

Dissertation

**Role of adipose triglyceride lipase in macrophage
inflammation, cytoskeleton rearrangement and
cell survival**

Submitted by

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“Science progresses best when observations force us to alter our preconceptions”

Vera Rubin

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Date, ... Signature

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Summary

Infiltration of monocytes and macrophages into the site of inflammation is critical in the progression of inflammatory diseases such as atherosclerosis. Cell migration is dependent on the continuous organization of the actin cytoskeleton, which is regulated by members of the small Rho GTPase family (RhoA, Cdc42, Rac) that are also important for the regulation of signal transduction pathways.

Here we provide evidence that defective lipolysis in macrophages lacking adipose triglyceride lipase (ATGL), the major enzyme responsible for triacylglycerol (TG) hydrolysis, favors an anti-inflammatory M2 macrophage phenotype. Our data implicate that insufficient lipolysis influences macrophage polarization and actin polymerization, resulting in impaired macrophage migration. Sustained phosphorylation of focal adhesion kinase (due to inactivation of its phosphatase by elevated levels of reactive oxygen species (ROS)) results in defective Cdc42, Rac1 and RhoA activation and increased and sustained activation of Rac2. Inactivation of Rac1 and Cdc42 lead to defective polarization and directional movement toward the chemoattractants. Furthermore, RhoA inactivation may cause impaired migration and actin polymerization in the presence of chemoattractant. Inhibition of ROS production leads to focal adhesion kinase (FAK) activation transiently and restores the migratory capacity of *Atgl*^{-/-} macrophages.

Macrophage apoptosis has an important implication in definition of early and late atherosclerosis. We found reduced plaque formation in model low density lipoprotein receptor (*Ldlr*^{-/-}) transplanted with bone marrow from *Atgl*^{-/-} mice. To elucidate the reason of less plaque formation, programmed cell death was investigated in primary peritoneal *Atgl*^{-/-} macrophages. We observed elevated levels of cytosolic Ca²⁺ and ROS, stimulated cytochrome c release and nuclear localization of apoptosis inducing factor (AIF). Fragmented mitochondria prior to cell death were indicative of the mitochondrial apoptosis pathway being triggered as a consequence of defective lipolysis. Other typical markers of apoptosis, such as externalization of phosphatidylserine in the

plasma membrane, caspase 3 and poly (ADP-ribose) polymerase cleavage, were increased in *Atgl*^{-/-} macrophages. In addition, Ca²⁺ depletion from ER triggers activation of ATF4/CHOP pathway that defines ER stress induction in *Atgl*^{-/-} macrophages. An artificial increase of cellular TG levels by incubating wild-type macrophages with very low-density lipoprotein almost completely mimicked the apoptotic phenotype observed in *Atgl*^{-/-} macrophages. Results obtained during the present study define a novel pathway linking intracellular TG accumulation to mitochondrial dysfunction and ER stress leading to programmed cell death in macrophages.

Zusammenfassung

Das Einwandern von Monocyten und Makrophagen in Entzündungsherde ist ein kritischer Schritt im Entstehen von Entzündungskrankheiten wie Atherosklerose. Die Zellmigration ist abhängig von der Organisation des Aktinzytoskeletts, welche durch Mitglieder der Familie von kleinen Rho-GTPase (RhoA, Cdc42, Rac) reguliert wird. Diese sind auch wichtig für die Regulation von Signaltransduktionswegen.

Hier zeigen wir, dass eine defekte Lipolyse, hervorgerufen durch das Fehlen der „Adipose triglyceride lipase“ (ATGL), das hauptverantwortliche Enzym für die Triglyzeridhydrolyse, zu einem anti-inflammatorischen M2 Makrophagenphenotyp führt.

Unsere Daten zeigen, dass unzureichende Lipolyse die Makrophagenpolarisation, als auch die Aktinpolymerisation beeinflusst, was schlussendlich zu einer verminderten Makrophagenmigration führt. Anhaltende Phosphorylierung der fokalen Adhesion Kinase (aufgrund Inaktivierung ihrer Phosphatase durch hohe reaktive Sauerstoff Spezies (ROS) Konzentrationen) resultiert in einer defekten Cdc42, Rac1 und RhoA Aktivierung und zu einer erhöhten und anhaltenden Aktivierung von Rac2. Inaktivierung von Rac1 und Cdc42 führt zu einer defekten Polarisierung und defekten direkten Bewegung in Richtung chemischen Lockstoff.

Weiters führt in Gegenwart eines chemischen Lockstoffs die RhoA Inaktivierung zu einer verschlechterten Migration und Aktinpolymerisierung. Inhibierung der ROS-Produktion führt zu einer vorübergehenden Aktivierung der fokalen adhesion Kinase (FAK) und stellt die Migrationskapazität von ATGL -/- Makrophagen wieder her.

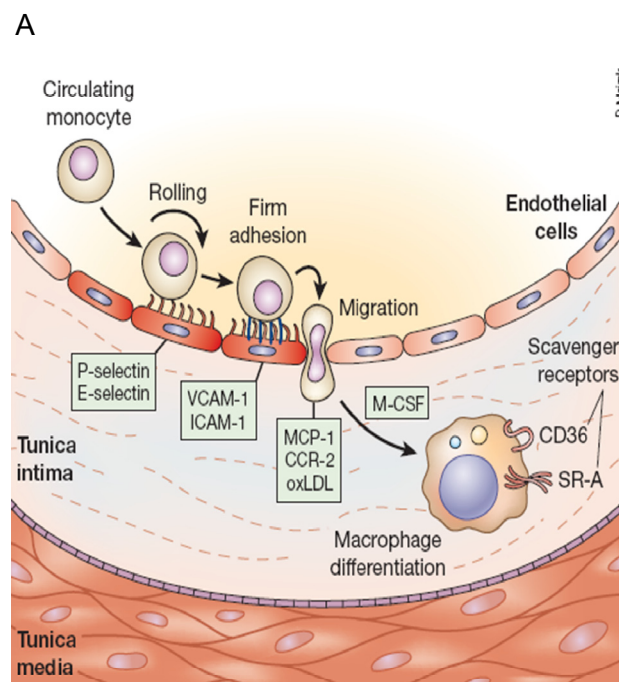
Die Apoptose von Makrophagen ist eine wichtige Implikation in der Definition von früher und später Atherosklerose. Wir fanden reduzierte Plaqueformation in Mäusen mit einem „low density lipoprotein receptor“ (Ldlr-/-) Hintergrund, denen das Knochenmark von Atgl-/- Mäusen transplantiert wurde.

Um herauszufinden, was der Grund für die reduzierte Plaqueformation ist, wurde der programmierte Zelltod von primären peritonealen Atgl^{-/-} Makrophagen untersucht. Wir beobachteten erhöhte Konzentrationen von cytosolischen Ca²⁺ und ROS, stimulierte Cytochrom C Freisetzung und nukleare Lokalisierung des „apoptosis inducing factors (AIF). Fragmentierte Mitochondrien bevor Eintreten des Zelltods ließen darauf schließen, dass der mitochondriale Weg der Apoptose aufgrund der defekten Lipolyse eingeschlagen wurde. Andere wichtige Marker der Apoptose wie Externalisierung von Phosphatidylserin in der Plasmamembran, Caspase 3 und Poly (ADP-ribose) polymerase Spaltung waren erhöht in Atgl^{-/-} Makrophagen. Weiters führt die Ca²⁺ -Entleerung vom ER zu einer Aktivierung des ATF4/CHOP Weges, was zu einer Induzierung von ER Stress in ATGL^{-/-} Makrophagen führt. Ein künstliches Erhöhen der zellulären TG Konzentration durch Inkubieren von Wild-Typ-Makrophagen mit „very low-density lipoprotein“ führt nahezu zum selben apoptotischen Phänotyp, wie jener der in ATGL^{-/-} Makrophagen zu beobachten war.

Die Resultate dieser Studie zeigen einen neuen Weg zwischen intrazellulärer TG Akkumulierung und mitochondrialer Dysfunktion sowie ER Stress, was schlussendlich zu programmierten Zelltod in Makrophagen führt.

1. Introduction

In atherosclerosis, monocytes initially are attached to lesion-prone sites. Monocytes adhere to endothelial cells by their adhesion molecules and migrate to the intima. Once they are at the site of inflammation they differentiate to macrophages. Macrophages take up modified lipids and differentiate to foam cells (Figure 1A). In early atherosclerotic lesion, phagocytosing of apoptotic cells (efferocytosis), is efficient and prompt the secretion of anti-inflammatory cytokines, preventing secondary necrosis (inducing early apoptosis) and decreasing plaque progression (Figure 1Ba). In advanced atherosclerosis, macrophages undergo late apoptosis or necrosis and efferocytosis does not occur properly. Therefore, inflammation resolution fails to occur. Moreover, in this condition the concentration of inflammatory cytokines like interleukin (IL)-6 increase, leading to formation of necrotic core plaque instability (Figure 1Bb).



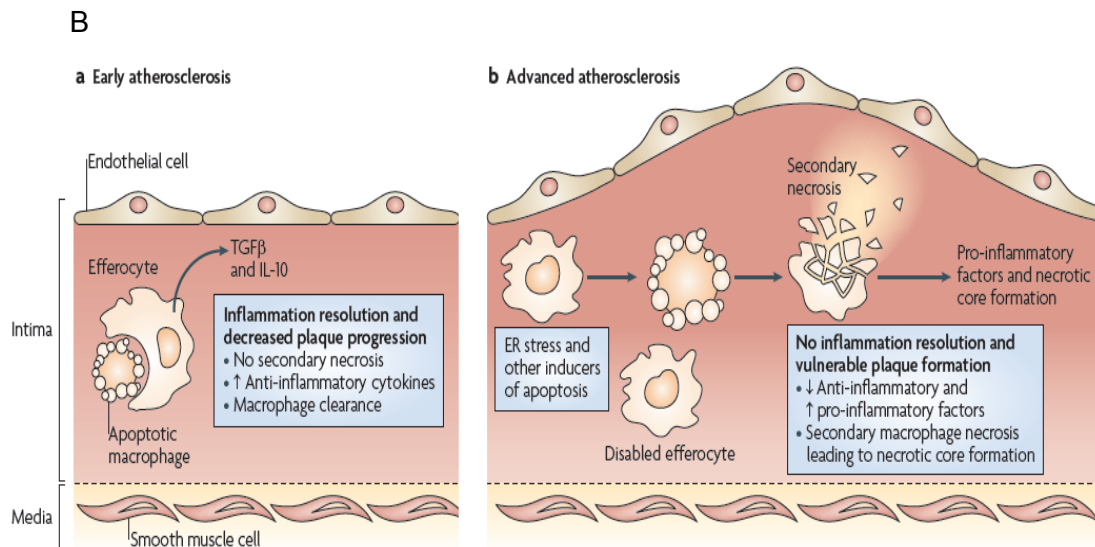


Figure 1. Role of macrophages in the progression of atherosclerosis. (A) Monocytes role and adhere on activated endothelial cells. They migrate through the gradient of chemoattractant into the site of inflammation and as soon as they are in the intima they differentiate to macrophages. **(B)** Depending on the apoptosis level, cytokine secretion and the efficiency of efferocytosis macrophages have a critical role on atherosclerosis progression (Tabas, 2010).

1.1 Macrophages and the immune system

Macrophages are widely distributed hematopoietic cells that play a vital role in cell homeostasis and defense. In innate immunity, tissue specific macrophages provide immediate defense against foreign antigens, coordinate leukocytes infiltration, and promote clearance of the tissues through phagocytosis and degradation of apoptotic and necrotic cells. Macrophages are a heterogeneous phenotypically polarized cell population consisting of classically activated (pro-inflammatory M1) macrophages, which promote IL-12-mediated Th1 inflammatory responses, and alternatively activated M2 macrophages which exert anti-inflammatory properties (Martinez et al., 2008).

Macrophage polarization is associated with distinctive gene signature. They differ in terms of receptor expression, function, chemokine and cytokine secretion (Table 1).

Classically activated macrophages are potent cells that kill microorganisms and tumor cells and produce pro-inflammatory cytokines. In contrast, M2 macrophages balance inflammatory responses and adaptive Th1

immunity, promote tissue remodeling and tissue repair (Mantovani *et al.*, 2003a).

Table.1 Essential properties of polarized macrophage population (modified from (Martinez *et al.*, 2008; Mantovani *et al.*, 2003b).

Phenotype	Classically activated (pro-inflammatory) M1 macrophages	Alternatively activated (anti-inflammatory) M2 macrophages
Receptors	TLR2, TLR4, FcγRI, II, III	Mrc1, SRA, SRB, CXCR1, CXCR2
Cytokines	IL-1, 6, 8, 12, TNFα, MCP-1, SDF-1, Grol	MCP-2, IL-4, IL-10, TGFβ, Sphk1
Chemokines	Ccl-2, 3, 5	Ccl-1
Effectors	ROS, iNOS	Arginase

1.2 Role of macrophages in progression of atherosclerosis

Atherosclerosis as a chronic inflammatory disease is characterized by abnormal thickening and hardening of the arterial walls with resulting loss of elasticity. Macrophages are critical cells in initiation and progression of atherosclerosis. Endothelial cells, as the first barrier between the blood and intima, play an important role in the recruitment of inflammatory cells by the expression of chemokines and adhesion molecules.

1.2.1 Leukocyte trans-endothelial migration

The initial step in atherosclerosis is endothelial dysfunction, followed by secretion of inflammatory cytokines and activation of monocytes. Monocytes adhere to endothelial cells via adhesion molecules. Leukocyte trans-endothelial migration is induced by soluble chemokine gradients (van Buul *et al.*, 2002), but the gradient will be rapidly washed away by the blood stream. Therefore it is known that leukocytes respond to the chemokines, which are immobilized on the surface of endothelial cells, such as stromal cell-derived factor-1 (SDF-1/CXCL12) (Cinamon *et al.*, 2001).

Initial attachment of leukocytes (tethering) to the endothelium is mediated by interaction of adhesion molecules (selectins) with their ligands. One of the most important selectins, which is expressed in most leucocytes, is L-selectin mediating rolling of leukocytes over the endothelium (McEver, 2002). P-selectin glycoprotein ligand-1 (PSGL-1) mediates leukocytes interaction with P- and E-selectin on endothelial cells and also supports their rolling (Figure 1a).

The next step of leukocyte extravasation is the firm attachment (arrest) on the surface of activated endothelial cells, the interaction of vascular cell-adhesion molecule 1 (VCAM1) with $\alpha 4\beta 1$ integrin (very late antigen 4, VLA4) and intracellular adhesion molecule 1 (ICAM1) with $\beta 2$ integrins (leukocyte function associated antigen-1, LFA1) occur on the leukocytes (Figure 1b).

In the final step of this process leukocytes must transmigrate between or across the endothelial cells (Garrido-Urbani *et al.*, 2008) and successfully cross the junctions they have to pass or block the adhesions. Connection of endothelial cell-to-cell junctions included a large number of proteins like vascular endothelial (VE)-cadherins, CD99 and platelet endothelial cell adhesion molecule (PECAM-1) (Figure 2c).

Moreover, it is known that reactive oxygen species (ROS), one of the most important factors which help leukocytes to cross the endothelial barrier, trigger loss of cell-to-cell junction (van Buul & Hordijk, 2004). Inflammatory cytokines enhance the permeability of the endothelial monolayer by altering intracellular junctions and cell attachments to the extracellular matrix (Campos *et al.*, 2009). At this step, the presence of macrophage colony stimulating factor (M-CSF) induces monocytes differentiation into macrophages.

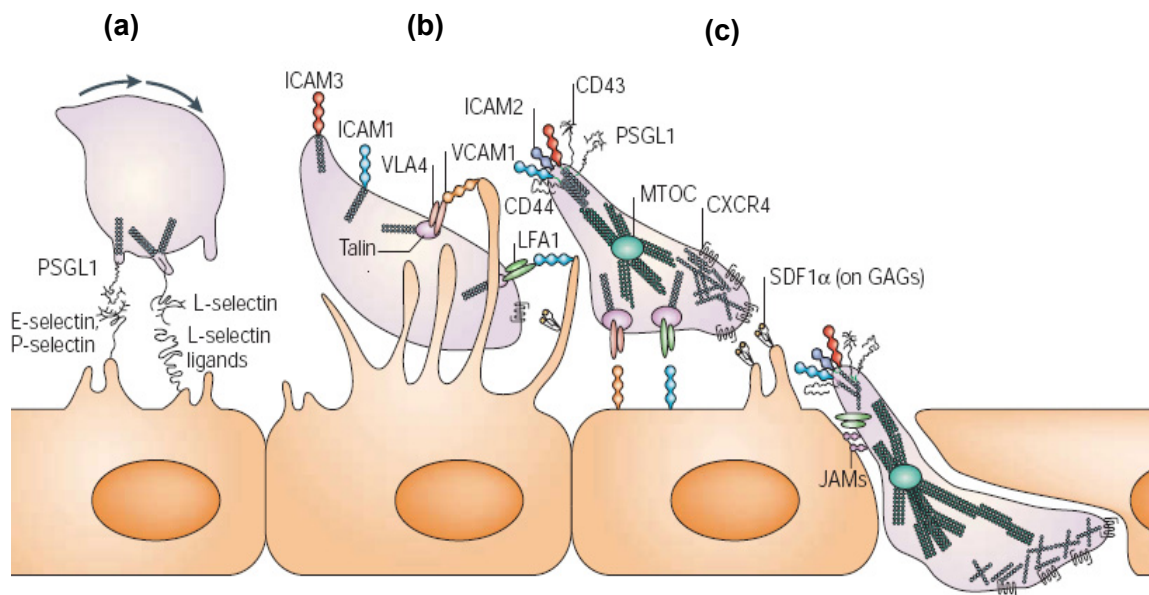


Figure2. Leukocytes extravasation into tissues. Tethering/rolling of leukocytes over the endothelial cells occurs through interaction between L-selectin and PSGL1 with their ligands on the activated endothelial cells. Before firm adhesion VLA-4 and LFA-1 interact with their ligands VCAM1 and ICAM1 in the endothelial cells (Vicente-Manzanares & Sanchez-Madrid, 2004).

1.2.2 Macrophage spreading and migration

Monocytes/macrophages alter their morphological feature from the round shape to the spreading form. The intracellular signaling pathway in macrophage spreading, however, is poorly understood. Migration following by spreading is defined by making new adhesions under leading edge, having stable adhesions under the cell body and breaking adhesions at the trailing edge (Moissoglu & Schwartz, 2006) (Figure 3).

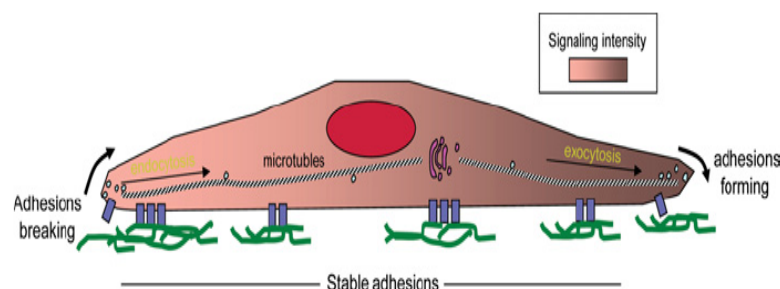


Figure 3. Persistence migration in macrophages. Migrating cells make new adhesion under leading edge, have stable adhesions under the cell body and break adhesions at the trailing edge.

Integrins also act as receptors for extracellular matrix proteins, transduce biochemical signals and regulate spreading and migration. Focal adhesion kinase (FAK) is known as an important protein involved in cell spreading (Cary et al., 2002). FAK is an integrin-binding non-receptor tyrosine kinase, which upon integrin ligation autophosphorylates its Y397 residue thereby allowing binding of Src family kinases. The consequence of activated FAK is the phosphorylation of several tyrosine residues of FAK like Y576 and Y577 (Parsons, 2003; Brown *et al.*, 2005). The major regulator of FAK phosphorylation dynamic is Src homology-2 domain (SHP-2), which is a tyrosine phosphatase. Phosphorylation of SHP-2 at Tyr580 releases its autoinhibition, thereby stimulating its phosphatase activity (Lu et al., 2001).

Another important protein is paxillin; its phosphorylation localizes FAK to focal adhesion. Paxillin plays additional roles downstream of FAK in the regulation of cell spreading (Cary *et al.*, 2002). Also, activation of FAK with various small GTPase proteins enables changes in FAK activity leading to alterations in the polymerization and stabilization of actin and microtubule filaments.

Microfilaments (F-actin) control membrane plasticity and cell motility. Actin polymerization leads to the initial extension of the plasma membrane at the cell front, thereby regulating the cell morphology through contraction and relaxation and generating a mechanical force required for moving the cell body forward (Lauffenburger & Horwitz, 1996). As regulators and coordinators of actin and microtubule cytoskeletal dynamics and adhesion, Rho GTPases have essential roles in the movement of phagocytic cells, such as macrophages. Rho GTPases included Rho, Rac and Cdc42. These GTPase proteins cycle between GDP-bound inactive and GTP-bound active states (Figure 4).

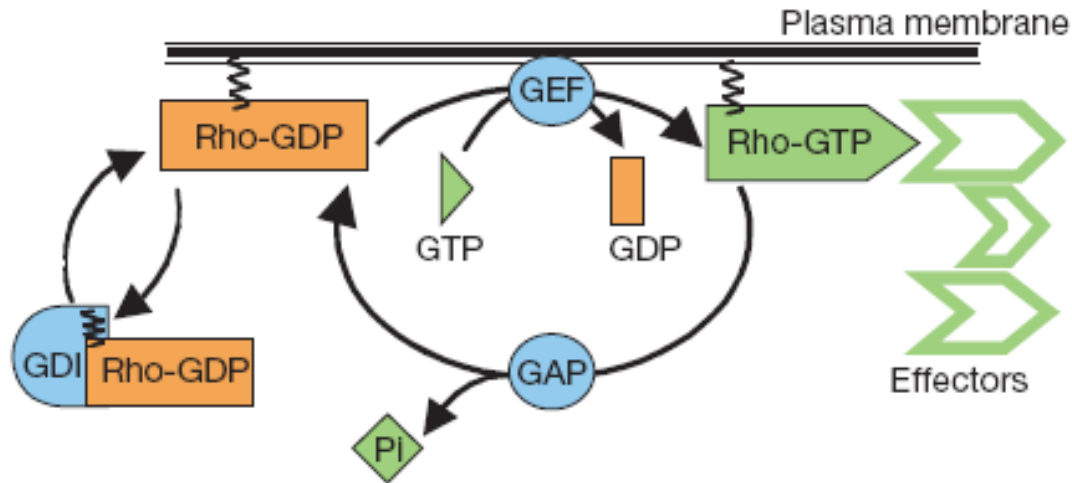


Figure 4. The Rho GTPase regulatory cycle. Rho GTPase activation cycles are modulated by three class proteins:

- i. GEFs (nucleotide exchange factors) assist the exchange of depleted nucleoside diphosphate to fresh nucleoside triphosphate.
- ii. GDIs (GDP dissociation inhibitors) sequester the GDP-bound GTPase and prevent dissociation of GDP and their interaction with the target.
- iii. GAPs (GTPase activating proteins) stimulate GTP hydrolytic activity of GTPases resulting in their conversion to the inactive form (Etienne-Manneville & Hall, 2002).

1.2.2.1 Rho GTPase proteins

Cdc42 is one of the most important GTPase proteins within leukocytes. Different studies have been shown that inhibition of Cdc42 by using dominant-negative mutants does not inhibit migration, however, cells neither polarize nor move directionally toward a gradient of chemoattractant (Jones *et al.*, 1998). Moreover, Cdc42 is required to sense the gradient of chemoattractants, cell polarization and maintaining the cell at the leading edge (Li *et al.*, 2003).

Rac1 proteins play a central role in cell migration by inducing the lamellipodia extension. Three isoforms of Rac (Rac1, Rac2 and Rac3) have been described. Rac3 is not expressed in macrophages. Rac1 is ~4-fold more expressed than Rac2 in murine macrophages (Yamauchi *et al.*, 2004)

Rho GTPase proteins include three isoforms in mammalian cells: Rho A, B and C. Rho proteins are important for cell migration by regulating actomyosin contractility and actin polymerization. Constitutively active RhoA leads to formation of stress fibers. A high level of stress fibers suppress migration because the cell contact and lamellipodium extension is inhibited (Ridley, 2008) (Figure 5).

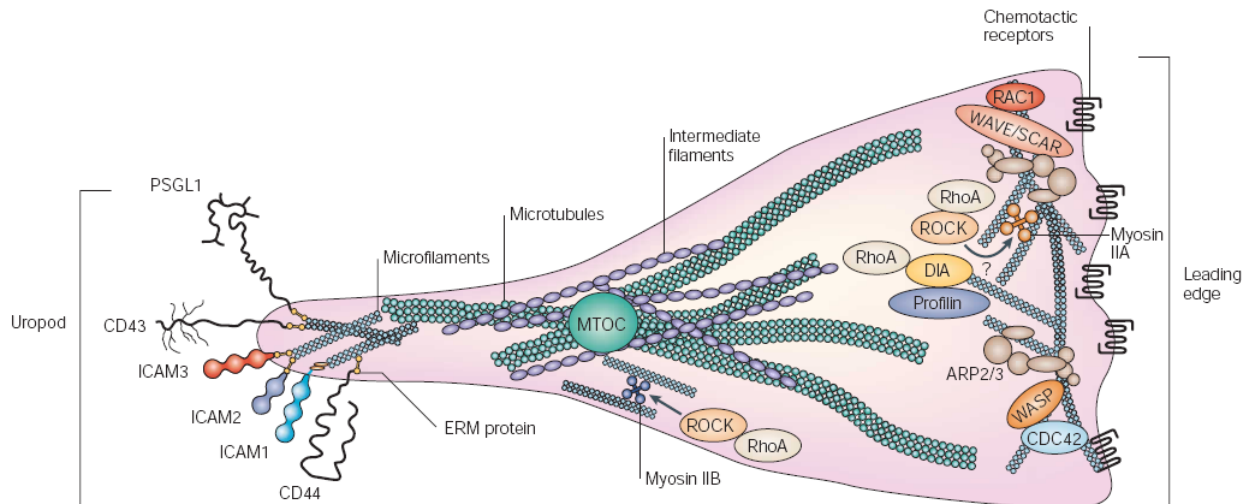


Figure 5. Chemoattractant signal transduction in leukocytes polarization and migration.

After binding of the chemoattractants to their G-coupled protein receptors phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) production is stimulated by phosphatidylinositol (3,4,5)-trisphosphate kinase (PI₃K) in the membrane. Accumulation of PIP₃ leads to activation of Rho GTPase protein Rac1 at the leading edge. Rac1 activation regulates the remodeling of the actin cytoskeleton to allow cell protrusion. In this pathway Cdc42 is also activated and regulates cell polarization. Moreover, Cdc42 is important to localize RhoA at the trailing edge which is required for uropod contraction (Vicente-Manzanares & Sanchez-Madrid, 2004; Barber & Welch, 2006).

1.2.2.2 Rac2 in phagocyte cells

Phagocytic cells including neutrophils and macrophages play critical roles in host defence, contributing to the innate immune system. Neutrophils and macrophages migrate chemotactically to the site of microbial infection and ingest invading bacteria by phagocytosis. ROS are produced during this process via activation of NADPH oxidase, which kills the microorganism alone or with the help of other proteases. In the presence of chemoattractant and phagocytic cup, NADPH oxidase assembles at the membrane and becomes active. It is known that murine *Rac2*^{-/-} neutrophils (Roberts *et al.*, 1999) and macrophages (Yamauchi *et al.*, 2004) show significant reduction of superoxide production after stimulation with phorbol esters as chemoattractants. These data indicate the importance of Rac2 as the crucial “molecular switcher”, which regulates ROS production during the innate immune response (Figure 6).

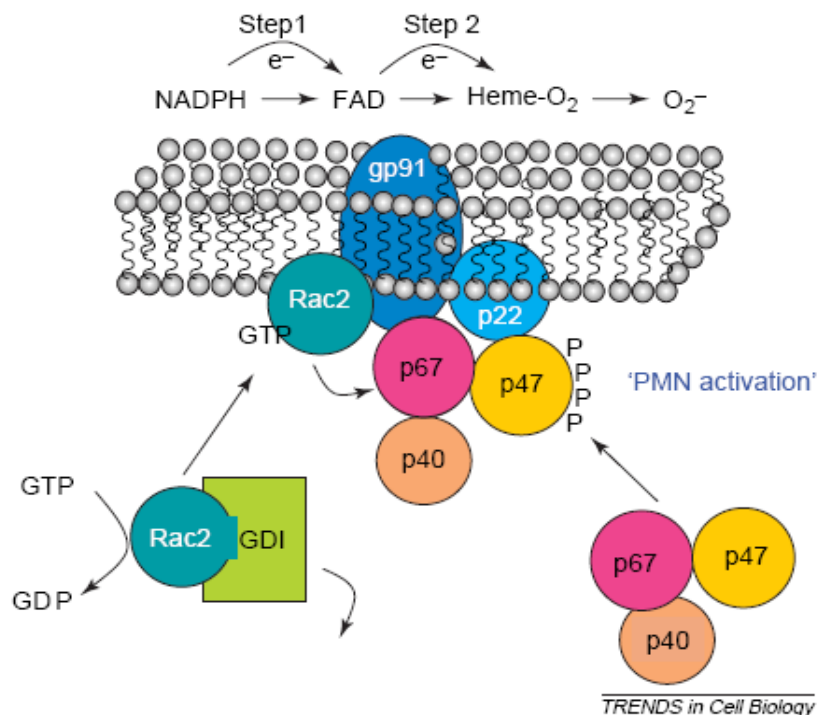


Figure 6. NADPH oxidase assembly in phagocytic cells. When phagocytosis is activated, the cytosolic proteins (Rac2, p47_{phox} and p67_{phox}) assemble with membrane-associated cytochrome b (cyt b) to form the active site of the enzyme. p67_{phox} binds to Rac2, whereas Rac2 interacts with cyt b. The Rac2 induces NADPH oxidase activity and coordinates translocation of the p47_{phox} - p67_{phox} complex (Bokoch & Zhao, 2006).

Taken together, Rac2 redistributes from the cytosol and perinucleus to the cell periphery, where it localizes just close to F-actin with little overlap.

1.3 Macrophage activation in the site of inflammation

Once monocytes contact with M-CSF, they differentiate to macrophages. Macrophages subsequently take up the modified-low density lipoprotein (LDL) like oxidized (ox-LDL) or acetylated-LDL (ac-LDL) via their scavenger receptors, SRA and CD36. Afterwards ox-LDL is delivered to the lysosome, where its cholesterol ester (CE) is hydrolyzed to free cholesterol (FC) and free fatty acids (FFA). It is known that FC is toxic in macrophages (Li & Glass, 2002). FC undergoes re-esterification by acyl coenzyme A:acylcholesterol acyl transferase (ACAT) and its function is opposed by neutral cholesterol esterases (NCEH), which hydrolyze CE (Li & Glass, 2002). Lipid-laden macrophages are called foam cells. These foam cells are filled with lipid droplets (LDs), which represent the dynamic organelles for the storage of neutral lipids (Guo *et al.*, 2009). Both FA and FC are mobilized from LDs by intracellular lipases. When

lipids over-accumulate in nonadipose tissues, FA enter dangerous pathways, such as ceramide production and apoptosis induction. Apoptosis in foam cells has pivotal effects in the progression of atherosclerosis. Increased macrophage apoptosis in early lesion is associated with smaller lesion size and less plaque progression (Babaev *et al.*, 2008).

1.3.1 Lipolysis in macrophages

Lipases are involved in adipocytes lipolysis; are adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacyl glycerol lipase (MGL) (Figure 6). Here we have introduced a model, which is not completely known in macrophages.

Adipose triglyceride lipase (ATGL) catalyzes the initial step of triglyceride (TG) hydrolysis in most tissues, generating diacylglycerol (DG) and FFA (Zimmermann *et al.*, 2004). ATGL deficiency in mice results in 80 % reduction in TG hydrolase activity and consequently in TG accumulation in cardiomyocytes leading to heart failure and premature death (Haemmerle *et al.*, 2006). It is known that CGI58 works as a cofactor of ATGL in adipocytes. Moreover, a mutation in the human ATGL gene has been found to cause neutral lipid storage disease with myopathy (Fischer *et al.*, 2007). Notably, the same mutation was observed in a patient who underwent cardiac transplantation because of cardiomyopathy (Hirano *et al.*, 2008). We have shown that the absence of ATGL leads to accumulation of TG-rich LDs and impaired phagocytosis in macrophages (Chandak *et al.*, 2010b).

Hormone sensitive lipase (HSL) is an enzyme, which plays an important role in the hydrolysis of CE stores in macrophages. Also it is known that HSL is a rate-limiting enzyme for (DG) hydrolysis (Haemmerle *et al.*, 2002)

Monoacyl glycerol lipase (MGL) is an important enzyme, which hydrolyzes DG to MG.

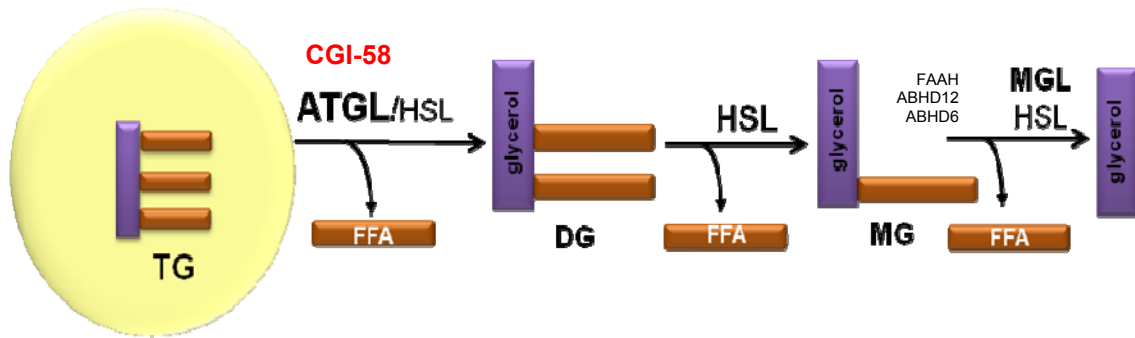


Figure 7. Lipases in macrophages Our hypothesized model of macrophage lipolysis includes in 3 steps; In the initial step, TG is mainly hydrolyzed by ATGL releasing of DG and FFA. Second step is regulated by HSL, which hydrolyses DG and produces another FFA. The last FFA and glycerol are released by a MG hydrolase, most possibly MGL. These metabolites are released and transported to the circulation for delivery to other tissues. The liberated FA are transported into many cell types where they undergo several fates including β -oxidation for ATP production and stored as phospholipids.

1.3.2 Sphingomyelin / Ceramide pathway

FFA which are produced in foam cells, because of increase in lipolysis, may enter another pathway to produce ceramide. Ceramide is synthesized by 3 pathways (Figure 8): In the *de novo* pathway, where 3-ketosphinganine is synthesized from L-serin and palmitoyl-CoA by serine palmitoyltransferase (SPT) and ceramide is produced by saturation of dihydroceramide. In the salvage, pathway ceramide is synthesized by ceramide synthase (Lass family proteins) from sphingosine. A third pathway includes sphingomyelinases (acid, neutral or alkaline) (SMase), which hydrolyzes sphingomyelin yielding ceramide. In sphingolipid biosynthetic pathway ceramide can be also phosphorylated by ceramide kinase (CerK).

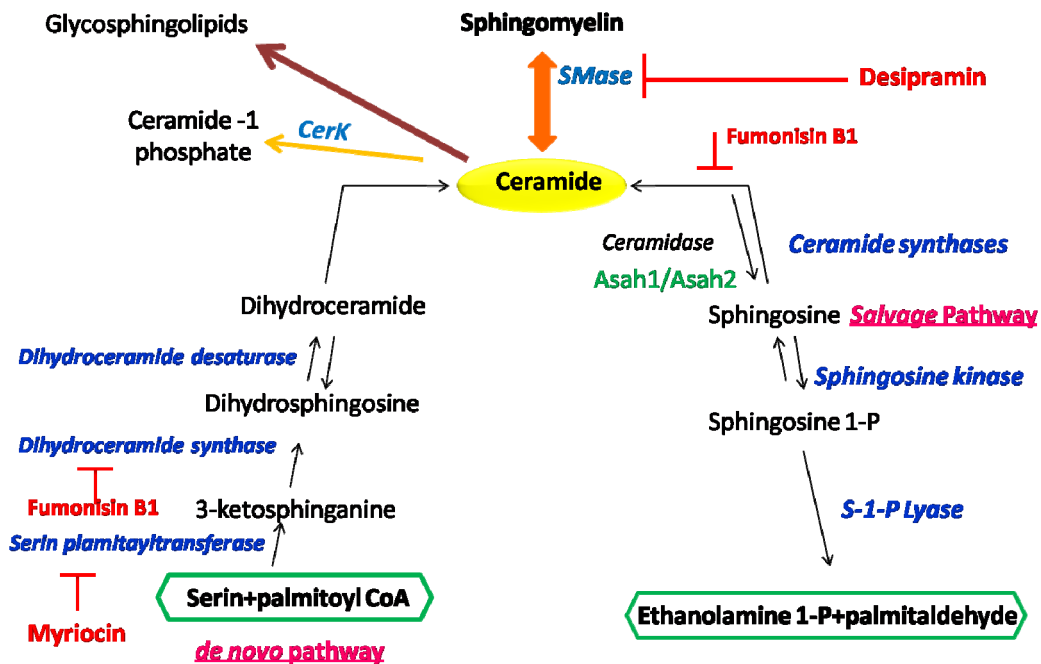


Figure 8. Ceramide biosynthesis via *de novo*, salvage and SM pathways. (Liu *et al.*, 2006)

Till date six different ceramide synthases (known as Lass family proteins) have been identified to catalyze the generation of dihydroceramides or ceramides. These ceramide synthases show distinct bias for the different FA-CoA substrates and thereby, they generate different ceramides with unique *N*-linked FA. For instance, CerS1 (Lass1) regulates the synthesis of C18-ceramide, whereas the CerS5 and 6 regulate the production of C16-ceramide and CerS 2,4 result in formation of C24-ceramide but CerS3 which is specific in testis manage the production of C18 ceramide. (Pewzner-Jung *et al.*, 2006).

1.3.3. Subcellular localization of sphingolipids

De novo synthesis of ceramide occurs in endoplasmic reticulum (ER), and then ceramide is transported to the Golgi by ceramide transfer protein (CerT), which is the site of synthesis of sphingomyelin, glycosphingomyelin and importantly ceramide-1 phosphate. Deliveries of sphingomyelin and glucosphingolipids to the plasma membrane appear to occur by vesicular particles. Acid SMase is present in the outer membrane of the leaflet and neutral SMase is in the inner leaflet of the bilayer and metabolizes sphingomyelin to ceramide. Membrane sphingolipids proceed through the

endosomal pathway where they are degraded by the action of SMases and form ceramide. Ceramide is hydrolyzed by acid ceramidase to produce sphingosine, which can leave the lysosome. Therefore, it is suggested that the sphingosine kinase (SK1) at or near the lysosomes is required to trap the sphingosine for phosphorylation. Subsequently, dephosphorylation by s1p phosphatase in the ER would recycle the salvaged sphinganine to ceramide (Hannun & Obeid, 2008) (Figure 9).

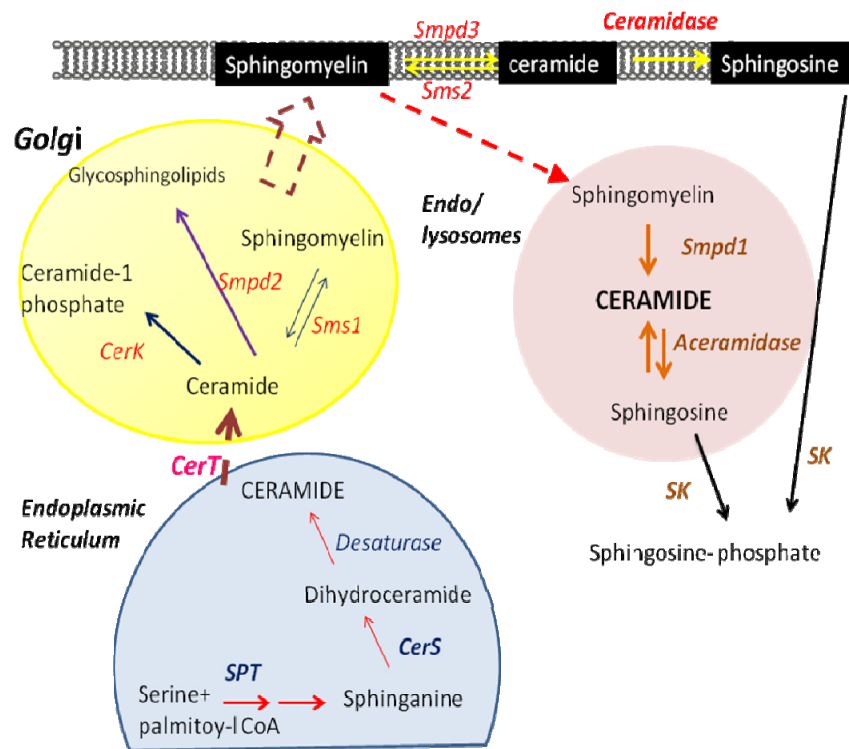


Figure 9. Subcellular localization of bioactive sphingolipids (Nikolova-Karakashian & Rozenova, 2010).

1.4 Macrophage apoptosis

Apoptosis consists of a well-organized sequence of morphological events. The cell first undergoes condensation of nucleus and cytoplasm with blebbing of the plasma membrane. Ceramide is known as a central molecule in sphingolipid metabolism, which mediates anti-proliferative responses such as growth inhibition and apoptosis. Ceramide, sphingosine and sphingosine-1-phosphate became known as important bioactive lipids and acid ceramidase plays a critical role in keeping a balance between their pro- or anti-apoptotic functions (Ogretmen & Hannun, 2004b).

1.4.1 Apoptosis via mitochondrial dysfunction

Mitochondria is an important membrane-closed organelle, which generates most of the source of chemical energy used as adenosine triphosphate (ATP). During apoptosis via the mitochondrial apoptosis pathway, the process of mitochondrial outer membrane permeabilization (MOMP) seems to be very important. MOMP: (a) releases pro-apoptotic proteins (b) destroys mitochondrial function (c) induces ROS production as a consequence of (a) and (b). Kuwana *et al.* have shown that MOMP by Bcl-2 family proteins requires neither the mitochondrial matrix inner membrane nor other proteins like Bid, which can activate Bax to open the membrane and release high molecular weight dextran. These data explain the high rate of protein translocation from mitochondria during apoptosis (Kuwana *et al.*, 2002).

An important factor in ATP production and mitochondrial protein import is the mitochondrial transmembrane potential ($\Delta\Psi_m$). The disruption of $\Delta\Psi_m$ suggests that the inner membrane permeability has been affected during cell death. Moreover, a decreased rate of electron transfer leads to the reduction of mitochondrial pyruvate consumption. In the absence of oxidative phosphorylation pyruvate is converted to lactate and results in the acidification of cytoplasm, leading to cell death (Gottlieb *et al.*, 1996). Also the $\Delta\Psi_m$ reduction during apoptosis is suggested to inhibit the import of proteins synthesized in the cytosol, release of Ca^{2+} and ATP synthesis, leading to increased production of superoxide anions (Ricci *et al.*, 2003).

Respiration in mitochondria is associated with ROS production. ROS is generated by incomplete reduction of molecular oxygen during the process of oxidative phosphorylation. This is related to the activity of respiratory complex I and complex III. Figure 10 shows the electron transport chain, ATP production and ROS generation.

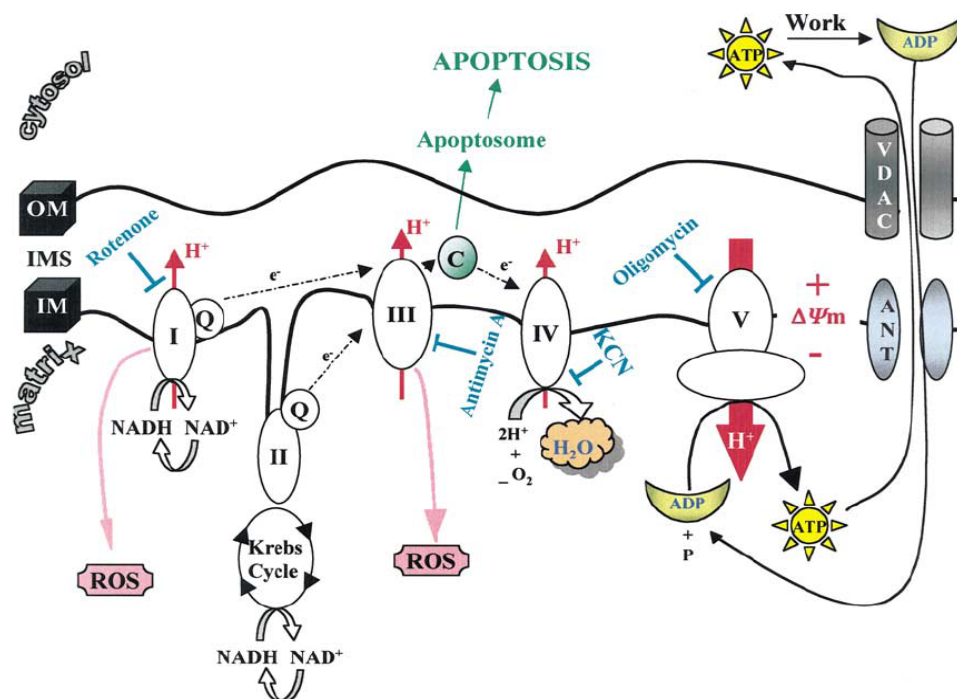


Figure 10. ATP production and ROS generation mechanism. Electron transport chain complexes (I-V) are localized in the inner membrane of mitochondria. Electrons enter complex I and II. Complex II converts the succinate to fumarate (produced by krebs cycle) and transfers an electron to coenzyme Q (Q). Cytochrome C (C) transfers that electron from complex III to complex IV. Complex IV then converts H^+ and O_2 to H_2O . Complexes I to IV (except II) pump H^+ out of the matrix to the intermembrane space (IMS). This charge distribution ($\Delta\Psi_m$) is used by complex V (ATP synthase) to convert ADP to ATP, which then is released into the cytosol.

1.4.1.1 Role of ceramide in mitochondrial dysfunction

Due to intrinsic hydrophobicity ceramide is trapped within the lysosome, unless it is broken by acid ceramidase to sphingosine, which can leave the acidic compartment. It is known that the acidic aspartate protease cathepsin D is a direct target for endosomal ceramide. After the activation of cathepsin D (32 kDa active form) by ceramide, cathepsin D is released to the cytosol and triggers mitochondrial dysfunction by cleavage of Bid (pro-apoptotic protein) thus elevating release of cytochrome C (Schutze *et al.*, 1992a) (Figure 11). Moreover, ceramide, which is generated by N-SMase, is activated by ROS produced in mitochondria. This ceramide can activate protein phosphatases-1 (PP1) and PP2A. PP2A dephosphorylates BCL2 (an important anti-apoptotic protein) and Akt, leading to apoptosis. Furthermore, PP1 acts on the pro-apoptotic protein Bid and results in release of cytochrome C (Figure 11) (Ruvolo *et al.*, 1999).

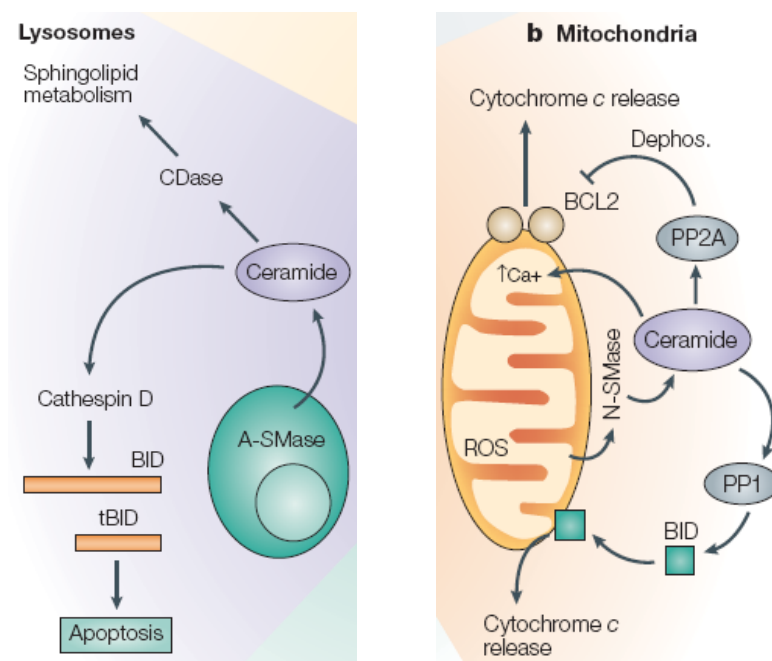


Figure 11. Effect of ceramide on mitochondrial dysfunction This figure shows the effect of ceramide in cell death in different compartment. Ceramide is produced by the action of acid SMase (A-SMase) in lysosomes or by the action of neutral SMase (N-SMase) in mitochondria. Both processes lead to activation of pro-apoptotic proteins and result in mitochondrial dysfunction (Ogretmen & Hannun, 2004a)

On the other hand, ceramides, which are produced by *de novo* or salvage pathway, are involved in ROS production. Ceramide induces the production of ROS in human endothelial cells (Li *et al.*, 2002). Some studies show that the cell permeable ceramide triggers a direct effect on mitochondria, from inhibition of respiratory chain (ROS production) to increased mitochondrial permeability (Garcia-Ruiz *et al.*, 1997). Also it has been shown that ceramide induces cytochrome C release and causes a decrease in mitochondrial membrane potential (Ghafourifar *et al.*, 1999).

1.4.2. ER stress induction in macrophages

Any disturbance occurring in Ca^{2+} storage and signaling (like folding and modifying protein synthesis, which is the main function of the ER in mammalian cells) is called ER stress. Ca^{2+} overload or depletion can result in changes in the protein machinery, protein folding and ER stress. Changes in protein folding capacity in the ER might outcome with activation of two different pathways:

First, induction of the unfolded protein response (UPR) pathway leads to activation of ER chaperons like Bip, which is known also as glucose regulated protein-78 (Grp78), Grp94, calreticulin, and the transcription factor CHOP/GADD153.

Second, the ER overload response leads to the production of cytokines through activation of NFkB (Kaufman, 2002). These pathways help to remove the unfolded proteins from the ER. In response to severe stress these pathways also contribute to eliminate apoptosis.

In mammalian cells, three ER-associated proteins are critical during the UPR; PKR-like eukaryotic initiation factor 2 α kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor-6 (ATF6). In comparison to IRE1 and PERK, which are responsible for the response to ER stress, ATF6 cleavage is required for UPR transcriptional induction. Bip negatively regulates these pathways by interaction with ATF6 and prevents its transport to the Golgi compartment. When unfolded proteins increase, they bind to Bip and reduce the available Bip to inhibit PERK, IRE1 and ATF6. Bip, which is released from ATF6, permits the transport to the Golgi apparatus. In the Golgi, ATF6 is cleaved by proteases and the fragments migrate to the nucleus and activate the transcription of XBP1 (Kaufman, 2002) (Figure 12a).

In addition, Bip is released from IRE1 and causes dimerization of IRE1 to activate its kinase and RNase activities, which lead to spliced XBP-1 mRNA (Figure 12b) (Todd *et al.*, 2008). Moreover, Bip release leads to dimerization of PERK and its phosphorylation causes eIF2 α phosphorylation to reduce the frequency of AUG initiation codon recognition and the general rate of translation is decreased. However, ATF4 mRNA is selectively translated under these conditions (Figure 12c) (Ron & Walter, 2007).

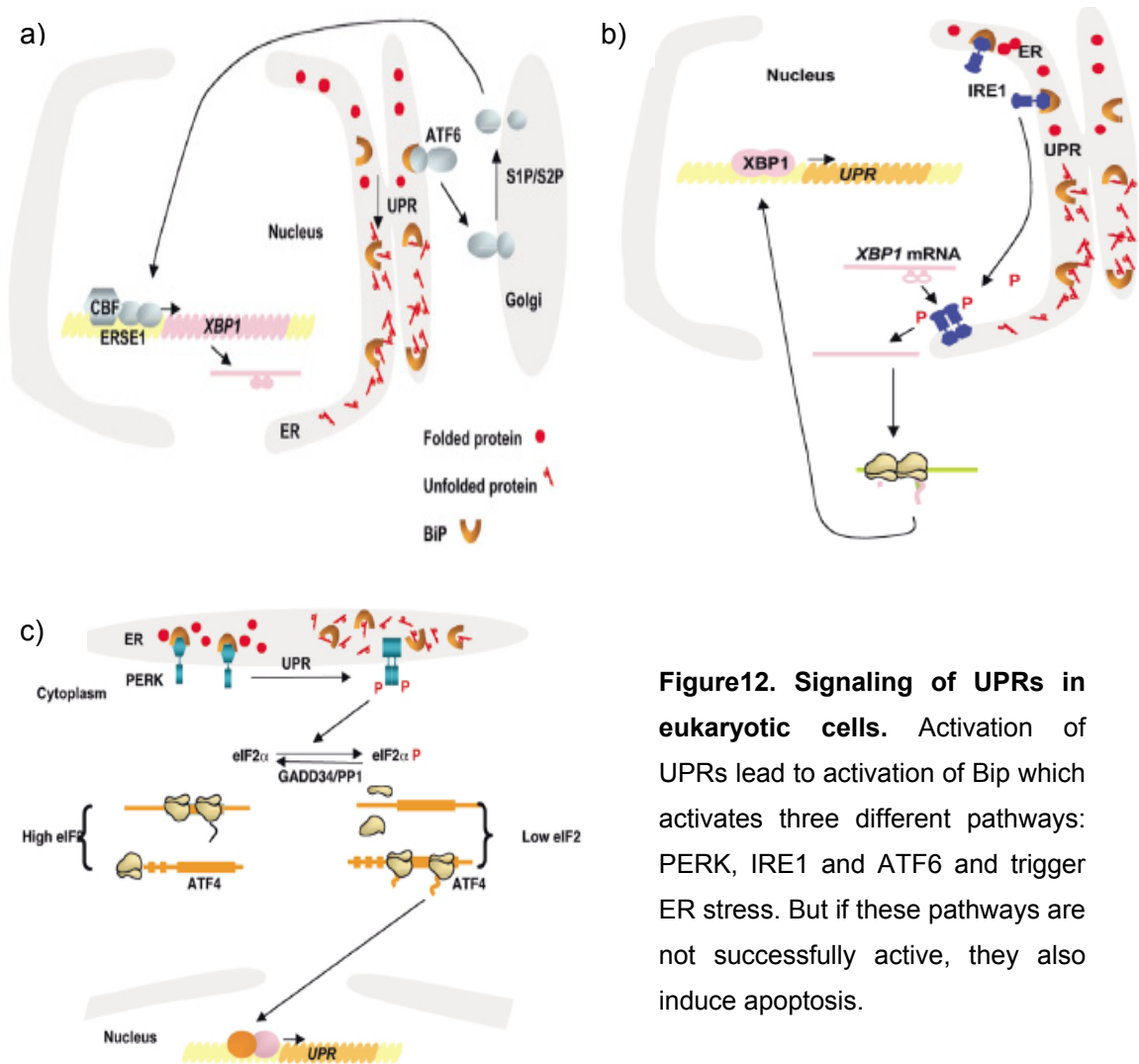


Figure12. Signaling of UPRs in eukaryotic cells. Activation of UPRs lead to activation of Bip which activates three different pathways: PERK, IRE1 and ATF6 and trigger ER stress. But if these pathways are not successfully active, they also induce apoptosis.

All these UPR arms work together to reduce protein degradation and increase folding capacity to resolve ER stress. If these pathways are not successfully active, they may also induce apoptosis. When macrophages, undergo apoptosis, PERK activation leads to an increase in CHOP level. Ca^{2+} depletion is another important factor to induce CHOP activation (Hotamisligil, 2010).

1.4.2.1 ER stress response in immunity

During ER stress, IRE1 triggers a key inflammatory signaling pathway by activation of c-Jun N-terminal kinases (JNK), which regulates transcription of many inflammatory genes (Urano *et al.*, 2000). IRE1 activation leads to the recruitment of tumor necrosis factor- α (TNF- α)-receptor associated factor-2

(TRAF-2) and this complex activates JNK and I κ B kinase (IKK) as inflammatory kinases. The only known substrate for IRE1 is XBP-1. XBP-1 was first identified because of its ability to bind to cyclic-AMP response element (CRE) in the genes encoding MHC class II HLA. The endoribonuclease activity of IRE1 leads to XBP-1 splicing. This transcriptional mRNA processing is unique and it does not use the traditional mRNA splicing mechanisms (Yoshida *et al.*, 2001).

It is known that the atherosclerosis involves inflammatory pathways, in which, macrophages play a critical role in the progression of atherosclerosis. Recent studies suggest that the UPR may have important role in atherosclerosis (Hansson, 2005). It has been shown that elevation of inflammatory cytokines like IL-6 in atherosclerotic lesions has been associated with the activation of the mitogen activated protein (MAP) kinase p38, ERK1/2 and JNK. Activation of these kinases induces the ATF4 and XBP-1 UPR pathway (Li *et al.*, 2005) (Figure 13).

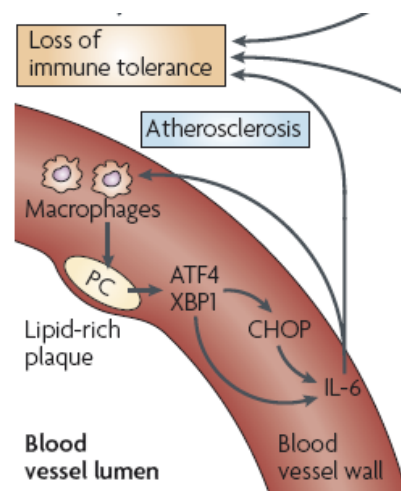


Figure 13. Defective UPR pathways in vascular epithelium lead to atherosclerosis.

2. Aims and objectives

Macrophages are important hematopoietic cells within tissues. Lipolysis has an important impact on macrophage phenotype and their role in progression of inflammatory diseases like atherosclerosis. ATGL is the rate limiting enzyme catalyzing the first step of TG hydrolysis. Till to date many studies have been done in analyzing the effect of CE and FC accumulation in macrophages. Since the effect of TG accumulation in macrophages is unknown, we have used *Atgl*^{-/-} macrophages to assess defective lipolysis in survival and macrophage function.

With this background, the aim of this thesis was to address the:

- Influence of TG accumulation on macrophage cytokine secretion
- Migration and cytoskeleton rearrangement in TG-laden macrophages
- Impact of TG accumulation on macrophage apoptosis
- Role of ceramide in ER stress and mitochondrial dysfunction in TG-laden macrophages

3. Material and Methods

3.1 3% Thioglycollate medium

0.7 g agar, 0.25 g L-cystein, 6 g glucose, 17 g peptone¹⁴⁰, 3 g peptone¹¹⁰, 2.5 g sodium chloride, 0.1 g sodium sulfate, 0,5 g sodium thioglycollate and 1000 ml ddH₂O were mixed and boiled. The mix was allowed to cool down at room temperature and stored in 50 ml aliquots at -20°C.

3.2 Diethyl pyrocarbonate water

Four hundred µl diethyl pyrocarbonate (DEPC) were mixed with 400 ml ddH₂O and thereafter kept under the hood overnight and autoclaved.

3.3 Western blot buffers and reagents

3.3.1 Lysis buffer

One M dithiotheritol, 1 mM EDTA, 0.1 M KH₂PO₄, 1:2000 protease cocktail inhibitor (Sigma) and 0.25 M sacharose were mixed and pH 7 was adjusted with KOH.

3.3.2 10X running buffer

150.1g glycin, 10 g SDS and 30.3 g Tris were adjusted to 1000 ml ddH₂O.

3.3.3 Separating gel buffer (Buffer 1)

Four ml of 10% SDS, 18,2 g Tris and 80 ml ddH₂O were mixed and pH 8.8 was adjusted up to 100 ml with ddH₂O.

3.3.4 Stacking gel buffer (Buffer 2)

Six g Tris was dissolved in 90 ml ddH₂O. pH 6.8 was adjusted with HCl and the volume was adjusted up to 100 ml with ddH₂O.

3.3.5 SDS sample buffer

2.15 g SDS ,0.76 g Tris and 45 ml ddH₂O were mixed and pH 6.8 was adjusted with HCl. Thereafter, 10 ml glycerol (80 %) and a small quantity of bromo phenol blue were added.

3.3.6 10X transfer buffer

One mg/ml EDTA, 30 g glycine and 12.1 g Tris were mixed in 1000 ml ddH₂O.

3.3.7 10X washing buffer (TBS)

Ninety g NaCl, 5 g Tween 20, 100 ml of 1 M Tris-HCl (pH 7.4) were mixed in 1000 ml ddH₂O.

3.3.8 Separating gel

Reagents	10% SDS-gel
Acrylamide	2866.25 µl
Buffer 1	2712.5 µl
ddH ₂ O	5171.25 µl
10 % SDS	100µl
TEMED	4.4 µl
10% APS	7.6 µl
Final volume	87804.4 µl

3.3.9 Stacking gel

Reagents	10% SDS-gel
Acrylamide	326 µl
Buffer 1	500 µl
ddH ₂ O	1650 µl
10 % SDS	21.5µl
TEMED	1.25 µl
10% APS	19 µl
Final volume	2517.75 µl

3.4 Animals

Atgl^{-/-} mice were generated as described elsewhere (17). Studies were performed with *Atgl*^{-/-}, *Atgl*^{+/-} mice and Wt littermates backcrossed at least 7 times on a C57Bl/6 genetic background. The mice were kept on a standard chow diet (4% fat and 21% protein; ssniff, Soest, Germany) on a regular 12h dark/light cycle. Animal experiments were performed in accordance with the standards established by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments (Vienna, Austria).

3.5 Cell culture

8-10 week old *Atgl*^{-/-}, *Atgl*^{+/-} and Wt mice were injected with 3 ml 3% thioglycolate medium and 3 days after intraperitoneal injection macrophages were harvested from the peritoneum of males for apoptosis experiments and of females for studying inflammatory and migratory capacities. Cells were washed three times with PBS and cultivated in Dulbecco's modified Eagle's medium (DMEM), 10% lipoprotein-deficient serum (LPDS), 100 µg/ml penicillin/streptomycin in the absence and presence of 100 µg/ml acetylated (ac)-low density lipoprotein (ac-LDL) for 24 h plus ACAT-inhibitor 58035 (10 µg/ml) for the last 12 h (FC-loaded macrophages) (Sandoz, Kundl, Austria) and 150 µg very low density lipoprotein (VLDL) /ml for 24 h (VLDL-loaded Wt cells). LDL and VLDL were isolated from human plasma by density gradient ultracentrifugation.

For inflammatory studies study *Atgl*^{-/-} macrophages were serum starved for 3 h. Then the macrophages were incubated in DMEM containing MCP-1 (50 ng/ml) (Peprotech, Vienna, Austria), SDF-1 (60 ng/ml) (Peprotech, Vienna, Austria) and LPA (9 ng/ml) (R&D System, Vienna, Austria) for 0, 2, 5 and 10 min.

3.6 Preparation of acetylated LDL preparation

The native LDL fraction (3.2 ml) was mixed with equal volume of saturated NaCl. This mixture was kept on ice under continuous stirring. 5.4 µl of diethyl ether was added very slowly every 3 min until a total of 57.76 µl was added. When 5.4 µl diethyl ether was added the third time, 100 µl of 10x PBS were added to avoid precipitation. This mixture was kept stirring on ice for 45 min. Thereafter, ac-LDL was dialyzed for 2-3 h with sterile PBS at 4 °C. TC concentration was measured enzymatically.

3.7 measurement of Lipid parameters

Lipids were extracted from cells with 2 ml hexane/isopropanol (3:2, v:v) for 1 h at 4°C. Lipid extracts were dried under nitrogen, redissolved in 100 µl of freshly prepared 1 % Triton-X100 (in chloroform), dried under nitrogen and resuspended in 100 µl distilled water for 15 min at 37°C. Aliquots (30 µl) were

used for enzymatic measurements of TG (Diagnostic Systems, Holzheim, Germany), TC (Greiner Diagnostics AG, Bahlingen, Germany), and FC (Diagnostic Systems, Holzheim, Germany) concentrations. Protein was quantified using a Bradford or Lowry assay (BioRad Laboratories, Vienna, Austria) after dissolving the proteins from extracted cells in 2 ml 0.3 M NaOH for 1 h at room temperature, using bovine serum albumin as a standard.

3.8 RNA isolation from primary macrophages

Total RNA was isolated according to the protocol of the Qiagen RNeasy kit (Qiagen, Hilden, Germany). Primary macrophages were lysed by freshly added 1% β -mercaptoethanol and RLT buffer (350-500 μ l). Equal amount of RNase-free EtOH was added to the filtrate, transferred to RNasy Mini Spin column and centrifuged for 15-20 s at 14,000 rpm. The filtrate was discarded and 700 μ l RW1- buffer was added to each column. After another centrifugation the filtrate was discarded and 500 μ l RPE buffer was added two times and centrifuged at 14000 rpm for 15-20 s. 30 μ l RNase-free water was added upon transfer to an RNase free tube, kept for 10-15 min at room temperature and centrifuged at 14000 rpm for 2 min. The RNA concentration was measured at 260 nm using NanoDrop (Thermo Scientific, Wilmington, USA).

3.9 cDNA preparation

cDNA was prepared from RNA by reverse transcription. Two μ g RNA in a final volume of 10 μ l were reverse transcribed by using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, USA).

High-Capacity cDNA Reverse transcription kit's master mix composition

Component	Volume (μ l)/reaction
10 X Buffer	2
25 X dNTP Mix (100 mM)	0.8
10 X RT Random Primers	2
Multiscribe reverse transcriptase	1
RNase inhibitor	0.7
Nuclease-free water	3.5
2 μ g RNA/10 μ l water	10
Total volume / reaction	20

Thermal cycle conditions for reverse transcription

	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 s	∞

3.10 Real Time PCR

cDNA was diluted 1:50 in ddH₂O. Three µl of diluted cDNA and 1 µl from each primer (forward and reverse) were mixed with Quantifast Sybr Green PCR kit (Qiagen, Hilden, Germany). Quantitative real time PCR was performed on an ABI prism 7900 real time PCR instrument (Applied Biosystems, Vienna, Austria) and the data are displayed as expression ratios of target genes normalized to the expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) for cells and cyclophilin A for tissues as internal reference in each sample. Quantitative real-time PCR data were analyzed by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001)

3.11 Protein isolation and western blotting

3.11.1 Isolation of cytosolic fractions

Mitochondria- and nucleus-free cytosolic fractions were isolated from untreated, FC- and VLDL-loaded macrophages using Benchtop mitochondria isolation kit (Mitoscience, Oregon, Eugene) according to the manufacturer's instructions. Cytosolic fractions were subjected to electrophoresis and assayed for Bax, Bcl-2 and cytochrome c by Western blotting

3.11.2 Cytoplasmic and nuclear extract

Macrophages were washed two times with PBS and scraped in 1ml PBS-EDTA followed by centrifugation at 3,000 rpm for 5 min. Pellet was resuspended in 250 µl harvest buffer and incubated on ice for 5 min. Sample was centrifuged at 1000 rpm for 10 min at 4°C. Supernatant was transferred to a new tube (cytoplasmic fraction). Cytoplasmic fraction was used for PERK, IRE1α and eIF2α western blotting.

Pellet was washed in buffer A, followed by centrifugation at 1000 rpm for 5 min at 4°C. Four times buffer C was added to get more concentrated nuclear

extract. Sample was mixed by vortexing for 15 min at 4°C and centrifuged at 14000 rpm for 10 min at 4°C. Nuclear fraction (supernatant) was transferred to a new tube. Nuclear fraction was performed for detection of CHOP, ATF4 and ATF6 by immunoblotting.

Harvest Buffer	
10 mM HEPES pH 7.9	*1 mM DTT
50 mM NaCl	*10 mM tetrasodium pyrophosphate
0.5 M Sucrose	*100 mM NaF
0.1 mM EDTA	*17.5 mM β -glycerophosphate
0.5% Triton-X100	*1 mM PMSF
	*4 μ g/ml aprotinin
	*2 μ g/ml pepstatin A`

Buffer A	
10 mM HEPES pH 7.9	*1 mM DTT
10 mM KCL	*1 mM PMSF
0.1 mM EDTA	*4 μ g/ml aprotinin
0.1 mM EGTA	*2 μ g/ml pepstatin A

Buffer C	
10 mM HEPES pH 7.9	*1 mM DTT
500 mM NaCl	*4 μ g/ml aprotinin
0.1 mM EDTA	*1 mM PMSF
0.1 mM EGTA	*2 μ g/ml pepstatin A
0.1% IGEPAL(NP40)	

*Freshly added to the buffer just before use

3.11.3 Pull down assay

pGEX-3X-Rhotekin RBD (for RhoA) and pGEX-T-PAK N (for Rac1, Rac2, Cdc42) plasmids (kindly provided by Silvio Gutkind, National Institute of Health, Bethesda, USA) were fused to GST-fusion proteins which were expressed in *E. coli* and immobilized on glutathione-conjugated agarose beads. Macrophages grown in 5-cm dishes were serum-starved for 2 h in DMEM and stimulated with LPA (9 ng/ml) for 0, 2, 5 and 10 min. The reaction was stopped

by putting the dishes on ice and washing the cells with ice-cold PBS. Cells were lysed with 500 μ l **lysis buffer** (50 mM Tris-HCl, 200 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol (DTT)) and freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 5% glycerol and 1% Nonidet P-40) and then centrifuged at 14,000 rpm for 5 min at 4°C. The cell lysates were incubated with 30 μ g of pre-chilled RBD (for RhoA) or PAK (for Rac1, Rac2 and Cdc42) glutathione agarose beads for 30 min at 4°C on a rotary shaker. The cell lysates were used for determination of the total amount of each GTPase. Samples were separated by SDS-PAGE. Proteins were transferred onto PVDF membranes (Millipore, Billerica, USA) and were blocked in TBST buffer (1 mM CaCl₂, 136 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, 0.1% (v/v) Tween-20), containing 5% non-fat dry milk for 1 h. Membranes were incubated with anti-RhoA, -Rac1, -Rac2 or -Cdc42 antibodies at 4°C overnight. After washing blots were incubated for 2 h with either HRP-conjugated goat anti-rabbit (1:4000) or HRP-conjugated goat anti-mouse antibodies. Proteins were visualized using Pierce ECL Western Blotting Substrate (Thermo SCIENTIFIC, Rockford, USA). The ratio of the active GTP-bound GTPase to total GTPase was measured using ImageJ software (NIH, USA).

3.11.4 Immunoblotting

Forty μ g of protein extract were mixed with equal volume of SDS sample buffer (5 % β -mercaptoethanol was added freshly) and boiled at 95°C for 5 min. Samples were then resolved by SDS PAGE. Proteins were transferred to nitrocellulose or PVDF membranes. The membrane was blocked in 5 % skim milk in TBS or in 5 % BSA plus 0.1% Tween 20 in TBS for 2 h at room temperature. Afterwards, the blot was incubated with the first antibody in the blocking solution for overnight at 4 °C. Primary antibody was washed out three times with TBS and the blot was incubated with second respective antibody for 2 hrs at RT. The blot was developed after extensive washing (2 h every 15 min) by incubating with ECL plus (Amersham Biosciences, Piscataway, USA) for 5 min. The AGFA Gurix Ultra X Rat film was used to expose the blot. The list of all primary antibodies and second any antibodies together with their blocking solution are listed in the table below:

Antibody	Dilution	Blocking	2 nd antibody	Size (kDa)
ATGL	1:200	5% skim milk	anti-Rabbit	58
Caspase 3	1:100	5% skim milk	anti-Rabbit	35,17
PARP	1:800	5% skim milk	anti-Rabbit	116, 85
Bcl-2	1:500	5% BSA+0.1%tween 20	anti-Rabbit	26
Bax	1:500	5% BSA+0.1%tween 20	anti-Rabbit	20
Cytochrome C	1:100	5% BSA+0.1%tween 20	anti-Rabbit	14
pFAK(Tyr576/577)	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	125
FAK	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	125
pSrc(Tyr416)	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	60
Src	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	60
pERK1/2	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	41/42
ERK	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	41/42
pSHP2	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	72
SHP2	1:1000	5% BSA+0.1%tween 20	anti-Rabbit	72
Cdc42	1:1000	5% skim milk	anti-Rabbit	21
Rac1 *	1:4000	5% skim milk	anti-mouse	21
Rac2**	1:1000	5% skim milk	anti-mouse	21
RhoA**	1:1000	5% skim milk	anti-mouse	21
β-actin **	1:2000	5% skim milk	anti-mouse	21
No sign , Cell Signaling		*BD BioScience	**Santa Cruz	

3.12 Immunofluorescence

Macrophages were cultured on glass coverslips. Cells were washed 2 times with PBS and fixed with 4 % paraformaldehyde for 30 min at room temperature (RT). Cells were washed at least 4 times with PBS until the smell of paraformaldehyde was gone. Cells were blocked with 3 % skim milk and 0.5 % triton X-100 in water for 60 min and then they were incubated with the primary antibody for 1 h at RT. Macrophages were then incubated with Alexa

flour 595 or 488 for 1 h. After extensive washing with PBS (4 times every 10 min) cells were mounted by vectashield / DAPI (Vector, Burlingame, USA).

3.12.1 Phalloidin staining

Macrophages were cultured on glass coverslips coated with 25 µg/ml fibronectin. After treating macrophages with chemoattractant at different time points cells were permeabilized with 0.5% Triton X-100 for 5 min followed by blocking with 3% BSA for 25 min, then they were incubated with phalloidin Alexa Fluor® 568 (Molecular Probes, Invitrogen, Vienna, Austria) for 25 min. After washing with PBS cells were mounted by vectashield/DAPI (Vector, Burlingame, USA).

3.13 Cytokine secretion

Mouse peritoneal macrophages from Wt and *Atgl*^{-/-} mice were incubated in DMEM in the absence and presence of macrophage chemoattractant protein-1 (MCP-1) (5 ng/ml) for 24 h. Secretion of IL-6, IL-10 and TGFβ from the cells into the medium was quantified by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol.

3.14 Adhesion and spreading assays

Macrophages from Wt and *Atgl*^{-/-} mice were plated onto fibronectin-coated coverslips (25 µg/ml) and adhered for 30 min. Thereafter, SDF-1 (40 ng/ml), LPA (9 ng/ml) and MCP-1 (50 ng/ml) were added for 0, 2, 5 and 10 min. Cells were fixed with 4% formaldehyde and stained with phalloidin AlexaFluor-568 to visualize F-actin. Spread cells were counted using a Leica SP5 confocal microscope (Leica Microsystem, Vienna, Austria). The surface area of the cells was measured using MetaMorph software (Molecular Devices, Downingtown, USA).

3.15 Cell migration assays

Cell migration assays of monocytes and macrophages were performed using 24-well transwell plates (pore sizes: 3 µm and 5 µm, respectively; Corning, Vienna, Austria). Two million cells were added into the upper well. The

lower chamber contained DMEM with and without MCP-1 (50 ng/ml), SDF-1 (60 ng/ml) and LPA (9 ng/ml). Transwell plates were incubated at 37°C for 4 h. Macrophages were stained with anti-F4/80 antibody (eBioscience, Vienna, Austria). Macrophages (stained with F4/80 antibody) and monocytes were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, USA). To evaluate the effect of antioxidant N-acetyl-cystein (NAC) (20 mM) and NADPH oxidase inhibitor diphenyleneidonium (DPI) (2 μ M) (CalBiochem/EMD Bioscience, San Diego, USA) on macrophage migration, cells were pretreated with NAC and DPI for 30 min before loading into the migration chamber. Chemotactic indexes were calculated from the ratio of the cells that had migrated in the presence or absence of the respective chemoattractant.

3.16 ROS production.

Wt and *Atgl*^{-/-} macrophages were cultivated in black 96-well plates in DMEM in the absence (control) or presence of LPA (9 ng/ml) or SDF-1 (60 ng/ml) at 37°C for 5 and 10 min or pretreated with NAC (20 mM) and DPI (2 μ M) for 30 min. The cells were washed and thereafter incubated with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFDA, 50 μ M) (Sigma-Aldrich, Vienna, Austria) for 10 min. Fluorescence (excitation: 488 nm; emission: 535 nm) was measured in a FlexStationTM II device (Molecular Devices, Downingtown, PA).

Intracellular ROS production in control and loaded macrophages was measured using 2.5 μ g/ml dihydroethidium (Sigma, Vienna, Austria) in PBS. Cells were incubated at RT for 10 min. O₂^{•-} production was determined by fluorescence microscopy using a rhodamine filter (Zeiss Axioskop; Carl Zeiss MicroImaging GmbH, Vienna, Austria).

3.17 Apoptosis assay

Apoptosis in macrophages, FC- and VLDL-loaded cells was assayed by annexin V and propidium iodide (PI) co-staining using Annexin-V-FLUOS Staining Kit (Roche, Vienna, Austria). After incubation, the cells were gently washed once with PBS and incubated for 15 minutes at room temperature with

120 µl annexin-binding buffer (25 mmol/L HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% BSA) containing 10 µl FITC–conjugated annexin V and 1 µl PI (100 µg/ml). The staining mixture was then removed and replaced with 120 µl annexin-binding buffer. The cells were immediately viewed at room temperature by fluorescence microscopy (Zeiss Axioskop; Carl Zeiss GmbH, Vienna, Austria) equipped with a 10X objective and filters appropriate for fluorescein (annexin V) and rhodamine (PI). For quantification, three fields of cells (~700 cells per field) for each condition were counted. The number of annexin V/PI positive and PI positive cells in each field is expressed as the percentage of these cells per total cells counted.

3.18 Cell survival or MTT assay

Macrophages from Wt and *Atgl*^{-/-} mice were cultivated in black 96-well plates in DMEM supplemented with 10 % LPDS. *Atgl*^{-/-} macrophages were treated with 10 µM fumonisin B1 (FB1) for 12 h or. Cells were washed carefully with PBS. MTT (5 mg/ml in PBS) (Sigma, Vienna, Austria) was added and cells were incubated at 37°C for 3 h. Afterwards, cells were washed two times with cold ddH₂O. Cells were lysed at RT for 15-20 min in acidic isopropanol (0.1 N HCl in absolute isopropanol). OD was measured at 560 nm and background wavelength was measured 630nm.

3.19 Caspase 3/7 activity assay

Macrophages from Wt and *Atgl*^{-/-} mice were cultivated in white 96 well plates in DMEM/10% LPDS. The combined caspase 3 and 7 activities were measured using Caspase- GLO[®]3/7 assay (Promega, Vienna, Austria) according to the manufacturer's instructions. Luminescence was determined on a TopCount NXT luminometer (Packard Instruments, Meriden, USA).

3.20 Mitochondria and lipid droplet staining

Mitochondria of untreated, FC-, VLDL- and FB1 loaded macrophages were stained using 0.5 µM MitoTracker Green (Molecular Probe, Invitrogen, Vienna, Austria) (37°C, 5 min). LDs were visualized by Nile red (2.5 µg/ml)

staining. Z-stacks were recorded using a Leica SP5 AOBS confocal microscope (Leica Microsystems, Vienna, Austria).

3.21 Oxygen consumption

Macrophages from Wt and *Atgl*^{-/-} mice cultivated in DMEM/10% LPDS were analyzed by intact cell respirometry in different states of mitochondrial respiration *in situ* by using an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria). Basal cellular O₂ consumption was recorded without metabolic inhibitors or uncouplers. ATP synthase was inhibited with 2 µg/ml oligomycin, followed by uncoupling of the respiratory chain from oxidative phosphorylation by stepwise titration with carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), to achieve maximal O₂ consumption. Oxygen consumption rates were normalized to basal values obtained by inhibiting mitochondrial O₂ consumption by the addition of 1M KCN to inhibit cytochrome c oxidase.

3.22 Cytosolic Ca²⁺ measurements

Cells grown on coverslips in 6-well dishes were loaded with 4 µM fura-2 (Molecular Probes, Eugene, OR) and sulphhinpyrazone (250 µM) (Sigma-Aldrich, Vienna, Austria) at RT for 30 min as previously described (10). After 100s, 15 µM of the sarcoplasmic/endoplasmic reticulum Ca²⁺ (SERCA) inhibitor 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ) was added to empty endoplasmic Ca²⁺ pools into the cytosol. Fluorescence images (510 nm emission after alternate 340 and 380 nm excitation) of ~ 300 cells were collected by fluorescence microscopy. Each image was corrected for background fluorescence and the 340:380 nm fluorescence ratio of individual cells was calculated.

3.23 Electron microscopy (performed by Dagmar Kolb)

Macrophages grown on a Melinex Film (Gröpl, Tulln, Austria) were fixed in 0.06 M phosphate buffer (pH 7.2) containing 2.5% glutardialdehyde for 90 min at room temperature. Macrophages were rinsed twice in 0.06 M phosphate buffer for 10 min and post-fixed in 1% osmium tetroxide in the same buffer for 1

h. Cells were rinsed four times for 10 min in 0.06 M phosphate buffer and dehydrated in a series of 50%, 70%, 90%, and 100% cold acetone, for 20 min each. Cells were infiltrated by 2:1, 1:1, 1:2 mixtures of 100% acetone and agar 100 epoxy resin (Gröpl, Tulln, Austria) and pure agar 100 epoxy resin for 4 h. Finally, cells were placed in agar 100 epoxy resin at room temperature for 8 h, transferred into embedding molds and polymerized at 60°C for 48 h. Ultrathin sections (75 nm) were cut with a Reichert Ultracut S Ultramicrotome and stained with lead citrate for 5 min and with uranyl acetate for 15 min. Images were taken on a Zeiss EM 902 transmission electron microscope equipped with a ProScan slow scan CCD camera.

3.24 FFA analysis (GC-MS was performed by Andreas Uellen)

For the preparation of pentafluorobenzyl (PFB)-ester derivatives, cellular lipids were extracted twice in hexane/isopropanol (3:2, v:v) in the presence of 2.5 µg stearic-d35-acid (C18:0-d35, Isotec) as internal standard. One hundred µl of 1 ml lipid extract were converted to the corresponding PFB-ester derivatives in 50 µl acetonitrile containing 2% (v/v) PFB bromide (Sigma-Aldrich, Vienna, Austria) and 2% (v/v) N,N diisopropylethylamine (Sigma-Aldrich). After 30 min at 25°C samples were dried under vacuum, redissolved in 100 µl hexane, transferred to autosampler vials and stored at -20°C. For GC-MS analysis, a Thermo Scientific Trace GC coupled to a DSQII mass spectrometer was used. The GC was fitted with a SGE BPX5 capillary column (30 m, I.D: 0.25 mm inner diameter, 0.25 µm methyl silicone film coating).

The injector was operated in the splitless mode 3 at 230°C. Helium was used as carrier gas at a flow rate of 1.5 ml/min. Initial column temperature was 120°C for 2 min, followed by an increase of 20°C/min to 160°C, an isothermal hold of 2 min, a second increase at 12°C/min to 300 °C, an isothermal hold for 2 min and increased by 10°C/min to 320°C with a hold for 2 min. The transfer line was kept at 300°C and the ion source was 225°C. Negative ion chemical ionization was performed with electron energy of 70 eV, and an emission current of 100 µA. Methane (2.5 ml/min) was used as reagent gas. Quantitation was performed by peak area comparison along with a standard calibration

curve consisting of unlabeled primary FA standards (Sigma-Aldrich) mixed with the stable isotope labeled standard.

3.25 Ceramide extraction

Macrophages from Wt, Wt VLDL-loaded (150 µg/ml), *Atgl*^{-/-} and *Atgl*^{-/-} treated with FB1 (10 µM for 12 h) or myriocin were cultured in 12 well plate (80 % confluence) in DME media and in the absence of exogenous lipid. Cells were washed and scrapped in 500 µl cold PBS. After centrifugation 5 min in 5000 rpm, pellet was resuspended in organic solvent including chloroform-methanol-water-pyridin (60:30:6:1, v:v:v:v) Extraction was performed by incubating samples in 37°C for 24 h in a water bath by continuous stirring. Denatured proteins were removed by passing the samples through cotton wadding, and the solvent was evaporated under a stream of nitrogen. Extracts were dissolved in 2.5 ml methanol and sonicated for 5 min. Afterwards, the sodium hydroxide (4 M ion in stock solution in ddH₂O) was added to a final concentration of 100 mM. After shaking for 2 h at 37°C, samples were neutralized by the addition of 10 µl concentrated acetic acid. Finally the solvent was evaporated under a stream of nitrogen. The extract was dissolved in 50 µl chloroform.

(This part was performed by Harald Koefeler) After lipid extraction the organic solvent was evaporated under a stream of nitrogen and lipids were redissolved in chloroforme:methanol (1:1, v:v) for HPLC-MS/MS analysis.

Ceramides were analyzed by a precursor ion scan of m/z 264 at 30 eV collision energy using a Quantum TSQ (Thermo Electron) coupled to an Accela UPLC system (Thermo Electron). For quantitation Cer 17:0 was used as internal standard and ratios of the respective analyses versus the internal standard were calculated.

4. Results

4.1 Effects of TG accumulation on macrophage polarization and migration

4.1.1 Macrophages lacking ATGL accumulate TG

We have investigated the role of ATGL in macrophages, which is the major enzyme responsible for TG hydrolysis in many tissues, including macrophages (MPM)(Chandak *et al.*, 2010c). Since absence of ATGL causes TG accumulation (Figure 14A), we used MPM of *Atgl*^{-/-} mice to examine the effect of TG accumulation on macrophage function. TG concentrations were 3.5-fold increased in *Atgl*^{-/-} compared to Wt and *Atgl*^{+/-} macrophages, whereas FC and CE levels were comparable between macrophages from all genotypes (Figure 14A). In order to confirm the expression of *Atgl*^{-/-} macrophages, western blot analysis was performed in Wt and *Atgl*^{-/-} macrophages by using a specific antibody against ATGL. Figure 14B shows the 54 kDa band corresponding to ATGL to be present in Wt but not in *Atgl*^{-/-} macrophages. Moreover, ATGL expression was increased in Wt VLDL-loaded macrophages. Bone marrow-derived macrophages (BM-MQ) isolated from Wt and *Atgl*^{-/-} mice were investigated by Nile red staining to monitor lipid droplet (LD) accumulation. Whereas Wt macrophages lack lipid inclusions, *Atgl*^{-/-} macrophages accumulate LD even in the absence of exogenous lipid loading (Figure 1C). Furthermore, the localization of ATGL was investigated by immunofluorescence staining. Anti-ATGL antibody and Bodipy as specific dye for neutral lipids were used to define LDs and ATGL. Figure 1D illustrates that ATGL is increased around the LDs in some cells.

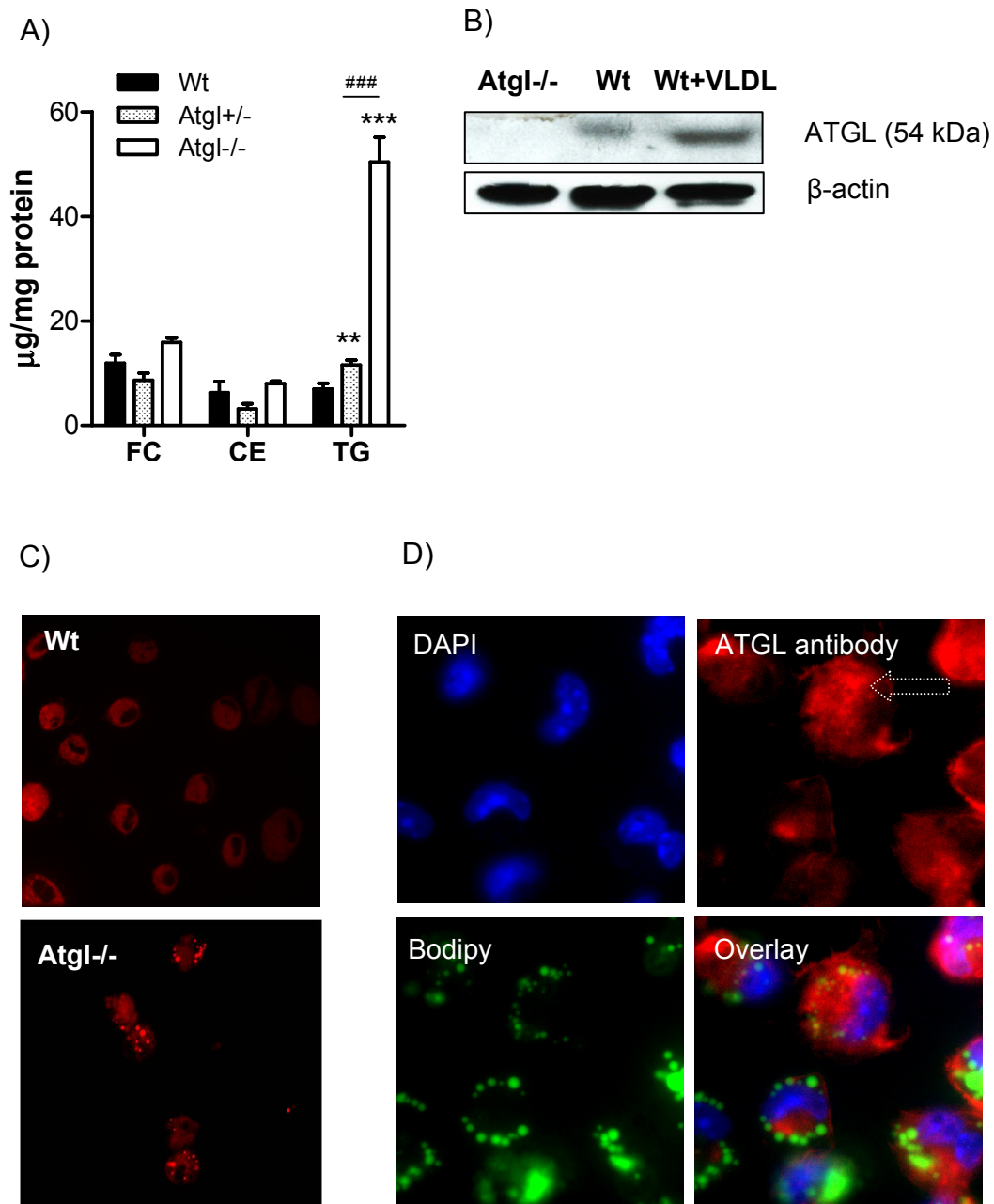


Figure 14. Triglyceride accumulation in *Atgl*^{-/-} macrophages. (A) Lipid parameters were measured in Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages. (B) Lysates from Wt and *Atgl*^{-/-} macrophages were resolved by SDS PAGE. (C) Lipid droplets were stained using nile red in BM-MQ. (D) Wt and *Atgl*^{-/-} peritoneal macrophages were fixed and stained with anti-ATGL, green bodipy was used as specific dye for LDs. Pictures were recorded using confocal microscopy (Nipkow, Heidelberg, Germany).

4.1.2 *Atgl*^{-/-} macrophages exhibit an anti-inflammatory phenotype

One of the most important roles of macrophages is the secretion of different chemokines or cytokines at the site of inflammation. Therefore, we first determined whether ATGL deficiency alters the cytokine expression pattern in

macrophages. Production of pro-inflammatory IL-6 was 30% decreased in *Atgl*^{-/-} compared to Wt macrophages, whereas the release of the anti-inflammatory cytokines IL-10 and TGF- β were increased by 1.6- and 3.6-fold, respectively (Figure 15A). Level of PGE₂ was comparable in both genotypes. mRNA for the M1 marker genes *Ccl5* and *Gro1* were almost totally absent in *Atgl*^{-/-} macrophages. In contrast, increased mRNA expression of anti-inflammatory genes such as *Mcp2*, *Sphk1*, *Clec7a*, *Mrc*, and *Arg1* were observed in *Atgl*^{-/-} macrophages (Figure 15B). *Chi3l3* and *Jag1* mRNA levels were comparable in macrophages of *Atgl*^{-/-} and Wt mice. This finding indicates that *Atgl*^{-/-} macrophages resemble features of anti-inflammatory M2 macrophages.

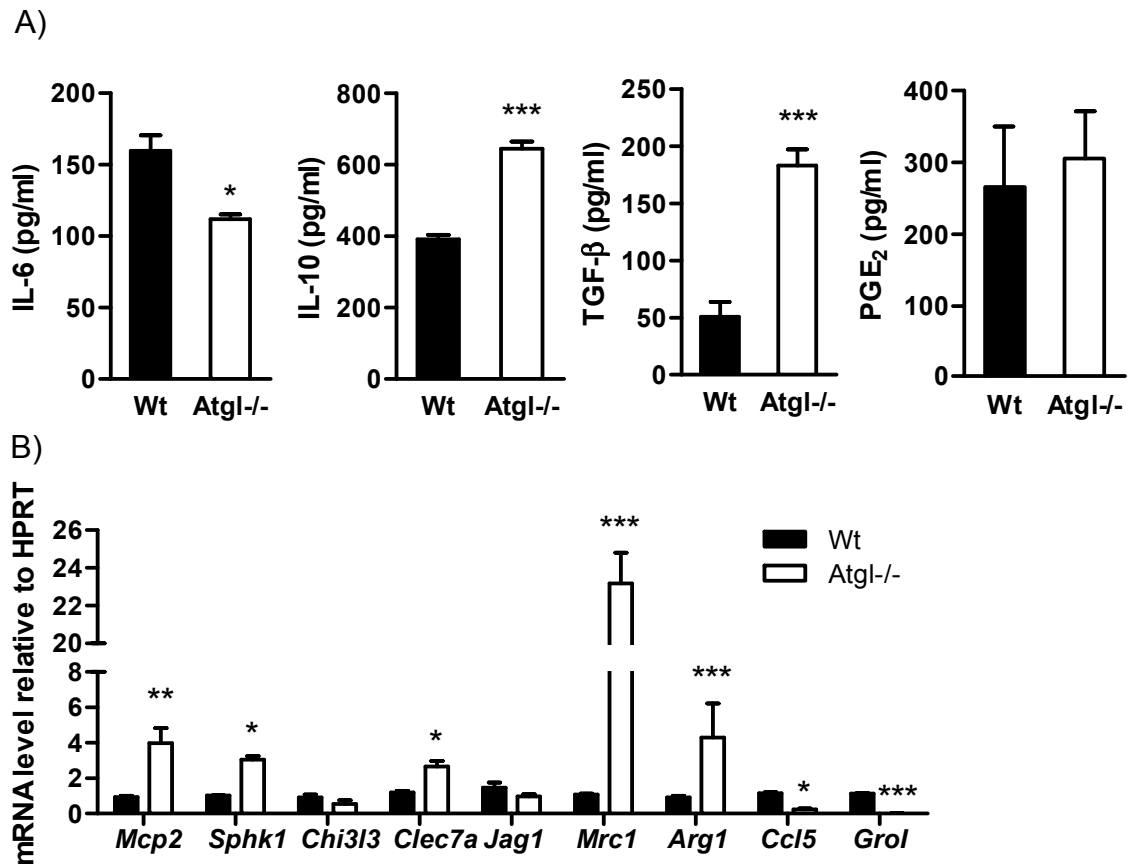


Figure 15. *Atgl*^{-/-} macrophages exhibit phenotype of alternatively activated macrophages. (A) Wt and *Atgl*^{-/-} macrophages were incubated in DMEM and cytokine secretion was determined by ELISA. Data represent mean values (n=6) $\pm$ SEM measured in triplicate. (B) Quantitative real time PCR analyses of macrophage from control and *Atgl*^{-/-} mice; *Mcp2*, macrophage chemoattractant protein 2; *Sphk1*, sphingosine kinase 1; *Chi3l3*, chitinase 3-like 3; *Clec7a*, dectin-1; *Jag1*, Jagged1; *Mrc1*, mannose receptor 1; *Arg1*, arginase 1; *Ccl5*, C-C chemokine ligand 5 (Rantes). Data represent mean (n=3) $\pm$ SEM measured in triplicate. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.3 Migration is diminished in *Atgl*^{-/-} macrophages.

Cytokines play important roles in macrophage migration in chemoattractant gradients to sites of inflammation. Therefore, we examined the effect of ATGL deficiency on macrophage migration *in vivo* after an i.p. thioglycolate injection. After 72 h, macrophage recruitment to the peritoneal cavity of *Atgl*^{-/-} mice was reduced by 64% compared to Wt mice (Figure 16A). These data indicate a central role for ATGL in macrophage recruitment in peritoneal inflammation. Next we determined the capacity of *Atgl*^{-/-} macrophages to migrate toward different chemoattractants *in vitro*. We found that the migration of macrophages toward MCP-1, stromal cell-derived factor-1 (SDF-1) and lysophosphatidic acid (LPA) was markedly reduced in *Atgl*^{-/-} compared to Wt macrophages (56%, 69% and 63%, respectively) (Figure 16B). Therefore, we determined mRNA levels of adhesion molecules in *Atgl*^{-/-} macrophages, monocytes and aortas. *Atgl*^{-/-} macrophages showed drastically reduced mRNA levels of VLA-4 (ligand for vascular cell adhesion molecule 1) and LFA-1 (ligand for intercellular adhesion molecule 1) compared to Wt macrophages (93% and 88%, respectively) (Figure 16C). Moreover, we observed a 54% reduction in P-selectin mRNA levels in *Atgl*^{-/-} compared to Wt macrophages, whereas E-selectin mRNA expression was not significantly changed. We also determined the expression of the corresponding adhesion molecules in the aortas of *Atgl*^{-/-} and Wt mice. ICAM-1 mRNA was 84% downregulated in *Atgl*^{-/-} aortas, whereas VCAM-1 mRNA was unchanged (Figure 16D). We then investigated whether differences in the migration capacity were already established in monocytes from *Atgl*^{-/-} mice. As observed in macrophages, migration to all three chemoattractants was markedly diminished in *Atgl*^{-/-} monocytes (Figure 16E). mRNA levels of VLA-4 and P-selectin were also decreased in *Atgl*^{-/-} monocytes, whereas LFA-1 and E-selectin mRNA levels were comparable in monocytes of both genotypes (Figure 16F). In summary, these data indicate that ATGL deficiency inhibits monocyte and macrophage migration *in vivo* and *in vitro*.

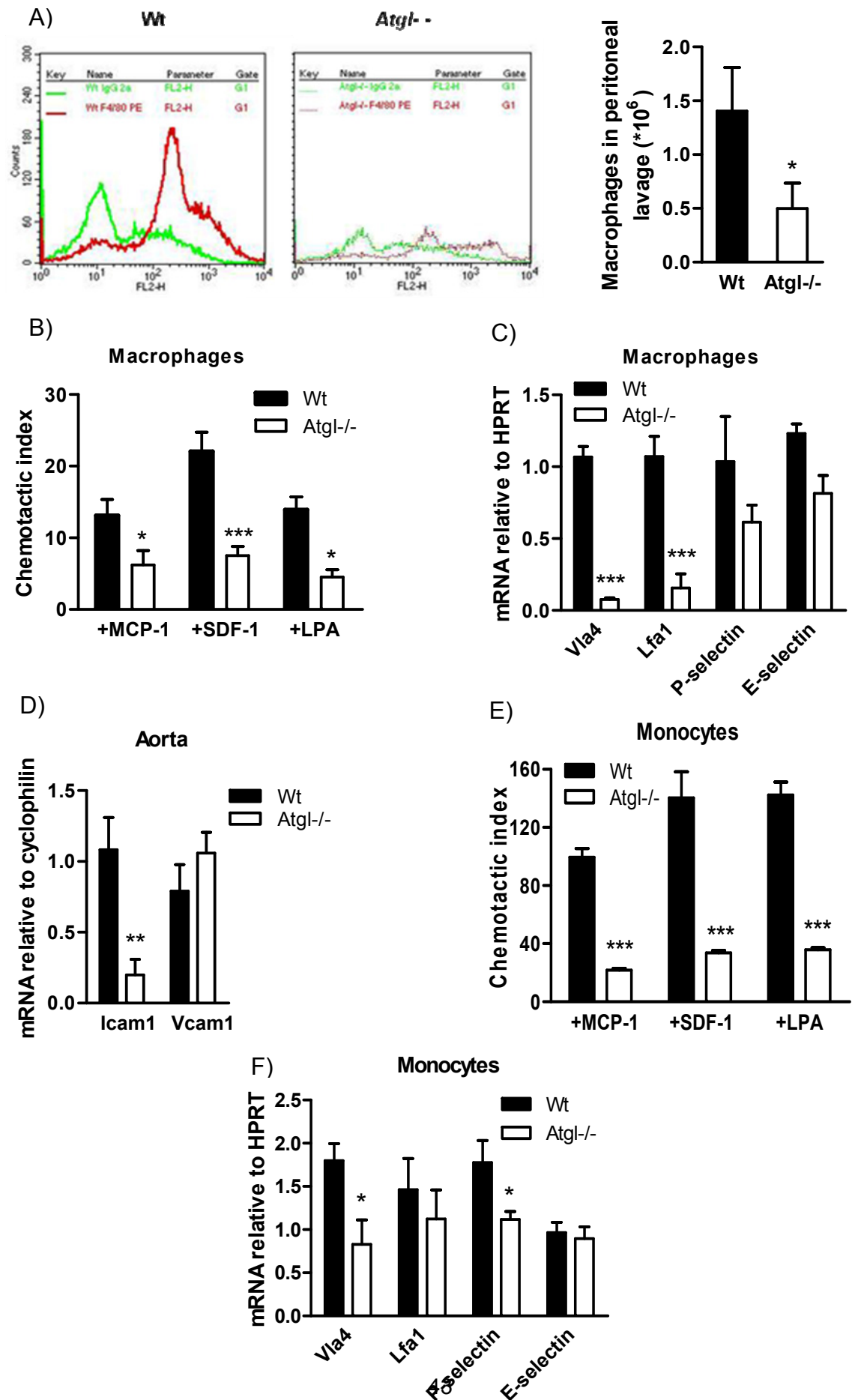
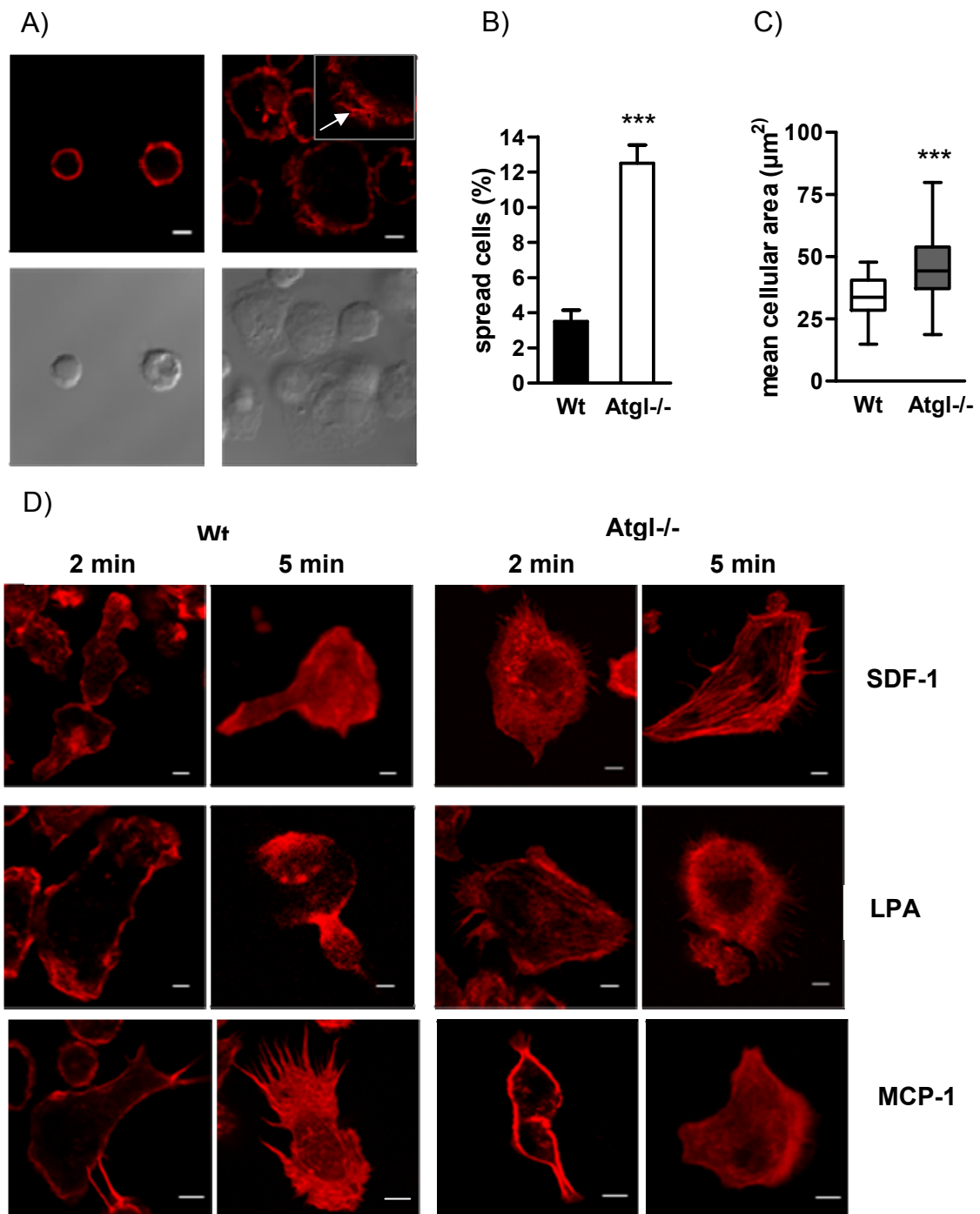


Figure 16. Migration is reduced in *Atgl*^{-/-} macrophages. (A) Wt and *Atgl*^{-/-} mice were challenged with 3% thioglycolate by intraperitoneal injection. Peritoneal lavage was collected 3 days after injection and the macrophage specific marker F4/80 was measured by flow cytometry. (B) Mouse peritoneal macrophages and monocytes from Wt and *Atgl*^{-/-} mice were added to the upper chamber of transwell plates and were allowed to migrate through the membrane (pore size: (macrophages) 5 μ m and (monocytes) 3 μ m) into the lower chamber containing DMEM in the absence or presence of macrophage chemotactant protein-1 (MCP-1; 50 ng/ml), stromal cell-derived factor-1 (SDF-1, 60 ng/ml) or lysophosphatidic acid (LPA, 9 ng/ml) at 37°C for 4 h. Migrated cells were counted by flow cytometry. Chemotactic indexes were calculated from the ratio of the cells that had migrated in the presence or absence of the respective chemoattractant. (C,D, F) Total RNA was isolated from Wt and *Atgl*^{-/-} (C) macrophages and (D) aorta and (F) monocytes reverse transcribed and mRNA expression of (C,F) *Vla4*, *Lfa1*, *P-selectin*, *E-selectin* and (D) *Vcam1* and *Icam1* were determined by real-time PCR including normalization to (C) hypoxanthine guanine phosphoribosyl transferase (HPRT) and (D) cyclophilin A. Data are expressed as mean values of 2 independent experiments $\pm$ SEM performed in triplicate repeats. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.4 Reduced actin polymerization and increased cell spreading in *Atgl*^{-/-} macrophages

The rearrangement of actin filaments is a prerequisite for leukocyte adhesion and migration. We therefore determined the consequences of ATGL deficiency on actin polymerization and cell spreading. When plated on fibronectin-coated coverslips and stained by phalloidin, *Atgl*^{-/-} immediately spread, which was not observed in Wt macrophages. *Atgl*^{-/-} macrophages formed broad protrusions, whereas Wt macrophages remained round-shaped and smaller (Figure 17A). Quantification revealed a 3.5-fold increased number of cells with spread shape (Figure 17B) and a 1.4-fold increased cellular mean area in *Atgl*^{-/-} compared to Wt macrophages (Figure 17C). Cell spreading requires actin polymerization to form lamellipodia and therefore we evaluated whether SDF-1, LPA and MCP-1 affected actin polymerization differently in Wt and *Atgl*^{-/-} macrophages. After 2 and 5 min of incubation, all three chemoattractants induced the reorganization of actin filaments in Wt cells, which appeared to be flawed in *Atgl*^{-/-} macrophages (Figure 17D). After 5 min of exposure to LPA, SDF-1 or MCP-1 *Atgl*^{-/-} macrophages had broad lamellopodia in different directions and no apparent leading edge-uropod morphology. In contrast, Wt macrophages showed an extending head and a retracting tail with F-actin rich protrusions (Figure 17D). The percentage of spread cells was increased considerably in *Atgl*^{-/-} macrophages upon stimulation with chemoattractants (Figure 17E). Accordingly, the mean cellular

area was also substantially higher in *Atgl*^{-/-} macrophages treated with SDF-1, LPA and MCP-1 (Figure 17F). Using flow cytometry we confirmed that the amount of polymerized actin was increased in Wt macrophages but unchanged in *Atgl*^{-/-} macrophages after the addition of LPA (Figure 4G).



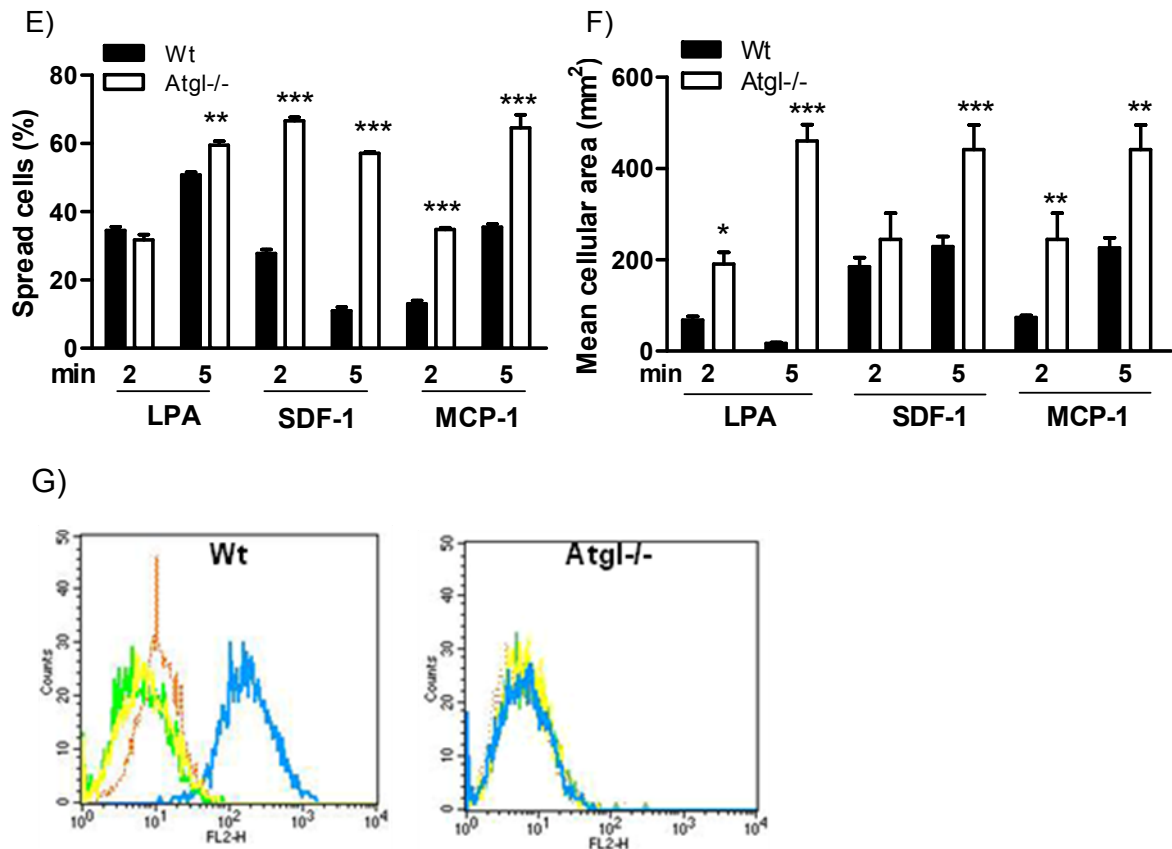


Figure 17. Accelerated cell spreading in *Atgl*^{-/-} macrophages. (A,D) Macrophages from Wt and *Atgl*^{-/-} mice were plated on fibronectin-coated glass coverslips for 10 min (A) in the absence or (D) presence of SDF-1 (60 ng/ml) and LPA (9 ng/ml) for 2 and 5 min. Adherent cells were fixed and stained with phalloidin AlexaFluor-586. Images were taken using a Leica AP5 AOBs confocal microscope (Leica Microsystems, Vienna, Austria). Scale bars: 5 μ m. (B,E) Cell spreading was calculated by counting the percentage of spread cells, and (C,F) mean cellular area was measured using MetaMorph software (Molecular Devices, Downingtown, PA). Data from 2 independent experiments are expressed as mean values $\pm$ SEM from > 650 cells per genotype. (G) Fluorescence intensity of phalloidin AlexaFluor-586-stained Wt and *Atgl*^{-/-} macrophages was measured by flow cytometry. The histograms show untreated cells (red) and macrophages treated with LPA for 2 min (green), 5 min (yellow) and 10 min (blue). * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.1.5 Sustained activation of FAK due to inactivation of its phosphatase in *Atgl*^{-/-} macrophages

To investigate possible pathways that underlie the enhanced cell spreading of *Atgl*^{-/-} macrophages, real-time PCR was performed for FAK, which plays an important role in integrin-mediated cell adhesion, spreading and focal adhesion formation (Cary et al., 2002). In *Atgl*^{-/-} macrophages, FAK expression was 3.5-fold up-regulated compared to Wt macrophages (Figure 18A). Immunoblotting revealed that only a very small portion of FAK was transiently

activated in Wt macrophages (Figure 18B). Phosphorylated FAK was increased after 5 min and decreased to the basal level after 10 min treatment with LPA and SDF-1 (Figure 18B). The increase in activated FAK after 5 min was more pronounced by SDF-1 compared to LPA treatment. In *Atgl*^{-/-} macrophages, we observed a marked increase in phosphorylation of FAK in the absence and presence of chemoattractants and FAK remained activated even after 10 min (Figure 18B). Since tyrosine phosphorylation of FAK is regulated by Src family kinases (Calalb et al., 1995), we determined Src activation. Activated Src was gradually increased in Wt macrophages after stimulation with LPA and SDF-1 for 2, 5 and 10 min, whereas it was significantly reduced in *Atgl*^{-/-} macrophages (Figure 18C). To assess whether FAK acts as a Src kinase substrate we pretreated *Atgl*^{-/-} macrophages with the Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP2) for 30 min. As expected, inhibition of Src kinase was associated with ineffective phosphorylation of Src in the absence and presence of LPA or SDF-1 (Figure 18D). In addition, Src kinase inhibition resulted in impaired FAK activation suggesting that FAK phosphorylation in *Atgl*^{-/-} macrophages occurs in a Src kinase-dependent manner. Moreover, we observed a drastically reduced ERK2 activation in *Atgl*^{-/-} macrophages upon treatment with LPA (Figure 18E), which may explain the defect in focal contact turnover in these cells.

The sustained phosphorylation of FAK in *Atgl*^{-/-} macrophages prompted us to investigate the role of tyrosine phosphatase SHP-2 as the major regulator of FAK phosphorylation dynamics (Yu et al., 1998). Phosphorylation of SHP-2 at Tyr580 releases its autoinhibition, thereby stimulating its phosphatase activity (Lu et al., 2001). In contrast to gradually increased phosphorylation of SHP-2 in Wt macrophages, we found reduced phosphorylation of SHP-2 in *Atgl*^{-/-} macrophages in response to LPA (Figure 18F). These findings indicate that sustained phosphorylation of FAK is a consequence of inactivation of SHP-2 in *Atgl*^{-/-} macrophages.

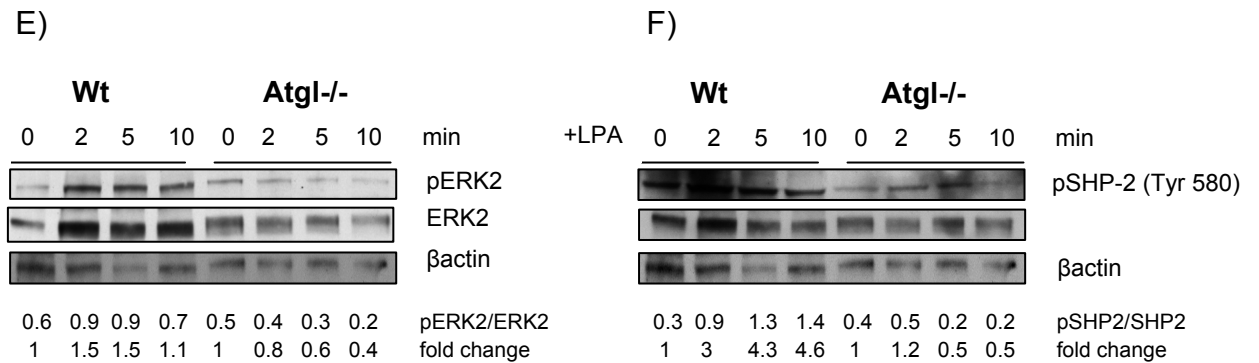
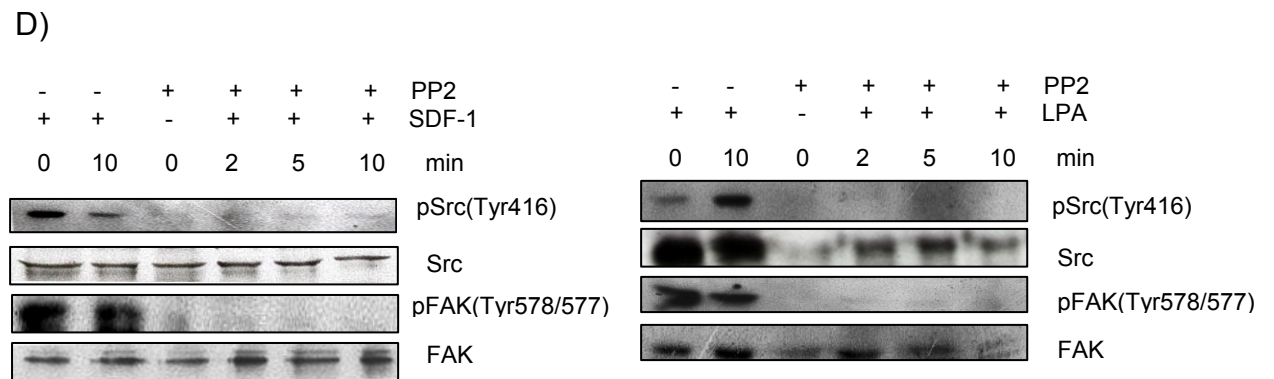
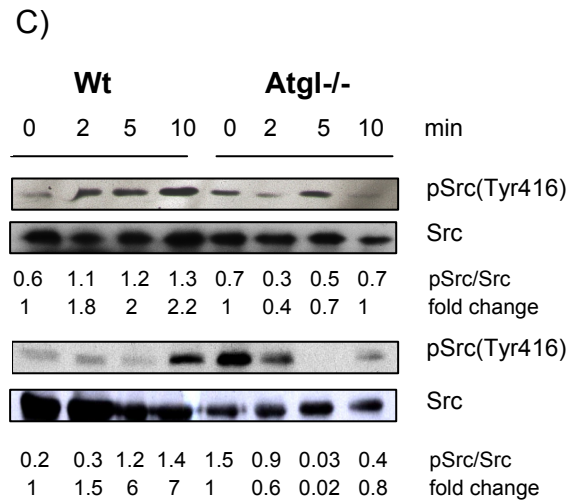
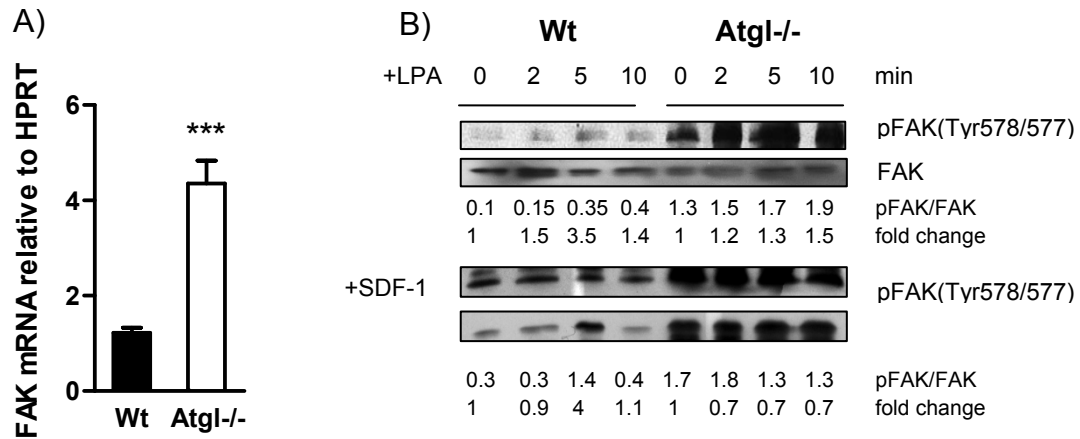


Figure 18. Sustained FAK phosphorylation mediates spreading in *Atgl*^{-/-} macrophages in a Src kinase-dependent manner. (A) Total RNA was isolated from Wt and *Atgl*^{-/-} macrophages, reverse transcribed and mRNA expression of FAK was determined by real-time PCR including normalization to hypoxanthine guanine phosphoribosyl transferase (HPRT). (B-F) Wt and *Atgl*^{-/-} macrophages were incubated with LPA (9 ng/ml) for 0, 2, 5 and 10 min. (B) The lysates were subjected to western blot analysis using antibodies specific for total and phosphorylated (p)FAK (Tyr576/577) and (C) total and pSrc (Tyr416). (D) *Atgl*^{-/-} macrophages were preincubated with the Src kinase inhibitor 4-amino-5(4chlorophenyl)7(βbutyl)pyrazolo[3,4-d] pyrimidine (PP2, 10 μM) for 30 min and then exposed to SDF-1 (60 ng/ml) for 0, 2, 5 and 10 min. Western blot analysis was performed using the above mentioned antibodies. (E,F) The lysates were subjected to western blot analysis using antibodies specific for (E) total and pERK2 and (F) total and pSHP2 (Tyr580). Ratios were calculated by dividing band intensities of phosphorylated to total protein expression. Fold changes were calculated relative to the ratios of unstimulated cells. Representative blots from 2 independent experiments are shown.

4.1.6. Defective Cdc42, Rac1 and Rho signaling in *Atgl*^{-/-} macrophages.

To investigate the mechanism of defective actin polymerization and cell polarization in *Atgl*^{-/-} macrophages we analyzed the activation of Rho GTPase proteins by pull-down assays. Activated GTP-bound Cdc42 was comparable in Wt macrophages treated with LPA for 0, 2 and 5 min but was almost abolished after 10 min of treatment (Figure 19A). In *Atgl*^{-/-} macrophages, Cdc42 activation in untreated and treated cells was markedly decreased compared to Wt cells (Figure 19A). In the absence of LPA the level of activated Rac1 was comparable in Wt and *Atgl*^{-/-} macrophages (Figure 19B). LPA treatment induced Rac1 after 2 min in Wt macrophages, whereas in *Atgl*^{-/-} macrophages significant Rac1 activation was not observed until 10 min of LPA treatment.

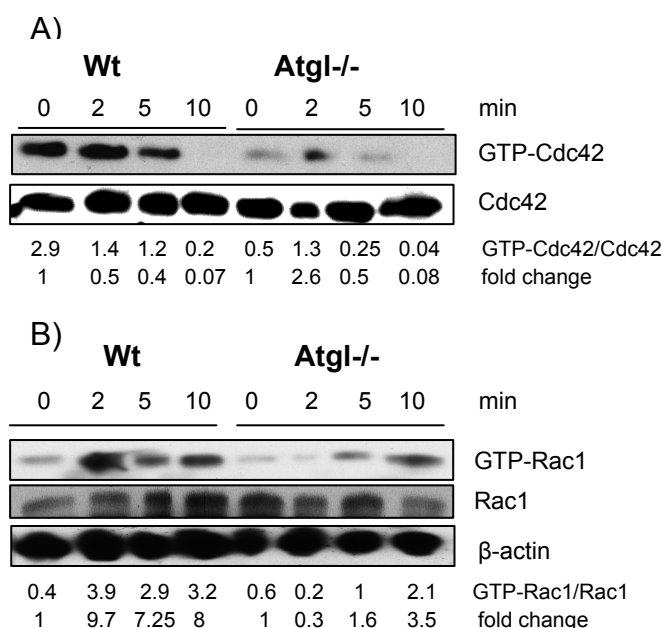
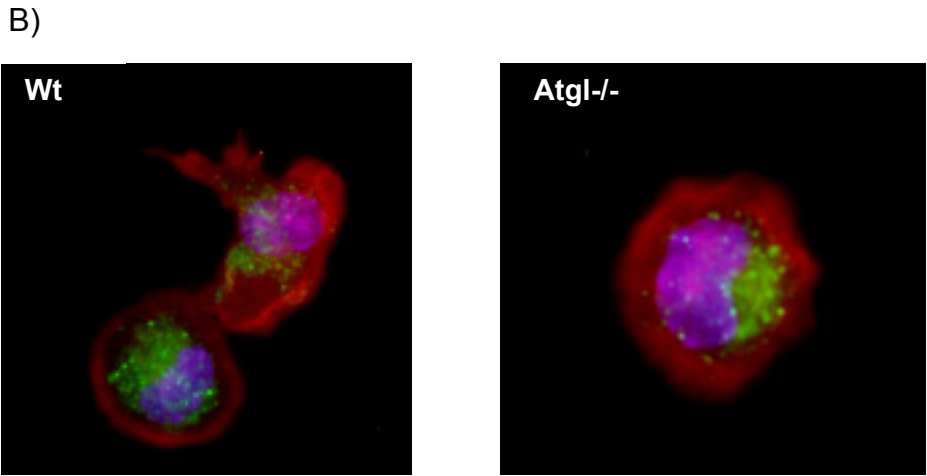
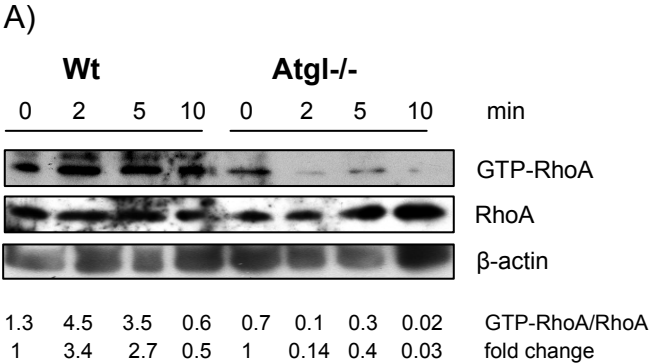


Figure 19. Defective Cdc42 and Rac1 activation in *Atgl*^{-/-} macrophages. Wt and *Atgl*^{-/-} macrophages were serum-starved for 3 h and then incubated in DMEM containing 9 ng/ml LPA. Cell lysates were incubated with glutathione-sepharose beads complexed with GST-PBD fusion proteins for GTP-bound Rac1 and Cdc42. Precipitates were resolved by SDS-PAGE in parallel with whole cell lysates (total Cdc42/Rac1). Protein expression of **(A)** Cdc42 and GTP-Cdc42, **(B)** Rac1 was determined by western blot analysis. Band intensities were quantified using Image J software. Ratios were analyzed by dividing band intensities of activated to total protein expression. Fold changes were calculated relative to the ratios in unstimulated cells

Next, we determined the activation and translocation of RhoA in Wt and *Atgl*^{-/-} macrophages. In Wt macrophages, RhoA activation was increased upon LPA stimulation for 2 and 5 min, whereas treatment of *Atgl*^{-/-} macrophages with LPA drastically reduced GTP-bound RhoA expression (Figure 20A). In Wt and *Atgl*^{-/-} macrophages, RhoA was predominantly localized around the nucleus in the absence of any chemoattractant (Figure 20B). After the addition of LPA or SDF-1, RhoA translocated to the leading edge and rear of Wt cells. In contrast, RhoA remained localized close to the nucleus in *Atgl*^{-/-} macrophages even in the presence of LPA or SDF-1 (Figure 20C).



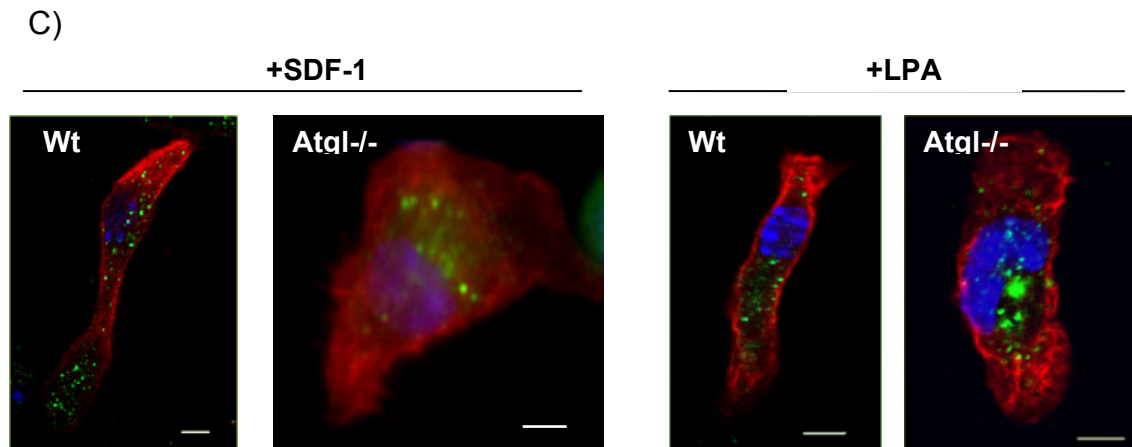


Figure 20. Defective RhoA activation in *Atgl*^{-/-} macrophages. (A) Wt and *Atgl*^{-/-} macrophages were serum-starved for 3 h and then incubated in DMEM containing 9 ng/ml LPA. Cell lysates were incubated with glutathione-sepharose beads complexed with GST-PBD fusion proteins for GTP-bound RhoA. Precipitates were resolved by SDS-PAGE in parallel with whole cell lysates (total RhoA). Protein expression of (A) RhoA and GTP-RhoA was determined by western blot analysis using specific antibodies. Band intensities were quantified using Image J software. Ratios were analyzed by dividing band intensities of activated to total protein expression. Fold changes were calculated relative to the ratios in unstimulated cells. (B-C) Wt and *Atgl*^{-/-} macrophages were plated on fibronectin-coated coverslips and serum-starved for 2 h. Cells were incubated in the absence (control) (B) and presence (D) of SDF-1 (60 ng/ml) and LPA (9 ng/ml) for 10 min at 37°C. Thereafter, cells were fixed and stained with anti-RhoA and AlexaFluor-586 phalloidin to visualize F-actin. Pictures were recorded using an Olympus FSX100 fluorescence microscope. Scale bar: 5 μ m.

4.1.7 Sustained Rac2 activation and increased ROS production in *Atgl*^{-/-} macrophages

Activated Rac2 was present in unstimulated Wt macrophages, increased by the addition of LPA after 5 min and was almost completely absent after 10 min of LPA treatment (Figure 21A). In *Atgl*^{-/-} macrophages, we observed sustained and even increased Rac2 activation after 10 min of LPA loading (Figure 21A). Immunofluorescence revealed that similar to RhoA Rac2 was localized near the nucleus in Wt macrophages lacking any overlay with F-actin (Figure 21B). In contrast, we found localization of Rac2 in the cytosol and near the cell periphery in *Atgl*^{-/-} macrophages. Additionally, we observed colocalization of Rac2 and F-actin in these cells.

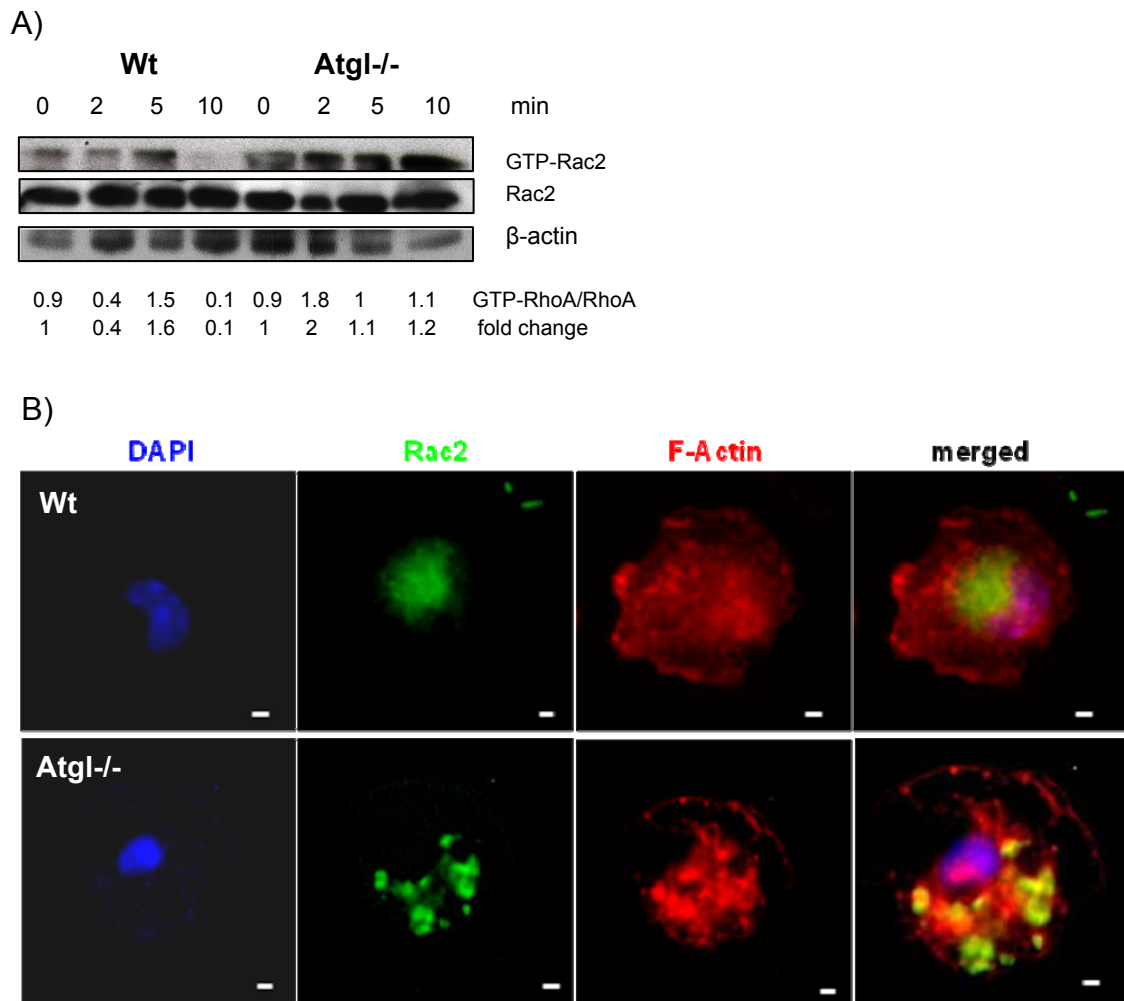


Figure 21. Increased ROS production in *Atgl*^{-/-} macrophages. (A) Wt and *Atgl*^{-/-} macrophages were incubated in DMEM containing LPA (9 ng/ml) for 0, 2, 5 and 10 min. Cell lysates were incubated with glutathione-sepharose beads complexed with GST-PBD fusion protein to active Rac2. Precipitates were resolved by SDS-PAGE in parallel with whole cell lysates (total Rac2). Band intensities were quantified using Image J software. (B) Wt and *Atgl*^{-/-} macrophages were plated on fibronectin-coated coverslips for 10 min. the cells were fixed and stained with anti-Rac2 antibody and phalloidin AlexaFluor-586. Pictures were taken on an Olympus FSX100 fluorescence microscope. Scale bars: 5 μ m.

GTP-Rac2 regulates NADPH oxidase activator, an enzyme catalyzing the NADPH-dependent one electron reduction of O_2 to superoxide anion ($O_2^{\cdot-}$). Hence, we examined ROS production in Wt and *Atgl*^{-/-} macrophages in the absence and presence of LPA and SDF-1. We found highly increased intracellular ROS concentrations, as monitored by the conversion of dichlorodihydrofluorescein diacetate (DCFDA) to fluorescent DCF, in *Atgl*^{-/-} compared to Wt macrophages (Figure 22). ROS concentrations, however, were independent of LPA and SDF-1 treatment of macrophages from both genotypes.

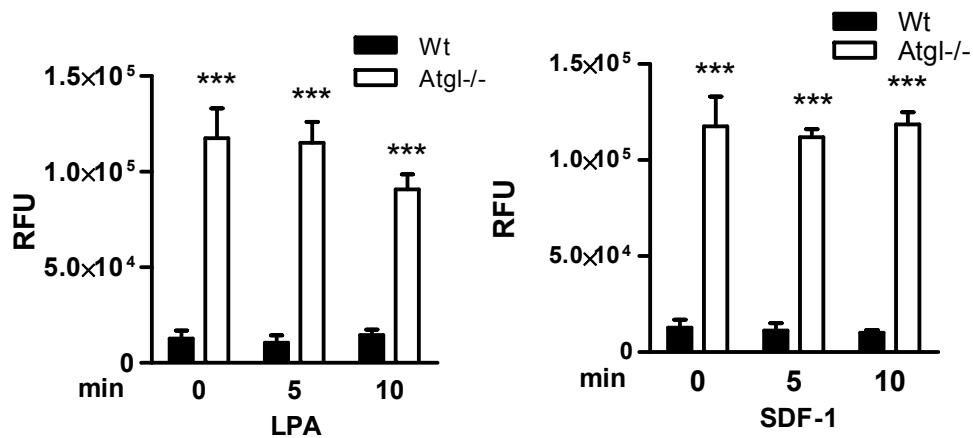


Figure 22. Increased ROS production in *Atgl*^{-/-} macrophages. Wt and *Atgl*^{-/-} macrophages were loaded with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFDA, 50 μ M) for 10 min and then treated with LPA or SDF-1 for 0, 5 and 10 min. Fluorescence was measured in a FlexStation device (excitation: 488 nm; emission: 535 nm).

4.1.8 Inhibition of ROS production in *Atgl*^{-/-} macrophages eliminates sustained phosphorylation of FAK and defective cell migration

To investigate whether ROS production affects macrophage migration, we used N-acetyl-cystein (NAC) as an anti-oxidant and diphenyleneidonium (DPI) as an NADPH oxidase inhibitor. *Atgl*^{-/-} macrophages pre-incubated with NAC or DPI showed a 72% inhibition of ROS concentration (Figure 23A). Since it is known that ROS formation inactivates SHP-2 (Meng et al., 2002), we investigated the activation of FAK in *Atgl*^{-/-} macrophages pre-incubated with NAC and DPI. *Atgl*^{-/-} macrophages pretreated with DPI and NAC and then stimulated with LPA showed transient phosphorylation of FAK, which was almost abolished after 10 min of LPA treatment (Figure 23B). To analyze whether reduced FAK phosphorylation affects migration, we incubated *Atgl*^{-/-} macrophages for 10 min with DPI and NAC and determined chemotaxis to LPA. As shown in Figure 23C, we found that migration of *Atgl*^{-/-} macrophages were restored using the same concentrations of NAC and DPI which dephosphorylated FAK (Figure 23C). However, higher concentration of DPI (20 μ M) did not restore the migration in *Atgl*^{-/-} macrophages. Taken together, these data show that ROS production leads to sustained FAK activation, which causes impaired migration.

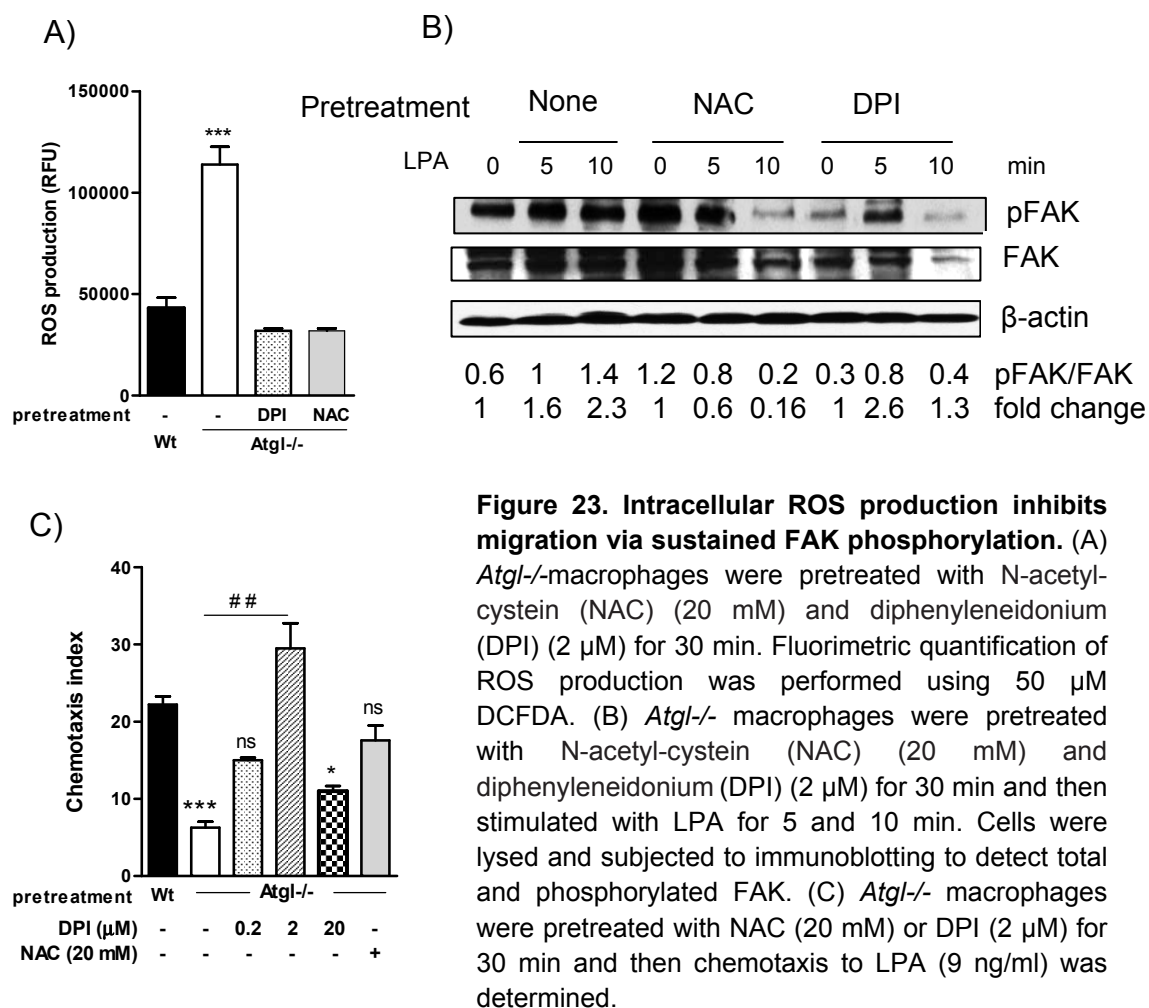
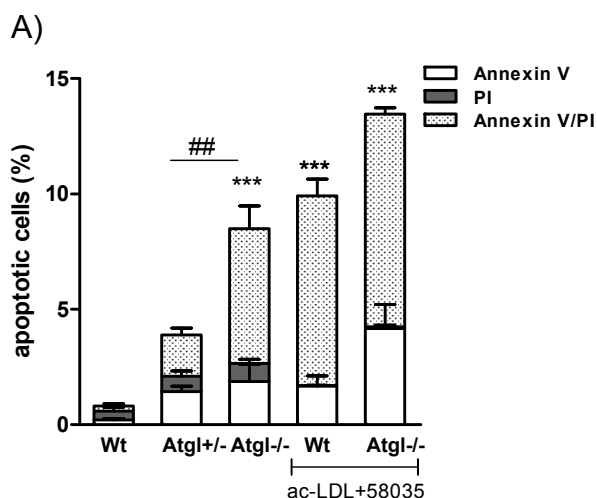


Figure 23. Intracellular ROS production inhibits migration via sustained FAK phosphorylation. (A) *Atgl*^{-/-} macrophages were pretreated with N-acetyl-cystein (NAC) (20 mM) and diphenyleneidonium (DPI) (2 μ M) for 30 min. Fluorimetric quantification of ROS production was performed using 50 μ M DCFDA. (B) *Atgl*^{-/-} macrophages were pretreated with N-acetyl-cystein (NAC) (20 mM) and diphenyleneidonium (DPI) (2 μ M) for 30 min and then stimulated with LPA for 5 and 10 min. Cells were lysed and subjected to immunoblotting to detect total and phosphorylated FAK. (C) *Atgl*^{-/-} macrophages were pretreated with NAC (20 mM) or DPI (2 μ M) for 30 min and then chemotaxis to LPA (9 ng/ml) was determined.

4.2) Triglyceride accumulation activates the mitochondrial apoptosis pathway

4.2.1 *Atgl*^{-/-} macrophages display defining features of apoptosis

To investigate whether cell viability was impaired upon TG accumulation in *Atgl*^{-/-} macrophages, various markers of apoptotic and necrotic cell death were analyzed. Macrophages loaded with acLDL in the presence of the ACAT inhibitor 58035 (FC-loaded macrophages) were used as a positive control for cells which undergo apoptosis. To monitor macrophage cell death, we compared annexin V and PI staining in macrophages isolated from *Atgl*^{+/-}, *Atgl*^{-/-} and Wt mice (Figure 9). Quantification of the annexin V/PI co-stainings revealed that only 1 % of Wt cells exhibited externalization of phosphatidylserine (annexin V positive), indicative of cells undergoing apoptosis, under the experimental conditions (Figure 9). In contrast, the 3.5-fold increased number of annexin V-positive cells in the population of *Atgl*^{+/-} and 8-fold increase in *Atgl*^{-/-} macrophages indicated higher susceptibility of these cells to apoptosis. In all genotypes we considered both annexin V single positives (early apoptotic cells) and annexin V/PI double positives (late apoptotic cells) as cells that died primarily from apoptosis. Incubation of Wt and *Atgl*^{-/-} cells with FC resulted in elevated levels of annexin V/PI-staining showing a similar induction of apoptosis in cells from both genotypes (Figure 9). Take together, apoptosis in TG-rich *Atgl*^{-/-} macrophages are triggered spontaneously and without any exogenous treatment.



B)

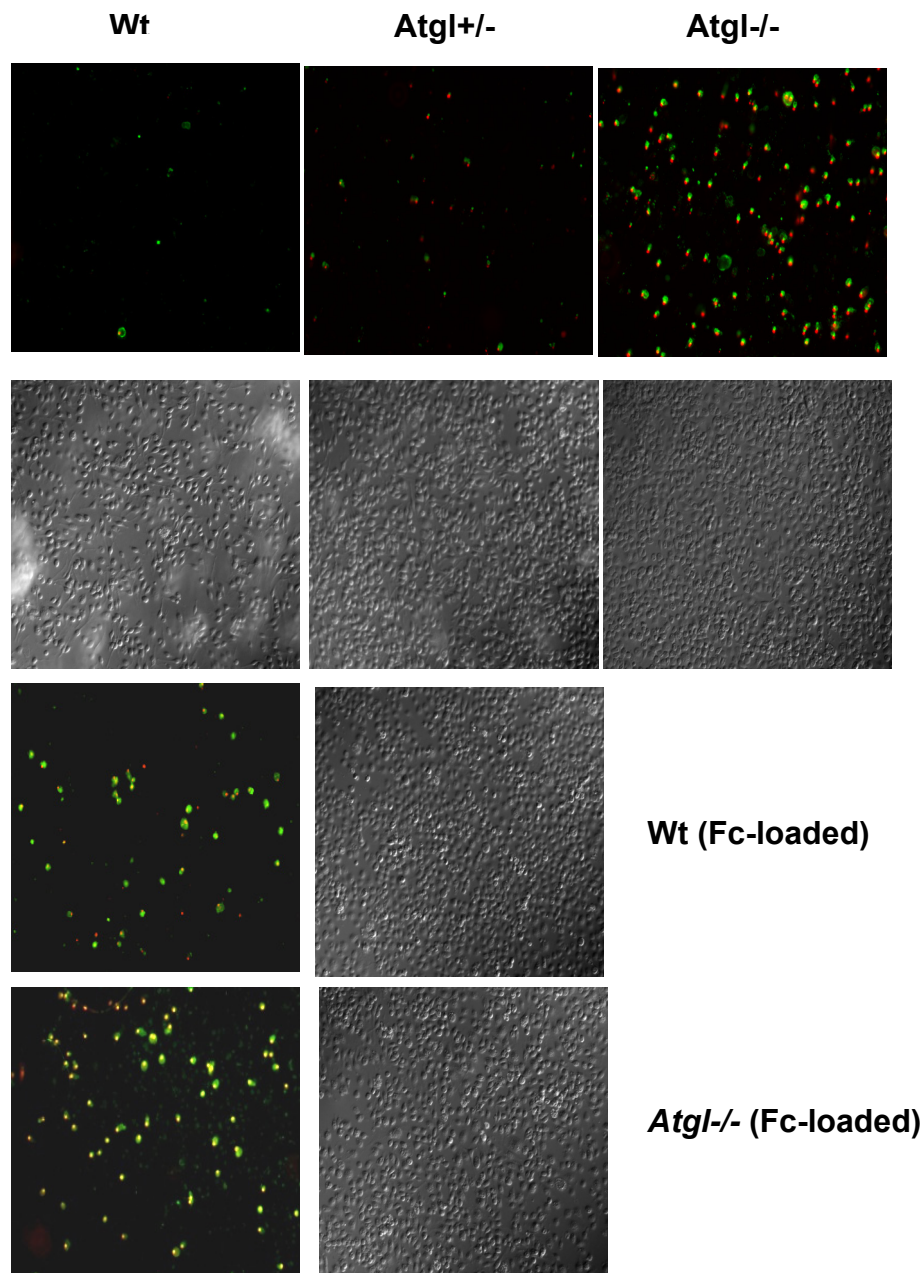


Figure 24. Induction of apoptosis in *Atgl*^{-/-} macrophages (A) Macrophages were isolated from 3 Wt, *Atgl*^{+/-} and *Atgl*^{-/-} mice and cultivated in DMEM/10% LPDS or treated for 24 h with acetylated low-density lipoprotein (acLDL) (100 µg/ml) in the presence of the ACAT inhibitor 58035 (10 µg/ml) for the last 12 h resembling FC loading. (B) Apoptosis was assessed after co-staining with FITC-conjugated annexin V (green) and propidium iodide (PI) (red), respectively, by fluorescence microscopy. Original magnification, x10. Seven fields of cells with ~ 700 cells per field were counted for each condition. Data are expressed as the percent of total cells that stained with annexin V and PI. Quantification of the total amount of annexin V/PI-positive, annexin V-positive and PI-positive cells are shown as means ± SEM of 4 independent experiments performed in duplicate. *** $p \leq 0.001$; ## $p \leq 0.01$ by ANOVA followed by Bonferroni's post-hoc test.

To validate the results obtained by annexin V/PI co-staining, we performed immunoblotting to test for cleavage of caspase 3 and poly (ADP-ribose) polymerase (Parp). Whereas caspase 3 cleavage was not detectable in Wt and *Atgl*^{+/-} macrophages, incubation with FC resulted in prominent caspase 3 cleavage (Figure 25A). In contrast, caspase 3 cleavage was already considerably observed in unloaded *Atgl*^{-/-} macrophages. Consistently, the combined caspase 3 and 7 activities *in vitro* were 2.6-fold increased in untreated *Atgl*^{-/-} macrophages compared to the Wt controls (Figure 25B). Loading of Wt and *Atgl*^{-/-} macrophages with FC resulted in similarly elevated levels of caspase 3 cleavage as observed in untreated *Atgl*^{-/-} macrophages, indicating that the FC loading in *Atgl*^{-/-} cells does not further stimulate caspase 3 processing. Moreover, essentially all cellular Parp was present in the cleaved form in *Atgl*^{-/-} macrophages (87%), which was largely absent in *Atgl*^{+/-} and Wt macrophages (4 and 3%)(Figure 25C). Based on the analysis of apoptotic markers it is evident that macrophages derived from *Atgl*^{-/-} mice show highly increased susceptibility to apoptotic cell death.

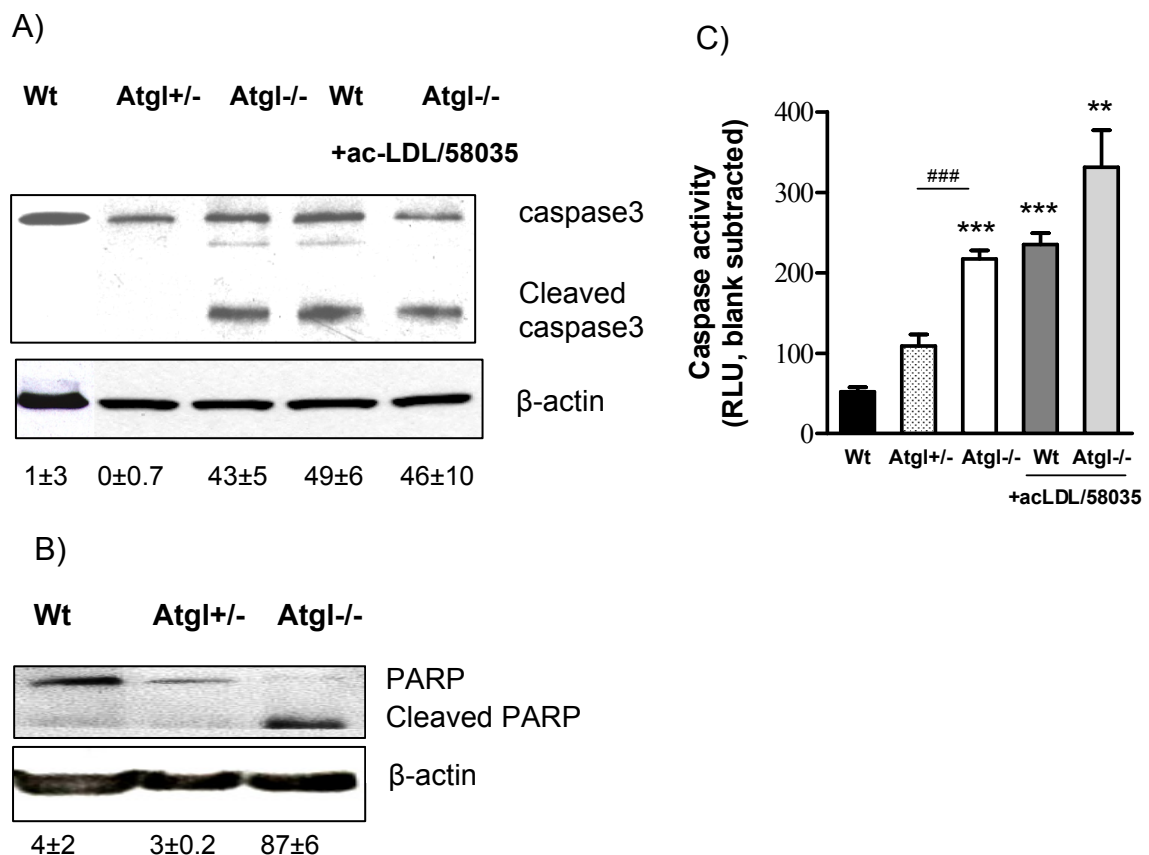
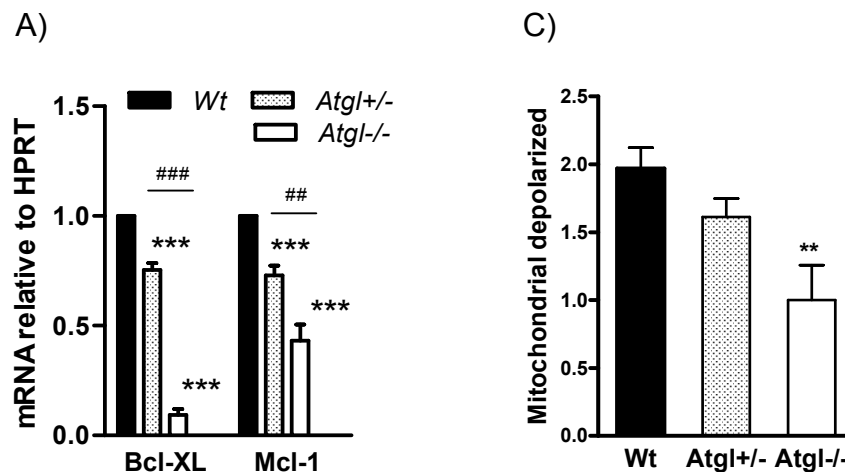


Figure 25. Caspase 3 and PARP activation in *Atgl*^{-/-} macrophages. (A,B) Cell lysates of Wt, *Atgl*^{+/-} and *Atgl*^{-/-} mice (40 µg protein/lane) were separated by SDS-PAGE. Protein expression of (A) cleaved caspase 3 and (B) Parp was analyzed by Western blotting experiments. Expression of the cleaved proteins was quantified relative to the expression of uncleaved proteins. Data are presented as means of 2-3 independent experiments ± SEM. (C) Combined caspase 3 and 7 activities were measured in cell lysates from Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages and FC-loaded cells. Data are presented as mean values (n=4) ± SEM. *** $p \leq 0.001$; #### $p \leq 0.001$ by ANOVA followed by Bonferroni's post-hoc test.

4.2.2. Activation of mitochondrial apoptosis pathway

The apoptotic phenotype in *Atgl*^{-/-} macrophages prompted us to investigate the expression of anti- and pro-apoptotic proteins in these cells. Anti-apoptotic Bcl-XL mRNA was reduced by 91% compared to Wt macrophages and the mRNA level of Mcl-1 was decreased by 57% in *Atgl*^{-/-} macrophages. *Atgl*^{+/-} macrophages showed 25-27% reduced mRNA levels of Bcl-XL and Mcl-1 (Figure 26A). The protein expression of Bcl-2 was slightly reduced in *Atgl*^{+/-} macrophages and decreased by 59% in *Atgl*^{-/-} compared to Wt macrophages. We could not find any difference in Bax protein level between Wt and *Atgl*^{+/-} but more than 3-fold increase in *Atgl*^{-/-} macrophages. Since Bax is proposed to induce the opening of outer mitochondrial membrane channels resulting in the release of pro-apoptotic factors, determined the cytochrome C release into the cytosol. Cytochrome C release was slightly increased in *Atgl*^{+/-} compared to Wt and readily detectable in the cytosol from untreated *Atgl*^{-/-} macrophages (Figure 26B). JC-1 assay was performed to determine the mitochondrial membrane potential ($\Delta\Psi_m$) of *Atgl*^{-/-} macrophages. We observed a 50% decrease in JC-1 red/green fluorescence ratio in *Atgl*^{-/-} compared to *Atgl*^{+/-} and Wt macrophages, suggesting a decrease in the $\Delta\Psi_m$ (Figure 26C).



B)

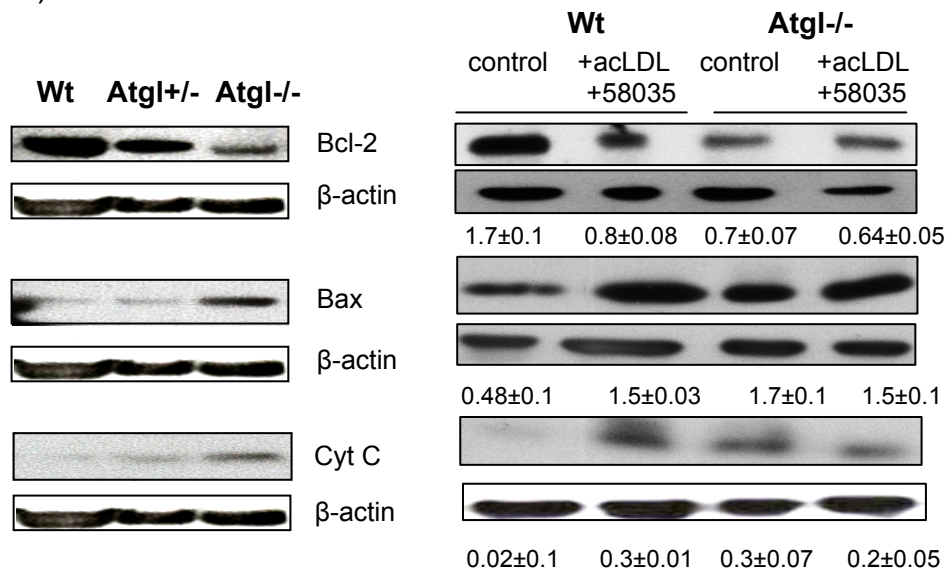


Figure 26. Activation of mitochondrial apoptosis in *Atgl*^{-/-} macrophages. Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages were cultivated in DMEM/10% LPDS or incubated with 100 µg/ml acetylated low-density lipoprotein (acLDL) (24 h) plus ACAT inhibitor 58035 (10 µg/ml) for the last 12 h resembling FC loading. **(A)** Total RNA was isolated from Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages and Bcl-XL and Mcl-1 mRNA levels including normalization to hypoxanthine-guanine phosphoribosyl transferase (HPRT) were determined by real time PCR. Data are expressed as means (n=6) of 2 independent experiments ± SEM. **(B)** Cells were lysed and subjected to cell fractionation. Cytosolic fractions were separated by SDS-PAGE and analyzed for Bcl-2, Bax and cytochrome c expression by Western blotting. Expression of the respective proteins from 3 blots was quantified relative to β-actin expression. Data are presented as means ± SEM. **(C)** Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages were plated in black 96 well plates and treated with JC1 (4µM) and for 40min. The ratio of red and green fluorescence measured for mitochondrial depolarization. *** $p \leq 0.001$; ## $p \leq 0.01$, ### $p \leq 0.001$.

4.2.3. Mitochondrial dysfunction in *Atgl*^{-/-} macrophages

Fluorescence and electron microscopy revealed that mitochondria in Wt and *Atgl*^{+/-} macrophages showed an intact ultrastructure with unfragmented mitochondria. Mitochondria of *Atgl*^{-/-} macrophages, however, were substantially fragmented (Figure 27A), which is a defining feature of apoptosis. Additionally, the size of mitochondria in *Atgl*^{-/-} macrophages ($0.37 \pm 0.16 \mu\text{m}$) was drastically reduced compared to *Atgl*^{+/-} ($0.58 \pm 0.24 \mu\text{m}$) and Wt mitochondria (vs $0.71 \pm 0.28 \mu\text{m}$) (this experiment was performed by Dagmar Kolb) (Figure 27B).

A)

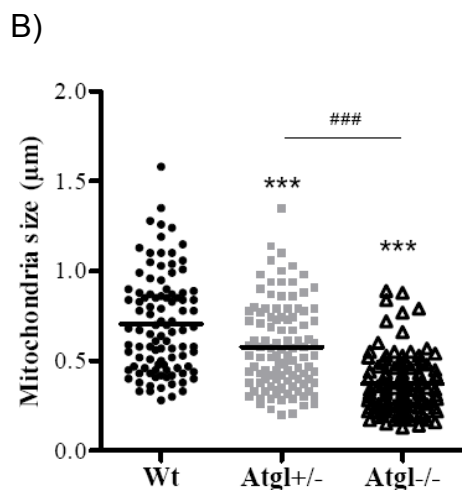
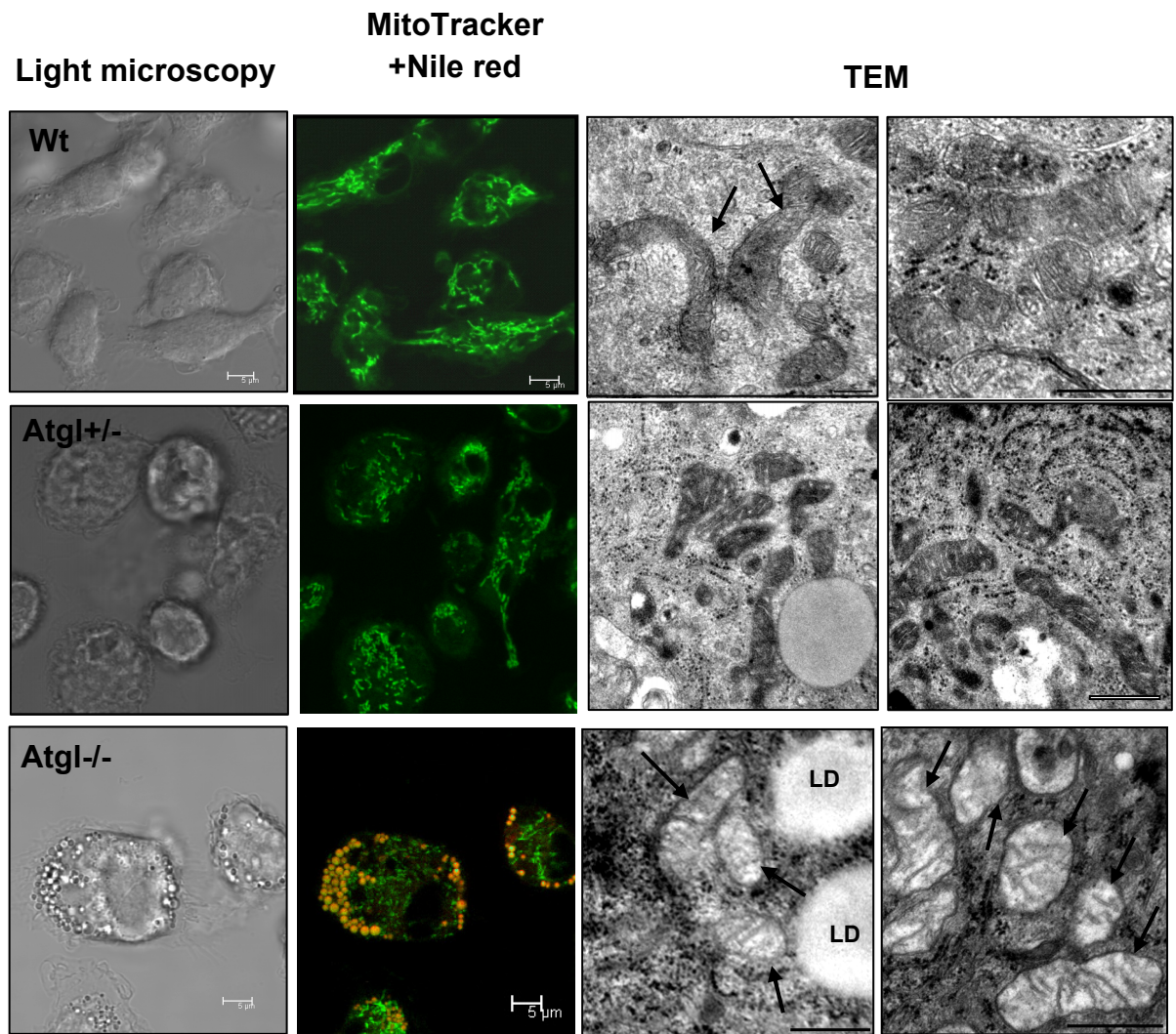


Figure 27. Mitochondrial dysfunction in *Atgl*^{-/-} macrophages. (A) Cells were stained with MitoTracker Green (0.5 μM) and Nile Red (2.5 μg/ml) for 5 min. Representative confocal laser scanning microscopy images are shown. Scale bars: 5 μm. Representative electron micrographs of mitochondria (indicated by arrows) in Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages (done by Dagmar Kolb). LD, lipid droplet. Scale bars: 0.5 μm. (B) The size of mitochondria in electron micrographs of Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages was measured using Olympus iTEM 5.1 soft imaging solution (this experiment was performed by Dagmar Kolb). Data from > 100 mitochondria and the mean values are presented. *** $p \leq 0.001$, ### $p \leq 0.001$ by ANOVA and Bonferroni's multiple comparison test.

4.2.4 Mitochondrial dysfunction leads to ROS production

To delve further insight into the molecular basis of mitochondrial dysfunction in *Atgl*^{-/-} macrophages, we determined mRNA levels of genes important in mitochondrial electron transport system. The mRNA level of NADH:ubiquinone oxidoreductase (complex I) was reduced by 87% in *Atgl*^{-/-} macrophages and NADPH oxidase activator (Noxa1), which triggers the generation of O₂^{•-}, was 4.8-fold increased (Figure 28A). We failed to detect any significant changes in mRNA level of complex I and Noxa1 in *Atgl*^{+/-} macrophages. Intracellular ROS production, as monitored by the conversion of DCFDA to fluorescent DCF, was 9.6-fold higher in *Atgl*^{-/-} compared to Wt macrophages. However we did not observe significant differences in ROS production between *Atgl*^{+/-} and Wt macrophages (Figure 28B). Deficiency of ATGL was also accompanied by significantly reduced mitochondrial O₂ consumption. Measurement of basal respiration demonstrated that *Atgl*^{-/-} macrophages consumed O₂ at a 65% reduced rate compared to Wt macrophages (Figure 28C). This difference was mainly due to a strong reduction in the oligomycin-sensitive respiration (i.e. oxidative phosphorylation). The maximal O₂ consumption, determined by uncoupling the respiratory chain with carbonyl cyanide p(trifluoromethoxy) phenylhydrazone (FCCP), was reduced by 70% in *Atgl*^{-/-} compared to Wt macrophages. Accordingly, the spare respiratory capacity, which is calculated as the difference between the mitochondrial O₂ consumption under basal conditions and the maximal O₂ consumption, was reduced by 73% in *Atgl*^{-/-} compared to Wt macrophages. Importantly, mitochondria are considered the most critical organelle for energy production inside the cell. Therefore ATP assay was performed in Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages. ATP concentration was reduced in *Atgl*^{-/-} macrophages, while it was comparable in Wt and *Atgl*^{+/-} macrophages (Figure 28D). Moreover, the source of ATP in mitochondria is FFA, which undergoes beta oxidation for energy production. FFA concentrations in freshly isolated macrophages without any exogenous stimulus were slightly but significantly reduced in *Atgl*^{+/-} and *Atgl*^{-/-} compared to Wt macrophages (Figure 28E) (measurement of FFA concentration was done by Andreas Uel, Ien). In different

composition of FFA level of C14, C16 and C18 were decreased significantly in both *Atgl*^{+/-} and *Atgl*^{-/-} compared to Wt macrophages.

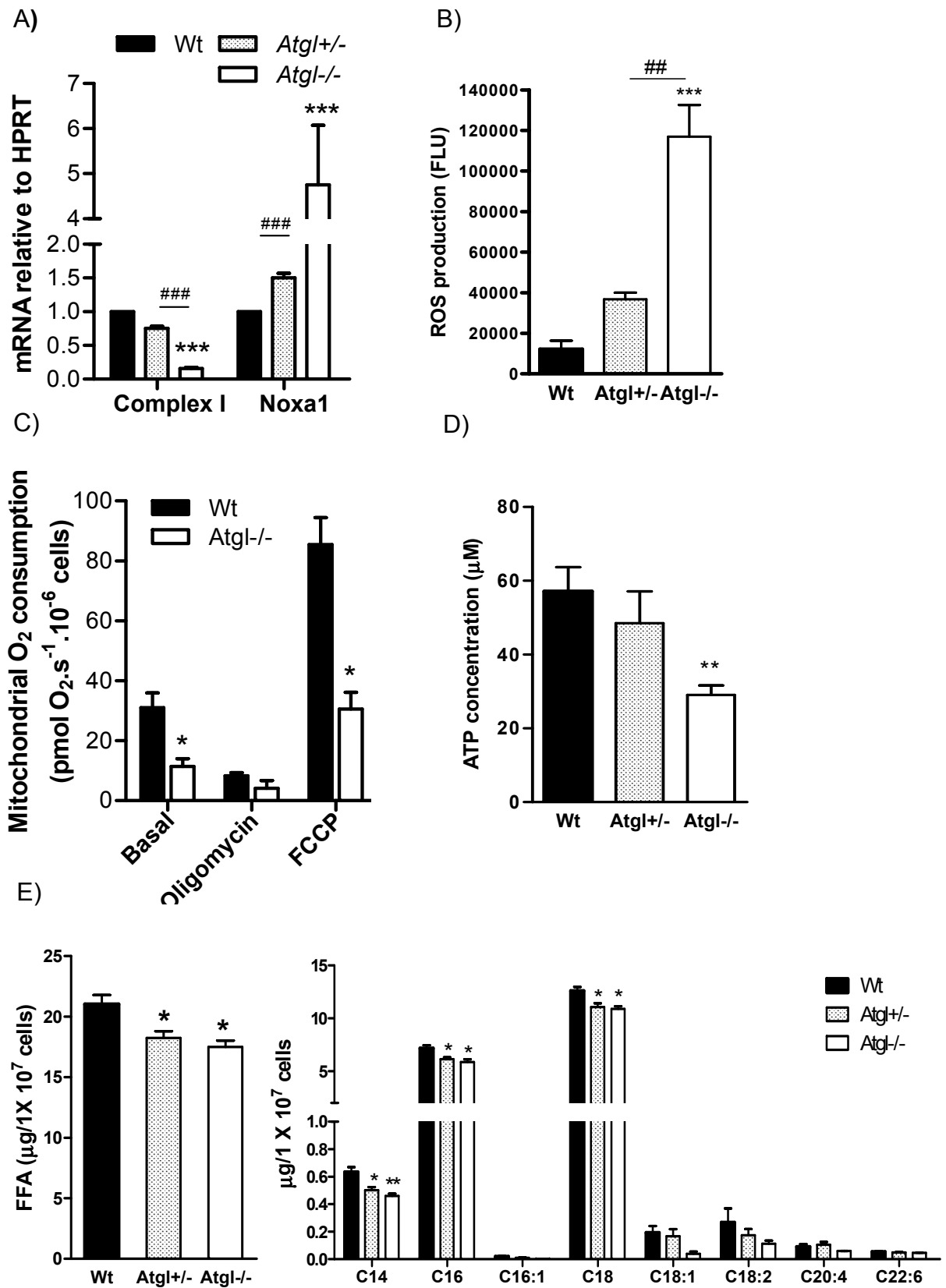


Figure 28 Mitochondrial dysfunction leads to increased ROS and reduced ATP production in *Atgl*^{-/-} macrophages. (A) NADH:ubiquinone oxidoreductase (complex I) and NADPH oxidase activator (Noxa1) mRNA levels including normalization to HPRT were determined in Wt and *Atgl*^{-/-} macrophages by real time PCR. Data are expressed as mean values (n=6) ± SEM. (B) Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages were stained with 50 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (DCFDA) for 10 min. Intracellular ROS production in macrophages was determined by the conversion of DCFDA to fluorescent DCF (excitation: 488 nm; emission: 535 nm) measured in a FlexStationTM II device (Molecular Devices, Downingtown, PA). (C) Basal respiration was determined in untreated Wt and *Atgl*^{-/-} macrophages. ATP synthase was inhibited by 2 µg/ml oligomycin, followed by uncoupling of the respiratory chain from oxidative phosphorylation by a stepwise titration with carbonyl cyanide p(trifluoromethoxy)phenylhydrazone (FCCP) to achieve maximal O₂ consumption. (D) ATP concentrations in Wt, *Atgl*^{+/-} and *Atgl*^{-/-} macrophages were determined after cultivating the cells for 24 h in DMEM containing 25 mM glucose and 10% LPDS using EnzyLight ATP assay (BioAssay Systems, Hayward, CA). Data are expressed as means (n=3-5) measured in triplicate ± SEM. (E) FFA concentrations (sum of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:4, C22:6) are presented as means (n=3-4) ± SEM (Experiment was done by Andreas Uellen). * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, #### $p \leq 0.001$ by ANOVA followed by Bonferroni's post-hoc test.

4.2.5 Triglyceride accumulation leads to elevation of cytosolic Ca²⁺

One of the most important protein which shown to be involved in caspase independent cell death is apoptosis inducing factor (AIF). This protein is anchored in the mitochondrial inner membrane. AIF manifests NADH oxidase activity and is important for oxidative phosphorylation (Susin *et al.*, 1999). AIF is synthesized as a 67 kDa protein in the cytosol. After import to the mitochondria it is matured to 62 kDa. In the process of apoptosis, AIF need to be cleaved by Ca²⁺ dependant calpain, which occurs as a consequence of increased intracellular Ca²⁺ (Norberg *et al.*, 2008b). Therefore, first we measured the cytosolic Ca²⁺ in *Atgl*^{-/-} macrophages to investigate the effect of increased ROS production in mitochondrial dysfunction via a caspase- independent pathway. After incubation of macrophages with fura-2 we observed a clear increase of the basal F340/F380 ratio in *Atgl*^{-/-} compared to Wt macrophages, indicating that the cytosolic Ca²⁺ concentration ([Ca²⁺]_{cyto}) is significantly elevated in cells lacking ATGL (Figure 29A). To test whether or not the endoplasmic reticulum Ca²⁺ content is affected in *Atgl*^{-/-} macrophages [Ca²⁺]_{cyto} signals were recorded upon Sarcoplasmic/endoplasmic reticulum Ca²⁺ (SERCA) inhibition with 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ) in the absence of extracellular Ca²⁺. This yields to an elevation of [Ca²⁺]_{cyto} due to a passive Ca²⁺ leak from the endoplasmic reticulum. As shown in Figure 29A and 29B, *Atgl*^{-/-}

macrophages displayed a markedly reduced response to BHQ compared to Wt cells, implicating that the endoplasmic reticulum Ca^{2+} content was decreased in *Atgl*^{-/-} macrophages. Increased intracellular Ca^{2+} levels are a prerequisite step for processing and release of apoptosis inducing factor (Aif). Indeed, we observed a nuclear localization of Aif in *Atgl*^{-/-} macrophages, which is generally associated with apoptosis (Figure 29C).

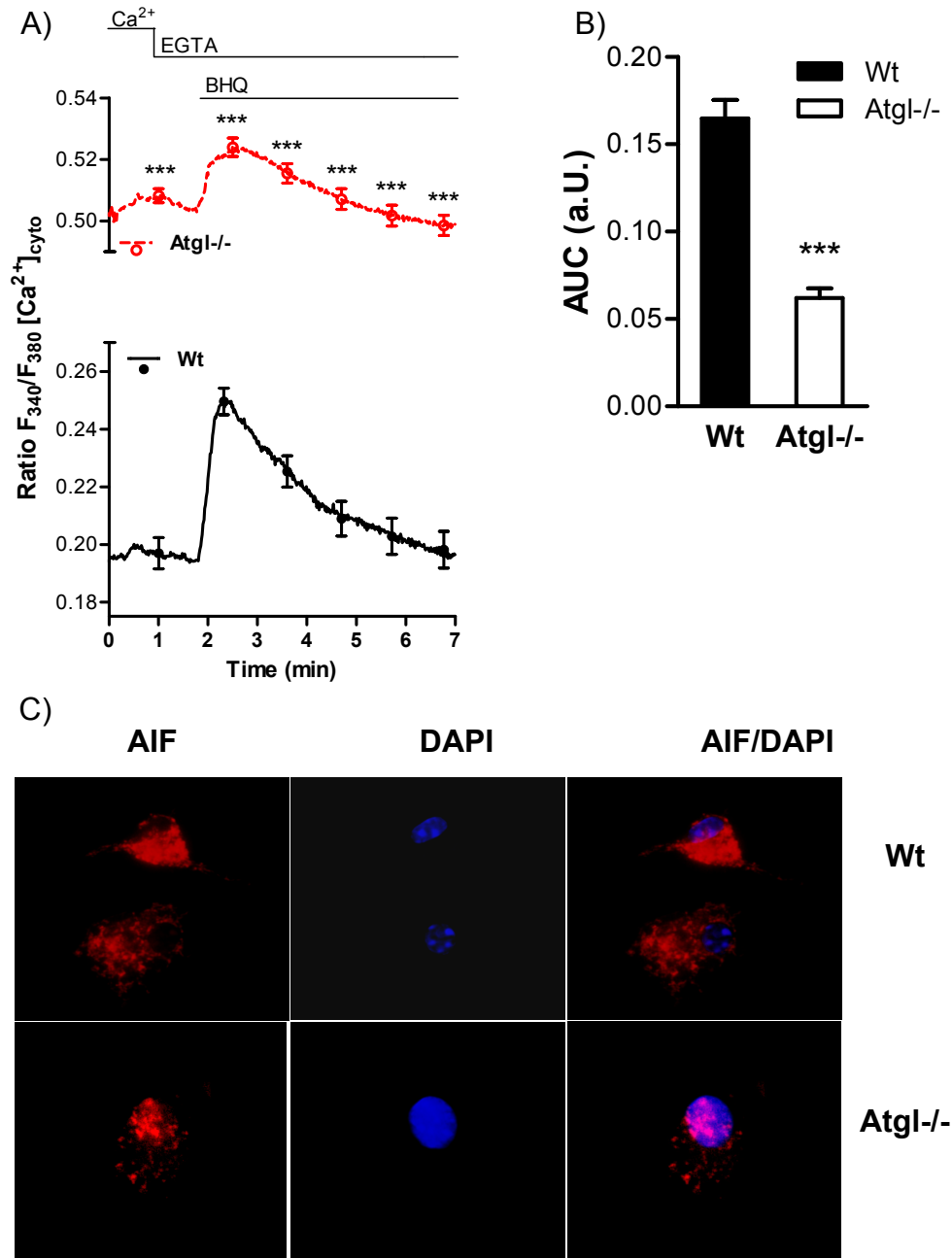


Figure 29. : Elevation of cytosolic Ca^{2+} and nuclear localization of Aif in *Atgl*^{-/-} macrophages. Wt and *Atgl*^{-/-} macrophages were plated on coverslips in DMEM/10% LPDS.

(A) The fura-2 fluorescence ratio (340/380 nm) was determined in single macrophages before and after the addition of 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ, 15 mM). Data are expressed as mean values of ~300 cells per genotype of 3 independent experiments $\pm$ SEM. (B) The area under the curve was calculated after the addition of BHQ in Wt and *Atgl*^{-/-} macrophages. Data are presented as means $\pm$ SEM. *** $p \leq 0.001$ determined by Student's *t*-test. (C) Wt and *Atgl*^{-/-} macrophages were fixed and incubated with anti-AI. To visualize AIF cells were then incubated with anti-rabbit AlexaFluor-594 and mounted in Vectashield/DAPI to visualize the nucleus. Representative images taken by fluorescence microscopy are shown

4.2.6. VLDL loading increases apoptosis in Wt macrophages

Mitochondrial dysfunction and apoptosis in *Atgl*^{-/-} macrophages may be due to increased levels of TG or reduced lipolysis and release of FFA as substrates of β -oxidation. To discriminate between these possibilities, we examined whether VLDL-induced TG loading of macrophages can phenocopy the consequences of genetically-induced TG accumulation by ATGL deficiency. We incubated Wt macrophages with VLDL (150 μ g/ml) for 24 h to achieve similar TG loading as observed in *Atgl*^{-/-} macrophages (Figure 30A). In accordance to the findings in *Atgl*^{-/-} macrophages, we found an increased number of annexin V/PI-stained cells upon incubation of Wt macrophages with VLDL (Figure 30B). Quantification of the stained cells revealed that VLDL loading increased the number of annexin V-positive cells (indicative of early apoptosis) and the number of annexin V/PI-positive cells (late apoptosis) (Figure 30C). Similar to *Atgl*^{-/-} macrophages, caspase 3/7 activity was 4.3-fold increased in VLDL-loaded compared to untreated macrophages (Figure 30D). mRNA levels of Bcl-XL and Mcl-1 were reduced in VLDL-treated macrophages by 64% and 45%, respectively (Figure 30E). Additionally, we observed cleaved caspase 3 and Parp in Wt cells after loading with VLDL (Figure 30F). Bax expression was 1.8-fold increased and cytosolic cytochrome c, which was undetectable in untreated Wt macrophages, was induced (Figure 30G). Accordingly, we found decreased protein levels of anti-apoptotic Bcl-2. These phenotypes are highly similar to *Atgl*^{-/-} macrophages and clearly confirm increased TG concentrations as profound inducers of apoptosis.

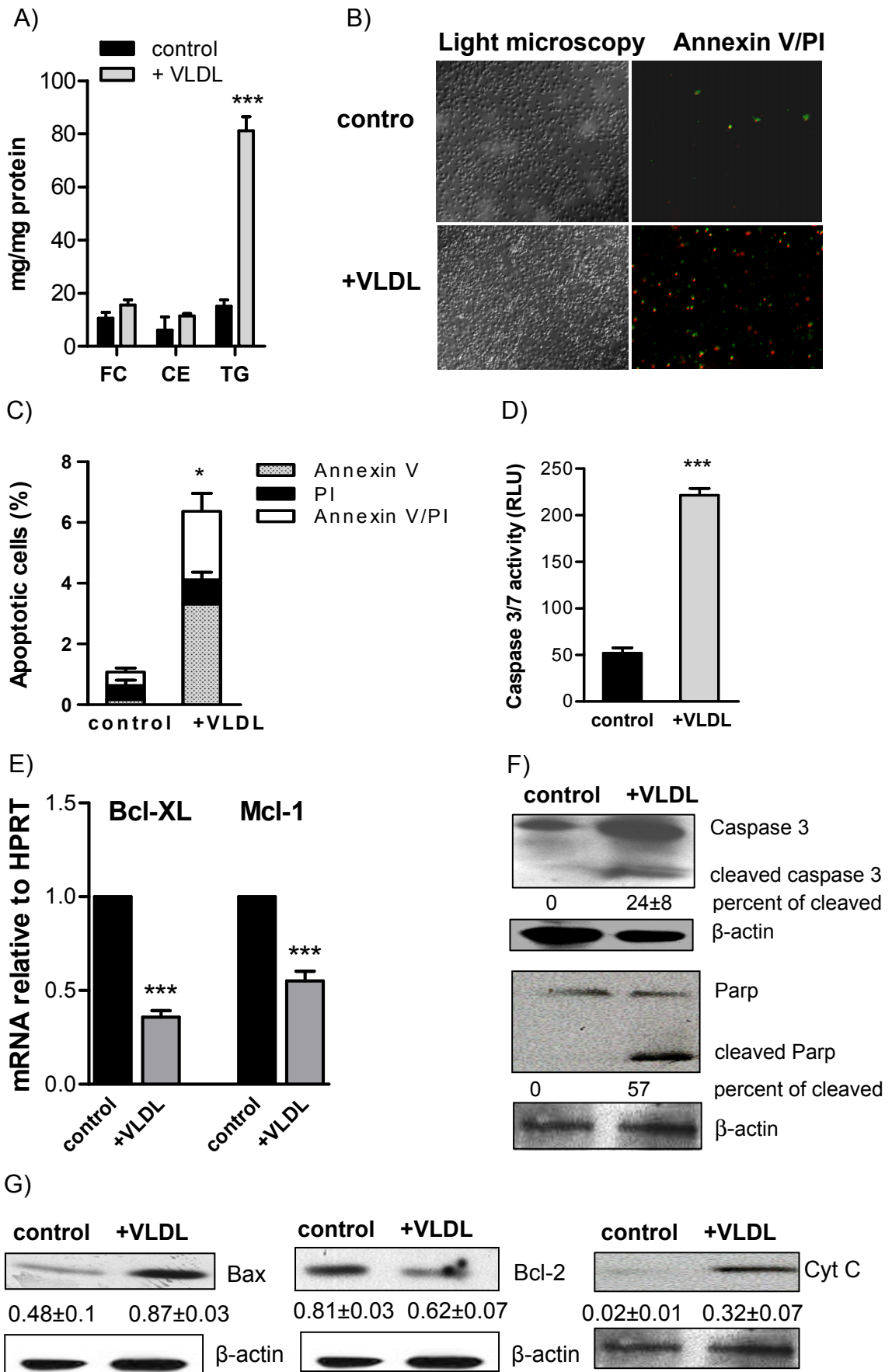


Figure 30. Induction of apoptosis in VLDL-loaded Wt macrophages. Wt macrophages were cultivated in DMEM/10% LPDS in the absence (control) or presence of VLDL (150µg/ml, 24 h). **(A)** Intracellular free cholesterol (FC), cholesterol ester (CE) and triacylglycerol (TG) concentrations were measured enzymatically. *** $p \leq 0.001$. **(B)** Apoptotic cells were determined by FITC-conjugated annexin V (green) and propidium iodide (PI) (red) co-staining and images were taken by fluorescence microscopy. Original magnification, x10. **(C)** Quantification of fluorescence signals from three fields of cells (~ 650 cells per field) for each condition is shown. Data are expressed as the percent of cells stained with annexin V and PI (means $\pm$ SEM from 3 independent experiments performed in duplicate). **(D)** Combined caspase 3 and 7 activities were measured in cell lysates from untreated and VLDL-loaded Wt macrophages. Data are presented as mean values (n=4) $\pm$ SEM. **(E)** Total RNA was isolated from untreated and VLDL-loaded Wt macrophages and Bcl-XL and Mcl-1 mRNA levels including normalization to hypoxanthine-guanine phosphoribosyl transferase (HPRT) were determined by real time PCR. Data are expressed as means $\pm$ SEM of 3 independent experiments performed in triplicate. **(F,G)** Cell extracts (40 µg protein/lane) were resolved by SDS-PAGE and protein expression of **(F)** caspase 3, Parp, and **(G)** Bax, Bcl-2, and cytochrome c (Cyt C) was analyzed by Western blotting. Expression of the respective proteins was quantified relative to β -actin expression. Data are presented as means of 3 independent experiments $\pm$ SEM. All statistical significances were determined by Student's *t*-test. * $p < 0.05$, *** $p \leq 0.001$

4.2.7 VLDL loading results in mitochondrial dysfunction of Wt macrophages

Similar to *Atgl*^{-/-} macrophages, VLDL-loading of Wt macrophages revealed a 12.6 ± 3 % decreased red/green fluorescence ratio in the JC-1 assay compared to unloaded macrophages (Figure 31B), indicative of a reduced membrane potential. Fluorescence and electron micrographs of VLDL-loaded Wt macrophages showed that the mitochondria show features of fragmentation (Figure 31A), very similar to the structure of mitochondria in *Atgl*^{-/-} macrophages (Figure 31A). mRNA levels of NADH:ubiquinone oxidoreductase (complex I) were reduced by 44% in VLDL-loaded Wt cells compared to untreated macrophages (Figure 14C), whereas Noxa1 mRNA levels were 10.8-fold increased. VLDL loading also substantially increased ROS production in Wt cells by 10-fold (Figure 31D).

In analogy to *Atgl*^{-/-} macrophages, basal respiration was strongly reduced by 71% upon VLDL loading compared to untreated Wt macrophages (Figure 31E). The maximal O₂ consumption after FCCP treatment was also reduced in VLDL-loaded macrophages, yielding a 71% decreased spare respiratory capacity in VLDL-loaded compared to untreated Wt macrophages (Figure 31E).

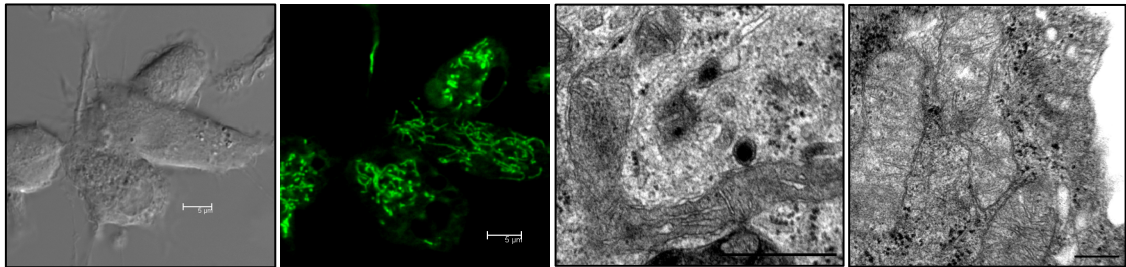
A)

Light microscopy

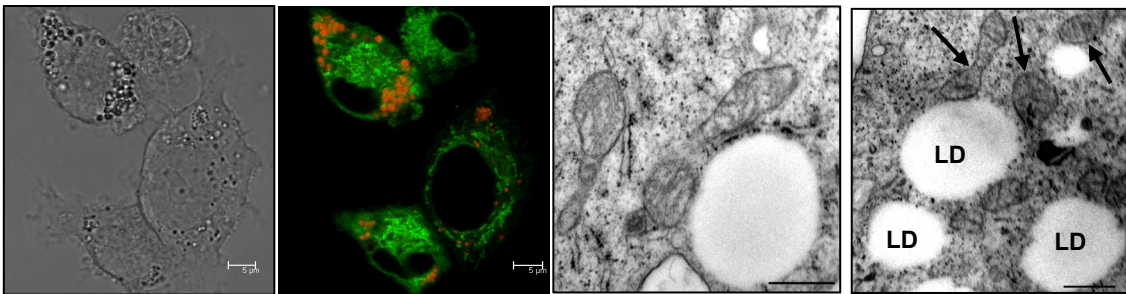
MitoTracker +
Nile Red

TEM

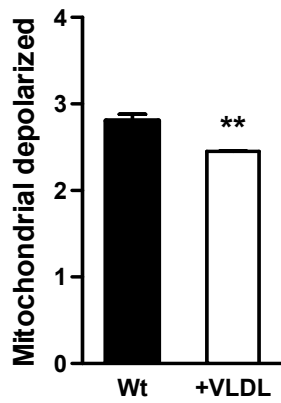
Wt



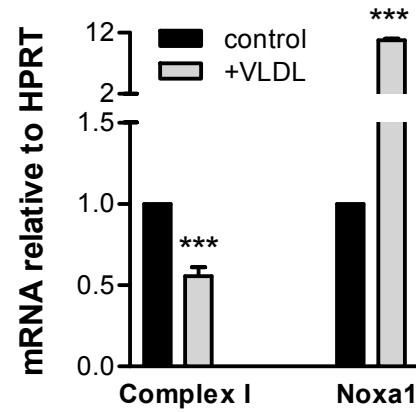
+VLDL



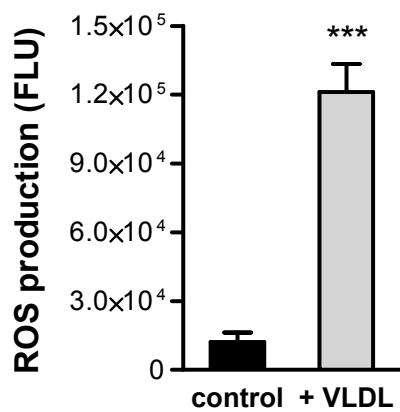
B)



C)



D)



E)

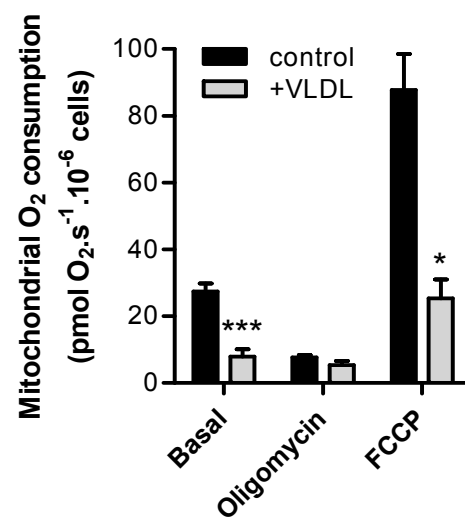


Figure 31. Mitochondrial dysfunction in VLDL-loaded Wt macrophages. Wt macrophages were cultivated in DMEM/10% LPDS in the absence (control) or presence of VLDL (150 µg/ml, 24 h). **(A)** Cells were stained with MitoTracker Green (0.5 µM) and Nile Red (2.5 µg/ml) for 5 min. Representative confocal laser scanning microscopy images are shown. Scale bars: 5 µm. Representative electron micrographs (TEM) of mitochondria (indicated by arrows) in untreated (control) and VLDL-loaded Wt macrophages. LD, lipid droplet. Scale bars: 0.5 µm. **(B)** Wt, Atgl^{+/-} and Atgl^{-/-} macrophages were plated in black 96 well plates and treated with JC1 (4µM) and for 40min. The ratio of red and green fluorescence measured for mitochondrial depolarization. **(C)** NADH:ubiquinone oxidoreductase (complex I) and NADPH oxidase activator (Noxa1) mRNA levels including normalization to hypoxanthine-guanine phosphoribosyl transferase (HPRT) were determined by real time PCR. Data are expressed as mean values (n=3) performed in triplicate ± SEM. **(D)** Cells were stained with 50 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (DCFDA) for 10 min. Intracellular ROS production was determined by the conversion of DCFDA to fluorescent DCF in a FlexStationTM II device. **(E)** Oxygen consumption in mitochondria from untreated and VLDL-loaded Wt macrophages. Basal respiration, oxygen consumption after ATP synthase inhibition with 2 µg/ml oligomycin and maximal O₂ consumption by uncoupling of the respiratory chain from oxidative phosphorylation with carbonyl cyanide p(trifluoromethoxy) phenylhydrazone (FCCP) were determined. Data are expressed as means (n=6-10) ± SEM. * $p < 0.05$, *** $p \leq 0.001$. All statistical significances were determined by Student's *t*-tes.

4.3. TG accumulation triggers ER stress via ATF4/CHOP pathway

4.3.1 TG accumulation induces activation of ATF4/CHOP pathway

Concerning the Ca^{2+} depletion in *Atgl*^{-/-} and Wt-VLDL loaded macrophages, we checked the key regulators of ER stress in these samples. Bip mRNA expression as one of the most important UPR was increased 2-fold in *Atgl*^{-/-} and 2.5-fold in Wt VLDL-loaded macrophages (Figure 32A), which led to phosphorylation of PERK, while PERK phosphorylation failed to occur in Wt macrophages (Figure 32B). Phosphorylation of the PERK substrate eIF2 α was also increased 2.9-fold in *Atgl*^{-/-} and 2.6-fold in VLDL-loaded Wt macrophages, respectively (Figure 32C). Followed by eIF2 α phosphorylation, ATF4 expression was also increased in nuclear fraction of *Atgl*^{-/-} and VLDL-loaded Wt macrophages (Figure 32C). Moreover, ATF4 was translocated from the cytosol to the nucleus in these cells (Figure 32D).

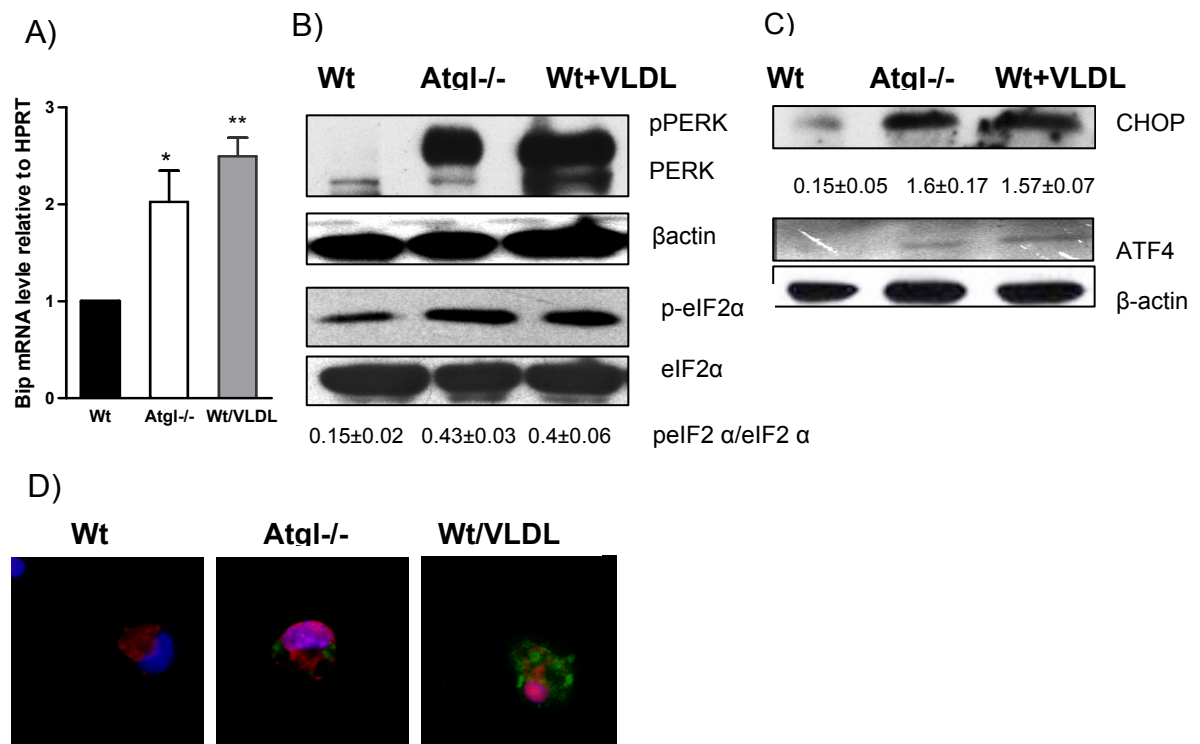


Figure 31. TG accumulation triggers ER stress via ATF4/CHOP pathway. (A) Bip mRNA level including normalization to HPRT was determined by real-time PCR. **(B)** Cytosolic fraction from Wt, *Atgl*^{-/-} and VLDL-loaded Wt macrophages was resolved by SDS-PAGE and protein expression was measured by western blotting. **(C)** Nuclear fraction from Wt, *Atgl*^{-/-} and VLDL-loaded Wt macrophages were subjected to SDS-PAGE and expression of CHOP and ATF4 was measured by western blotting. **(D)** Cell from Wt, *Atgl*^{-/-} or VLDL-loaded Wt macrophages

were fixed and incubated with anti-ATF4 antibody for 1 h. To visualize ATF4 cells were then incubated with anti-rabbit AlexaFluor-594 and mounted in Vectashield /DAPI to visualize the nucleus. Representative fluorescence micrographs are shown.

4.3.2 IRE-1 α pathway failed to be activated in *Atgl*^{-/-} macrophages

Upregulation of Bip as an important UPR causes activation of IRE1 α in *Atgl*^{-/-} and VLDL-loaded Wt macrophages (Figure 32A). IRE1 α phosphorylation activates its kinase and RNase activities; therefore we sought to check the XBP-1 splicing in *Atgl*^{-/-} and VLDL-loaded Wt macrophages. Surprisingly, we could not detect any splicing of XBP-1 (Figure 32B). Moreover, the expression of XBP-1 is abolished in *Atgl*^{-/-} and VLDL-loaded Wt macrophages. Therefore, the ATF6 cleavage, which induces the expression of XBP-1 after its translocation to the nucleus, was checked. Figure 32A illustrates that ATF6 cleavage was absent in *Atgl*^{-/-} and VLDL-loaded Wt macrophages. Immunofluorescence staining confirmed the lack of ATF6 translocation to the nucleus (Figure 32C).

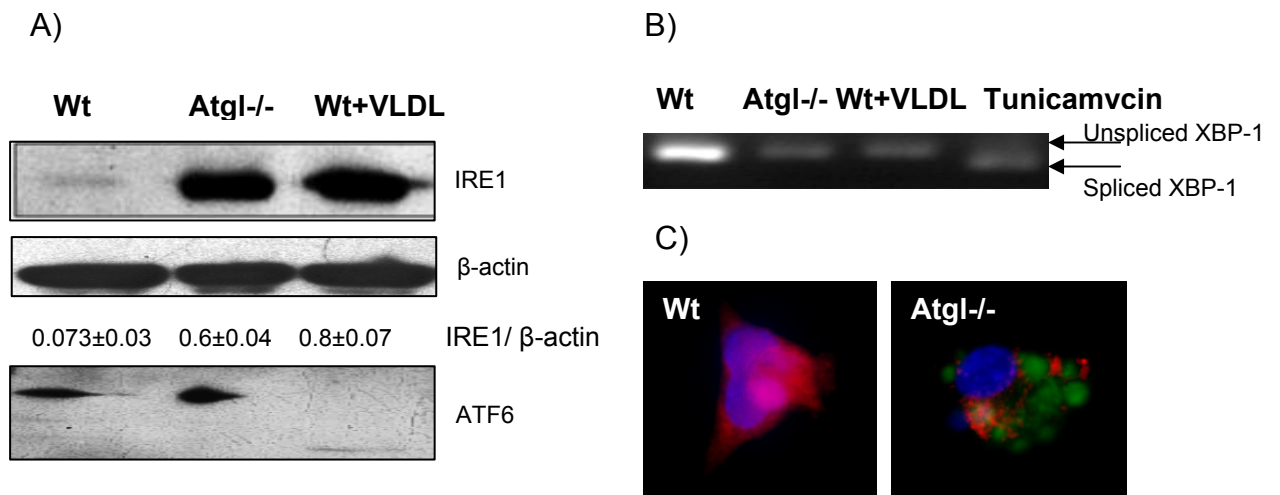
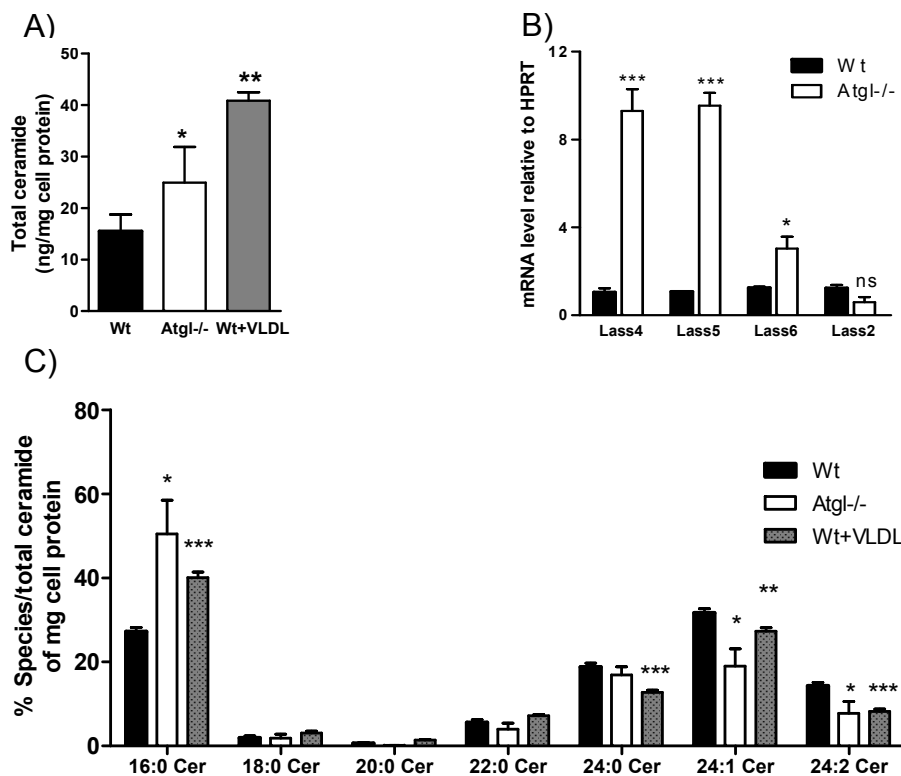


Figure 32. Lack of IRE1 α activation in *Atgl*^{-/-} macrophages. (A) Cytosolic fraction and nuclear fraction were subjected to SDS-PAGE and expression of IRE1 α or ATF6 were measured by western blotting. (B) RNA from Wt, *Atgl*^{-/-} and VLDL-loaded Wt macrophages were isolated and reverse transcribed. PCR for XBP-1 was performed for these samples. (C) Wt and *Atgl*^{-/-} macrophages were fixed and to visualize ATF6 cells were incubated with anti-rabbit AlexaFluor-594 and mounted in Vectashield/DAPI. Bodipy staining was performed to detect lipid droplets. Representative fluorescence micrographs are shown.

4.3.3 ATGL deficiency induces ceramide accumulation in macrophages

To find out the reason of mitochondrial dysfunction in *Atgl*^{-/-} or VLDL-loaded Wt macrophages, ceramide concentration was measured in freshly isolated *Atgl*^{-/-} (without exogenous lipid) and Wt macrophages loaded with 150 µg/ml VLDL. Total ceramide was increased 1.6-fold in *Atgl*^{-/-} and 2.6-fold in VLDL-loaded Wt macrophages (Figure 33A). Moreover, measuring different species of ceramide showed that C16:0 ceramide increased significantly in *Atgl*^{-/-} and VLDL-loaded Wt macrophages (Figure 33C). In addition, C24:0, C24:1 and C24:2 were significantly decreased in *Atgl*^{-/-} and VLDL-loaded macrophages. To confirm the accumulation of C16:0 ceramide in *Atgl*^{-/-} macrophages, quantitative PCR was performed to measure the expression of different ceramide synthases. Figure 33C shows that Lass 4 and 5 expressions were increased 9-fold and Lass6 increased 2.4-fold in *Atgl*^{-/-} macrophages, while Lass2 expression was decreased 50% in these cells and Lass1 was not expressed in macrophages (Figure 33B). Ceramide accumulation has an impact in the MAPK pathway. Therefore, I determined p38, Akt and JNK phosphorylation in Wt, *Atgl*^{-/-} and VLDL-loaded Wt macrophages. Inhibition of p38, Akt and JNK phosphorylation was observed in *Atgl*^{-/-} and VLDL-loaded Wt macrophages (Figure 33D).



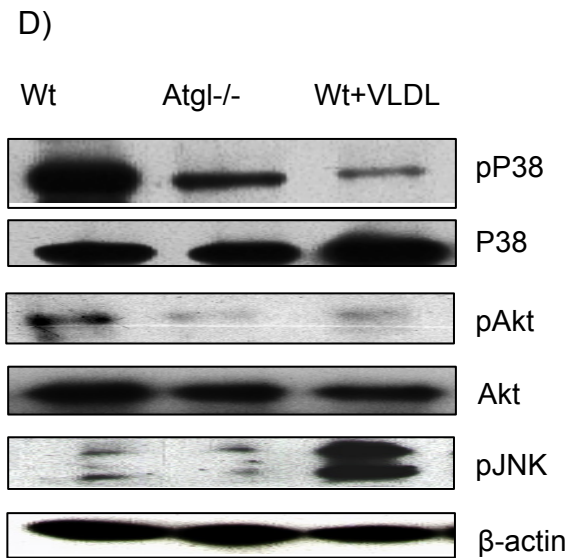


Figure 33. ATGL deficiency in macrophages leads to ceramide accumulation. (A,C) Macrophages from Wt, *Atgl*^{-/-} and Wt VLDL-loaded were plated on 12 well plate and ceramide were isolated immediately after culture. Different species of ceramide was measured by HPLC-MSMS by Harald Kofeler. **(B)** RNA from Wt and *Atgl*^{-/-} macrophages was isolated and expressions of LASS family proteins were measured using real-time PCR. Data are represented from the mean of 2 different experiments. **(D)** Proteins from Wt, *Atgl*^{-/-} and Wt VLDL loaded macrophages were resolved in SDS PAGE and activation of p38, Akt and JNK were investigated using specific antibodies

4.3.4 Inhibition of ceramide accumulation rescues mitochondrial dysfunction in *Atgl*^{-/-} macrophages

We have shown that ceramide accumulation induces apoptosis in *Atgl*^{-/-} macrophages. To inhibit ceramide synthesis, *Atgl*^{-/-} macrophages were treated with fumonisin B1 (FB1) and MTT assay was performed to measure cell viability. Cell viability was decreased 27 % in *Atgl*^{-/-} macrophages respectively while incubating these cells with FB1, rescued 10 % *Atgl*^{-/-} macrophages (figure 34A). As FB1 inhibits *de novo* and salvage pathway, levels of different ceramide species were investigated by HPLC-MS/MS. Figure 34B illustrates that C16:0 ceramide level was 44 % decreased in FB1-treated *Atgl*^{-/-} compared to untreated *Atgl*^{-/-} macrophages and reached the level of Wt macrophages. C24:0, C24:1 and C24:2 levels were increased by FB1 treatment and were comparable to the levels in Wt macrophages.

Thereafter, we checked whether FB1 has also an effect on mitochondrial dysfunction of *Atgl*^{-/-} macrophages. For this purpose protein expressions of pro-apoptotic Bax and anti-apoptotic Bcl-2 was identified by immunoblotting. Bax expression was decreased in FB1-treated *Atgl*^{-/-} macrophages and reached the level of Wt cells (Figure 34C). Moreover, Bcl2 expression level was determined in FB1-treated *Atgl*^{-/-} macrophages and was comparable with the expression of Bcl2 in Wt macrophages (Figure 34B). MitoTracker staining showed significant decrease in fragmented mitochondria compared to *Atgl*^{-/-}

macrophages (Figure 34D), indicating that the inhibition of ceramide synthesis eliminates mitochondrial dysfunction in *Atgl*^{-/-} macrophages.

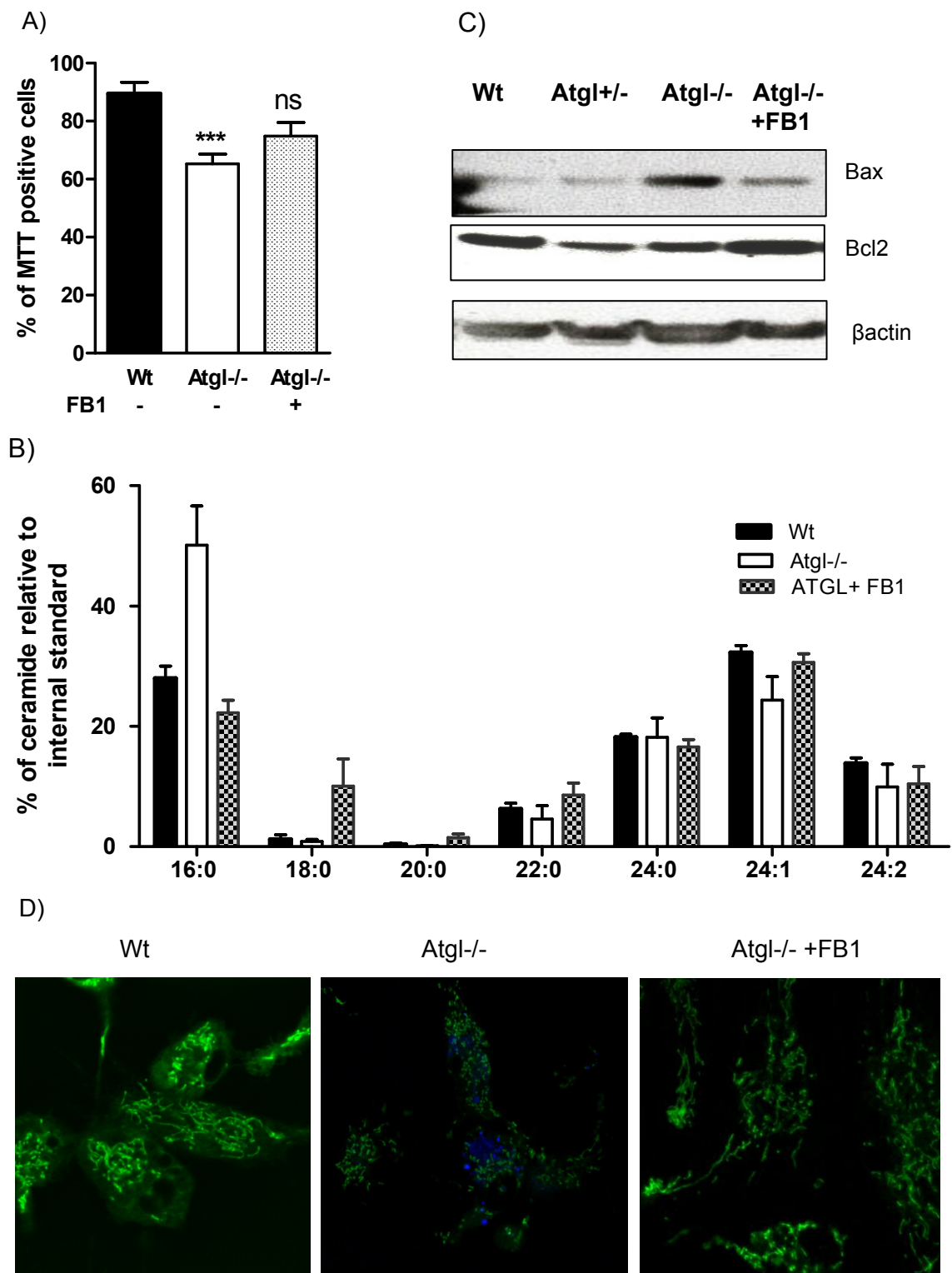


Figure 34. Ceramide accumulation induces apoptosis via mitochondrial dysfunction. (A) Effect of FB1 (10 μ M, 12 h) on *Atgl*^{-/-} macrophages was measured by MTT assay. **(B)** Expression of Bax and Bcl-2 was investigated from cytosolic fraction of Wt, *Atgl*^{+/-}, *Atgl*^{-/-} and

Atgl^{-/-} treated with FB1 by immunoblotting. **(C)** Cells were stained with MitoTracker Green (0.5 μ M) for 5 min. Representative confocal laser scanning microscopy images are shown. **(D)** Different ceramide species were measured by MS/MS.

4.3.5 Inhibition of ceramide accumulation has no effect in inhibition of ER stress in *Atgl*^{-/-} macrophages

We have shown that *Atgl*^{-/-} or *VLDL*-loaded Wt macrophages induce ER stress via ATF4/CHOP pathway. Moreover, as we showed ceramide accumulation in these macrophages, inhibition of ceramide synthesis with FB1 was performed to investigate the ER stress at this condition. Figure 35A illustrates that phosphorylation of eIF2 α and IRE1 α was not inhibited by using FB1. In addition, ATF6 was translocated from the cytosol to the nucleus which was not observed in *Atgl*^{-/-} macrophages (Figure 35B).

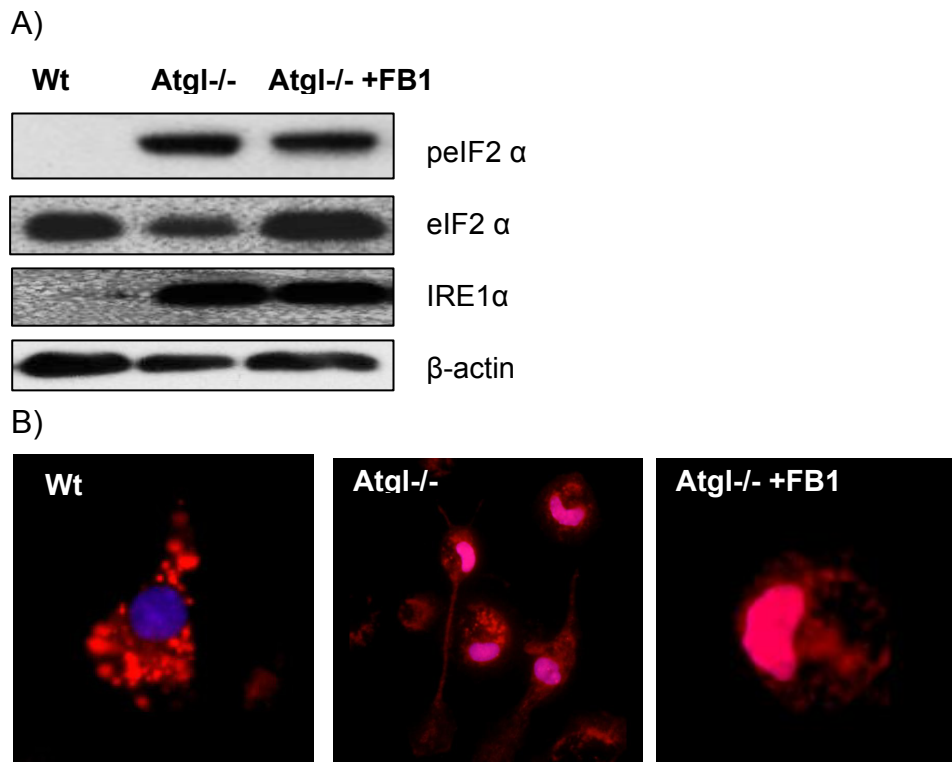


Figure 35. Inhibition of ceramide accumulation does not rescue the ER stress in *Atgl*^{-/-} macrophages. **(A)** Expression of eIF2 α and IRE1 α was investigated from Wt, *Atgl*^{-/-} and FB1 treated *Atgl*^{-/-} macrophages. **(B)** Wt, *Atgl*^{-/-} and FB1-treated *Atgl*^{-/-} macrophages were fixed and to visualize ATF6 cells were incubated with anti-rabbit AlexaFluor-594 and mounted in Vectashield/DAPI. Representative fluorescence micrographs are shown.

5. Conclusion

Macrophages are versatile cells that fulfill various functions in response to environmental changes. Macrophage-derived foam cells play an important role in the progression of inflammatory diseases like atherosclerosis. We have recently reported that *Atgl*^{-/-} macrophages, which accumulate TG-rich lipid droplets, show impaired phagocytosis due to a reduced availability of FFA as energy substrate (Chandak et al., 2010). Moreover, we found markedly decreased atherosclerosis in (*Ldlr*^{-/-} mice after transplantation of *Atgl*^{-/-} bone marrow, compared to Wt bone marrow-transplanted *Ldlr*^{-/-} mice (Lammers et al., 2011). The attenuation of lesion formation in these mice might be (at least in part) due to decreased leukocyte infiltration into the arterial wall. Recruitment of monocytes and macrophages to the site of inflammation and the secretion of cytokines are critical steps in the inflammatory response and necessitates cytoskeleton rearrangements. In *Atgl*^{-/-} monocytes and macrophages chemotaxis was markedly reduced. Our data provide evidence that decreased chemotaxis of *Atgl*^{-/-} macrophages is a consequence of (i) reduced expression of adhesion molecules, (ii) decreased expression of pro-inflammatory but increased expression of anti-inflammatory cytokines resulting in an M2 macrophage phenotype, and (iii) defective actin polymerization.

For active chemotaxis, actin polymerization and depolymerization is a prerequisite. To accomplish efficient migration, leukocytes (including macrophages) extend a front F-actin rich lamellipodium constituting the leading edge, and the uropod, in which both the microtubule and intermediate-filament network are retracted during migration (Vicente-Manzanares and Sanchez-Madrid, 2004). Thus, cell migration needs actin polymerization, which drives lamellipodia extension, disruption of existing and formation of new focal contacts (Stossel, 1994). We therefore hypothesize that defective actin polymerization in *Atgl*^{-/-} macrophage is the cause of reduced cell migration.

In migrating cells, dephosphorylation of FAK is temporally associated with disruption of focal adhesions and increased motility (Matsumoto et al.,

1994; Park et al., 2009). *Atgl*^{-/-} macrophages, however, show increased and sustained phosphorylation of FAK suggesting that hyperphosphorylation of FAK is one of the factors involved in impaired migration and enhanced spreading. Mitra et al suggested that FAK-Src activation and signaling to ERK2 promote FAK release from existing focal contacts and then trigger FAK re-binding and activation at different focal contacts in a migrating cell (Mitra et al., 2005). We therefore conclude that impaired activation of SHP-2, the major phosphatase for FAK, and decreased ERK2 activation are involved in sustained FAK phosphorylation of *Atgl*^{-/-} macrophages. Direct phosphorylation of FAK by Src kinase family members is an important step in the formation of an active FAK signaling complex (Calalb et al., 1995). Consequently, inhibition of Src abolished FAK phosphorylation in *Atgl*^{-/-} macrophages implying that Src is a substrate for FAK also in these cells. The regulated balance between cytoskeletal assembly and disassembly, which has to be maintained to ensure cell movement, is impeded in *Atgl*^{-/-} macrophages.

The signaling pathways required for actin polymerization, which are associated with FAK phosphorylation, include Rho GTPases (in particular RhoA, Rac1 and Cdc42) and their regulator and effectors proteins (Etienne-Manneville and Hall, 2002). Cdc42 acts at the front of the macrophage to control the cell's orientation toward an extracellular stimulus resulting in head extension and tail retraction and eventually in the activation of Rac1 (Allen et al., 1998; Nobes and Hall, 1995)(Nobes & Hall, 1995). RhoA regulates the adhesion required for normal tail retraction during leukocytes migration. In *Atgl*^{-/-} macrophages, RhoA activation is almost abolished in the presence of chemoattractants. RhoA localizes close to the nucleus in *Atgl*^{-/-} macrophages and fails to translocate to the side or rear upon chemoattractant treatment. Activated RhoA is distributed predominantly at the sides and rear in polarized neutrophils (Xu et al., 2003), which we also observed in Wt macrophages. We therefore conclude that impaired RhoA activation diminishes RhoA translocation from the nucleus resulting in a severe defect in macrophage polarization. Since Rho activity must be precisely regulated to allow efficient cell migration (Allen et al., 1998), this defect on cell polarity via its inability to

stimulate contractility likely contributes to impaired chemotaxis in *Atgl*^{-/-} macrophages. In stimulating the formation of filopodia, Cdc42 initiates gradient detection and cell polarization. Inhibition of Cdc42 and Rac1 prevents macrophage polarization and directional movements toward chemoattractants. Cdc42 inhibition renders macrophages to random movement instead of directional migration, whereas inhibition of Rac1 blocks cell movement (Allen et al., 1998; Jones et al., 1998). In line with the reduced motility of *Atgl*^{-/-} macrophages, we observed a marked reduction of activated GTP-bound Cdc42 and Rac1 in *Atgl*^{-/-} macrophages in response to LPA. In this context, it is important to note that the chemokine Ccl5/Rantes was shown to induce lamellipodia formation (Di Marzio et al., 2005) and to activate Rac1 in macrophages (Weiss-Haljiti et al., 2004). As a pro-inflammatory cytokine, Ccl5/Rantes plays an active role in recruiting leukocytes into inflammatory sites. In *Atgl*^{-/-} macrophages, Ccl5/Rantes and Gro1 mRNA levels were almost absent. The impact of Gro1 on small Rho GTPase activation has not been investigated so far. We hypothesize that together with the lack of RhoA activation and translocation, impaired Cdc42 and Rac1 activation and reduced Ccl5/Rantes expression account for the reduced migration capacity of *Atgl*^{-/-} macrophages.

Oxidative inactivation of tyrosine phosphatases such as SHP-2 is induced by intracellular ROS production (Meng et al., 2002). The production of ROS through the multiprotein enzyme complex NADPH oxidase is crucial for the immune response. ROS accumulation in macrophages leads to sustained activation of FAK, inactivation of SHP-2 and fast spreading (Park et al., 2009). To address the impact of ROS on FAK activation, we determined ROS concentrations, which were drastically increased in *Atgl*^{-/-} compared to Wt macrophages in the absence of chemoattractants. Inhibition of ROS production in *Atgl*^{-/-} macrophages by NAC and DPI triggered the loss of sustained FAK phosphorylation. As a consequence, the phenotype of defective migration of *Atgl*^{-/-} macrophages was eliminated. Rac2 regulates NADPH oxidase activator and ROS concentrations in intact phagocytes, most importantly in neutrophils (Bokoch and Zhao, 2006). In macrophages, Rac1 protein expression is ~4-fold

higher compared to Rac2 (Yamauchi et al., 2004). A role of Rac2 in macrophage function, however, was assessed in *Rac2*^{-/-} macrophages, which exhibit defects in NADPH oxidase activation resulting in reduced ROS formation (Yamauchi et al., 2004). Activated Rac2 expression was increased in *Atgl*^{-/-} macrophages and we observed Rac2 localization in the cytosol and near the cell periphery, whereas in Wt macrophages Rac2 was localized in and around the nucleus. This finding indicates that Rac2, which is activated in *Atgl*^{-/-} macrophages, translocates to the membrane as observed during NADPH oxidase activation in stimulated neutrophils. In these cells, the translocation of Rac2 to the membrane during NADPH oxidase activation exhibits kinetics and stoichiometries consistent with a role of Rac2 in NADPH oxidase regulation (Knaus et al., 1991). Rac1 also contributes to NADPH oxidase activation in macrophages (Miyano et al., 2009). Since Rac1 activation was rather decreased than increased we hypothesize that the sustained activation of FAK in *Atgl*^{-/-} macrophages results from increased ROS concentrations due to activated Rac2.

Data from the present study provide strong evidence that ATGL deficiency leads to an increased secretion of anti-inflammatory cytokines accompanied by the development of M2 macrophages. We conclude that reduced atherosclerosis in *Ldlr*^{-/-} mice transplanted with *Atgl*^{-/-} bone marrow (Lammers et al., 2011) might be due to altered cytoskeletal dynamics, reduced trafficking and a shift from pro- to anti-inflammatory cytokine secretion from the macrophages. Defective activation of the small Rho GTPases RhoA, Cdc42 and Rac1 in *Atgl*^{-/-} macrophages causes disturbances in actin polymerization and cell polarization. In addition, activation of Rac2 in *Atgl*^{-/-} macrophages leads to increased ROS production and impaired dephosphorylation of FAK, resulting in enhanced spreading and impaired migration. Our findings implicate macrophage ATGL as a promising target to reduce inflammation and macrophage infiltration and thereby attenuate atherosclerosis.

In atherosclerosis apoptosis is a critical factor, which defines the progression of lesions. Therefore, cell survival was investigated in ATGL

deficient macrophages. It is well established that FC but not CE accumulation, results in macrophage apoptosis that represents a hallmark in the appearance of late atherosclerosis lesions (Tabas, 2002). In particular, FC accumulation is toxic for macrophages and they undergo apoptosis when safety mechanisms to increase FC accumulation fail (Yao & Tabas, 2000; Yao & Tabas, 2001). 9-15 % apoptotic cells were observed in FC-loaded macrophages *in vitro* (Feng *et al.*, 2003; Yao & Tabas, 2001)

In the present study, we have investigated the effect of TG accumulation on macrophage cell death by using two different models: i) *Atgl*^{-/-} macrophages with defective TG hydrolysis, which accumulate cytosolic LDs even in the absence of exogenous stimuli, and ii) Wt macrophages loaded with VLDL to increase intracellular TG concentrations that mimic TG levels observed in *Atgl*^{-/-} macrophages. As a positive control, we used FC-loaded Wt macrophages. Macrophages lacking only one functional allele of ATGL show 1.7-fold increased intracellular TG concentrations compared to Wt macrophages but no significant increase in annexin V-positive cells. In contrast, macrophages lacking ATGL, which exhibit 4.3-fold higher TG levels compared to *Atgl*^{+/-} macrophages, display externalization of phosphatidylserine, a hallmark of an apoptotic event. Due to the importance of mitochondria in apoptosis pathway, we focused on mitochondrial dysfunction (Green & Reed, 1998). Indeed, we observed spontaneous caspase 3 and PARP cleavage in untreated *Atgl*^{-/-}, but not in *Atgl*^{+/-} and Wt macrophages. To achieve a similar effect in Wt macrophages, the cells had to be exogenously triggered with FC or VLDL. The proteolytic processing of the inactive pro-caspase 3 to the active caspase 3 represents an essential step in the mitochondria-dependent apoptotic cascade (Yao & Tabas, 2000). Moreover, Cyt C was present in the cytosol of *Atgl*^{-/-} macrophages. PARP cleavage is required for the translocation of AIF from mitochondria to nucleus (Yu *et al.*, 2002). In *Atgl*^{-/-} macrophages, we observed Aif localization in the nucleus. Both Aif and cytochrome c are important for cell viability when they are located in mitochondria, but when either is released they activate death programs. To investigate whether an additional boost potentiates apoptotic cell death of *Atgl*^{-/-} macrophages we also included FC-loaded *Atgl*^{-/-}

macrophages in some experiments. Since apoptotic features were comparable between untreated and FC-loaded *Atgl*^{-/-} macrophages we hypothesize that programmed cell death by ATGL deficiency and FC loading converge on the same pathway. Most of the apoptotic features we observed in *Atgl*^{-/-} macrophages were also found in VLDL-loaded Wt macrophages, suggesting that TG accumulation triggers programmed cell death. We have previously reported that FFA taken up by macrophages first have to be esterified to TG (and CE) for the subsequent usage as energy substrate by ATGL hydrolysis (Chandak *et al.*, 2010a) We observed reduced ATP and increased ADP/ATP ratios in *Atgl*^{-/-} macrophages. In *Atgl*^{+/-} macrophages, ATP concentrations were comparable to Wt macrophages (Figure 28D). Decreased acetyl-CoA levels indicated defective β -oxidation and less NADH and FADH₂ being available for oxidative phosphorylation to drive ATP synthesis in *Atgl*^{-/-} macrophages. In VLDL-loaded Wt macrophages, however, lipolysis is intact and the TG accumulation itself likely triggers the apoptotic response. Release of cytochrome c is mostly associated with a permanent loss of $\Delta\Psi_m$ (Heiskanen *et al.*, 1999). $\Delta\Psi_m$ was significantly reduced in *Atgl*^{-/-} macrophages and VLDL-loaded Wt cells. However, the release of cytochrome c was also reported in cells whose mitochondria have an apparently normal or even increased $\Delta\Psi_m$ (Szalai *et al.*, 1999; Green & Reed, 1998).

Partial disruption of the $\Delta\Psi_m$ by genetic or metabolic stress causes elevated cytoplasmic Ca²⁺ as a consequence of the inability of mitochondria to participate in Ca²⁺ uptake (Arnould *et al.*, 2002). During the process of apoptosis, Aif needs to be cleaved by Ca²⁺-dependent calpain, for which elevation of intracellular Ca²⁺ is a prerequisite (Norberg *et al.*, 2008a). We therefore conclude that increased basal cytosolic Ca²⁺ levels in *Atgl*^{-/-} macrophages might (at least in part) be responsible for Aif localization in the nucleus, contributing to apoptotic cell death. In addition, pro-apoptotic Bax was increased, whereas antiapoptotic Bcl-XL, Mcl-1 and Bcl-2 were decreased in *Atgl*^{-/-} and VLDL-loaded Wt macrophages, resulting in fragmented mitochondria and reduced O₂ consumption. Fragmented mitochondria are defining features of apoptosis. Several mitochondria in TG-rich macrophages are clustered

around LDs and have aberrant cristae providing evidence of considerable mitochondrial dysfunction. Impaired mitochondria generate ROS, which are capable to act as second messengers that mediate multiple cellular responses, including apoptosis. Consistently, ROS production was increased in *Atgl*^{-/-} macrophages and VLDL-loaded Wt cells.

It has been postulated that TG accumulation increases ceramide synthesis and causes cell death (Aronis *et al.*, 2005). Ceramide species in different cell types behaved differently. It is known that ceramide accumulation induces cell death via the mitochondrial pathway (Schutze *et al.*, 1992b). Importantly, it has been found that C16-ceramide was elevated in lymphoma cells through *de novo* synthesis (Kroesen *et al.*, 2001). Therefore, to find out whether apoptosis in *Atgl*^{-/-} macrophages is the consequence of altered ceramide content, we measured ceramide concentrations in these cells. Interestingly, we found an increase in ceramide accumulation with a relative increase in C16:0 ceramide and a decrease in C24:0, C24:1 and C24:2 levels. Moreover it is suggested that C16 ceramide is toxic in hematopoietic cells. Using FB1 as an inhibitor for ceramide synthesis rescued *Atgl*^{-/-} macrophages from mitochondrial dysfunction. The level of C16:0 ceramide in FB1-treated *Atgl*^{-/-} macrophages was comparable to Wt macrophages leading to a decrease in Bax and increase in Bcl-2 expression. Recently the role of ceramide in the induction of ER stress and apoptosis have been reported in various human cancer cells (Lei *et al.*, 2008). However, the mechanisms by which ceramides regulate ER stress are still enigmatic. Accordingly, *de novo* ceramide synthesis occurs in ER. Hence we conclude that activation of PERK, eIF2 α and CHOP in *Atgl*^{-/-} macrophages can be the consequence of Ca^{2+} depletion from the ER because of triglyceride accumulation. Translocation of ATF4 from the cytosol to the nucleus confirms the ER stress in *Atgl*^{-/-} macrophages via the CHOP/ATF4 pathway. IRE1 α is a protein, whose activation results in splicing of XBP-1 to initiate ER stress. IRE1 α activation was increased but we failed to detect XBP-1 splicing. In addition, ATF6, another transcription factor, was not cleaved in the Golgi and was failed to translocate to nucleus. From these observations we

conclude that activation of XBP-1 requires both IRE1 α phosphorylation and ATF6 cleavage.

One important issue arising from this study is the potential physiological consequence of intracellular TG accumulation in macrophages on atherosclerotic lesion formation. Apoptotic cell death was detected in atherosclerotic plaques, with increasing frequency as the plaque develops (Lutgens *et al.*, 1999) However, controversies exist about the implication of macrophage apoptosis on early and late atherosclerotic lesion development. In accordance with the data presented in this study, we observed increased intraplaque apoptosis in *Ldlr*^{-/-} mice transplanted with *Atgl*^{-/-} bone marrow compared to *Ldlr*^{-/-} mice transplanted with bone marrow from Wt animals. Macrophage count and plaque development was markedly reduced in sections from *Ldlr*^{-/-} mice lacking ATGL arguing for an attenuation of atherosclerosis by macrophage apoptosis (Lammers *et al.*, 2011).

Taken together this study shows the pivotal role of ATGL in macrophage lipolysis. The absence of ATGL in macrophages favors an anti-inflammatory M2 macrophage phenotype. Impaired migration and polarization of *Atgl*^{-/-} macrophages might be due to inactivation of Rho GTPase proteins. I demonstrated that sustained phosphorylation of FAK due to inactivation of its phosphatase (SHP2) by elevated level of ROS production causes fast spreading. Inhibition of ROS production restores macrophage migration by transient activation of FAK. Furthermore, TG accumulation because of ATGL inhibition (defective lipolysis) or VLDL loading induces mitochondrial dysfunction and ER stress via the CHOP/ATF4 pathway. I conclude that ATGL inhibition has an enormous impact on macrophage functions and survival.

6-Reference

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7-Abbreviations

ACAT	Acyl coenzyme A:acylcholesterol transferase
AIF	Apoptosis inducing factor
Arg1	Arginase1
a-SMase	Acid sphingomyelinase
ATF-6	Activating transcription factor-6
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BHQ	2,5-di-t-butyl-1,4-benzohydroquinone
Ca ²⁺	Calcium
CCL5	C-C chemokine ligand 5
CE	Cholesterol ester
CerK	Ceramide kinase
CerS	Ceramide synthase
CerT	Ceramide transfer
Chi3l3	Chitinase 3-like 3
CRE	cAMP response element
DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
DEPC	Diethyl pyrocarbonate
DG	Diacylglycerol
DMEM	Dulbeco's modified Eagle's medium
DPI	Diphenyleneidonium
DTT	Dithiothreitol
EOR	ER overload response
ER	Endoplasmic reticulum
FA	Fatty acids
FAK	Focal adhesion kinase
FB1	Fumonosine B1
FC	Free cholesterol
FCCP	carbonyl cyanide p(trifluoromethoxy) phenylhydrazone
FFA	Free fatty acid
Grp78	Glucose regulated protein-78

HSL	Hormone sensitive lipase
ICAM-1	Intracellular adhesion molecule 1
IL	Interleukins
IMS	Intermembrane space
iNOS	inducible nitric oxide synthase
IRE-1	Inositol-requiring enzyme-1
Jag1	Jagged-1
JNK	c-Jun N-terminal kinases
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LDs	Lipid droplets
Lfa-1	Leukocyte function associated antigen-1
LPDS	Lipoprotein deficient serin
MAPK	Mitogen activated protein kinase
MCP1	Manocyte chemoattractant protein-1
MCP2	Manocyte chemoattractant protein-2
M-CSF	Macrophage-colony stimulating factor
MGL	Monoacyl glycerol lipase
MOMP	Mitochondrial outer membrane permeabilization
Mrc1	Mannose receptor 1
NAC	Antioxidant N-acetyl cystein
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NCEH	Neutral cholesterol esterase
Noxa1	NADPH oxidase activator
n-SMase	Neutral sphingomyelinase
Ox-LDL	Oxidased- low density
PERK	PKR-like eukaryotic initiation factor 2 α kinase
PIP ₃	phosphatidylinositol (3,4,5)-trisphosphate
PMSF	Phenylmethanesulfonsylfluoride
PP1	Protein phosphatase-1
PSGL-1	P-selectin glycoprotein ligand-1
ROS	Reactive oxygen species
SDF-1	stromal cell-derived factor-1

SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}
SK-1	Sphingosine kinase
SMase	Sphingomyelinase
SPHK1	Sphingosine kinase-1
SPT	Serine palmitoyltransferase
SRA/B	Scavenger receptor A/B
TG	Triglyceride
TG	Triglyceride
TLRs	Toll-like receptors
TRAF-2	Tumor necrosis factor- α (TNF- α)-receptor associated factor-2
UPR	Unfolded protein response
Vcam1	Vascular cell-adhesion molecule 1
Vla-4	very late antigen 4
VLDL	Very low density lipoprotein
$\Delta\Psi_m$	Mitochondrial transmembrane potential