Direct and indirect effects of microorganisms on autophagy

Summary of Ph.D. Thesis

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1. Introduction

1.1 the Interaction of cytokines and viral infections with Autophagy

1.1.1 Autophagy

Autophagy is an intracellular metabolic process in which cytoplasmic target molecules are transferred to the lysosome for degradation and recycling. Depending on the type of cargo delivery, three different forms of autophagy can be distinguished: (i) macroautophagy, (ii) microautophagy, and (iii) chaperon-mediated autophagy. The difference among these types is how the constituents to be degraded are transported to the lysosome. The major element in the regulation of autophagy is the mechanistic target of rapamycin (mTOR) pathway. The serine/threonine-protein kinase mTOR can be found in two distinct functional complexes, the mTOR complex 1 (mTORC1) and mTORC2. Only mTORC1 can directly govern autophagy. Autophagy inducers inhibit mTORC1 activity, leading to consecutive activation of the ULK1 (autophagy-related gene 1 (atg1) homolog] complex and class III phosphatidylinositol 3-kinase (PI3KC3) complex, containing Beclin-1 protein. The activated P13KC3 induces local production of phosphatidylinositol-3-phosphate (P13P) at the endoplasmic reticulum membrane and thereby promotes the generation of an omegasome from which the isolation membrane is generated. PI3P attracts WD repeat domain phosphoinositide-interacting proteins (WIP1 and WIPI2), and WIPI2 then recruits a ubiquitin-like conjugation system, the ATG12-ATG5 ATG16L1 complex, to the omegasome. A second conjugation system, consisting of ATG7 and ATG3, forms a covalent bond between the membrane-resident phosphatidylethanolamine and the ATG8 family member proteins, such as the microtubule-associated protein 1 light chain 3 (LC3) proteins. The ATG8 proteins are essential for the elongation and closure of the phagophore membrane, trafficking of the autophagosomes, and their fusion with lysosomes. The autophagosomes eventually fuse with lysosomes, the content of the autophagic cargo is degraded and made available for reuse.

1.1.2. Interaction between Autophagy and the pro-inflammatory cytokines

The ubiquitous process of autophagy is strongly associated with immune and inflammatory activity. There is a two-way interaction between autophagy and pro-inflammatory cytokines; many cytokines modulate the autophagy process, and autophagy regulates many pro-inflammatory cytokines. In face of a threat, pro-inflammatory cytokines, which are synthesized by adaptive and innate immune cells, play a key role in the functional responses of the immune and inflammatory systems, and the survival of the immune cells. The activity of the adaptive and innate immune systems might be regulated at a fundamental level by the relationship between autophagy and cytokines. The level of the autophagic activity is determined by several cytokines, such as interferon (IFN)-γ, which initiates autophagic responses to kill pathogens, like *Chlamydia* and mycobacteria. Other cytokines that trigger autophagy include interleukin IL-1, IL-6, IL-17 and tumour necrosis factor (TNF)-α; to counter these activators of autophagy, IL-4, IL-10, IL-13 and IL-33 inhibit the process. Autophagy simultaneously regulates the synthesis and secretion of some cytokines. The interactions

between autophagy and pro-inflammatory cytokines and their significant effect upon the pathophysiology of disease, have become a point of research interest, with much being learned about the interactions. The pro-inflammatory cytokine, IFN-γ, which plays a key role in adaptive and innate immunity, is secreted by activated CD4+ and CD8+ T cells, and natural killer cells. It achieves this by activating multiple immunomodulatory molecules; as such, IFN-γ contributes to several inflammatory and autoimmune disorders. Evidence suggests that autophagy is boosted by IFN-γ, facilitating the presentation of antigens, the clearance of microbes and cellular proliferation. IFN-γ secretion is stimulated by the activation of autophagy, initiating a positive feedback loop. Cell death can be triggered, and invading pathogens can be destroyed by IFN-γ-stimulated autophagy. IFN-γ promotes the autophagic elimination of intracellular pathogens by stimulating macrophages, which heightens the innate immune system's receptor-mediated phagocytosis and microbe-killing defensive processes. IFN-γ may activate macrophages via a pathway involving the family M member 1 GTPase Irgm1/IRGM1, leading to maturation of mycobacteria-containing phagosomes. Studies of concanavalin A (Con A) in hepatoma cell lines show that cell death was increased by the IFN-γ stimulation of autophagy. it has been reported IFN-γ elevated concanavalin A (Con A)-induced autophagic flux in hepatoma cell lines. Inhibiting Irgm1 suppressed IFN-γ/ConA-mediated lysosomal membrane permeabilization and hepatocyte death. The pathway involved in IFN-γ-induced autophagy is dependent on mitogen-activated protein kinase (MAPK) 14 but does not require signal transducer and activator of transcription 1 (STAT1). In macrophages, the autophagy initiated by IFN-γ involves JAK1/2, p38 MAPK and PI3K signaling.

1.1.3. The IL-1 family and autophagy

Immune and inflammatory processes are regulated by pro-inflammatory interleukin cytokines (IL-1α, IL-1β, IL-18, IL-33 and IL-36). Also known as "alarmins", reflecting their ability to send out an alert calling for inflammatory responses, these cytokines are 'first line responders', being secreted in the earliest stages of inflammation. Acting in a self-regulatory manner, autophagy, which can be initiated by IL-1α and IL-1β, regulates the secretion of IL-1α, IL-1β, and IL-18 and their destruction. Synthesized mainly by macrophages and monocytes, IL-1β, is considered the 'master' pro-inflammatory cytokine. Inflammatory cascades are initiated by the binding of IL-1α and IL-1β to IL-1 receptor 1. Acting as the 'master', IL-1β can influence inflammatory responses by initiating the synthesis of IL-1α and IL-23. IL-1β acts as a natural adjuvant, prompting antigen-specific immune responses. IL-1α and IL-1β have also been identified as being capable of causing the formation of autophagosomes. Furthermore, these proinflammatory cytokines might limit inflammatory responses by stimulating autophagy as part of a negative feedback loop and support cytokine-mediated anti-microbial defense mechanisms. ERS was activated by IL-1β in mouse pancreatic acinar cells, stimulating the release of Ca^{2+} into the cytosol, culminating in autophagy. It was noted that trypsin was activated by impaired autophagic flux, resulting in damage to the pancreas. However, protective effects were cast by mitochondrial signals stimulated by IL-1β-initiated autophagy.

1.1.4. Interleukin-36α, β, γ, and IL-36Ra

Interleukin-36α (IL-36α), IL-36β, IL-36γ, and IL-36 receptor antagonist (IL-36Ra) belong in the IL-36 subfamily of the IL-1 cytokine family. The IL-36 subfamily includes three

agonist cytokines (IL-36α/β/γ) as well as the natural antagonist of IL-36 (IL-36Ra). Expression of IL-36α can be observed at low levels in many different tissues most notably in the skin, esophagus, tonsil, lung, gut, and brain. IL-36α can also be secreted by the immune cells including monocytes/macrophages and T cells. IL-36α/β/γ are highly induced in response to several stimuli including cytokines, Toll-like receptor agonists, bacteria, viruses, and various pathological conditions. The truncated IL-36α/β/γ bind to the IL-36R (IL-1Rrp2) and use the IL-1 receptor accessory protein (IL-1RAcP) as a co-receptor. Following ligand binding, the Toll/IL-1 receptor (TIR) domain—located in the intracellular portion of the IL-36R:IL-1RAcP heterodimer—recruits myeloid differentiation primary response 88 (MyD88) adaptor protein, which in turn interacts with IL-1 receptor-associated kinases (IRAKs) and tumor necrosis factor receptor-associated kinase 6 (TRAF-6). IL-36 subtypes stimulate the production of several cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IL-22, IL-23, TNFα, HB-EGF, and IFN-γ), colony-stimulating factors (GM-CSF and G-CSF), chemokines (CCL1-3, CCL20, CXCL1-3, CXCL5, CXCL10, CXCL12), and cell adhesion molecules (VCAM-1, ICAM-1) in various cell types. Furthermore, IL-36 cytokines increase the intracellular level of antimicrobial peptides (beta-defensins 2 and 3, LL37, and protein S100-A7) and elevate the expression of major histocompatibility complex class 2 and clusters of differentiation 14 (CD14), CD40, CD80/CD86, and CD83. IL-36α/β/γ thereby activate innate immune cells and induce inflammation. The pro-inflammatory IL-36 subfamily members also modulate the adaptive immune responses by stimulating TH-cell proliferation and promoting CD4+ T lymphocyte differentiation toward TH1, TH17, and TH9 phenotypes. IL-36γ was shown to activate natural regulatory T-cells (Tregs). and inhibit the generation of induced Tregs. IL-36 cytokines play important roles in various diseases including asthma, chronic obstructive pulmonary disease, ankylosing spondylitis, rheumatoid arthritis, psoriasis, systemic lupus erythematosus, glomerulonephritis, diabetes, and obesity.

1.1.5. Lipopolysaccharide of Gram-negative bacteria

Lipopolysaccharide (LPS) is a powerful immunomodulatory molecule that contributes to the pathogenesis and clinical symptoms of infections caused by Gram-negative bacteria. LPS is the major structural component of the outer bacterial membrane and is composed of O antigen, poly/oligosaccharide core, and lipid A, termed endotoxin. LPS is a pathogen-associated molecular pattern (PAMP) detected by sensor molecules located in the cytoplasmic and endosomal membranes as well as in the cytoplasm of cells. Extracellular LPS is sensed and extracted from the bacterial outer membrane by the LPS-binding protein (LBP) and CD14. CD14 transfers monomeric LPS molecules to the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD-2) heterodimer leading to a conformational change and crosslinking of TLR4/MD2 receptor complexes. In the cytoplasmic membrane, the dimerized TIR domain in the cytoplasmic portion of TLR4 consecutively recruits TIR domain-containing adaptor protein (TIRAP), MyD88, and IRAK proteins, thus leading to the assembly of a supramolecular complex termed the myddosome. The LPS-TLR4/MD2 complexes can also be internalized into endosomes. The TIR domain of endosomal TLR4 binds TRIF-related adaptor molecule (TRAM), which attracts TIR domain-containing adaptor-inducing interferon-β (TRIF) resulting in the formation of another complex called the triffosome. Myddosomes and

triffosomes recruit TRAF6 or TRAF3 and activate AP-1, NF-κB, CREB, and interferon regulatory factor 3 (IRF-3) via MAPKs, IKK, and class I phosphatidylinositol 3-kinase (PI3K). In addition to TLR4, another group of cytoplasmic membrane receptors, the transient receptor potential cationic channels can also bind LPS. Additionally, intracellular LPS within the cytoplasm of cells directly binds to the caspase activation and recruitment domains of caspase-4/5 in humans and caspase-11 in mice, which in turn leads to activation of inflammatory caspases, secretion of IL-1β and IL-18, as well as induction of pyroptosis. In localized infections caused by Gram-negative bacteria, LPS-mediated activation of the immune response is protective by restricting bacterial invasion whereas the exaggerated inflammation seen in systemic infections is of pivotal pathogenetic and prognostic importance.

1.2. COVID-19: SARS-CoV-2 and its cellular effects on GIT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) recently emerged as a highly virulent respiratory pathogen that is known as the causative agent of coronavirus disease 2019 (COVID-19). SARS-CoV-2 enters the human body through the airways and multiplies in the lungs. This novel coronavirus causes mild, severe, and critical respiratory disease in 81%, 14%, and 5% of cases, respectively. It may also enter the bloodstream, which results in viremia and systemic spread throughout the body. In the severe and critical clinical manifestations of COVID-19, atypical pneumonia leading to progressive respiratory failure develops.

In addition to the airways, the virus can multiply in the gastrointestinal (GI) tract (GIT), urinary tract, and central nervous system. The infection elicits an intemperate immune response characterized by a life-threatening cytokine storm and a corrupted interferon (IFN) system, which is unable to eliminate the pathogen effectively. As a result, a systemic inflammatory response syndrome occurs. In the severe and critical clinical manifestations of COVID-19, atypical pneumonia leading to progressive respiratory failure develops.

Initially, the S protein of SARS-CoV-2 binds to its corresponding cell-surface receptor, angiotensin-converting enzyme type 2 (ACE2). The S protein has two subunits: S1 and S2. The S1 subunit has a receptor-binding domain and is responsible for receptor engagement, whereas the S2 subunit is involved in the fusion process. Following ACE2 binding, cellular proteases such as transmembrane protease/serine subfamily member 2 (TMPRSS2), TMPRSS4, and cathepsin L cleave S protein into S1 and S2 subunits, and the virus enters the host cell by receptor-mediated endocytosis. These enzymes may also facilitate entry or expand the tissue tropism of SARS-CoV-2.

GIT involvement is frequent in COVID-19 patients and includes anorexia, nausea, vomiting, diarrhea, and abdominal pain. Among the specific GI symptoms, diarrhea is the most common. Based on different studies, the prevalence of diarrhea might range from 2% to 49.5%. The reason why GI symptoms occur in only a subset of COVID-19 patients is currently unknown. A study conducted by Jin *et al* [166] revealed that the rate of chronic liver disease in COVID-19 patients with GI symptoms is much higher than among those without GI symptoms. Moreover, the incidence of COVID-19 with GI symptoms displays familial clustering[166].

Based on these interesting observations, it is reasonable to infer that genetic, immunological, and epidemiological factor are involved in the development of COVID-19-associated diarrhea.

The treatment of COVID-19 is based on the phase of infection or the severity of the case. At the first phases, anti-inflammatory drugs are applied for 7-8 days such as chloroquine or hydroxychloroquine. Although numerous antiviral medications have been considered, such as lopinavir/ritonavir and remdesivir have gained considerable attention. The first antiviral medication, lopinavir/ritonavir, was used to treat HIV and SARS in 2003. Another antiviral agent is remdesivir, which is a promising pharmaceutical drug and is currently being examined by several clinical trials as a possible COVID-19 medication. On February 15, 2020. Favipiravir , an antiviral drug made by the Japanese pharmaceutical company Fujifilm Toyama Chemical, was authorized for the treatment of new influenza in China, and clinical studies are now underway. According to preliminary data from 80 individuals, favipiravir exhibited a more powerful antiviral impact than lopinavir/ritonavir and even had fewer adverse effects.

2. Aims

Our specific aims were:

Aim 1: Investigation of the pro-autophagic effect of IL36α and lipopolysaccharide in the THP-1 cells line.

Our aim was to examine the (*i*) the levels of LC3B-I and LC3B-II, (*ii*) the autophagic flux, (*iii*) the subcellular localization of LC3B and Beclin-1 and (*iv*) the signaling pathways activated in THP-1 cells treated with IL-36α and LPS alone or in combination.

Aim 2: Collection of literature data on the main cellular effect of SARS-CoV-2 on GIT.

In our review study, we aimed to summarize the effects of SARS-CoV-2 on the autophagic activity, viability, ion secretion, and inflammatory response of enterocytes. Furthermore, we also collected literature data on the role of the cellular effects exerted by SARS-CoV-2 in the development of COVID-19 gastrointestinal manifestation.

3. Materials and Methods

3.1.Chemical compounds

Human recombinant IL-36α (Biomol GmbH, Hamburg, Germany) was prepared in sterile distilled water and used at 10 ng/ml concentration in all experiments. Human recombinant IL-36Ra (Sigma–Aldrich, St. Louis, MO, USA) was prepared in sterile dis-tilled water and used at 20-fold molar excess. A stock solution of autophagy inhibitor bafilomycin A1 BFLA (Santa Cruz Biotechnology, Dallas, TX, USA) was prepared in dimethyl sulfoxide. BFLA was used at a concentration of 100 nM in all experiments.

3.2.Cell culture

The THP-1 human pro-monocytic cell line was grown in Dulbecco's modified Eagle's minimal essential medium (Sigma–Aldrich) supplemented with 10% fetal calf serum (Lonza, Verviers, Belgium) and 1% of an antibiotic/antimycotic (AB/AM) solution (Lonza) at 37 °C in a 5% CO2 atmosphere.

3.3.Indirect Immunofluorescence assay

Cytospin cell preparations were fixed in methanol acetone (1:1) for 10 minutes at -20 °C. The cells were treated with 1% bovine serum albumin in PBS for 30 min at 37 °C to block non-specific binding of the antibodies. To detect LC3B, the slides were stained with a 1:150 dilution of rabbit polyclonal antibody to LC3B (Sig-ma-Aldrich) for 1 h at 37 °C. To detect Beclin-1, the slides were stained with a 1:100 dilution of rabbit polyclonal antibody to Beclin-1 (Sigma-Aldrich) for 1 h at 37 °C. After washing with PBS, the samples were reacted with a 1:300 dilution of CF488A-conjugated anti-rabbit antibody (Sigma-Aldrich) for 1 h at 37 °C. The cells were visualized by confocal microscopy using an Olympus FV1000 confocal laser scanning microscope using UPLSAPO 60X (N.A. 1.35) oil immersion objective and 488 nm laser excitation with 500-600 nm detection range. LC3B-positive vacuoles were automatically quantified for each field after subtraction of the background level and establishment of an intensity threshold using Image J software. The numbers of the LC3B-positive puncta were normalized by the numbers of cells in each field. An average of 500 cells was analyzed for each condition. The fluorescence intensity of LC3B was determined using the surface plot functions of the Image J software. The mean fluorescence intensity (MFI) method was used to quantify the fluorescent signal intensities of cells. ImageJ software was used to draw an outline around each cell, and the MFI was measured. The corrected total cell fluorescence (CTCF) was calculated via the following formula: CTCF = integrated density – (area of selected cell × mean fluorescence of background readings).

3.4.Western blot assays

The cells were homogenized in CytoBuster lysis buffer (Merck KGaA, Darmstadt, Germany), and the mixture was then centrifuged at 10,000 g for 10 min to remove cell debris. Protein concentrations of cell lysates were determined using the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). Supernatants were mixed with Laemmli sample buffer and boiled for 3 min. Aliquots of the supernatants were resolved by SDS-PAGE and electrotransferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Inc.). The membranes were blocked in PBS containing 0.05% Tween 20, and 5% dried non-fat milk (Difco Laboratories Inc., Detroit, MI, USA). The pre-blocked blots were probed with the appropriate antibodies for 4 h in PBS containing 0.05% Tween 20, 1% dried non-fat milk and 1% bovine serum albumin (Sigma-Aldrich). Rabbit anti-LC3B (Sigma-Aldrich) and rabbit anti-β-actin (Sigma-Aldrich) primary antibodies were used at a 1:1,000 dilution. Blots were then incubated for 2 h with peroxidase-conjugated anti-rabbit antibody (Sigma). Membranes were developed using a chemiluminescence detection system (GE Healthcare, Chicago, IL, USA). The autoradiographs were scanned with a GS-800 densitometer

(Bio-Rad Laboratories Inc.), and the relative band intensities were quantified using ImageJ software.

3.5.Phospho-kinase array analysis

A human phospho-kinase array (R&D Systems Inc., Minneapolis, MN, USA) was used to measure the relative phosphorylation levels of 43 signaling molecules. Control cells and cultures treated with IL-36α and LPS alone or in combination for 30 min were homogenized in lysis buffer and centrifuged for five min at 14,000 × g. Protein concentrations of the supernatants were determined using a Bio-Rad protein assay (Bio-Rad Laboratories Inc.); after blocking, 300 μg of protein was incubated with each array overnight at 4 °C. After washing, the arrays were reacted with a cocktail of phospho-site-specific biotinylated antibodies for two hours at room temperature, carefully washed again, and incubated with streptavidin–peroxidase for 30 min at room temperature. Signals were developed using a chemiluminescence detection system and recorded on autoradiography film. Spot densities of phospho-proteins were quantified using ImageJ software. and normalized to those of positive controls on the same membrane after subtraction of background values.

3.6.Statistical analysis

Statistical significance was analyzed by one-way ANOVA followed by Tukey's or Sidak's multiple comparison post-hoc tests. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc.), and P values less than 0.05 were considered statistically significant.

4. Results

4.1. The effects of IL-36α and LPS on the subcellular localization of LC3B in the THP-1 cell line

To elucidate how IL-36α and LPS affect the basal autophagy, we treated THP-1 cells with IL-36α and LPS alone or in combination and measured the subcellular localization of LC3B. Indirect immunofluorescence assays could determine the intracellular localization of LC3B at the 6-h time point and demonstrated that the control cells and the cultures treated with LPS have faint cytoplasmic LC3B staining. Accordingly, the 3D surface plots revealed a few peaks of low height. Cells treated with IL-36α displayed staining patterns characterized by faint, punctate LC3B staining. In contrast, the cells treated with the combination of IL-36α and LPS displayed very bright LC3B staining, and the 3D surface plot consisted of numerous robust peaks. This result indicates that IL-36α and LPS cooperatively increased the accumulation of LC3B-positive vacuoles. IL-36 receptor antagonist (IL-36Ra) was used to investigate the role of IL-36α in the synergistic activation of autophagy elicited by the combined treatment with IL-36α and LPS. The cultures were pre-treated with IL-36Ra for 30 min, and then IL-36α or a double combination of IL-36α and LPS were added. The cultures treated either with IL-36Ra alone or in combination with IL-36α displayed a faint cytoplasmic LC3B staining. Cells treated

with the triple combination of IL-36α–IL-36Ra–LPS likewise displayed staining patterns characterized by faint, punctate LC3B staining. Accordingly, the 3D surface plots revealed a few peaks of low height. To investigate the effects of IL-36α and LPS on autophagosome formation, the abundances of LC3B-positive vacuoles were determined at the 6-h time point. The average numbers of LC3B-positive vacuoles per cell in the control, IL-36α-, or LPS-treated cultures were 4.72, 6.73, and 2.8, respectively. The average numbers of LC3B-positive vacuoles per cell in cultures treated with a combination of IL-36α and LPS were significantly higher than that observed in the control cultures (the average number of autophagosomes in the cultures treated with IL-36α–LPS was 10.97 versus 4.72 in the control, $p < 0.0001$). Moreover, the cells treated with the triple combination of IL-36α–IL-36Ra–LPS exhibited significantly lower numbers of LC3B-positive vacuoles per cell than in the cultures treated with the IL-36α-LPS combination: The average number of autophagosomes in the cultures treated with IL-36α–IL-36Ra–LPS was 7.58 vs 10.97 in cells treated with IL-36α–LPS, $p < 0.05$. Thus, although IL-36α and LPS alone do not cause a significant alteration in the number of LC3B-positive vacuoles, the combination of IL-36α and LPS does significantly stimulate the accumulation of autophagosomes.

4.2. The effects of IL-36α and LPS on the levels of LC3B-I and LC3B-II

The effects of IL-36α and LPS on the levels of LC3B-I and LC3B-II were determined by western blot analysis. The control THP-1 cells displayed endogenous expression of both the lipidated and the non-lipidated forms of LC3B at each time point. IL-36α-treated cells exhibited slightly higher LC3B-II levels at the 0.5-, 2-, and 6-h time points than controls. However, these alterations were not statistically significant. Likewise, there were no significant alterations in LPS-treated cells versus controls. In contrast, the simultaneous treatment of cells with IL-36α and LPS triggered a significant increase in the level of LC3B-II as compared with the controls at the 0.5-, 6-, and 24-h time points the fold increases of LC3B-II levels in cells treated with IL-36α–LPS combination were 3.52, $p < 0.01$, 3.0, and 3.02, $p < 0.05$ for both, respectively). These data suggest that IL-36α and LPS alone do not increase the level of LC3B-II whereas combined IL-36α and LPS treatment cooperatively stimulates the lipidation of LC3B.

4.3. The effects of IL-36α and LPS on the autophagic flux

Bafilomycin A1 (BFLA) is an inhibitor of autophagosome-lysosome fusion and lysosomal hydrolase activity and was used to investigate the autophagic flux. The cultures were incubated with IL-36α and LPS alone or in combination for 2 h and then treated with BFLA for another 4-h period just before the preparation of cell lysates. Compared with the control, BFLA increased the level of LC3B-II. The elevated LC3B-II level of the BFLA-treated cells indicates that this drug efficiently blocked the autophagic flux under the experimental conditions used. In the presence of BFLA, IL-36α triggered a higher increase in the level of LC3B-II than in the corresponding drug control. However, this alteration was not statistically significant. In contrast, compared with the BFLA control, LPS acting singly or in combination with IL-36α elicited a significant increase in the levels of LC3B-II of cells incubated in the presence of

BFLA (the fold increases of LC3B-II levels in cells treated either with LPS alone or the IL-36α–LPS combination in the presence of BFLA were 6.22, and 6.47, $p < 0.05$ for both). In the presence of BFLA, the cells treated with the IL-36α–LPS combination exhibited higher increases in the level of LC3B-II than cultures stimulated only with LPS. These data indicate that combined IL-36α and LPS treatment cooperatively stimulates the autophagic flux.

4.4. The effects of IL-36α and LPS on the level of Beclin-1

The effects of IL-36α and LPS on the level and intracellular localization of Beclin-1 were determined by indirect immunofluorescence assay at the 6-h time point. The control cells showed a faint cytoplasmic Beclin-1 staining. Accordingly, the 3D surface plots revealed a few peaks of low height. In contrast, the cells treated with IL-36α and LPS alone or in combination displayed very bright Beclin-1 staining, and the 3D surface plots consisted of numerous robust peaks. Measurement of the staining intensities showed that IL-36α and LPS acting singly or in combination elicited significant increases as compared with the control (the CTCF values in cells treated with IL-36α, LPS, or IL-36α–LPS combination were 1.35, 1.66, or 1.8 *vs* 1.0 in the control, $p < 0.0001$ for all, respectively). Measurement of the abundances of Beclin-1-positive vacuoles likewise revealed that IL-36α and LPS acting singly or in combination triggered significant increases as compared with the control (the average numbers of Beclin-1-positive puncta in cells treated with IL-36α, LPS or IL-36α–LPS combination were 5.86, 8.18, or 13.55 *vs* 2.72 in the control, $p < 0.01$, $p < 0.0001$ and $p < 0.0001$, respectively). These data indicate that IL-36α and LPS cooperatively elevate the level of Beclin-1.

4.5. The effects of IL-36α and LPS on cellular signaling in the THP-1 cell line

A phospho-kinase array that detects the phosphorylation levels of 43 major protein kinases was used to investigate the effect of IL-36α and LPS on the activation level of signaling pathways implicated in autophagy regulation. IL-36α led to the activation of a subset of kinases. Compared with the control, the most significant effect was an increase in Ak strain transforming factor 1/2/3 (Akt1/2/3) (S473) phosphorylation. The phosphorylation levels of the proline-rich Akt substrate of 40 kDa (PRAS40) (T246) and mechanistic target of rapamycin (mTOR) (S2448) two signaling molecules downstream of Akt1/2/3) were also increased. IL-36α triggered phosphorylation of with no lysine kinase 1 (WNK1) (T60), some steroid receptor coactivator (Src) family kinases including Src (Y419) and Lyn (Y397), and signal transducer and activator of transcription (STAT) family members such as STAT2 (Y689), STAT3 (S727) and STAT5a/b (Y694/Y699). Compared with the control, LPS increased the levels of phospho-Akt1/2/3 (S473), phospho-Src (Y419), and STAT5a/b (Y694/Y699); it decreased phosphorylation of adenosine monophosphate-activated protein kinase α1 (AMPKα1). Compared with the control, IL-36α and LPS combined treatment increased phosphorylation of STAT5a/b whereas the levels of phospho-Yes (Y426), phospho-focal adhesion kinase (FAK) (Y397), and phospho-WNK1 (T60) were decreased. Thus, IL-36α, LPS, and the combined treatment elicit distinct phosphorylation patterns of signaling molecules.

5. Discussion

5.1. IL-36α and LPS cooperatively induce Autophagy

Compelling evidence indicates that cellular autophagic and immune processes are highly intertwined and that their coordinated functioning is essential for the efficient protection of the human body against pathogenic Gram-negative bacteria. During infections, cytokines and molecules defined as pathogen-related molecular patterns (PAMP) act simultaneously to activate partially overlapping signaling pathways. The combined effect may differentially regulate cellular autophagic activity. Thus, this study investigated the impact of IL-36α upon endogenous and LPS-induced autophagy.

To study the autophagic activity of THP-1 cells treated with IL-36α and LPS alone or in combination, we determined the intracellular distribution of LC3B and measured LC3B lipidation as well as the autophagic flux. These experiments demonstrated that the cells treated with IL-36α alone displayed increased abundances of autophagic vesicles, elevated endogenous LC3B-II levels, and stimulated autophagic flux; these differences, however, were not statistically significant. Recent observations indicated that IL-36β and IL-36γ activate the autophagic process in primary murine $CD4^{+}CD25^{+}$ Treg cells. and human macrophages, respectively. There may be several explanations for the weaker pro-autophagic effect of IL-36α revealed in our present study such as differences between IL-36 subtypes and different sensitivities of various cell types to this cytokine. Consistent with previous findings. our results demonstrated that LPS significantly increased autophagosome synthesis. The combination of IL-36α and LPS raised the intensity level of LC3B staining and cooperatively stimulated the translocation of this protein into autophagic vesicles. Supporting this observation, we found that IL-36Ra significantly inhibited the pro-autophagic effect of the IL-36α/LPS combined treatment. The IL-36α–LPS combination elevated LC3B-II and decreased LC3B-I levels indicating that the lipidation of LC3B is highly stimulated. Finally, our experiments showed that in cultures treated with the combination of IL-36α and LPS, the autophagic flux is increased considerably by this cytokine/PAMP combination. These results suggest that IL-36α and LPS cooperatively stimulate autophagy.

To investigate the effect of IL-36α and LPS on the activation of some signaling pathways, we determined the phosphorylation levels of protein kinases implicated in autophagy regulation. Our studies have shown that IL-36α increased the phosphorylation of Akt1/2/3 (S473), PRAS40 (T246), mTOR (S2448), WNK1 (T60), and some Src as well as STAT family kinases such as Src, Lyn, STAT2, STAT3, and STAT5a/b. PRAS40 is a negative regulator of mTORC1. Akt- and mTORC1-mediated phosphorylation of PRAS40 resulting in its dissociation from mTORC1 that in turn alleviates inhibition of mTORC1 and blocks induction of the autophagic cascade. An interesting recent study revealed that IL-36β stimulates mTORC1 via the PI3K/Akt, IκB kinase and MyD88 pathways. Our experiments demonstrate that, like IL-36β, IL36α activates mTORC1 via the Akt/PRAS40/mTOR pathway. Other previous studies indicated that WNK1 acts as an autophagy inhibitor by interfering with the activation of AMPK and PI3KC3. STAT3 has been shown to regulate autophagy in

localization- and context-dependent manners and can elicit both pro-autophagic and anti-autophagic effects. In light of these observations, our data suggest that in the early phase of IL-36α signaling, both anti- and pro-autophagic pathways are activated. Our observations show that LPS increases phosphorylation of Akt1/2/3, Src as well as STAT5a/b and decreases the level of phospho-AMPKα1 in THP-1 cells; this is fully consistent with previous reports. Interestingly, the phosphorylation pattern of cells incubated with IL36α and LPS differed from the signatures detected either in IL36α- or LPS-treated cells. We found that the IL36α/LPS combined treatment increased phosphorylation of STAT5a/b, had minimal effect on the Akt/PRAS40/mTOR pathway, and reduced the levels of phospho-Yes, phospho-FAK, and phospho-WNK1. Thus, the combined treatment of IL-36α and LPS appears to dampen PI3K/Akt/mTOR, FAK and WNK1 signaling. The TLR4 signal transduction network is known to be kept under strict control by multiple mechanisms including positive and negative crosstalk regulations that maintain the integrity of immune cells by preventing excessive inflammation. The negative regulators acting through the activation of transcription play a primary role in the late phase of TLR and IL-36 signaling; their role in the early stage thus can be excluded. Important studies, however, revealed that PI3K has an essential role in the safety mechanism controlling the early-stage of TLR4-mediated signaling. PI3K can suppress TLR4 signaling by altering the availability of phosphatidylinositol-(4,5)bisphosphate (PIP_2) at the cytoplasmic membrane and hence can modulate the intracellular localization of adaptors, the magnitude of activation, and signal output. Based on these observations, we suggest that the combinatorial effect of IL-36α/LPS may exert an excessive PI3K activation, which while not suspending the inhibition of downstream events of autophagy—can significantly reduce it by decreasing the Akt-mediated activation of mTORC1.

Previous studies have demonstrated that Beclin-1 plays a pivotal role in the autophagic process. Beclin-1 was shown to interact with Bcl-2 and Bcl-XL, which suppresses autophagosome biogenesis. The release of Beclin-1 is a prerequisite for the formation of a functional PI3KC3 complex. Phosphorylation events and TRAF6-mediated ubiquitination of Beclin-1 may destabilize Beclin-1–Bcl-2/Bcl-XL association and abrogate Beclin-1-Bcl-2/Bcl-X_L interaction. Some IL-36α and LPS signaling intermediates have the potential to regulate the functional activity of Beclin-1. Thus, we investigated the effect of IL-36α and LPS on Beclin-1 protein. Our results showed that IL-36α and LPS acting singly or in combination elevated the staining intensities and increased the abundances of Beclin-1-positive vacuoles. The IL-36α/LPS treatment was again more efficient than IL-36α or LPS alone. These data further support the notion that IL-36α and LPS cooperatively promote the autophagic process because increased Beclin-1 levels were shown to correlate with enhanced autophagy.

Our results suggest a hypothetical model for the mechanism of the enhanced pro-autophagic effect observed in cells treated with IL-36α and LPS simultaneously. The IL-36α/LPS combination reduces the activation level of the PI3K/Akt/mTORC1 axis by triggering rapid depletion of PIP_2 at the cytoplasmic membrane. As a result, mTOR-mediated inhibition of autophagy is alleviated. Some components of the IL-36α and LPS signaling networks are known to induce autophagy. MyD88 binds directly whereas TRAF3 and TRAF6 ubiquitinate the Beclin-1 protein, thus disrupting the interaction between Beclin-1 and Bcl-2. This results in increased oligomerization of Beclin-1, activation of the PI3KC3 complex, and initiation of

autophagosome formation. The IL-36α/LPS combination increases the activation level of PI3KC3 complex directly and subsequently stimulates autophagy. Thus, this cytokine/PAMP combination triggers pro-autophagic biased signaling by several mechanisms and thereby cooperatively stimulates the autophagic cascade. Previous studies have shown that bacteria affect the autophagic cascade and, conversely, autophagy influences the infection process. The bacteria studied so far all interact with the autophagic machinery but in different ways. The structural components, PAMPs, and exotoxins of several bacteria induce autophagy. However, some bacteria can effectively prevent autophagic recognition, inhibit autophagy initiation and maturation of autophagosomes or block the fusion of lysosomes with autophagosomes, while others hijack the autophagic compartment to support their intracellular survival. Our data indicate that cytokines may modify the pro-autophagic effect of bacterial PAMPs. An increased xenophagic activity of innate immune cells exposed to IL-36α and LPS—functioning as part of the cell-autonomous defense system—may play a protective role in the pathogenesis of infections caused by Gram-negative bacteria.

5.2. the cellular effect of SARS-CoV-2 on GIT

During multiplication, SARS-CoV-2 modulates several cellular aspects, including signaling, transcription, translation, cell division, the IFN system, autophagy, and apoptosis, as well as the biogenesis, function, and morphology of mitochondria and intracellular vesicles. Phosphoproteomic profiling has revealed that SARS-CoV-2 infection affects the activity of 97 kinases. The activities of several members of the p38 pathway and the guanosine monophosphate-dependent protein kinases are upregulated, while cell cycle kinases (CDK1/2/5), cell growth-related signaling pathway kinases (AKT1/2), and regulators of the cytoskeleton are down-regulated. functional changes in the signal transduction pathways have been shown to play an important role in SARS-CoV-2-induced cytoskeletal damage, cytokine production, and slow-down in cell proliferation at the S/G2 transition phase. Transcriptomic profiles of SARS-CoV-2-infected primary human bronchial epithelial cells, lung biopsy, and bronchoalveolar lavage fluid samples of COVID-19 patients have demonstrated upregulated expression of genes implicated in metabolism, immunity, and the stress responses of the endoplasmic reticulum and mitochondria. It has been shown that the M protein, Nsp7, and ORF9c stimulate lipogenesis, while Nsp7, Nsp12, and ORF8 trigger endoplasmic stress response, and Nsp7 induces mitochondrial dysfunction. Moreover, the M and E proteins, along with Nsp3a, Nsp6, Nsp8, Nsp10, and Nsp13, were shown to be able to modify the structure and function of the endomembrane system and vesicle trafficking, thereby facilitating several steps of viral multiplication. Interestingly, the expression of genes involved in the humoral immune response and innate immune response-activating signal transduction are increased, whereas genes implicated in cytokine-mediated signaling pathways are down-regulated. A multiplex gene expression analysis showed that the genes involved in type I IFN signaling were highly up-regulated, whereas the expression of IFN-stimulated genes (*ISGs*) was decreased in severe COVID-19 patients. The levels of pro-inflammatory cytokines measured in sera of COVID-19 patients were highly increased in a pattern corresponding to a cytokine storm. Consistent with this observation, transcriptional activation of pro-inflammatory cytokine genes was also

detected in peripheral blood mononuclear cells and bronchoalveolar lavage fluid. The sera and lung tissue samples of patients have shown IL-1β, IL-6, IL-10, IL-18, IL-33, transforming growth factor-β, IFN-γ, CSF2/GM-CSF, CSF3/G-CSF, CC chemokines [CCL2/MCP-1, CCL3/MIP-1A, CCL4/MIP-1B, CCL5/RANTES, CCL8, CCL3L1] and CXC chemokines [CXCL1, CXCL2 and CXCL10/IP10]. However, during SARS-CoV-2 infection, the production of type I and III IFNs is decreased. Thus, these data clearly demonstrate that SARS-CoV-2 infection alters both the transcriptional and translational patterns in cells profoundly. Other observations indicate that SARS-CoV-2 could trigger several cell-death processes, including apoptosis, necrosis, pyroptosis, and anoikis, depending on the type of cell. The death of infected cells may contribute to tissue damage and induce an inflammatory reaction.

It has also been revealed that SARS-CoV-2 Orf3a stimulates the formation of the autophagic Beclin-1-Vps34-Atg14 complex while simultaneously inhibiting the Beclin-1 complex containing the UVRAG adaptor protein. Orf 3a thereby exerts a dual effect on the autophagic process manifesting in the induction of the initial steps and a block in the fusion of the autophagosomes with lysosomes.

ACE2, the cellular receptor of SARS-CoV-2, is widely expressed in many types of cells and tissues of the GIT, including the esophagus, stomach, small intestine, colon, rectum, pancreatic exocrine glands and islets, and gallbladder. The expression level of ACE2 in the GIT is highest in the ileum epithelial cells, especially in the absorptive enterocytes. It has also been demonstrated that ACE2 is co-expressed with TMPRSS2/4 proteases in the GIT, with the highest level in the ileum. These observations indicate that several cell types in the GIT are potentially susceptible to SARS-CoV-2 infection. SARS-CoV-2 infection in the lungs and GIT seems to display some different tissue-specific features. The production of type I and III IFNs is more efficient in the GIT than in the lungs. The antiviral IFNs may restrict viral replication in the GIT to some extent, which may allow the development of a less cytopathogenic or persistent form of infection in this anatomical region. SARS-CoV-2-mediated dysregulation of the ACE2:B0AT1 complex may modify the biological response of cells to the infection, and in enterocytes, it may contribute to the development of diarrhea by inducing amino acid starvation, which can decrease Na+ uptake. These effects are not seen in the lungs, however, as ACE2 does not form a complex with B0AT1 in this organ.

SARS-CoV-2 infection of the GIT is of pivotal epidemiological significance, but further studies are needed to assess the extent of this risk.

6. Conclusion

Our results demonstrate that IL-36α alone does not stimulate the cellular autophagic activity, whereas LPS increases autophagosome synthesis significantly. The IL-36α and LPS combination synergistically elevate the level of LC3B-II, increase the autophagic flux and stimulate the intracellular redistribution of LC3B and Beclin-1. The phospho-kinase array results indicate that IL-36α, LPS, and the combined treatment elicit distinct phosphorylation

patterns of signaling molecules. The IL-36α/LPS combination triggers pro-autophagic biased signaling by several mechanisms and thereby stimulates the autophagic cascade cooperatively in the THP-1 cell line. However, some limitations of this investigation, such as the need for additional experimental evidence that can corroborate the synergistic effect of this cytokine/PAMP combination in other cell types, have yet to be addressed. Moreover, further studies are needed to investigate the effect of increased autophagic activity on the functions of innate immune cells treated with IL-36α and LPS simultaneously.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replicates in enterocytes, triggers ionic imbalances, activates the NLRP3 inflammasome pathway, induces apoptosis, and exerts a dual effect on the autophagic process. These effects of SARS-CoV-2 lead to the development of leaky gut. Increased permeability triggers the absorption of LPS into the circulation, further exacerbating inflammation induced by viral infection. In addition to drugs that affect the inflammatory response and viral replication, agents targeting autophagy and apoptosis appear to be potentially suitable for the treatment of COVID-19. The fecal-oral route of SARS-CoV-2 transmission calls for strict and more consistent adherence to hygiene rules to prevent the spread of COVID-19.

7. Summary

Autophagy is an intracellular catabolic process that controls infections both directly and indirectly via its multifaceted effects on the innate and adaptive immune responses. It has been reported that LPS stimulates this cellular process, whereas the effect of IL-36α on autophagy remains largely unknown. We therefore investigated how IL-36α modulates the endogenous and LPS-induced autophagy in THP-1 cells. The levels of LC3B-II and autophagic flux were determined by western blotting. The intracellular localization of LC3B was measured by immunofluorescence assay. The activation levels of signaling pathways implicated in autophagy regulation were evaluated by using a phosphokinase array. Our results showed that combined IL-36α and LPS treatment cooperatively increased the levels of LC3B-II and Beclin-1, stimulated the autophagic flux, facilitated intracellular redistribution of LC3B, and increased the average number of autophagosomes per cell. The IL36α/LPS combined treatment increased phosphorylation of STAT5a/b, had minimal effect on the Akt/PRAS40/mTOR pathway, and reduced the levels of phospho-Yes, phospho-FAK, and phospho-WNK1. Thus, this cytokine/PAMP combination triggers pro-autophagic biased signaling by several mechanisms and thus cooperatively stimulates the autophagic cascade. An increased autophagic activity of innate immune cells simultaneously exposed to IL-36α and LPS may play an important role in the pathogenesis of Gram-negative bacterial infections.

SARS-CoV-2 can infect and replicate in esophageal cells and enterocytes, leading to direct damage to the intestinal epithelium. The infection decreases the level of angiotensin converting enzyme 2 receptors, thereby altering the composition of the gut microbiota. SARS-CoV-2 elicits a cytokine storm, which contributes to gastrointestinal inflammation. The direct cytopathic effects of SARS-CoV-2, gut dysbiosis, and aberrant immune response result in

increased intestinal permeability, which may exacerbate existing symptoms and worsen the prognosis. By exploring the elements of pathogenesis, several therapeutic options have emerged for the treatment of COVID-19 patients, such as biologics and biotherapeutic agents. However, the presence of SARS-CoV-2 in the feces may facilitate the spread of COVID-19 through fecal-oral transmission and contaminate the environment. Thus, gastrointestinal SARS-CoV-2 infection has important epidemiological significance. The development of new therapeutic and preventive options is necessary to treat and restrict the spread of this severe and widespread infection more effectively.

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Publications related to the subject of the Thesis:

1. Zaid I. I. Al-Luhaibi, Áron Dernovics, György Seprényi, Ferhan Ayaydin, Zsolt Boldogkői, Zoltán Veréb and Klára Megyeri,* IL-36α and lipopolysaccharide cooperatively induce autophagy.

Biomedicines Oct,2021. Impact factor: 6.081, Q1

2. Klara Megyeri, Áron Dernovics, **Zaid I I Al-Luhaibi**, András Rosztóczy. COVID-19-associated diarrhea.

World J Gastroenterol Jun,2021. Impact factor: 5.742, Q1

Abstracts related to the subject of the Thesis:

1. ESCCMID 30th 2020, Paris, France (Abstract only). **Zaid I.I Al-Luhaibi[1]** ,Áron Dernovics[1], Klára Megyeri[1*] and György Seprényi[2] . *Investigation the pro-autophagic effect of IL-36α and lipopolysaccharide in THP-1 cell line.*

2. MED-PÉCS 2021(Abstract and Poster), **Zaid I.I Al-Luhaibi[1]** ,Áron Dernovics[1], Klára Megyeri[1*] and György Seprényi[2] . *Investigation the pro-autophagic effect of IL-36α and lipopolysaccharide in THP-1 cell line.*