proposed that RASSF2 regulates osteoblast and osteoclast differentiation by inhibiting NF-κB signalling via restricting IKK activity [171].

RASSF5

RASSF5 (also called NORE1 or RAPL) was the first member of RASSF proteins to be cloned[172]. It has 3 isoforms (A, B and C) and the longest form of RASSF5A has 40% amino acid similarity with RASSF1A. RASSF5A associates with cytoskeletal proteins through its RA domain and inhibits growth via the ERK pathway [173]. In addition, RASSF5A can associate with MST1 kinase upon TNF-alpha and TNF-related apoptosis- inducing ligand (TRAIL) stimulation to induce apoptosis [174]. The *Rassf5a*$^{-/-}$ mice are recently generated but do not have an overt phenotype nor evidence of tumor formation as they age [174]. However, cells from the *Rassf5a*$^{-/-}$ mice are resistant to TNF-alpha and TRAIL-dependent cell death. Recently, studies demonstrated that *Rassf5c*$^{-/-}$ mice have impaired lymphocyte trafficking and lymphoid organ abnormalities indicating its essential role in inflammation [175].

RASSF6

A few years after the discovery of RASSF1, a new Ras effector protein, RASSF6 was characterized and mapped [176, 177]. Three isoforms (A–C) for RASSF6 have been identified [166]. RASSF6 behaves like a tumour suppressor protein and is epigenetically silenced in childhood leukaemias and neuroblastomas [178, 179]. Overexpression of RASSF6 in HeLa cells, induces apoptosis via a signalling mechanism associated with Bax activation and cytochrome c release [180]. Similar to RASSF1A and RASSF2, RASSF6 can inhibit NF-kB activity and possibly inflammation but the molecular mechanism is still unknown [176]. To date, no known *Rassf6*$^{-/-}$ mouse has been generated.

RASSF7, RASSF8, RASSF9 and RASSF10

They are called N-terminal RASSF group due to the N-terminal localization of their RA domains. Furthermore, these 4 members lack the SARAH domain otherwise present in RASSFs [181] .

RASSF7

RASSF7 is ubiquitously expressed in many tissues, specifically in brain, lung and human cell lines and is up regulated in several carcinomas such as islet cell tumours [182] endometrial cancer [183] and ovarian clear cell carcinomas [184]. No promoter methylation of the RASSF7 gene was found in 57 cancer cell lines [185].

RASSF8

The tumour suppressor role of RASSF8 was initially proposed due to reduced transcript levels of RASSF8 in lung adenocarcinomas [186]. Further studies demonstrated that transient expression of RASSF8 in lung carcinoma cells attenuated cell growth in soft agar [186], supporting that RASSF8 has a role in tumour development.

RASSF9

In 2011, Chang's group reported that RASSF9 is commonly expressed in epithelial tissue[187]. RASSF9-deficient mice exhibited signs of senescence (biological aging) including increased alopecia, shorter life expectancy and growth retardation, suggesting its essential role in epidermal development [187].

RASSF10

RASSF10 is expressed in bone marrow as well as thyroid, brain, prostate and kidney [179]. RASSF10 appears to be hypermethylated in cancer conditions such as thyroid carcinoma and childhood leukemias, suggesting the tumour suppressor activity of RASSF10 [179, 188]. More recently, the tumour suppressor activity of RASSF10 was investigated in gastric cancer cells, demonstrating that reintroduction of RASSF10 in gastric cell lines induces apoptosis. Additionally, RASSF10 may play an essential role in the regulation of mitotic progression due to its localization to centrosomes and microtubule association [188].

1.3 The Hippo pathway

1.3.1 Core components of the Hippo pathway

The Hippo pathway has a major role in limiting organ size, by restricting cell proliferation upon cell-cell contact and tumorigenesis by inhibiting cell proliferation and inducing cell death [53], [54],[189]. The Hippo pathway were originally recognized in Drosophila [190]. In mammals, the core components of the Hippo pathway consist of the serine/threonine kinases MST1/2 (mammalian STE20-like protein kinase 1/2) (homologs of Hippo/Hpo in Drosophila), Lats1/2 (large tumour suppressor1/2) (homologs of Warts/Wts), adaptor proteins Sav1(Salvador homologue 1) (homolog of Salvador/Sav) and Mob, MOB kinase

activator 1A and 1B (MOBKL1A and MOBKL1B; homologs of Mats) [191, 192]. The final components of the Hippo tumor suppressor pathway are the transcription co-activators YAP (Yes-associated protein) and its paralog TAZ (transcriptional co-activator with PDZ-binding motif) (homologs of Yorkie/Yki).

The Hippo signalling phosphorylates and inhibits YAP/TAZ/Yki, inducing cytoplasmic retention, degradation and inhibiting their growth induction function. The TEAD family transcription factors were indicated as essential partners of YAP/TAZ which promote the oncogenic potential of YAP/TAZ by activating target genes involved in proliferation and anti-apoptosis [189, 193]. The main function of the Hippo pathway is to regulate the activity of YAP/TAZ transcription co-activators. Lats1/2 phosphorylates YAP, phosphorylation of YAP S127 induces 14-3-3 binding which results in cytoplasmic retention of YAP, restricting its association with nuclear transcription factors and target gene promoters [189, 194]. Therefore, YAP is inactive when it is phosphorylated on S127.

In addition, previous studies have suggested an essential role for c-Abl driven tyrosine 357 phosphorylation of YAP in response to DNA damage, which is increased during intestinal inflammation injury stimulated by DSS [162, 163]. This will induce pY-YAP/p73 complex to enhance pro-apoptotic gene expression, especially Bax and induction of cell death. Furthermore, the loss of Mst1/Mst2 induces YAP/TEAD dependent proliferation, increased organ growth and resistance to apoptosis [195-197].

1.3.2 Hippo pathway and intestinal homeostasis

In the small intestinal and proximal colonic epithelium of the normal mouse, YAP is found in the crypts together with active Mst1 , so the function of YAP is mainly inactive [198].

Although YAP can be demonstrated in the nucleus of colon crypt cells but removal of YAP does not decrease the abundance of Ki-67＋ cells (a cellular marker for proliferation), indicating that YAP is not inducing the proliferation of these cells [198]. Furthermore, under normal conditions YAP deletion causes no obvious intestinal defects [53, 198]. These findings suggest that under normal conditions YAP has little or no role in intestinal epithelial homeostasis. On the other hand, inhibiting the Hippo pathway induces dysregulation of intestinal epithelial homeostasis. Conditional knockout of Mst2 kinase in intestinal epithelial cells resulted in increase of stem-like undifferentiated cells and complete lack of all secretory lineages [198]. Deficiency of Mst1/2 in the intestine decreases phosphorylation of YAP (Ser127 and Ser384) and induces an increase in YAP levels, nuclear localization and decrease in YAP cytoplasmic retention. Mst1null/Mst2ff/villin-Cre mouse demonstrated the same phenotype as transgenic mice overexpressing YAP (Ser127Ala) in the small intestinal, there was intestinal dysplasia and decrease in goblet and Paneth cells [199]. Surprisingly, the increase in proliferation and loss of differentiation induced by the MST1/2 deficiency can be completely reversed by loss of a single YAP allele in Mst1null/ Mst2ff/villin-Cre mouse [198]. Also, the loss of another Hippo pathway component, Salavador/ WW45 in mouse intestinal epithelial cells caused increase in levels of YAP and crypt overgrowth in the affected mice but less than Mst1null/Mst2ff/ villin-Cre mice. The hyperplasia of Savl1-deficient crypts can also be completely reversed by YAP deletion.

Exposure to chemicals, pathogens, mechanical injuries or acute inflammation can cause injury to the intestinal tissue therefore; it has to rapidly shift from normal epithelial turnover to regeneration. This transition is very critical and has to be very well regulated or it could result in pathological conditions. If the transition is insufficient, it could result in tissue atrophy and

collapse. On the other hand, excessive or improper regeneration may facilitate tumour growth. Inhibition of the Hippo-signalling pathway appears to be critical for the intestinal regenerative response [200]. Levels of YAP protein is significantly increased in the crypts of the recovery phase from the DSS treated mice. Following DSS treatment, the conditional knock out of YAP with villin-Cre mouse demonstrated significant increase in disease severity and dramatic decrease in survival rate [54]. These findings suggest that YAP has no major role in the normal mouse intestinal homeostasis, however it has an essential and critical role in tissue regeneration caused by injury as in IBD patients [53, 200].

1.4 Autophagy

1.4.1 Overview

Autophagy is a highly regulated and controlled process which results in the formation of autophagosome (double membraned vesicles containing cytosolic components) followed by lysosomal degradation and recycling of the degraded components to be used by the cell [201]. There are three major types of autophagy have been identified. Macroautophagy (the main form of autophagy) is characterized by the formation of a double-membrane organelle called the autophagosome, microautophagy, involves lysosomal engulfment of cytoplasmic materials by inward invagination of the lysosomal membrane and chaperone-mediated autophagy, mediated by the chaperone hsc70, co-chaperones and the lysosomal-associated membrane protein type 2A [202, 203].

In this thesis I will focus on macroautophagy, hereafter termed autophagy, as it is the main form of autophagy and molecules related to its pathway are involved in IBD.

Autophagy has developed as a physiological response to stress providing nutrients in times of cellular starvation or removing protein aggregates, damaged organelles and intracellular microbes. Autophagy has an essential role in cellular homeostasis for cell survival response or if the cellular stress is high, it will trigger and promote cell death. Recent studies have shown that autophagy also has an important role in the innate immunity by the response to different intracellular pathogens and clearing them. Interestingly, autophagy is impaired in Crohn's disease patients [204-206]. The autophagic process is characterized by highly controlled steps which are mediated by activation of protein kinases and ubiquitin-like machinery. Most of the components of autophagy were initially identified and characterized in yeast model systems, leading to the discovery of over 32 autophagy-related (*ATG*) genes. Many of the mammalian *ATG* homologues have been identified but not all of them. This highly ordered and conserved process can be divided into four steps initiation, elongation, cargo selection, and maturation [201, 207, 208].

Initiation

Various autophagic stimuli will activate the ULK (uncorordinated-51-like kinases) kinase complex to initiate the formation of an isolation membrane. Once, the ULK complex is activated it will recruit other autophagy proteins to the membrane site, including class III phosphatidylinositol 3-kinase (PI3K) complex involving, Beclin-1(ATG6), Vps34 (vacuolar protein sorting 34), the serine/threonine kinase p150 and Barkor/mAtg14. The activation of the PI3K complex generates phosphoinositides that will recruit other proteins to transport membrane to a phagophore [207, 208].

Elongation

The elongation of the isolation membrane needs two ubiquitin-like conjugation systems. The first system is controlled by Atg7 and Atg10 which covalently link Atg12 to Atg5. ATG16L1 will interact with the Atg12-5 conjugate and this complex will be localised to the phagophore. The second conjugation system consists of Atg4, Atg7 and Atg3, which will modify the cytosolic LC3 (microtubule-associated protein light chain 3) protein by attaching a phosphoethanolamine lipid anchor and subsequent insertion into the autophagosomal membrane. It has been demonstrated that the Atg12-5/16L1 complex promotes LC3 lipidation and also determines the site of insertion of the modified LC3 (LC3-II). Cargo recruitment and autophagosomal closure are promoted by LC3 insertion. Since the autophagosomal membrane is mainly associated with LC3 and the ability to detect LC3-II by immunoblotting therefore, LC3 is the most commonly used marker of autophagy. Changes in LC3 localization; monitoring levels of conversion of LC3-I to LC3-II is a reliable method to detect autophagic activity. LC3-II levels correlate with autophagosome formation, due to its association with the autophagosome membrane [207, 208].

Cargo Selection

Specific adaptor proteins interacting with LC3 regulate the identification and targeting of protein aggregates, damaged organelles and intracellular pathogens to autophagosomes.

Two main autophagosome adaptors are p62/sequestosome 1 (SQSTM1) and NBR1 (neighbour of BRCA1 gene 1), which directly bind ubiquitinated proteins and target them to the autophagosome by binding with LC3. Sequestosome1 (p62) protein links ubiquitinated proteins to the autophagic machinery for their further degradation in the lysosome and p62 is also degraded by autophagy. Since p62 accumulates when autophagy is inhibited, and decreased

levels can be observed when autophagy is induced, p62 is used as a marker to study the autophagic flux [208].

Autophagosome maturation

Autophagosome maturation is considered to be complete when it fuses with lysosomes to cause degradation of its contents. The mutual action of the small GTPase Rab7 and lysosome-associated membrane proteins, LAMP-1 &-2 regulates the fusion of the autophagosome with the lysosome. The autophagosome cargo is degraded and broken down into small molecules by the action of lysosomal hydrolases such as the cathespsins B, D, and L as well as lysozyme. Degraded molecules are then transported to the cytosol for recycling or to be presented as antigens to activate the adaptive immune response [208].

1.4.2 Autophagy and IBD

Many genes that are genetically or functionally related to autophagy have IBD-associated single nucleotide variants. These molecules include proteins involved in the detection of autophagic triggers IRGM (Immunity-related GTPase family M protein) [209-211], NOD2 [204-206], VDR(Vitamin D receptor) [212] and DAP (Death associated protein) [213, 214], autophagosome formation, ULK1 **(**Unc-51 Like Autophagy Activating Kinase 1**)** [215] and ATG16L1(Autophagy related 16 like1) [216, 217] or in autophagosome maturation, LRRK2 (Leucine-rich repeat kinase 2) [218]. The most specific IBD susceptibility genes are NOD2, ATG16L1 and IRGM and they are presented in IBD patients as single nucleotide polymorphisms.

ATG16L1

In 2007, a single nucleotide polymorphism (SNP) in the coding region of the *ATG16L1* gene has been identified to be associated with an increased risk of developing CD (rs2241880) [43], [217]. Individuals homozygous for the risk allele have two-fold increase in disease risk and this variant to IBD is stronger in CD patients. Further studies confirmed its association primarily with CD [219, 220]. This SNP is very common where there is about 45–50% of healthy individuals carrying the risk allele, confirming the multi-factorial nature of IBD where genetics, microbiome and environment all contribute to disease apperance.

Atg16L1 is an essential protein for autophagosme formation as it plays an essential role in guiding molecules of the autophagic machinery responsible for autophagosome formation [221]. Furthermore; ATG16L1 forms a complex with Atg12-Atg5 to induce LC3 lipidation and autophagosome formation [222, 223].

Recent studies have demonstrated that the interaction between Atg16L1 and FIP200 is important for the localization of the Atg12-Atg5-Atg16L1 complex to the autophagosome formation site [224, 225]. FIP200 [FAK-family interacting protein of 200kDa] also known as Rb1cc1, a ULK-interacting protein, required for autophagosome formation in mammalian cells. Targeting of intracellular bacteria to the autophagic vacuoles is markedly decreased by knocking down *ATG16L1* expression in cell lines, suggesting its main role in the anti-bacterial function of autophagy [217]. Crohn's disease-associated *ATG16L1* variant results in a threonine to alanine amino acid change at position 300 (T300A) within the C-terminal tightly conserved WD repeat domain. However, studies showed that the Atg16L1 WD repeat domain is not essential for autophagic activity [225, 226]. Furthermore, studies with healthy individuals and CD patients demonstrated that Atg16L1 expression pattern at both protein and mRNA levels were not affected either by *ATG16L1* genotype or the state of inflammation in the intestine [227].

Mutations studies on *ATG16L1* confirmed that this region is not essential for starvation-induced autophagy in fibroblasts [221]. Other studies showed that antibacterial autophagy mediated by the *ATG16L1* T300A variant is severely impaired in a cell type dependent manner [205, 228, 229]. These findings suggest that the impaired interaction of *ATG16L1* T300A mutation with other protein partners essential for the autophagic machinery might contribute to the pathogenesis of CD in humans.

To explore the role of ATG16L1 *in vivo*, two mouse models have been generated. Mice with functional knockout of the ATG16L1 protein (*Atg16L1*KO) was generated by a group [230]. Autophagy-deficient mice die within 24 hours of birth, indicating that autophagy is essential for survival of newborn mice [230, 231], so fetal liver chimeric mice were generated resulting in wild type mice with the hematopoietic system of *Atg16L1*KO mice to study the impact of ATG16L1 deficiency on immune cell function. Autophagy could not be activated in *ATG16L1KO* macrophages but they had increased IL-18 and IL-1β production after stimulation with (Lipopolysaccharide [LPS] or lipid A) or other activators of the inflammasome. These mice are very sensitive to DSS and they show exacerbated signs of colitis, associated with lymphocytic infiltration and increased ulceration which correlated with high serum levels of the pro-inflammatory cytokines IL-1β, IL-18 and IL-6. Interestingly, peripheral blood mononuclear cells from CD patients and healthy volunteers genotyped for the *ATG16L1* risk variant have hyper-production of IL-1β [232]. These findings suggest that in response to bacterial stimulation autophagy and ATG16L1 play a major role in the regulation of pro-inflammatory cytokine production.

Mouse lines with the expression of Atg16L1 reduced to ~30% of normal levels (*Atg16L1* hypomorph; *Atg16L1HM*) have been generated by another group [233]. The morphology and

architecture of the intestine and the colon was not affected in this mouse model also the mice has no signs of spontaneous inflammation but with further investigations, Paneth cells of the small intestine demonstrated significant changes including, transcriptional alterations, morphological abnormalities and functional changes. Paneth cells are highly secretory cells at the base of the crypts of Lieberkühn which play an important role in mucosal defence through the secretion and production of anti-microbial lysozyme, peptides and inflammatory mediators into the gut. Transcriptionally, high levels of the immunoregulatory adipocytokines, leptin and adiponectin are produced from Paneth cells of *Atg16L1HM* mice both of which have been linked to a pathologic hallmark of CD termed "creeping fat"[234]. Obvious packaging and exocytosis of antimicrobial granules have been demonstrated in Paneth cells. Surprisingly, similar Paneth cell abnormalities were also observed in CD patients homozygous for the *ATG16L1* risk allele, indicating that this mouse model may reflect some of the pathological changes that are associated with human disease and highlights the importance of animal models in studying IBD [233]. Furthermore, these mice are resistant to DSS-induced intestinal injury as wild-type mice, indicating that reduction of ATG16L1 expression (or autophagy) alone is not sufficient for the pathologic changes found in IBD patients. These findings support the multi-factorial nature of CD development, where carriage of a genetic risk factor alone is not sufficient for disease appearance.

NOD-like receptors (NLRs)

Rapid induction of host defence mechanisms following infection with a pathogen is supported by innate immune system. The actions of several groups of proteins that recognize conserved microorganism-associated molecular patterns (MAMPs) that are present in different microbial groups activate this response. One of the most essential classes of intracellular innate

immune proteins are NOD-like receptors (NLRs). Two members of this group, NOD1 (nucleotide-binding oligomerization domain-containing protein 1) and NOD2 (nucleotide-binding oligomerization domain-containing protein 2) are essential for innate immune responses to certain bacterial infections. NOD1 and NOD2 are cytosolic proteins that respond to intracellular fragments of bacterial peptidoglycan and that activate mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB)-dependent gene transcription, resulting in the release of pro-inflammatory mediators and anti-microbial products such as nitric oxide [235]. NOD1 detects D-glutamyl-meso-diaminopimelic acid (iE-DAP), which is a dipeptide that is present in a peptidoglycan that is primarily found in Gram-negative bacteria but also in some groups of Gram-positive bacteria, including Bacillus spp and Listeria spp [236-238]. NOD2 detects muramyl dipeptide (MDP) that is commonly present in bacterial peptidoglycan of Gram-positive and Gram-negative bacteria [237, 239, 240].

Domain architecture of NOD1 and NOD2

NOD1 and NOD2 proteins are comprised of a series of carboxy-terminal leucine-rich repeats (LRRs), a central nucleotide-binding domain (NBD; also known as a NOD domain) which contains NACHT, winged helix and superhelical subdomains [241]. In addition, the amino-terminal domain of NOD1 contains a single caspase recruitment domain (CARD), while NOD2 contains tandem CARDs. The LRR domains of the NODs are essential for ligand sensing [242, 243] and the recognition of MDP by NOD2 or of iE-DAP by NOD1. When NODs are stimulated conformational changes occur in these proteins promoting NBD-mediated oligomerization of the NOD proteins. Recruitment of the downstream adaptor receptor-interacting protein 2 (RIP2; also known as RIPK2) is promoted by the oligomerization of NOD1 or NOD2 through CARD–CARD interactions to form a large signalling platform [244-246].

RIPK2 has an essential role in both the NOD1 and NOD2 response, RIPK2-deficient mice do not respond to NOD1 or NOD2 ligands and they have decreased chemokines production after stimulation with MDP [245] [247]. Studies indicated that following activation of NOD signalling through infection or direct stimulation, RIPK2 is polyubiquitinated at K209 and this polyubiquitination is essential for NOD mediated NF-κB activation. RIPK2 clearly has kinase activity; it was classified as a serine–threonine kinase and its kinase domain is essential for is its polyubiquitination at K209 [248]. Interestingly, a study in 2010 demonstrated that RIPK2 also has tyrosine kinase activity, it undergoes auto-phosphorylation on Tyr 474 (Y474) [249]. Interestingly, this phosphorylation event does not occur in the presence of Crohn's disease-associated *NOD2* allele and it is necessary for effective NOD2 signalling to activate NF-κB [249].

Activation of RIPK2 leads to the recruitment and activation of the TAK1 (TGFβ-activated kinase 1), TAB2 (TAK1-binding protein 2) and TAB3 complex, which drives IκB kinase (IKK) complex activation and subsequent NF-κB inhibitor-α (IκBα) phosphorylation and degradation [250]. Pharmacologic inhibition of RIPK2 tyrosine kinase activity by gefitinib (an established EGFR inhibitor, used in cancer therapy, IC_{50} for EGFR = 33nM and for Ripk2= 10nm -1μM) blocks downstream activation of NOD2 signalling required for NF-κB activation [249]. Since RIPK2 has a central importance in the NOD signalling and it distinguishes NOD signalling from similar Toll-like receptor (TLR) mediated signalling so RIPK2 could be a novel therapeutic target for NOD signalling pathway regulation in IBD patients.

NOD2

A frameshift mutation resulting from a cytosine insertion after amino acid 1007 (L1007fs, rs41450053) and two missense mutations, arginine 702 to tryptophan (R702W,

rs2066844) and glycine 908 to arginine (G908R, rs2066845) are the three *NOD2* variants most commonly associated with CD [251]. A significant reduction or loss of NOD2 function is associated with these main variants. However, the common CD-linked NOD2 SNP13, 3020insC is a frameshift mutation leading to truncation of NOD2, a gain of function mutation and decrease in IL-10 production at the transcriptional level [252, 253]. European carriers of heterozygous NOD2 risk allele have 2.4 fold increases in the risk of having CD and 17.1 fold increases was found in carriers of homozygous or compound heterozygous NOD2 risk allele [251]. Interestingly, in African or Asian populations no association of *NOD2* variants has been observed [251]. It was found that carrying *NOD2* variants is not enough to cause CD and this proves the multifactorial nature of IBD onset but the disease is characterized by early occurrence, ileal location and stricture formation [254]. A study in 2014 investigated the presence of the three main NOD2 mutations in 200 patients with UC and in 202 healthy controls in Portugal. It was found that NOD2 mutations were present in 28 patients with UC (14.0 %) and in 27 controls (13.4 %). It was concluded that NOD2 mutations is in the Portuguese population are not involved with increased risk of UC but are associated with a more aggressive course [255].

NOD2 deficiency in mice resulted in increased susceptibility to pathogens, reduction in the expression of anti-microbial defensin [115, 256] and change in the amount and type of commensal bacteria [257]. Interestingly, reduction of β-defensin expression and alteration of bacterial microbiome have been seen in individuals with *NOD2*-associated CD [258-260]. Studies by three different groups demonstrated that autophagy could be triggered by NOD2 [204-206] via its interaction with ATG16L1 and therefore these studies highlighted that IBD susceptibility might be due to dysregulation in a central pathway rather than impairment of

function of a certain gene. In multiple cell lines, it has been proved that autophagy induced by NOD2 is dependent on ATG16L1 expression [204, 205]. In epithelial cells, interaction of NOD2 with ATG16L1 is essential for guiding ATG16L1 to pathogens entry sites to the plasma memebrane to activate autophagy [206]. Recently, it was demonstrated that the NOD2 L1007fs risk variant does not localize to the plasma membrane unlike the wild-type NOD2 [261, 262] suggesting that in Crohns disease patients the inability of NOD2 to localize to the cell membrane might be one of the contributing factors for disease appearance. Additionally, other studies showed that NOD2 L1007fs expressing cells cannot induce the formation of bacteria targeted autophagosomes and localization of NOD2 and ATG16L1 to the plasma membrane is impaired, indicating that NOD2 and ATG16L1 membrane localization is essential for autophagic clearance of bacteria [206]. Furthermore, studying NOD2-mediated autophagy in human dendritic cells demonstrated that this process is also essential in MHC class II antigen presentation to T lymphocytes [204].

In 2010, Homer; et al indicated that in epithelial cells autophagy may play a role in the activation of NOD2 signalling pathways [205]. It was found that activation of NF-κB by MDP stimulation was significantly reduced when autophagy was blocked by chemical inhibitors or by knockdown of ATG16L1 expression [205]. These results suggest that autophagy is an additional and novel mechanism that can regulate NOD2 activation during intracellular bacterial infection. However, this effect depends on cell type and function, as NF-κB activation by MDP stimulation in human epithelial cells and macrophages was similar to cells expressing wild-type ATG16L1 but bacterial killing enhanced by MDP in epithelial cells was impressively impaired. In contrast, Sorbara et al, 2013 revealed that ATG16L1 is negatively regulating NOD driven inflammatory responses. ATG16L1 knockdown without ATG5 or ATG9a knockdown, promotes NOD-driven

cytokine production [263]. This suggests that ATG16L1 has an inhibitory role in NF-κB activation via NOD receptors. Therefore, there is contradiction between the two studies described above, suggesting more investigations have to be done to confirm the role of autophagy related proteins specifically ATG16L1 in regulating NOD driven inflammatory responses. NOD2 stimulation by bacteria and activation of autophagy provides a functional link between NOD2 and ATG16L1 both of which are related to IBD. Furthermore, it highlights that the impairment of NOD2 and autophagy signalling might influence intestinal inflammation and contributes to IBD pathogenesis.

IRGM

Immunity related guanosine triphosphatases (IRGs) are a family of proteins important for immunity against intracellular pathogens [264]. Mice have 21 IRG family members while, humans carry only two IRG genes (IRGC and IRGM) with only IRGM related to host defence. IRGM is ubiquity expressed in different human cell lines and also expressed in primary cells from small intestine, peripheral blood leukocytes and monocytes and the colon. The relation between IRGM and autophagy was developed depending on its role in eliminating Mycobacterium tuberculosis [265]. Furthermore, recent studies demonstrated that IRGM is essential for autophagy induction and maturation of the autophagosome [265] [266].

Multiple CD-associated SNPs have been identified around the IRGM locus, with the strongest replication related to the two SNPs (rs13361189 and rs4958847) flanking the coding region of IRGM [210, 267]. Interestingly, no amino acid sequence changes are related to these disease-associated SNPs and this suggest that it might affect IRGM expression, splicing or translational rate. Reduction of IRGM expression resulted in increased survival of intracellular bacteria such as Salmonella typhimurium, M. tuberculosis [229, 265, 268]. Overexpression of

IRGM induce autophagic bacterial clearance, but high levels of expression resulted in intracellular bacterial accumulation due to increase in the lysosomal machinery [268], indicating that autophagy is controlled by a critical threshold of IRGM expression and dysregulation of IRGM expression could result in dysfunction of the autophagic machinery.

1.4.3 Autophagy modulation in IBD therapy

Studies mentioned above strongly suggest a relation between impairment in the autophagic machinery regulation and IBD susceptibility. Genes involved in autophagy such as ATG16L1, IRGM and NOD2 are associated with increased risk of IBD and loss of their function is associated with increased bacterial infection and dysfunction of the innate immune response suggesting the essential role of autophagy in IBD. Autophagy modulating compounds could play a major role in IBD treatment. Autophagy is already becoming a therapeutic target for several other diseases such as cancer, Alzheimer's disease, myopathy, cardiac disease, pancreatitis, liver disease, stroke, type 2 diabetes, Parkinson's disease and Huntington's disease [269-271]. Additionally, there are several FDA-approved drugs which target autophagy [272, 273] and bioactive food components have been introduced in autophagy modulation [274]. Many of these compounds target mTOR, (mammalian target of rapamycin, a- serine/threonine kinase), an essential inhibitor of autophagy and can be inhibited by different stimuli such as nutrient starvation and endoplasmic reticulum stress.

Autophagy activating compounds such as rapamycin have been introduced for IBD treatment and they might be beneficial in treating and saving IBD patients. The effect of autophagy activating compounds in animal colitis models, clinical IBD trials and CD case reports and are promising but limited and need more investigations. A study demonstrated that

everolimus (an mTOR inhibitor) significantly decreased the severity of chronic colitis in *IL-10*$^{-/-}$ mouse model [275]. Furthermore, in another two case reports showed that treatment with mTOR inhibitors (everolimus and sirolimus) resulted in significant amelioration of disease in refractory CD patients [276, 277]. However, when everolimus was tested on CD patients with moderate-to-severe disease in a larger scale, everolimus therapy was not effective and only 22% of patients showed steroid-free remission [278]. Interestingly, studies examined the effect of vitamin D (a dietary autophagy inducer) on experimental colitis and in CD clinical trials. Vitamin D has been known to be an autophagy modulator in the autophagic process, by regulation of autophagy gene expression and calcium fluxes. A study in 2010, demonstrated that the expression of NOD2 is stimulated by VDR (vitamin D receptor) activation [279]. In addition, studies demonstrated that polymorphisms in *VDR* (vitamin D receptor) are associated with susceptibility to IBD (both CD and UC) [212, 280, 281]. Furthermore; other studies demonstrated that vitamin D deficiency as a risk factor for CD [282]. Administration of vitamin D or a VDR agonist (BXL062) resulted in reduction of signs and symptoms of colitis in DSS colitis model [283, 284] and mice fed on vitamin D deficient diet were sensitive to DSS-induced colitis [19]. The use of safe and natural compounds such as vitamin D to regulate autophagy is interesting, as their side effects are less and they might be promising and safe for long term treatment of chronic diseases such as IBD.

Chloroquine, a lysosomotropic compound that inhibits autophagy by increasing the lysosomal pH thereby, inhibiting the fusion of the lysosome with the autophagosome and inhibiting lysosomal protein degradation [285]. In addition, chloroquine accumulates inside the acidic parts of the cell (such as endosomes and lysosomes) leading to inhibition of lysosomal enzymes that need an acidic pH and inhibits fusion of endosomes and lysosomes [286, 287]. Chloroquine and hydroxyl-chloroquine (plaquenil) are commonly used as anti-malarial drugs,

but recently they are prescribed for the treatment of chronic autoimmune diseases especially for systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and because of their ability to inhibit antigen processing by antigen presenting cells. Furthermore, these drugs have anti-inflammatory properties including, inhibition of polymorphonuclear leukocyte migration, inhibition of cytokine production by macrophages and they partially inhibit the cycloxygenase pathway [288]. How chloroquine modulates inflammation and immunity is not fully understood and need more investigation [289, 290]. However, it has been demonstrated that the chloroquine can restrict the innate immune response via endosomal Toll-like receptor (TLR) signalling. Furthermore, a study suggested that it has an essential role in T cell responses [289]. Since chloroquine has anti-inflammatory properties, it has been proposed for IBD clinical trials. In 1990 a small pilot study (18 UC patients) showed a high response to plaquenil [291]. In a larger scale study by the same group with 93 patients in a placebo-controlled trial vs. hydroxychloroquine (400 mg/daily), it was not efficient in a four week period, although 15% of patients in the plaquenil group went into remission. Unfortunately, there were no specific parameters that defined this subpopulation. Recently, a group studied the effect of chloroquine in a DSS induced colitis mouse model. Interestingly, treatment with chloroquine significantly reduced body weight loss, tissue damage and infiltration of inflammatory cell. Furthermore, they showed that chloroquine significantly suppress the TLR2 as well as TLR9 signalling both in vitro and vivo experiments, while it has no effect on TLR4 pathway. Also they proved that chloroquine can suppress the T cell cytokine and proliferative responses [290]. Unfortunately, this group focused mainly on analysing the anti-inflammatory properties of chloroquine and they did not investigate the effect of chloroquine on the autophagic machinery in the context of DSS-induced inflammation injury.

Autophagy is a physiological protective response to bacterial infection and essential in activating innate immunity but at a certain point it has to be shut down. Uncontrolled and excessive autophagic response might be detrimental to epithelial healing after inflammation-induced injury. Unpublished data from our lab suggest that *Rassf1a* knockout mice have increased autophagic response after DSS treatment, which coincides with increased cell death and decreased healing. Currently, our lab is studying the potential effect of 3-MA (3-methyladenine), autophagy inhibitor on DSS-treated *Rasssf1a* knockout mice. Autophagy inhibitor, 3-MA blocks autophagosome formation via the inhibition of type III Phosphatidylinositol 3-kinases (PI-3K) [292, 293]. Interestingly, the autophagy inhibitor, 3-MA can fully protect *RASSF1A* and *IL-10* knockout mice from DSS-induced inflammation injury and ameliorates the signs of colitis. Furthermore, chloroquine partially reversed the damaging effect of inflammation- induced by DSS and increases the survival rate of *Rassf1a* knockout mice from 20% to 40%. These findings suggest that pharmacological modulation and/or pharmacological inhibition of the autophagic response might be a novel therapeutic approach for IBD treatment to inhibit inflammation of the gut, enhancing repair and healing after inflammation induced injury, furthermore; protecting IBD patients from predisposing to cancer later in life.

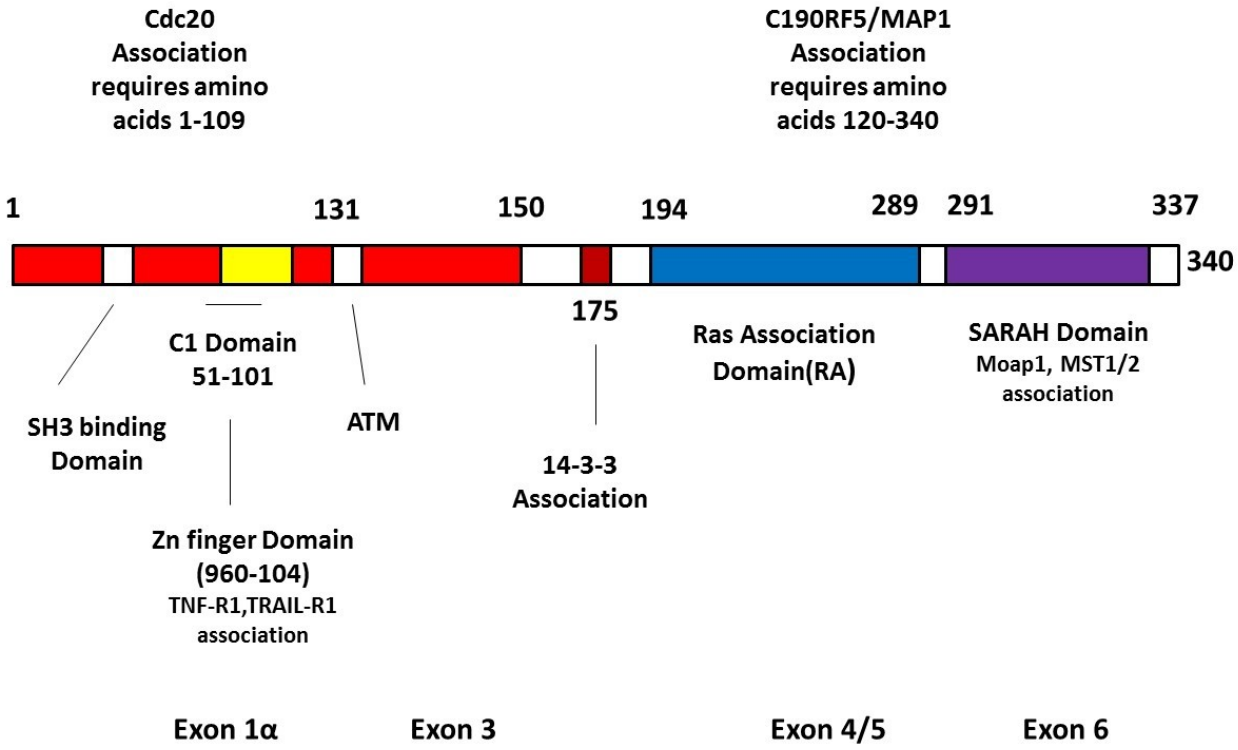


Figure 1.1: Schematic diagram of RASSF1A and its functional domains showing, the SH3 binding domain of RASSF1A which is conserved in mouse and human forms of RASSF1A, the C1 zinc finger domain (essential for TNF-R1 and TRAIL-R1 association), the ATM (Ataxia telangiectasia mutated) phosphorylation site, ATM is a serine/threonine protein kinase that is activated upon double strand breaks and apoptosis and the binding sites for several RASSF1A effector proteins are shown. The Ras association domain (RA) may potentially associate with the Ras family of oncogenes. The SARAH domain associates with the sterile-20 like kinases, MST1 and MST2. Positions of exons and amino acids are also indicated. Adapted from El-Kalla, et al; 2010

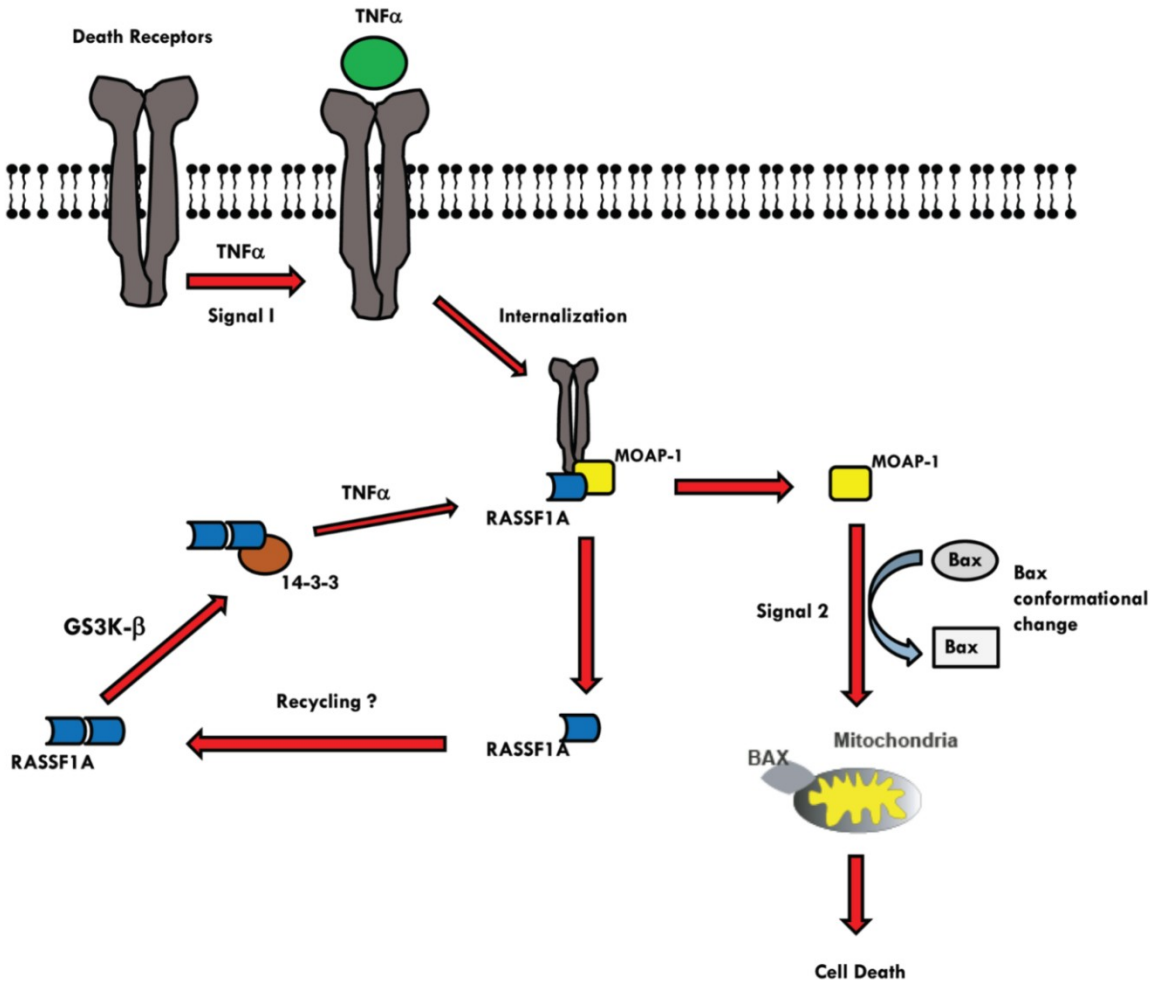


Figure 1.2: Model for RASSF1A modulated apoptosis. Once a death receptor is activated, such as TNF-R, it recruits Moap-1 to form a complex (TNFR-Moap-1) and this allows Moap-1 to interact with Rassf1a to form a trimeric complex. Rassf1a-Moap-1 interaction causes conformational changes in Moap-1, facilitating Moap-1 binding with the pro-apototic protein, Bax. Rassf1a indirectly regulates Bax activation by Moap-1 by inducing conformational changes in Bax that allows it to insert itself in the mitochondrial membrane and activate cell death events. Gordon, et al; 2011.

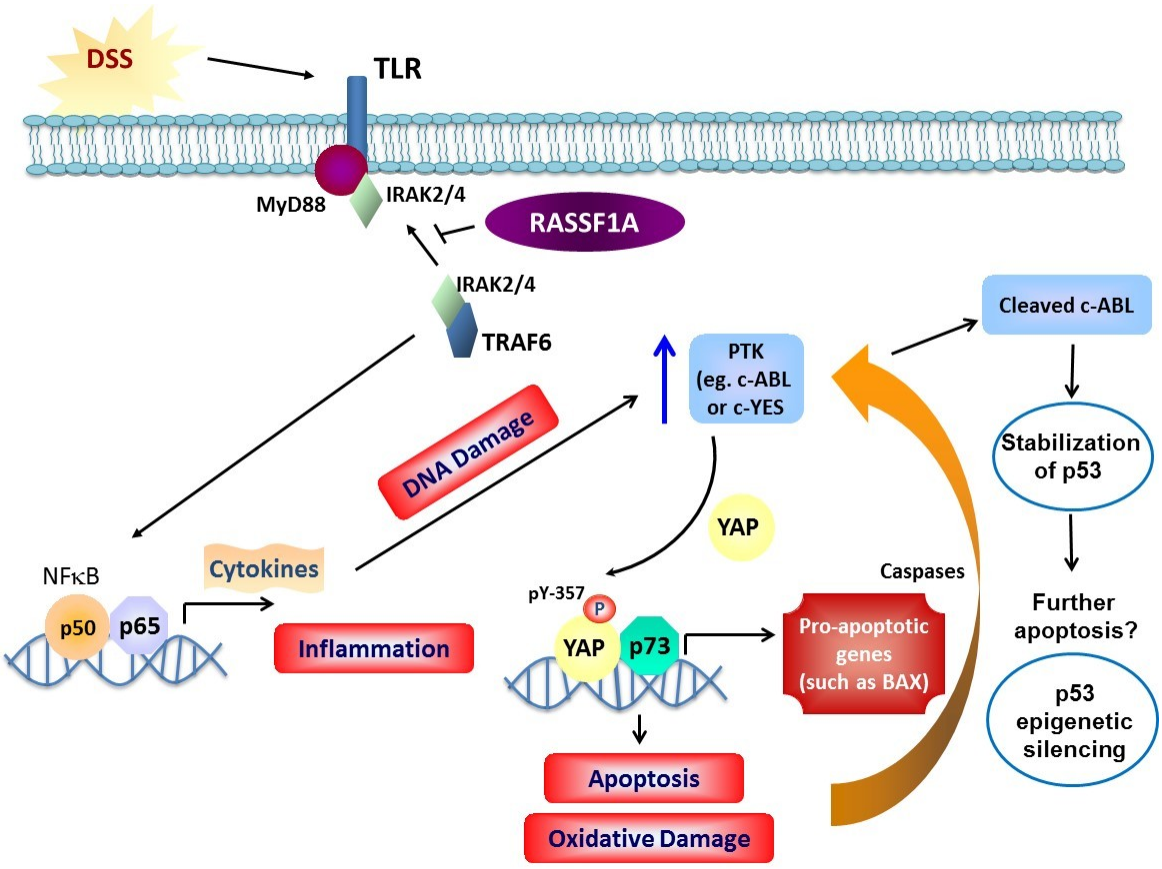


Figure 1.3: Model for RASSF1A regulation of intestinal inflammation.Rassf1a restricts NF-κB activation via interfering with the ability of membrane proximal (TLR/Myd88/Traf6/Irak2/4) to activate NF-κB. Restriction of Rassf1a to NF-κB will lead to the decrease in PTK activity (such as c-abl or c-yes). In the absence of Rassf1a, there is increase in PTK activity that would drive pY 357 Yap and the formation of pY-Yap/p73 complex to drive transcriptional upregulation of pro-apoptotic genes such as Bax. The increase in levels of Bax will lead to increased cell death, intestinal inflammation and DNA damage from the increased levels of Bax. Sustained inflammation and cell death will promote c-abl cleavage by caspases to enhance p53 stabilization and accumulation. Accumulated p53 can induce more cell death, epithelial damage and poor recovery after inflammation insult. The model is also showing drugs used in this thesis project. Gordon et al; 2013.

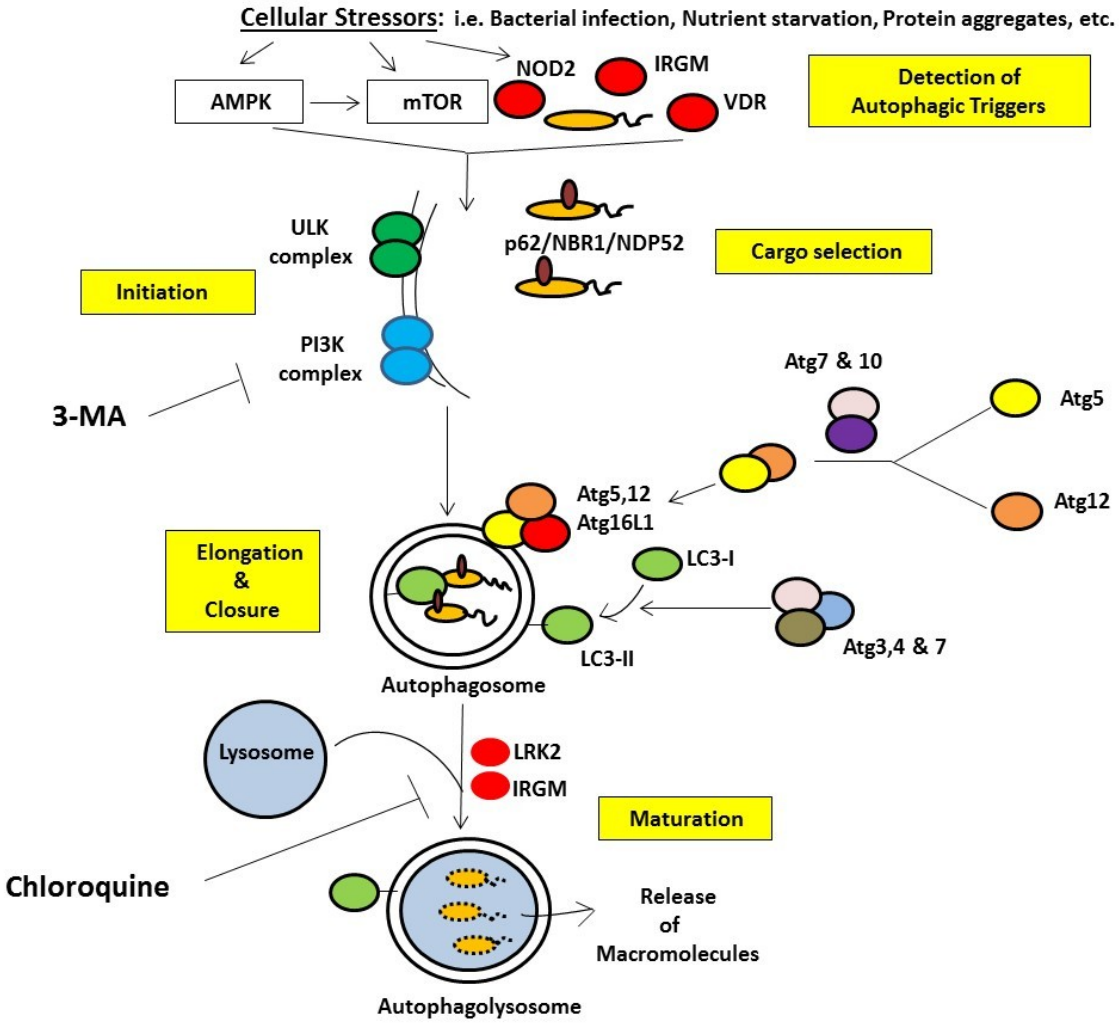


Figure 1.4: Model for autophagy signalling pathway. Autophagy is a highly conserved process which can be activated by several cellular stressors to activate molecules to initiate autophagy (eg AMPK, NOD2, etc.). Once autophagy is activated it will result in the formation of doubled membrane vesicle (autophagososme), encapsulation of targeted cytoplasmic structures and fusion of the autophagosme with lysosome for degradation and subsequent recycling of degraded components. Autophagy can be divided into four main steps including, initiation, elongation, cargo selection, and maturation. Proteins in red are genes that have been associated with increased risk of IBD. The model is also showing anti-autophagy drugs used in this thesis project and their targets. Adapted from Kabi et al; 2012.

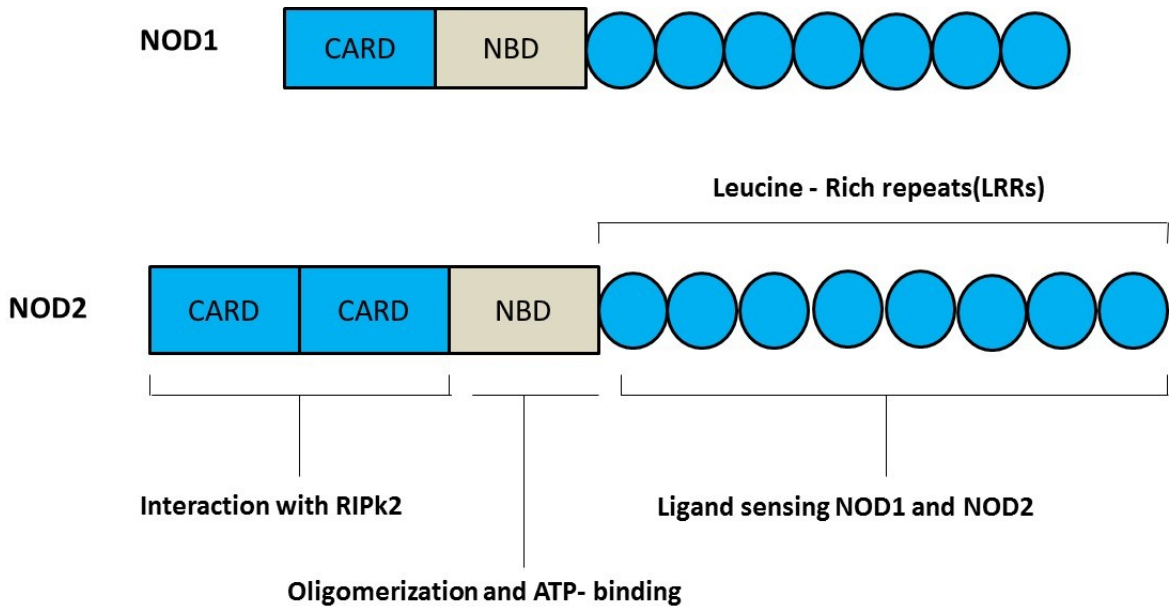


Figure 1.5: Schematic diagram for NOD1 and NOD2. NOD1 (nucleotide-binding oligomerization domain-containing protein 1) contains a single amino-terminal caspase recruitment domain (CARD), and NOD2 (nucleotide-binding oligomerization domain-containing protein 2) contains tandem N-terminal CARDs that interact with the CARD of receptor-interacting protein 2 (RIPK2). Both NOD1 and NOD2 contain a central nucleotide-binding domain (NBD), which binds ATP and mediates NOD oligomerization and carboxy-terminal leucine-rich repeats (LRRs), which are essential for ligand sensing. Adapted from Philpott et al; 2013.

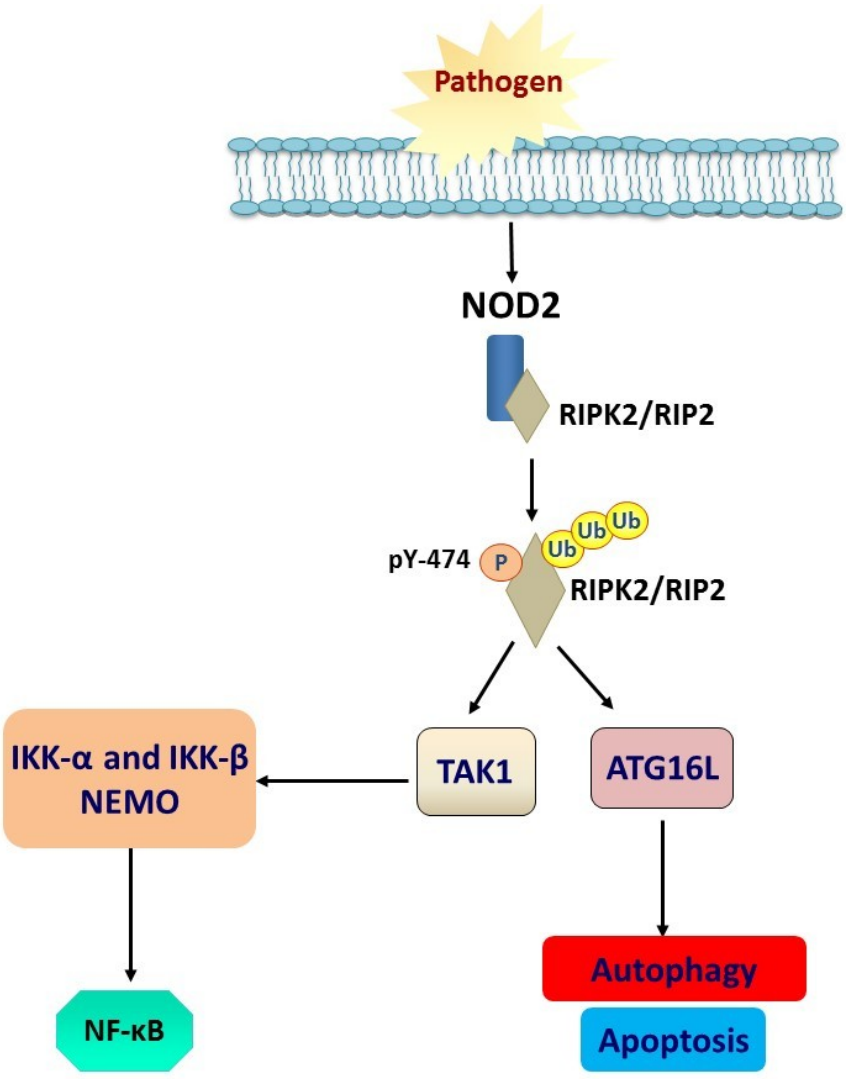


Figure 1.6: Model for NOD2 signalling. NOD2 is an intracellular pattern recognition receptor which is stimulated by MDP (muramyl didpeptide), a peptidoglycan from Gram +ve and Gram –ve bacteria. Once NOD2 is stimulated by its specific ligand (MDP) it recruits Ripk2 which undergoes auto-phosphorylation on Tyrosine 474 (Y474) and then it is polyubiquitinated at K209. Activation of RIPK2 promotes the recruitment and activation of the TAK1 and /or Atg16L1. Activation of TAK1 and Atg16L1 will probably lead to activation of NF-κB and autophagy/apoptosis respectively. Adapted from Baksh lab.

Chapter 2

Materials and methods

2.1 Materials

All chemicals were used according to the manufacturer's specifications as well as in accordance with the protocols set out by the Environmental Health and Safety of the University of Alberta and work Hazardous Materials Information System (WHMIS).

2.1.1 Chemicals, Reagents and other Materials

Acrylamide	Invitrogen
Ammonium persulfate (APS)	Biorad
Aprotonin	Sigma
β-mercaptoethanol	BioShop
DMEM medium	Fisher Scientific
Dextran sulphate (DSS)	MP Biomedical
DMSO (Dimethylsulfoxide)	Fisher Scientific
Ethanol	Commercial Alcohols
Enhanced Chemiluminescence (ECL)	Homemade
Ethylenediaminetetraacetic acid (EDTA)	EMD
Ethylene glycol tetra acetic acid (EGTA)	EMD
Glycine	Fisher Scientific
Glycerol	Anachemia
Hydrochloric acid	Caledon
MDP	Sigma

Methanol	Fisher Scientific
(Polyvinylidene Fluoride)PVDF membrane	Millipore
PEI	Polysciences, USA
PMSF	Sigma
Protein G Sepharose	GE Healthcare
Sodium dodecyle sulphate (SDS)	BioRad
Tris	Fisher Scientific
Trypsin	Fisher Scientific
T-PER	Thermoscientific
Tween 20	Fisher Scientific
Z-fix	Anatech LTD

2.1.2 Buffers

4x Separating Buffer: 1.5M Tris, pH 8.7 and 0.4% SDS.

4x Stacking buffer: 0.5M Tris, pH 6.8 and 0.4% SDS.

4x SDS-Page loading Buffer: For 50mL: 13ml Glycerol, 2.5g SDS, 8.7mL of 1M Tris/HCL pH 6.8 and 10mg Bromophenol blue.

10x SDS Running Buffer: For 1L: 30.2g Tris, 144g Glycine and 10g SDS.

SB Lysis Buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1.5 mM EDTA, 0.5% Triton X-100, 20 mM β-glycerolphosphate, 100 mM NaF and 0.1 mM PMSF.

1x wet Transfer Buffer: For 4L: 12g Tris, 57.6g Glycine, and 800mL methanol and then bring to volume with water.

Semidry transfer buffer: 50mM Tris, 380mM Glycine, 0.1% SDS and 20% methanol.

Phosphate Buffered saline(PBS): 137mM NaCL, 2.7mM KCL, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 and pH 7.4.

Stripping buffer: 52mm Tris pH 6.8 and 2% SDS.

2.1.3 Antibodies

Primary antibodies

Mouse anti-HA	In house produced
Rabbit anti -NOD2	Santa Cruz sc-30199
Rabbit anti-ATG16L1	Cell signalling-D6D5
Rabbit anti-RIPK2	Santa Cruz sc-22763
Rabbit anti-PARP	Cell signalling-9542
Rabbit anti-p62	Enzo scientific BML-PW9860-0100
Mouse anti –Flag	Sigma F1804
Anti-Human PCNA	Caltag Laboratories- PC10
Mouse anti-p53 for immunoblotting	Abcam Pab1801
Mouse anti-p53 for immunoprecipitation	Santa Cruz sc-126
Mouse anti-Ubiquitin	Santa Cruz sc-8015
Rabbit anti-ERK1	Santa Cruz sc-93
Rabbit anti-ERK2	Santa Cruz sc-154

Secondary antibodies

Anti-mouse IgG Horseradish Peroxidase-Linked Whole antibody	GE Heathcare UK
Anti-Rabbit IgG Horseradish Peroxidase-Linked Whole antibody	GE Heathcare UK

2.1.4 Drugs

Imatinib mesylate (Gleevec): Purchased from Selleckchem (S1026). Imatinib mesylate was dissolved in sterile water to prepare a stock solution (10mg/ml). Imatinib was administrated intraperitoneally (60-80 ug/g body weight). Usually the dose is 400 mg- 600 mg/day for patients with leukemia. Imatinib, IC_{50} has been determined in both leukemic patients and cancer cell lines [294, 295] (Fig. 2.1).

3-Methlyadenine (3-MA): Purchased from Santa Cruz (sc-205596). Autophagy inhibitor, 3-MA was dissolved in sterile water to prepare a stock solution (3mg/ml). Autophagy inhibitor,3-MA was administrated intraperitoneally (20 ug/g body weight) (Fig. 2.1).

Chloroquine diphosphate salt: Purchased from Sigma (c6628). Chloroquine diphosphate was dissolved in sterile water to prepare a stock solution (10mg/ml). Chloroquine was administrated via oral gavage (60µg/g body weight) (Fig. 2.1).

Gefitinib (Iressa): Purchased from Selleckchem (S1025). Gefitinib was dissolved in 1ml of 100% DMSO to prepare a stock solution (100mg/ml). The stock solution was diluted in sterile water containing 1% methylcellulose according to the desired volume and concentration for administration via oral gavage (Fig. 2.1).

Amlexanox: Purchased from R and D Tochris (4857). Amlexanox was dissolved in 1ml of 100% DMSO to prepare a stock solution (100mg/ml). The stock solution was diluted in sterile

water containing 1% methylcellulose according to the desired volume and concentration for administration via oral gavage.

Rebamipide: Purchased from Selleckchem (S2032). Rebamipide was dissolved in 1ml of 100% DMSO to prepare a stock solution (100mg/ml). The stock solution was diluted in sterile water containing 1% methylcellulose according to the desired volume and concentration for administration via oral gavage.

BML-190: Purchased from Selleckchem (S2854). BML-190 was dissolved in 1ml of 100% DMSO to prepare a stock solution (100mg/ml). The stock solution was diluted in sterile water containing 1% methylcellulose according to the desired volume and concentration for administration via oral gavage.

2.2 Cell line

HEK-293 cells (human embryonic kidney fibroblast) from ATCC were maintained in DMEM medium plus 10% bovine growth serum (BGS) and incubated in a 37^0C with 5% CO2 incubator. Transfections were carried out using the linear 25 kDa polymer, polyethyleneimine (PEI) obtained from Polysciences, USA (Catalog #23966-2). PEI transfections were carried out by mixing PEI/DNA in a ratio of 4µl PEI/1 µg DNA.

2.3 Mice

All animal experiments/husbandry have been approved and follow the guidelines of the Canadian Animal Care and Use Committee and the animal ethics board at the University of Alberta (permit numbers # 219 and 218). *Rassf1a*$^{+/+}$, *Rassf1a*$^{+/-}$ and *Rassf1a*$^{-/-}$ were (10-14 weeks old) on the C57BL/6 background. *Rassf1a* $^{\text{IEC-KO}}$ and *Rassf1a* $^{\text{IEC-WT}}$ were (10-14 weeks old) on

the C57BL/6-129 background. *IL-10*$^{+/+}$ and *IL-10*$^{-/-}$ mice were (4-5 months old) on the 129S1/SvlmJ background and were obtained from Madsen Lab, University of Alberta.

2.4 Methods

2.4.1 Intraperitoneal injection

Mice were restrained and tilted so that the head is facing downwards to prevent movement and traumatizing organs during injection. The needle (25 gauges) was injected into the abdomen with 30-degree angle. We did aspiration to be sure that the needle is not penetrating blood vessels or the intestines. The maximum volume to be administrated via intraperitoneal injection was 200µl.

2.4.2 Oral gavage

Mice were restrained and maintained in upright position (vertical). A bulb tipped gastric gavage needle attached to a syringe was used to deliver drugs into the stomach. The needle was passed along the side of the mouth towards the oesophagus and the stomach. If there was any resistance we had to change the needle position as we might be entering the trachea. After passing the needle to the correct length the drug was slowly injected. The maximum volume to be administrated via for oral gavage was 10ml/kg.

2.4.3 Cardiac puncture

Blood was collected from mice by cardiac puncture. Mice were anesthetized using ether. The blood was drained from the heart using 23 gauge needles. Mice were then sacrificed by CO2 asphyxiation. Blood was collected in 1.5 ml eppendorf tubes and left to sit on ice for 1 hour until coagulation. After blood coagulation, blood was spinned down at 10000 rpm for 10 minutes. Serum was then collected and stored in -80°C for cytokine analysis.

2.4.4 Colon lysates preparation

Selected mice were sacrificed by CO2 asphyxiation. Skin over the colon was cut and the colon was removed. The colon was flushed with1X PBS using a syringe to remove any faeces or blood remnants. The whole colon was transferred to 1.5 ml eppendorf tube with 1ml T-PER (Tissue protein extraction reagent) containing 2µl of 0.1mM PMSF and 1µl of o.5 mg/ml aprotonin. The whole colon was homogenized for 1-2 minutes using tissue homogenizer and then centrifuged for 10 minutes at max speed. The supernatant is transferred to another 1.5 ml tube and stored in -80^0C for protein analysis.

2.4.5 Acute Dextran Sodium Sulphate (DSS) treatment

Selected mice were separated in individual cages. The following day mice were administered 3% w/v DSS (#160110, molecular weight of 10000, MP Biomedicals) in the drinking water for 7 days followed by fresh water for recovery for 7 days. Mice were monitored for tremors, piloerection, bloatednesss, lack of movement, rectal bleeding, stool consistency and weight loss (all on a scale of 0–5 with 5 being very severe, adapted from Madsen et al [296]. Mice were euthanized once rectal bleeding became grossly apparent. For weight loss, a score of 0 for no weight loss, 1 if, 5% loss, 2 for 5– 10% loss, 3 for 10 – 15% loss, 4 for 15 – 20% loss and a score of 5.20% loss in initial body weight. Disease activity indices (DAI) were the sum of all individual scores. All animals were about 25 g in body weight at the beginning of the experiment.

2.4.6 Cell culture, Transfection, Lysis and Immunoprecipitation

HEK-293 cells were cultured in DMEM media containing 10% bovine growth serum and kept at 37°C in an incubator with 95% oxygen and 5% CO2. Fourty percent confluent cells were used

for transfection in a 6 well dish. The cells were transfected using polyethyleneimine (PEI). PEI transfections were carried out by mixing PEI/DNA in a ratio of 4 ul PEI/1 µg DNA in 400 µl of serum. The mixture was allowed to incubate for 15 minutes. While incubating, cells were washed 3 times with serum free media and 2 ml of complete media added after the washes to each well. The PEI/DNA mixture was added to the cells, mixed gently and grown overnight. Media was changed the following day with 1.5 ml of complete media. MDP (0.04µM/1.5ml) was added the day after according to the experiment followed by cell lysis for further analysis. Cells were then transferred to 1.5 ml eppendorf tube and washed 2 times with 1X PBS to ensure that no further DMEM media containing bovine growth serum was still present which may interfere with later steps for detection of proteins by western blot. Following cells washing, 1000 µl of SB-lysis buffer containing 2µl of (0.1 mM PMSF) and 1µl of (0.5mg/ml Aprotonin) is added to the cells.

Lysis buffer containing cells are then vortexed at high speed followed by incubation at 4°C for 15 minutes to ensure complete lysis. After incubation for 15 minutes, supernatant are transferred to another 1.5 ml tubes for immunoprecipitation. Immunoprecipitation are carried out by 1-2 ug of anti-NOD2 antibody which then incubated overnight. After overnight incubation, samples were added to 50% slurry of protein G Sepharose affinity beads (15-20 µl volume) for 1-1.5 hours followed by two washes with 1XPBS and bound proteins resolved by SDS-PAGE. For whole cell lysate (WCL) and colon lysates immunoblots, ~ 70µg/well and 40µg/well were used respectively. Colon lysates immunoprecipitation is carried out by using 500-600µg proteins.

2.4.7 Western Blotting

Proteins carried out by SDS-PAGE separation were transferred to PVDF membrane (MilliPore) in a transfer apparatus. Following a successful transfer, the membrane was blocked with 10%

skim milk for 30 minutes at room temperature. The membrane was then incubated with the primary antibody (diluted in 2% milk) overnight at 4°C on a shaker. Subsequent washing of the membrane with 3X with 1XTBS-T for 5 minutes is essential to get rid from any unbound primary antibody. This wash is followed by the incubation of the membrane with the secondary antibody (diluted in 2% milk) for 2 hours at room temperature on a shaker. Following the incubation, the membrane was washed again 3X using 1XTBS-T for 5 minutes. The membrane is then incubated with homemade enhanced chemiluminescence (ECL) detection reagent for 2 minutes followed by developing using X- ray films.

2.4.8 Enzyme-linked immunosorbent assay (ELISA)

We purchased mouse pro-inflammatory multi-spot 96 well -7 spot plate, detecting (IL-1, IL-12, IFN-γ, KC/Gro, IL-10, IL-6 and TNF-α) from MSD (Meso scale discovery) (Catalog#: N75012B-1). Diluent 4 was added to each well (25ul) and the plate was sealed with an adhesive plate and incubated for 30 minutes at room temp with vigorous shaking. After incubation we dispensed 25 ul of each serum sample in separate wells and the plate was sealed and incubated for another 2hours at room temp with vigorous shaking. Following incubation, the plate was washed 3 times with PBS-T (phosphate buffered saline plus 0.05 % tween -20). After the 3 washes we dispensed 25ul of 1X detection antibody solution into each well and the plate was sealed and incubated for 2 hours at room temperature with vigorous shaking. Following incubation, the plate was washed again 3 times with PBS-T and then we added 150ul of 2X reading buffer T to each well. The plate was analysed on the sector Imager of the department of pharmacology, University of Alberta.

2.4.9 Tissue histology

The descending colon was isolated, fixed in z-Fix (Anatech Ltd) and paraffin-embedded. Longitudinal sections of the colon were cut followed by Hematoxylin and Eosin staining using the animal histology core facility of the Alberta Diabetes Institute. All inflammation scores were obtained utilizing blinded scoring by a gastrointestinal pathologist (Dr.Aducio Thiesen) based on infiltration of enterocytes, neutrophils, lamina propria cellularity, crypt structure and epithelial hyperplasia (scored as 0 – 2 where 2 = maximal injury) [296].

2.4.10 Immunohistochemistry

PCNA staining for colon sections were carried out in the histology core facility of Alberta Diabetes Institute, using anti-Human PCNA antibody (Biotin conjugated).

2.5 Statistics

Statistical analysis was performed using one-way ANOVA and Students t-test (two-tailed), using the GraphPad Prism 5 software. Statistics for individual experiments are indicated with the graph or figure legends.

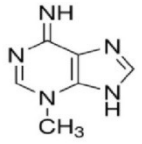
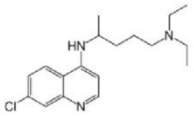
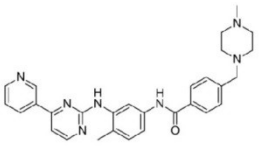
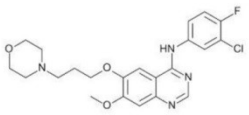
Compound Name	Target	Chemical structure	IC_{50}	Drug action
3-Methyladenine (3-MA)	PI3Kinases		25-60μM	Autophagy inhibitor (Early stage autophagic events inhibitor)
Chloroquine diphosphate salt (Chloroquine)	Autophagosome		unknown	Anti-malarial and Autophagy inhibitor (Late stage autophagic events inhibitor)
Imatinib (Gleevec)	c-Abl,cKit and PDGFR		0.6 μM, 0.1 μM and 0.1 μM, respectively	Protein tyrosine kinase inhibitor
Gefitinib(Iressa)	EGFR- Ripk2		33nM and 10nm-1μM respectively	Protein tyrosine kinase inhibitor

Figure 2.1: Table with main drugs used in this thesis project showing their targets, chemical structure, drug action and IC_{50} (The half maximal inhibitory concentration IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or bio-chemical function. This quantitative measure indicates how much of a particular drug or inhibitor is needed to inhibit a given biological process by half). IC_{50} has been determined in both leukemic patients and cancer cell lines [297-300].

Chapter 3

The effect of PTK inhibitor, imatinib mesylate on DSS-treated *Rassf1a* knockout mice

3.1 Introduction and Rationale

RASSF1A (1A) is a tumour suppressor gene silenced in human cancers resulting in its functional inactivation. Our lab has demonstrated that the genetic loss of 1A in *Rassf1a*$^{-/-}$ or *Rassf1a*$^{+/-}$ mice resulted in clinical symptoms of colitis including increased intestinal permeability, enhanced cytokine/chemokine production, elevated NF-κB activity, severe colonic epithelial cell injury and poor recovery following dextran sulphate sodium (DSS)-induced inflammation injury, DSS is a chemical inducer of colitis in mice and a potent activator of innate immunity [82]. Furthermore, epithelial repair was decreased in the absence of RASSF1A with reduction of several markers of proliferation including Yes-associated protein (YAP)-driven proliferation. Surprisingly, tyrosine phosphorylation of YAP was detected in colon lysates that coincided with increased Bax expression, increased epithelial cell death and poor survival of DSS-treated mice in the absence of *Rassf1a* [82]. Previous studies, have suggested a major role for c-Abl driven tyrosine 357 phosphorylation of YAP in response to DNA damage, which is prominent during intestinal inflammation injury in our DSS-treated *Rassf1a* knockout mouse [161, 162]. This induces the formation of a pY-YAP/p73 complex to drive pro-apoptotic gene expression, such as Bax. Enhanced Y357 YAP phosphorylation has been detected in *Rassf1a* $^{-/-}$ that coincided with increased Bax expression and cell death [82]. In this chapter we explored the potential effect of inhibiting this abnormal phosphorylation of YAP using the c-Abl class of PTK inhibitors (imatinib mesylate/gleevec),a tyrosine-kinase inhibitor used in the treatment of chronic myeloid leukemia [301]. We speculate that pharmacological inhibition of pY-YAP in DSS treated *Rassf1a* knockout mouse could enhance epithelial regeneration and increase healing after inflammation induced injury thus reversing the damaging effects caused by pY-YAP. We

utilized our unique animal model missing *1A* to explore if the PTK inhibitor, imatinib mesylate, can inhibit inflammation of the gut and increase epithelial regeneration after DSS-treatment. We speculate that the loss of function of 1A will not only lead to uncontrolled inflammation but to cancer if the inflammation is not properly regulated. Pharmacological inhibition of tyrosine phosphorylation of YAP might be a novel therapeutic to increase recovery, repair and healing after inflammation-induced injury in IBD.

3.2 Results

3.2.1 *Rassf1a*$^{+/-}$ mice are sensitive to DSS treatment

Rassf1a$^{+/-}$ mice (10-14 weeks) were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Mice were monitored for signs of colitis including weight loss, rectal bleeding, piloerection, bloatedness, hunching, movement, tremors and stool consistency. The phenotypic characteristics were assigned a score of 0–5 with 5 being very severe and animals were euthanized once rectal bleeding became grossly apparent. Interestingly, the *Rassf1a*$^{+/-}$ mice demonstrated similar symptoms and survival rate to *Rassf1a*$^{-/-}$ mice. In addition, *Rassf1a*$^{+/-}$ mice showed clinical symptoms of colitis including enhanced cytokine/chemokine production, elevated NF-κB activity, severe colonic epithelial cell injury and poor recovery following dextran sulphate sodium (DSS)-induced inflammation injury [82] (Fig. 3.1).

Both *Rassf1a*$^{+/-}$ and *Rassf1a*$^{-/-}$ mice have the similar responses to DSS, demonstrating haploinsufficiency at the *Rassf1a* loci. Thus, DSS-treated *Rassf1a*$^{+/-}$ mice might be a promising model to study IBD.

3.2.2 The effect of PTK inhibitor, imatinib on DSS treated *Rassf1a* $^{-/-}$ *and Rassf1a*$^{+/-}$ mice

Imatinib mesylate/gleevec (herein referred to imatinib) was developed to specifically inhibit c-Abl (IC50 of 25 nM) [302] and has been successfully utilized to treat leukemia patients. However, it can also inhibit c-Kit and PDGF-R at 400 nM [302]. Imatinib (60µg/gram body weight) was administrated by intraperitoneal injections on day 3 and 6 as to start treatment before the clinical onset of the disease which is around day 4 and to help in recovery before

changing to fresh water on day 7. Imatinib treatment for *Rassf1a*$^{+/-}$ mice resulted in >80% survival and low disease activity indices (Fig 3.2). In addition, *Rassf1a*$^{+/-}$ mice demonstrated reduced IL-6, low histopathological scoring, reduced DNA and oxidative damage, reduced cell death, regained normal crypt architecture and increased PCNA staining (increased proliferation) [82]. Furthermore, imatinib significantly reduced pY-YAP indicated by immunoblotting and immunohistochemical staining [82]. In contrast, imatinib did not protect DSS-treated *Rassf1a* $^{-/-}$ mice (Fig. 3.3). However, the clinical onset of disease was delayed by 2 – 3 days when compared to DSS-treated *Rassf1a* $^{-/-}$ mice. Imatinib treated *Rassf1a* $^{-/-}$ mice had similar disease activity indices to imatinib-treated *Rassf1a*$^{+/-}$ mice until day 8, but by days 9 – 10, *Rassf1a* $^{-/-}$ mice revealed higher DAI (Disease activity index) than *Rassf1a*$^{+/-}$ mice.

3.2.3 p53 analysis in colon lysates

It has been demonstrated that p53 is upregulated in crypt cells of chronic UC patients suggesting a critical role for p53 in IBD [303]. To investigate the influence of p53 on cell death and mice recovery, we explored levels of p53 in colon lysates by immunoblotting.

DSS-treated and untreated wild type mice almost revealed undetectable levels of p53 whereas DSS-treated *Rassf1a*$^{+/-}$ or *Rassf1a*$^{-/-}$ mice showed highly elevated levels of p53 (as early as day 3, especially for DSS treated *Rassf1a* $^{-/-}$) (Fig. 3.4). Accumulation of p53 was more apparent in day 9 and increased in imatinib-treated mice. p53 was not only accumulated but appeared to be modified to a slower migrating form, around 75 kDa (might be one of the phosphorylated p53 residues) [82]. It has been known that p53 stabilization and turnover is a dynamic process which occurs frequently and fast [304], so we decided to explore p53 stabilization in DSS-treated *Rassf1a* knockout mice with/without imatinib treatment to investigate the ability of imatinib to

interfere with p53 dependent ubiquitination and how it could enhance p53 stabilization. Colon lysates from DSS-treated *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice with /without imatinib were immunoprecipitated with anti-p53 antibody and immunoblotted for ubiquitin.

Interestingly, imatinib interfered with p53 ubiquitination and we saw decreased levels of ubiquitinated p53 in imatinib treated mice, that was even more apparent in imatinib treated *Rassf1a* $^{-/-}$ mice, suggesting that imatinib can enhance p53 stabilization specially in *Rassf1a* $^{-/-}$ mice (Fig. 3.4) [82]. Previous, studies suggest that caspase cleaved c-abl can phosphorylate the p53 inhibitor/E3 ligase, Mdm2, to inhibit dependent degradation of p53 and allowing it to accumulate [163]. Indeed further analysis, revealed increased c-Abl cleavage in DSS-treated *Rassf1a* $^{-/-}$ mice that was highly elevated in imatinib treated *Rassf1a* $^{-/-}$ mice [82]. Thus, we speculate that in the absence of RASSF1A, DSS induced inflammation injury can result in accumulated p53 and therefore increased levels of cell death.

3.3 Conclusion

Both *Rassf1a* $^{-/-}$ and *Rassf1a* $^{+/-}$ mice have similar responses to DSS, demonstrating haploinsufficiency at the *Rassf1a* locus. DSS treatment for *Rassf1a* $^{-/-}$ and *Rassf1a* $^{+/-}$ mice, resulted in clinical signs of colitis with reduction of several markers of proliferation such as YAP driven proliferation. Furthermore, tyrosine phosphorylation of YAP was detected in colon lysates that coincided with increased epithelial cell death and poor recovery. Inhibiting this abnormal phosphorylation of YAP using, imatinib reversed the damaging effects of DSS induced intestinal inflammation but only on a *Rassf1a*$^{+/-}$ background. Imatinib partially reversed the damaging effects of DSS in a *Rassf1a*$^{-/-}$ mice, due to robust accumulation and stabilization of p53 by day 9, possibly leading to increased apoptosis and poor recovery. Thus we showed that 1A has a novel

regulatory role during the recovery phase post inflammation-induced injury by restricting YAP transcription activity to inhibit inflammation and promote efficient epithelial repair. Pharmacological inhibition of pY-YAP during inflammation induced injury in DSS-treated *Rassf1a* knockout mouse could be a novel approach for IBD treatment.

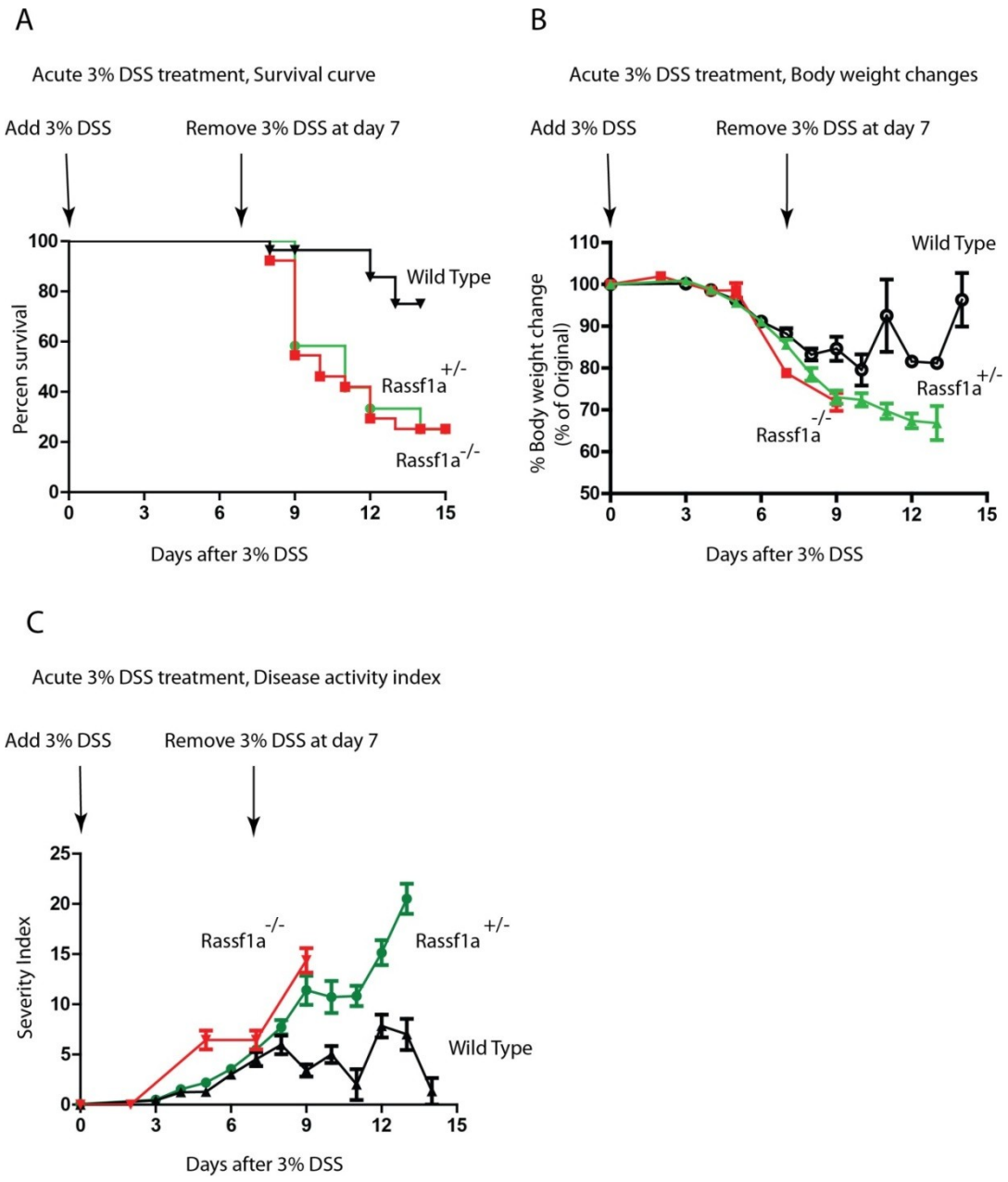


Figure 3.1: *Rassf1A*$^{+/-}$ mice are sensitive to DSS treatment. Mice were administrated 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. (A) p value is < 0.0001 WT vs. *Rassf1a*$^{-/-}$ (or *Rassf1a*$^{+/-}$), (B) p value = 0.0232 (WT vs *Rassf1a*$^{-/-}$ or *Rassf1a*$^{+/-}$) and (C) p value = 0.1203 (WT vs *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$) n=16-20 for all. Gordon et al; 2013.

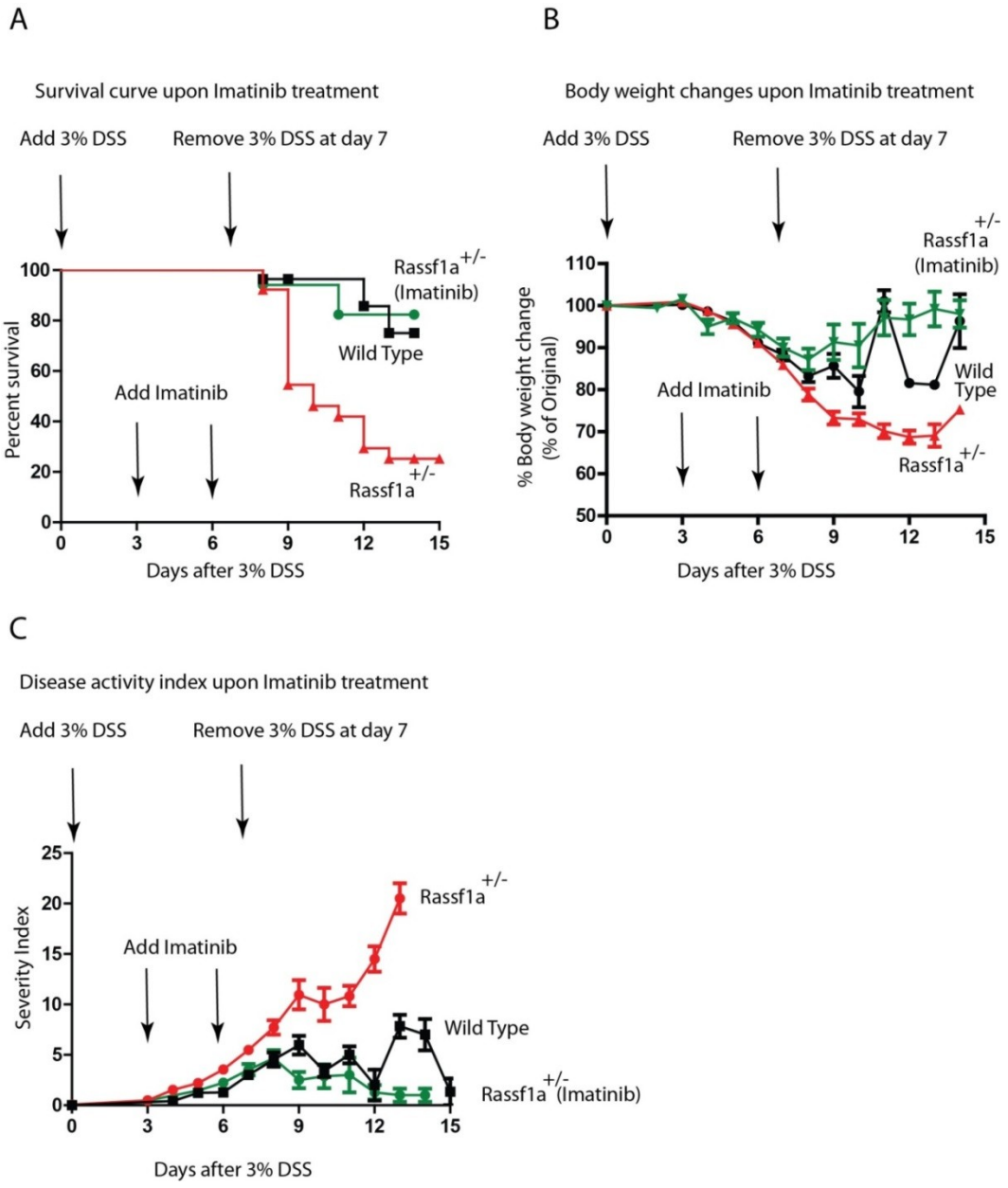


Figure 3.2: PTK inhibitor, imatinib, protected DSS-treated *Rassf1a*$^{+/-}$ from DSS inflammation-induced injury. Imatinib (60µg/gram body weight) was injected intraperitoneally on day 3 and 6. (A) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (- /+ imatinib treatment) = 0.0100, (B) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ imatinib treatment) = 0.0008 and (C) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ imatinib treatment)=0.0064. n=17-22 for all. Gordon et al; 2013.

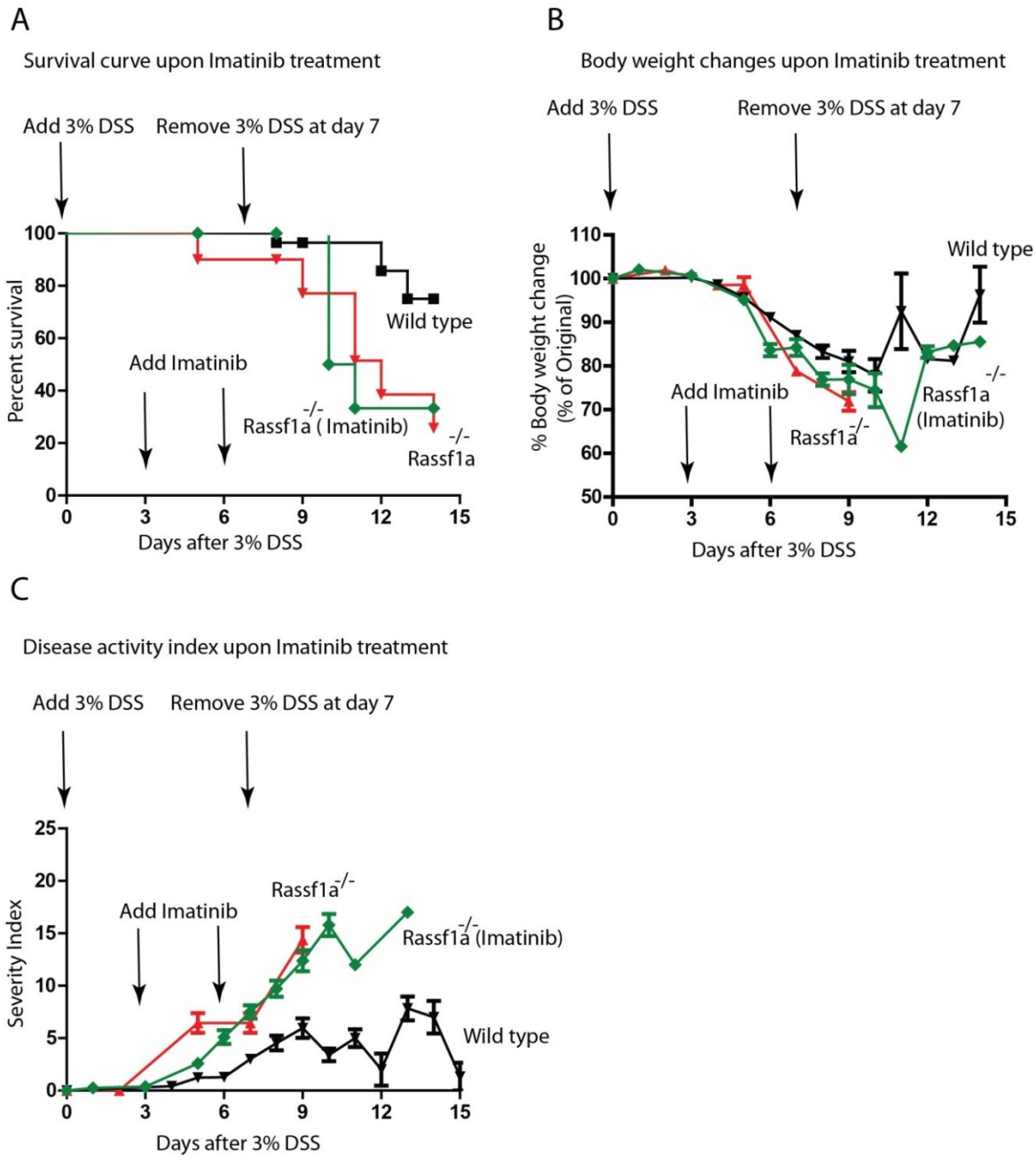
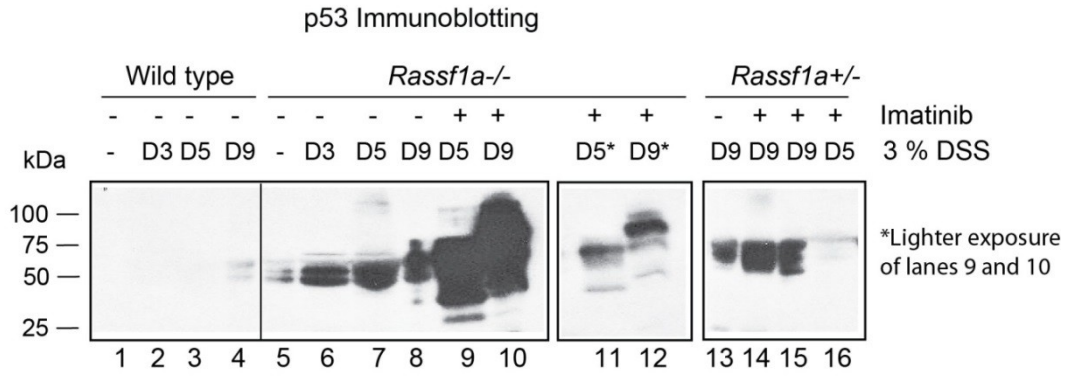


Figure 3.3: PTK inhibitor, imatinib, did not protect DSS-treated *Rassf1a*$^{-/-}$ from DSS inflammation- induced injury. Imatinib (60µg/gram body weight) was injected intraperitoneally on day 3 and 6. (A) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (- /+ imatinib) = 0.38 , (B) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (-/+ imatinib)=0.139 and (C) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (-/+ imatinib)= 0.4189. n= 22 for all. Gordon et al; 2013.

A



B

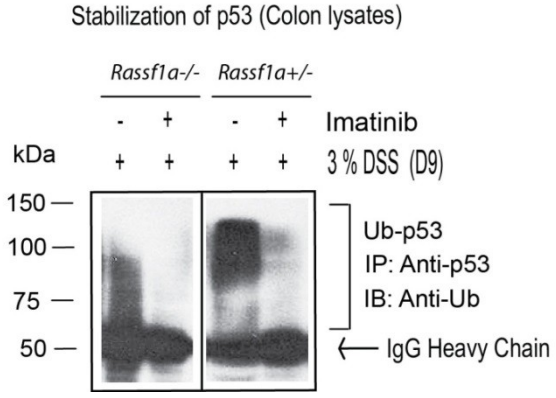


Figure 3.4: Colon lysates from different genotypes and treatments were immunobloted for p53 (upper panel). The loss of RASSF1A results in accumulation and stabilization of p53. DSS-treated and untreated wild type animals revealed almost undetectable levels of p53 whereas DSS-treated *Rassf1a*$^{+/-}$ or *Rassf1a*$^{-/-}$ mice revealed highly elevated or accumulated levels of p53. The accumulation of p53 was more apparent by day 9 and increased in imatinib-treated animals whereby, p53 levels not only accumulated but p53 appears to be modified to a slower migrating form, around 75 kDa. In the lower panel, colon lysates were immunoprecipitated with p53 antibody and immnuoblotted for ubiquitin, to analyse p53 stabilization with/without imatinib. Imatinib interfered with p53 ubiquitination and p53 ubiquitination is increased without imatinib treatment. Gordon et al; 2013.

Chapter 4

The effect of anti-autophagy drugs on DSS-treated *Rassf1a* knockout mice

4.1 Introduction and Rationale

Epithelial regeneration was reduced in the absence of RASSF1A in DSS-treated *Rassf1a* knockout mice with reduction of Yes-associated protein (YAP)-driven proliferation[82]. Furthermore, tyrosine phosphorylation of YAP and increase in the autophagic response was detected in colon lysates that coincided with increased epithelial cell death and poor survival of DSS-treated mice in the absence of RASSF1A. In chapter 3, we explored the potential effect of inhibiting the abnormal tyrosine phosphorylation of YAP using the c-Abl class of PTK inhibitors (imatinib mesylate/gleevec) and in this chapter we will discuss the potential effect of inhibiting the autophagic response that might be detrimental to epithelial repair and healing, using autophagy inhibitors, 3-methyl-adenine (3-MA) and chloroquine.

Autophagy is a basic catabolic mechanism, in which dysfunctional or non-essential cellular components are degraded through the actions of lysosomes. Targeted cytoplasmic components are isolated in a double-membrane vesicle known as autophagosome. The autophagosome then fuses with a lysosome and the targeted cytoplasmic components are degraded and recycled [201]. Many genes that have either genetic or functional links to autophagy have IBD-associated genetic variants, including *NOD2, ATG16L1* and *IRGM* [251]. As we showed before epithelial regeneration was decreased in the absence of RASSF1A with subsequent increase in the autophagic response (detected by immunoblotting for p62, a marker for autophagic flux) in colon. In addition, NOD2 (an intracellular pattern recognition receptor) polymorphisms are associated with childhood onset of Crohn's disease and NOD2 is known to be involved in autophagy via recruiting ATG16L1 [204, 205]. Interestingly, $1a^{-/-}$ $Nod2^{-/-}$ mice are resistant to DSS induced inflammation injury when compared to $1a^{-/-}$ mice, suggesting that the damage from DSS might be through the active NOD2 signaling (unpublished data from Baksh

lab). Furthermore, our unpublished data demonstrated that RASSF1A forms robust association with NOD2 upon MDP treatment in colon cancer cell line (HCT-116), indicating that RASSF1A might negatively regulate NOD2 signaling after MDP stimulation.

The data mentioned above, suggest that upon DSS treatment the lack of RASSF1A might result in uncontrolled autophagic response via NOD2 that might be detrimental to epithelial repair and results in poor recovery. Moreover, we think that active autophagy is an efficient response to DSS treatment and after a certain point; the autophagic response needs to be shut down. However, in the absence of 1A this does not occur and the response continues to be uninhibited. In this chapter we explored the potential effect of autophagy inhibitors, 3-methyladenine (3-MA) and chloroquine on DSS-treated mice to inhibit the autophagic response (probably induced by NOD2 signaling) that might be detrimental to epithelial repair and healing after inflammation-induced injury. Autophagy inhibitor, 3-MA inhibits early stages of autophagic cascades via inhibition of type III Phosphatidylinositol 3-kinases (PI-3K) [292, 293]. Chloroquine inhibits late stage autophagic events by inhibiting autophagosme fusion with lysosome leading to autophagosome accumulation[285]. We speculate that the pharmacological inhibition of the autophagic response could be a novel therapeutic approach to enhance recovery from inflammation-induced injury and be useful in treating and saving IBD patients from increased risk of getting cancer later in life.

4.2 Results:

4.2.1 The effect of autophagy inhibitor, 3-MA on DSS-treated *Rassf1a* $^{-/-}$, *Rassf1a* $^{+/-}$ and *Rassf1a* $^{IEC\text{-}Ko}$ mice

Rassf1a$^{-/-}$, *Rassf1a*$^{+/-}$ and *Rassf1a* $^{IEC\text{-}Ko}$ (intestinal epithelial cell (IEC) specific knockout) mice (10-14 weeks old) were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Autophagy inhibitor, 3-MA (20μg/gram body weight) was administrated by intraperitoneal injections on day 3 and 6. To explore the role of RASSF1A in intestinal inflammation an intestinal epithelial cell specific knockout to RASSF1A, *Rassf1a* $^{IEC\text{-}Ko}$ mice were generated by mating *Rassf1a-LoxP* conditional mice with the Villin-Cre transgenic mouse. Villin is an intestinal protein that is associated with microvilli. Both *Rassf1a* $^{IEC\text{-}Ko}$ and *Rassf1a*$^{-/-}$ mice had similar responses to DSS treatment suggesting that RASSF1A is essential for protection from intestinal inflammation [82] (*Rassf1a* $^{IEC\text{-}Ko}$ mice have been generated by Baksh lab).

Interestingly, the anti-autophagy drug 3-MA significantly increased the survival rate of DSS-treated *Rassf1a*$^{+/-}$ mice to >70% (normally, DSS-treated *Rassf1a*$^{+/-}$ mice have survival rate about 25%) (Fig.4.1). For DSS-treated *Rassf1a*$^{IEC\text{-}Ko}$, 3-MA treatment also significantly increased the survival rate to 75% (normally, DSS-treated *Rassf1a*$^{IEC\text{-}Ko}$ have survival rate about 30%) (Fig.4.2). In addition, there was significant decrease in DAI for both DSS-treated *Rassf1a*$^{+/-}$ and *Rassf1a* $^{IEC\text{-}Ko}$ .In contrast, 3-MA did not protect DSS-treated *Rassf1a*$^{-/-}$ mice and they had the same survival rate and disease severity as mice treated only with DSS (Fig.4.2). It is possible that in *Rassf1a*$^{-/-}$ mice the loss of both alleles has multiple and diverse damaging effects caused by molecular pathways away from the autophagic signalling and thus not inhibited with 3MA. Therefore, inhibiting the damaging effects caused by autophagy in *Rassf1a*$^{-/-}$ mice using 3-MA is not enough to protect from DSS insult.

4.2.2 The effect of autophagy inhibitor, chloroquine on DSS-treated *Rassf1a* $^{+/-}$ mice

In the previous experiment we explored the use of autophagy inhibitor, 3-MA and in this experiment we explored autophagy inhibition by chloroquine, which inhibits late stage autophagic events via the inhibiton of autophagosme fusion with the lysosome and prevents autophagosome maturation. *Rassf1a* $^{+/-}$ mice (10-14 weeks old) were given 3% DSS in drinking water for 7 days followed by fresh water for another 7 days for recovery. Chloroquine was administrated orally (60 µg/g body weight) on days 2, 4, 6 and 8. Chloroquine partially protected DSS-treated *Rassf1a* $^{+/-}$ mice, the survival rate increased from 20% up to 40% with chloroquine treatment (Fig.4.3). Interestingly, there was significant decrease in disease severity indices. Inhibiting the autophagic response, using chloroquine was not sufficient for a full recovery from DSS induced- inflammation insult. However, there was significant decrease in disease severity; but unfortunately, it was not enough to enhance repair and recovery from inflammation induced injury.

4.2.3 The effect of 3-MA on DSS-treated Rassf1a$^{IEC\text{-}WT}$ and Wild type mice

Rassf1a $^{IEC\text{-}WT}$ and Wild type (WT) mice (10-14 weeks old) were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Autophagy inhibitor, 3-MA was injected intraperitoneally (20 µg/g body weight) on day 3 and 6. Surprisingly, treatment with 3-MA did not protect DSS-treated *Rassf1a* $^{IEC\text{-}WT}$ (C57BL/6-129 background) and Wild type (C67BL/6 background) mice. Furthermore, 3-MA worsened DSS-treated *Rassf1a* $^{IEC\text{-}WT}$ and WT mice, the survival rate dramatically decreased and the disease severity dramatically increased (data not shown). The survival rate of DSS-treated *Rassf1a* $^{IEC\text{-}WT}$ mice decreased from 93.3% to 20% and for the DSS-treated WT mice from 75% to 0% , after treatment with 3-MA (Fig.4.4).

We speculate that active autophagy is an efficient response to DSS treatment and after a certain point the autophagic response needs to be shut down. We speculate that since 1A can robustly associate with NOD2 and NOD2 can drive autophagic signalling, the absence of 1A would result in the failure of proper shut down of the autophagic response. In DSS-treated *Rassf1a* $^{IEC\text{-}WT}$ and *WT* mice the autophagic response is properly regulated due to the presence of RASSF1A (Fig.4.10). Therefore additional inhibition of autophagy with 3-MA might be detrimental to DSS-treated *Rassf1a* $^{IEC\text{-}WT}$ and WT.

4.2.4 Biomarkers analysis for DSS-treated *Rassf1a* knockout mice with anti-autophagy drugs

4.2.4.1 Histopathological scoring

Histopathological scoring helps in assessment of disease severity and tissue damage in IBD mice models. To explore the histopathological scoring in our mice, descending colons from Wild type and *Rassf1a* knockout mice with different treatments were isolated at day 8, fixed in z-Fix, paraffin-embedded, cut longitudinally and H &E stained (using standard techniques). All inflammation scores were obtained utilizing blinded scoring by a gastrointestinal pathologist (Dr.Aducio Thiesen) based on infiltration of enterocytes, neutrophils, lamina propria cellularity, crypt structure (scored as 0 – 2 where 2 = maximal injury).

Unfortunately, there was no significant decrease in histopathological scores for 3-MA treated *Rassf1a*$^{+/-}$, *Rassf1a*$^{-/-}$ mice and chloroquine treated *Rassf1a*$^{+/-}$.The average inflammation score for 3-MA treated *Rassf1a*$^{+/-}$ mice was 5, for chloroquine treated *Rassf1a*$^{+/-}$ mice it was 6 and for 3-MA treated *Rassf1a*$^{-/-}$ mice it was 4.5 (Fig.4.5). Normally, DSS-treated *Rassf1a*$^{+/-}$ and *Rassf1a*$^{-/-}$ mice have histopathological scoring of 6.3 and they have severe colonic disruption

with crypt damage and increase in immune cells infiltration and DSS-treated WT reveal minimal colonic damage and significantly less infiltration of immune cells. The data presented above suggest that anti-autophagy drugs cannot decrease the inflammation scoring and inflammatory cells infiltration.

4.2.4.2 Colon length analysis

Analysing colon length helps in exploring the extent of inflammation and regeneration status in inflammation induced-injury models. Usually, mice with severe clinical symptoms of colitis are associated with shortening in colon length due to colonic erosion by cell death. Interestingly, we demonstrated significant increase in colon length of DSS-treated *Rassf1a* $^{-/-}$ mice with 3MA (>7.2 cm), DSS-treated *Rassf1a*$^{+/-}$ mice with 3-MA (> 6.6cm) and DSS-treated *Rassf1a*$^{+/-}$ with chloroquine (>6.1 cm) when compared to *Rassf1a* knockout mice treated only with DSS (Fig.4.5). Normally, DSS-treated *Rassf1a*$^{-/-}$ and *Rassf1a* $^{+/-}$ have shortened colon with average length < 4.5 cm due to decreased repair and healing. These results suggest that inhibiting the autophagic response enhances repair and healing and helped in maintaining colon length.

4.2.4.3 Serum cytokine analysis

We analysed serum cytokines for wild type and 3-MA treated *Rassf1a*$^{+/-}$ and *Rassf1a*$^{-/-}$ mice and chloroquine treated *Rassf1a* $^{+/-}$ mice (harvested at day 8**).** There was significant decrease in pro-inflammatory cytokines production (IL-6, IL-12, KC/GRO and IFN-γ) in DSS-treated *Rassf1a* $^{+/-}$ with 3-MA and DSS treated *Rassf1a* $^{+/-}$ mice with chloroquine (Fig.4.6). For DSS treated *Rassf1a* $^{-/-}$ mice with 3-MA there was significant decrease in IL-12 and IFN -γ cytokine production and there was decrease in IL-6 and KC/Gro cytokine production (Fig.4.7). Normally, DSS-treated *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice have increased pro-inflammatory cytokines production.

Data presented above suggest that anti-autophagy drugs reduced NF-κB regulated pro-inflammatory cytokine production and the decrease in disease severity especially in DSS-treated *Rassf1a*$^{+/-}$ mice with 3-MA and chloroquine might be due to the decrease in damaging effects caused by inflammatory cytokines.

4.2.4.4 Proliferation analysis

PCNA (Proliferating cell nuclear antigen) is widely used as marker for proliferation. PCNA encircles the DNA, where it acts as a scaffold to recruit proteins involved in DNA repair, chromatin remodelling and DNA replication[305]. We did immunohistochemical staining for PCNA on descending colons DSS-treated *Rassf1a* $^{+/-}$ mice with 3-MA and chloroquine and *Rassf1a* $^{-/-}$ mice with 3-MA. Colons were isolated at day 8, fixed in z-Fix, paraffin embedded, cut longitudinally and stained with anti-PCNA antibody (using standard technique). We did quantitation analysis for the number of positive PCNA stained cells per crypt. There was significant increase in PCNA staining (proliferation) for DSS-treated *Rassf1a* $^{+/-}$ with 3-MA and chloroquine when compared to *Rassf1a* $^{+/-}$ mice treated only with DSS, the number of positive PCNA stained cells/crypt were 10.3 and 6.2, respectively. Normally, DSS-treated *Rassf1a* $^{-/-}$ and *Rassf1a* $^{+/-}$ have decreased PCNA staining (proliferation), the number of positive PCNA stained cells /crypt for DSS-treated *Rassf1a* $^{-/-}$ and *Rassf1a* $^{+/-}$ mice were 5.5 and 3.5, respectively (Fig.4.7). Surprisingly, DSS-treated *Rassf1a* $^{-/-}$ mice with 3-MA had dramatic decrease in PCNA staining and there was less PCNA-positive cells/crypt compared to mice treated with DSS only (Fig.4.8).

Data presented above suggest that, anti-autophagy drugs (3-MA and chloroquine) can reverse the damaging effects caused by increased autophagic response and can enhance repair and healing, but only in the *Rassf1a* $^{+/-}$ background. In contrast, inhibition of the autophagic

response by 3-MA in the *Rassf1a*$^{-/-}$ background did not enhance proliferation. Furthermore, 3-MA caused severe reduction of cell proliferation which might explain why DSS-treated *Rassf1a*$^{-/-}$ are not protected by 3-MA. We speculate that the loss of both alleles is inducing more damaging and unfavourable effects caused by pathways other than autophagy that might be detrimental to epithelial repair and healing. Unfortunately, these damaging effects cannot be reversed by 3-MA.

4.2.4.5 Cell death analysis

To analyse cell death, colon lysates from different genotypes and treatments (harvested at day 8) were immunoblotted for PARP (Poly ADP ribose polymerase) to explore levels of cleaved p85 PARP (85kDa), a marker for late apoptosis. PARP can be activated in cells under DNA damage and/or stress and PARP can induce programmed cell death, via the production of PAR [Poly (ADP) Ribose Polymer], which stimulates mitochondria to release apoptosis inducing factor [306, 307]. During active apoptosis, PARP (116kDa) is cleaved by caspase-3 and possibly other caspases into an 85 kDa fragment [307].

There was significant decrease in levels of cleaved p85 product of PARP in colon lysates upon 3-MA treatment (Fig.4.9). Normally, there is increased levels of cleaved p85 PARP (cell death) in DSS-treated *Rassf1a*$^{-/-}$ mice, accompanied by decreased recovery and repair after inflammation-induced injury [82]. We speculate that 3-MA might enhance repair and healing, by decreasing autophagy induced- cell death. However, DSS-treated *Rassf1a*$^{-/-}$ mice are not protected by 3-MA, indicating that restricting cell death in DSS-treated *Rassf1a*$^{-/-}$ mice by anti-autophagy drugs is essential but not sufficient for full recovery from DSS inflammation insult and damaging events other than cell death have to inhibited.

4.2.4.6 Autophagic flux analysis

p62 [sequestosome 1 (SQSTM1)] is a protein that binds to ubiquitin and co-localizes with ubiquitinated protein aggregates in many liver and neurodegenerative diseases. SQSTM1 (p62) protein is degraded by autophagy and serves to link ubiquitinated proteins to the autophagic machinery (autophagosome) for their degradation in the lysosome. Since p62 accumulates when autophagy is inhibited and decreased levels can be observed when autophagy is induced, p62 is used as a marker to study the autophagic flux [308]. The term "autophagic flux" indicates, autophagosome formation, fusion of the autophagosome with lysosome and degradation of autophagic substrates inside the lysosome. Some studies consider p62 as a more reliable indicator for autophagic activity than monitoring the number of autophagosomes (by immunoblotting for LC3-II) [309]. Colon lysates were immunoblotted for p62 to assess levels of p62 and to monitor the autophagic flux. DSS-treated *Rassf1a* $^{+/-}$ mice revealed decreased levels of p62 and DSS-treated *Rassf1a*$^{-/-}$ mice revealed almost undetectable levels of p62, indicating the increase in the autophagic flux (autophagic activity). In contrast, DSS-treated *Rassf1a* $^{+/-}$ mice with 3-MA and chloroquine and DSS-treated *Rassf1a*$^{-/-}$ mice with 3-MA, revealed elevated levels of p62, indicating that autophagy inhibitors can decrease the autophagic activity in DSS-treated *Rassf1a* knockout mice. Furthermore, we demonstrated significant increase in the autophagic activity in untreated *Rassf1a*$^{-/-}$ mice when compared to untreated *Rassf1a* $^{+/-}$ mice, indicated by decreased levels of p62 in colon lysates (Fig.4.10).

These results suggest that the loss of both alleles in *Rassf1a*$^{-/-}$ mice, lead to increased and uncontrolled autophagic response even without DSS treatment, but in *Rassf1a* $^{+/-}$ mice, the loss of only one allele lead to increase in the autophagic response only under acute inflammation

condition (DSS treatment). As mentioned before, we speculate that under DSS treatment, the loss of RASSF1A in *Rassf1a* $^{-/-}$ and *Rassf1a* $^{+/-}$ mice will lead to uncontrolled autophagic response via NOD2 that might be detrimental to epithelial repair and healing.

4.2.5 RASSF1A can restrict the association of NOD2 with RIPK2 after stimulation with MDP

NOD2 is an intracellular pattern recognition receptor required in response to certain bacterial invasion. When NOD2 is activated after stimulation with its specific ligand, MDP (muramyl didpetide) it recruits the downstream receptor-interacting serine-threonine kinase 2 (RIPK2) to promote activation of ATG16L1 (to drive autophagy) and/or NF-κB (to drive an inflammatory response) [244, 245]. RIPK2 is crucial to NOD2 response, RIPK2-deficient mice do not respond to NOD2 ligand [245, 247]. RIPK2 also goes by the name RIP2 or RICK1. Herein, it will be referred as RIPK2.

As mentioned before, we have unpublished data to prove that RASSF1A forms robust association with NOD2 after stimulation with MDP, suggesting that RASSF1A might restrict NOD2 signaling, therefore, we decided to explore the ability of RASSF1A to physically interfere with the association of NOD2 with RIPK2 and how it could interfere with NOD2 signalling to inhibit activation of NF-κB and /or autophagy after stimulation with MDP. We transfected HEK -293 cells (human embryonic kidney cells) with and without *RASSF1A,* cells were stimulated with different time courses using MDP. Cells were harvested, lysed, immunoprecipitated with anti-NOD2 antibody and immunoblotted for RIPK2. We observed that overexpression of *RASSF1A* restricted the association of NOD2 with RIPK2 but, at 5hrs we lose this restriction and

we can see association between NOD2 and RIPK2 (Fig.4.11). Data presented above, suggest that RASSF1A can down regulate NOD2 signalling after stimulation with MDP via restricting NOD2 association with RIPK2, required for activation of NF-κB and/or autophagy.

4.2.6 RASSF1A can restrict the association of NOD2 with ATG16L1 after stimulation with MDP

HEK -293 cells were transfected with/without *HA-RASSF1A* and with *FLAG-ATG16L1,* cells were stimulated with MDP (0, 3 hrs) (Fig. 4.12). Cells were harvested, lysed, immunoprecipitated with anti-NOD2 antibody and immunoblotted for ATG16L1. We demonstrated that, RASSF1A can interfere with the association of NOD2 with ATG16L1 at 3hrs, suggesting that RASSF1A can negatively regulate NOD2 pathway after stimulation with MDP to inhibit ATG16L1 recruitment to activate autophagy (Fig.4.12). Similar results were obtained in HCT116 colon cancer cells (data not shown). We speculate that upon DSS treatment the lack of RASSF1A in *Rassf1a* knockout mice resulted in uncontrolled autophagic response via NOD2 and ATG16L1 that might be detrimental to epithelial repair and recovery.

4.3 Conclusion

Upon DSS treatment, the loss of RASSF1A in *Rassf1a* knockout mice lead to increased and uncontrolled autophagic response via NOD2 that might be detrimental to epithelial repair and healing. However, in the presence of RASSF1A, there was robust association with NOD2 after MDP stimulation and NOD2 pathway was negatively regulated by restricting NOD2 association with its partners required for NF-κB and/or autophagy activation. Inhibition of the autophagic response using 3-MA protected only DSS-treated *Rassf1a* $^{+/-}$ and *Rassf1a* ^{IEC} mice.

Treatment with 3-MA resulted in low DAI, reduced pro-inflammatory cytokines production, increased colon length, low histopthological scoring and increased epithelial cell proliferation in DSS-treated *Rassf1a*$^{+/-}$ mice. In contrast, inhibition of autophagic response using 3-MA did not protect DSS treated *Rassf1a*$^{-/-}$ mice. However, 3-MA reduced pro-inflammatory cytokine production, histopathological scoring and there was significant decrease in cell death, but at the same time there was dramatic decrease in proliferation, indicating that inhibition of the autophagic response is essential but it is not enough to protect *Rassf1a* $^{-/-}$ mice. The loss of both alleles in *Rassf1a* $^{-/-}$ mice is inducing damaging effects caused by unknown molecular pathways away from the autophagic signalling and thus not inhibited with 3-MA. RASSF1A has a novel regulatory role during the recovery phase post inflammation-induced injury by restricting the NOD2 induced autophagic response to inhibit inflammation and promote efficient epithelial repair. Pharmacological inhibition of autophagy during inflammation induced injury could be a novel therapeutic approach for IBD treatment and so preventing patients from predisposing to cancer later in life.

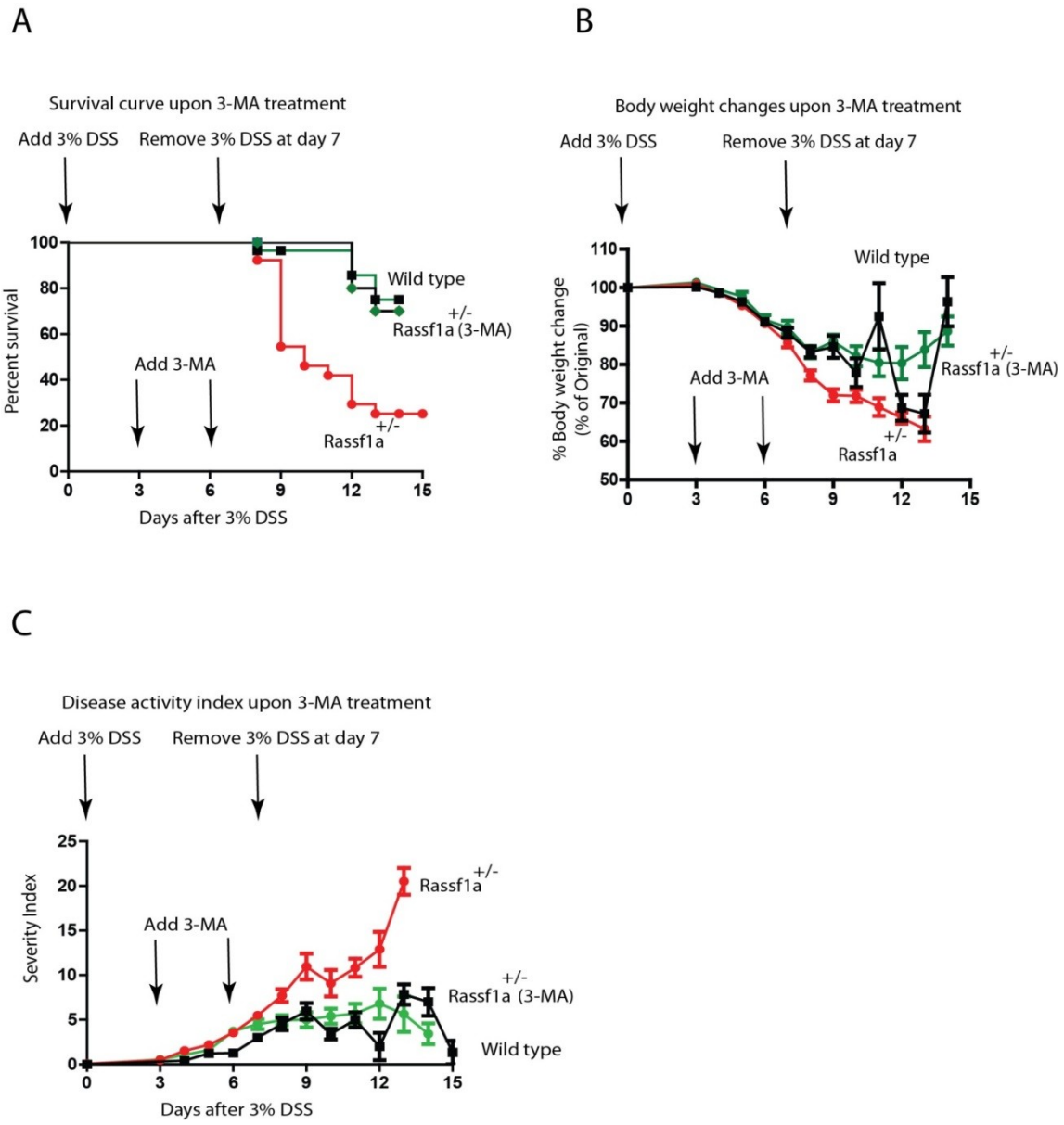


Figure 4.1: Autophagy inhibitor, 3-MA, protected DSS-treated *Rassf1a*$^{+/-}$ from DSS inflammation- induced injury. Autophagy inhibitor, 3-MA (20µg/gram body weight) was injected intraperitoneally on day 3 and 6. (A) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (- /+ 3-MA) = 0.0002 , (C) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ 3-MA)= 0.0966 and (C) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ 3-MA)= 0.0533. n=18-22 for all.

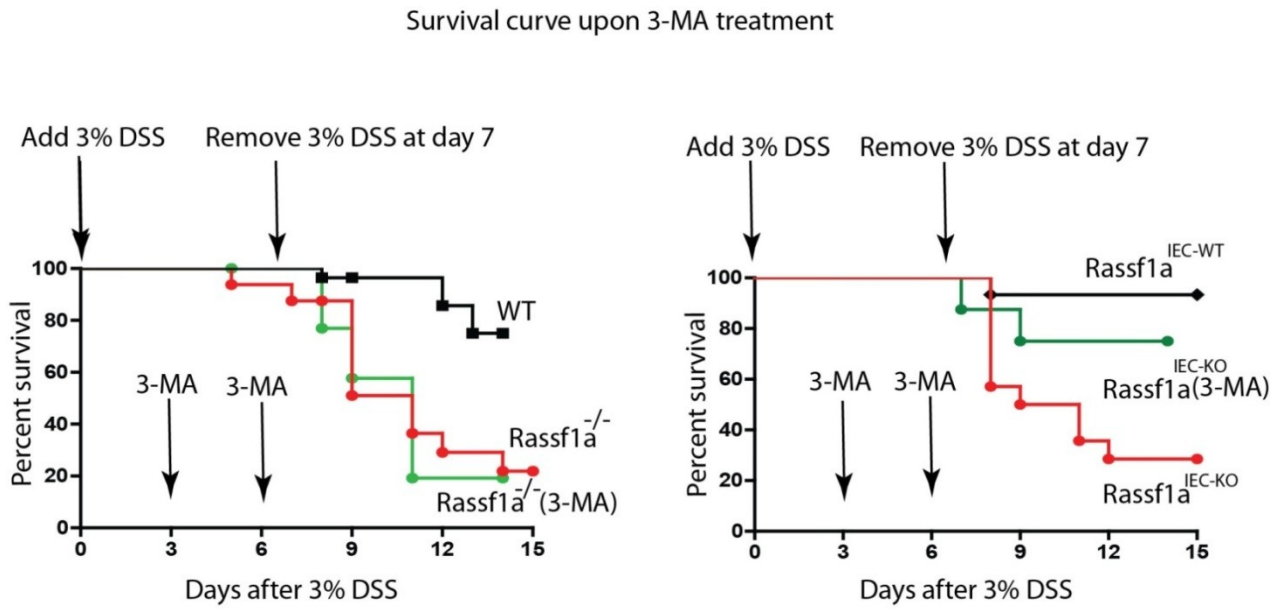


Figure 4.2: Autophagy inhibitor, 3-MA, did not protect DSS-treated *Rassf1a*$^{-/-}$ (left panel) from DSS inflammation- induced injury, but on the other hand it protected DSS-treated *Rassf1a*$^{IEC\text{-}KO}$ (right panel). 3-MA (20µg/gram body weight) was injected intraperitoneally on day 3 and 6. p value for DSS-treated *Rassf1a*$^{-/-}$ (left panel) (- /+ 3-MA)= 0.08 , p value for DSS- treated *Rassf1a* $^{IEC\text{-}KO}$ (right panel) (-/+ 3-MA)=0.018. n=16 for left panel and n=8-12 for right panel.

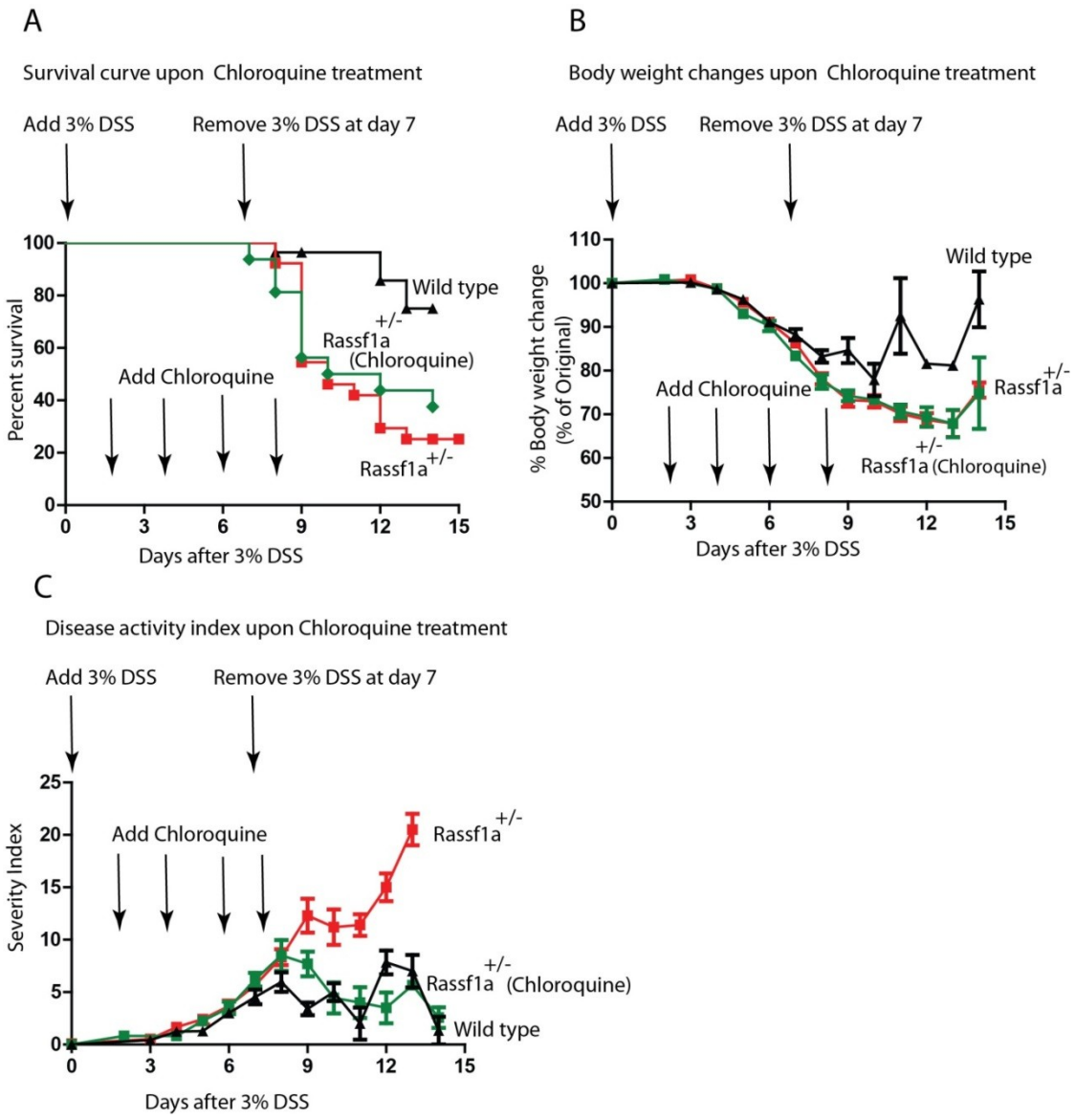


Figure 4.3: Chloroquine partially protected *Rassf1a*$^{+/-}$ from DSS inflammation-induced injury. Chloroquine (60µg/gram body weight) was administrated orally on days 2, 4, 6 and 8. (A) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (- /+ chloroquine) = 0.1163, (B) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ chloroquine)=0.47 and (C) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ chloroquine)= 0.0337. n=18-22 for all.

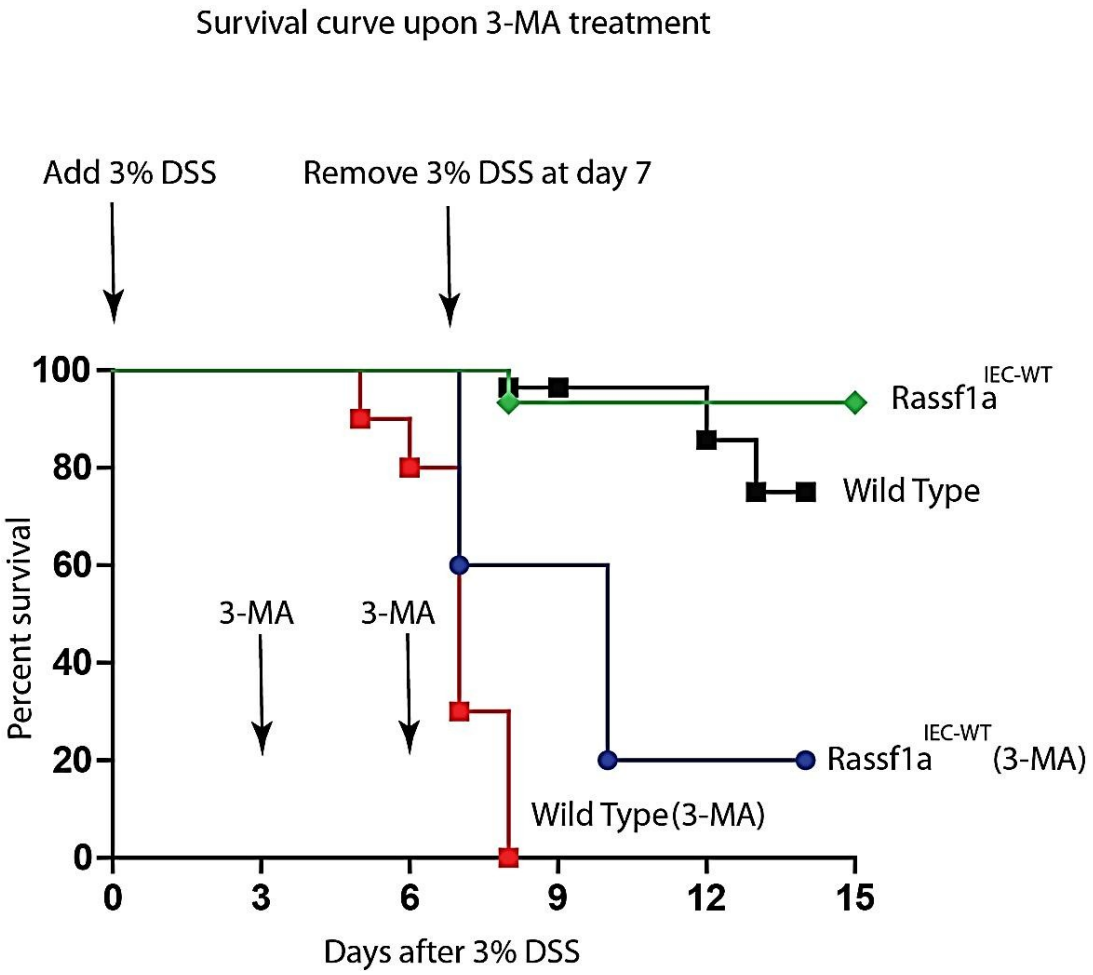


Figure 4.4: Autophagy inhibitor, 3-MA, did not protect DSS-treated Wild type and DSS-treated *Rassf1a*$^{IEC\text{-}WT}$ from inflammation- induced injury. Autophagy inhibitor, 3-MA (20µg/gram body weight) was injected intraperitoneally on day 3 and 6. p value for DSS-treated *Rassf1a*$^{IEC\text{-}WT}$ (-/+ 3-MA) = 0.0001. n=10-15 for Wild type and n=5-10 for *Rassf1a*$^{IEC\text{-}WT}$.

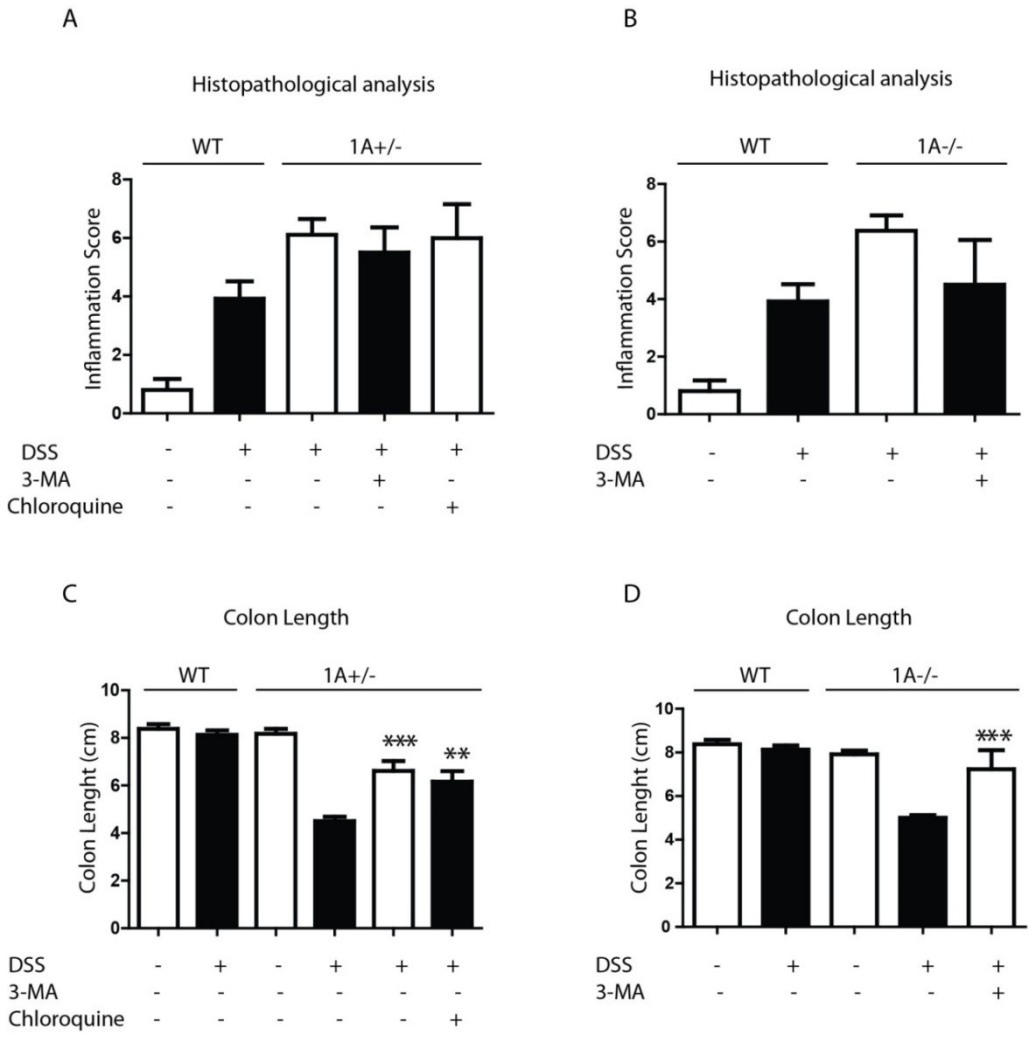


Figure 4.5: Descending colons (harvested at day 8) were isolated from *Rassf1a* knockout and WT mice with different treatments. Fixed in z-Fix, paraffin-embedded, cut longitudinally and H &E stained (using standard techniques). For upper panels: (A) p value for DSS-treated $1a^{+/-}$ (-/+ 3-MA) = 0.193 and (-/+ chloroquine) =0.37. n= 8-12 for $1a^{+/-}$ (-/+ 3-MA) and n= 3-16 for (-/+chloroquine), (B) p value for DSS-treated $1a^{-/-}$ (-/+ 3-MA) = 0.089.) and n= 6-10. Colon length of DSS-treated *Rassf1a* knockout mice and WT were analysed. For lower panels, (C) p value for DSS-treated $1a^{+/-}$ (-/+ 3-MA) = 0.0002 and for $1a^{+/-}$ (-/+ chloroquine) = 0.0012. n=8-13 for $1a^{+/-}$ (-/+3-MA), n= 3-6 for $1a^{+/-}$ (-/- chloroquine) (D) p value for DSS-treated $1a^{-/-}$ (-/+ 3-MA) = 0.0001 and n=3-6. (*) mean significant p value.

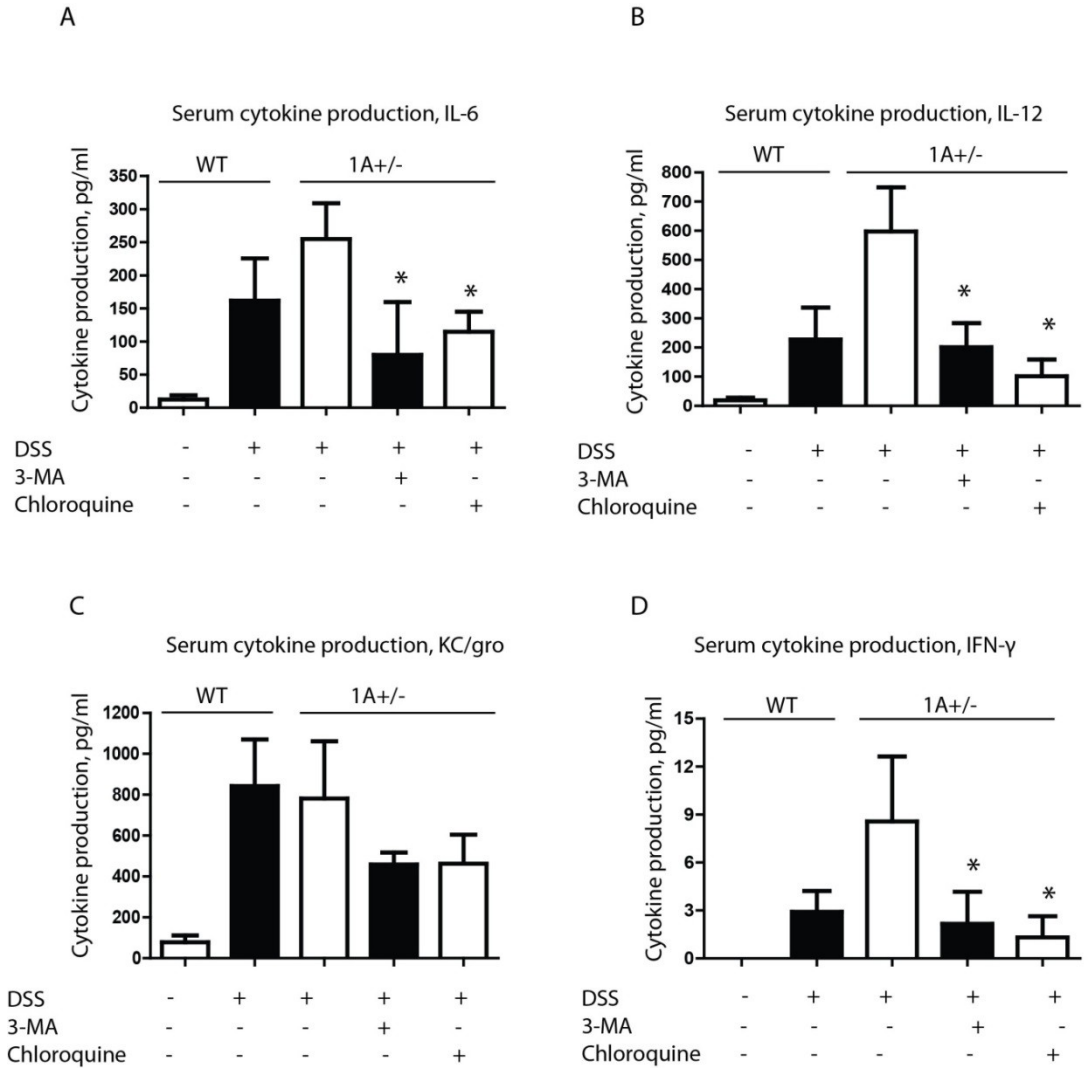


Figure 4.6: Serum cytokine analysis for *Rassf1a*$^{+/-}$ mice with different treatments (harvested at day 8). DSS-treated *Rassf1a*$^{+/-}$ mice with anti-autophagy drugs revealed decrease in inflammatory cytokine production when compared to *Rassf1a*$^{+/-}$ mice treated only with DSS. (A) p value for DSS-treated *Rassf1a*$^{+/-}$ (-/+ 3-MA)= 0.0242 and (-/+ chloroquine)=0.0722. (B) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ 3-MA)=0.0222 and (-/+ chloroquine)=0.0188. (C) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ 3-MA) = 0.094 and(-/+ chloroquine)= 0.1183. (D) p value for DSS-treated *Rassf1a*$^{+/-}$ mice (-/+ 3-MA)= 0.079 and (-/+ chloroquine)= 0.082. n= 3-5 for (-/+3-MA) and 3 for (-/+chloroquine). (*) mean significant p value.

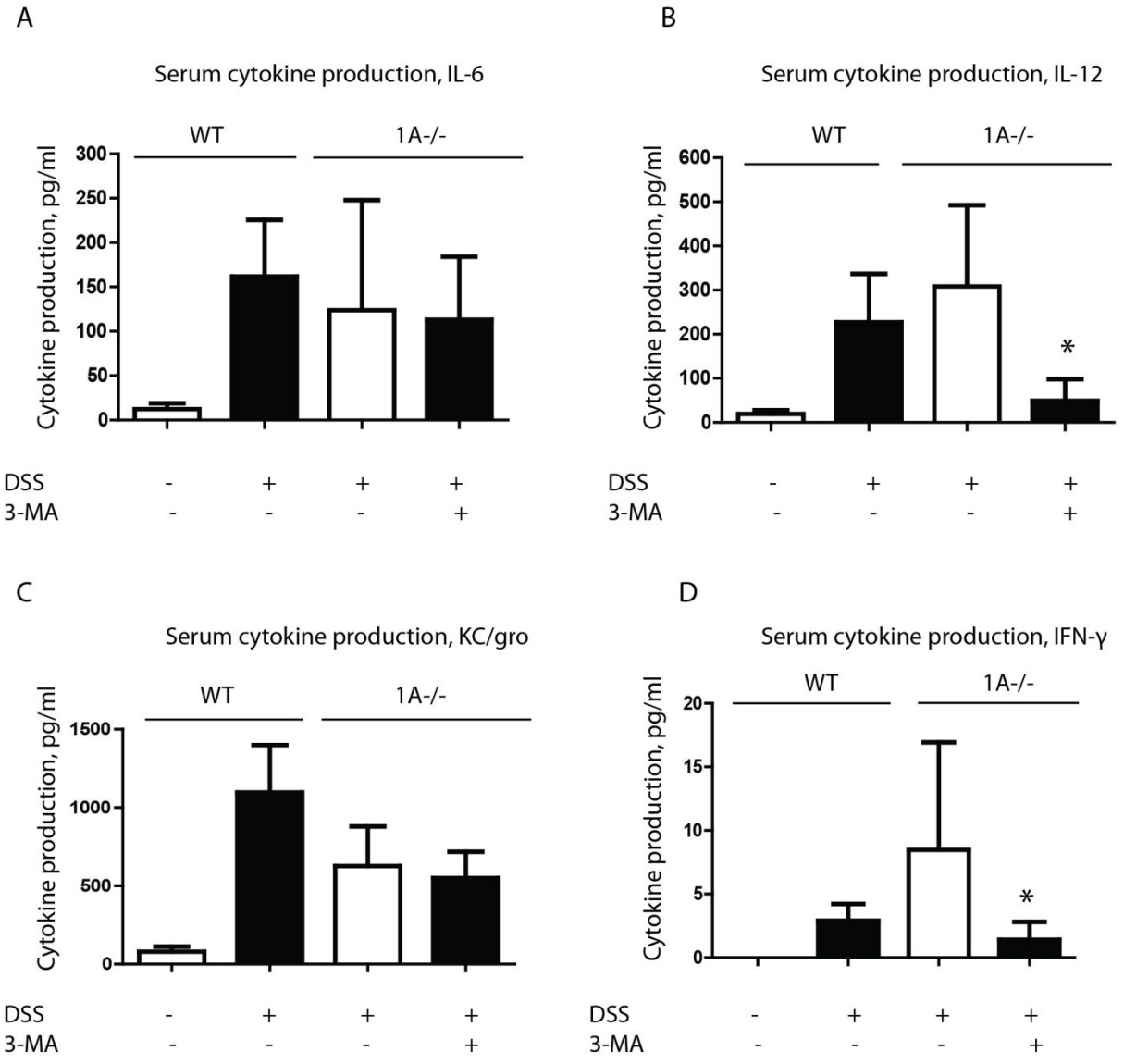


Figure 4.7: Serum cytokine analysis for WT and *Rassf1a*$^{-/-}$ mice with different treatments (harvested at day 8). DSS-treated *Rassf1a*$^{-/-}$ mice with 3-MA revealed decrease in inflammatory cytokine production versus *Rassf1a*$^{-/-}$ mice treated only with DSS. (A) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (-/+ 3-MA)=0.46, (B) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (-/+ 3-MA)=0.08, (C) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (-/+ 3-MA)=0.4.(D) p value for DSS-treated *Rassf1a*$^{-/-}$ mice (-/+ 3-MA)=0.18. n= 3-5 for (-/+3-MA). (*) mean significant p value.

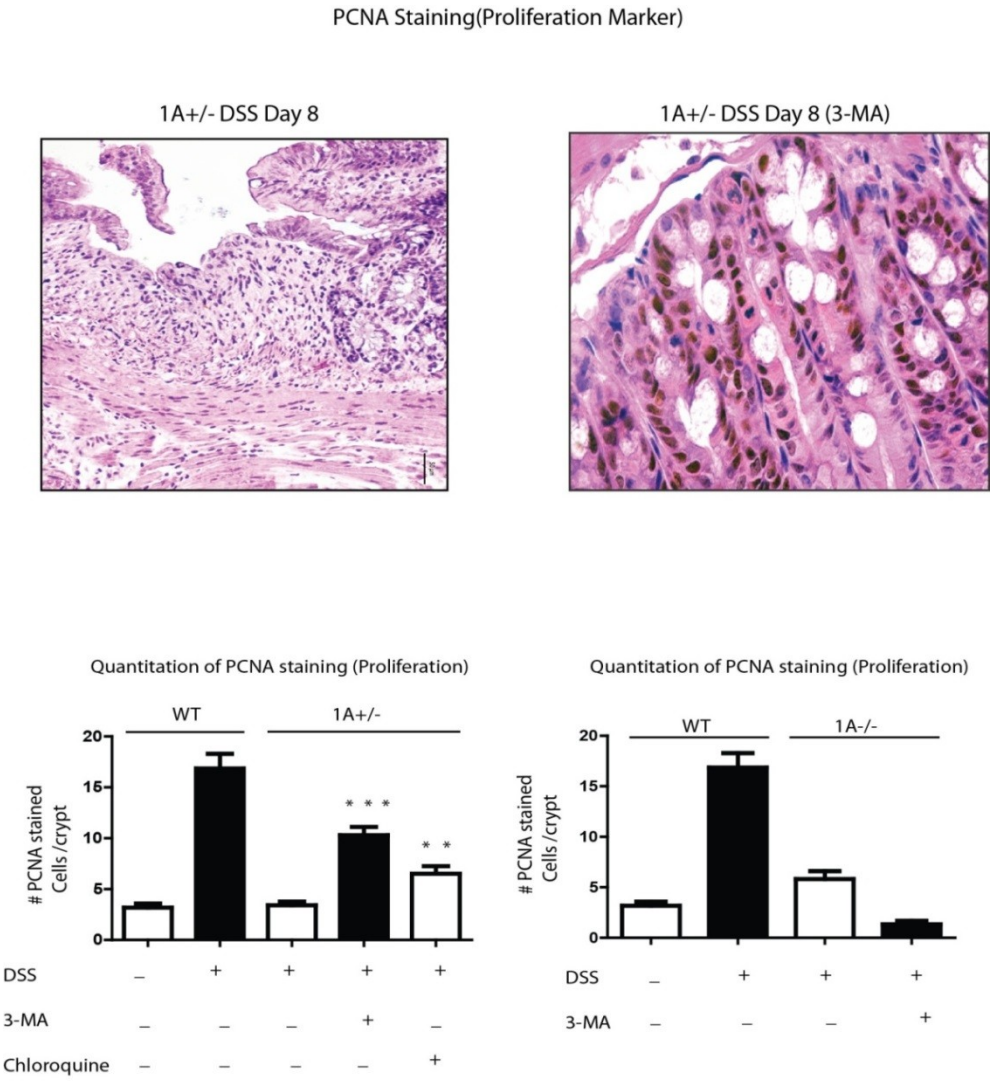


Figure 4.8: Biotinylated PCNA staining for colon sections (harvested at day 8) was detected using diaminobenzidine (DAB) streptavidin. HRP appears as a brown precipitated over the H&E stained sections (upper panels). Quantitation analysis for number of positive PCNA stained cells/crypt (proliferation) in colonic sections (lower panels). DSS-treated $1a^{+/-}$ with anti-autophagy drugs (3-MA and chloroquine) have increased crypt cells proliferation but DSS - treated $1a^{-/-}$ have decreased crypt cells proliferation when compared to mice treated only with DSS. p value for DSS-treated $1a^{+/-}$ (-/+ 3-MA) = 0.0001 and for DSS-treated $1a^{+/-}$ (-/+ chloroquine) = 0.0012. n = 3-5 for all. (*) mean significant p value.

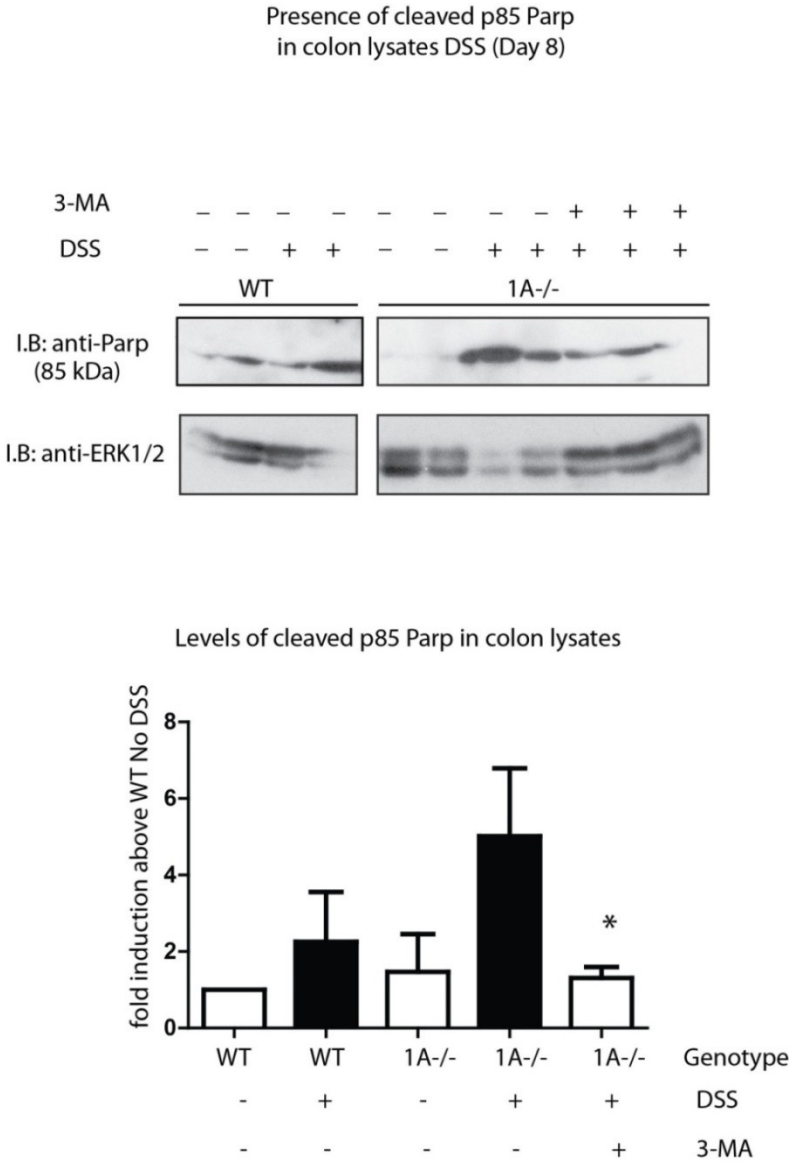


Figure 4.9: Colon lysates (harvested at day 8) from WT and *Rassf1a*$^{-/-}$ mice with different treatments were immunoblotted for Parp to detect levels of cleaved p85 Parp (late marker of apoptosis). Normally, DSS-treated *1a*$^{-/-}$ mice have high levels of cell death (cleaved p85parp) but DSS -treated *1a*$^{-/-}$ mice with 3-MA had decreased cell death, indicated by low levels of cleaved p85 Parp in colon lysates (upper panel). We did graph analysis for fold induction for immunoblotting results (lower panel). p value for DSS-treated *1a*$^{-/-}$ (-/+ 3-MA)= 0.036. n=4-6 for all. (*) mean significant p value.

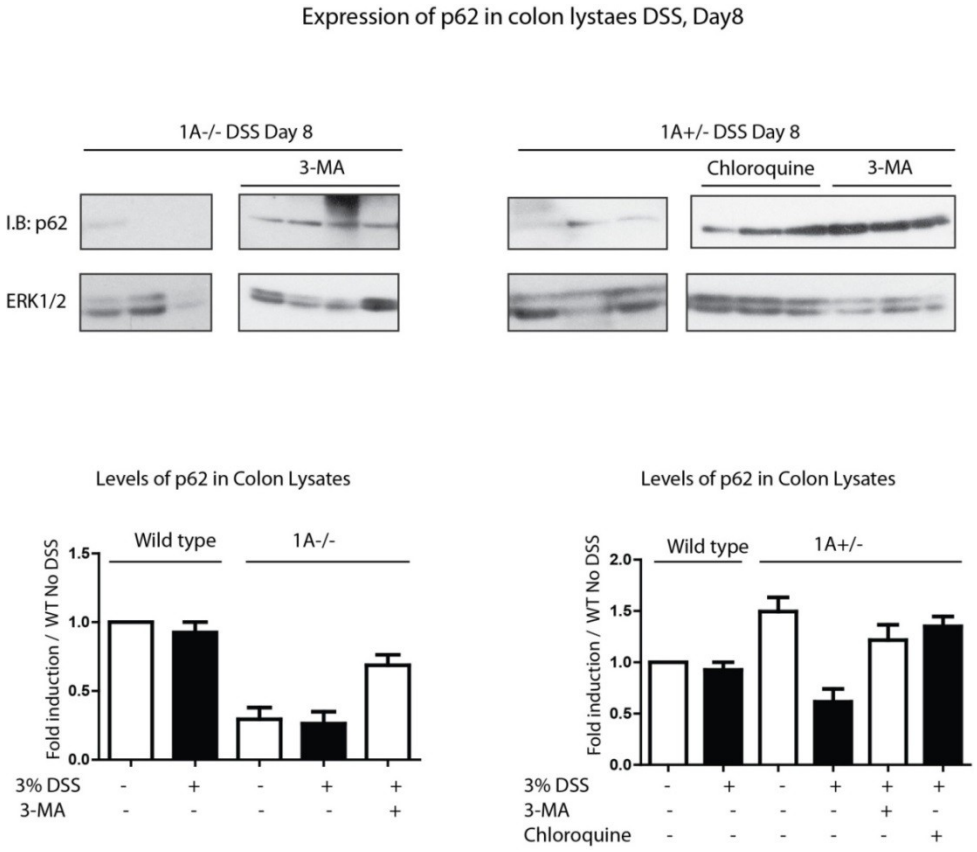


Figure 4.10: Colon lysates (harvested at day 8) from *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice with different treatments were immunoblotted for p62 (upper panels). There was increased autophagic flux in DSS-treated *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$, detected by decreased levels of p62 in colon lysates. In contrast, DSS-treated *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice with 3-MA and DSS-treated *Rassf1a*$^{+/-}$ mice with chloroquine have decreased autophagic flux, detected by increased levels of p62 in colon lysates. In addition, untreated *Rassf1a*$^{-/-}$ mice have increased autophagic flux when compared to *Rassf1a*$^{+/-}$ mice, indicated by western blotting for p62 (data not shown). We did graph analysis for fold induction of the immunoblotting results (lower panels). p value for DSS-treated *1a*$^{+/-}$ (-/+ 3-MA)= 0.0108 and (-/+ chloroquine)= 0.0010. n=4-5 for all. p value for DSS-treated *1a*$^{-/-}$ (-/+ 3-MA) =0.0106. n= 4-5 for all. (*) mean significant p value.

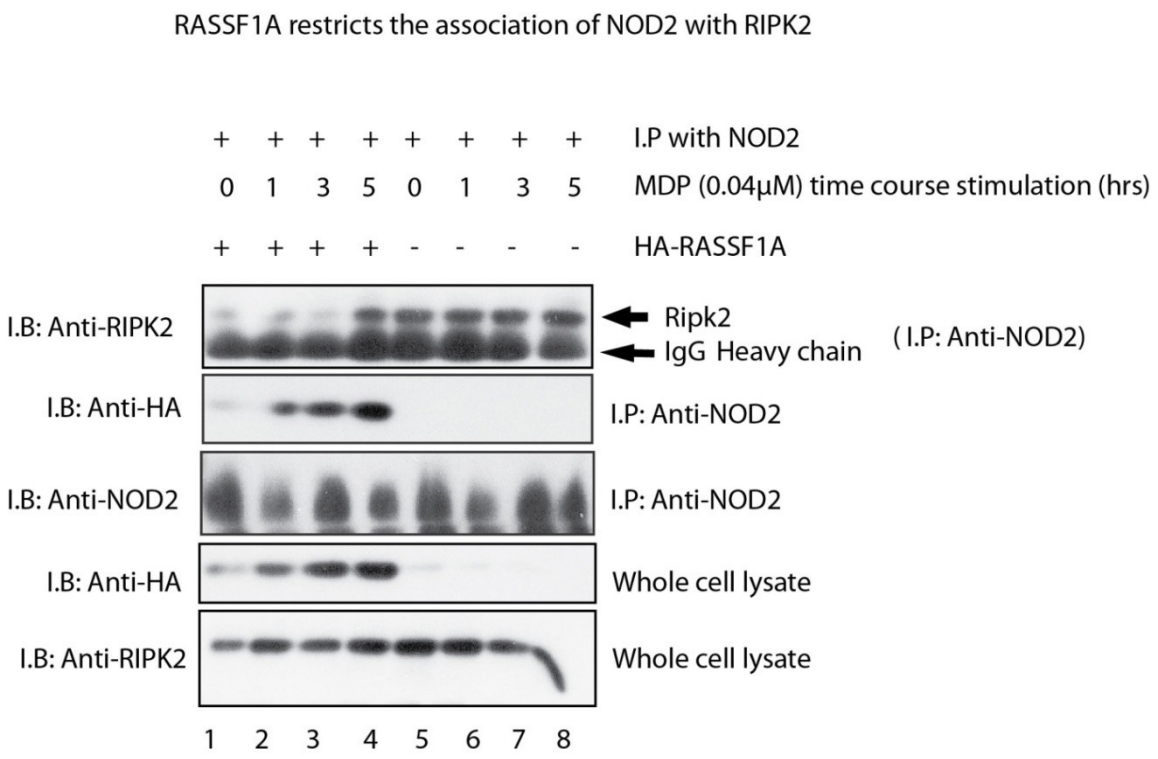


Figure 4.11: HEK-293 cells were transiently transfected with/or without *HA-RASSF1A*, stimulated with MDP (0.04μM) with different time courses (0, 1, 3 and 5 hrs), immuno-precipitated with anti- NOD2 antibody and immunoblotted as indicated. RASSF1A restricted the association of NOD2 with RIPK2, detected by immunoblotting for RIPK2. At 5hrs we can see association between RIPK2 and NOD2, suggesting that at 5 hrs RASSF1A loses its ability to interfere with this association.

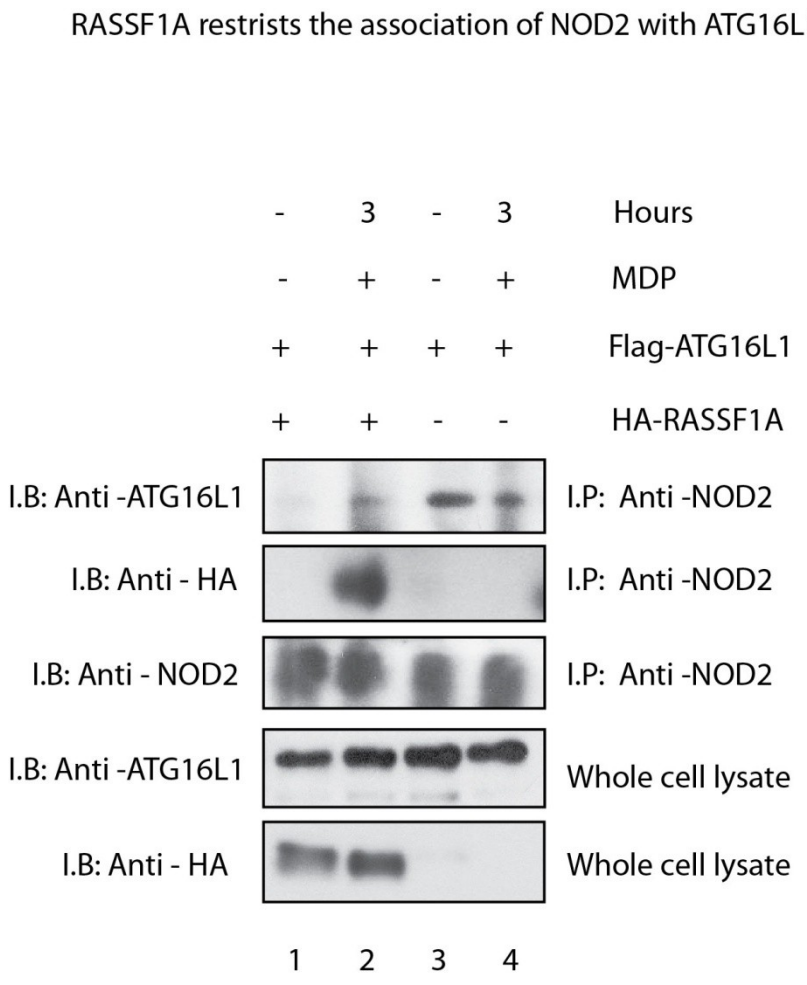


Figure 4.12: HEK-293 cells were transiently transfected with *Flag-ATG16L1* and with/without *HA-RASSF1A*, stimulated with MDP, 0.04μM (0 and 3 hrs) and immuno-precipitated with anti-NOD2 antibody and immunoblotted as indicated. RASSF1A restricted the association of NOD2 with ATG16L1, detected by immunoblotting for ATG16L1.

Chapter 5

The effect of PTK inhibitor, imatinib mesylate and the autophagy inhibitor, 3-MA on the survival of DSS-treated *IL-10*$^{-/-}$ mice

5.1 Introduction and Rationale

In chapters three and four, we explored the effect of imatinib, 3-MA and chloroquine on our mouse model missing RASSF1A. In this chapter we investigated the potential effects of 3-MA and imatinib on a reliable and established IBD mouse model, the *IL-10*$^{-/-}$ mice to support the effectiveness of inhibiting autophagy and c-Abl kinase activity. IL-10 is an anti-inflammatory cytokine essential for immune-regulation of the GIT, produced by dendritic cells, thymocytes, B cells, macrophages and T cells [310]. IL-10 regulates both adaptive and innate immunity by restricting macrophage function and inhibiting Th1 responses. IL-10 plays a major role in maintaining regulation of inflammatory responses to beneficial bacteria and normal antigens [311]. IL-10 deficient mice spontaneously develop a transmural enterocolitis by 2-3 months of age similar to CD in humans [312] and they are very sensitive to DSS treatment. Furthermore, patients with early-onset enterocolitis are associated with mutations in genes encoding *IL-10R* (IL-10 receptor), leading to exaggerated inflammatory responses in the intestine and increase in pro-inflammatory cytokine production such as TNF-alpha [313]. In addition, patients with Crohn's disease are ameliorated by treatment with recombinant interleukin-10 producing bacteria, demonstrating the importance of IL-10 for counteracting the hyperactive immune response in IBD patients [105]. All of these findings suggest that *IL-10*$^{-/-}$ mouse is an interesting model for IBD drug study.

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5.2 Results

5.2.1 The effect of autophagy inhibitor, 3-MA on DSS-treated *IL-10* $^{-/-}$

Autophagy inhibitor, 3-MA protected DSS-treated *Rassf1a*$^{+/-}$ mice from damaging effects caused by the increased autophagic response therefore, we decided to explore the effectiveness of inhibiting autophagy using 3-MA on DSS-treated *IL-10*$^{-/-}$ mice. *IL-10*$^{-/-}$ mice (4-5 months old) were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Anti-autophagy, 3-MA (20 µg/g body weight) was injected intraperitoneally on day 4 and 7. We speculated that inhibiting the autophagic response as early as day 3 might be detrimental to DSS-treated *IL-10*$^{-/-}$ mice as their autophagic response should be properly regulated due to the presence of RASSF1A. In addition, the clinical onset of the disease is delayed 1-2 days in DSS-treated *IL-10*$^{-/-}$ mice when compared to DSS-treated *Rassf1a* knockout mice, so we decided to begin 3-MA treatment on day 4 instead of day 3. Autophagy inhibitor, 3-MA significantly protected DSS-treated *IL-10* $^{-/-}$ mice and there was significant increase in survival rate (from 0% to 60 %) and significant decrease in disease severity (Fig.5.1). Normally, *IL-10*$^{-/-}$ mice are very sensitive to DSS treatment and they have to be euthanized between day 7 and day 10 due to massive rectal bleeding and increased disease indices.

Data presented above, suggest that 3-MA significantly reversed the damaging effects of DSS induced intestinal inflammation in *IL-10*$^{-/-}$ mice by inhibiting the autophagic response that might be detrimental to epithelial repair and healing. More investigations have to be done to analyse the autophagic response in *IL-10* $^{-/-}$ mice by exploring levels of p62 (marker of autophagic flux) and LC3-II (a marker of autophagosome formation) in colon lysates.

5.2.2 The effect of PTK inhibitor, imatinib (gleevec) on DSS-treated *IL-10*$^{-/-}$ mice

Imatinib protected *Rassf1a*$^{+/-}$ mice from DSS inflammation-induced injury by inhibiting the c-abl kinase activity and probably inhibiting tyrosine phosphorylation of YAP, so we decided to explore the effect of inhibiting c-abl kinase activity using imatinib on DSS-treated *IL-10*$^{-/-}$ mice. *IL-10*$^{-/-}$ mice (4-5 months old) were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Imatinib (80 µg/g body weight) was injected intraperitoneally on days 2, 4, 6 and 8. Unfortunately, imatinib did not fully protect DSS-treated *IL-10* $^{-/-}$ mice, the survival rate increased from 0% to 33.3% and there was insignificant decrease in disease severity (Fig.5.2).

These results suggest that inhibiting the c-abl kinase activity and probably decreasing cell death using imatinib is essential but not sufficient for complete recovery from inflammation-induced injury. More investigations have to be done to analyse cell death and several markers of proliferation including Yes-associated protein (YAP)-driven proliferation in *IL-10*$^{-/-}$ mice.

5.2.3 The effect of (3-MA+ imatinib) on DSS-treated *IL-10* $^{-/-}$

IL-10$^{-/-}$ mice(4-5 months old) were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Anti-autophagy, 3-MA was injected intraperitoneally on days 3 and 6 and PTK inhibitor, imatinib (80 ug/g body weight) was injected on days 2, 4, 6 and 8. In previous experiments we inhibited the c-abl kinase activity (via imatinib) and the autophagic response (via 3-MA) separately. So we decided to explore the outcome of inhibiting both (the autophagic response and c-abl kinase activity) at the same time using (3MA+imatinb), on DSS-treated *IL-10*$^{-/-}$ mice.

There was a significant increase in survival rate (from 0% to 66.6 % survival), the p value = 0.074 and there was significant decrease in disease severity, the p value = 0.024 (Fig.5.3). DSS

treated IL-10$^{-/-}$ mice are significantly protected when they are treated with 3-MA suggesting that the damage from DSS might be through the active autophagic response and inhibiting the c-abl kinase activity only is essential, but not enough to fully protect DSS treated mice and it has to be combined with anti-autophagy drugs (3-MA) to reverse the damaging effects caused by the autophagic response under DSS treatment. The overall survival rate of *IL-10*$^{-/-}$ mice treated with 3-MA was suprisingly comparable to *IL-10*$^{-/-}$ mice treated with 3-MA + imatinib but Fig. 5.lA and 5.2A reveal that these drug combinations have different kinetics of effectiveness that we are just beginning to understand.

5.2.4 Cytokines analysis for (3MA + Imatinib) treatment for DSS-treated *IL-10* $^{-/-}$ mice

We analysed serum cytokines for WT and *IL-10* $^{-/-}$ mice with different treatments (harvested at day 8). There was significant decrease in pro-inflammatory cytokine production (IFN-γ and IL-12) under (3MA+ imatinib) treatment for DSS-treated *IL-10* $^{-/-}$ mice vs *IL-10* $^{-/-}$ mice treated only with DSS (Fig.5.4).

These results suggest that treatment with (3-MA + imatinib) might restrict NF-κB induced pro-inflammatory cytokine production, thereby, decreasing inflammation, enhancing repair and healing. The decrease in inflammatory cytokines production under treatment with (3-MA+ imatinib) might be one of the main contributing factors that aid in decreasing disease severity and increasing recovery after DSS treatment in *IL-10* $^{-/-}$ mice.

5.2.5 Proliferation analysis for *IL-10* $^{-/-}$ mice

We carried out immunohistochemical staining for PCNA on descending colons from WT and *IL-10* $^{-/-}$ mice with different treatments (mice were harvested at day 8).

There was significant increase in PCNA staining for DSS-treated *IL-10* $^{-/-}$ mice with 3-MA and with (3-MA+ imatinib) versus *IL-10* $^{-/-}$ mice treated only with DSS. The average number of positive PCNA stained cells/crypt for DSS-treated *IL-10* $^{-/-}$ mice was 3 and increased to be 14.3 with 3-MA and 9.5 with (3-MA + imatinib) (Fig. 5.5).

Data proposed above suggest that treatment with 3-MA or (3-MA+ imatinib) enhanced repair by inhibiting the damaging effects caused by the autophagic response or the autophagic response and c-abl kinase activity that might be detrimental to proliferation and repair.

5.3 Conclusion

Inhibiting the autophagic response (using 3-MA) or the autophagic response and c-abl kinase activity (using 3-MA+ imatinib) reversed the damaging effects of DSS inflammation injury in DSS treated *IL-10*$^{-/-}$ mice and there was significant increase in survival rate, significant decrease in disease severity, decrease in pro-inflammatory cytokines production and increase in epithelial cells proliferation. In contrast, DSS treated *IL-10*$^{-/-}$ with imatinib were partially protected from DSS induced- inflammation injury. Based on data presented in this chapter we suggest that the DSS damage in *IL-10*$^{-/-}$ mice might be through active autophagic response, indicated that DSS treated *IL-10* $^{-/-}$ mice are fully protected only when the autophagic response is inhibited by 3-MA. Inhibiting the c-abl tyrosine kinase activity and probably decreasing cell death using imatinib are essential but not sufficient for complete recovery from inflammation-induced injury. DSS treated *IL-10*$^{-/-}$ mouse is an interesting mouse model for IBD drug study and pharmacological inhibition of the autophagic response with/without the inhibition of c-abl kinase activity might be a novel therapeutic approach for IBD treatment.

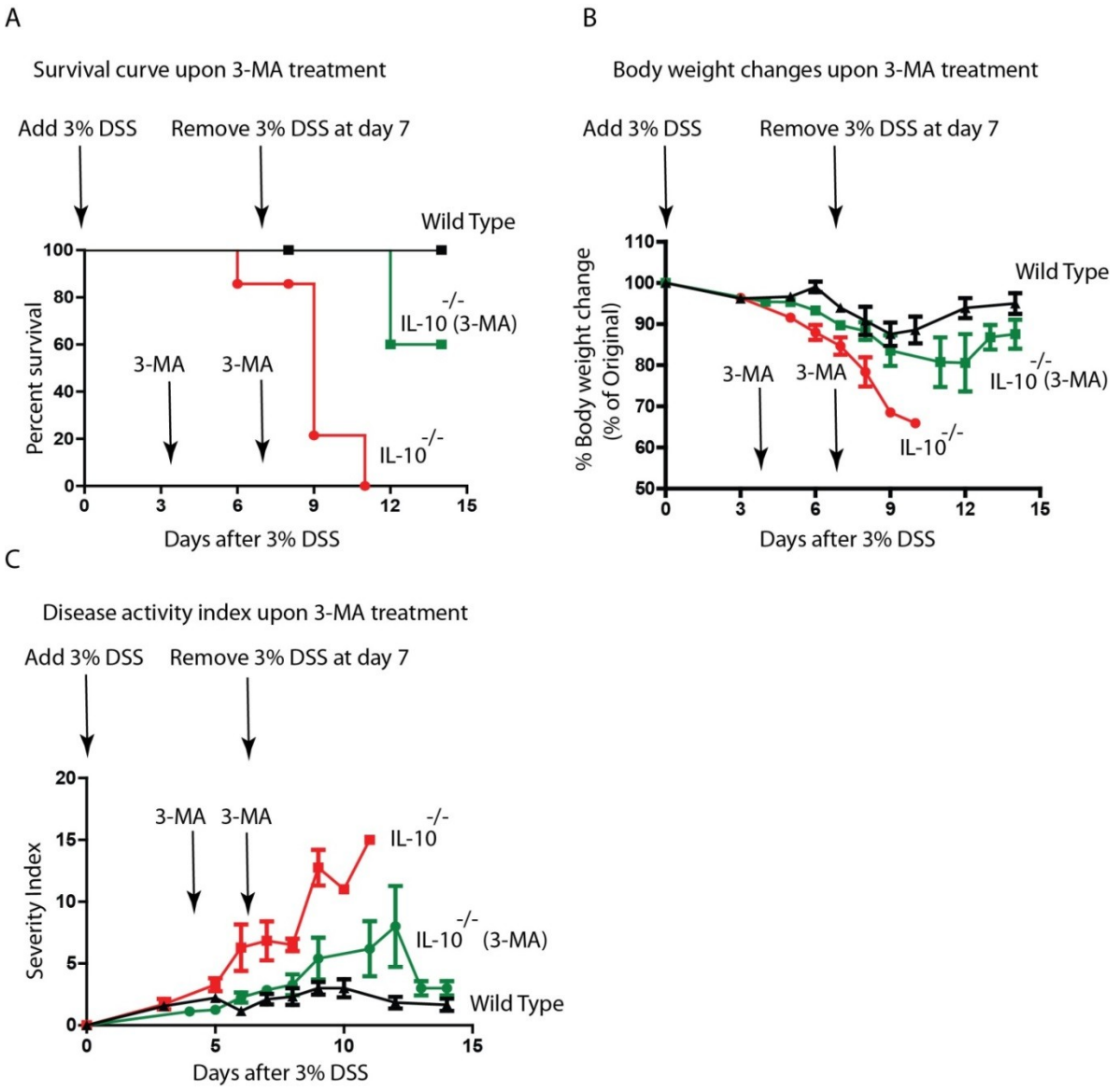

Figure 5.1: Autophagy inhibitor, 3-MA, protected DSS-treated *IL-10* $^{-/-}$ mice from inflammation-induced injury. Autophagy inhibitor, 3-MA (20 µg/gram body weight) was injected intraperitoneally on day 3 and 6. (A) p value for DSS-treated *IL-10* $^{-/-}$ mice (- /+ 3-MA) = 0.063, (B) p value for DSS-treated *IL-10* $^{-/-}$ mice (-/+ 3-MA) = 0.12 and (C) p value for DSS-treated *IL-10* $^{-/-}$ mice (-/+ 3-MA)= 0.0213. n=7-10 for all.

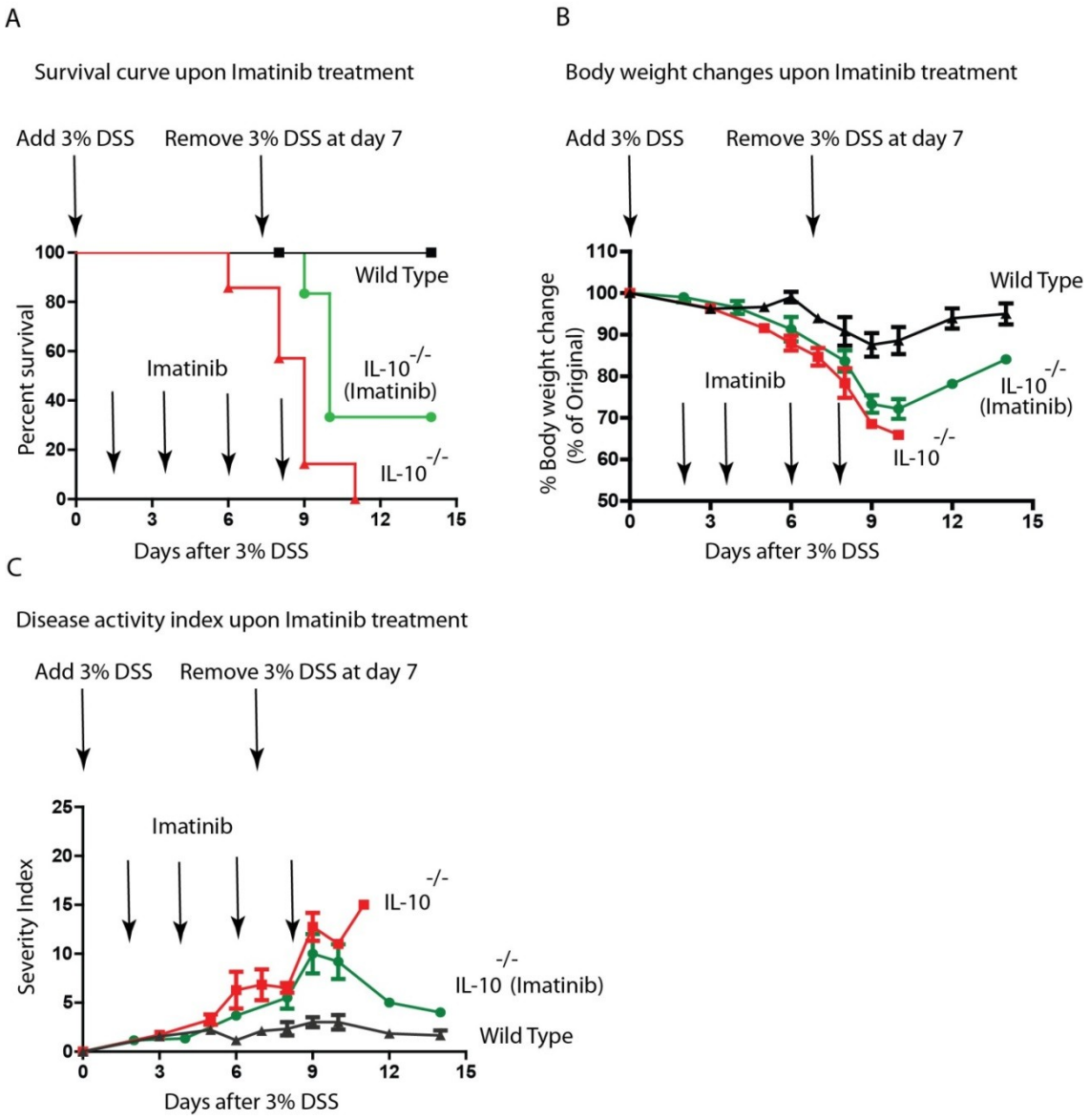

Figure 5.2: PTK inhibitor, imatinib partially protected DSS-treated *IL-10* $^{-/-}$ mice from inflammation –induced injury. Imatinib (80 µg/g body weight) was injected intraperitoneally on days 2, 4, 6 and 8. p value for DSS-treated *IL-10* $^{-/-}$ mice (survival rate) (-/+ imatinib) = 0.34 and p value for DSS treated *IL-10* $^{-/-}$ mice (DAI) (-/+ imatinib)= 0.109. n=7-10 for all.

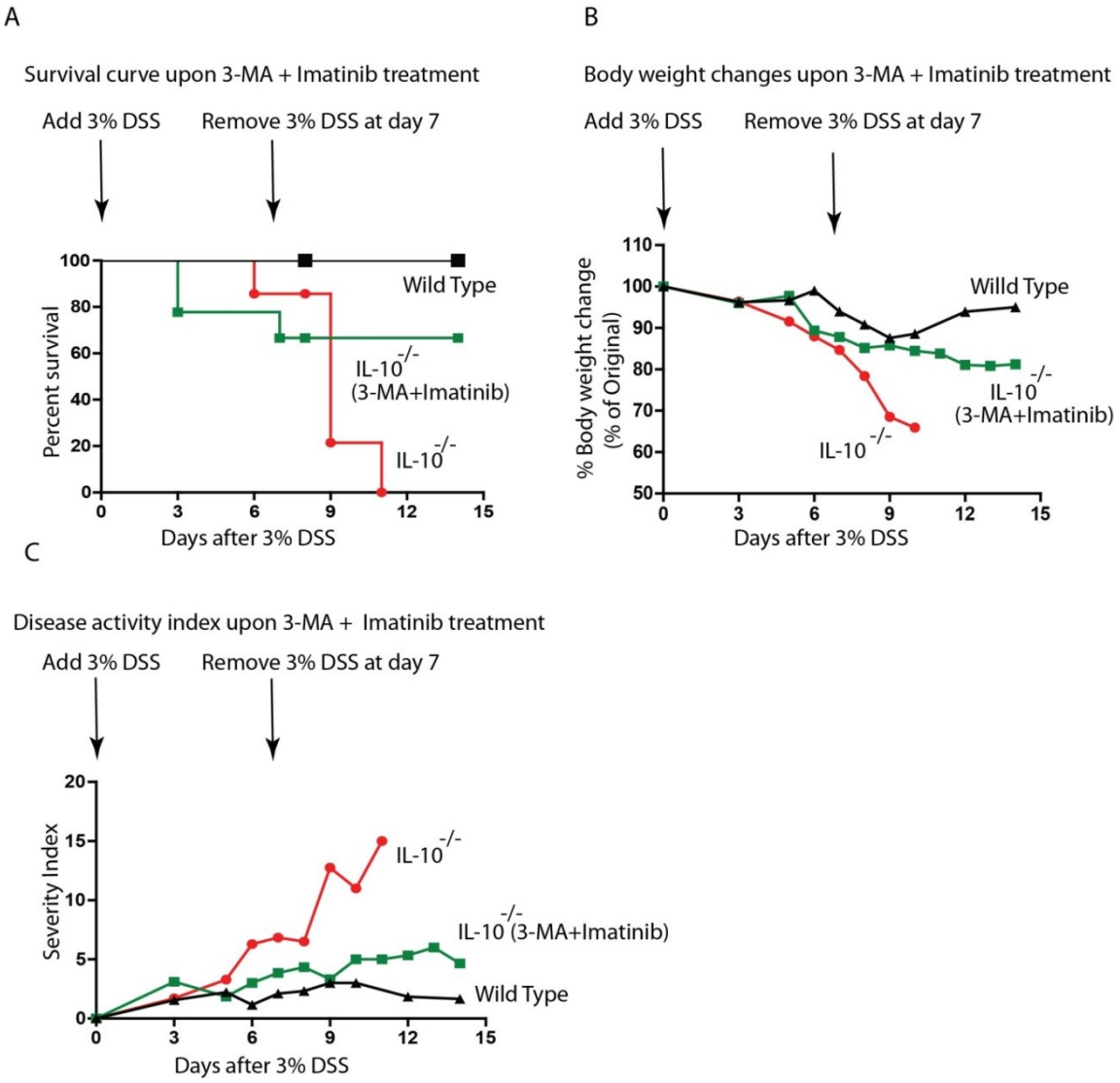

Figure 5.3: PTK inhibitor, imatinib + autophagy inhibitor, 3-MA protected DSS-treated *IL-10* $^{-/-}$ mice from inflammation induced injury and there was significant increase in survival rate. Imatinib (80µg/g body weight) and 3-MA (20 µg/g body weight) were injected intraperitoneally on days (2, 4, 6 and 8) and days (3 and 6) respectively. (A) p value for DSS treated *IL-10* $^{-/-}$ [-/+ (3-MA + Imatinib)] = 0.074. (B) p value for DSS-treated *IL-10* $^{-/-}$ mice = 0.204 [-/+ (3-MA + Imatinib)] and (C) for DSS-treated *IL-10* $^{-/-}$ mice [-/+(3-MA + Imatinib)] =0.024. n= 7-10 for all.

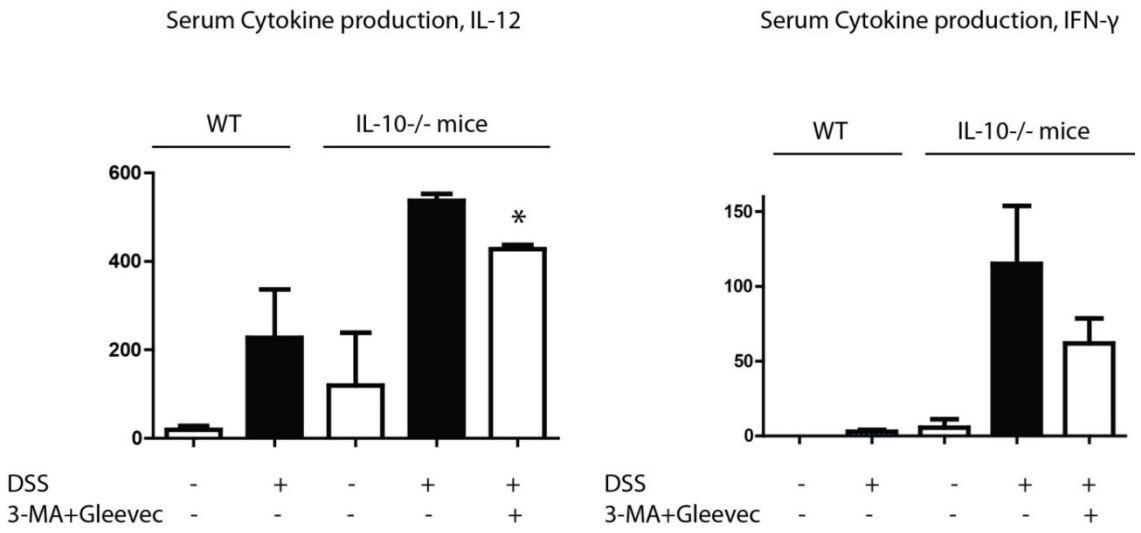

Figure 5.4: Serum cytokine analysis for WT and *IL-10* $^{-/-}$ mice with different treatments. DSS-treated *IL-10* $^{-/-}$ mice with (3-MA+ imatinib) revealed decreased levels of IL-12 and IFN-γ vs *IL-10* $^{-/-}$ mice treated only with DSS. p value for IL-12 [-/+(3-MA + imatinib)] = 0.0130. p value for INF-gamma [-/+(3-MA + imatinib)]= 0.16. n= 3-5 for all. (*) mean significant p value.

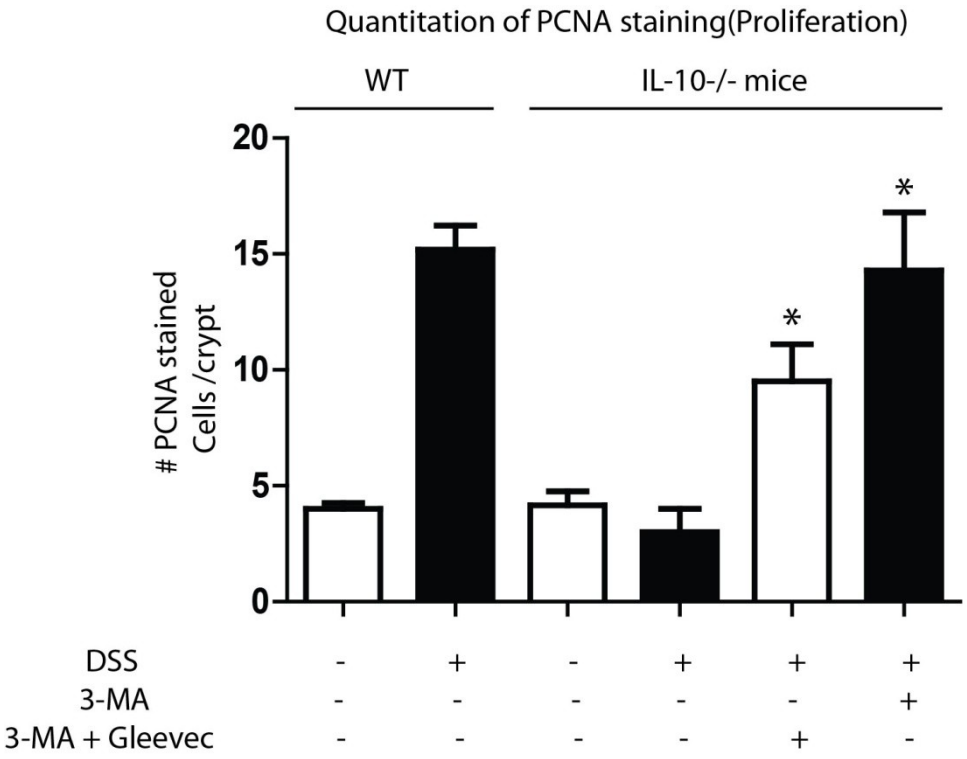

Figure 5.5: Quantitation analysis for number of positive PCNA stained cells/crypt (proliferation) in colon sections. DSS-treated *IL-10* $^{-/-}$ mice with 3-MA and with (3-MA+ gleevec) have increased proliferation versus *IL-10* $^{-/-}$ mice treated only with DSS. p value for DSS-treated *IL-10* $^{-/-}$ [-/+ (3-MA + Gleevec)] = 0.0375 and for DSS-treated IL-10-/- (-/+ 3-MA)= 0.0262. n= 3-4 for all. (*) mean significant p value.

Chapter 6

Discussion, future experiments and summary

6.1 Discussion

Recently, over 160 susceptibility genes have been identified as IBD susceptible genes [33]. Knockout mice models helped in understanding the pathophysiological mechanism that may be responsible for disease initiation and progression but unfortunately, there is no single animal model can explain all the pathophysiology behind IBD. Linkage studies have implicated several genomic regions containing IBD susceptibility genes including linkages on 3p21 that involve macrophage stimulating1 gene (MST1) [44] and Ras-association domain family member 1A gene (RASSF1A or 1A) [47, 49]. Recently, our lab proposed *Rassf1a* knockout mouse as a novel model for IBD, acute treatment of *Rassf1a*$^{-/-}$ mice with DSS resulted in symptoms of experimental colitis including, increased NF-κB activity, increase levels of serum inflammatory cytokines, significant loss of colon length, decreased intestinal epithelial repair and decrease in survival rate. Surprisingly, the *Rassf1a*$^{+/-}$ mice also demonstrated similar symptoms and survival rate to *Rassf1a*$^{-/-}$ mice suggesting haploinsufficiency at the *Rassf1a* locus [82]. We speculate that DSS-treated *Rassf1a*$^{+/-}$ mouse could be an interesting model for IBD drug study, as the epigenetic loss of *1A* occurs early during chronic inflammation and an effective therapy may be needed in heterozygotes to suppress any abnormal pathways that may be differentially regulated due the loss of one allele of 1A. Further analysis for DSS-treated *Rassf1a* knockout mice, revealed reduction in markers of proliferation (especially YAP- driven proliferation) with increase in pY-YAP levels in colon lysates, coincided with increased Bax expression, increased cell death and poor recovery [82]. Previous studies have suggested a major role for c-Abl driven tyrosine 357 phosphorylation of YAP in response to DNA damage, which is prominent during intestinal inflammation injury stimulated by DSS [161, 162]. This induces the formation of a pY-

YAP/p73 complex to drive pro-apoptotic gene expression, such as Bax [165]. Furthermore, there was significant increase in the autophagic response (detected by immunoblotting for p62) in colon lysates from DSS-treated *Rassf1a* knockout mice associated with decreased repair and poor recovery. Several lines of evidence support an involvement of 1A and autophagy in disease appearance in IBD: (i) a SNP in the autophagy gene, *ATG16L1*, was found in IBD patients and *Atg16*$^{-/-}$ hematopoietic cells have increased levels of inflammatory cytokines IL-1β and IL-18 [43, 217, 232] (ii) Polymorphisms in *NOD2* have been found in CD patients and NOD2 polymorphisms are associated with childhood onset of CD. Furthermore, NOD2 is known to be involved in autophagy via recruiting ATGL16L1[206, 314] (iii) The 1A interacting protein, MAP1S/C19ORF5 was found to associate with LC3 (a mammalian homolog of yeast Atg8) and act as a trigger for autophagy in order to suppress genome instability [141, 315] (iv) increase in the autophagic response (decreased levels of p62) was detected in colon extracts from DSS-treated *Rassf1a*$^{-/-}$ mice (v) Our lab performed microarray analysis on descending colon snips from DSS-treated *Rassf1a* $^{-/-}$ mice, revealed upregulation of autophagy related genes such as Atg4c (8 fold increase), Atg2b (2.02 fold increase) and Laptm5 (2.6 fold increase) (data not shown) (vi) As mentioned before, *1A*$^{-/-}$*Nod2*$^{-/-}$ mice have higher survival rates and decreased disease severity upon DSS treatment when compared to *1A*$^{-/-}$ mice to suggest that NOD2-driven inflammation induces damage and this why *Rassf1a*$^{-/-}$ animals are sensitive to DSS-induced inflammation injury. (vii) Lastly, 1A forms robust association with NOD2 and 1A can negatively regulate NOD2 signaling by interference with NOD2 associations with RIPK2 and ATG16L1. Based on the above data we speculate that upon DSS treatment, the lack of RASSF1A resulted in uncontrolled autophagic response via NOD2 that may be detrimental to epithelial repair and results in poor recovery from inflammation insults.

Therefore, RASSF1A has a novel regulatory role during the recovery phase post inflammation induced injury by restricting both pY-YAP and autophagy to inhibit inflammation and promote efficient epithelial repair. In this thesis, we investigated the Hippo pathway co-transcriptional activator, YAP, the importance of NOD2-driven inflammation and autophagic response in promoting poor survival in DSS-treated *1A*$^{-/-}$ animals and mechanistically how 1A can modulate NOD2 signaling. More importantly, we have identified two therapeutic targets that may alleviate the detrimental effects of acute and chronic inflammation, protein tyrosine kinase inhibitors (such as gleevec) and autophagic inhibitors (such as 3-MA and chloroquine.).

Interestingly, the use of imatinib/gleevec had promising results in reversing the damaging effects caused by DSS inflammation –induced injury in *Rassf1a*$^{+/-}$ background but not in the *Rassf1a*$^{-/-}$ background. It appears that differential effects on p53 may explain this difference as the modified and accumulated p53 may have resulted in more cell death and poor recovery. Investigations have to be done to identify this p53 modification and if unique it might be a novel marker for IBD prognosis.

Furthermore, treatment with 3-MA, reversed the damaging effects caused by DSS induced-inflammation but only in *Rassf1a*$^{\text{IEC-KO}}$ and *Rassf1a*$^{+/-}$ background. In a *Rassf1a*$^{-/-}$ background, 3-MA did not protect the mice and there was poor recovery after DSS treatment that might be due to the dramatic decrease in epithelial cell proliferation. We speculate that the loss of both alleles is causing unfavourable events induced by pathways other than autophagy that cannot be inhibited by 3-MA. However, 3-MA treated *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice revealed reduction in pro-inflammatory cytokine production. This might be explained by the inhibitory effect of 3-MA on autophagy as it has been demonstrated that MDP-stimulated activation of NF-

κB was significantly reduced when autophagy was blocked by chemical inhibitors or knockdown of Atg16L1 expression [205]. Therefore, we speculate that inhibiting autophagy (or ATG16L1) with 3-MA might aid in restricting NF-κB induced pro-inflammatory cytokine production via the upstream inhibition of PI3K and deactivating ATG16L1. On the other hand, chloroquine partially reversed the damaging effects caused by DSS- induced inflammation in *Rassf1a*$^{+/-}$ mice; this might be due to the inhibitory effect of chloroquine on TLR2 and TLR9 signalling. Recently, a study demonstrated that chloroquine can significantly suppress the TLR2 as well as the TLR9 signalling, in both vitro as well as in vivo experiments [290]. Studies showed that TLR2 and TLR9 are essential for intestinal epithelial barrier defence and defects in TLRs signalling specially TLR2 might increase susceptibility to intestinal mucosa injury and colitis [316, 317].

Since we had promising results with 3-MA and imatinib in DSS-treated *Rassf1a*$^{+/-}$ mice so we decided to support our hypothesis by exploring the effect of inhibiting c-abl kinase activity (using imatinib) and /or the autophagic response (using 3-MA) in an established IBD mouse model like *IL-10* $^{-/-}$. Interestingly, inhibiting the autophagic response (using 3-MA) or the autophagic response and c-abl kinase activity (using 3-MA+ imatinib), reversed the damaging effects of DSS intestinal inflammation. In contrast, inhibiting the c-abl kinase activity only (using imatinib) partially reversed the damaging effects of DSS induced inflammation. Indicating, that the damage from DSS in *IL-10*$^{-/-}$ mice might be through the autophagic response and inhibiting the c-abl kinase activity is essential but not enough for a full recovery from DSS inflammation. More investigations have to be done to explore the autophagic response and YAP-driven proliferation in *IL-10*$^{-/-}$ mice

In this thesis project we presented data suggesting that imatinib, 3-MA and chloroquine could be novel therapeutic approaches to enhance recovery from inflammation-induced injury and be useful in treating and saving IBD patients from increased risk of getting cancer later in life.

6.2 Future experiments

Based on results from this thesis, future experiments will include: (1) Detailed analysis of the molecular association of RASSF1A and NOD2. This can be accomplished by exploring the interaction between different constructs of both RASSF1A and NOD2 by immunoprecipitation (I.P) experiments, utilizing 1A mutations and deletions constructs that include mutants are within the TNF-R1 interacting zinc finger domain, Ras binding domain, ATM phosphorylation site, 14-3-3 association sites and the SARAH domain at the C-terminus[123, 318]. In addition, NOD2 mutants within the N terminal CARD domain, C-terminal leucine rich repeat and nucleoside-triphosphatase (NTPase) domain will be also used [249]. Understanding 1A/NOD2 association may allow locking NOD2 in an inactive conformation and inhibit downstream activation of autophagy and/or NF-κB activation by using a small molecule design of peptide sequences to match the interacting surface of 1A/NOD2 (interaction mimetics). (2) Studying NOD2 associations with RIPK2 or ATG16L1 *in vivo* using colon lysates from untreated and DSS-treated *Rassf1a* knockout and Wild type mice to explore the associations in absence and presence of RASSF1A. This will help in understanding the activity of NOD2 pathway under different treatments and how NOD2 pathway could enhance disease appearance in DSS treated mice. (3) Exploring RIPK2 activity in (1and 2) using a phosphotyrosine (pY) specific antibody to pY-474 RIPK2 and ubiquitination will be also monitored using an anti-ubiquitin antibody. Following

NOD2 stimulation by MDP, RIPK2 is tyrosine phosphorylated and polyubiquitinated for its optimum kinase activity [249, 319]. Tracking RIPK2 activity is essential for monitoring autophagy and /or NF-κB activity via NOD2 pathway [320]. (4) Exploring the inhibition of NOD2 pathway in *vivo* using DSS-treated *Rassf1a* knockout mice. We speculate that NOD2-driven inflammation induces damage and this why *Rassf1a*$^{-/-}$ animals are sensitive to DSS treatment. Exploring NOD2 pathway inhibition in DSS treated *Rassf1a* knockout mice will be accomplished by two approaches. The first approach by pharmacological inhibition of RIPK2 using drugs (such as gefitinib) [249] and the second approach by direct inhibition of NOD2 using biologics, mouse-anti NOD2 to neutralize NOD2 and to interfere with downstream activation of autophagy and/or NF-κB activation. Inhibition of the downstream signalling of NOD2 will give a brief picture on the impact of NOD2 signalling activity in *Rassf1a* knockout mice. Pharmacological inhibition of NOD2 pathway might be a novel therapeutic approach for IBD. (5) Other future experiments will include biomarkers analysis for DSS-treated *IL-10*$^{-/-}$ mice. Autophagy analyses will be accomplished by immunoblotting and immunohistochemical staining for p62 (marker for autophagic flux) and LC3-II (marker for autophagosome formation), cell death analysis by immunoblotting for (p53, Bax and Parp) and analysis of YAP-driven proliferation by immunoblotting and immunohistochemical staining for pY-Yap. Biomarkers analysis will help in understanding how 3-MA and imatinib reversed the damaging effects of DSS in *IL-10*$^{-/-}$ mice. (6) Studying the role of RASSF1A in *IL-10* $^{-/-}$ mice by exploring levels of *Rassf1a* expression in *IL-10* $^{-/-}$ mice with different time courses (6, 12, 17 and 20 weeks of age) using real time PCR. We speculate that epigenetic loss of *Rassf1a* by promoter specific methylation might enhance disease appearance in *IL-10*$^{-/-}$ mice.

6.3 Summary

Both DSS-treated *Rassf1a*$^{-/-}$ *and Rassf1a*$^{+/-}$ mice are promising models to study IBD. RASSF1A has a novel regulatory role during the recovery phase post inflammation-induced injury by restricting both pY-YAP and NOD2-dependent autophagy to inhibit inflammation and promote efficient epithelial repair. Pharmacological inhibition of pY-YAP and/or autophagy reversed the damaging effects of DSS in *Rassf1a*$^{+/-}$ mice only. Imatinib and 3-MA might be novel therapeutic approaches to enhance recovery from inflammation-induced injury and be useful in treating and saving IBD patients from increased risk of getting cancer later in life.

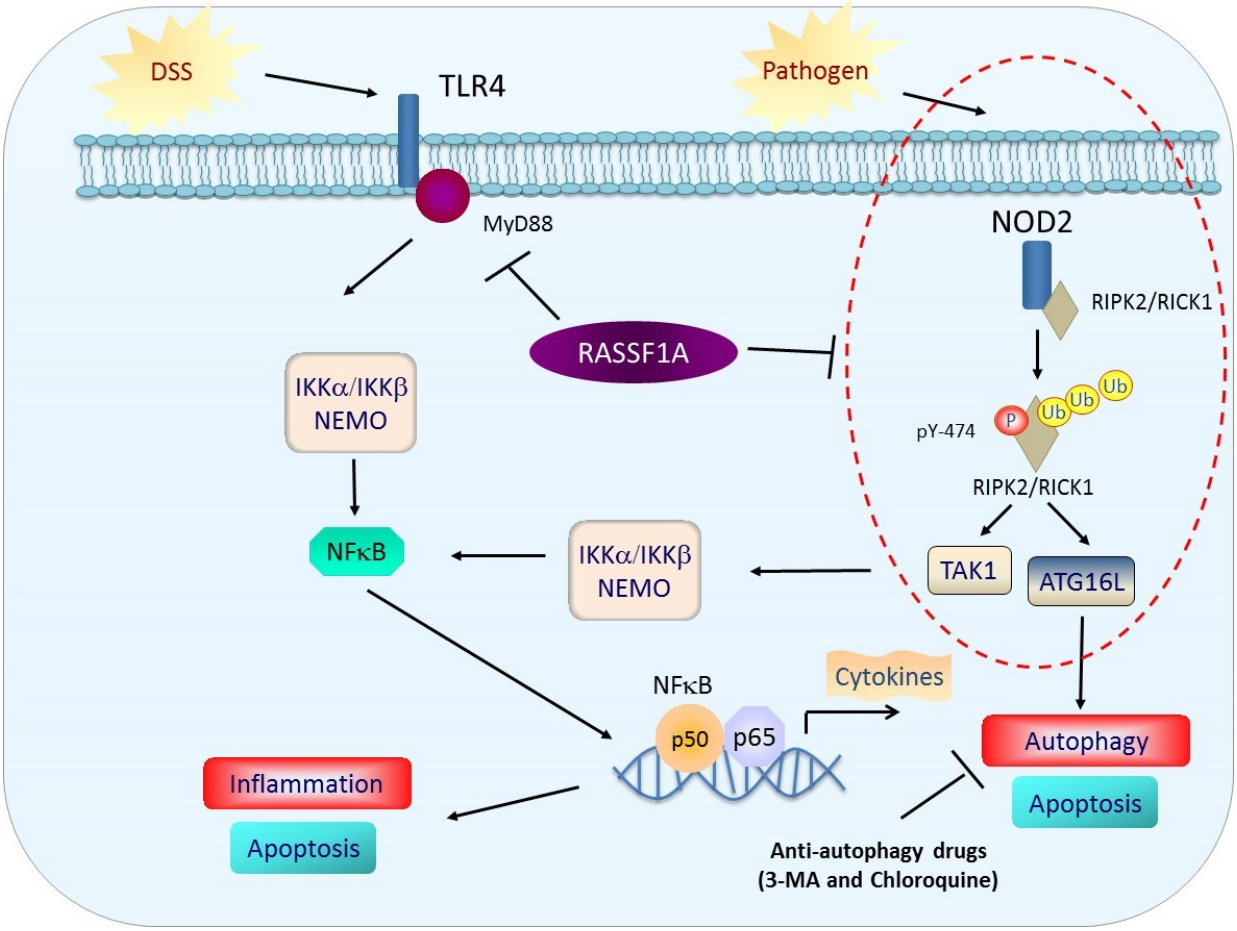

Figure 6.1: Final model for intestinal inflammation involving RASSF1A and NOD2. RASSF1A restricts NF-κB activity by interfering with Toll receptor (TLR) activation of NF-κB. High levels of NF-κB transcriptional activity can result in intestinal inflammation and abnormal activation of apoptosis leading to inflammation induced damage. In addition, the presence of pathogens can also result in the activation of another pattern recognition receptor, NOD2 to result in NF-κB activation and initiation of the autophagic response (the circled right side pathway). The model is also showing drugs used to inhibit NOD2-induced autophgy. Adapted from Baksh lab.

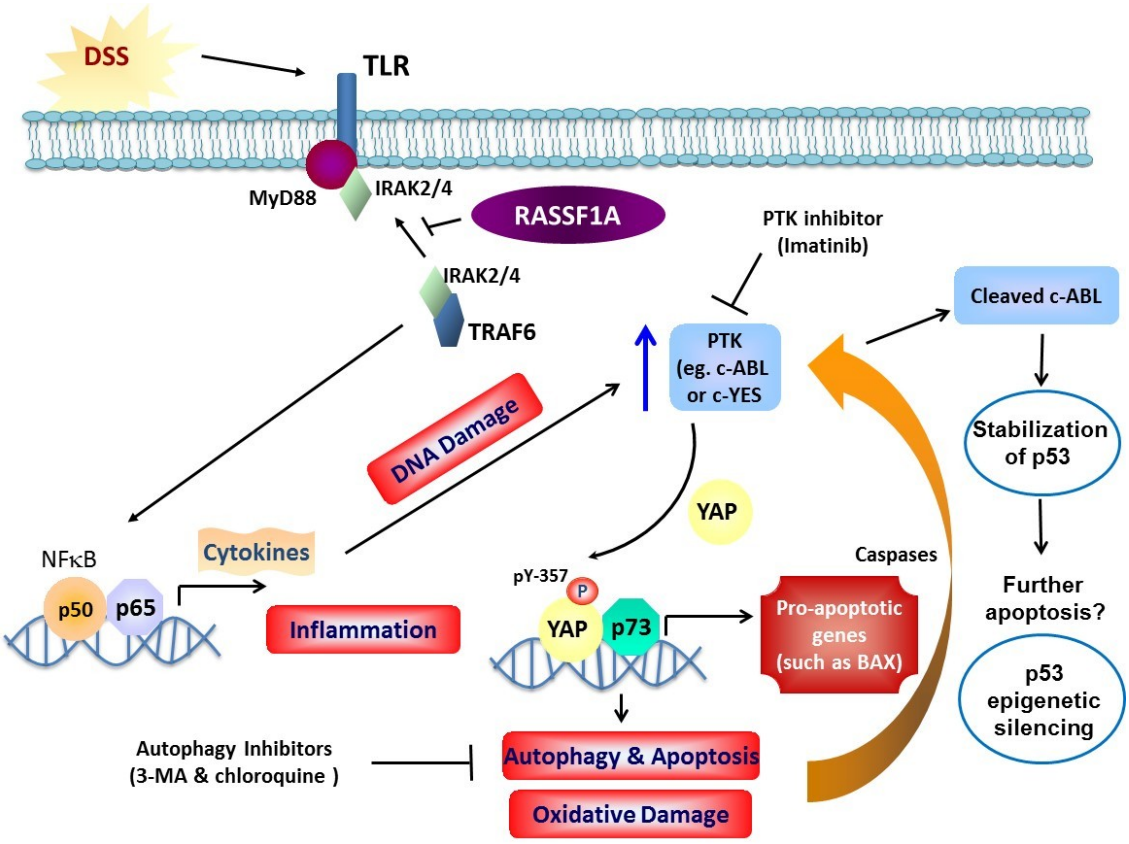

Figure 6.2: Final model for RASSF1A regulation of intestinal inflammation, showing drugs usedin this thesis project and their targets. Gordon et al; 2013.

Appendix

Introduction

The pathophysiology of IBD is complicated by multiple and diverse pathways such as inflammatory, cell death and autophagic pathways. In this thesis we have explored pharmacological inhibiton of the phosphotyrosine signalling and/or autophagic response, to decrease cell death, enhance repair and healing after DSS inflammation-induced injury, using our mouse model missing *Rassf1a*. We had promising results using PTK inhibitor, imatinib and autophagy inhibitor 3-MA but only in DSS-treated *Rassf1a*$^{+/-}$ mice. We also explored alternative drugs for IBD treatment, targeting different pathways and having different mechanism of action; using DSS-treated *Rassf1a*$^{+/-}$ mouse model. Unfortunately, these drugs did not protect DSS-treated *Rassf1a* $^{+/-}$ from inflammation- induced injury, but the data is presented for future optimizations of the use of these drugs (Figures 7.1-7.4).

Gefitinb

Gefitinib (Iressa), a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which restricts signalling through the epidermal growth factor receptor (EGFR) in target cells and is effective only in cancers with mutated and hyperactive EGFR [321]. Recently, Gefitinb was found to inhibit both RIPK2 tyrosine phosphorylation and MDP (muramyl dipeptide)-induced cytokine release via NOD2 pathway [249]. RIPK2, is an essential protein for NOD2 signalling to activate NF-κB and /or autophagy. Therefore, inhibiting RIPK2 activity via Gefitinb might be a novel therapeutic approach to restrict NOD2 signalling after stimulation with

MDP to inhibit activation of NF-κB and /or autophagy, thereby, protecting DSS treated mice from the damaging effects caused by hyperactive NOD2 signalling. We already have unpublished results showing that *Rassf1a*$^{-/-}$*Nod2*$^{-/-}$ are resistant to DSS inflammation –induced injury versus *Rassf1a*$^{-/-}$ indicating that inhibiting NOD2 signalling might be a novel approach for IBD patients to decrease inflammation –induced injury and increase repair and healing. IC_{50} for Gefitinib = 30-50nM [300].

Amlexanox

Amlexanox (Aphthasolis) an anti-inflammatory and anti-allergic drug used for recurrent aphthous ulcers (canker sores) treatment [322]. Amlexanox is an active ingredient in common topical treatment for recurrent aphthous ulcers of the mouth as it helps in reducing time required for healing and reduces pain [322]. It has been demonstrated that amlexanox has the ability to inhibit, the non-canonical, IκB kinase (IKK-ε) and TANK-binding kinase 1 (TBK1) [323].Therefore, it might gain its anti-inflammatory effect from the inhibition of these two kinases, thereby, inhibiting the activation of NF-κB and pro-inflammatory cytokine production.

Recently, studies demonstrated that IKKε has an effect on cell proliferation and transformation, and it is classified as an oncogene [324]. In addition, knockout studies with IKK-ε, have illustrated that IKK-ε has an essential and major role in inflammatory and metabolic diseases [325]. Inhibiting NF-κB activation via the inhibition of the non-canonical, IκB kinase (IKK-ε) and TANK-binding kinase 1 (TBK1) using amlexanox might be a novel therapeutic approach to protect IBD patients from inflammation induced injury by decreasing inflammation, enhancing repair and healing. IC_{50} for Amlexanox =1-2μM [326].

Rebamipide

Rebamipide, (amino acid derivative of 2-(1H) - quinolinone) is a drug used for gastric mucosa protection, healing of gastroduodenal ulcers, and treatment of gastritis. It works by activating genes encoding cyclooxygenase-2, enhancing mucosal defence and scavenges free active radicals [327]. Rebamipide is used in a number of Asian countries including Japan, South Korea, and China, but it is not approved to be used in the United States. The mechanisms of how rebamipide ameliorates inflammation, have not been fully understood and more investigation have to be done, studies suggest that it may involve inhibition of reactive oxygen species production, suppression of neutrophil accumulation, decrease permeability of the epithelium and induction of hepatocyte growth factor expression(essential for wound healing and regeneration) [328]. We speculate that rebamipide could be a novel therapeutic approach for IBD patients by decreasing inflammation and ROS generation which is prominent DSS-treated *Rassf1a*$^{-/-}$ mice [82]. IC_{50} for rebamipide =37.7μM [329].

BML-190

BML-190 (Indomethacin morpholinylamide) is a drug used in scientific research, it is a selective CB2 (cannabinoid receptor type 2) inverse agonist[330]. BML-190 is structurally derived from the NSAID indomethacin but has complete different biological activity [331].
Studies illustrated that the activity produced by BML-190 considers it as a CB_2 agonist rather than an inverse agonist [332, 333]. Furthermore, CB2 receptor ligands, such as BML-190, could be useful anti-inflammatory agents as they inhibit the secretion of IL-1beta and tumour necrosis factor-alpha (TNF-α) in THP-1 cells (human acute monocytic leukemia cells) after stimulation with LPS and IFN-γ [331].

We speculate that BML-190 could be an interesting therapeutic approach for IBD treatment by its ability to decease inflammatory cytokine production, thereby decreasing their damaging effects and enhancing healing and repair after inflammation –induced injury. IC_{50} for BML-190= 435 nM(K_i) [330].

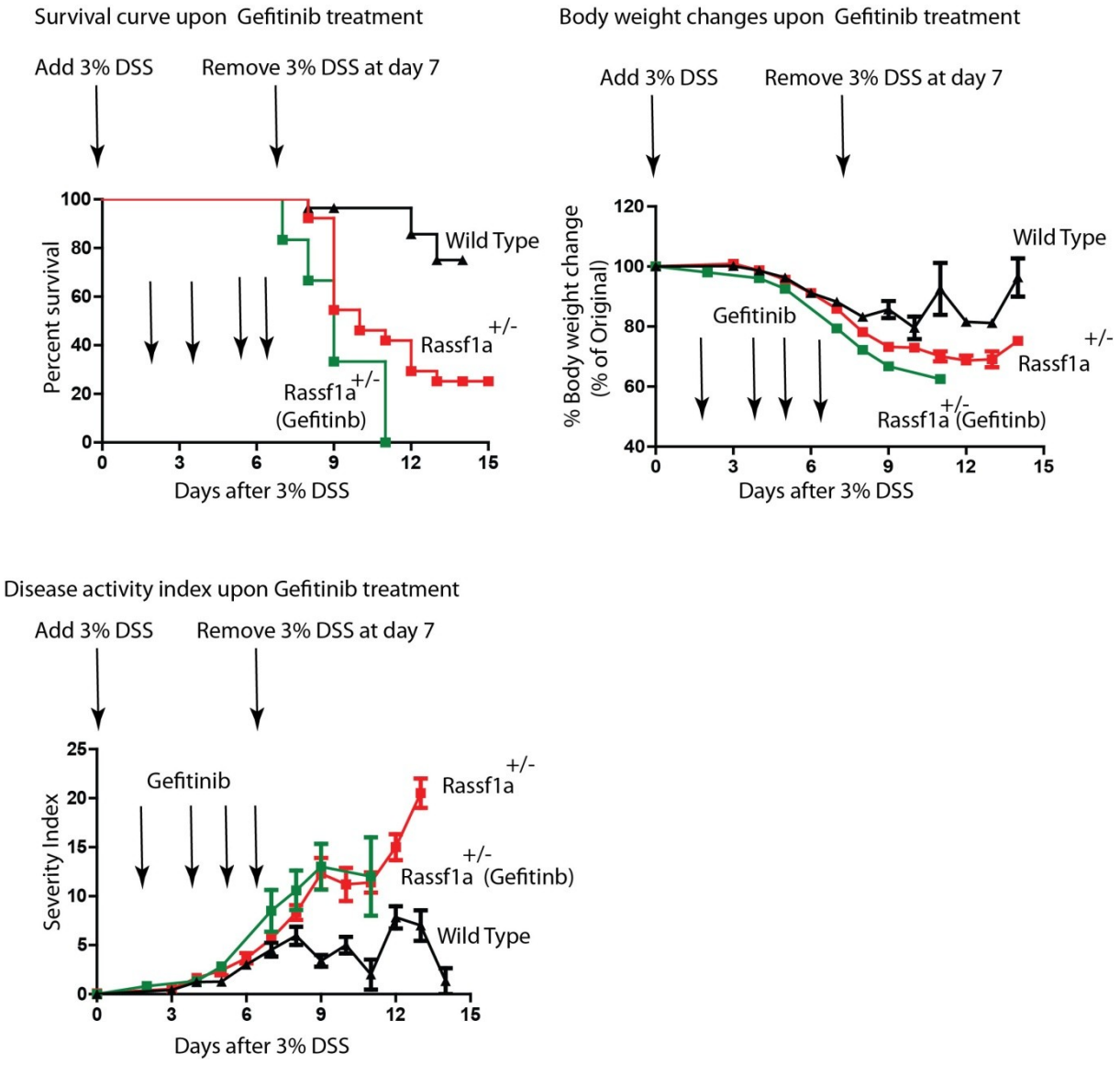

Figure 7.1: The effect of gefitinib on DSS-treated *Rassf1a* $^{+/-}$ mice. DSS-treated *Rassf1a*$^{+/-}$ micewere given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Gefitinib was administrated orally (100µg/g body weight) on days 2, 4, 5and 7. Gefitinib did not protect DSS-treated *Rassf1a*$^{+/-}$ from inflammation induced injury. n= 6-10 for all.

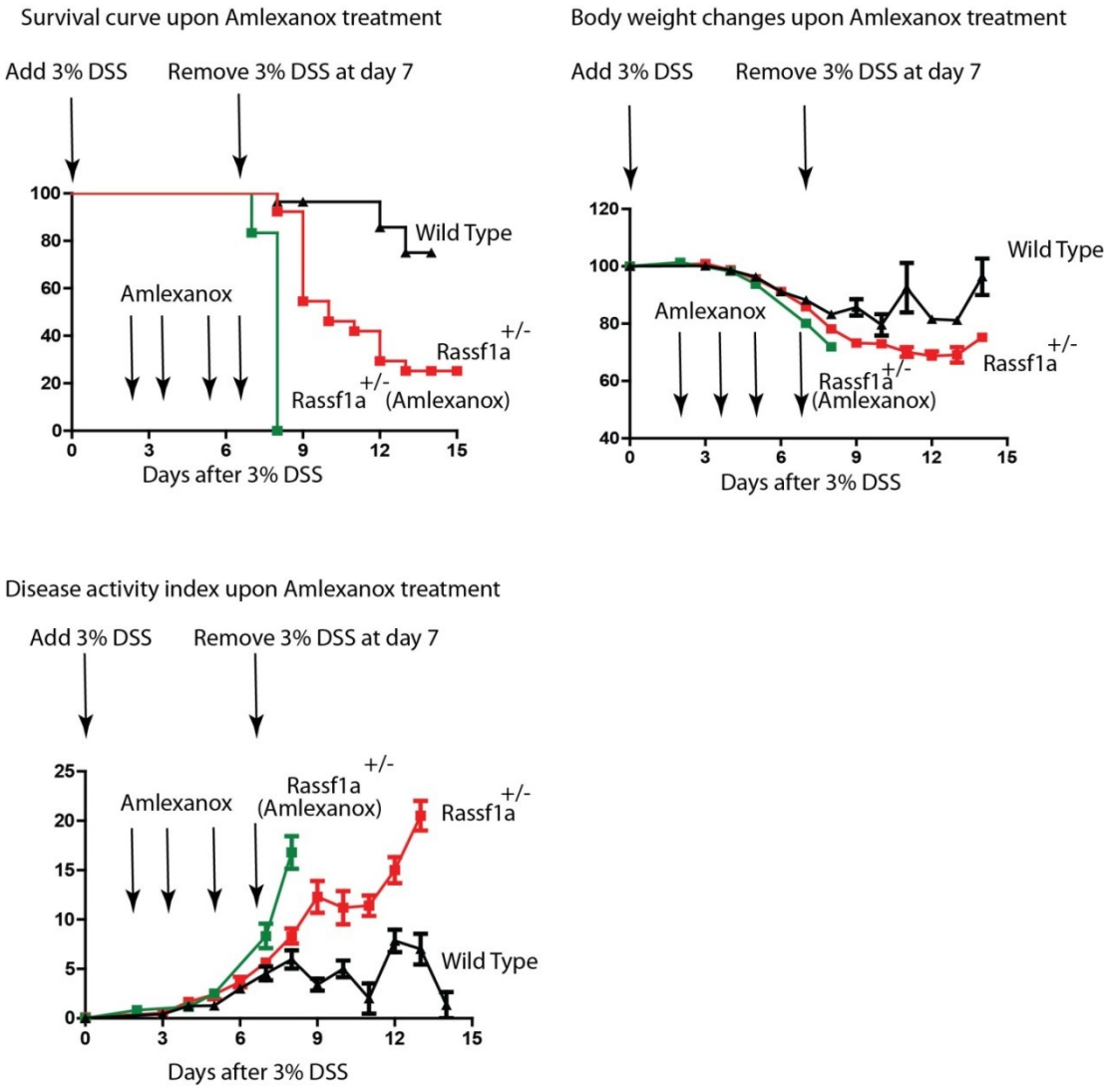

Fig.7.2: The effect of amlexanox on DSS-treated *Rassf1a* $^{+/-}$ mice. DSS-treated *Rassf1a*$^{+/-}$ mice were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Amlexanox was administrated orally (60µg/g body weight) on days 2, 4,5and 7. Amlexanox did not protect DSS-treated *Rassf1a*$^{+/-}$ from inflammation induced injury. n= 6-10 for all.

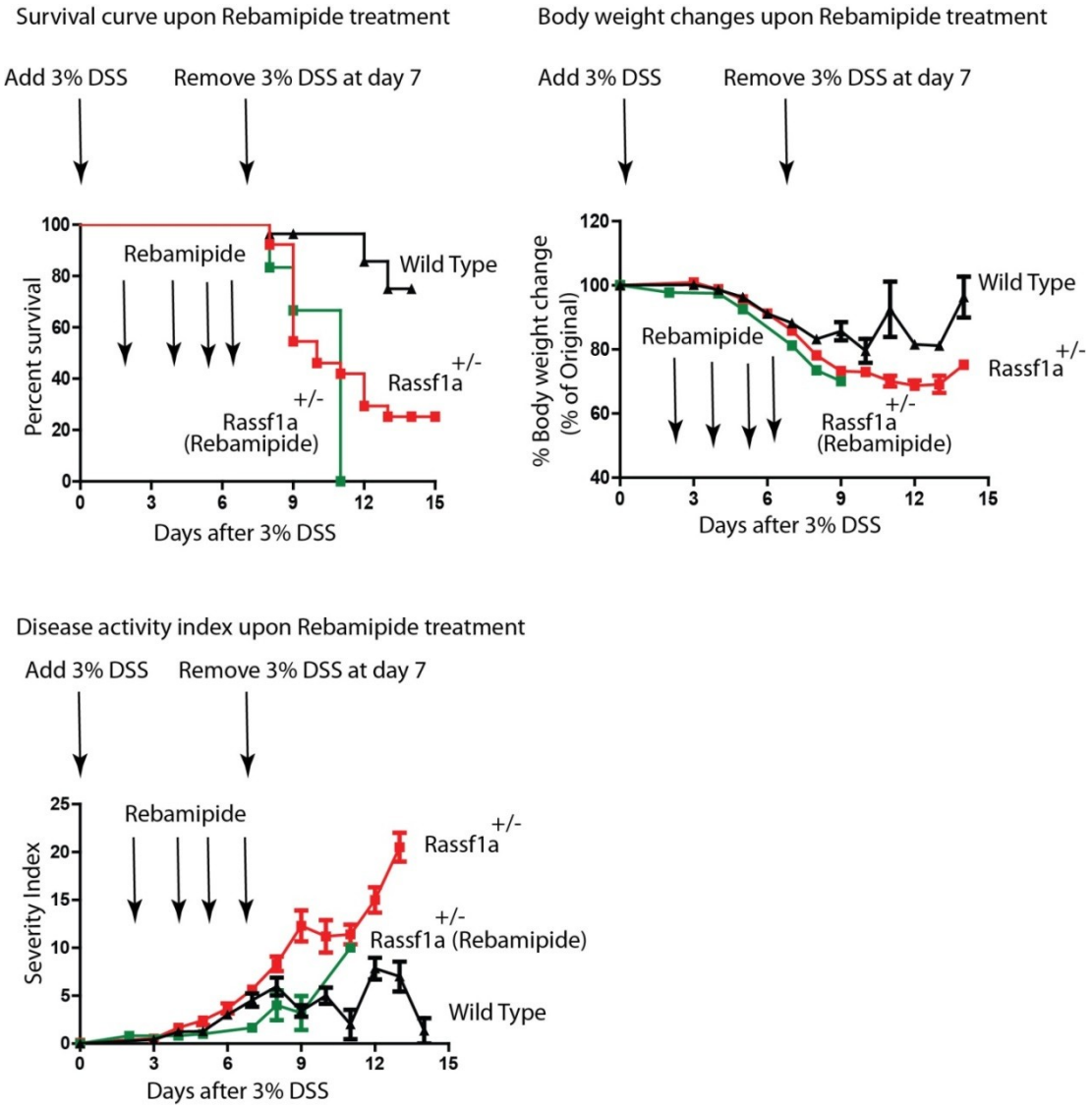

Fig.7.3: The effect of rebamipide on DSS-treated *Rassf1a* $^{+/-}$ mice. DSS-treated *Rassf1a*$^{+/-}$ mice were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. Rebamipide was administrated orally (100µg/g body weight) on days 2, 4,5and 7. Rebamipide did not protect DSS-treated *Rassf1a*$^{+/-}$ from inflammation induced injury. n= 6-10 for all.

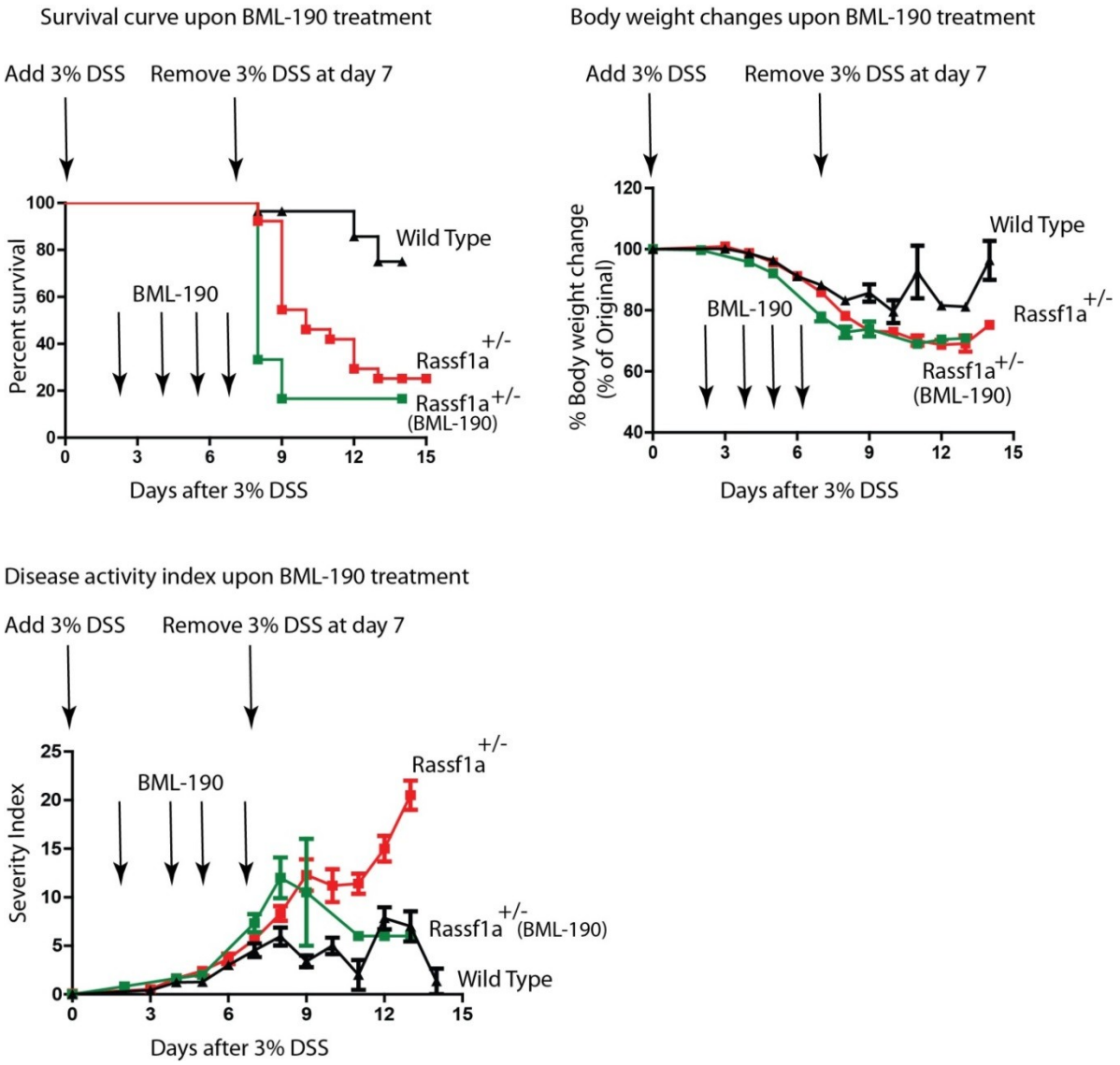

Fig.7.4: The effect of BML-190 on DSS-treated *Rassf1a* $^{+/-}$ mice. DSS-treated *Rassf1a*$^{+/-}$ mice were given 3% DSS in drinking water for 7 days followed by fresh water for recovery for another 7 days. BML-190 was administrated orally (100µg/g body weight) on days 2, 4, 5and 7. BML-190 did not protect DSS-treated *Rassf1a*$^{+/-}$ from inflammation induced injury. n= 6-10 for all.

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