



# Lysosomal autophagy in the treatment of inflammatory bowel disease

Sruthi Vijaya Retnakumar

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Discipline/ Spécialité : Immunologie

**L'autophagie lysosomale, une nouvelle  
cible thérapeutique dans les maladies  
inflammatoires de l'intestin**

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## LIST OF ABBREVIATIONS

6-MP	6-Mercaptopurine
ADA	Antidrug antibodies
AE	Adverse effects
<i>AIEC</i>	<i>Adherent invasive E. coli</i>
AMBRA	Autophagy and beclin 1 regulator 1
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APC	Antigen-presenting cells
ASA	Aminosalicylate
ASC	Adapter protein apoptosis-associated speck-like protein
ASCAs	Anti- <i>Saccharomyces cerevisiae</i> antibodies
ATG	Autophagy-related
ATP	Adenosine triphosphate
AZA	Azathioprine
CARD	Caspase recruitment domain-containing protein
CASA	Chaperone-assisted selective autophagy
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
CDI	<i>Clostridium difficile</i> infections
ClC	Chloride channel
CLR	C-type lectin receptor
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CRP	C-reactive protein
CT	Computed tomography
CTLA	Cytotoxic lymphocyte antigen
DAI	Disease Activity Index
DC	Dendritic cell
DFCP	Double FYVE-containing protein
DSS	dextran sulphate sodium
EBI	Epstein-Barr virus-induced
<i>E. coli</i>	<i>Escherichia coli</i>

EIM Extraintestinal manifestation  
EMA European Medicines Agency  
eMI Endosomal microautophagy  
EPG5 Ectopic p-granules autophagy protein 5 homolog  
ERK Extracellular signal-regulated kinase  
ESR Erythrocyte Sedimentation Rate  
FCGR2A Fc Fragment of IgG Receptor IIa  
FDA Food and Drug Administration  
FIP200 Focal adhesion kinase family interacting protein of 200 kDa  
FKBP12 FK506-binding protein 12  
Foxp3 forkhead box protein P3  
FYCO FYVE and coiled-coil domain-containing  
GABARAP GABA type A receptor-associated protein  
GAS Group A *Streptococcus*  
GBA Gut-brain axis  
GDP Guanosine diphosphate  
GEF Guanine nucleotide exchange factor  
GF Germ-free  
GWAS Genome-wide association studies  
gp130 Glycoprotein130  
GTP Guanosine-5'-triphosphate  
HBI Harvey–Bradshaw Index  
HCQ Hydroxychloroquine  
HLA Human leukocyte antigen  
HOPS Homotypic fusion and protein sorting  
HSPA8 Heat shock protein family A (Hsp70) member 8  
HSP90AA1 Heat shock protein 90 alpha family class A member 1  
HSV-I Herpes simplex virus type I  
IBD Inflammatory bowel disease  
IEC Intestinal epithelial cell  
IFN Interferon  
IL Interleukin  
IRGM Immune-related GTPase M  
JAK Janus kinase

JAM Junction adhesion molecule  
KIR Keap 1-interacting region  
KO Knockout  
KRAS Kirsten rat sarcoma virus  
LAMP Lysosomal associated membrane protein  
LAP LC3-associated phagocytosis  
LIMP Lysosomal integral membrane protein  
LIR LC3 interacting region  
LKB1 Liver kinase B1  
LP Lamina propria  
LPS Lipopolysaccharide  
LRRK2 Leucine-rich repeat kinase 2  
mAb Monoclonal antibody  
MAdCAM Mucosal addressin cell adhesion molecule  
MAP *Mycobacterium avium subsp. Paratuberculosis*  
MAP1LC3B Microtubule-associated proteins 1A/1B light chain 3B  
MDP Muramyl dipeptide  
MHC Major histocompatibility complex  
MLN Mesenteric lymph nodes  
MMX Multi matrix  
MRI Magnetic resonance imaging  
MS Multiple sclerosis  
MTMR3 Myotubularin-related protein 3  
mTNF Membrane-bound TNF  
mTOR Mammalian target of rapamycin  
NEMO NF-kappa-B essential modulator  
NET Neutrophil extracellular trap  
NLR NOD-like receptor  
NLRP NOD leucine-rich repeat and pyrin domain-containing  
NOD2 Nucleotide-binding oligomerization domain-containing 2  
NP Nanoparticle  
NSAID Non-steroidal anti-inflammatory drug  
nTreg Natural Treg  
PAMP Pathogen-associated molecular pattern

pANCA Perinuclear antineutrophil cytoplasmic antibody  
PAS Phagophore assembly site  
PB1 Phox and Bem1  
PD Programmed cell death  
PE Phosphatidylethanolamine  
PI3KC3 Phosphatidylinositol 3-kinase complex  
PINK Phosphatase and tensin homolog induced kinase  
PLEKHM1 Pleckstrin homology domain-containing family M member 1  
PMCI Partial Mayo Clinic Index  
PML Progressive multifocal leukoencephalopathy  
PP Payers patch  
PRDM PR domain zinc finger protein  
PRR Pattern recognition receptors  
PtdIns4P Phosphatidylinositol 4-phosphate  
PTPN2 Protein tyrosine phosphatase non-receptor type 2  
PUCAI Paediatric Ulcerative Colitis Activity Index  
PUFA Polyunsaturated fatty acid  
QOL Quality of life  
RAB Ras-related protein in brain  
RAR Retinoic acid receptor  
RILP Rab interacting lysosomal protein  
RLR RIG-I-like receptor  
ROR $\gamma$  Retinoic acid receptor-related orphan nuclear receptor gamma  
SAE Serious adverse effect  
SCCAI Simple Colitis Clinical Activity index  
SCFA Short-chain fatty acid  
SCID Severe combined immunodeficient  
SIP Sphingosine-1-phosphate  
SLE Systemic lupus erythematosus  
SMAD Mothers against decapentaplegic homolog  
SNAP29 Synaptosomal-associated protein 29  
SNARE Soluble N-ethylmaleimide-sensitive factor attachment protein receptor  
SNP Single nucleotide polymorphism  
SQSTM1 Sequestosome

STAT Signal transducer and activator of transcription  
 sTNF Soluble TNF  
 STX Syntaxin17  
 TACE TNF-converting enzyme  
 TCR T cell receptor  
 TECPR1 Tectonin beta-propeller repeat-containing protein 1  
 TFEB Transcription factor EB  
 TGF transforming growth factor  
 TJ Tight junction  
 TLR Toll-like receptor  
 TNBS Trinitrobenzene sulphonic acid  
 TNF Tumor necrosis factor  
 TNFSF TNF superfamily member  
 Treg T regulatory cell  
 TREM-1 Triggering receptor expressed on myeloid cells-1  
 TRP Transient receptor potential  
 TSLP Thymic stromal lymphoprotein  
 UBA Ubiquitin associated domain  
 UBL Ubiquitin-like protein  
 UC Ulcerative colitis  
 ULK Unc-51 like autophagy activating kinase  
 VAMP8 Vesicle associated membrane protein 8  
 vATPase Vacuolar-type proton adenosine triphosphatase  
 VPS Vacuolar protein sorting  
 WIPI WD repeat domain, phosphoinositide interacting  
 WT Wild-type

# RÉSUMÉ DE THÈSE EN FRANÇAIS

## 1. Introduction

Les maladies inflammatoires de l'intestin (MICI) désignent un groupe de troubles graves, chroniques et récidivants affectant le tractus gastro-intestinal. Bien que les MICI aient été initialement décrites comme des maladies du monde occidental, leur incidence augmente régulièrement dans les pays en développement d'autres régions du monde, probablement en raison de l'évolution des modes de vie et de l'alimentation. Les MICI résultent d'une combinaison de facteurs génétiques, microbiens et environnementaux entraînant un dérèglement des systèmes immunitaires innés et adaptatifs de l'intestin. La nature complexe et polygénique de la maladie reste un obstacle au développement de thérapies sûres et ciblées, et les traitements actuellement disponibles restent essentiellement symptomatiques et visent à améliorer la qualité de vie du patient. De plus, leur utilisation efficace est largement limitée par leurs effets secondaires indésirables et au fait que certains patients s'avèrent réfractaires aux médicaments.

L'autophagie est un processus vital d'auto-digestion dans lequel le contenu cytoplasmique cellulaire est acheminé vers les lysosomes pour y être dégradé. Il s'agit d'une voie dynamique et multifonctionnelle qui intervient dans une variété de processus cellulaires, notamment la croissance cellulaire, la différenciation et les réponses immunitaires. Par conséquent, la modulation pharmacologique des processus d'autophagie est apparue comme une stratégie thérapeutique potentielle dans une pléthore de troubles humains. Dans ce contexte, l'autophagie constitue une voie potentielle pour le traitement des MICI, car plusieurs gènes liés à l'autophagie (par exemple ATG16L1 et la GTPase M liée à l'immunité) ont été associés au risque de MICI. Des études approfondies ont été menées sur des modèles animaux de colite pour démontrer les rôles fonctionnels de la voie de l'autophagie dans la pathogenèse des MICI.<sup>1</sup>

Le P140 est un peptide thérapeutique développé par notre équipe qui cible sélectivement les processus d'autophagie. Dans le lupus, il a été démontré que ce peptide inhibe les processus d'autophagie qui sont hyperactivés et interfère avec la présentation anormale de l'antigène dans les cellules B. L'effet "correcteur" de P140 est un facteur important dans la régulation du développement de la maladie.<sup>2</sup> L'effet "correcteur" du P140 sur l'autophagie entraîne une signalisation plus faible des cellules T et B autoréactives, ce

qui conduit à une amélioration significative des conditions physiopathologiques. Le peptide s'est révélé sûr et non immunogène et est actuellement évalué dans le cadre d'essais cliniques de phase III pour le traitement de patients atteints de lupus. Le potentiel thérapeutique prometteur du peptide P140 a également été démontré dans des modèles murins d'autres affections inflammatoires telles que le syndrome de Sjögren,<sup>3</sup> la polyneuropathie inflammatoire démyélinisante chronique<sup>4</sup> et l'asthme,<sup>5</sup> dans lesquels on a constaté que les processus d'autophagie étaient dérégulés. Par conséquent, le mécanisme d'action de ce peptide via l'autophagie nous a conduit à l'hypothèse que le traitement par P140 pourrait être efficace dans les MICI également.<sup>6</sup>

Dans cette étude, nous avons cherché à analyser les effets thérapeutiques du P140 dans des modèles animaux pertinents de MICI. En raison de la complexité de la maladie et de l'absence d'un modèle animal parfaitement représentatif, nous avons évalué l'efficacité du P140 dans trois modèles murins distincts mais complémentaires, selon des protocoles différents. Des études rigoureuses ont été menées aux niveaux clinique et moléculaire dans deux modèles induits chimiquement - le modèle induit par du dextran sulfate de sodium (DSS) et le modèle induit par de l'acide trinitrobenzène sulfonique (TNBS). Les résultats obtenus ont été renforcés par une évaluation clinique dans un modèle génétiquement induit qui développe spontanément une inflammation intestinale chronique en raison d'une double mutation des gènes *il10* et *rhomboid 2* (*iRhom2*).<sup>7</sup> Sur le plan mécanistique, nous avons également constaté que le P140 corrige les défauts d'autophagie chez les souris atteintes de colite. Les principales expériences qui ont permis de démontrer l'efficacité du peptide et les principaux résultats obtenus sont décrits ci-dessous.

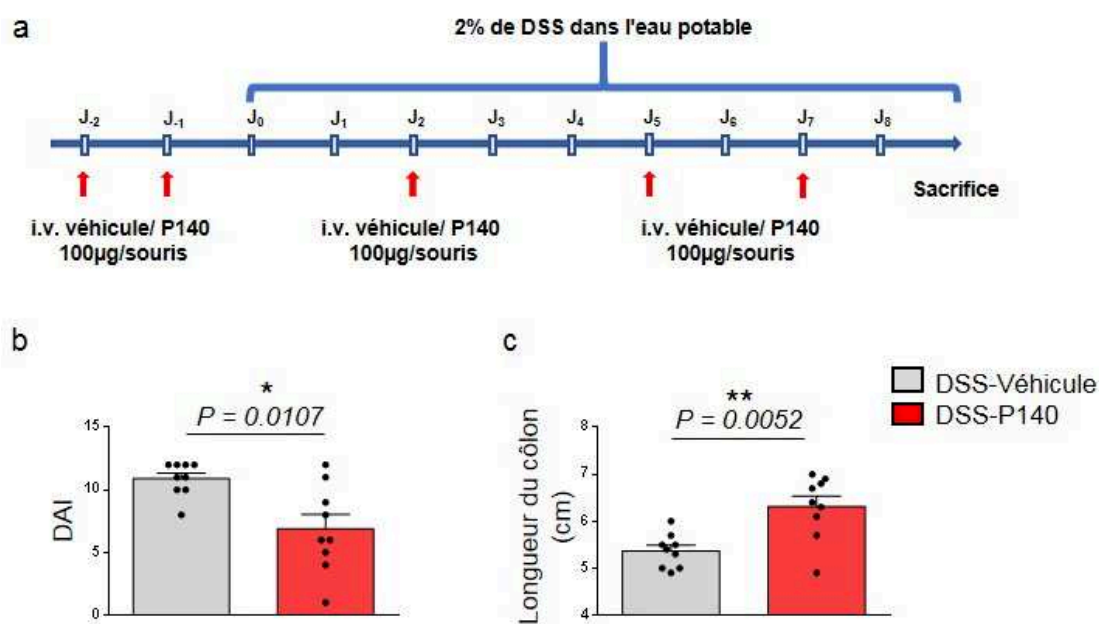
## **2. Résultats**

### **2.1. Effets thérapeutiques de P140 dans le modèle de colite DSS**

Tout d'abord, nous avons étudié l'efficacité du peptide P140 dans le modèle de colite DSS qui est établi par l'administration orale du produit chimique dissous dans l'eau de boisson à des souris de type sauvage. Le produit chimique exerce une toxicité sur les cellules épithéliales intestinales, ce qui compromet la fonction de barrière et induit une inflammation dans le côlon. Dans cette expérience, on a administré à des souris mâles C57BL/6 2% de DSS pour induire la maladie, et le P140 a été injecté (par voie intraveineuse) avant et après l'induction de la maladie, combinant ainsi un schéma



préventif et thérapeutique. Les souris ont été sacrifiées au jour +9 pour une analyse post-mortem. La **Figure 1. a** présente une représentation schématique du protocole expérimental et des schémas de traitement appliqués. Le groupe de souris ayant reçu du P140 a montré une diminution significative du score de l'indice d'activité de la maladie (DAI), classiquement calculé comme la somme de la perte de poids corporel, de la consistance des selles et de la présence de sang dans les selles, par rapport aux souris ayant reçu le véhicule témoin (**Figure 1. b**). Le raccourcissement de la longueur du côlon, qui est un symptôme caractéristique de la colite, a également été inversé par le traitement au P140 (**Figure 1. c**).



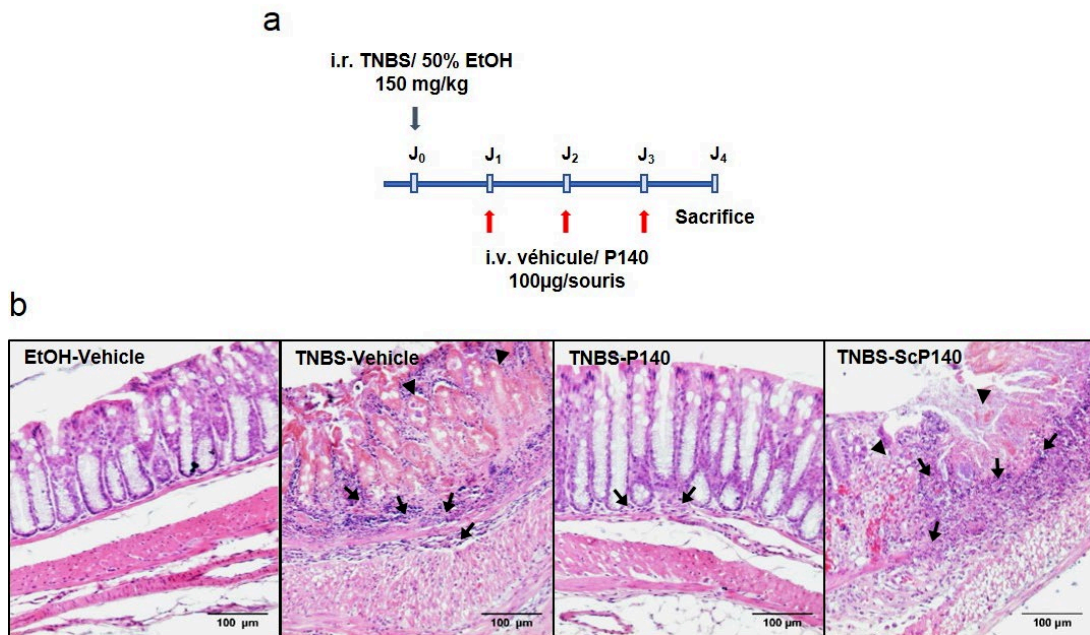
**Figure 1: Effets thérapeutiques du P140 dans la colite DSS**

a) Plan expérimental ; b) DAI ; c) Longueur du côlon. Véhicule, NaCl 0,9 % p/v. Le % DSS est exprimé en p/v (test U de Mann Whitney).

## 2.2. Effets thérapeutiques de P140 dans le modèle de colite TNBS

Pour démontrer plus avant le potentiel curatif du P140, nous avons utilisé le modèle de colite TNBS. Pour établir ce modèle, le produit chimique est dissous dans de l'éthanol et administré par voie intra-rectale. L'éthanol perturbe légèrement la barrière intestinale pour permettre l'entrée du TNBS dans la lumière. Le TNBS est une molécule haptène qui forme des complexes avec les protéines coliques ou celles du microbiote pour les rendre immunogènes. L'expérience a été réalisée chez des souris mâles C57BL/6 où le TNBS a été injecté à une dose de 150 mg/kg, et l'injection de P140 a été effectuée en

suivant strictement un schéma thérapeutique. Dans un autre groupe de souris, l'analogue à séquence brouillée du peptide P140 (ScP140) a été injecté à la même dose, ce qui sert de contrôle négatif dans ces expériences. Les souris ont été sacrifiées au jour +4 et des échantillons ont été collectés pour nos analyses ultérieures (**Figure 2. a**). L'effet thérapeutique du peptide est représenté ici par une amélioration marquée des dommages histomorphologiques du côlon (**Figure 2. b**).



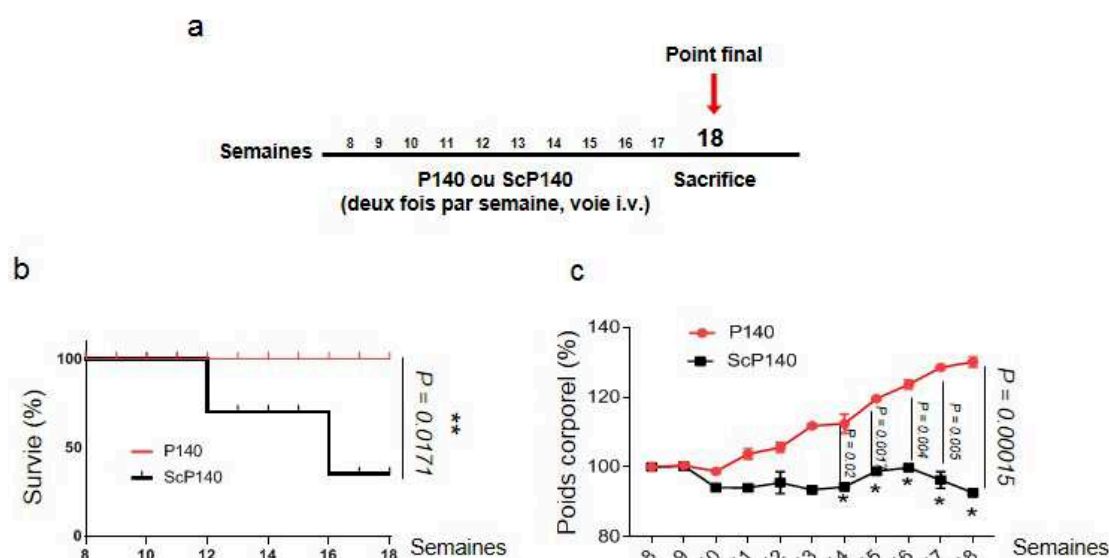
**Figure 2: Effets thérapeutiques du P140 dans la colite TNBS**

a) Plan expérimental ; b) Images représentatives de la coloration à l'hématoxyline et à l'éosine. Les flèches noires indiquent l'infiltration de cellules inflammatoires et les pointes de flèches indiquent les sites où l'épithéliale est perturbée ; Véhicule, NaCl 0,9% w/v. EtOH, éthanol. Barres d'échelle 100µm.

### 2.3. Effets thérapeutiques du P140 dans le modèle de colite spontanée *il10<sup>-/-</sup>/iRhom2<sup>-/-</sup>*

Les souris *il10<sup>-/-</sup>/iRhom2<sup>-/-</sup>* développent une colite spontanée dans les 8 à 12 semaines suivant la naissance. L'interleukine (IL)-10 est une cytokine anti-inflammatoire très importante dans la pathogenèse des MICI. *iRhom2* est un régulateur de la sécrétion du facteur de nécrose tumorale - alpha (TNF- $\alpha$ ) dans les cellules immunitaires. Outre sa pertinence clinique, ce modèle a surtout permis une étude à long terme qui est similaire à la pathogenèse des MICI chroniques humaines. Dans cette expérience, le traitement par P140 a été initié à l'âge de 8 semaines (début de la maladie). Le P140 / ScP140 a été administré par injection i.v., deux fois par semaine, pendant 11 semaines, puis les souris ont été sacrifiées pour recueillir des échantillons (**Figure 3. a**). On a constaté une nette

amélioration du taux de survie et du poids corporel des souris dans le groupe traité par P140 par rapport au groupe traité par ScP140 (Figure 3. b, c).

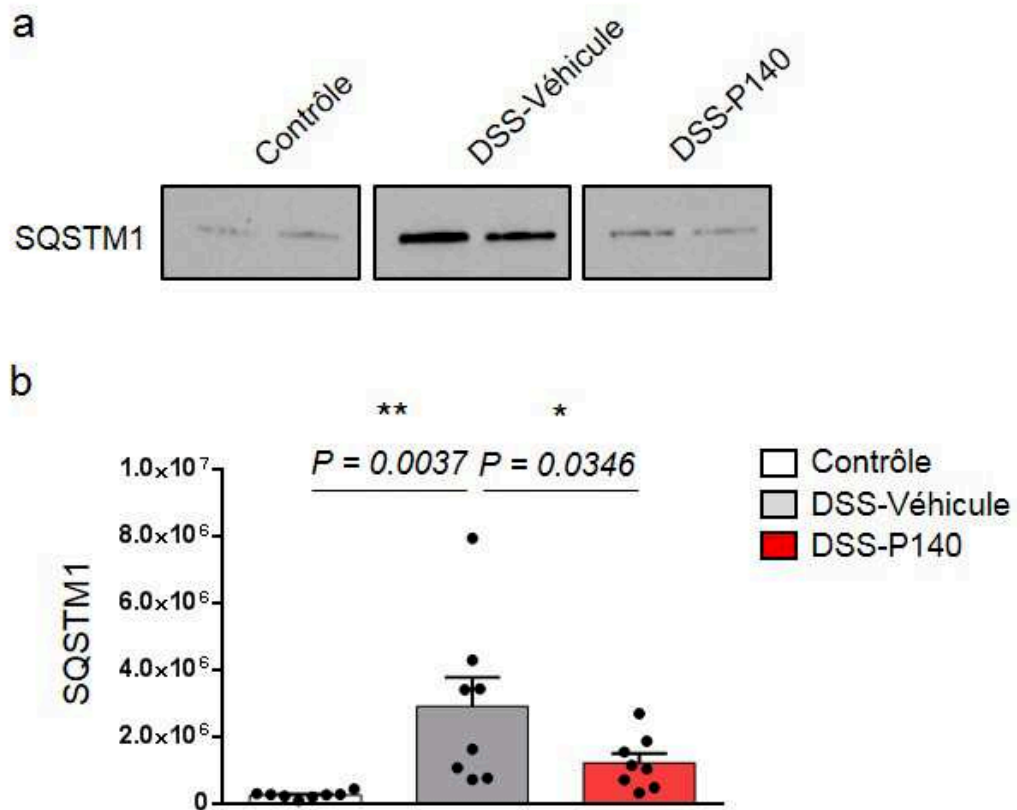


**Figure 3: Effets thérapeutiques du P140 chez les souris *il10<sup>-/-</sup>/iRhom2<sup>-/-</sup>***

a) Plan d'expérience; b) survie; c) variation du poids corporel en pourcentage (des comparaisons par paires post-hoc ont été effectuées par correction de Bonferroni).

## 2.4. Évaluation des processus d'autophagie

Les niveaux d'expression protéique des marqueurs clés de l'autophagie ont été évalués dans les tissus du côlon par des méthodes biochimiques. SQSTM1/p62 est un adaptateur de cargaison autophagique classique qui cible les substrats autophagiques vers les autophagosomes. Comme la protéine elle-même est dégradée dans les lysosomes avec les substrats au cours du processus, une accumulation de la protéine SQSTM1 indique une altération de l'autophagie. Renforçant les résultats publiés précédemment dans des modèles de colite, nous avons montré que SQSTM1 s'accumule dans le tissu du côlon des souris DSS par rapport aux souris contrôles saines. Ce phénomène a été corrigé par le traitement au P140, ce qui suggère une restauration de l'autophagie déficiente comme conséquence directe ou indirecte de l'effet du P140 (Figure 4. a-b).



**Figure 4: Effet de l'autophagie de P140 sur les marqueurs du côlon**

a) Image représentative de SQSTM1 par immunoempreinte ; b) Quantification des niveaux de protéine SQSTM1. Véhicule, NaCl 0,9 % p/v (voie unique suivie de comparaisons multiples).

### 3. Conclusions

En utilisant trois modèles murins indépendants, nous avons montré que le peptide thérapeutique P140 exerce des effets protecteurs sur la colite aux niveaux clinique et moléculaire. Les processus d'autophagie qui sont défectueux chez les souris atteintes de colite ont été corrigés par le traitement au P140. Les mécanismes moléculaires par lesquels le P140 module l'autophagie dans les MICI doivent encore être étudiés. Cependant, en conclusion, nos résultats suggèrent fortement que le phosphopeptide P140, modulateur de l'autophagie, pourrait être une option thérapeutique prometteuse pour traiter les patients atteints de MICI, seul ou en association avec d'autres médicaments existants.

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# INTRODUCTION

This section is based on the review article titled “Pharmacological Autophagy Regulators as Therapeutic Agents for Inflammatory Bowel Diseases” Retnakumar, S.V., and Muller, S. *Trends Mol Med.* 2019;25:516-537, which we have updated with the latest published data.



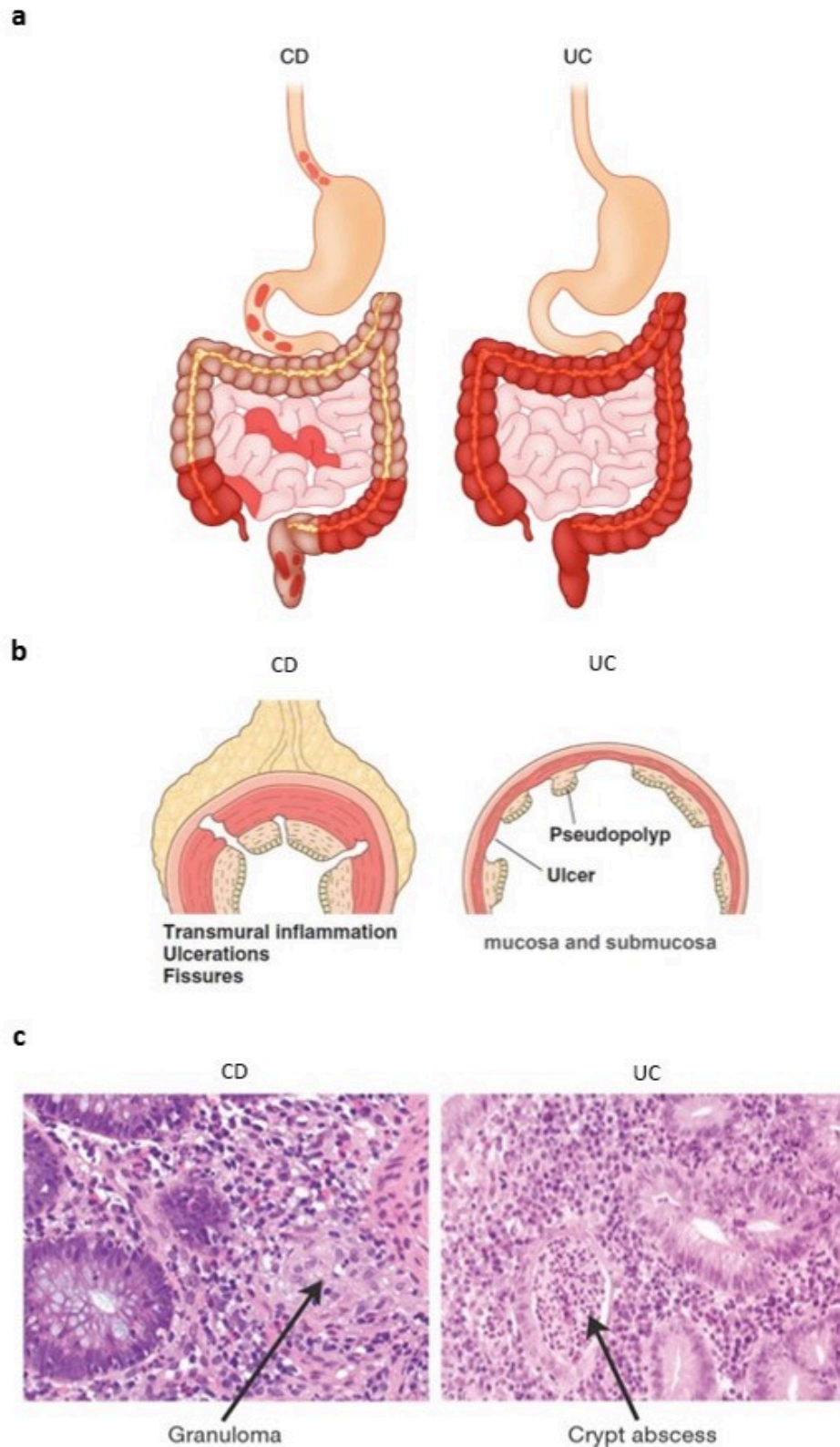
## 1. INFLAMMATORY BOWEL DISEASES

Inflammatory bowel disease (IBD) is a collective term used to refer to a group of heterogeneous, chronic, relapsing disorders affecting the gastrointestinal tract. Though vague descriptions about several forms of gut inflammation and chronic diarrhea date back to ancient times, the exact clinical descriptions about IBDs started since 1859. Sir Samuel Wilks, a British physician, has first used the term “ulcerative colitis” (UC) in a case report of a 42-year old woman who died after several months of diarrhea and fever, where he demonstrated inflammation in her colon and terminal ileum by autopsy.<sup>8</sup> Another major form of IBDs that we characterise as Crohn’s disease (CD) today, was coined as a separate entity in 1932 from a study of 14 patients, which was then called “regional ileitis”, but later named after the first author of the publication Dr. Burrill B. Crohn.<sup>9,10</sup> But, a century later, the case described by Sir. Samuel Wilks was identified as a case of CD.<sup>11</sup> Instead, another case report of Wilks and Moxon in 1875<sup>12</sup> describing ulceration and inflammation of the entire colon in a young woman who died of severe bloody diarrhea was an early description of UC.<sup>13</sup>

### 1.1. Crohn’s disease and ulcerative colitis

As described above, CD and UC are the major forms of IBDs. Though they share several common clinical symptoms, they have distinct features concerning their pathology and origin. A comparison showing the similarities and differences between both forms of IBDs is summarised in **Figure 1** and **Table 1** and detailed in the next subsections.<sup>14,15</sup>





**Figure 1: Comparison between CD and UC**

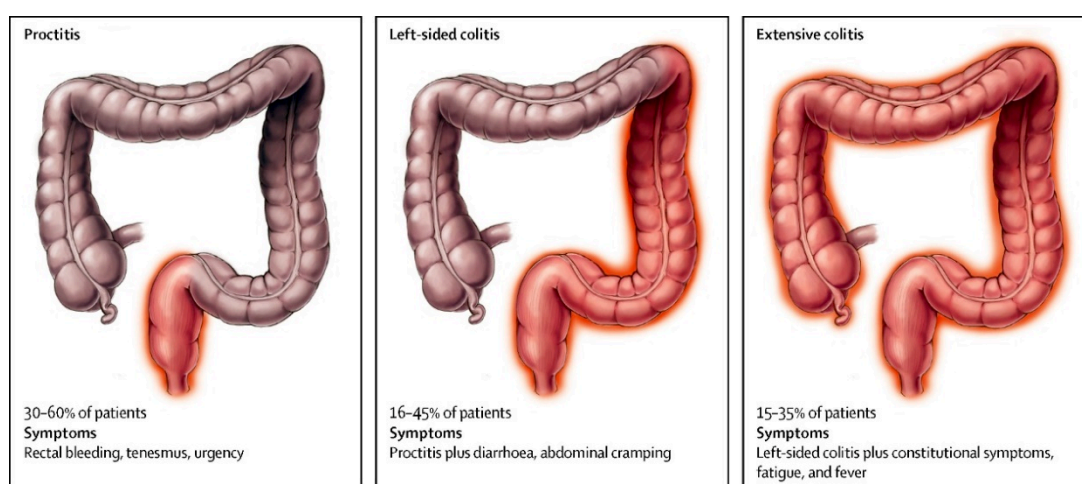
a. Anatomical location of inflammation; b. involvement of bowel wall; c. histological hallmarks.  
*Figure taken from Xavier RJ, Podolsky DK. Nature. 2007;448(7152):427-434 with permission. See subsection 1.2 for detailed information.*

**Table 1: Comparison between CD and UC**

<b>Features</b>	<b>Crohn's disease</b>	<b>Ulcerative colitis</b>
Incidence	3.1 to 20.2 cases/ 100,000 individuals/ year.	2.2 to 19.2 cases/ 100,000 individuals/ year.
Location	Inflammation affects any part of the gastrointestinal tract, more commonly in distal ileum and colon ( <b>Figure 1. a</b> ).	Affects the colon only ( <b>Figure 1. a</b> ).
Pathology	Discontinuous, patchy distribution of inflammation with skip lesions ( <b>Figure 1. a</b> ).	Continuous inflammation beginning from the anorectal margin extending proximally until the rectum (proctitis), sigmoid colon (proctosigmoiditis/ left sided colitis), or the whole colon (pancolitis/extensive colitis) ( <b>Figure 1. a</b> ).
Histology	Transmural inflammation involving all layers of the intestinal tissue. Histologically characterized by the presence of intestinal epithelioid granulomas which is formed by the aggregation of macrophages. They are composed of compact macrophages, epithelioid cells (derivatives of activated macrophages resembling epithelial cells) and giant cells (formed by the fusion of epithelioid cells) ( <b>Figure 1. b, c</b> ).	Inflammation is restricted to the mucosal layer with severe inflammatory cell infiltration and widespread mucosal distortion (shortened and branching crypts). Activity can be recognized by the presence of significant number of neutrophils infiltrating the wall of some crypts forming crypt abscesses. Goblet cell depletion is also common in UC ( <b>Figure 1. b, c</b> ).
Symptoms	Diarrhoea, abdominal cramping, fever, anaemia, weight loss, fatigue.	Bloody diarrhoea, abdominal cramps, anaemia, weight loss, fatigue.

## 1.2. Symptoms

IBDs are characterised by the occurrence of frequent and chronically relapsing flares leading to severe symptoms such as abdominal pain, diarrhea, rectal bleeding, fatigue, malnutrition, and weight loss, in general. The symptoms and their severity are largely dependent on the localization of the disease and the frequency of symptoms can be subject to the populations studied. UC mostly presents visible blood in the feces in more than 95 % of the cases, along with the urgency of defecation and rectal tenesmus (feeling of incomplete defecation). The clinical symptoms of UC can be classified based on the anatomical extent of the disease (described in **Table 1**, **Figure 2**).<sup>16</sup> CD has more variability in the symptoms due to its wavering disease localisation, yet the most common symptoms are chronic diarrhea, abdominal pain, and weight loss. Intermittent fevers, tachycardia, fatigue, and subfertility are also commonly associated with CD, while these symptoms are usually restricted to severe cases in UC.<sup>17,18</sup>

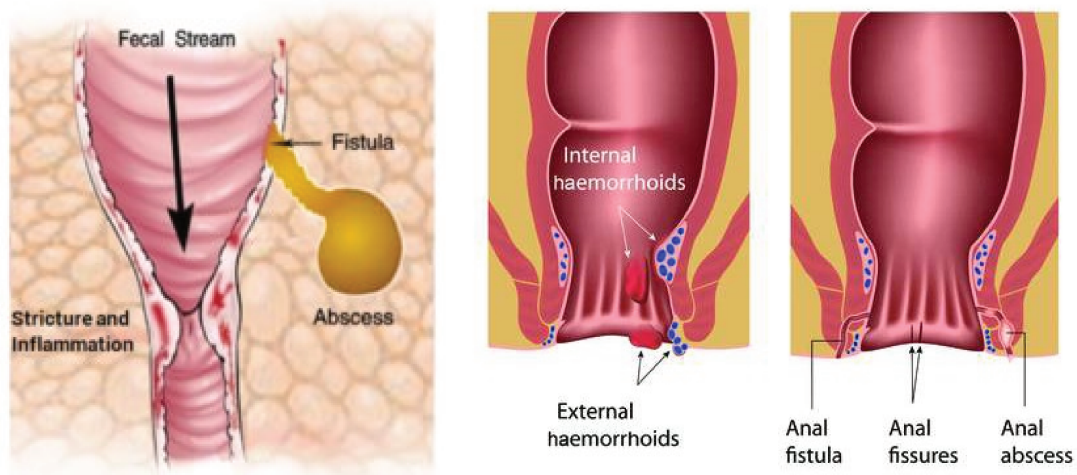


**Figure 2: Clinical presentation of UC phenotypes depending on the extent of the disease**

Figure taken from Ungaro R, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel J-F. *Lancet*. 2017;389(10080):1756-1770 with permission.

Certain gastrointestinal or perianal complications are also reported in IBD patients. A frequently observed complication is the formation of strictures, which causes narrowing of parts of the intestine due to the build-up of fibrous tissue on the intestinal wall. Fistulas are narrow tunnels formed between parts of the intestine or to the skin or other internal organs. Sometimes fluid accumulates in these fistulas to develop infection, which is then called abscesses. The most common type of fistulas is peri anal fistulas formed around the anus. In addition, anal fissures (small tears or splits formed at the

end of the anal canal), perianal skin tags (narrow growth that sticks out of the skin), and hemorrhoids (swollen veins in the lower rectum and anus) are some of the perianal complications associated with IBDs (**Figure 3**). These complications are more frequently observed in CD patients than in UC.<sup>19</sup>



**Figure 3: Common gastrointestinal and perianal complications in IBD**

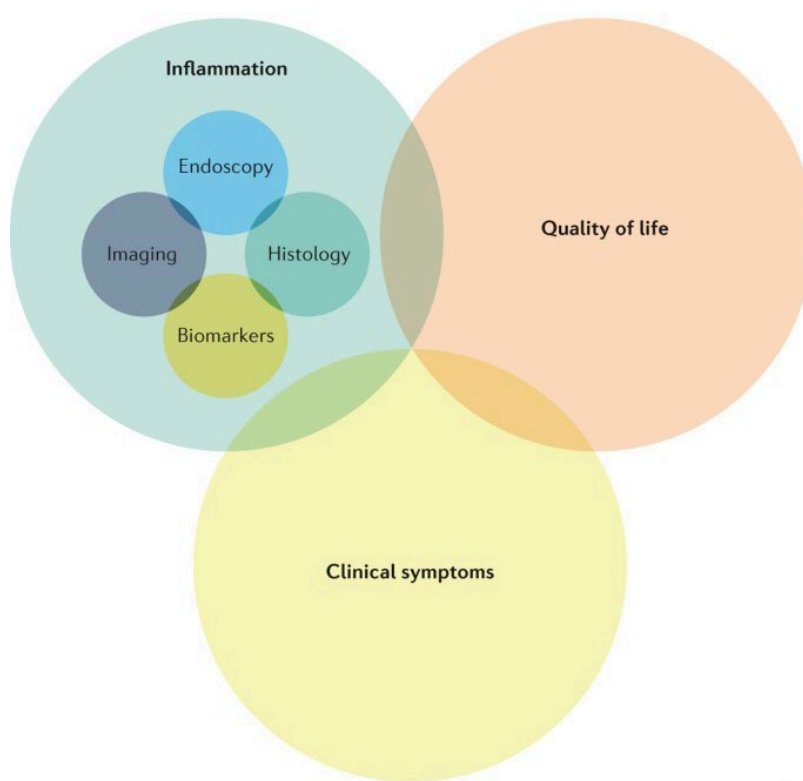
Figure taken from <https://consultqd.clevelandclinic.org/new-needle-knife-application-leads-endoscopic-innovations-digestive-disease-week/>, <https://ueq.eu/a/173>.

More than one-third of the patients with IBDs present extraintestinal manifestations (EIMs) which include musculoskeletal, ophthalmological, neurological and pulmonary manifestations, and anaemia, further increasing the disease burden on the individuals. EIMs have been mechanistically defined as, “an inflammatory pathology in a patient with IBD that is located outside the gut and for which the pathogenesis is either dependent on extension/translocation of immune responses from the intestine, or is an independent inflammatory event perpetuated by IBD or that shares a common environmental or genetic predisposition with IBD”.<sup>20</sup> A strong impact of these complications on the quality of life (QOL) of the patients (see section 1.3) points to the requirement of diagnosing and treating them along with the classical symptoms. The most commonly occurring EIMs are arthropathies affecting 35% of IBD patients, which are caused by the underlying disease as well as the corticosteroids, immunosuppressants and anti-tumor necrosis factor (TNF) therapies. *Erythema nodosum*, which is characterised by the formation of painful nodules on the skin, is observed in 10-15% of the patients, whereas other skin complications include drug-induced cutaneous lesions, psoriasis and rarely, skin cancers. Chronic intestinal blood loss and loss of appetite

leading to insufficient dietary intake are some direct causes that lead to anemia in IBD patients. Moreover, intestinal inflammation affects its ability to absorb iron from the food, causing iron deficiency anemia.<sup>21,22</sup>

### 1.3. Diagnosis

Despite the revolutionary advances in modern medical technologies, diagnosis of IBDs and distinction between CD and UC are still challenging. Since there is no single test that can accurately detect the disease, diagnosis of IBDs is generally carried out by combining various practices.<sup>23,24</sup> Standard disease severity indices are used to quantitatively assess the prognosis of the disease and to guide therapy and clinical trials of IBD patients. The disease activity measurements span multiple domains including assessment of clinical symptoms, evaluation of patient's QOL, and objective quantification of inflammation using endoscopic, histological, and radiological diagnostic tools and relevant biomarkers (**Figure 4**). A number of indices have been developed in each domain of assessment and they have been modified over time to improve their validity and feasibility.<sup>25</sup>



**Figure 4: Domains of disease activity assessment in IBD**

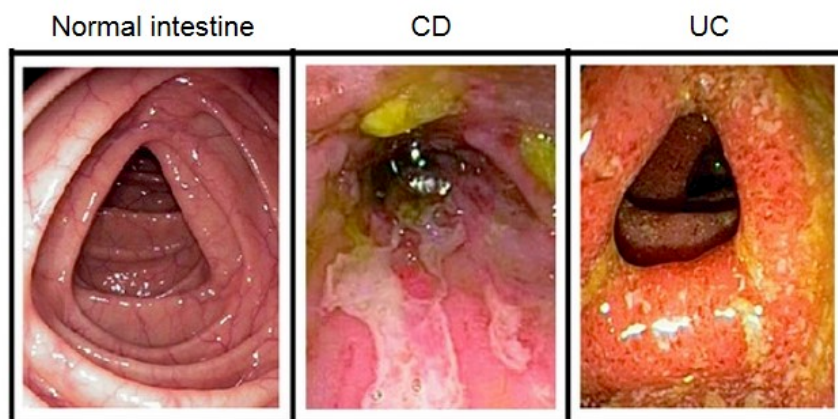
Figure taken from Walsh AJ, Bryant R V, Travis SPL. *Nat Rev Gastroenterol Hepatol.* 2016;13(10):567 with permission.

The Simple Colitis Clinical Activity index (SCCAI),<sup>26</sup> the Partial Mayo Clinic Index (PMCI), and the Paediatric Ulcerative Colitis Activity Index (PUCAI)<sup>27</sup> are commonly used indices for assessing the clinical symptoms of UC. The SCCAI is widely used in clinical practice since the evaluation can be completed by patients without the help of a physician's global assessment, and is sufficient to discriminate remission from active disease. The PMCI is currently the most accepted index in adult clinical trials which includes subjective measurement from clinicians. The PUCAI is a validated index developed by paediatric gastroenterologists and widely accepted in clinical practice and clinical trials for children.<sup>25</sup> The Crohn's disease activity index (CDAI)<sup>28</sup> and the Harvey–Bradshaw Index (HBI)<sup>29</sup> are commonly used for the clinical assessment of CD. Though the CDAI is the most widely used one in trial designs, it involves complex calculations with a 7-day patient assessment and is therefore not preferred in clinical practice. The HBI, on the other hand, involves much simpler data collection and calculations and is found to correlate well with CDAI scores.<sup>29</sup>

Apart from clinical symptoms, the evaluation of the QOL of patients is salient since it depicts their social and emotional welfare. The patients are provided with standard QOL questionnaires to have a valid, reproducible measurement acceptable to the patients. Clinical symptoms and QOL are most important to patients to help them achieve their physical and psychological well-being, while in a clinician's perspective, objective measures of inflammation are essentially required to make suitable decisions.<sup>25</sup>

Endoscopic techniques have currently become the gold standard for diagnosis of IBDs allowing direct visualisation of the colon and collection of mucosal biopsies (**Figure 5**).<sup>30,31</sup> Endoscopic disease activity indices set mucosal healing as the therapeutic goal in clinical trials.





**Figure 5: Endoscopy images of large intestine**

Representative images from healthy individuals or patients with IBD. *Figure taken from Marsal J, Agace WW. J Intern Med. 2012;272(5):411-429 with permission.*

Histopathological assessment of biopsies by routine staining methods provides a better picture of microscopic inflammation. In fact, endoscopic remission can still be associated with persistent microscopic inflammation whereas histological healing represents a complete disease remission in UC. However, endoscopic evaluation and histological assessment are of less importance in CD due to the transmural and discontinuous nature of the inflammation. Radiological imaging techniques encompassing computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography aid in scenarios beyond the reach of endoscopy which is particularly useful in CD. The advantage of these techniques includes the simultaneous assessment of luminal and extraluminal complications of CD, such as the formation of strictures, fistulae, and abscesses, as well as the tracking of EIMs of IBDs. Although CT has its limitations associated with radiation exposure risk, MRI and ultrasonography help to overcome these problems with similar sensitivity and accuracy.<sup>25,32</sup>

The use of biomarkers provides an objective and non-invasive measurement of disease activity. Certain serum antibodies such as perinuclear antineutrophil cytoplasmic antibodies (pANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) are predominantly present in UC and CD patients, respectively, allowing their differential diagnosis. However, their wide usage is limited by their low sensitivity.<sup>33,34</sup> C-reactive protein (CRP) is a serum marker of acute phase response produced by the liver under inflammatory conditions. The production of CRP by the hepatocytes is stimulated by the cytokine interleukin (IL)-6 and the elevation is more pronounced in CD than in UC. Erythrocyte Sedimentation Rate (ESR) - the rate at which red blood cells migrate

through the plasma over the period of 1 hour - is another widely used marker of acute phase response in IBD. However, CRP levels will increase in other inflammatory conditions (e.g., various cancers, diabetes, cardiovascular diseases) and ESR in response to inflammation, infection, anemia, pregnancy, and with aging, hence both are not specific to IBDs (**Table 2**). Nonetheless, they have been widely used in clinical practice to monitor the treatment response and to predict the disease evolution, supplementing clinical indices.<sup>33–35</sup>

Faecal biomarkers are also popularly used in the diagnosis of IBDs. The advantage of fecal biomarkers is the ease of access to patient stool samples and their specificity to gastrointestinal inflammation. Most of them have also shown high specificity in differentiating IBD from other intestinal disorders as well. Nevertheless, they do not discriminate between CD and UC. A number of neutrophil-derived proteins have been stably found in high levels in the stools of IBD patients. Faecal calprotectin, lactoferrin, lipocalin-2, and S100A12 are some of the proteins released upon neutrophil activation and migration, which are currently in use or tested for future applications in IBD diagnosis (**Table 2**).<sup>33,34</sup>

**Table 2: Current biomarkers used in the diagnosis and clinical management of IBDs**

Sl No.	Biomarkers	Source	Specificity for IBD (Yes/No)	Distinguish CD vs UC (Yes/No)
1	pANCAs	Serum	Yes	Yes
2	ASCAs	Serum	Yes	Yes
3	CRP	Serum	No	No
4	ESR	Blood	No	No
5	Calprotectin	Faeces	Yes	No
6	Lactoferrin	Faeces	Yes	No
7	Lipocalin-2	Faeces	Yes	No
8	S100A12	Faeces	Yes	No

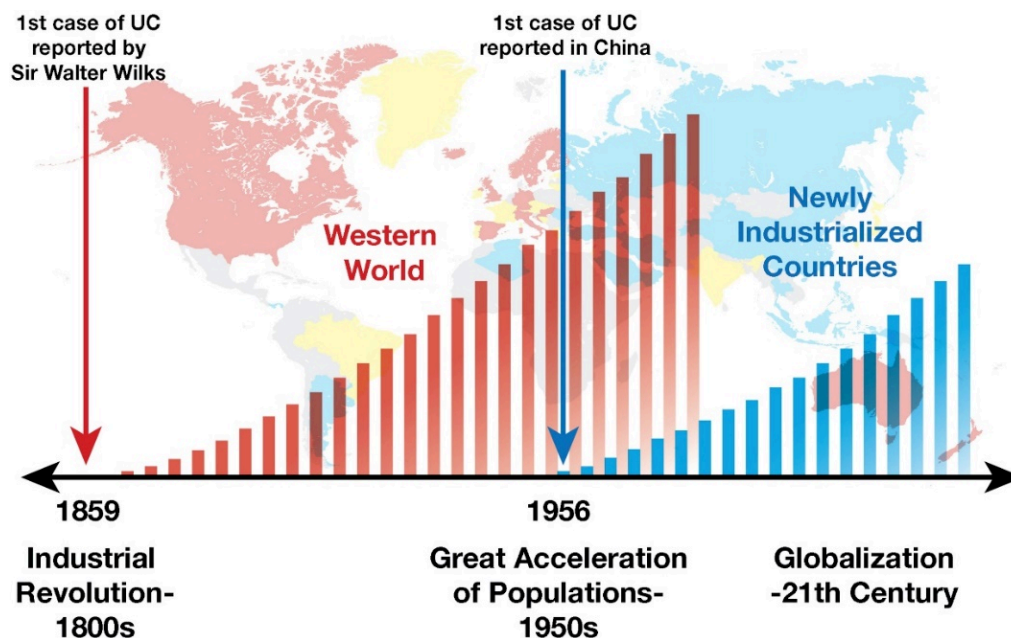
With the development of high-throughput analysis systems, current research focuses on developing biomarker signatures based on transcriptomic, proteomic, metabolomic, and microbiota profiling to enable a more specific, sensitive, and responsive diagnosis of the disease. For example, protein profiles from the serum of IBD patients have been studied by several groups as an attempt to generate models that predict treatment outcomes in response to anti-TNF therapy.<sup>36</sup> Multi-omics technologies have also greatly



contributed to the recent developments in the use of microbiome-based biomarkers in patient stratification and predicting clinical responses.<sup>37</sup> The current developments in biomarker signature studies progress towards personalised precision medicine by generating a systems biology approach that uses multi-omics derived data to create predictive models of disease progression and response to therapy.<sup>38</sup>

#### 1.4. Epidemiology

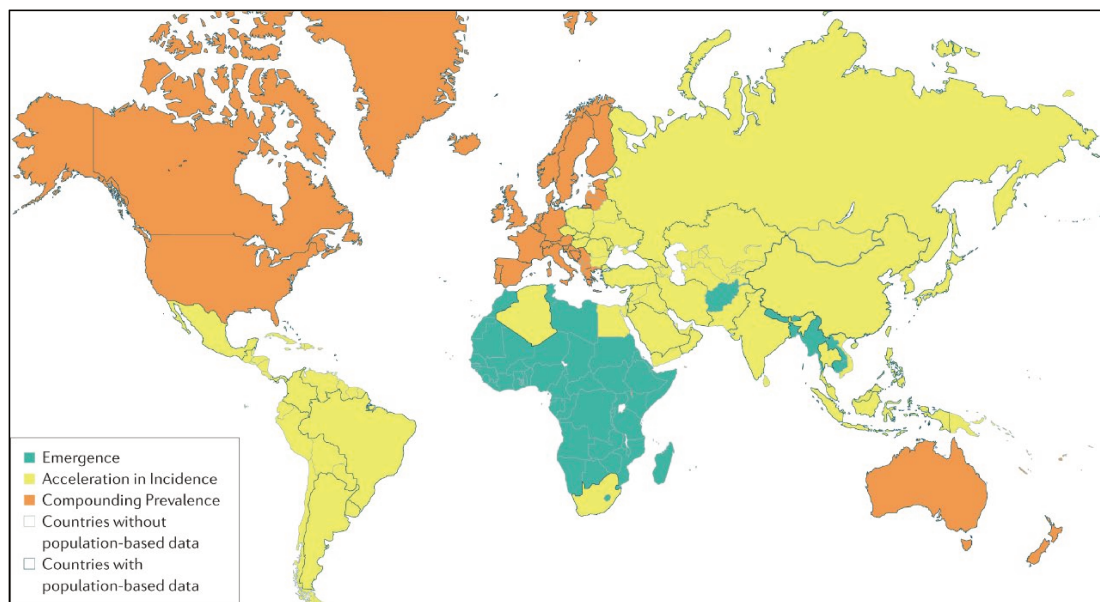
In epidemiological terms, the incidence of a disease refers to the number of new cases developed in a population during a particular period, while, prevalence refers to the number of existing cases of a disease in a population at a given time. Since the first reported cases in Western Europe in the 18<sup>th</sup> century, the prevalence of IBDs continues to increase in Western countries with the highest number of cases in Europe (2 million) and North America (1.5 million), which led to a previously held belief that it is a Western world disease affecting people of Caucasian descent. This has been contradicted by the observation that, newly industrialised countries in other parts of the world are also now following a similar trend with a steady increase in the rate of incidence (**Figure 6**). Currently, 6.8 million individuals are estimated to live with IBDs worldwide.<sup>39</sup>



**Figure 6: Increasing trend of IBDs in the western world and newly industrialised countries**

*Figure taken from Kaplan GG, Ng SC. Gastroenterology. 2017;152(2):313-321 with permission.*

Kaplan et. al stratifies the global evolution of IBDs into four epidemiological stages (**Figure 7**). According to this classification, in 2020, developing countries are in the ‘emergence stage’ (shown in green) in which sporadic cases begin to be identified in the population. Whereas, newly industrialised countries are in the ‘acceleration in incidence stage’ (shown in yellow) where there is a dramatic increase in the incidence, but the prevalence remains low. Western countries are in the ‘compounding prevalence stage’ (shown in orange) in which the incidence rate stabilises but the slope of prevalence continues to increase. It is postulated that western regions will gradually transition into a fourth ‘prevalence equilibrium stage’. In this predictive view, the incidence of IBDs approximates the mortality rate, possibly due to the advancing age of the IBD population and an unexpected rise in mortality due to the coronavirus disease 2019 (COVID-19) pandemic in 2020-2021, causing prevalence to stabilize.<sup>40</sup>



**Figure 7: Global map showing the epidemiological evolution of IBD in 2020**

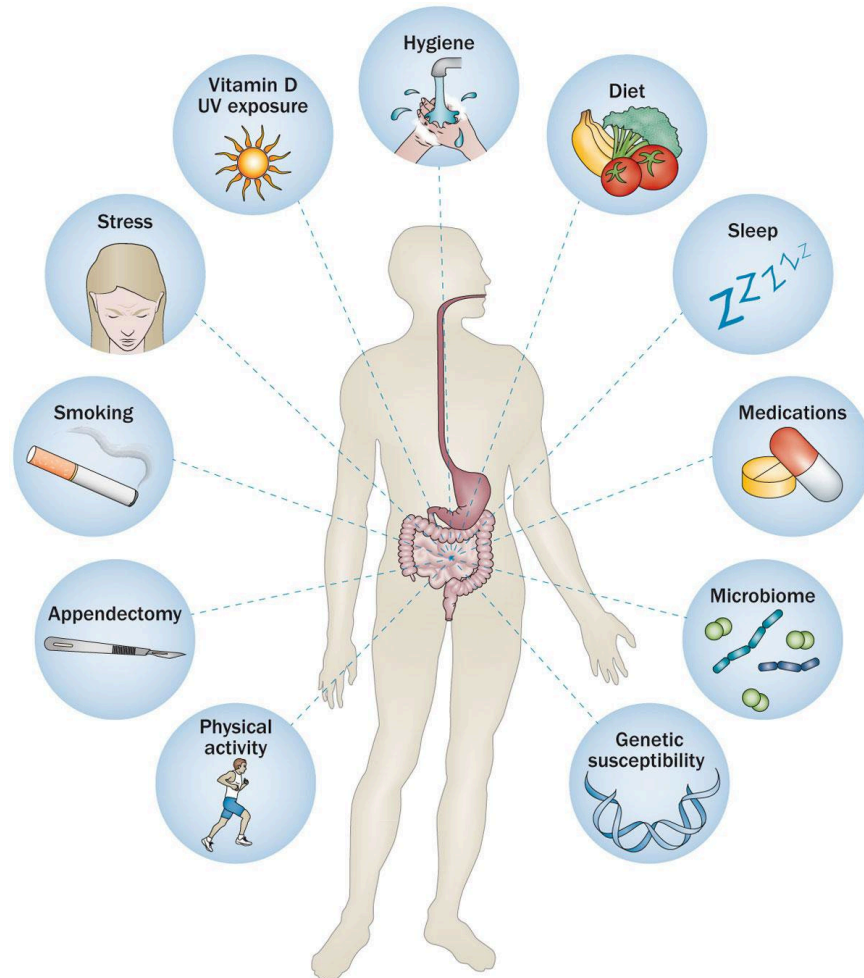
Global organisation of regions into current epidemiological stages of IBD evolution according to United Nations development classifications (2020). *Figure taken from Kaplan GG, Windsor JW. Nat Rev Gastroenterol Hepatol. 2021;18(1):56-66 with permission.*

The increasing incidence of IBDs especially in newly developed countries can be attributed to many factors including environmental exposures (see section 1.5.3) related to the westernisation of societies (especially diet), increased awareness about the disease, improved access to healthcare facilities, and improved disease diagnosis systems.<sup>41</sup>

IBDs are primarily diagnosed in young individuals between 18-35 years of age with a very low mortality rate. Paediatric onset IBDs are also diagnosed. As a matter of fact, approximately 20-30% of patients with IBDs have the onset of symptoms before 18 years of age.<sup>42</sup> There are no clear differences observed in the incidence rates of IBDs in male and female populations. In a systematic literature search carried out by Molodecky et al. 2012, incidence rates stratified by sex were reported in 50 UC and 59 CD studies. According to this analysis, some studies showed more incidence in males or others showed vice versa, and few others found no difference between males and females. The female to male incidence ratio largely varied from 0.51 to 1.58 for UC studies and 0.34 to 1.65 for CD studies. This inconsistency possibly indicates that the diagnosis of IBDs is not sex-specific.<sup>43</sup>

## 1.5. Aetiology

The aetiology of IBDs involves complex multifactorial events which combine genetic, microbial, and environmental factors (**Figure 8**).<sup>44</sup>



**Figure 8: Interplay of genetic, microbial and environmental factors in IBDs**

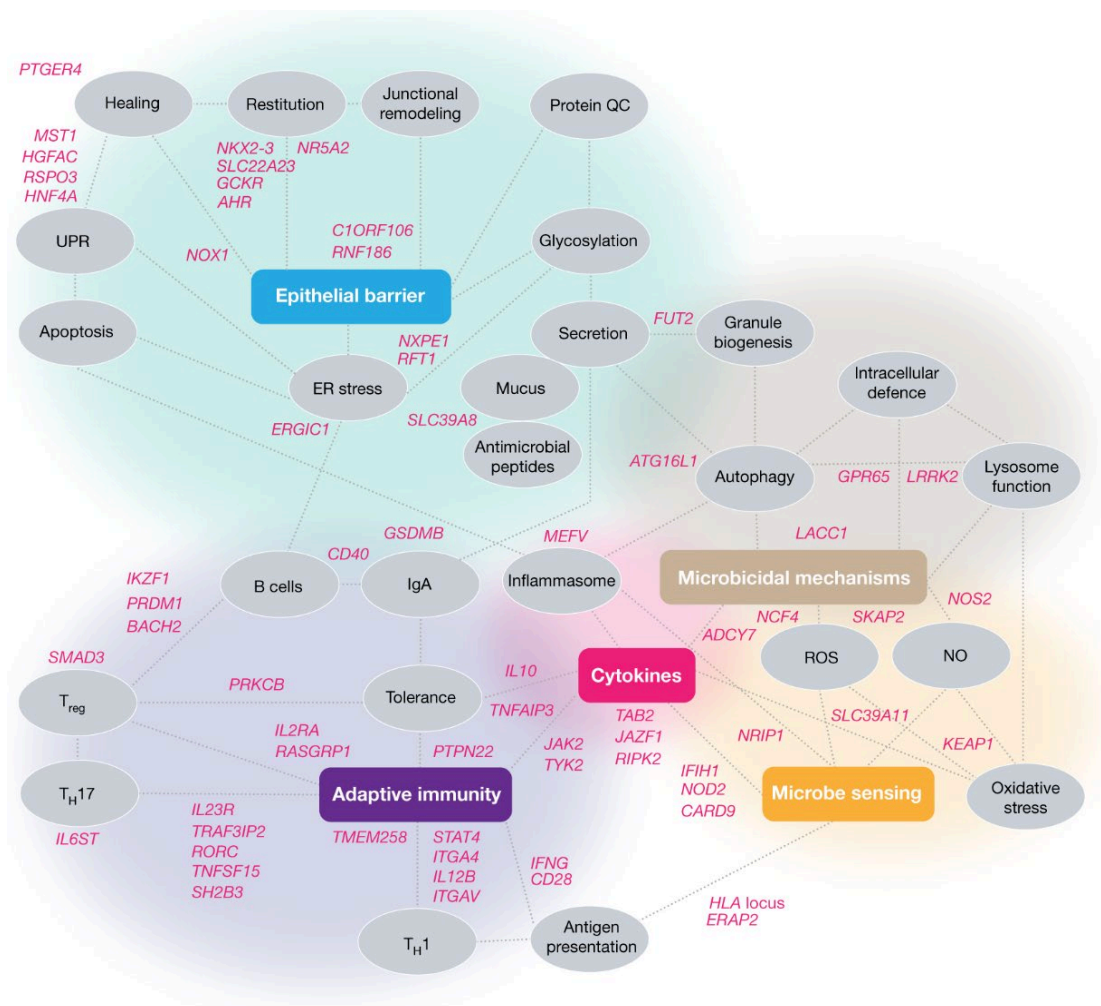
IBDs results from a complex interaction between genetic predispositions, gut dysbiosis and environmental influences. None of these factors alone is sufficient to induce the development of the disease. *Figure taken from Ananthakrishnan AN. Nat Rev Gastroenterol Hepatol. 2015;12(4):205-217 with permission.*

### 1.5.1. Genetic factors

The first genome-wide linkage analysis in IBD has identified a CD susceptibility locus (IBD1) on chromosome 16 in 1996.<sup>45</sup> Later, in 2001, the intracellular pattern recognition receptor (PRR) nucleotide-binding oligomerization domain-containing (NOD)2 has been discovered as a major susceptibility gene for CD within the IBD1 locus by positional cloning strategy. This study has identified three independent genetic

associations for CD including a frameshift variant and two missense variants of NOD2<sup>46</sup>. Genome-wide association studies (GWAS), a method which investigates the whole genome to associate genetic variations with specific diseases, have revolutionised our understanding about complex polygenic disorders. The first GWAS in CD was published in 2005 and it has found that single nucleotide polymorphisms in the *TNF superfamily member (TNFSF) 15* gene, which is a novel TNF-like factor expressed in endothelial cells, confer increased CD risk.<sup>47</sup> To date, more than 240 risk loci are found to be associated with IBDs.<sup>48–51</sup>

Apart from demonstrating the polygenic nature of the disease, GWAS have provided insights into the disease pathology revealing numerous interconnected functional pathways associated with IBDs (**Figure 9**).<sup>52</sup>



**Figure 9: IBD risk genes are involved in a complex network of interconnected pathways**

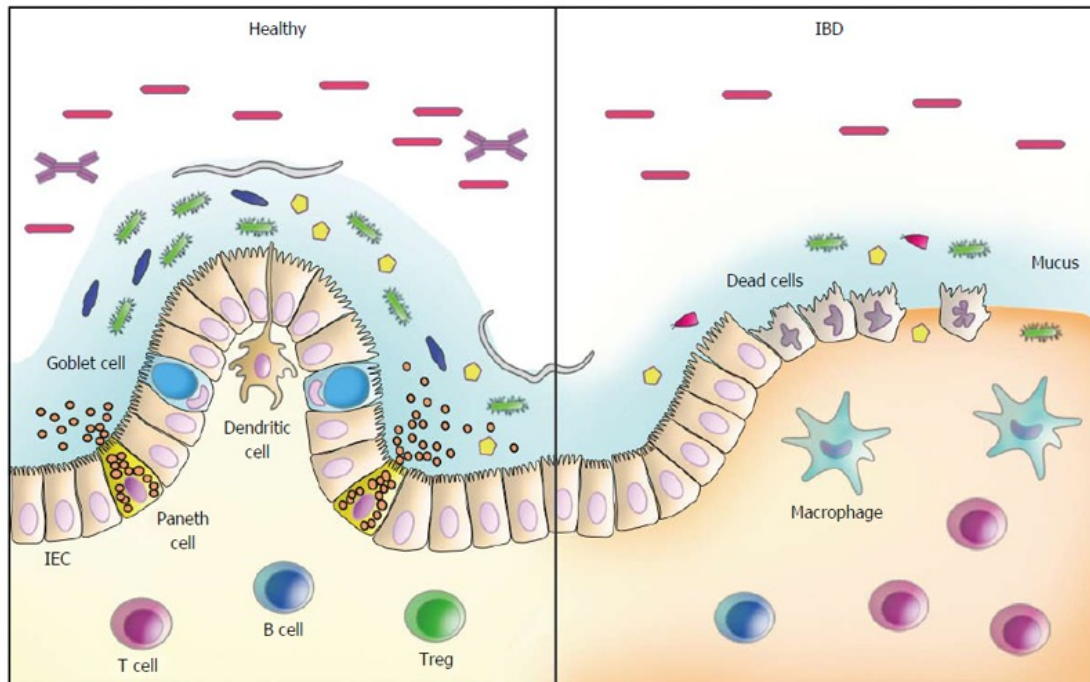
IBD risk genes regulate several overlapping biological functions depending on their cell-type specific activities and multifunctional nature. *Figure taken from Graham DB, Xavier RJ. Nature. 2020;578(7796):527-539 with permission.*

Identification of *NOD2* as the first susceptibility gene shed light on the importance of innate immunity in providing defence against pathogens invading the intestinal mucosa, which was further underlined by the association of other genes involved in innate mucosal defence, such as *caspase recruitment domain-containing protein (CARD)9* and *Fc gamma receptor IIa (FCGR2A)*.<sup>50</sup> In 2007, identification of genetic polymorphisms in the autophagy-related (ATG) genes *ATG16 like 1 (ATG16L1)* and *Immunity related GTPase M (IRGM)* to be associated with IBD risk, was a major milestone in the history of IBD genetics, revealing an unexpected role of the autophagy pathway - a vital cellular degradation machinery - in the pathogenesis of IBDs.<sup>1,6,53</sup> Although *NOD2* and autophagy were thought to independently influence IBD pathogenesis, subsequent studies have established a link between these pathways by discovering the interaction between *NOD2* and *ATG16L1* in an autophagy-dependent manner. *NOD2-ATG16L1* interaction is implicated in antibacterial autophagy as well as in autophagy-mediated major histocompatibility complex (MHC) antigen presentation.<sup>54</sup> Moreover, the multifunctional nature of *ATG16L1* interconnected autophagy with several other pathways such as inflammasome activation and Paneth cell functions in the disease pathogenesis of IBDs.<sup>55,56</sup> Together, these associations also point to the role of genetic determinants in shaping the local microbial environment and thereby promoting a healthy gut microbiome. IBD genetics also contributed immensely to establishing the complex cytokine networks associated with the disease pathology. Various elements of adaptive immune cell responses are also integrated into these genetic associations such as T-cell and B-cell regulation, activation, tolerance, etc. Genetic studies in IBDs have also revealed several regulators of intestinal epithelial barrier functions to be associated with disease risk (**Figure 10**).<sup>50,52</sup>

Besides, the extent to which genetic studies have contributed to the current developments in the diagnosis and therapeutics of IBDs has been remarkable. The early therapeutic interventions in IBDs were largely focused on treating inflammation. The new insights generated from IBD genetics help to better define the mechanisms of



action for therapeutic interventions as well as provide potential avenues for developing targeted treatments (see section 1.8).<sup>52</sup>



**Figure 10: Schematic illustration of the intestinal mucosa (healthy vs IBD)**

Figure taken from Rapozo DCM, Bernardazzi C, de Souza HSP. *World J Gastroenterol.* 2017;23(12):2124-2140 with permission.

### 1.5.2. Microbial factors

Gut dysbiosis, which is described as the alterations in the composition of intestinal microflora, is a major factor in the pathogenesis of IBDs. Interestingly, many animal models of IBDs including genetically induced models (see section 1.7) do not develop the disease under germ-free (GF) conditions emphasising the critical role of microbes in triggering the immune response. The gut microbiome of each individual is shaped by genetics as well as by environmental exposures during childhood such as mode of delivery, diet, hygiene, etc. These factors are obviously not identical among individuals, explaining the creation of personal microbiomes. For example, a baby born through a vaginal delivery acquires vaginal microbes while a cesarean section imprints a microbiota similar to that of human skin.<sup>57</sup> The dynamic composition of this microbial colonisation during early childhood, which becomes more stable with increasing age, is important in the development and maturation of the immune system by establishing a balance between tolerance and protective immunity against pathogens.<sup>58</sup>

### **1.5.3. Environmental factors**

#### **1.5.3.1. Smoking**

The earliest environmental risk factor found to be associated with IBDs was cigarette smoking. Smoking increases the risk of CD by 2-fold compared to the people who never used tobacco products. However, in UC, smoking seems to exert a protective effect, a mystery that is currently unresolved.<sup>59,60</sup> Cessation of smoking significantly increases the risk of developing UC, and in smokers with established UC, it increases the severity of the disease.<sup>61,62</sup> The effect of smoking on IBDs is thought to be driven mainly by altering microbiome composition. In support of this hypothesis, Allais et al. 2016 have shown that exposure of mice with smoke for 24 weeks shifts the gut bacterial community structure and strongly increases the activity of *Lachnospiraceae* sp.<sup>63</sup>

#### **1.5.3.2. Appendectomy**

Similar to cigarette smoking, appendectomy appears to have opposite effects on CD and UC. Patients who have undergone appendectomy for an inflammatory condition like appendicitis and mesenteric lymphadenitis before the age of 20 years are found to have a low risk of UC. On the contrary, patients who underwent appendectomy for nonspecific abdominal pain did not show this effect.<sup>64,65</sup> However, appendectomy increases the risk of CD and this association persists up to 20 years after the appendectomy.<sup>66</sup>

#### **1.5.3.3. Diet**

Extensive studies have been carried out to determine the role of diet in IBD pathogenesis. Though diet has emerged as a key determinant factor in the disease, the relationship seems to be complex and the exact pathophysiological aspects remain to be elucidated. Diet is thought to directly affect the gut microbiome composition in many ways. Diet can also alter the production of metabolites by the commensal microflora. In addition, dietary antigens can sometimes trigger an immune response.<sup>67</sup>

Breast milk, which is the first dietary exposure of humans, exerts protective effects on paediatric IBDs compared to formula milk, by altering the composition of the gut microbiota.<sup>68</sup> A high-fat diet is shown to increase the susceptibility to colitis in experimental mice, independent of obesity.<sup>69</sup> n-6 Polyunsaturated fatty acids (PUFA) can activate innate immune receptors, whereas n-3 PUFA can inhibit them. Therefore,



a high ratio of n-6:n-3 fatty acids is associated with an increased risk of developing IBDs.<sup>67</sup> According to a prospective study, a high intake of dietary fibre was associated with a 40% reduced risk of CD.<sup>70</sup> Although the precise mechanism is not known, several studies regarding dietary fibre intake support this observation. For example, the fermentation of fibres by the intestinal microbes produces a large amount of short-chain fatty acids (SCFA) as by-products. SCFAs are found to have immunoregulatory functions by increasing the development of T regulatory cells (T regs) and are also important in epithelial barrier function as the main energy source for the colonic epithelial cells.<sup>71</sup> Supplementation of certain amino acids such as glutamine, arginine, tryptophan, and threonine was found to reduce symptoms of colitis in experimental mice due to their immune regulatory functions. In contrast, an iron sulfate containing diet was found to change the microbial composition and promote intestinal inflammation.<sup>72</sup> The breakage of redox regulatory balance and oxidative stress are key features of IBD pathogenesis. The reactive species levels in the gut can be nutritionally modulated by the supply of antioxidant substances (e.g., vitamins C and E, polyphenols, or uric acid). A diet rich in plant-derived foods (fruits and vegetables) contains several sources of antioxidant micronutrients thereby protecting from IBD risk.<sup>73</sup>

#### **1.5.3.4. Vitamin D**

IBD incidence is associated with reduced UV exposure, a major source of vitamin D. In addition to the effect on disease activity, IBD patients with low plasma vitamin D are found to have an increased risk of colorectal cancer, and *Clostridium difficile* infection (CDI).<sup>74</sup> Knockout (KO) of vitamin D receptor in mice models of colitis was shown to be associated with increased disease susceptibility and administration of vitamin D reduced this phenotype.<sup>59</sup> The protective immunomodulatory functions of vitamin D have been demonstrated in many other inflammatory disorders/autoimmune diseases (AIDs) as well, with potential therapeutic implications.<sup>75,76</sup>

#### **1.5.3.5. Hygiene**

The ‘hygiene hypothesis’ proposed by David Strachan in 1989 related high levels of environmental hygiene with reduced exposure to microbial infections during early childhood with the rise in allergic diseases such as asthma and hay fever during the 20th century.<sup>77</sup> Later, it has also been linked to the increase of other inflammatory conditions/AIDs in developed countries.<sup>78</sup> In accordance with this hypothesis, high-

income countries with higher levels of hygiene have been associated with increased IBD risk possibly due to reduced gut microbial diversity. However, it may not apply to all the populations worldwide and the data are limited by confounding factors. The association is found to be more relevant in newly developed countries and populations migrating from less to high-income countries, especially in the second-generation migrants born in the high-income country. This correlation is no longer significant in the countries living in high standards for several generations. Nevertheless, in developing countries, low hygiene levels and increased exposure to infections are found to be associated with an increased risk of developing IBDs.<sup>79</sup>

#### **1.5.3.6. Medications**

Antibiotics are useful in treating several infections, yet excessive use of antibiotics may dramatically alter the gut microbial composition, especially in early childhood. Consequently, several studies, including a recent large population-based study by Nguyen and colleagues, have made a correlation between increased IBD risk and high exposure to antibiotics, especially treatments with a broad spectrum of microbial coverage.<sup>80</sup> The effect of non-steroidal anti-inflammatory drugs (NSAIDs) has also been extensively investigated in relation to IBDs. Mouse models of colitis administered with NSAIDs develop more severe colitis due to a reduction of cyclooxygenase-mediated prostaglandin synthesis in the gut. Prostaglandins have important roles in regulating the mucosal immune responses and intestinal epithelial growth.<sup>81</sup> The use of oral contraceptives is also weakly associated with increased IBD risk, but the mechanisms are currently unknown.<sup>59</sup>

#### **1.5.3.7. Lifestyle-stress, sleep, physical activities**

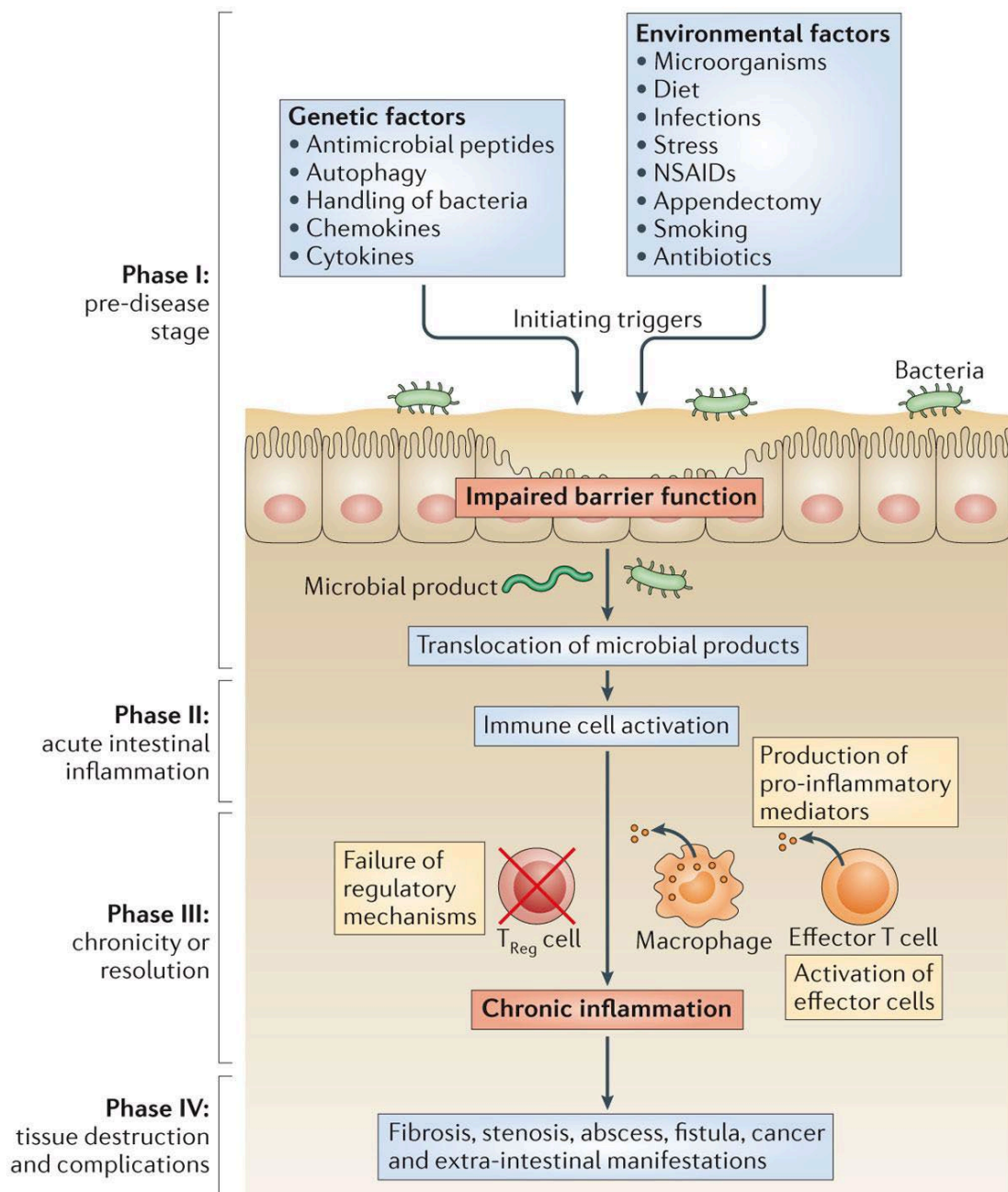
IBD patients are often diagnosed with depression and anxiety disorders. However, pre-existing conditions of stress, depression, or anxiety can increase the risk of IBDs. These factors can also be associated with an increased rate of disease relapses. Moreover, both increased or decreased sleep and reduced sleep quality have been associated with higher disease risk and relapses in IBD patients. Mice induced with stress develop more severe colitis, and interestingly, it is reversed by the administration of antibiotics, pointing to a mechanism driven through gut microbiota modulation by stress.<sup>59</sup>

The existence of a gut-brain axis (GBA), consisting of bidirectional communication between the gastrointestinal tract and the central nervous system (CNS), has been well described. This complex interaction network includes the central and peripheral nervous systems, endocrine, immune and metabolic pathways. The CNS plays an important role in modulating gut functions such as intestinal motility, secretion, and the gut immune system in response to psychosocial stressors. The crucial role of gut microbiota has been demonstrated in influencing these interactions and thus the concept of a microbiome GBA has now emerged.<sup>82</sup> Gut dysbiosis has been associated with major neurological diseases including Parkinson's disease, Alzheimer's disease, multiple sclerosis (MS), autism and major depressive disorder.<sup>83</sup> Studies on GF animals have shown that microbial colonisation of the gut is essential for the development of CNS and the absence of microbes is associated with the altered expression of several neurotransmitters.<sup>84</sup> On the other hand, there are also evidences that psychological stressors can potentially modulate the composition and total biomass of the gut microbiota. The presence of neurotransmitter receptors has been reported in bacteria such as *Escherichia coli* (*E. coli*) and *Pseudomonas fluorescens* (*P. fluorescens*). Besides, hormones released from the CNS have the potential to modulate intestinal permeability allowing bacterial antigens to penetrate and induce immune responses.<sup>82,85</sup>

Studies have also been conducted to investigate the relationship between physical activity and IBD risk, which showed that sedentary occupations increased the risk of disease whereas heavy labour occupations were inversely associated with disease risk.<sup>59</sup>

## **1.6. Pathology**

A combined effect of the IBD risk factors described above leads to disruption of the intestinal epithelial barrier, exposing luminal bacterial antigens of the commensal gut microbiota to the lamina propria immune cells. In genetically susceptible individuals, this causes aberrant activation of the immune cells and excessive cytokine production resulting in acute mucosal inflammation. A failure to resolve this acute inflammation by anti-inflammatory mechanisms or other regulatory systems further leads to chronic intestinal inflammation and associated extra-intestinal complications (**Figure 11**).<sup>86</sup>



**Figure 11: Conceptual framework for the pathogenesis of IBDs**

Genetic and environmental factors induce barrier dysfunctions resulting in the translocation of commensal microbes into the gut wall and leads to the activation of gut immune system and cytokine production. A failure to resolve acute inflammatory responses eventually leads to chronic intestinal inflammation and tissue destruction. *Figure taken from Neurath MF. Nat Rev Immunol. 2014;14(5):329-342 with permission.*

### 1.6.1. Gut microbiota

The general notion regarding the interaction between the gut microbiome and IBDs is the loss of tolerance to commensal microbiota. Increased T cell and antibody (Ab) responses against microbial antigens are reported in IBD patients. One of the early studies in this setting was conducted by Pirzer and colleagues to demonstrate that intestinal T lymphocytes derived from IBD patients which are otherwise unresponsive to microbial antigens *in vitro*, proliferate in response to a range of commensal bacterial antigens.<sup>87</sup> Circulating antibodies against microbes, such as *saccharomyces cerevisiae* (ASCA; IgA and IgG), *E. coli* outer membrane protein C (Omp-C; IgA), anti-flagellin (CBir1; IgG), and anti-*P. fluorescens* (IgA) have been found in CD patients.<sup>88</sup> Although the role of such antibodies in the disease pathogenesis is not clear, it points to enhanced adaptive immune responses to the commensal microflora in IBD patients.<sup>89</sup>

The gut microbiota of healthy humans is composed of four major bacterial phyla: an abundant population of *Firmicutes*, *Bacteroidetes*, and lower amounts of *Proteobacteria* and *Actinobacteria*. In IBD patients, an increased abundance in *Actinobacteria* and *Proteobacteria* families and a decrease in the abundance of *Firmicutes* and *Bacteroidetes* have been found.<sup>90,91</sup> Although it is well established that changes in the gut microbiota composition and a decreased bacterial diversity are hallmarks in IBD patients, it is not clear whether dysbiosis is a primary or secondary phenomenon in IBDs. The alterations in microbial composition can be a cause that can potentially trigger immune responses or a consequence of the series of events that changes the gut physiology, which will then contribute to perpetuating the inflammation. There are evidences to support both these arguments. Genetically induced models of colitis such as IL-10<sup>-/-</sup> mice do not develop inflammation when they are housed under gnotobiotic conditions, but when they are transferred to conventional conditions, they spontaneously develop the disease.<sup>92</sup> However, there are also ample evidences to show that microbial communities can shift as a consequence of alterations in genes linked to mucosal barrier functions and antimicrobial defence mechanisms in the gut.<sup>89,93</sup>

Apart from global shifts in the bacterial communities, the contribution of some specific bacteria or pathogens has been widely investigated in the pathogenesis of IBDs. Some species belonging to the family *Enterobacteriaceae*, especially *adherent invasive E. coli* (AIEC) are enriched in the intestinal mucosa. Around 40% of patients with ileal CD

were found to have higher colonisation of *AIEC* compared to healthy controls.<sup>94</sup> Although these species are part of the commensal flora, they act as opportunistic pathogens which modulate the host immune barrier to favour their growth in genetically susceptible individuals.<sup>95</sup> Another obligate pathogenic species that is frequently associated with IBD pathogenesis is *Mycobacterium avium subsp. paratuberculosis* (MAP). MAP/MAP-DNA levels are found to be high in mucosal tissues from CD patients.<sup>96</sup> CDI are also found in up to 10% of IBD patients, which can further exacerbate the immune responses or cause reactivation of IBDs. However, the reason for the increased development of CDI is mainly thought to be the drugs used for IBD treatment, especially repeated administration of antibiotics which can favour their colonisation in the mucosa.<sup>97</sup>

### **1.6.2. Intestinal epithelial barrier**

The key role of intestinal epithelium - a monolayer of columnar epithelial cells connected by tight junctions (TJ) - is to form a tightly regulated barrier to prevent excessive contact of luminal food-borne and microbiota-derived antigens with the underlying immune cells, and to allow a selective entry of antigens to educate the gut immune system and thereby develop tolerance against self-antigens. Moreover, specialised epithelial cells such as Paneth cells and goblet cells provide multiple layers of protection by secreting antimicrobial peptides and mucins.<sup>98</sup> Failures in many of these functions are strongly implicated in the pathogenesis of IBDs (**Figure 10**).<sup>99</sup>

Several members of the TJ protein families claudins, occludin, and junction adhesion molecule (JAM) family were found to be dysregulated in IBDs. Zeissig et al. 2007 have shown that changes in the distribution of claudin-2, 3, 5, and 8 lead to barrier dysfunctions in active CD patients. An increase in the expression of pore-forming claudin-2 and downregulation and redistribution of sealing claudins 3, 5, and 8 was observed, resulting in increased paracellular permeability.<sup>100</sup> JAM-A, a TJ localised protein that controls leucocyte migration into the tissues is significantly downregulated in IBD patients as well as in experimental colitis.<sup>101</sup> The mRNA expression level of Occludin was found to be reduced in the colonic mucosa of UC patients.<sup>102</sup> The consequences of these alterations in the TJ proteins would be an increased diffusion of ions and water from blood to lumen, a phenomenon known as leaky flux diarrhea, and increased exposure to luminal antigens leading to excessive inflammatory responses.<sup>99</sup> Interestingly, even the patients with quiescent IBD and first-degree relatives of CD

show increased intestinal paracellular permeability<sup>103</sup> suggesting that altered barrier permeability can precede the disease onset.

The epithelium is renewed every 4-5 days with cells shedding into the lumen and the crypt base stem cells proliferate to compensate for the cell loss. Excessive death of intestinal epithelial cells by apoptosis or necroptosis is consistently linked to disrupted barrier integrity and consequently to the severity of IBDs (**Figure 10**). The histomorphological damages including erosions and crypt loss observed in the mucosa of IBD patients are largely due to intestinal epithelial cell (IEC) damage.<sup>99</sup> The pro-inflammatory cytokine TNF- $\alpha$  is directly involved in inducing epithelial cell apoptosis and cell shedding. In support of this view, anti-TNF- $\alpha$  treatments in IBD patients (see section 1.8.2.1) are shown to reverse the increased epithelial apoptosis rates and epithelial barrier dysfunctions, whereas, the levels of TJ proteins were unaffected.<sup>104</sup>

Obviously, excessive cell death can eventually lead to the loss of specialised epithelial cells and their functional involvement in keeping the barrier defence. Apart from that, several genetic polymorphisms associated with IBD risk are implicated in goblet cell and Paneth cell development and functions. The *NOD2* and *ATG16L1* variants involved in antimicrobial peptide secretion by Paneth cells and *Mucin2* gene variant associated with mucus secretion by goblet cells are important examples for this scenario (see section 3.2.1).

### **1.6.3. Innate immune cells**

#### **1.6.3.1. Macrophages**

Intestinal lamina propria comprises a variety of mononuclear phagocyte cell subsets including dendritic cells, monocytes, and tissue macrophages. Macrophages are the most abundant mononuclear phagocytes in the intestine constituting one-fifth of all leukocytes. The typical intestinal resident macrophages lie beneath the epithelial monolayer in the lamina propria and lack the innate immune receptor CD14. They do not produce pro-inflammatory cytokines but maintain their phagocytic activity by immediately capturing the bacteria that cross the epithelial barrier avoiding an immune response. They also produce anti-inflammatory cytokines, such as IL-10 and transforming growth factor-beta (TGF- $\beta$ ).<sup>105</sup>

In IBD patients, CD14<sup>+</sup> macrophages have also been reported in high numbers in the inflamed mucosa which are capable of producing abundant pro-inflammatory cytokines

(IL-23 and TNF- $\alpha$ ) in response to bacterial antigens compared to typical resident macrophages. These CD14<sup>+</sup> macrophage-derived cytokines trigger the production of interferon-gamma (IFN- $\gamma$ ) by lamina propria mononuclear cells, which in turn induces the differentiation of the IL-23 hyperproducing macrophage phenotype in a feedback loop contributing to the pathogenesis of IBDs.<sup>106</sup> In contrast, another study with macrophages derived from peripheral blood mononuclear cells from IBD patients, has found an impaired secretion of pro-inflammatory cytokines in response to *E. coli* and toll-like receptor (TLR) ligation. The levels of intracellular TNF- $\alpha$  was also found to be diminished in these macrophages.<sup>107</sup> These results suggest that both enhanced pathogenic responses and inadequate protective responses by macrophages to enteric microbiota can contribute to the pathogenesis of IBDs.<sup>89,108</sup>

### **1.6.3.2. Dendritic cells**

Dendritic cells (DCs) are bone marrow-derived antigen-presenting cells (APCs) that display antigens to T and B cells, hence acting as a connecting link between innate and adaptive immune systems. They are the most potent APCs and are found throughout the gastrointestinal tract, including lamina propria (LP), Payers' patches (PP), mesenteric lymph nodes (MLNs), and lymphoid follicles.<sup>109</sup> It is established that they play crucial roles in intestinal homeostasis by regulating both immunity and tolerance. Immature DCs exist in a phagocytic state in LP or PP where they continuously acquire foreign and self-antigens from the intestinal lumen through various mechanisms. Then they mature and migrate to the MLNs to present the antigens to naïve T cells to trigger a protective immune response against pathogens or induce a tolerogenic response by inducing T regs to self-antigens. Besides, a crosstalk between epithelial cells and DCs exists as the IEC-derived thymic stromal lymphoprotein (TSLP) keeps the DCs in a T helper (Th)2-like phenotype producing less IL-12.<sup>110</sup>

In IBD patients, mucosal DCs express increased levels of TLR2 and TLR4, show higher levels of CD40 receptor, and produce more amount of IL-12 and IL-6 cytokines.<sup>111</sup> In healthy individuals, migration of DCs to MLNs is induced by binding of the lymphoid chemokine ligands CCL19 and CCL21 to the chemokine receptor CCR7 expressed on mature DCs. However, high expression levels of CCL19 and CCL21 are observed in the colonic mucosa of IBD patients which probably create a similar chemokine microenvironment usually present in lymph nodes, causing the matured DC to be



trapped in the inflammatory sites of the mucosa.<sup>112</sup> In addition to these observations, The IECs isolated from CD patients express significantly low or undetectable levels of TSLP mRNA and lose control over the IL-12 production by DCs polarising a Th1 response and thereby contributing to IBD pathogenesis.<sup>89,113</sup>

#### **1.6.4. Adaptive immune cells**

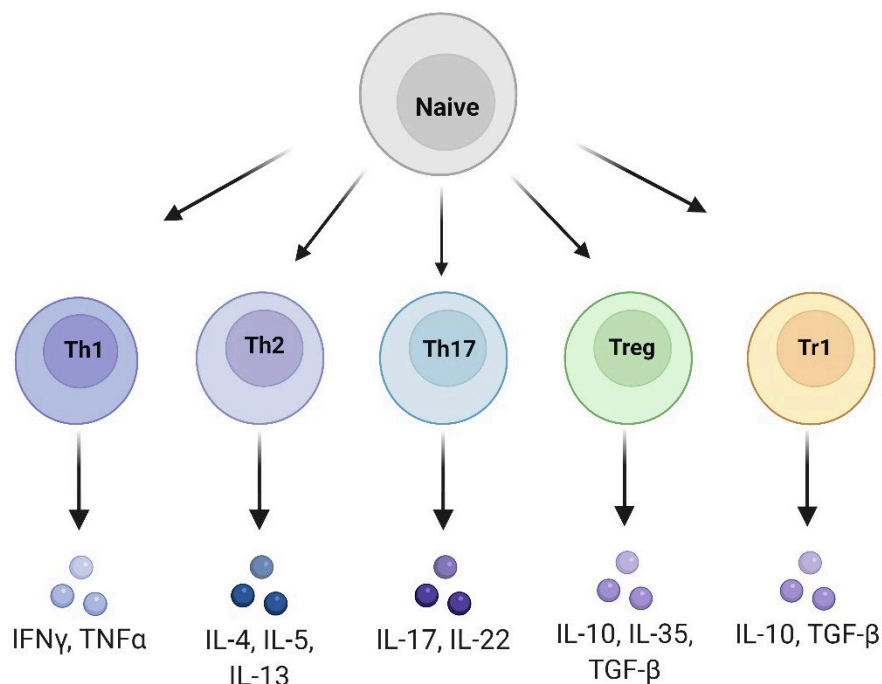
##### **1.6.4.1. T cells**

A tight balance between the inflammatory and regulatory T cell subpopulations is essential to maintain intestinal homeostasis by preventing unwanted inflammatory responses to self-antigens. In a healthy intestine, the regulatory mechanisms outweigh the inflammatory signals, but this can be disrupted by epithelial barrier disruption to increase the exposure to pro-inflammatory stimuli or defective immune mechanisms leading to enhanced immune reactivity.<sup>114</sup> Massive infiltration of inflammatory CD4<sup>+</sup> T cells in intestinal tissue is a characteristic feature of chronic intestinal inflammation and depletion of CD4<sup>+</sup> cells with an anti-CD4 Ab has shown to be effective in treating IBD patients.<sup>115</sup>

Effector T cells: The major effector CD4<sup>+</sup> T cell subsets relevant to IBDs are Th1, Th2, and Th17. Th1 cell polarisation is driven by IL-12 cytokine *via* signal transducer and activator of transcription (STAT)4 signalling to stimulate the production of IFN- $\gamma$  and TNF- $\alpha$  by these cells. Th1 cells typically respond to intracellular pathogens and activate innate immune cells. Th2 cells are driven by IL-4 *via* STAT6 signalling to produce the cytokines IL-4, IL-5, and IL-13, and are activated primarily in response to parasitic helminth infections. The transcription factor GATA-3 is an important regulator of Th2 differentiation. Th17 cells differentiate in response to IL-6, IL-23, and TGF- $\beta$  cytokines *via* STAT3 and retinoic acid receptor-related orphan nuclear receptor gamma (ROR $\gamma$ t) signalling leading to the production of Th17 cytokines IL-17 and IL-22. Although Th17 cells have an important contribution in driving autoimmunity, they have both protective and pro-inflammatory roles in mucosal epithelial barriers.<sup>116</sup> IL-17 induces the production of CXC chemokines and granulocyte colony-stimulating factors resulting in the recruitment of inflammatory cells to fight against the pathogens invading mucosal surfaces. However, excessive immune cell infiltration can further lead to inflammation and tissue damage at the mucosa. On the other hand, IL-17 and IL-22 act on epithelial cells to promote barrier repair functions and induce the production of antimicrobial

peptides to kill extracellular pathogens.<sup>116,117</sup> Previously, the inflammatory responses associated with CD were thought to be mediated by Th1 associated IL-12/IFN- $\gamma$  axis. But recent evidences suggest an involvement of a Th17 associated IL-23/IL-17 axis. The emerging view from this observation is that the early lesions of CD are characterised by a Th1 signature while later stages are mediated by Th1/Th17-like responses.<sup>118</sup> Whereas, the pathogenesis of UC is classically attributed to a Th2 response. The transcription factor GATA-3 driving Th2 response is increased in colonic tissues of UC patients compared to CD patients and normal healthy controls.<sup>119</sup>

Regulatory T cells: Tregs are specialised subset of T cells characterised by the expression of the biomarker forkhead box protein P3 transcription factor (Foxp3). The regulatory function of Tregs occurs through multiple mechanisms such as IL-2 scavenging, production of regulatory cytokines (IL-10, IL-35, TGF- $\beta$ ), and high expression of co-inhibitory receptors (cytotoxic lymphocyte antigen 4, CTLA-4; programmed cell death, PD-1).<sup>116</sup> Tr1 is another distinct population of immunosuppressive T cells that do not express Foxp3. They also secrete IL-10 and TGF- $\beta$  mainly in the small intestine (**Figure 12**).<sup>120</sup>



**Figure 12: Major effector and regulatory T cell subsets implicated in IBD pathogenesis**

*Image created with biorender.*

In line with the immunosuppressive role of Tregs, adoptive transfer of Tregs is a largely exploited strategy in treating experimental colitis in mice.<sup>121</sup> However, the role of Tregs in human IBD pathogenesis is currently unclear. The number of Tregs in the intestinal tissue was found to be higher in IBD patients.<sup>122</sup> Hence, the lack of Tregs-mediated immune suppression is not due to an insufficient number of Tregs, rather there is a possible impairment of Tregs function or insensitivity of the effector T cells to Tregs.<sup>123</sup> Though Tregs isolated from IBD patients exhibit suppressive activity during *in-vitro* suppression assays,<sup>124</sup> it is difficult to demonstrate if they sustain the activity in the inflammatory environment. Supporting the latter argument, a previous study has shown that the mucosal CD4<sup>+</sup> T cells isolated from CD patients are resistant to Tregs-mediated suppression due to overexpression of mothers against decapentaplegic homolog (SMAD)7, a factor which controls TGF- $\beta$  anti-inflammatory signalling. This resistance to suppression could be reversed by SMAD7 antisense treatment.<sup>125,126</sup> Recent data also suggests impaired trafficking of Tregs from peripheral blood to the intestinal tissues in CD patients due to reduced expression of the gut-homing molecule  $\alpha 4\beta 7$ .<sup>127</sup> Deeper investigations are needed to establish the exact relation between Tregs and IBD pathogenesis.

#### **1.6.4.2. B cells**

The B cells of the intestine are activated in the lymphoid follicles and mesenteric lymph nodes and subsequently migrate to the lamina propria to become differentiated into plasma cells. The histological features of IBDs include the presence of lymphoid follicles and lymphoplasmacytic infiltrate in the inflamed intestine suggesting a role of B cells in the pathogenesis of IBDs.<sup>128</sup> In the normal gut, the plasma cells predominantly secrete IgA antibodies, which provide protection against the microbes invading the mucosa, but poorly activate the complement system and antagonise the inflammatory effect of other Igs. However, in IBD patients, Ig response in the mucosa is predominantly IgG-mediated, which leads to enhanced complement activation and abnormally elevated immune responses to pathogens.<sup>129</sup>

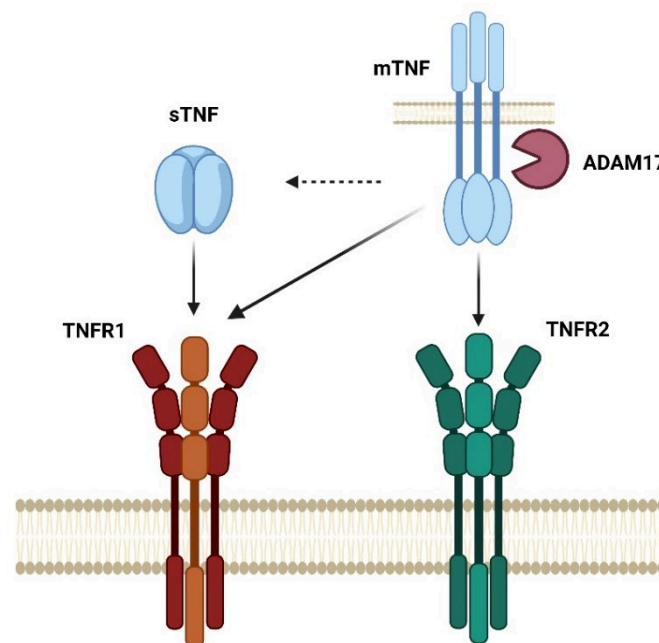
B cells can also serve as APCs to mediate the activity of T cells. A previous study has demonstrated the role of the interaction between B cells and CD8<sup>+</sup> T cells in controlling colitis. This experiment carried out in a genetically induced mouse model (Gai2<sup>-/-</sup>, see **Table 3**) of colitis suggests that efficient induction of CD8<sup>+</sup> Tregs requires direct B

cell-mediated MHC I antigen presentation.<sup>130</sup> There are also evidences to suggest that B cells can activate the expansion of Tregs. B cell deficiency in dextran sulphate sodium (DSS)-induced colitis resulted in a more severe phenotype and a significantly reduced number of Tregs. Adoptive transfer of B cells into these mice attenuated colitis with a simultaneous increase of Tregs.<sup>131</sup> Further understanding of the role of B cells in IBD pathogenesis is yet to be demonstrated in patients.<sup>128</sup>

### 1.6.5. Cytokines networks in IBDs

#### 1.6.5.1. TNF- $\alpha$

TNF- $\alpha$  is a pleiotropic cytokine produced by a wide range of cell types. It is produced as a transmembrane protein and further cleaved by a metalloprotease TNF- $\alpha$  converting enzyme (TACE, also known as ADAM metalloproteinase domain 17 or ADAM17) to produce the soluble TNF- $\alpha$  (sTNF- $\alpha$ ). The signalling is mediated by two receptors TNFR1 and TNFR2. The soluble TNF- $\alpha$  binds to TNFR1 whereas the membrane-bound TNF- $\alpha$  (mTNF- $\alpha$ ) binds to both the receptors (**Figure 13**). TNFR1 is constitutively expressed in most of the cells, whereas, TNFR2 is expressed specifically in immune cells, epithelial cells, and endothelial cells. Differential signalling through both these receptors regulates multiple functions such as cell death, proliferation, and gene activation.<sup>132,133</sup>



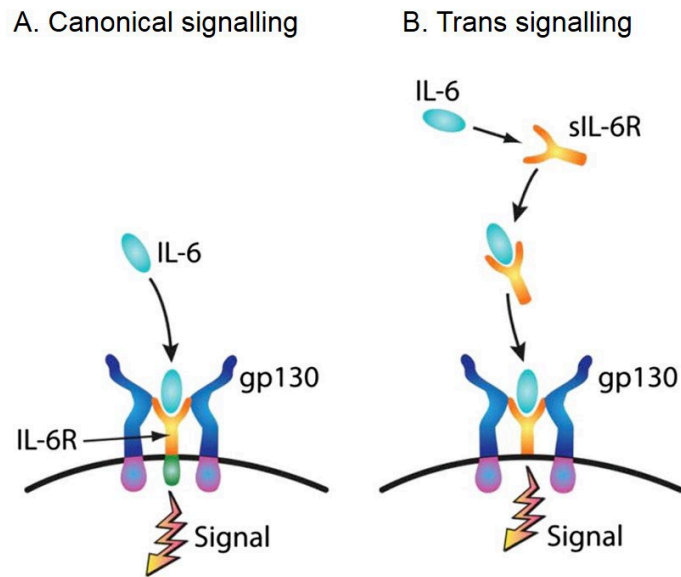
**Figure 13: Schematic of TNF signalling via two receptors**

The mTNF- $\alpha$  is cleaved by the metalloprotease ADAM17 to produce sTNF- $\alpha$ . sTNF- $\alpha$  binds to TNFR1 whereas, mTNF- $\alpha$  binds to both TNFR1 and TNFR2. *Image created with biorender.*

CD14<sup>+</sup> macrophages, adipocytes, fibroblasts and T cells isolated from IBD patients have been found to produce high levels of membrane-bound and soluble TNF- $\alpha$ .<sup>86</sup> TNF- $\alpha$  signalling drives a variety of functions in colitis including pro-inflammatory cytokine production by macrophages, cell death of IECs and Paneth cells resulting in impaired barrier functions, and T cell resistance to apoptosis. Recent evidences present more attention to mTNF- $\alpha$  rather than sTNF- $\alpha$ , since treatments that neutralise both mTNF- $\alpha$  and sTNF- $\alpha$  (e.g., infliximab) or mTNF- $\alpha$  alone was effective in T-cell mediated experimental colitis as well as in patient clinical trials, but specific neutralisation of sTNF- $\alpha$  (e.g., etanercept) alone was not effective.<sup>86,134,135</sup>

#### 1.6.5.2. IL-6

The serum IL-6 levels, as well as colonic mucosal mRNA levels, are found to be elevated in IBD patients and these levels correlate very well with the disease activity.<sup>136,137</sup> Canonical IL-6 signalling is initiated by binding of IL-6 to the membrane-bound form of the IL-6-specific receptor alpha subunit (IL-6R alpha), which then triggers its association with the signal-transducing glycoprotein (gp)130 receptor subunit. This is limited to a small fraction of cells that express IL-6R. However, IL-6 can also exert a trans signalling pathway in cells that express gp130, but lack IL-6R. It is achieved through the generation of a soluble form of IL-6R (sIL-6R), which then forms the IL-6-sIL-6R complex and stimulates the gp130 surface molecule on its target cells. Since gp130 is ubiquitously expressed, trans signalling allows a wide range of cells to be activated by IL-6 (**Figure 14**).<sup>110</sup>



**Figure 14: IL-6 signalling pathways**

A) In the canonical pathway, binding of IL-6 to its transmembrane receptor IL-6R induces its association with gp130. B) In the trans-signaling pathway, IL-6 binds sIL-6R. The IL-6-sIL-6R complex activates cells expressing gp130 alone. *Figure taken from Lee SY, Buhimschi IA, Dulay AT, et al. J Immunol. 2011;186(5):3226–3236 with permission.*

IL-6 is the principal cytokine inducing the production of CRP in the liver during acute phase responses (described above in section 1.3). It was previously demonstrated that CRP serves as a physiological activator of IL-6R shedding to form IL-6-sIL-6R complex and enhance the effect of IL-6 activity in a feedback loop.<sup>138</sup> The IL-6-sIL-6R complex stimulation on lamina propria T cells causes IL-6 dependent STAT3 overexpression and nuclear translocation to induce anti-apoptotic genes Bcl-2 and Bcl-xl to provide resistance to apoptosis. This observation was supported by the fact that treatment of purified lamina propria T cells from IBD patients with sIL-6R neutralizing Ab induced enhanced T cell apoptosis *in vitro*.<sup>139</sup>

### 1.6.5.3. IL-12 family

The IL-12 family consists of a group of heterodimeric cytokines with shared subunits including IL-12 (p35/p40), IL-23 (p19/p40), IL-35 (p35/EBI3), and IL-27 (EBI3/p28).<sup>140</sup> IL-12 plays an important role in Th1 T cell differentiation through activation and phosphorylation of STAT4.<sup>141</sup> The APCs such as dendritic cells and macrophages showed an increased production of IL-12 in CD, but not in UC, justifying a Th1 mediated cytokine response in CD. Later studies have found that these cells also

produce augmented levels of IL-23, a cytokine involved in Th17 cell responses and suppression of Tregs.<sup>86</sup> IL-23R has been identified as a risk gene for IBDs by GWAS.<sup>142</sup> Several experimental studies in animal models of colitis also suggest a more prominent role for IL-23 than IL-12 in driving the inflammation.

IL-27 is found to have both pro-inflammatory and anti-inflammatory activities. A study carried out by Wirtz and colleagues has shown that deficiency of Epstein-Barr virus-induced (EBI)3 gene resulted in more severe colitis in mice compared to IL-27-p28 deficiency. Moreover, the administration of recombinant IL-35 attenuated DSS-induced colitis.<sup>143</sup> However, the role of these IL-12 family cytokines in human IBD pathogenesis remains to be investigated.

#### **1.6.5.4. IL-17 family**

The IL-17 cytokine family consists of six ligands from IL-17A to IL-17F and they are produced by Th17 cells in response to IL-23 stimulation.<sup>144</sup> However, in contrast to the pro-inflammatory effect of IL-23 and the therapeutic efficacy of IL-23 antagonists in IBD patients, IL-17 is found to have major protective roles in IBD (see section 1.6.4.1). IL-17A antagonists were successful in clinical trials for treating psoriasis, psoriatic arthritis, and ankylosing spondylitis, but paradoxically, IL-17 inhibition leads to exacerbation of colitis symptoms in some IBD patients.<sup>145</sup> In addition, genetic polymorphisms in IL-17A and IL-17F genes are associated with increased susceptibility to UC.<sup>146</sup>

#### **1.6.5.5. IL-1 family**

The IL-1 family cytokines consist of 11 members including agonists (IL-1 $\alpha$ , IL-1 $\beta$ , IL-18, IL-36 $\alpha$ , IL-36 $\beta$ , IL-36 $\gamma$ , IL-33, IL-37), and receptor antagonists (IL-1Ra, IL-36Ra, IL-38).<sup>147</sup> Several members of this family, including IL-1 $\beta$ , IL-18, and IL-33, have been upregulated in mucosal tissues of IBD patients as well as in mice models of colitis.<sup>148</sup> IL-1 $\beta$  and IL-18 are found to have prominent roles in intestinal inflammation as effector cytokines produced in response to inflammasome activation. KO of IL-1 $\beta$  converting enzyme (caspase-1), which cleaves pro-IL-1 $\beta$  and IL-18 into active cytokines, reduces colitis symptoms in DSS-induced colitis.<sup>149</sup> Besides, the ratio of the receptor antagonist IL-1Ra to IL-1 has been significantly decreased in the intestinal mucosa of IBD patients indicating an activation of the IL-1 signalling pathway.<sup>150</sup> Treatment with recombinant IL-1Ra suppressed acute immunocomplex-induced colitis in rabbits.<sup>151</sup> Another study

has shown that blockade of IL-1 signalling using IL-1R antagonising drugs is found to ameliorate colitis in IL-10R KO mice as well as in some patients with IL-10R deficiency (see section 1.6.5.6 below), indicating the potential relevance of IL-1 targeting in IBD therapy.<sup>152</sup>

IL-33 cytokine is mainly derived from the IECs and mesenchymal cells and functions as an endogenous danger signal or alarmin in response to tissue injury. It has been linked to intestinal inflammation by having a pro-inflammatory role in acute colitis while it was found to be protective in the chronic phase of the inflammation.<sup>153</sup> This is supported by the observation that in acute DSS and trinitrobenzene sulfonic acid (TNBS) colitis models, blockade of the IL-33 receptor ST2 protected against colitis.<sup>154</sup> On the other side, IL-33 promotes Treg differentiation and accumulation in inflammatory sites in adoptive T cell transfer colitis.<sup>155</sup>

#### **1.6.5.6. IL-10 family**

The IL-10-related cytokine family includes several members, out of which IL-10 and IL-22 are the best-characterized members.<sup>156</sup> IL-10 is an anti-inflammatory cytokine highly relevant to IBD pathogenesis. Both IL-10 and IL-10R knockout mice spontaneously develop colitis upon aging. GWAS has identified polymorphisms in IL-10 and IL-10R in severe infantile (very early onset) colitis, and since IL-10 acts on hemopoietic and immune cells, allogeneic hematopoietic stem cell transplantation was shown to induce remission in patients with IL-10R deficiency.<sup>157</sup> IL-22 also exerts protective effects specifically in the epithelial cells and stromal cells due to the restricted expression of IL-22R in these cells. A local targeted delivery of IL-22 gene in DSS-induced colitis model was shown to ameliorate intestinal inflammation through STAT3 activation and enhanced mucus production by goblet cells.<sup>158,159</sup>

### **1.7. Animal models of IBDs**

As in several other diseases, the development of suitable animal models has contributed greatly to the understanding of the disease pathology, and in developing diagnostic and therapeutic tools for IBDs. More than 65 different animal models have been established so far, which can be largely classified as genetically engineered, adoptive cell-transfer, or chemically induced models (**Table 3**). None of these models can completely represent the human IBD criteria which emphasise the necessity of testing the efficacy of new treatment or diagnostic tools in several independent animal models.



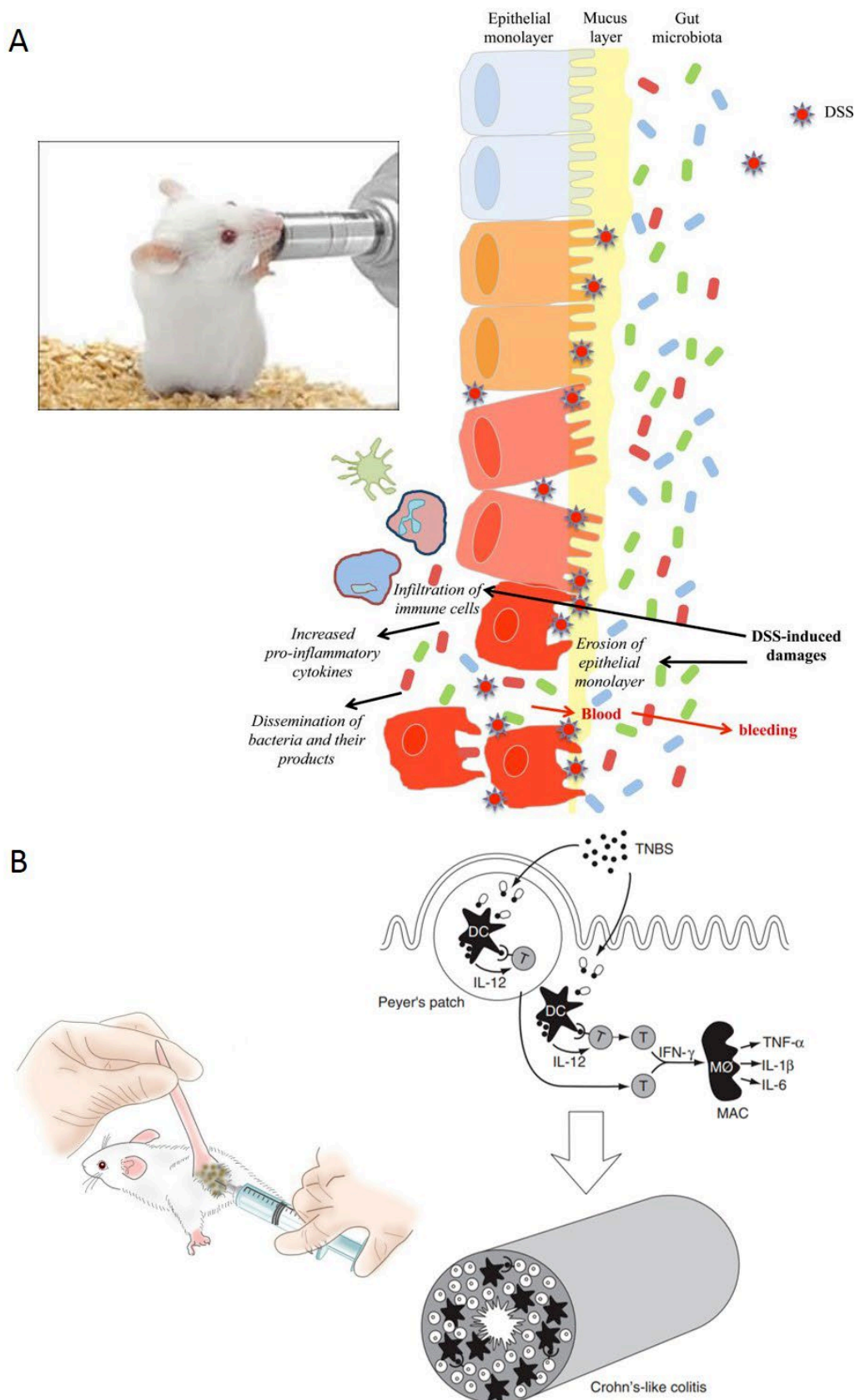
The first experimentally induced animal model was introduced in 1957 by Kirsner and colleagues by sensitizing rabbits with egg albumin prior to exposure of the colon to formalin.<sup>160</sup> Since then, many other chemically-induced models were established. In 1994, Dr. Powrie and colleagues came up with an adoptive T-cell transfer system to induce colitis in immunodeficient mice.<sup>161</sup> In the same year, another team has demonstrated that transgenic rats carrying human leukocyte antigen (HLA)-B27 gene develop colitis,<sup>162</sup> kick-starting an array of genetically-engineered models of IBDs.<sup>163</sup>

### **1.7.1. Chemically-induced models**

Chemically-induced models are established by introducing certain chemicals to mimic an IBD-like intestinal inflammation. Due to the rapid onset of the disease, low cost, and relatively easy experimental setup, they have been widely exploited for therapeutic studies. Though a number of chemicals have been used for this purpose over time, currently, the most commonly used ones are DSS and TNBS colitis models.

#### **1.7.1.1. DSS-induced model**

The model is established by the oral administration of DSS, a negatively charged sulfated polysaccharide, in drinking water, in an acute or chronic method. Though the underlying mechanism of DSS-induced colitis is not clearly understood, the toxicity of the chemical to the intestinal epithelia is believed to cause epithelial barrier disruption and increased intestinal permeability. This in turn results in the exposition of pro-inflammatory luminal contents such as bacteria to the underlying tissue eliciting an excessive inflammation characterized by mucosal erosions/ulcers, loss of crypts, and infiltration of granulocytes (**Figure 15A**).<sup>164</sup> The inflammation in DSS model is restricted to the colon and the resulting clinical and histological features resemble more closely to that of human UC.<sup>165</sup> The acute DSS-induced inflammation occurred in immunodeficient mice such as severe combined immunodeficient (SCID) mice, similar to wild-type (WT) mice, suggesting that innate immune cell-derived cytokines are sufficient to produce the inflammation, rather, the T or B cell-mediated adaptive immunity is not required.<sup>166</sup> Hence, the acute DSS model is particularly useful for studying the contribution of epithelial barrier and innate immune system to the development of intestinal inflammation.<sup>165</sup> However, T cells have been found to accumulate in the chronic DSS model over time, perpetuating the intestinal inflammation.<sup>167,168</sup>



**Figure 15: Chemically-induced animal models of colitis**

A) DSS-induced model. B) TNBS-induced model. Figure taken from Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. *Curr Protoc Immunol.* 2014;104(1):15-25 and Neurath M, Fuss I, Strober W. *Int Rev Immunol.* 2000;19(1):51-62 with permission.

### **1.7.1.2. TNBS-induced model**

The TNBS model involves the rectal administration of the chemical along with ethanol. The function of ethanol is to disrupt the epithelial barrier allowing TNBS to penetrate the bowel wall, where it haptenizes the intracolonic proteins containing a trinitro phenyl moiety (**Figure 15B**). In contrast to DSS model, the resulting inflammation and clinical manifestations are more similar to human CD. It is characterised by a massive transmural infiltration of T cells and macrophages across the intestinal wall along with weight loss, severe diarrhea, and rectal prolapse. Studies by Neurath and colleagues have shown that the inflammation associated with TNBS model is mainly Th1 mediated.<sup>169,170</sup> However, TNBS-induced colitis can also be generated in SCID and recombination activating gene (RAG)1 KO mice that lack both T and B cells with an effect superficially similar to that of WT mice with infiltrating granulocytes or monocyte (CD11b<sup>+</sup> cells),<sup>171</sup> suggesting that innate immune system is also involved. TNBS colitis models have been widely exploited for studying the immunological aspects of the disease such as cytokine profiles as well as for therapeutic studies.<sup>164</sup>

### **1.7.2. Genetically-engineered models**

The extreme genetic complexity of IBDs has resulted in the development of a large number of knockout or transgenic models, over time. IL-10 KO mouse is widely used as a genetic model of IBDs which develop spontaneous colitis after 3 months of age with an inflammation driven by a Th1 response.<sup>172</sup> T cell receptors (TCR) composed of  $\alpha$  and  $\beta$  subunits are involved in the recognition of antigens presented by MHC and subsequent activation of adaptive immune responses. Both TCR $\alpha^{-/-}$  or TCR $\beta^{-/-}$  mice develop a spontaneous Th2 mediated colitis at the age of 6 months.<sup>173</sup> IL-7 cytokine is a risk gene associated with UC and found to be upregulated in UC patients. Transgenic expression of IL-7 leads to spontaneous development of colitis at 1-3 weeks of age.<sup>174</sup> Apart from these models, a growing list of several other genetically-induced models has also been described.<sup>7,163,175</sup>

### **1.7.3. Adoptive cell-transfer models**

Adoptive transfer colitis models have revolutionised our understanding about the role of T cells in the pathogenesis of IBDs, both in terms of the induction or suppression of the disease. The method originally developed by Powrie and colleagues involves the transfer of naïve T cells (CD4<sup>+</sup>CD45RB<sup>high</sup>) to immunodeficient SCID mice.<sup>161</sup>

Interestingly, co-transfer of both CD4<sup>+</sup>CD45RB<sup>high</sup> and CD4<sup>+</sup>CD45RB<sup>low</sup> T cells prevents the induction of colitis,<sup>161</sup> which later shed light on the role of natural Tregs (nTregs), of which the CD4<sup>+</sup>CD45RB<sup>low</sup> T cells are a subset, in suppressing the inflammation associated with colitis.<sup>176</sup>

**Table 3: Classification of major animal models of colitis**

<b>Animal models of IBD</b>	<b>Characteristics/ origin</b>
<b>Chemically-induced models</b>	
DSS	Luminal toxin, epithelial injury, acute or chronic models
TNBS	Hapten, T-cell mediated immune response, acute/chronic models
Oxazolone	Hapten, T-cell mediated immune response, acute/chronic models
Acetic acid	Epithelial or mucosal necrosis and transient inflammation, acute model
<b>Genetically engineered models</b>	
Conventional KO	IL-10 <sup>-/-</sup> , IL-2 <sup>-/-</sup> , TCRα <sup>-/-</sup> , TCRβ <sup>-/-</sup> , NOD2 <sup>-/-</sup> , Gαi2 <sup>-/-</sup> , TGF-β <sup>-/-</sup> , A20 <sup>-/-</sup> , WASP <sup>-/-</sup>
Conditional KO	XPB1 <sup>-/-</sup> , NEMO <sup>-/-</sup>
Conventional Tg	IL-7 Tg, STAT4, HLA-B27
Conditional Tg	SOCS1 Tg, DNN-cadherin Tg
<b>Adoptive transfer models</b>	
CD45RB <sup>high</sup> transfer	Chronic T-cell mediated colitis

Abbreviations not used in the text. WASP; Wiskott-Aldrich syndrome protein, Tg; transgenic, SOCS1; Suppressor of cytokine signaling 1, DNN-cadherin; dominant negative N-cadherin; NEMO; NF-kappa-B essential modulator, XPB; X-box binding protein.<sup>163,177,178</sup>

## 1.8. Current treatment options for IBDs

### 1.8.1. First-line therapies

The first drugs used to treat IBDs with some efficacy were immunosuppressants such as aminosalicylates, corticosteroids, and thiopurines (**Table 4**). Sulfasalazine, an aminosalicylate, which is a class of anti-inflammatory compounds acting mainly as oxygen scavengers, showed some potent effects. This discovery led to the development of a range of drugs in this class of compounds, such as mesalazine (**Table 4**). Corticosteroids were also found to be remarkably effective in both CD and UC. However, the long-term toxicity, steroid dependency, and refractoriness to treatment that occurred in some patients necessitated discontinuation or restricted use in some cases.<sup>179–181</sup> Therefore, ongoing research is focused on the identification of molecules that have fewer deleterious secondary effects.

This has led to the development of several molecules, especially budesonide (Entocort or Mikicort), an oral glucocorticoid, which is quickly metabolized by the liver, thereby reducing corticosteroid-related adverse effects (AEs). It is used in the management of asthma, allergic rhinitis, and various skin disorders, and has been extended to CD.<sup>182</sup> Although budesonide appears significantly less effective than conventional steroids for inducing remission in active CD, it displays fewer AEs.<sup>183</sup> Other immunomodulators such as thiopurines, methotrexate, and calcineurin inhibitors were also explored alone or concomitantly with other drugs as treatment options for IBDs (**Table 4**). Thiopurines are incorporated into nucleotides and suppress T cell function by decreasing the expression of pro-inflammatory cytokines. Methotrexate (e.g., Imeth, Novatrex, Methotrexate Bellon, or Metoject) is effective in steroid-dependent CD (effective for induction and maintenance of remission in CD, but not in UC<sup>184</sup>), while cyclosporine A and tacrolimus/FK-506 calcineurin inhibitors, which are strong immunosuppressive compounds, decrease pro-inflammatory lymphokine production in UC.<sup>185,186</sup> A previous study that included a large cohort of CD patients demonstrated that coadministration of 5-aminosalicylate (5-ASA; mesalazine) and azathioprine (AZA) or 6-mercaptopurine (6-MP) was not more effective than AZA or 6-MP alone in terms of the requirement for rescue medications such as steroids and anti-TNF agents.<sup>187</sup> The cumulative probabilities of hospitalization and intestinal resection were similar between the groups of patients on either regimen. Although these molecules and peptides are often effective as primary or first-line therapy for IBDs, their long-term use is hindered

by serious ailments such as myelosuppression, multiple infections, pancreatitis, and in some cases sensorineural hearing loss and tinnitus (**Table 4**), justifying the introduction of more selective therapeutic strategies.

### **1.8.2. The era of therapeutic antibodies and cell modulators for treating IBDs**

A number of Abs have been developed against cytokines and adhesion molecules, which are key players in the pathogenesis of IBDs (**Table 4; Figure 16**). In patients with IBDs, cytokines produced by intestinal mucosa largely contribute to the activation and migration of inflammatory cells such as monocytes and neutrophils (described in section 1.6.5).<sup>89,188–190</sup> Cytokines are therefore especially targeted for treating IBD patients.

#### **1.8.2.1. Anti-TNF antibodies**

The use of anti-TNF drugs has been a significant breakthrough in the treatment of IBDs.<sup>180,190–192</sup> Several anti-TNF- $\alpha$  Abs are currently approved for treating patients with IBDs. In the case of CD, these include infliximab (Remicade), which is a chimeric human/mouse Ab (and its biosimilars Inflectra, Remsima, and Flixabi), adalimumab (Humira), a fully human IgG1 mAb [and its biosimilars Hulio (Mylan), Cyltezo (Boehringer Ingelheim), Imraldi (Samsung Bioepis), Hyrimoz (Sandoz), and Amgevita (Amgen)], and certolizumab pegol (Cimzia), a humanized antigen-binding fragment (Fab0) of a mAb that has been conjugated to polyethylene glycol. For UC treatment, infliximab, adalimumab, and golimumab (Simponi), a fully human IgG mAb (**Table 4**), have been tested. Few studies have demonstrated the efficacy of golimumab in anti-TNF-refractory CD patients.<sup>193,194</sup> At this stage, however, further studies are awaited in CD to formally assess the efficacy of golimumab in a randomized controlled trial and to establish the optimal dosing regimen.

Altogether, TNF-targeting Abs have been claimed to induce clinical response in about 60% of CD and UC patients; a result that is remarkable in the context of these severe and heterogeneous diseases.<sup>191,195,196</sup> It is however pertinent to remember the well-characterized serious AEs (SAEs) induced in certain patients by TNF blockers when given for long periods of treatment. Two major concerns with these drugs include the risk of serious infections and malignancies.<sup>197–200</sup>

**Table 4: Therapeutic strategies currently approved and/or in use for the treatment of IBDs**

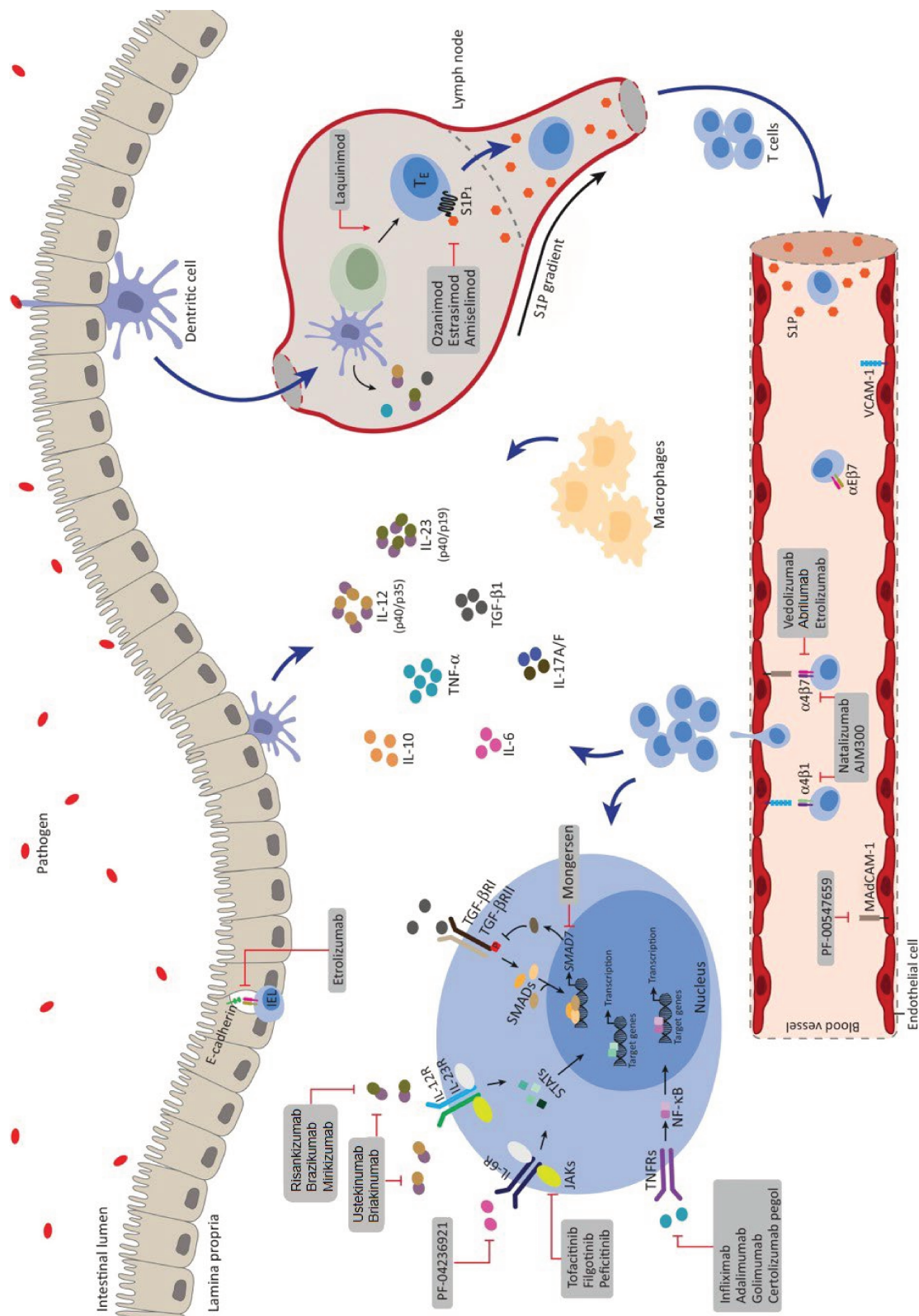
Drug	Mechanism of action /target	Efficacy (significant results)	SAEs	Clinical status	Refs.
<b>Small molecules (corticoids and immunosuppressants)</b>					
Amino salicylates (sulfasalazine, mesalazine, 4-aminosalicylic acid, balsalazine, olsalazine)	Free radical scavengers, 5-lipoxygenase inhibition, effects on leucocyte function and production of cytokines	Clinical remission rates of 40–70% have been reported with mesalazine over 6–8 weeks in UC	Nephrotoxicity, agranulocytosis, alveolitis, pancreatitis, abdominal pain, flatulence, nausea, dyspepsia	Common use	201,202
Prednisone, 6-methylprednisolone, budesonide MMX	Binds to high affinity intracellular cytoplasmic receptors	Clinical remission at 8 weeks (17.4%-Budesonide MMX 9 mg vs. 4.5%-placebo) in UC	Diabetes, osteoporosis, moon face, and acne, growth retardation in children, psychosis, hepatic steatosis	Common use	179–182
Thiopurines (6-mercaptopurine, azathioprine)	Incorporates into nucleotides	Maintain remission in moderate to severe CD/UC	Myelosuppression, non-Hodgkin lymphoma	Common use	203
Methotrexate	Inhibits enzymes in folic acid metabolic pathway (for high doses used in oncology/ hematology; mode of action is unknown for low doses used in CD)	Clinical remission at 16 weeks (39%-25 mg vs. 19%-placebo; 65%-15 mg vs. 39%) in CD	Dyspnoea, nausea, vomiting, and neutropenia	Common use	185,204
Cyclosporine	Calcineurin inhibitor	Cyclosporine (4 mg/kg) showed 82 % response rate vs. placebo (P < 0.001) in 7 days in UC	Renal failure, bacterial pneumonia, Pneumocystis jirovecii pneumonia, venous catheter infections	Common use	205
Tacrolimus	Calcineurin inhibitor	Clinical remission at 2 weeks (9.4% vs. 0.0%-placebo) in UC	Tremor, paraesthesia, insomnia, hot flush, alopecia, hyperglycaemia, hypomagnesaemia, hypertension, hepatotoxicity, nephrotoxicity	Common use	206
<b>Therapeutic antibodies</b>					
Generic name	Trade name/ synonym				
Infliximab	Remicade	TNF- $\alpha$ antagonist	Drug-induced lupus, infusion reactions, hypersensitivity reactions, demyelination, reactivation of latent tuberculosis, Non-Hodgkin's lymphoma	Approved by FDA since 2007 for CD/UC	207,208

**Table 4 (continued)**

Drug	Mechanism of action /target	Efficacy (significant results)	SAEs	Clinical status	Refs.
Adalimumab	TNF- $\alpha$	Clinical remission at week 56 (36%-400 mg eow, 41%-400 mg weekly, vs. 12%-placebo) in CD	Congestive heart failure, lupus-like syndrome, lymphoma, cytopenia, MS/neurological disease, pancytopenia	Approved by FDA since 2007 for CD/UC	196
Certolizumab Pegol	TNF- $\alpha$	Clinical response at 10 weeks (52.8%-400 mg vs. 30.1%-placebo) in CD	Injection site reaction, Infections, lupus-like syndrome	Approved by FDA since 2008 for CD	195,209,210
Golimumab	TNF- $\alpha$	Clinical remission at 54 weeks (27.8%-100 mg vs. 15.6%-placebo) in UC	Erythema, tuberculosis, rectal, thyroid, and lung adenocarcinoma	Approved by FDA and EMA since 2013 for UC	211,212
Ustekinumab	IL-12/ IL-23	Clinical remission at 44 weeks (53.1%-90 mg every 8 weeks and 48.8%-90 mg every 12 weeks vs. 35.9%- placebo) in CD	Nasopharyngitis upper respiratory tract infections, diverticulitis, cellulitis, pneumonia	Approved by FDA since 2016 for CD	213-216
Natalizumab	$\alpha$ 4 integrin	Clinical remission at 8 weeks (26%-300 mg vs. 16%-placebo) in CD	Pharyngitis, urinary tract infection, urticaria, cephalgia, arthralgia, PML	Approved by FDA since 2004 for CD	217
Vedolizumab	$\alpha$ 4 $\beta$ 7 integrin	Clinical remission at 52 weeks (44.8%-300 mg vs. 29.1%-placebo) in CD	Gastrointestinal and respiratory tract infections, hepatic steatosis	Approved by FDA since 2014 for CD/UC	218-220
<b>Other small molecules</b>					
Tofacitinib	Pan-JAK inhibitor	Clinical remission at 8 weeks (18.5%-10 mg vs 8.2%-placebo) in UC	Herpes zoster infection, upper respiratory tract infections, headache, diarrhea, nasopharyngitis	Approved by FDA since 2018 for UC	221

Abbreviations not used in the text: EMA, European Medicines Agency; eow, every other week; MMX, multi matrix.





**Figure 16: Targets of the major medications used for the treatment of IBD**

Figure adapted from Coskun M, Vermeire S, Nielsen OH. Trends Pharmacol Sci. 2017;38(2):127-142 with permission.

### 1.8.2.2. Other cytokine biological therapies

Apart from TNF- $\alpha$ , other cytokines are also used as targets in emerging therapeutic strategies.<sup>222</sup> Ustekinumab (Stelara) is a human IgG1 mAb that targets the p40 subunit of IL-12 and IL-23 by inhibiting the binding to their receptors (**Table 4**). This mAb, which was approved by the FDA in 2016, is effective in CD patients with moderate-to-active disease.

Other biologics, for example, Abs that target IL-23 by binding to its P19 subunit, such as risankizumab (BI-655066 or ABBV-066), brazikumab (AMG 139 or MEDI2070), briakinumab (ABT-874), and mirikizumab (LY3074828) are currently being evaluated for their potential efficacy (**Table 5**).

Briakinumab is a human mAb that was initially developed for treating rheumatoid arthritis (RA), MS, and IBDs. In November 2009, a Phase III clinical trial for plaque psoriasis was completed and a Phase II clinical trial for MS was announced. A Phase II clinical trial for CD was also carried out.<sup>223</sup> Head-to-head comparisons were made with regard to etanercept (Enbrel), a dimeric fusion protein targeting TNF- $\alpha$ , and placebo, in double-blind trials. The results gained with briakinumab were promising in psoriasis (81–82% of patients under briakinumab, 40–56% under etanercept, and 7% under placebo reached a Psoriasis Area Severity Index reduction of at least 75%). However, in January 2011, the withdrawal of the briakinumab application was announced in favour of other strategies.

Migration of leukocytes to mucosal lesions is important in the pathogenesis of IBDs, and this trafficking process is actively mediated by integrins. Hence, targeting integrins has emerged as another potential therapy. The first attempt in this area was based on natalizumab (Tysabri), a human IgG4 Ab targeting the  $\alpha$ 4 integrin subunit (**Table 4**; **Figure 16**). Its use, however, was preferable for short-term treatment. In some rare cases, due to inhibition of leukocyte migration into the CNS, it was found to promote reactivation of John Cunningham (JC) virus in the brain, resulting in the development of progressive multifocal leukoencephalopathy (PML), an SAE that precluded its indication.

Vedolizumab (Entyvio) is an IgG1 mAb, which also blocks the  $\alpha$ 4 $\beta$ 7 integrin subunit but on account of its gut selectivity, it was not associated with PML (**Table 4**). In Phase III clinical trials, vedolizumab was found to be safe and efficient in the induction and

maintenance phases of therapy in CD and UC patients. Although patients receiving vedolizumab presented more frequently with SAEs and infections compared with patients treated with placebo, the promising data generated with this Ab led to growing interest in developing other anti-integrin Abs, such as etrolizumab (rhuMAb  $\beta$ 7), abrilumab (AMG 181), and Ontamalimab (PF-00547659), which are currently being evaluated in clinical trials (**Table 5**, and references therein). Ontamalimab is a fully human mAb that binds to human mucosal addressin cell adhesion molecule (MAdCAM), which is predominantly expressed on the cell surface of high endothelial venules of organized intestinal lymphoid tissues (Peyer's patches and mesenteric lymph nodes). It was found to selectively reduce lymphocyte homing to the intestinal tract. Although compared with placebo, this mAb did not meet the primary endpoint of clinical response in moderate-to-severe CD, it raised great hopes as it presented some appreciable pharmacological effects, which remain to be analysed further.<sup>224</sup> The long-term safety and efficacy of Ontamalimab were demonstrated in phase II clinical trials for moderate to severe UC, supporting a phase III testing.<sup>225</sup>

### **1.8.2.3. Adverse effects of biologics**

Several clinical trials and meta-analyses have verified the efficacy and safety of biologic-based therapies. The risk of SAEs associated with these therapies is lower compared with other conventional (immunosuppressive) treatments, and some biologics have proven to be beneficial in the induction and maintenance of clinical remission and response.<sup>226,227</sup> However, cases of SAEs, including hypersensitivity reactions, injection site reactions, skin cancers, drug-induced lupus, psoriasis, reactivation of latent tuberculosis, hepatotoxicity, lymphomas, and solid tumours have been reported (**Tables 4 and 5**). Compared to anti-TNF drugs developed initially, other approved biologics showed higher safety profiles. The increased risk of PML has limited the use of Natalimumab, while the other anti-adhesion therapy vedolizumab showed the best safety profile among current biologics, owing to its gut selective mechanism of action. Despite the limited safety data for ustekinumab in IBD patients, long-term studies in other indications of this drug have demonstrated appreciable tolerability.<sup>228</sup> Nevertheless, the high production cost of therapeutic mAbs remains a hurdle in maintaining the cost-effectiveness of these drugs.<sup>229,230</sup>

Another serious issue that is encountered with certain biologics is the generation of anti-drug Abs (ADAs) that makes at least 40% of the patients receiving anti-TNF drugs secondary nonresponders. This loss of responsiveness mostly occurs in the case of patients receiving episodic therapy or in the presence of lower levels of ADAs against other anti-TNF agents received earlier (including biosimilars).<sup>231</sup> According to previous studies, the formation of Abs against infliximab occurs in 61% of patients receiving episodic treatment, and 44% of patients losing response to adalimumab were found to have developed Ab to adalimumab. It is a general observation and a source of concern that more and more cases of ADAs are reported in the literature,<sup>232–236,237</sup> influencing the efficacy of treatment and the potential clinical improvement of patients under biotherapy. Sensitive assays have been developed to detect ADAs that are produced early in certain individuals and can dramatically affect the results of clinical trials and the efficacy of current treatments in patients.<sup>232,238,239</sup> High serum concentrations of anti-TNF drugs are associated with improved clinical outcomes in IBD patients. In contrast, low concentrations have been shown to frequently associate with the formation of ADAs. Thus, careful monitoring of the serum concentrations of the drugs and ADA levels are essential in predicting loss of response and optimising biologic therapies.<sup>240–242</sup>

#### 1.8.2.4. Small molecules for treating IBDs

In terms of small molecules, apilimod mesylate {N-[(E)-(3-methylphenyl)methylideneamino]-6-morpholin-4-yl-2-(2-pyridin-2-ylethoxy)pyrimidin-4 amine; formerly STA-5326}, which inhibits IL-12/IL-23, was evaluated in clinical trials including patients with CD.<sup>243</sup> Up to 700 subjects have been treated with mild-to-moderate AEs. However, apilimod did not meet the primary endpoints in Phase II inflammatory disease indications.<sup>244</sup> This molecule is currently being evaluated in other indications.

ABX464 {8-chloro-N-[4-(trifluoromethoxy)phenyl]quinolin-2-amine} is a small molecule for oral administration identified from a chemical library screen targeting human immunodeficiency virus (HIV) replication. It is found to induce the expression of microRNA (miR)-124 in human immune cells, a negative regulator of intestinal inflammation. It has shown strong inflammatory effects in the DSS colitis model and met the safety and efficacy endpoints in phase IIa clinical trials for UC.<sup>245</sup> It is currently being evaluated in Phase IIb clinical trials for UC and CD patients.<sup>246</sup>

**Table 5: Therapeutic strategies currently under clinical evaluation for IBDs**

Drugs		Mechanism of action /target	Efficacy (significant results)	SAEs	Clinical status	Refs.
Therapeutic antibodies						
Generic name	Trade name/ synonym					
Golimumab	Symponi	TNF- $\alpha$	Retrospective analysis in 115 CD patients: Clinical response 55.8% in 4 months	Infections, drug-induced lupus	No formal trials have been done for CD	212,247
Risankizumab	BI-655066 or ABBV-066	IL-23(p19)	Clinical remission at 12 weeks (31% vs. 15%-placebo) in CD	Arthralgia, headache, abdominal pain, nausea, and pyrexia, worsening of underlying CD	Phase III trials for CD/UC	248,249
Brazikumab	AMG 139 or MEDI2070	IL-23(p19)	Clinical remission at 8 weeks (49.2% vs. 26.7%-placebo) in CD	Headache, nasopharyngitis	Phase II trials for CD/UC	250
Briakinumab	ABT-874	IL-12/23(p40)	Clinical remission at 24 weeks (48%-400 mg, 57%-700 mg vs. 29%-placebo) in CD	Respiratory tract infection, nausea, abdominal pain, headache, cardiovascular events	Phase II trials for CD	251,252
Mirikizumab	LY3074828	IL-23 (p19)	Clinical remission at 12 weeks (31% vs. 4.8%-placebo) in UC	No SAEs reported	Phase III trials for CD/UC	224
Etolizumab	rhuMab $\beta 7$	$\beta 7$ subunit of integrins $\alpha 4\beta 7$ and $\alpha E\beta 7$	Clinical remission at 10 weeks (21%-100mg vs. 10%-placebo) in CD	Exacerbation of UC, headache, fatigue, abdominal pain, dizziness, nasopharyngitis, nausea, arthralgia, urinary tract infection	Withdrawn from UC trials due to mixed results, phase III trials for CD	253–256
Ontamalimab	PF-00547659	MAdCAM-1	Clinical remission at 12 weeks (16.7%-22.5 mg vs. 2.7%-placebo) in UC	No SAEs reported	Phase II trials for CD/UC	257,258
Abrilumab	AMG181	$\alpha 4\beta 7$ integrin	Clinical remission at 12 weeks (30.8%-210 mg vs. 17.6%-placebo) in CD	Upper respiratory tract infection, headache	Phase II trials for CD/UC	259

**Table 5 (Continued)**

Drugs	Mechanism of action /target	Efficacy (significant results)	SAEs	Clinical status	Refs.
Small molecules					
Generic name	Trade name/ synonym				
Filgotinib	GLPG0634	JAK-1	Clinical remission at 10 weeks (47% vs. 23%-placebo) in CD	No SAEs reported	Phase III trials for CD/UC 260,261
Upadacitinib	ABT-494	JAK1	Clinical remission at 8 weeks (19.6%-45 mg vs. 0%-placebo) in UC	Headache, non-melanoma skin cancer	Phase III trials for CD/UC 262,263
Ozanimod	RPC1063	SIP	Clinical remission at 10 weeks (18.4%-1 mg, 6%-placebo) in UC	Headache, anemia, nasopharyngitis, urinary-tract infections	Phase III trials for UC 264
Mongersen	GED 0301	TGF-β1	Clinical remission at day 15 (55%-40 mg, 65%-160 mg vs 10%-placebo) in CD	No serious side-effects reported	withdrawn after interim analysis of a phase III trial for CD 265,266
Carotegrast Methyl	AJM300	α4 integrin	Clinical remission at 8 weeks (23.5%-960 mg vs. 3.9%-placebo) in UC	Potential risk of PML	Phase III trials for UC 267
Laquinimod	ABR-215062 or TV-5600	Inhibitory effect on APCs and T cells	Clinical remission at 8 weeks (48.3%-0.5 mg vs. 15.9%-placebo) in CD	Headache, CD exacerbation	Phase II trials for CD 268
	ABX464	Induce the expression of miR-124 in immune cells	50 mg, daily for 2 months, clinical remission 35% vs 11% placebo in UC	Headaches, nausea and vomiting (not considered as treatment-limiting effects)	Phase II trial for UC/CD 245,246
	Etrasimod	SIP	2 mg, once daily for 52 weeks , clinical remission at week 12 ( 46.9% vs 9.5% placebo) in UC	Anaemia, worsening of UC	Phase III trial for UC 269

Laquinimod (ABR-215062 or TV-5600; developed by Active Biotech and Teva) is another oral drug that has inhibitory effects on APCs and T cells, resulting in reduced pro-inflammatory cytokine production. In randomized controlled trials laquinimod was efficacious for CD (**Table 5**). Head-to-head studies with existing treatments and longer-term safety data are however needed at this stage of investigation.

The Janus kinase (JAK)/STAT pathway is a major signalling cascade downstream from the cytokine and growth factor receptors, and hence JAK inhibition has been shown to be potentially therapeutic in IBDs. Tofacitinib (Xeljanz) is a pan-JAK inhibitor currently available for the treatment of UC.<sup>221</sup> Other molecules such as filgotinib and upadacitinib (JAK1 inhibitors) are undergoing clinical trials for CD and UC.

Mongersen (GED-0301), an oral oligonucleotide drug containing an anti-SMAD7 oligonucleotide has proved to be able to restore signalling by the mucosal anti-inflammatory cytokine TGF- $\beta$ 1. Although positive results were obtained in Phase II trials for CD with a clinical remission rate of 72% after 2 weeks of treatment, this drug was withdrawn from clinical studies in November 2017 due to disappointing results from an interim analysis of a Phase III study.<sup>265,266,270</sup>

Small molecules targeting leukocyte trafficking are also being currently investigated. One of them is the  $\alpha$ 4-integrin antagonist carotegrast methyl (AJM300), an oral phenylalanine derivative, which is presently evaluated in Phase III clinical trials for UC (**Table 5**). Amiselimod (MT1303; Biogen), ozanimod (RPC1063; Celgen), and etrasimod (APD334; Arena Pharmaceuticals) are other molecules that act as sphingosine-1-phosphate (SIP) receptor modulators, which lead to lymphocyte sequestration in lymph nodes and reduce the migration of lymphocytes to the gastrointestinal tract. The development of amiselimod, which was in Phase II clinical trials for CD has been halted, as Biogen is currently focusing on other drugs from its portfolio. A Phase III clinical trial of ozanimod in patients with moderate-to-severe UC is ongoing. Etrasimod is also currently being tested in phase III trial for UC. The long-term safety and efficacy of etrasimod was demonstrated in a Phase II, randomised, double-blind trial in patients with moderately-to-severely active UC for up to 52 weeks.<sup>269</sup>

Besides their lower production costs, small molecules present promising pharmacological advantages over biologics. Most of the small molecules described

above can be taken orally or subcutaneously, avoiding the need for hospital visits for intravenous administrations. The small molecular weight of these medications allows easy diffusion through the cell membrane compared to large molecular weight antibodies. They tend to have a shorter serum half-life favouring their rapid elimination, and the lack of immunogenicity prevents ADA formation and the resulting loss of response.<sup>271,272</sup>

Alternatives to pharmacological therapies including stem cell transplant and faecal microbiota transplant are also emerging as exciting future additions to the list of IBD treatments.<sup>273,274</sup> However, extensive studies are needed to find standardised protocols, donor selection criteria, and appropriate mode of delivery for these methods to be widely implemented in clinical practice. In addition, the cost and technical challenges associated with these approaches limit their large-scale and long-term use in IBD patients.<sup>275</sup>

#### **1.8.2.5. Pros and cons: how can we progress?**

Although the currently available therapeutic options greatly help to maintain middle-term remission and improve the IBDs patients' QOL to a certain extent, we must recognize that patients remain mostly in symptomatic remission and the therapies do not address the root genetic causes.<sup>89,180,189,276,277</sup> Besides, their high cost, severe impacts, and the SAEs of some of these treatments in the long-term, necessitate the development of cost-effective small molecule drugs that are disease-specific. In this context, several elements of the autophagy pathway might be key targets for novel therapeutic options.



## 2. AUTOPHAGY PATHWAY

The concept of autophagy originated with the discovery of lysosomes by Christian de Duve in 1955, as an organelle with lytic function. In 1957, Sam L. Clark observed bilayer lipid vesicles engulfing amorphous materials including mitochondria in renal epithelial cells by electron microscopy, which was then called ‘dense bodies’. Similar structures were subsequently reported by others, and based on these findings, Christian de Duve proposed the term “Autophagy” (the Greek term for ‘self-eating’) in 1963, for the delivery of cytoplasmic cargo *via* single or bilayer membrane vesicles known as ‘autophagosomes’ to the lysosomes for degradation.<sup>278</sup>

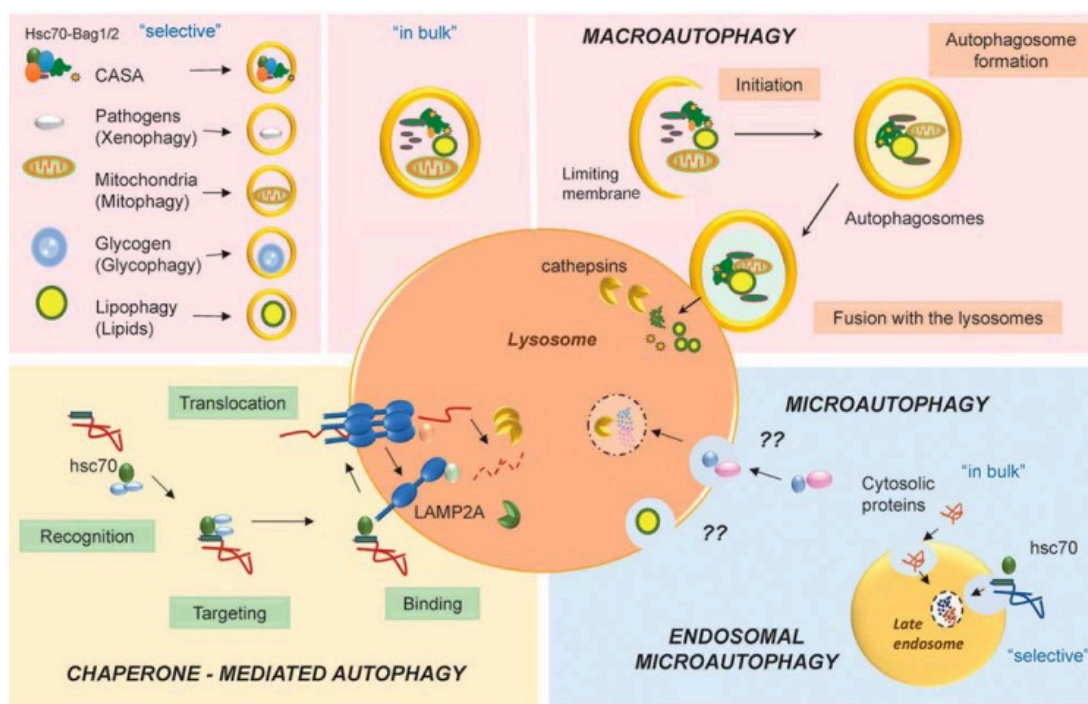
It is an evolutionary conserved catabolic process in eukaryotes which continuously clears unnecessary or dysfunctional cellular components (damaged organelles, abnormally folded proteins, or proteins produced in excess). The stimuli for the autophagic process include various forms of stress such as nutrient deprivation, hypoxia, endoplasmic reticulum (ER) stress, infection, and primarily helps the organism to adapt itself to the stressful conditions by maintaining cellular homeostasis.<sup>279</sup>

Initial concepts on autophagy were developed based on morphological and biochemical studies while the molecular machinery was unknown. A breakthrough in understanding the molecular mechanisms of autophagy came in with a yeast genetic screen carried out by Yoshinori Ohsumi which lead to the identification of several ATGs. This Nobel prize (Physiology or Medicine, 2016) winning discovery has made revolutionary advancements in detecting, analysing, and genetically manipulating the process. The current developments in the field of autophagy assign it numerous important functions in human health and disease making it more than a degradative process.<sup>280,281</sup>

### 2.1. Types of autophagy pathways

Depending on the molecular mechanisms involved, mainly three different types of autophagic pathways have been described (**Figure 17**).<sup>282</sup> The first identified form of autophagy involves the delivery of cytoplasmic cargo inside double-membrane vesicles (autophagosomes) into the lysosomes and is currently known as the **macroautophagy** pathway. However, later studies have identified that the cytosolic materials can reach the lysosomes by other means also. In the 1980s, Mortimore and colleagues studied the ultrastructure of liver lysosomes and proposed that the lysosomal membrane can invaginate to form vesicles that internalise small parts of the cytoplasm, a process

known as **microautophagy**. In 1985, Dice et al. demonstrated the selective degradation of ribonuclease A in the lysosomes with the help of chaperones, in a process known as the **chaperone-mediated autophagy (CMA)** pathway.<sup>278</sup>



**Figure 17: Types of autophagy pathways**

Figure taken from Tekirdag K, Cuervo AM. *J Biol Chem.* 2018;293(15):5414-5424 with permission.

### 2.1.1. Macroautophagy

In this highly genetically controlled, canonical autophagy process, a double-membrane sequestering compartment, termed a phagophore, is formed and expands encapsulating cytoplasmic cargos. The resulting sealed, double-membrane autophagosomes, subsequently fuse with hydrolytic enzyme-rich lysosomes to form autolysosomes in which the cellular cargos that have been engulfed are degraded. The resulting compounds that are cleaved by hydrolases are released back into the cytosol for reuse (recycling). This degradation mechanism which is evolutionarily conserved in all eukaryotes from yeast to humans exists at a basal level in all cell types, but it is upregulated upon stress conditions such as starvation. Macroautophagy can be in bulk (non-selective macroautophagy) or selective, depending on the cargo sequestered. Various selective forms of macroautophagy pathways have been identified for the degradation of pathogens (xenophagy), mitochondria (mitophagy), glycogen (glycophagy), and lipids (lipophagy). Chaperone-assisted selective autophagy (CASA)

involves the selective, ubiquitin-dependent degradation of chaperone-bound proteins (**Figure 17**).<sup>283,284</sup>

### **2.1.2. Microautophagy**

Microautophagy doesn't involve autophagosomes, instead, the non-selective cargo is directly engulfed by the lysosomes (mammals) or vacuoles (plants and fungi). Microautophagy functions in maintaining organelle size, membrane homeostasis, and cell survival under nitrogen starvation. Microautophagy can also selectively uptake cargos including peroxisomes (micropexophagy), mitochondria (micromitophagy), lipids (microlipophagy), or portions of the nucleus (piecemeal microautophagy). The studies in microautophagy were largely limited to yeast and cell-free systems due to the difficulty to detect the invaginations in lysosomes and the lack of conserved functions for yeast microautophagy genes in mammals. A similar process termed endosomal microautophagy (eMI) has been demonstrated in mammals where these invaginations are formed in late endosomes/multivesicular bodies instead of lysosomes, for the bulk degradation of cytosolic proteins. Some proteins can also be selectively degraded by eMI with the help of chaperones (**Figure 17**).<sup>284,285</sup>

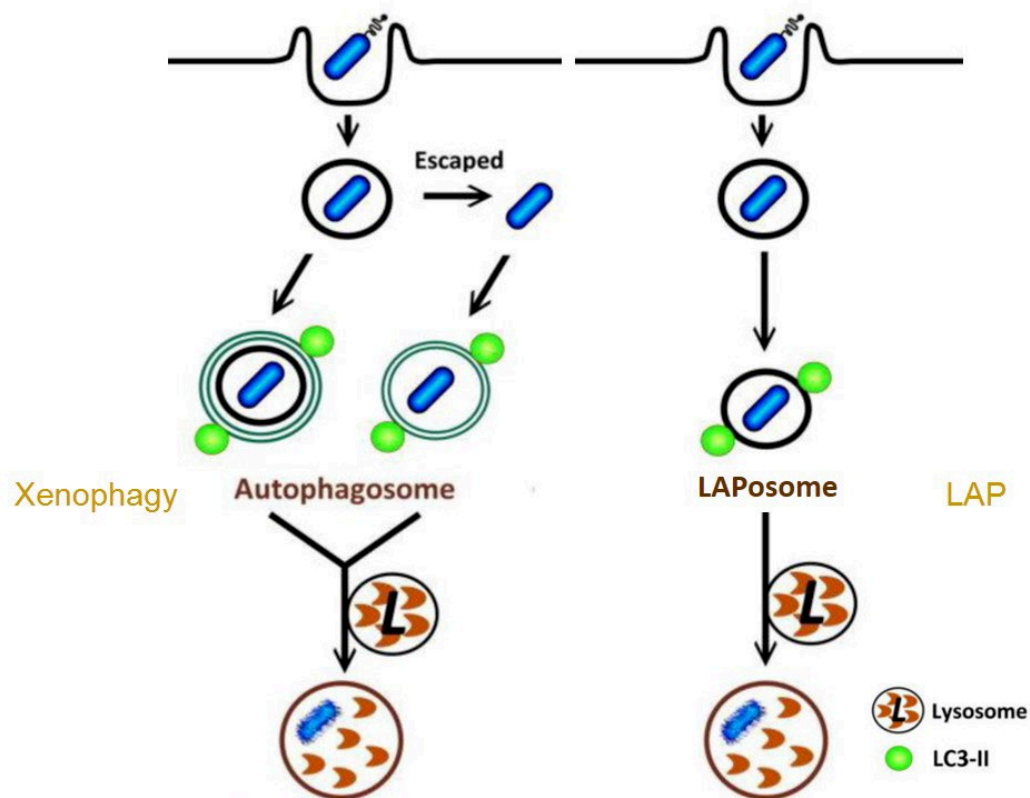
### **2.1.3. CMA**

CMA is a selective form of autophagy that also doesn't involve an autophagosome vesicle, instead, specific cytosolic proteins are targeted to the lysosomes with the help of chaperone proteins (**Figure 17**). Similar to macroautophagy, a basal level of CMA activity exists in many cell types, but it is maximally activated upon general stress conditions such as starvation and hypoxia. During starvation, macroautophagy is first activated, but prolonged starvation forces the cell to switch to CMA to mediate selective degradation of non-essential proteins to generate amino acids required for the synthesis of essential proteins.<sup>286,287</sup> The key role of CMA in antigen presentation has also been demonstrated. Overexpression of lysosomal-associated membrane protein (LAMP)2A was associated with enhanced cytoplasmic antigen presentation while its reduced expression showed vice versa.<sup>288</sup>

### **2.1.4. Xenophagy and LC3-associated phagocytosis**

Xenophagy is a selective autophagy process used to eliminate invading pathogens. The first evidence of xenophagy was observed in 1984 when autophagosomes were formed

in neutrophils infected with *Rickettsia conorii*.<sup>289</sup> Xenophagy typically follows the same steps of macroautophagy except the triggering signals are TLR/ NOD-like receptors (NLR)-mediated cytoplasmic recognition of pathogen-associated molecular patterns (PAMPs). Intracellular pathogens that are either inside the cytosol or in pathogen-containing vacuoles (phagosomes) are surrounded by isolation membranes, engulfed into autophagosomes, and degraded inside lysosomes (**Figure 18**).<sup>290</sup> In this setting, autophagy acts as an innate immune response against bacterial infections. Xenophagy is shown to restrict the growth of several pathogens including bacteria and viruses in different animal models (see section 2.4). It plays a particularly important role in maintaining intestinal mucosal homeostasis since the intestinal epithelium resides in continuous interaction with potentially pathogenic bacteria.<sup>291</sup>



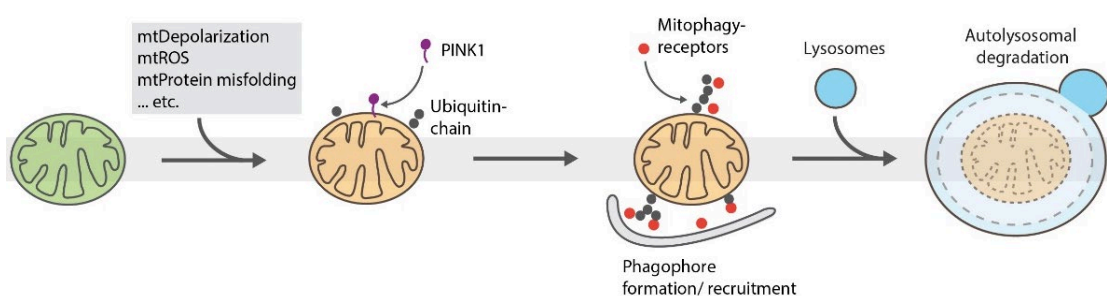
**Figure 18: Selective autophagic responses against invading pathogens**

The bacteria engulfed by the phagosomes can be degraded through xenophagy. Some bacteria can escape from the phagosome and further encaptured by autophagosomes (shown on the left). Bacteria captured in phagosomes can also be degraded by LAP (shown on the right). *Figure taken from Lai S, Devenish RJ. Cells . 2012;1(3) with permission.*

LC3-associated phagocytosis (LAP) is another recently emerging pathway involved in pathogen elimination. LAP doesn't involve the formation of double-membrane autophagosomes. Instead, it uses the canonical autophagy genes to conjugate the family of microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3, see **Table 6**) proteins to the single membrane phagosomes containing the pathogens. The resulting vesicles are called LAPosomes which then fuse with the lysosomes for degradation. The molecular mechanisms underlying LAP is not clearly understood. However, the major role of LAP is to promote the fusion of phagosomes with lysosomes enhancing the degradation of pathogens (**Figure 18**).<sup>292</sup>

### 2.1.5. Mitophagy

Removal of damaged mitochondria through selective autophagy is termed mitophagy. Along with the production of adenosine triphosphate (ATP) by oxidative phosphorylation, reactive oxygen species are also formed in mitochondria which can cause cell toxicity and cell death. Therefore, it is essential to keep a healthy population of mitochondria by timely turnover of damaged and aged mitochondria. Though several mechanisms have been suggested for the selective recognition of mitochondria, the ubiquitin-dependent phosphatase and tensin homolog induced kinase (PINK)1-Parkin pathway is the most characterised one (**Figure 19**).



**Figure 19: Mitophagy pathway**

The damaged mitochondria gets ubiquitinated by the PINK1-Parkin mediated pathway, and are subsequently targeted to the autophagosomes to be degraded in lysosomes. *Figure taken from <https://biochem2.com/files/2021-07/mitophagy-header-01.jpg?84762ecf3>.*

The unhealthy mitochondria lose their membrane potential and become depolarised. Subsequently, the mitochondrial kinase PINK1 is stabilised and recruits the E3 ubiquitin ligase Parkin to ubiquitinate outer mitochondrial membrane proteins, which

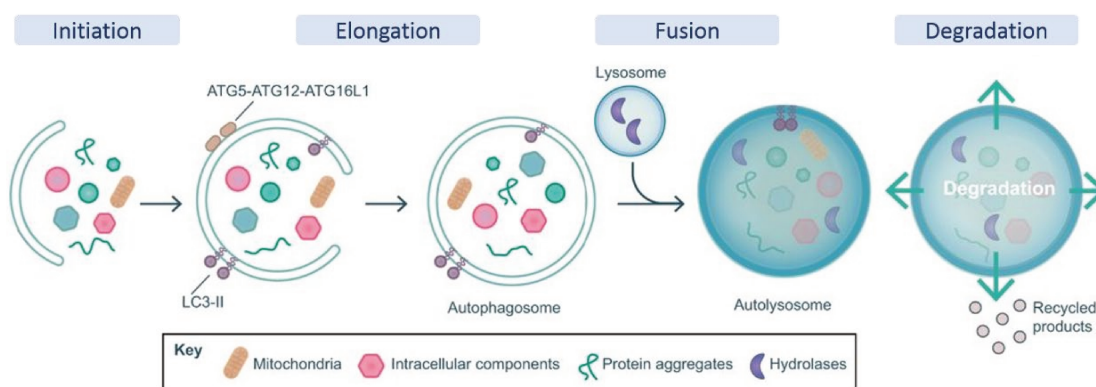
in turn recruits the autophagic cargo receptors to initiate autophagy. A defect in mitophagy is strongly implicated in Parkinson's disease by loss of function mutations in PINK1 and Parkin.<sup>293</sup>

## 2.2. The molecular machinery of mammalian autophagy

In this section, we will focus on the molecular mechanisms of macroautophagy and CMA.

### 2.2.1. Macroautophagy

Macroautophagy typically follows a multistep process involving initiation, elongation, fusion, and degradation (**Figure 20**).<sup>294</sup>



**Figure 20: Schematic diagram showing the key steps of macroautophagy pathway**

Macroautophagy typically follows the steps i) initiation, ii) elongation, iii) fusion and iv) degradation. *Figure adapted from Boya P, Codogno P, Rodriguez-Muela N. Autophagy in stem cells: repair, remodelling and metabolic reprogramming. Development. 2018;145(4). with permission.*

Each step is regulated by a complex network of proteins. The core proteins involved in the autophagy machinery and their key functions are listed in **Table 6**.<sup>279,295</sup>

**Table 6: Core autophagy proteins involved in the macroautophagy pathway**

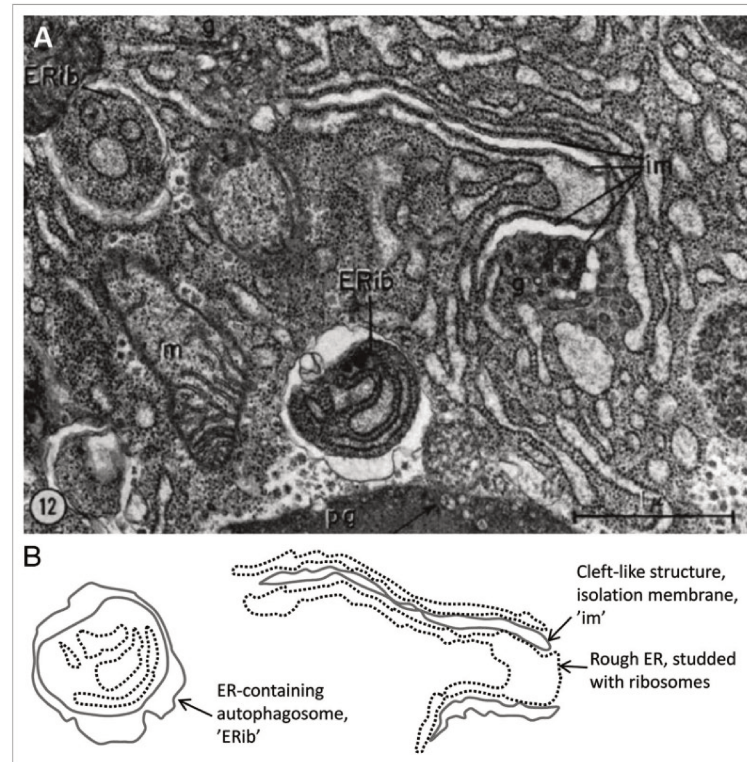
<b>Protein</b>	<b>Function</b>
ULK1/2	Serine/threonine kinase which phosphorylates components of autophagy machinery.
FIP200	Component of ULK1 complex.
ATG13	Component of ULK1 complex; enhances ULK1 kinase activity.
ATG101	Component of ULK1 complex; stabilizes ATG13.
VPS34	Catalytic component of PI3KC3 complex I.
Beclin1	Core subunit of the PI3KC3 complex I.
ATG14	Core subunit of the PI3KC3 complex I.
AMBRA1	Core subunit of the PI3KC3 complex I.
ATG4	A cysteine protease that processes pro-ATG8s and cleaves lipidated ATG8.
ATG7	E-1 enzyme for ubiquitin-like proteins ATG12 and ATG8.
ATG3	E2-like covalent binding of PE to ATG8-like proteins.
ATG9	Membrane delivery to the phagophore.
ATG10	E2-like enzyme that catalyzes the conjugation of ATG12 to ATG5
ATG12-ATG5- ATG16L1	E3-like complex that facilitates the conjugation of PE to the activated Atg8.
MAP1LC3	The lipid-modified form of LC3, referred to as LC3-II, is believed to be involved in autophagosome membrane expansion and fusion events.
SQSTM1/p62	Autophagic cargo receptor.
RAB7	Involved in autophagosome-lysosome fusion.
ATG14	Promotes membrane tethering of SNAREs.
STX17	A component of the SNARE complex involved in the direct control of autophagosome membrane fusion with the lysosome membrane, interacts with ATG14.
VAMP8	A component of the SNARE complex.
LAMP1/2	Lysosomal membrane proteins.

Abbreviations not mentioned in the text above: ULK, Unc-51 like autophagy activating kinase; FIP200, focal adhesion kinase family interacting protein of 200 kD; VPS, vacuolar protein sorting; PI3KC3, phosphatidylinositol 3-kinase; AMBRA, autophagy and beclin-1 regulator; PE, phosphatidylethanolamine; SQSTM, sequestosome; RAB, ras-related protein in brain; STX, syntaxin; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein.



### 2.2.1.1. Induction and phagophore nucleation

The initiation step of autophagy is induced by various stress signals that lead to the stepwise activation of several protein complexes and finally results in the formation of a small flattened membrane structure called the 'isolation membrane' or 'phagophore'. This process is called phagophore nucleation (**Figure 21**).<sup>296</sup>



**Figure 21: The first published picture of phagophores**

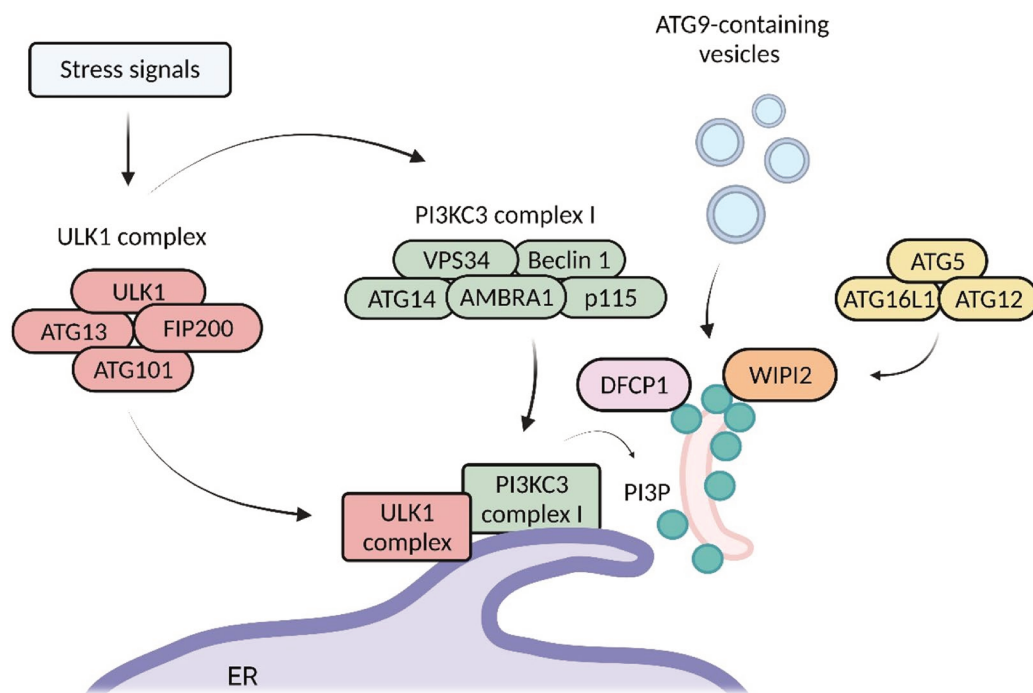
TEM image taken from a fat body cell of a butterfly larva. Scale bar, 1  $\mu\text{m}$ . *Figure taken from Eskelinen E-L, Reggiori F, Baba M, Kovács AL, Seglen PO. Autophagy. 2011;7(9):935-956 with permission.*

The stress signals that potentially activate the autophagy process converge to the ULK1 initiation complex which consists of ULK1, FIP200, ATG13, and ATG101. The serine/threonine kinase mammalian target of rapamycin (mTOR) is a master regulator of cell growth and proliferation. mTOR is part of two protein complexes mTORC1 and mTORC2, out of which mTORC1 is a direct regulator of autophagy. Under normal conditions, mTORC1 inhibits the activity of the ULK1 complex by phosphorylating and binding to ULK1 and ATG13. Nutrient starvation - the most common trigger for autophagy - leads to the inactivation of mTOR releasing the ULK1 complex free. As a result, ULK1 undergoes autophosphorylation and phosphorylates ATG13 and FIP200.<sup>297</sup> Energy limitation, such as glucose starvation can also be a signal for



autophagy sensed by the ATP: adenosine monophosphate (AMP) ratio through the serine-threonine protein kinase liver kinase B (LKB)1 and AMP-activated protein kinase (AMPK) which can, in turn, inhibit mTORC1.<sup>279</sup> AMPK-mediated autophagy can also happen independently of mTOR by directly phosphorylating ULK1, VPS34, and beclin-1.<sup>298</sup>

The activated ULK1 complex then phosphorylates components of the PI3KC3 complex consisting of VPS34, Beclin-1, ATG14, and AMBRA1.<sup>279</sup> The phagophore nucleation is triggered in response to the activation and translocation of the ULK1 and PI3KC3 complexes to phagophore assembly sites (PAS) which determines the site of phagophore nucleation. It takes place at the ER to generate 'Ω' shaped structures called omegasomes rich in phosphatidylinositol 3-phosphate (PI3P) produced by the PI3KC3 complex I. PI3P then recruits effectors, including zinc-finger FYVE domain-containing protein 1 (DFCP1) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) through interaction with their PI3P binding domains, which then recruit factors that control phagophore formation. WIPI2 is shown to interact with ATG16L1, mediating the recruitment of the ATG5-ATG12-ATG16L1 complex. The ULK1 complex also associates with ATG9-containing vesicles in a PI3KC3 complex I-dependent manner.<sup>299</sup> ATG9 is the only transmembrane protein in the core autophagic machinery which regulates the delivery of membrane material from donor organelles such as Golgi apparatus, endosomes, mitochondrial membrane, or plasma membrane to the PAS. These ATG9-containing vesicles are the major sites of PI3P generation and membrane sources for phagophore formation (**Figure 22**).<sup>300</sup>

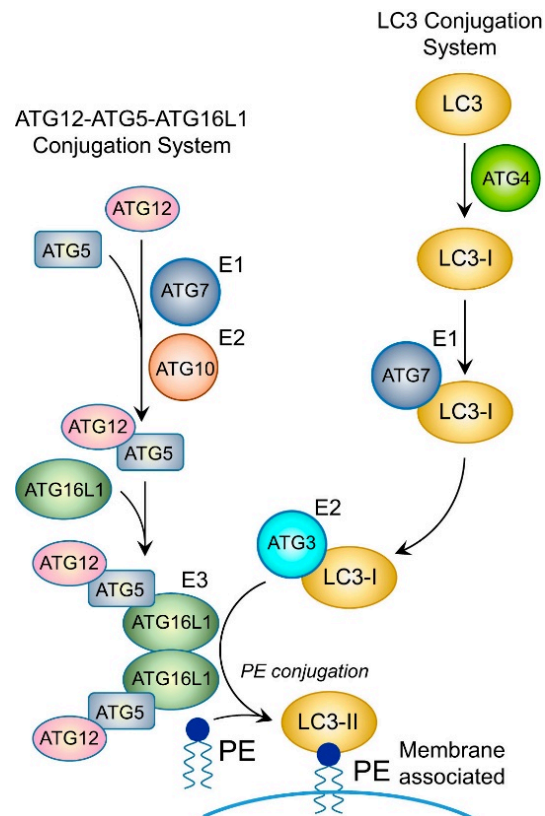


**Figure 22: Initiation machinery of autophagy**

Autophagy induction by various stress signals release the ULK1 complex free. The ULK1 complex activates the PI3KC3 complex I which produce PI3P. PI3P recruits effector proteins such as DFCP1 and WIPI2 to promote the formation of isolation membrane from the ER. WIPI2 interacts with ATG16L1 to recruit the ATG5-ATG12-ATG16L1 complex. ATG9 containing vesicles are the membrane sources for phagophore formation. *Image created with Biorender.*

### 2.2.1.2. Elongation

Elongation and maturation of autophagosomes from the omegasomes involve two ubiquitin-like conjugation systems (**Figure 23**).<sup>301</sup>



**Figure 23: Conjugation systems involved in LC3 lipidation**

Two ubiquitin-like conjugation systems are involved in the lipidation of LC3. The assembly of ATG12-ATG5-ATG16L1 complex is facilitated by the activity of E1-like ATG7 and E2-like ATG10. The LC3 conjugation system involves the cleavage of LC3 by ATG4 to form mature LC3-I. LC3-I is activated by the E1-like ATG7 and then transferred to E2-like ATG3 to facilitate the conjugation of LC3-I with PE to form LC3-II. *Figure taken from O'Grady SM. Am J Physiol Cell Physiol. 2019;316(1):C16-C32 with permission.*

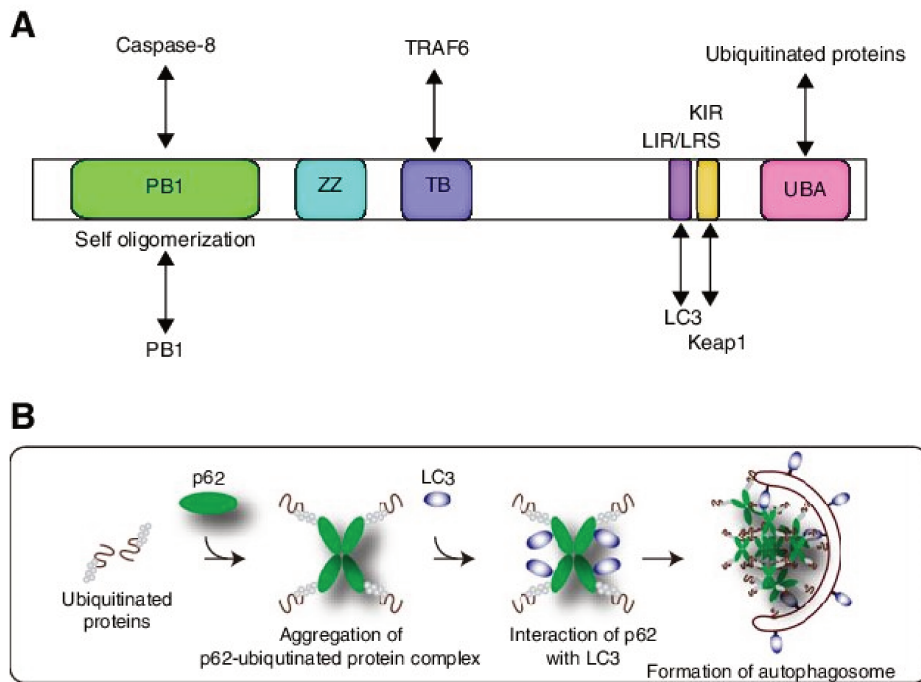
- i) The ubiquitin-like ATG12 conjugates to ATG5 with the combined action of the E1-like enzyme ATG7 and E2-like enzyme ATG10. The ATG12-ATG5 conjugate then non-covalently binds with ATG16L1 and forms a homodimer of 800 kDa.
- ii) The ubiquitin-like ATG8 family of proteins includes MAP1LC3 (MAP1LC3A, MAP1LC3B, MAP1LC3C) and gamma-aminobutyric acid receptor-associated proteins (GABARAP, GABARAPL1, and GABARAPL2) subfamilies. They are produced as inactive Pro-ATG8s. The cysteine protease ATG4 cleaves the pro-ATG8s exposing a glycine residue at the C-terminus that is required for its conjugation to PE. These processed ATG8s are then activated by the E1-like enzyme ATG7. Further, the E2-like

enzyme ATG3 conjugates the mature cytoplasmic ATG8 (MAP1LC3I) to membrane-associated PE to form the lipidated membrane-bound form MAP1LC3II. The E3-like conjugate ATG12~ATG5 assists in this process.

The ATG12~ATG5 conjugate forms a dimeric complex with ATG16L1, which binds to WIPI2, localising its activity to the PAS.<sup>279</sup> Conjugation of PE to ATG8s promotes phagophore expansion as well as the recruitment of cargo receptors bound to the autophagic substrates.

### **2.2.1.3. Cargo recognition and binding**

Cargo adaptors serve as connecting bridges between autophagic substrates and autophagosomes. SQSTM1/p62 was the first and most well-described mammalian autophagic cargo adaptor, involved in linking polyubiquitinated protein aggregates to autophagic machinery.<sup>302</sup> The domain structure of SQSTM1 contains an N-terminal Phox and Bem (PB)1 domain to facilitate self-oligomerisation, intermediate domains that mediate binding of SQSTM1 to other proteins, and a C-terminal ubiquitin-associated (UBA) domain (**Figure 24A**).<sup>303</sup> SQSTM1 binds to LC3/GABARAP proteins through a short linear sequence known as LC3 interacting region (LIR) with the formula Q-X-X-G, where Q is an aromatic amino acid (W/F/Y), G is hydrophobic (L/I/V), and X can be any amino acid. It interacts with the ubiquitinated proteins through its UBA domain and undergoes self-oligomerization through its PB1 domain to form aggregates. These SQSTM1-ubiquitinated protein complexes are delivered to the autophagosomes through their interaction with LC3 and degraded along with the autophagic cargo (**Figure 24B**).<sup>303</sup> Inhibition of autophagy results in the accumulation of SQSTM1 protein aggregates, and therefore the accumulation of SQSTM1 is often used as a reliable marker for impairment of autophagy.



**Figure 24: The structure and function of SQSTM1**

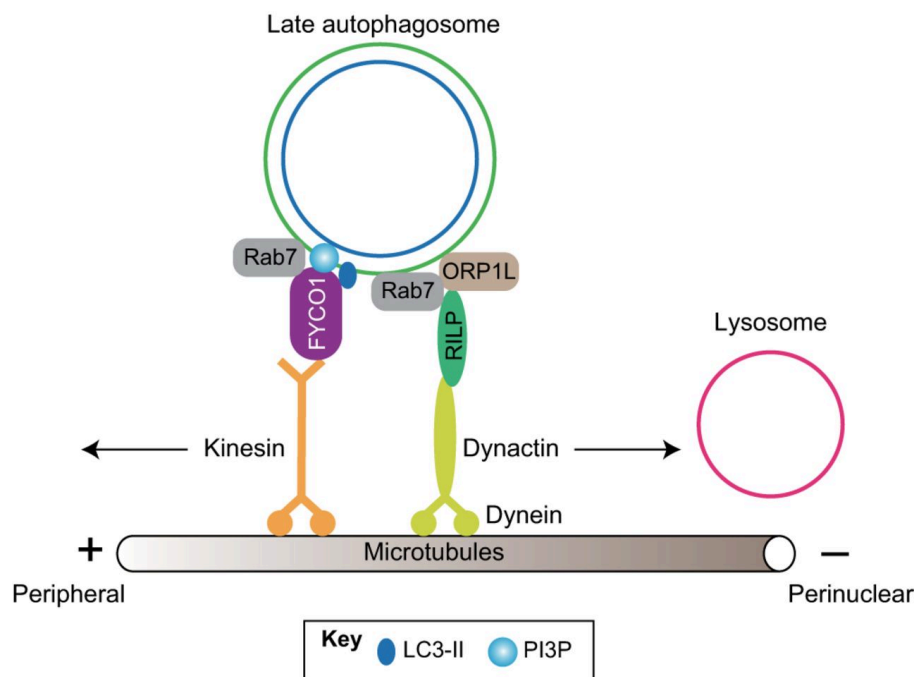
A) The cargo receptor SQSTM1 contains an N-terminal PB1 domain, ZZ zinc finger domain, TB (TRAF6 binding) domain, the LIR/LRS (LC3 recognition sequence), the KIR (Keap1-interacting region), and UBA domain. B) p62 interacts with poly-ubiquitinated proteins via the UBA domain to undergo self-oligomerisation. The complex is sequestered in the autophagosomes through the interaction of LIR/LRS–LC3. *Figure taken from Ichimura Y, Komatsu M. Semin Immunopathol. 2010;32(4):431-436 with permission.*

#### 2.2.1.4. Maturation and transport

Maturation of autophagosomes is characterised by the removal ATGs from the autophagosomal membrane with the action of PI3P phosphatases and members of the ATG4 protease family.<sup>304</sup> It was shown previously that depletion of the PI3P phosphatase YMR1 in yeast or MTM3 in *Caenorhabditis elegans* causes persistence of ATG proteins on autophagosomal membranes. It possibly prevents the recruitment of the machinery for the next step and inhibits subsequent fusion with vacuoles or lysosomes leading to accumulation of autophagosomes in the cytoplasm.<sup>305,306</sup>

Since autophagosomes are randomly formed throughout the cytoplasm, they need to be transported to the perinuclear region where late endosomes and lysosomes accumulate under starvation conditions. Microtubules which are the main components of the cytoskeleton have two polar ends, the plus end directed to the periphery of the cell and the minus end directed to the centrosome. They are responsible for the plus end and minus end directed movement of autophagosomes with the help of two motors proteins

kinesin and dynein respectively. The small GTPase Rab7 (see section 2.2.1.5) links the autophagosomes to dynein-dynactin motor complex through Rab-interacting lysosomal protein (RILP) and the cholesterol sensor ORP1L to facilitate the movement towards the perinuclear region to fuse with the lysosomes, whereas, it is opposed under normal conditions by binding to kinesin through Rab7-FYVE and coiled-coil domain-containing (FYCO)1 interaction (**Figure 25**). Several evidences also suggest the involvement of actin filaments in this transport through myosin family of motor proteins.<sup>307</sup>

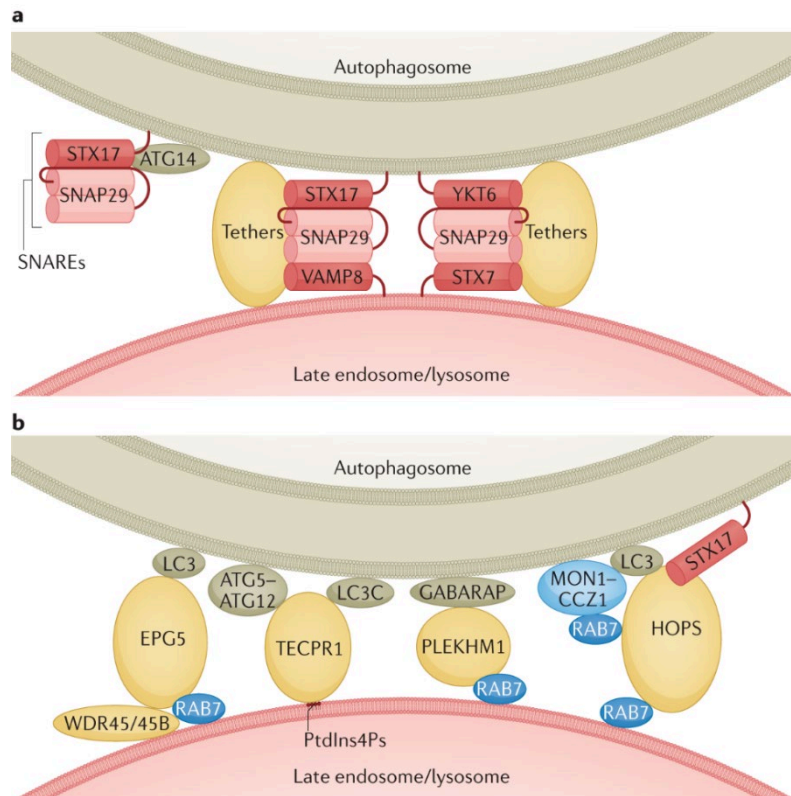


**Figure 25: Transport of autophagosomes**

Rab7 GTPase links autophagosomes to a microtubule motor through FYCO1 to mediate kinesin-driven movement towards the cell periphery. Rab7 also binds to RILP and ORP1L in order to mediate dynein and/or dynactin-driven movement towards the perinuclear region. *Figure taken from Nakamura S, Yoshimori T. J Cell Sci. 2017;130(7):1209-1216 with permission.*

### 2.2.1.5. Fusion

The machinery of autophagosome-lysosome fusion involves the concerted action of SNAREs complexes, membrane tethering complexes, phosphoinositides, and Rab GTPase family proteins (**Figure 26**).<sup>308</sup>



**Figure 26: SNAREs, tethers and RAB proteins mediate autophagosome maturation**

a) Autophagosome-lysosome fusion is mediated by the activity of two sets of SNARE complexes: i) the autophagosomal Qa-SNARE STX17, Qbc-SNARE SNAP29 and endolysosomal R-SNARE VAMP8; and ii) autophagosomal R-SNARE YKT6, SNAP29 and endolysosomal Qa-SNARE STX7. ATG14 interacts with STX17 to promote the formation of STX17-SNAP29 subcomplex. b) Multiple tether proteins are also involved in bridging the two fusing membranes. Rab7 is localized to the membranes to facilitate the fusion. *Figure taken from Zhao YG, Codogno P, Zhang H. Nat Rev Mol Cell Biol. 2021 with permission.*

**Rabs:** The Rab family of proteins is a member of the Ras superfamily of small GTPases. They act as molecular switches with an inactive guanosine diphosphate (GDP)-bound form and are activated by guanine nucleotide exchange factors (GEFs) by converting to a guanosine triphosphate (GTP)-bound form to interact with their effectors. A member of this family, Rab7 is localized on late endosomes and lysosomes and is also recruited to late autophagosomes.<sup>307</sup>

**SNAREs:** Structurally, SNAREs are divided into two classes, Q-SNAREs (which have subclasses Qa, Qb, Qc) and R-SNAREs. These SNAREs form a four-helix bundle to bridge the two fusing membranes. Two such SNARE complexes are formed during autophagosome-lysosome fusion:

- i) the autophagosomal Qa-SNARE STX17, Qbc-SNARE SNAP29, and endolysosomal R-SNARE VAMP8.
- ii) autophagosomal R-SNARE YKT6, SNAP29, and endolysosomal Qa-SNARE STX7.

IRGM helps in the translocation of STX17 from the cytosol to autophagosomes upon starvation.<sup>309</sup> It was also shown recently that STX17 is involved in the formation of isolation membrane by binding to and recruiting ATG14.<sup>310</sup> Besides, ATG14L binds to and stabilizes the binary complex STX17-SNAP29 emphasizing the crucial role of STX-ATG14 interaction in multiple steps of the autophagy pathway.<sup>308</sup>

Tethering factors: Tethers are another group of factors that facilitate the bridging of the fusing membranes and stimulate the assembly of SNARE complexes. Homotypic fusion and protein sorting (HOPS) complex, ectopic p-granules autophagy protein 5 homolog (EPG5), pleckstrin homology domain-containing family M member 1 (PLEKHM1), and tectonin beta-propeller repeat-containing protein 1 (TECPR1) are the tether complexes involved in the fusion process. HOPS complex is a prominent tethering complex which acts as a GEF for Rab7 and interacts with STX17 and LC3 on autophagosomes. EPG5 and PLEKHM1 are other Rab7 effectors which bind to LC3 and GABARAPs respectively on autophagosomes.<sup>308</sup> The tectonin beta-propeller repeat-containing protein 1 (TECPR1) interacts with LC3 and ATG5-ATG12 conjugate on autophagosomes and phosphatidylinositol 4-phosphate (PtdIns4P) on lysosomes, thus strongly facilitating the tethering of autophagosomes with lysosomes.<sup>311</sup>

#### **2.2.1.6. Degradation**

The degradation process takes place within the highly acidic compartments of the lysosomal vesicles by the activity of hydrolytic enzymes present inside. The lysosomal vesicles are characterised by the presence of highly glycosylated lysosomal membrane proteins forming a glycocalyx-like coating on the inner surface of the membrane possibly to withstand the acidic environment inside the lysosomal lumen. Although all the lysosomal membrane proteins are not well characterised, the LAMPs (LAMP1, LAMP2) and lysosomal integral membrane proteins (LIMP1/CD63 and LIMP2) constitute more than 50% of all the membrane proteins on lysosomes.<sup>312</sup> Attempts have been made to characterise the specific functions of LAMPs by mutation studies in mice. The deficiency of LAMP1 or LAMP2 alone in mice keeps them viable while their



double mutation provides them an embryonically lethal phenotype. The deficiency of LAMP2 alone caused a more severe phenotype than that of LAMP1 alone and resulted in the accumulation of autophagic vacuoles, suggesting that LAMP2 has more specific functions than LAMP1. Moreover, the deficiency of LAMP1 was compensated by an upregulation of LAMP2 expression.<sup>313</sup>

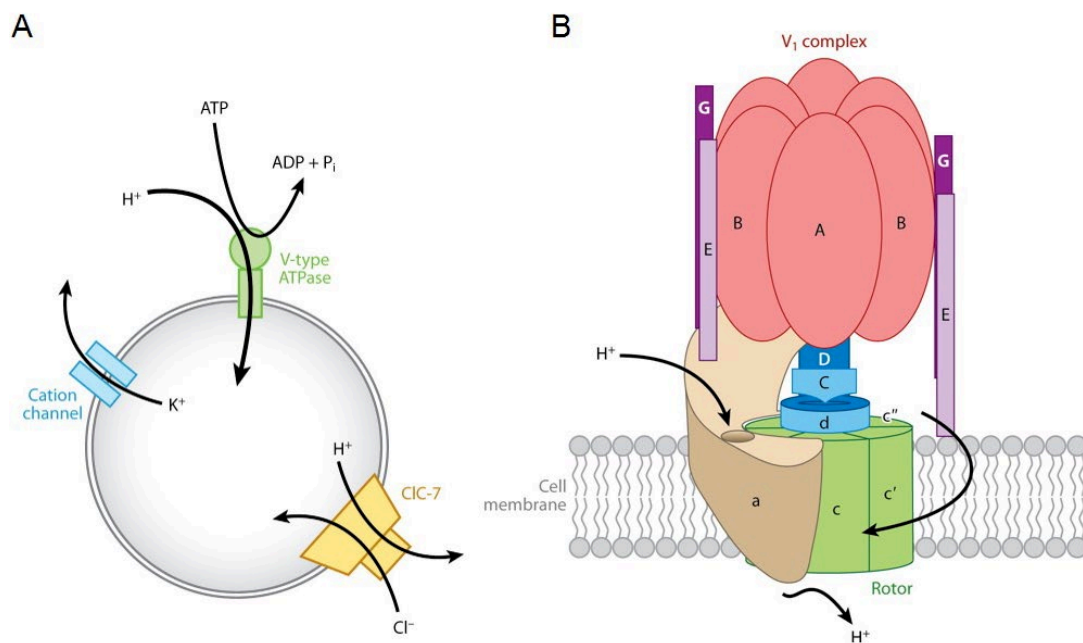
During normal conditions, lysosomes are heterogeneous in size, number, and distribution. However, these parameters adapt themselves during autophagy induction, based on the nutrient availability sensed through mTORC1 inhibition.<sup>314</sup> Upon nutrient starvation, they translocate to the perinuclear region where the autophagosome-lysosome fusion takes place, the number of lysosomes decreases, but the size increases sharply due to the fusion of multiple lysosomes. Further, lysosomal biogenesis is activated through the transcription factor TFEB (transcription factor EB) to restore the lysosomal quantity.<sup>315</sup>

Lysosomes contain more than 60 different hydrolytic enzymes (nucleases, proteases, phosphatases, lipases, sulfatases, etc), most of which need optimal acidic pH to be active (see section 2.2.1.7 below). The degradation process starts with the disruption of the inner autophagosomal membrane with the help of an unidentified lipase, after which the lysosomal enzymes gain access to the autophagic substrates. However, the outer membrane is somehow resistant to the activity of this lipase. The catabolites generated from the degradation of autophagic cargo are exported to the outside through numerous transporters on the lysosomal membrane for reutilising in the biosynthetic pathways.<sup>316</sup>

#### **2.2.1.7. Lysosomal acidification mechanisms**

The highly acidic environment inside the lysosomes (less than pH 5.0) is maintained by the activity of vacuolar-type ATPase (v-ATPase), a type of proton pump that uses energy from ATP hydrolysis to drive translocation of protons into the lysosomal lumen. The v-ATPase is a multisubunit complex consisting of an extrinsic  $V_1$  domain and a membrane-integral  $V_0$  domain. The  $V_1$  domain is composed of 8 subunits from A-H and it is functionally responsible for ATP hydrolysis. The  $V_0$  domain contains 6 subunits (a, c, c', c'', d and e) and coordinates with the  $V_1$  subunit to transport the protons generated through ATP hydrolysis (**Figure 27**). They are structurally similar to the  $F_0F_1$  ATPase present in mitochondria. However, the  $F_0F_1$  ATPase can synthesis and hydrolyse ATP, while v-ATPase is optimised only for ATP hydrolysis.<sup>317,318</sup>

However, this proton pumping generates a voltage difference across the lysosomal membrane which in turn inhibits further transport. Therefore, a counterion movement must accompany the proton transport to dissipate this voltage. This could be achieved by the movement of a cation out of the lysosomal lumen, an anion moving into the lysosomal interior, or by both (**Figure 27**). A number of channels and transporters have been proposed to be involved in the counterion pathway, however, their identities remain controversial. A member of the chloride channel (ClC) family of chloride ion ( $\text{Cl}^-$ ) transporters, ClC-7 was the first proposed  $\text{Cl}^-/\text{H}^+$  antiporter in this process.<sup>319</sup> Other potential candidates are the transient receptor potential (TRP) channels, which are similar to the voltage-gated  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels. A member of this family (mucolipin transient receptor potential, TRPML1) is a  $\text{Ca}^{2+}$  channel implicated in lysosomal storage disorders.<sup>320</sup>

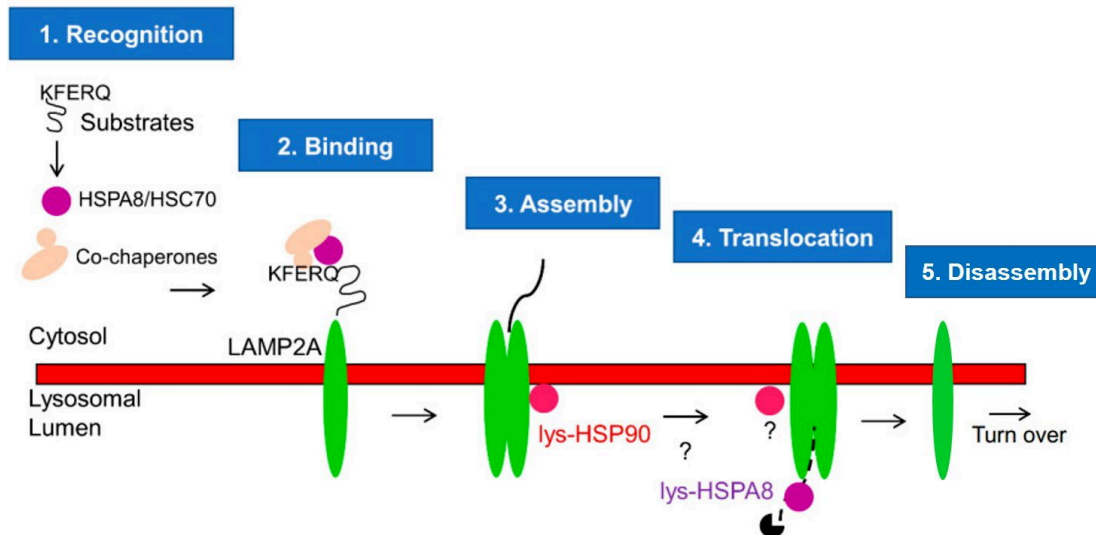


**Figure 27: Mechanisms involved in lysosomal pH homeostasis**

A. The v-ATPase uses the energy generated from ATP hydrolysis to drive protons into the lysosomal lumen. The voltage thus created is dissipated by counterion transport through various transporters. B. The structure of v-ATPase. *Figure taken from Mindell JA. Annu Rev Physiol. 2012;74(1):69-86 with permission.*

### 2.2.2. CMA

Similar to macroautophagy, CMA is also a multi-step process as illustrated in **Figure 28**.



**Figure 28: Scheme of different CMA steps**

The cytosolic proteins containing the specific CMA-targeting motif are recognised by HSPA8 and targeted to the lysosome. The substrate-chaperone complex then binds to the LAMP2A receptor at the lysosomal membrane inducing LAMP2A oligomerisation to form the translocation complex. HSP90AA1 is involved in stabilising this complex. Through the translocation complex the substrate protein is unfolded and transported into the lysosomal lumen with the assistance of lys-HSPA8. Once the substrates are fully translocated, LAMP2A disassembles and is degraded by cathepsin A mediated cleavage. *Figure adapted from Wang F, Tasset I, Cuervo AM, Muller S. Cells. 2020;9(10):2328 with permission.*

All CMA substrates are characterized by the presence of a pentapeptide motif similar to Lys-Phe-Glu-Arg-Gln (KFERQ) in their amino acid sequence, known as the CMA target motif.<sup>321</sup> According to this criterion, ~30% of the cytosolic proteins are putative substrates for CMA (**Table 7**). A group of chaperones and co-chaperones present in the cytosol recognizes the proteins containing this motif and targets them to the lysosomes. The heat shock protein family A member 8 (HSPA8/HSC70) is the major player in this process, which directly binds to the KFERQ motif.<sup>322,323</sup>

**Table 7: List of proteins experimentally validated as CMA substrates**<sup>322–326</sup>

Symbol	Protein full name	Symbol	Protein full name
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	HK2	Hexokinase-2
TP53	Tumor protein P53	MDM2	Mouse double minute 2 homolog
MLLT11	MLLT11 transcription factor 7 cofactor	c-Myc	MYC proto-oncogene
CHK1	Checkpoint Kinase 1	Vav1	Vav guanine nucleotide exchange factor 1
NCOR1	Nuclear receptor corepressor 1	PED	Phosphoprotein enriched in diabetes
RND3	Rho family GTPase 3	TFEB	Transcription factor EB
GAL3	Galectine-3	RKIP	Raf kinase inhibitor protein
Bcl2-L10	Bcl2 like 10	MEF2D	Myocyte enhancer factor 2D
HBB	Hemoglobin ( $\beta$ -chain)	PGAM1	Phosphoglycerate mutase 1
HSPA8	Heat shock protein family A member 8	MAPT	Tau
ANXs	Annexins I, II, IV and VI	Fos	Fos proto-oncogene
SNCA	$\alpha$ -synuclein	RNase A	Ribonuclease A
RYR2	Ryanodine receptor 2	PAX2	Paired box 2
PKM	Pyruvate kinase M2	EGFR	Epidermal growth factor receptor
PUMA	P53 upregulated modulator of apoptosis	EPS8	EGFR pathway substrate 8
I $\kappa$ B $\alpha$	NF- $\kappa$ B inhibitor alpha	UBQLN1	Ubiquilin 1
HIF-1 $\alpha$	Hypoxia-inducible factor 1 alpha	AST	Aspartate aminotransferase
RCAN1	Regulator of calcineurin 1	ALDB	Aldolase B
LRRK2	Leucine-rich repeat kinase 2	UCHL1	Ubiquitin C-terminal hydrolase L1
TARDBP	TAR DNA binding protein	PLINs	Perilipins
ITCH	Itchy E3 ubiquitin protein ligase	HTT	Huntingtin
	Subunits of the 20S proteasome		C8 subunit (26S proteasome)

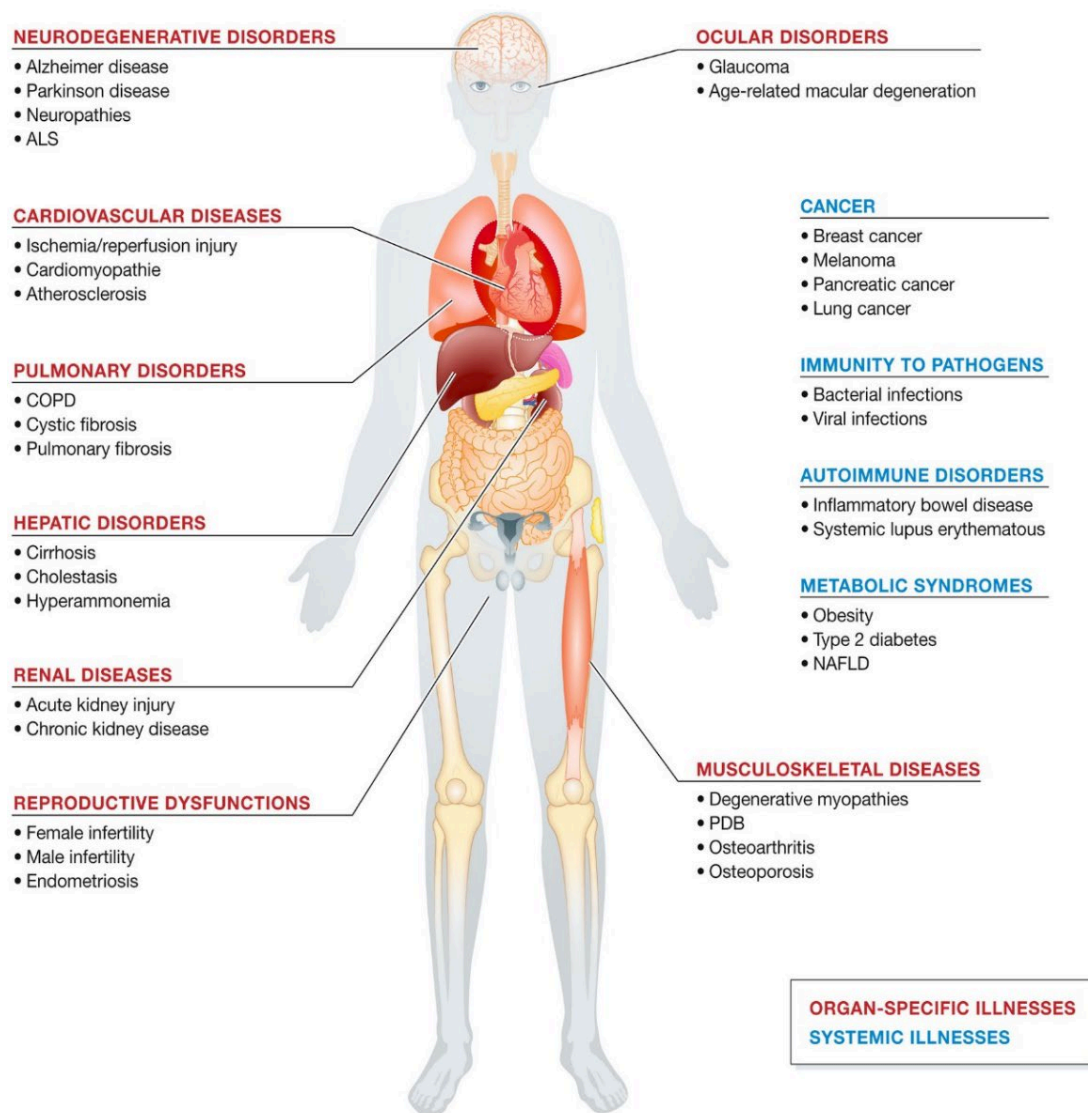
Once reaching the surface of lysosomes, the substrate-chaperone complex binds to the cytosolic tail of the single-span membrane protein LAMP2A, one among the three splice variants of LAMP2 protein (LAMP2A, LAMP2B, and LAMP2C). Being the unique receptor for CMA substrates, LAMP2A binding to substrate is thought to be the limiting step of this pathway.<sup>287</sup> Overexpression of LAMP2A alone increases the CMA activity in cells.<sup>327</sup>

The binding of substrates to monomeric LAMP2A induces conformation changes and its oligomerisation to form the 700 kDa substrate translocation complex. A lysosomal form of another chaperone heat shock protein 90 alpha family class A member 1 (HSP90AA1) stabilises the translocation complex. A luminal form of HSPA8 (lys-HSPA8) is also essential for the substrate unfolding and translocation.<sup>286</sup> The presence of lys-HSPA8 is a defining feature of the lysosomal population involved in CMA. Once the substrates reach the lysosomal lumen, the disassembly of the complex occurs, a step that is also assisted by the ATPase activity of HSPA8.<sup>328</sup> Along with the substrates, LAMP2A is subjected to degradation by cathepsin A.

### **2.3. Autophagy in human health and diseases**

More than a cellular quality control mechanism, the largely diverse physiological functions of autophagy are currently being discovered in the context of human health and diseases. The intersection of different autophagy processes with various developmental processes and immune-related mechanisms has been demonstrated. From an evolutionary perspective, autophagy primarily equips the cells to survive under nutrient starvation conditions, and consequently, autophagy competent cells gain an advantage over autophagy-deficient cells. But when the focus shifts from single-cell survival to fitness of the whole organism, the relationships become more complex and both deregulation and upregulation of autophagy can have unpredictable outcomes. Moreover, alterations in different stages of the autophagy pathway can have different consequences.<sup>329</sup>

Mutations in several ATG genes and physiological disturbances in different autophagy processes are implicated in the initiation and progression of major human pathologies ranging from neurodegenerative dysfunctions to immune system abnormalities (**Figure 29**).<sup>329</sup>



**Figure 29: Some examples of human diseases linked to dysregulated autophagy**

Representation of major organ-specific (red) and systemic (blue) human disorders in which autophagy plays a critical role in pathogenesis and progression. *Figure taken from Klionsky DJ, Petroni G, Amaravadi RK, et al. EMBO J. 2021;40(19):e108863 with permission.*

One of the early milestones in this context is the discovery of the ATG gene *beclin-1* as a tumour suppressor gene in 1999. The *beclin-1* gene maps to a tumour susceptibility locus on human chromosome 17q21 that is mono-allelically deleted in ovarian and breast carcinomas. *Beclin-1* gene overexpression in tumor cells inhibits their proliferation *in vitro*.<sup>330</sup> But other studies have also demonstrated the pro-tumorigenic role of autophagy especially in Kirsten rat sarcoma virus (KRAS)-driven cancers. The pro-tumorigenic effect is based on the ability of autophagy to promote the survival of tumor cells during metabolic stress.<sup>295</sup> Another well explored area of human diseases in

relation to autophagy is neurodegenerative disorders. Numerous studies have provided evidence that autophagosomes accumulate in the brain of patients with Alzheimer's disease, Parkinson's disease, and Huntington's disease as a protective mechanism to clear the misfolded proteins and damaged organelles.<sup>331,332</sup>

GWAS in patients as well as the implementation of autophagy-deficient animal models have greatly contributed not only to our understanding of the role of autophagy in disease progression but also, in finding potential targets to modulate autophagy processes to prevent or treat the disease.<sup>281,329,333</sup> In fact, several elements of autophagy have emerged as attractive therapeutic targets in many of these disorders. Pharmacological targeting of different autophagy processes has been demonstrated as an efficient treatment strategy in different animal disease models and some of them are in clinical use too (**Table 8**). Interestingly, the well-established effects of some of the old drugs or natural compounds used to treat diseases have now been explained through their potential modulation on autophagy processes. For example, aspirin is one of the oldest chemicals to be used in the treatment of pain, fever, or inflammation. Recent evidences suggest that the effects of aspirin depend on autophagy induction and the protective effect was not observed in mouse models of genetic autophagy deficiency.<sup>334</sup>

**Table 8: Some examples of autophagy modulating drugs approved or in clinical trials for various diseases**<sup>335–339</sup>

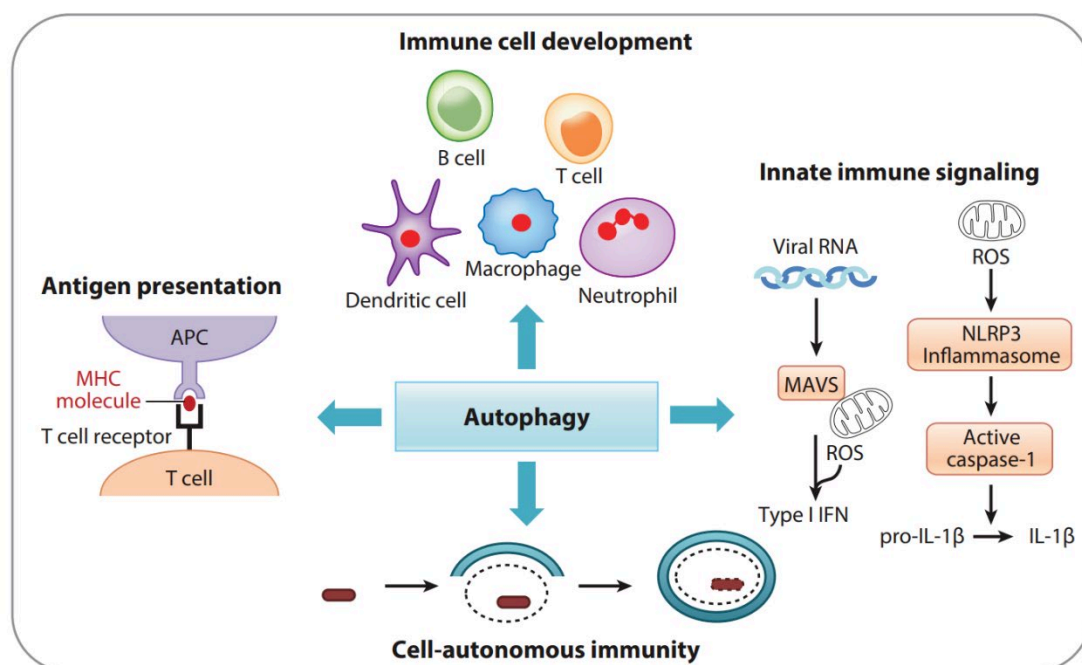
Drug	Mechanism of action	Clinical status	Major limitations
<b>Autophagy inducers</b>			
Rapamycin/ sirolimus	Inhibits mTORC1	Approved for organ transplant rejection and lymphangioleiomyomatosis (a rare pulmonary disease), in clinical trials for CD	Chronic exposure causes mTORC2 inhibition
Metformin	Activates AMPK	Approved for type 2 diabetes	Multiple off-target effects including inhibition of mitochondrial respiration
Carbamazepine	Reduces inositol and Ins(1,4,5)P <sub>3</sub> levels	Approved for epilepsy, bipolar disorder	Inhibits various neuronal functions
Everolimus	Inhibits mTORC1	Approved for cancer therapy	Immunosuppressive effects
Trehalose	Activates TFEB	In clinical trials for bipolar disorders, dry eye syndrome, and vascular aging	Enhances CDI
Resveratrol	Caloric restriction mimetic	Nutritional supplement, in clinical trials for several disorders	Causes nephrotoxicity at high doses
Retinoic acid	Autophagy mediated degradation of RARs	Approved for cancer therapy	Multiple targets
Simvastatin	AMPK activation	Approved for treatment of obesity	Myotoxicity
<b>Autophagy inhibitors</b>			
Chloroquine and Hydroxychloroquine	Lysosomal inhibition	Approved for malaria, SLE, and rheumatoid arthritis	Retinal toxicity
Azithromycin	Blocks autophagic flux	Approved for multiple bacterial infections	Deleterious effects of autophagic flux inhibition in certain conditions
LY294002	VPS34 inhibition	In clinical trials for refractory neuroblastoma	Non-specific inhibitor, binds to other PI3Ks

Abbreviations not used in the text above: RAR, retinoic acid receptor; SLE, systemic lupus erythematosus.



## 2.4. Autophagy in immunity and inflammatory disorders

The intersection between autophagy and immunity lies in various cellular functions including immune response against pathogens, immune cell development, innate immune signalling, and antigen presentation (**Figure 30**).<sup>340</sup>



**Figure 30: The role of autophagy in immunity**

Autophagy has critical functions in the development and function of immune cells, innate immune signalling, and cell-autonomous defence. *Figure taken from Matsuzawa-Ishimoto Y, Hwang S, Cadwell K. Annu Rev Immunol. 2018;36:73-101 with permission.*

As previously described (section 2.1.4), autophagy primarily serves as a first line of defence against pathogenic infections through xenophagy (also LAP). Numerous pathogens including bacteria (Group A *Streptococcus*; GAS, *Mycobacterium tuberculosis*, *Shigella flexneri*, *Salmonella enterica*, *Listeria monocytogenes*), viruses (herpes simplex virus type 1; HSV-1, Sindbis virus), and parasites (*Toxoplasma gondii*) are shown to be degraded by xenophagy.<sup>341</sup> However, many of these pathogens have also co-evolved mechanisms to evade the autophagy machinery or even manipulate the machinery for their survival. As an interesting example, in contrast to the GAS strains that were previously examined in the laboratory, the clinically isolated GAS strain MIT1 evades autophagy by expressing a protease that degrades the autophagic cargo adaptors.<sup>342</sup> Another first line of defence against pathogens employed by neutrophils is the formation of neutrophil extracellular traps (NETs) composed of DNA, histones, and

neutrophil antimicrobial peptides that can trap and kill various pathogens, a process termed NETosis. Remijsel et al. (2011) have first proposed the relationship between NETosis and autophagy by showing that pharmacological inhibition of autophagy reduced the formation of NETs in phorbol myristate acetate (PMA)-stimulated neutrophils.<sup>343</sup> However, a reverse relationship was also found in chronic kidney disease patients, where the levels of NETs were increased after autophagy inhibition.<sup>344</sup> The differential roles of autophagy on the formation of NETs seem to be dependent on the extent of autophagy and its mechanism of regulation.<sup>345</sup>

Beyond the degradation of pathogens, other innate immune defence mechanisms can also be activated through the detection of pathogens by PRRs such as TLRs, NLRs, RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs). The downstream signalling events can lead to the secretion of pro-inflammatory cytokines, reactive oxygen species generation, and inflammasome activation. Autophagy is found to play crucial roles in these pathways.<sup>346</sup> Canonical autophagy, as well as LAP, participates in the delivery of viral nucleic acids to endosomes for TLR-mediated innate immune activation and type I IFN secretion.<sup>347,348</sup> In contrast, the autophagic machinery negatively regulates RLR-mediated cytosolic nucleic acid sensing for type I IFN activation.<sup>349</sup> This negative regulation is partly due to efficient autophagic removal of the ligands for these receptors, and in addition, ATG proteins are found to directly inhibit the complexes involved in the type I IFN pathway.<sup>350</sup> Autophagy also exerts negative regulation at the level of inflammasomes to prevent their aberrant activation (see section 3.2.2).

With respect to adaptive immunity, autophagy pathway functionally participates in antigen presentation. Classically, endogenous antigens processed by proteasome are presented by MHC class I molecules to CD8<sup>+</sup> T cells whereas, MHC class II molecules are involved in presenting extracellular antigens taken up by endocytosis or phagocytosis to CD4<sup>+</sup> T cells. However, in a pathway called cross-presentation, APCs can process extracellular antigens for MHC class I presentation and some intracellular (nuclear or cytoplasmic) antigens can also be presented *via* MHC class II molecules.<sup>351</sup> The role of autophagy (macroautophagy and CMA) is well demonstrated in processing and delivering both extracellular and intracellular antigens to MHC class II antigen-presenting molecules.<sup>288,352,353</sup> Self-antigen presentation through autophagy is one of the mechanisms by which autophagy can contribute to the development of autoimmune

disorders. Although MHC class I antigens are mainly generated by the proteasome, autophagy sometimes provides an alternative pathway especially during herpes viral infections.<sup>354</sup>

Autophagy is indispensable for the development and differentiation of various immune cells.<sup>355,356</sup> Studies involving immune cell-specific deletion of autophagy genes have demonstrated the role of autophagy in maintaining the homeostasis of immune cell populations. Pua et al. 2006 has shown that chimeric mice transferred with ATG5-deficient fetal liver hematopoietic progenitor cells exhibited a reduced number of peripheral T and B cell populations. Furthermore, ATG5<sup>-/-</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells from these mice failed to undergo efficient proliferation upon TCR stimulation.<sup>357</sup> In another study, B cell-specific deletion of ATG5 in mice was associated with inefficient B cell development and a significant decrease in their numbers.<sup>358</sup> Defective autophagy-mediated mitochondrial clearance and the role of ATG5 in cytokine-driven differentiation or cell survival after growth factor withdrawal are the postulated explanations for these observations.<sup>351</sup> Moreover, the elimination of autoreactive T cells in the thymus is mediated by autophagy-dependent MHC class II antigen presentation by thymic epithelial cells. Deletion of ATG5 in thymus resulted in altered T cell specificities leading to severe colitis and multi-organ inflammation in mice.<sup>359</sup> Subsequently, several studies have demonstrated the essential roles of other ATG genes in lymphocyte development and survival.<sup>355,356</sup>

There are emerging genetic and functional evidences to support the role of autophagy in various immune-related and autoimmune disorders.<sup>360,361</sup> For example, polymorphisms in an intergenic region between PR domain zinc finger protein (PRDM)1 and ATG5 were linked to increased susceptibility to the autoimmune disorder SLE and correlated with increased ATG5 expression in B cells.<sup>362</sup> Similarly, ATG5 expression is found to be higher in a mouse model of autoimmune encephalomyelitis as well as in blood and brain tissues from MS patients.<sup>363</sup> However, the most strongly associated link described so far is between autophagy gene mutations and CD, which will be further described below.

### 3. AUTOPHAGY AND IBDs

#### 3.1. ATG genes associated with IBDs

As described above, GWAS have identified several genetic polymorphisms in ATG genes to be strongly associated with increased risk of IBDs. A summary of the important variants discovered is listed below (**Table 9**).

**Table 9: Main genetic variants related to IBDs and autophagy**<sup>364</sup>

Gene	Chromosomal site	Functional relation to autophagy
ATG16L1	2p37.1	A subunit of the autophagy-related ATG12-ATG5-ATG16L1 complex which acts as a scaffold to MAP1LC3 for lipidation and autophagosome formation.
IRGM	5q33.1	Belongs to the p47 immunity-related GTPase family. Implicated in autophagy induction and autophagosome maturation in response to intracellular pathogens.
LRRK2	12q12	A multifunctional kinase enzyme which is thought to be a regulator of macroautophagy and CMA.
NOD1/2	16q12.1	An intracellular PRR involved in bacterial sensing. Interacts with ATG16L1 to induce an autophagic response against bacteria.
ULK1	12q24.33	Part of the autophagy initiation complex regulated by mTORC1 and AMPK.

##### 3.1.1. ATG16L1

Among the nine genetic variants of *ATG16L1* that are associated with CD, the variant rs2241880, comprising a missense mutation resulting in threonine to alanine substitution at position 300 of the protein is most commonly associated with an increased risk of developing the disease. The T300A mutation is located in the cleavage site of caspase 3, and this mutation enhances the degradation of ATG16L1 by caspase 3 and hence diminishes autophagy.<sup>55</sup> Animal models with mutations in *ATG16L1* (e.g., *ATG16L1*<sup>T300A</sup> knock-in mice) have been used to reinforce our understanding of the importance of this gene in the development of IBDs.

### 3.1.2. IRGM

Multiple CD-associated polymorphisms have been found in the IRGM locus affecting the protein expression and splicing. It was previously shown that loss of IRGM in intestinal epithelial cells resulted in defective autophagy and increased the survival of AIEC bacteria.<sup>365</sup> A CD-associated exonic synonymous single nucleotide polymorphism (SNP) in IRGM alters the binding of miR-196 to the IRGM risk variant. MiR-196 is overexpressed in the inflammatory intestinal epithelia of individuals with CD and downregulates the IRGM protective variant but not the risk-associated allele. The subsequent loss of regulation of IRGM expression levels leads to defective autophagy-mediated clearance of AIEC bacteria.<sup>366</sup>

### 3.1.3. LRRK2

A member of the leucine-rich repeat kinase family localizing to endolysosomal compartments and specific membrane microdomains. It is thought to be a regulator of macroautophagy and CMA. *LRRK2* mRNA expression level is found to be higher in DCs of patients with CD. An increase in LRRK2 expression levels suppresses autophagy and LRRK2 transgenic mice overexpressing LRRK2 showed increased susceptibility to DSS colitis.<sup>367</sup>

### 3.1.4. NOD1/2

Intracellular PRRs expressed in intestinal Paneth cells and monocyte-derived immune cells. They act as muramyl dipeptide (MDP) sensors, which activate the downstream effector pathways in response to bacterial infection. NOD2 was the first gene to be identified as the CD risk gene. Around one-third of patients with CD harbour NOD2 mutations with a 17-fold increased risk of the disease. Three mutations within the ligand-binding domain at the C-terminal of NOD2 have been associated with CD.<sup>368</sup> Three polymorphisms have also been detected in NOD1 in UC patients of Northern India.<sup>369</sup> However, their interest in autophagy emerged with the discovery as interacting partners of ATG16L1.<sup>370</sup>

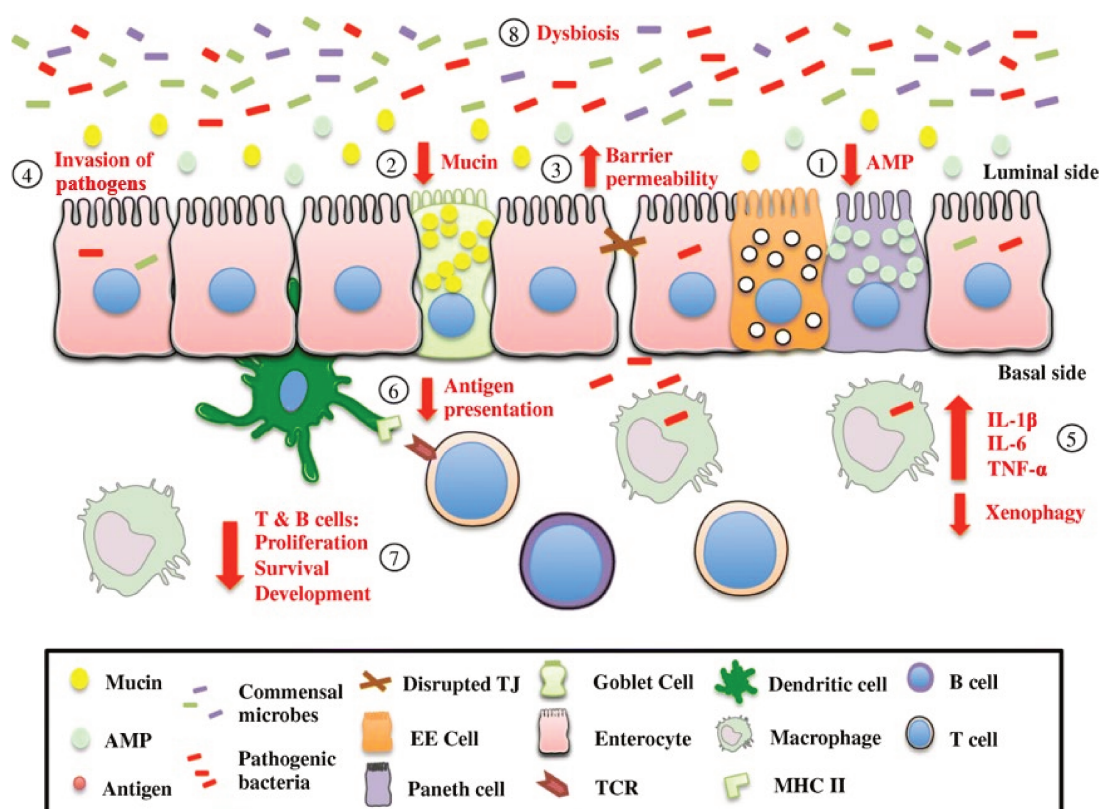
### 3.1.5. ULK1

Henckaerts and colleagues selected human homologs of 12 yeast autophagy-related genes according to their location in a known IBD locus or in a genomic region detected in a GWAS study or GWAS meta-analysis. This study has discovered a novel

association between one haplotype tagging SNP (rs12303764) in the ULK1 gene and CD risk.<sup>371</sup> However, the functional relationship between this variant and IBD pathogenesis is not known.

### 3.2. Autophagy and intestinal homeostasis

Several functional studies have been carried out in mouse models of IBD as well as in some patient samples, linking the genetic polymorphisms in ATG genes and pathophysiological hallmarks of IBDs, emphasising the indispensable role of autophagy in intestinal homeostasis functions (**Figure 31**).<sup>1,372,373</sup>



**Figure 31: Role of autophagy in maintaining intestinal homeostasis**

Defective autophagy leads to decreased antimicrobial peptide (AMP) (1) and mucin (2) secretion, increased barrier permeability (3), increased bacterial invasion (4), impaired cytokine production (5), reduced antigen presentation (6), reduced T and B cell survival and development (7) and gut dysbiosis (8). *Figure taken from Haq S, Grondin J, Banskota S, Khan WI. J Biomed Sci. 2019;26(1):19. with permission.*

#### 3.2.1. Autophagy and intestinal epithelium

The loss of intestinal barrier integrity or a leaky gut is a characteristic feature and an initial event in the pathogenesis of IBD leading to a cascade of inflammatory events

underlying the epithelia. Several mechanisms contribute together in this process including alterations in TJ protein expression, loss of epithelial cells by apoptosis, and severe abnormalities in the function of specialised epithelial cells.

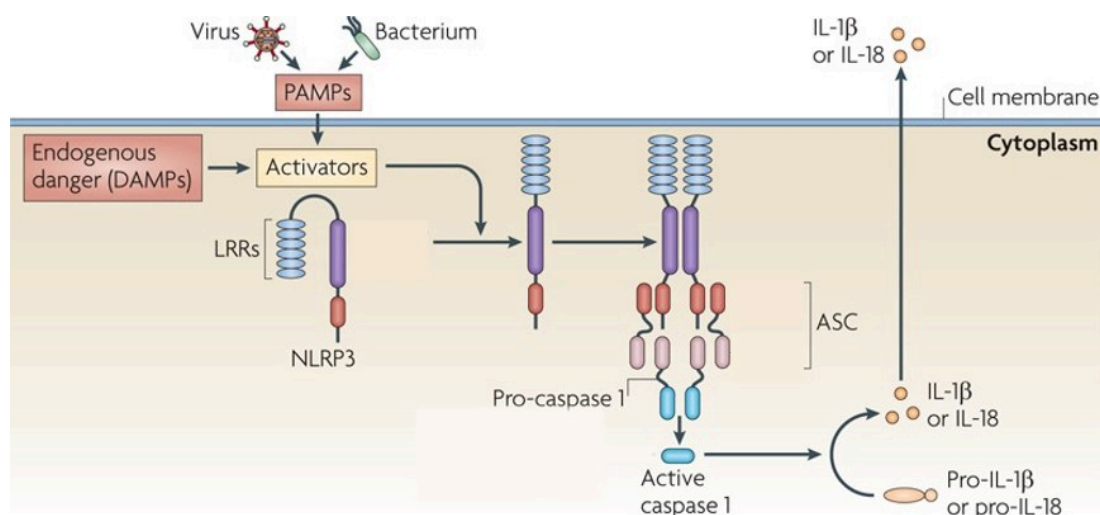
Autophagy in intestinal epithelial cells is found to be protective against TNF-induced apoptosis. IEC-specific deletion of ATG16L1 exacerbates chronic colitis induced by *Helicobacter hepaticus* infection in mice which was counteracted by TNF blockade.<sup>374</sup> A defective TJ barrier also contributes to the pathogenesis of IBDs by causing increased intestinal barrier permeability and antigen breaching. The interplay of autophagy and TJ barrier was first described by the degradation of the TJ barrier protein claudin-2 by autophagy. It was shown that in Caco-2 intestinal epithelial cells starvation-induced autophagy increases the degradation of claudin-2 resulting in reduced barrier permeability.<sup>375</sup>

Paneth cells are specialised cells of the intestinal epithelium that is majorly involved in antimicrobial peptide secretion against invading bacteria. The morphology and secretory functions of Paneth cells are largely affected by impairment in autophagy pathway. The role of ATG genes in the sorting of lysozymes to Paneth cell secretory granules has been highlighted by the discovery of a NOD2–LRRK2–RIP2–RAB2A linked pathway.<sup>376</sup> The deficiency of these genes resulted in lysosomal degradation of lysozyme. Certain pathogens like *Salmonella typhimurium* are found to disrupt the Golgi apparatus to affect the conventional ER-Golgi mediated secretion of lysozyme, which is then rerouted through an autophagy-based alternative secretory pathway. Mice harbouring *ATG16L1*<sup>T300A</sup> mutation exhibited disrupted ER-Golgi secretion pathway as well as defective secretory autophagy in response to *Salmonella typhimurium* infection.<sup>377</sup> In another study, *irgm*<sup>-/-</sup> mice showed defective autophagy in Paneth cells leading to alterations in Paneth cell location and granule morphology and increased susceptibility to DSS-induced colitis.<sup>378</sup> Goblet cells are another group of specialised intestinal epithelial cells involved in the secretion of mucins to form the mucus layer to protect against microbes. Loss of function of several ATG genes such as ATG5, ATG7 and MAP1LC3B in mice results in mucin granule accumulation in colonic goblet cells indicating a role of autophagy in mucin secretion.<sup>379</sup>

### 3.2.2. Autophagy and intestinal immune responses

Inflammasomes are multiprotein complexes of the innate immune system formed in response to activation of PRRs and induce the maturation and release of pro-inflammatory cytokines such as IL-1 $\beta$  and IL-18 (**Figure 32**). Several studies have shown that autophagy has a potential role in negatively regulating inflammasome activity, and consequently, defective autophagy can lead to aberrant inflammasome activation. Loss of ATG16L1 in macrophages leads to elevated secretion of IL-1 $\beta$  and IL-18 cytokines in response to lipopolysaccharide (LPS) stimulation. In addition, mice with myeloid-specific deletion of ATG16L1 gene also showed increased susceptibility to DSS-induced colitis.<sup>380</sup> Protein tyrosine phosphatase non-receptor type (PTPN) 2 is a CD susceptibility gene implicated in the regulation of autophagy. The presence of PTPN2 risk variant in human THP-1 monocytes (a human monocytic cell line derived from an acute monocytic leukemia patient) and IECs results in impaired autophagosome formation and elevated inflammasome activation in response to bacterial cell wall component MDP stimulation.<sup>381</sup> The CD-associated autophagy gene IRGM was shown to be a negative regulator of the NOD-like receptor family, pyrin domain-containing (NLRP)3 inflammasome activation. The NLRP3 inflammasome is composed of three components, including NLRP3 protein, adapter protein apoptosis-associated speck-like protein (ASC), and procaspase-1. Mechanistically, IRGM performs this regulation by the direct inhibition of NLRP3 oligomerisation or by promoting SQSTM1 mediated autophagic degradation of the inflammasome components NLRP3 and ASC. Moreover, the exacerbated colitis in response to DSS treatment in *irgm*<sup>-/-</sup> mice was alleviated by the pharmacological blockade of NLRP3 inflammasome activation.<sup>382</sup> CD-associated polymorphisms in another protein myotubularin-related protein 3 (MTMR3) also lead to impaired autophagy and thereby increase PRR-induced inflammasome activation.<sup>383</sup>





**Figure 32: Mechanism of NLRP3 inflammasome complex formation**

In response to PAMPs from microorganisms or DAMPs from endogenous danger signals, NLRP3 oligomerizes and recruits ASC and pro-caspase 1, triggering the activation of caspase 1 and the maturation and secretion of pro-inflammatory cytokines such as IL-1 $\beta$  and IL-18. LRRs, leucine-rich repeats; DAMPs, damage-associated molecular patterns. *Figure taken from Tschopp J, Schroder K. Nat Rev Immunol. 2010;10(3):210-215 with permission.*

The role of macroautophagy and CMA in antigen presentation has been well described as a way of delivering cytoplasmic antigens to MHC molecules (described in section 2.4). DCs from patients expressing CD-associated risk variants of NOD2 and ATG16L1 showed defective autophagy-mediated bacterial clearance in response to MDP stimulation and failed to generate MHC class II antigen-specific CD4<sup>+</sup> T cell responses.<sup>384</sup> Another study also supported this fact by showing that knockdown of ATG16L1 and IRGM in DCs leads to hyper stable interactions between DCs and T cells resulting in a Th17 mediated immune response.<sup>385</sup>

The essential role of ATGs in lymphocyte development and functions (see above, section 2.4) has been demonstrated in the context of intestinal inflammation as well. Selective deletion of ATG16L1 in T cells of mice resulted in a spontaneous intestinal inflammation characterised by an increased Th2 cell expansion and loss of Treg cells suggesting a role of autophagy in promoting Treg cell survival and restricting Th2 mediated inflammatory response.<sup>386</sup> In another study, B-cell expression of ATG5 was found to be indispensable for Ab secretion against intestinal parasitic (*Heligmosomoides polygyrus*) infection and DSS-induced colitis in mice.<sup>387</sup>

### 3.2.3. Autophagy and gut microbiota

As described above (section 3.1), the association between a number of ATG genes involved in antibacterial autophagy or xenophagy (ATG16L1, NOD2, IRGM) and IBD risk points to the importance of autophagy pathway in determining the gut microbial composition. Several studies have been carried out in this regard. For example, IEC-specific ATG7 conditional knockout mice showed increased susceptibility to experimental DSS colitis with an increased bacterial load in epithelial cells and abnormal fecal microbiota composition.<sup>388</sup> Similarly, IEC-specific deletion of ATG5 leads to decreased gut microbial diversity. More specifically, some families associated with the control of inflammation (e.g., *Akkermansia muciniphila* and members of the *Lachnospiraceae* family) decreased, while those of pro-inflammatory bacteria (e.g., *Candidatus arthromitus*) and potential pathogens (the *Pasteurellaceae* family) increased in ATG5<sup>-/-</sup> mice.<sup>389</sup> In another study, ATG16L1<sup>T300A</sup> mutant mice grown in GF conditions have been exposed to human stools, and later analysis found that mutant mice harboured a greater number of *Bacteroides* - a family that is found in higher numbers in IBD patients - and Th17 cells compared to WT mice.<sup>390</sup>

However, a reverse correlation has also been postulated by which how pathogens manipulate autophagy to favour their colonisation in the gut. For example, CD-associated AIEC infection in T84 cells and mouse enterocytes suppressed autophagy by upregulating the levels of miR-30C and miR-130A and enhanced their survival. An inverse correlation between the levels of these miRNAs and those of ATG5 and ATG16L1 was also observed in the ileal mucosa of CD patients in support of this finding.<sup>391</sup>

### 3.3. Autophagy as a therapeutic target for IBDs

In light of the above described evidences on the strong genetic links between the autophagy pathway and IBDs, and various functional roles of autophagy in regulating the immune and inflammatory responses associated with IBD pathogenesis, it becomes increasingly clear that modulation of autophagy has potential benefits in the treatment of IBDs. Consequently, investigations are ongoing to evaluate the therapeutic efficacy of autophagy modulation in IBD treatments.

Interestingly, some drugs currently in use for the treatment of IBDs affect the autophagy pathway indirectly and might be inducing potentially beneficial effects through this

method of action.<sup>392</sup> For example, thiopurine (see **Table 4**) treatment, in addition to its immunosuppressive effects, induce autophagy as a downstream pathway and probably serves as a mechanism to reverse adverse effects of the drug such as hepatotoxicity. In this scenario, autophagy is probably a compensatory response to protect the liver against the deleterious effects of thiopurines.<sup>393</sup> In another study, the bacterial conversion of thioguanine pro-drug to active metabolite was shown to increase autophagy in epithelial cells, resulting in increased intracellular bacterial killing and decreased intestinal inflammation and immune activation in colitis models.<sup>394</sup>

Many direct autophagy modulators, which are already in use for the treatment of human diseases are also tested for their efficacy in IBD patients (**Table 8**) or in colitis models (**Table 10**). A previous study has shown that rapamycin/sirolimus, a macrocyclic triene antibiotic which binds to the cytosolic 12-kDa tacrolimus-binding protein (FKBP12) and also inhibits the mTOR pathway, could represent a good candidate to treat CD patients.<sup>395</sup> In a retrospective analysis of patients treated with rapamycin, five out of eleven UC patients and all three CD patients achieved clinical remission. An additional two UC patients achieved clinical response. The remaining four UC patients did not respond to rapamycin treatment. Mucosal healing was achieved in five of eleven UC patients and two of three CD patients. Clinical response to treatment occurred at least 2 weeks after treatment was started. The only significant AE reported was minor gastrointestinal distress.<sup>395</sup> A recent pilot study also demonstrated the effectiveness of rapamycin in CD-related strictures in the upper gastrointestinal tract. The common AE reported in this study was mouth ulcers in 40% of the patients.<sup>396</sup> These reports confirm some data generated in TNBS-treated mice, that intestinal inflammation and colitis are ameliorated by rapamycin and trehalose.<sup>397</sup> In another experiment, an mTOR inhibitor molecule, namely a haloacyl aminopyridine-based molecule called P2281, was shown to be efficient in a murine model of DSS colitis by inhibiting T cell function.<sup>398</sup> Several independent studies have shown that metformin, a synthetic derivative of guanidine that acts as an inducer of autophagy ameliorates colitis. In an experimental model, administration of metformin reduced inflammation through the inhibition of phospho (p)-STAT3, IL-17, and p-mTOR expression and the increased expression of p-AMPK and Foxp3.<sup>399</sup> It has also been demonstrated that metformin limits DSS-induced intestinal barrier disruption by a mechanism involving the inhibition of c-Jun N-terminal kinase activation *via* an AMPK $\alpha$ 1-dependent signaling pathway.<sup>400</sup>

However, most of these classical molecules are known to act on multiple targets and therefore present several AEs and undesirable off-target effects (see **Table 8**). Many efforts have been made in recent years to identify more selective drug targets and to design molecules that are more specific with minimum AEs. One of the examples of this type of molecule developed for IBD treatment is a peptide known as LR12, which inhibits triggering receptor expressed on myeloid cells-1 (TREM-1). Pharmacological inhibition of TREM-1 using LR12 peptide significantly ameliorates colitis in DSS model and restores impaired autophagy in the colon of these mice.<sup>401</sup>

**Table 10: Examples of autophagy modulators demonstrated to work in experimental models of colitis**

<b>Autophagy modulators</b>	<b>Experimental model of Colitis</b>	<b>Mechanism of action</b>	<b>References</b>
P2281	DSS	mTOR inhibition	398
Rapamycin	TNBS, LPS-induced colitis	mTOR inhibition	397,402
AZD8055	LPS-induced colitis	mTOR inhibition	402
Trehalose	TNBS	Unknown	397
Metformin	DSS	AMPK activation	399,400
Evodiamine	DSS	Unknown	403
Celastrol	IL-10 <sup>-/-</sup>	Several targets including AMPK	404
LR12 peptide	DSS	TREM-1 inhibition	401

### 3.4. How to move forward?

Besides the pathophysiological interest of autophagy, the aforementioned results present potential pharmacological evidence that targeting autophagy using small molecules is sufficiently robust for future treatment options for IBD. Current autophagy regulators lack precise selectivity on their targets and most of them present AEs, which can potentially limit their usage as safe therapeutic drugs. Intense research is therefore devoted to the identification of small molecules and peptides to precisely up- or downregulate specific autophagy processes that are pathologically defective without interfering with other autophagy processes.

## SCIENTIFIC RATIONALE OF THE STUDY

As described in the introduction, IBDs are a major health problem with a continuously increasing incidence all over the world. Extensive research is ongoing in several directions to help decrease the disease burden on the affected individuals and improve their QOL. Although an array of biologics and small molecules have been developed so far, their long-term usage is limited by AEs and many patients develop drug refractoriness.

Our team has previously described a therapeutic peptide called P140 that selectively targets autophagy processes. This 21-mer linear synthetic phosphopeptide corresponding to the sequence H-RIHMOVYSKRSGKPRGYAFIEY-OH was first published in 2003.<sup>405</sup> The sequence is originally derived from the 131-151 amino acid sequence of the U1-70K spliceosomal protein, and it contains a phosphoserine residue in position 140, which is essential for its stability and activity. It was identified in a cellular screening assay using overlapping peptide fragments spanning the U1-70K protein and purified CD4<sup>+</sup> T cells from MRL/lpr lupus-prone mice. The synthetic peptide analogue thus created was found to have impressive therapeutic effects in MRL/lpr mice upon intravenous (i.v.) administration.<sup>405</sup> In phase I and phase II clinical trials, P140/Lupuzor was found to be safe and met its primary efficacy endpoints, confirming pre-clinical data generated in lupus mice.<sup>406</sup> Lupuzor is currently being evaluated in phase-III clinical trials in the US, Europe, and Mauritius. Subsequently, the protective effect of P140 was also demonstrated in other mouse models of chronic inflammatory demyelinating polyneuropathy (CIDP; a neurological disorder affecting the sciatic nerves)<sup>4</sup>, Sjögren's syndrome (SjS; a systemic disorder that affects the body's moisture-producing lacrimal and salivary glands),<sup>3,407</sup> and asthma.<sup>5</sup>

The autophagy modulating property of the P140 peptide was primarily postulated in the MRL/lpr lupus model when it was discovered that the peptide directly binds to the chaperone HSPA8, which is a key player in CMA.<sup>408</sup> Successive studies have demonstrated the inhibitory effect of this peptide on CMA and macroautophagy processes that is hyper-activated in the splenic B cells collected from MRL/lpr mice. Probably as a direct consequence, the MHC II-mediated antigen presentation and downstream pro-inflammatory signaling events such as B cell activation and T cell proliferation are also inhibited.<sup>2,335,409–412</sup> When the studies were extended to other

autoinflammatory disorders, more interesting findings were generated concerning autophagy. For example, in CIDP, CMA was found to be abnormally elevated in sciatic nerves, whereas, macroautophagy was downregulated. Both these defects were corrected in the P140-treated mice.<sup>4</sup> Experiments carried out in SjS mice showed that both CMA and macroautophagy are decreased in the salivary glands, while elevated macroautophagy was observed in the spleen of these mice. All of these defects were returned to basal levels in the mice administered with P140 peptide.<sup>413</sup> The exact target of action of P140 or the mechanism by which autophagy is directly or indirectly modulated in the latter models is unknown. Nevertheless, these observations revealed the fact that the P140 peptide is not a global inhibitor of autophagy, the effects on different autophagy processes are organ/cell type-specific and only the pathological defects are corrected.

P140's mechanism of action *via* autophagy modulation and the strong evidence of autophagy defects in IBD pathogenesis prompted us to postulate the potential therapeutic interest of this peptide in IBD. Hence, the main aim of my Ph.D. project was to evaluate the therapeutic effects of the P140 peptide in relevant animal models of colitis. Furthermore, we have evaluated the effect of P140 treatment on different autophagy pathways in the established models to see if the pathological dysfunctions are repaired.



# RESULTS



# 1. PUBLICATION

## **Targeting the endo-lysosomal autophagy pathway to treat inflammatory bowel diseases**

Sruthi Vijaya Retnakumar, Ramasatyaveni Geesala, Alexis Bretin, Julien Tourneur-Marsille, Eric Ogier-Denis, Thorsten Maretzky, Hang Thi Thu Nguyen and Sylviane Muller

### **1.1. Forward**

Murine models of colitis are the greatest tools used for pre-clinical studies in IBDs. A number of them are developed so far, but no animal model, whether it is chemically induced or genetically engineered, ideally represents all the human IBD pathological features. Animal models are carefully chosen depending on the context of the study and the mechanism explored. For therapeutic studies, a combination of multicenter studies in different animal models would be necessary to extrapolate them into patients.<sup>414</sup> Hence, we have exploited three salient mouse models of colitis having different mechanistic and pathological features to test the efficacy of the therapeutic tool P140 peptide we intend to use for IBD treatment.

DSS-induced colitis is one of the most commonly used chemically induced animal models in IBD studies due to its technical simplicity, rapidity, reproducibility, and controllability. Acute or chronic models can be set up using WT mice having C57BL/6 background, by varying the length and frequency of administration of DSS.<sup>165</sup> An acute DSS model is established by a single cycle of DSS administration, whereas a chronic model is induced by repeated cycles of DSS exposure with recovery phases in between. The replacement of DSS with normal drinking water allows full recovery of the mice from inflammation. So, this model is ideally suited for studying both the induction of inflammation as well as the intestinal healing process.<sup>415</sup> Acute models have the advantage of producing fast and reproducible results with relevant inflammatory features and therefore, are more frequently used for therapeutic studies. However, chronic models are better representative of human IBD pathology. We have first started our experiments in the acute DSS model in order to see any possible effects of the peptide. Three different protocols were tested in this model applying the peptide in different treatment regimens. In the initial experiment, we have followed a combination

of preventive and therapeutic schemes of administration of the peptide where the peptide is injected both before and after the induction of the disease by DSS, to maximise the efficacy of the peptide. Since in the real case scenario, our aim is to treat the disease, later, we have set up a model strictly following a therapeutic scheme of administration of the peptide where the peptide is injected only after the onset of the disease. In the third protocol, the mice were allowed to recover from the disease after 7 days of DSS administration. The peptide P140 was given only during this period of recovery.

The TNBS colitis model, on the other hand, is technically more challenging. The susceptibility to TNBS colitis varies between different strains of mice. The SJL/J mice (a strain widely used in MS research) are highly susceptible, BALB/c mice are susceptible, while C57BL/6 mice are more resistant to TNBS induction. However, most of the studies in TNBS colitis models exploited the common BALB/c and C57BL/6 strains.<sup>416</sup> Acute, established, or chronic protocols can be set up by varying the number of TNBS administrations. In majority of the studies, a single dose of TNBS administration is carried out, which results in an acute local inflammatory reaction characterised by the production of Th1 cytokines and CD4<sup>+</sup> T cell infiltration. This corresponds to the priming phase of the induction of a non-specific Th1 immune response. In an established TNBS model, the animal is pre-sensitised to TNBS at another site such as skin, a few days before the intrarectal administration which results in delayed-type hypersensitivity (DTH) response. In the initial sensitization phase of the DTH, Th1 cells are activated and clonally expanded by the exposure to foreign antigens in complex with MHC II molecules on the surface of antigen-presenting cells. The subsequent exposure of the sensitised Th1 cells to antigens results in a specific Th1 response perpetuating the inflammation. The chronic TNBS colitis is induced by repeated induction of DTH responses leading to intestinal fibrosis and Th1/Th17 cytokine profiles resembling human CD.<sup>416</sup> In this study, we have set up two acute protocols of TNBS colitis with the BALB/c or C57BL/6 strains to find the optimal disease induction parameters in which the therapeutic effects of the peptide can be observed.

The third model implemented in this study was a genetically engineered mice model of chronic colitis with a double mutation in *il10* and *iRhom2* genes.<sup>7</sup> *iRhom2* is identified as a crucial regulator of ADAM17, a metalloprotease causing the cleavage and secretion

of TNF- $\alpha$  in myeloid cells (described in section 1.6.5.1). A preliminary experiment was carried out in this model with P140 peptide administration starting with the onset of the disease.

The therapeutic effects of the peptide treatment were analysed with established parameters classically used to assess colitis symptoms. For experiments in which the preliminary outcomes are positive, further studies were carried out at the molecular level to see the effect of the peptide in the production of relevant pro-inflammatory mediators. We have also carefully looked at different autophagy processes (macroautophagy and CMA) in these models on the target organ (colon) as well as other lymphoid organs (spleen). With respect to the complexity and dynamic nature of autophagy pathways, different biochemical methods (gene and protein expression analysis) have been employed using validated markers to draw meaningful conclusions. In this manuscript, we describe the experimental designs and methods implemented to achieve our objectives and the results obtained from each of them in detail.

## **1.2. Targeting the endo-lysosomal autophagy pathway to treat inflammatory bowel diseases**



# Targeting the endo-lysosomal autophagy pathway to treat inflammatory bowel diseases

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## ABSTRACT

Inflammatory bowel disease (IBD) is a serious public health problem in Western society with a continuing increase in incidence worldwide. Safe, targeted medicines for IBD are not yet available. Autophagy, a vital process implicated in normal cell homeostasis, provides a potential point of entry for the treatment of IBDs, as several autophagy-related genes are associated with IBD risk. We conducted a series of experiments in three distinct mouse models of colitis to test the effectiveness of therapeutic P140, a phosphopeptide that corrects autophagy dysfunctions in other autoimmune and inflammatory diseases. Colitis was experimentally induced in mice by administering dextran sodium sulfate and 2,4,6 trinitrobenzene sulfonic acid. Transgenic mice lacking both *il-10* and *iRhom2* – involved in tumor necrosis factor  $\alpha$  secretion – were also used. In the three models investigated, P140 treatment attenuated the clinical and histological severity of colitis. Post-treatment, altered expression of several macroautophagy and chaperone-mediated autophagy markers, and of pro-inflammatory mediators was corrected. Our results demonstrate that therapeutic intervention with an autophagy modulator improves colitis in animal models. These findings highlight the potential of therapeutic peptide P140 for use in the treatment of IBD.

## 1. Introduction

IBD is a public health challenge with a high incidence in Western countries, but is also increasing sharply in newly-industrialized countries. Crohn's disease (CD) and ulcerative colitis (UC), the main forms of inflammatory bowel disease (IBD), were the first chronic disorders in which autophagy dysfunctions were suggested to play a potentially major etiopathogenic role [1,2]. Population-based studies provide

compelling evidence that genetic factors contribute to the pathogenesis of IBD, and many IBD risk loci have been identified. However, IBD has multifactorial triggers, including genetic, microbial, and environmental factors, causing dysregulation of the innate and adaptive immune system in the intestine [3–6].

IBD has high recurrence, and low cure rates [7], and we currently lack effective treatment options, primarily due to either limited efficacy or unsustainable side effects [8–10]. Today, therapies are largely limited

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to treatment of symptoms with the aim of improving the patient's quality of life. However, even with this limited scope, the effectiveness of treatment varies dramatically between patients [11–13]. More ambitious therapies, including cytokine blockers, such as therapeutic monoclonal antibodies (mAb) adalimumab and infliximab, directed against tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), or ustekinumab, targeting interleukin (IL)-12 and IL-23, were recently tested in patients with severe, active CD [14]. Therapeutic mAbs targeting integrins (e.g., vedolizumab, natalizumab) have also been tested. All these treatments produce heterogeneous patient responses [11], added to which, their cost and high potential for serious toxicity limit their long-term clinical use. Strategies based on small molecules, such as molecules in the Janus kinase (JAK) pathway, have also been explored, and numerous compounds, including herbal extracts are under clinical evaluation, alone or in combination [15,16]. Many have only shown limited effectiveness to date [9,10,17,18].

P140 is a 21-mer phosphopeptide derived from the cognate sequence 131–151 of the U1-70K spliceosomal protein [19]. It contains a phosphoserine residue at position 140 that is inserted during synthesis. Using lysosomes purified from the liver of untreated or P140-treated MRL/lpr lupus-prone mice, we previously showed that P140 regulates chaperone-mediated autophagy (CMA) – a process that contributes to degradation of intracellular proteins in lysosomes – at the lysosomal substrate uptake step [20]. P140 downregulates hyperactive autophagy processes and (probably as a direct consequence) decreases expression of major histocompatibility complex-II molecules, which is relevant in its action on lupus [20–22]. P140 has since been shown to be effective in murine models of primary and secondary Sjögren's syndrome [23,24], chronic inflammatory demyelinating polyneuropathy [25], and chronic house dust mite-induced airway inflammation [26].

The potential of P140 in the IBD context is based on its targeting of autophagy. Indeed, in addition to barrier functions and immune responses, numerous risk loci for IBD are situated in regions containing genes encoding proteins involved in autophagy [5,27–30]. Specifically, polymorphisms in autophagy-related (Atg) genes, such as *ATG16L1*, sequestosome (*SQSTM1*)/p62, serine/threonine-protein kinase *ULK1*, immunity-related GTPase M (*IRGM*), nucleotide-binding oligomerization domain 2 (*NOD2*)/*CARD15* are associated with an increased risk of developing CD [1,31–36] or IBD [8,37]. Functional studies [5] have emphasized the pivotal role played by dysfunctional autophagy in intestinal homeostasis functions, leading to pathogenic hallmarks of IBD. Autophagy was found to be indispensable in the maintenance of intestinal epithelial barrier integrity by protecting intestinal epithelial cells against TNF-induced apoptosis [38,39] or via the degradation of tight junction barrier proteins such as Claudin-2 [40]. Paneth and goblet cells are specialised cell types of the intestinal epithelium involved in the secretion of antimicrobial peptides and mucins, respectively, to protect against pathogenic microbes; autophagy deficiency in these cells severely impacts their secretory functions [41,42]. In addition, the loss of autophagy in immune cells has been associated with elevated inflammasome activation [43] as well as impaired antigen presentation responses against pathogens [44] and therefore an increased susceptibility to colitis. Various studies have also demonstrated that autophagy dysfunctions lead to altered gut microbial composition or gut dysbiosis [45,46]. We hypothesized that P140 could correct these defects, reducing the extent of molecular and cellular inflammation, and could delay the development of the disease.

We, therefore, evaluated the effectiveness of P140 in three distinct but complementary murine models of colitis – two chemically-induced models, and one that spontaneously develops chronic intestinal inflammation with UC-like features due to a double mutation in *il-10* and *rhomboid 2* (*iRhomb2*) genes [47]. Analysis of these three independent models revealed that treatment with P140 attenuates inflammation and disease at the clinical and histological levels. Expression levels for several pro-inflammatory mediators were significantly diminished in colonic tissues. Mechanistically, we found that P140 corrected

autophagy defects in the target tissues (colon) and spleens from mice with colitis.

## 2. Methods

### 2.1. Peptides

P140 (RIHMYVSKRpSGKPRGYAFIEY) and Sc140 (YVSRYFG-pSAIRHEPKMKIYR) phosphopeptide (where pS represents phosphoserine residues) were synthesized using classical N-[9-fluorenyl] methoxycarbonyl solid-phase chemistry, and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) [19,48]. Peptide homogeneity was checked by analytical HPLC, and their identity was assessed by liquid chromatography-mass spectrometry (LC-MS) on a Finnigan LCQ Advantage Max system (Thermo Fischer Scientific, Courtaboeuf, France).

### 2.2. Mouse models of colitis

Three independent experiments (A, B, C) were performed in the dextran sodium sulphate (DSS) model with distinct protocols (Fig. 1). Following these experiments, mice were sacrificed by exsanguination through direct cardiac puncture under isoflurane anesthesia. All mice were housed under specific pathogen-free conditions in the respective animal care facilities. Mice were weighed daily, and stool consistency, diarrhea, or blood in the stools were monitored to calculate the disease activity index (DAI).

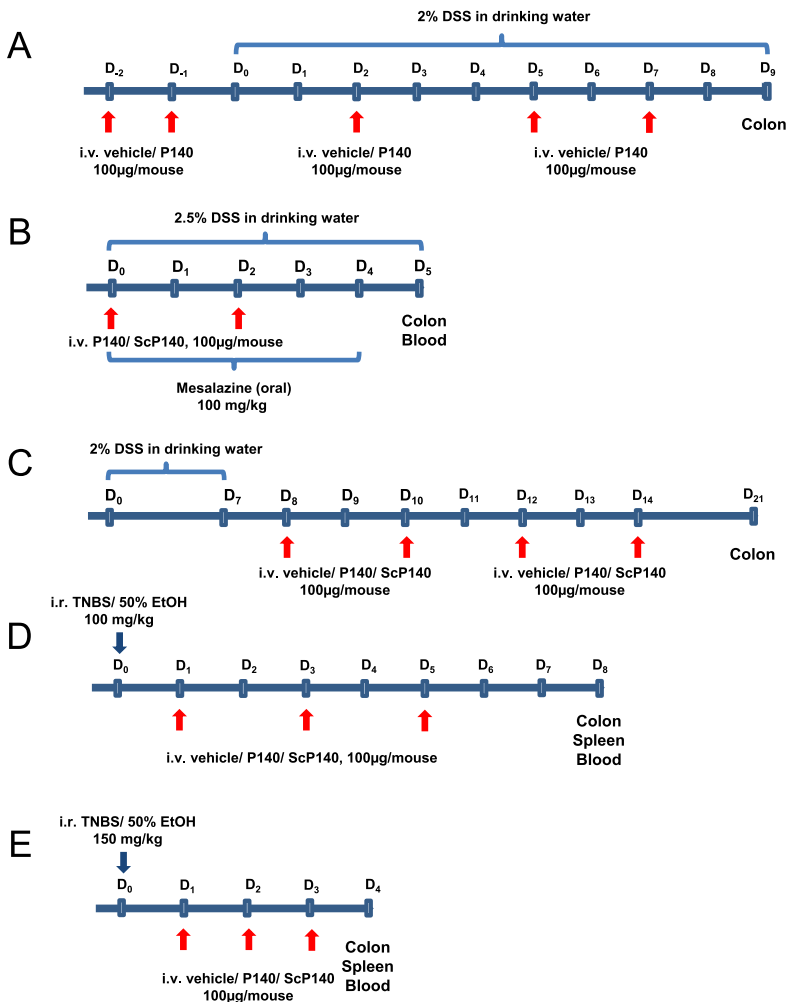
In Exp. A, 7-week-old C57BL/6 male mice (Charles River Laboratories, France) were induced with DSS from day 0 (2.0% w/v; MP Biomedicals 160110 added to the animals' drinking water). They were treated with either P140 (4 mg/kg mouse body-weight, intravenously (i.v.)) or vehicle (NaCl) on days –2 and –1, and then at days +2, +5 and +7 (preventive and curative experimental design). The control peptide ScP140 was not administered in this experiment. Mice were sacrificed on day +9.

In Exp. B, 8-week-old C57BL/6 female mice (Japan SLC, Japan) received 2.5% w/v DSS (MP Biomedicals) for 5 days [49]. They were treated with P140 and ScP140 peptides (i.v.) on days 0 and +2, and sacrificed on day +5. A group of DSS-induced mice was also treated orally with 100 mg/kg mesalazine (Kobayashi Kako) once daily for 4 days (day 0 to day +4). Total volume of mesalazine: 10 mL/kg in 0.5% w/v carboxymethyl cellulose [50].

In Exp. C, 6-week-old C57BL/6 male mice (Janvier Laboratories, France) received 2.0% w/v DSS (MP Biomedicals) for 7 days (replaced by sterile water on day +7). Peptides were administered i.v. in curative mode, every two days from day +7 to day +14, and mice were sacrificed on day +21.

Two distinct protocols (D, E) were evaluated with the 2,4,6-trinitrobenzene sulfonic acid (TNBS) model (Fig. 1). In Exp. D, 8-week-old BALB/c female mice (Janvier Laboratories) maintained in standard cages and fed with classic standard chow and tap water *ad libitum*, received TNBS (Sigma-Aldrich, 2508-19-2; 100 mg/kg mouse body-weight) intrarectally (i.r.) on day 0 (instillation of 50  $\mu$ L 50% v/v ethanol under anesthesia with 3% isoflurane *via* nose cone). P140, ScP140, or vehicle were administered i.v. on days +1, +3 and +5. Control mice received an intrarectal instillation of 50  $\mu$ L 50% ethanol. As above, clinical parameters were monitored daily. Mice were sacrificed on day +8 by isoflurane overdose followed by cervical dislocation.

Exp. E included C57BL/6 mice – less susceptible to TNBS than BALB/c mice [51]. Eight-week-old male mice (Charles River laboratories, France) were anesthetized with a subcutaneous injection of xylazine/ketamine, and TNBS (Sigma-Aldrich, 92822) dissolved in 50% ethanol (40  $\mu$ L) was administered intrarectally at 150 mg/kg mouse body-weight. Control mice received an intrarectal instillation of 40  $\mu$ L 50% ethanol. Mice were treated i.v. with P140, ScP140, or vehicle on days +1, +2 and +3 (4 mg peptide/kg body-weight). Animals were



Group No.	No. of mice	Inducer	Therapy
1	9	DSS	Vehicle
2	9	DSS	P140

Group No.	No. of mice	Inducer	Therapy
1	8	DSS	P140
2	8	DSS	ScP140
3	8	DSS	Mesalazine

Group No.	No. of mice	Inducer	Therapy
1	10	DSS	Vehicle
2	10	DSS	P140
3	10	DSS	ScP140

Group No.	No. of mice	Inducer	Therapy
1	20	Ethanol	Vehicle
2	20	TNBS	Vehicle
3	20	TNBS	P140
4	10	TNBS	ScP140

Group No.	No. of mice	Inducer	Therapy
1	5	Ethanol	Vehicle
2	20	TNBS	Vehicle
3	20	TNBS	P140
4	20	TNBS	ScP140

**Fig. 1.** Experimental protocols. Schematic representation of experimental protocols and treatment regimens applied with the DSS-induced (Exp. A, B and C) and TNBS (Exp. D and E) mouse models of colitis. A-E show Exp. A to E, as referenced in the text. ScP140 was not used in Exp. A. Vehicle was NaCl 0.9% w/v. % DSS is expressed as w/v. EtOH, ethanol.

sacrificed on day +4 by cervical dislocation. *il10<sup>-/-</sup>/iRhom2<sup>-/-</sup>* mice [47] were used as a genetic model of IBD in Exp. F. P140 therapy was initiated at 8 weeks of age (onset of the disease in these mice). P140, ScP140 (both at 100 µg peptide/mouse), or vehicle was administered i. v. twice a week for 11 weeks. Mice were sacrificed 11 weeks after initiating treatment. The scores used to evaluate the disease intensity are described in [Supplementary Tables 1 and 2](#). At the time of sacrifice, the gastrointestinal tract was collected and weighed, opened longitudinally, and washed several times according to standard procedures. Colon sections were isolated as shown in [Supplementary Figure 1](#).

2.3. Histological analysis

Colons were immediately fixed in 4% w/v paraformaldehyde and embedded in paraffin. 5-mm-thick tissue sections were stained with hematoxylin and eosin (H&E) and observed under a light microscope. Histological damage was assessed based on the criteria described in [Supplementary Table 3](#).

2.4. Immunofluorescence

Paraffin-embedded colonic tissues were cut into 7-mm-thick sections. Non-specific binding was blocked with phosphate-buffered saline containing 5% w/v bovine serum albumin and 0.2% Tween-20. Primary

antibodies were incubated at 4 °C overnight and the corresponding secondary antibodies at room temperature for 4 h. The following antibodies were used: SQSTM1/p62 (Abcam, ab109012), Alexa Fluor 488 goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody (Invitrogen, A-11008). Nuclei were labeled with Hoechst 33258 (Invitrogen, H1398). Slides were mounted with Fluoromount-G (ThermoFisher Scientific, 00-4958-02). Confocal (Carl Zeiss) images were acquired, and fluorescence intensity was measured using Fiji software.

2.5. Western blotting

Autophagy protein expression was measured in colon or spleen tissue homogenates by western blotting, as described [25,52]. Antibodies were: MAP1LC3B (Novus Biologicals, NB100-2220), SQSTM1/p62 (Abcam, ab109012), BECLIN1/BECN1 (Abcam, ab207612), ATG5 (Cell Signaling Technology, 12994S), lysosomal-associated membrane protein (LAMP)2A (Abcam, ab18528), and HSPA8 (Abcam, ab51052), HSP90 (Abcam, ab203126). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch, 115-035-008 and 111-035-008; 50 ng/mL). Signal was detected using Clarity western ECL Substrate (Bio-Rad, 1705061). Expression levels of autophagy markers were normalized by densitometry relative to the total protein level, using Image Lab (Bio-Rad) software.



## 2.6. MPO activity assay

Granulocyte infiltration into the colon was quantified by measuring myeloperoxidase (MPO) activity. Briefly, selected colon sections (Supplementary Figure 1) were homogenized using a Mixer Mill MM 400 (Retsch) and resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich, 57-09-0; 50 mg/mL). The homogenate was sonicated for 10 s and centrifuged at  $13,400 \times g$  for 6 min. An aliquot (25  $\mu$ L) of supernatant was used for the assay following appropriate dilution. The final reaction was visualized by adding  $H_2O_2$  as peroxidase substrate and 3,3',5,5'-tetramethylbenzidine as chromogen, and incubating for 30 min at 37 °C. The reaction was blocked by the addition of 1 M HCl, before measuring absorbance at 450 nm using a plate reader (Thermo Scientific Multiskan GO). Results were expressed as absorbance per mg of tissue.

## 2.7. qRT-PCR

Total RNA was extracted using the NucleoSpin RNA isolation kit (Macherey Nagel, 740955.250) from a portion of colon (Supplementary Figure 1) following homogenization in a Mixer Mill MM 400 (Retsch). RNA was reverse-transcribed using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, 172-5035). qRT-PCR was performed using Sso Advanced Universal SYBR Green Supermix (Bio-Rad, 172-5274) with the CFX C1000 Touch™ Real-Time PCR detection system (Bio-Rad, 1855195). Primers used are listed in Supplementary Table 4. A GeNorm study was performed using Bio-Rad's CFX Maestro 1.1 software with ten housekeeping genes (*Actb*, *B2m*, *G6pd*, *Gusb*, *Hprt1*, *Rpl13a*, *Rps18*, *Taf8*, *Tfrc*, *Ywhaz*), to determine which were the most stable in our conditions. The three most stable, or acidic ribosomal phosphoprotein P0 (*36b4*) in some experiments, were used for data normalization. Data were analyzed by the  $\Delta\Delta C_T$  method, as follows:  $\Delta\Delta C_T = (C_{T, target} - C_{T, reference})_{test} - (C_{T, target} - C_{T, reference})_{calibrator}$ , and the final data were derived from  $2^{-\Delta\Delta C_T}$ .

## 2.8. Ex-vivo colon culture and ELISA-based cytokine quantification

Segments of the distal colon (0.5 cm-long; Supplementary Figure 1) were dissected and washed in PBS containing penicillin and streptomycin. Segments were then placed in 24-well flat-bottomed culture plates containing 500  $\mu$ L complete RPMI 1640 medium and incubated at 37 °C under 5%  $CO_2$  for 24 h. Culture supernatants were harvested and assayed for keratinocyte-derived chemokines [53] using a commercial ELISA kit (KC/CXCL1; Mouse CXCL1/KC DuoSet ELISA, DY453, R&D Systems). Serum samples prepared from blood samples collected at the time of sacrifice were also assayed for cytokines/chemokines – IL-6 (DY406-05), IFN- $\gamma$  (DY485-05), IL-12 p70 (DY419-05), TNF- $\alpha$  (DY410-05), and IL-1 $\beta$  (DY401-05) – using commercial kits (R&D systems) according to the supplier's protocols. Standard curves were generated by titration with recombinant protein calibrators provided in commercial kits.

## 2.9. Statistical analysis

All values are expressed as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Prism (Version 8.0). Statistics are described in the corresponding figure legends. *P* values  $\leq$  0.05 were considered statistically significant.

## 2.10. Ethics statement

Experiment protocols involving animals were approved by the local Institutional Animal Care and Use Committee and the French ministry for higher education, research and innovation (APAFIS #20654-2019061116549343; APAFIS #2171-2019092716434837; APAFIS #26681-2020072115122312). In line with these agreements, and

taking into account the best European practices in the field (3-R rules), we took the necessary measures to avoid pain and minimize distress and pointless suffering of mice during experiments and at the time of sacrifice. Animals were maintained under controlled environmental conditions ( $20 \pm 2$  °C) in either specific pathogen-free or conventional husbandry conditions (as specified above). A 12 h/12 h light-dark cycle (lighting 7:00 a.m.-7:00 p.m.) was maintained. Mice were housed in large polycarbonate cages, with 8–10 mice per cage on bedding made from spruce wood chips (Safe) and enriched with play tunnels which were changed weekly. Mice monitored in Japan (Exp. B) were housed and cared for in accordance with the Japanese Pharmacological Society Guidelines for Animal use. Animals (a maximum of 4 per cage; TPX cages, CLEA Japan) were maintained under controlled environmental conditions of temperature ( $23 \pm 3$  °C), humidity ( $50 \pm 20\%$ ), and lighting (8:00 a.m.-8:00 p.m.). Experiments with *il10*<sup>-/-</sup>/*iRhom2*<sup>-/-</sup> mice (Exp. F) were conducted according to the institutional regulations for animal care and use of the University of Iowa.

## 3. Results

### 3.1. The autophagy modulator P140 ameliorates disease progression in three murine models of colitis

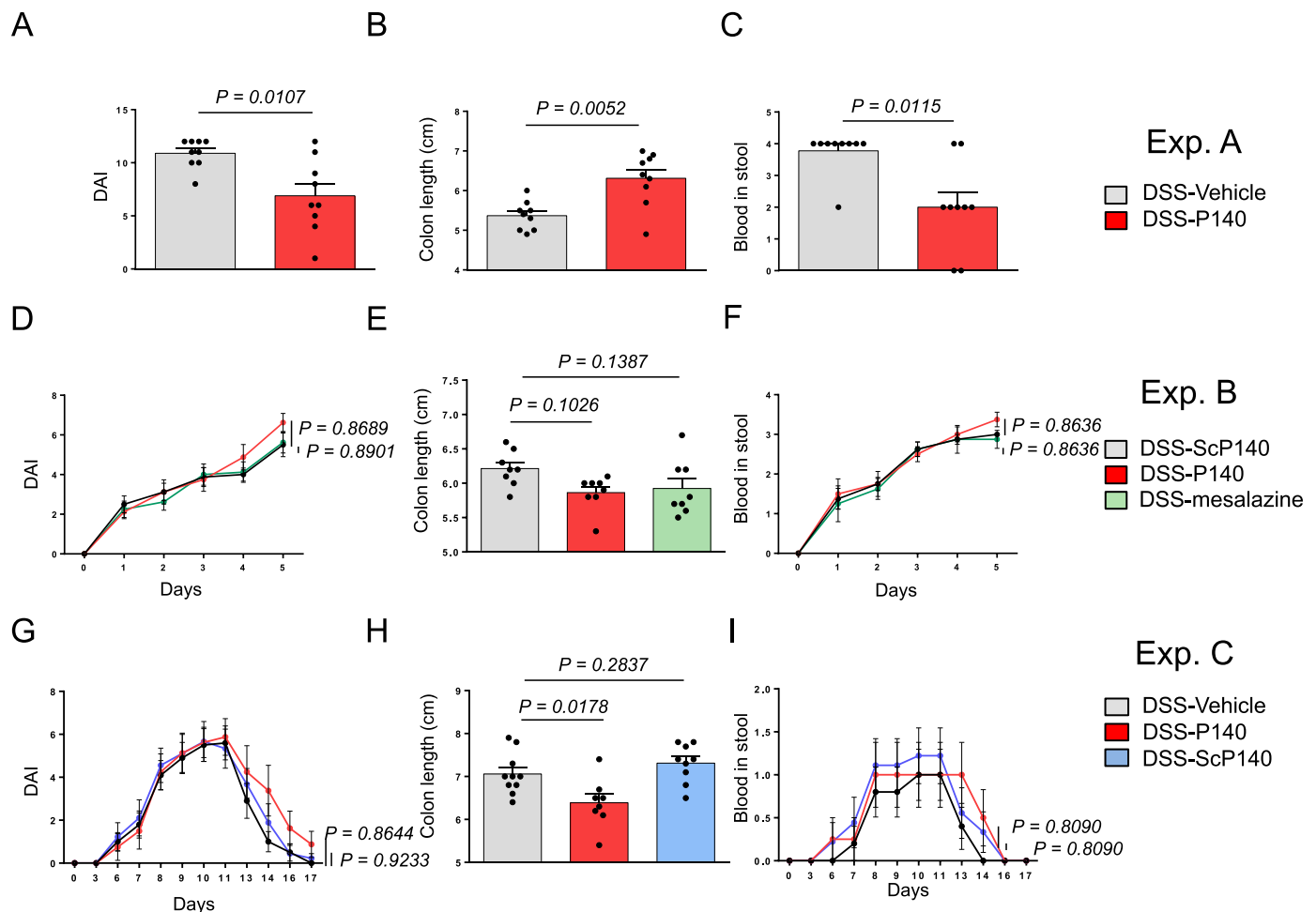
We first investigated the effect of P140 in a mouse model of DSS-induced colitis. In the three DSS protocols tested (Fig. 1; Exp. A, B, C), mice developed colitis, as indicated by the clinical parameters measured (Fig. 2; Supplementary Figures 2 and 3). No effect was observed in Exp. B (mesalazine that is commonly used to treat patients with IBD, was also inactive) or Exp. C (Fig. 2 and Supplementary Figures 2 and 3). However, beneficial clinical effects of P140 were observed in Exp. A, where P140 was administered according to both preventive and curative protocols, P140-treated mice had longer colons, less blood in stools, and decreased DAI scores (Fig. 2 A-C).

To further examine the curative potential of P140, we used another chemically-induced colitis model, the widely used TNBS mouse model. TNBS-induced mice developed colitis in the two protocols evaluated (Exp. D, E) (Fig. 3; Supplementary Figure 4). In Exp. D, P140 did not significantly improve any of the clinical and biochemical parameters assessed (Supplementary Figure 4). In Exp. E, although not significant, a trend for decreased mortality (with unexplained weight loss; Fig. 3A) was observed in the group of mice treated with P140 (13% mortality versus 39% of TNBS-induced mice treated with vehicle, and 40% of TNBS-induced mice treated with a scrambled (Sc) analogue of the P140 sequence; Fig. 3B). This effect was associated with improvement of colonic lesions at macroscopic and histological levels. Thus, P140 decreased the extent of inflammatory lesions by 35% according to the Wallace score, compared to only 15% in mice that received ScP140 (Fig. 3C). The Ameho score (grading on a scale from 0 to 6) – which considers the degree of the inflammatory infiltrate, the presence of erosion, ulceration or necrosis, and the depth and surface extension of the lesions – was decreased by 41% in the P140 group ( $3.13 \pm 0.63$  versus  $5.28 \pm 0.41$  in vehicle-treated TNBS-induced mice; Fig. 3D). Surprisingly, in this experiment, ScP140 also reduced inflammation in TNBS-induced mice, although less efficiently (Ameho scores =  $3.67 \pm 0.78$ , corresponding to a 31% improvement of inflammatory lesions).

As part of our analysis of inflammation, we also evaluated MPO activity. MPO is one of the best diagnostic biomarkers of inflammation and oxidative stress. This ancestor of cyclooxygenase helps to defend gut-associated lymphoid tissue against harmful enteric microbes, while tolerating harmless commensal bacteria and dietary antigens. MPO activity correlates with the severity of experimentally-induced colitis [54]. Activity of this enzyme in mice with TNBS-induced colitis was significantly reduced following P140 treatment compared to vehicle- or ScP140-treatment (Fig. 3E).

In the experiments described above, no sign of toxicity of P140 peptide was noticed, even under inflammatory conditions. Changes in





**Fig. 2.** Therapeutic effects of P140 on DSS-induced colitis. Animals were treated with P140, ScP140, or vehicle alone (Exp. A, B, C). The clinical parameters shown in the figure are DAI (A, D, G), colon length (B, E, H; post-mortem measurement) and the presence of blood in stools (C, F, I). In Exp. A histopathological score could not be defined. Histology is shown for Exp. B (Supplementary Fig. 2). Data are mean  $\pm$  SEM.  $P$  values were calculated using a Mann-Whitney  $U$  test (A, B, C), one-way (H), or two-way ANOVA (D, F, G, I) followed by Holm-Sidak's multiple comparisons; or a Kruskal-Wallis test followed by Dunn's multiple comparisons (E). Vehicle, NaCl 0.9% w/v; ns, non-significant.

body-weight can be used as an indirect marker of colonic lesions in mice with chronic colitis [55]. Although we studied acute colitis, this parameter was used to demonstrate homogeneity of groups before its induction, and to detect possible toxic effects of the treatments tested. Therefore, animals were weighed before inducing colitis and daily until sacrifice. No change in body weight with regard to the control groups was observed following treatment with P140 (4 mg/kg mouse body-weight for each injection in Exp. A-E) (see Fig. 3B; Supplementary Figures 2A, 3A, 3C, 4B).

In addition to the chemically-induced models, we also evaluated the effect of P140 in a genetic model of spontaneous IBD (Exp. F; Fig. 4). Mice lacking both *il10* and *iRhom2* (*il10*<sup>-/-</sup>*iRhom2*<sup>-/-</sup>) develop early intestinal inflammation followed by accelerated weight loss within 8–12 weeks of birth [47]. Using this novel mouse model, we extended our findings from the chemically-induced colitis models. At 6–8 weeks of age (onset of the disease), mice received either P140 or ScP140 twice per week for 10 weeks (Fig. 4A). Mortality was significantly decreased in the group of mice treated with P140, as compared to the ScP140 group and the group of *il10*<sup>-/-</sup>*iRhom2*<sup>-/-</sup> mice left untreated (Fig. 4B). P140-treated mice also showed improved weight gain (Fig. 4C) and reduced colon inflammation, as reflected by an increase in colon length (Fig. 4D and E). In addition, the size and weight of spleens from P140-treated mice were significantly decreased with regard to the two other groups of mice used as controls (Fig. 4F).

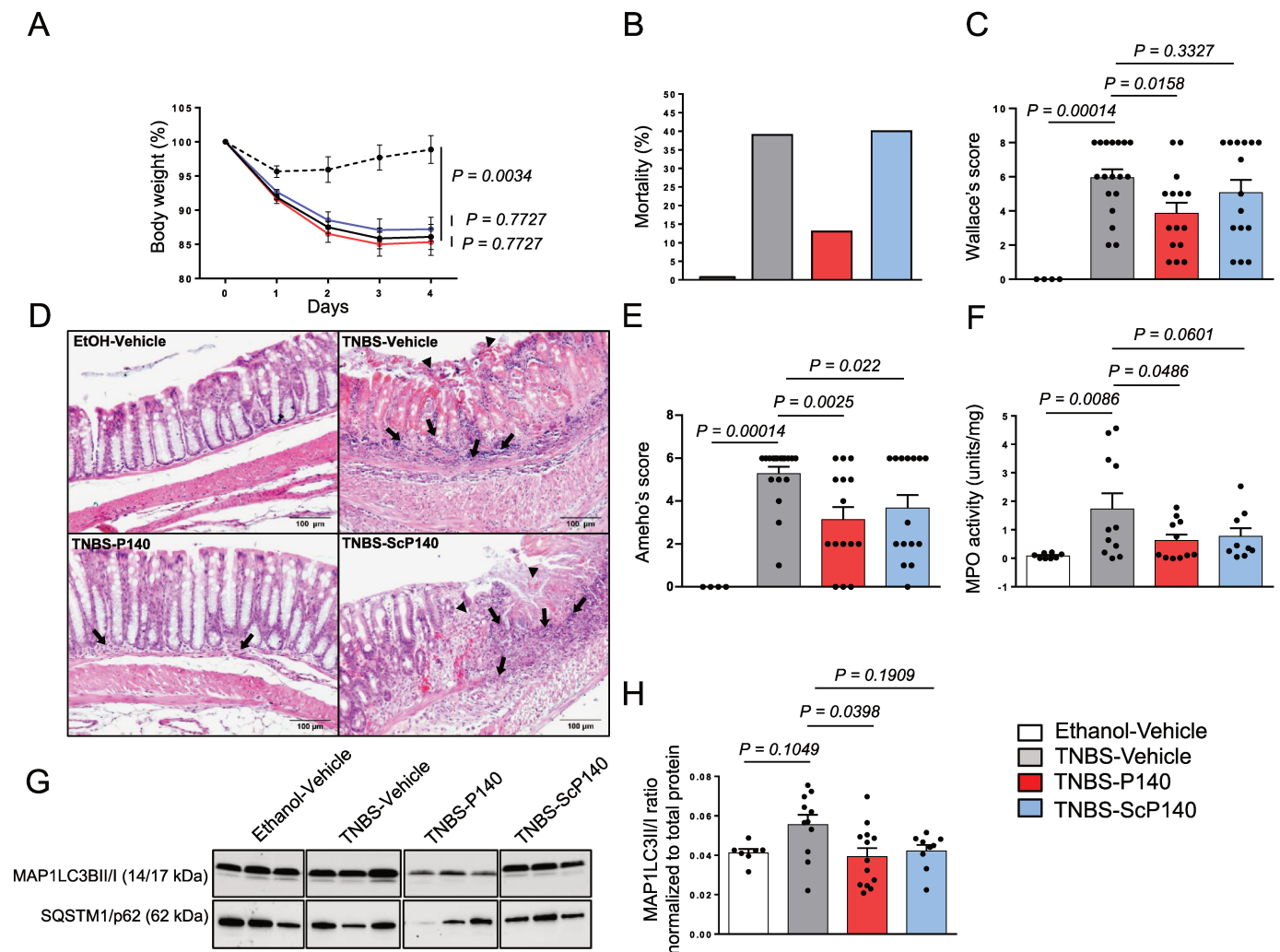
As the onset of colitis is strongly linked to inflammation and defects in autophagy, we next investigated the effect of P140 on these processes.

### 3.2. P140 treatment reduces the production of several pro-inflammatory mediators in colons from DSS-induced mice

Compared to mice with DSS-induced colitis treated with vehicle only, several genes involved in inflammation were significantly down-regulated in colons collected from mice treated with P140 (Fig. 5; Supplementary Figure 5; Exp. A). These genes included *Tnfa*, *Il6*, *Kc/Cxcl1*, *Mcp1/Ccl2* and *Il12a*. The level of secreted KC/CXCL1 protein remained unchanged in colon culture supernatants, as measured by ELISA (Supplementary Figure 5F). No change in *Il1b*, *Ifng*, *Il-17a*, *Gm-csf* and *Mip2* gene expression was detected (Supplementary Fig. 5A-E). Following P140 treatment, no change in gene expression levels was detected in colon tissues from mice with TNBS-induced colitis in Exp. E (Supplementary Figure 6), and circulating cytokine levels were below the sensitivity limit of the commercial ELISA kits used.

### 3.3. Autophagy processes are defective in colons from mice with colitis and are partially corrected by P140 peptide

The mRNA and protein expression levels of a series of markers characteristic of macroautophagy and CMA were evaluated by



**Fig. 3.** Therapeutic effects of P140 on TNBS-induced colitis. Animals were treated with P140, ScP140, or vehicle alone (Exp. E). A. body-weight percentage; B. Percent mortality; C. Wallace's score; D. Representative images of H&E staining. Black arrows indicate inflammatory cell infiltration and arrowheads indicate disrupted epithelium; scale bars 100  $\mu$ m. E. Ameho score; F. MPO activity. G. Representative Western blot images for MAP1LC3B and SQSTM1 in spleen tissue. H. Quantification of protein expression levels normalized relative to total protein. Data are shown as mean  $\pm$  SEM.  $P$  values were calculated using a one-way (F) or 2-way ANOVA (A) followed by Holm-Sidak's multiple comparisons, a permutation test for two independent samples (C, E), or a Kruskal-Wallis test followed by Dunn's multiple comparisons (H). Vehicle, NaCl 0.9% w/v; ns, non-significant. EtOH, ethanol.

quantitative reverse transcription PCR (mRNA) and western blot (protein). In terms of gene expression, no change was observed for several of them – including *Sqstm1*, *Lamp2*, or (at the limit of significance) *Map1lc3b* – in colon cells from mice with DSS-induced colitis. However, a statistically significant decrease was detected for others (*Mtor*, *Ulk1*, *Becn1*, *Atg14*, *Atg5*, *Atg12*, and *Atg16l1*). The altered expression of *Atg14*, *Atg5* and *Map1lc3b* was clearly compensated following treatment with P140 (Supplementary Fig. 7D, 7E, 7H).

At the protein level, in Exp. A, equivalent levels of ATG5-ATG12, MAP1LC3BII/I, BECN1, HSPA8, and HSP90 were measured in DSS-induced and control (healthy) mice. SQSTM1 and LAMP2A expression was increased in DSS-induced mice, but was corrected by P140 treatment (Fig. 5H and I; Supplementary Fig. 8). Results from Exp. B were less conclusive, with similar levels of protein markers ATG5-ATG12 and BECN1, but also SQSTM1, and LAMP2A detected in colon tissues from DSS-induced and healthy mice. In this experiment, expression of MAP1LC3BII/I and HSPA8 decreased upon exposure to DSS, but no compensation was observed following treatment with P140.

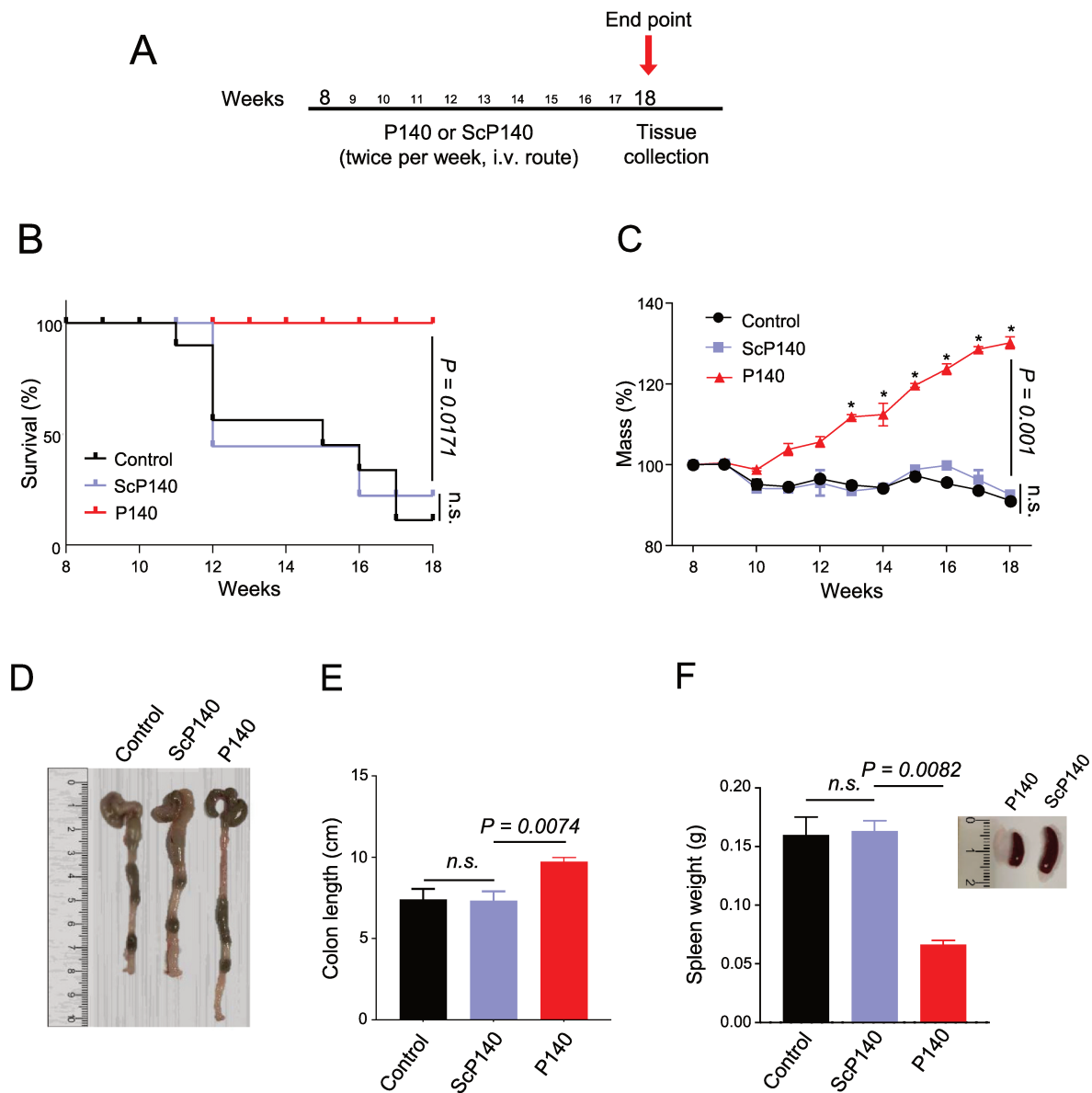
In colon tissues from TNBS-induced mice (Exp. E), no change in ATG5/12, BECN1, MAP1LC3B, SQSTM1, HSPA8, HSP90, or LAMP2A protein expression was detected. We also had access to spleens from

these animals. Once again, expression levels for ATG5/12, SQSTM1, HSPA8, and LAMP2A were equivalent in TNBS-induced and healthy mice. However, a trend for an increasing ratio between cytosolic MAP1LC3B-I and autophagosome membrane-bound MAP1LC3B-II (MAP1LC3B puncta) – indicating active autophagy – was observed in mice with colitis (Fig. 3F and G), and corrected upon treatment with P140, with a return to the basal ratio (Fig. 3G). No significant effect was observed with the control peptide ScP140.

#### 4. Discussion

The aim of this study was to determine whether the phosphopeptide P140, which has been shown to influence autophagy linked to several human diseases, could have a positive impact on IBD.

Using two chemically-induced mouse models of colitis and a genetic model that spontaneously develops early intestinal inflammation, we demonstrated for the first time the protective potential of P140 in an intestinal inflammatory disorder. In the three murine models evaluated, P140 treatment attenuated the clinical and histological severity of colitis and, in the DSS-induced model, expression levels for several pro-inflammatory mediators were decreased. From a mechanistic point of



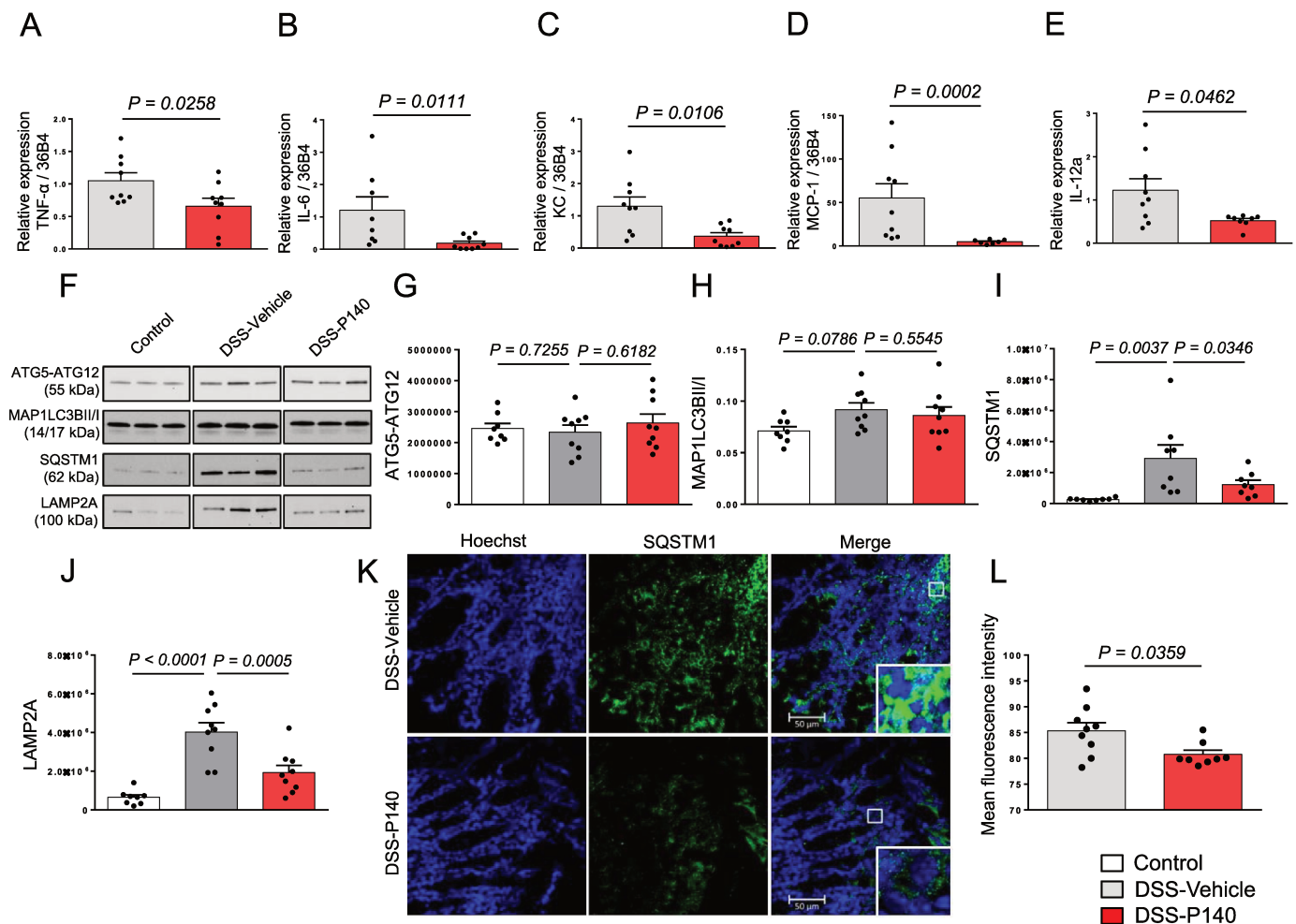
**Fig. 4.** Therapeutic effects of P140 treatment in *il10*<sup>-/-</sup>*iRhom2*<sup>-/-</sup> mice. Mice were left untreated or treated with either P140 or ScP140 for 11 weeks (twice per week); A. Experimental design; B. Survival (n = 4–8 per group); C. Body-weight measured over the course of treatment; D. Representative photomicrographs of colons from untreated and P140- and ScP140-treated *il10*<sup>-/-</sup>*iRhom2*<sup>-/-</sup> mice; E. Colon length. F. Spleen weight and representative images of spleens from P140 and ScP140-treated *il10*<sup>-/-</sup>*iRhom2*<sup>-/-</sup> mice. P values were calculated using log-rank (Mantel-Cox) test (B), Mann-whitney test (C) and two tailed unpaired Student's t-test (E, F).

view, our results confirmed that – as in MRL/lpr lupus-prone mice, where the mode of action of P140 was initially identified [20–22] – P140 affected macroautophagy and CMA in colon cells and splenocytes from mice with colitis, as indicated by decreased expression of biomarkers.

The chemically-induced models of colitis selected for this investigation are commonly used to assess the efficacy of candidate pharmacological treatments. DSS is a chemical colitogen with anticoagulant properties. It causes epithelial cell death, leading to compromised intestinal barrier function and inflammation [50,55,56]. TNBS promotes transmural colitis with severe diarrhea, weight loss, and rectal prolapse, as found in patients with CD [57,58]. The genetic model of colitis was important in this investigation due to its clinical relevance, particularly as it allows long-term study. IL-10 plays a key role in both innate and adaptive immunity, through its regulation of inflammatory responses in the intestine. A small proportion (5%) of mice lacking IL-10 spontaneously develop colitis at 2–8 months of age. Disease onset can be accelerated by exposing mice to piroxicam, a nonsteroidal anti-inflammatory

drug in the oxicam class. Histologically, colitis in *il10*<sup>-/-</sup> mice is similar to human IBD [59]. *iRhom2* is a crucial regulator of TNF- $\alpha$  secretion in myeloid cells. Recently, we reported that mice lacking both *iRhom2* and *IL10* developed severe colitis [47] at 8 weeks of age, with significantly increased colonic inflammation, gut dysbiosis and permeability.

The use of several distinct experimental mouse models strengthens the results presented here. Indeed, IBD is particularly difficult to study as models can be inconsistent, potentially presenting a high degree of variability from one experiment to another. This variability is linked to several parameters, e.g., the method used to induce colitis, doses administered and duration (acute or chronic model), the sex and strain of mice used, the conditions in which animals are housed. In addition, due to the severity of the disease and the associated pain, it is often necessary to limit the period over which animals are observed [50,57]. In addition to the different models, various induction designs (strain and sex of experimental animals, concentration of inducer, duration of the experiment until sacrifice) and treatment protocols (number of



**Fig. 5.** Effect of P140 on pro-inflammatory mediator production and selected autophagy markers in the colon from DSS-treated mice. Data are from Exp. A. A-E. mRNA expression levels for *Tnfa*, *Il6*, *Kc/Cxcl1*, *Mcp1/Ccl2* and *Il12a* measured by qPCR. Results were normalized relative to levels of housekeeping reference *36b4* or *Pgk1*, *Actb* and *Ywhaz*; n = 7–9 per group. Data are mean ± SEM. Statistical analysis: Mann-Whitney U test. F-J. Western blots and quantification of autophagy markers present in uninduced colon cells, or following DSS-induction and treatment with either vehicle or P140. F. Results from three representative mice. Signals were normalized relative to total protein content measured directly on the membrane (stain-free procedure). K, L. Representative immunofluorescence images, and quantification of SQSTM1 expression. Scale bars 50 μm. Results are expressed as mean ± SEM; Statistical analysis: one-way ANOVA followed by Holm-Sidak's multiple comparisons. Vehicle, NaCl 0.9% w/v; ns, non-significant.

injections, timeframe between peptide doses) were tested to fully explore the efficacy of P140, delivered via the route validated previously in several studies of inflammation [21,23–26].

In terms of the course of disease progression, our results show a clear favorable outcome following P140 treatment. Curative therapy was applied with the TNBS model; both prophylactic and curative treatments were used with the DSS model; and *il10*<sup>-/-</sup>/*iRhomb2*<sup>-/-</sup> mice were successfully treated preventively for 10 weeks. Translation to patients will require additional studies to precisely determine the optimal conditions for use, including prevention, but also treatment of established IBD conditions as part of disease control.

Through its disruption of the intestinal epithelial barrier, DSS allows entry of luminal bacteria or bacterial antigens into the mucosa, leading to enterocolitis [50,55,56]. Inflammation is known to be involved in the pathogenesis of IBDs, including through the recruitment (migration and infiltration) of monocytes/macrophages to inflamed intestinal tissues. Following P140 treatment, inflammation was significantly reduced in colonic mucosa from DSS-induced mice. In TNBS-induced mice, where an excessive cell-mediated immune response involving acute Th1 inflammation develops, P140 treatment reduced macroscopic and colon damage scores, as well as excessive MPO activity. In this model, no visible effect of P140 on cytokine/chemokine production was observed.

Differences in cytokine levels between the two colitis models have also been reported and discussed elsewhere [60–62]. However, this result could also be linked to the duration of the experiment (4 days), or the fact that several mice in this group died before the end of the experiment, and consequently tissue samples were unavailable.

In support of our results, orally-administered 5-aminosalicylic acid compounds such as mesalazine or olsalazine, as well as the corticosteroid budesonide have been reported to display limited efficacy in DSS-induced mice [50]. This lack of effect seems to be related to the pharmacokinetics and metabolism of these compounds, and their capacity to reach inflamed colon tissues before being metabolized in the liver.

Dysfunctional autophagy has been reported in intestinal biopsies from IBD patients, and other mouse models [5,63], and an important result from this investigation was the demonstration that autophagy is dysfunctional in colon cells and splenocytes from mice with colitis, and the evidence that it could be at least partially restored by P140. The autophagic flux is classically used to represent the dynamic process of autophagy, but due to the experiment design, we were unable to assess its intensity. In the two chemically-induced models of colitis used here, compared to control mice, several key autophagy markers were expressed at equivalent levels. These results could be due to experimental parameters, in particular the timing of tissue sampling, and/or to



inherent difficulties detecting autophagy markers in colon tissues (as highlighted by Klionsky et al. [64]). However, since colon samples were isolated following a precisely established dissection procedure, we are confident that biases were avoided.

In DSS-induced mice, the expression of other autophagy markers, including ATG14, ATG5, and ATG12 (mRNA level; decrease), as well as SQSTM1 and LAMP2A (protein level; increase), was altered.

SQSTM1 is a classical autophagic cargo adaptor which can itself become degraded during the autophagy process. Accumulation of this protein suggests that autophagy is either decreased or inhibited in DSS-induced colitis. As SQSTM1 transcription is known to be sensitive to many factors, including prolonged starvation and endoplasmic reticulum stress, it is important to verify its mRNA levels if its protein level is used as an indicator of autophagy [65]. We found no change in SQSTM1 transcript levels in mice with colitis. Increased accumulation of SQSTM1 protein combined with a decrease in ATG14, ATG5, and ATG12 transcription, suggests dysfunctional autophagy in the intestines of DSS-induced mice – like that observed in patients – P140 treatment restored basal expression levels for all five markers identified, suggesting that autophagy had recovered.

The functional role played by CMA is relatively poorly understood in IBD. LAMP2A acts as the receptor for CMA substrates at the lysosomal membrane, and determines the rate of CMA activity. In patients with colorectal cancer, increased LAMP2A expression has been described to promote proliferation of cancer cells [66]. The increased LAMP2A (protein) level observed here could reflect an increase in CMA activity in the colon of DSS-induced mice. Except in some specific circumstances such as oxidative stress, the lysosomal level of LAMP2A is regulated by decreasing its degradation rate, with transcription remaining stable [67]. As we only measured total LAMP2 transcription, we cannot conclude that there was no change in transcription of the LAMP2A isoform.

BECN1 plays a critical role in regulating autophagy, and is also involved in tumor and metastasis formation, particularly in colorectal cancer, through an autophagy-independent pathway [68]. BECN1 protein expression was unchanged here, despite a moderate decrease in its mRNA expression. However, autophagy-dependent phosphorylation of BECN1 is strongly dependent on ATG14, which promotes BECN1 translocation from the *trans*-Golgi network to autophagosomes while also enhancing phosphatidylinositol 3-kinase catalytic subunit type 3 activity in a BECN1-dependent manner [69]. ATG14 is therefore pivotal in autophagosome-endolysosome fusion [70]. Expression of ATG14 was strongly reduced in colon cells from DSS-induced mice.

## 5. Conclusions

In conclusion, our results show that P140 alleviates colitis in murine models, as characterized by decreased DAI or clinical and histological scores, reduced colon shortening, lower levels of MPO activity, and down-regulated pathogenic cytokine and chemokine expression in colonic mucosa. In the patho-physiologically relevant tissues (colon cells), P140 regulated markers of both macroautophagy (ATG14, ATG5, ATG12, SQSTM1) and CMA (LAMP2A), suggesting that as shown in lupus, it might affect both upstream and downstream elements of the endo-lysosomal autophagy pathway. The molecular mechanisms through which P140 controls autophagy in IBD will need to be further investigated, and head-to-head trials with drugs that are currently used to treat patients with IBDs or other proposed treatments [10,50,71–73] should be performed. Nevertheless, our results strongly suggest that the phosphopeptide P140 – which has been demonstrated to be safe in clinical trials involving patients with SLE [74,75] – could also be used, alone or in combination with other medication [15,16,63,76,77], to treat patients with IBD, either preventively, or as part of disease control.

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## Author contributions

SM, TM, and HN designed the experiments. SVR, RG, JTM, AB and HN performed experiments. SVR, RG, TM, HN, and SM analyzed the results. SM wrote the paper. All authors approved the manuscript.

## Declaration of competing interest

SM is named as co-inventor on CNRS-ImmuPharma patents relating to P140 peptide. TM holds a patent on a method to identify agents for use in combination with inhibitors of iRhoms. The other authors have no potential conflicts of interest to disclose. All the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data availability

Data will be made available on request.

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We thank Dominique Ciocca for assistance with animal colony management and animal handling; Catherine Godfraind for histological assessment.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2022.102814>.

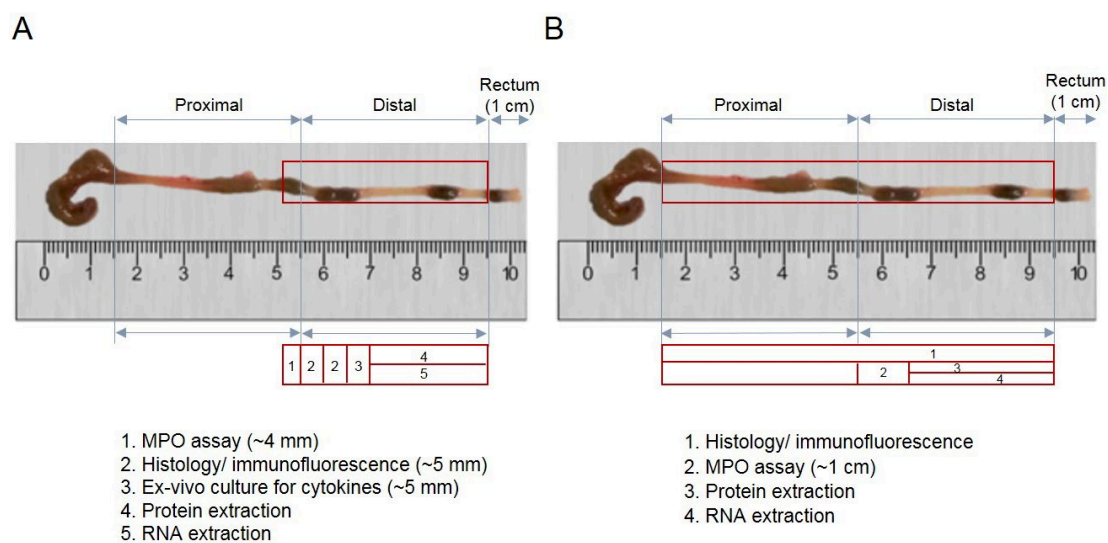
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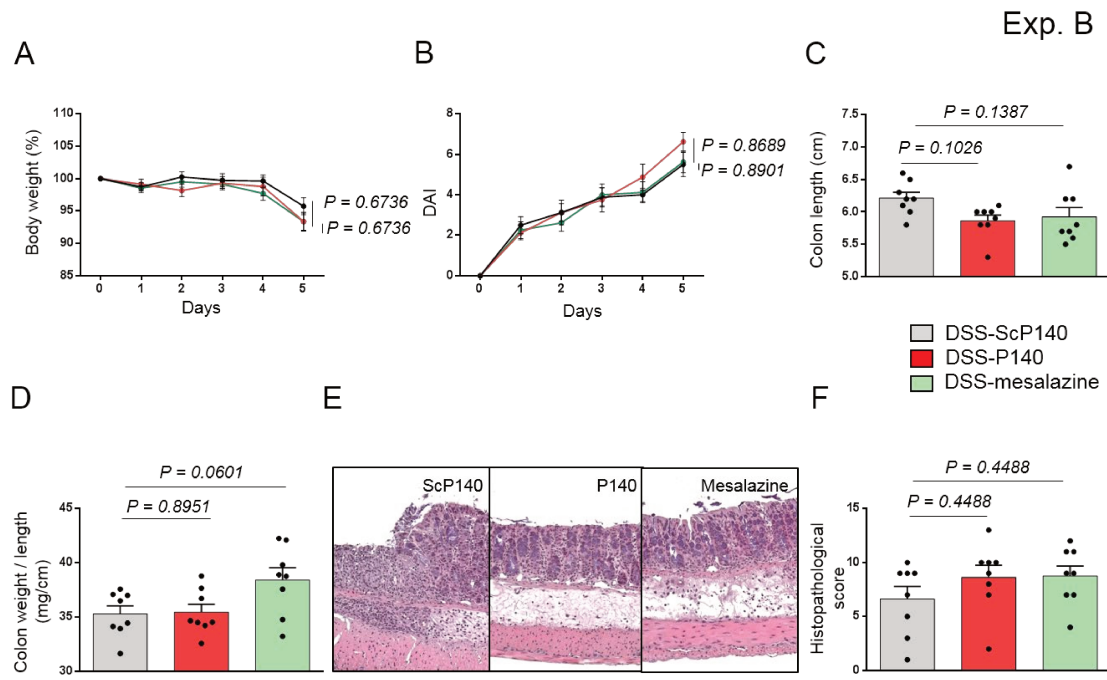
## Supplementary figures and legends



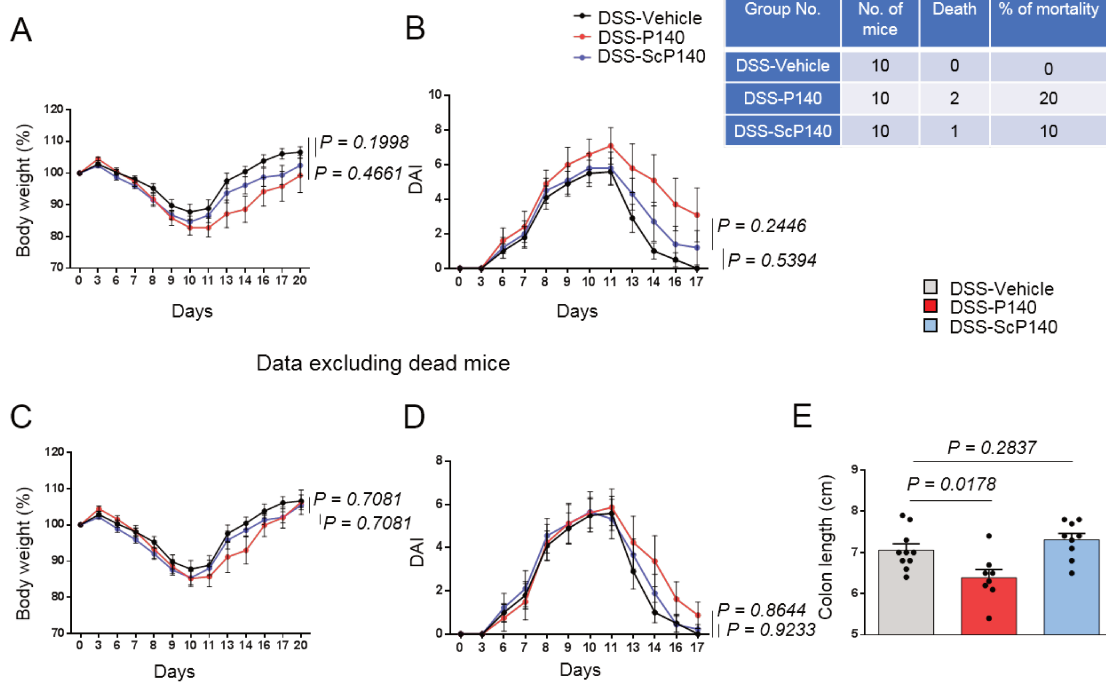
### Supplementary Figure 1. Colon sections selected to perform the different assays.

Colons were isolated from mice, opened longitudinally, and washed several times according to standard procedures. A, colon sections selected for the mice of Exp. A and B; B, colon sections selected for mice of Exp. C, D and E.

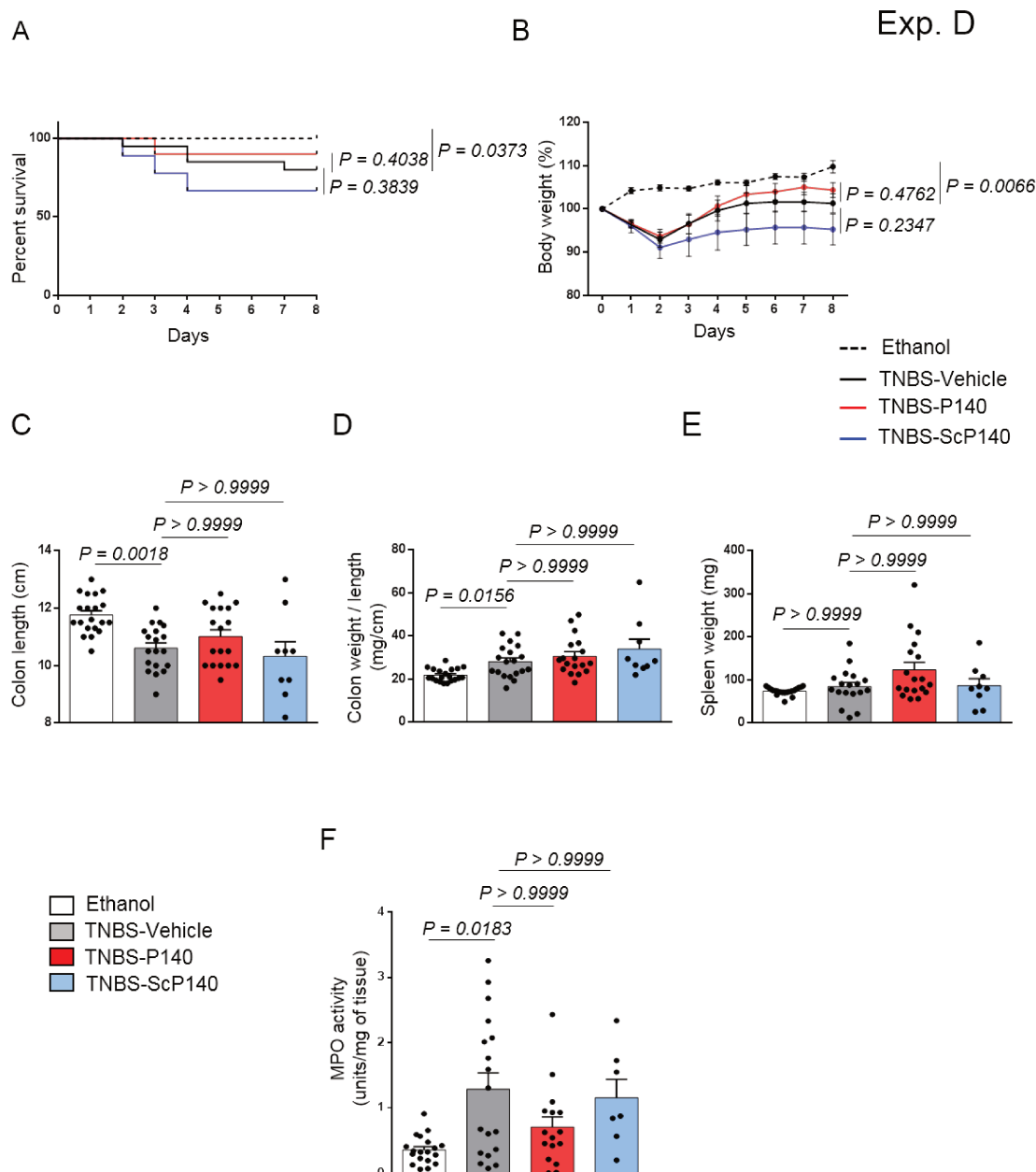




**Supplementary Figure 2. Therapeutic effects of P140 treatment in DSS colitis model.** A. body weight percentage; B. disease activity index score; C. length of colon; D. weight per length of colon; E. representative images of hematoxylin-eosin staining (magnification, X 200); F. histopathological score. Data shown are means  $\pm$  SEM.  $P$  values were calculated using two-way ANOVA followed by Holm-Sidak's multiple comparisons (A, B), Kruskal-Wallis test followed by Dunn's multiple comparisons (C) or one-way ANOVA followed by Holm-Sidak's multiple comparisons (D, F). ns, non-significant.

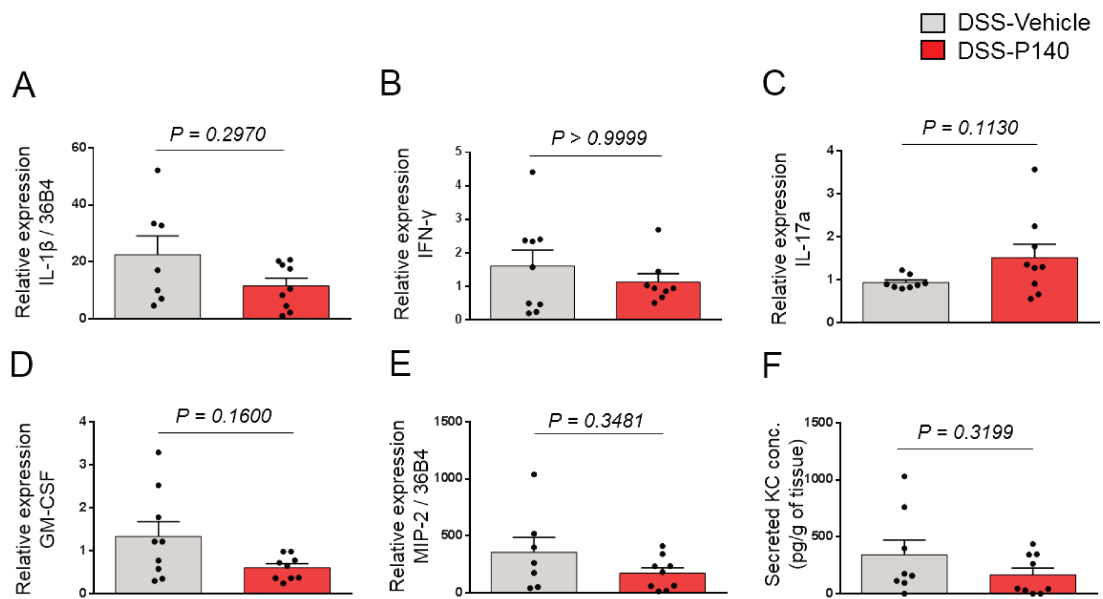


**Supplementary Figure 3. Therapeutic effects of P140 treatment in DSS colitis model.** The data are shown in including all mice (A, B) or in excluding dead mice (C, D). A, C. Body weight percentage; B, D. Disease activity index score; E. Length of colon. Data shown are means  $\pm$  SEM.  $P$  values were calculated using two-way (A-D) one-way or ANOVA (E) followed by Holm-Sidak's multiple comparisons. ns, non-significant.



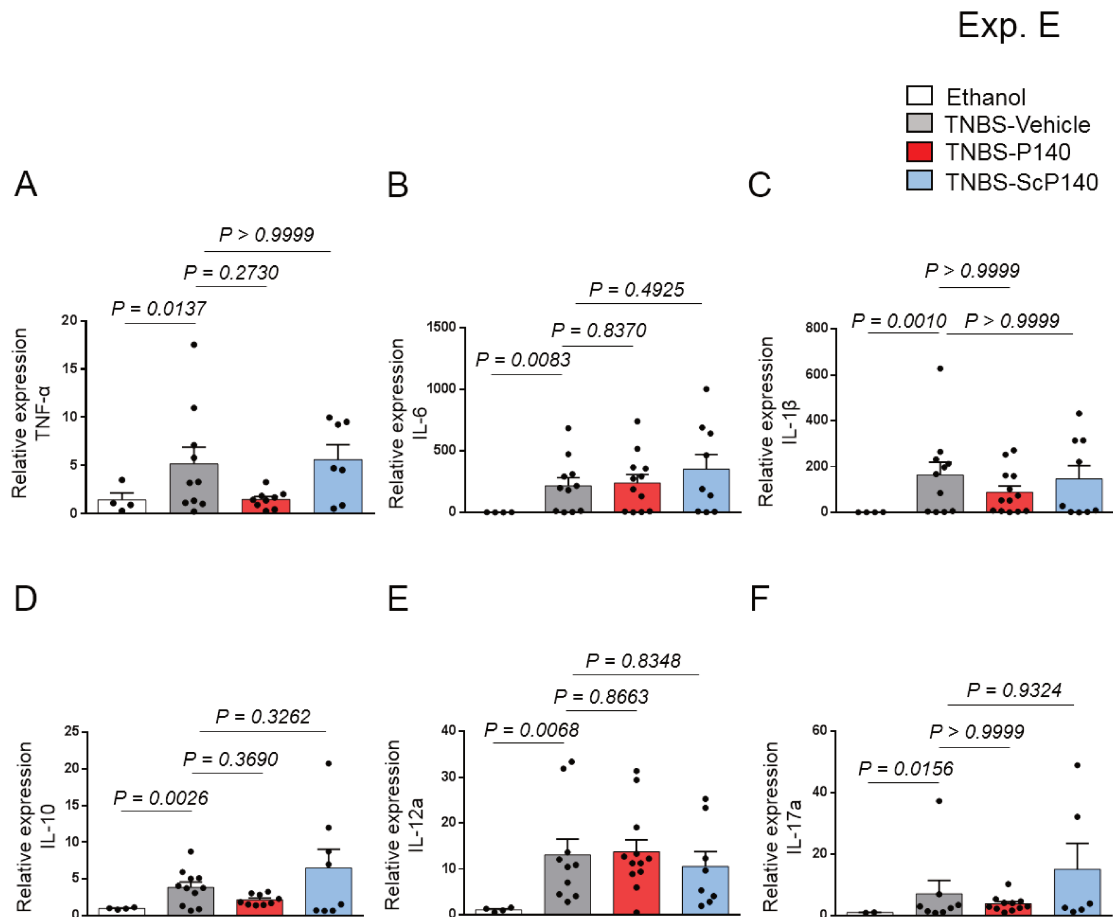
**Supplementary Figure 4. Therapeutic effects of P140 treatment in TNBS colitis model.** A. survival rate; B. body weight percentage; C. length of colon; D. weight per length of colon; E. spleen weight; F. myeloperoxidase activity.  $n = 7-20$  per group. Data shown are means  $\pm$  SEM.  $P$  values are calculated from log-rank (Mantel-Cox) test (A), 2-way ANOVA followed by Holm-Sidak's multiple comparisons (B) or Kruskal-Wallis test followed by Dunn's multiple comparisons (C, D, E, F). Vehicle, NaCl 0.9% w/v; ns, non-significant.

## Exp. A

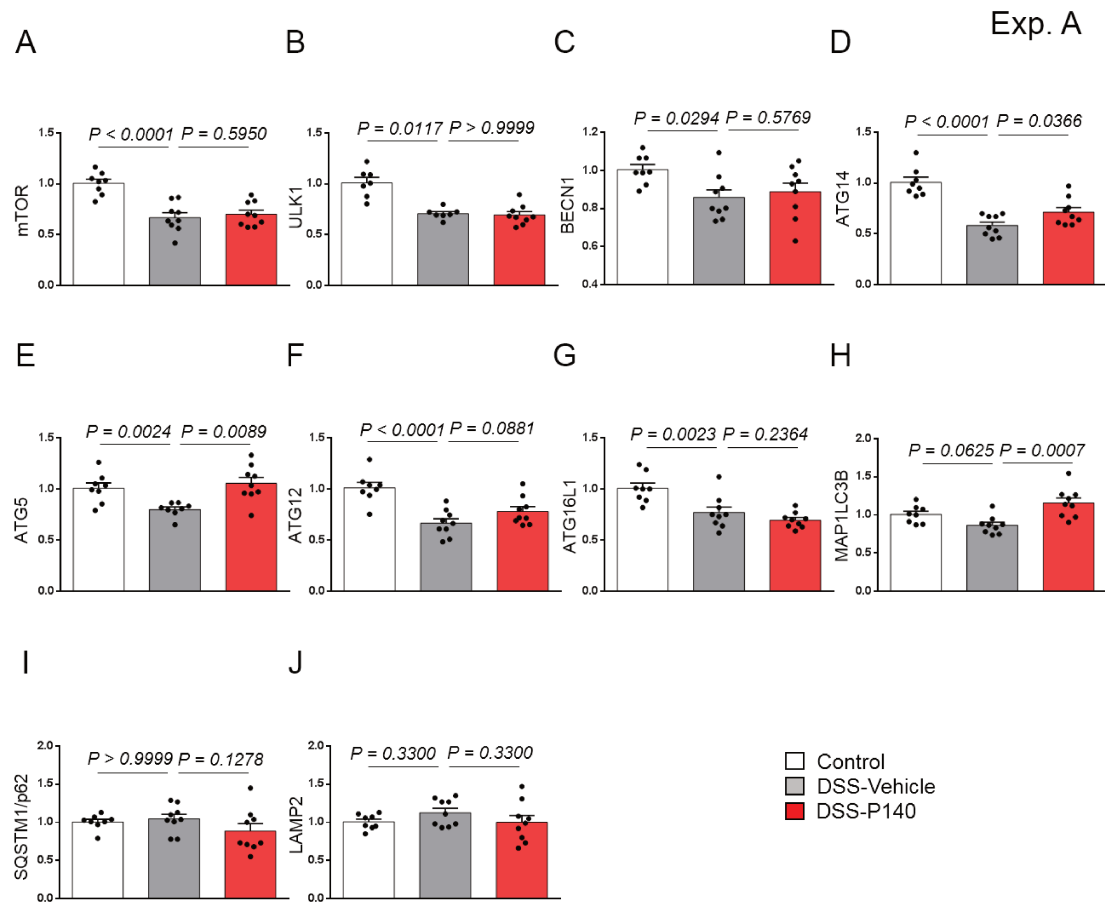


**Supplementary Figure 5. Effect of P140 on pro-inflammatory mediator production in colon in DSS colitis model.**

**Exp A:** A-E. mRNA expression levels of IL-1 $\beta$ , IFN- $\gamma$ , IL-17a, GM-CSF and MIP-2 measured by qPCR. The results are compared to the expression of 36B4, or Pgk1, Actb and Ywhaz used as housekeeping reference; F. Secreted levels of KC in colon culture supernatants measured by ELISA.  $n = 7-9$  per group. Data shown are means  $\pm$  SEM.  $P$  values are calculated from Mann-Whitney test. Vehicle, NaCl 0.9% w/v; ns, non-significant.

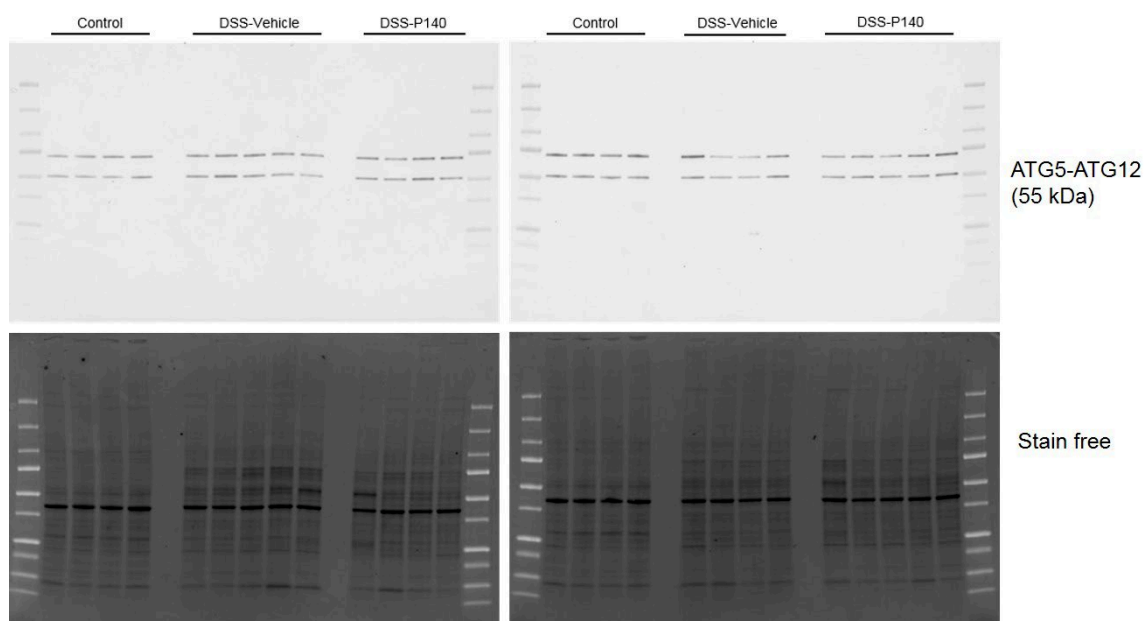


**Supplementary Figure 6. Effect of P140 on pro-inflammatory mediator production in colon in TNBS colitis model.** A-F. mRNA expression levels of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, IL-12a, IL-17a measured by qPCR;  $n = 4-13$  per group. The results are compared to the expression of Rps18, Hprt and Rpl13a, used as housekeeping references. Data shown are means  $\pm$  SEM.  $P$  values between control vs TNBS-vehicle groups are calculated from one-sample Wilcoxon signed-rank test (A, F) or one-sample t-test (B, C, D, E).  $P$  values between TNBS treated groups were calculated from Kruskal-Wallis test followed by Dunn's multiple comparisons (A, F) or one-way ANOVA followed by Holm-Sidak's multiple comparisons (B, C, D, E). Vehicle, NaCl 0.9% w/v; ns, non-significant.

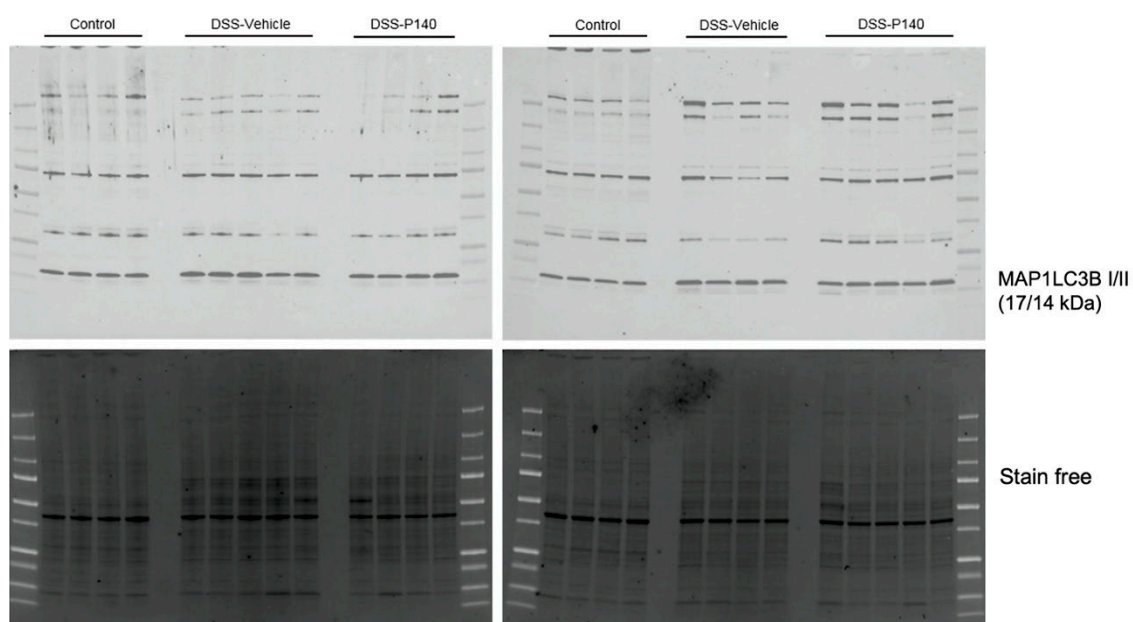


**Supplementary Figure 7. Effect of P140 on autophagy markers in colon in DSS colitis model.** A. A-J. mRNA expression levels of mTOR, ULK1, BECN1, ATG14, ATG5, ATG12, ATG16L1, MAP1LC3B, SQSTM1/p62, LAMP2 in colon tissue measured by qPCR. The results are compared to the expression of Pgk1, Actb and Ywhaz, used as housekeeping reference genes;  $n = 8-9$  per group. Data shown are means  $\pm$  SEM.  $P$  values are calculated from one-way ANOVA followed by Holm-Sidak's multiple comparisons (A, C, D, F, G, H, J) or Kruskal-Wallis test followed by Dunn's multiple comparisons (B, E, I). Vehicle, NaCl 0.9% w/v; ns, non significant.

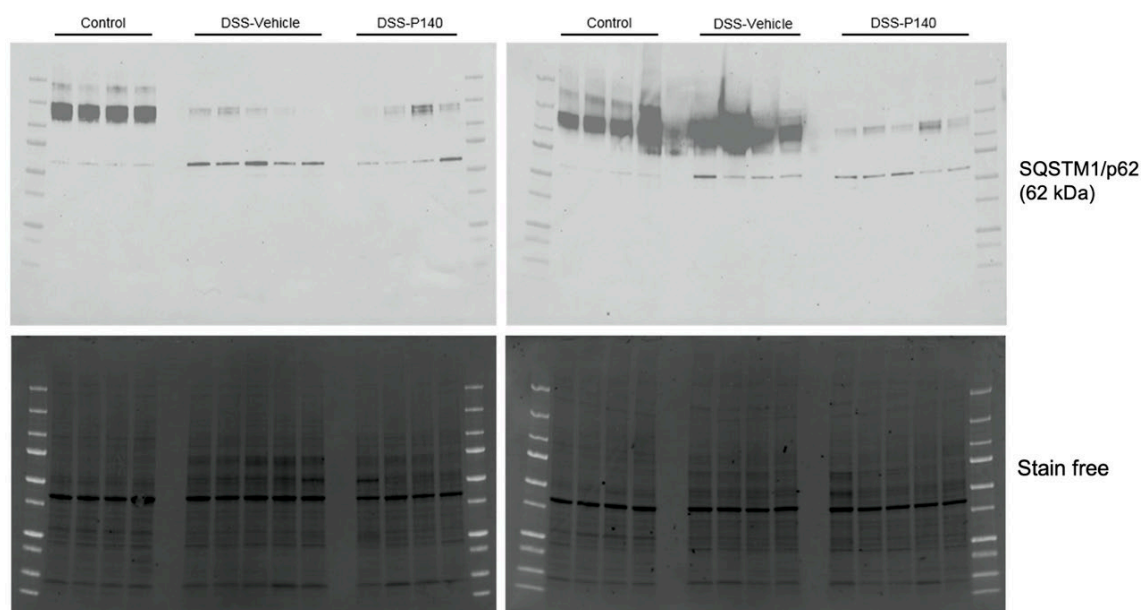
# A. ATG5-ATG12 (Exp.A)



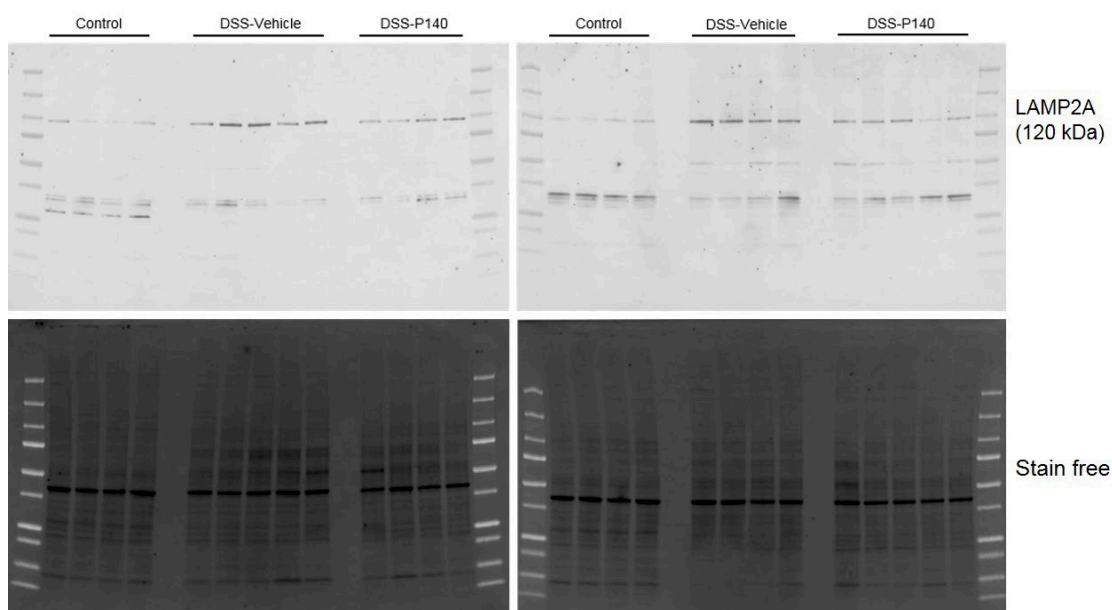
# B. MAP1LC3B (Exp. A)



C. SQSTM1/p62 (Exp. A)



D. LAMP2A (Exp. A)



**Supplementary Figure 8. Images of stain-free blots used for protein quantification.**

A-D, Membranes revealed with antibodies to ATG15-ATG12, MAP1LC3B, SQSTM1 and LAMP2A (shown as examples). To avoid quantification mistakes resulting from the fact that a loading control protein could represent a substrate for autophagy, the expression levels of autophagy markers were done using stain-free technology (total protein lane content) using ImageJ or Image Lab softwares.



**Supplementary Table 1: Disease Activity Index (DAI)**

<b>Score</b>	<b>Body weight loss</b>	<b>Stool consistency</b>	<b>Occult blood positivity</b>
<b>0</b>	None	Normal	No bleeding
<b>1</b>	1-5%	-	Occult blood test (+)
<b>2</b>	6-10%	Loose	Occult blood test (++)
<b>3</b>	11-15%	-	Occult blood test (+++)
<b>4</b>	>15%	Diarrhea	Gross bleeding from the anus

DAI is calculated as the sum of scores of body weight loss, stool consistency and occult blood positivity.

**Supplementary Table 2: Wallace's score**

Score	Criteria of macroscopic evaluation
0	No Inflammation
1	Hyperemia without ulcerations
2	Hyperemia with thickening of the mucosa without ulcerations
3	1 ulceration without thickening of the colonic wall
4	2 or more ulcerative or inflammatory sites
5	2 or more ulcerative and inflammatory sites with an extent > 1cm
6	Ulcerative or inflammatory site > 2cm
7	Ulcerative or inflammatory site > 3cm
8	Ulcerative or inflammatory site > 4cm
9	Ulcerative or inflammatory site > 5cm
10	Ulcerative or inflammatory site > 6cm

The Wallace's score rates macroscopic lesions on a scale from 0 to 10, based on features reflecting inflammation, such as hyperemia, thickening of the bowel, and extent of ulceration.

**Supplementary Table 3: Ameho's score**

<b>Score</b>	<b>Criteria of histologic evaluation</b>
<b>0</b>	No alterations
<b>1</b>	Middle mucosal and/or sub-mucosal inflammatory infiltrates with oedema. Few mucosal erosions. Integrity of the muscularis mucosae
<b>2</b>	Same criteria as score 1 but >50% of the section
<b>3</b>	Large inflammatory infiltrate with ulceration area through all the colonic wall
<b>4</b>	Same criteria as score 3, >50% of the section
<b>5</b>	Wide ulcerations with cellular necrosis
<b>6</b>	Wide ulcerations with cellular necrosis >50% of the section

On a scale from 0 to 6 the Ameho's score takes into account the degree of inflammation infiltrate, the presence of erosion, ulceration, or necrosis, and the depth and surface extension of lesions.

**Supplementary Table 4: List of primers used for qPCR\***

Primer	Reference/Sequence (5'-3')
<i>36b4</i>	FP-TCCAGGCTTTGGGCATCA RP-CTTTATCAGCTGCACATCACTCAGA
<i>Actb</i>	qMmuCED0027505
<i>Atg5</i>	qMmuCID0013019
<i>Atg12</i>	qMmuCID0016287
<i>Atg14</i>	qMmuCID0015163
<i>Atg16l1</i>	qMmuCID0011303
<i>Becn1</i>	qMmuCID0005981
<i>B2m</i>	qMmuCID0040553
<i>G6pdx</i>	qMmuCID0023829
<i>Gm-CSf</i>	qMmuCED0044875
<i>Gusb</i>	qMmuCED0004608
<i>Hprt</i>	qMmuCID0005679
<i>Ifng</i>	qMmuCID0006268
<i>Il6</i>	qMmuCID0005613
	FP-ACAAGTCGGAGGCTTAATTACACAT RP-TTGCCATTGCACAACCTCTTTTC
<i>Il10</i>	qMmuCID0015452
<i>Il12a</i>	qMmuCID0015668
<i>Il17a</i>	qMmuCID0026592
<i>Il1b</i>	qMmuCID0005641
	FP-TCGCTCAGGGTCACAAGAAA RP-CATCAGAGGCAAGGAGGAAAAC
<i>Kc</i>	FP-TTGTGCGAAAAGAAGTGCAG RP-TACAAACACAGCCTCCCACA
<i>Lamp2</i>	qMmuCID0011408
<i>Mcp-1</i>	FP-ACTGAAGCCAGCTCTCTTCCTC RP-TTCCTTCTTGGGGTCAGCACAGAC
<i>Mip-2</i>	FP-CACTCTCAAGGGCGGTCAAA RP-TACGATCCAGGCTTCCCGGGT

<i>Pgk1</i>	qMmuCED0060973
<i>Rpl13a</i>	qMmuCED0040629
<i>Rps18</i>	qMmuCED0045430
<i>Tfrc</i>	qMmuCID0039655
<i>Tnf</i>	qMmuCED0004141
	FP-AGGCTGCCCCGACTACGT RP-GACTTTCTCCTGGTATGAGATAGCAAA
<i>Ywhaz</i>	qMmuCED0027504

\* All from Biorad, FP; Forward primer, RP; Reverse primer

### 1.3. Comments

In this study, we have exploited pertinent murine models of colitis to demonstrate the protective potential of an autophagy modulating peptide in IBD. Among the three protocols applied with the DSS-induced colitis model (Exp. A, B, and C), Exp. A - which followed a combination of preventive and therapeutic administration of P140 peptide - showed promising results at the clinical and molecular levels. However, Exp. B, in which the peptide administration was carried out along with the onset of the disease presented no effects of the treatment. The reason for the failure of this experiment is not clear. One limitation of the protocol applied in this experiment was the very short time span (5 days) even for an acute setting in DSS colitis. Moreover, female mice were chosen for this experiment which is known to be less susceptible to DSS colitis than male mice. Consequently, the clinical course of the colitis observed from this experiment were much lighter compared to Exp A (~5% body weight loss in Exp. B vs ~20% loss in Exp. A; DAI~5 in Exp. B vs DAI~10 in Exp. A) at the time of sacrifice. Similarly, results obtained from Exp C, wherein the peptide administration started at the resolution phase of the inflammation, were also negative. The intestinal inflammation in colitis starts with an induction phase, characterized by pro-inflammatory immune responses against harmful stimuli such as exposure to luminal contents. This pro-inflammatory response must be controlled by a resolution phase to restore the tissue homeostasis once the stimuli are removed, which also is a crucial event involving multiple players. The mechanisms underlying the induction phase and the resolution stage of intestinal inflammation are completely different.<sup>417</sup> Combining the positive results from Exp. A and negative results from Exp. C, we may postulate that the peptide is active when administered in the induction phase of the inflammation but may not help when applied only in the resolution phase of the inflammation. However, more detailed studies need to be carried out in this regard, to determine the appropriate time course for peptide administration (active disease phase and/or quiescent phase) when translated into patients.

Two protocols were evaluated in the TNBS-induced colitis model in the therapeutic regimen. The results obtained from the first protocol (Exp. D) carried out with BALB/c mice, turned out to be disappointing. In this experiment, after a single dose of TNBS administration, several mice died within 2-4 days, and the remaining mice started to resolve from the disease as observed from the survival and bodyweight curves. Thus,

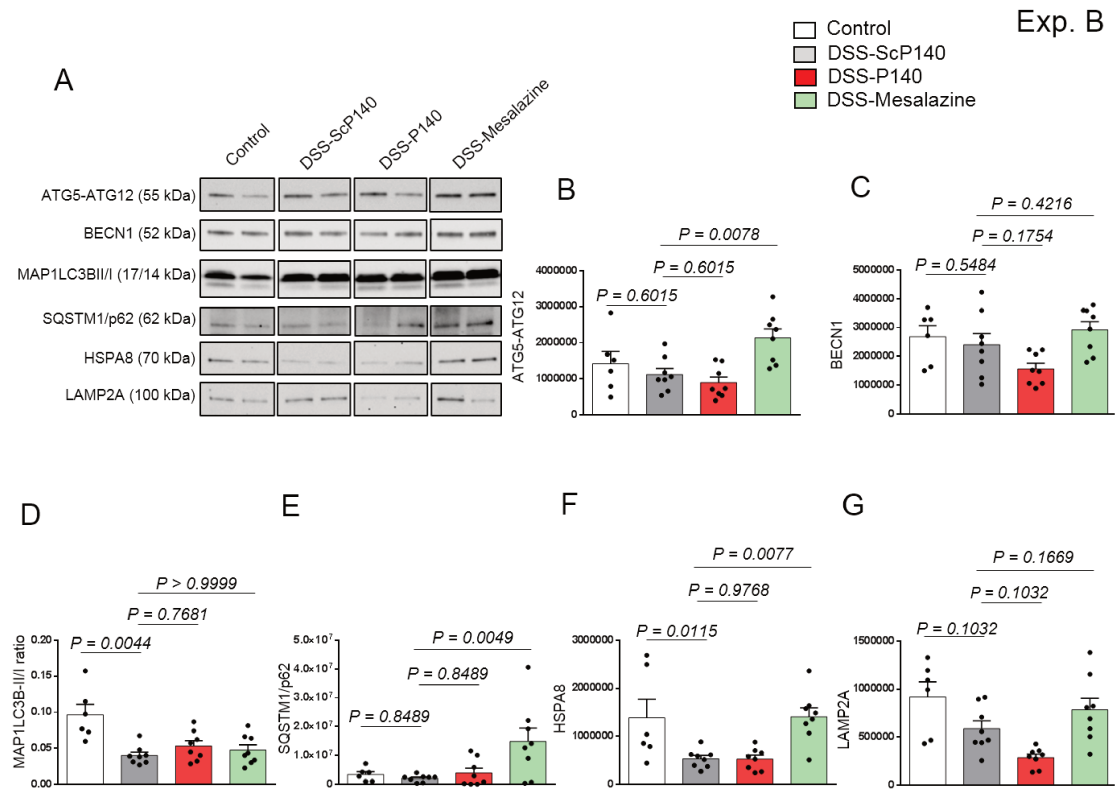
the 8-day protocol implemented in this acute model of TNBS seems too long for this type of study. Subsequently, a more refined experimental design (Exp. E) was applied with C57BL/6 male mice which are more resistant than BALB/c mice to TNBS-induced colitis (lower mortality) with a shorter time span of 4 days. A clear favourable effect of the P140 peptide treatment was observed in this experiment.

Finally, we have strengthened our results from the acute chemically-induced colitis models with a genetically relevant chronic model which is more representative of human IBD pathology. The experiment conducted with the *il10<sup>-/-</sup>iRhom2<sup>-/-</sup>* mouse model generated very promising clinical outcomes in all the parameters tested. Further analysis needs to be carried out at the histological and molecular levels in this model.

As previously described in the literature, alterations in several autophagy markers were detected in the colon tissues collected from Exp. A, and they were found to be restored upon P140 treatment. The defects in the autophagy pathway were not observable in the colon of TNBS colitis mice at least under the conditions that we have applied, despite a possible effect detected in the spleen of these mice. Autophagy is a dynamic multistep process involving numerous players at each step and therefore, measuring the changes in this pathway with static measurements is not conclusive enough. Autophagy flux measurements in distinct live cell populations would be necessary to validate these results. Due to the experimental settings and tissue sampling conditions, our results are limited in this aspect, which needs to be addressed in the future.

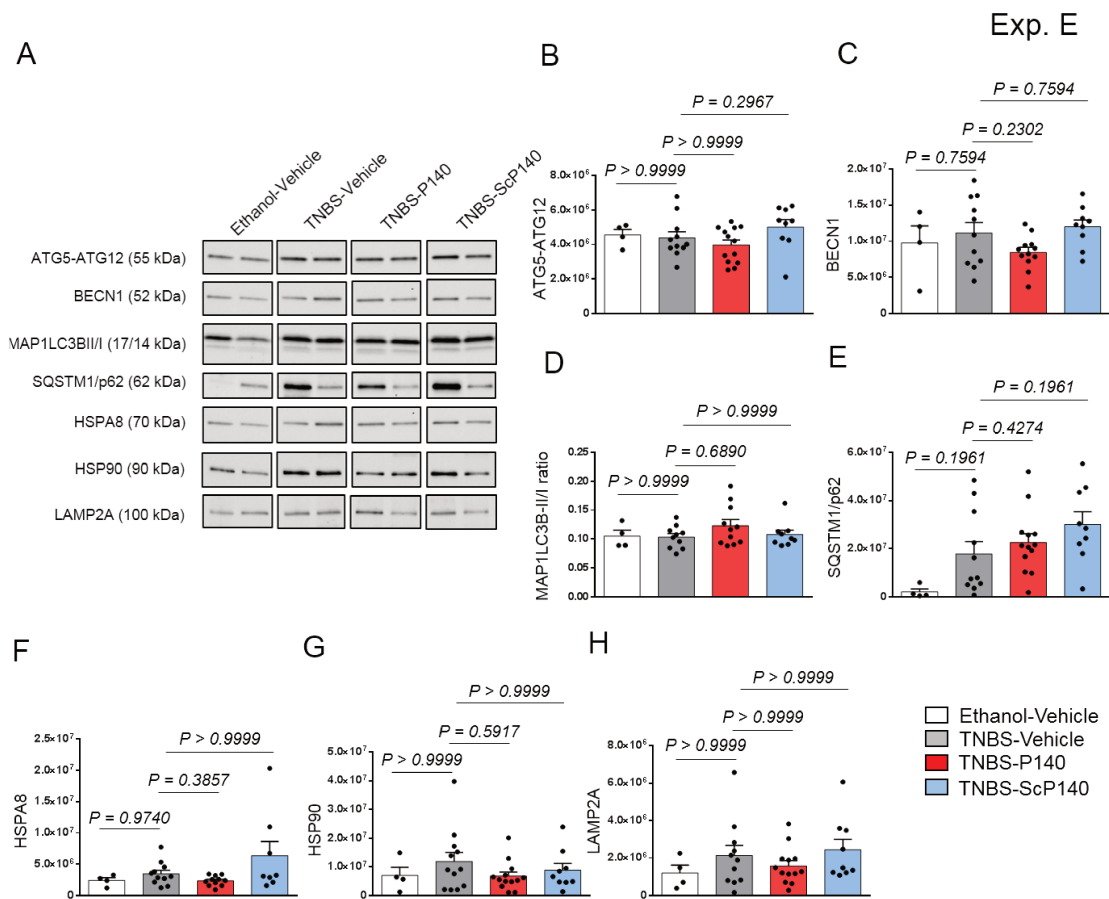
To summarise, our results demonstrate for the first time, the beneficial effects of an autophagy modulator peptide P140 in animal models of colitis. Further, some evidences are generated to support the restoration of defective autophagy processes in colitis models by P140 treatment. The fine molecular mechanisms behind the activity of the peptide in IBD remain to be explored.

## Unsubmitted data



**Supplementary Fig. 9. Effect of P140 on autophagy markers in colon in DSS colitis model.** A. Representative images of western blot for ATG5-ATG12, BECN1, MAP1LC3B, SQSTM1/p62, HSPA8 and LAMP2A in colon tissue. B-G. Quantification of protein expression levels normalised to total protein.  $n = 6-8$  per group. Data shown are means  $\pm$  SEM.  $P$  values are calculated from one-way ANOVA followed by Holm-Sidak's multiple comparisons (B, C, E, F, G) or Kruskal-Wallis test followed by Dunn's multiple comparisons (D). ns, non-significant.





**Supplementary Fig. 10. Effect of P140 on autophagy markers in colon in TNBS colitis model.** A. Representative images of western blot for ATG5-ATG12, BECN1, MAP1LC3B, SQSTM1/p62, HSPA8, HSP90 and LAMP2A in colon tissue. B-G. Quantification of protein expression levels normalised to total protein. n = 4-13 per group. Data shown are means  $\pm$  SEM. *P* values are calculated from one-way ANOVA followed by Holm-Sidak's multiple comparisons (C, E, H) or Kruskal-Wallis test followed by Dunn's multiple comparisons (B, D, F, G). Vehicle, NaCl 0.9% w/v; ns, non-significant.

**DISCUSSION  
AND  
FINAL COMMENTS**

## 1. LIMITATIONS OF OUR STUDY AND PERSPECTIVES

While the results generated from our study are decisively promising, they open numerous questions and create potential avenues for future exploration. A large panel of studies ought to be undertaken to address these questions and to be able to translate these results into patients safely and effectively.

The experiments initiated with the chemically induced models provided a kick start for our investigation, demonstrating the efficacy of the peptide P140 in short-term protocols. The last and foremost results generated in the long-term genetically-induced model (*il10<sup>-/-</sup>iRhom2<sup>-/-</sup>*) are currently limited to measurements of clinical symptoms. These studies need to be extended to the cellular and molecular levels using principal biomarkers. In this experiment, P140 peptide treatment started with the onset of colitis in *il10<sup>-/-</sup>iRhom2<sup>-/-</sup>* mice. The efficacy of the peptide, when administered in later stages of the disease (strictly therapeutic scheme) in this model, remains unknown. It would be important to generate such data in a genetically-induced chronic model when the disease is already established to predict the outcomes while treating the patients. It was not yet done because of the very long duration of this evaluation (> 1 year).

The major drug delivery routes used for IBD treatment are oral, injectable, and rectal routes of administration. The mode of administration of the P140 peptide used in this study was through the i.v. route as previously validated in other models of inflammation. We do not know the possible impact of a local intrarectal administration of the peptide directed to the target organ (colon). Several advantages have been described for the rectal route of administration in IBD treatment. The oral drug absorption rate is dependent on physiological factors within the gut such as gastric emptying rate, intestinal motility, and pH variations of the gastrointestinal fluids. The rectal route of delivery helps to avoid these complications associated with oral drug pharmacokinetics. It also allows a site-specific delivery into the inflamed sites at high doses.<sup>418</sup> However, patient compliance with oral administration is higher than rectal administration in IBD treatment as the patients suffer from severe diarrhea.

The use of encapsulated nanoparticle (NP) delivery systems can protect peptide-based drugs from the harsh environment of the GI tract. Compared to free drugs, encapsulated systems increases the bioavailability of the drugs and improve their retention time at the inflammation sites. Moreover, site-specific targeting modifications are possible with NPs, in which they are attached to some ligands that have an affinity to specific markers

at the inflammation sites.<sup>418</sup> Attempts are in progress in our laboratory in Strasbourg to create NP conjugated systems of the P140 peptide. A hyaluronic acid NP formulation of P140 peptide has been tested in MRL/lpr lupus-prone through intra-duodenal route.<sup>419</sup> This serves as a first step for the future formulation of P140 peptide for oral administration.<sup>420</sup> It can in turn provide possibilities to try more efficient methods of administration of P140 or its encapsulated formulations in colitis models aimed at improving its bioavailability and biodistribution in the long run.

In order to overcome the suboptimal efficacy of current IBD drugs, a combination therapy approach is currently being evaluated.<sup>421</sup> Combination therapy involves the rational use of two medications to exert a synergistic effect and therefore higher efficiency than the individual drugs. A successful example in the case of IBD treatment is the combined use of infliximab and AZA (**Table 4**). In this case, AZA was found to increase the bioavailability of infliximab and prevented the formation of ADAs.<sup>422</sup> Combination therapies are predicted to work the best when the two medications have complementary mechanisms of action. For instance, drugs that target the immune pathways could be combined with autophagy modulators or microbiota modulators in IBD treatment. In order to find the appropriate combination of drugs, their individual drug mechanisms and their potential impact on the downstream biological pathways needs to be deeply investigated.<sup>423</sup> In our studies, we haven't tested yet if the P140 peptide treatment could have some beneficial effect on the microbial imbalance in the gut. These types of studies need to be carried out to see if the combination of the peptide with any microbiota modulators could provide some additive benefits.

The precise target of action of P140 and the molecular mechanisms by which the peptide exerts protective effects in colitis remains unknown. In MRL/lpr lupus mice, the receptor of P140 peptide was identified to be HSPA8 overexpressed in B cells. Further, the peptide inhibits the CMA pathway and possibly interfere with the CMA-mediated antigen presentation. A similar result was observed in our experiments with the colitis model, wherein P140 reduces the over-expression of a CMA marker (LAMP2A) in diseased mice. This observation needs to be confirmed by checking the expression of LAMP2A specifically in isolated lysosomes rather than the whole tissue. Moreover, the functional role of the CMA pathway in IBD pathogenesis needs to be investigated to demonstrate the significance of this result. The *in vivo* biodistribution of P140 in MRL/lpr mice, when administered *via* i.v. route, was examined previously in our

team.<sup>409</sup> Several organs were analysed in this study to find out that P140 mostly accumulates in the spleen and lungs. This type of experiment can be extended to colitis models with a focus on the colon, and relevant lymphoid organs as a first step to identify the target of action of P140.

In the experiment designs we have followed throughout this study, samples were collected and immediately preserved for several experiments at the same time. The downstream biochemical and molecular analyses on the collected organs were thus performed in the frozen whole tissue homogenates. The intestinal mucosa is a multilayered complex environment containing many different cell types. The functional roles of these cells are variable in many processes. Especially, when evaluating autophagic processes, the activity can be variable and even opposing in different cell types. Also, the number and distribution of immune cells and epithelial cells can change during colitis. So, it is important to make sure that the changes that are observed in different biomarkers at the tissue level are not due to the alterations in cell numbers and type of cells. Methods have been established to efficiently separate the epithelial cells and lamina propria immune cells from the intestine.<sup>424</sup> Hence, isolating different cell types to conduct these types of mechanistic studies could provide greater scope and more accurate results. Due to the same technical reasons, experiments in live cells could not be performed. Rigorous flow cytometric analysis on live cells isolated from the colon or other lymphoid organs should be carried out to discover the immune cell types that are particularly affected in colitis upon P140 treatment. Live cell experiments will also enable us to assess the autophagic flux in the presence or absence of lysosomal inhibitors in different cell types.

As described in the introduction (section 1.6.1), gut microbiota plays an important role in the pathogenesis of IBDs and emerged as a potential target of intervention for IBD therapeutics. The commensal microbiota inhabiting different parts of our body can influence multiple physiological functions such as immune responses, metabolism, and behavior. Microbial alterations have been demonstrated in other autoimmune disorders as well (SLE, MS, SjS, etc.).<sup>425</sup> It is a future point of consideration whether P140 peptide indirectly exerts some beneficial effects on the microbiota composition in the diseases tested. The fecal microbiota composition of colitis mice treated with the controls and the P140 peptide should be compared in this context to see if the microbial balance is

restored or not and if the combined use of some probiotics or antibiotics could enhance the beneficial effects of the peptide.

In this thesis, we have focused on the autophagy pathway with respect to the mechanism of action of the P140 peptide derived in lupus mice. However, we do not know in IBD whether the changes we have observed in the autophagy pathway are a direct consequence of the activity of the peptide. It will also be interesting to assess other relevant signalling pathways involved in the pathogenesis of IBDs such as pro-inflammasome activation, antigen presentation, ER stress, and so forth.

## **2. GENERAL DISCUSSION**

### **2.1. Animal models of IBDs**

Using animal models for studying the pathologies that are exclusively present in humans raises an inevitable question: how these models can be correlated with human IBD pathogenesis? In general, an optimal animal model should exhibit inflammation and morphological alterations of the gut, in addition to clinical symptoms similar or identical to human IBD.<sup>414</sup> The mechanistic origin of mucosal inflammation in each of these animal models can be different, and sometimes completely unrelated to human IBD etiology, as in the case of chemically-induced animal models. Despite that, the final common pathways of mucosal inflammation and the basic immunologic abnormalities leading to these pathways in experimental colitis models adequately reflect human IBD pathways. An excessive IL-12-driven Th1-mediated inflammation and cytokine profile are observed in many of the murine models (e.g., TNBS colitis) similar to that observed in human CD.<sup>426</sup> Thus, animal models remain a valuable source of information regarding the pathology and therapeutics of IBDs, and novel models are being added to this array with the help of latest genetic engineering technologies to overcome the limitations of existing models.<sup>427</sup>

### **2.2. Therapeutic peptide P140**

A stumbling block in the long-term application of the currently available treatments for immune disorders is their immunosuppressive property and the associated side effects. In this context, the ability of P140-treated mice to respond to viral antigens was previously tested in MRL/lpr mice. The mice were able to mount an efficient B and T cell immune response to influenza virus antigens and resist infection, suggesting that P140 behave as an immunomodulator rather than as an immunosuppressor.<sup>428</sup> When

P140/Lupuzor was evaluated in patients, in Phase 1 and Phase II clinical trials, the peptide was found to be safe and well-tolerated. A subcutaneous administration of 3 doses (200 µg each), 4 weeks apart, has significantly reduced the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and other markers of inflammation.

As the safety of P140 peptide is already established in clinical trials, a mechanism-based repurposing of this drug candidate into other chronic inflammatory diseases was a perceptive strategy. Given the high cost, and slow pace of developing a new therapeutic tool, repositioning of existing drugs for novel clinical uses outside the scope of its original medical validation is becoming an attractive approach these days. The method typically follows three steps. Hypothesis-driven selection of a candidate drug for a given disease (1), pre-clinical testing of the drug in relevant models (2), and evaluating the efficacy of the drug in Phase II clinical trials for the proposed condition (3), assuming that the safety data generated from Phase I studies for its original medical indication is sufficiently robust. This strategy has resulted in the identification of several successful examples of candidate drugs in a much shorter time frame, and many others are in the pipeline for common and rare diseases.<sup>429</sup>

### **2.3. Future of peptide-based therapies**

During the initial phase of its discovery, the use of peptide-based drugs was limited as hormone analogues to treat metabolic disorders. Insulin was the first peptide hormone analogue to get approved for clinical use in 1920. In addition to the use of natural or synthetic analogues of endogenous peptides as replacement therapies, peptide therapeutics gained substantial momentum with the development of candidate peptide drugs to interfere with protein-protein interactions and inhibit specific protein targets which are key to many fundamental processes inside an organism including immune response events.

Peptide-based drugs have several advantages over other small molecules or biologics. Peptides are short amino acid sequences containing less than 50 amino acids in length, but they can mimic the function of protein molecules despite their small molecular weight. In fact, most of the synthetic peptides are derived from cognate sequences of functional proteins. They have higher activity per unit mass, greater storage stability, and weaker immunogenicity due to their low molecular weight and distinct biochemical properties.<sup>430</sup> Peptides can be designed to interfere with proteins or protein complexes

with a high specificity over other compounds and thus emerged as a unique class of pharmacological tools capable of precisely modulating biological processes.<sup>431,432</sup>

One of the factors that limited the efficacy of peptide-based drugs was their poor *in vivo* instability due to the degradation by omnipresent proteolytic enzymes in our body or quick elimination *via* other excretory mechanisms. The short half-life of peptides is advantageous to some extent since they do not accumulate in the body and create toxicity. In an otherwise disadvantageous scenario, several chemical modification strategies are currently employed to protect them from the activity of peptidases and enhance their stability. Another problem of naturally occurring peptides, in general, is their membrane impermeability, which hindered their application to intracellular targets. However synthetic peptides offer possibilities to modulate their hydrophobicity and electrostatic charges and thereby enhance their cellular uptake. Another strategy to overcome this limitation is the conjugation of the candidate drug peptides to a cell-penetrating peptide, which are specialised carrier peptides designed to facilitate the uptake of other molecules through cell membranes. The third shortcoming of peptide-therapeutics is their poor oral bioavailability which necessitates their injectable administration. As oral administration is the most patient-compliant method of drug delivery, research is underway to improve the oral bioavailability of peptides using carriers or NP delivery systems.<sup>420,430–433</sup> Another non-invasive route that has shown success in peptide administration, so far, is transcutaneous delivery, where the drug is applied to bare skin with a patch or in solution allowing its diffusion through the skin into the systemic circulation.<sup>434,435</sup>

A broad range of diverse chemical and biological applications is currently possible with a careful design of synthetic peptides and newer strategies are being introduced to overcome their existing limitations. With the technical advances in the synthesis of peptides and the lowering of prices of raw materials, peptide-based therapy has become a cost-effective treatment strategy compared to biological therapies. Thus, peptide-based therapies have secured a promising future in the treatment of various diseases. Other than the conventional use of peptides as replacement therapies in metabolic disorders, another area of application of peptide-based materials that has grown rapidly is cancer immunotherapy, as vaccines as well as therapeutic drugs. Numerous protein targets including transcription factors, structural proteins, and receptor tyrosine kinases have been identified for potential modulation by peptides in cancer, which are not easy



to be targeted by other small molecules. These peptides exert their therapeutic effects by various mechanisms such as by disrupting protein-protein interactions involved in tumor progression, inducing apoptosis of cancer cells, or modulating the immune responses in the tumour microenvironment.<sup>436</sup> The applications of peptide therapies are also being expanded to inflammatory disorders/AIDs for their ability to mimic or inhibit pathways or mediators involved in inflammatory responses.<sup>437</sup>

With the recent developments in computational biology approaches, it is now possible to carry out high throughput screenings of combinatorial peptide libraries to identify new peptide leads that can efficiently target protein complexes. Computational modeling methods also facilitate the discovery of peptide drugs by accurately predicting protein interaction surfaces and the structural effects of peptide binding.<sup>438</sup> Hence, the range of peptide-based pharmaceuticals will possibly continue to expand to newer targets in various indications with higher success rates than ever.

#### **2.4. Closing note**

Complex, polygenic immune disorders remain a major clinical challenge in our society for centuries and significant efforts have been made by the medical and scientific community to understand their etiopathogenesis and to find effective cures for these disorders. Collective efforts have optimized several strategies to help the affected patients lead a normal life, yet they are limited by various downsides. The quest for new therapeutic targets and more precisely targeted treatment modalities and effective delivery methods continues to expand with the help of the most recent innovative technologies and interdisciplinary approaches, which gives immense hopes for the future.

# **ANNEXE**

## **1. REVIEW**

### **Pharmacological autophagy regulators as therapeutic agents for inflammatory bowel diseases**

Sruthi Vijaya Retnakumar and Sylviane Muller (2019)

## Feature Review

## Pharmacological Autophagy Regulators as Therapeutic Agents for Inflammatory Bowel Diseases

Sruthi Vijaya Retnakumar<sup>1</sup> and Sylviane Muller<sup>1,2,\*</sup>

**The arsenal of effective molecules to treat patients with chronic inflammatory bowel diseases (IBDs) remains limited. These remitting-relapsing diseases have become a global health issue and new therapeutic strategies are eagerly awaited to regulate the course of these disorders. Since the association between autophagy-related gene polymorphism and an increased risk of Crohn's disease (CD) has been discovered, a new domain of investigation has emerged, focused on the intracellular degradation system, with the objective of generating new medicines that are safer and more targeted. This review summarizes the drugs administered to IBD patients and describes recently emerged therapeutic agents. We compile evidence on the contribution of autophagy to IBD pathogenesis, give an overview of pharmacological autophagy regulators in animal models of colitis, and propose novel therapeutic avenues based on autophagy components.**

### Inflammatory Bowel Diseases: A Group of Chronic, Relapsing Disorders That Depend on Environmental, Genetic, Microbial, and Immunological Factors

Inflammatory bowel diseases (IBDs) have an increased incidence in developed countries, affecting 0.1% of the Western population. They adversely affect several million people worldwide, with the highest rate of incidence in Europe and North America, probably as a result of diet, lifestyle, and sanitation [1–3]. IBDs, which cause inflammation of the lining of the digestive tract, are commonly detected in young people between 18–25 years of age, and so far, they are incurable [4]. Patients complain of frequent and chronically relapsing flares, which can lead to abdominal pain, diarrhea, rectal bleeding, fatigue, malnutrition, and weight loss. The major types of IBDs are exemplified by Crohn's disease (CD) and ulcerative colitis (UC). Although they share several clinical symptoms, CD and UC have markedly distinct features concerning their pathology and origin [5–7]. CD can affect any part of the gastrointestinal tract, most commonly the terminal ileum and colon, and can involve all of the layers of intestinal tissues. UC affects the large intestine only, and the inflammation is restricted to the mucosal layer [8]. CD and UC are associated with increased intestinal permeability, which involves paracellular passage regulated through tight junctions (TJs) [3,9]. SNPs located in genes encoding interacting TJ proteins and changes in the expression of these proteins have been described [10–14]. Several studies have shown the role of autophagy (Box 1) in the defects observed in the intestinal TJ barriers that occur in IBDs [15].

The etiology of IBDs involves complex genetic factors and environmental elements. Genome-wide association studies identified more than 200 confirmed genetic risk loci associated with this set of diseases. These loci are notably involved in common, albeit central, cellular

## Highlights

Autophagy, a vital intracellular degradation system that delivers cytoplasmic constituents to the lysosome is deregulated in numerous chronic inflammatory diseases.

An association has been described between *ATG16L1* autophagy gene polymorphism and an increased risk of CD development.

Any therapeutic strategy focused on key elements of autophagy pathways might be highly beneficial in the immunoregulation of IBDs.

Devising molecules correcting autophagy deficits without influencing other survival/death pathways remains of prime importance.

Newly designed molecules, such as the P140 peptide or others, which directly act on chaperone-mediated autophagy may offer unique effective precision therapy.

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## Box 1. Autophagy

Autophagy is a vital cellular process in which a cytoplasmic cargo is delivered to lysosomes for degradation and recycling. This evolutionarily conserved intracellular pathway is finely regulated by a large family of genes [195,196]. It continuously clears unnecessary or dysfunctional cellular components (damaged organelles, abnormally folded proteins, or proteins produced in excess). Autophagy is crucial for cell adaptation to the environment and maintenance of cell homeostasis, especially under stress conditions (nutrient deprivation, hypoxia, oxidative stress, or changes in intracellular calcium levels). Three main forms of autophagy have been described: macroautophagy, microautophagy and CMA. Besides these defined types, other forms of selective autophagy also operate, for example, mitophagy (selectively disrupts damaged mitochondria through autolysosomal degradation), xenophagy, and others [195]. One of the major mechanisms by which autophagy affects the pathogenesis of IBDs is through the regulation of pathogen clearance. Autophagy in Paneth cells, macrophages, and goblet cells in the intestinal wall targets invading pathogens for degradation or helps in secretion of antimicrobial peptides. In addition to pathogen clearance, autophagy plays a critical role in the adaptive immune response through MHCII-dependent antigen presentation, as substrates of autophagy can be loaded onto MHC. Mice lacking ATG5 in thymic epithelium develop severe colitis implicating that autophagy is required by the adaptive immune response in protection against IBDs [123,124].

**Macroautophagy:** in this highly genetically controlled canonical autophagy process, a double-membrane sequestering compartment, termed a phagophore, is formed and expands encapsulating cytoplasmic cargos. The resulting sealed, double-membrane vacuoles termed autophagosomes, subsequently fuse with hydrolytic enzyme-rich lysosomes to form autolysosomes in which the cellular cargos that have been engulfed are degraded. The resulting compounds that are cleaved by hydrolases are released back into the cytosol for reuse (recycling).

**Microautophagy:** this dynamic form of autophagy is characterized by direct engulfment of cytoplasmic cargos by lytic organelles (lysosomes in mammals and vacuoles in plants and fungi).

**CMA:** a selective form of autophagy that, in contrast to macroautophagy and microautophagy, does not involve vesicles, but instead utilizes chaperone proteins to directly target specific proteins to the lumen of lysosomes. CMA-targeted cargos are soluble cytoplasmic proteins, which contain a KFERQ-related pentapeptide motif that is recognized by HSPA8. Once docked on the outside of the lysosomal membrane, the targeted protein begins to unfold before it is internalized into the lysosomal lumen with the help of other chaperones and cochaperones, including lysosomal proteins HSPA8 and HSP90. LAMP2A plays a crucial role in the translocation process. Proteases and hydrolases that optimally function at low pH in the lysosome lumen degrade the selected unfolded cargo and recycle critical amino acid residues.

**Xenophagy:** this selective autophagy process is used to eliminate invading pathogens. Intracellular pathogens that are either inside the cytosol or in pathogen-containing vacuoles are surrounded by isolation membranes, engulfed into autophagosomes, and degraded inside autolysosomes.

pathways, such as autophagy, cytokine signaling, intestinal barrier regulation, and microbial recognition [16–18]. Recent studies have hypothesized that IBDs result from chronic abnormal immune responses against enteric bacteria or gut flora that develop in genetically susceptible individuals. IBDs are therefore a consequence of both autoimmune and immune-mediated phenomena. For example, autoreactive antibodies (Abs) and autoreactive T cells coexist with cytotoxic leukocytes for colonic epithelial cells, and serum Abs against colonic epithelium, but are also crossreactive with *Escherichia coli* antigens. Among the large diversity of Abs occurring in patients' serum, some may serve to differentiate between various forms of IBDs and can be used as predictors for disease activity [19–21]. Abnormalities affecting the innate and adaptive immune system are largely reported in IBDs [6]. A particular subset of dendritic cells (DCs) expressing CD11b and CD103 surface markers appear as a major source of interleukin (IL)-23 during colitis development [22–25]. The closely related IL-23 and IL-12 cytokines, as well as their major downstream components, including IL-17, play important roles in the regulation of mucosal inflammation, especially in the gut. These cytokines control autophagosome formation and autophagic flux. The **regulatory T cell (Tregs, see Glossary)** compartment is also deeply impacted in IBDs, as discussed elsewhere [5,26,27].

The importance of gut microbiota is central to many vital functions of the body and is not solely directly linked to intestinal functioning as originally thought. The gut microbiota is comprised of

## Glossary

**Hydroxychloroquine (HCQ):**

hydroxylated analog of chloroquine; this potent autophagy inhibitor prevents lysosomal acidification, thereby interfering with a key step in the autophagic process. Also acts as a TLR7/9 inhibitor.

**Immune-related GTPase M**

**(IRGM):** belongs to the p47 immunity-related GTPase family. Implicated in autophagy induction and autophagosome maturation. Reduced expression of IRGM increases the survival of the CD-associated adherent-invasive *E. coli* strain and correlates with decreased autophagy-mediated bacterial clearance. Multiple CD-associated polymorphisms have been found in the IRGM locus affecting the protein expression and splicing. A CD-associated exonic synonymous SNP alters the binding of miRNA-196 to the IRGM risk variant. miRNA-196 is overexpressed in inflamed intestinal tissue resulting in the loss of regulation of IRGM expression levels and hence defective autophagy-mediated clearance of adherent-invasive *E. coli* bacteria.

**Leucine-rich repeat kinase 2**

**(LRRK2):** a multifunctional kinase localizing to endolysosomal compartments and specific membrane microdomains. Thought to be a regulator of macroautophagy and CMA. Its expression level is higher in colon biopsy specimens of patients with CD.

**Nucleotide-binding oligomerization domain 2 (NOD2/CARD15):**

an intracellular pattern-recognition protein expressed in intestinal Paneth cells and monocyte-derived immune cells. Around one-third of patients with CD harbor NOD2 mutations with a 17-fold increased risk of the disease. Three mutations within the leucine-rich repeat region have been associated with CD. Acts as a muramyl peptide sensor, which activates the nuclear factor (NF)- $\kappa$ B pathway in response to bacterial infection. Interacts with ATG16L1 to induce an autophagic response against bacteria. Its stimulation leads to autophagy-dependent upregulation of MHCII molecules and generation of antigen-specific CD4<sup>+</sup> T cell responses.

thousands of diverse microbial species whose interactions with the host exert decisive regulating effects that are linked to immune, metabolic (e.g., in the regulation of systemic glucose metabolism), and neurological functions. It is only recently that the eminent role of the microbiota–gut–brain axis has emerged and that a connection between the gut microbiome and autophagy has been highlighted [28–31]. In IBDs, it is well documented that the microbial composition, diversity, and richness of microbiota is dramatically altered [32,33]. This microbial imbalance (known as gut dysbiosis) has been reported both at the mucosal and fecal level, and systematic studies have shown that on average, IBD-affected patients display 25% fewer microbial genes (a reflection of microbiota) than healthy individuals [34,35].

**Regulatory T cells (Tregs):** subset of CD4<sup>+</sup> T helper cells that express transcription factor Foxp3 and potentially suppress many immune responses.

A better knowledge of fundamental aspects involved in the loss of tolerance of immune functions affecting patients with IBDs and a finer understanding of the key elements prevailing in the tropism of organs, tissues, or cells that typify these patients, remains a central challenge in our quest for adapted specific treatments and personalized medicine, for these complex disorders. The focus of this Feature Review is on the potential disease interventions linked to autophagy defects that have recently emerged. A brief overview of preclinical data obtained with pharmacological autophagy regulators obtained in animal models of IBD-mimicking colitis is also provided.

### Animal Models of IBDs

It has long been recognized that the furtherance of new treatments is closely tied to the pertinence of relevant animal models. In the case of IBDs, more than 65 different animal models have been established, which can be classified as genetically engineered, congenic mutants, cell-transfer, or chemically induced models [36]. This multiplicity of experimental *in vivo* models crucially illustrates that none of them completely represents the criteria of human IBDs. Therefore, it is necessary to use several independent experimental animal models to demonstrate the efficacy of a newly developed treatment or diagnostic tool, or to study IBDs mechanistically.

The first experimentally induced colitis model was introduced as early as 1957 in rabbits [37]. Since then, many other genetically modified animal models have been generated, which have largely contributed to the understanding of the disease pathology and genetic features of IBDs. Genetically engineered animal models used to study IBDs include IL-10, signal transducer and activator of transcription (STAT)3, X-box binding protein 1, IL-2R $\alpha$ , IL-23R, transforming growth factor (TGF)- $\beta$ , and tumor necrosis factor (TNF)- $\alpha$  knockout mice, and TNF superfamily member (SF)-15, IL-7, IL-17, and IL-23-expressing transgenic mice [23,38,39]. Transgenic CEABAC10 mice expressing human carcinoembryonic antigen-related cell adhesion molecule 6, a receptor for some *Escherichia coli* strains, are also commonly exploited [40,41]. Using these mice models, a major role for CXCR1<sup>+</sup> mononuclear phagocyte-derived, TNF-like ligand 1A in driving IL-22 production in a subset of innate lymphoid cells that are central to the regulation of mucosal homeostasis has been demonstrated [39].

Some models are induced by chemicals that generate IBD-like inflammation in the intestine of normal mice or rats. These models of acute or chronic colitis are notably based on the use of dextran sulfate sodium (DSS), trinitrobenzene sulfonic acid (TNBS), oxazalone, or polyI:C. These chemicals are commercially available and are routinely used in research [42–44]. The resultant chemical models are relatively simple to set up with an appreciable reproducibility and present many similarities with human colitis. Several adoptive transfer colitis models have also been exploited [38,45]. In these models, recipient animals are given an intraperitoneal injection of cells (e.g., CD45RB<sup>high</sup> T cells or CD62L<sup>+</sup>/CD44<sup>−</sup> T cells) or monoclonal Abs (mAbs) (e.g.,

anti-CD40 Ab), and are then evaluated daily (as in the other models of colitis listed above) for survival, body weight, evidence of bloody stools, and diarrhea. Video endoscopy completes this follow-up, providing a daily visual assessment of the severity of colitis and monitoring of mucosal healing after treatment.

## Current Treatments for IBDs

### First-Line Therapies

The first drugs used to treat IBDs with some efficacy were immunosuppressants such as aminosalicylates, corticosteroids, and thiopurines (Table 1, and references therein [46–72]). Sulfasalazine, an aminosalicylate, which is a class of anti-inflammatory compounds acting mainly as oxygen scavengers, showed some potent effects. This discovery led to the development of a range of drugs in this class of compounds, such as mesalazine (Table 1). Corticosteroids were also found to be remarkably effective in both CD and UC. However, the long-term toxicity, steroid dependency, and refractoriness to treatment that occurred in some patients necessitated discontinuation or restricted use in some cases [48,68,70]. Therefore, ongoing research is focused on the identification of molecules that have fewer deleterious secondary effects. This has led to the development of several molecules, especially budesonide (Entocort or Mikicort), an oral glucocorticoid, which is quickly metabolized by the liver, thereby reducing corticosteroid-related adverse effects (AEs). It is used in the management of asthma, allergic rhinitis, and various skin disorders, and has been extended to CD [47]. Although budesonide appears significantly less effective than conventional steroids (e.g., mesalazine) for inducing remission in active CD, it displays fewer AEs [73]. It was not found to be effective, however, for maintaining remission at 12 months in CD.

Other immunomodulators such as thiopurines, methotrexate, and calcineurin inhibitors were also explored alone or concomitantly with other drugs as treatment options for IBDs (Table 1). Thiopurines are incorporated into nucleotides and suppress T cell function by decreasing the expression of proinflammatory cytokines. Methotrexate (e.g., Imeth, Novatrex, Methotrexate Bellon, or Metoject) is effective in steroid-dependent CD (effective for induction and maintenance of remission in CD, but not in UC [74]), while cyclosporine A and tacrolimus/FK-506 calcineurin inhibitors, which are strong immunosuppressive compounds, decrease proinflammatory lymphokine production in UC [63,75].

A recent study that included a large cohort of CD patients demonstrated that coadministration of 5-aminosalicylate (5-ASA; mesalazine) and azathioprine (AZA) or 6-mercaptopurine (6-MP) was not more effective than AZA or 6-MP alone in terms of the requirement for rescue medications such as steroids and anti-TNF agents [76]. The cumulative probabilities of hospitalization and intestinal resection were similar between the groups of patients on either regimen. Thus, although these molecules and peptides are often effective as primary or first-line therapy for IBDs, their long-term use is hindered by serious ailments such as myelosuppression, multiple infections, pancreatitis, and in some cases sensorineural hearing loss and tinnitus (Table 1), justifying the introduction of more selective therapeutic strategies.

### Era of Therapeutic Antibodies and Cell Modulators for Treating IBDs

A number of Abs have been developed against cytokines and adhesion molecules, which are key players in the pathogenesis of IBDs (Table 1; Figure 1). In patients with IBDs, cytokines produced by intestinal mucosa largely contribute to the activation and migration of inflammatory cells such as monocytes and neutrophils [5–7,16]. Cytokines are therefore especially targeted for treating IBD patients.

Table 1. Therapeutic Strategies Currently Approved and/or in Use for the Treatment of IBDs

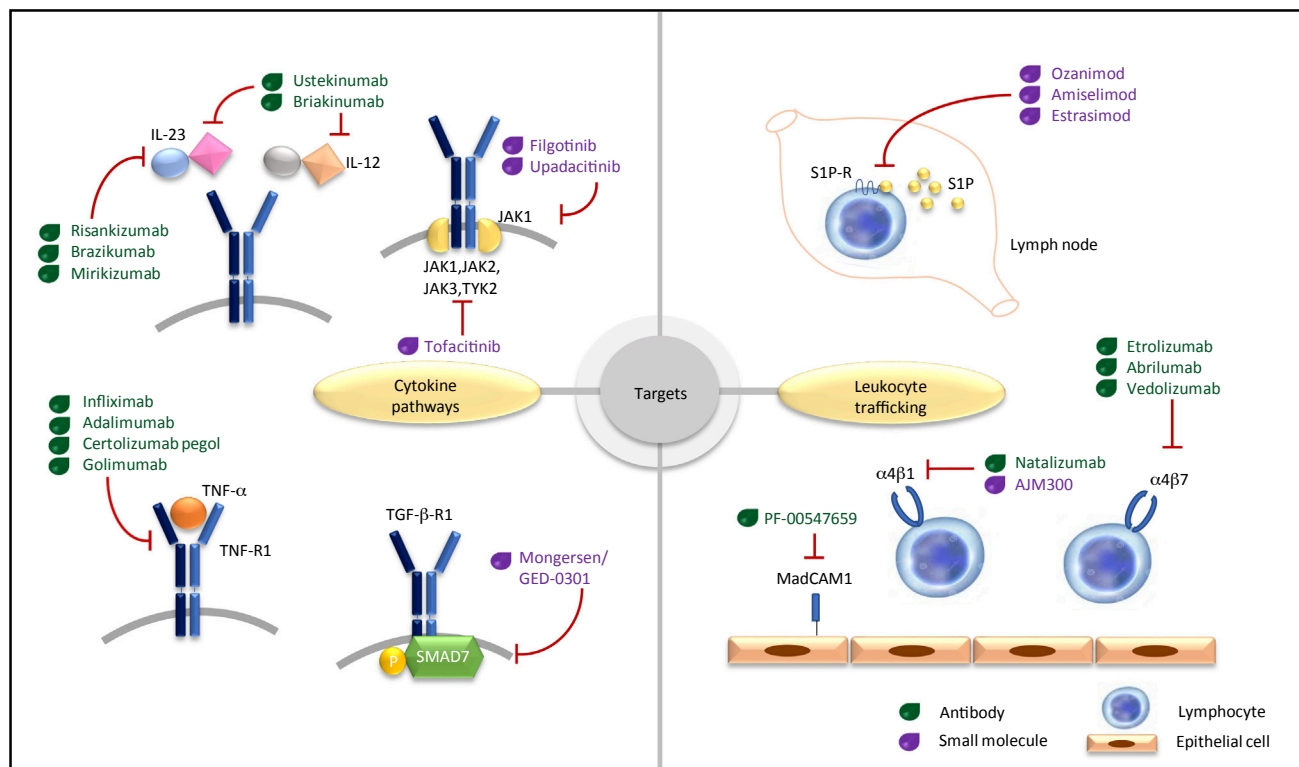
Drug		Mechanism of action/target	Efficacy (significant results)	SAEs	Clinical status	Refs
<b>Small molecules (corticoids and immunosuppressants)</b>						
Aminosalicylates (sulfasalazine, mesalazine, 4-aminosalicylic acid, balsalazide, olsalazine)		Free radical scavengers, 5-lipoxygenase inhibition, effects on leucocyte function and production of cytokines	Clinical remission rates of 40–70% have been reported with mesalazine over 6–8 weeks in UC	Nephrotoxicity, agranulocytosis, alveolitis, pancreatitis, abdominal pain, flatulence, nausea, dyspepsia	Common use	[57,59]
Prednisone, 6-methylprednisolone, budesonide MMX		Binds to high affinity intracellular cytoplasmic receptors	Clinical remission at 8 weeks (17.4% – budesonide MMX 9 mg vs. 4.5% – placebo) in UC	Diabetes, osteoporosis, moon face, and acne, growth retardation in children, psychosis, hepatic steatosis	Common use	[47,48,68,70]
Thiopurines (6-mercaptopurine, azathioprine)		Incorporates into nucleotides	Maintains remission in moderate to severe CD/UC	Myelosuppression, non-Hodgkin's lymphoma	Common use	[61]
Methotrexate		Inhibits enzymes in folic acid metabolic pathway (for high doses used in oncology/hematology; mode of action is unknown for low doses used in CD)	Clinical remission at 16 weeks (39% – 25 mg vs. 19% – placebo; 65% – 15 mg vs. 39% – placebo) in CD	Dyspnea, nausea, vomiting, and neutropenia	Common use	[63,64]
Cyclosporine		Calcineurin inhibitor	Cyclosporine (4 mg/kg) showed 82% response rate vs. placebo ( $P < 0.001$ ) in 7 days in UC	Renal failure, bacterial pneumonia, <i>Pneumocystis jiroveci</i> pneumonia, venous catheter infections	Common use	[46]
Tacrolimus		Calcineurin inhibitor	Clinical remission at 2 weeks (9.4% vs. 0.0% – placebo) in UC	Tremor, paresthesia, insomnia, hot flush, alopecia, hyperglycemia, hypomagnesemia, hypertension, hepatotoxicity, nephrotoxicity	Common use	[56]
<b>Therapeutic antibodies</b>						
Generic name	Trade name/synonym					
Infliximab	Remicade	TNF- $\alpha$ antagonist	Clinical remission at 30 weeks (35.8% – 10 mg vs. 15.7% – placebo) in UC. Clinical remission at 10 weeks (57.5%) in CD	Drug-induced lupus, infusion reactions, hypersensitivity reactions, demyelination, reactivation of latent tuberculosis, non-Hodgkin's lymphoma	Approved by FDA since 2007 for CD/UC	[49,65]
Adalimumab	Humira	TNF- $\alpha$	Clinical remission at week 56 (36% – 400 mg eow <sup>a</sup> , 41% – 400 mg weekly, vs. 12% – placebo) in CD	Congestive heart failure, lupus-like syndrome, lymphoma, cytopenia, MS and other demyelinating diseases, pancytopenia	Approved by FDA since 2007 for CD/UC	[62]

Table 1. (continued)

Drug		Mechanism of action/target	Efficacy (significant results)	SAEs	Clinical status	Refs
Certolizumab Pegol	Cimzia	TNF- $\alpha$	Clinical response at 10 weeks (52.8% – 400 mg vs. 30.1% – placebo) in CD	Injection site reaction, infections, lupus-like syndrome	Approved by FDA since 2008 for CD	[50,51,54]
Golimumab	Symponi	TNF- $\alpha$	Clinical remission at 54 weeks (27.8% – 100 mg vs. 15.6% – placebo) in UC	Erythema, tuberculosis, rectal, thyroid, and lung adenocarcinoma	Approved by FDA and EMA since 2003 for UC	[69,72]
Ustekinumab	Stelara	IL-12/IL-23	Clinical remission at 44 weeks (53.1% – 90 mg every 8 weeks and 48.8% – 90 mg every 12 weeks vs. 35.9% – placebo) in CD	Nasopharyngitis upper respiratory tract infections, diverticulitis, cellulitis, pneumonia	Approved by FDA since 2016 for CD	[53,58,66,71]
Natalizumab	Tysabri	$\alpha$ 4 integrin	Clinical remission at 8 weeks (26% – 300 mg vs. 16% – placebo) in CD	Pharyngitis, urinary tract infection, urticaria, cephalgia, arthralgia, PML	Approved by FDA since 2004 for CD	[52]
Vedolizumab	Entyvio	$\alpha$ 4 $\beta$ 7 integrin	Clinical remission at 52 weeks (44.8% – 300 mg vs. 29.1% – placebo) in CD	Gastrointestinal and respiratory tract infections, hepatic steatosis	Approved by FDA since 2014 for CD/UC	[55,60,67]
<b>Other small molecules</b>						
Tofacitinib	Xeljanz	Pan-JAK inhibitor	Clinical remission at 8 weeks (18.5% – 10 mg vs 8.2% – placebo) in UC	Herpes zoster infection, upper respiratory tract infections, headache, diarrhea, nasopharyngitis	Approved by FDA since 2018 for UC	[121]

<sup>a</sup>Abbreviations: EMA, European Medicines Agency; eow, every other week; MMX, multi matrix.





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**Figure 1. Targets of the Major Medications Indicated for Treatment of Inflammatory Bowel Diseases.** Existing drugs and compounds under development commonly target two large areas of regulation; namely, inhibition of cytokine signaling pathways (left) and inhibition of leukocyte trafficking to the gut mucosa (right). The targets of therapeutic antibodies (green) and small molecules (violet) are shown. Most if not all of these regulatory compounds are inhibiting/blocking agents. Abbreviations:  $\alpha 4\beta 1/7$ , integrin  $\alpha 4\beta 1/7$ ; IL, interleukin; JAK, Janus kinase; TYK, tyrosine kinase; MadCAM1, mucosal addressin cell adhesion molecule 1; TGF- $\beta$ , transforming growth factor  $\beta$ ; TNF, tumor necrosis factor; S1P-R, sphingosine-1-phosphate receptor; SMAD7, mothers against decapentaplegic homolog 7.

### Anti-TNF Antibodies

The use of anti-TNF drugs (Box 2) has been a significant breakthrough in the treatment of IBDs [7,68,77,78]. Several anti-TNF- $\alpha$  Abs are currently approved for treating patients with IBDs. In the case of CD, these include infliximab (Remicade), which is a chimeric human/mouse Ab (and its biosimilars Inflectra, Remsima, and Flixabi), adalimumab (Humira), a fully human IgG1 mAb [and its biosimilars Hulo (Mylan), Cyltezo (Boehringer Ingelheim), Imraldi (Samsung Bioepis), Hyrimoz (Sandoz), and Amgevita (Amgen)], and certolizumab pegol (Cimzia), a humanized antigen-binding fragment (Fab') of a mAb that has been conjugated to polyethylene glycol. For UC treatment, infliximab, adalimumab, and golimumab (Simponi), a fully human IgG mAb (Table 1) have been tested. A recent study has demonstrated the efficacy of golimumab in anti-TNF-refractory CD patients [79]. At this stage, however, further studies are awaited in CD to formally assess the efficacy of golimumab in a randomized controlled trial and to establish the optimal dosing regimen.

Altogether, TNF-targeting Abs have been claimed to induce a clinical response in about 60% of CD and UC patients; a result that is remarkable in the context of these severe and heterogeneous diseases [50,62,77]. It is however pertinent to remember the well-characterized serious AEs (SAEs) induced in certain patients by TNF blockers when given for long periods of

**Box 2. Mechanisms of Action of Anti-TNF Abs**

The mechanism of action of anti-TNF Abs can be many, including simple neutralization of the TNF- $\alpha$  ligand, modulation of the immune system, outside-to-inside signaling, and the induction of direct or indirect apoptosis [197,198] (and references in Table 1). Infliximab, which was the first anti-TNF- $\alpha$  mAb reported to be successful for the treatment of IBDs in 1993, binds with high affinity to soluble and transmembrane TNF- $\alpha$  but not to lymphotoxin- $\alpha$  (also called TNF- $\beta$ ). Adalimumab neutralizes the activity of TNF- $\alpha$  by inhibiting its interaction with p55 and p75 cell surface TNF- $\alpha$  receptors. Its clinical efficacy was proven for maintaining remission in moderate-to-severe CD through 56 weeks. Its safety and efficiency were also demonstrated in patients with secondary loss of response. Similar to infliximab and adalimumab, certolizumab pegol binds and neutralizes TNF- $\alpha$ . However, because it does not contain an Fc region, an important structural difference with regard to infliximab and adalimumab, certolizumab pegol does not mediate complement-dependent cytotoxicity and Ab-dependent cell-mediated cytotoxicity, and hence emerges as an attractive alternative anti-TNF drug. Although observed in a small proportion of patients, anti-TNF therapy displays wide-ranging effects on the immune system, resulting in a spectrum of potential AEs. Many efforts have been developed to minimize these complications [80,81,83].

treatment. Two major concerns with these drugs include the risk of serious infections and malignancies [80–83].

*Other Cytokine Biological Therapies*

Apart from TNF- $\alpha$ , other cytokines are also used as targets in emerging therapeutic strategies [84]. Ustekinumab (Stelara) is a human IgG1 mAb that targets the p14 subunit of IL-12 and IL-23 by inhibiting binding to their receptors (Table 1; Figure 1). This mAb, which was approved by the FDA in 2016, is efficacious in CD patients with moderate-to-active disease.

Other biologics, for example, Abs that target IL-23 by binding to its P19 subunit, such as risankizumab (BI-655066 or ABBV-066), brazikumab (AMG 139 or MEDI2070), briakinumab (ABT-874), and mirikizumab (LY3074828), are currently being evaluated for their potential efficacy (Figure 1; Table 2) [72,85–103].

Briakinumab is a human mAb that was initially developed for treating rheumatoid arthritis (RA), multiple sclerosis (MS), and IBDs. In November 2009, a Phase III clinical trial for plaque psoriasis was completed and a Phase II clinical trial for MS was announced. A Phase II clinical trial for CD was also carried out [104]. Head-to-head comparisons were made with regard to etanercept (Enbrel), a dimeric fusion protein targeting TNF, and placebo, in double-blind trials. The results gained with briakinumab were promising in psoriasis (81–82% of patients under briakinumab, 40–56% under etanercept, and 7% under placebo reached a Psoriasis Area Severity Index reduction of at least 75%). However, in January 2011, the withdrawal of the briakinumab application was announced in favor of other strategies.

Migration of leukocytes to mucosal lesions is important in the pathogenesis of IBDs, and this trafficking process is actively mediated by integrins. Hence, targeting integrins has emerged as another potential therapy. The first attempt in this area was based on natalizumab (Tysabri), a human IgG4 Ab targeting the  $\alpha$ 4 integrin subunit (Table 1). Its use, however, was preferable for short-term treatment. In some rare cases, due to inhibition of leukocyte migration into the central nervous system, it was found to promote reactivation of JC virus in the brain, resulting in the development of progressive multifocal leukoencephalopathy (PML); an SAE that precluded its indication.

Vedolizumab (Entyvio) is an IgG1 mAb, which also blocks the  $\alpha$ 4 $\beta$ 7 integrin subunit but on account of its gut selectivity, it was not associated with PML (Table 1). In Phase III clinical trials, vedolizumab was found to be safe and efficient in the induction and maintenance phases of therapy in CD and UC patients. Although patients receiving vedolizumab presented more

Table 2. Therapeutic Strategies Currently under Clinical Evaluation for IBDs

Drugs		Mechanism of action/target	Efficacy (significant results)	SAEs	Clinical status	Refs
Generic name	Trade name/ synonym					
<b>Therapeutic antibodies</b>						
Golimumab	Symponi	TNF- $\alpha$	Retrospective analysis in 115 CD patients: Clinical response 55.8% in 4 months	Infections, drug-induced lupus	No formal trials have been undertaken for CD	[72,95]
Risankizumab	BI-655066 or ABBV-066	IL-23 (p19)	Clinical remission at 12 weeks (31% vs. 15% – placebo) in CD	Nausea, worsening of underlying CD	Phase III trials for CD	[94]
Brazikumab	AMG 139 or MEDI2070	IL-23 (p19)	Clinical remission at 8 weeks (49.2% vs. 26.7% – placebo) in CD	Headache, nasopharyngitis	Phase II trials for CD	[99]
Briakinumab	ABT-874	IL-12/23 (p40)	Clinical remission at 24 weeks (48% – 400 mg, 57% – 700 mg vs. 29% – placebo) in CD	Respiratory tract infection, nausea, abdominal pain, headache, cardiovascular events	Phase II trials for CD	[85,90]
Mirikizumab	LY3074828	IL-23 (p19)	Clinical remission at 12 weeks (31% vs. 4.8% – placebo) in UC	None reported	Phase II trials for CD/UC	[103]
Etrolizumab	rhuMAb $\beta$ 7	$\beta$ 7 subunit of integrins $\alpha$ 4 $\beta$ 7 and $\alpha$ E $\beta$ 7	Clinical remission at 10 weeks (21% – 100 mg vs. 10% – placebo) in CD	Exacerbation of UC, headache, fatigue, abdominal pain, dizziness, nasopharyngitis, nausea, arthralgia, urinary tract infection	Phase III trials for CD/UC	[88,92]
	PF-00547659	MAdCAM-1	Clinical remission at 12 weeks (16.7% – 22.5 mg vs. 2.7% – placebo) in CD	None reported	Phase II trials for CD/UC	[98,101]
Abrilumab	AMG181	$\alpha$ 4 $\beta$ 7 integrin	Clinical remission at 12 weeks (30.8% – 210 mg vs. 17.6% – placebo) in CD	Upper respiratory tract infection, headache	Phase II trials for CD/UC	[87]
<b>Small molecules</b>						
Filgotinib	GLPG0634	JAK-1	Clinical remission at 10 weeks (47% vs. 23% – placebo) in CD	None reported	Phase III trials for CD/UC	[100]
Upadacitinib	ABT-494	JAK-1	Clinical remission at 16 weeks (27% – 6 mg vs. 11% – placebo) in CD	Headache, non-melanoma skin cancer	Phase II trials for CD/UC	[97]
Ozanimod	RPC1063	sphingosine-1-phosphate receptor	Clinical remission at 32 weeks (21% – 1 mg, 26% – 0.5 mg vs 6% – placebo) in UC	Headache, anemia, nasopharyngitis, urinary-tract infections	Phase III trials for UC	[96]
Mongersen	GED-0301	TGF- $\beta$ 1	Clinical remission at day 15 (55% – 40 mg, 65% – 160 mg vs 10% – placebo) in CD	None reported	Withdrawn after interim analysis of a Phase III trial for CD	[89,102]
	AJM300	$\alpha$ 4 integrin	Clinical remission at 8 weeks (23.5% – 960 mg vs. 3.9% – placebo) in UC	Potential risk of PML	Phase III trials for UC	[91]
Laquinimod	ABR-215062 or TV-5600	Inhibitory effect on antigen presenting cells and T cells	Clinical remission at 8 weeks (48.3% – 0.5 mg vs. 15.9% – placebo) in CD	Headache, CD exacerbation	Phase II trials for CD	[86]
	ABX464	Triggers IL-22 secretion in macrophages	50 mg, daily for 2 months, clinical remission 35% vs 11% placebo in UC	Headaches, nausea and vomiting (not considered as treatment-limiting effects)	Phase II trial for UC	[93]

frequently with SAEs and infections compared with patients treated with placebo, the promising data generated with this Ab led to growing interest in developing other anti-integrin Abs, such as etrolizumab (rhuMAb  $\beta 7$ ), abrilumab (AMG 181), and PF-00547659, which are currently being evaluated in clinical trials (Table 2, and references therein). PF-00547659 is a fully human mAb that binds to human mucosal addressin cell adhesion molecule (MAdCAM), which is predominantly expressed on the cell surface of high endothelial venules of organized intestinal lymphoid tissues (Peyer's patches and mesenteric lymph nodes). It was found to selectively reduce lymphocyte homing to the intestinal tract. Although compared with placebo, this mAb did not meet the primary endpoint of clinical response in moderate-to-severe CD, it raised great hopes as it presented some appreciable pharmacological effects, which remain to be analyzed further [98].

#### *Adverse Effects of Biologics*

Several clinical trials and meta-analyses have verified the efficacy and safety of biologic-based therapies. The risk of SAEs associated with these therapies is lower compared with other conventional (immunosuppressive) treatments, and some biologics have proved to be beneficial in the induction and maintenance of clinical remission and response [105,106]. However, cases of SAEs including hypersensitivity reactions, injection site reactions, skin cancers, drug-induced lupus, psoriasis, reactivation of latent tuberculosis, hepatotoxicity, lymphomas, and solid tumors have been reported (Tables 1 and 2). In addition, the high production cost of therapeutic mAbs remains a hurdle in maintaining the cost-effectiveness of these drugs [107,108].

Another serious issue that is encountered with certain biologics is the generation of anti-drug Abs (ADAs) that makes at least 40% of the patients receiving anti-TNF drugs secondary nonresponders. This loss of responsiveness mostly occurs in the case of patients receiving episodic therapy or in the presence of lower levels of ADAs against other anti-TNF agents received earlier (including biosimilars) [109]. According to previous studies, the formation of Abs against infliximab occurs in 61% of patients receiving episodic treatment and 44% of patients losing response to adalimumab were found to have developed Ab to adalimumab. It is a general observation and a source of concern that more and more cases of ADAs are reported in the literature [110–114], influencing the efficacy of treatment and the potential clinical improvement of patients under biotherapy. Sensitive assays have been developed to detect ADAs that are produced early in certain individuals and can dramatically affect the results of clinical trials and the efficacy of current treatments in patients [110,115,116]. A careful follow-up of patients throughout their treatment should be performed. High serum concentrations of anti-TNF drugs are associated with improved clinical outcomes in UC patients. In contrast low concentrations have been shown to frequently associate with the formation of ADAs [117,118].

#### *Small Molecules for Treating IBDs*

In terms of small molecules, apilimod mesylate {N-[(E)-(3-methylphenyl)methylideneamino]-6-morpholin-4-yl-2-(2-pyridin-2-ylethoxy)pyrimidin-4 amine; formerly STA-5326}, which inhibits IL-12/IL-23, was evaluated in clinical trials including patients with CD [119]. Up to 700 subjects have been treated with mild-to-moderate AEs. However, apilimod did not meet the primary endpoints in Phase II inflammatory disease indications [120]. This molecule is currently being evaluated in other indications.

ABX464 {8-chloro-N-[4-(trifluoromethoxy)phenyl]quinolin-2-amine} is a small molecule that induces IL-22 production in macrophages, which may act on intestinal inflammation. Laquinimod (ABR-215062 or TV-5600; developed by Active Biotech and Teva) is another oral drug

that has inhibitory effects on antigen-presenting cells and T cells, resulting in reduced proinflammatory cytokine production. In randomized controlled trials laquinimod was efficacious for CD (Table 2). Head-to-head studies with existing treatments and longer-term safety data are however needed at this stage of investigation.

The Janus kinase (JAK)/STAT pathway is a major signaling cascade downstream from the cytokine and growth factor receptors, and hence JAK inhibition has been shown to be potentially therapeutic in IBDs. Tofacitinib (Xeljanz) is a pan-JAK inhibitor currently available for treatment of UC [121]. Other molecules such as filgotinib and upadacitinib (JAK1 inhibitors) are undergoing clinical trials for CD and UC.

Mongersen (GED-0301), an oral oligonucleotide drug containing an anti-SMAD7 (mothers against decapentaplegic homolog 7) oligonucleotide has proved to be able to restore signalling by the mucosal anti-inflammatory cytokine TGF- $\beta$ 1. Although positive results were obtained in Phase II trials for CD with a clinical remission rate of 72% after 2 weeks of treatment, this drug was withdrawn from clinical studies in November 2017 due to disappointing results from an interim analysis of a Phase III study [89,102].

Small molecules targeting leukocyte trafficking are also being currently investigated. One of them is the  $\alpha$ 4-integrin antagonist AJM300, an oral phenylalanine derivative, which is presently evaluated in Phase III clinical trials for UC (investigated in a small cohort of patients until now; Table 2). Amiselimod (MT1303; Biogen), ozanimod (RPC1063; Celgen), and etrasimod (APD334; Arena Pharmaceuticals) are other molecules that act as sphingosine-1-phosphate receptor modulators, which lead to lymphocyte sequestration in lymph nodes and reduce the migration of lymphocytes to the gastrointestinal tract. The development of amiselimod, which was in Phase II clinical trials for CD has been halted, as Biogen is currently focusing on other drugs from its portfolio. A Phase III clinical trial of ozanimod in patients with moderate-to-severe UC is ongoing. Etrasimod is also being tested in a Phase II trial in UC [96].

#### Pros and Cons: How Can We Progress?

Although the advent of all these therapeutic options greatly helps to maintain middle-term remission and improve the IBDs patients' quality of life to a certain extent, we must recognize that patients remain mostly symptomatic and the therapies do not address the root genetic causes [4–6,17,68]. Besides, their high cost, severe impacts, and the SAEs of some of these treatments in the long-term, there is a need for the development of cost-effective small molecule drugs that are disease specific. In this context, several elements of the autophagy pathway might be key targets for novel therapeutic options.

### Autophagy, an Emerging Element in the Regulation of IBDs and a Novel Therapeutic Option

#### IBDs and Autophagy

Autophagy is a crucial intracellular pathway that continuously degrades, recycles, and clears unnecessary or dysfunctional cellular components (e.g., damaged organelles, or proteins abnormally folded or produced in excess). It is a finely gene-regulated and evolutionarily conserved process. Autophagy is prominent in the adaptation of cells to their environment and in the maintenance of cell homeostasis, especially under stress conditions (nutrient deprivation, hypoxia, oxidative stress, or changes in intracellular levels of calcium). It is thus a central actor in cellular processes, such as development, lineage differentiation, and immunity.

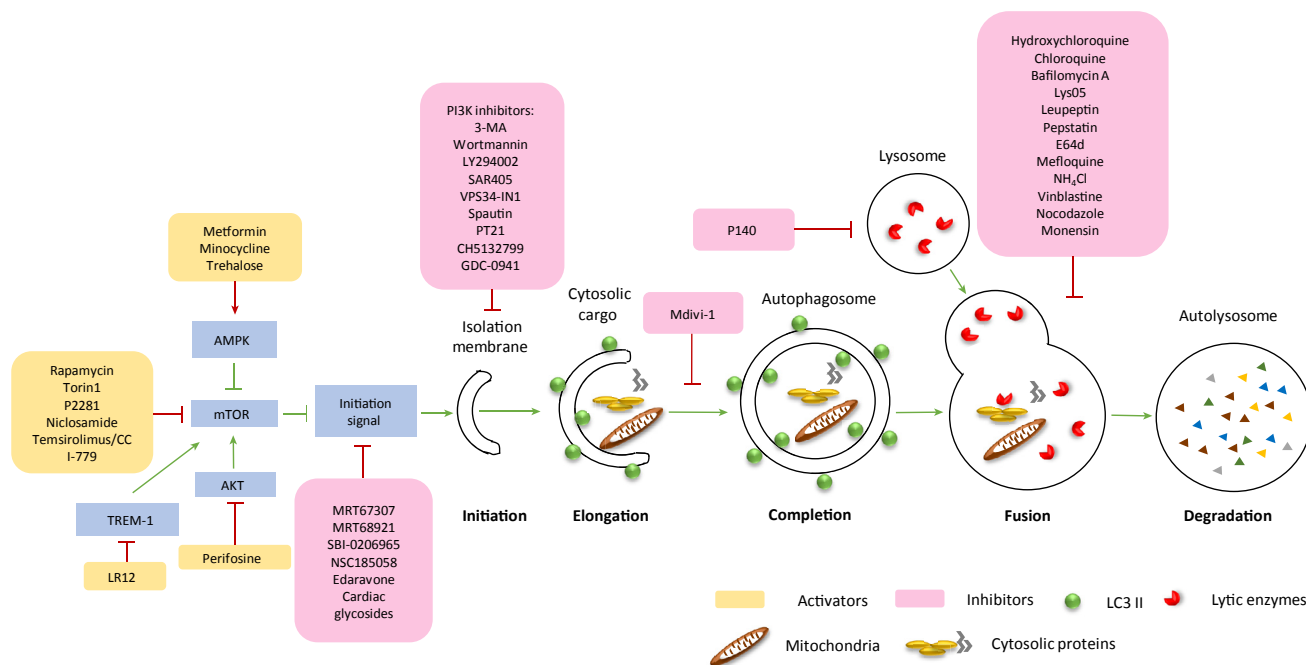
Among the pathways that have been associated with so-far-identified IBD risk loci, autophagy seems to be significant. Since the identification of the autophagy-related gene (ATG) 16L1 (Box 3) as a major player in IBD genetics in 2006 [122], various studies have repeatedly established a link between IBDs, ATG16L1, and the process of autophagy [123–130]. It has been shown that ATG16L1 modulates ubiquitination of the adaptor protein sequestosome 1 (SQSTM1)/p62 through the neddylation of cullin-3 (a core element of a complex known as an E3 ubiquitin ligase), leading thus to the suppression of IL-1 $\beta$  signaling [131]. Animal models with mutations in *Atg16l1* (e.g., *Atg16l1* T300A knock-in mice and especially *Atg16l1/Xbp $\Delta$ IEC* mice) have been used to reinforce our understanding of the importance of this gene in the development of IBDs [132,133]. Other ATGs such as genes encoding **immune-related GTPase M (IRGM)** [134–137], **leucine-rich repeat kinase 2 (LRRK2)** [138], and **nucleotide-binding oligomerization domain (NOD)2/CARD15** [125,139], have also been closely associated with CD and UC [140–142].

Some alterations – either upregulation or downregulation – in several autophagy pathways, including macroautophagy, chaperone-mediated autophagy (CMA; Box 1), mitophagy, and other forms of autophagy, have been implicated in numerous (auto)immune and inflammatory disorders, such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), RA, psoriasis, some neuroinflammatory and neurodegenerative diseases including MS, chronic inflammatory demyelinating polyneuropathies, amyotrophic lateral sclerosis (ALS), and Huntington's, Alzheimer's, and Parkinson's diseases [126,141,143–151]. Hence, components of this central metabolic system have recently emerged as particularly attractive, and even key therapeutic targets in many of these diseases [152–159]. A previous study has shown, for example, that a mammalian target of rapamycin (mTOR) inhibitor molecule (Figure 2), namely a haloacyl aminopyridine-based molecule called P2281, was efficient in a murine model of DSS colitis by inhibiting T cell function [160]. Another report also concluded that rapamycin/sirolimus, a macrocyclic triene antibiotic, which binds to the cytosolic 12-kDa tacrolimus-binding protein (FKBP12) and also inhibits the mTOR pathway, could represent a good candidate to treat CD patients [161]. In a retrospective analysis of patients treated with rapamycin, five of 11 UC patients and all three CD patients achieved clinical remission. An additional two UC patients achieved clinical response. The remaining four UC patients did not respond to rapamycin treatment. Mucosal healing was achieved in five of 11 UC patients and two of three CD patients. Clinical response to treatment occurred at least 2 weeks after treatment was started. The only significant AE reported was minor gastrointestinal distress [161]. This report confirms some data generated in TNBS-treated mice, that intestinal inflammation and colitis are ameliorated by rapamycin and trehalose [162]. P2281, rapamycin, and trehalose all affect the macroautophagy pathway (Figure 2). Besides their pathophysiological interest, the aforementioned results present potential pharmacological evidence that targeting autophagy using small molecules is sufficiently robust for future treatment options.

### Box 3. *ATG16L1*, an Autophagy-Related Gene That Is Associated with Risk of IBDs

ATG16L1 is an essential component of autophagy [199]. It undergoes self-multimerization and forms a heterocomplex with ATG5 and ATG12, which acts as a scaffold to MAP1LC3 for lipidation. Among the nine genetic variants of *ATG16L1* that are associated with CD, the variant rs2241880, comprising a missense mutation resulting in threonine to alanine substitution at the amino acid position 300 is associated with an increased risk of developing the disease. The T<sup>300</sup>A mutation is located in the cleavage site of caspase 3, and this mutation enhances the degradation of ATG16L1 by caspase 3, and hence diminishes autophagy. Loss of function of ATG16L1 inhibits autophagy in intestinal Paneth cells, resulting in a decreased production of antimicrobial peptides. In addition, *ATG16L1* risk variants are defective in the generation of MHCII antigen-specific CD4<sup>+</sup> T cell responses in DCs.





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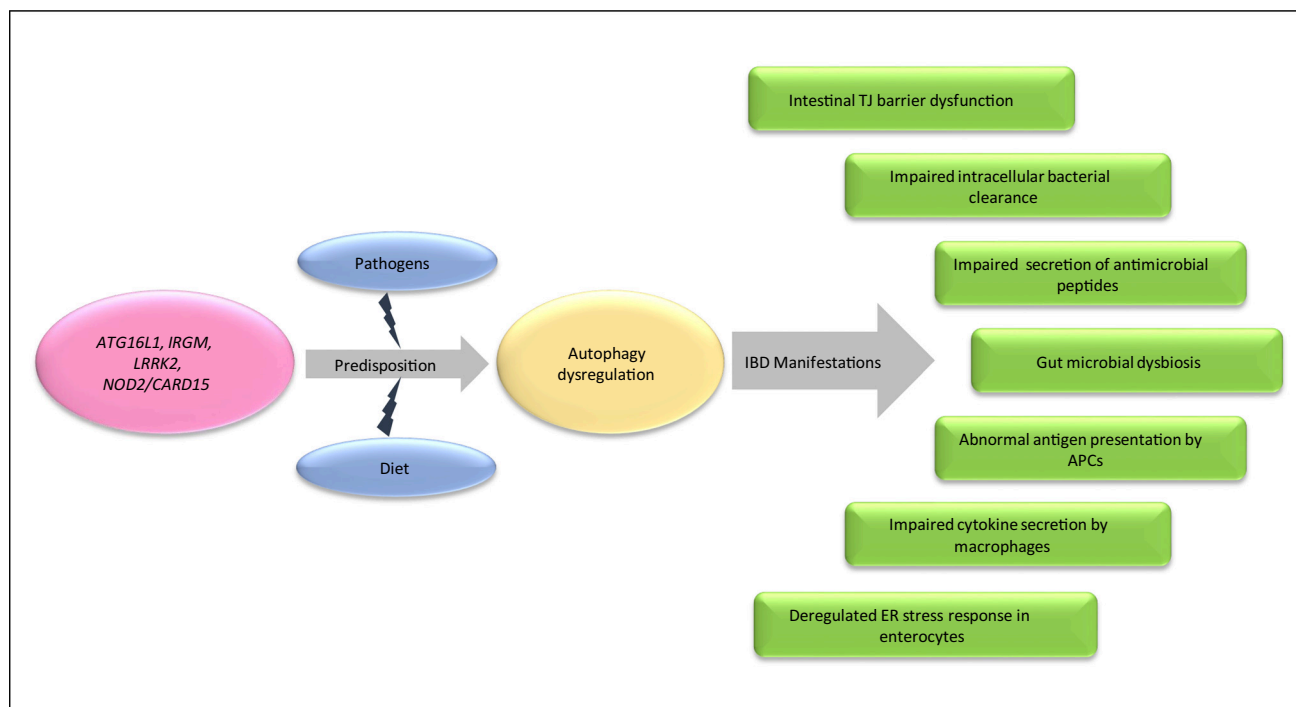
**Figure 2. Signaling Pathways That Control the Macroautophagy Machinery and Sites of Action of Past and Novel Autophagy-Regulating Pharmacological Tools.** Autophagy can be activated by inhibitors of mTOR (rapamycin, torin1, P2281, niclosamide, temsirolimus/CCI-779), AKT (perifosine), TREM-1 (LR12 peptide), as well as by activation of AMPK (metformin, minocycline, and trehalose). Inhibitors of autophagy include PI3K inhibitors (3-MA, wortmannin, LY294002, SAR405, VPS34-IN1, spautin, PT21, CH5132799, and GDC-0941), initiation inhibitors (MRT67307, MRT68921, SBI-0206965, NSC185058, edaravone, and cardiac glycosides), a phagophore elongation inhibitor Mdivi-1 and autophagosome-lysosome fusion inhibitors (e.g., bafilomycin A, vinblastine, nocodazole, and monensin). The final degradation step of autophagy can also be inhibited by compounds such as hydroxychloroquine (HCQ), chloroquine (CQ), bafilomycin A, lys05, leupeptin, pepstatin, E64d, mefloquine, and NH<sub>4</sub>Cl. In lupus, administration of P140 peptide significantly decreases the overexpression of LAMP2A and HSPA8, which are key factors of chaperone-mediated autophagy (CMA). Abbreviations: 3-MA, 3-methyladenine; AMPK, AMP-activated protein kinase; Mdivi-1, mitochondrial division inhibitor; mTOR, mammalian target of rapamycin; TREM-1, triggering receptor expressed on myeloid cells 1.

### Autophagy Pathways: Novel Options to Treat Patients with IBDs

As described above, autophagy plays multiple roles in IBD pathogenesis. Under the control of genes such as *ATG16L1*, *IRGM*, *LRRK2*, and *NOD2/CARD15*, the expression of which appears deregulated in susceptible patients, several vital functions assumed by autophagy processes are severely altered. For example, intracellular bacterial killing, antimicrobial peptide secretion by Paneth cells, goblet cell functions, proinflammatory cytokine production by macrophages, antigen presentation and processing by antigen-presenting cells (DCs, B cells, and macrophages), and the endoplasmic reticulum stress response in enterocytes are all affected as a result of deregulation in these genes (Figure 3) [4,5,17,142,144]. Thus, the first line of defense against pathogenic infection and many other aspects of the innate and adaptive immune response are profoundly unbalanced. Therefore, elements of autophagic pathways represent targets of choice to probe in novel therapeutic options for IBDs.

### Multiplicity of Targets of Current Antiautophagy Regulators

Developing an effective treatment targeting specific components of autophagic pathways requires identification of the elements of the said pathways that are crucial, whose expression is specifically modified (activated or repressed) as a result of the pathophysiological context, and for which we possess tools (existing drugs or newly developed molecules) that reach their



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**Figure 3. Role of Autophagy in the Pathogenesis of IBDs.** Genetic predispositions in autophagy-related genes (*ATG16L1*, *IRGM*, *LRRK2*, and *NOD2/CARD15*) are largely reported in IBDs. Upon environmental triggers, such as pathogens and unhealthy diet, defects in multiple steps of autophagy may lead to altered functions of the intestinal barrier and abnormal intestinal immune responses, which can collectively manifest as IBDs. Abbreviations: APC, antigen-presenting cell; ER, endoplasmic reticulum; IBDs, inflammatory bowel diseases; TJ, tight junction.

target (preferably one) without generating AEs in unrelated metabolic circuits. Small molecules possess several pharmacological receptors, and targeting some of them may generate unwanted reactions that hamper or limit their use due to their deleterious influence on vital functions.

A number of activators and inhibitors of autophagy have been described (Figure 2) [153–155,157,158,163–168], and a growing list of patents have been filed. They notably include phytochemicals and antioxidants (e.g., polyphenol, curcumin, and resveratrol) [169]. In almost all cases, their precise specificity is not known. Few of them, if any, interact with one single target of a specific autophagic process (e.g., macroautophagy, microautophagy, CMA, mitophagy, or lipophagy), and the fact that these pharmacological molecules may cause several effects, via distinct cell receptors and intracellular pathways, can render them harmful to health. An illustrative example is **hydroxychloroquine (HCQ)** (Plaquenil, Axemal, Dolquine, and Quensyl). HCQ is a potent autophagy inhibitor, which affects the lysosomal pH (Figure 2) but also, inhibits CXCL12/CXCR4 signaling, resulting in reduced phosphorylation of extracellular signal-regulated kinases (ERKs) and STAT3. The main concern regarding HCQ is its retinal toxicity that requires regular ophthalmic follow-up to evaluate the extent of eventual retinopathy in susceptible individuals [156,170]. Identifying the retinal target of HCQ should help in generating a class of molecules that retain their primary efficacy without causing secondary deleterious effects.



Rapamycin (discussed above) is another example of a molecule that exerts potent effects on different targets. It has been shown to acutely inhibit the mTOR complex (mTORC1), whereas chronic exposure to rapamycin can also inhibit mTORC2; two functionally distinct complexes in mammalian cells [171]. These complexes share some protein components, but their distinctive activities are defined by their unique components, namely Raptor (mTORC1), and Rictor and mSin1 (mTORC2). Rapamycin forms a tripartite complex with mTOR and FKBP12 (see above) that leads to mTORC1 inhibition. The complexity of its effects on mTORC2 is not completely resolved [172].

Another example of this complexity emerges with metformin (Glucophage, among other names; Box 4); a synthetic derivative of guanidine that acts as an inducer of autophagy but also displays several other effects via different targets. Several independent studies have shown that metformin ameliorates IBDs [173–175]. In an experimental model, administration of metformin reduced inflammation through the inhibition of phospho (p)-STAT3, IL-17, and p-mTOR expression and the increased expression of phospho-AMP-activated protein kinase (p-AMPK) and Foxp3 [173]. It has also been demonstrated that metformin limits DSS-induced intestinal barrier disruption by a mechanism involving the inhibition of c-Jun N-terminal kinase activation via an AMPK $\alpha$ 1-dependent signaling pathway [174].

#### Selective Regulators of Autophagy

In recent years, much effort has been made to identify more selective drug targets, in particular, based on interactome or metabolomics studies, and accordingly to redesign some molecules that were discarded to render them more selective of the chosen target. This kind of investigation is especially important in IBDs to adapt treatment in a frame of personalized and precision medicine that aims at optimizing treatment practices with significantly reduced SAEs.

One example of this new family of molecules is the peptide LR12 [176] of sequence H-LQEEDTGEYGCV-NH<sub>2</sub> that inhibits the triggering receptor expressed on myeloid cells 1 (TREM-1). LR12 has been shown to correct the severity of colitis clinically, endoscopically, and histologically in a DSS-induced mouse model of colitis [177]. TREM-1 is expressed on the majority of innate immune cells and to a lesser extent on parenchymal cells. The frequency of TREM-1-expressing neutrophils and recruited macrophages has been found to be higher in inflamed than in noninflamed biopsies from patients with UC and CD [178]. Injection of LR12 peptide in DSS-induced model mice generated a significant increase of macroautophagy (ATG1/ULK-1, ATG13, ATG5, ATG16L1, and MAP1LC3-II) and CMA (HSPA8 and HSP90AA1) protein expression. This impressive effect of the LR12 peptide was confirmed genetically using *Trem-1* knockout mice [177]. TREM-1 inhibition prevented dysbiosis.

#### Box 4. Metformin, a Molecule That Displays Pleiotropic Effects on Autophagy

Metformin (or 1,1-dimethyl biguanide) is widely given to patients with type 2 diabetes. Recent investigations led to the discovery that this synthetic derivative of guanidine displays a larger spectrum of properties than expected and could, therefore, be advantageously used for other indications, such as autoimmune diseases, certain cancers (breast, ovarian, and glioblastoma), and in aging [200,201]. Metformin is an inducer of autophagy that acts in an AMPK-dependent manner, which phosphorylates the Unc-51 like autophagy activating kinase-1 (ULK-1/ATG1) and BECLIN 1. Metformin interacts with several receptor molecules and, directly or indirectly, interferes with several cellular pathways that are vital in cell metabolism and regulation, notably in immune cells. The mitochondrial respiratory-chain complex 1 (OCT1) is presented as the primary target of metformin [202]. The preferential action of metformin in hepatocytes is due to the predominant expression of OCT1, which has been shown to facilitate cellular uptake of metformin. It seems that metformin does not directly target AMPK but activates AMPK in a process that is secondary to its effect on the mitochondria; the primary target of the drug. Regarding the effect of metformin on the insulin receptor, it acts through inhibition of PTP-1B, a phosphatase that inhibits the tyrosine kinase activity of the insulin receptor.

To our knowledge, the cell-permeable transactivator of transcription (TAT)-coiled-coil, moesin-like BCL2-interacting protein (BECLIN)-1 peptide construct (YGRKKRRQRRRGGTNVFNAT-FEIVHDGEFGT) [179], which has shown some promise in several neurological, infectious, and tumoral settings and is now commercialized, has not been evaluated in experimental models of IBDs. TAT-BECLIN peptide might have interesting applications since it has been proposed that BECLIN-1 regulates TJ barrier function via endocytosis of occludin (a 65-kDa tetraspan integral membrane protein) in an ERK- and mTORC2-dependent way [180].

P140 (Rigerimod or IPP-201101) is another peptide that selectively targets autophagy processes, and more especially CMA. This 21-mer phosphopeptide corresponding to the sequence H-RIHMYSKRSGKPRGYAFIEY-OH (residues 131–151) was described in 2003 [181]. It was initially spotted in a cellular screening assay using overlapping peptides covering the whole spliceosomal U1-70K protein and CD4<sup>+</sup> T cells collected from MRL/lpr mouse lymph nodes. A number of analogs have been produced and the one that possesses the most favorable properties contains a phosphoserine residue at position 140, which is crucial for its activity and stability [182–185]. P140 is not immunogenic [185], it is safe, and displays no immunosuppressive activity in mice and humans [186–190]. It directly interacts with HSPA8 [182,191] and inhibits the chaperone activity of the latter [182,183]. It also alters HSPA8 shuttling between the cytoplasm and the nucleus/nucleoli in case of stress [192]. It has been shown *in vitro* and *in vivo* that P140 enters MRL/lpr spleen B cells via a clathrin-dependent pathway and accumulates in lysosomes [148]. Notable effects associated with different components of the autophagy process were identified after treating cells and autoimmune mice with P140. The levels of HSPA8 and LAMP2A, which are overexpressed in MRL/lpr B cells, are corrected after P140 treatment. P140 has no direct effect on B cell receptor signaling in memory, naïve, mature, transitional, or B1 human cells, suggesting that it does not alter B cell survival and maturation in these B cell subsets [190]. However, likely as a matter of consequence resulting from its interaction with HSPA8, it strongly reduces the overexpression of MHC class II molecules on lupus B cells acting as antigen-presenting cells, and hampers peptide–MHC molecule loading in late lysosomal vesicles [143,148,183,190]. This impressive effect has been shown in mice and humans, and decelerates the complex signaling cascade, leading to the final production of pathogenic auto-Abs. P140 effectively downregulates T cell activation [187] and consequently reduces the differentiation of human B cells into plasma cells and IgG secretion [190]. Altogether, these results indicate that by interfering with overactivated autophagy processes, P140 peptide efficiently affects the processing of endogenous (auto)antigens, the peptide loading to MHCII molecules, and the entire downstream deleterious proinflammatory events. It must be emphasized that the normal immune system is not affected in this scheme, and that experimental MRL/lpr mice are still capable of developing cellular and humoral immune reactions towards a pathogen [187]. In a multicenter, randomized, placebo-controlled Phase IIb study for lupus, P140/Lupuzor was found to be safe and met its primary efficacy endpoints, confirming preclinical data generated in MRL/lpr lupus-prone mice [189]. Lupuzor is currently being evaluated in Phase III clinical trials in the USA, Europe, and Mauritius. An open-labeled trial including several hundred lupus patients worldwide is planned. P140 is also evaluated in the context of other autoimmune or inflammatory conditions and has shown some promise in preclinical studies including experimental animal models [143,150,151]. Preliminary data have tended to show that P140 is also a valuable tool for treating IBDs. Future investigations based on chemically and genetically induced murine models, organoids, and cells collected from patients with CD and UC are warranted to determine if P140 could be exploited as a potent drug in affected patients.

### Clinician's Corner

Genetic susceptibility, environmental factors, microbial flora, and alterations affecting both the innate and adaptive immune systems are common components that are recognized as major contributors to the complex set of IBDs. Most importantly, the cytokine imbalance of proinflammatory and favorable regulating cytokine responses is thought to be critically involved. The current strategies consider that these elements and some immunosuppressive drugs, corticoids, and biologics have shown efficacy in reducing, at least transiently, disease progression.

Some of the current drugs display harmful effects that can generate even more dramatic health status. Thus, deciphering further the molecular and cellular elements giving rise to IBDs is necessary.

The objective of novel therapeutic strategies is to replace disease-modifying medications by mechanism-driven therapies, which will be more targeted and specific than the current ones and should therefore prove to be safer for the patients. Such targeted therapies could be personalized if appropriate biomarkers of responsiveness can be identified, avoiding thus the use of medications that are ineffective in individual patients.

Due to the diversity of symptoms in IBDs, and the extent and location of inflammation, it is unlikely that a single drug will correct all of the issues in the millions of patients affected by CD and UC. Combination therapy, or polytherapy, should help to control these aspects. In these cases, however, particular caution should be taken to avoid administration of molecules with opposite properties that could adversely affect certain individuals.

## Concluding Remarks

Research for therapeutic options to treat IBDs has identified new compounds targeting elements involved in maintaining intestinal homeostasis, and has identified biomarkers allowing detection of inter- and intrapersonal variations in patients (see Clinician's Corner). In the pipeline of new possible treatments, elements of the autophagy process are particularly indicated. In this context, a crucial aspect that has largely hampered the clinical applications of autophagy-based therapeutic strategies is that until now, among the large set of existing activator/inhibitor molecules, few are strictly selective for one autophagy pathway and one target. In general, molecules such as rapamycin, HCQ, trehalose, metformin, perifosine (inhibitor of protein kinase B or AKT), minocycline (a semisynthetic tetracycline derivative with dual properties on autophagy processes), or niclosamide (inhibitor of mTORC1) interact with several targets and receptors [193]. This favors SAEs and therefore limits their use as drugs. Intense research is therefore devoted to identification of small molecules and peptides to precisely up- or downregulate specific autophagy processes that are pathologically defective without interfering with other autophagy processes.

Another aspect that further complicates the design of new strategies based on deregulated autophagy is that in a single individual, autophagic activity can be raised in certain organs or tissues, and diminished in others [143,194], in an order that can vary from patient to patient [194]. More research is therefore needed to understand the interplay between the different autophagic pathways that are supposed to protect cells and ensure cell homeostasis, and the effects of counterbalance between them in the same organ, and in cells of different organs (see Outstanding Questions). We should not have a reductionist definition of phenomena, and just claim that autophagy, as a whole, is exacerbated or compromised in a particular illness. Instead, we would be well advised to more precisely define the type of autophagy pathway, and in which organ and cell subtype these defaults occur [168]. Further investigation is also needed to discover valid predicting markers of drug responsiveness. This complete set of information is crucial in order to direct rescuing molecules to specific sites of autophagy dysregulation, and to design more personalized and safe therapeutic options. Finally, new directions taking into account the specific infectious facet of IBD-affected patients should lead to the development of new precision medicine based on molecules that selectively target xenophagy, which would also contribute to eliminating invading pathogens.

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## Disclaimer Statement

S.M. has the following conflicts of interest to disclose: research funding (paid to institution) and past consultant for ImmuPharma; co-inventor of CNRS-ImmuPharma patents on P140 peptide. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Outstanding Questions

Have we accumulated enough robust data to claim that targeting autophagy pathways is an effective way of intervention in IBD? What are the best animal models to progress?

Is it possible to devise therapeutic tools able to correct the faults in one autophagy pathway without affecting the other cell death/survival pathways?

What are the criteria to design and efficiently deliver smart drugs that ensure optimal therapeutic responses and safety?

Will strategies targeting autophagy processes be more efficient than current medications given to patients with IBDs?

Taking into account the specific infectious facet of IBD-affected patients, should we pay more attention to xenophagy, an autophagy pathway that specifically involves pathogens?

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# L'autophagie lysosomale, une nouvelle cible thérapeutique dans les maladies inflammatoires de l'intestin

## Résumé

Les maladies inflammatoires de l'intestin (MICI) sont des troubles chroniques idiopathiques du tractus gastro-intestinal qui touchent principalement la population jeune. On attend toujours des médicaments ciblés sur la maladie, tolérables et rentables pour traiter les MICI. La découverte de l'implication cruciale de la voie de l'autophagie dans la prédisposition génétique des MICI a ouvert une nouvelle voie d'exploration pour la thérapie des MICI. L'autophagie est un processus vital qui joue un rôle central dans le maintien de l'homéostasie cellulaire et la régulation pharmacologique de l'autophagie s'est avérée bénéfique dans plusieurs maladies. Nous avons donc évalué l'efficacité du P140 - un phosphopeptide thérapeutique connu pour moduler les processus d'autophagie dans d'autres conditions auto-immunes et inflammatoires - dans des modèles murins de MICI. Nous avons démontré que le peptide exerce des effets protecteurs sur les modèles de colite et corrige les dysfonctionnements pathologiques dans différentes voies d'autophagie. Ainsi, après l'ère des médicaments classés comme thérapeutiques "modificatrices de la maladie", les nouveaux produits pharmaceutiques "guidés par les mécanismes" semblent très prometteurs pour le traitement des maladies inflammatoires.

**Mots clés :** • Maladie inflammatoire de l'intestin • autophagie • thérapie à base de peptides • peptide P140 • colite expérimentale

## Résumé en anglais

Inflammatory bowel diseases (IBD) are chronic, idiopathic disorders of the gastrointestinal tract that affect mainly the young population. Disease-targeted, tolerable, cost-effective medications to treat IBD are still awaited. The discovery of the crucial involvement of the autophagy pathway in the genetic predisposition of IBD opened a novel avenue for exploration in IBD therapeutics. Autophagy is a vital process that plays a central role in maintaining cellular homeostasis and pharmacological regulation of autophagy has proven to be beneficial in several diseases. Hence, we evaluated the efficacy of P140 - a therapeutic phosphopeptide known to modulate autophagy processes in other autoimmune and inflammatory conditions - in murine models of IBD. We have demonstrated that the peptide exerts protective effects on colitis models and corrects the pathological dysfunctions in different autophagy pathways. Thus, after the era of drugs classified as "disease-modifying" therapeutics, emerging "mechanism-guided" pharmaceuticals seem to hold a lot of promises for treating inflammatory diseases.

**Keywords:** Inflammatory bowel disease • autophagy • peptide-based therapy • P140 peptide • experimental colitis