



Transcriptome Modifications in Porcine Adipocytes via Toll-Like Receptors Activation

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Adipocytes are the most important cell type in adipose tissue playing key roles in immunometabolism. We previously reported that nine members of the Toll-like receptor (TLR) family are expressed in an originally established porcine intramuscular pre-adipocyte (PPI) cell line. However, the ability of TLR ligands to modulate immunometabolic transcriptome modifications in porcine adipocytes has not been elucidated. Herein, we characterized the global transcriptome modifications in porcine intramuscular mature adipocytes (pMA), differentiated from PPI, following stimulation with Pam3csk4, Poly(I:C) or LPS which are ligands for TLR2, TLR3, and TLR4, respectively. Analysis of microarray data identified 530 (218 up, 312 down), 520 (245 up, 275 down), and 525 (239 up, 286 down) differentially expressed genes (DEGs) in pMA following the stimulation with Pam3csk4, Poly(I:C), and LPS, respectively. Gene ontology classification revealed that DEGs are involved in several biological processes including those belonging to immune response and lipid metabolism pathways. Functionally annotated genes were organized into two groups for downstream analysis: immune response related genes (cytokines, chemokines, complement factors, adhesion molecules, and signal transduction), and genes involved with metabolic and endocrine functions (hormones and receptors, growth factors, and lipid biosynthesis). Differential expression analysis revealed that *EGR1*, *NOTCH1*, *NOS2*, *TNFAIP3*, *TRAF3IP1*, *INSR*, *CXCR4*, *PPARA*, *MAPK10*, and *C3* are the top 10 commonly altered genes of TLRs induced transcriptional modification of pMA. However, the protein-protein interaction network of DEGs identified *EPOR*, *C3*, *STAR*, *CCL2*, and *SAA2* as the major hub genes, which were also exhibited higher centrality estimates in the Gene-Transcription factor interaction network. Our results provide new insights of transcriptome

modifications associated with TLRs activation in porcine adipocytes and identified key regulatory genes that could be used as biomarkers for the evaluation of treatments having immunomodulatory and/or metabolic functional beneficial effects in porcine adipocytes.

Keywords: transcriptome, adipocytes, microarray, TLRs (Toll-like receptors), immunometabolism, pig

INTRODUCTION

The innate immune system recognizes infectious microbial pathogens through germ line-encoded patterns recognition receptors (PRRs), such as Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (1). These receptors interact with the evolutionarily conserved microbial structures known as microbial associated molecular patterns (MAMPs), including lipopolysaccharides (LPS), lipoteichoic acids (LTA), peptidoglycan (PGN), and double stranded viral RNA, which are essential for the survival of microorganisms (2). In addition, PRRs also recognize endogenous damage-associated molecular patterns (DAMPs) derived from dead cells or injury (3), such as free fatty acids, cholesterol, high glucose concentration, ceramides, and urate crystals (4). Although low levels of DAMPs are beneficial during tissue repair, excessive amounts induce chronic low-grade inflammation in various tissue including adipose tissues. Deregulated inflammation in the adipose tissue is involved in the development metabolic disorders like obesity, atherosclerosis, and type-2 diabetes mellitus (5). Therefore, elucidation of the cellular transcriptome modifications in adipocytes associated with the activation of their PRRs is of great importance to understand in more depth the physiopathological mechanisms involved in the metabolic diseases with an inflammatory component and to propose alternatives to prevent them.

TLRs are an important family of PRRs with capacity to sense several types of MAMPs and thereby trigger inflammatory responses (6). Depending on the cellular localization TLRs can be categorized into two subgroups: trans-membrane (such as TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) and intracellular (such as TLR3, TLR7, TLR8, and TLR9) receptors (6, 7). Early in the immune response TLR ligation induces gene transcription leading to inflammation, tissue repair and initiation of adaptive immunity (6, 8, 9). Previous studies reported that Pam3csk4 is recognized at the cell membrane by TLR1/2 as a mimic of bacterial lipopeptides (10). Poly(I:C), a synthetic double-stranded RNA is recognized in the endosome by TLR3 (11), while LPS is recognized by TLR4 sequentially at the cell membrane and endosome (8, 12). Upon binding to respective ligands, TLRs recruit a set of specific adapter molecules such as myeloid differentiation primary response gene 88 (MyD88), Toll/interleukin-1 receptor (TIR) domain-containing adapter protein (TIRAP), TIR-domain-containing adapter-inducing interferon- β (TRIF), or TRIF-related adapter molecule (TRAM) to initiate the downstream signal transductions that lead to the activation of different transcriptional factor such as nuclear factor-kappa B (NF- κ B), activator protein-1 (AP-1), and interferon regulatory factor (IRF) (2, 6). The transcription factors specifically signal the cells to secrete proinflammatory cytokines

and chemokines, type-I interferon, and antimicrobial peptides (6), which coordinately induce inflammatory responses.

The adipose tissue is a highly active organ capable of integrating metabolic, endocrine, and immune functions into a single entity that plays a crucial part on systemic homeostasis (4, 13). In addition to its role as an endocrine gland with pleiotropic function in the metabolism (14), adipose tissue is increasingly becoming recognized as part of the innate immune system (15, 16). Adipose tissue contains several distinct groups of cells including mature adipocytes, pre-adipocytes, fibroblasts, M1/M2 macrophages; neutrophils, dendritic cells, eosinophils, and endothelial cells (17). Pre-adipocytes have the ability to differentiate into mature adipocyte according to the energy balance (17, 18). Morphologically, adipocytes are spherical cells with a single large lipid droplet formed by triglycerides that account >90% of the cell's volume (19). Mature adipocytes are functionally the most important cell type in adipose tissue and play roles in storing triglycerides and systemic energy balance (20), as well as in antigen presentation (21). Upon stimulation by MAMPs and/or DAMPs, mature adipocytes secrete a wide variety of cytokines and other mediators, which are able to contribute to the generation of both local and systemic immune responses (13, 22).

Different transcriptomic analytic approaches have been employed to identify key regulatory genes in response to multi-TLR activation in white blood cells (23) and macrophages (24). Transcriptomic studies aimed to elucidate the gene expression changes after *in vitro* stimulation with different MAMPs have been also performed in human adipocytes (15, 25). However, there is no detailed information on immunotranscriptomic responses following TLRs activation in the porcine adipocytes. Pigs are considered as one of the closest approximate animal models for studying human diseases because of their anatomical, physiological and immunological similarities with humans (26). By using a porcine intramuscular pre-adipocyte (PIP) cell line originally established by our group (27), we recently demonstrated that nine TLRs (TLR 1-9) are expressed in PIP and differentiated porcine mature adipocytes (pMA) (28). Importantly, TLR2 and TLR4 showed significantly higher expression in pMA as compared to PIP while TLR3 showed higher expression in PIP than that of pMA (28). We therefore, aimed herein to characterize the global transcriptome modifications downstream of TLR2, TLR3, and TLR4 activation in pMA. We obtained a global overview of the modulation of the transcriptomic response in pMA and its association with immune, metabolic and endocrine responses. Results of the present study indicated that several immune genes including *EGR1*, *NOTCH1*, *NOS2*, *TNFAIP3*, *TRAF3IP1*, *INSR*, *CXCR4*, *PPARA*, *MAPK10*, and *C3* were differentially modulated in pMA cells after TLR ligation. Our results provide new insights of

transcriptome modifications associated with TLRs activation in porcine adipocytes and indicate that pMA cells are an interesting tool to study *in vitro* the immune responses triggered by TLR2, TLR3, or TLR4 in this cell population.

MATERIALS AND METHODS

Cell Line, Culture Condition, and Differentiation

Porcine intramuscular pre-adipocyte (PIP) cell line was previously established by our group (27), which was derived from marbling muscle tissue of the musculus longissimus thoracis from female Duroc pig. Culture conditions for inducing adipogenesis were performed according to our previous work (28). In brief, PIP cells (between the 26th and 35th passages) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco™, Paisley, Scotland, UK) supplemented with 10% (v/v) fetal calf serum (FCS; Sigma-Aldrich, Tokyo, Japan), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco™ 15140122, Life Technologies) as a growth medium. The PIP cells were plated at density of $2.5 \times 10^4/\text{cm}^2$ in 6-well cell culture plates (BD Falcon, Tokyo, Japan), and incubated at 37°C in a humidified atmosphere of 5% CO₂. The 4-day post-confluent PIP cells were fed with differentiation medium for another 4 days to yield the differentiated adipocyte. The differentiation medium composed with DMEM containing 10% FCS and 50 ng/ml insulin (swine, Sigma-Aldrich, Tokyo, Japan), 0.25 µM dexamethasone (Sigma-Aldrich), 2 mM octanoate (Wako, Osaka, Japan), 200 µM oleate (Ardorich, Milwaukee, WI, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every day. The differentiation of PIP into functionally matured porcine mature adipocyte (pMA) was confirmed by detecting the presence of intracellular lipid droplets with Oil red O staining according to our previous publication (28). Briefly, cells were rinsed three times in Dulbecco's Phosphate-Buffered Saline and then fixed in 10% (v/v) formaldehyde for 30 min. Subsequently, the fixed cells were rapidly rinsed with MiliQ water. Finally, 0.5% Oil red O (Sigma-Aldrich, Tokyo, Japan) in isopropanol was added to the cells for 5 min to visualize lipid droplets stained red. The cytosolic triglyceride content was analyzed using LabAssay™ triglyceride kit (FUJIFILM Wako Chemicals USA, Corp.) according to the manufacturer's protocol. In addition, qRT-PCR-based (method followed as described later in this section) expression of specific marker genes were also evaluated for adipocyte maturation.

Stimulation to Porcine Mature Adipocyte (pMA) by TLR Ligands

Synthetic analogs for three Toll-like receptors: TLR2, TLR4 and TLR3; were used to mimic the inflammatory response induced by gram positive bacteria, gram negative bacteria, and by virus infection, respectively. Pam3csk4, Poly(I:C), and LPS were used as ligands for TLR2, TLR3, and TLR4, respectively. For optimizing the dose of ligands, we first stimulated pMA cells ($2.5 \times 10^4/\text{cm}^2$) with serial dilutions of each ligand and evaluated CCL2 expression. The optimal doses were selected according to

their ability to increase CCL2 expression at least five-folds as compared to that of control (data not shown). The pMA cells were seeded at density of $2.5 \times 10^4/\text{cm}^2$ in 6 well or 12 well plates (BD Falcon, Tokyo, Japan). The 4-day post-confluent pMA cells were stimulated either with Pam3csk4 (10 ng/ml), Poly(I:C) (0.1 µg/ml), or LPS (0.1 µg/ml) at 37°C with 5% CO₂ for 12 h.

RNA Isolation and Quality Control

Total RNA was isolated from the ligand-treated and control pMA cells using PureLink RNA Mini Kit (Life Technology Inc., USA) along with on-column DNase treatment. RNA integrity, quality and quantity were evaluated with microcapillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) using the Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA integrity number (RIN) of >8 were used for this gene expression study.

Microarray Hybridization

The microarray hybridization was performed with Porcine Gene Expression Microarray 4 × 44 K oligonucleotide slide (v2.0, Agilent Technologies, Santa Clara, CA, USA) containing 43,803 probes for the identification of known genes of the porcine transcriptome. The microarray experiment was conducted at Hokkaido System Science Co., according to the one-color Microarray-based Gene Expression Analysis protocol v6.7 (Agilent Technologies, Santa Clara, CA, USA). For each samples, 200 ng of total RNA was converted into cDNA by reverse transcription. The cDNA was subsequently transcribed into cRNA and labeled with cyanine 3 (Cy3). About 1.65 µg of labeled cRNA was mixed with hybridization buffer and hybridized on microarray slide (4 samples in each slide) for 17 h at 65°C with constant rotation. After hybridization, microarrays were cleaned with Gene Expression wash buffer and scanned with High-Resolution Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA). The Feature Extraction software (v10.7.3.1, Agilent Technologies, Santa Clara, CA, USA) was used for detailed analysis of scanned images including filtering the outlier spots, background subtraction from features and dye normalization. The spot intensity data for individual sample were extracted for statistical analysis.

Statistical Analysis of Microarray Data

The normalization and differential expression analysis of microarray data were performed with GeneSpring GX software (v13.1, Agilent Technologies, USA). The log₂ transformed expression values of probes were normalized based on 75 percentile shifts. In order to determine the TLR-ligand induced differential expression of genes, an unpaired *t*-test was performed between untreated control and TLR-ligand stimulated samples. The pairwise comparisons were performed between control and each of the three TLR-ligand stimulations to detect the differentially expressed genes. Benjamini and Hochberg (B-H) adjustment method was applied for multiple test correction. Significant differentially expressed genes were selected on the basis of two criteria: an adjusted *p*-value (FDR, false discover rate) of <0.05, and a cutoff in fold change of at least 1.5. The human orthologs gene symbols of DEGs were determined using dbOrtho

panel of the bioDBnet tool (29) which were used for downstream functional analyses.

Gene Ontology (GO) and Pathway Analyses

For biological interpretation of differential gene expressions, GO enrichment and pathway analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8) (30). Human orthologous symbols of DEGs were uploaded to the DAVID web portal and the official gene symbol was chosen as identifier. Then enriched biological themes, particularly GO terms and KEGG pathways were extracted. In the analysis, GO terms and KEGG pathways with an FDR-adjusted $p < 0.05$ were retained.

Network Enrichment Analyses

In order to visualize TLR ligands-induced transcriptional network as well as to identify the regulatory genes, the sub-network enrichment analysis was performed using NetworkAnalyst online tool (31). This tool uses the InnateDB protein-protein interaction datasets comprised of 14,755 proteins and 145,955 literature-curated interactions for human (32). Human orthologous gene symbols of the common DEGs from all three stimulation were uploaded into the NetworkAnalyst to construct the interaction network based on Walktrap algorithm taking only direct interaction of seed genes. The network was depicted as nodes (circles representing genes) connected by edges (lines representing direct molecular interactions). Two topological measures such as degree (number of connections to the other nodes) and betweenness (number of shortest paths going through the nodes) centrality were taken into account for detecting highly interconnected genes (Hubs) of the network. Nodes having higher degree and betweenness were considered as potentially important hubs in the cellular signal trafficking. In addition, a gene regulatory network focusing the adipose tissue specific gene-transcription factor (Gene-TF) interaction network was also constructed using the NetworkAnalyst tool (31). For constructing the Gene-TF network, transcription factor and gene target data derived from the ENCODE ChIP-seq data were used. Only peak intensity signal < 500 and the predicted regulatory potential score < 1 were included based on BETA Minus algorithm (33).

Validation of Microarray Expression by qRT-PCR

Two-step real-time PCR (qRT-PCR) was performed to confirm the microarray results by quantifying expression of selected mRNAs in pMA. Primer sequences are presented in **Table 1**. Total RNA was isolated from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by treated with gDNA Wipeout Buffer (Qiagen, Tokyo, Japan). All cDNAs were synthesized using the Quantitect reverse transcription Kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. The qRT-PCR was performed using 7300 real-time PCR system (Applied Biosystems, Warrington, UK) using the TaqMan[®] gene expression assay kit (Life Technologies) and TaqMan[®] Universal Master Mix II, with UNG (Applied Biosystems, Warrington, UK). The PCR cycling conditions were 2 min at 50°C, followed by 10 min at 95°C, and then 40 cycles

of 15 s at 95°C, 1 min at 60°C. The reaction mixtures contained 2.5 μ l of sample cDNA, 1 μ l gene expression assay and 10 μ l TaqMan[®] Universal Master mix II, with UNG, and 6.5 μ l distilled water. According to the minimum information for publication of quantitative real-time PCR experiments guidelines, Beta actin (ACTB) was used as a house-keeping gene because of its high stability across various porcine tissues (34, 35). Relative index was calculated as the ratio of target mRNA expression to ACTB. Then, raw data were transferred from the mean Ct values of replicated samples to copy number of the established standard curve.

Statistical Analysis of qRT-PCR Data

The raw data were log-transformed followed by checking the normality by Kolmogorov-Smirnov test. Comparisons between mean values were carried out using one-way ANOVA and Fisher's least significant difference test. For every cases, $p < 0.05$ was considered significant. The Pearson's correlation coefficient between the expression values obtained microarray and qRT-PCR were calculated to explore the linear relationship between the microarray and qRT-PCR results.

RESULTS

Differentiation of Adipocytes From PIP

Differentiation of functionally matured adipocytes from pre-adipocytes is an essential biological process. In order to obtain porcine mature adipocytes (pMA), we cultured the porcine intramuscular pre-adipocyte (PIP) cells (25) supplemented with differentiation medium for 4 days. The maturation of adipocytes was confirmed by the detection of lipid droplets in cell cytoplasm under microscopy using Oil red O staining as well as triglyceride assay. The PIP cells at the day of seedling showed no lipid droplets (**Figure 1A**). Following 1 day of culture, some cells started to exhibit flattened cellular morphology (**Figure 1B**). Finally, after 4 days of culture more than 95% of the PIP cells exhibited more lipid droplets indicated their differentiation into fully mature adipocytes (**Figure 1C**). A significant ($p < 0.05$) increased lipid accumulation within the cells indicated their maturation into adipocytes (**Figure 1D**).

In order to further confirm the adipocyte maturation, we quantified the expressions of some differentiation marker genes using qRT-PCR (**Figure 2**). The expression of peroxisome proliferator-activated receptor γ (PPAR γ) and insulin receptor (INSR) were significantly increased in PIP after 4 days of culture with differentiation medium while cytochrome P540 receptor 3A (CYP3A46) and free fatty acid receptor 2 (FFAR2) were down regulated. The glutamine fructose-6-phosphate transaminase 1 (GFPT1) showed a decreasing trend but activin receptor type 1B (ACVR1B) and PPAR α showed an increasing trend of expression (**Figure 2**).

Differentially Expressed Transcripts in pMA After TLRs Activation

Stimulation of pMA with TLRs induced changes in a total of 1,575 genes. Among them, 702 (44.57%) and 873 (55.43%) genes were up- and down-regulated, respectively (**Table 2**). The

TABLE 1 | Sequences of the primers used for qRT-PCR study.

Gene symbol	Primer sequence (3'-5')	Size (bp)	Accession no
ACTB	F: CAT CAC CAT CGG CAA CGA R: GCG TAG AGG TCC TTC CTG ATG T	144	XM_003124280.5
INSR	F: AGA GCG GAT CGA GTT TCT CA R: CCA TCC CAT CAG CAA TCT CT	245	XM_021083943.1
PPAR γ	F: ACA CCG AGA TGC CGT T R: CGA CAG GTC CAC AGA G	56	XM_005669788.3
PPARA	F: CGA CCT GGA AAG CCC GTT AT R: GGA TCC ATC TGA TCC CGG AC	148	NM_001044526.1
ACVR1B	F: CAT CGA GGG GAT GAT CAA GT R: GGC AAT GTC AAT GGT GTC AG	211	NM_001195322.1
CYP3A46	F: ACA GCA TTT GGA GTG AAC GTC R: CCA CTC GGT GCT TTT GTG TAT	250	NM_001134824.1
GFPT1	F: ATG CTC TTC AGC AGG TGG TT R: TCT ACG GTT ACC GAT TTG GC	232	XM_005662490.3
FFAR2	F: TCA TGG GTT TCG GCT TCT AC R: AAC GAT GAA CAC GAC AGT GC	191	XM_021093196.1
TLR2	F: ACA TGA AGA TGA TGT GGG CC R: TAG GAG TCC TGC TCA CTG TA	109	XM_005653577.3
TLR3	F: TAG AGA CAT GGA TTG CTC CC R: AAC TTC TGG AAT GCA GGT CC	435	NM_001097444.1
TLR4	F: CTC TGC CTT CAC TAC AGA GA R: CTG AGT CGT CTC CAG AAG AT	322	NM_001293316.1
EPCAM	F: GCG ATA GCG ATT GTT GCT GG R: CCC TAT GCA TCT CGC CCA TC	106	NM_214419.1
SELL	F: GTG ATG CAG GGT ACT ACG GG R: AGA ACT TGC CCA AAG GGT GA	108	NM_001112678.1
SAA2	F: AGA GCC TAC TCG GAC ATG AGA GA R: CCC CGG GCA TGG AAG TAC	65	NM_001044552.1
CCL5	F: CCA GCA GCA AGT GCT CCA T R: ACA CCT GGC GGT TCT TTC TG	60	NM_001129946.1
CXCL2	F: CCG GGA CCC CAC TGT GA R: CAACTTCCTGACCATTCTTGAGA	62	NM_001001861.2
CFB	F: CCT CGG GCT CCA TGA ATA TC R: TGC CCC AAT GCT GTC TGA T	56	NM_001101824.1
C3	F: CCA ACA GGG AGT GCA ACG A R: TGA CTC CGT GTC TGG GAC TTG	70	NM_214009.1
CSF1	F: CCA ACA GGG AGT GCA ACG A R: TGA CTC CGT GTC TGG GAC TTG	147	NM_001244523.1
TGFB3	F: TTG CTA AAT GCT CCA GCC AG R: GCC TCC GCC TGT AGA ACA AG	90	NM_214198.1
TNFAIP3	F: CCC TGG GGC ATT ATG GGT TT R: CCT CAC ACG TTG TAG CAC CT	60	NM_001267890.1
CCL2	F: CCT CCC TGG AAA GCC AGA A R: GTG CCA CAA GCT TCC TCA CTT	58	NM_214214.1
TNF α	F: CGA CTC AGT GCC GAG ATC AA R: CCT GCC CAG ATT CAA AG	58	JF831365.1
IL8	F: GCT CTC TGT GAG GCT GCA GTT R: TTT ATG CAC TGG CAT CGA AGT T	62	NM_213867.1
IL6	F: TGG ATA AGC TGC AGT CAC AG R: ATT ATC CGA ATG GCC CTC AG	109	NM_214399.1
IL1 α	F: AGA ATC TCA GAA ACC CGA CTG TTT R: TTC AGC AAC ACG GGT TCG T	62	NM_214029.1
IL1 β	F: GCC CTG TAC CCC AAC TGG TA R: CCA GGA AGA CGG GCT TTT G	61	NM_001302388.2

F, Forward; R, Reverse; bp, base pair.

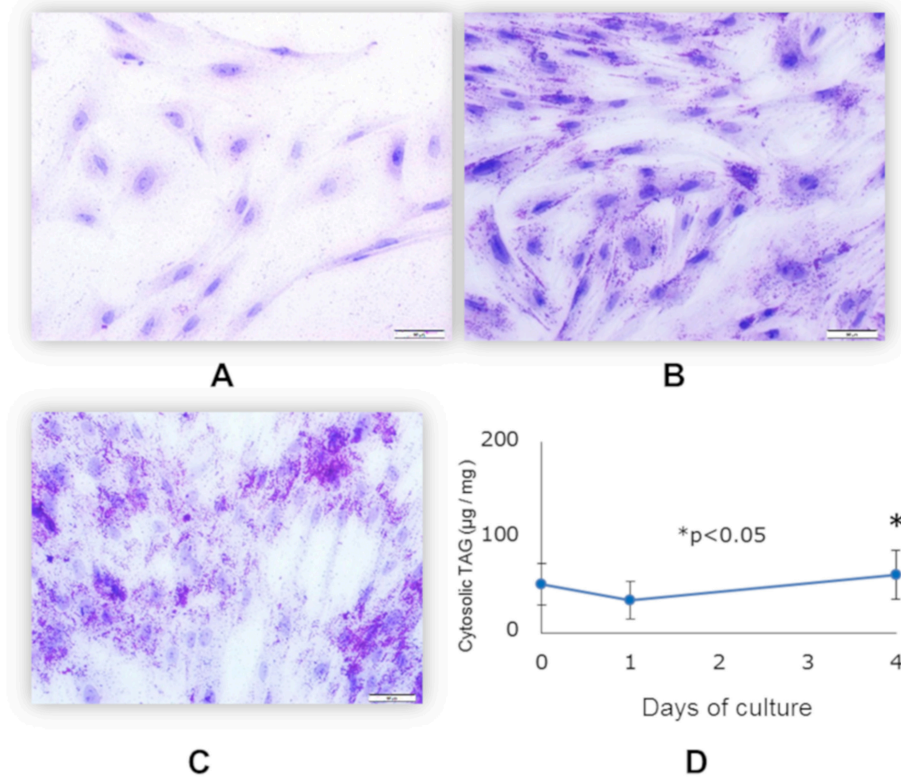


FIGURE 1 | Differentiation of mature porcine adipocytes (pMA) from porcine intramuscular pre-adipocyte (PIP). Oil-red O stained images display the cellular morphology at day 0 (A), day 1 (B), and day 4 (C) of culture of PIP cells with differentiation media. Fat accumulation was determined by triglyceride assay (D). Data (Mean ± SD) presented are the average of 3 independent experiments performed in triplicates.

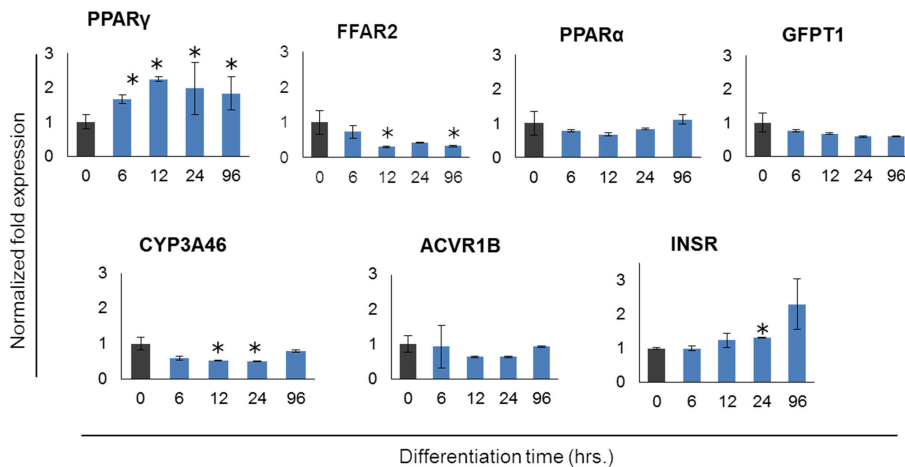


FIGURE 2 | Expression profiles of marker genes for adipocyte differentiation in porcine intramuscular pre-adipocyte (PIP) and porcine mature adipocytes (pMA). The asterisk (*) indicates Statistical differences with significant levels of $P < 0.05$. Data (Mean ± SD) presented are the average of 3 independent experiments performed in triplicates.

TLR2 ligand Pam3csk4 differentially regulated 530 transcripts in pMA, being 218 and 312 transcripts up- and down-regulated, respectively. After TLR3 stimulation 245 transcripts were up-regulated while 275 transcripts were down-regulated in

pMA. In addition, stimulation of pMA with LPS up-regulated 239 transcripts and down-regulated 286 transcripts (Table 2). The list of significantly (adjusted $p < 0.05$) up-regulated and down-regulated genes are presented in Supplementary Tables S1–S3.

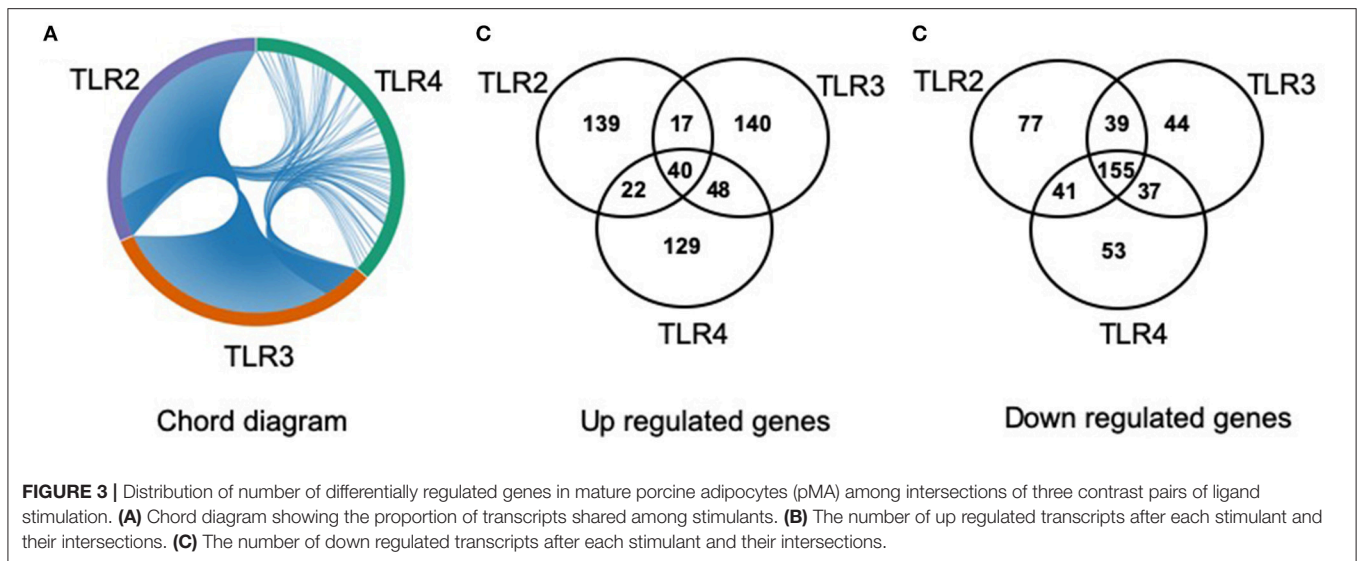


TABLE 2 | Number of differentially regulated genes in the porcine intramuscular adipocytes after Pam3csk4, Poly(I:C) and LPS stimulation.

	Pam3csk4 vs. control	Poly(I:C) vs. control	LPS vs. control
Up	218	245	239
Down	312	275	286
Total	530	520	525

Intersections of TLR Ligand-Induced Differentially Regulated Transcripts

The lists of differentially regulated transcripts obtained from three pairwise contrasts were overlapped to assess the cross-talk among the three TLR ligands. We observed that there were 195 differentially expressed transcripts shared by the three stimulations. Among them, 40 were up- and 155 were down-regulated transcripts (Figure 3). A total of 251 differentially expressed transcripts were common between TLR2 and TLR3 stimulation, 57 and 194 were up- and down-regulated, respectively. In addition, 280 differentially expressed transcripts were common between TLR3 and TLR4 stimulation. A total of 258 transcripts were shared by pMA stimulated with TLR2 or TLR4. Among them, 62 were up- and 196 were down-regulated transcripts (Figure 3).

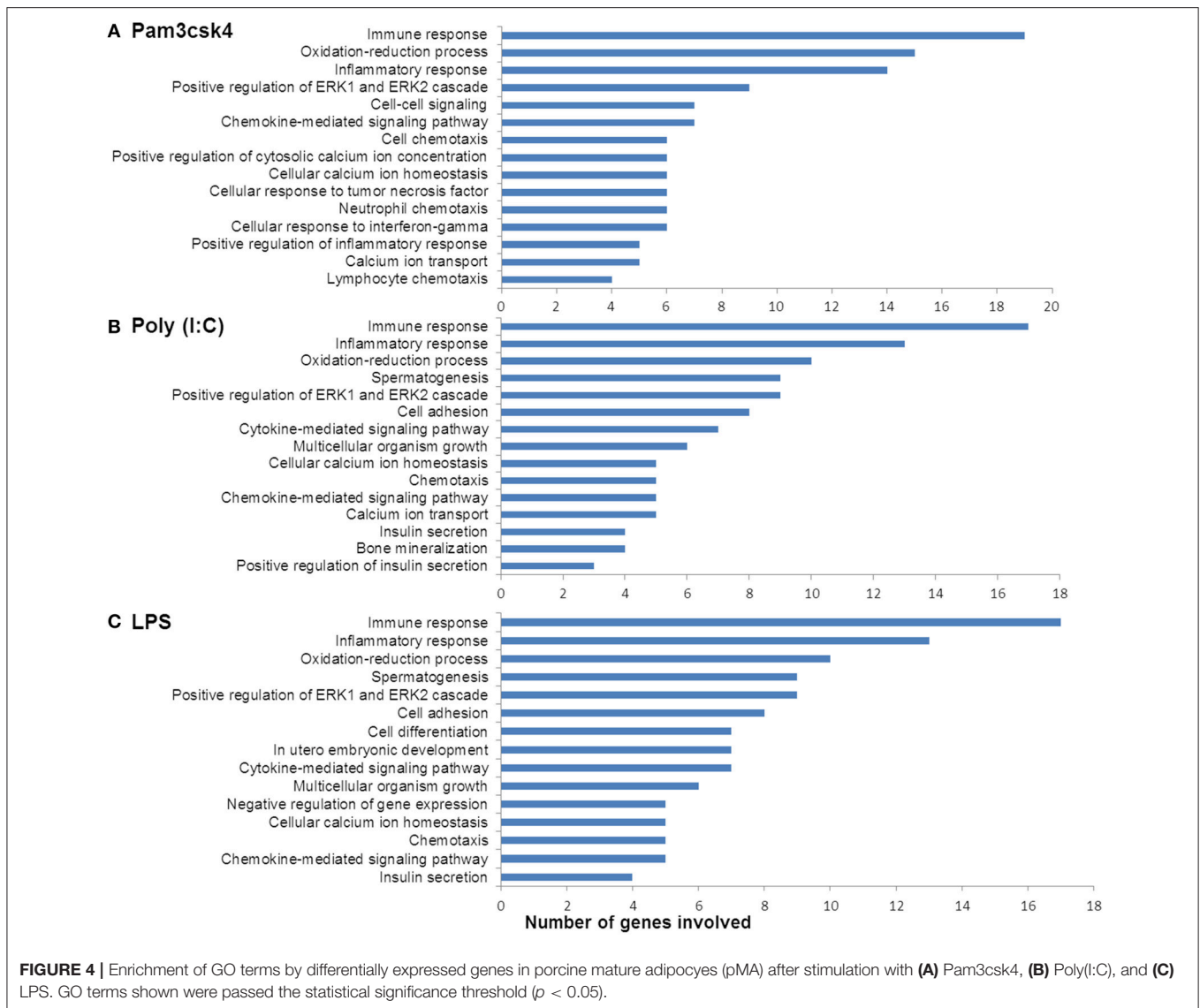
Gene Ontology Classification

In order to characterize the biological implications of the differentially expressed genes in pMA after TLRs stimulations, we performed the GO and pathway enrichment analysis using DAVID online tool. Based on the ascending order of adjusted *p*-value, the top 15 GO biological process significantly enriched with the differentially expressed genes following

Pam3csk4, Poly(I:C), and LPS stimulation are presented in Figure 4. There were some common GO terms enriched following each of the three TLR treatments including immune response (GO: 0006955), inflammatory responses (GO: 0006954), oxidation-reduction process (GO: 0055114), chemokine-mediated signaling (GO: 0070098), and positive regulation of ERK1 and ERK2 cascade (GO: 0070374). Cell adhesion (GO: 0007155), chemotaxis (GO: 0006935), and insulin secretion (GO: 0030073) were commonly enriched GO biological process between Poly(I:C) and LPS stimulation, while enrichment of calcium ion transport (GO: 0006816) was shared between Pam3csk4 and Poly(I:C) stimulation. The full list of enriched GO and pathways are presented in Supplementary Table S4.

Transcript Abundances Associated With Immune Response Function

The expression patterns of differentially modulated immune related genes in pMA following TLR activation are presented in Figure 5. The mRNAs of cytokine and cytokine receptors including *IL1 β* , *IL1 α* , *IL13RA2*, *IL1RAPL1*, *IL9*, *IL10*, *IL15*, *IL6R*, *IL12B*, *IL18A*, *IL20RB*, *IL23R*, *IFNB1*, and *IFN-omega5* showed differential expression. The expression of chemokine and chemokine receptors including *CCL2*, *CCL20*, *CCL8*, *IL8*, *CX3CR1*, *CXCL9*, *CSF3*, *CXCL12*, *CXCR4*, *CCL5*, *CCL3L1*, *CCR1*, *CCR7*, *CXCR6*, *CCR9*, and *CXCL1* were differentially regulated. The expression of complement factors including *C3*, *CFB*, *CFH*, *F9*, and *THBD*; and adhesion molecules including *EPCAM*, *ITGA2*, *SELE*, *SELL*, and *MCAM* were differentially modulated following TLR activation in pMA. The signal transduction molecules including *TNFAIP3*, *TNFSF4*, *TRAF3IP1*, *MAPK*, *NOTCH1*, *NF- κ B*, *NKAPL*, *MALL*, and *CRABP1* showed differential expression in pMA after 12 h of TLR activation (Figure 5).

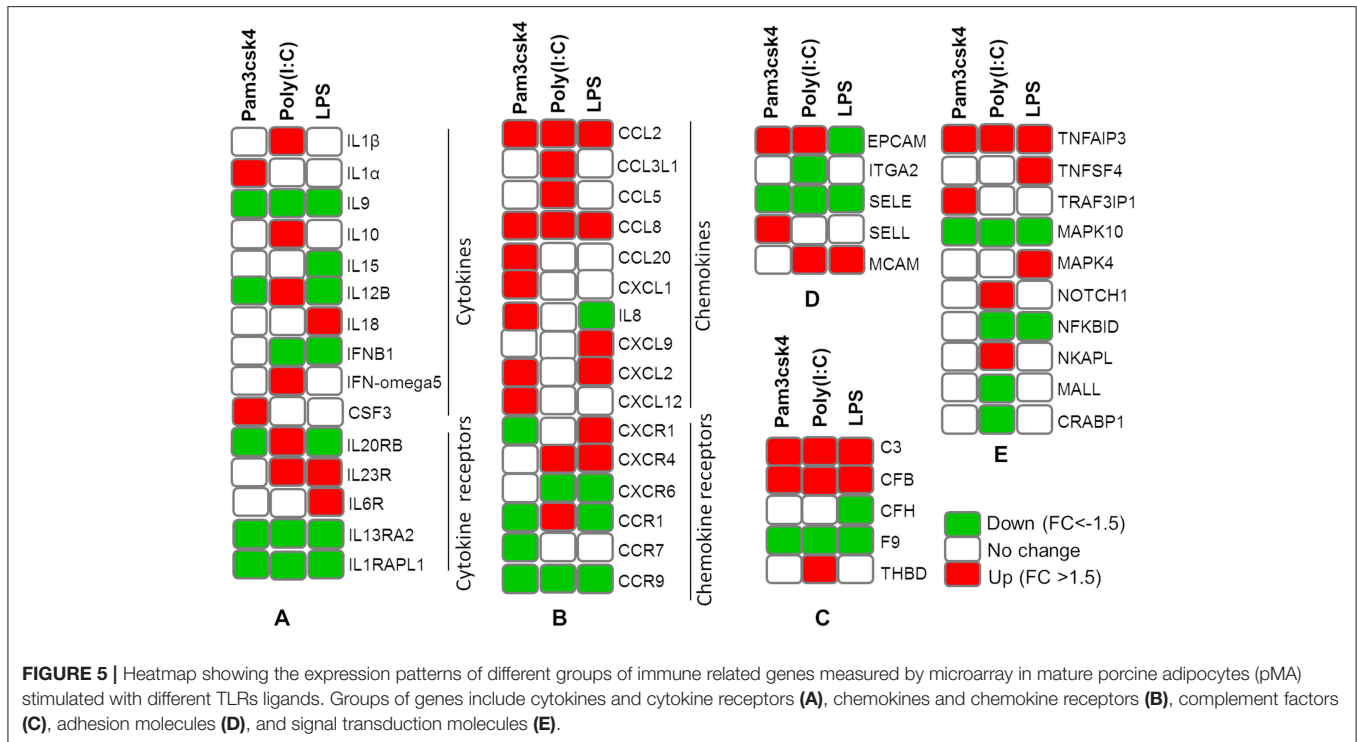


Transcripts Abundance Associated With Metabolic and Endocrine Functions

Differentially regulated transcripts encoded for proteins involved in metabolism and endocrine functions are summarized in **Figure 6**. Among them, genes involved in lipid metabolism included *FFAR2*, *APOB*, *APOM*, *ADIPOQ*, *CYP8B1*, *CYP2C49*, *CYP2025*, *STAR*, *CYP21A2*, *ACACB*, *AKR1C1*, *CYP4A24*, *CYP2B22*, *FFAR1*, *PPARA*, *CYP27B1*, *CYP3A46*, *CYP2A19*, *CYP4F2*, and *CYP19A3*. The mRNAs for hormones and receptor including *ADRA1B*, *ADRA2B*, *ADRA1D*, *CCK*, *CHRM2*, *CHRN2*, *DRD1*, *EPOR*, *ESRRB*, *GHRH*, *HTR1F*, *HTR2C*, *INSR*, *IRS1*, *P2RY1*, *P2Y12R*, *PC2*, *PRLR*, and *PTH1R* showed differential expressions in pMA after TLR activation. In addition, TLR activation resulted in a differential expression of some growth factors including *EGF*, *EGR1*, *EGR3*, *EGFR4*, *EGF17*, *EGF23*, *KLF1*, *SCG5*, *RETN*, *ITLN2*, and *GHSR* in the porcine adipocytes (**Figure 6**).

Gene Expression Measured by qRT-PCR

In order to confirm the microarray expression results, we quantified mRNAs of several differentially expressed genes as well as some other genes which are known to be involved in immune and metabolic functions by using qRT-PCR. The Pearson's correlation coefficient ($r = 0.9064$, $p < 0.001$) indicated that microarray expression results were strongly correlated with that obtained from qRT-PCR (**Figure 7A**). Among the genes quantified by qRT-PCR, *SAA2* was significantly up regulated and *EPCAM* was down regulated after all the ligand stimulation (**Figure 7B**). The expression of *SELL*, *PPARA*, *INSR*, and *ADIPOQ* were up regulated after both Pam3csk4 and Poly(I:C) stimulation but down regulated after LPS stimulation. *GLP2R* expression was increased after Pam3csk4 and LPS stimulation but *CCL5* was increased only after Poly(I:C) stimulation (**Figure 7B**). Among the immune response related genes, up regulation of *CXCL2*, *C3*, *CSF1*, *TNFAIP3*, *CCL2*, *TNF α* , *IFN β* , *IL8*, *IL6*,



and *IL1 α* were noticed relatively more in case of Pam3csk4 and LPS stimulation than Poly(I:C) stimulation (Figure 7C). The expression of CFB was increased after Pam3csk and Poly(I:C) stimulation but decreased after LPS stimulation. *IL1 β* was increased after Poly(I:C) stimulation, but decreased after Pam3csk4 and LPS stimulation. Expression of *TGFB3* was remained stable following all three stimulation (Figure 7C).

Protein-Protein Interaction (PPI) Network

The PPI network is a hierarchical structure, where the hubs play a central role in directing cellular response to a given stimulus. To identify the hub genes involved in the regulation of transcriptome modification of pMA following TLRs ligation, we have constructed and visualized the PPI network of differentially expressed transcripts (Figure 8). The hub nodes of the network were selected based on the values of two centrality measures interpreted serially, first “degree” then “betweenness.” Accordingly, *RELA*, *HNF4A*, *SP1*, *EPOR*, *C3*, *STAR*, *CCL2*, and *SAA2* were identified as major hub genes of the TLRs induced transcriptional network in the pMA (Figure 8). Though *RELA*, *HNF4A*, and *SP1* were not differentially expressed in the TLR ligand-treated pMA but predicted to be involved in the regulation of PPI-network. The centrality measures of the network nodes/interactome are presented in Supplementary Table S5.

Gene-Centered Transcription Factor Network

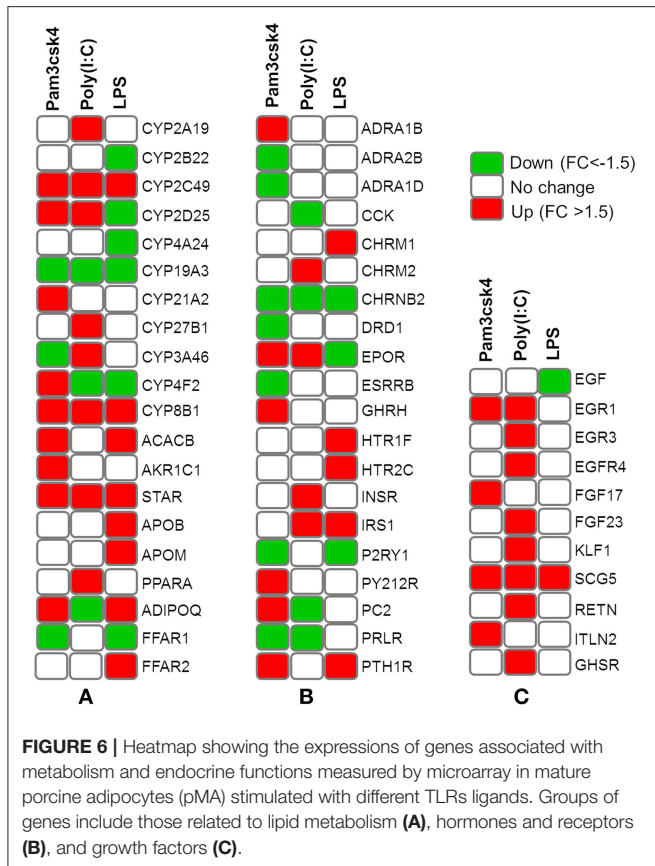
Transcription factors (TF) are potential regulators of differential gene expression. We constructed a gene-TF interaction network in order to explore the involvement of transcription factors

in TLR ligand-induced differential gene expression in porcine adipocytes using NetworkAnalyst. Gene-TF network illustrated that potential hub genes of PPI network also exhibited higher centrality estimates in the Gene-TF interaction network indicating their possible regulation by transcription factors. The transcriptional factor binding site (TFBS) analysis revealed that promoter regions of *C3*, *TNFAIP3*, *CFB*, *CXCL2*, *STAR*, *ADIPOQ*, *CYP8B1*, *SOD2*, *EPCAM*, *EPOR*, *SAA2*, and *CCR9* genes have DNA-binding sites of transcription factors, which are likely contributing pMA transcriptional modification (Figure 9). In particular, *TNFAIP3*, *CXCL2*, *STAR*, and *ADIPOQ* are regulated by *NF- κ B* transcription factor. The network centrality measures of potential genes are presented in Supplementary Table S6.

DISCUSSION

In recent years, it became clearer that along with the metabolic and endocrine functions, the adipose tissue exerts multiple roles in the generation and regulation of immune responses (4, 13). In this work, we performed a microarray-based global transcriptome profiling of pMA after *in vitro* stimulation with TLR ligands in order to evaluate the immune and metabolic responses of porcine adipocytes triggered by the activation of these PRRs.

The differentiation and maturation of adipocytes is crucial for their proper physiological functions and for the prevention of metabolic disorders (36, 37). Apart from those in the main subcutaneous and visceral fat depots, adipogenic differentiation has also been described in other locations, including skin, bone marrow, and skeletal muscle (36). A recent microarray



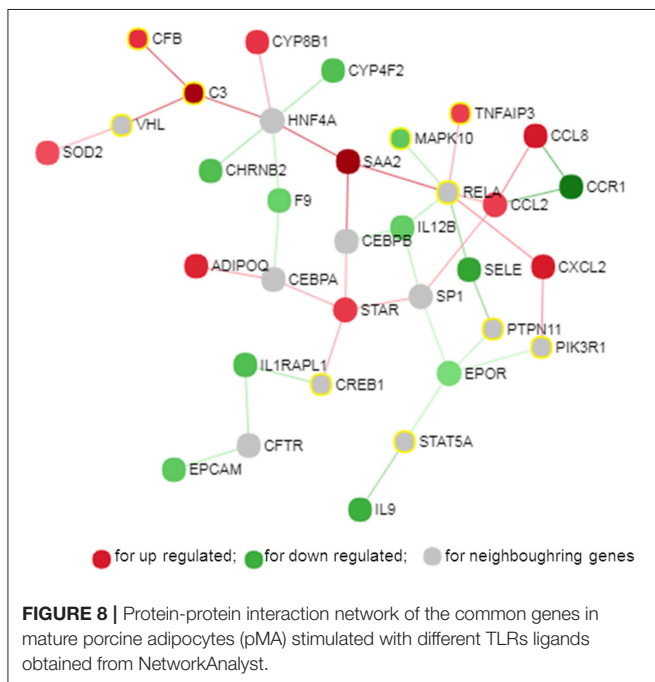
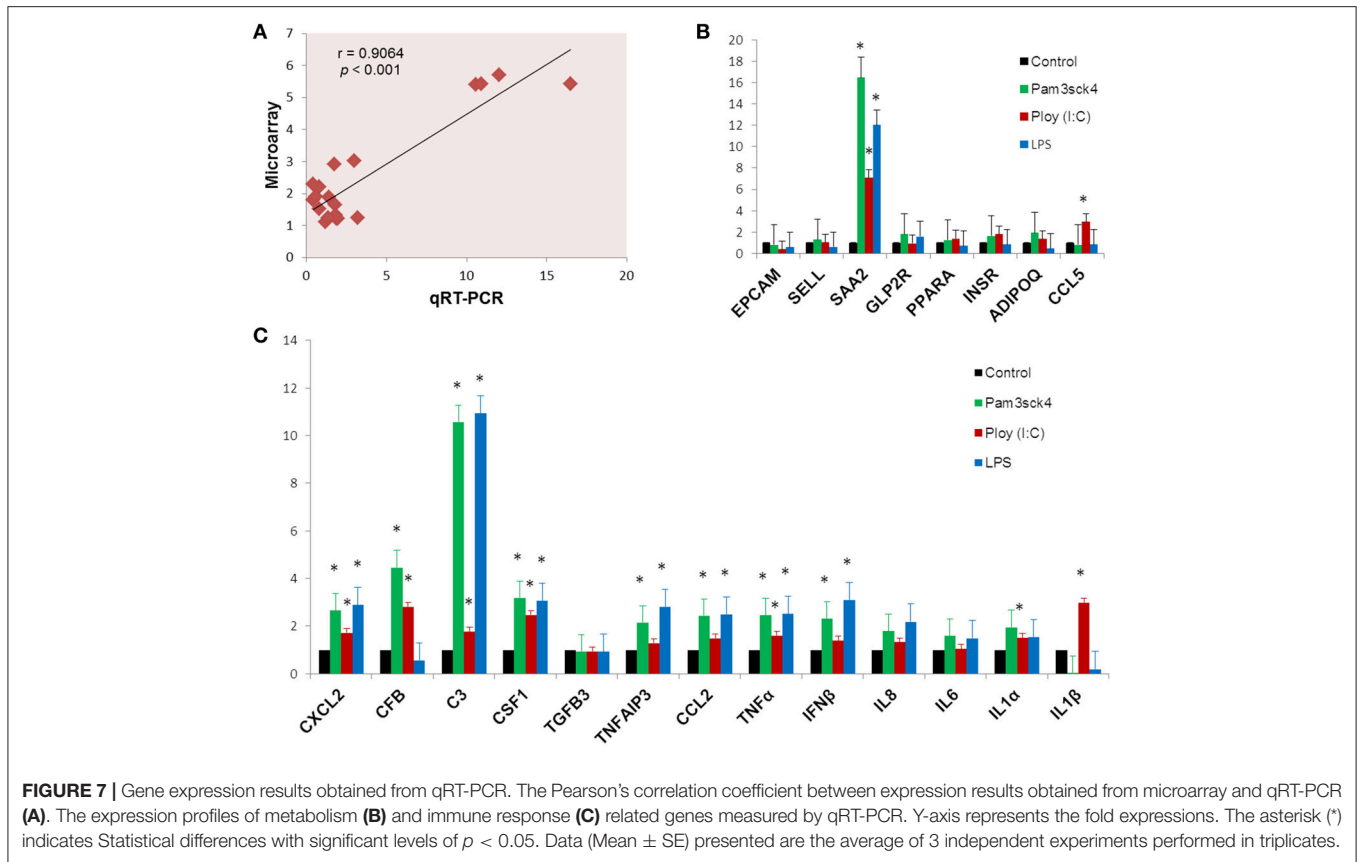
resulted in a shift from anti- to pro-inflammatory cytokines in the adipose tissues transcriptome profile clearly demonstrating the role of adipocytes in the development of immune response (40). To the best of our knowledge, no transcriptional analysis has been performed in porcine adipocytes in response to TLR2 activation. Similar to human and murine adipocytes, we observed that pMA stimulated with Pam3csk showed a major transcriptome alteration, and that the differentially regulated transcripts were known to be involved in the positive regulation of inflammatory responses, including cytokines (*IL1 α* , *IL12B*), chemokines (*CXCL1*, *CXCL2*, *CCL20*, *CSF3*), and adhesion molecules (*SELL*) (Figure 10). This result indicates the ability of porcine adipocytes in participating in immune responses triggered by TLR2 activation.

On the other hand, the LPS from gram-negative bacteria is a well-known innate immune stimulant of TLR4 activation (6). The responsiveness of human adipocytes to LPS stimulation has been widely documented (15, 25); therefore, we speculated that porcine adipocytes would also have the ability to respond to LPS in a similar way. LPS/TLR4 interaction induces signaling pathways through the adaptor molecule MyD88, which results in the activation of transcription factor such as NF- κ B and AP-1 (6, 9, 41). MyD88 recruits different interleukin-1 receptor associated kinase (IRAK) family proteins and TNF receptor-associated factors 6 (TRAF6) (42). This complex activates TGF-activated kinase 1 (TAK1), leading to activation of NF- κ B and mitogen activated protein kinases (MAPKs) (43), which in turn induce the expression of factors participating in inflammatory responses. In agreement, we observed that several signal transduction molecules including members of TNF, MAPK, and NF- κ B pathways were found to be differentially expressed in pMA following the activation of TLR4. Of note, pMA stimulated with LPS showed an up-regulation of *CXCL2* and *CXCL9* that are strongly chemotactic for lymphocytes. Similar to our results, LPS up-regulated the expression of *CCL2* and *CCL8* in human adipocytes (44), and *CCL2* in mouse adipocytes (15). Of note, although microarray results showed down-regulation of *IL8* and no effect on *IL6* expression after LPS stimulation (Figure 5), we checked the expression of both cytokines by qRT-PCR because of their up-regulation is well-reported in LPS responses of human (44), mouse (15), and pig (45) adipocytes. Although it was statistically insignificant, but both *IL6* and *IL8* expression showed an upward trend after LPS stimulation (Figure 7C). In addition, an increase in the expression of *IL18*, which is a cytokine belongs to the *IL1* superfamily and has the ability to stimulate the cellular immune response through the activation of natural killer cells (NK) cells and T cells, was observed after TLR4 activation in pMA (Figure 10). These results are in line with study of Vielma et al. (46) who used fibroblast-derived adipocytes and spleen lymphocytes in order to evaluate whether adipose cells were able to modulate the function of immune cells. The work demonstrated that adipocyte-conditioned medium was able to activate spleen lymphocytes and stimulate their production of the inflammatory cytokines *IL6*, *IL9*, *IFN γ* , and *TNF α* .

Interestingly, TLR3 activation in pMA also led to the development of a complex transcriptomic response. TLR3 ligation lead to the activation of NF- κ B and MAPKs to induce

study based on primary cells of intramuscular preadipocytes obtained from Landrace pigs has reported gene expression changes associated with adipocyte differentiation (38). Among other genes, pattern recognition receptors were differentially expressed when preadipocytes were compared with pMA (38), which was in line with our previous findings (28). Then, we focused our attention in pMA. Here, we confirmed the differentiation of intramuscular preadipocytes cell line obtained from Duroc pigs by the evaluation of fat accumulation, and expression of several marker genes. Thus, adipocytes used for this transcriptome analysis were functionally mature. In addition, in our previous study we demonstrated that members of the TLR family including TLR2, TLR3, and TLR4 focused in the present study are expressed in pMA (28).

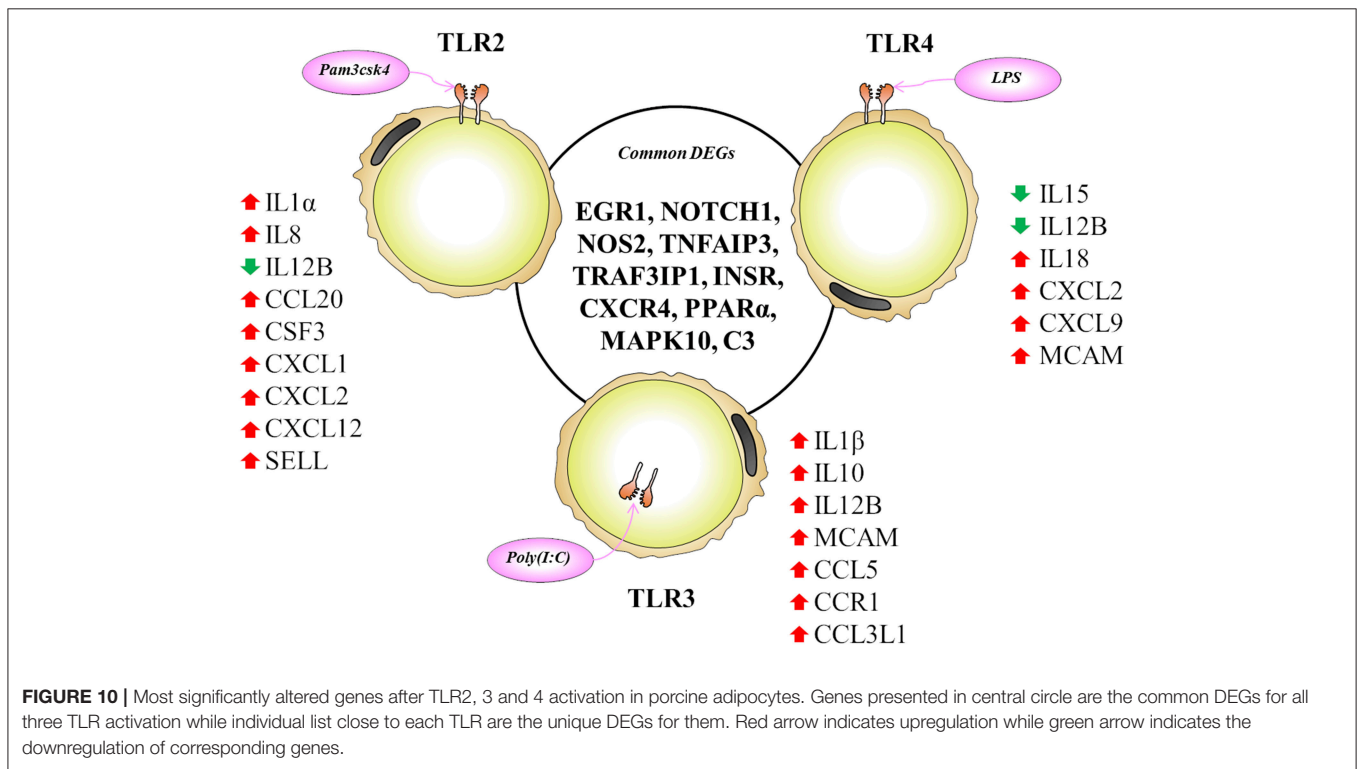
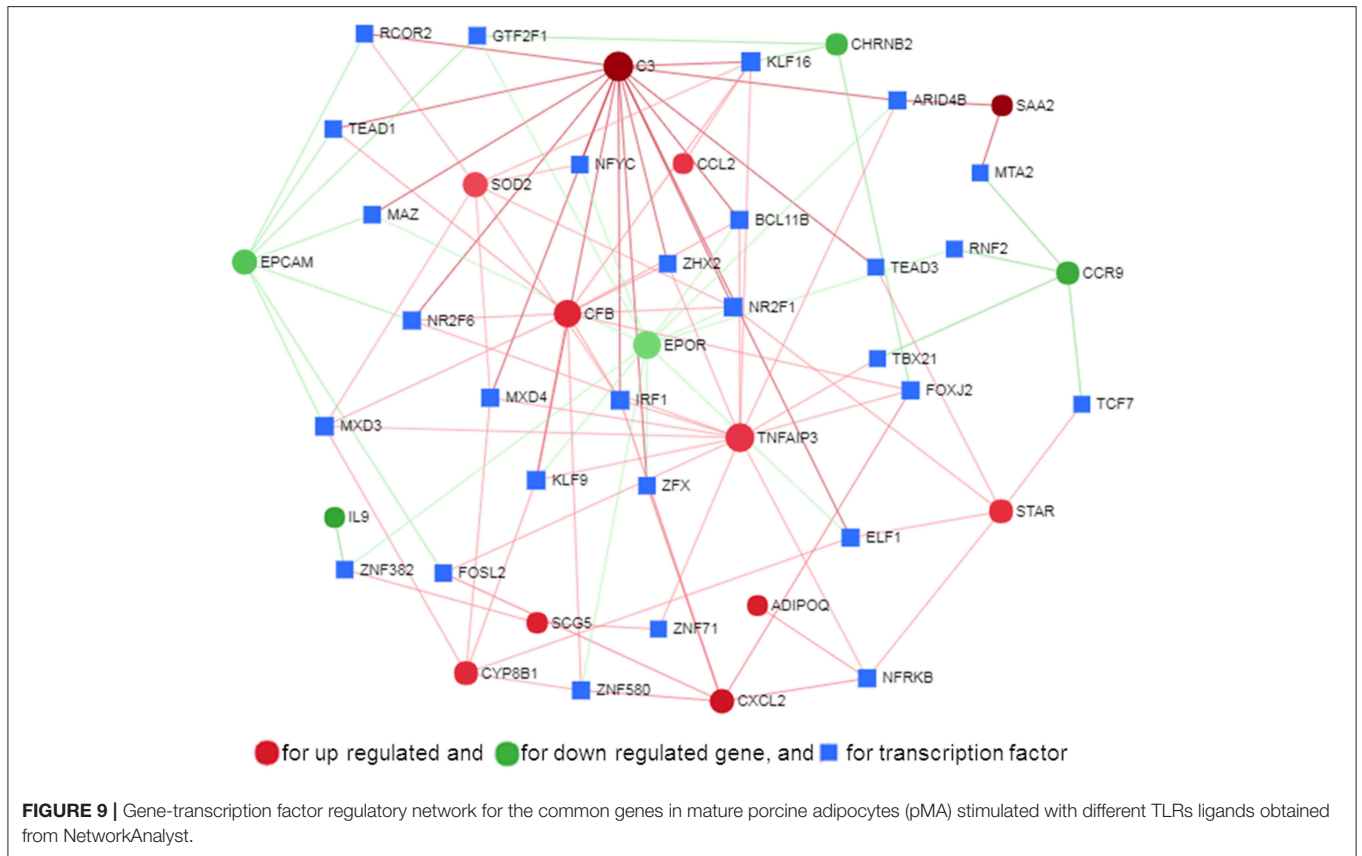
The TLR2 is activated by the lipopeptides/peptidoglycan present in the cell wall of bacteria. It has been reported that human and murine adipocytes express TLR2 (15, 25, 39). Stimulation of adipocytes from humans and mice with the TLR1/2 ligand Pam3Csy or the TLR2/6 ligand MALP-2 differentially modulated the release of the proinflammatory factors including *IL6*, *IL8*, and *CCL2* in those cells (15, 39), whereas resistin was either not affected or even down regulated by both ligands (15). The role of the adipose tissue in the innate immune response induced by local or systemic infection with *Staphylococcus aureus* has been studied *in vivo* in a rodent model (40). The work reported that systemic bacterial infection



and functionally active in human (48) and mouse (49) adipocytes. Stimulation of human and mouse adipocytes with Poly(I:C) is able to induce the expression of pro-inflammatory factors such as *TNF α* , *IL6*, *IL8*, and *CCL2* as well as IFN- α/β and multiple anti-viral proteins including 2'5'-oligoadenylate synthetase and Mx GTPase 1 (48, 49), indicating that adipose cells are able to trigger innate antiviral responses. Here, we observed that TLR3 activation in pMA increased the expression of several inflammatory genes (*CCL2*, *CCL8*, *CCL5*, *CCL3L1*, *IL1 β* , *IL12B*, and *MCAM*) that participate in the antiviral inflammatory responses resembling human and mice adipocytes (Figure 10). However, our transcriptomic analysis was no able to detect increases in the expression of IFN- α/β or IFN-induced antiviral genes. Deeper kinetic and dose-response studies are necessary to establish whether this difference between porcine and human/mice adipocytes are due to differences related to the species or are an effect of the experimental conditions.

Activation of human adipocytes by multiple TLRs ligands was reported to induce pro-inflammatory and pro-diabetic responses through the phosphorylation of extracellular signal-induced kinase and c-Jun N-terminal kinase pathways (25). Human adipocytes have shown the ability to synthesize and secrete complement factors, C1q/TNF-related proteins (CTRPs) (15, 16), cytokines, chemokines, and pro- and anti-inflammatory adipokines including resistin, visfatin, leptin, and adiponectin; as well as antibacterial peptides such as lipocalin-2 and cathelicidin in response to multiple TLRs ligands (25, 50). In addition,

pro-inflammatory cytokines (43), and the phosphorylation and activation of IRF3, leading to IFN γ (42) and type I interferons (47) production. It was described that TLR3 is highly expressed



taking into consideration that in most cases microorganisms carry more than one TLR-ligand and that infections with multiple pathogens, such as virus-bacteria superinfections, have become a common disease scenario; we focused in the transcripts showing differential expression after the activation with the three TLRs in pMA. These commonly differentially regulated genes (**Figure 10**) are involved in two major biological functions: inflammatory response and insulin mediated metabolism, in agreement with previous reports that evaluated TLRs activation in human adipocytes (15, 16, 25, 48). In fact, immune response, cytokine-mediated signaling, inflammatory responses, oxidation-reduction process, positive regulation of ERK1/ERK2 and lipid metabolisms are commonly enriched GO terms in pMA following TLR2, TLR3, or TLR4 activation.

Here we report that *EGR1*, *NOTCH1*, *NOS2*, *TNFAIP3*, *TRAF3IP1*, *INSR*, *CXCR4*, *PPARA*, *MAPK10*, and *C3* are the top 10 (based on fold expression) commonly altered genes of TLRs induced transcriptional modification in pMA (**Figure 10**). It is well-known that *TNFAIP3*, *CXCR4*, *MAPK4*, *MAPK10*, and *IL8* among others are involved in the generation of innate immune responses (2, 6, 8, 9). Other common DEGs like *EGR1* and *INSR* are known to be involved in insulin and glucose metabolism (35). *PPARA* and *NOS2* participate in adipocyte maturation (35, 37). However, the immune response and the lipid metabolism are complex traits, not regulated only by a particular gene but rather by networks of complex molecular interactions (51). Therefore, network analysis based on larger immune-specific and metabolic-specific gene databases is considered to be more effective strategy for identification of regulatory genes (31). The PPI network predicted *RELA*, *HNF4A*, *SPI*, *EPOR*, *C3*, *STAR*, *CCL2*, and *SAA2* as the major regulatory hub genes of TLRs-induced transcriptional network in porcine adipocytes. Interestingly, *C3* is the hub gene that overlapped with top 10 common DEGs. The *C3* is the central component of complement system and plays a vital role in the innate immune response, which was up-regulated in pMA stimulated with TLRs ligands. It was reported that the agonists for TLR2, TLR4, and TLR9 are able to activate the complement-TLR crosstalk, which signals through MyD88 pathway and mediated inflammatory responses (52). We also observed that the hub genes of PPI network also exhibited higher centrality estimates in the gene-centered transcription factor interaction network indicating their potential to regulate the TLR-induced transcriptional modifications in the porcine adipocytes. A long-term goal of our laboratory is to establish an adipocyte-based *in vitro* evaluation system for the selection of beneficial microbes able to differentially modulate immune responses in the adipose tissue for their application in the prevention of immunometabolic diseases in human and animals. We have been successful in a similar approach before by establishing a porcine intestinal epithelial (PIE) cells model for the efficient selection of anti-diarrheal immunomodulatory probiotic bacteria (53).

The physiological roles including metabolic and secretory function of adipocytes are distinguished by their tissue origin, appearance and location of the body (54, 55). The intramuscular adipocytes play pivotal role in the control of systemic energy balance (56) and immuno-metabolic homeostasis (57). However, due to their particular location in close vicinity with muscle

fibers, the biology of intramuscular adipocytes may differ from those originated from other location. Among the hub genes identified in this study, *EPOR*, *STAR*, and *SAA2* are known to be involved in lipid metabolism. Serum amyloid A (SAA) including *SAA2*, have been reported to function in metabolic homeostasis and healthy adipose development through accompanying with retinoic acid, a potential regulator of lipid metabolism (58). In addition, though the lipolysis pathway was not induced after TLRs ligation in intramuscular derived pMA, the intracellular calcium homeostasis and positive regulation of insulin secretion pathways were significantly enriched, which indicates that the TLR-induced transcriptional modification is linked to metabolic changes. These are in line with finding of Gardan et al. (55) who reported that porcine intramuscular adipocytes display a relatively lower lipogenic activity compared with adipocytes isolated from subcutaneous or visceral adipose tissue. Besides the subcutaneous and visceral fat depots, adipogenic differentiation appears to be beneficial in the skin; however, adipogenesis in the skeletal muscle is associated with pathology (36). Therefore, the results obtained in this work indicate that pMA are a useful tool for the *in vitro* study of the transcriptomic changes associated not only with the innate immune response, but also with inflammatory disorders linked to lipid metabolism and hormones resulting from TLRs ligation in porcine adipocytes.

CONCLUSION

This study reported for the first time the global transcriptome modifications in porcine adipocytes following activation of TLR2, TLR3, or TLR4. We demonstrated that the activation of TLRs in porcine mature adipocytes induced modifications of transcripts involved not only in the immune response, but also in cellular lipid metabolism. Sub-network enrichment analysis suggested that *EPOR*, *C3*, *STAR*, *CCL2*, and *SAA2* are the major hub genes of TLRs-mediated transcriptional network responses of porcine adipocytes. In addition, gene-transcription factor interaction network analysis revealed the potentials of hub genes to regulate the TLRs-induced transcriptional modification. Therefore, we identified key regulatory genes that could be used as candidates for the evaluation of immunomodulators (e.g., immunomodulatory probiotic bacteria) having functional beneficial effects (e.g., anti-inflammatory capacity) in porcine adipocytes.

DATA AVAILABILITY

The MIMAE (minimum information about a microarray experiment) standard raw microarray dataset have been submitted to the NCBI-GEO database under the access number GSE124171.

AUTHOR CONTRIBUTIONS

MI, MAI, JV, and HK designed the study. MI, AT, and MT did the cell culture and qRT-PCR experiments, and prepared the microarray samples. KM, KY, and FH arranged the kit, chemicals, and reagents. MAI analyzed the expression data and

wrote the manuscript. LA contributed to data analysis and results interpretation. AK, WI-O, HA, and HT reviewed the manuscript. HK and JV approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01180/full#supplementary-material>

Supplementary Table S1 | Top 40 significantly (adjusted $p < 0.05$) differentially expressed genes in pMA after Pam3csk stimulation (.xlsx).

Supplementary Table S2 | Top 40 significantly (adjusted $p < 0.05$) differentially expressed genes in pMA after Poly(I:C) stimulation (.xlsx).

Supplementary Table S3 | Top 40 significantly (adjusted $p < 0.05$) differentially expressed genes in pMA after LPS stimulation (.xlsx).

Supplementary Table S4 | GO terms and pathways enriched by TLR ligand-induced DEGs in adipocytes obtained from DAVID analysis (.xlsx).

Supplementary Table S5 | Centrality estimates of protein interaction network of TLR ligand-induced DEGs in adipocytes obtained from NetworkAnalyst (.xlsx).

Supplementary Table S6 | Centrality estimates of Gene-transcription factor interaction network of TLR ligand-induced DEGs in adipocytes obtained from NetworkAnalyst (.xlsx).

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Conflict of Interest Statement: KM, KY, and FH are employed by Takanashi Milk Products Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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