UNIVERZA V LJUBLJANI

FAKULTETA ZA FARMACIJO

EMA SEVER

AVTOFAGIJA V ČREVESNIH EPITELIJSKIH CELICAH

(AUTOPHAGY IN INTESTINAL EPITHELIAL CELLS)

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJE

Ljubljana, 2016

The laboratory work was carried out at the Institute of Pharmacology at the University of Bern, with collaboration of the Faculty of Pharmacy, University of Ljubljana. I worked under the supervision of Prof. Dr. Hans-Uwe Simon and home mentorship of Prof. Dr. Irena Mlinarič-Raščan.

Acknowledgments:

First, I would like to thank Prof. Dr. Hans-Uwe Simon who gave me the opportunity to perform my Master thesis at the Institute of Pharmacology, to work in his research group and who gave me access to the laboratory and research facilities. His guidance helped me in all the time of research and writing of this thesis. I very much appreciated his advice.

Special thanks goes to my advisor Dr. Christiane Sokollik for the continuous support, her patience, motivation and encouragement. I felt very welcomed in her little group. She did not hesitate with sharing her knowledge with me and taught me that one can achieve anything, if one works hard. She truly was my inspiration.

I am very thankful to Prof. Irena Mlinarič-Raščan who supported and advised me after my return from Bern. She was always available to answer my questions.

I would like to thank also all members at the institute who created pleasant environment where I felt very welcomed. In particular, I am grateful to Flavia Büchler, who helped me with practical work.

Last but not least, I would like to thank my family for supporting me throughout writing this thesis and all my years of study. This thesis is dedicated to you.

Statement

Hereby, I testify having performed the experiments to the best of my knowledge and having written this thesis independently under guidance of my supervisors: Prof. Dr. Irena Mlinarič-Raščan and Prof. Dr. Hans-Uwe Simon.

Ljubljana, September 2016 Ema Sever

President of the Thesis defence committee: Prof. Dr. Julijana Kristl

Member of the Thesis defence committee: Assist. Prof. Dr. Mojca Lunder

TABLE OF CONTENTS

TABLE OF FIGURES

ABSTRACT

Intestinal epithelial cells represent the barrier between the antigen-filled gut lumen and the host's immune system. Through cytokine secretion and adhesion molecule expression, these cells perform also immunoregulatory function. Both tasks are defective in inflammatory bowel disease (IBD). One major characteristic of IBD is a disrupted barrier function with an increased level of proinflammatory cytokines and adhesion molecules.

Autophagy is a cell recycling mechanism that has recently received attention in relation to IBD as genetic studies have identified several susceptibility loci involved in autophagic machinery. Thus, our investigations focused on the potential involvement of autophagy and its effect on three parameters that are altered in the inflamed gut of IBD patients: tight junction integrity, adhesion molecule expression and proinflammatory cytokine secretion.

Autophagy inducers and blockers were used for autophagy manipulation in cytokine treated Caco-2 cells, thus simulating a proinflammatory environment similar to that found in the inflamed gut. Western blot, immunofluorescence staining, transepithelial resistance measurement, permeability assay, neutrophil adhesion assay, ELISA and flow cytometry were performed for the purpose of our investigation.

Enhancing autophagy did not alter the effects induced by proinflammatory cytokine treatment in regards to tight junction structure, tight junction function and adhesion molecule expression. Disrupting autophagy, on the other hand, diminished cytokine-induced intercellular adhesion molecule-1 expression and significantly reduced interleukin-1-beta-mediated interleukin-8 secretion. Importantly in functional studies a reduction in neutrophil adherence was found. No effect was observed on barrier integrity, neither with manipulation of autophagy nor with sole cytokine treatment. We propose that additional stimuli are needed for barrier dysfunction. Our results suggest that disrupting autophagy may influence the inflammatory response in IBD patients. If this reaction is beneficial and whether it is important for the normal function of intestinal epithelial cells are further research questions of our group.

Keywords: *autophagy, intestinal epithelial cells, inflammatory bowel disease*

RAZŠIRJENI POVZETEK

Avtofagija je kataboličen proces, ključen za preživetje, diferenciacijo, razvoj in ohranjanje ravnovesja v celici. V normalnih razmerah ima pomembno vlogo pri odstranjevanju odsluženih in neželenih celičnih sestavin. Pri tem se ustvari dvojna membrana, obkroži tarčni del citoplazme in razgradi vsebino z lizosomalnimi encimi. Proces je ojačan v stresnih razmerah. Med stradanjem okrepljena avtofagija razgradi citoplazemske proteine in organele ter tako zagotovi energijo in gradnike, ki so nujno potrebni za celično preživetje. Avtofagija je vpletena tudi v obrambo pred patogeni, njeno disregulacijo pa povezujejo s številnimi patološkimi stanji.

Črevesni epitelij predstavlja največjo pregrado med človeškim telesom in zunanjim okoljem. Predstavlja ga tanka plast črevesnih epitelijskih celic, ki vršijo dve pomembni nalogi. S pomočjo tesnih stikov, ki se nahajajo med celicami in zapirajo paracelularni prostor, tvorijo bariero med, z antigeni bogatim, lumnom črevesa in pretežno sterilnim spodaj ležečim tkivom. Preko izločanja citokinov in ekspresije adhezijskih molekul na svoji površini opravljajo tudi imunoregulatorno funkcijo.

Obe funkciji črevesnih epitelijskih celic sta okvarjeni pri bolnikih s kronično vnetno črevesno boleznijo *(Inflammatory bowel disease-IBD)*, kamor sodita Crohnova bolezen in ulcerativni kolitis. Zanju je značilna povečana prepustnost črevesnega epitelija in iztirjen, prekomerno delujoč imunski sistem v gastrointestinalnem traktu. V črevesni sluznici bolnikov najdemo povišane vrednosti provnetnih citokinov in povečano število nevtrofilcev, ki se vežejo na medcelično adhezijsko molekulo ICAM-1. Z izločanjem dodatnih citokinov ti nevtrofilci še poslabšujejo vnetno stanje in s kemotaktičnimi molekulami privabljajo nove nevtrofilce. S tem je začarani krog sklenjen. Vzrok bolezni ni znan, predvidevajo, da gre za kombinacijo prekomerne aktivnosti imunskega sistema, mikrobioloških in okoljskih dejavnikov ter genetske nagnjenosti.

S pomočjo genetskih študij so znanstveniki identificirali več genomskih označevalcev, vpletenih v dovzetnost za IBD. Medtem ko, pričakovano, večina genov kodira za proteine, vpletene v signalizacijo citokinov in v uravnavanje prepustnosti epitelija, so nekateri pomembni za proces avtofagije. S tem odkritjem se je povečal interes za povezavo med tem reciklažnim procesom in IBD.

Ker naj bi bile motnje v avtofagiji vpletene v patogenezo IBD in ker sta za le-to značilna okvarjena barierna funkcija in prekomerna aktivnost imunskega sistema v črevesnih epitelijskih celicah, smo želeli preveriti njen vpliv na tri parametre, ki so v črevesu bolnikov značilno spremenjeni: integriteta tesnih stikov, ekspresija ICAM-1 in izločanje provnetnih citokinov. Naše raziskave so bile opravljene na Caco-2 celicah. Te pogosto služijo kot model črevesne bariere, saj spontano diferencirajo v enterocitom podobne celice in tvorijo tesne stike. Z uporabo interferona gama (IFN-γ), tkivo nekrotizirajočega dejavnika alfa (TNF-α) in interlevkina 1 beta (IL-1β) smo simulirali provnetno okolje, značilno za IBD. Obenem smo celice podvrgli bodisi induktorju, bodisi inhibitorju avtofagije in spremljali morebitne spremembe v vseh treh parametrih. Oba pristopa smo ubrali zato, ker ni jasno, ali je pri bolnikih avtofagija povečana ali zmanjšana.

Sprva smo se osredotočili na indukcijo avtofagije in uspešnost spremljali preko ugotavljanja količine njenega kazalca, proteina LC3-II, s prenosom Western. Provnetni citokini so že sami po sebi predstavljali stres za celico in okrepili avtofagijo. Tudi uporaba farmakološkega induktorja rapamicina je inducirala avtofagijo, ob hkratni uporabi s citokini pa jo močno ojačila.
Ko smo dokazali, da so Caco-2 celice sploh sposobne avtofagije, smo preverili njen vpliv na tesne stike. Osredotočili smo se na tretiranje s TNF-α, saj študije dokazujejo njegovo ključno vlogo v IBD. Imunofluorescenčno smo označili dva transmembranska proteina, ki tvorita tesne stike: okludin in ZO-1. Do sprememb v njuni lokalizaciji je prišlo po 72 urah izpostavitve TNF-α. Sočasna uporaba rapamicina ni vplivala na spremembe, povzročene s citokinom. Sledila sta dva funkcijska testa: merjenje transepitelne električne upornosti in test permeabilnosti, ki pa nista pokazala sigifikantnih sprememb v primerjavi s kontrolo. Zaključili smo, da je struktura Caco-2 celic po daljšem tretiranju s TNF-α spremenjena, a funkcija ohranjena. Domnevamo, da je za njihovo okvaro potreben dodaten dražljaj. Ojačitev avtofagije z rapamicinom situacije ni spremenila.

S prenosom Western smo preverili še vpliv na ekspresijo ICAM-1, adhezijske molekule, na katero se vežejo nevtrofilci. Provnetni citokini so, pričakovano, povečali njen nivo, sam rapamicin in kombinacija s citokini pa ne. Zaključili smo, da okrepitev avtofagije nima vpliva niti na ekspresijo adhezijske molekule in se v nadaljevanju osredotočili na njeno inhibicijo.

Uporabili smo farmakološki inhibitor klorokin. Zopet smo najprej dokazali, da lahko v Caco-2 celicah avtofagijo blokiramo. Nato smo z uporabo fluorescenčne mikroskopije, merjenja transepitelne električne upornosti in testa permeabilnosti spremljali vpliv na tesne stike. Tretiranje je trajalo le 24 ur, saj je daljša izpostavitev klorokinu toksična. Tudi v tem primeru manipulacija avtofagije ni imela vpliva na strukturo ali funkcijo tesnih stikov.

Do pomembnih ugotovitev smo prišli pri spremljanju vpliva na ICAM-1 s prenosom Western in pretočno citometrijo. Inhibicija avtofagije s klorokinom je zmanjšala s citokini inducirano ekspresijo te adhezijske molekule. Ker je slednja pomembna za adhezijo nevtrofilcev, smo le-te obarvali s fluorescenčnim barvilom in z mikroskopom ugotavljali njihovo vezavo na različno tretirane celice. Rezultati so potrdili naše ugotovitve, saj je bilo, v primerjavi s samim tretiranjem s citokini, število vezanih nevtrofilcev manjše pri sočasni uporabi klorokina in citokinov. Zaradi spodbudnih ugotovitev smo preverili še vpliv inhibirane avtofagije na izločanje provnetnih citokinov iz Caco-2 celic. Sočasno tretiranje s klorokinom je signifikantno znižalo z IL-1β inducirano sekrecijo IL-8.

Naše ugotovitve nadgrajujejo izsledki drugih študij in vključujejo transkripcijski faktor NF-κB. Ta je v neaktivnem stanju vezan na svoj inhibitor IκB in tako ujet v citoplazmi. Ko se IκB razgradi, je NF-κB prost in lahko inducira transkripcijo ICAM-1, IL-8 ter drugih tarčnih genov. Novejše raziskave kažejo na povezavo med razgradnjo inhibitorja IκB in procesom avtofagije. Z inhibicijo avtofagije s klorokinom smo torej po tej teoriji preprečili razgradnjo inhibitorja in s tem onemogočili transkripcijo analiziranih molekul.

Na osnovi rezultatov lahko zaključimo, da tretiranje s citokini poveča avtofagijo in ekspresijo ICAM-1 v črevesnih epitelijskih celicah. S sočasno uporabo klorokina se nivo ICAM-1 zmanjša, v primeru IL-1β pa tudi sekrecija IL-8. Sklepamo, da z inhibicijo potencialno povečanega procesa avtofagije lahko vplivamo na vnetni odgovor bolnikov z IBD. Nadaljnje študije naše skupine bodo slonele na genetskih pristopih za inhibicijo avtofagije in poskušale osvetliti, če je ta odziv koristen in dognati, kako pomemben je za normalno delovanje črevesnih epitelijskih celic.

Ključne besede: *avtofagija, črevesne epitelijske celice, kronična vnetna črevesna bolezen*

LIST OF USED ABBREVIATIONS

AMPK	AMP-activated protein kinase
ATG	Autophagy-related
BCA	Bicinchoninic acid
CD	Crohn's disease
CLB	Cell lysis buffer
Cq	Chloroquine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Diaminoethane-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IEC	Intestinal epithelial cells
IF	Immunofluorescence
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta

IL-6	Interleukin-6
IL-8	Interleukin-8
JAK	Janus-activated kinase
JAM	Junctional adhesion molecule
LC3	Microtubule-associated protein light chain 3
MAPK	p38 mitogen-activated protein kinase
MLCK	Myosin light-chain kinase
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
PBS	Phosphate-buffered saline
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PRR	Pattern recognition receptors
Rapa	Rapamycin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT	Signal transducers and activators of transcription
TBS	Tris-buffered saline
TEER	Transepithelial electrical resistance
TJ	Tight junction
TNF-α	Tumor necrosis factor alpha

UC	Ulcerative colitis
ULK1	Unc-51 like autophagy-activating kinase 1
ZO	Zonula occludens

1.) INTRODUCTION

1.1. Autophagy

Autophagy, literally meaning »self-eating«, is an evolutionarily conserved catabolic process that occurs in all eukaryotic cells [1, 2]. It is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis [3]. At base level activity it has an important role in removing exhausted, redundant or unwanted cellular components. By performing protein and organelle turnover it protects cells against damage caused by toxic macromolecules and damaged organelles. Autophagy is upregulated as a response to extracellular stress conditions. It is strongly induced during nutrient starvation, whereby cytoplasmic proteins and organelles are degraded and re-used for energy supply and the synthesis of components essential for survival. Hypoxia, overcrowding and high temperature are also known autophagy inducers [1, 4]. In addition to its role in cell survival, autophagy is involved in defense against invading microbes and, if exaggerated, it can result in a nonapoptotic cell death. Deregulation of autophagy has been proposed to play a role in certain diseases, including cancer, liver disease, cardiomyopathy, and muscular, neurodegenerative, and inflammatory diseases. Thus, autophagy plays a crucial role in human health and disease [3, 4].

Three forms of autophagy, which differ in respect of their physiological functions and the model of cargo delivery to the lysosome, have been identified: chaperone-mediated autophagy, microautophagy and macroautophagy [3]. In this work, I will focus on macroautophagy, herein referred to as autophagy.

1.1.1. The autophagic pathway

Although a first morphological description of autophagy in mammalian cells was reported in 1950s, a more detailed understanding of this recycling process was achieved in 1990s after the identification of autophagy-related (ATG) genes [5].

Autophagy is characterized by the sequestration of cytosolic proteins and organelles into double-membrane vesicles called autophagosomes. These autophagosomes maturate through fusion with lysosomes, leading to the degradation of the protein content by

lysosomal enzymes [6]. This general autophagy process can be divided into four stages: initiation, elongation, maturation, and degradation. Autophagic pathway is shown in Figure 1.

At initiation an autophagic double membrane starts with surrounding of cytoplasmic and organelle portions of the cell [7]. One of the key regulators of autophagy is mammalian target of rapamycin (mTOR), protein kinase, which is activated by class I phosphoinositide 3-kinase (PI3K)/Akt signaling molecules [3]. In nutrient-rich conditions, mTOR is activated, which results in negative regulation of a down-stream protein complex that contains the autophagic protein unc-51 like autophagy-activating kinase 1 (ULK1). During nutrient starvation, activation of AMP-activated protein kinase (AMPK) phosphorylates ULK1 and blocks the inhibitory effect of mTOR. Phosphorylation of the ULK1 results in translocation of PI3K class III to the isolation membrane, also called phagophore, and promotes the generation of phosphatidylinositol 3-phosphate (PI3P), which is essential for recruitment of a number of ATG proteins [2, 8].

Initiation is followed by elongation. Two ubiquitin-like conjugation systems, the ATG12-ATG5-ATG16L1 conjugation system and the microtubule-associated protein light chain 3 (LC3) conjugation system, are involved in this step. Both require participation of a range of ATG-proteins, such as ATG3, ATG4B, ATG7, and ATG10, which act as proteases or ligases, thus mediating formation of conjugation complexes. ATG12-ATG5-ATG16L1 complex is needed for the conjugation of the processed form of pro-LC3, LC3-I, to phosphatidylethanolamine (PE), resulting in formation of LC3-II, which is stably membrane-associated and thought to be required for the closure of the expanding autophagosomal membrane. The LC3-II on the outer surface of the completed autophagosome is cleaved from PE and released back into the cytosol, along with other ATG proteins. The LC3-II that is present on the concave surface remains associated with the membrane and can be detected biochemically or microscopically, thus allowing detection of the autophagic pathway [5, 7, 8].

At the final step, the outer membrane of the mature autophagosome fuses with the lysosome, forming the so called autolysosome. This creates an acidic environment that results in the degradation of the cargo as well as of the inner membrane [4].

Autophagy can be enhanced or inhibited by pharmacological inducers and inhibitors.

Rapamycin (sirolimus), an allosteric inhibitor of mTOR, is the most commonly used autophagy inducer. It is also well established in clinical practice being an immuno-

suppressant in organ transplantations [9]. On the other hand, autophagy can be pharmacologically inhibited by different approaches. 3-methyladenine (3-MA) targets the class III PI3K and enables autophagosome formation, while inhibitors of the lysosomal proton pump such as bafilomycin A1 or lysosomotropic alkalines such as chloroquine (Cq) target the fusion of the autophagosomes with the lysosomes [10]. However, as true with all pharmacological agents there is a lack of specificity for a certain pathway. Therefore genetic approaches to inhibit autophagy, such as knockout of ATG genes, are becoming more important [3].

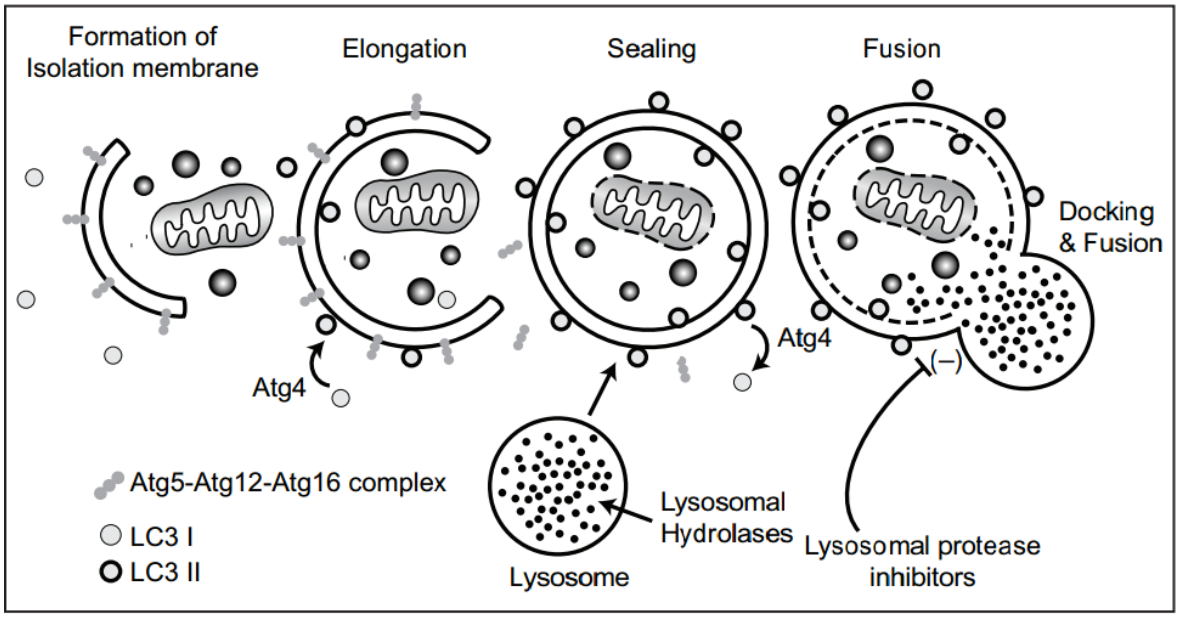


Figure 1: Autophagic pathway. Autophagy process can be divided into four stages: initiation, elongation, maturation, and degradation. At initiation an autophagic double membrane starts with surrounding of the cellular contents. Two conjugation systems: the ATG12-ATG5-ATG16L conjugation system and LC3 conjugation system are involved in elongation step. ATG4 controls the lipidation and recycling of LC3. Sealing of the two tips leads to the closure and maturation of the autophagosome. At the final step, mature autophagosome fuses with the lysosome, forming so called autophagolysosome. Result is the degradation of the cargo as well as of the inner membrane (adapted from [11]) .

1.2. Intestinal epithelial cells

The intestinal epithelium represents the largest frontline to the outer-body environment, which counts up to at least 32 m^2 in humans [12, 13]. It is only ~20 μm thick and completely renewed every 3-5 days. It is lined by a monolayer of intestinal epithelial cells (IECs), which form the barrier that separates the microbe-filled and nutrient-filled gut lumen from the largely sterile underlying tissue [14]. The intestinal epithelium has a

difficult task: it must balance the needs for a barrier against a hostile environment, while also allowing the absorption of water and nutrients essential to life [15, 16]. Additionally, not all antigens to which the intestinal mucosa is exposed are pathogenic; the presence of commensal bacteria in the lumen is needed as these bacteria significantly contribute to nutrient digestion, vitamin synthesis, immune regulation and tissue maturation. The epithelium therefore has to sense both beneficial and harmful microbes, modulate immune responses, and in this way maintain intestinal homeostasis [14, 17]. The intestinal epithelium performs two different but important functions: a barrier and an immunosecretory function which together maintain the integrity of the intestinal epithelial barrier. To fulfill such diverse tasks, the epithelium is composed of different cell types, which can be divided into two main groups: absorptive and secretory cells [16]. The first group which is the largest and less studied due to its diverse functions is represented by enterocytes on which this thesis focuses. The other cells are antimicrobial peptide-secreting Paneth cells, mucus-producing goblet cells and enteroendocrine cells. Each epithelial sub-type can produce specific immune modulating factors, driving innate immunity to pathogens as well as preventing autoimmunity [18].

1.2.1. Epithelial barrier function

The apical and basal plasma membranes of closely linked epithelial cells form the majority of the barrier surface. Specific pumps, channels and transporters facilitate transport of water, nutrients, and solutes through the cells, thus performing transcellular transport [19, 20].

However, the space between individual IECs, that is, the paracellular space, also needs to be sealed. This function is carried out by three protein complexes located at the apical end of the lateral membrane, collectively termed the apical junctional complex. This includes tight junctions, adherent junctions, and desmosomes. The last two are involved in communication between neighboring epithelial cells and important to establish the cell-cell adhesion force, thus ensuring structural integrity of the cell layer [19]. On the other hand, tight junctions (TJs) form a selectively permeable seal between adjacent IECs and are essential in maintaining the intestinal barrier while regulating paracellular permeability of

ions, nutrients, and water [21]. They are also important for cell polarity: with an intact TJ, apical membrane proteins cannot diffuse to the basolateral side [22].

Tight junction structure and function

TJs are the most apical components of epithelial intracellular cleft. The complex consists of transmembrane proteins and their intracellular adapter and scaffold proteins (Figure 2). Until today, four integral transmembrane proteins and protein families have been identified: occludin [23], claudins, junctional adhesion molecule (JAM) [24], and tricellulin [25].

Occludin is a 65 kDa transmembrane protein [23]. Its function is not yet fully understood, but as studies showed that knockdown induces an increase in paracellular permeability to macromolecules, occludin is supposed to have a crucial role in the TJ structure and permeability in the intestinal epithelia [26]. Claudins represent a multigene family with at least 24 members in humans that modulate the paracellular movement of ions based on their charge and size. Some isoforms tighten and some create pores in the epithelium. Therefore the ratio of different claudins in the TJ determines its functional property as either leaky or tight [27]. JAM proteins belong to the Ig superfamily and have a role in the formation of TJs and in cell-cell adhesion [21]. Tricellulin is, as the name says, specifically concentrated at tricellular contacts, where three cells join together [25].

With their extracellular domain, these transmembrane complexes prevent the leakage through the paracellular pathway. The intracellular domain, on the other hand, interacts with cytosolic scaffold proteins. Latter anchor the transmembrane proteins to the actin cytoskeleton. The most important and first discovered scaffold proteins are zonula occludens (ZO) proteins, which are linked with their C-terminal region to the actin cytoskeleton through numerous linker proteins. Three ZO proteins, ZO-1, -2, and -3, have been identified to date [21]. Among them, 220 kDa ZO-1 is the most studied one [28]. Although intensive efforts have been made to clarify its role, the precise function has not been determined. Recent studies have shown that ZO-1 deficient cells could still form normal TJ structures and show normal permeability under normal conditions [29]. However, a change in Ca^{2+} concentration leads to a retardation of occludin recruitment in

ZO-1 deficient cells. Unfortunately functional consequences were not investigated in this study.

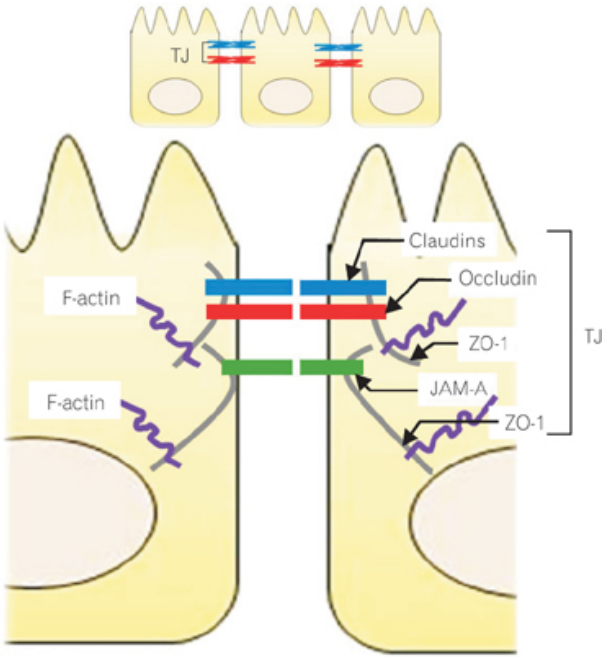


Figure 2: TJ structure. Extracellular domains of occludin, claudins, JAM proteins and tricellulin (not shown) prevent leakage through the paracellular route. Their intracellular domains are attached to cytosolic scaffold proteins, such as ZO-1. Latter anchor the transmembrane proteins to actin (adapted from [21]).

1.2.2. Immuno- and secretory function

As mentioned before, the epithelium that lines the gut comes in contact with a variety of foreign substances and organisms such as bacteria and viruses that can cause injury upon contact or invasion. Thus, IECs developed several mechanisms to perform effective immunity against pathogenic intruders. Rapid renewal of the epithelium and consequently shedding of infected cells represents the first line of defense against pathogens. Next, the mucus layer forms a physico-chemical barrier against bacterial invasion. Besides hindering bacteria from coming in close proximity to the epithelium, mucus is enriched with several peptides that possess antibacterial activity. The best known such peptide is defensin, secreted by the Paneth cells. If pathogens reach the monolayer, IECs express a variety of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns, evolutionary conserved structural motifs produced by microorganisms. This way IECs can distinguish between commensal and pathogenic bacteria. Most known PPRs are Toll-like receptors. Activation of PPRs facilitates transcriptional epithelial responses which

lead to induction of cytokine secretion. Secreted cytokines and microbiota itself induce expression of major histocompatibility complex molecules and adhesion molecules expression on IECs that directly interact with lymphocytes, thus enhancing immune response [14, 30].

1.2.2.1. Cytokine secretion

The term cytokine is a general reference to many families of soluble regulating agents of inflammation and immunity, and includes the interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), chemokines and transforming growth factors (TGFs), to mention a few [31]. Proinflammatory cytokines play a critical role in recognizing and destroying intestinal pathogens through activation of intestinal immune responses [32], while anti-inflammatory cytokines limit excessive inflammatory reaction. The right balance between pro- and anti-inflammatory cytokines is therefore essential for immune homeostasis in the gut. Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines. They are often produced in a cascade, as one cytokine stimulates its target cells to produce additional cytokines [33]. IECs secrete many different cytokines, among which interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) are the most important.

IL-1β is a potent proinflammatory cytokine. It is crucial for the host's defense responses to infections and injuries and is involved in cellular recruitment. IL-1β is produced as an inactive precursor, termed pro-IL-1β, which is cleaved by the proinflammatory protease caspase-1. The latter is a part of a big multi-protein complex, called inflammasome. After processing of pro-IL-1β, mature IL-1β is secreted from the cell and plays a major role in the initiation and amplification of many inflammatory conditions [34]. According to a recent study, autophagy may regulate the production of IL-1β in macrophages by targeting pro-IL-1β for autophagosomal degradation [35].

IL-6 is a glycoprotein with pro- as well as anti-inflammatory activity, whose expression, under physiological conditions, is important for the host response to a number of infections [36].

IL-8 is a chemokine, chemoattractive cytokine that attracts monocytes, basophils, and especially neutrophils, causing them to migrate toward the site of infection. It has also been named neutrophil activating protein-1 as it stimulates release of neutrophil granules [37].

After being released by IECs, cytokines bind to their receptors, which can be situated on neighboring IEC or on leukocytes (paracrine activity) or on the same IEC, by which it is secreted (autocrine activity). Binding leads to the activation of many pathways, involving activation of Janus-activated kinases (JAK), signal transducers and activators of transcription (STAT), p38 mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) [36, 38, 39].

1.2.2.2. Adhesion molecule expression

One of the effects of cytokine secretion by IECs is the induction of adhesion molecule expression on their apical surface. Adhesion molecules are important for binding neutrophils, which are short-lived immune cells that are packed with secretory granules and represent the first line of defense during acute inflammation. The antimicrobial activity of neutrophils is achieved through the release of extracellular traps, phagocytosis and degranulation of cytokines, chemokines, myeloperoxidase, gelatinase or matrix metalloproteinases [40].

To perform such actions, neutrophils must first be recruited from the blood stream along a chemotactic gradient to the sites of infected intestinal mucosa or other tissue injury [30]. Neutrophils extravasate from blood vessels by moving between endothelial cells, which is followed by migration through the interstitium [41]. After being recruited from the circulation, neutrophils meet and interact with the basal surface of IECs with adhesion molecule beta-2 integrin (CD11b/CD18), expressed on neutrophil surface and fucosylated receptors on the epithelium. Next, neutrophils move into the paracellular space between IECs and disrupt adherent junctions by releasing protease elastase. Little is known how they cross TJs. One theory states that neutrophil-expressed junctional adhesion molecule-like protein (JAM-L) binds to epithelial expressed Coxsackie adenovirus receptor (CAR), while others hypothesis suggest that JAM-L connects with claudins, JAM proteins, and occludin [42]. After successfully transmigrating across the epithelium, neutrophils arrive at the luminal side of the membrane, where they can interact with previously mentioned adhesion molecules. Such molecules are CD44v6, CD11b/CD18 and intercellular adhesion molecule-1 (ICAM-1) [30].

ICAM-1 (CD54) is a membrane-bound glycoprotein that belongs to the immunoglobuline superfamily. It is composed of five Ig domains, a transmembrane domain and a short cytoplasmic tail that interacts with the actin cytoskeleton. ICAM-1 is expressed on all types of leukocytes, endothelial cells, platelets, glial cells and many other cells, only in epithelial cells its expression is still detectable under non-inflammatory conditions. Upon inflammatory stimuli, ICAM-1 is strongly upregulated. Cytokines, especially tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and IL-1β are potent ICAM-1 inducers [43].

1.3. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a complex, life-long illness that has a significant impact on quality of life of affected individuals [17, 44]. IBD, which is often diagnosed in childhood or early adulthood, has been most evident in western civilizations; however, its prevalence in developing countries has increased in recent years [45]. Crohn's disease (CD) and ulcerative colitis (CD) constitute the majority of IBD cases and are characterized by chronic, relapsing immune activation and inflammation within the gastrointestinal tract. While UC is restricted to the colon and is confined to the mucosa, CD can affect the entire gastrointestinal tract and any layer of the bowel wall [46]. Symptoms include abdominal pain, weight loss, fever and diarrhea with the passage of blood, mucus, or both [44].

Though the exact etiology of IBD remains unclear, it is thought to be a combination of genetic susceptibility, immune dysregulation, microbial flora (dysbiosis) and environmental factors (food, smoking, drugs). The intestinal epithelium constitutes at the interface between all four elements [12] (Figure 3).

Currently no cure for IBD exists. The therapy is directed at resolving symptoms and subsequently maintaining symptom-free periods [44]. As CD and UC are immune-mediated conditions, general immunosuppressive treatment and anti-inflammatory agents were the first introduced therapies. Antibiotics are commonly prescribed as they decrease the concentrations of bacteria in the gut lumen. With the progress in understanding IBD pathogenesis, new possibilities for therapeutic targets have emerged [46].

1.4. Cytokines, tight junctions and adhesion molecules in IBD

A typical feature in patients suffering from IBD is a defective intestinal epithelial barrier with increased intestinal permeability. It is not clear whether the primary cause are changes in TJs, leading to abnormal epithelial barrier integrity and the enhanced immune responses, or whether the inflammation itself causes the alterations in TJ expression and distribution [27]. Some evidence indicate that dysregulation of mucosal immunity in the gut causes overproduction of proinflammatory cytokines that, in association with diminished levels of anti-inflammatory cytokines, induce neutrophil recruitment into the bowel wall and lumen. Migrated neutrophils impair the epithelial barrier function by secreting new cytokines that disrupt TJs [47]. It was shown that TNF-α and IFN-γ, cytokines typically secreted from neutrophils, increase TJ permeability by activating myosin light-chain kinase (MLCK) [48]. Phosphorylation of MLC by MLCK leads to contraction and tension of scaffold proteins bound actin. An alternative mechanism of TJ rupture is the dysregulation of TJ protein expression such as occludin by these two cytokines [49]. Disrupted epithelial barrier allows bacterial invasion and enhances the exposure of neutrophils to foreign antigens. This leads to their activation and release of cytotoxic mediators, such as reactive oxygen and nitrogen species. Besides, neutrophil secreted cytokines alter leukocyte interactions with epithelia through changes in expression of adhesion molecules and attract other neutrophils that secrete more cytokines, thus perpetuating the vicious circle [47,30]. In fact, neutrophil accumulation in luminal spaces is a hallmark of mucosal inflammation and directly correlates with the severity of IBD [30,50,45]. Mechanisms of barrier disruption are presented in figure 3.

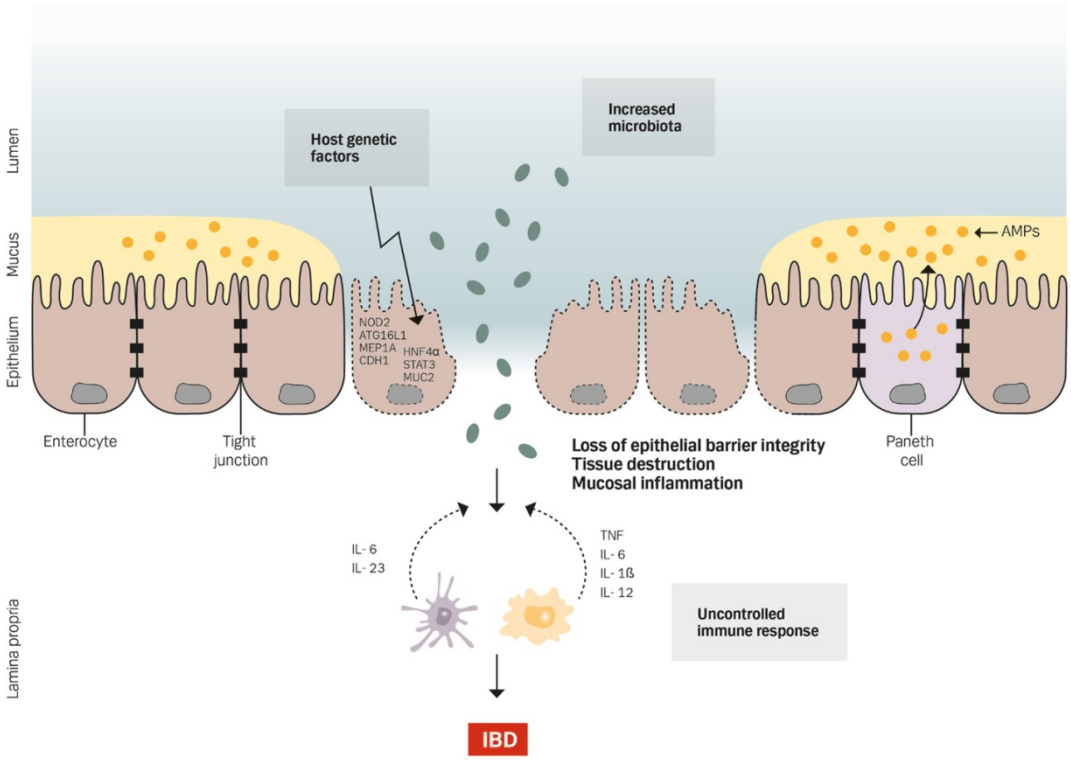


Figure 3: Intestinal barrier dysfunction. Genetics, immune dysregulation, dysbiosis and environmental factors (not shown) contribute to the etiology of IBD. Enterocytes form the majority of epithelial barrier. TJs seal the paracellular space between epithelial cells. It is not clear if TJ disruption is the primary cause or a consequence of exaggerated immune response. Defective barrier allows bacterial invasion and exposes neutrophils to foreign antigens. Activated neutrophils release cytotoxic mediators and cytokines. Latter induce adhesion molecule expression and attract additional neutrophils, thus perpetuating the vicious circle (adapted from [51]).

Cytokine involvement in the pathogenesis of IBD was confirmed with successful introduction of anti-TNF-α monoclonal antibodies in therapy. However, almost a third of patients do not respond to anti-TNF therapy or lose response in time. Consequently, the need for new, more potent, and targeted molecules is obvious. Novel therapeutic approaches include targeting dysbiosis, the disrupted epithelial barrier, proinflammatory cytokines, leukocyte trafficking and genetics [46, 52].

1.5. Autophagy in IBD

Application of high-throughput genomic analysis has resulted in the identification of several susceptibility loci for IBD, the majority of them being shared by both CD and UC. Most are involved in cytokine signaling, barrier function and also in the autophagic

machinery [53]. Thus, the interest in the link between autophagy and IBD appeared and led to several studies on how this recycling process could affect the gastrointestinal tract.
Genetic associations with CD have been reported in the following autophagy-related genes: ATG16L1 (autophagy-related 16-like 1), NOD2 (nucleotide-binding oligomerization domain-containing 2), IRGM (immunity-related GTPase family, M), PTPN2 (protein tyrosine phosphatase, non-receptor type 2), XBP1 (X-box binding protein 1), LRRK2 (leucine-rich repeat kinase 2) and ULK1.

Polymorphisms in the autophagy gene ATG16L1 are among the most investigated CD susceptibility gene variants. ATG16L1 deficiency affects granule exocytosis pathway in Paneth cells [54], enhances secretion of proinflammatory cytokine IL-1β after stimulation with lipopolysaccharide in myeloid cells [55] and weakens autophagy-mediated clearance of several pathogens [56, 57].

On the other hand, UC has been associated with increased levels of beclin-1, which is involved in the formation of the autophagosome [58]. Nonetheless, polymorphisms in genes that regulate barrier function [53] and ICAM-1 overproduction seem to be more tangled in etiology of UC [59].

2.) OBJECTIVES

Impaired autophagy seems to be involved in the pathogenesis of IBD, which is characterized by a defective epithelial cell barrier and an exaggerated immune activity in intestinal epithelial cells. Thus, we want to determine the effect of manipulating autophagy on three parameters that are altered in the inflamed gut of IBD patients: tight junction integrity, ICAM-1 expression and proinflammatory cytokine secretion. Our investigations were performed on Caco-2 cells, which although originally derived from a human colon adenocarcinoma, are still able to undergo spontaneous differentiation into enterocyte-like cells. Therefore, Caco-2 cells form confluent monolayers consisting of well polarized cells with tight junctions that have been extensively used as a model of the intestinal barrier [60]. Several inflammatory mediators are thought to be implicated in the development of IBDs. In vitro research on intestinal epithelial cells has focused on the involvement of three major cytokines: TNF-α, IFN-γ and IL-1β as their concentrations are significantly elevated in the intestinal tissue of IBD patients [61]. We exposed Caco-2 cells to these three cytokines, thus simulating a proinflammatory environment similar to that in IBD patients and evaluated the contribution of enhanced or disrupted autophagy on those three parameters: tight junction integrity, adhesion molecule expression and cytokine secretion.

3.) MATERIALS AND METHODS

3.1. Materials

Table I: Equipment

Name	Type	Company
Aspirating pipette	2 mL	Greiner Bio-One
Autoradiography film	Amersham Hyperfilm ECL	GE Healthcare
Cell culture plates	6-, 24- and 96-well plates	Greiner Bio-One
Centrifuge	Heraeus Multifuge 3SR	Thermo Fisher Scientific
Centrifuge	Heraeus Multifuge 40R	Thermo Fisher Scientific
Confocal microscope	LSM 5 Exciter	Carl Zeiss
Counting chamber	Neubauer chamber	Marienfeld
Cover glasses	Φ 12 mm	Thermo Scientific
Cryogenic vial	Nunc CryoTubes; 1.8 mL	Sigma
Electronic analytical balance	XP205	Mettler Toledo
Electronic balance	PM4000	Mettler Toledo
Electrophoresis machine	Powerpac 3000	BioRad
Electrophoresis system	XCell SureLoc Mini-Cell	Life Technologies
Epihelial voltohmmeter	EVOM^2	WPI
Falcon tubes	15 mL, 50 mL	Greiner Bio-One
Filters	Filter paper	GE Healthcare
Imaging system	X-Omat 2000 processor	Kodak
Incubator	Heraeus HERAcell 150i	Thermo Fisher Scientific
Light microscope (inverted fluorescent)	Zeiss Axiovert 35	Carl Zeiss
Micro Test Tube	3810X; 1,5 mL	Eppendorf
Microcentrifuge	Centrifuge 5415 D	Eppendorf
Microcentrifuge	Centrifuge 5417 R	Eppendorf
Petri dishes		Milian
PVDF Transfer membrane	Immobilon – P	EMD Millipore
Serological pipettes	2mL, 5mL, 10mL, 25mL	Eppendorf
Spectrometer	SpectraMax M2	Molecular Devices
Sysmex	Sysmex KX – 21	Sysmex Digitana AG
Transwell® Permeable Supports (0.33cm^2)		Corning

Table II: Chemicals

Name	Cat. Number	Company
Alexa Fluor 680-Dextran, MW=3kDa	D34681	Invitrogen
Amersham ECL Prime Western Blotting Detection Reagent	RPN2232	GE Healthcare

Amersham Hyperfilm™ECL	KNO90029	GE Healthcare
Bovine serum albumin (BSA) Fraction V Solution 7,5%	A-8412	SIGMA
Calcein-AM	C3099	Molecular Probes
Chloroquine diphosphate salt	C6628	SIGMA
DMEM + GlutaMAX (Dulbecco's Modified Eagle Medium)	31966-021	Life Technologies
DMSO	D2650	SIGMA
DPBS	17-512F	Satorius
DTT (Dithithreitol)	197 777	Roche
Fetal calf serum	A15-104	GE Healthcare
Glycin	50049	Fluka
Human IL-1β ELISA MAX™ Deluxe Set	437004	BioLegend
Human IL-6 ELISA MAX™ Deluxe Set	430504	BioLegend
Human IL-8 ELISA MAX™ Deluxe Set	431504	BioLegend
Interferon gamma (IFN-γ)	285-IF	R&D Systems
Interleukin 1 beta (IL-1β)	201-LB	R&D Systems
Lucifer Yellow, MW=457.3g/mol	L0259	SIGMA
Magnesium chloride	M-8266	SIGMA
Methanol	1.06009.2511	Merck
Milk powder		»Commercial«
Novex®Sharp Pre-Stained Protein Standard	LC5800	Invitrogen
NuPAGE LDS Sample Buffer (4x)	NP0007	Invitrogen
Pancoll human	P04-60500	Pan Biotech
Paraformaldehyde (PAF) extra pure	16005	Riedel-de-Häen
Phenylmethylsulfonylfluoride (PMSF)	837 091	Roche
Phosphate-buffered saline (PBS) pH 7.4	P3813	Sigma
Pierce BCA Protein Assay Kit	23255	Thermo Scientific
Pierce™ECL Plus Western Blotting Substrate	32132	Thermo Scientific
ProLong Gold Antifade Reagent with DAPI	8961	Life Technologies
Proteases inhibitor cocktail (PIC)	P-8340	SIGMA
Rapamycin (Sirolimus)	BML-A275-0005	Enzo
Restore™ Western Blot Stripping Buffer	21059	Thermo Scientific

RunBlue 12% SDS PAGE Precast Gel	NXG01212K	Expedeon
RunBlue SDS Running Buffer (20x)	NXB50525	Expedeon
Sodium azide (N3Na)	71289	Fluka
Sodium chloride	1.06404.1000	Millipore
Sodium dodecyl sulfate (SDS 10% solution)	71736	Sigma
Streptomycin/Penicillin	15140-122	Invitrogen
Sulfuric acid (2N)	35276	Fluka
Recombinant human TNF-alpha (TNF-α)	210-TA-020	R&D Systems
Trizma Base (TRIS)	T-6066	SIGMA
Trypsin-EDTA		Life Technologies
Tween® 20	P2287	SIGMA

Table III: Prepared solutions

Solution	**Composition**
Assay medium for permeability assay	HBSS (Ca^{2+}+Mg^{2+} ,no phenol red), 5%FCS, 25nM Hepes
Blocking buffer for immunofluorescent staining	5% milk powder in TBST
Blocking buffer for Western blot	5% milk powder in TBST
FACS buffer	7,5% BSA (0,4%), 0,5M EDTA in PBS
FACS blocking buffer	10% FCS, 10% polyvalent IgG in PBS
Lysis buffer	5% skimmed milk, 0.3% Triton X-100, 0.04% NaN3 in TBS, set to pH7.4
TBS 10x	0.20 M Tris, 1.50 M NaCl [pH=7.6]
TBS 1x	10 times diluted TBS 10x
TBST	0.1% Tween 20 in TBS
Transfer buffer 10x	0.25M Tris, 1.87M Glycine
Transfer buffer 1x	20% MeOH + 10% Transfer buffer (10x) + ddH2O
Working solution for permeability assay	7,5 µL Lucifer Yellow + 15 µL Dextran + assay medium up to 3000 µL

Table IV: Antibodies

Target protein	**Cat. Number**	**Company**	**Source**	**Conc. Western**	**Conc. IIF**	**Conc. FACS**
Anti-LC3B	NB600-1384	Novusbio	Rabbit	1:2000		
Anti-CD54/ICAM-1	4915	Cell signaling	Rabbit	1:1000		

Anti-SQSTM1	sc-28359	Santa Cruz	Mouse	1:500		
Anti-ZO-1	61-7300	Invitrogen	Rabbit	1:1000	1:100	
Goat anti-rabbit IgG	NA934V	GE Healthcare	Goat anti-rabbit IgG	1:10000		
Sheep anti-mouse HRP	LNXA931	GE Healthcare	Sheep anti-mouse HRP	1:10000		
Actin (pan actin)	AAN01-A	Cytoskeleton	Rabbit	1:2000		
Anti-occludin	OC-3F10	ThermoFisher	Mouse	1:1000	1:100	
IgG F(ab')2 fragm.	711-136-152	Jackson	donkey anti-rabbit		1:100	
IgG F(ab')2 fragm.	711-116-152	Jackson	donkey anti-rabbit		1:100	
IgG F(ab')2 fragm.	A-11070	Mol. Probes	goat anti-rabbit		1:100	
PE anti-human CD54	322708	BioLegend				1:100
PE mouse IgG1 (isotype control)	400114	BioLegend				1:100

3.2. Methods

3.2.1. Cell culture

3.2.1.1. Caco-2 cell line maintenance and subculturing

Human Caco-2 HTB-37 intestinal epithelial cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % FCS and 1% P/S (10000 units/mL of penicillin and 10000 µg/mL of streptomycin). Cells were grown in Petri dishes and maintained at 37 °C in a 5% CO_2 atmosphere. Medium was changed every two days.

When cells reached the subculturing density of 50-60% confluence, the medium was removed with a vacuum pump and cells were washed twice with 5 mL PBS (Phosphate Buffered Saline). Then the PBS was replaced with 3 mL of trypsin-EDTA solution for 30

seconds. Trypsin solution was removed and the dish was left in the incubator for 5 minutes to completely detach the cells. Trypsin action was stopped by addition of 2 mL of complete medium and cells were dispersed by pipetting, gently, over the surface of the monolayer until a single cell suspension was obtained. The latter was transferred into a 50 mL Falcon tube. After counting, the appropriate amount of cells was resuspended and placed in a new Petri dish, already containing 8 mL of fresh DMEM. The cells were then kept in the incubator for a new growing phase.

3.2.1.2. Cell counting

Cells were mixed well with a serological pipette in a 50 mL Falcon tube and 10 µL of cell suspension were added to 90 µL of Trypan blue in an Eppendorf tube. A Neubauer chamber was used for cell counting. A coverslip was placed in the middle of a chamber and 10 µL of diluted cells (1:10) were added. The chamber was placed under the microscope with a 10x objective. The chamber contained four corner squares and cells were counted in each of them. For each corner cells that were overlapping the top and left lines were included in counting and cells that were overlapping the bottom and right lines were not included.

Cells per millilitre were calculated using the following equation:

$Cells/ml = average\ count\ per\ square \times dilution\ factor\ (10) \times 10^4$

3.2.1.3. Cell freezing

First cryovials were labeled with the name of cell line, passage number and date. Cells were detached from the Petri dish and resuspended in 2 mL culture medium as described above. Then they were transferred to a 15 mL Falcon tube and centrifuged at 400g for 5 minutes at 20°C. The supernatant was carefully removed with a vacuum pump. The cell pellet was resuspended in 1 mL of complete medium and cells were counted. 4 million cells per mL were mixed in the ratio 1:1 with ice-cold freezing buffer (Table III). 1 mL of cell suspension was dispensed into each cryovial and kept at -80°C overnight. Then the cryotubes were transferred to liquid nitrogen tank for undetermined storage.

3.2.1.4. Cell thawing

Cryotubes containing cells were transferred from liquid nitrogen or -80°C freezer to a 37°C water bath in order to thaw the cells more rapidly. Cells were then transferred to a 50 mL Falcon tube which contained few mL of complete medium. The cells were centrifuged at 400g for 5 minutes at room temperature. The supernatant was carefully removed with a vacuum pump. Cells were resuspended in 1 mL of complete medium and transferred to a Petri dish, already containing 8 mL of fresh medium.

3.2.2. Protein concentration measurement

Protein concentrations were determined using a Pierce BCA protein assay kit and a SpectraMax M2 machine. A 96-well plate was used; the first 18 wells were reserved for 25 µL of BCA protein standards (8 concentrations) and blank sample (all in duplicates). Supernatants of lysed samples were taken from the -20°C freezer and put on ice to thaw. Then they were diluted with double distilled water at a ratio of 1:10 (6 µL of sample + 54 µL of ddH2O) and pipetted (25 µL) in duplicates into the wells of a 96-well plate. A mixture of BCA reagent A and B was prepared in the ratio 50:1 and 200 µL were added to each filled well. After 20 minutes incubation at 37°C, absorbance was measured at 540 nm. From this data, the volumes of lysates that contained 25-50 µg of proteins were calculated. To these volumes cell lysis buffer (CLB) (1x) was added to reach a total volume of 17 µL, which resulted in the same protein concentration in all samples.

3.2.3. Western blotting

3.2.3.1. Sample preparation

Calculated amounts of thawed samples and CLB (1x) were pipetted to new Eppendorf tubes. 8.5 µL loading buffer (1 µL 10% SDS, 1 µL 1M MgCl2, 0,5 µL DTT, and 6 µL of loading buffer) were added to each diluted sample. Samples were vortexed, heated at 90°C for 10 minutes and spun down at 13000 rpm for 10 minutes at room temperature.

3.2.3.2. Running

Running buffer (Table III) and chamber for electrophoresis were prepared. 12% SDS-PAGE running gel was rinsed with double distilled water before inserting it in the chamber and filling it with 800 mL of running buffer. The comb wells were first rinsed with running buffer by pipetting up and down, then 8 µL of molecular weight marker was added into the first well and 25.5 µL of centrifuged samples were loaded in the others wells. Gel was run for 25 minutes at 80 V, followed by 1.5h at 120 V. The electrophoresis was stopped when blue markers from the loading buffer reached the bottom of the gel.

3.2.3.3. Transfer

Transfer buffer (1x) was prepared (Table III) and poured into a box containing two filter papers, six blotting sponge pads, cathode and anode. The box was placed in the fridge at 4°C for at least 20 minutes. Just before use, a polyvinylidene difluoride (PVDF) membrane was activated in methanol for 1 minute and rinsed with double distilled water. The running gel was carefully taken out of the two plastic plates and parts without proteins (bottom and top) were removed with a gel knife. A sandwich was created in the following order - cathode, 2 blotting pads, filter paper, gel, membrane, filter paper, 4 blotting pads. Before closing with anode, bubbles were taken off by gentle rolling over with a glass stick. This sandwich like transfer stack was put in the transfer chamber and transfer buffer (1x) was poured over it. Membrane transfer was run at 30 V for 1 hour at 4°C.

3.2.3.4. Antibody treatment

After transfer, membrane was blocked with blocking buffer for 1 hour at room temperature with shaking on a shaker. Then weight markers were labelled with a pen and the membrane was incubated overnight at 4°C on a rotor in a 50 mL Falcon tube with the primary antibodies (see table III for concentration) in 5 mL of blocking buffer. For detection of proteins with different sizes the membrane was cut according to their sizes and incubated separately with the respective primary antibody. After the first use 50 µL of NaN_3 were added to 5 mL of primary antibody solution in order to prevent spoilage and to allow multiple use.

After incubation with primary antibodies the membrane was washed five times with TBST, shaking for 5 minutes each. Next, HRP-conjugated secondary antibodies were added (see table IV for concentration) in 15 mL of blocking buffer. Incubation time was 1 hour at room temperature on shaker. Then the membrane was washed again with TBST on a shaker for at least four times in one hour, to remove unbound secondary antibodies.

After washing the membrane was put on a paper napkin for five seconds and placed on a glass plate. A light sensitive mixture of ECL Plus reagent A and B for the chemiluminescent detection of horseradish peroxidase was prepared at a ratio of 40:1. For one membrane 2 mL of solution were needed. The membrane was covered with this solution for 1 minute and then dried very well on paper napkins. Completely dried membrane was then transferred into a cassette with the protein side facing up. The cassette was closed and transported to the dark room. A film was placed on the membrane for different amount of times. The film was developed using a X-Omat 2000 processor.

3.2.3.5. Reuse of membrane

The membrane was washed once with TBST and stripped with 5 mL of stripping solution for 25 minutes at 50°C in a stripping oven. Next it was washed for four times with TBST, blocked with blocking buffer (1 hour, room temperature) for using with the next primary antibody solution.

3.2.4. Transepithelial electrical resistance measurement

Transepithelial electrical resistance (TEER) was measured with EVOM2, Epithelial Volt/Ohm Meter for TEER (World precision instruments, Sarasota, FL) [62]. Cells were placed in room temperature for 10 minutes. Electrodes for measurement were first immersed in ethanol, washed with PBS and then adjusted in DMEM. For every insert, three repeated measurements were performed and average resistance calculated. For electrode maintenance they were placed for 10 minutes in ethanol for disinfection after each experiment, than dried in room air and stored in the dark.

3.2.5. Permeability assay

For determination of apical-to-basal flux rates two paracellular markers of different sizes were used: 3 kDa Dextran and 457 Da Lucifer Yellow. First, 600 µL of assay medium (Table III) were added into wells of incubation plate and saved at 37°C for 30 minutes. In the meantime, working solution (Table III) and standard curve solutions for both markers were prepared and pipetted into wells of a 96-well plate. Transwell inserts were washed with assay medium (600 µL in lower and 100 µL in upper compartment), which was aspirated carefully with vacuum pump. Then, 100 µL of working solution were added on each filter. Inserts were carefully and quickly transferred into first wells of prepared incubation plate and incubated at 37°C. Every 10 minutes filters were put into new wells, always in the same order. After 40 minutes, they were put in normal medium, liquid from every well of incubation plate was mixed gently with a pipette and 20 µL of each well was collected and transferred in duplicates in wells of the 96-well plate. Fluorescence was measured at two different wavelengths directly, or the plate was kept at -80°C until measurement.

Paracellular marker	Excitation wavelength	Emission wavelength
3 kDa Dextran	679 nm	702 nm
Lucifer Yellow	425 nm	538 nm

3.2.6. Immunofluorescence staining

3.2.6.1. Cells grown on transwell inserts

Membranes of transwell inserts were first washed twice with TBS (1x). Then, cells were fixed for 10 minutes with 100 µL of 4% PAF per insert. Membranes were washed again three times with TBS (1x), and 150 µL of blocking buffer (Table III), containing permeabilization agent Triton X-100, were added in each upper compartment. After 20 minutes of incubation, blocking buffer was replaced with 100 µL of primary antibody solution (Table IV) for 1 hour. Two different antibodies, produced in two different species, were in this mixture. Then inserts were washed for three times with TBS (1x). 100 µL of secondary antibody solution (Table IV) were added; again with two different antibodies,

labeled with different colours and originated from two different species. After 1 hour incubation, nuclei were stained with 100 µL of H solution (Table III) for 5 minutes. Then inserts were washed again three times with TBS (1x). Fixed and stained cells were removed from the transwell insert by carefully cutting around the membrane edges with a scalpel. Membrane was placed on a cytoslide, cell side facing up, and one drop of mounting media Prolong Gold was placed on top of it before sealing it with a coverslip. Prepared slides were dried and stored protected from light at room temperature until microscopy. Visualization was done with LSM Exciter confocal laser-scanning microscope (Zeiss) and images were analysed with LSM Image Browser 2.1.

3.2.6.2. Cells grown on coverslips

For the purposes of some of our experiments, just nuclei were stained. Cells were washed, dried, fixed and washed again (as described above), before adding H-solution for 5 minutes. After washing, coverslips were dried and mounted in 4 µL of mounting media, turned up-side-down. Slides were dried and stored in the same way as mentioned before.

3.2.7. Neutrophil isolation

15 mL of Pancoll were poured in a 50 mL Falcon tube. 10 mL of fresh human blood were taken in a heparinised sterile tube, mixed with 20 mL of PBS and gently added on top of Pancoll, without mixing the phases. The tube was spun down for 20 minutes (800 g, acc. 4, dec. 4) at room temperature. After completing centrifugation the granulocytes and the red blood cells were both found at the bottom of the vial. Plasma sample layer, PBMC's layer and Pancoll layer, which were above, were carefully removed with a vacuum pump. The bottom red fraction was transferred to a new 50 mL Falcon tube and filled up to 50 mL with ice-cold lysis buffer (Table III). The tube was incubated on ice for 11 minutes and inverted every 2-3 minutes. Afterwards, the tube was centrifuged at 4°C for 7 minutes (1400 rpm). Supernatant with lysed erythrocytes was removed with the pump and the white pellet with the neutrophils was washed with a mixture of PBS and 2% FCS. Centrifugation was performed at 4°C for 7 minutes (1400 rpm) and the supernatant was removed again. If there was still some red colour in neutrophil fraction, this step was repeated for one more time. Purity was tested with Sysmex blood analysis machine.

3.2.8. Staining neutrophils with calcein

10 µL of calcein-AM was mixed with 2 mL of PBS to obtain working concentration of 5 µM. Neutrophil pellet was resuspended in calcein solution and incubated for 30 minutes in the dark at 37°C in a 50 mL Falcon tube. Afterwards, calcein-labelled neutrophils were washed with addition of 10-20 mL of PBS and centrifugated (5 minutes, 1350 rpm, RT). Supernatant was removed and the pellet was resuspended again in 1 mL of medium. Neutrophils were counted with a Sysmex machine and diluted to a final concentration of 1 million per mL.

3.2.9. Fluorescence-activated cell sorting

Cells were harvested by trypsinisation and washed with FACS buffer (Table III). Cells were resuspended in 90 µl blocking buffer (Table III) and incubated on ice for 10 minutes before adding primary antibodies for 45 minutes at 4°C in the dark. Cells were washed twice with FACS buffer and resuspended in 500 µl FACS buffer for acquisition.

3.2.10. Enzyme-linked immunosorbent assay

Before performing enzyme-linked immunosorbent assay (ELISA) the ELISA plate had to be covered with capturing antibodies. 100 µL of diluted capture antibody solution were added to each well of a 96-well plate, which was then sealed and incubated overnight between 2°C and 8°C.

On day 2, the plate was washed four times with washing buffer, blocked by adding the required amount of diluted assay diluent A, sealed and incubated at room temperature for one hour with shaking on a plate shaker. Afterwards, the plate was washed again for four times with washing buffer, and the wells were filled with the required amounts of prepared standards, samples and, in some cases, additional reagents. The plate was covered and incubated at room temperature for 2 hours while shaking. After incubation, the plate was washed for four times, and 100 µL of diluted detection antibody solution was added to each well before sealing it. Incubation lasted for 1 hour while shaking. Before adding 100 µL of diluted Avidin-HRP solution to each well, the plate was washed as usual, sealed and incubated at room temperature for 30 minutes with shaking. Afterwards, the plate was

washed five times, soaking for 30 seconds to 1 minute per wash. 100 µL of substrate solution were added to every well and the plate was incubated for the required time in the dark. The reaction was stopped by adding 100 µL of 2N sulfuric acid to each well. The absorbance was read at 450 nm and 570 nm within 15 minutes. The absorbace at 570 nm was substracted from the absorbance at 450 nm.

3.2.11. Treatment regimes

3.2.11.1. Cytokine and pharmacological treatment

100.000 cells were seeded per well in a 6-well plate and grown to confluency (5 days). Caco-2 monolayers were then washed twice with PBS and 2 mL of appropriate treatment solution was applied to each well.

Treatment	**Concentration**
Interferon gamma (IFN-γ)	25 ng/mL
Interleukin 1 beta (IL-1β)	25 ng/mL
Tumor necrosis factor alpha (TNF-α)	50 ng/mL
Chloroquine (Cq)	3 µM
Rapamycin (Rapa)	10 µM

The plate was incubated for 4 hours at 37°C. Control wells contained 2 mL of complete medium. At the end of the treatment, 1 mL of each supernatant was collected and stored at -20°C. The remaining supernatant was removed, cells were washed twice with ice-cold PBS and lysed with 100 µL of lysis buffer per well. After 1 minute of lysis on ice, cells were scraped with a scraper, transferred to Eppendorf tubes and spun down at 13200 rpm for 10 minutes at 4℃. Supernatants were collected into new Eppendorf tubes and stored at -20°C. To determine the effect of each treatment on our cells, protein concentration measurement and Western blot were performed, as described above. For ICAM-1 detection also FACS was performed.

3.2.11.2. TJ integrity

100.000 Caco-2 cells were seeded on 0.4 μm pore size, 6,5-mm diameter transwell inserts. 600 μL of medium was added to each well of a 24-well plate well and 100 μL of cell containing medium to the insert. From the third day onwards, TEER was measured every day, as described above. To determine the effect of rapamycin and TNF-α on TJ integrity, cells were treated with TNF-α alone or in combination with rapamycin for up to 72 hours after having differentiated into a monolayer of polarized epithelial cells (usually around day 11). Treatment was performed for different time periods (also 48 hours and 24 hours). One compartment was treated with rapamycin alone for 24 hours and one control well was left untreated (just medium with cells). The same concentrations as listed above were used. At the end of the treatment, permeability assay was performed, followed by IF staining (both described above). The same protocol was followed with Cq.

3.2.11.3. Neutrophil adhesion assay

5 days before the experiment, 300.000 Caco-2 cells were seeded on coverslips in wells of a 24-well plate, filled with 800 μL of medium. Medium was changed every two days. On the day of treatment, cells were first washed twice with PBS, which was gently added with glass pipette, to prevent the rupture of Caco-2 monolayer. Then 600 μL of appropriate treatment was added to every well. Cells were treated with Cq, TNF-α, TNF-α plus IFN-γ, TNF-α plus Cq and TNF-α plus IFN-γ plus Cq. The same concentrations as for the other experiments were used. Cells were incubated at 37°C for 4 hours. After treatment, coverslips were carefully washed twice with PBS and 300 μL of calcein-labelled neutrophil solution was added to every well. The plate was transferred into a 37°C incubator for 45 minutes. IF staining, which is described above, followed immediately after.

3.2.11.4. Cytokine secretion measurements

Cytokine concentrations were determined using specific ELISA kits for IL-1β, IL-6 and IL-8. Supernatants, collected after 4 hour cytokine and pharmacological treatment, were our samples.

3.2.12. Statistical analysis

Analysis of all data was performed by the GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Results were analysed using Student's t-test or ANOVA as appropriate. A p-value < 0.05 was considered statistically significant.

4.) RESULTS

4.1. Enhancing autophagy

In order to verify if Caco-2 cells are autophagy competent, Western blotting of LC3-I and LC3-II was performed (Figure 4). LC3-II level, which is a marker of autophagic activity, was increased after 4 hour treatment with the pharmacological autophagy inducer rapamycin and after 4 hour stimulation with proinflammatory cytokines TNF-α, IFN-γ and IL-1β, that are typically found in IBD. Moreover, when rapamycin was added to cytokine treatment the LC3-II fraction was further increased.

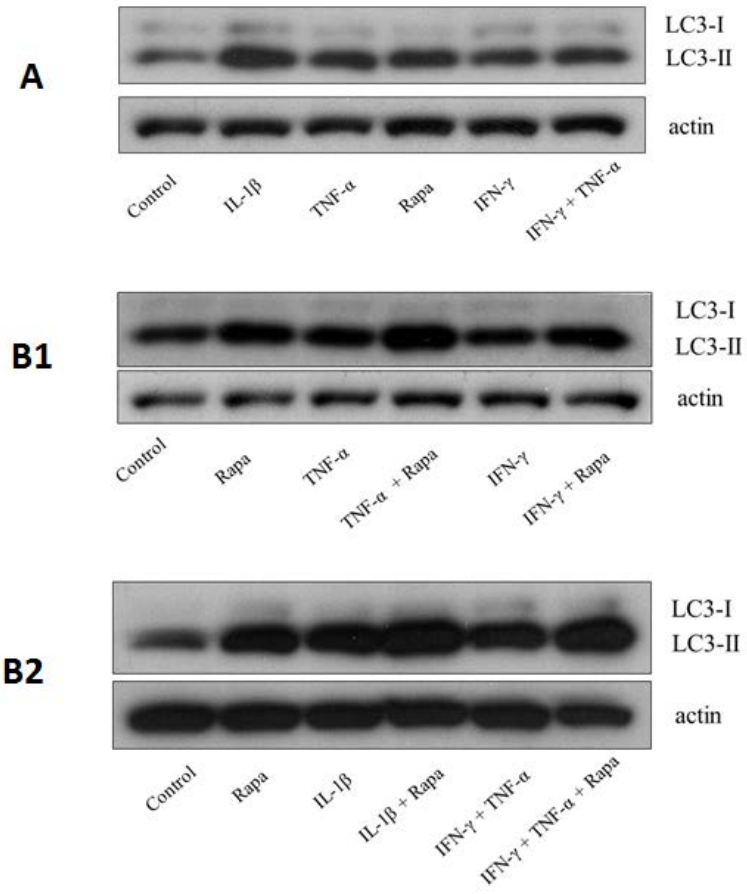


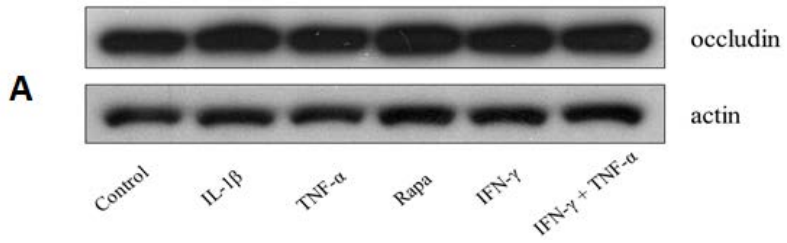
Figure 4. Caco-2 cells are autophagy competent A.) Pharmacological autophagy inducer rapamycin can enhance autophagy. Cytokines interleukin-1β (Il-1β), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) which are typically found in inflammatory bowel disease enhance autophagy. B1, B2.) Pharmacological inducer further enhance cytokine induced autophagy. LC3-I and LC3-II protein expression levels were examined by Western blot analysis. Cells were treated for 4 hours with 25 ng/mL IL-1β, 50 ng/mL TNF-α, and 25 ng/mL IFN-γ, alone or in combination. Rapamycin concentration was 10 μM. Control was left untreated. Extracted protein samples were subjected to 12% SDS-PAGE and transferred onto PVDF membrane. Actin levels served as an internal control.

4.1.1. Effects of enhanced autophagy on TJ integrity

A typical feature in patients suffering from CD is a defective intestinal epithelial barrier with increased intestinal permeability [63]. Our aim was to evaluate if enhanced autophagy is involved in the mechanisms of altered intestinal barrier.

4.1.1.1. Effects on TJ structure

To determine whether the level of autophagy regulates the expression of TJ protein occludin, Western blotting was performed. No significant changes were observed upon 4 hour stimulation with proinflammatory cytokines or rapamycin (Figure 5A). To exclude potential delocalization of the protein from cell borders to the cytoplasm and in order to ascertain if more time is needed to disrupt the epithelial barrier we performed IF staining of occludin and additional TJ protein ZO-1 (Figure 5B), and prolonged the treatment up to 72 hours (Figure 5C). For IF stainings TNF-α was used as recent data suggest that TNF-α-induced dysregulation of the intestinal barrier may be a critical component of IBD. IF of TJ proteins showed no changes after 24 hours and 48 hours. After 72 hours of TNF-α treatment irregularities became obvious. Formerly smooth edges turned into wrinkled cell membranes and condensation of TJ proteins was observed. There was no rescue with rapamycin of this phenotype.



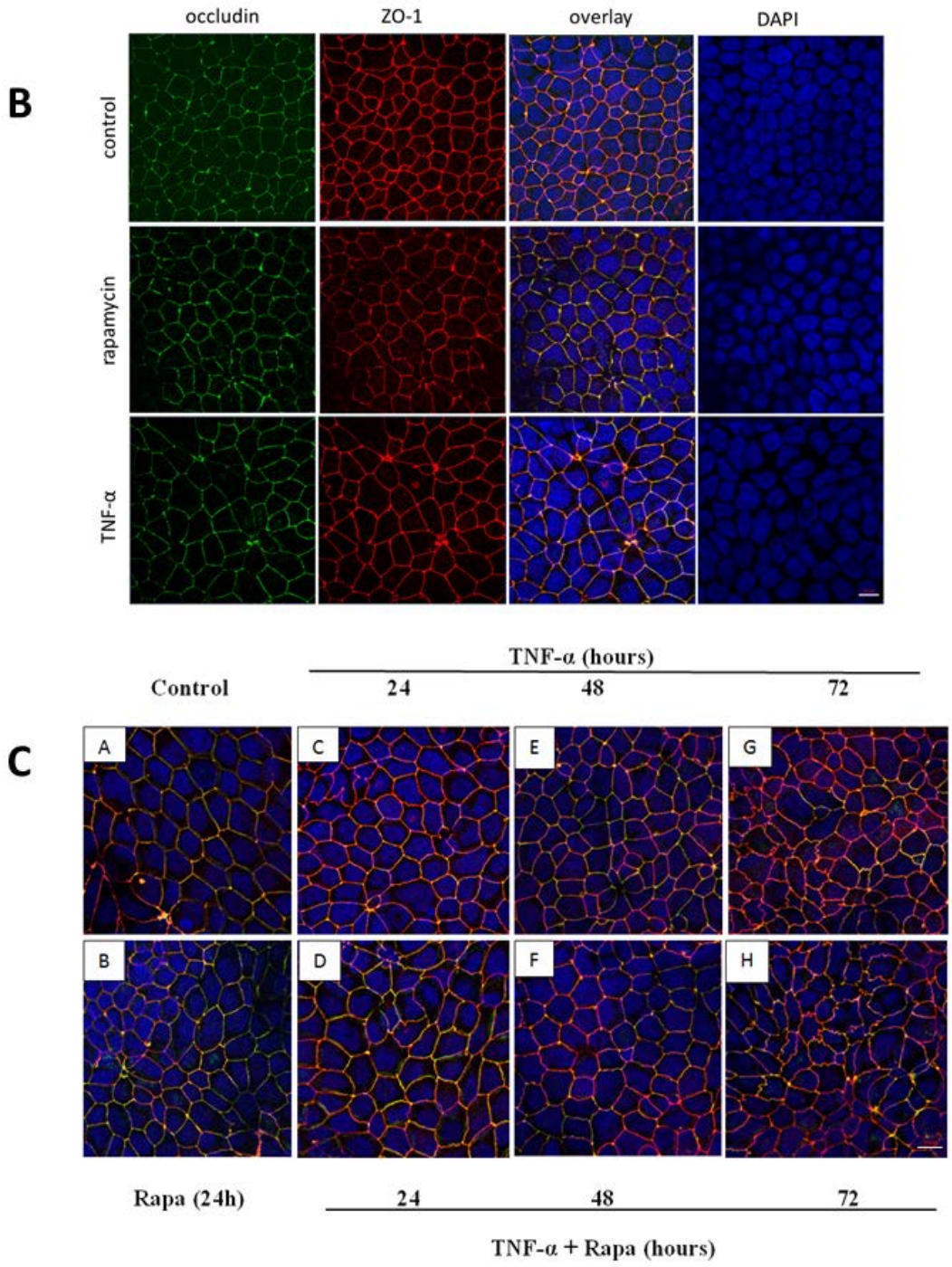


Figure 5: Proinflammatory cytokine or rapamycine treatment alters TJ structure in Caco-2 cells just after 72 hour stimulation. A.) Western blot for occludin after 4 hour treatment with 50 ng/mL TNF-α, 25 ng/mL IL-1β, 25 ng/mL IFN-γ and 10 µM rapamycin. Actin was used as a loading control. B.) Immunostaining of Caco-2 cells for ZO-1 (red) and occludin (green) after 24 hour treatment with 10 µM rapamycin or 50 ng/mL TNF-α. Nuclei were stained with DAPI. Scale bar: 10 µm. C.) Confocal IF of ZO-1 (red) and occludin (green) at different time points. After 24 hour and 48 hour treatment with TNF-α alone (images C and E), or in combination with rapamycin (D and F), no disruptions of TJs were seen. Merged images reveal that, both ZO-1 and occludin, exhibited a well-defined localization around cell boarders, indicating the presence of intact TJs. After 72 hour treatment with TNF-α (G) irregularities were seen. This phenotype could not be rescued with rapamycin treatment. (H). TNF-α and rapamycin were always used in the same concentration of 50 ng/mL and 10 µM. Nuclei were stained with DAPI (blue). Scale bar: 10 µm

4.1.1.2. Effects on TJ function

To confirm our findings on TJ proteins and to determine if there is a link between their structure and functional role, two additional functional tests were performed: transepithelial electrical resistance (TEER) measurement and permeability assay. The latter was carried out by measuring apical-to-basal flux of two macromolecules of different sizes - dextran 3kDa and Lucifer Yellow 457 Da.

As shown in Figure 6A, no significant differences in TEER values were observed upon 24 hour and 48 hour stimulation with TNF-α, rapamycin, or combination of both. 72 hour TNF-α treatment alone also had no significant effect. However, 72 hour stimulation with combination of TNF-α and rapamycin caused slight increase in TEER difference, suggesting that autophagy inducer enhanced TJ barrier function. Interestingly, after 24 hours of combined treatment, rapamycin seemed to decrease TEER value and thereby may weaken TJ functional integrity, but the differences were not statistically significant.

Figure 6B represents the results of permeability assay. Compared to the control all treatments lead to a small, however, statistically not significant decrease in flux of both macromolecular probes, independent of the duration of the exposure. Treatment with the autophagy inducer rapamycin did not influence the difference in flux further. These findings seemed to be more pronounced for small molecules as the permeability for Lucifer Yellow decreased around one third. It can be assumed that with increased resistance the permeability decreases. However, despite an increase in TEER, differences in flux of dextran and Lucifer Yellow were not significant even after 72 hour stimulation with cytokine alone or in combination with rapamycin. We could show slight differences in resistance (TEER) and permeability (flux) as marker of TJ function in Caco-2 cells with different treatment regimes, however these changes were minimal and statistically not significant.

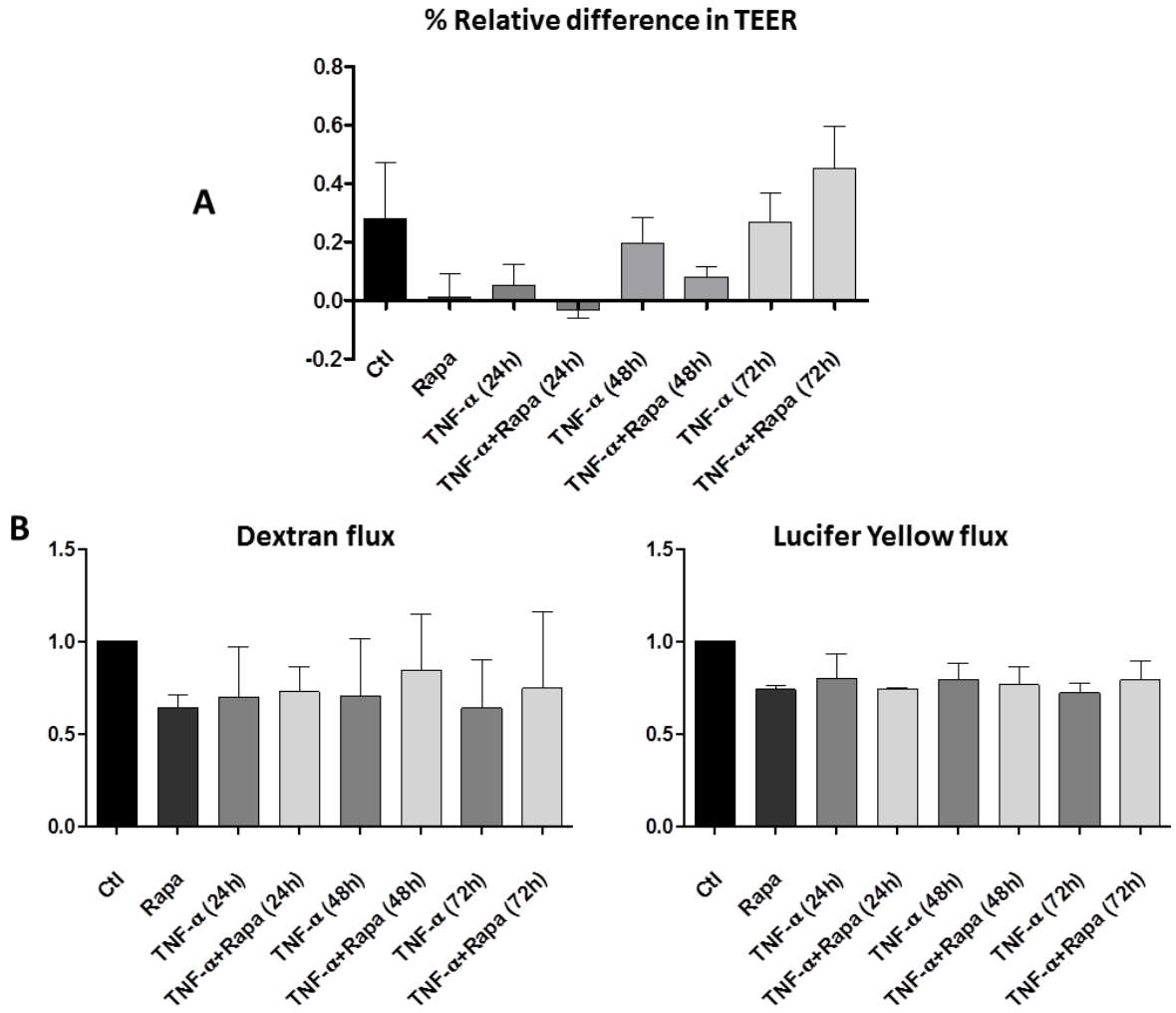


Figure 6: Stimulation with TNF-α and rapamycin does not affect the epithelial barrier function of filter-grown Caco-2 monolayers. Cells were cultured on inserts until differentiation and TNF-α or rapamycin treatments were applied during 72, 48 and 24 hours. A.) Measurement of TEER in Caco-2 cells. Measurements were performed from day 3 on, respectively, at 3 different areas of the filter inserts and out of 3 independent experiments. Relative change in TEER was calculated by substracting the TEER before treatment from the TEER after treatment and dividing the difference by TEER before treatment of the same transwell. B.) Permeability was measured by dextran and Lucifer Yellow flux through the Caco-2 monolayer. Data represent means ± SEM of 3 independent experiments and are normalized to control.

4.1.2. Effects of enhanced autophagy on ICAM-1 expression

Next we wanted to determine if enhancing autophagy effects adhesion molecule expression. An increase in ICAM-1 could be shown in intestinal tissue of UC patients and is hypothesized to be involved in the pathogenesis of IBD.

Immunoblotting for ICAM-1 (Figure 7) revealed that adhesion molecule expression is increased after stimulation with proinflammatory cytokines. IL-1β and the combination of TNF-α + IFN-γ seemed to have a stronger effect on ICAM-1 overexpression compared to TNF-α and IFN-γ alone. An additive effect of TNF-α and IFN-γ is suggested. Enhancing autophagy with rapamycin did not alter ICAM-1 expression.

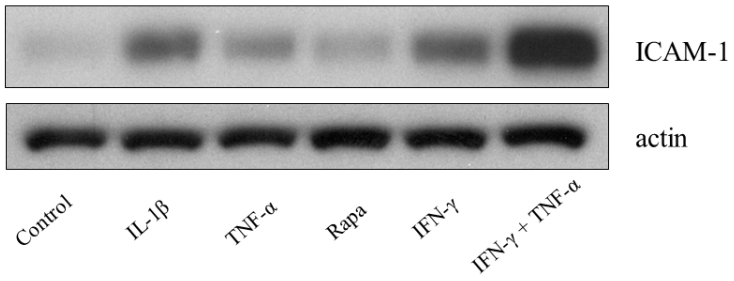


Figure 7: **Cytokines induce cell surface ICAM-1 expression in Caco-2 cells.** Western blot for ICAM-1 after 4 hour treatment with 25 ng/mL IL-1β, 50 ng/mL TNF-α, and 25 ng/mL IFN-γ, alone or in combination. Rapamycin concentration was 10 μM. Control was left untreated.

4.2. Blocking autophagy

Next we assessed the effect on autophagy disruption on IEC function. Cq is a commonly used autophagy blocker. It diffuses into acidic vesicles, becomes protonated, gets trapped, and thereby raises the pH in the autophagolysosome and inhibits the autophagic flux at a late stage [64].

To verify the modulation of autophagy, Western blotting of LC3-II was performed. After 4 hour stimulation with proinflammatory cytokines and Cq LC3-II level increased. Combination of proinflammatory cytokines and Cq further increased LC3-II level confirming that autophagy was increased after cytokine treatment and that autophagy was disrupted at the level of the autophagolysosome. The raise in LC3-II also suggested that proinflammatory cytokines really induce autophagy and that the increase in LC3-II with sole cytokine treatment was not due to a cytokine-induced prevention of degradation of LC3-II.

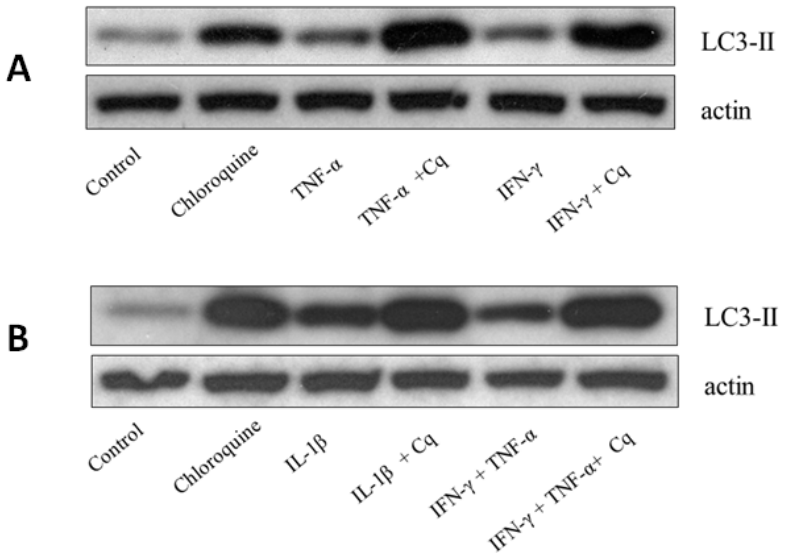


Figure 8: Cq disrupts autophagy in Caco-2 cells. LC3-II protein expression of IECs was determined by Western blotting upon 4 hour stimulation with different combinations of proinflammatory cytokines (25 ng/mL IL-1β, 50 ng/mL TNF-α, 25 ng/mL IFN-γ) and Cq treatment. Cq concentration was 3 µM.

4.2.1. Effects of disrupted autophagy on TJ integrity

4.2.1.1. Effects on TJ structure

Blocking cytokine-induced autophagy at the final step with Cq showed no change in occludin expression compared to the control (Figure 9A) upon 4 hour stimulation. The same could be shown for ZO-1 (Figure 9B). Stimulation with the cytokines TNF-α or IFN-γ alone and in combination with Cq had no effect of occludin expression either. IF staining of ZO-1 (Figure 9C) showed no alterations in TJ structure after TNF-α, Cq or combined treatment, despite a prolonged exposure (24 hours) to proinflammatory stimuli.

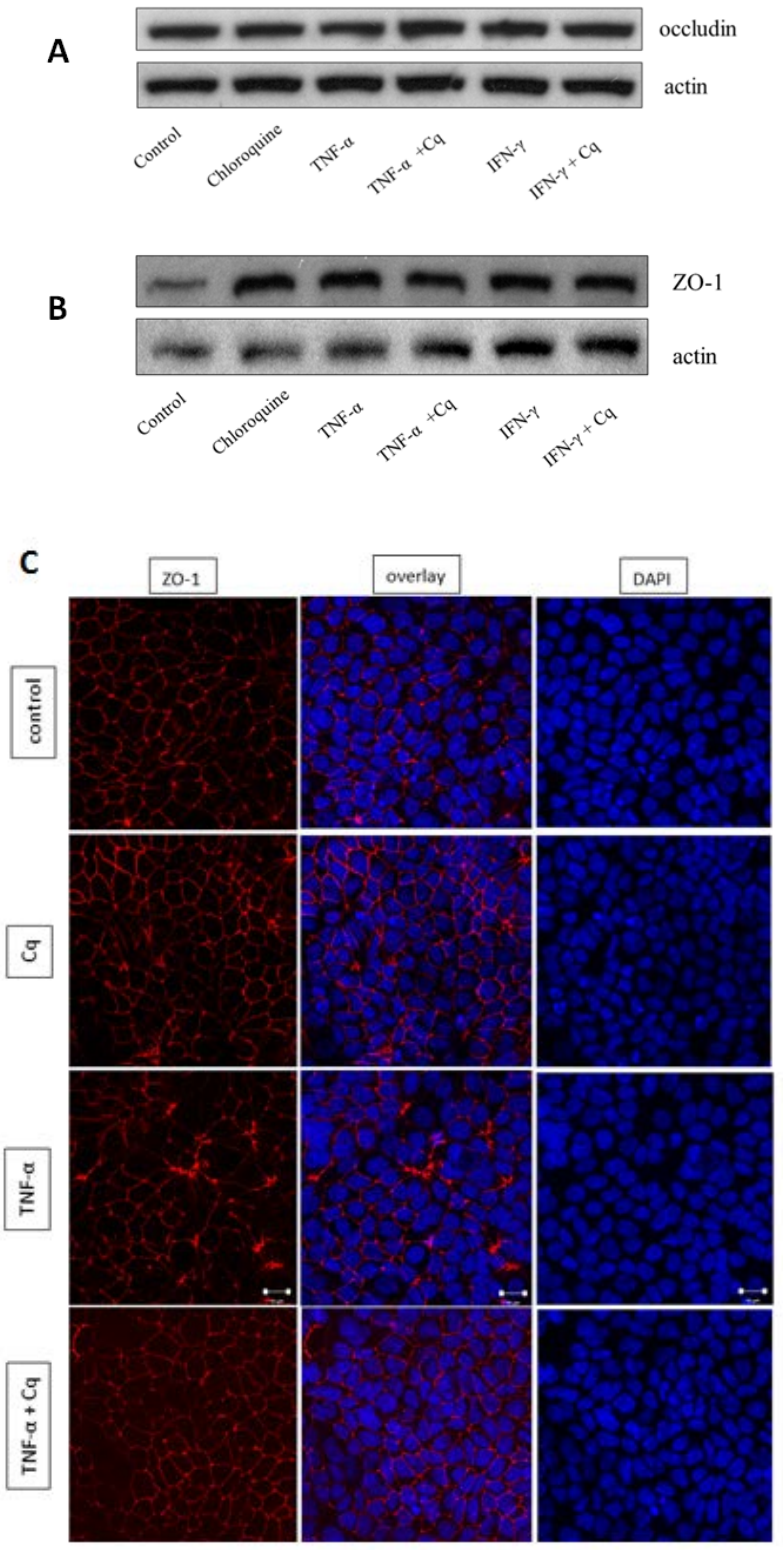
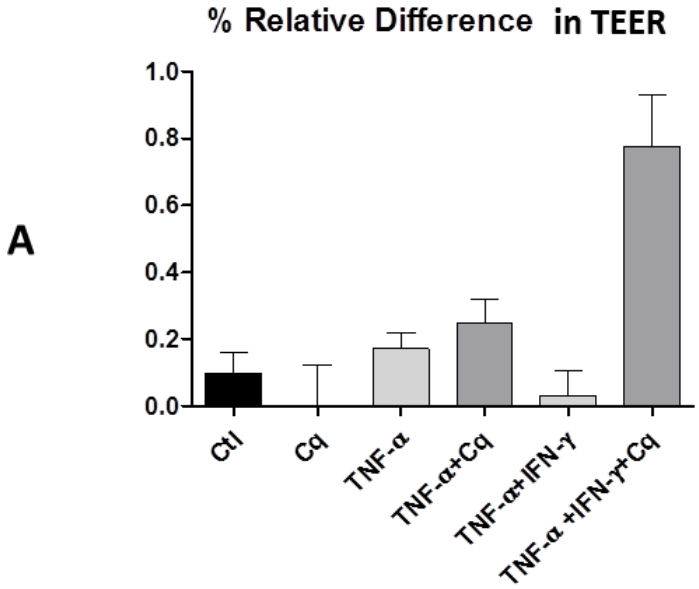


Figure 9: Disrupting AP with Cq does not alter TJ protein level and TJ structure in cytokine-treated Caco-2 cells. A, B.) Cells were stimulated with proinflammatory cytokines (50 ng/mL TNF-α, 25 ng/mL IFN-γ, 25 ng/mL IL-1β) and Cq (3 µM) for 4 hours. Cellular extracts were immunoblotted for occluding and ZO-1. C.) IF staining of Caco-2 cells for ZO-1 (red) after 24 hour Cq, TNF-α and TNF-α + Cq treatment. TNF-α and Cq were used in the concentration of 50 ng/mL and 3 µM. Nuclei were stained with DAPI. Scale bar: 10 µm.

4.2.1.2. Effects on TJ function

Effect on TJ function was evaluated by TEER and permeability measurement after 24 hour stimulation. Cq treatment alone did not alter TEER (Figure 10A) and flux of both molecular probes (Figure 10B) compared to control. The same was found for TNF-α and confirmed previously obtained results (Figure 6). Addition of Cq to TNF-α treatment had no significant effect on both parameters. TNF-α and IFN-γ cytomix had no influence on TEER but increased permeability of dextran and Lucifer Yellow. The latter suggested IFN-γ's role in TJ disruption. Addition of Cq to cytomix slightly increased the relative percentual change in TEER, decreased dextran permeability by half and lightly increased Lucifer Yellow diffusion. However, all changes were statistically insignificant, leading to the conclusion that disrupting autophagy with Cq had no effect on TJ function.



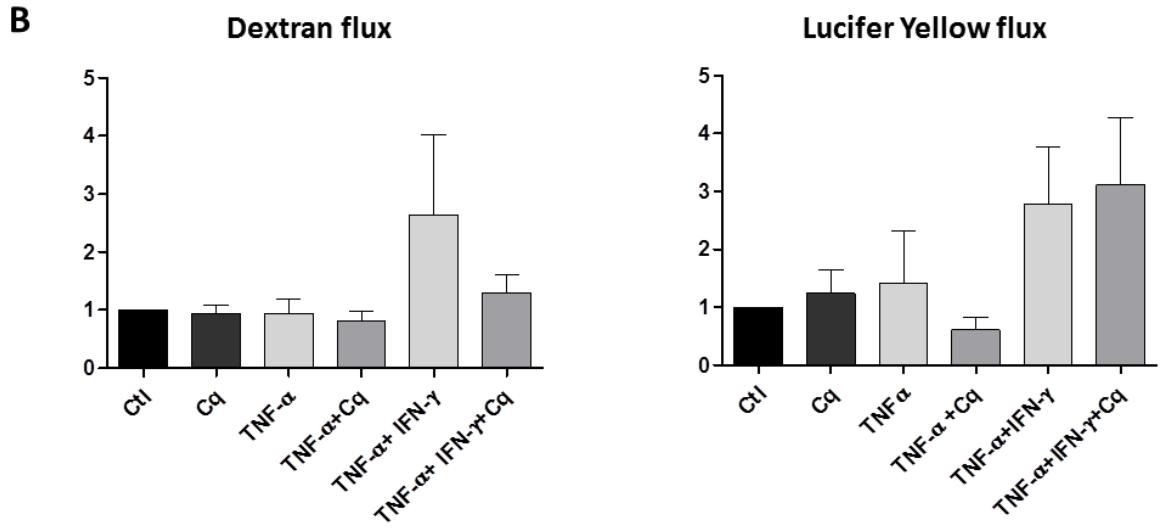


Figure 10: Stimulation with Cq and proinflammatory cytokines does not affect the epithelial barrier function of filter-grown Caco-2 monolayers. Cells were cultured on inserts until differentiation and treated for 24 hours with 3 µM Cq, 50 ng/mL TNF-α and 25 ng/mL IFN-γ alone or in combination. A.) Measurement of TEER in Caco-2 cells. Measurements were performed from day 3 on, respectively, at 3 different areas of the filter inserts and out of 3 independent experiments. Relative change in TEER was calculated by subtracting the TEER before treatment from the TEER after treatment and dividing the difference by TEER before treatment of the same transwell. B.) Permeability was measured by dextran and Lucifer Yellow flux through the Caco-2 monolayer. Data represent means ± SEM of 3 independent experiments and are normalized to control.

4.2.2. Effects of disrupting autophagy on ICAM-1 expression

4.2.2.1. Disrupting autophagy with Cq altered cytokine induced cell surface ICAM-1 expression in Caco-2 cells

Proinflammatory cytokines are known to increase ICAM-1 expression on the cell surface of IEC. Disrupting autophagy with Cq diminished this increased ICAM-1 expression upon 4 hour TNF-α and IL-1β stimulation (Figures 11A and B). This effect could not be shown with sole IFN-γ and combination of TNF-α and IFN-γ treatment. With combination of TNF-α and IFN-γ, Cq's ability of reducing ICAM-1 expression was limited on protein expression in Western blot (Figure 11B). Using FACS there was a trend for reduced ICAM-1 expression with Cq for all cytokine treatments (Figure 11C). This data suggested

that disrupting cytokine induced autophagy may prevent ICAM-1 overexpression and therefore may have beneficial effects in IBD patients.

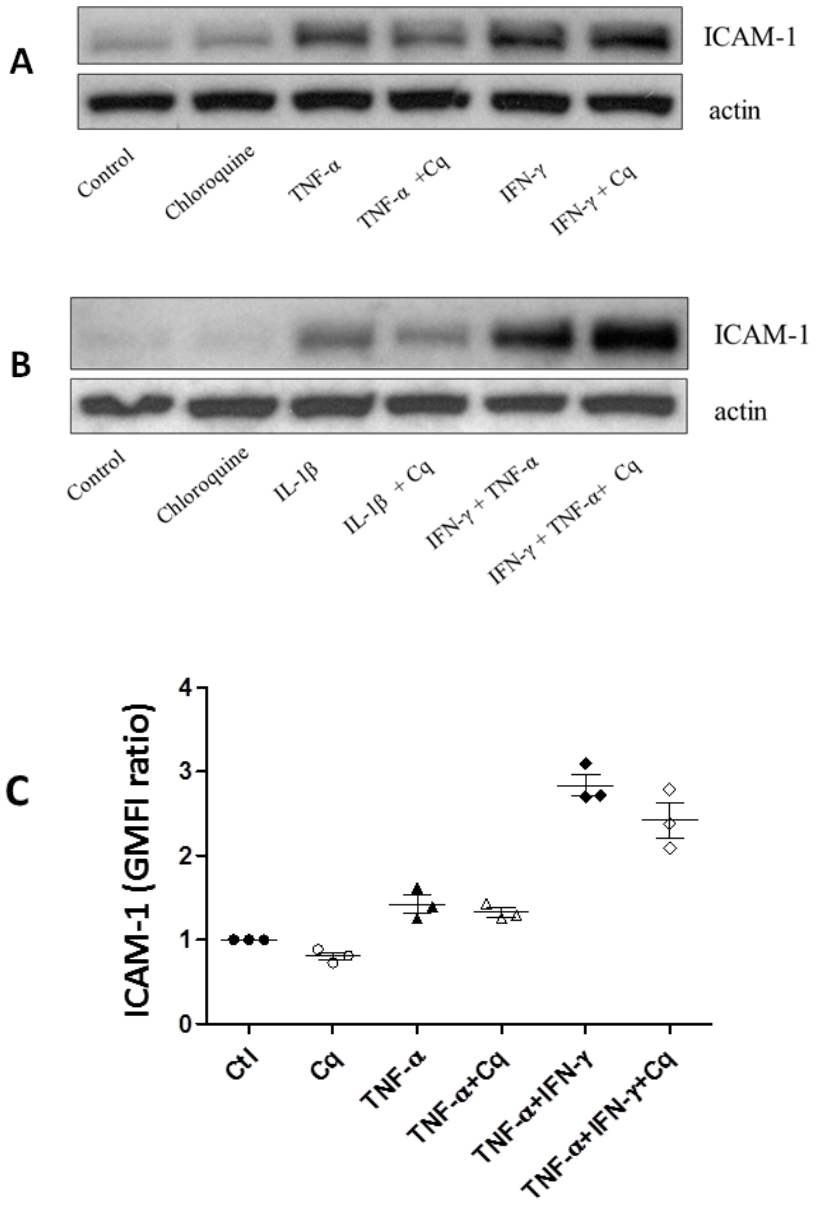


Figure 11: Disrupting autophagy at the final step with Cq alters ICAM-1 expression in cytokine treated Caco-2 cells. A and B.) Western blot analysis of ICAM-1 in lysates of Caco-2 cells stimulated with proinflammatory cytokines and Cq. C.) Demonstration of ICAM-1 expression in differently treated Caco-2 cells by measurement of geometrical mean fluorescence intensity (GMFI) by flow cytometry. The resulting GMFIs were plotted as a ratio of ICAM-1 to control. The scatter dot plot shows three experiments with bar graphs indicating mean ± SEM. For both experiments 4 hour treatments (50 ng/mL TNF-α, 25 ng/mL IL-1β, 25 ng/mL IFN-γ, 3 μM Cq) were applied.

4.2.2.2. Disrupting autophagy with Cq altered neutrophil adhesion to Caco-2 cells

ICAM-1 is important for neutrophil adhesion. Accumulation of large number of neutrophils in the intestine is a hallmark of the active disease phase of IBD. For this reason we performed additional functional test and evaluated neutrophil adhesion to differently stimulated Caco-2 cells. We focused on TNF-α and TNF-α + IFN-γ combination, as first showed a decrease and second opposing results in ICAM-1 expression after blocking autophagy with Cq.

Neutrophil adhesion assay results are shown on Figure 12. Fluorescence images (Figure 12A) and associated graph (12B) clearly showed that a negligible number of neutrophils bound to untreated or Cq treated cells. TNF-α treatment increased neutrophil attachment to Caco-2 cells. Concomitant treatment with Cq resulted in significantly decreased adhesion in comparison to TNF-α treated cells. This is consistent with previous obtained results, of decreased ICAM-1 after TNF-α and Cq combination as neutrophils bind to ICAM-1 (Figure 11). Adhesion was highest with combination of TNF-α and IFN-γ treatment. Addition of Cq slightly, but insignificantly decreased neutrophil adhesion to TNF-α and IFN-γ stimulated cells, which was more consistent with the results of FACS analysis.

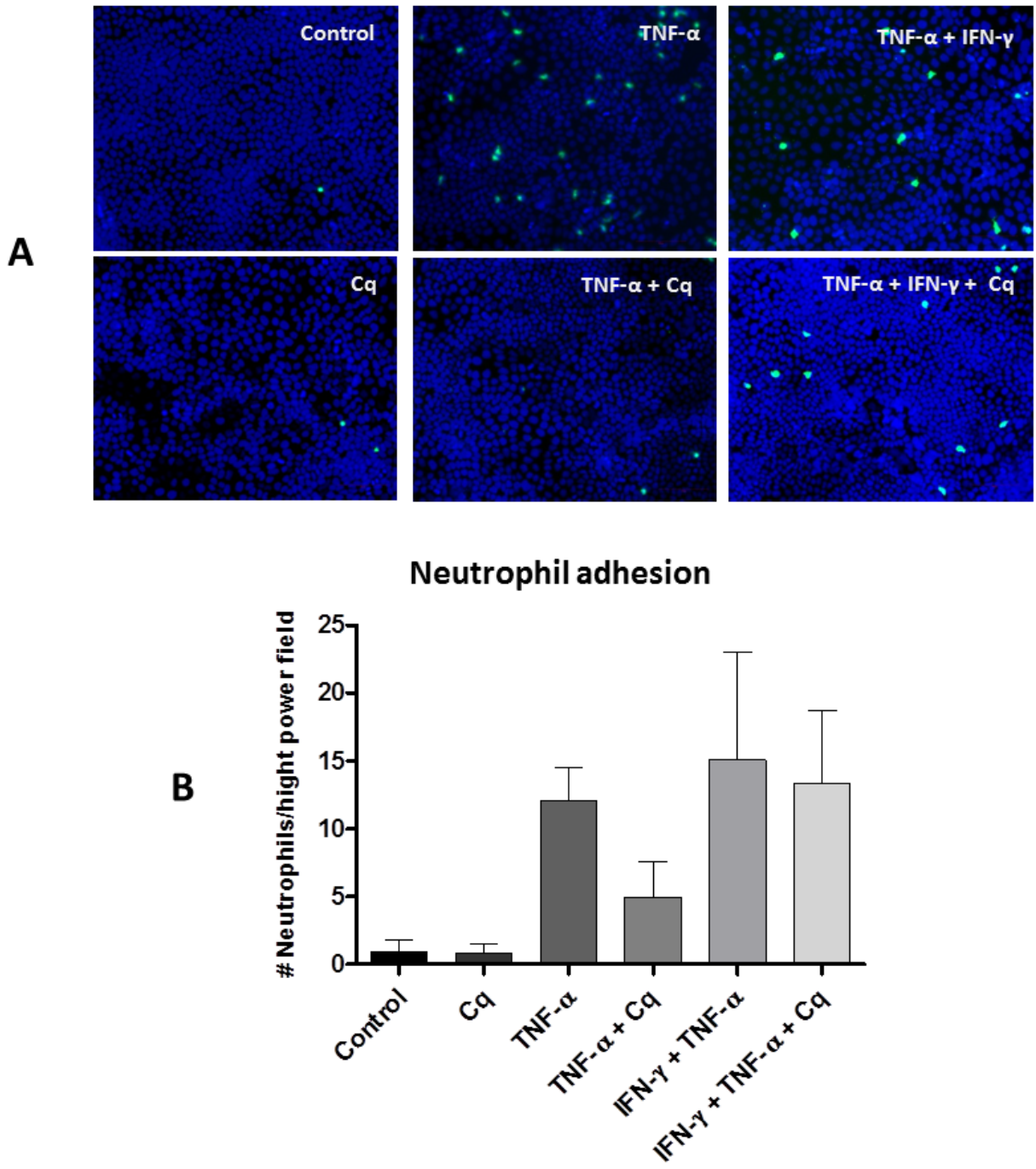


Figure 12: **Disrupting autophagy with Cq affects neutrophil adhesion in cytokine treated Caco-2 cells.** A.) Neutrophils (green) were isolated from blood, stained with calcein-AM and their adhesion to Caco-2 cells (blue) was evaluated by fluorescence microscopy. Scale bar: 100 µm. B.) Ten pictures of each treatment were randomly taken and adhered neutrophils were counted on every image. Graph represents average number of adhered neutrophils per high-power field depending on a treatment. Three independent experiments were performed.

4.2.3. Effect of disrupting autophagy on cytokine secretion

A third function of IEC is cytokine production. Therefore we performed ELISA on cell supernatant to determine the influence on proinflammatory cytokine production of Caco-2 cells. Secretion of IL-1β, IL-6 and IL-8, three proinflammatory cytokines which are involved in the pathogenesis of IBD, was measured by specific ELISA.

Caco-2 cells did not secrete measurable amounts of IL-6 after different treatments and stimulation. There was minimal induction of IL-1β secretion when stimulated with TNF-α, IFN-γ plus Cq, IFN-γ plus TNF-α, or IFN-γ plus TNF-α and Cq (data not shown).

Figure 13 shows that IL-8 secretion was induced by TNF-α, IFN-γ plus TNF-α and especially by IL-1β treatment, with or without Cq. Statistical analysis revealed that only IL-1β induced significant secretion of IL-8. IFN-γ treatment did not cause IL-8 secretion. As the increase of the IL-8 level after TNF-α and IFN-γ plus TNF-α stimulation was similar, it may be suggested that TNF-α and not IFN-γ is mainly responsible for the induction of IL-8 secretion. Addition of Cq did not alter IL-8 secretion in TNF-α, IFN-γ, or combined treatment. For IL-1β, adding Cq to disrupt autophagy significantly reduced IL-8 secretion.

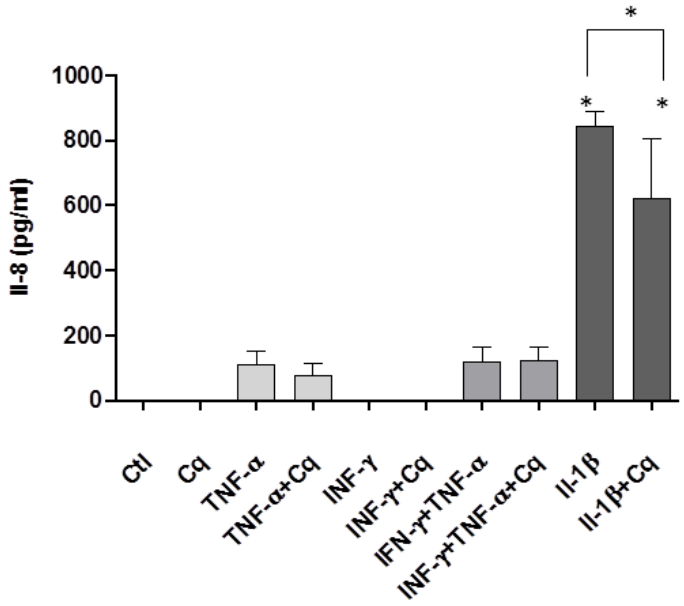


Figure 13: TNF-α and IL-1β induce IL-8 secretion, only the latter being significant. Disrupting autophagy with Cq significantly reduces IL-8 secretion in IL-1β treated Caco-2 cells. Cytokine secretion was measured in supernatants of differently treated cells by specific ELISA. Data are presented as means ± SEM; n=2 for IL-1β experiments and n=3 for the remaining; $p<0.05$.

5.) DISCUSSION

The aim of this study was to characterise the role of autophagy in intestinal epithelial cells (IECs) in regards to tight junction (TJ) integrity, adhesion molecule expression and cytokine secretion.

It is known that autophagy plays an important role in health and disease. There are many examples of the conflicting effects of autophagy. For example in cancer, autophagy may initially prevent tumor growth by targeting damaged proteins and DNA. Later it promotes tumor growth by providing energy in its hypoxic centre. There are only sparse data about the role in IECs. Therefore, we wanted to elucidate the role of autophagy in IECs in an inflammatory environment similar to that found in IBD. First we investigated the effect of enhancing autophagy. We could confirm that the Caco-2 clone HTB-37, which we used in our study, is autophagy competent. After 4 hour stimulation with the autophagy inducer rapamycin we could demonstrate an induction of autophagy. Next we simulated the proinflammatory environment found in IBD. Cells were treated with the proinflammatory cytokines TNF-α, IFN-γ, and IL-1β. All three cytokines induced autophagy. Kathiria et al. showed a similar effect of TNF-α and IFN-γ treatment in the Caco-2 clone BBE. [65] We could also show a cumulative autophagy induction when treating cells with proinflammatory cytokines and the autophagy inducer rapamycin.

Next, we wanted to determine the effect of enhanced autophagy on TJ structure. Proinflammatory cytokines are known TJ disrupters, but no investigations have been made with contemporarily rapamycin treatment. TJ consists of multiple proteins which use ZO-1 as anchor and connection to the actin cytoskeleton. We focused on occludin which together with claudins represents the main component of TJ. Ou and colleagues demonstrated that TNF-α, IL-1β and IFN-γ treated Caco-2 cells show changes in mRNA expression of several proteins already after 4 hour stimulation [66]. Therefore we examined if 4 hour treatment was enough to show any changes in occludin levels. As no alterations were observed, we wanted to exclude that changes in protein levels need more time and prolonged the treatment up to 72 hours. We enhanced autophagy by single compounds as well as by combination treatments. We focused on TNF-α as recent studies suggested its crucial role in barrier dysregulation. Irregularities were observed just after 72 hour treatment with TNF-α, which was delayed compared to other studies in Caco-2 cells. Ma

and colleagues [67] and Chen et al. [68] observed wrinkled appearance and punctate at points of multiple cellular contact in TJ proteins already after 48 hour TNF-α stimulation. In both studies TNF-α was used in concentration of 10 ng/mL, which was lower in comparison with our experiments. In accordance with our results, in murine models of intestinal inflammation TNF-α appears to maintain the integrity of the epithelial barrier during acute inflammation. Pagnini et al. reported that multiple probiotic formulation prevented the onset of intestinal inflammation by increased production of epithelial-derived TNF-α in mouse model of CD [69]. Blockade of TNF-α by monoclonal antibody treatment given concomitantly with probiotic administration completely abrogated the beneficial effects of the probiotic mixture in this model. On the other hand, probiotic treatment did not affect established disease. These data are also reassuring that findings in our *in vitro* model may be translatable into *in vivo* studies. Addition of rapamycin in our model showed no effect on this phenotype, however if pharmacological induction of autophagy in a disease with defective autophagy such as IBD has a beneficial effect is not known.

Although TJ structure was affected after 72 hour stimulation with TNF-α and rapamycin, TJ function was not altered according to TEER and permeability measurements. Our results are in contrast with findings of other groups as TNF-α treatment usually increases permeability and decreases TEER of Caco-2 cells within 48 hours [66, 67, 69]. The only difference compared to our experiment was the application of the treatments. While others applied it basolaterally, we placed treatments in apical compartments. However, IECs come in contact with proinflammatory cytokines and express cytokine receptors also on their apical side [36, 70]. Neutrophils recruited from blood stream perform their effects on IECs from luminal through their apical side. Furthermore, Sumagin et al. [41] stimulated T84 cells, another cancer cell line used to simulate the epithelial layer of the gut, with another important proinflammatory cytokine IFN-γ and found no changes in TEER, although applied basolaterally. However, after adding neutrophils the TEER declined already after 24 hours. They hypothesized that ICAM-1 crosslinking by neutrophils was responsible for the disruption of the TJ. Therefore, cytokines alone may be insufficient to disrupt TJ and a second stimuli is needed.

One limitation of TEER measurements is that different cell lines and cell clones seem to have different baseline values and dynamics during differentiation, polarization, and

treatments [72]. Therefore precaution is required, when comparing the results of various studies.

We observed differences in the permeability of molecules with different sizes. As Lucifer Yellow flux seemed to be more affected upon TNF-α stimulation than dextran flux, permeability of smaller molecules should be investigated in future. Nighot and colleagues [73] showed in their cell culture study, that under starvation condition the permeability for small sized molecules is reduced. They claim that autophagy may selectively target the small-size, cation-selective, "pore" pathway without affecting the large-size, non-charge selective "leak" pathway by lysosomal degradation of the TJ protein claudin-2. A similar effect of pharmacological induction of autophagy could explain why we found no significant changes in the permeability of differently treated Caco-2 cells in our study.

Next we investigated the potential involvement of enhanced autophagy in adhesion molecule expression, focusing on ICAM-1. ICAM-1 is the main adhesion molecule for neutrophil adhesion in IECs. Proinflammatory cytokine treatment resulted in ICAM-1 overexpression. Whereas rapamycin treatment alone had no effect on ICAM-1 expression and combination of proinflammatory cytokines and rapamycin treatment had no further effect on the increase in ICAM-1 expression (data not shown) that was seen with proinflammatory cytokine treatment.

In conclusion: enhancing autophagy did not alter the effects induced by proinflammatory cytokine treatment in regards to TEER, TJ and adhesion molecule expression of healthy IECs in our study.

In the second part of our study we focused on blocking autophagy. Increased LC3-II levels clearly showed that Cq, which blocks autophagy at the stage of autolysosomes, successfully disrupted autophagy in Caco-2 cells. When Cq was added to cytokine treatment, there was further increase in LC3-II level, confirming that proinflammatory cytokines induced autophagy.

Investigating the TJ structure after 4 hour treatment with proinflammatory cytokines or Cq showed that ZO-1 and occludin levels were not altered. To determine long-term impact of TNF-α and Cq treatment on Caco-2 cells, we prolonged stimulation up to 24 hours. Effect of TNF-α and IFN-γ stimulation alone or in combination with Cq was also examined. Longer time periods were not considered as Cq exhibits toxic effects upon prolonged

exposure. IF staining of ZO-1 indicated that TJ anchoring remained intact also after disrupting autophagy for a prolonged time period. TEER and permeability measurements also showed no significant changes.

Interesting findings were obtained while delineating the role of blocked autophagy on adhesion molecule expression. Disrupting autophagy with Cq diminished cytokine increased ICAM-1 expression upon TNF-α and IL-1β stimulation according to Western blot results. To confirm our findings and to better analyse single cells, we also performed FACS. Here a trend for reduced ICAM-1 expression when disrupting autophagy with Cq for all cytokine treatments could be shown. Treede and colleagues [74] reported that ICAM-1 gene transcription was higher with basolateral TNF-α treatment than with apical. Therefore, additional FACS experiments were performed comparing basolaterally and apically applied treatments. However, in our cell line no differences in ICAM-1 expression were observed independent of the direction of application. This finding confirmed also our previous presumption that there are no significant differences between exposing cells to proinflammatory stimuli on the apical or basolateral side. With FACS we focused on the detection on membrane-bound ICAM-1. We were able to not only see a reduction in ICAM-1 expression after TNF-α plus Cq, but also after TNF-α plus IFN-γ plus Cq treatment.

Massive neutrophil infiltration is one of the main features of IBD. For their adhesion to IECs neutrophils require ICAM-1 as binding partner on IECs. Thus, we performed a neutrophil adhesion assay to elucidate a possible clinical relevance of the observed increased ICAM-1 expression. As expected, the cytokines induced ICAM-1 expression resulted in an increased number of bound neutrophils. The cytomix of TNF-α and IFN-γ caused a larger increase in the number of bound neutrophils compared to sole TNF-α treatment. Therefore, cytokines seem to synergise in ICAM-1 induction. Disrupting cytokine induced autophagy with Cq decreased the neutrophil adhesion to Caco-2 cells. This was consistent with a decreased ICAM-1 expression. Interestingly, Cq reduced neutrophil adhesion more in TNF-α then in TNF-α plus IFN-γ treated cells. This could be explained by different mechanisms involved in the cellular signalling of both cytokines. Indeed, TNF-α and IL-1β are thought to exhibit their effects through NF-κB signalling, while IFN-γ primarily signals through the JAK-STAT pathway [75].

As disrupting autophagy provided promising results on ICAM-1 expression, we further investigated its effect on cytokine secretion from Caco-2 cells. IL-8 secretion was induced by TNF-α and IL-1β, only the latter being significant. IFN-γ failed to induce IL-8 secretion. Our data confirm the findings of Van De Walle and colleagues [61] who investigated effects of different inflammatory stimuli on Caco-2 cells. Also in their case IL-1β caused the largest increase in IL-8 production, while TNF-α induced it to a lesser extent. As in our study, IFN-γ alone failed to upregulate IL-8 secretion in their study although they even used a higher concentration of 50 ng/mL and applied it for a longer time period (24 hours). The same study showed that Caco-2 cells secrete IL-6 upon IL-1β stimulation. In our case, there was no secretion of IL-6 with either treatment. One explanation for the different findings may be that their treatments lasted for 24 hours. Disrupting autophagy with Cq significantly reduced IL-8 secretion in IL-1β treatment.

IL-8 and ICAM-1 transcription has been shown to be regulated by NF-κB [36, 75]. The latter is typically overactivated in inflamed intestinal mucosa [36]. Interestingly, recent studies suggested that TNF-α and IL-1β induced autophagy could be involved in the degradation of the NF-κB inhibitor IκBα. IκBα binds NF-κB in the cytoplasm and prevents its translocation to the nucleus [76, 77] and so NF-κB is unable to act as a transcription factor. Based on these findings, a possible explanation for a reduced ICAM-1 expression upon concomitant cytokine and Cq treatment could be that Cq prevents autophagy-mediated IκBα degradation. The same could apply for IL-1β-mediated IL-8 secretion. Supporting this theory is the finding that IFN-γ treatment did not show the same effect as other pathways are involved in its signalling.

In summary, we hypothesize, that the proinflammatory cytokines IL-1β and TNF-α induce autophagy which degrades IκB-α. Liberated NF-κB can translocate to the nucleus where it acts as a transcription factor and upregulates expression of ICAM-1 and, in case of IL-1β, IL-8. Disrupting autophagy with Cq prevents this upregulation. As manipulated autophagy and stimulation with proinflammatory cytokines seems to have no effect on TJ structure and function, we hypothesize that additional stimuli are needed for inducing barrier dysfunction. If the functional consequence of decreased neutrophil adhesion is beneficial in the setting of IBD is not known. Knock down of ATG genes, which would prevent the initiation of autophagy instead of blocking the last step in the autophagy pathway may give here some answers.

6.) CONCLUSION

Our study elucidates the role of autophagy in IECs and its effect on TJ, adhesion molecule expression and cytokine secretion. The main finding is that blocking autophagy pathway at the last step can decrease adhesion molecule expression and neutrophil adhesion in an inflammatory environment. Blocking a potentially excessively activated autophagy process in IECs may have beneficial effects in IBD patients. The next research step should use genetic approaches to inhibit autophagy in Caco-2 cells to confirm that dysrupted autophagy was responsible for our findings. Additionally the NF-κB pathway should be investigated.

It should be noted that our model included cells with normal autophagic function. The situation and responses may be different in the inflamed gut of IBD patients.

In general our study showed that autophagy is undoubtedly important for a normal function of IECs.

7.) REFERENCES

[1] B. Levine and D. J. Klionsky, “Development by Self-DigestionMolecular Mechanisms and Biological Functions of Autophagy,” *Dev. Cell*, vol. 6, no. 4, pp. 463–477, 2004.

[2] T. Johansen and T. Lamark, “Selective autophagy mediated by autophagic adapter proteins,” *Autophagy*, vol. 7, no. 3, pp. 279–296, 2011.

[3] Beth Levine and Guido Kroemer, “Autophagy in the Pathogenesis of Disease,” *Cell.*, vol. 132, no. 1, pp. 27–42, 2008.

[4] V. Todde, M. Veenhuis, and I. J. van der Klei, “Autophagy: Principles and significance in health and disease,” *Biochim. Biophys. Acta - Mol. Basis Dis.*, vol. 1792, no. 1, pp. 3–13, 2009.

[5] M. Salem, M. Ammitzboell, K. Nys, J. B. Seidelin, and O. H. Nielsen, “ATG16L1: A multifunctional susceptibility factor in crohn disease,” *Autophagy*, vol. 11, no. 4, pp. 585–594, 2015.

[6] T. O. Crişan, T. S. Plantinga, F. L. van de Veerdonk, M. F. Farcaş, M. Stoffels, B. J. Kullberg, J. W. M. van der Meer, L. A. B. Joosten, and M. G. Netea, “Inflammasome-independent modulation of cytokine response by autophagy in human cells,” *PLoS One*, vol. 6, no. 4, 2011.

[7] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, and T. Yoshimori, “Erratum: LC3, a mammalian homolog of yeast Apg8p, is localized in autophagosome membranes after processing (EMBO Journal (2000) 19 (5720-5728)),” *EMBO J.*, vol. 22, no. 17, p. 4577, 2003.

[8] R. C. Russell, H.-X. Yuan, and K.-L. Guan, “Autophagy regulation by nutrient signaling.,” *Cell Res.*, vol. 24, no. 1, pp. 42–57, 2014.

[9] D. Benjamin, M. Colombi, C. Moroni, and M. N. Hall, “Rapamycin passes the torch: a new generation of mTOR inhibitors,” *Nat. Rev. Drug Discov.*, vol. 10, no. 11, pp. 868–880, 2011.

[10] D. C. Rubinsztein, J. E. Gestwicki, L. O. Murphy, and D. J. Klionsky, “Potential therapeutic applications of autophagy.,” *Nat. Rev. Drug Discov.*, vol. 6, no. 4, pp. 304–312, 2007.

[11] C. Sokollik, M. Ang, and Nicola L Jones, “Autophagy : A primer for the gastroenterologist/hepatologist,” *Can J Gastroenterol*, vol. 25, no. 12, pp. 667–674, 2011.

[12] R. Okamoto and M. Watanabe, “Role of epithelial cells in the pathogenesis and treatment of inflammatory bowel disease,” *J. Gastroenterol.*, vol. 51, no. 1, pp. 11–21, 2016.

[13] H. F. Helander and L. Fändriks, “Surface area of the digestive tract - revisited.,” *Scand. J. Gastroenterol.*, vol. 49, no. 6, pp. 681–9, 2014.

[14] K. Zhang, M. W. Hornef, and A. Dupont, “The intestinal epithelium as guardian of gut barrier integrity,” *Cell. Microbiol.*, vol. 17, no. 11, pp. 1561–1569, 2015.

[15] J. Turner, “Molecular Basis of Epithelial Barrier Regulation: From Basic Mechanisms to Clinical Application,” *Am. J. Pathol.*, vol. 169, no. 6, pp. 1901–1909, 2006.

[16] A. Negroni, S. Cucchiara, and L. Stronati, “Apoptosis, necrosis, and necroptosis in the gut and intestinal homeostasis,” *Mediators Inflamm.*, vol. 2015, 2015.

[17] S. Randall-Demllo, M. Chieppa, and R. Eri, “Intestinal epithelium and autophagy: Partners in gut homeostasis,” *Front. Immunol.*, vol. 4, no. SEP, pp. 1–14, 2013.

[18] J. J. Worthington, “The intestinal immunoendocrine axis: novel cross-talk between enteroendocrine cells and the immune system during infection and inflammatory disease.,” *Biochem. Soc. Trans.*, vol. 43, no. 4, pp. 727–33, 2015.

[19] L. Shen, “Tight junctions on the move: Molecular mechanisms for epithelial barrier regulation,” *Ann. N. Y. Acad. Sci.*, vol. 1258, no. 1, pp. 9–18, 2012.

[20] K. E. Cunningham and J. R. Turner, “Myosin light chain kinase: Pulling the strings of epithelial tight junction function,” *Ann. N. Y. Acad. Sci.*, vol. 1258, no. 1, pp. 34–42, 2012.

[21] S. H. Lee, “Intestinal Permeability Regulation by Tight Junction: Implication on Inflammatory Bowel Diseases,” *Intest. Res.*, vol. 13, no. 1, p. 11, 2015.

[22] C. Will, M. Fromm, and D. Müller, “Claudin tight junction proteins: novel aspects in paracellular transport,” *Perit. Dial. Int.*, vol. 28, no. 1, pp. 577–584, 2008.

[23] M. Furuse, T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita, “Occludin: A novel integral membrane protein localizing at tight junctions,” *J. Cell Biol.*, vol. 123, no. 6 PART 2, pp. 1777–1788, 1993.

[24] I. Martin-Padura, S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, and E. Dejana, “Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration,” *J. Cell Biol.*, vol. 142, no. 1, pp. 117–127, 1998.

[25] J. Ikenouchi, M. Furuse, K. Furuse, H. Sasaki, S. Tsukita, and S. Tsukita, “Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells,” *J. Cell Biol.*, vol. 171, no. 6, pp. 939–945, 2005.

[26] R. Al-Sadi, K. Khatib, S. Guo, D. Ye, M. Youssef, and T. Ma, “Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier.,” *Am. J. Physiol. Gastrointest. Liver Physiol.*, vol. 300, no. 6, pp. G1054–G1064, 2011.

[27] J. Landy, E. Ronde, N. English, S. K. Clark, A. L. Hart, S. C. Knight, P. J. Ciclitira, and H. O. Al-Hassi, “Tight junctions in inflammatory bowel diseases and inflammatory bowel disease associated colorectal cancer,” *World J. Gastroenterol.*, vol. 22, no. 11, pp. 3117–3126, 2016.

[28] B. R. Stevenson, J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough, “Identification of ZO-1: A high molecular weight polypeptide associated with the tight junction (Zonula Occludens) in a variety of epithelia,” *J. Cell Biol.*, vol. 103, no. 3, pp. 755–766, 1986.

[29] K. Umeda, T. Matsui, M. Nakayama, K. Furuse, H. Sasaki, M. Furuse, and S. Tsukita, “Establishment and characterization of cultured epithelial cells lacking expression of ZO-1.,” *J. Biol. Chem.*, vol. 279, no. 43, pp. 44785–44794, 2004.

[30] J. D. Matthews, C. M. Weight, and C. A. Parkos, “Leukocyte-epithelial interactions and

mucosal homeostasis.," *Toxicol. Pathol.*, vol. 42, no. 1, pp. 91–8, 2014.

[31] A. W. Stadnyk, "Intestinal epithelial cells as a source of inflammatory cytokines and chemokines.," *Can. J. Gastroenterol.*, vol. 16, no. 4, pp. 241–246, 2002.

[32] G. Bamias, D. Corridoni, T. T. Pizarro, and F. Cominelli, "New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation," *Cytokine*, vol. 59, no. 3, pp. 451–459, 2012.

[33] Jun-Ming Zhang and Jianxiong An, "Cytokines, Inflammation and Pain," *Int Anesth. Clin.*, vol. 45, no. 2, pp. 27–37, 2007.

[34] G. Lopez-Castejon and D. Brough, "Understanding the mechanism of IL-1b secretion," *Cytokine Growth Factor Rev.*, vol. 22, no. 4, pp. 189–195, 2011.

[35] J. Harris, M. Hartman, C. Roche, S. G. Zeng, A. O'Shea, F. A. Sharp, E. M. Lambe, E. M. Creagh, D. T. Golenbock, J. Tschopp, and H. Kornfeld, "Autophagy controls IL-1?? secretion by targeting Pro-IL-1?? for degradation," *J. Biol. Chem.*, vol. 286, no. 11, pp. 9587–9597, 2011.

[36] L. Wang, B. Walia, J. Evans, A. T. Gewirtz, D. Merlin, and S. V. Sitaraman, "IL-6 Induces NF- B Activation in the Intestinal Epithelia," *J. Immunol.*, vol. 171, no. 6, pp. 3194–3201, 2003.

[37] D. C. T. Palomino and L. C. Marti, "Chemokines and immunity," *Einstein*, vol. 13, no. 3, pp. 469–473, 2015.

[38] A. Kelso, "Cytokines: Principles and prospects," *Immunol. Cell Biol.*, vol. 76, no. 4, pp. 300–317, 1998.

[39] L. E. Neuder, J. M. Keener, R. E. Eckert, J. C. Trujillo, and S. L. Jones, "Role of p38 MAPK in LPS induced pro-inflammatory cytokine and chemokine gene expression in equine leukocytes," *Vet. Immunol. Immunopathol.*, vol. 129, no. 3–4, pp. 192–199, 2009.

[40] R. Lyck and G. Enzmann, "The physiological roles of ICAM-1 and ICAM-2 in neutrophil migration into tissues.," *Curr. Opin. Hematol.*, vol. 22, no. 1, pp. 53–9, 2015.

[41] R. Sumagin, A. Z. Robin, A. Nusrat, and C. A. Parkos, "Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment.," *Mucosal Immunol.*, vol. 7, no. 4, pp. 905–15, 2014.

[42] K. Zen, Y. Liu, I. C. McCall, T. Wu, W. Lee, B. A. Babbin, A. Nusrat, and C. A. Parkos, "Neutrophil Migration across Tight Junctions Is Mediated by Adhesive Interactions between Epithelial Coxsackie and Adenovirus Receptor and a Junctional Adhesion Molecule-like Protein on Neutrophils," *Mol. Biol. Cel*, vol. 16, no. 2, pp. 2694–2703, 2005.

[43] T. N. Ramos, D. C. Bullard, and S. R. Barnum, "ICAM-1: Isoforms and Phenotypes," *J. Immunol.*, vol. 192, no. 10, pp. 4469–4474, 2014.

[44] P. Kawalec, "Indirect costs of inflammatory bowel diseases: Crohn's disease and ulcerative colitis. A systematic review," *Arch. Med. Sci.*, vol. 2, pp. 295–302, 2016.

[45] J. Brazil, N. Louis, and C. Parkos, "The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease," *Inflamm Bowel Dis.*, vol. 19, no. 7, pp. 1556–1565, 2013.

[46] G. Bamias, T. T. Pizarro, and F. Cominelli, “Pathway-based approaches to the treatment of inflammatory bowel disease,” *Transl. Res.*, vol. 167, no. September, pp. 1–12, 2015.

[47] M. Bosani, S. Ardizzone, and G. B. Porro, “Biologic targeting in the treatment of inflammatory bowel diseases.,” *Biologics*, vol. 3, pp. 77–97, 2009.

[48] F. Wang, W. V. Graham, Y. Wang, E. D. Witkowski, B. T. Schwarz, and J. R. Turner, “Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression.,” *Am. J. Pathol.*, vol. 166, no. 2, pp. 409–19, 2005.

[49] J. Mankertz, S. Tavalali, H. Schmitz, a Mankertz, E. O. Riecken, M. Fromm, and J. D. Schulzke, “Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma.,” *J. Cell Sci.*, vol. 113 (Pt 1, pp. 2085–2090, 2000.

[50] A. N. and C. P. R Sumagin, AZ Robin, “Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment.” pp. 905–915, 2014.

[51] M. Coskun, “Intestinal epithelium in inflammatory bowel disease.,” *Front. Med.*, vol. 1, no. August, p. 24, 2014.

[52] T.-E. M. Manuc, M. M. Manuc, and M. M. Diculescu, “Recent insights into the molecular pathogenesis of Crohn’s disease: a review of emerging therapeutic targets.,” *Clin. Exp. Gastroenterol.*, vol. 9, pp. 59–70, 2016.

[53] C. W. Lees, J. C. Barrett, M. Parkes, and J. Satsangi, “New IBD genetics: common pathways with other diseases,” *Gut*, vol. 60, no. 12, pp. 1739–1753, 2011.

[54] K. Cadwell, J. Y. Liu, S. L. Brown, H. Miyoshi, J. Loh, J. K. Lennerz, C. Kishi, W. Kc, J. A. Carrero, S. Hunt, C. D. Stone, E. M. Brunt, R. J. Xavier, B. P. Sleckman, E. Li, N. Mizushima, T. S. Stappenbeck, and H. W. Virgin, “A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells.,” *Nature*, vol. 456, no. 7219, pp. 259–63, 2008.

[55] T. Saitoh, N. Fujita, M. H. Jang, S. Uematsu, B.-G. Yang, T. Satoh, H. Omori, T. Noda, N. Yamamoto, M. Komatsu, K. Tanaka, T. Kawai, T. Tsujimura, O. Takeuchi, T. Yoshimori, and S. Akira, “Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 beta production,” *Nature*, vol. 456, no. 7219, pp. 264–U68, 2008.

[56] P. Kuballa, A. Huett, J. D. Rioux, M. J. Daly, and R. J. Xavier, “Impaired autophagy of an intracellular pathogen induced by a Crohn’s disease associated ATG16L1 variant,” *PLoS One*, vol. 3, no. 10, pp. 1–8, 2008.

[57] F. Balzola, G. Cullen, G. T. Ho, and R. Russell, “T300A variant of autophagy ATG16L1 gene is associated with decreased antigen sampling and processing by dendritic cells in pediatric Crohn’s disease: Commentary,” *Inflamm. Bowel Dis. Monit.*, vol. 14, no. 2, p. 61, 2014.

[58] X. Hao, B. Yang, X. Liu, H. Yang, and X. Liu, “Expression of Beclin1 in the colonic mucosa tissues of patients with ulcerative colitis,” vol. 8, no. 11, pp. 21098–21105, 2015.

[59] B. Vainer, “Intercellular adhesion molecule-1 (ICAM-1) in ulcerative colitis: Presence, visualization, and significance,” *Apmis*, vol. 118, no. SUPPL. 129, pp. 1–46, 2010.

[60] M. Natoli, B. D. Leoni, I. D'Agnano, F. Zucco, and A. Felsani, "Good Caco-2 cell culture practices," *Toxicol. Vitr.*, vol. 26, no. 8, pp. 1243–1246, 2012.

[61] J. Van De Walle, A. Hendrickx, B. Romier, Y. Larondelle, and Y. J. Schneider, "Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation," *Toxicol. Vitr.*, vol. 24, no. 5, pp. 1441–1449, 2010.

[62] Epithelial Voltohmmeter: https://www.wpiinc.com/products/top-products/evom2-evom2-epithelial-volt-ohm-meter-for-teer/.

[63] V. Spindler, M. Meir, B. Vigh, S. Flemming, K. Hütz, C.-T. Germer, J. Waschke, and N. Schlegel, "Loss of Desmoglein 2 Contributes to the Pathogenesis of Crohn's Disease," *Inflamm. Bowel Dis.*, vol. 21, no. 10, p. 1, 2015.

[64] M. L. Applebury, "Variations in retinal degenerations," *Curr Biol*, vol. 2, no. 3, pp. 113–115, 1992.

[65] A. S. Kathiria, L. D. Butcher, L. A. Feagins, R. F. Souza, C. R. Boland, and A. L. Theiss, "Prohibitin 1 modulates mitochondrial stress-related autophagy in human colonic epithelial cells," *PLoS One*, vol. 7, no. 2, 2012.

[66] G. Ou, V. Baranov, E. Lundmark, S. Hammarström, and M. L. Hammarström, "Contribution of intestinal epithelial cells to innate immunity of the human gut - Studies on polarized monolayers of colon carcinoma cells," *Scand. J. Immunol.*, vol. 69, no. 2, pp. 150–161, 2009.

[67] T. Y. Ma, G. K. Iwamoto, N. T. Hoa, V. Akotia, A. Pedram, M. a Boivin, and H. M. Said, "TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation.," *Am. J. Physiol. Gastrointest. Liver Physiol.*, vol. 286, no. 3, pp. G367–G376, 2004.

[68] S. Chen, J. Zhu, G. Chen, S. Zuo, J. Zhang, Z. Chen, X. Wang, J. Li, Y. Liu, and P. Wang, "1,25-Dihydroxyvitamin D3 preserves intestinal epithelial barrier function from TNF-α induced injury via suppression of NF-kB p65 mediated MLCK-P-MLC signaling pathway," *Biochem. Biophys. Res. Commun.*, vol. 460, no. 3, pp. 873–878, 2015.

[69] C. Pagnini, R. Saeed, G. Bamias, K. O. Arseneau, T. T. Pizarro, and F. Cominelli, "Probiotics promote gut health through stimulation of epithelial innate immunity.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 1, pp. 454–9, 2010.

[70] T. C. Contreras, E. Ricciardi, E. Cremonini, and P. I. Oteiza, "(À) -Epicatechin in the prevention of tumor necrosis alpha-induced loss of Caco-2 cell barrier integrity," *Arch. Biochem. Biophys.*, vol. 573, pp. 84–91, 2015.

[71] E. P. Molmenti, T. Ziambaras, and D. H. Perlmutter, "Evidence for an acute phase response in human intestinal epithelial cells.," *J. Biol. Chem.*, vol. 268, no. 19, pp. 14116–24, 1993.

[72] S. Tavelin, J. Gråsjö, J. Taipalensuu, G. Ocklind, and P. Artursson, "Applications of epithelial cell culture in studies of drug transport," *Epithel. Cell Cult. Protoc.*, vol. 188, pp. 233–272, 2002.

[73] P. K. Nighot and C. A. Hu, "Autophagy Enhances Intestinal Epithelial Tight Junction Barrier Function by Targeting Claudin-2 Protein Degradation *," vol. 290, no. 11, pp. 7234–7246, 2015.

[74] I. Treede, A. Braun, P. Jeliaskova, T. Giese, J. Füllekrug, G. Griffiths, W. Stremmel, and R. Ehehalt, “reduced by phosphatidylcholine in intestinal epithelial cells,” vol. 11, pp. 1–11, 2009.

[75] K. Schroder, P. J. Hertzog, T. Ravasi, and D. A. Hume, “Interferon- y : an overview of signals , mechanisms and functions,” *J. Leukoc. Biol.*, vol. 75, no. February, pp. 163–189, 2004.

[76] D. J. J. Waugh and C. Wilson, “The Interleukin-8 Pathway in Cancer,” vol. 8, no. 21, pp. 6735–6742, 2008.

[77] A. Colleran, A. Ryan, A. O’Gorman, C. Mureau, C. Liptrot, P. Dockery, H. Fearnhead, and L. J. Egan, “Autophagosomal IκBα degradation plays a role in the long term control of tumor necrosis factor-α-induced nuclear factor-κB (NF-κB) activity,” *J. Biol. Chem.*, vol. 286, no. 26, pp. 22886–22893, 2011.

[78] R. Al-Sadi, D. Ye, H. M. Said, and T. Y. Ma, “IL-1beta-induced increase in intestinal epithelial tight junction permeability is mediated by MEKK-1 activation of canonical NF-kappaB pathway.,” *Am. J. Pathol.*, vol. 177, no. 5, pp. 2310–22, 2010.