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Lipid droplets participate in modulating innate immune genes in *Ctenopharyngodon idella* kidney cells

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ABSTRACT

Lipid droplets (LDs) are increasingly being recognized as important immune modulators in mammals, in addition to their function of lipid ester deposition. However, the role of LDs in fish immunity remains poorly understood. In this study, the function of LDs in the innate immune response of *Ctenopharyngodon idella* kidney (CIK) cells, which are the equivalent of myeloid cells in vertebrates, was investigated. LD number and TG content significantly increased in the CIK cells following exposure to lipopolysaccharide (LPS), peptidoglycan (PGN), and polyriboinosinic-polyribocytidylic acid (Poly [I: C]) for 24 h, accompanied by increases in the relative expression of several innate immune genes. However, fatty acid compositions of the triglycerides were not changed after treatment with these three pathogenic mimics. LPS, PGN, and Poly (I: C) did not alter the relative expressions of lipogenic (*FAS*, *SCD*, and *DGAT*) and lipid catabolic (*PPARα*, *ATGL*, and *CPT-1*) genes. However, these treatments did increase the mRNA levels of lipid transportation genes (*FATP/CD36*, *ACSL1*, and *ACSL4*), and also decreased the non-esterified fatty acid level in the medium. To further explore the role of LDs in the immune response, CIK cells were incubated with different concentrations (0, 100, 200, 300, 400, 500 μM) of exogenous lipid mix (LM; oleic acid [OA]:linoleic acid [LA]:linolenic acid [LNA] = 2:1:1), and were then transferred to a lipid-free medium and incubated for 24 h. LD size and number increased with the increase in lipid levels, and this was accompanied by increased expression of innate immune genes, including *MyD88*, *IRF3,* and *IL-1β*, which were expressed at their highest levels in 300 μM exogenous lipid mix. Interestingly, after incubating with different fatty acids (LM, OA, LA, LNA, arachidonic acid [ARA], and docosahexaenoic acid [DHA]; 300 μM), ARA and DHA were more potent in inducing LD formation and innate immune gene expression in the CIK cells. Finally, atglistatin, an ATGL inhibitor, effectively attenuated the expression of most genes upregulated by ARA or DHA, suggesting that lipolysis may be involved in the regulation of immune genes at the transcriptional level. Overall, the findings of this study demonstrate that LDs are functional organelles that could act as modulators in the innate immune response of CIK cells. Additionally, long-chain polyunsaturated fatty acid enriched LDs play a unique role in regulating this process.

1. Introduction

Lipid droplets (LDs) are ubiquitous and evolutionarily conserved organelles found in almost all organisms [1]. LDs consist of a neutral lipid core, predominantly composed of triglycerides and sterol esters, surrounded by a monolayer of phospholipids and a variety of proteins [2]. Previously, LDs were perceived as static fat particles in cells. The role of LDs in lipid storage has been elucidated. Sufficient energy in the environment results in the formation of LDs; these stored lipids are broken down by lipolysis or lipophagy when energy is required by organisms [1,3]. Recently, LDs have been shown to play important roles in the inflammatory processes of mammalian immune cells, particularly

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Abbreviations

ACSL1	acyl-CoA synthetase long-chain family member 1
ACSL4	acyl-CoA synthetase long-chain family member 4
ARA	arachidonic acid
ATGL	adipose triglyceride lipase
CIK	ctenopharyngodon idellus kidney
CPT-1	carnitine palmitoyltransferase 1
DGAT	diacylglycerol *O*-acyltransferase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAS	fatty acid synthase
FATP/CD36	fatty acid translocase protein
IFN-I	type I interferon
IL	interleukin
IPS-1	IFN-β promoter stimulator 1
IRF3	IFN regulatory factor3
IRF7	IFN regulatory factor7
LC-PUFA	long chain polyunsaturated fatty acid
LD	lipid droplets
LPS	lipopolysaccharide
PGN	peptidoglycan
Mx1	myxovirus resistance 1
MyD88	myeloid differentiation factor 88
NEFA	non-esterified fatty acid
Poly (I:C)	polyriboinosinic-polyribocytidylic acid
PPARα	peroxisome proliferator activated receptor α
SCD	stearoyl-CoA desaturase
TLR	toll-like receptor
TNFα	tumour necrosis factor α

in myeloid cells [4]. Pathological events, such as microbial infection, can have a major impact on the quantity and quality of LDs in the immune cells [5,6]. Similarly, increased LD accumulation has also been observed in immune cells of *Aedes aegypti* in response to bacteria and Dengue virus [7]. These findings suggest that different types of LDs may be intrinsically associated with distinct cellular functions and/or cell types [5]. For instance, unlike adipocyte LDs, leukocyte LDs store arachidonic acid (ARA), an essential component for the production of inflammatory mediators such as eicosanoids [5]. Nevertheless, the function of LDs in the immune response of fish is relatively unexplored, since most studies on LDs in fish have focused on the tissues that easily accumulate fat, such as liver and adipose tissues, and the mechanisms that regulate the LDs [8–11].

The immune system of fish is physiologically similar to that of higher vertebrates, despite certain differences. The thymus, kidney, and spleen are the largest lymphoid organs in teleosts. Among them, the kidneys are the equivalent of the bone marrow in vertebrates and are the largest site of haematopoiesis until adulthood [12]. In fish, the innate response has been considered an essential component in combating pathogens due to their limited adaptive immune system, poikilothermic nature, limited repertoire of antibodies, and due to the slow proliferation, maturation, and memory of their lymphocytes [13]. The innate immune response in fish is primarily composed of a system of receptors proteins, which identify molecular patterns that are typical of pathogenic microorganisms such as polysaccharides, lipopolysaccharide (LPS), peptidoglycan (PGN), bacterial DNA, viral RNA. [14]. Following receptor-ligand binding, signal transduction initiates a complex cascade of reactions, which leads to the production of one or more of a wide array of effector molecules, eventually resulting in the elimination or inactivation of the pathogen [15]. For example, toll-like receptors (TLRs) play a major role within this group of host receptors [15]. The signaling pathways of TLRs can be largely classified as either myeloid differentiation factor 88 (MyD88)-dependent pathways, which drive the induction of inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferon (IFN-I) as well as for inflammation [16]. However, there have been few studies on the regulatory mechanisms underlying fish immune signaling pathways compared to those on mammals, and studies focusing on the role of LDs in the immune response have been rare.

It has been widely proved that tissue lipid levels or fatty acid composition reflect those in the diet [17,18]. Studies on fish have shown that exogenous lipids or specific fatty acids impact the regulation of immunity. Proper dietary lipid supplementation enhance the immune response but excess or low dietary lipids may elevate liver oxidation rates in grass carp [19,20]. Long-chain fatty acids, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and ARA, have unique roles in the improvement of the immunity in the kidneys of fish such as large yellow croaker [21,22], Japanese seabass [23], and grass carp [24,25]. However, the mechanisms behind these roles need to be further addressed. Therefore, in this study, we used *Ctenopharyngodon idella* kidney (CIK) cells to describe the role of LDs in the modulation of the immune response of grass carp *in vitro*.

2. Materials and methods

2.1. Cell culture

CIK cells were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). Cells were cultured in M199 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA), 100 U/ml penicillin (GIBCO, USA), and 100 U/ml streptomycin (GIBCO, USA). The cells were incubated at 28 °C in a 5% CO_2 humid atmosphere. The medium was changed every two days.

2.2. Oil red O staining

Experiments involving oil red O staining were performed in six-well plates with CIK cells seeded at 1.2×10^6/well for 24 h. After treatment, the medium was removed and cells were washed twice with phosphatic buffer solution (PBS), and then fixed in 10% formalin for 30 min. After rinsing twice with PBS, cells were stained using oil red O solution (Jiancheng Biotech Co.) for 30 min. Hematoxylin solution (Jiancheng Biotech Co.) was used to stain the nucleus (5 min) after removing the oil red O by washing the cells three times with PBS. Finally, after removing the hematoxylin solution and washing with PBS, the cells were observed using an inverted microscope (Olympus BX41, Japan).

2.3. Electron microscope

Cells used for electron microscopy were plated in 25 cm plastic bottles at a plating density of 1.0×10^6/well. After treatments, the medium was removed and cells were washed twice with PBS. The cells were then detached from the flasks using 0.25% trypsin-EDTA (GIBCO, USA). Afterwards, the cells were collected for centrifugation, and were then fixed in 2.5% glutaraldehyde in 1% agarose. After rinsing three times using PBS (pH 7.4), the cells were fixed in 1% osmium tetroxide in 0.1 M PBS for 2 h, and were then washed again using PBS. After step-by step ethanol dehydration and embedding in 812 medium (SPI Supplies, West Chester, PA, USA), samples were desiccated at 37 °C for 24 h and then at 60 °C for 48 h. Finally, the samples were cut on a Leica

UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and were stained. Cells were observed using a transmission electron microscope (HT7700, Hitachi).

2.4. Triglycerides (TGs) and non-esterified fatty acids (NEFAs) contents assays

TG assays were performed in six-well plates with CIK cells seeded at 1.2 × 10^6/well 24 h prior to treatments, with three replicates per assay. Cells were lysed and collected for TG assays using an enzymatic TG assay kit (Applygen, Beijing, China). For NEFA assays, cells were plated in 96-well plates at 5 × 10^4/well for 24 h before treatment, with four replicates per assay. NEFAs were measured using a NEFA assay kit (Jiancheng Biotech Co. Nanjing, China).

2.5. Cell activities assay

Cells were seeded in 96-well plates at 5 × 10^4/well for 24 h before treatment, with 10 replicates per assay. Cell activity was assayed using the Cell Counting Kit-8 (CCK-8, MCE, USA), according to the manufacturer's instruction.

2.6. Fatty acid composition

CIK cells were seeded in 25 cm plastic bottles at a plating density 1.0 × 10^6/well for 24 h prior to treatment. Thereafter, cells were harvested using 0.25% trypsin-EDTA and were washed twice with PBS. Chloroform/methanol (2:1, v/v) was then added for 30 min to extract the lipids based on the method described by Folch et al. (1957) [26]. Extracts were vacuum dried, and the tissue lipids were dissolved in chloroform. Non-polar lipids (NPL) were separated using Silica Cartridges according to the method recorded in Juaneda & Rocquelin (1985) [27]. A volume of 20 ml of chloroform was used to elute the NPL fraction. Extracts were then vacuum dried again. The preparation of fatty acid methyl esters (FAME) was performed based on the method previously described [28–30]. In brief, 1 ml of hexane was added to dissolve the lipid fractions, and methyl esterification was performed for 1 h after adding 1 ml of potassium hydroxide methanol (0.4 M). Then, 2 ml of distilled water was added to separate the mixture into two layers. The upper layer was separated and used for GC analysis. The FAME were determined by an Agilent 7820a Series GC (Agilent Technologies) equipped with a flame ionization detector and capillary column (HP-88, length 100 m, internal diameter 0·25 mm, film thickness 0.20 μm; Agilent Technologies). Individual methyl esters were identified through comparison with known standards (47015-U; Sigma-Aldrich, Inc.). The results of identified fatty acids were presented as percentage of total fatty acids.

2.7. Real-time quantitative RT-PCR

Experiments involving real-time quantitative RT-PCR were performed in 24-well plates with CIK cells seeded at 2.5 × 10^5/well 24 h prior to treatment. When cells were harvested, cells from four wells were pooled and each treatment was replicated at least three times. Total RNA was extracted from cells using Trizol reagent (life Technologies Inc. USA) according to the manufacturer's instructions. After removing the DNA in the total RNA, cDNA was synthesized with the use of the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA). Real-time PCR assays were performed in triplicate using the Applied Biosystems Step-One-plus real-time PCR system (Applied Biosystems) with a final volume of 20 μL consisting of: 2.0 μL primer (2.5 μM), 2.0 μL diluted first strand cDNA product, 10 μL 2 × Power SYBR™ Green PCR Master Mix (Thermo Scientific, USA), and 6.0 μL sterilized double-distilled water. The cycling parameters were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, and finally 60 °C for 1 min. After the PCR reaction, the melting curve was analyzed over a range of 72–95 °C (in 1 °C/20 s steps) to confirm a single product. A relative quantification method was used to calculate the gene expression values using the comparative CT method ($2^{-\Delta\Delta Ct}$) described in previous studies [31,32]. The primer sequences for β-actin, fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), diacylglycerol *O*-acyltransferase (DGAT), peroxisome proliferator activated receptor α (PPARα), adipose triglyceride lipase (ATGL), carnitine palmitoyltransferase 1 (CPT-1), fatty acid translocase protein (FATP/CD36), acyl-CoA synthetase long-chain family member 1 (ACSL1), acyl-CoA synthetase long-chain family member 4 (ACSL4), TLR22, MyD88, IFN-β promoter stimulator 1 (IPS-1), IFN regulatory factor 3 (IRF3), IFN regulatory factor 7 (IRF7), IFN-I, myxovirus resistance 1 (Mx1), interleukin-8 (IL-8), interleukin-1β (IL-1β), and tumor necrosis factorα (TNFα) are listed in Table 1.

Table 1
Primers used in real-time quantitative PCR.

Target genes	Accession no.	Forward (5′-3′)	Reverse (5′-3′)
Fatty acid synthase (FAS)	GQ466046	CCTCAGCTTACAGCAGAATC	CTCTTCAGCAAGGGAGTTTAG
Stearoyl-CoA desaturase (SCD)	AJ243835	GCCTTCCAGAATGACATCTAC	GCCGATGTGAGCAAAGAA
Diacylglycerol *O*-acyltransferase (DGAT)	KY800895	CACCTTCCAAGTACCTTCTG	AGATCCCACTCGCCTATT
Peroxisome proliferator activated receptor α (PPARα)	FJ623265	CGCTGAGGTTCGGATATTT	ACGTCACCTGGTCATTTAAG
Adipose triglyceride lipase (ATGL)	HQ845211	TCGTGCAAGCGTGTATATG	GCTCGTACTGAGGCAAATTA
Carnitine palmitoyltransferase 1 (CPT-1)	JF728839	GCATCCATGACACGTTTATTC	GAAGTTTCTCTTCTCTCGTCTC
Fatty acid translocase protein (FATP/CD36)	KU361231	GTGTCCAAGGGCTTCAATGA	GTGGTAAAGGGGTAAACAGTCATA
Acyl-CoA synthetase long-chain family member 1 (ACSL1)	KP262348	GGGAGAGGTGTGTGTGAAAGGA	CAGTGTGCAGCCAGCCGTC
Acyl-CoA synthetase long-chain family member 1 (ACSL4)	KU878102	CGTAAGAAGGACCTGGTAAAG	GGCATAGACGCAGATGTTAT
Toll-like receptor 22 (TLR22)	FJ547498	TCGTTTGTCTGGCATTTTCTG	CAAGGCTCGTCTTCGGTGT
Myeloid differentiation factor 88 (MyD88)	FJ843088	CAAATGATGGACTTTACCTACCTG	ACATCTTTCCTTTCGGCTTT
IFN-β promoter stimulator 1 (IPS-1)	GQ483645	GACCGTAAGAAGTCAGCCTCC	CCTGAATAACTCTTGATAGCCCTC
IFN regulatory factor3 (IRF3)	KC898261	ACTTCAGCAGTTTAGCATTCCC	GCAGCATCGTTCTTGTTGTCA
IFN regulatory factor7 (IRF7)	GQ141741	CGCCTGTGTTCGTCACTCGT	GGTGGTTGGAAAGCGTATTGG
Type I IFN (IFN-I)	DQ357216	AAGCAACGAGTCTTTGAGCCT	GCGTCCTGGAAATGACACCT
Myxovirus resistance1 (Mx1)	HQ245104	CTGGGGAGGAAGTAAAGTGTTCT	CAGCATGGATTCTGCCTGG
Interleukin-8 (IL-8)	JN255694	AGGTCTGGGTGTAGATCCACGCTG	TTAGTGTGAAAACTAACATGATCTCT
Interleukin-1β (IL-1β)	JX014320	GCCAAGTAGCCGAATCACAGA	AAGCCCAAGATATGCAGGAGTC
Tumour necrosis factorα (TNFα)	JQ670915	AACCAGGACCAGGCTTTCT	GCATAACTGCGTGGCTCATA
β-actin	DQ211096	TCCACCTTCCAGCAGATGTGGATT	AGTTTGAGTCGGCGTGAAGTGGTA

2.8. Statistical analysis

All data are expressed as means ± S.D. (standard deviation). Percentage data were arcsine-transformed prior to analysis. One-way analysis of variance was used to compare differences between the experimental groups, followed by LSD post hoc tests. All analyses were performed using PASW Statistics 18 (SPSS, Chicago, IL, USA). Statistical significances are denoted with asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Results

3.1. LDs are induced by analogous pathogenic invasion in CIK cells

In order to explore whether pathogenic invasion could induce LDs formation in grass carp myeloid cells, CIK cells were treated with different concentration of LPS (1, 10, 20 μg/ml, components of gram-negative bacteria) for 24 h. As can be seen in Fig. 1 A-B, the number of LDs and TG contents both increased as LPS concentration increased, as was shown by staining neutral lipids with Oil Red O and by quantification of TGs. Electron micrographs also clearly showed LD formation in the cells after exposure to 10 μg/ml LPS (Fig. 1C). We thus sought to investigate whether other pathogenic invasion mimics could also influence LD quantity in CIK cells. To this end, cells were further treated with PGN (components of gram-positive bacteria) and polyriboinosinic-polyribocytidylic acid (Poly [I: C], components of virus). Similarly, 10 μg/ml of both PGN and Poly (I: C) also increased LD contents in the CIK cells (Fig. 1D–E), suggesting that LD induction is a widespread phenomenon under pathological invasion. However, the fatty acid composition of the TGs was not significantly changed after LPS, PGN, or Poly (I: C) incubation, although 18:2n-6 and 20:4n-6 had a numerical increase in the three treatment groups (Fig. 1F). Finally, LPS, PGN, and Poly (I: C) significantly increased the mRNA levels of the innate immune marker genes TLR22, IRF3, IFN-I, IL-1β, and TNFα (Fig. 1G).

3.2. LD formation induced by analogous pathogenic invasion accompanied by decreased NEFA levels in the medium

To explore the potential mechanisms behind LD formation, several lipid metabolism genes were tested. Interestingly, although SCD expression was significantly increased after PGN exposure ($P < 0.05$), the mRNA levels of the lipogenic genes FAS, SCD, and DGAT were not increased after treatment with any of the pathogen mimics (Fig. 2A). The genes related to lipid catabolism, such as PPARα, ATGL, and CPT-1, were also not obviously regulated, with the exception of ATGL, which showed significantly lower levels with LPS treatment ($P < 0.05$) (Fig. 2B). Interestingly, fatty acid transportation related genes, such as FATP/CD36, ACSL1, and ACSL4 had significantly increased expression in response to the three pathogenic analog treatments (Fig. 2C). These results suggest that LD formation induced by pathogenic invasion mimics might not be mainly caused by the improvement in *de novo* synthesis and reduction in lipolysis of lipids in the CIK cells, but may be due to the absorption of exogenous lipids. To address this, we investigated the NEFA content in the medium after analogous pathogenic invasion. Total NEFA levels decreased over time after LPS, PGN, and Poly (I: C) treatments in comparison with the control (Fig. 2D), indicating that exogenous fatty acids probably participate in LD formation after triggering the immune response in CIK cells.

3.3. Exogenous lipids induced LDs formation and increased expression of innate immune genes in CIK cells

The result that analogous pathogenic invasion tends to induce fatty acid absorption and LD formation suggests that LDs might play an important role in the immune response of CIK cells, similar to that in mammals. To address this, CIK cells were incubated with different concentrations (0, 100, 200, 300, 400, 500 μM) of exogenous lipids (LM, oleic acid [OA]: linoleic acid [LA]: linolenic acid [LNA] = 2: 1: 1) for 24 h to increase LD number in the cells, then these cells were transferred to a non-added lipid medium for another 24 h. As expected, the number and size of LDs increased with the increased concentration of lipid mix as shown by oil red O staining and transmission electron microscopy (TEM) observations (Fig. 3A). Lipid mix had no influence on the cell activities measured by MTT assays. However, the mRNA levels of innate immune marker genes MyD88, IPS-1, IRF3, IFN-I, Mx1, IL-8, and IL-1β were significantly upregulated after LD levels in the cells increased (Fig. 3B), suggesting that increasing the LDs in CIK cells improves the immunity capacity. Furthermore, mRNA expression of several genes, such as MyD88, IRF3, Mx1, and IL-1β, increased from 0 to 300 μM lipid mix but declined from 300 to 500 μM.

3.4. Long chain polyunsaturated fatty acid (LC-PUFA)-induced LDs play a unique role in regulating expression of immune genes in CIK cells

In addition to the quantity, the quality of LDs also plays a unique role in the immune response of mammals. To further investigate the role of LDs in innate immune gene expression in fish, CIK cells were incubated with various fatty acids (LM, OA, LA, LNA, ARA, and DHA) at a concentration of 300 μM for 24 h, after which cells were transferred to non-added lipid medium for another 24 h. The quantity and size of LDs both increased after incubating with these fatty acids, as shown by the TEM observations and TG contents (Fig. 4A and B). Specifically, ARA and DHA had unique roles in the increase of LDs in the CIK cells (Fig. 4A and B), suggesting that CIK cells may preferentially absorb LC-PUFA. Thereafter, the fatty acid composition of the TGs was tested. Although cells were incubated with a variety of single fatty acids, OA was the most abundant fatty acid in the cells. In addition to OA, the proportions of the exogenous acids LA, LNA, ARA, and DHA in the TGs of the cells increased significantly (Fig. 4C). These results suggest that the quality of LDs was successfully modified in the CIK cells.

We further tested the innate immune genes. The relative expressions of the genes TLR22, MyD88, IPS-1, IRF3, IRF7, IFN-I, Mx1, IL-8, IL-1β, and TNFα showed similar trends after treatment with different fatty acids. LM, OA, LA, and LNA slightly increased the expression of these genes ($P < 0.05$). However, ARA and DHA drastically upregulated these genes ($P < 0.001$) (Fig. 4D).

3.5. Lipolysis of LDs induced by LC-PUFA participates in the modulation of innate immune gene expression

LDs are dynamic structures that have complex interactions with other organelles. Lipid mobilization is one of the main biological processes of LDs, during which ATGL and lipophagy play important roles in fat degradation. To explore the mechanism by which LC-PUFA induced LDs significantly increase innate immune gene expression, cells were treated with ARA or DHA for 24 h, after which cells were incubated with non-added lipid medium supplemented with or without atglistatin (ATGL inhibitor; 50 μM) or 3-Methyladenine (3M, autophagy inhibitor; 5 mM) for 24 h. The results showed that atglistatin significantly attenuated the induction of 6 of the 10 (including TLR22, MyD88, IPS-1, IRF7, IL-1β and TNFα) ARA-dependent immune genes, and nine of the 10 (including TLR22, MyD88, IPS-1, IRF, IRF7, Mx1, IL-8, IL-1β, and TNFα) DHA-dependent immune genes. 3M blocked the induction of 4 of the 10 (IPS-1, IFN-I, IL-8, and TNFα) ARA-dependent immune genes, and 2 of the 10 (TLR22 and IRF7) DHA-dependent immune genes (Fig. 5). These results suggest that both ATGL and lipophagy are responsible for the increased immune capacity induced by LC-PUFA, and it seems that ATGL contributes more than lipophagy, especially for DHA.

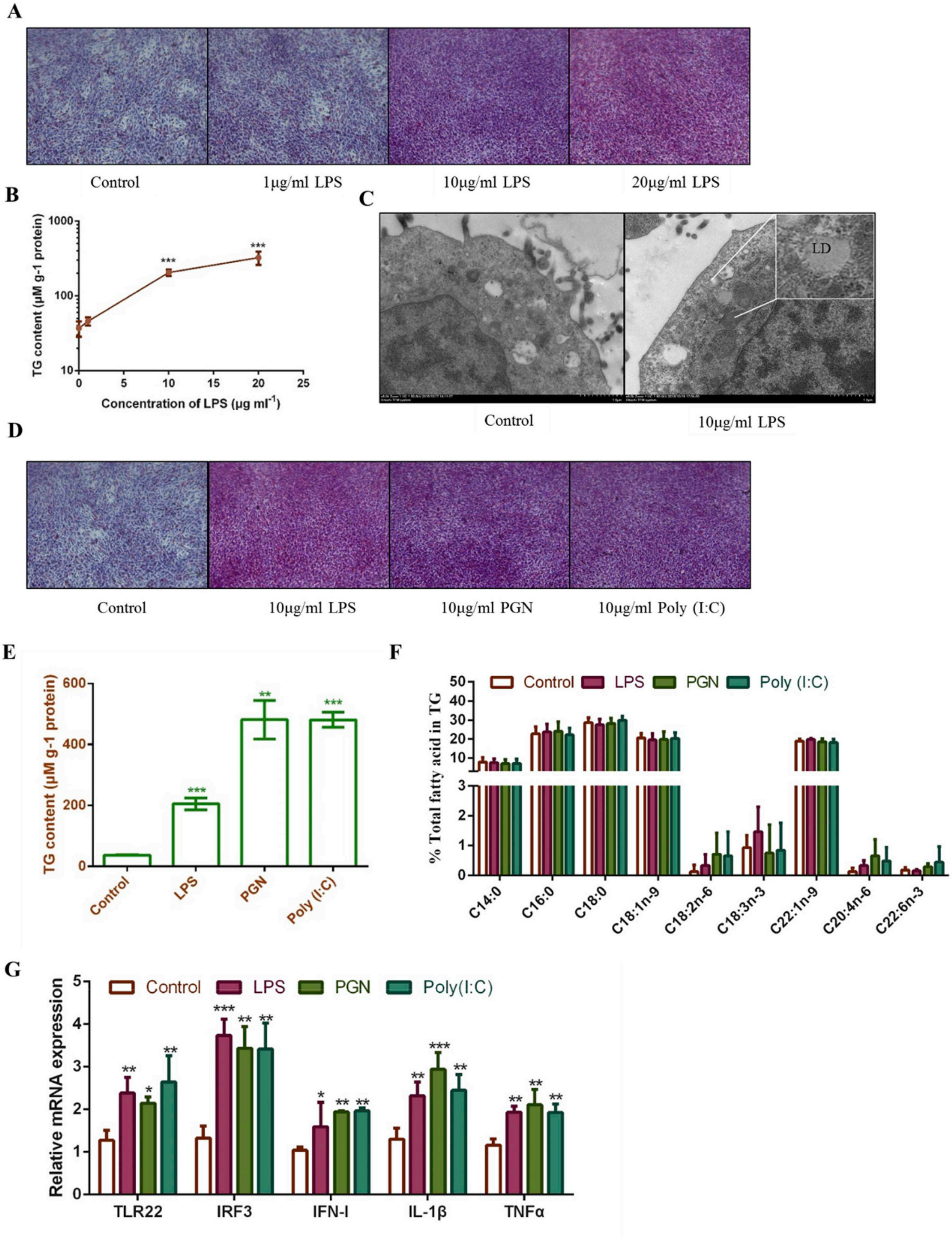

Fig. 1. Effects of pathogenic mimics on the lipid droplets (LDs), triglyceride (TG) contents, fatty acid composition, and expression of innate immune genes in CIK cells. A, Oil red O staining of CIK cells after treatment with different concentrations (1, 10, 20 μg/ml) of lipopolysaccharide (LPS) for 24 h. B, TG contents of CIK cells upon treatment with different concentrations (1, 10, 20 μg/ml) LPS for 24 h (n = 3). C, Electron micrographs of CIK cells upon treatment with 10 μg/ml LPS for 24 h. D, Oil red O staining of CIK cells upon treatment with 10 μg/ml LPS, peptidoglycan (PGN), or polyriboinosinic-polyribocytidylic acid (Poly [I: C]) for 24 h. E, TG contents of CIK cells upon treatment with 10 μg/ml LPS, PGN, or Poly (I: C) for 24 h (n = 3). F, Fatty acid composition of the TGs in CIK cells after incubation with 10 μg/ml LPS, PGN, or Poly (I: C) for 24 h (n = 3). G, Relative expression of innate immune genes in CIK cells after incubation with 10 μg/ml LPS, PGN, or Poly (I: C) for 24 h (n = 3). All results are presented as the mean ± SD (error bars). Statistically significant differences are denoted with asterisks as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

4. Discussion

In mammals, LDs were initially recognized as an inert store of excess lipids, but are increasingly described as being dynamically engaged in various cellular functions, with important roles not only in diseases such as diabetes and cancer, but also in immune regulation [2,5]. However, though immune function may be impacted by the quantity or quality exogenous lipids [19,20,22,33], understanding of the role of

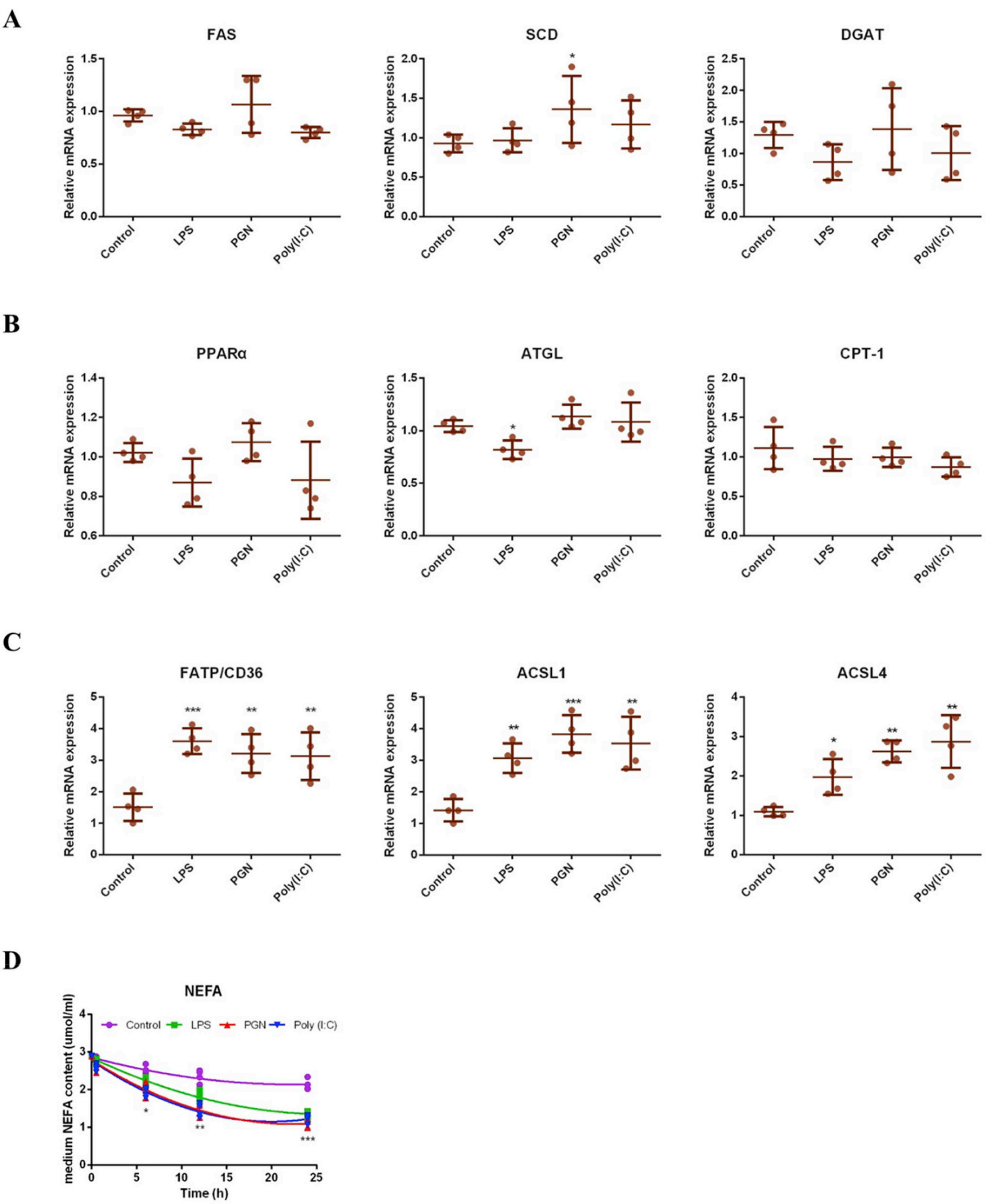

Fig. 2. The relative expressions of genes involved in lipid metabolism in CIK cells, and non-esterified fatty acid (NEFA) levels in medium in response to different pathogenic mimics. CIK cells were incubated with 10 μg/ml lipopolysaccharide (LPS), peptidoglycan (PGN), and polyriboinosinic-polyribocytidylic acid (Poly [I: C]) for 24 h, after which qRT-PCR was performed. All results are presented as the mean ± SD (error bars; for qRT-PCR, n = 4; for NEFA, n = 3). Statistically significant differences are denoted with asterisks as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

LDs in the immune response of fish was limited. This study clearly shows that pathogenic mimics LPS, PGN, and Poly (I: C) induced LD accumulation in CIK cells, and that NEFA levels in the medium decreased and lipid transportation genes were upregulated. Increased LD levels in the CIK cells caused by exogenous lipids were accompanied by an increase in the expression of innate immune related genes, especially when the cells were incubated with ARA or DHA. Moreover, the ATGL inhibitor atglistatin effectively blocked the increased gene expression induced by ARA or DHA enriched LDs. Collectively, these data demonstrate the indispensable function of LDs in the regulation of innate immune genes.

Pathogenic invasion is often reported to be accompanied by LD induction, and this phenomenon is widely pervasive from mosquitos to mammals [5,7,34]. However, studies like this in teleosts remain rare. This study showed that LD levels increased in CIK cells in response to LPS, PGN, and Poly (I: C), which provides evidence for the existence of

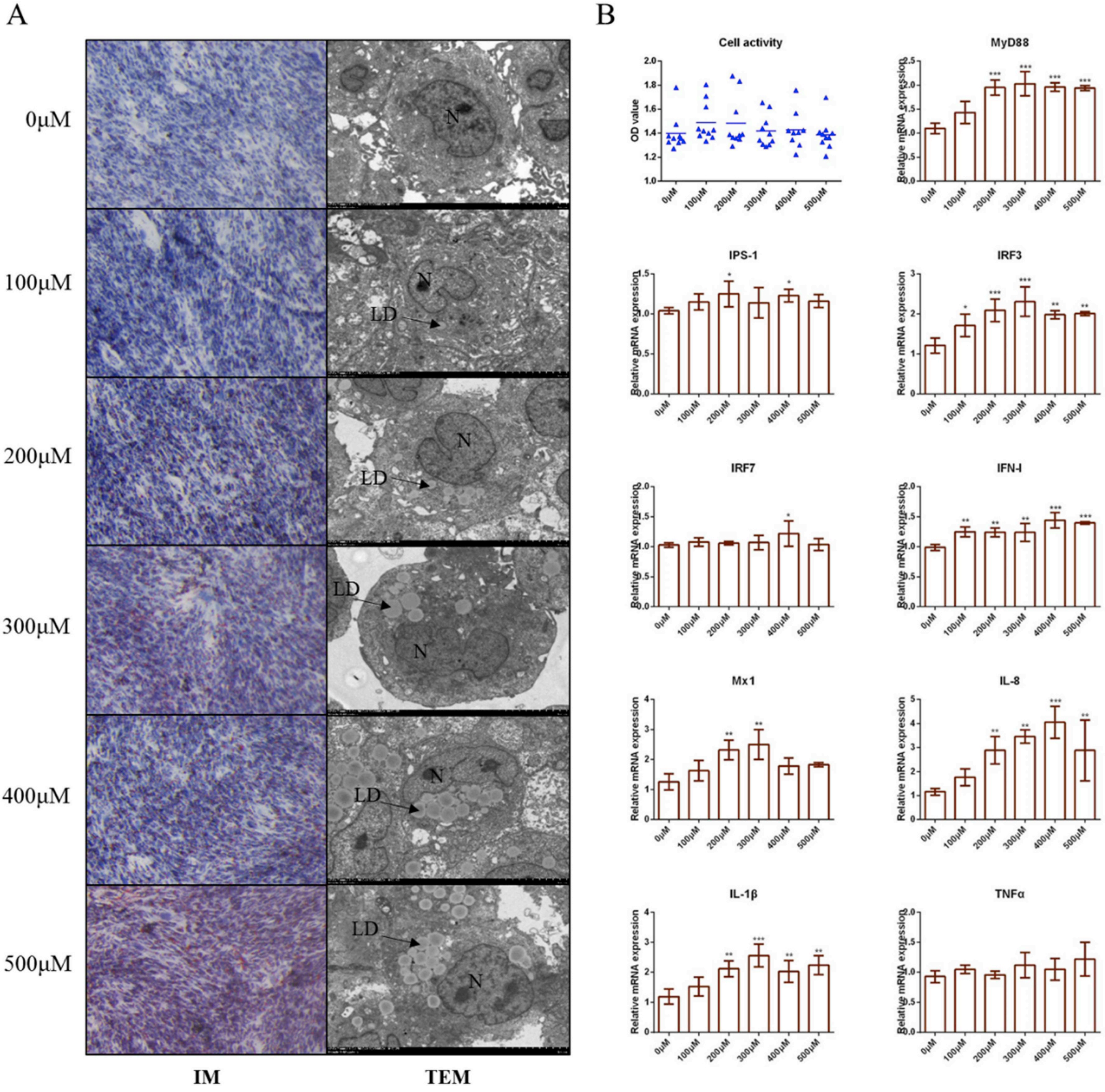

Fig. 3. Effect of different concentrations of lipid mix on lipid accumulation and innate immune gene expression in CIK cells. CIK cells were incubated with different concentrations (0, 100, 200, 300, 400, 500 μM) of lipid mix (LM; oleic acid [OA]: linoleic acid [LA]: linolenic acid [LNA] = 2: 1: 1) for 24 h, and were then transferred to lipid-free medium for 24 h. A, Oil red O staining and transmission electron microscope image of the CIK cells. B, Cell activity and relative expression of innate immune genes in CIK cells (n = 10 for cell activity, n = 3 for gene expression). All results are presented as the mean ± SD (error bars). Statistically significant differences are denoted with asterisks as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

this biological phenomenon in fish, suggesting conservative processes of LD formation in response to pathological events. It has been suggested that pattern recognition receptors (PRRs) are responsible for the lipid accumulation, especially those belonging to the TLR family, which detect the presence of so-called "pathogen-associated molecular patterns" (PAMPs), such as LPS or lipopeptides [16]. TLR2, TLR3, TLR4, or TLR7 agonists have been reported to increase LD numbers and proteins important for LD biogenesis, indicating that LD formation is the result of a host defense mechanism orchestrated by PRR triggering [35,36]. Thus, it is possible that LD formation induced by LPS, PGN, and Poly (I:C) in CIK cells was due to the activation of the TLR family. Up-regulation of TLR22 in CIK cells after treatment with the three pathogenic mimics can be considered as indirect evidence of this hypothesis. In dendritic cells, TLR activation induced glycolytic flux supports *de novo* synthesis of lipids for LD biogenesis [37]. Moreover, selective autophagy of LDs (termed lipophagy) and lipolysis also control the number of

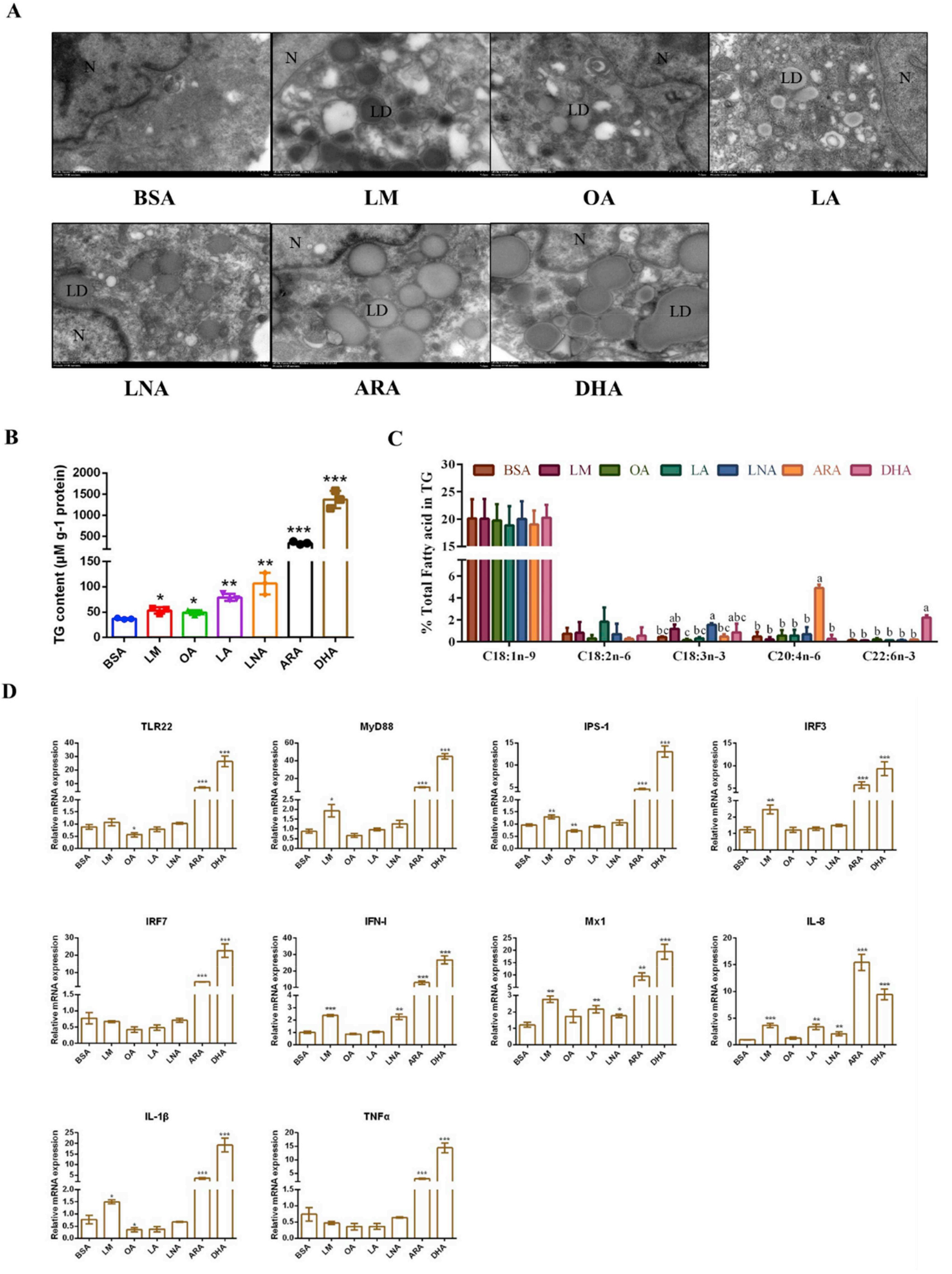

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Fig. 4. Effects of different fatty acids on the lipid accumulation, fatty acid composition, and relative expression of innate immune genes in CIK cells. CIK cells were incubated with BSA, lipid mix (LM), oleic acid (OA), linoleic acid (LA), alpha-linolenic acid (LNA), arachidonic acid (ARA), and docosahexaenoic acid (DHA) at 300 μM for 24 h, and were then transferred to lipid-free medium for 24 h. A, Transmission electron microscope image of CIK cells. B, TG contents of CIK cells (n = 3). C, Fatty acid composition of the triglycerides (TGs) in the CIK cells (n = 3). D, Relative mRNA levels of innate immune genes (n = 3). All results are presented as the mean ± SD (error bars). Statistically significant differences are denoted with asterisks as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. For fatty acid composition, different symbols denote significant differences ($P < 0.05$).

LDs in fibroblast cells [38,39]. In the present study, lipid synthesis- (FAS, SCD, and DGAT) and catabolism- (PPARα, ATGL, and CPT-1) related genes were not obviously altered in CIK cells after exposure to pathogenic mimics, but the possibility that the protein levels of these genes were modified should not be excluded. Interestingly, genes involved in lipid transportation, including FATP/CD36, ACSL1, and ACSL4, were significantly increased after treatment with the three pathogenic mimics, and this was accompanied by decreased NEFA levels in the medium. These data indirectly demonstrate the activation of lipid transport from the medium into the cells, which might further contribute to LD synthesis. The differences in the raw components of LDs between mammals and fish may be due to their differing abilities to use carbohydrates, as it has been suggested that fish preferentially use lipids over carbohydrates [40]. In mammals, LDs in the immune cells, for example in leukocytes, store ARA, an essential component for the production of inflammatory mediators such as eicosanoids [5]. In the present study, although there was a numerical increase in the ARA level, the fatty acid composition of pathogenic mimic-induced LDs was not obviously different from the LDs in the control cells. This could be attributed to the fact that the fatty acid composition of the LDs was largely influenced by the exogenous lipids.

After incubation with lipid mix, LD number increased with increased lipid concentration in CIK cells, similar to in other cells such as adipocytes and hepatocytes [11,41], suggesting that CIK cells are susceptible to exogenous lipids and are easily able to store LDs. We then transferred cells to a non-supplemented lipid medium to eliminate the interference of exogenous free fatty acids and established the mechanisms behind LD-containing CIK cells. The CIK cells containing LDs exhibited higher gene expression in genes related to the innate immune response, and their expression was highest in CIK cells exposed to exogenous lipids at a concentration of 300 μM. It seems to decrease these genes when cells were incubated with lipid mix up to 400 μM, similar to an *in vivo* study, which reported that proper dietary lipid level enhanced the immune response in grass carp [19]. Moreover, the immune genes with increased expression triggered by LDs were similar to those that experienced increased expression when cells were exposed to pathogenic mimics, which further indirectly proves the role of LDs induced by pathogenic mimics on the up-regulation of innate immune genes.

The results of the present study showed that CIK cells absorbed and retained different fatty acids types to different extents. For example, it seemed that LC-PUFAs were easier to transport across the cell membrane as suggested by the fact that the proportions of ARA and DHA were obviously higher than other fatty acids, even though all fatty acids were incubated at the same concentrations. Previous studies of grass carp and Songpu mirror carp have proven that immune organs, such as the kidneys and the spleen, easily deposit ARA and DHA [24,28]. Interestingly, OA was the main fatty acid component of the LDs regardless of the different types of incubated fatty acids. This indicates the fatty acids, such as ARA or DHA, could promote other fatty acids to be esterified into the TGs (mainly OA). Studies focusing on the adipocytes of grass carp have also shown that ARA and DHA can promote cell differentiation and lipid accumulation after a short-term (three-day) treatment [30,42]. This might be due to the trait of TGs that monounsaturated fatty acids are preferentially located in the sn-1 and sn-3 positions, whereas polyunsaturated fatty acids are preferentially located in the sn-2 position [43]. Moreover, ARA, DHA, and their metabolites are the natural ligands for PPARγ, which regulates several genes such as lipoprotein lipase (LPL), fatty acid binding protein (FABP), acetyl-CoA carboxylase 1 (ACC1), and FAS, contributing to TG synthesis and to LD formation [8,42]. The findings of the present study further demonstrate that ARA- and DHA-enriched LDs play a unique role in the upregulation of the expression of innate immune genes. Several studies have shown that LC-PUFAs, including ARA, EPA, and DHA, may improve immunity in some fish species including grass carp [24,44], Japanese seabass [23], and rabbitfish [45]. Interestingly, after infestation with parasites, large yellow croakers had a higher survival rate when fed n-3 LC-PUFA, suggesting an important role of LC-PUFAs in the diet when fish are infected with disease [22,46]. Overall, our findings might provide further evidence that LC-PUFAs impact immunity. However, LC-PUFA-enriched LD formation *in vivo* should be further investigated. Notably, although LD number and size increased in the cells, ARA- and DHA-induced increases in immune-related genes may not be entirely due to lipid contents, but may instead be due to the changes in fatty acid composition, because cells incubated with excess amounts of lipids had reduced expression of these genes.

In mammals, fatty acid composition-induced differences in LDs may be intrinsically connected to distinct cellular functions and/or cell types [47]. In leukocytes, LDs are well-known for their unique capacity to generate eicosanoids, which are synthesized *de novo* from ARA by lipoxygenase and cyclooxygenase localized specifically to LDs of activated immune cells [48,49]. In *Mycobacterium leprae*-infected Schwann cells, mycobacterium-induced LD biogenesis is correlated with increased production of prostaglandin E_2 and IL-10 [50]. In the present study, ARA-enriched LDs in CIK cells increased the expression of immune genes, which is probably due to the increase in eicosanoids in the cells. One of the main interactions between LDs and the extracellular cytoplasm is the release of FFA, during which ATGL and lipophagy play important roles in fat degradation [51]. The present study further demonstrated that most genes in ARA-enriched LDs were blocked by an ATGL inhibitor but were not blocked by an autophagy inhibitor, suggesting that lipolysis-induced FFA participated in the upregulation of immune gene expression. In Tian et al. (2017) an *in vivo* study, ARA-induced up-regulation of TLR22 and MyD88 could be blocked by the cyclooxygenase inhibitor ASA in the kidneys [25], suggesting that PGs play an important role in the modulation of innate immunity in grass carp. However, DHA-enriched LDs also had a large capacity to induce innate immune gene expression, and similar to ARA, this effect could be efficiently blocked by an ATGL inhibitor. Interestingly, DHA and its derivate, resolvins, have been revealed to regulate immune cell functions and to be involved in potent anti-inflammatory actions [52,53]. Nevertheless, ARA- or DHA-enriched LDs and their metabolites seem to expand the immune response in fish and the mechanisms behind this should be further explored. Overall, the ATGL inhibitor mostly abolished the upregulation of several immune-related genes, further demonstrating the important role of LDs in the modulation of immunity.

5. Conclusion

In conclusion, this study provides novel insights into the function of LDs in the innate immune response of CIK cells. We demonstrated that LDs can be formulated following exposure to pathogenic mimics,

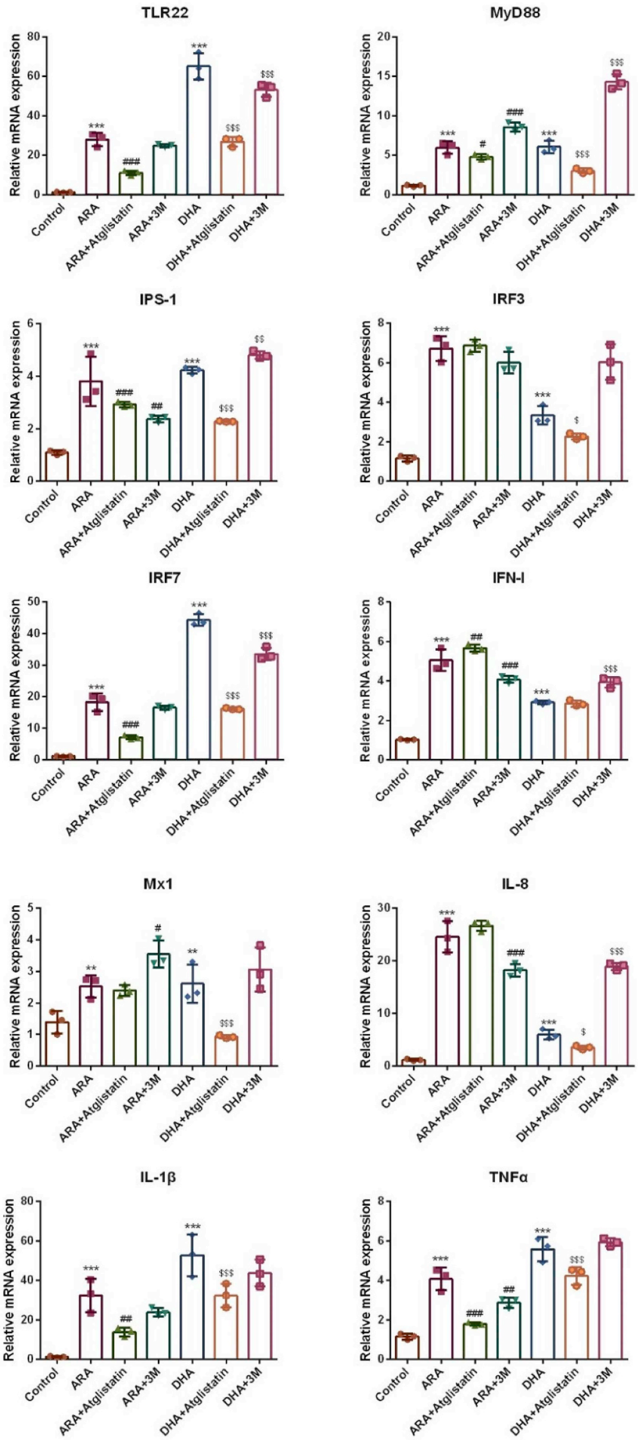

Fig. 5. Effects of arachidonic acid (ARA) or docosahexaenoic acid (DHA) administered with or without atglistatin or 3-methyladenine (3M) on innate immune gene expression. CIK cells were incubated with 300 μM ARA or DHA for 24 h, and were then incubated with non-added lipid medium supplemented with or without atglistatin (50 μM) or 3M (5 mM) for 24 h, after was qRT-PCR was performed (n = 3). All results are presented as the mean ± SD (error bars). Statistically significant differences are denoted as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs ARA; $^{\$}P < 0.05$; $^{\$\$}P < 0.01$; $^{\$\$\$}P < 0.001$ vs DHA.

possibly due to absorption of exogenous fatty acids. LDs induced by exogenous lipids, especially ARA and DHA, could act important modulators in the expression of innate immune genes, and lipolysis may play a key role in this biological function.

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