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THE MOLECULAR BASIS OF HUMAN DISEASE

Master thesis

[CONTRIBUTION OF AKT KINASES IN MACROPHAGE AUTOPHAGY]

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Abstract

Autophagy is a defence mechanism of the cell that is activated under conditions of stress or due to environmental or nutrition changes of the cell. During this process, the cell gets rid of damaged or dysfunctional organelles or invading pathogens that are engulfed in a double-membrane vesicle which is known as autophagosome. Autophagosome fuses with the lysosome and as a result its entire content is degraded and recycled. In this way, autophagy promotes cell survival. Autophagy is one of the many cell functions that are controlled by the PI3K-Akt-mTOR intracellular signaling pathway whose key components are Akt kinases. Akt kinases through mTORC1 complex inhibit autophagy, therefore we presume that their absence should lead to autophagy induction. The purpose of this study was to identify the effect of Akt1 and Akt2 loss on macrophage autophagy on cells treated with different stimuli including LPS, IL4, IFNγ and insulin. The results showed that LPS treatment of both Akt1 and Akt2 deficient macrophages did not induce the expression of LC3 autophagy marker. Serum starvation did not affect LC3-II levels in Raw cells and there was a small induction of autophagy after 6h and 24h of IFNγ or IL4 treatment in both peritoneal macrophages and Raw264.7. Moreover, treatment with rapamycin, an inhibitor of mTOR, did not increase LC3 above basal levels. Quantitative real-time PCR was used to evaluate the expression levels of two important autophagy genes, Atg5 and Atg7. The results indicated an increase in both Atg5 and Atg7 after 24h of LPS treatment on Raw264.7 under starvation conditions. In the presence of serum, Atg7 expression was decreased after 6h and 24h of LPS treatment of both WT and Akt1-/- macrophages and Atg5 expression was increased 24h following LPS treatment of both WT and Akt1-/- cells. Finally, both genes were increased after 6h and 24h of IFNγ treatment in Akt1-/- cells compared to WT controls and only Atg7 levels were higher after simultaneous treatment with LPS and IFNγ on Raw macrophages. As for the mitophagy marker Pink-1, its expression levels varied in response to different stimuli. Specifically, absence of Akt1 resulted in increased basal mitophagy. While LPS suppressed mitophagy in WT macrophages this suppression was not

observed in Akt1-/- macrophages 24 hours following stimulation. At 48 hours of treatment or at serum starvation conditions these differences were not observed. We can therefore conclude that Akt1 differentially controls Atg5 and Atg7 expression in macrophages and that absence of either Akt1 or Akt2 does not affect LC3-dependent autophagy, suggesting a potential redundancy between the two Akt isoforms.

Key words: Autophagy, macrophages, Akt1, Akt2, LC3, autophagosome, stimuli

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ ΣΤΗΝ ΜΟΡΙΑΚΗ ΒΑΣΗ ΤΩΝ ΝΟΣΗΜΑΤΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

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Περίληψη

Η αυτοφαγία είναι ένας μηχανισμός άμυνας του κυττάρου, ο οποίος ενεργοποιείται σε καταστάσεις πίεσης ή σαν απόκριση σε περιβαλλοντικές ή θρεπτικές αλλαγές. Κατά την διάρκεια της αυτοφαγίας, το κύτταρο ξεφορτώνεται κατεστραμμένα και δυσλειτουργικά οργανίδια ή παθογόνα, τα οποία μέσω αυτής της διαδικασίας, εσωκλείονται σε κυστίδιο με διπλή μεμβράνη, το αυτοφαγόσωμα. Το αυτοφαγόσωμα συντήκεται με το λυσόσωμα με αποτέλεσμα όλο το περιεχόμενο να αποικοδομηθεί και να ανακυκλωθεί και με αυτόν τον τρόπο η όλη διαδικασία συμβάλλει στην επιβίωση του κυττάρου. Η αυτοφαγία είναι μία από τις πολλές κυτταρικές λειτουργίες που ελέγχονται από το PI3K-Akt-mTOR ενδοκυττάριο σηματοδοτικό μονοπάτι που οι βασικές του συνιστώσες είναι οι Akt κινάσες που μέσω του mTORC1 συμπλόκου καταστέλλουν την αυτοφαγία. Επομένως, υποθέτουμε ότι η απουσία αυτών των κινασών θα πρέπει να οδηγεί σε επαγωγή της αυτοφαγίας. Ο σκοπός της παρούσας εργασίας, ήταν να προσδιορίσουμε την επίδραση της έλλειψης των Akt1 και Akt2 κινασών στην αυτοφαγία των μακροφάγων, υπό την επίδραση διαφορετικών ερεθισμάτων όπως LPS, IL4, IFNγ και ινσουλίνης. Τα αποτελέσματα έδειξαν ότι η προσθήκη LPS στα μακροφάγα με έλλειψη είτε στην Akt1 ή στην Akt2 κινάση (Akt1-/-, Akt2-/- μακροφάγα αντίστοιχα), δεν οδήγησε σε έκφραση του LC3 δείκτη αυτοφαγίας. Η απουσία ορού από το θρεπτικό υλικό των κυττάρων δεν επηρέασε τα επίπεδα του LC3-II στα Raw264.7 κύτταρα και υπήρχε μια μικρή επαγωγή της αυτοφαγίας μετά από 6h και 24h από την προσθήκη IFNγ ή IL4 τόσο στα περιτοναϊκά μακροφάγα όσο και στα Raw. Επιπλέον, η προσθήκη rapamycin, ενός αναστολέα του mTOR, δεν αύξησε τα επίπεδα του LC3 πάνω από τα βασικά επίπεδα. Ποσοτική ανάλυση με αλυσιδωτή αντίδραση πολυμεράσης πραγματικού χρόνου (real-time PCR) πραγματοποιήθηκε για να εκτιμηθούν τα επίπεδα έκφρασης δύο σημαντικών γονιδίων αυτοφαγίας, των Atg5 και Atg7. Τα αποτελέσματα έδειξαν αύξηση στα επίπεδα και των δύο γονιδίων μετά από 24h από την προσθήκη LPS στα Raw264.7 κύτταρα σε συνθήκες απουσίας ορού από το θρεπτικό. Παρουσία του ορού, η έκφραση του Atg7 μειώθηκε μετά από 6h και 24h από την προσθήκη LPS τόσο στα WT όσο και στα Akt1-/-

μακροφάγα, ενώ η έκφραση του Atg5 αυξήθηκε 24h μετά την προσθήκη LPS τόσο στα WT όσο και στα Akt1-/- μακροφάγα. Επιπρόσθετα, η έκφραση και των δυο γονιδίων αυξήθηκε μετά από 6h και 24h από την προσθήκη IFNγ στα Akt1-/- κύτταρα, σε σύγκριση με τα αγρίου τύπου WT, ενώ μόνο τα επίπεδα του Atg7 ήταν υψηλότερα στα Raw κύτταρα μετά από ταυτόχρονη προσθήκη με LPS και IFNγ σε σύγκριση με τα κύτταρα ελέγχου (untreated). Όσον αφορά τον δείκτη μιτοφαγίας Pink-1, τα επίπεδα έκφρασης του ποίκιλλαν σε απόκριση στα διαφορετικά ερεθίσματα. Συγκεκριμένα, η απουσία του Akt1, οδήγησε σε αυξημένα βασικά επίπεδα μιτοφαγίας. Παρόλο που το LPS κατέστειλε την μιτοφαγία στα WT μακροφάγα, αυτή η καταστολή δεν παρατηρήθηκε στα Akt1-/- μακροφάγα 24h μετά την διέγερση. Στις 48h μετά την προσθήκη LPS και στις συνθήκες απουσίας του ορού από το θρεπτικό, αυτές οι διαφορές δεν παρατηρήθηκαν. Επομένως, μπορούμε να συμπεράνουμε ότι η Akt1 κινάση ελέγχει με διαφορετικό τρόπο την έκφραση των Atg5 και Atg7 γονιδίων στα μακροφάγα και η απουσία είτε της Akt1 ή της Akt2 δεν επηρεάζει την LC3-εξαρτώμενη αυτοφαγία, υποδηλώνοντας έναν πιθανό πλεονασμό μεταξύ των δύο Akt ισομορφών.

Λέξεις κλειδιά: Αυτοφαγία, μακροφάγα, Akt1, Akt2, LC3, αυτοφαγόσωμα, ερέθισμα

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1. Introduction

1.1 The importance of autophagy in infections and inflammatory diseases

Autophagy is a defence mechanism of the cell that is activated in fasting conditions or in order for the cell to get rid of damaged or dysfunctional organelles or invading microorganisms and pathogens. It is a lysosomal degradation pathway conserved from yeast to primates and aims to prevent cell death and keep the cell functional [1],[2]. There are three main types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy. During microautophagy and chaperone-mediated autophagy, small portions of the cytosol are engulfed directly in the lysosomal lumen and in the latter case, chaperones are responsible for the transport of the cargo intended for degradation to the lysosomes. Macroautophagy is responsible for the engulfment and degradation of larger portions of cytoplasm or for the turnover of proteins and organelles through a double-membrane structure known as autophagosome. The autophagosome then fuses with the lysosome and form the autophagolysosome which finally degrades its content [3],[4],[5]. The autophagic machinery consists of many autophagy proteins (Atg) and other complexes essential for the process. Atg1 (ULKI and ULK2 in mammals) is required for the induction of autophagy together with Vps34 (also referred as PI3K) and their interacting proteins [5]. The proteins Atg6, Atg14, Vps34, and Vps15 mediate vesicle nucleation, Atg8 and Atg12 conjugation systems mediate vesicle expansion, Atg2, Atg9, Atg18, are responsible for the dissociation of Atg proteins from the mature autophagosomes and also permeases that permit the release of amino acids from autophagosomes [1], [6]. Currently, the LC3 (mammalian homolog of Atg8) is the highly specific and universally used marker for autophagy, that undergoes an appropriate C-terminal modification that enables it to convert to LC3-II which translocates from cytosol to the phagosomal membranes and thus by interacting with p62 that recognizes ubiquitylated protein aggregates, participates in the autophagic process

[7],[8]. The pathway of autophagy with the main proteins and molecules contributing to it is depicted in the below diagram.

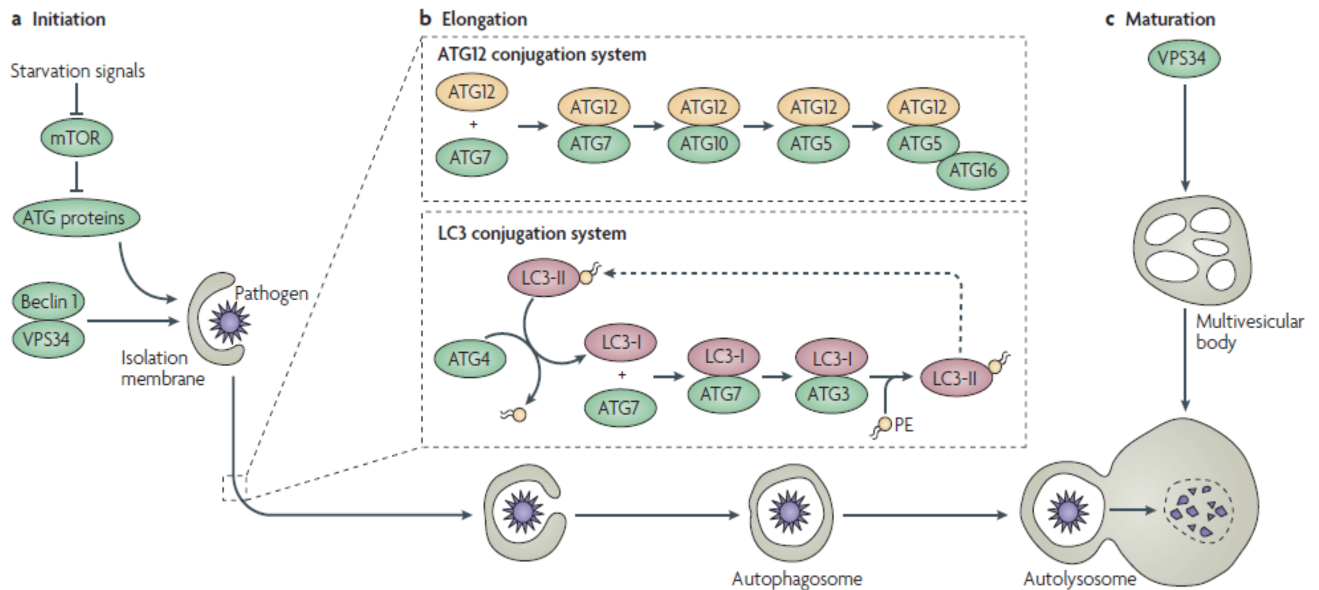

Fig.1: Autophagy pathway with its contributing factors (Levine and Deretic, Nat Rev Immunology, 2007)

Autophagy through the mechanism of xenophagy, functions as a defence mechanism against pathogens and invading microorganisms which in turn have adapted various mechanisms to avoid clearance from autophagy. Pathogens are recognized by PRRs (Pathogen Recognition Receptors) such as NOD-like receptors and TLRs and then their phagocytosis by macrophages follows [2]. Autophagy proteins have an essential role in the defense against bacteria, virus and protozoans. In plants, genetic deletion or knock down of autophagy genes prevents infection by pathogens while in drosophila and in mice, mutations and knock out of autophagy genes respectively, increase the prossibility of infection by bacteria, viruses or other microorganisms. Furthermore, autophagy regulators have been proved to play an important role in the defence against Mycobacterium tuberculosis in human cells [9]. As far as inflammatory diseases are concerned, the autophagy-related genes ATG16L1 and IRGM are associated with the development of Crohn’s disease which is a chronic inflammatory disorder of the small intestine that results from defective

recognition or clearance of the commensal bacteria and thus leading to intestinal inflammation. A polymorphism in ULK1 is also related to Crohn's disease. Furthermore, SNPs (Single Nucleotide Polymorphisms) on ATG5 gene are linked with SLE predisposition, a heterogeneous disease characterized by autoimmune responses against self-antigens, generated by dying cells and also mouse embryos that lack ATG5 are more prone to tissue inflammation due to defective clearance of apoptotic cells [1] .ATG7 is also implicated with inflammatory diseases, because its suppression on the mouse liver results in increased ER stress and insulin resistance. Finally, mice that lack the autophagy adaptor p62 are prone to develop mature-onset diabetes and insulin resistance [2], [9].

1.2 The role of macrophages and the importance of autophagy on macrophages

Macrophages originate from circulating peripheral blood mononuclear cells (PBMCs) that migrate into tissues either on physiological conditions or in response to inflammation and constitute a phagocytic population of cells whose main function is to clear the cellular environment from extracellular substances. Macrophages are also responsible for the removal of cell debris originated from tissue remodeling and are capable of efficiently clear cells that have undergone apoptosis [10]. Macrophages have two subtypes that are activated by different cytokines, growth factors and environmental agents, the M1 and M2. The M1 or classically activated macrophages, which are activated by IFN-γ, LPS or other pro-inflammatory cytokines, protect the organism against pathogen invasion and tumors, while the M2 or alternative macrophages which are activated by stimuli such as IL-4,IL-10, glucocorticoids etc, fight against parasitic infections, promote tissue repair and encounter of inflammation. Although both types of macrophages if not properly regulated can lead to inflammatory diseases, inflammation and tumor growth (M1) or cause fibrosis, impair immune responses or exploited by pathogens for intracellular survival [6],[11],[12],[13]. The PI3K-AKT-mTOR pathway contributes to polarization of macrophages and macrophages with enhanced expression of the mTOR pathway tend to

express M2 markers and exhibit this type of polarization while inhibition of mTORC1 which is a complex of the pathway enhances the M1 polarization phenotype. Furthermore, AKT kinases which are important factors of the PI3K-AKT-mTOR pathway, differentially contribute to macrophages polarization according to their isoform and more specifically macrophages deficient of AKT1 display an M1 phenotype, while AKT2 deficiency leads to an M2 type [6]. Autophagy plays an important role on macrophages defense against pathogens including *Mycobacterium tuberculosis, Salmonella enterica, Shigella flexneri, Listeria monocytogenes and Streptococcus pyogenes.* Induction of autophagy follows after recognition of these pathogens through specific PRRs and also under in-vitro conditions it seems like autophagy protects the macrophages from death signals through limiting the ROS and IL-1β and thus restricting inflammation [14]. Another important stimulous for autophagy with equal or even stronger effect than that of starvation-induced autophagy is the cytokine IFN-γ that activates macrophages, degrades proteins with long lifespan and have a protective role against *Mycobacterium tuberculosis* [8]. Furthermore, autophagy has cytoprotective roles and promotes cell survival under starvation conditions because by degrading proteins, organelles or other cytoplasmic materials, supply the cell with amino acids, fatty acids and other metabolic substrates which maintain the necessary energy for the survival of the cell. Through degradation of misfolded and aggregated proteins, autophagy also prevents their toxic effects and their contribution to the development of various neurodegenerative diseases such as Parkinson's and Huntigton's disease and also prevents the apoptotic pathway to be activated[15],[16]. Although autophagy as a self-limiting survival mechanism can lead to cell death if not reserved and also cell death can result from intracellular damage caused by virus infection, toxins, chemotherapeutic agents or hypoxia [17]. Moreover, in the case in which the macrophages are infected with *Toxoplasma gondi,* the parasite triggers CD40 receptor which in turn induces the fusion of the phagosome containing the parasite with autophagosomes, thus causing macrophages to exert their antimicrobial functions that result in lysosomal degradation of the pathogen [18]. Autophagy also contributes to organismal survival during nutrient deprivation and this is confirmed by studies in

various animal models such as in mice that are either Atg5 or Atg7 deficient, that are born normally but die within hours due to their inability to adapt to early starvation conditions. In the below picture are depicted some of the basic functions of autophagy during the autophagic process.

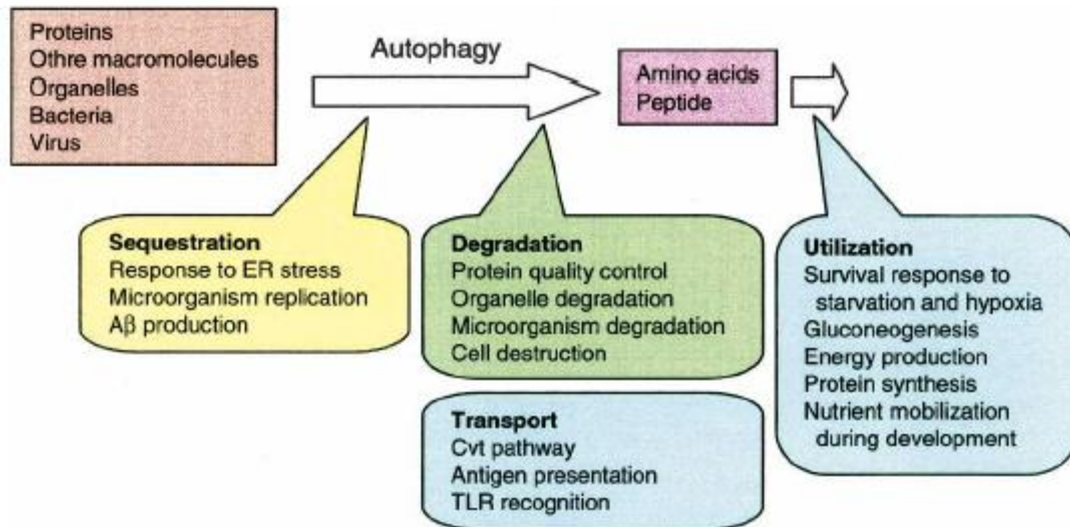

Fig.2: Basic functions of autophagy (Mizushima, Genes&Development,2007)

Another important function of autophagy is that it can limit chromosomal instability and DNA damage because in autophagy defective cells, deregulated turnover of centrosomes, failure to remove damaged mitochondria that result in toxic effects for the cells and other problems concerning defects in clearing DNA repair proteins, likely contribute to genomic instability [1]. One of the remarkable questions concerning autophagy is how the autophagic targets are recognized by the cell and targeted for degradation given the variety of targets from organelles and protein aggregates to macromolecules. In the case of endogenous targets and especially for protein aggregates, the signal for autophagic degradation is the ubiquitylation, while exogenous pathogens are recognized by PRRs like TLRs, Nod-like receptors (NLRs) etc [19],[20].

1.3 Pathways and mechanisms of autophagy regulation

Two of the most important pathways through which autophagy is regulated are the mTOR and AMPK that sense environmental factors such as energy, oxygen and nutrient stress and in turn activate the serine/threonine kinases ULK1 and ULK1, thus providing a mechanism for the control of autophagy process and autophagosome formation [11],[5]. This happens because ULK1 and ULK2 are located on the isolation membrane which is the starting point for autophagosome formation and as a result they contribute to its maturation [21]. In particular, one of the two complexes of mTOR pathway, the mTORC1 complex, detects growth factors and amino acids that are required for activation of mTORC1 kinase which in turn through phosphorylation of ULK1 inhibits autophagy in yeast. In mammals, mTORC1 appears to suppress ULK1 in presence of amino acids. Although, nutrient starvation activates ULK1 through AMPK mediated phosphorylation and subsequent inhibition of mTORC1. A series of downstream events follows including phopshorylation of Beclin 1 and activation of the ATG14-containing Vps34, UVRAG-containing Vps34 and Ambra-containing Vps34 autophagic complexes that result in autophagy initiation [22]. The importance of ULK1 (and ULK2) kinases for cell survival in cases of nutrient deprivation has also been proved by the fact that simultaneous deficiency of ULK1 and ULK2 results in apoptosis and cell death [23]. The importance of PI3K-Akt-mTOR pathway in autophagy regulation can be also assessed by the role of inhibitors of this pathway, such as wortmannin which inhibits autophagy and rapamycin (an mTOR inhibitor) that induces autophagy [24].

Furthermore, the mechanism of inhibition through mTORC1 is an important defence mechanism of mouse macrophages against bacteria such as *Mycobacterium tuberculosis* and *Salmonella enterica* because invasion of these pathogens leads to the expression of a specific miRNA (miR-155) which binds to RHEB mRNA and results in suppression of mTORC1, activation of autophagy and thus in increased intracellular clearance of the pathogens. However, there are microorganisms that have developed mechanisms that either suppress autophagy or avoid it and as a result they manage to survive inside the cell. Finally, inhibition of mTORC1 prevents

atherosclerotic plaques, formation of foam cells and generally inflammation on macrophages[5],[25],[26]. AMPK kinase is a more sensitive detector of energy status compared to mTOR because it detects the ratio AMP/ATP and is activated in order to restore metabolism and ATP levels [23]. In response to energy starvation, AMPK phosphorylates and activates TSC2 (Tuberous Sclerosis Complex 2) which negatively regulates cell growth by inhibiting mTOR mediated protein synthesis and thus maintaining homeostasis and survival. Overall, TSC2 combines signals from various pathways to regulate cell size and apoptosis and the pathway TSC-mTOR is essential for prevention of cell death under energy starvation conditions [27].

In addition to sensing growth factors and stress signals, autophagy in multicellular organisms can also be triggered by developmental signals such as hormones, in order to control cell remodeling and promote organism development [5]. Other ligands that positively regulate autophagy include cytokines such as IFN-γ, TNF and CD40-CD40L while the Th2 cytokines IL-4 and IL-13 suppress autophagy. According to the signaling molecule, there is a specific response for autophagy in the cell, like in the case of IFN-γ and TNF activation that protect macrophages from pathogens and mycobacteria. It is also possible that Th1 immune responses are related to autophagy induction and as a result protect the cell from invaders, while Th2 responses impair the autophagy process and complicate pathogen confrontation [7]. Indeed, IFN-γ is secreted by type 1 T helper cells and NK cells in order to exert its function through a signaling cascade that is activated by JAK kinases that phosphorylate STAT which in turn dimerizes, binds to the response elements and induces their expression. Except for JAK-STAT, IFN-γ also induces gene expression through other pathways such as PI3K, p38 MAPK and Myd88. The main targets of IFN-γ on macrophages include antimicrobial proteins, inflammatory cytokines and major histocompatibility complexes I and II (MHC I, II) that contribute to protection against pathogens [28]. Additionally, autophagy as an immune defense mechanism is not only induced by IFN-γ but is also indispensable for the IFN-γ mediated cellular and inflammatory responses and the early signal that appears to be necessary for this induction comes from JAK 1 and JAK 2 kinases.

Moreover, possible autophagic regulators induced by IFN-γ, include the immunity-related GTPases, Irgm1 and Irga6 and other factors such as protein kinase R and eukaryotic initiation factor (eIF)-2a kinase. As mentioned above, IFN-γ also induces other pathways that contribute to autophagy activation such as PI3K and p38 MAPK. In case of PI3K pathway, class I PI3K through phosphorylation of PIP2, generates PIP3 and activates mTOR and finally this results in recruitment of Beclin 1- Atg6 to stimulate autophagy. The mTOR pathway can have distinct function in response to environmentan signals and starvation conditions. Finally, IFN-γ through the p38 MAPK pathway, contributes to host defence, activates Erk2 and regulates the binding of p38IP to Atg9 that results in its enclosure to autophagosomes [28],[29]. One important inducer is the TLR4 agonist Lipopolyssacaride (LPS) that has been found to activate autophagy on Raw264.7 cells through a TRIF-dependent and Myd88-independent pathway with downstream targets RIP1 and p38-MAPK [30]. Other pathways that affect autophagy independently of mTOR signaling include PERK (protein kinase R-like endoplasmatic reticulum kinase) /eIF2a which lead to increased stimulation of the complex Atg5-Atg12-Atg16 in response to protein aggregation, and PKR (double-stranded RNA dependent protein kinase) that has been found to trigger autophagy during starvation and viral infection. The Ras pathway also regulates autophagy in response to growth factors and amino acids deprivation. In the first case, Ras-PI3K signaling cascade inhibits autophagy while in absence of amino acids Ras pathway through Raf-1/MAP kinase and ERK1/2 induces autophagy, so the effects are dependent on the environmental conditions and nutrients availability [31].

In addition to post-translational regulation, autophagy is also arranged at the transcription level and there are some transcription factors responsible for this control. One of those factors is E2F, which is a member of retinoblastoma pathway and participates in cell cycle regulation, progression and in cellular responses to DNA damage stress. Under such conditions, E2F has been found to activate the autophagy genes Atg1, Atg5 and LC3 which are required for autophagy induction and vesicle formation

respectively and thus promoting autophagy [32]. Another epigenetic regulator of autophagy is the methyltransferase G9a which is abundant in somatic cells and forms a complex with G9a-like protein (GLP) that function as repressors of gene expression through methylation of CpG islands [33]. Under normal conditions, G9a suppresses the expression of the autophagy genes involved in autophagosome formation, however in the presence of signals that induce autophagy such as starvation, G9a is released from the target gene promoters, leads to chromatin remodeling events-such as decrease in repressive methylation marks- and promotes an increase expression of LC3-II, p62, Atg9B and other autophagy-related gene. In response to T-cell activation, c-Jun contributes to the expression of LC3-II and p62 through the JNK pathway and reverses the inhibitory effect of G9a [33]. Finally, among the important transcription factors that are necessary for autophagy induction is FOXO3 that acts alongside with mTOR pathway-both are targets of IGF-1-insulin-PI3K/Akt pathway- and stimulates the expression of many autophagy genes such as ULK2, Beclin 1, LC3-II, Atg12 and Vps34 [31].

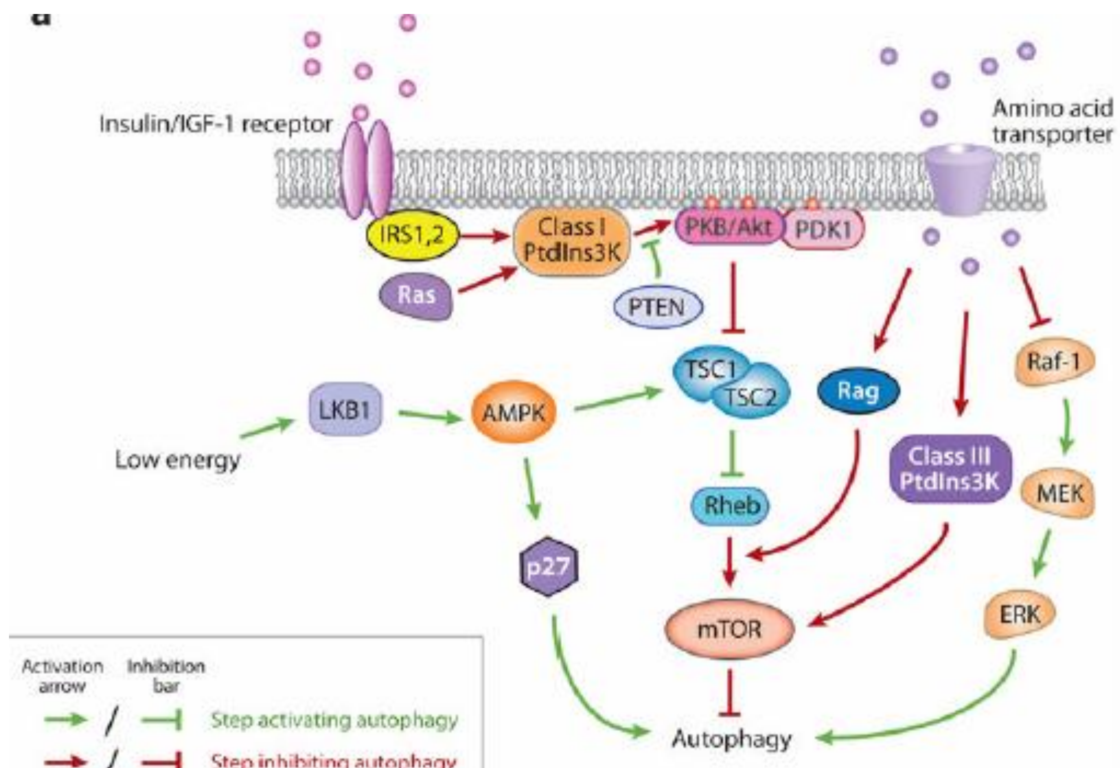

Fig.3: Regulatory pathways of autophagy by amino acids, hormones and energy in mammals (Congcong and Klionsky. Annu Rev Genet. 2009)

1.4 Autophagy and Endotoxin tolerance

Endotoxin tolerance is a term used to describe cells, animals or humans that are hyporesponsive to an increased dose of endotoxin, if they had been previously exposed to minimum amounts of this toxin and are rendered unable to respond to further stimulations. Immune cells that can develop this condition are mostly macrophages and monocytes and after becoming tolerant they can result in impaired responses that could lead to immunosuppresion and even fatality [34],[35]. In vitro studies have demonstrate that endotoxin tolerance affects a variety of immune response molecules and factors either by their downregulation, such as in the case of inflammatory cytokines (TNFα, IL-6, IL-1β etc) or by enhancing the expression of anti-inflammatory cytokines and other regulators [34],[36],[37]. Furthermore, it is intriguing that the phenotype of tolerant macrophages or monocytes resembles that of M2-type macrophages that have also decrease expression of inflammatory cytokines and increased levels of the anti-inflammatory ones [38],[12]. Endotoxin tolerance emerges as a negative feedback mechanism in response to inflammation malfunction and is regulated by multiple factors that include chemokines and cytokines that act through different signaling pathways, various positive and negative regulators and also miRNAs that result in gene reprogramming [34],[39]. One of the most important categories of receptors that mediate inflammation in response to pathogens are TLRs, like TLR4, which recognizes lipopolysaccharide (LPS) and activates pro-inflammatory cytokines resulting in induction of macrophage's immune response. Given the fact that increased expression of TLR4 promotes inflammation and disease, its suppression is essential for developing endotoxin tolerance for the control of macrophage's response to LPS and for limiting the negative outcomes that would result from the secondary exposure to LPS [40]. A neuropeptide that promotes this endotoxin tolerance on macrophages is VIP (vasoactive intestinal peptide) that inhibits TLR4 expression through PI3K and specifically Akt1 isoform, exerts its anti-inflammatory properties and has a protective role in autoimmune disorders such as rheumatoid arthritis and Crohn's disease [40].

Autophagy, as it has already been mentioned, is a defence mechanism of the organism against invading pathogens that confer resistance and protection by destroying them. The importance of autophagy in conferring tolerance was demonstrated by the fact that deficiency in Atg16L1 that is one of the major contributors in autophagosome formation that catalyzes the maturation process of LC3-I to LC3-II, leads to decreased autophagy and resulted in an increased lethality of mice after infection with a specific strain of *Staphylococcus aureus* [41]. Another interesting example is in the case of *Cryptococcus neoformans* (Cn) which is a pathogen responsible for pulmonary infections. Autophagy has an important contribution in dealing with this pathogen because a mutant type of Cn that is deficient in Vps34- an autophagy protein- not only showed a dramatically decreased toxicity in the infected mouse models but also this mutant strain was rapidly cleared from the infected lungs after macrophage phagocytosis in contrast with the WT strain. This was due to defective tolerance of starvation and formation of autophagosomes [42].

Finally, Akt1 kinase has been found to be important mediator for macrophage's response to LPS through regulation of specific microRNAs. Particularly, LPS can stimulate negative feedback signals that either promote the expression of genes that suppress the response to LPS such as suppressor of cytokine signaling 1(SOCS 1) or inhibit the expression of genes that are required for the response such as TLR4. These genes are regulated by miRNAs which in turn are under the control of Akt1 and microarrays, in silico analysis and transfection studies revealed that LPS-induced Akt1-/- macrophages suppressed let-7e miRNA expression while increased miR155 miRNA expression in contrast to Akt1+/+ macrophages. Due to the fact that let-7e controls TLR4 and miR155 controls SOCS1, the suppression of the former in Akt1-/- macrophages and the induction of miR55, results in expression of TLR4 and suppression of SOCS1 accordingly and thus Akt1-/- macrophages are hyperresponsive to LPS and does not develop endotoxin tolerance [43].

1.5 Mitophagy as a specialized form of autophagy

Mitophagy is a category of cargo-specific autophagy that concerns the removal of damaged mitochondria by engulfment into LC3-coated vesicles for degradation. Prior to this engulfment. mitochondria are teared apart into pieces of proper size in order to be encapsulated for mitophagy [44],[45]. In yeast, an important protein of mitophagy is the autophagic Atg32 that consists of a small carboxy-terminal domain in the interior of mitochondria and another ~40KDa domain that spans into the cytosol. This protein interacts with Atg11 which recruits cargoes to autophagosomes by interacting with Atg8. This indirect interaction of Atg32 with Atg8 is thought to guide mitochondria to autophagosomes for degradation. In mammalian cells two proteins are the basic players on the pathway of mitophagy induction, PINK-1 and parkin. Parkin is an E3 ubiquitin ligase that is expressed in many tissues such as brain, heart, liver and skeletal muscle and is able to track and translocate to impaired mitochondria without affecting the healthy ones in the same cell. Parkin is recruited to uncoupled mitochondria by another kinase, PINK-1(phosphatase and tensin homolog (PTEN)-incuded kinase 1) which undergoes proteolysis on healthy mitochondria and is accumulated only on damaged mitochondria where the proteolysis is inhibited and therefore recruits parkin to them [46]. Mitophagy as a process is fundamental for cell homeostasis because it regulates the number of mitochondria and controls metabolic demands of the cell, however mitochondria can also harm the organism either by releasing ROS or inflammatory signals or even through the permeabilization of their membrane [47].Furthermore, TNFa activated macrophages exhibit mitophagy as it has been confirmed by quantitative proteomics analyses, flow cytometry, biochemical assays and immunofluorescence experiments that indicated a downregulation of mitophagy genes in response to TNFa triggering. One of the mechanisms that is reclaimed by the cell to target mitochondria for mitophagy includes AMPK kinase which activates ULK1 which as an initiator of autophagy, marks the induction of mitophagy. Finally, another study showed that mice which are heterozygous for ATG5 on macrophages of the heart have abnormal mitophagy that leads to increased

mitochondrial ROS production triggering NF-kB activation, infiltration of macrophages and production of pro-inflammatory cytokines that result to cardiac injury and thus providing a key role for ATG5 efficiency for normal mitophagy and resolution of inflammation in heart tissue [48].

1.6 The contribution of Akt kinases on macrophages function

Macrophages consist a fundamental category of immune cells as they are responsible for maintaining tissue integrity and homeostasis. They are antigen presenting cells, able to discriminate between self and foreign invaders and their main function is to protect the organism by eliminating damaged or aging cells, organelles and pathogens and thus preventing the toxic effects that would result from their death [49],[50]. AKT kinases through a series of specific phosphorylation events can promote and control the expression of various cytokines such as IFN-β and NO that is necessary molecule for the destruction of invading bacteria [50]. Furthermore, macrophages in order to come up against their phagocytic targets, they need to migrate and Akt has been found to act as an upstream signaling regulator for this chemotactic process. One such evidence is that suppression of Akt2 expression resulted in impairment in migration of THP-1 monocytic cells and peritoneal macrophages and also the siRNA for Akt2, reduced actin polymerization and phosphorylation of PKCζ and LIMK/cofilin which are important events for chemotaxis [51],[52]. Macrophages mostly express Akt1 and Akt2 isoforms while the Akt3 isoform is predominantly found on brain. Akt kinases during inflammatory responses on macrophages mediate TLR signaling cascades and also LPS tolerance which is strengthened by the fact that Akt1deficient macrophages are hyperesponsive to LPS and this effect is modulated by miRNAs [51],[43].

1.7 The effect of AKT deficiency in insulin resistance

The protein kinase Akt is activated in response to various signals including insulin and both the expression and translocation of glucose transporters are regulated by Akt [53]. Specifically, the Akt2 isoform is considered to be an essential signaling molecule that mediates glucose homeostasis in fat tissue and muscle, it seems to be abundant in insulin-responsive tissues and has been found to be involved in the metabolic actions of insulin. The loss of Akt2 results in hyperglycemia that is accompanied by hyperinsulinemia and peripheral insulin resistance as evidenced by the elevated levels of insulin in the plasma. In other words, insulin in mice deficient of this kinase, is less able to lower blood glucose due to defective action of insulin in liver and skeletal muscle [54]. Furthermore, in Akt2 deficient males, insulin resistance progressively leads to diabetes and pancreatic β cells failure as opposes to female mice lacking Akt2 that have a mild form of hyperinsulinemia and hyperglycemia without developing diabetes [53]. Finally, another study showed that in db/db mice that is a genetic model of obesity, insulin resistance and type 2 diabetes, there is decreased activation of Akt in response to insulin and impaired GLUT4 translocation that results in dysfunctional glucose uptake and insulin resistance [55].

2. Materials and Methods

2.1 Buffers and solutions

- Freezing medium: 80% FBS+20% DMSO
- DMEM (Dulbecco's Modified Eagle medium) 1x+GlutaMAX^{TM} [+] 1g/L D-Glucose, [+] pyruvate supplemented with 10% FBS and 1% P/S
- Running Buffer: 144gr Glycine, 30gr Tris-Base, 10gr sds
- Transfer Buffer: 5x Tris/Glycine (3,02gr Tris, 18,8 gr Glycine), methanol, H_2O
- Western Blocking buffer: 5% BSA in PBS-Tween
- PBS/PBS-T: 8g/L NaCL, 0,2g/L KCL, 1,42g/L Na_2HPO_4, 0,24g/L KH_2PO_4 / + 0,1% Tween-20
- Ripa lysis buffer: 5M NaCL, 0,5M EDTA pH 8, 1M Tris Ph 8, NP-40, 10% sodium deoxycholate, 10% SDS, dH_2O
- Chemiluminescent HRP (Reagent A + Reagent B) from GenScript (cat. No L00221V500)
- IF fixation solution: 4% formaldehyde
- IF blocking buffer: 2% BSA/0,1% saponin/PBS
- Wash buffer (for eliza): 0,05% Tween-20 in PBS
- Reagent Diluent: 1% BSA in PBS
- Substrate solution: 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)
- Stop solution: 2N H_2SO_4

2.2 Mice models

For the conducted experiments, both male and female wild type C57BL-6 and specific AKT1, AKT2 knock-out mice (AKT1-/-,AKT2-/- respectively) were used. The knock-out strains resulted with the cre-loxP recombination system. In order to achieve the specific knock-out with this system, the one parent contains the Cre DNA sequence and the other the "target" recognition site, so as a result through mating, Cre recombinase function by recognizing the two loxP sites at each side of the target sequence and

delete it. All mice were housed in a specialized Animal House facility at IMBB-FORTH and Medical School at University of Crete. Before proceeding to macrophages isolation, the mice were injected intraperitoneally with 4% thioglycollate. After 5 days, mice were sacrificed and peritoneal macrophages were collected and cultured in the appropriate medium (DMEM) for further treatments.

2.3 Cell culture and treatments

For this study, both cell line and primary cells were used. The cell line was RAW264.7 cells that are macrophage-like, leukemia virus transformed cell line derived from BALB/c mice and the primary cells was peritoneal macrophages derived from specific mouse strains. Both cell types are able to adhere to the plastic of cultured flasks and plates and were cultured at 37^0C / 5% CO_2. Cell scrapper was used for their subsequent detachment from the flask.

Culture flasks: $25cm^2$ and $75cm^2$ from SARSTEDT or Corning

Culture plates: 6-well plates, 12-well plates and 24-well plates from SPL life sciences

Macrophages were treated with different ligands such as LPS, insulin rapamycin, IL4 and IFNγ at different timepoints in order to investigate their effect on LC3 expression and autophagy induction.

For the LPS treatments, macrophages were treated with 100ng/ml Lipopolysaccharides from Escherichia Coli (SIGMA®, Saint Luis, Missouri, USA) for various timepoints (30min, 6h, 24h and 48h).

Insulin was used at a final concentration of 0,1U/ml for 24h and 48h. Due to reduced half-life, insulin had to be added to cells every day during treatment course.

Macrophages were also treated with 20nM rapamycin for 24h and 48h, 1μg/ml IL4 for 6h and 24h and 100ng/ml IFNγ for 6h and 24h.

2.4 Protein Detection Techniques

2.4.1 Cell lysis for protein extraction

After the timepoints treatment, the medium from the 6-well or 12-well plates was removed and 1ml PBS/well was added. Cells were then transferred with cell scrapper into eppendorf tubes, centrifuged at 1500rpm for 10min at 4^0C and after that the supernatant was discarded and each pellet was resuspended at RIPA+ protease inhibitor solution. Samples were kept at -80^0C until further use.

2.4.2 BCA Protein Determination Assay

For the measurement of total protein concentration from cultured macrophages, the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Rockford, USA) was used.

In this assay, standards with specific concentrations raging from 200-2000μg/ml were prepared and 10μl of each one (of the 6 standards) was added to a microplate well (Thermo Scientific™ Pierce™ 96 -Well Plates, Product No. 15041). At the last two wells of the column 10μl PBS and 5μl Ripa solution were added as blanks because the standards are diluted in PBS and the samples in Ripa. After addition of samples (5μl) to the plate, the total number of wells had to be calculated in order to determine the amount of Working Reagent (WR) needed. For the calculation we took into consideration: (n of standards+ Ripa+ PBS + n of samples) x (volume of WR per sample) = total volume of WR required. For the Working Reagent, BCA Reagent A and B were mixed in a ratio of 50:1. Then 200μl of WR was added to each standard and sample and the plate was covered with aluminum foil and incubated for 30min at 37^0C. Finally the concentration was measured at a microplate reader at 550nm.

2.4.3 Western Blot

For the samples preparation: An appropriate amount from each sample (depended on its concentration) from the supernatant was transferred into a

new eppendorf tube together with the appropriate amount of 6x loading dye (in order to be 1x final concentration). The samples were boiled for 10min at 100^0C to break all disulfide bonds, spinned down and were ready to load into the gels.

For the western blot analysis, both 12% and 14% polyacrylamide gels were prepared. The materials needed for the preparation were:

- For the **separating gel**: H2O, 30% acrylamide, 1,5 M Tris (pH 8,8),10% SDS, 10% ammonium persulfate, TEMED
- For the **staking gel**: the same solutions as separating except of using 1M Tris (pH 6,8)

The amounts of each solution depended on the percentage of gel that was prepared each time and the number of gels needed.

After running the gel(s) at 120V for about 1,5hour, they were transferred to a PVDF (Polyvinylidene difluoride) membrane by using a wet transfer apparatus. For the transfer, two blotting papers were placed above the sponge, the gel was placed above the papers, followed by the membrane, another two blotting papers and the other sponge. The sponges, the gel and the blotting papers were first incubated at transfer buffer and the membrane was first activated at methanol and then incubated at transfer buffer. The apparatus was set at 400mA for 1hr on ice. After the transfer process, the membrane was incubated into blocking buffer (5% BSA in 1X PBS-Tween), for 1hr at room temperature. The blocking was followed by 3 washes (5-10min each) with 1X PBS-T. After the washes, the membrane was incubated with the primary antibodies - a-LC3 (1:5000 in 5% BSA in PBS-T), and a-actin (1:1000 in 5% BSA IN PBS-T) - overnight at 4^0C. The next day, membrane was washed 3-4 times (10min each) with 1X PBS-T and then incubated with the secondary antibodies- a-mouse (1:5000 in blocking buffer) for a-actin and a-rabbit (1:10000 in blocking buffer) for a- LC3, for 2hrs at RT. For the detection,

2.5 ELISA

For the detection of the cytokines IL-6 and TNF-a that were produced from the cultured macrophages, Enzyme Linked ImmunoSorbent Assay (ELISA) was performed according to the corresponding protocols: DuoSet® ELISA Development Systems for mouse IL-6 by R&D Systems (Cat. No: DY406-05 or DY406) and Mouse TNF-a ELISA MAX™ DELUXE from BioLegend.

According to protocol, after diluting the Capture Antibody to the working concentration in PBS, a 96-well plate was coated with 100μl/well of the diluted Capture Antibody, sealed and incubated overnight at RT. The next day, the capture antibody was aspirated from each well and the wells were washed 3 times with 150μl of Wash Buffer. After the last wash any remaining Wash Buffer was aspirated and then the plate was blocked by adding 200μl Reagent Diluent/well and incubated for1hr at RT. The wash step was repeated and then 100μl of samples as well as standards (diluted according to protocol in Reagent Diluent) were added per well. The plate was sealed and incubated for 2hrs at RT. After incubation the wash step was repeated and then 100μl of Detection Antibody (diluted in Reagent Diluent) were added to each well and the plate was sealed and incubated for another 2hrs in RT. Another wash step followed and then 100μl of working dilution of Streptavidin-HRP were added to each well, the plate was covered and incubated for 20min at RT in the dark. The plate was again washed 3 times with Wash Buffer and then 100μl of Substrate Solution were added per well and the plate was left for 20min at RT in the dark. Finally, the reaction was stopped by adding 50μl/well of Stop Solution and the optical density of each well was determined with a microplate reader set at 450nm and 570nm. Values at 570 nm were subtracted from those of 450 nm and data were analyzed by Graph Pad Prism 6 software.

2.6 RNA Extraction

For the isolation of RNA from the cultured macrophages, the TRIzol^R Reagent (ambion by life technologies) protocol was performed as following:

The medium from 24-well plates was aspirated and the cells were washed once with 500μl PBS. Afterwards, 200μl of Trizol/well were added and the plate was incubated for 5min at room temperature. The dissociated cells with the Tizol were collected in eppendorf tubes, vortexed and incubated at room temperature for 5 minutes. Then the cells were centrifuged at 11000 rpm for 5’ at 4^0C for removal of cell debris. The supernatant was transferred in new tubes and 40μl of chloroform was added in each sample. Samples were then shaked vigorously by hand for 15 seconds, incubated for 2-3min at RT and centrifuged at 12000rpm for 15min at 4^0C. Then the upper aqueous phase from each sample (containing the RNA) was transferred to new eppendorf tube and 100μl of 100% isopropanol were added to the aqueous phase. Samples were incubated for 10min at RT and then centrifuged at 12000rpm for 10min at 4^0C. The supernatant was discarded and the pellet was washed with 200μl of 75% ethanol. The samples were vortexed briefly and centrifuged at 7500rpm for 5min at 4 ^{0}C. After that, the wash was discarded and the RNA pellet left to dry on air for 5-10min. Finally, it was resuspended in 20μl RNase-free water, incubated at heat block set at 55^0C for 10min and then stored at -20^0C or used for further applications.

2.7 cDNA Synthesis

In order to synthesize the first strand of cDNA from the isolated RNA, the PrimeScript^{TM} 1st strand cDNA Synthesis Kit by TaKaRa was used.

Protocol:

1. The first mix that was prepared contained the following reagents:

Reagent (per reaction)	Volume
Random 6mers (50μM)	0,5μl
dNTP mixture (10mM each)	0,5μl
Template RNA + RNase free H_20	6,25μl (RNA conc =500-1000ng)
Total	**7,25μl**

2. The mix was incubated for 5min at 65^{0}C and then cooled immediately on ice. After the incubation, the reaction mixture was prepared and added to the first mixture.
3. Reaction mixture:

Reagent (per reaction)	Volume
5x PrimeScript Buffer	2μl
RNase Inhibitor (40U/μl)	0,25μl
PrimeScript RTase (200U/μl)	0,5μl
Template RNA primer mixture (from step 1)	7,25μl
Total	**10μl**

4. Then the reaction mix was incubated to the following conditions:

- 30^{0}C for 10min
- 42^{0}C for 30-60min
- 95^{0}C for 5min to inactivate the enzyme

Finally the mix was incubated on ice.

2.8 Quantitative Real-Time PCR

To detect the expression levels of the target genes, a quantitative PCR was performed according to KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal by KAPA Biosystems.

After the cDNA synthesis, all cDNAs were diluted in 10μl WFI and then 1,5μl of the diluted cDNA was used as a template for the real-time PCR.

In the MicroAmp® Optical 8-Cap Strip the following mix was made (for the reference gene and the target gene(s) and the amounts were adjusted according to the number of samples):

Reagent	**Final Concentration**	**Master mix volume per 10μl reaction**
2X KAPA SYBR® FAST qPCR Master Mix2 Universal	1X	5μl
Forward primer (10μM)	200nM	0,5μl
Reverse primer (10μM)	200nM	0,5μl
Template DNA	<10ng	1,5μl
Water		2,5μl
Total		**10μl**

Then, the qPCR was performed according to the following parameters:

- Enzyme activation: 95 ^{0}C for 3min
- Denaturation: 95 ^{0}C for 1-3 sec
- Annealing/Extension: 60 ^{0}C for $\geq$ 20sec

(Denaturation and Annealing/Extension: 40 cycles)

2.9 Immunofluorescence

For the detection of autophagy induction indicated by LC3 puncta in confocal microscopy, immunofluorescence staining for LC3 was used.

The coverslips on which the cells were cultured, were poly-L-lysine treated. For this treatment, coverslips were incubated for 1hr at 100ml of 10% poly-L-lysine solution in a UV sterilized hood and then washed with WFI or dH_2O.

Protocol: The medium from a 24-well plate was aspirated and the cells (on coverslips) were washed with 1ml PBS and then fixed with 4% formaldehyde for 15min at RT. Then, the cells were washed twice with PBS and the plate was incubated at 4 ^{0}C until the staining. Afterwards, the cells were permeabilized with ice cold methanol for 10min at -20 ^{0}C and then washed once with PBS. Blocking was performed with PS blocking buffer (2% BSA/0,1% saponin/PBS) for 15min at RT. After the blocking, the coverslips were placed in a numbered petri dish and were covered with 100μl PS (from the wells) and then the cells were incubated with 100μl of the 1st antibody a-LC3 (diluted 1:20 in PS) for 1hr at RT. The coverslips were then washed 3 times with PS (100μl each) and then incubated with the secondary antibody Alexa Fluor 488 (1:500 in PS), for 1hr at RT at dark. Another 3 washes with PS followed and then the coverslips were covered with 100μl TOPRO (1:500 in PS) each. Finally, the coverslips were washed 2 times with PS and once with PBS and they were mounted on slides with αMowiol mounting solution in order to preserve the staining and the fluorescence. The coverslips were sealed with nail varnish.

Antibodies used for immunofluorescence:

Antibody	Host	Manufacturer	Dilution
LC3	rabbit	Nanotools	1:20
AlexaFluor 488 or FITCH	rabbit	Sigma Aldrich	1:500

- For staining of nuclei, TO-PRO-3 (monomeric cyanine nucleic acid stains) from Invitrogen (cat.no. T3605) was used.

3. Results

3.1 LPS treatment decreases the expression of LC3-autophagy marker on both primary WT and Raw264.7 macrophages.

It has been shown that LPS induces autophagy and the formation of autophagosomes [56] and also another study suggested that after LPS treatment there was an increase in Beclin 1 as well as LC3-II and the induction of autophagy was dose-dependent and time-dependent [57]. In order to ascertain how LPS treatment affects the expression of LC3, we treated both primary peritoneal macrophages from WT mice and Raw264.7 macrophages with LPS (100ng/ml) at different time points as it is shown in the immunoblots below.

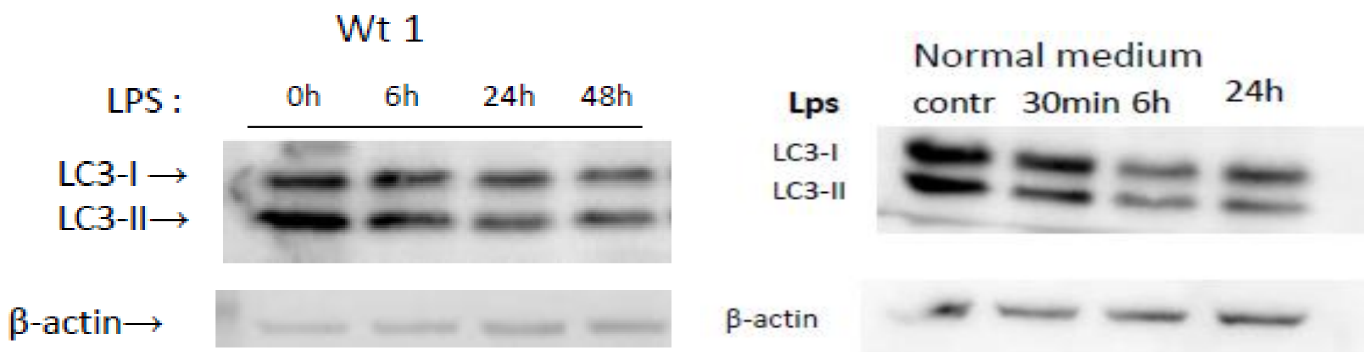

Fig.3.1: Primary and Raw264.7 macrophages treated with LPS (100ng/ml) for 6h, 24h and 48 hours. LC3 expression was detected with western blot.

As it is obvious from the above immunoblots, in both types of macrophages, LC3 expression was decreased with LPS compared to untreated controls. The graph representation of LC3-II/Actin ratio for both cases is shown below.

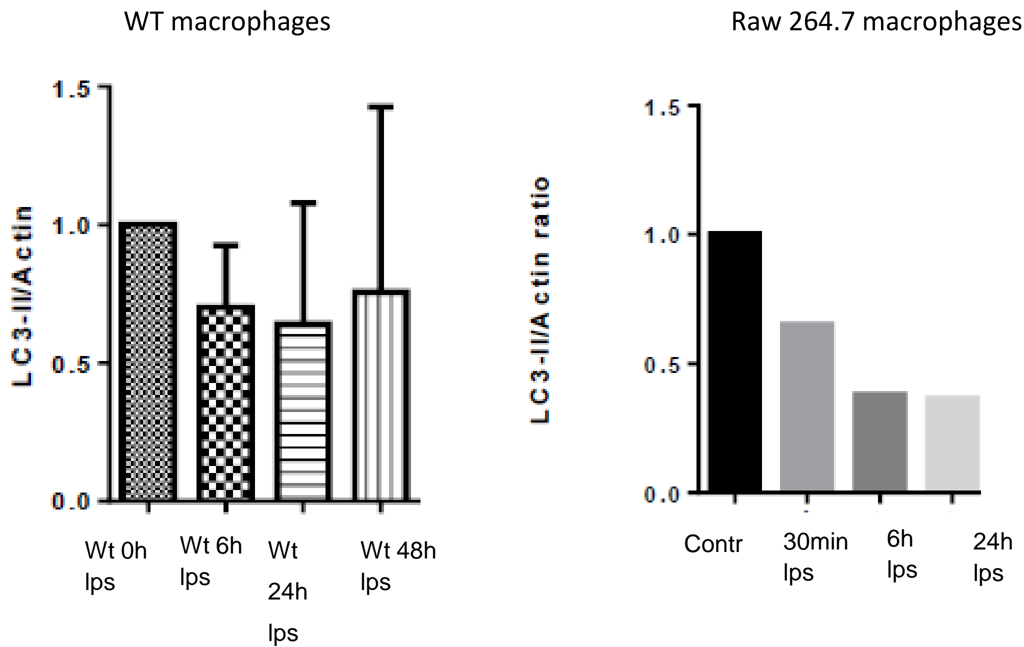

Fig.3.2: LC3-II to Actin ratio for the indicated timepoints in both WT and Raw macrophages. In both types of macrophages LC3 expression is decreased compared to controls

3.2 Starvation conditions in combination with LPS treatment does not affect LC3-II levels in Raw264.7 cell line

Another important condition that is thought to induce autophagy is nutrient starvation as it has been indicated by various studies. One of those studies showed that upon starvation, autophagy is activated in order to provide cells with the necessary components for their survival and this induction is dependent on Ulk1 dephosphorylation and especially a particular single amino acid mutation on Ulk1 that leads to dissociation from AMPK and increased the LC3-II levels upon starvation [58]. Another study indicated that there was an up to 3-fold increase in LC3-mRNA levels in response to starvation [59].

In order to test whether serum starvation induces the expression of LC3 and subsequently autophagy, we cultured Raw264.7 cells with LPS (100ng/ml) at starvation conditions (without FBS) for the timepoints indicated below.

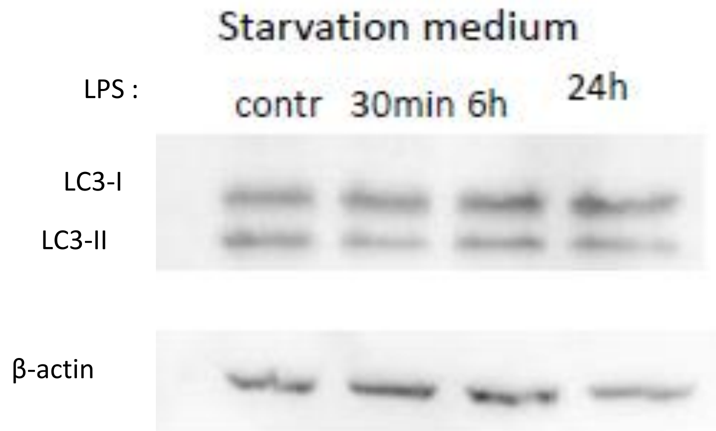

Fig.3.4: Raw264.7 cells were treated with LPS (100ng/ml) at 30min, 6h and 24hours at starvation conditions and LC3-II levels were detected with western blot

As it is obvious from the above western blot, starvation medium not only did not induce the expression of LC3 but its expression remained unchanged during different timepoints of LPS stimulation. This almost stable expression is also apparent from the graph of LC3-II to actin ratio.

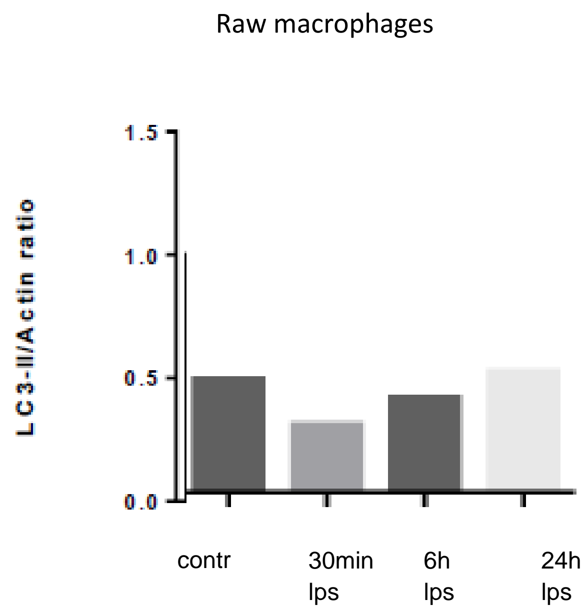

Fig.3.5: LC3-II to actin ratio for the indicated LPS timepoints. As it is shown from the expression bars there is almost stable expression at all times after lps treatment

Furthermore, we tested by RT-PCR the mRNA expression levels of two important autophagy genes, Atg5 and Atg7, to estimate whether their expression was affected in starved cells. In both normal and starvation medium, the Atg5 mRNA expression was increased more than two-fold only after 24 hours of LPS but this increase was statistically significant only in starvation conditions.

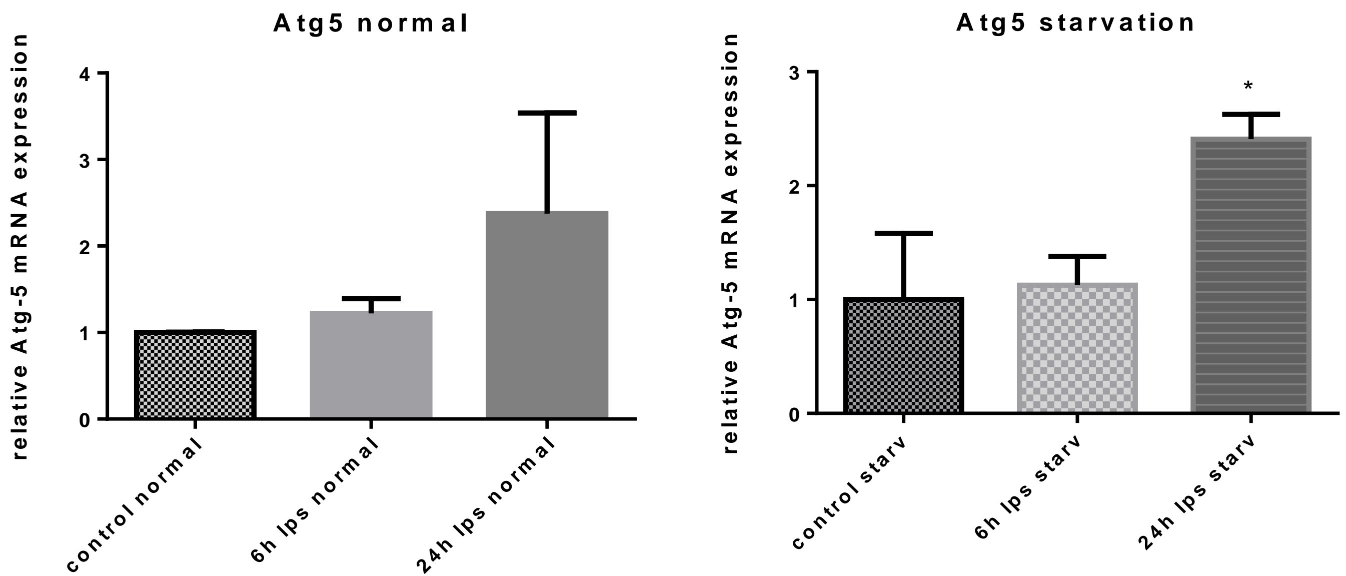

Fig.3.6: Atg5 mRNA expression levels on Raw264.7 cells were detected by RT-PCR. Increased expression in both cases was detected after 24h of LPS and was statistically significant on starved cells

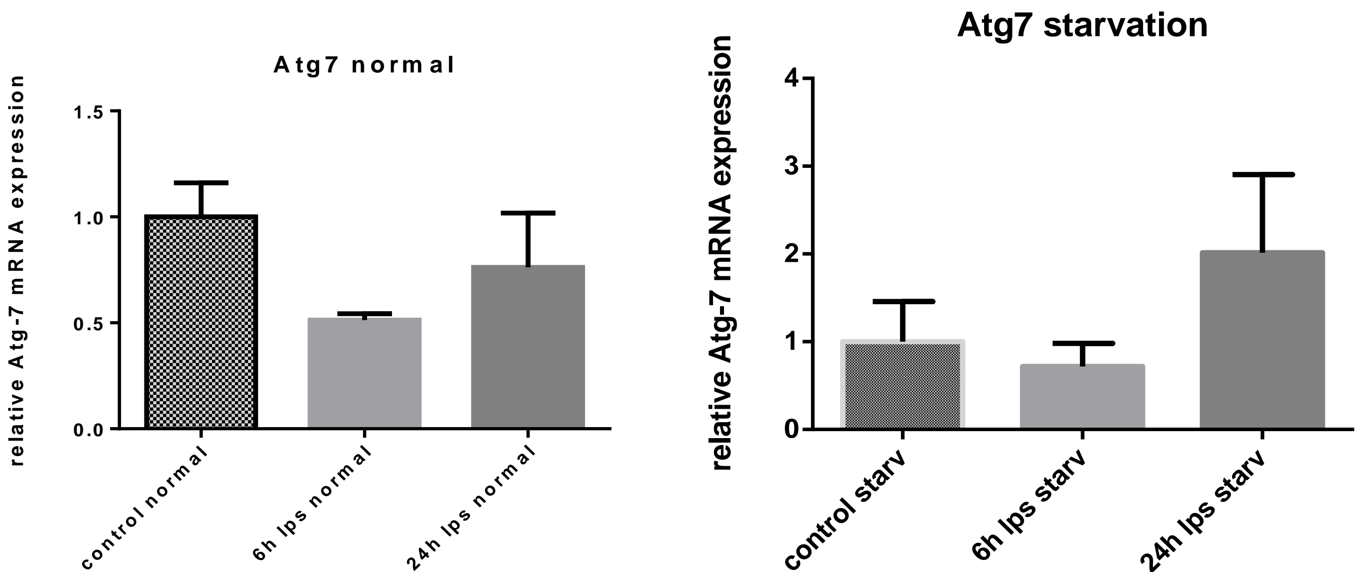

Fig.3.7: Atg7 mRNA expression levels on Raw264.7 cells at both complete and starvation conditions were detected by RT-PCR. Only at starved cells Atg7 expression after 24h of LPS appeared to be higher compared to control cells but this increase was not statistically important.

3.3 LC3 expression is also decreased in both Akt1-/- and Akt2-/- macrophages after LPS treatment.

Next, we wanted to check whether macrophages that are deficient of Akt1 and Akt2 kinases show an increase in the expression of the autophagy marker LC3. We expected the macrophages from mice that lack these two kinases to have greater induction of autophagy because their deficiency leads to dysfunctional mTOR pathway which is known to inhibit autophagy.

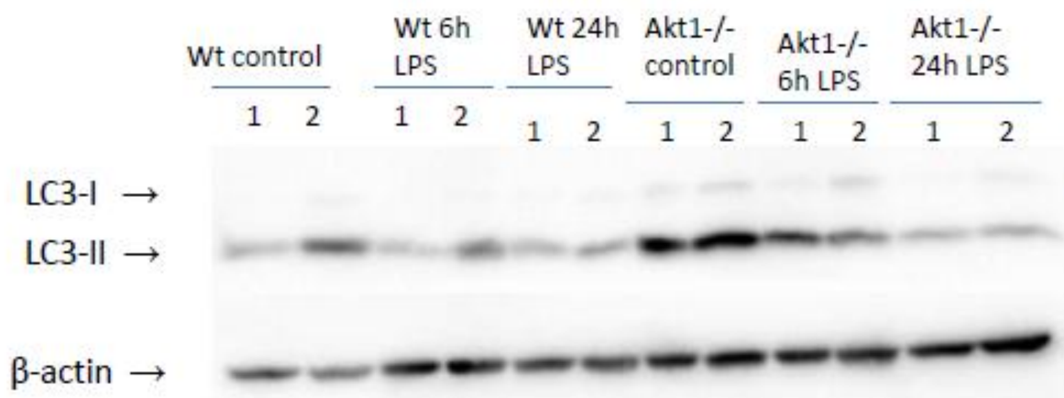

Fig.3.8: LC3 expression on WT and Akt1-/ - macrophages on basal conditions and after 6h and 24h of LPS treatment. Increased LC3 expression is observed only at Akt1-/- control cells with a tendency to decrease after the indicated LPS timepoints

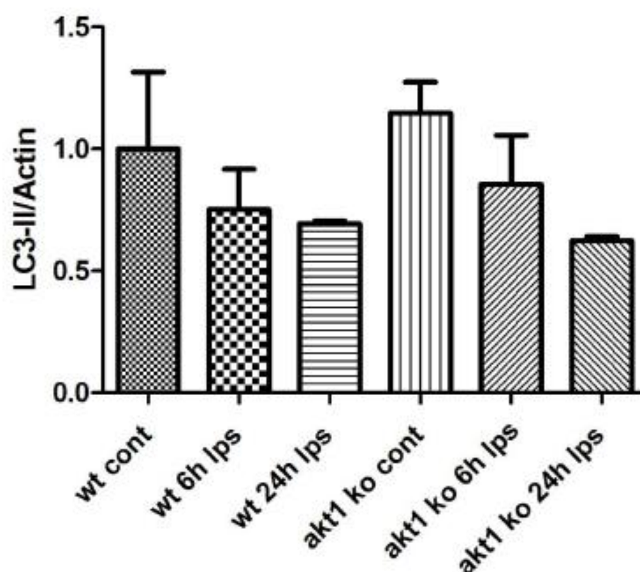

Fig.3.9: Graph representation of LC3-II to actin ratio for WT and Akt1-/- macrophages for the indicated timepoints. Akt1-/- control cells have the greater ratio as it was confirmed by the immunoblot

Next we checked with quantitation Real-time PCR the expression of Atg5 and Atg7 in order to note whether their expression was affected by the elimination of Akt1 kinase. The results from the analysis are shown in the below diagrams.

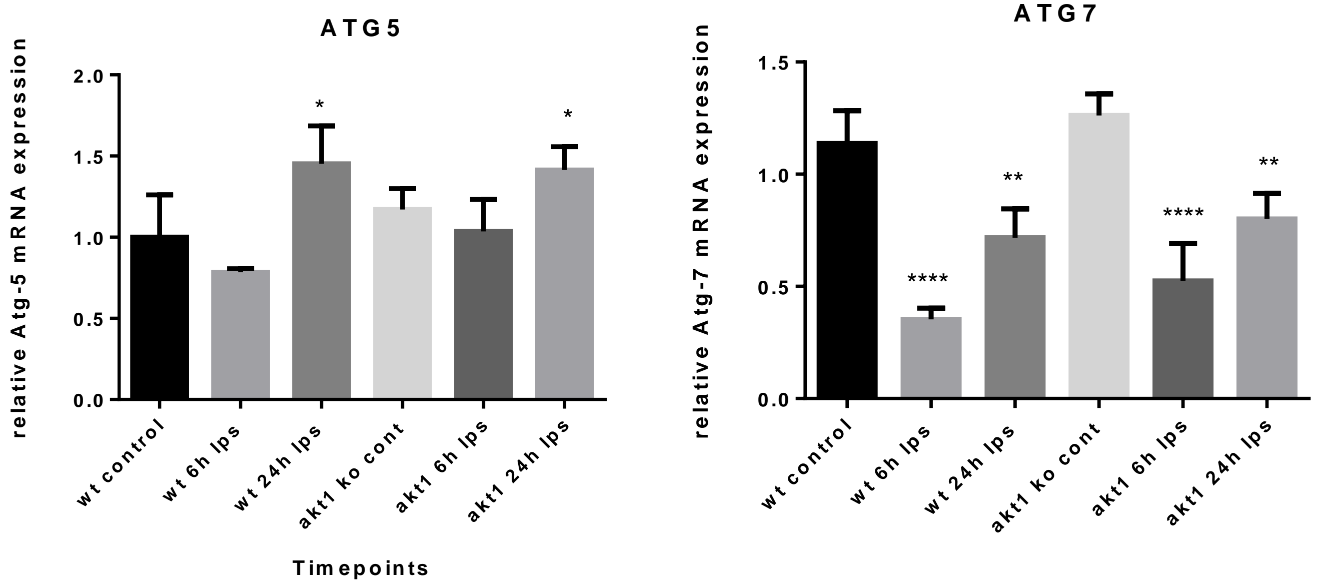

Fig.3.10: The levels of autophagy genes Atg5 and Atg7 for WT and Akt1 ko macrophages for the indicated timepoints were measured by qPCR. The increase on Atg5 mRNA expression was statistically significant after 24h of LPS on both WT and Akt1 ko macrophages while the expression of Atg7 was significantly less both at WT and Akt1 ko cells after 6h and 24h of lps compared to corresponding controls ((* p≤0.05, ** p≤ 0.01, *** p≤0.001, ****p≤0.0001)

The western blots for 2 different pairs of WT and Akt2-/- mice are shown below

Fig.3.11: Macrophages from WT and Akt2-/- mice were treated with LPS (100ng/ml) for 6h, 24h and 48hours. More intense bands of LC3-II expression were detected at basal levels (without lps) in both mice and there was not an important induction among the different

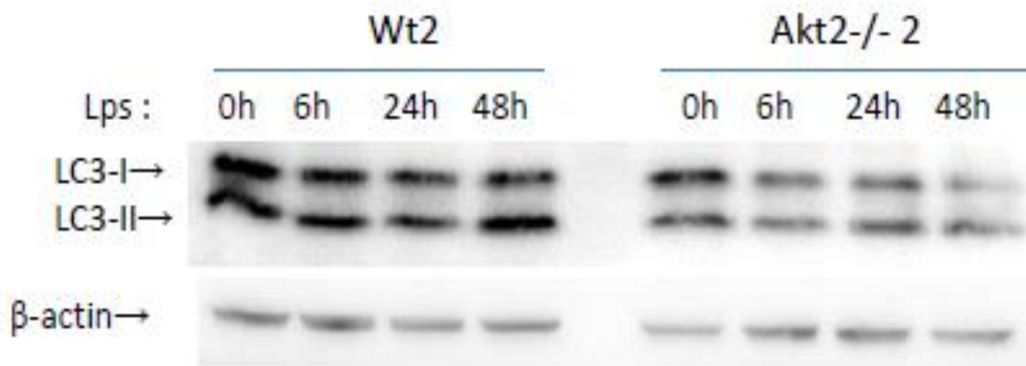

Fig.3.12: Macrophages from another pair of WT and Akt2-/- mice were treated with LPS (100ng/ml) for 6h, 24h and 48hours. Again LC3-II levels did not differ between timepoints in the Akt2-/- mice and in the WT mice, except a slightly more intense band of LC3-II after

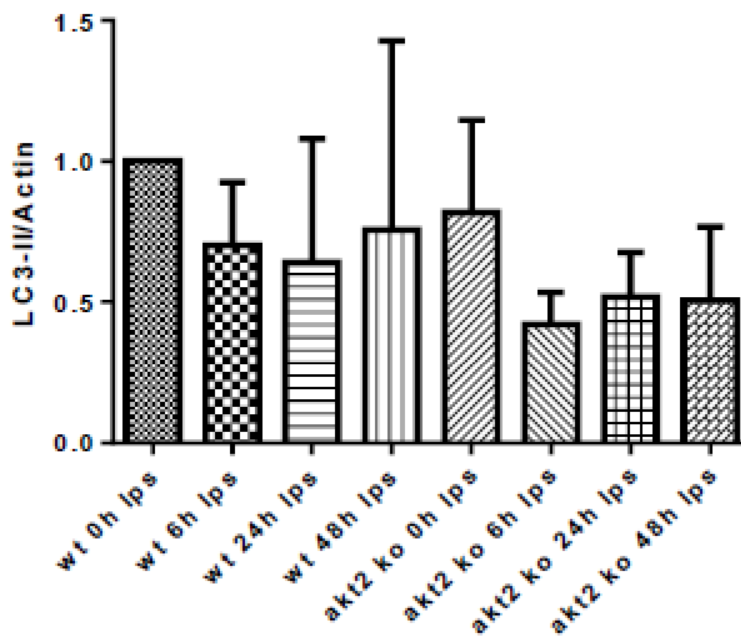

Fig.3.13: LC3-II to Actin ratio for WT and Akt2-/- macrophages

Therefore, from the above immunoblots it is clear that LPS at these timepoints did not induce LC3-II expression and autophagy. Specifically in the case of macrophages from the Akt1-/- mice, LPS treatment reduced the expression of LC3 compared to controls and so did in the WT macrophages,

while in Akt2-/- macrophages LC3 expression appears to be the same between timepoints.

3.4 The effect of IFNγ and IL4 on macrophage's autophagy

As it was previously shown [28], IFNγ promotes the maturation of autophagosomes and their fusion with lysosomes in order to form the autolysosome and this process is peaked after 2hours of stimulation with IFNγ and reaches the peak point after 4hours post-stimulation. This IFNγ-induced autophagy is mediated by JAK1 and 2 kinases but in a pathway independent of STAT1 and Irgm1. Apart from this pathway, IFNγ also induces autophagy through two other important pathways, p38MAPK and PI3K that control some of the crucial components of the autophagic process [28]. Furthermore, autophagy and IFNγ synergistically promote proinflammation, antiviral replication, defense and elimination of microbes such as *Mycobacterium tuberculosis* [29],[8],[19]. In some cases the effect of IFNγ in activation of autophagy can be even stronger than that of starvation. On the other hand, T helper 2 cytokines, such as IL4, inhibits the induction of IFNγ induced or starvation induced autophagy and as a result it favors the survival of mycobacteria in the infected macrophages. IL4 exerts its function by inhibiting starvation-induced autophagy through activation of Akt signaling pathway and the opposing results on autophagy between IFNγ and IL4 indicate the antagonistic effect of Th1 and Th2 cytokines in response to invading pathogens [60].

Moreover, we treated primary peritoneal macrophages from WT and Akt1-/- mice and Raw264.7 macrophages with IFNγ (100ng/ml), IL4(1μg/ml) and LPS (100ng/ml) to compare the LC3-II expression between timepoints and to check whether our results agree with the bibliography. The immunoblots of LC3 expression from the primary macrophages for two different pairs of WT and Akt1-/- mice are shown below.

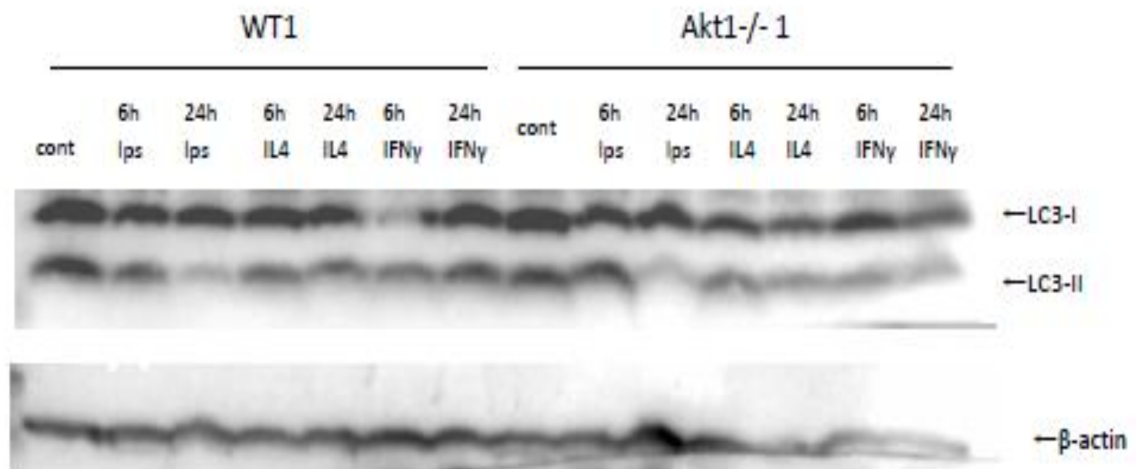

Fig.3.14: LC3 expression on primary macrophages from WT and Akt1 ko mice after the indicated timepoints. LC3-II expression is more intense after 24h of IFNγ stimulation on WT macrophages and not on Akt1 ko ones.

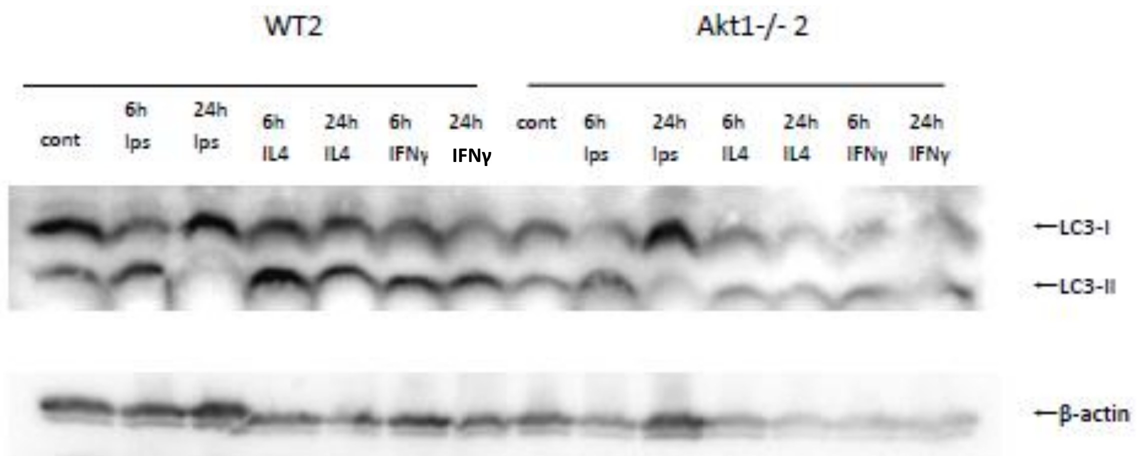

Fig.3.15: LC3 expression on macrophages from another pair of WT and Akt1 ko mice. In this case, LC3-II appears to have greater expression after 6h of IL4 on WT cells and decreasing expression at all timepoints on Akt1 ko cells

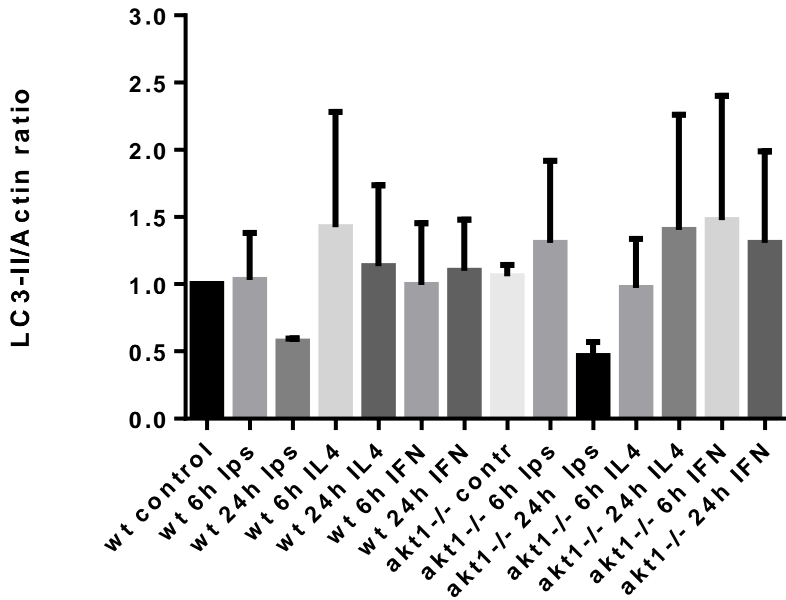

Fig.3.16: Graph representation of LC3 to actin ratio. Almost half decrease in 24h of lps in both WT and Akt1-/- macrophages compared to controls and a small increase after 6h of IL4 for WT and after 24h of IL4 and 6h of IFNγ for Akt1-/- macrophages.

The results from Raw264.7 cells are shown in the immunoblot below

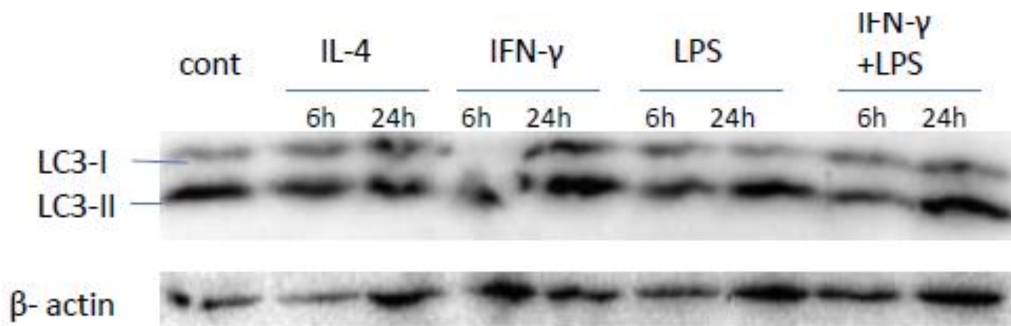

Fig.3.17: LC3 expression on Raw264.7 macrophages More intense expression of LC3-II is observed after 24h of IFNγ stimulation

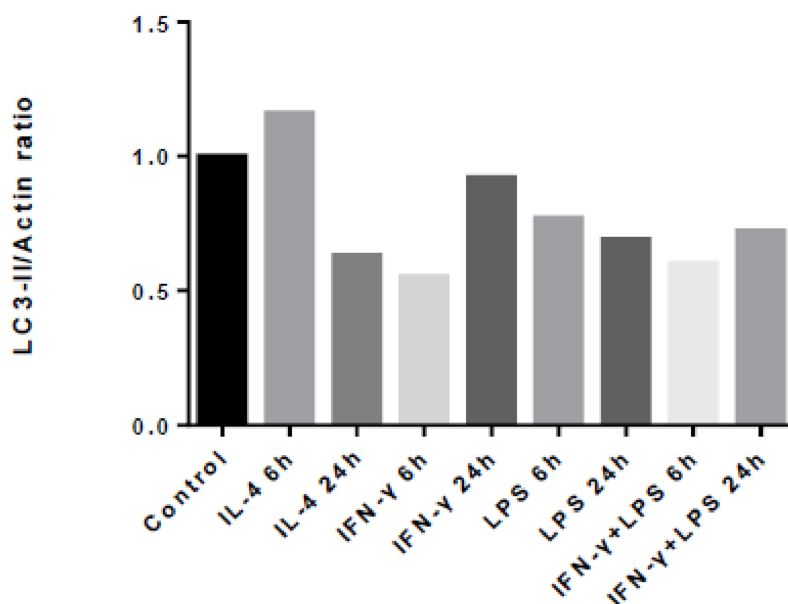

Fig.3.18: LC3-II to actin ratio. Increased expression is observed 6 hours post IL-4 stimulation

Conclusively, our results are somehow different from those reported on bibliography indicating that IFNγ increases autophagy and IL4 decreases it, because the above immunoblots demonstrate a slight increase of LC3-II expression after 24hours of IFNγ on WT1 macrophages that is not greater than control cells (Fig.3.14) and also increased expression after 6h and 24h of IL4 on WT2 (Fig.3.15), something that comes in contrast with reports from other studies.

Next, we performed real-time PCR to estimate the mRNA expression levels of the autophagy genes Atg5 and Atg7 for each pair of WT and Akt1-/- macrophages as it is depicted in the graphs that follow.

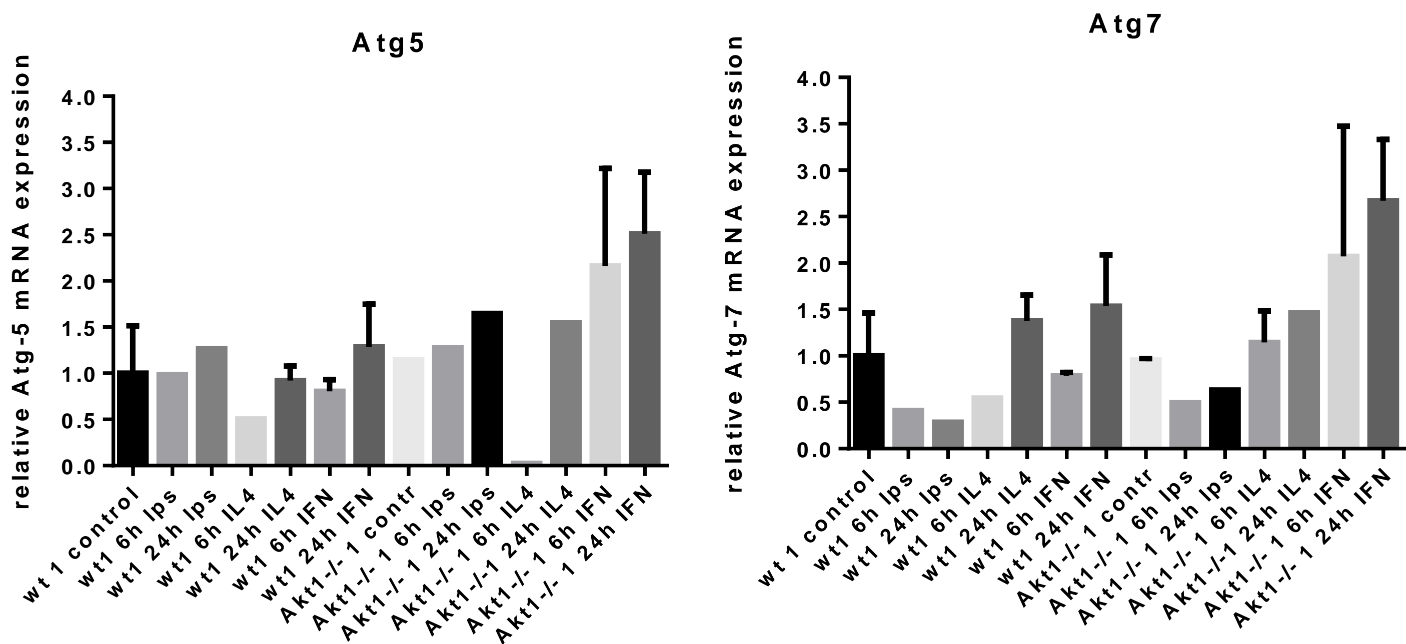

Fig.3.19: mRNA expression levels of Atg5 and Atg7 for WT1 and Akt1-/- macrophages. Increased levels on both genes are observed after 6h and 24h of IFNγ stimulation on Akt1-/- cells and also after 24h of IFNγ on WT cells but differences are not statistically significant

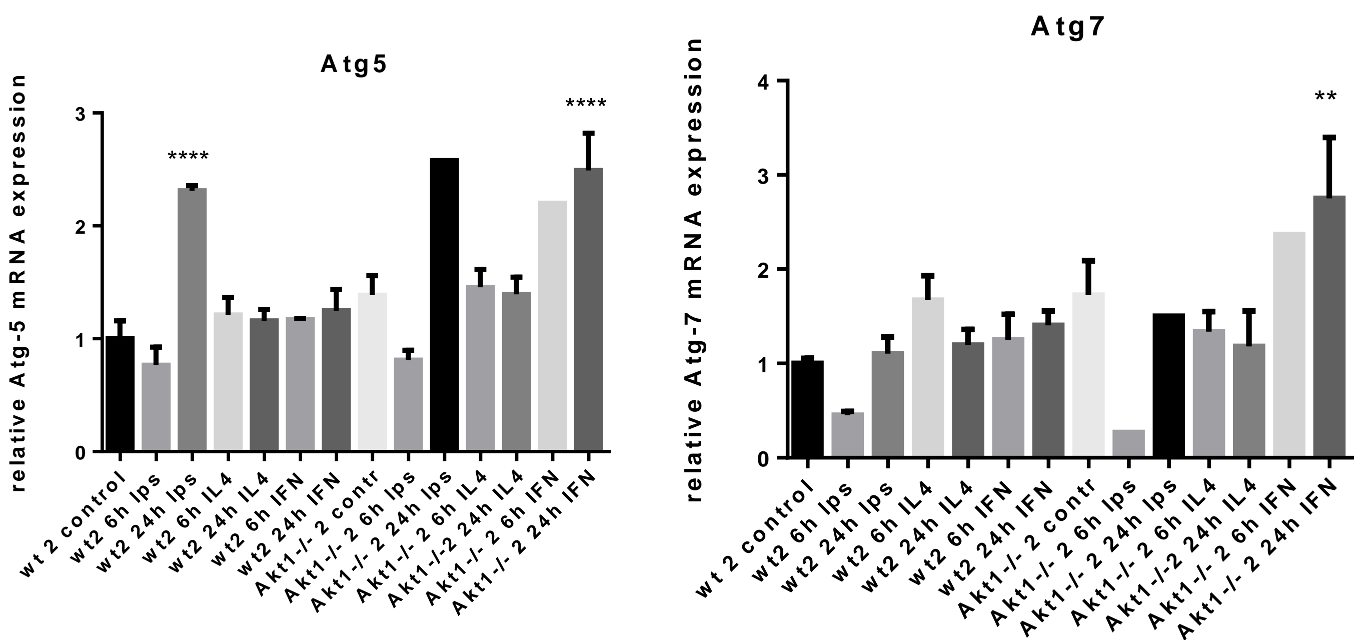

Fig.3.20: Atg5 and Atg7 expression levels for WT2 and Akt1-/- 2 macrophages. Atg5 mRNA expression is significantly higher after 24h of lps on WT2 macrophages and 24h post IFNγ stimulation, while in the case of Atg7, statistically important difference is observed 24h after IFNγ treatment (* p≤0.05, ** p≤ 0.01, *** p≤0.001)

The results of mRNA expression for Atg5 and Atg7 genes from Raw264.7 macrophages are depicted in the diagrams below

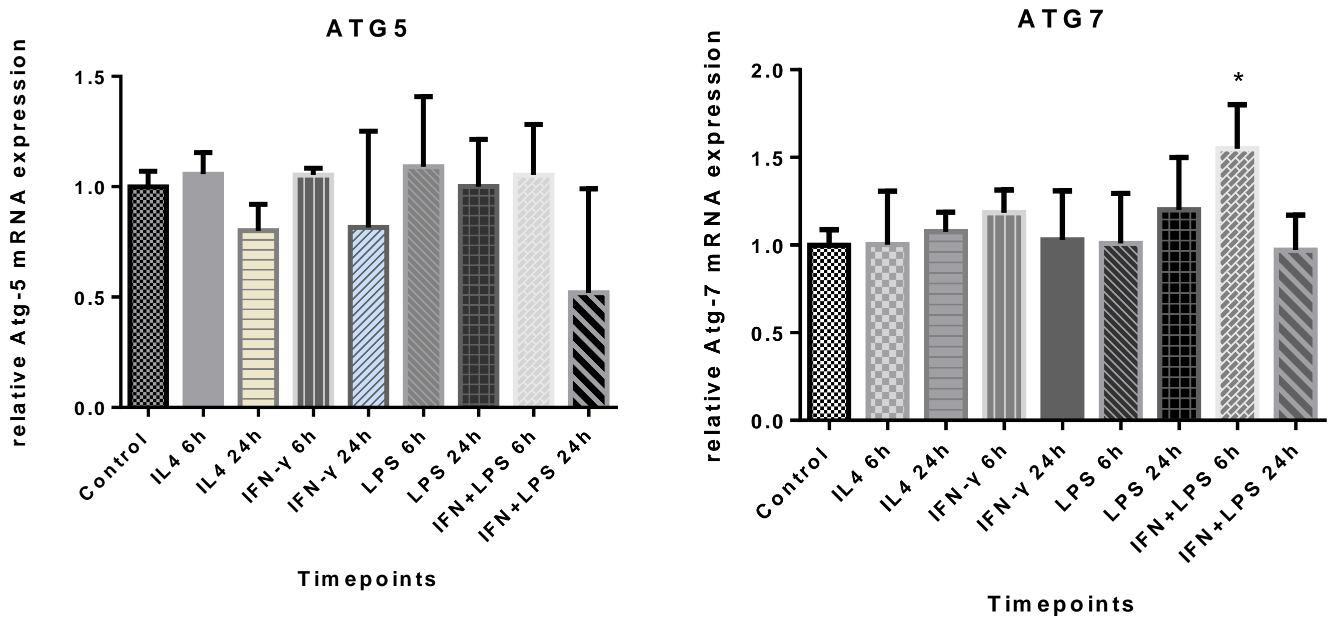

Fig.3.21: Atg5 and Atg7 mRNA expression on Raw264.7 macrophages was measured by qPCR and only after combined treatment with IFNγ and LPS for 6h, the levels of Atg7 expression was significantly higher compared to control cells

Peritoneal macrophages from WT and Akt1-/- mice were also stained with the specific antibody for LC3 (1st ab: a-LC3, 2nd ab: alexa fluor 488 or FITCH a-rabbit) in order to detect with confocal microscopy the levels of LC3 fluorescence on the cells. The images for all the timepoints and the average intensity that corresponds to each treatment are shown below.

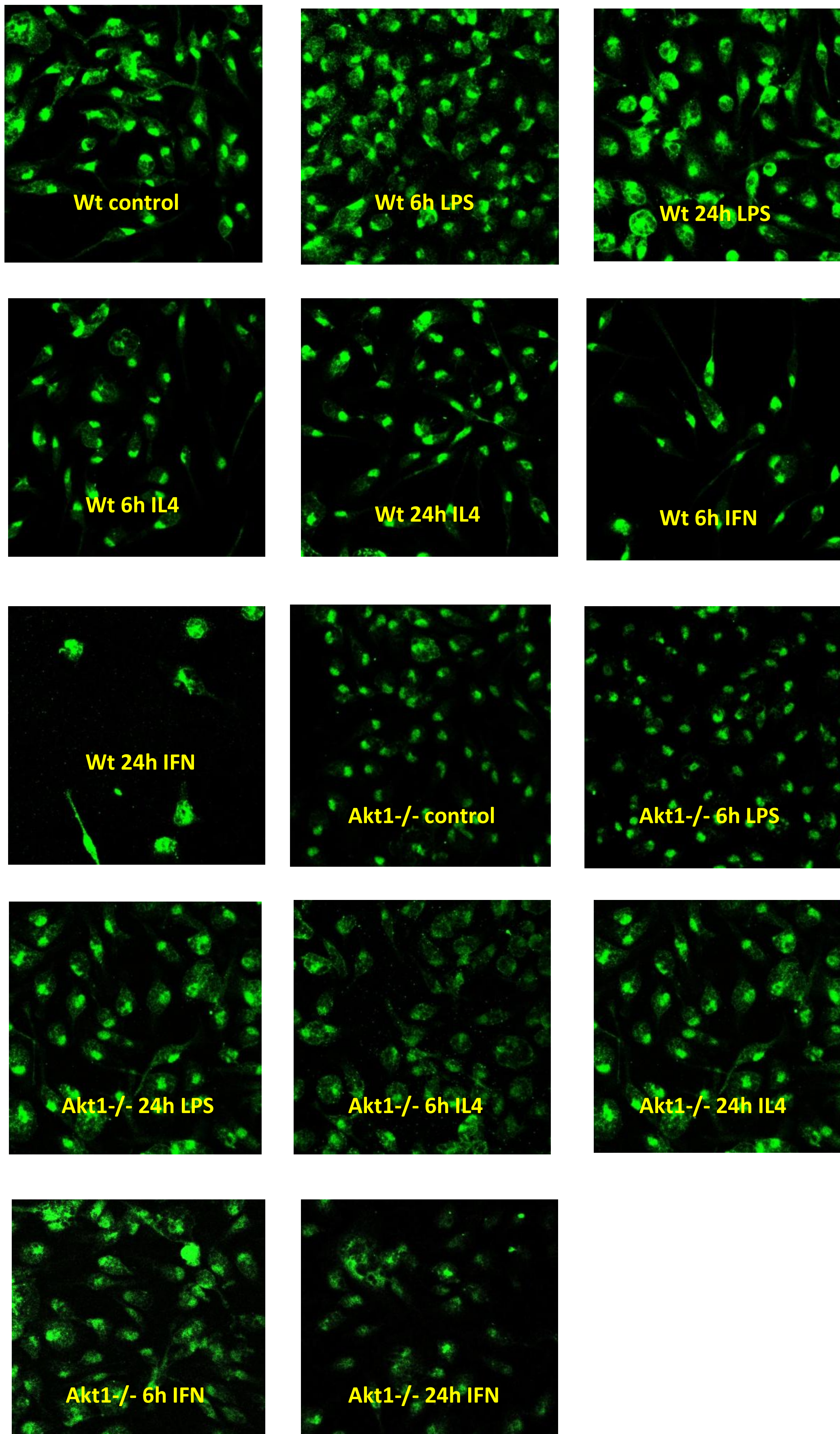

Fig.3.22: Macrophages from WT and Akt1-/- mice were stained for detecting LC3 fluorescence with confocal microscopy (40x magnitude).

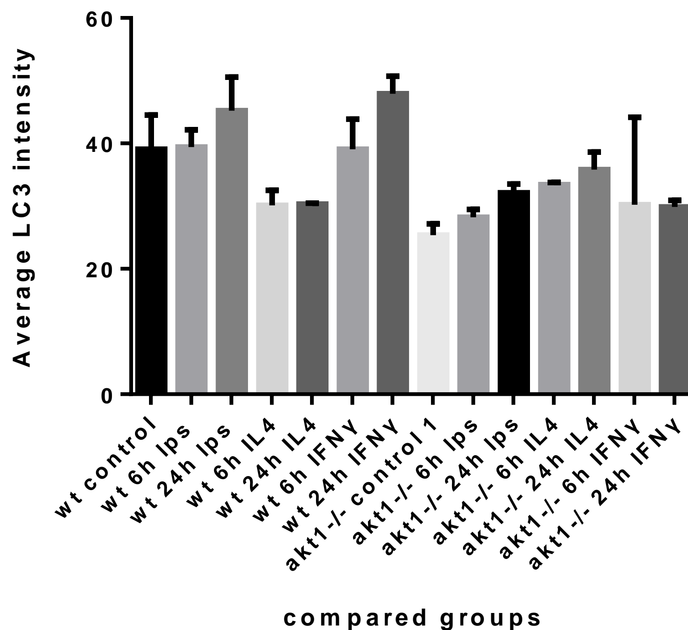

Fig.3.23: Average of LC3 intensity was calculated by measuring 8 different cells from a specific scanned area. 2 different areas from each slide were scanned in each condition

3.5 Prolonged treatment with insulin and rapamycin decreased LC3 expression on Raw 264.7 cells

As it has already been mentioned, mTOR negatively regulates autophagy but rapamycin can reverse this effect and actually induce autophagy. Rapamycin is a macrocyclic antibiotic produced by the bacterium *Streptomyces hygroscopicus* that was found on Easter island and was named after the native name of the island, Rapa Nui. Rapamycin was discovered as antifungal factor and now is very useful clinical drug and a potent autophagy inducer in a variety of cells from yeast to mammals [61],[62]. It has been proposed that rapamycin inhibits mTOR by destabilizing the connection between mTOR and raptor and thus affecting mTORC1 while the other important complex of mTOR, mTORC2 remains unaffected by rapamycin. Furthermore, while in yeast there is a robust

activation of autophagy by rapamycin, the latter affects only partially the autophagic process in mammals [61], [63]. Moreover, in contrast to the effects of rapamycin on autophagy, Naito and colleagues found that insulin inhibited autophagy induction in the muscle of insulin-treated mice and that this inhibition could also be regulated by other pathways apart from mTORC1 pathway in the muscle [64]. Another study supported that the effect of insulin on autophagy depends on the origin of macrophages. More specifically, spleen macrophages from mice treated with insulin showed lower levels of autophagy compared to control cells, M1 macrophages derived from bone marrow had also lower LC3 levels in contrast to M2 macrophages (from diabetic and insulin treated rats) that had higher autophagy levels compared to control group and finally, macrophages from bronchoalveolar lavage of mice treated with insulin showed increased expression of LC3 [65].

Based on the above, we checked the effect of rapamycin (20nM) and insulin (0,1U/ml) on Raw264.7 macrophages as it is depicted in the immunoblot below.

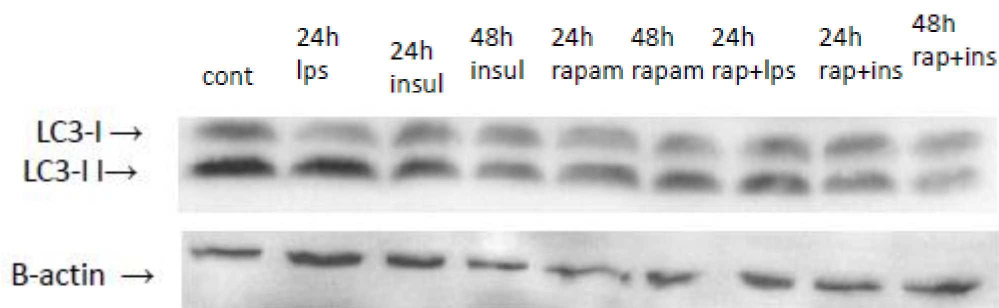

Fig.3.24: LC3 expression on Raw264.7 macrophages after 24h and 48h treatments with lps, rapamycin and insulin. LC3-II expression is more intense at control cells and after 24h of lps , insulin and combined stimulation with rapamycin and lps.

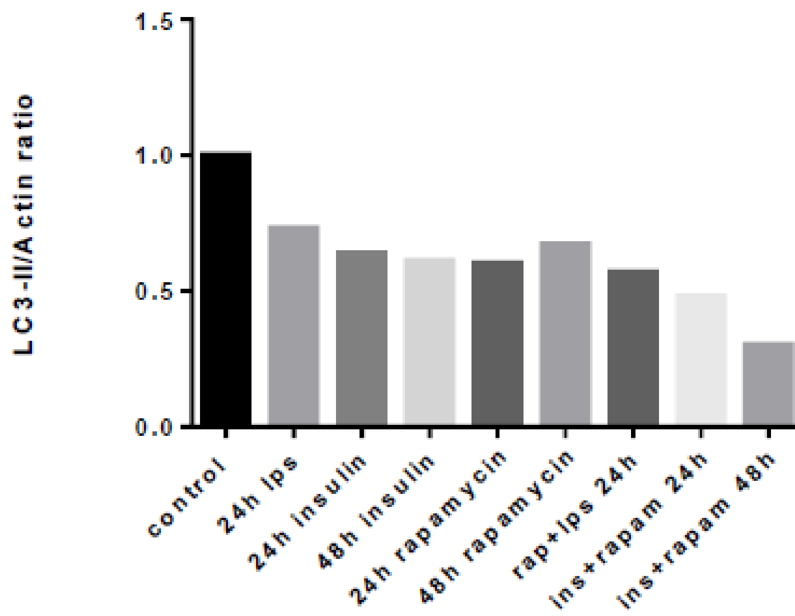

Fig.3.25: Column bars for LC3-II to actin ratio. None of the stimuli managed to increase LC3-II expression above basal levels

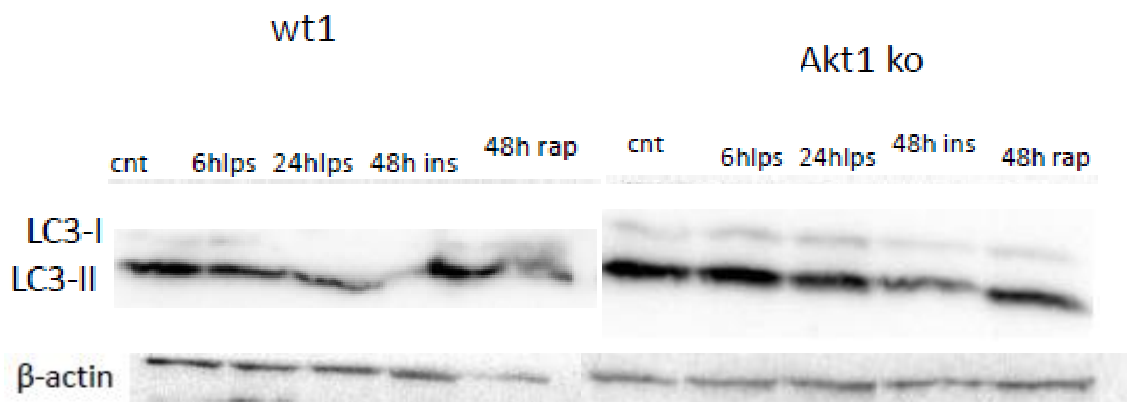

Fig.3.26: LC3-II expression on WT cells was more intense after 48h of insulin in contrast with Akt1-/- cells where increased expression is observed at 6h of lps

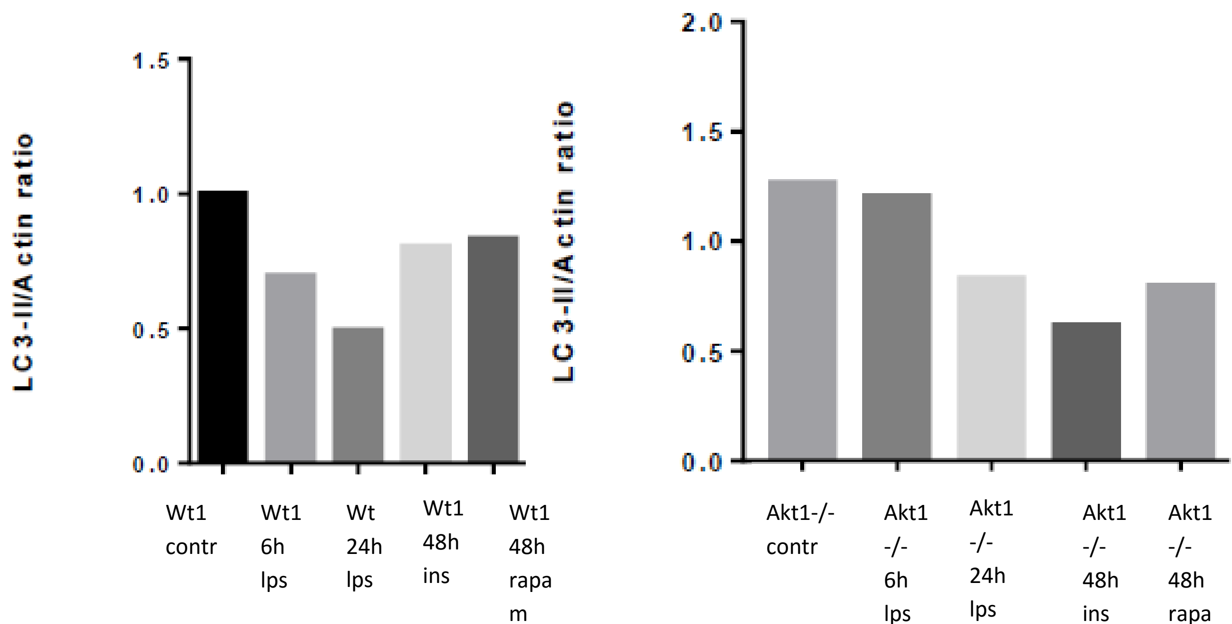

Fig.3.27: LC3-II to actin ratio indicating that none of the treatments increased LC3-II expression above the basal levels

According to our results, rapamycin on Raw cells did not have the anticipated outcome of increasing LC3 expression, instead its levels was decreased compared to control cells and more or less the same happened with WT and Akt1-/- cells. As for the effect of insulin, it also lowered the expression of LC3 but this was consistent with previous reports.

The mRNA expression of Atg5 and Atg7 was analyzed with quantitative real-time PCR and the results are shown in the below diagrams.

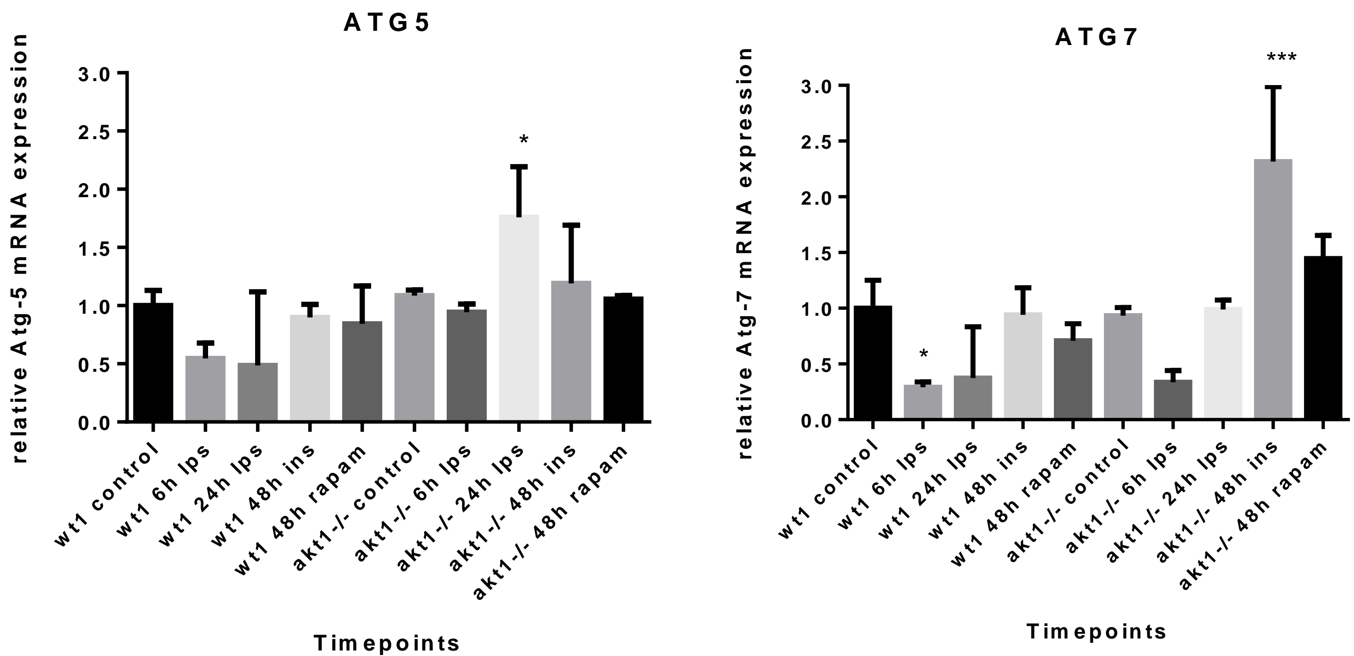

Fig.3.28: The expression of Atg5 was significantly increased after 24h of lps on Akt1-/- macrophages, while the Atg7 mRNA expression levels were decreased more than half compared to control wt cells and significantly higher levels of Atg7 are observed after 48h of insulin (* p≤0.05, ** p≤ 0.01, *** p≤0.001)

Finally, we also checked the effect of LPS stimulation on WT and Akt2-/- macrophages, that were cultured under starvation conditions, the effect of short treatment (30min) of lps and insulin on LC3 expression.

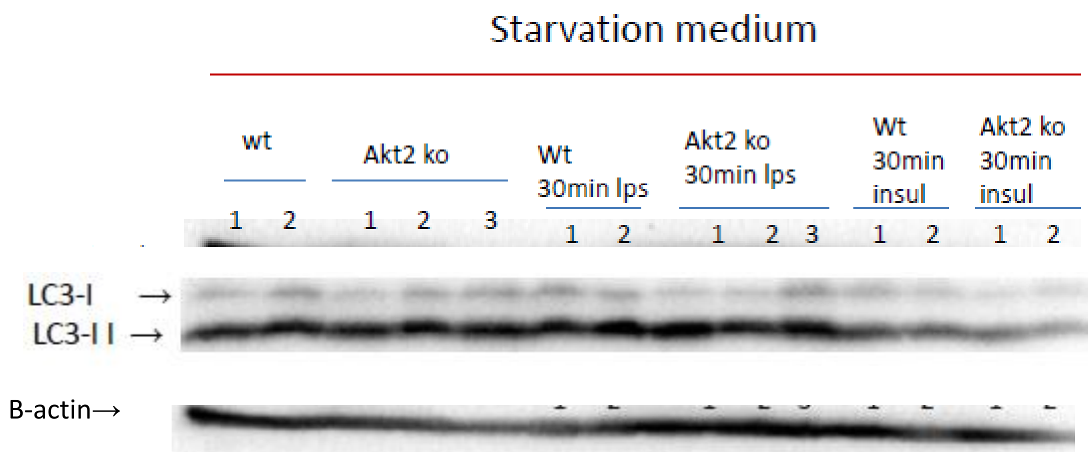

Fig.3.29: LC3-II expression appears to be increased on Wt and Akt2-/- macrophages after 30min of lps treatment and significantly decreased after stimulation with insulin

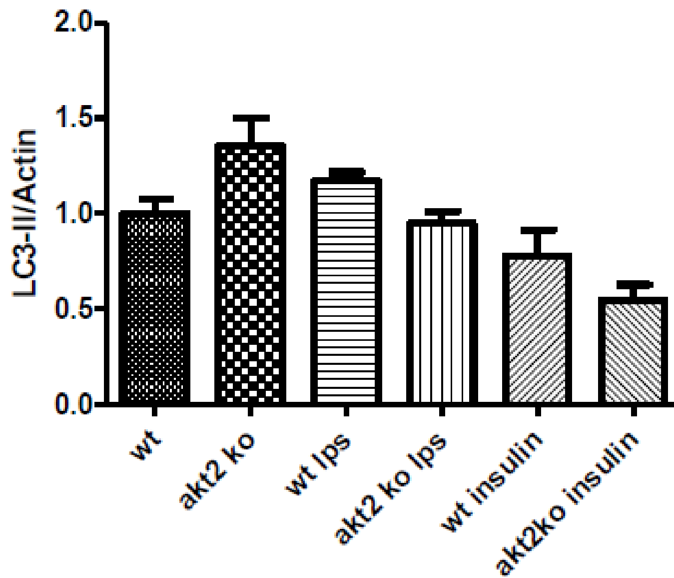

Fig.3.30: LC3-II to actin ratio for WT and Akt2-/- macrophages

3.6 Variability in mRNA expression of the mitophagy marker Pink-1 in response to different stimuli

As it has been mentioned on the introduction, mitophagy is a specific form of autophagy that targets and degrades damaged mitochondria [44], [45]. Pink-1 is a serine/threonine kinase that recruits parkin, which is an E3 ubiquitin ligase and together they give the signal to initiate mitophagy.

In order to check whether the expression of Pink-1 is induced either on primary or Raw264.7 macrophages after stimulation with ligands that some of which are thought to promote autophagy, we performed quantitative real-time PCR and the results are shown in the diagrams that follow.

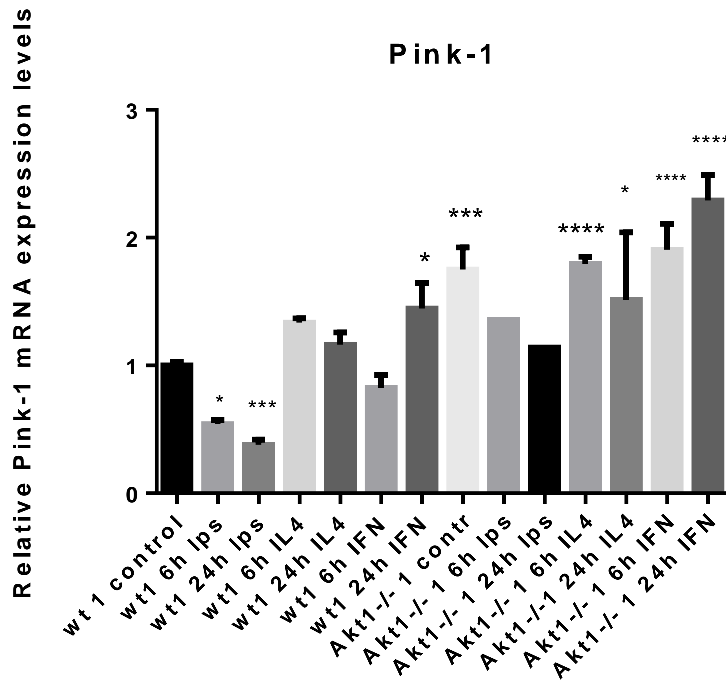

Fig.3.31: Pink-1 mRNA expression was decreased after 6h and 24h of LPS while its levels were significantly elevated at Akt1-/- control cells and after 6h of IL-4 and 6h of IFN and 24h of IFN (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

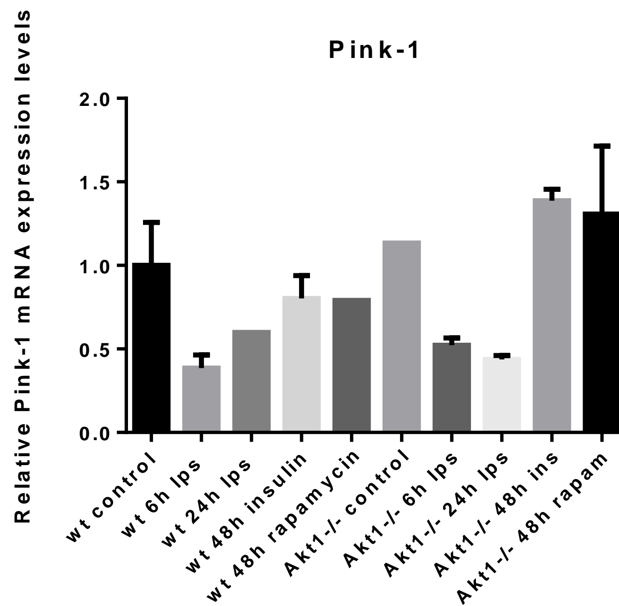

Fig.3.32: In this case, Pink-1 expression was decreased in all timepoints except a small but not significant increase after 48h of insulin and rapamycin on Akt1-/- macrophages

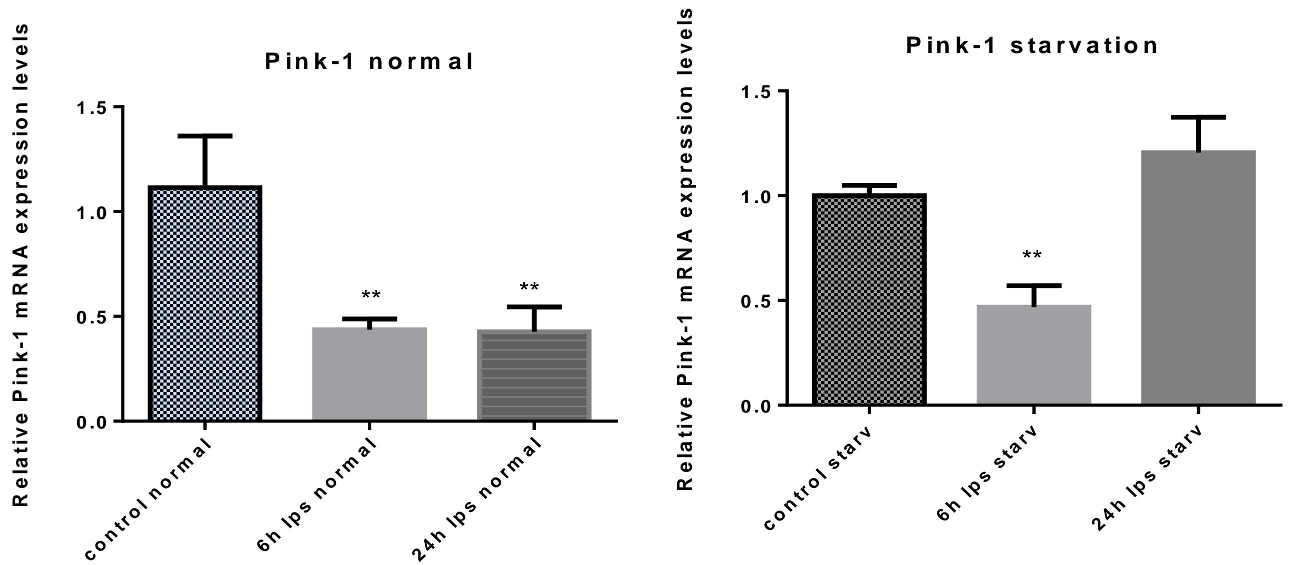

Fig.3.33: In serum containing media, the expression of Pink-1 on Raw264.7 was decreased more than half after 6h and 24h of lps treatment and the same happened after 6h of lps on starvation medium, but 24h post lps stimulation there was a small increase of Pink-1 expression (** $p \leq 0.01$)

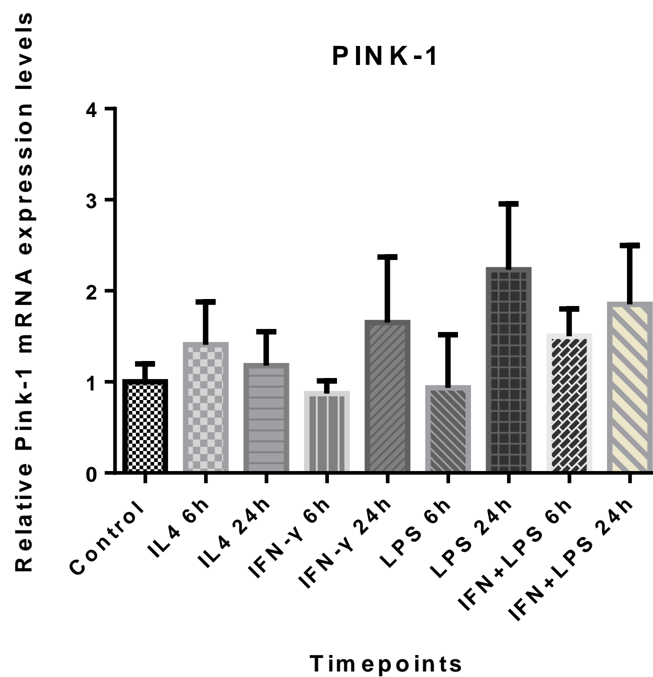

Fig.3.34: Pink-1 mRNA expression on Raw264.7 did not change following 24h of IFNγ and LPS treatment

4. Discussion

Autophagy is a mechanism of self-degradation, in which cell components, organelles and other no longer needed or dysfunctional materials are engulfed in double-membrane vesicles, the autophagosomes, that fuse with the lysosomes and target their content for degradation. Autophagy can be activated by environmental or intracellular signals such as ER stress, starvation and pathogen infection and is considered to be an essential process for maintaing cell survival and homeostasis, because malfunction in the autophagic process can lead to various diseases such as cancer, neurodegenerative disorders and cardiovascular diseases [31]. Furthermore, autophagy is regulated by various signaling pathways depending on the signal such as mTOR pathway in response to nutrients, Ras/PKA pathway, insulin and growth factor pathways and AMPK pathway in response to energy. In general it is a process that ensures a balance between rates of organelle's and protein synthesis and turnover [66].

Macrophages are the most important cells for host defense that contribute to innate immune responses and to pathogen recognition through a variety of recognition receptors for many ligands and pathogens that activate signaling pathways that finally result in their confrontation and elimination and thus to organism protection [56]. Macrophages have the ability to interchange between different states in response to activation by growth factors, cytokines, microbes and other agents. The basic states of macrophage's activation are termed M1 and M2 and were originated in the early 1990s due to the observations that IL4 had different effects on macrophage's status and gene expression in comparison with IFNγ or LPS. The two states had to do with macrophage's transition from inflammation to restoration and healing. More specifically, M1 macrophages mediate the defense against various pathogens and tumors and contribute to chronic inflammatory diseases and autoimmunity, while M2 macrophages, promote resolution of inflammation, tissue repair, are implicated in the defense against parasites but also can promote tumor growth [11], [38].

Macrophages utilize autophagy in order to fight against pathogens such as *Mycobacterium tuberculosis* and *Salmonella enteric*. Autophagy is induced after recognition of these pathogens through specific receptors on macrophages, the PRR (pattern recognition receptors), and is thought to have protective role and to promote cell survival not only by eliminating those pathogens but also during adverse conditions for the cell [14]. During such conditions, like starvation, autophagy provides amino acids and other metabolic substrates necessary for cell survival by degrading organelles, misfolded proteins or other cytoplasmic material [15],[16].

In this study, we showed that LPS treatment alone decreased LC3 levels at all time points studied (30min, 6h, 24h and 48h) in both WT peritoneal macrophages and Raw264.7 macrophages. Our conclusions come in contrast with a study indicating that LPS treatment of Raw264.7 cells increased the LC3-II levels and thus the formation of autophagosomes as it was obvious from their punctuate staining [67]. These results was consistent with the ones conducted by Waltz and colleagues who found that LPS demonstrated the strongest induction in LC3 expression compared to other TLR ligands tested and also increased LC3 proteins levels on peritoneal macrophages from HeOuJ mice [30]. Another group of researchers confirmed the LPS-induced LC3-II expression on peritoneal mesothelial cells (HMrSV5) in a dose dependent and time dependent way [57].

In our peritoneal macrophages isolated from Akt1-/- mice, we found that LPS decreased LC3 levels compared to controls, while in Akt2-/- macrophages, its expression was similar between timepoints, although one would expect an induction in autophagy in these types of macrophages due to dysfunctional mTOR pathway. In Raw cells cultured on starvation medium, the presence of LPS did not manage to elevate LC3 levels despite studies indicating that upon starvation, autophagy is activated in order to provide the cell with all the necessary factors for its survival. The mRNA levels of Atg5 and Atg7 were increased after 24h of lps on starved cells while in Akt1-/- and WT primary macrophages only Atg5 was increased after 24h of LPS and Atg7 was significantly less at this timepoint.

IFNγ is a known inducer of autophagy because it promotes the maturation of autophagosomes and their fusion with lysosomes while IL4 inhibits autophagy by activating Akt signaling pathway [28],[29],[60]. Our results demonstrated slight increase in LC3 levels after 24h of IFNγ on the one WT mouse (WT1) and also a small induction in LC3 after 6h and 24h of IL4 on the other WT mouse (WT2) but their levels did not overcome the ones of the corresponding controls. Furthermore, significantly higher mRNA levels were observed for both Atg5 and Atg7 after 24h of IFNγ on Akt1-/- 2 macrophages. Finally, treatments of Raw264.7 macrophages with rapamycin and insulin did not enhance LC3 levels something that was in agreement with previous studies in the case of insulin but not for rapamycin that is considered an mTOR inhibitor and thus an inducer of autophagy.

As far as mitophagy is concerned, we studied the mRNA expression levels of Pink-1, the serine-threonine kinase that accumulates on dysfunctional mitochondria and its function is required for recruiting Parkin which in turn ubiquitinates outer membrane mitochondrial proteins to initiate mitophagy [68]. Our results indicate a statistically significant increase on Pink-1 mRNA expression levels on Akt1-/- control macrophages and after 6h and 24h of treatment with IL-4 and IFNγ. Furthermore, increased but not significant levels were observed 48h post insulin and rapamycin treatment on Akt1-/- macrophages. Raw264.7 cells on serum containing media, had a more than half decrease on Pink-1 expression after 6h and 24h of LPS treatment compared to untreated cells and decreased expression of Pink-1 was also noticed on starved cells after 6h of LPS. Finally, IFNγ and IL-4 did not induce Pink-1 mRNA expression to a significant extent on Raw cells.

Future perspectives

Autophagy is a fundamental process for the survival of the cell not only because it degrades no longer needed cell components but also due to its importance as a defence mechanism against invading pathogens and its contribution to innate immunity against intracellular bacteria. However, many pathogens exploit components of the autophagic pathway for their benefit and in order to replicate. Future studies could deal with the molecular mechanisms that some pathogens use in order to avoid autophagy and how this can be prevented by specific drugs that induce autophagy. The field of drug discovery that aims at any stage of the autophagic pathway by either inducing or inhibiting autophagy would be really promising for the control and maybe treatment of inflammatory diseases. Moreover, other TLR ligands except from LPS, such as imiquimod, ssRNA or CpG oligonucleotides could be used alone or in combination with mTOR inhibitors that act as autophagy inducers like rapamycin, everolimus and metformin in macrophage cell lines or primary macrophages in order to examine their effects on LC3 expression and autophagy. Furthermore, we noticed that in both Akt1-/- macrophages and Akt2-/- macrophages there wasn't a substantial difference on LC3-II levels compared to WT macrophages and this may be due to the fact that the presence of the one Akt kinase compensates for the loss of the other. For this reason, double knock out macrophages that lack both Akt1 and Akt2 kinases could be used to check whether they express LC3. Alternatively, the function of Akt kinases could be inhibited by Akt inhibitors either ATP-competitive inhibitors (GSK690693,GDC-0068) or allosteric pan-Akt inhibitors such as MK-2206 and perifosine that targets the plextrin domain of Akt and thus preventing its translocation to the plasma membrane that is required for activation. Conclusively, except for the effect of these inhibitors on autophagy, the fact that PI3K-Akt pathway is also involved in other cell functions such as cell growth, migration and survival makes them promising drug candidates for cancer immunotherapy.

5. Acknowledgements

This project was the result of team work among all members of Clinical Chemistry Laboratory and it couldn't be accomplished without the excellent collaboration we had.

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References

1. Levine, B. and G. Kroemer, *Autophagy in the pathogenesis of disease.* Cell, 2008. **132**(1): p. 27-42.
2. Deretic, V., T. Saitoh, and S. Akira, *Autophagy in infection, inflammation and immunity.* Nat Rev Immunol, 2013. **13**(10): p. 722-37.
3. Cecconi, F. and B. Levine, *The role of autophagy in mammalian development: cell makeover rather than cell death.* Dev Cell, 2008. **15**(3): p. 344-57.
4. Schmid, D. and C. Munz, *Innate and adaptive immunity through autophagy.* Immunity, 2007. **27**(1): p. 11-21.
5. Das, G., B.V. Shravage, and E.H. Baehrecke, *Regulation and function of autophagy during cell survival and cell death.* Cold Spring Harb Perspect Biol, 2012. **4**(6).
6. Mizushima, N., T. Yoshimori, and B. Levine, *Methods in mammalian autophagy research.* Cell, 2010. **140**(3): p. 313-26.
7. Levine, B. and V. Deretic, *Unveiling the roles of autophagy in innate and adaptive immunity.* Nat Rev Immunol, 2007. **7**(10): p. 767-77.
8. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages.* Cell, 2004. **119**(6): p. 753-66.
9. Levine, B., N. Mizushima, and H.W. Virgin, *Autophagy in immunity and inflammation.* Nature, 2011. **469**(7330): p. 323-35.
10. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
11. Weichhart, T., M. Hengstschlager, and M. Linke, *Regulation of innate immune cell function by mTOR.* Nat Rev Immunol, 2015. **15**(10): p. 599-614.
12. Ghesquiere, B., et al., *Metabolism of stromal and immune cells in health and disease.* Nature, 2014. **511**(7508): p. 167-76.
13. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
14. Mihalache, C.C. and H.U. Simon, *Autophagy regulation in macrophages and neutrophils.* Exp Cell Res, 2012. **318**(11): p. 1187-92.
15. Moreau, K., S. Luo, and D.C. Rubinsztein, *Cytoprotective roles for autophagy.* Curr Opin Cell Biol, 2010. **22**(2): p. 206-11.
16. Boya, P., et al., *Inhibition of macroautophagy triggers apoptosis.* Mol Cell Biol, 2005. **25**(3): p. 1025-40.
17. Lum, J.J., et al., *Growth factor regulation of autophagy and cell survival in the absence of apoptosis.* Cell, 2005. **120**(2): p. 237-48.
18. Codogno, P., M. Mehrpour, and T. Proikas-Cezanne, *Canonical and non-canonical autophagy: variations on a common theme of self-eating?* Nat Rev Mol Cell Biol, 2012. **13**(1): p. 7-12.
19. Delgado, M.A., et al., *Toll-like receptors control autophagy.* EMBO J, 2008. **27**(7): p. 1110-21.
20. Delgado, M.A. and V. Deretic, *Toll-like receptors in control of immunological autophagy.* Cell Death Differ, 2009. **16**(7): p. 976-83.

21. Jung, C.H., et al., *mTOR regulation of autophagy.* FEBS Lett, 2010. **584**(7): p. 1287-95.
22. Russell, R.C., H.X. Yuan, and K.L. Guan, *Autophagy regulation by nutrient signaling.* Cell Res, 2014. **24**(1): p. 42-57.
23. Egan, D.F., et al., *Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy.* Science, 2011. **331**(6016): p. 456-61.
24. Noguchi, M., N. Hirata, and F. Suizu, *The links between AKT and two intracellular proteolytic cascades: ubiquitination and autophagy.* Biochim Biophys Acta, 2014. **1846**(2): p. 342-52.
25. Deretic, V., *Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors.* Curr Opin Immunol, 2012. **24**(1): p. 21-31.
26. Zhai, C., et al., *Selective inhibition of PI3K/Akt/mTOR signaling pathway regulates autophagy of macrophage and vulnerability of atherosclerotic plaque.* PLoS One, 2014. **9**(3): p. e90563.
27. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): p. 577-90.
28. Matsuzawa, T., et al., *IFN-gamma elicits macrophage autophagy via the p38 MAPK signaling pathway.* J Immunol, 2012. **189**(2): p. 813-8.
29. Chang, Y.P., et al., *Autophagy facilitates IFN-gamma-induced Jak2-STAT1 activation and cellular inflammation.* J Biol Chem, 2010. **285**(37): p. 28715-22.
30. Waltz, P., et al., *Lipopolysaccaride induces autophagic signaling in macrophages via a TLR4, heme oxygenase-1 dependent pathway.* Autophagy, 2011. **7**(3): p. 315-20.
31. He, C. and D.J. Klionsky, *Regulation mechanisms and signaling pathways of autophagy.* Annu Rev Genet, 2009. **43**: p. 67-93.
32. Polager, S., M. Ofir, and D. Ginsberg, *E2F1 regulates autophagy and the transcription of autophagy genes.* Oncogene, 2008. **27**(35): p. 4860-4.
33. Artal-Martinez de Narvajas, A., et al., *Epigenetic regulation of autophagy by the methyltransferase G9a.* Mol Cell Biol, 2013. **33**(20): p. 3983-93.
34. Biswas, S.K. and E. Lopez-Collazo, *Endotoxin tolerance: new mechanisms, molecules and clinical significance.* Trends Immunol, 2009. **30**(10): p. 475-87.
35. West, M.A. and W. Heagy, *Endotoxin tolerance: a review.* Crit Care Med, 2002. **30**(1 Suppl): p. S64-73.
36. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-8.
37. Mages, J., H. Dietrich, and R. Lang, *A genome-wide analysis of LPS tolerance in macrophages.* Immunobiology, 2007. **212**(9-10): p. 723-37.
38. Murray, P.J., et al., *Macrophage activation and polarization: nomenclature and experimental guidelines.* Immunity, 2014. **41**(1): p. 14-20.

39. Baltimore, D., et al., *MicroRNAs: new regulators of immune cell development and function.* Nat Immunol, 2008. **9**(8): p. 839-45.
40. Arranz, A., et al., *Vasoactive intestinal peptide suppresses toll-like receptor 4 expression in macrophages via Akt1 reducing their responsiveness to lipopolysaccharide.* Mol Immunol, 2008. **45**(10): p. 2970-80.
41. Maurer, K., et al., *Autophagy mediates tolerance to Staphylococcus aureus alpha-toxin.* Cell Host Microbe, 2015. **17**(4): p. 429-40.
42. Hu, G., et al., *PI3K signaling of autophagy is required for starvation tolerance and virulenceof Cryptococcus neoformans.* J Clin Invest, 2008. **118**(3): p. 1186-97.
43. Androulidaki, A., et al., *The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs.* Immunity, 2009. **31**(2): p. 220-31.
44. Youle, R.J. and D.P. Narendra, *Mechanisms of mitophagy.* Nat Rev Mol Cell Biol, 2011. **12**(1): p. 9-14.
45. Kim, I., S. Rodriguez-Enriquez, and J.J. Lemasters, *Selective degradation of mitochondria by mitophagy.* Arch Biochem Biophys, 2007. **462**(2): p. 245-53.
46. Narendra, D.P., et al., *PINK1 is selectively stabilized on impaired mitochondria to activate Parkin.* PLoS Biol, 2010. **8**(1): p. e1000298.
47. Green, D.R., L. Galluzzi, and G. Kroemer, *Mitochondria and the autophagy-inflammation-cell death axis in organismal aging.* Science, 2011. **333**(6046): p. 1109-12.
48. Zhao, W., et al., *Atg5 deficiency-mediated mitophagy aggravates cardiac inflammation and injury in response to angiotensin II.* Free Radic Biol Med, 2014. **69**: p. 108-15.
49. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-55.
50. Zhang, Y., et al., *Kinase AKT controls innate immune cell development and function.* Immunology, 2013. **140**(2): p. 143-52.
51. Lee, Y.G., et al., *Functional role of Akt in macrophage-mediated innate immunity.* Front Biosci (Landmark Ed), 2011. **16**: p. 517-30.
52. Zhang, B., et al., *Akt2 is required for macrophage chemotaxis.* Eur J Immunol, 2009. **39**(3): p. 894-901.
53. Garofalo, R.S., et al., *Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKBβ.* Journal of Clinical Investigation, 2003. **112**(2): p. 197-208.
54. Cho, H., et al., *Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta).* Science, 2001. **292**(5522): p. 1728-31.
55. Shao, J., et al., *Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Leprdb/db mice.* J Endocrinol, 2000. **167**(1): p. 107-15.
56. Vural, A. and J.H. Kehrl, *Autophagy in macrophages: impacting inflammation and bacterial infection.* Scientifica (Cairo), 2014. **2014**: p. 825463.
57. Wang J, F.X., Zeng Y, Fan J, Wu J, Li Z, Liu X, Huang R, Huang F, Yu X, Yang X, *Lipopolysaccharide (LPS)-induced autophagy is*

involved in the restriction of Escherichia coli in peritoneal mesothelial cells. BMC microbiology, 2013.

58. Shang, L., et al., *Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK.* Proc Natl Acad Sci U S A, 2011. **108**(12): p. 4788-93.
59. Karim, M.R., H. Kawanago, and M. Kadowaki, *A quick signal of starvation induced autophagy: transcription versus post-translational modification of LC3.* Anal Biochem, 2014. **465**: p. 28-34.
60. Harris, J., et al., *T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis.* Immunity, 2007. **27**(3): p. 505-17.
61. Ballou, L.M. and R.Z. Lin, *Rapamycin and mTOR kinase inhibitors.* J Chem Biol, 2008. **1**(1-4): p. 27-36.
62. Sarkar, S., et al., *Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies.* Cell Death Differ, 2009. **16**(1): p. 46-56.
63. Thoreen, C.C., et al., *An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1.* J Biol Chem, 2009. **284**(12): p. 8023-32.
64. Naito, T., A. Kuma, and N. Mizushima, *Differential contribution of insulin and amino acids to the mTORC1-autophagy pathway in the liver and muscle.* J Biol Chem, 2013. **288**(29): p. 21074-81.
65. Sunahara, K.K., et al., *Insulin influences autophagy response distinctively in macrophages of different compartments.* Cell Physiol Biochem, 2014. **34**(6): p. 2017-26.
66. Klionsky, D.J., *Autophagy as a Regulated Pathway of Cellular Degradation.* Science, 2000. **290**(5497): p. 1717-1721.
67. Xu, Y., et al., *Toll-like receptor 4 is a sensor for autophagy associated with innate immunity.* Immunity, 2007. **27**(1): p. 135-44.
68. Pickrell, A.M. and R.J. Youle, *The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease.* Neuron, 2015. **85**(2): p. 257-73.