AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT OF DIFFERENT SELECTIVE INHIBITORS OF AUTOPHAGY ON INFLAMMATION IN MACROPHAGES

by
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AN ABSTRACT OF THE THESIS OF

Ghina Farouk Tleys for Master of Biomedical sciences
Major: Biochemistry

Title: Assessment of different selective inhibitors of autophagy on inflammation in macrophages

Autophagy is a self-eating process responsible for the sequestering and lysosomal degradation of damaged organelles and proteins in response to stress. Accumulating evidence indicates that defects in autophagy lead to increased inflammatory responses. Statins are hydroxy-3-methylglutaryl-coenzyme A or HMG Co-A reductase inhibitors that have many beneficial effects on cells including anti-inflammatory and anti-oxidant roles. Intensive studies have shown that autophagy is involved in different inflammatory conditions such as infectious diseases, neurodegenerative disorders, and cancer. Our goal is to investigate the mechanisms involved in the anti-inflammatory effects of Rho kinase (ROCK) specific inhibitor Y27632. We aimed to develop in culture a cellular system of macrophages where autophagy is diminished using potential autophagy inhibitors.

Methods. Murine macrophages were prepared from bone marrow of C57BL/6j mice activated with LPS. Enzyme linked immunosorbent assay (ELISA) was used to determine the levels of proinflammatory cytokines, IL-6 and TNF-α. Prostaglandin E_2 (PGE_2) was measured using enzyme linked immunoassay (EIA). Western blotting was performed to detect the expression of LC3, a known autophagy marker. Quantitative polymerase chain reaction (qPCR) was conducted to detect the expression of anti-inflammatory genes.

Results. Bone marrow derived macrophages (BMDM) were prepared from C57BL/6j mice and tested for their response. Lipopolysaccharide (LPS) increased IL-6 and TNF-α by 132 and 200-fold, respectively, compared to the control ($p<0.05$). Under these conditions, inhibitors of ULK (unc-51-like) 1/2, an important kinase in the initiation steps of macroautophagy decreased the levels of proinflammatory cytokines in response to LPS. These data suggest that inhibition of ULK in BMDM has an additional effect to the

inhibition of autophagy. In contrast, inhibitors of VPS34, a PI3K-III isoform important in the phagophore formation, showed an increase in IL-6 by 7.6-fold and TNF-α by 7.8-fold compared to LPS ($p<0.05$, unpaired t-test). LC3 shift to the lipidated form (LC3-II) was diminished using these inhibitors, supporting an effective inhibition of autophagy. These molecules were further used to test the effect of any anti-inflammatory drugs including atorvastatin, a hypocholesterolemic drug, and inhibitors of RhoA/C -activated kinase, Y27632. Pretreatment of cells with 10 µM atorvastatin or 10 µM Y27632 did not significantly inhibited IL-6, TNF-α and PGE_2 formation in response to LPS. However, pretreatment of BMDM with VPS inhibitors prior to the addition of Y27632 and LPS resulted in a strong inhibitory effect of the ROCK inhibitor compared to LPS.

Conclusion. The identification of potential inhibitors of autophagy constitutes a crucial step for the analysis of the mechanistic behind anti-inflammatory effects. This will allow us to investigate the implication of autophagy in different inflammatory contexts.

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CHAPTER ONE

INTRODUCTION

A. Inflammation

1. General aspects

Inflammation is a physiological process that occurs in response to various harmful stimuli such as pathogens, physical injury or chemicals leading to body damage. Inflammation occurs immediately to defend the body by initiating a healing process to restore tissue homeostasis. Upon injury or infection, an acute inflammatory response is activated and a panel of molecules recognized by cellular sensors is triggered contributing to the recruitment of mediators acting on specific target tissues. Resolution of inflammation occurs at the end of this highly regulated process and tissue homeostasis is successfully restored. However, disturbance or prolongation of the inflammatory response can prevent the resolution of inflammation resulting in chronic inflammatory state (Medzhitov 2008, Gordon and Martinez-Pomares 2017).

Inflammatory reactions involve many cell types involved both in innate and immune response. Acute and chronic inflammation differs by many factors like the period and the type of infiltrating cells. Acute inflammation is characterized by a short-term response resulting in healing mediated by the recruitment of leukocytes to the damaged region for repair. This process of leukocyte migration involves different steps to reach the healing process. The initial step in leukocyte migration is the formation of interactions

between leukocytes and endothelial cells of venules that are close to damaged tissues. This leads to the stimulation of leukocytes by the different players present on the luminal side of blood vessels priming the process of leukocyte migration that starts with rolling, followed by adhesion of leukocytes mediated by endothelial cells adhesion molecules such as intercellular adhesion molecule-I (ICAM-1) and vascular adhesion molecule (VCAM-1), and finally leukocytes can breach the endothelial wall and reach the target tissues in a process referred to as diapedesis. This series of sequential and overlying events is mediated by two major adhesion receptor families: selectins (E-selectin and P-selectin) and integrins (ICAM-1 and VCAM-1) (Nourshargh and Alon 2014) (Figure 1).

In some cases, if the resolution of inflammation cannot occur for any reason this leads to a persistent inflammatory state known as chronic inflammation. Many players control the resolution of inflammation including neutrophils and macrophages. Neutrophils are the most abundant type of white blood cells that constitute 60-70% of the circulating leukocytes (Nourshargh and Alon 2014). Neutrophils are the first cells to participate in the early stages of acute inflammation. The life span of a circulating human neutrophil is about 5.4 days (Pillay, den Braber et al. 2010).

Then neutrophils die by apoptosis and are cleared by macrophages in a process known as efferocytosis. When macrophages uptake apoptotic neutrophils this contributes to a resolving phenotype mediated by macrophages and restoring tissue homeostasis (Ortega-Gomez, Perretti et al. 2013)**.** If any disturbance occurs, this can prevent the resolution phase and lead to chronic inflammation characterized by prolonged period and the additional recruitment of macrophages to the site of injury and interaction with different molecules

dictating the fate of macrophages being either proinflammatory or anti-inflammatory macrophages.

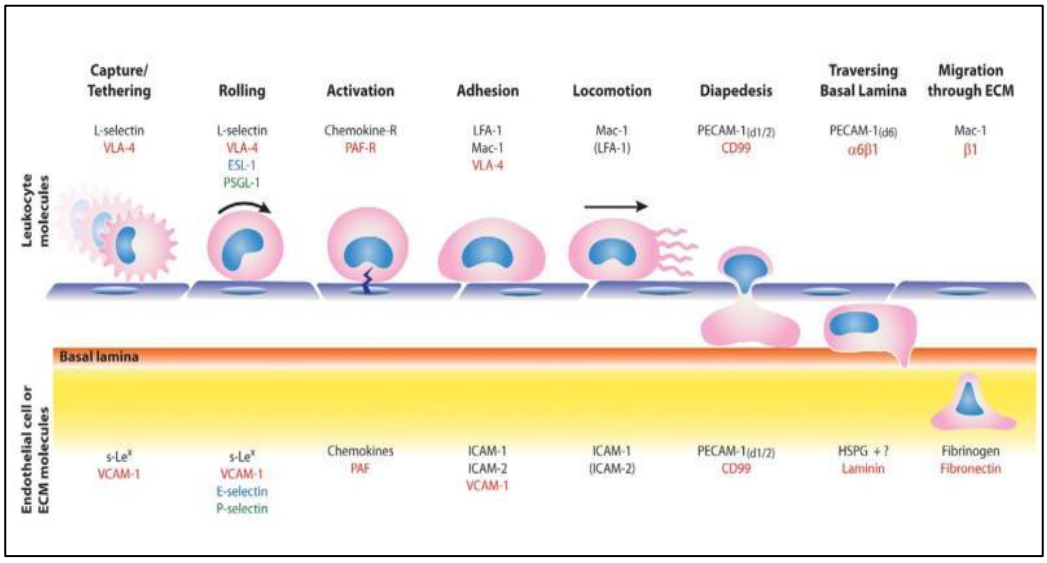

Figure 1: Leukocyte migration during inflammation (Muller 2013).

2. Macrophage, an important cellular player in inflammation

Macrophages were first discovered by Metchnikoff in 1908 (Wang, Wang et al. 2017) Macrophages constitute a heterogeneous population of immune cells derived from monocytes originating from the bone marrow. Macrophages can exhibit different phenotypes. It can be present as resident macrophages in some tissues such as Kupffer cells in liver, microglia in brain, osteoclasts in bones or alveolar macrophages in lung. They can be recruited mainly from the circulation and bone marrow to the different sites upon injury (infiltrating macrophages) (Shi and Pamer 2011)((Murray 2017).

The functions of macrophages are various and essential in immune responses. In addition to their defensive role accomplished by phagocytosis of parasites, microbes and apoptotic cells, they have an antigen-presenting role and participate in the activation of B

and T lymphocytes (Elhelu 1983, Murray and Wynn 2011) as well as in wound healing. During inflammation, macrophages are activated and monocytes are recruited from blood or the bone marrow and trans-endothelial migration occurs for the macrophages to reach the target tissues and exhibit their functions (as discussed earlier). Macrophages are characterized by high plasticity due to their ability to acquire different phenotypes depending on their interaction with different stimuli. The resulting type of macrophage being either proinflammatory or anti-inflammatory (or intermediate) depends on the type of cytokines encountered. Cytokines are proteins that influence cell behavior and participate in the initiation or attenuation of the inflammatory response depending on their type.

Depending on different stimuli, macrophages are classified as "classically activated", "wound-healing" or "regulatory" macrophages. When macrophages are stimulated by interferon-gamma, they are recruited to phagocytose invading microbes and are known as classically activated macrophages (proinflammatory macrophages previously known as M1 macrophages). The identification of subclasses of macrophages results in various and improved classification (Figure 2).

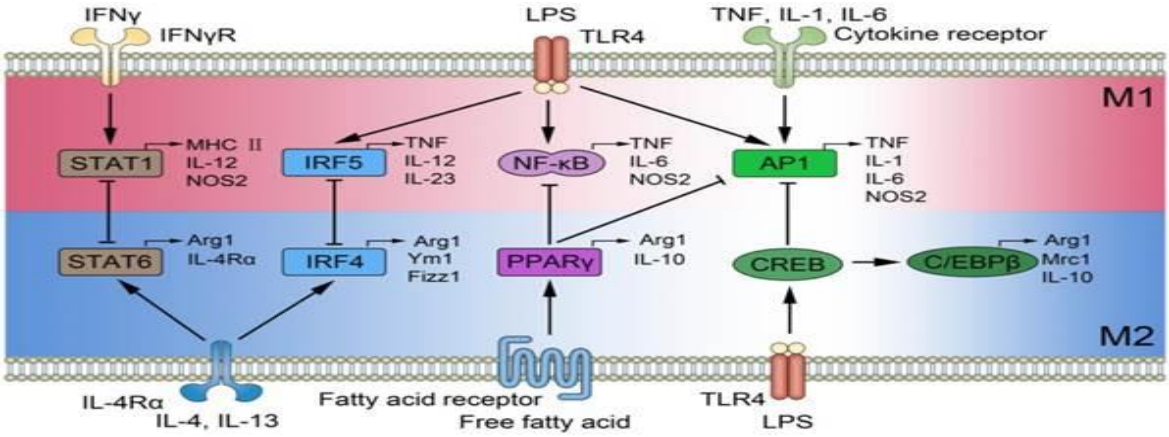

Figure 2: Differentiation of macrophages into M1 and M2 classes upon different signals (Liu, Zou et al. 2014).

In general, when the stimulus is interleukin-4 (IL-4) macrophages are known as wound-healing macrophages that are activated upon tissue injury to participate in wound healing. Under stress conditions, glucocorticoids released by adrenal cells inhibit inflammatory function of macrophages through the inhibition of the transcription of proinflammatory cytokines genes resulting in a population of regulatory macrophages (Mosser and Edwards 2008).Macrophages display a various cluster of surface receptors involved in different functions like phagocytosis, differentiation, growth and survival (Table 1)

Table 1: Classification of monocyte binding molecules and their function in monocyte trafficking (Shi and Pamer 2011) [a]

Monocyte-expressed molecules	Binding partners	Functions in monocyte recruitment
Chemokines and their receptors		
CCR2	CCL2,CCL7 and CCL12	Emigration of LY6C monocytes from the bone marrow
CXCR1	CXCL1	Patrolling: recruitment to splenic sites of bacterial
CCR1 and CCR5	Various, including the shared ligands CCL3 and CCL5	Recruitment into or within inflamed tissues
CCR6	CCL20	Possible role in the migration or function of LY6C monocytes in inflamed tissues
CCR7 and CCR8	CCL19 and CCL1, respectively	Migration of monocyte-derived DCs from the skin to lymph nodes
CXCR2	MIF	Arrest in atherosclerotic arteries of mice
Adhesion molecules		
L-selectin	Glycoproteins, including CD34, GLYCAM1 and MADCAM1	Tethering and rolling: recruitment during thioglycollate-induced peritonitis ; migration to lymph nodes
PSGL1	P-selectin and E-selectin	Migration through inflamed dermal venules ;tethering and rolling on atherosclerotic endothelium
LFA1	ICAM1	Patrolling during the steady state; not involved in the early recruitment of inflammatory monocytes
MAC1	ICAM1	Adhesion during acute inflammation
VLA4	VCAM1	Adhesion to inflamed endothelium
PECAM1	Endothelial PECAM1	Transendothelial migration

[a)] CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CX3CL1, CX3C-chemokine ligand 1; CX3CR1, CX3C-chemokine receptor 1; CXCR2, CXC-chemokine receptor 2; DC, dendritic cell; GLYCAM1, glycosylation-dependent cell adhesion molecule 1; ICAM1, intercellular adhesion molecule 1; LFA1, lymphocyte function-associated antigen 1; MAC1, macrophage receptor 1; MADCAM1, mucosal addressin cell adhesion molecule 1; MIF, macrophage migration inhibitory factor; PECAM1, platelet endothelial cell adhesion molecule; PSGL1, P-selectin glycoprotein ligand 1; VCAM1, vascular cell adhesion molecule 1; VLA4, very late antigen 4.

These receptors recognize a wide range of endogenous and exogenous ligands and their responses are central to mediate the functions of macrophages in tissue homeostasis, host defense as well as inflammation. (Stavitsky 2007, Koyama, Ishii et al. 2008, Gmiterek, Klopot et al. 2016).

B. Autophagy

Autophagy is a term originating from Greek "eating of self" was first discovered by Christian De Duve over forty years ago in yeast (Saccharomyces cerevisiae) and 32 autophagy (Atg) genes were recognized, most of them having homologous genes in mammals. Autophagy is a lysosomal degradation pathway that can be either selective or non-selective arbitrating the degradation of damaged cellular organelles such as mitochondria, peroxisomes, endoplasmic reticulum and irregular protein aggregates that escape proteasomal degradation (Levine, Mizushima et al. 2011). In addition, autophagy plays a housekeeping role in clearing pathogens, viruses and bacteria. These functions explain the essential role of autophagy in maintaining tissue homeostasis.

1. General aspects

Autophagy is a highly conserved process that occurs in nearly all eukaryotic cells (Bento, Renna et al. 2016, Zhong, Sanchez-Lopez et al. 2016). Any misregulation in this

process may lead to various disease conditions including diabetes, aging, inflammatory diseases and cancer. In fact, there are at least three types of autophagy: macroautophagy, micro-autophagy (selective or non-selective) and chaperone mediated autophagy (selective) (Figure 3). These types mediate lysosomal degradation but they differ by the way of delivering them. During macroautophagy, the components to be degraded are engulfed by a double-membrane vesicle called autophagosome, which then fuses with a lysosome for degradation. However, the components in micro-autophagy are directly degraded by the lysosome. Chaperone-mediated autophagy is a selective type of autophagy by which only targeted proteins that form a complex with chaperone proteins that bind specific receptors on the lysosome are subsequently unfolded to be finally degraded (Saftig, Beertsen et al. 2008). The resulting metabolites are recycled and used to fulfill the needs of the cells making autophagy an important recycling mechanism in the body (Boya, Reggiori et al. 2013).

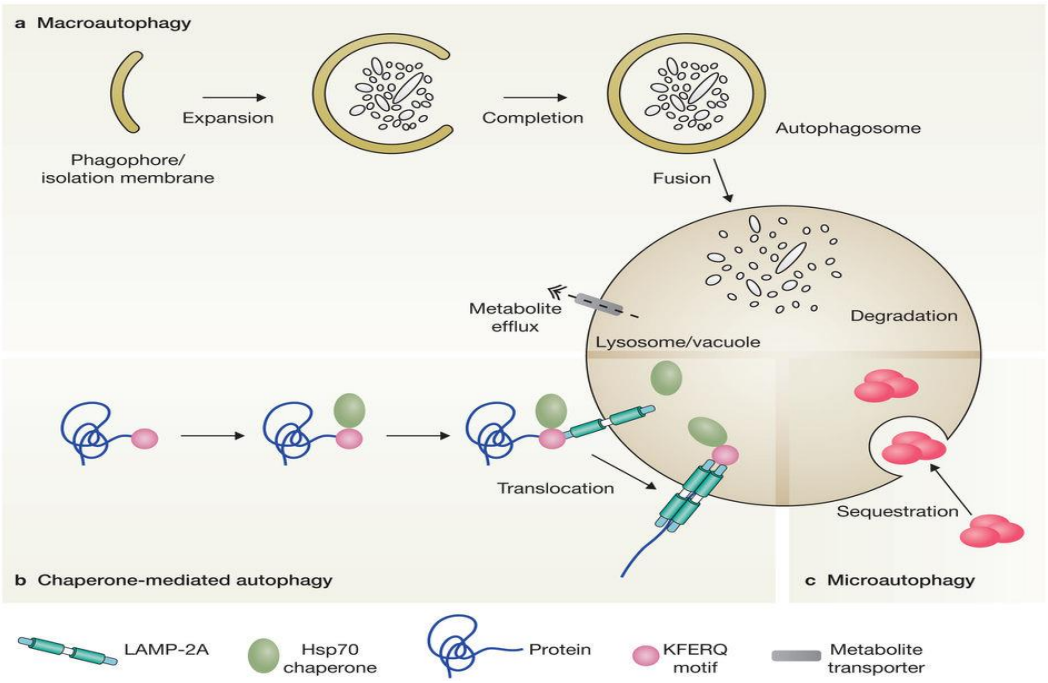

Figure 3: Different types of autophagy (Boya, Reggiori et al. 2013).

2. Autophagy: a multi-step process

Autophagy is a multi-step process that involves five essential key steps that start with a) the phagophore formation, b) conjugation of Atg-5 and Atg-12, c) elongation of the phagophore mediated by LC3 insertion, d) capture of targets for degradation, and e) the fusion of the autophagosome with the lysosome (Figure 5), followed by proteolytic degradation of the engulfed molecules mediated by lysosomal proteases (Mizushima 2007, Axe, Walker et al. 2008, Simonsen and Tooze 2009, Glick, Barth et al. 2010).

The phagophore formation constitutes the initial step for the autophagy machinery to occur. Studies concerning the origin of the phagophore are controversial, showing that the phagophore is derived from a lipid bilayer originating either from the endoplasmic reticulum or the trans-golgi apparatus. (Axe, Walker et al. 2008, Simonsen and Tooze

2009). The essential role of the phagophore, a double membrane called autophagosome, is to engulf various targets such as protein aggregates, organelles and ribosomes. Then the fusion of the phagophore with a lysosome forms the autophagolysosomal complex responsible for lysosomal degradation of the sequestered components. After degradation, the components present in the cytoplasm can recycle and serve for building macromolecules and metabolism. Therefore, autophagy can serve as a recycling machinery that eliminate damaged proteins and organelles as well as to generate energy (Mizushima 2007).

3. Regulation of the initial step of autophagy (phagophore formation)

The formation of the autophagosome is the most complex step and requires the recruitment of multiple Atg proteins. This step is initially mediated by the class III phosphatidylinositol 3-kinase (PtdIns3K) complex which is composed of the PI-3 Kinase VPS34 (vesicular protein sorting 34), a serine/threonine kinase VPS15 (p150 in mammalian cells), Atg14 (mAtg14 in mammalian cells) and Atg6/VPS30 (Beclin 1 in mammalian cells) (Kihara, Noda et al. 2001, Itakura, Kishi et al. 2008) (Liang, Jackson et al. 1999, Sun, Fan et al. 2008) (Figure 4).

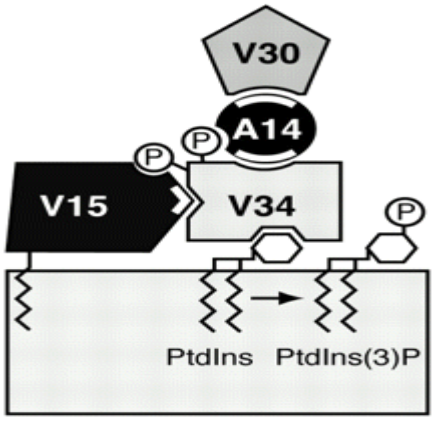

Figure 4: Class III phosphatidylinositol 3-kinase (PtdIns3K) complex formation during autophagy (Kihara, Noda et al. 2001).

The interaction between PI-3 kinase VPS34 and Beclin constitutes a key step for the inhiation of autophagy in mammalian systems. VPS34 is involved in different functions in the cell but when it binds to Beclin-1 and other Atg proteins it becomes selectively involved in autophagy (Backer 2008). VPS34 is a PI-3 kinase that generates phosphatidyl inositol triphosphate (PI3P) which is essential for phagophore elongation and recruitment of other Atg proteins to the phagophore (Xie and Klionsky 2007).

When Beclin-1 interacts with VPS34, it promotes its catalytic activity and increases the levels of PI3P thus enhancing the autophagic machinery. Studies have shown that Beclin-1 is mono-allelically deleted in some cancers such as human breast, ovarian and prostate cancer, suggesting that autophagy may have anti-tumorogenic properties (Liang, Jackson et al. 1999).

Additional regulatory proteins complex with VPS34 and Beclin-1 at the endoplasmic reticulum and nucleated phagophore to either promote autophagy, such as UVRAG, BIF-1, Atg14L and (Liang, Feng et al. 2006, Fimia, Stoykova et al. 2007) or to

inhibit autophagy, such as Rubicon and Bcl-2 (B-cell lymphoma 2) (Matsunaga, Saitoh et al. 2009).

A well-recognized regulatory mechanism is the interaction of Beclin-1 with Bcl-2 which is an antiapoptotic protein that prevents the interaction of Beclin-1 with VPS34 (Pattingre, Tassa et al. 2005). The dissociation of Beclin-1 from Bcl-2 is required to induce autophagy.

Thus Bcl-2 constitutes an autophagy inhibitor that is only disrupted from beclin-1 upon phosphorylation of Bcl-2 by c-Jun N-terminal kinases Jnk-1 in response to starvation - mediated signaling thus leading to autophagy activation (Wei, Pattingre et al. 2008).

4. Regulation of autophagy

Autophaghy plays a housekeeping role in most cells types where it is active at basal levels and maintains the integrity of organelles and proteins (Jin 2006). 5'-AMP activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) are main regulators of autophagy. AMPK is a key energy sensor that is involved in the regulation of cellular metabolism and in maintaining energy homeostasis that promotes autophagy by controlling the earliest step of phagophore formation. Under low energy (decrease ATP/AMP), AMPK is activated. ULK1 (Unc-1 like kinase) is a main player in the phagophore formation, that is activated upon phosphorylation by AMPK at ser 317 and ser 777 under starvation conditions, thus activating autophagy. Under stress conditions or low nutrients (high AMP/ATP), autophagy becomes strongly induced and acts as a mechanism that promotes survival until nutrients are available again. However, when nutrients are available, the mammalian target of rapamycin (mTOR) is activated leading to

autophagy inhibition. mTOR is another important regulator of autophagy which acts as an essential cell-growth regulator that integrates growth factor and nutrient signals. mTOR is a control point downstream different signals including growth factor receptor signaling, insulin signaling, hypoxia and ATP levels. When mTOR is activated upon PI3K/Akt signaling, nutrients availability or under low energy levels, autophagy is downregulated (Sabatini 2006, He and Klionsky 2009). Studies have shown that when nutrients are available mTOR inhibits autophagy by phosphorylation of ULK-1 at ser 757 and preventing the interaction of ULK-1 with AMPK thus ULK-1 is not activated and autophagy is inhibited by mTOR when nutrients are available (Vural and Kehrl 2014).

Under stress conditions, mTOR activity is reduced and autophagy is induced when low cytosolic ATP levels are sensed by AMPK. AMPK then inhibits mTOR activity through phosphorylation of TSC1/2 complex, stimulating autophagy (Inoki, Zhu et al. 2003).

Several signaling pathways regulate autophagy and both deficient and additional autophagy in the cell can be damaging (Mizushima, Levine et al. 2008, He and Klionsky 2009).

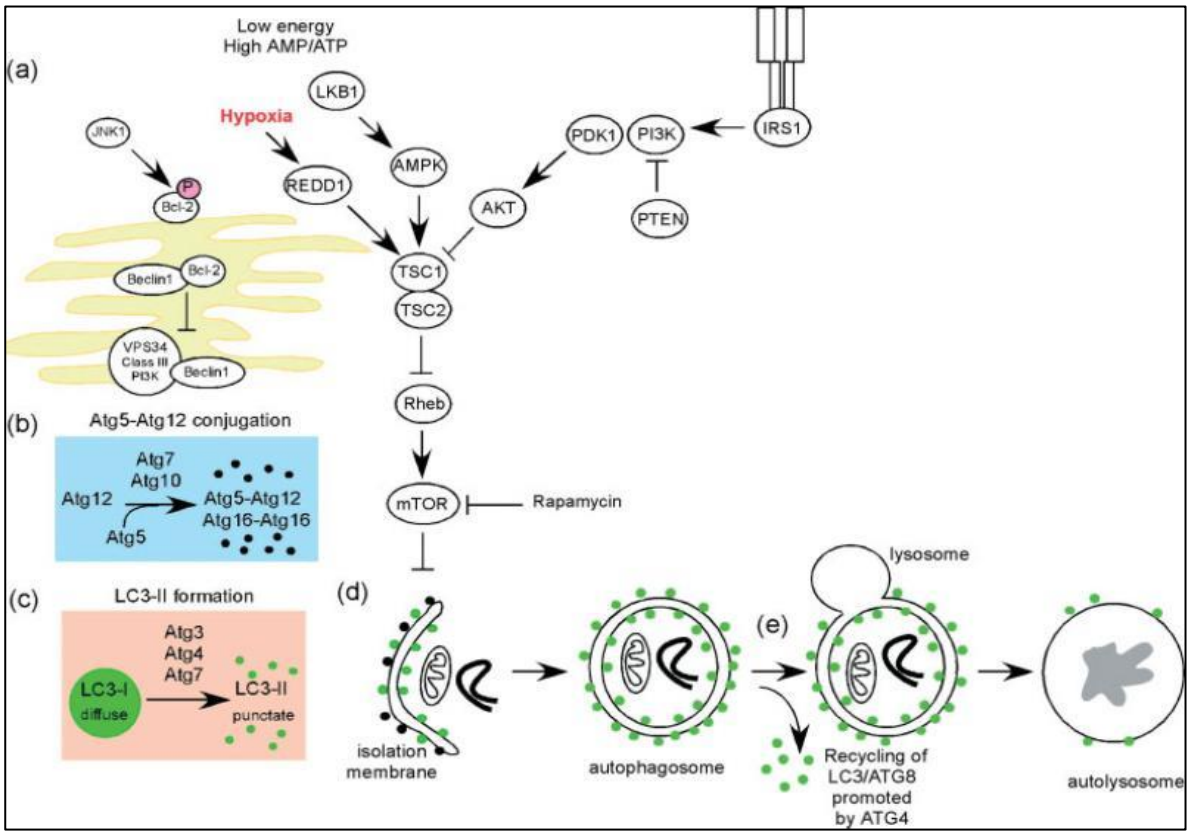

Figure 5: Signaling pathways regulating autophagy (Glick, Barth et al. 2010)

C. Statins and inflammation

Statins are known cholesterol-lowering drugs that inhibit the mevalonate pathway by blocking the enzyme 3-hydroxy 3-methylglutaryl CoA (HMG-CoA) reductase. This enzyme catalyzes a rate-limiting step in the mevalonate pathway essential for cholesterol biosynthesis (Bang and Okin 2014). Since the discovery of the first statin 30 years ago, statins are the most effective class of drugs prescribed to reduce cholesterol levels. In addition, studies have shown that statins can reduce the risk of cardiovascular diseases (Bang and Okin 2014). Studies have shown in the last two decades that statins have additional effects beyond the cholesterol-lowering role. Many pleiotropic effects of statins

were described including anti-inflammatory, anti-oxidant, neuroprotective, and improving endothelial function in addition to many others.(Cohn, Quyyumi et al. 2004, Grines 2006) (Takemoto and Liao 2001).

Induction of the endothelial NO synthase isoform in the endothelium improving endothelial function (Endres, Laufs et al. 2004, Liao and Laufs 2005) inhibition of NADPH oxidase in inflammatory cells as well as oxidative stress both *in vivo* and *in vitro* have been demonstrated. Previous studies by our group have shown a regulation of pro-inflammatory genes and cytokines by statins in monocytes (Habib, Shamseddeen et al. 2007) and as well as increase in the anti-inflammatory and anti-oxidant enzyme, heme-oxygenase-1, in parallel to a decrease of the synthesis of pro-inflammatory cytokines (Mouawad et al, 2013; Mrad et al, 2012). These studies have been confirmed *in vivo* (El Achkar et al, in preparation, 2017). The inhibition of members of the family of the small G-protein Rho was the major regulation described in these studies.

These beneficial effects of statins may help to treat many devastating diseases. Many of these pleiotropic effects are mediated through the inhibition of Rho and its downstream targets: Rho associated coiled-coil-containing protein kinase (ROCK) (Liao and Laufs 2005, Bedi, Dhawan et al. 2016).

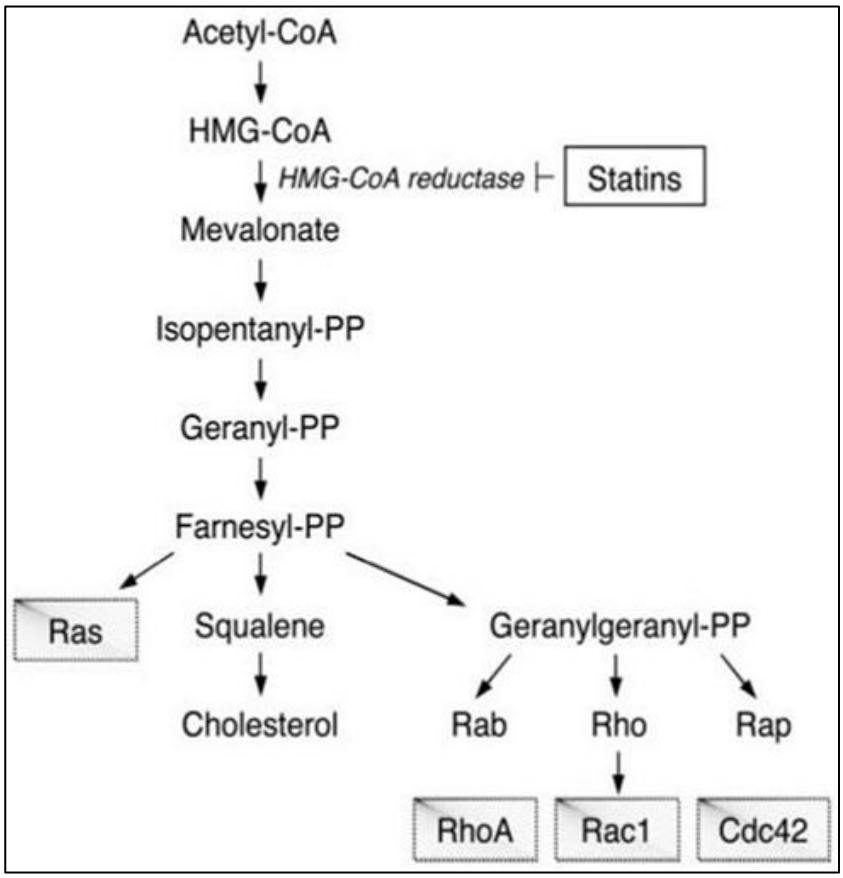

Figure 6: Mevalonate pathway (Rikitake and Liao, Circ Res,2005).

Recently, few studies addressed the relation between statins and autophagy (Araki et al 2012) have shown that statins induced autophagy in human rhabdomyosarcoma (Araki, Maeda et al. 2012) whereas Zhang et al (2013) have demonstrated that statins can have anti-metastatic effects mediated through the induction of autophagy (Zhang, Yang et al. 2013).

Deletion of autophagy genes such as ATG5 from the myeloid lineage which includes neutrophils and macrophages was recently shown to increase the inflammatory response (Lodder et al, 2015). Thus, the investigation of the role of the autophagy pathway in the anti-inflammatory effects of statins gained interest.

CHAPTER II

AIMS OF THE STUDY

Since statins and the inhibitors of the downstream effectors such as Rho Kinases are anti-inflammatory and since autophagy has anti-inflammatory functions, we aimed to assess the effect of statins and Rho kinase inhibitors when autophagy is inhibited in macrophages.

AIM 1

Develop a way to block autophagy in macrophages examining its role in inflammation.

AIM 2

Investigate the role of autophagy in the regulatory effects of atorvastatin and a downstream inhibitor of the mevalonate pathway, and small G-protein Rho, on inflammation.

CHAPTER III

MATRIALS AND METHODS

A. Cell culture

L929 cells produce macrophage colony stimulating factor (M-CSF) which is required to induce hematopoietic cell differentiation into macrophages. L929 cells were grown to confluence in complete Dulbecco's Phosphate Buffered Saline (DMEM) containing 10 % FBS at 37 °C, 5% CO_2. Culture media were collected after 10 days and centrifuged at 1000 rpm for 5 min and stored at -80 °C.

B. Bone marrow-derived macrophages (BMDM) preparation

The experiments were performed at the American University of Beirut and the protocol for animal handling was approved (16-09-M379).

Male C57BL/6J mice, age 10-17 weeks (grown in Animal Facility at AUB), were used to prepare bone–marrow derived macrophages as described in the next paragraphs. C57BL/6J mice were sacrificed in CO_2 chamber. An incision was made at the top of each hind leg, then the skin was pulled down towards the foot to expose the muscle. The hind legs were cut off and cleaned, femur and tibia were separated at the knee joint and kept in 1X Phosphate Buffer Saline (PBS). Under the hood, bones were cut and flushed using RPMI culture media (Sigma-Aldrich RNBF5228) and cells were flushed, collected and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 2 ml red blood lysis (RBL) (Sigma-Aldrich RNBF1541) medium 10% in PBS (Sigma-Aldrich RNBF8385), incubated for 5 min at room temperature. 5 ml RPMI

were added for neutralization and centrifuged at 1500 rpm for 5 min, and the obtained cell pellet was resuspended in RPMI complete media containing 20% L929 media. Culture plates were incubated at 37 °C, and bone marrow cells were allowed to proliferate and differentiate.

Differentiated macrophages (referred to as BMDM) were obtained after 5-6 days of incubation in the presence of M-CSF. Cells are scrapped using a cell scraper, counted by Trypan Blue using improved new Bauer hematocytometer and plated in 6 well or 12-well plates.

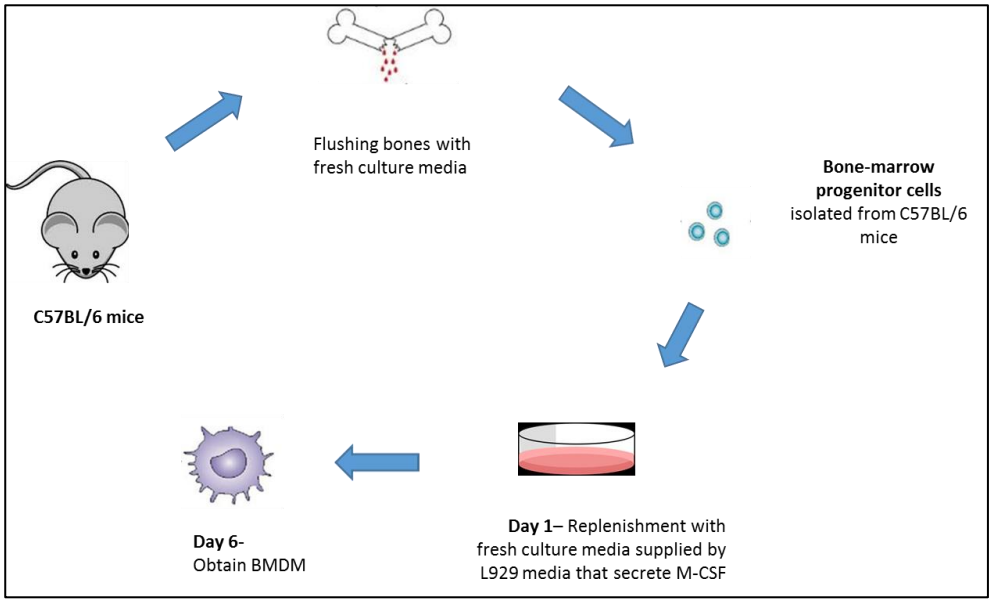

Figure 7: Bone marrow-derived macrophages preparation

C. Treatment of macrophages

Cells were pre-treated with inhibitor prior to the addition of lipopolysaccharide (LPS, serotype Escherichia coli 0111: B4, Sigma-Aldrich, L4391). Each well contained one of the following treatments: inhibitors (ULK or VPS34), atorvastatin or Y27632, LPS and control groups. Supernatants were collected for cytokines, PGE_2 and nitrite measurement.

Cells were lysed in RIPA lysis buffer for protein extraction or with QIAzol (QIAGEN, 79306) for mRNA extraction.

D. RNA extraction, reverse transcription and real-time polymerase chain reaction

1. RNA Extraction

500 µL QIAsol (QIAGEN, 79306) was added to each well and total RNA was extracted according to the Manufacturer instructions. Total RNA was resuspended into 20 µL RNAase, DNAase free water and quantified using a Nanodrop (Thermo Fisher Sciemtific)

2. Reverse Transcriptase (RT) & Real-Time PCR

cDNA synthesis was performed according to the manufacturer's instructions (Thermo Fischer Scientifics, 00407363) and proceed as follows: 10 min at 25°C, 2 hours at 37°C followed by 5 min at 85°C.

The cDNA samples were stored at -20°C. Reverse transcriptase reaction was performed using 1 µL of total RNA in a final volume of 20 µL. The cDNA samples were stored at -20°C.

Real-Time PCR was performed: 2.5 µL of the cDNA was used in each well. Annealing of primers reaction proceeded in a PCR machine (Real-time PCR Biorad CFX384) (Bio-Rad Laboratories, California, USA) as follows: one cycle at 94°C for 15 minutes, 50 cycles at 94°C for 15 seconds, 56°C for 9 seconds each, and finally one cycle at 72°C for 30 minutes. Melt curve analysis was done and the results were analyzed. The primers used are for 18S rRNA, Forward primer: 5'-AAC TTT CGA TGG TAG TCG CCG T-3 Reverse primer: 5'-TCC TTG GAT GTG GTA GCC GTT T-3'; for Mgl-1: Forward

primer: 5'-TGG CCT GAA GCT GAC AAG TA-3', Reverse primer: 5'-AGG CCG ATC CAA CTA ACC ACA TT-3'; for ATG5: Forward primer: 5'-AAG TCT GTC CTT CCG CAG TC-3'; Reverse primer: 5'-GAA GAA AGT TAT CTG GGT AGC TCA-3'; for Beclin: Forward primer: 5'-ATT ACT TAC CAC AGC CCA GG-3'; Reverse primer: 5'-CCC GAT CAG AGT GAA GCT AT-3'

E. Protein extraction and quantification

After 4 hours treatment, supernatants were aspirated. Cells in each well were washed with 2 ml PBS (1x) with Ca^{2+} and Mg^{2+}. After removing PBS, 250 µL of lysis buffer was added followed by quenching on ice for 10 min. Lowry (Bio-Rad, 500-0115) Protein quantification was done using Lowry method using BSA as standard (λ=750 nm).

F. Western blot analysis of LC3 protein

Total proteins were separated by gel electrophoresis using a 13% SDS-polyacrylamide gel followed by transfer of the proteins from the gel into 0.22 □m nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature using 5% non-fat milk in TBST buffer solution and incubated with 1/1000 LC3B (Bio-Techne, NB100-2220) and 1/5000 β-actin (Sigma-Aldrich, A5441). Signals were developed using chemiluminescence reagent (Bio-Rad, 170-5061).

G. ELISA assay, nitrite measurement and enzyme immune assay

1. ELISA Assays for IL-6 and TNF-alpha

To perform ELISA assays (TNF-α, Thermo Fischer Scientifics, 88-7324-88; IL-6, Thermo Fischer Scientifics, 88-7064-22) supernatants were collected after 6 hours of incubation and stored at -20˚C. Assays were performed according to the manufacturer

instructions. Plates were read via spectrophotometer using the ELISA Plate reader at λ=450nm.

2. Griess assay for nitrite measurement

Nitrite level was measured using Griess reagent. The supernatant was collected from BMDM cells treated with LPS (10 ng/ml) with or without VPS inhibitors for 24 hours. 100 µL of cell culture supernatant was mixed with 100 µL of Griess reagent. The absorbance was measured at 540 nm.

3. Enzyme immune-assay (EIA)

To perform EIA, supernatants were collected after 24 hours of incubation and stored at -20 ˚c, PGE_2 was measured as described previously (Habib et al, 2007). Plates were read via spectrophotometer using the ELISA Plate reader at λ=414 nm.

H. Statistical Analysis

Data was analyzed by one-way ANOVA test for multiple comparisons, using "GraphPad Prism" software. Values were considered significant for P value ≤ 0.05. Error bars on graphs represent the standard error mean for each sample.

CHAPTER IV

RESULTS

A. BMDM characterization

First, we prepared and characterized bone-marrow derived macrophages to test the role of autophagy in inflammation, by blocking autophagy using different selective autophagy inhibitors.

Bone-marrow cells were obtained from C57BL/6J mice and allowed to differentiate in culture for 5 days in presence of fresh culture media containing 20% of L929 media that secretes macrophage colony stimulating factor (M-CSF). Then cells were treated in presence and absence of 10 ng/ml LPS. Lipopolysaccharide (LPS), which is an endotoxin derived from gram-negative bacteria that binds to the extracellular domain of TLR4 and induces a complex signal transduction cascade leading to the formation of pro-inflammatory cytokines.

After 6 hours, supernatants were harvested and cytokines were measured to test for the presence of IL-6 (Figure 8 A) TNF-α (Figure 8 B) and 24 hours for the measurement of PGE_2 (Figure 8 C) to assess the effect of LPS. After performing ELISA, LPS increased IL-6 synthesis by 4 folds compared to the control. TNF-α synthesis was also increased in presence of LPS by 2 folds and PGE_2 3 folds. These results revealed the inflammatory effect of LPS on BMDM in culture.

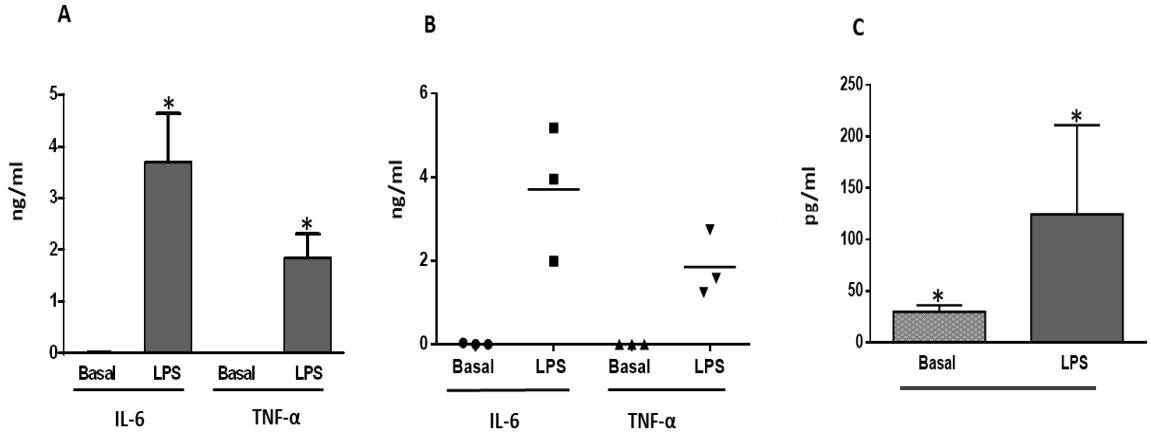

Figure 8: Effect of LPS on IL-6, TNF-α and PGE_2 formation in BMDM. Bone-marrow derived macrophages (BMDM) were seeded in 12-well plates and treated in absence or presence of 10 ng/ml LPS for 6 hours for IL-6 and TNF-α measurement and for 24 hours for PGE_2. Supernatants were collected and IL-6 and TNF-α were measured by ELISA. C) PGE_2 measured by enzyme immunoassay (EIA). Data are represented as histograms (A) and scatter plot (B) and are expressed as mean ± SEM of 3 experiments performed with 5 replicates. * P value < 0.05, versus LPS-treated cells, unpaired *t*-test

B. Inhibition of autophagy

1. Inhibitors of VPS34

Confirming that the BMDM are functional and reply to LPS, cells were treated with different autophagy inhibitors to test the effect of autophagy inhibition on the inflammatory response. Cells were treated with 1 □M VPS34IN-1 (Cayman Chemical, Ann Arbor, Michigan, USA) 15 min prior to the addition of 10 ng/ml LPS. VPS34IN-1 inhibits the initial step of autophagy and prevents the formation of VPS and beclin, which is a crucial step for the phagophore formation.

The inhibition of autophagy by VPS34-IN1 in presence of LPS for 6 hours increased the inflammation as shown by a 3 fold increase in IL-6 compared to LPS alone (Figure 9).

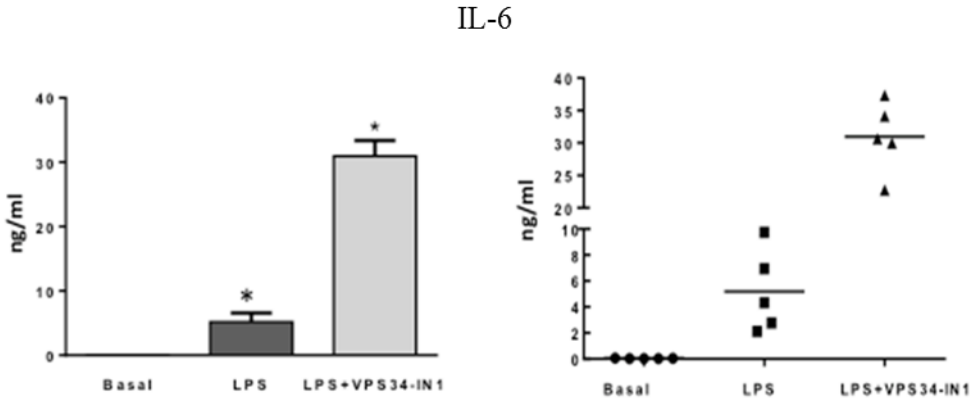

Figure 9: Effect of VPS34-IN1 on IL-6 formation in BMDM. BMDM were pretreated in absence or presence of 1 μM VPS34-IN1 or vehicle for 15 min prior to the addition of 10 ng/ml LPS. After 6 hours supernatants were harvested and IL-6 secretion was measured by ELISA as described in the materials and methods section. Data are represented as histograms and scatter plot and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results. * P value < 0.05, versus LPS-treated cells, unpaired *t*-test

Similarly, the treatment of cells by VPS34-IN1 elevated the levels of TNF-α by 2 folds more than LPS alone when measured after 6 hours of treatment (Figure 10).

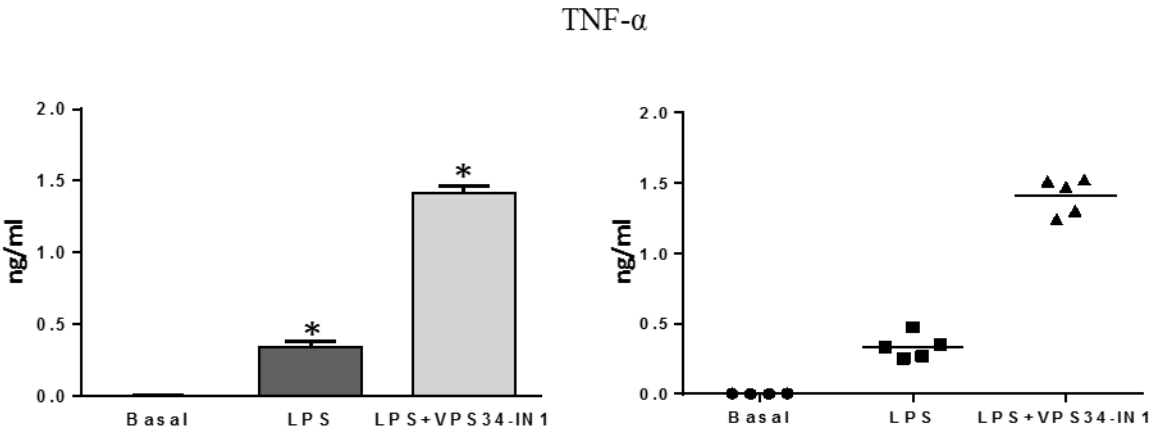

Figure 10: Effect of VPS34-IN1 on TNF-α formation in BMDM. BMDM were treated as described in the legend for figure 2. TNF-α was measured by ELISA. Data are represented as histograms and scatter plot and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results. * P value < 0.05, versus LPS-treated cells, unpaired *t*-test

An alternative autophagy inhibitor (SAR) (Medchem Express, Monmouth Junction,NJ) that inhibits VPS-Beclin complex was also used to assess the effect of autophagy inhibition on inflammation in BMDM. Cells were treated with 1 μM SAR for 15 min prior to the addition of 10 ng/ml LPS. The supernatants were harvested after 6 hours and IL-6 levels were measured. In presence of SAR, there was a 3 folds rise in IL-6 levels compared to LPS alone (Figure 11).

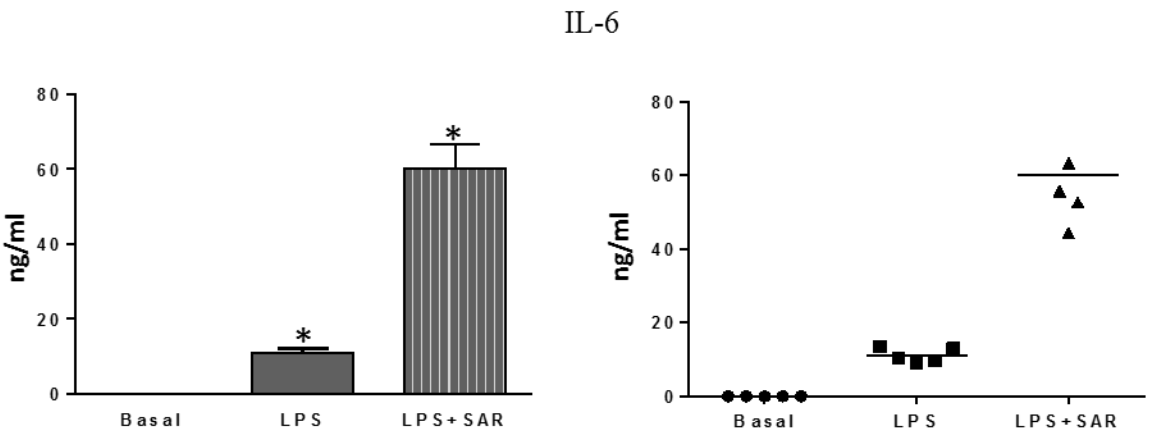

Figure 11: Effect of SAR inhibitor on IL-6 formation in BMDM. BMDM were pretreated in absence or presence of 1 μM SAR or vehicle for 15 min prior to the addition of 10 ng/ml LPS. After 6 hours supernatants were harvested and IL-6 secretion was measured by ELISA as described in the materials and methods section. Data are represented as histograms and scatter plot and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results. * P value < 0.05, versus LPS-treated cells, unpaired *t*-test

The same result was shown concerning the levels of TNF-α which were doubled in presence of LPS and SAR compared to LPS alone (Figure 12).

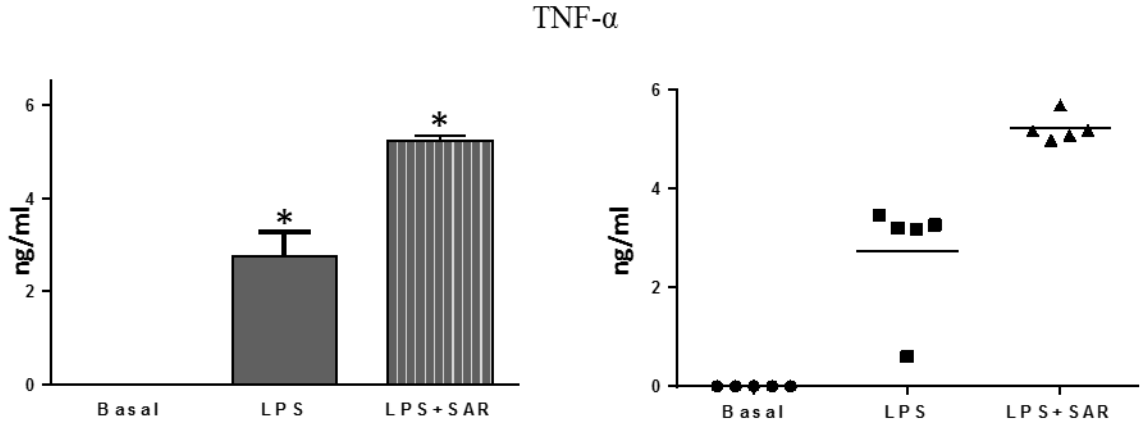

Figure 12: Effect of SAR inhibitor on TNF-α formation in BMDM. BMDM were treated as described in the legend for figure 8. TNF-α was measured by ELISA. Data are represented as histograms and scatter plot and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results. * P value < 0.05, versus LPS-treated cells, unpaired *t*-test

Prostaglandin E_2 levels were measured in BMDM treated with SAR and VPS34IN-1. Cells were treated with 1 μM SAR or VPS34IN-1 for 15 min prior to the addition of 10 ng/ml LPS for 24 hours. As shown in the diagram (Figure 13), PGE_2 levels were increased by 2 folds upon SAR treatment compared to LPS alone and by 5 folds with VPS34IN-1.

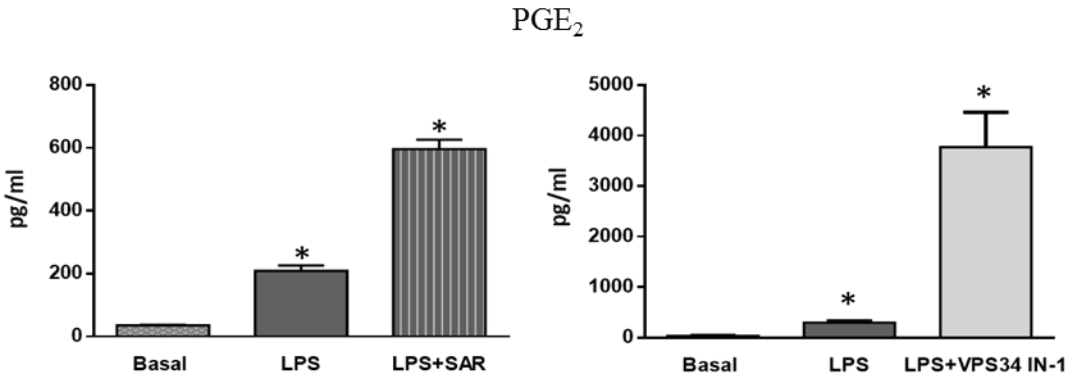

Figure 13: Effect of SAR inhibitor and VPS34-IN1 on PGE_2 formation in BMDM. BMDM were treated as described in the legend for figure 8. PGE_2 was measured by EIA. Data are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 2 experiments with similar results* P value < 0.05versus LPS-treated cells, unpaired t-test

These results show that inhibition of autophagy by two different inhibitors of the initiation of autophagy lead to an increased pro-inflammatory state in BMDM compared to LPS alone, which means that autophagy is an essential mechanism playing an important role in maintaining the inflammatory state of the cells.

We also assessed the effect of VPS34IN-1 on nitrite formation in BMDM. In presence of VPS34IN-1, nitrite secretion was doubled compared to LPS alone (Figure 14).

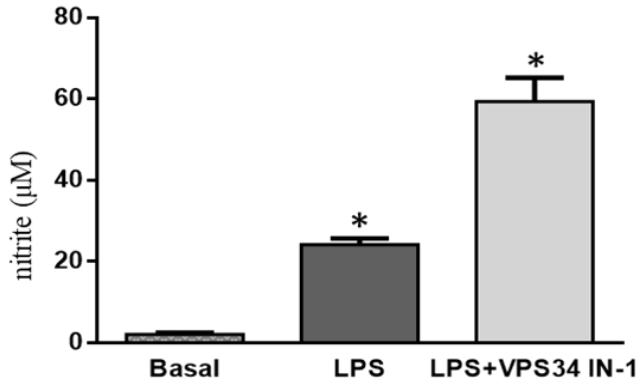

Figure 14: Effect of VPS34 IN-1 on nitrite formation in BMDM. BMDM were treated as described in the legend for figure 8. After 24 hours, nitrite was measured by Griess Assay. Data are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 2 experiments with similar results. * P value< 0.05, versus LPS-treated cells, unpaired t-test.

After examining the effect of the autophagy inhibitors on cytokines, we wanted to make sure that autophagy was inhibited by (SAR and VPS34IN-1). Western blot analysis of microtubule-associated protein light chain 3 (LC3) was performed to check the inhibition of autophagy at the protein level. Cells were incubated in normal media or starved by incubation for 4 hours in EBSS without serum. LC3 is a pro-peptide that is cleaved to form LC3-I. Phosphatidylethanolamine is added to LC3-I at C terminus causing its conversion to LC3-II. LC3-II participates in the elongation of autophagosomes and is used as a maker for autophagosome formation or accumulation when autophagy is completed. However, if

autophagy is inhibited, LC3 lipidation is decreased and LC3-I is not completely converted to LC3-II. This is reflected on western blots by the formation of a doublet of LC3. In starved condition, LC3 II is formed compared to normal culture media (not shown), and further increased in presence of CQ incubated in EBSS starvation media .When autophagy is inhibited by SAR, a faint LC3-I band appears and LC3-II appears with a band of higher intensity (Figure 15). Antibodies against LC3: one of the definitive markers of autophagy was used. Similar results were obtained in the laboratory for VPS34IN-1 tested on Hela cells and BMDM (data not shown).

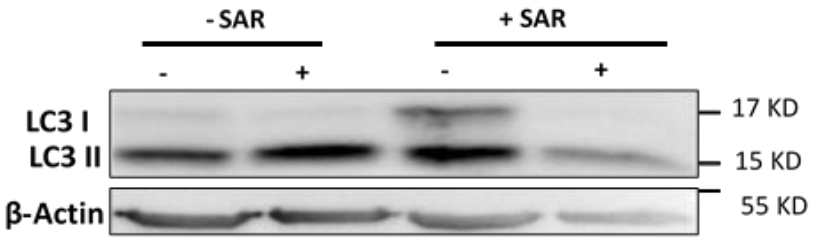

Figure 15: Effect of SAR on LC3 II in BMDM under starvation conditions. Western blot analysis- Cells were treated with 30 μM chloroquine (CQ) for 15 min prior to the addition of 1 μM SAR. After 4 hours, supernatants were harvested and total proteins were extracted. Immunoblot analysis was performed using LC3 Ab (1/1000) and β-actin (1/5000) as described in the materials and methods section.

2. Inhibitors of ULK

SBI is an ULK inhibitor. It was assessed on cytokines: IL-6 (Figure 16 A) and TNF-α (Figure 16 B), and showed an increase in IL-6 levels by 3 folds compared to LPS alone (A). Conversely, in presence of SBI, TNF-α levels were lower compared to LPS alone. In addition, the effect of SBI on nitrite formation was assessed (Figure 16 C). Nitrite levels were decreased in presence of SBI compared to LPS. Moreover, SBI was unable to shown the increase in LC3-I compared to LC3-II in western blot analysis (data now shown).

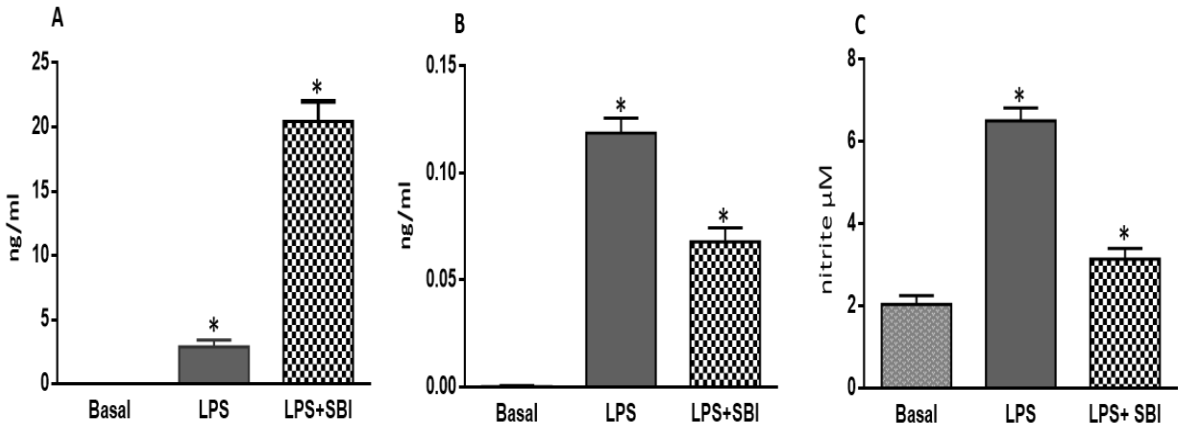

Figure 16: Effect of SBI on IL-6 (A), TNF-α (B), and Nitrite (C) formation in BMDM. BMDM were pretreated in absence or presence of 1 µM SBI or vehicle for 15 min prior to the addition of 10 ng/ml LPS. After 6 hours supernatants were harvested and IL-6 (A) and TNF-α (B) secretion was measured by ELISA and after 24 hours nitrite (C) formation was measured by Griess reagent as described in the materials and methods section. Data are represented as histograms and scatter plot and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results. * P value < 0.05, versus LPS-treated cells, unpaired *t*-test.

Based on these results we elected to assess the effect of the inhibitors of VPS. Table 2 summarizes the results obtained with VPS34IN-1 and SAR.

In our study, we used two autophagy inhibitors: SAR and VPS34IN-1, which were both shown to inhibit autophagy in bone marrow-derived macrophages and to stimulate an additional pro-inflammatory condition when compared to LPS. The table 2 shows the percentage of LPS of IL-6 and TNF-α formation in cells treated with VPS34IN-1 and SAR. The results showed that VPS34IN-1 has a greater efficiency in increasing IL-6, TNF-α, PGE_2 and nitrite formation in BMDM compared to SAR.

Table 2: Percentage of LPS of IL-6 and TNF-α formation in cells treated with VPS34IN-1 and SAR inhibitors

	SAR	VPS34 IN-1
IL-6	401.3 ± 97.76 %	659.3 ± 142.3 %
TNF-α	185.7 ± 14.31 %	262.3 ± 78.34 %
PGE_2	596.3 ± 30.00 %	3771 ± 693.8 %
Nitrite	38.79 ± 1.453 %	59.39 ± 2.611 %

(a) Data are expressed as **percentage** of LPS-treated macrophages and correspond to the % mean ± SEM (n=3) of 3 experiments. * P value < 0.05, versus LPS-treated cells, unpaired t-test.

a)

C. The role of statins and Y27632 in inflammation and effect of autophagy inhibition

Statins are cholesterol-lowering drugs that target HMG-CoA reductase and reduce the risk of coronary diseases. In addition to this, statins were shown to have anti-inflammatory effects. Y27632 is a selective ROCK inhibitor, a downstream target of the Rho GTPase that has anti-inflammatory effects.

1. Effect on anti-inflammatory and autophagy gene expression

First, we assessed the effects of both atorvastatin and Y27632 on Mgl-1, which is anti-inflammatory marker. This was done by qPCR and results showed that in presence of atorvastatin, Mgl-1 expression was increased by 2 folds compared to the control. However, Y27632 was shown to have no effect on Mgl-1 expression compared to the control (Figure 17).

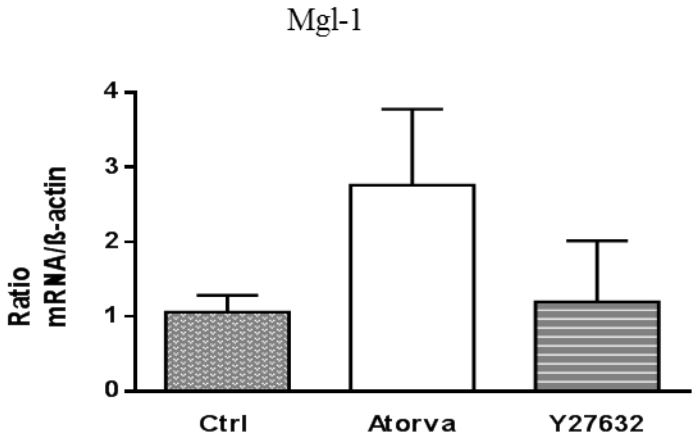

Figure 17: Effect of atorvastatin and Y27632 on Mgl-1 gene was assessed by q-PCR. BMDM were treated with 10 μM atorva or 10 μM 27632. After 24 hours supernatants were collected and the effect of atorva and Y27632 on Mgl-1 was assessed by q-PCR

Then we assessed the effects of atorvastatin and Y27632 on the expression of autophagy markers such as Beclin (Figure 18 A) and Atg5 (Figure 18 B) by qPCR. Results showed that atorvastatin and Y27632 has no significant effect on the expression of both autophagy genes. Further experiments should be done in the future.

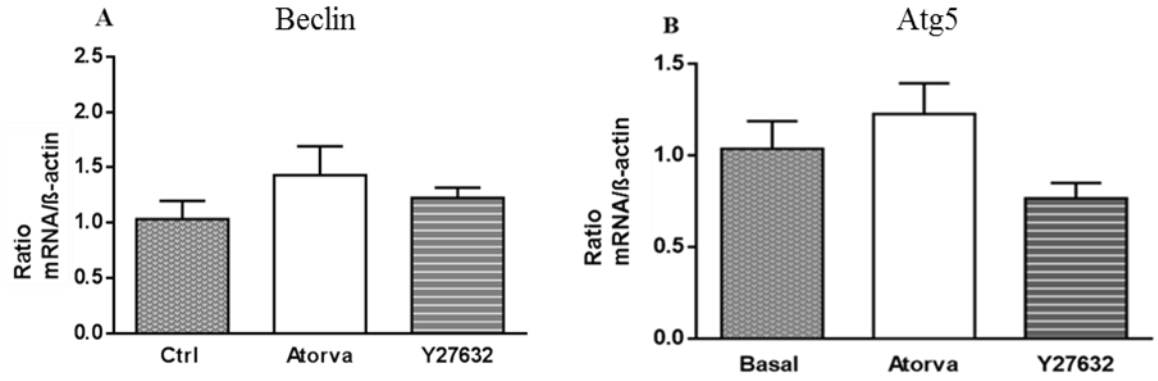

Figure 18: Effect of atorva and Y27632 on Beclin and Atg5 autophagy genes assessed by q-PCR. BMDM were treated with 10 μM atorvastatin (atorva) or 10 μM Y27632. After 24 hours supernatants were collected and the effect of atorva and Y27632 on Beclin (A) and Atg5 (B) was assessed by q-PCR

The effect of atorvastatin and Y27632 on IL-6 and TNF-α was measured using ELISA.

The effects of atorvastatin on IL-6 (Figure 19 A) and TNF-α (figure 19 B) was not significant.

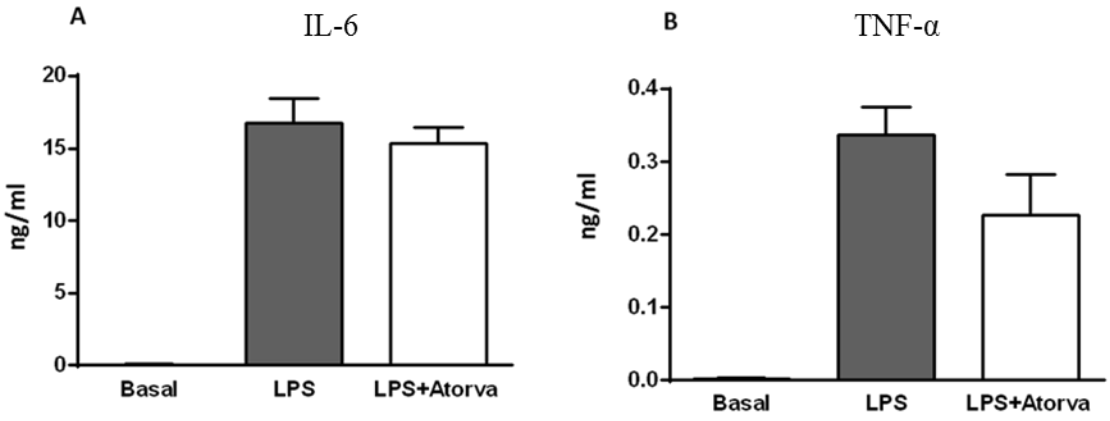

Figure 19: Effect of atorva on IL-6 and TNF-α. BMDM were pretreated in absence or presence of 10 μM atorva or vehicle for 15 min prior to the addition of 10 ng/ml LPS. After 6 hours supernatants were harvested and IL-6 (A) and TNF-α (B) secretion was measured by ELISA as described in the materials and methods section. Data are represented as histograms and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results.

Similarly, Y27632 had no significant change on IL-6 (Figure 20 A) and TNF-α (Figure 20 B) secretion. More experiments should be done to determine the effect of atorvastatin in inflammation.

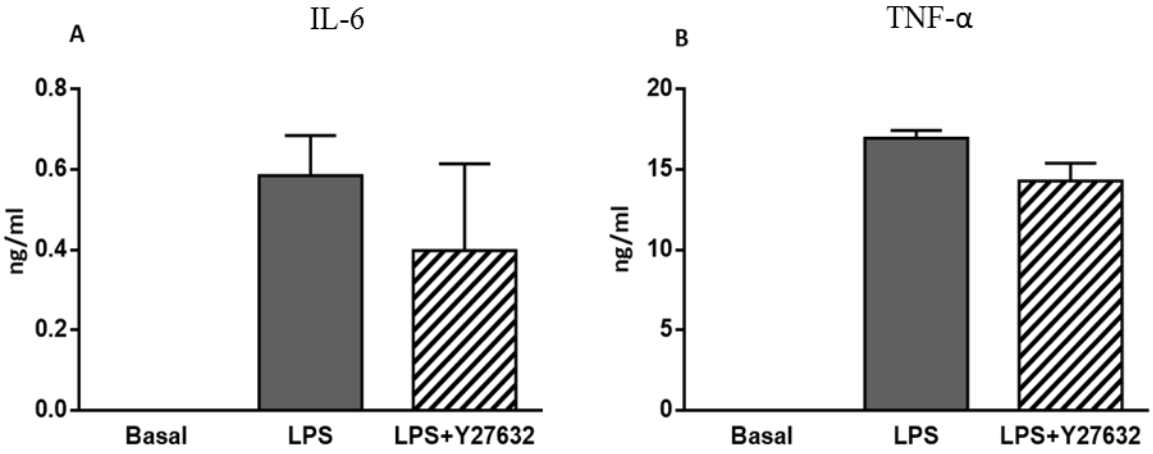

Figure 20: Effect of Y27632 on IL-6 and TNF-α formation in BMDM. BMDM were pretreated in absence or presence of 10 μM Y27632 or vehicle for 15 min prior to the addition of 10 ng/ml LPS. After 6 hours supernatants were harvested and IL-6 (A) and TNF-α (B) secretion was measured by ELISA as described in the materials and methods section. Data are represented as histograms and are expressed as mean ± SEM of one experiment performed with 5 replicates and is representative of 3 experiments with similar results.

After these results, we wanted to examine the effects of both inhibitors and statins together on the inflammation in BMDM. Y27632 showed no significant decrease in IL-6 (Figure 20 A) nor TNF-α (figure 20 B) in the presence of LPS. However, when BMDM were treated with SAR in presence of LPS and Y27632 the results showed an interesting decrease in IL-6 levels (Figure21 A) and TNF-α (Figure 21 B) in presence of SAR and Y27632 compared to SAR and LPS alone. This suggests that the anti-inflammatory effect of Y27632 was more effective and significant when inflammation was stronger. More experiments should be done in the future to examine the anti-inflammatory effects of Y27632 in BMDM and in relation to autophagy or PI3K inhibition.

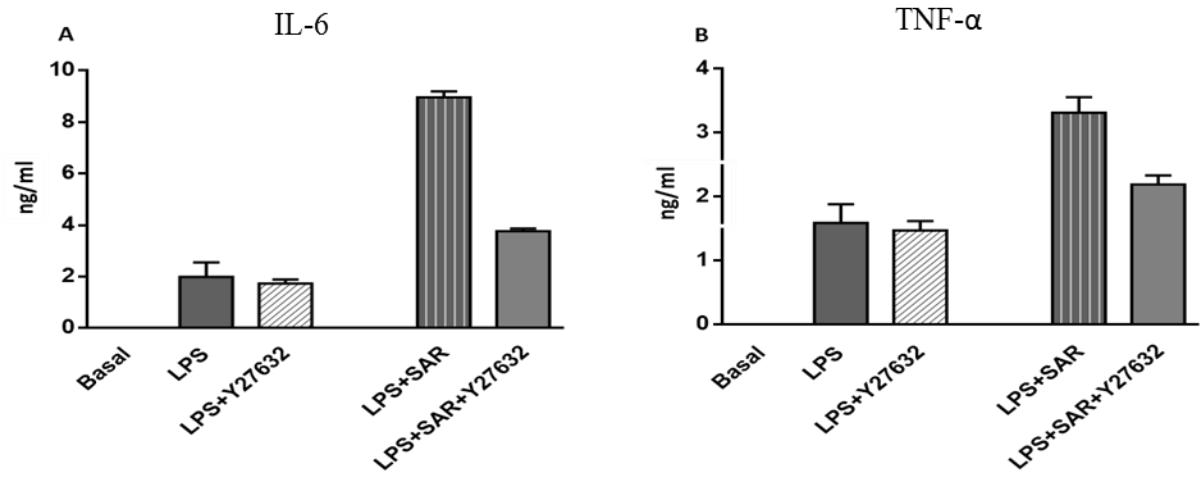

Figure 21: Effect of SAR and Y27632 on IL-6 and TNF-α formation in BMDM. BMDM were pretreated in absence or presence of 1 μM SAR or vehicle for 15 min prior to the addition of 10 μM Y27632 for 15 min followed by 10 ng/ml LPS. After 6 hours supernatants were harvested and IL-6 (A) and TNF-α (B) secretion was measured by ELISA as described in the materials and methods section. Data are represented as histograms and are expressed as mean ± SEM of one experiment performed with 5 replicates.

CHAPTER V

DISCUSSION

In the current work, we studied the effect of autophagy inhibition in macrophages (BMDM) using selective autophagy inhibitors. We elected to use as a model of murine primary macrophages bone-marrow derived macrophages in culture. Different types of primary macrophages are generally tested to assess mechanisms in inflammation. These include elicited peritoneal macrophages, blood monocyte-derived macrophages, or bone-marrow derived macrophages. During inflammation (except in the brain), the blood and the bone-marrow are the predominant source of macrophages during inflammation. Except the brain, they infiltrate the different tissues (Schilling, Besselmann et al. 2003). Our results show that LPS created a pro-inflammatory state in the cultured cells where many inflammatory mediators such as IL-6, TNF-α, PGE_2 and nitrite were increased. Previous studies have shown that 3-methyladenine, a non-selective inhibitor of PI3Kinase, increased IL-6 and TNF-α levels in macrophages induced by lipopolysaccharide (LPS) where autophagy was inhibited by 3-methyladenine (Ren, Zhang et al. 2017). However, in our system, preliminary experiments showed the opposite with an inhibition of cytokines by 3-methyladenine. Thus, a need for other inhibitors was urgent when assessing the involvement of autophagy. Recently, selective inhibitors of VPS34 were made available as blockers of autophagy.

Our results show a pro-inflammatory state in macrophages activated with LPS, demonstrated by an additional increase in the inflammatory mediators (IL-6, TNF-α, PGE_2 and nitrite) in presence of VPS inhibitors (VPS34IN-1 and SAR). They pointed towards the

relation between autophagy and cytokine secretion. In parallel to our results, several recent studies have demonstrated that autophagy plays a key role in cytokines secretion (Harris 2011, Deretic, Jiang et al. 2012) and that autophagic proteins can regulate the inflammatory mediators and affect cytokine production in macrophages (Hosogi, Kusuzaki et al. 2014). Indeed, we cannot rule out a non-selective effect of the 2 inhibitors, modulating regulatory systems or transcription factors involved in blocking the synthesis of these proinflammatory cytokines but also cyclooxygenase and NO synthase expression. Mice with selective deletion of ATG5 in the myeloid lineage showed an increase of the inflammatory status in isolated macrophages in the presence of LPS and *in vivo* as assessed by liver inflammation and fibrosis (Lodder et al, 2015). All these results support that autophagy has an important anti-inflammatory function.

In correlation with this, several studies have indicated that autophagy plays an essential role in acute and chronic inflammatory diseases (Qian, Fang et al. 2017). Zhang et al. and Yuan et al. Have demonstrated that macro-autophagy plays a crucial role in chronic obstructive pulmonary disease (COPD) which is a chronic airway inflammatory disease that deteriorates lung function progressively (Wang 2016). This was determined by increased expression of LC3-II autophagy marker in COPD patients compared to non-COPD patients (Zhang, Yin et al. 2016, Yuan, Chang et al. 2017). In our study, we also assessed the effect of autophagy inhibitors (SAR and VPS34IN-1) using western blot analysis, the appearance of LC3-I band indicated that autophagy was diminished in treated cells. In parallel, cytokines levels were increased in these cells, which confirms the anti-inflammatory role of autophagy.

In this study, we demonstrated that autophagy plays an essential role in controlling the inflammatory state of macrophages, which are essential players in the immune system. Since statins, known cholesterol-lowering drugs, are anti-inflammatory and since autophagy has anti-inflammatory functions, we tested the effect of atorvastatin and Y27632, another inhibitor of small G-protein RhoA, a downstream player of the mevalonate pathway, on inflammation in BMDM. Several studies have shown that statins have cholesterol independent effects termed pleiotropic effects. These include: decreasing oxidative stress, increasing the stability of atherosclerotic plaques through enhancing the endothelial function caused by reactive oxygen species, and many other functions. In our study, atorvastatin increased the expression of Mgl-1, which is a known anti-inflammatory marker. However, Y27632 didn't affect its expression. When we assessed the effects of atorvastatin and Y27632 on the expression of autophagy markers: Beclin-1 and Atg5 there was no significant effect on the expression of both autophagy genes. A recent study has shown that atorvastatin, when given in vivo, has increased autophagy which was shown by a decrease in Beclin-1 gene expression in addition to other markers (Sabe, Elmadhun et al. 2014). We also investigated the effects of atorvastatin and Y27632 on cytokines levels (IL-6 and TNF-α), and we found that atorvastatin had no significant effect in lowering cytokines compared to the control. Similarly concerning the effect of Y27632. These results may be due to the pro-inflammatory state of the cells already induced by LPS or due to other factors related to animal's conditions. Our laboratory has shown an anti-inflammatory effect of statins *in vivo* in mice in an inflammation model (G. A. El-Achkar, M.F. Mrad, A.A. Jaffa, R. Motterlini, E. Hamade, A. Habib, Heme oxygenase-1 – dependent anti-inflammatory effects of atorvastatin in zymosan-injected subcutaneous air pouch in

C57BL/6 mice. Manuscript in preparation). Conversely, another study has shown that another statin (simvastatin) has significantly decreased the levels of IL-6 and TNF-α in air pouch granuloma model (Hassan, Al-Gayyar et al. 2014). This indicates that statins may be more effective *in vivo* and can exert their anti-inflammatory effects through enhancing autophagy, which can be masked when assessed *in vitro* where cells are isolated from their milieu and where interactions with other cells is absent.

Our work should be completed in the future by using an animal model to be able to give a clear explanation about the anti-inflammatory role of atorvastatin in relation to autophagy. If atorvastatin decreases the levels of IL-6 and TNF-α *in vivo*, it may be an effective way to reverse or decrease diseases related to aging such as type 2 diabetes, atherosclerosis, dementia and Alzheimer's and others where inflammation is higher (Omoigui 2007). Studies addressing the effect of aging on the inflammatory response have highlighted the importance of several cytokines mainly IL-6 and TNF-α (Huang, Patel et al. 2005). These pro-inflammatory cytokines play an essential role in arbitrating cellular as well as physiological responses and were found to serve as markers for cardiovascular diseases (Cesari, Penninx et al. 2003).

Our result on the effect of Y27632 together with SAR autophagy inhibitor on inflammation on BMDM demonstrated a strong reduction of inflammation by Y27632 when the inflammation was stronger, i.e. in the presence of SAR. These results need to be confirmed with VPS34 or in macrophages from ATG5 knockout. Even though the treatment by SAR has partially revealed the anti-inflammatory effect of Y27632, more experiments are needed in this context to identify the molecular mechanism underlying Y27632 function and its relation to autophagy. Moreover, we cannot exclude the non-

selectivity effects of Y27632. The use of more selective ROCK inhibitors will be performed soon.

In overall our study has given the opportunity to study the role of autophagy in inflammation in BMDM using different inhibitors, which offers a great tool to study autophagy *in vitro* and to assess the role of other drugs in the future.

As future perspectives, ATG5 knockout model would be an important tool to get more specific results concerning the role of statins and ROCK inhibitors in autophagy. In addition, we are interested to assess the role of autophagy and inflammation in aging *in vivo* in animal models.

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