



C1q and TNF related protein 1 regulates expression of inflammatory genes in vascular smooth muscle cells

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Abstract

Background C1q and TNF related protein 1 (C1QTNF1) is known to be associated with coronary artery diseases. However, the molecular function of C1QTNF1 on the vascular smooth muscles remains to be investigated.

Objective This study was therefore undertaken to investigate the effect of C1QTNF1 on gene expression of human smooth muscle cells and to reveal potential molecular mechanisms mediated by C1QTNF1.

Methods Vascular smooth muscle cells were incubated with recombinant C1QTNF1 for 16 h, followed by determining any change in mRNA expressions by Affymetrix genechip. Gene ontology (GO), KEGG pathway, and protein–protein interaction (PPI) network were analyzed in differentially expressed genes. In addition, validation of microarray data was performed using quantitative real-time PCR.

Results The mRNA expressions of annotated 74 genes were significantly altered after incubation with recombinant C1QTNF1; 41 genes were up-regulated and 33 down-regulated. The differentially expressed genes were enriched in biological processes and KEGG pathways associated with inflammatory responses. In the PPI network analysis, IL-6, CCL2, and ICAM1 were identified as potential key genes with relatively high degree. The cluster analysis in the PPI network identified a significant module composed of upregulated genes, such as IL-6, CCL2, NFKBIA, SOD2, and ICAM1. The quantitative real-time PCR results of potential key genes were consistent with microarray data.

Conclusion The results in the present study provide insights on the effects of C1QTNF1 on gene expression of smooth muscle cells. We believe our findings will help to elucidate the molecular mechanisms regarding the functions of C1QTNF1 on smooth muscle cells in inflammatory diseases.

Keywords CTRP1 · C1QTNF1 · Gene expression · Regulation · Microarray

Introduction

C1q and TNF related protein 1 (C1QTNF1) is an adipokine of the C1q/TNF family, characterized by variable collagen-like region and conserved C-terminal globular domain (Wong et al. 2008). It was initially identified as a vascular wall protein that regulates collagen-induced platelet activation and aggregation (Lasser et al. 2006). C1QTNF1 is

involved in diverse processes under various physiological and pathological conditions. C1QTNF1 stimulates aldosterone production in the zona glomerulosa of the adrenal cortex (Jeon et al. 2008). C1QTNF1 increases fatty acid oxidation by activating the AMP-activated protein kinase (Peterson et al. 2012). It is involved in chondrocyte proliferation and maturation through the activation of ERK1/2 signaling pathway (Akiyama et al. 2013). Recently, C1QTNF1 was found to exert anti-apoptotic and anti-inflammatory responses through the S1P and cAMP signaling pathways (Yuasa et al. 2016). C1QTNF1 is expressed in primary macrophages and is responsible for the induction of proatherogenic factors (Wang et al. 2016b).

Accumulating evidences suggest that C1QTNF1 is associated with coronary artery diseases such as atherosclerosis. C1QTNF1 expression are increased in atherosclerosis plaque, and recombinant C1QTNF1 promotes atherogenesis

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in ApoE-knockout mice (Lu et al. 2016). Serum levels of C1QTNF1 are also increased in coronary artery disease patients (Tang et al. 2015; Wang et al. 2016a; Yuasa et al. 2014). C1QTNF1 is upregulated in macrophages in response to oxidized LDL in a PPAR- γ -dependent manner, and enhances the secretion of proatherogenic factors (Wang et al. 2016b). A recent study has reported it to be a mechano-sensitive proinflammatory factor that promotes endothelial cell dysfunction under disturbed flow (Liu et al. 2017). Conversely, C1QTNF1 prevents the progression of pathological vascular remodeling after artery injury (Kanemura et al. 2017). Thus, the molecular mechanism by which C1QTNF1 affects atherogenesis remains uncertain and needs to be further investigated.

Vascular smooth muscle cells play important roles in the initiation and progression of atherosclerosis through production of extracellular matrix proteins, cell surface receptors for lipid uptake, cell adhesion molecules, and cytokines (Braun et al. 1999; Falk 2006; Raines and Ferri 2005; Rong et al. 2003). In the present study, we investigated changes in gene expression of smooth muscle cells regulated by C1QTNF1. Our results indicate that C1QTNF1 regulates the expression of inflammatory genes such as interleukin-6 (*IL6*), C–C chemokine 2 [*CCL2*, as referred in Monocyte chemoattractant protein 1 (*MCP-1*)], and intercellular adhesion molecule 1 (*ICAM1*). Our findings therefore provide information on the effects of C1qTNF1 in smooth muscle cell function.

Materials and methods

Cell culture

Human aortic smooth muscle cells (CC-2571) were purchased from Ronza and grown in SmGM-2 smooth muscle growth medium (Ronza) supplemented with 5% fetal bovine serum (FBS). 293FT cells were purchased from Invitrogen and maintained in DMEM supplemented with 10% FBS and antibiotics (100 units/ml of penicillin and 100 μ g/ml of streptomycin).

Purification of recombinant C1QTNF1

The DNA fragments corresponding to the coding sequences of human C1QTNF1 and C1QTNF5 genes were amplified by polymerase chain reaction (PCR) using human spleen cDNA (Clontech) with the following primers: C1QTNF1 (forward), 5'-AAA AGG ATC CCG GTG CAA GAG CCA CTA C-3'; C1QTNF1 (reverse), 5'-AAA ACT CGA GGG GCT CGG TGG CGT GCT T-3'; C1QTNF5 (forward), 5'-AAA AGA ATT CAC TGC GAG AGG ACC CCG-3'; C1QTNF5 (reverse), 5'-AAA ACT CGA GAG CAA AGA

CTG GGG AGC TG-3'. The PCR products for C1QTNF1 and C1QTNF5 were cloned into the *Bam*HI/*Xho*I and *Eco*RI/*Xho*I restriction sites of the pcDNA3.1 vector with FLAG tag, respectively. Using Lipofectamine 2000 (Invitrogen), 293FT cells plated in 100-mm dishes were transfected with the plasmid encoding human C1QTNF1 or C1QTNF5, in accordance with manufacturer's instruction. After 24 h, the medium was refreshed with growth medium (DMEM supplemented with 10% FBS) and incubated for further 48 h, after which the culture medium was collected and secreted C1QTNF1 or C1QTNF5 proteins were isolated using anti-FLAG M2 affinity gel (Sigma).

Affymetrix cDNA microarray

Vascular smooth muscle cells were seeded in a 6-well dish at a density of 1×10^6 cells/well. After 24 h, the cells were incubated with 5 μ g/ml recombinant C1QTNF1 proteins or vehicle for 16 h, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.5% FBS. Total RNAs isolated from three independent experiments using the RNeasy mini kit (Qiagen) were pooled and used for gene expression analysis by GeneChip human gene 1.0 ST arrays (Affymetrix). Briefly, 300 ng of total RNA was mixed with T7-(N)6 Primers/Poly-A RNA control solutions. The synthesis of first-cycle, first and second strand cDNA was performed using the GeneChip™ WT cDNA synthesis kit. The first-cycle cRNA was synthesized using the GeneChip WT cDNA amplification kit and the GeneChip sample cleanup module. The second-cycle, single-strand cDNA was synthesized using the GeneChip WT cDNA synthesis kit and was purified using the GeneChip sample cleanup module. Fragmentation and labeling of single strand cDNA were performed using the GeneChip WT terminal labeling kit. Sample hybridization, washing, and staining were performed using the GeneChip hybridization, wash and stain kit. The samples were washed and stained on the GeneChip Fluidics Station 450, and arrays were scanned using the GeneChip Scanner 3000 7G controlled by the GeneChip Operating Software (GCOS, Affymetrix Inc.).

Data processing

The microarray scan data were analyzed using the GeneChip operating software, expression console software (Affymetrix Inc., USA), and GenPlex v3.0 (ISTECH, Inc., Korea). To compare the results of different hybridization experiments, the signal intensity of each gene in an array was normalized with respect to the total intensity of all genes in the array. The corresponding normalized signals of different arrays were compared. Genes with relative gene expression changes of > 1.5-fold and normalized signal (\log_2) of > 4 were considered to represent up- or down-regulation.

Gene ontology (GO) analysis

Gene ontology (GO) analysis was conducted for differentially expressed genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8), an online analysis resource (<http://david.abcc.ncifcrf.gov/>). The GO terms were divided into three categories, such as biological process (BP), cellular component (CC), and molecular function (MF). The GO terms with P value < 0.05 were considered as significant. The GO terms for differentially expressed genes were ranked by the $-\log(P \text{ value})$.

KEGG pathway analysis

The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway was analyzed using DAVID Bioinformatics Resources 6.8. The pathways with number of different genes ≥ 2 and P value < 0.05 were considered as significant.

Construction of protein–protein interaction (PPI) network

To evaluate the interactive relationships for differentially expressed genes, the PPI network was constructed using the online Search Tool for the Retrieval of Interacting Genes database (STRING, version 10.5) (<http://string-db.org>) (Szkarczyk et al. 2015). The interactions with a confidence score > 0.4 (default setting) were selected as significant. The analysis result of PPI network from STRING was imported into Cytoscape software (version 3.6.1; <http://www.cytoscape.org>) (Shannon et al. 2003). The Network Analyzer

plug-in of Cytoscape was used to investigate the topological properties of PPI network, including node degree, clustering coefficient, and closeness centrality. The nodes with high degree score was selected as important genes (named ‘hub genes’). To detect densely connected regions in the PPI network, the clustering in the PPI network was analyzed by the Molecular Complex Detection (MCODE) plug-in of Cytoscape with default setting (a degree cut-off = 2, node score cut-off = 0.2, k-core = 2, and max. depth = 100).

Quantitative real-time PCR

cDNA synthesis was performed in a final volume of 30 μl , comprising of 2 μg of total RNA, 200 ng of oligo(dT)₁₅ primer, 1 \times reverse transcription buffer, 0.5 mM deoxynucleotide triphosphate (dNTP) mixture, 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen), and diethylpyrocarbonate (DEPC)-treated water. After incubation at 42 °C for 50 min, reverse transcription was terminated by heating at 70 °C for 15 min. To verify changes of gene expression by genechip analysis, the cDNA was diluted fivefold, and used to amplify target genes by a LightCycler 480 (Roche Applied Science) using SYBR green master mix (Roche Applied Science), with beta-actin (ACTB) as the reference gene. Real-time PCR amplification was carried out as follows: initial denaturation at 95 °C for 5 min, 45 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s), 1 cycle of melting curves (95 °C for 5 s, 65 °C for 1 min, and 97 °C continuous) followed by a final cooling step at 40 °C for 30 s. The primers used in this study are presented in Table 1. qRT-PCR results were analyzed using

Table 1 Primers used for quantitative real-time PCR

Identity	Nucleotide sequences	Product size (bp)
IL-6	Forward: 5'-TAGTGAGGAACAAGCCAGAG-3' Reverse: 5'-ATGCTACATTTGCCGAAGAG-3'	238
C3	Forward: 5'-GAAGAACGGCTGGACAAGGC-3' Reverse: 5'-CCAGAAATCGGAGGAGAGAC-3'	249
SOD2	Forward: 5'-GCACCACAGCAAGCACCACG-3' Reverse: 5'-GAGCCTTGGACACCAACAGATG-3'	288
CCL2	Forward: 5'-CTTCTGTGCCTGCTGCTCATAG-3' Reverse: 5'-CTTCTTTGGGACACTTGCTG-3'	169
ICAM1	Forward: 5'-TGTCATCATCACTGTGGTTTC-3' Reverse: 5'-GTGGCTTGTGTGTTCCGGTTTC-3'	147
NFKBIA	Forward: 5'-GACCTGGTGTCACTCCTGTTG-3' Reverse: 5'-TCGTCTCTGTGAACTCCGTG-3'	215
TNFAIP3	Forward: 5'-CAAAAGGACTACAGTAGAGGC-3' Reverse: 5'-AATGAGGCAGCAGCAAAATG-3'	296
CCL4	Forward: 5'-GCTTCCTCGCAACTTTGTGGTAG-3' Reverse: 5'-GGTCATACACGTACTCCTGGAC-3'	140
β -Actin	Forward: 5'-TCACCCACACTGTGCCCATCTACGA-3' Reverse: 5'-GGATGCCACAGGACTCCATGCCCA-3'	351

the comparative cycle threshold (C_T) method, as previously described (Park et al. 2005). Briefly, relative expressions for any given gene (expressed as fold induction over the control) were calculated by determining the difference between the C_T of the target gene and the C_T of the reference gene (ACTB) using the formula $2^{-\Delta\Delta C_T}$.

Statistical analysis

All statistical analyses were carried out using the SPSS software (v23). Comparisons between two groups were performed using the Mann–Whitney U test (for non-normally distributed data). Results are expressed as means \pm s.d. P values < 0.05 are considered statistically significant.

Results

C1QTNF1 regulates gene expression in vascular smooth muscle cells

Vascular smooth muscle cells play important roles in the initiation and progression of atherosclerosis through production of extracellular matrix proteins, cell surface receptors for lipid uptake, cell adhesion molecules, and cytokines (Braun et al. 1999; Falk 2006; Raines and Ferri 2005; Rong et al. 2003). To assess the effect of C1QTNF1 on human vascular smooth muscle cells, recombinant human C1QTNF1 proteins were isolated from the supernatant of 293FT cell culture transfected with plasmid encoding human C1QTNF1 (Fig. 1a). C1QTNF1 expression is shown to be increased in atherosclerosis plaque (Lu et al. 2016) and in sera of coronary artery disease patients (Tang et al. 2015; Wang et al. 2016a; Yuasa et al. 2014), suggesting that C1QTNF1 might have a prolonged effect on progression of coronary artery

diseases. Thus, we incubated vascular smooth muscle cells in the presence of the recombinant human C1QTNF1 protein (5 $\mu\text{g/ml}$) or vehicle for 16 h and analyzed gene expression changes using an Affymetrix genechip. The results revealed that annotated 74 genes and 11 pseudogenes were significantly regulated by C1qTNF1: 41 and 33 genes were up-regulated or down-regulated, respectively, in response to recombinant C1QTNF1 protein, barring the 11 pseudogenes. The majority of differentially expressed genes exhibited less than twofold changes, whereas 15 genes (4 up and 11 down) are differentially regulated by more than twofold (Fig. 1b). All the up- and down-regulated genes (> 1.5 -fold) are presented in Supplementary Table 1. Expression level of C1QTNF1 gene was not changed by treatment of recombinant C1QTNF1 (fold change: 1.015).

Genes differentially regulated by C1QTNF1 are enriched in biological processes and pathways related to inflammatory responses

The differentially expressed 74 genes were assigned into BP, CC, and MF categories based on GO annotations. In the category of BP, genes differentially regulated by C1QTNF1 were enriched in 21 GO terms. According to P value, top 5 GO terms were cellular response to interleukin-1 (GO:0071347), cellular response to amino acid (GO:0043200), cellular response to tumor necrosis factor (GO:0071356), cellular response to lipopolysaccharide (GO:0071222), and inflammatory response (GO:0006954) (Fig. 2a). This result indicates that differentially expressed genes were enriched in GO terms associated with inflammation. Genes involved in these GO terms are for IL6, CCL2, chemokine (C–C motif) ligand 4 (CCL4), ICAM1, Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3, as also referred in A20), Complement component 3 (C3), and Rac

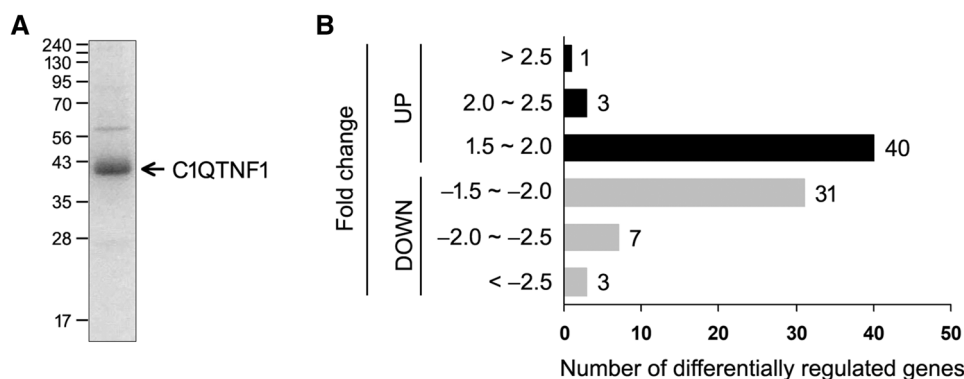


Fig. 1 C1QTNF1 regulates gene expression in smooth muscle cells. **a** Recombinant C1QTNF1 protein was subjected to SDS-PAGE and stained with Coomassie blue. Arrow indicates C1QTNF1 protein. **b** Vascular smooth muscle cells were incubated with 5 $\mu\text{g/ml}$ recom-

binant C1QTNF1 proteins for 16 h, and global gene expression was analyzed by Affymetrix genechip. Distribution of the fold changes shown by genes regulated by C1QTNF1 is presented

Fig. 2 Genes differentially regulated by C1QTNF1 are enriched in biological processes associated with inflammatory responses. The 74 annotated genes regulated by C1QTNF1 were assigned into biological process (a), cellular component (b), and molecular function (c) categories based on gene ontology annotations. *BP* biological process, *CC* cellular component, *MF* molecular function

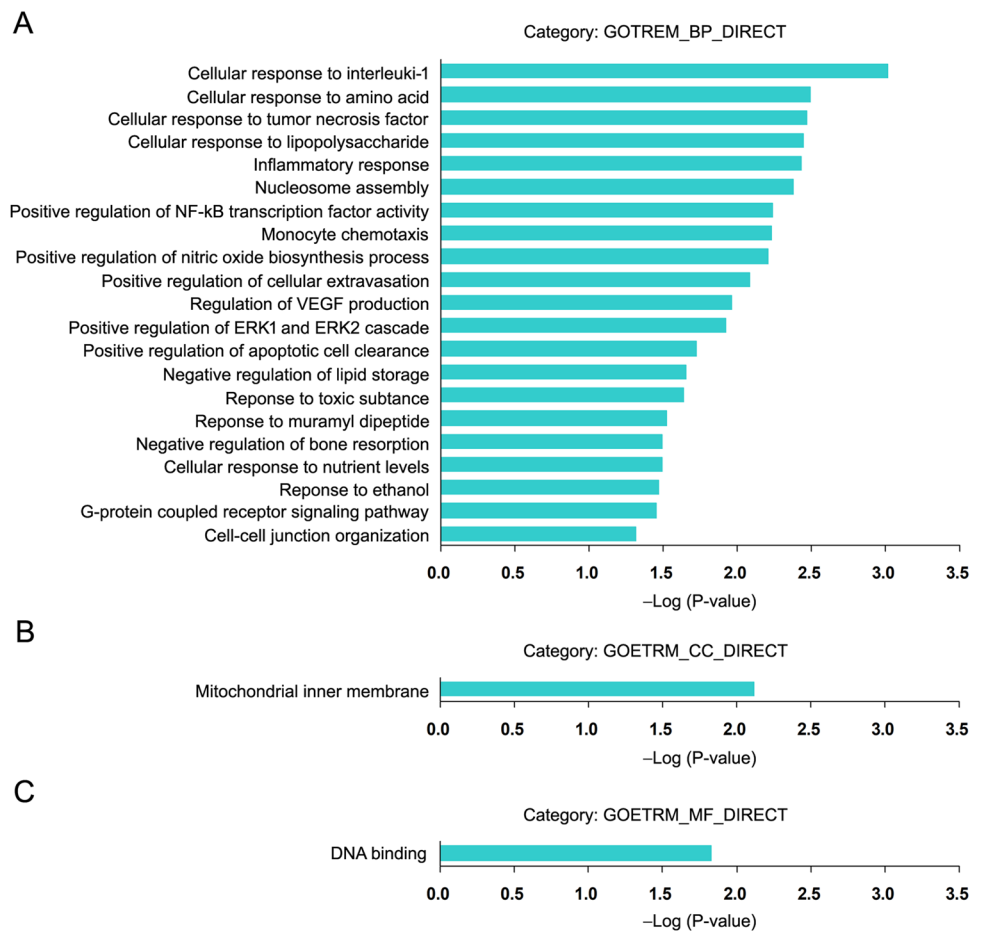


Table 2 DEGs included in top 5 GO terms (biological process)

Symbol	Gene name	Regulation	Fold
IL6	Interleukin-6	Up	2.08
C3	Complement component 3	Up	1.82
RAC1	Rac family small GTPase 1	Up	1.78
CCL2	Chemokine (C–C motif) ligand 2	Up	1.72
ICAM1	Intercellular adhesion molecule 1	Up	1.69
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	Up	1.52
CCL4	Chemokine (C–C motif) ligand 4	Down	– 2.41

family small GTPase 1 (RAC1) (Table 2; Supplementary Fig. 1). Of these, IL6, CCL2, ICAM1, TNFAIP3, C3, and RAC1 were upregulated, whereas CCL4 was downregulated. In the category of CC and MF, mitochondrial inner membrane (GO:0005743) and DNA binding (GO:0003677) were GO terms enriched in differentially expressed genes, respectively (Fig. 2b, c). To further analyze the function of differentially expressed genes, we analyzed the Kyoto encyclopedia of genes and genome (KEGG) pathways using the DAVID online tool (<http://david.abcc.ncifcrf.gov/>). As

shown in Table 3, differentially expressed genes were closely associated with pathways related to inflammatory responses, including TNF signaling, NOD-like receptor signaling, NFκB signaling, and toll-like receptor signaling pathways, suggesting that C1QTNF1 may modulate the functions of vascular smooth muscle cells under inflammatory diseases.

CCL2, IL6, and ICAM1 are key genes regulated by C1QTNF1

We constructed PPI network for differentially expressed genes using the STRING online tool. The network consisted of 57 nodes (proteins produced by DEGs) and 25 edges (protein–protein associations) with an average local clustering coefficient of 0.219, a PPI enrichment P value of 0.0416 and an average node degree of 0.877 (Fig. 3). In PPI network, the degree of a node represents the number of protein linked with the node. The nodes with high degree are known to play an important role in maintaining the PPI network. DEGs encoding nodes with a high degree are considered as important genes in the network, named ‘hub genes’. The nodes with degree > 5 were IL6 (degree 8), ICAM1 (degree 7), and CCL2 (degree 6), indicating that these genes are key

Table 3 The pathway analysis of genes differentially regulated by CTPR1 ($P < 0.05$)

KEGG pathway	Genes	P value
TNF signaling pathway	CCL2, IL6, ICAM1, NFKBIA, TNFAIP3	4.4×10^{-4}
Legionellosis	IL6, C3, HSPA6, NFKBIA	8.1×10^{-4}
NOD-like receptor signaling pathway	CCL2, IL6, NFKBIA, TNFAIP3	8.6×10^{-4}
Influenza A	CCL2, IL6, ICAM1, HSPA6, NFKBIA	2.8×10^{-3}
NF-kappa B signaling pathway	NFKBIA, TNFAIP3, ICAM1, CCL4	3.2×10^{-3}
Chagas disease	CCL2, IL6, C3, NFKBIA	5.3×10^{-3}
Toll-like receptor signaling pathway	IL6, NFKBIA, RAC1, CCL4	5.6×10^{-3}
Measles	IL6, NFKBIA, TNFAIP3, HSPA6	1.0×10^{-2}
Malaria	CCL2, IL-6, ICAM1	1.2×10^{-2}
Non-alcoholic fatty liver disease	NDUFAB1, COX7B, IL6, RAC1	1.5×10^{-2}
Cytosolic DNA-sensing pathway	IL6, NFKBIA, CCL4	2.0×10^{-2}
Herpes simplex infection	CCL2, IL6, C3, NFKBIA	2.5×10^{-2}
Chemokine signalling pathway	CCL2, NFKBIA, RAC1, CCL4	2.6×10^{-2}
Epstein–Barr virus infection	ICAM1, NFKBIA, TNFAIP3, HSPA6	2.7×10^{-2}
Virual carcinogenesis	H2BFM, NFKBIA, C2, RAC1	3.3×10^{-2}
Salmonella infection	CCL2, IL6, ICAM1	3.3×10^{-2}
Rheumatoid arthritis	CCL2, IL6, ICAM1	3.7×10^{-2}

genes in the PPI network regulated by C1QTNF1. Next, we performed module analysis in the PPI network using the MCODE plug-in of Cytoscape and found a module composed of 5 nodes and 10 edges (Fig. 4). The genes included in this module were CCL2, IL6, NF κ B inhibitor alpha (NFKBIA), superoxide dismutase 2 (SOD2) and ICAM1, which were upregulated in response to C1QTNF1. The most associated KEGG pathway with these genes is TNF signaling pathway.

Validation of differentially expressed genes by quantitative real-time PCR

To confirm the genes differentially regulated by recombinant C1QTNF1 protein, we selected eight candidate genes among genes involved in top five GO terms and in a significant module of the PPI network. Expression of these genes was analyzed by quantitative real-time PCR. In agreement with our microarray results, IL-6, C3, SOD2, CCL2, ICAM1, NFKBIA, and TNFAIP3 were up-regulated, and CCL4 was down-regulated (Fig. 5). Furthermore, fold changes of these genes as determined by microarray and quantitative real-time PCR were similar (Table 4). To address the possibility that increased expression of inflammatory genes is due to non-specific effects derived from recombinant protein, we generated recombinant human C1QTNF5 protein and examined the effect of C1QTNF5 on expression of IL-6 and CCL2 which are representative proatherogenic genes regulated by C1QTNF1. A significant change in mRNA expression of these genes was not observed in vascular smooth muscle cells treated with C1QTNF5 protein (Supplementary Fig. 2).

Discussion

C1QTNF1 is a relatively uncharacterized adipokine and associated with coronary artery diseases, such as atherosclerosis (Lu et al. 2016; Tang et al. 2015; Wang et al. 2016a; Yuasa et al. 2014). However, the effect of C1QTNF1 on functions of smooth muscle cells has not been determined. Microarray analysis is a powerful tool for examining changes in gene expression in response to specific stimuli. In this study, we generated recombinant human C1QTNF1 proteins from mammalian cells and investigated the effect of C1qTNF1 on gene expression of human vascular smooth muscle cells using the Affymetrix genechip.

GO enrichment and KEGG pathway analysis from differentially expressed genes revealed that genes regulated by C1QTNF1 are enriched in BP and pathway associated with inflammatory responses. Using the PPI network analysis, IL6, CCL2, and ICAM1 were identified as potential key genes regulated by C1QTNF1. These genes were upregulated by C1QTNF1, and their expression was verified by quantitative real-time PCR. Of them, IL-6 and CCL2 are known as proatherogenic factors that contribute to pathogenesis of atherosclerosis. IL-6 is a proinflammatory cytokine expressed in atherosclerotic plaques from coronary artery disease patients and hyperlipidemic animals (Ikeda et al. 1992; Lu et al. 2016) and promotes migration and proliferation of vascular smooth muscle cells (Lee et al. 2012; Morimoto et al. 1991). Likewise, treatment of ApoE-deficient mice with recombinant IL-6 and high-fat diet markedly increased atherosclerotic lesions (Lu et al. 2016). CCL2 is a C–C chemokine having potent chemotactic activity toward monocytes (Rollins 1991). It is reported to accelerate

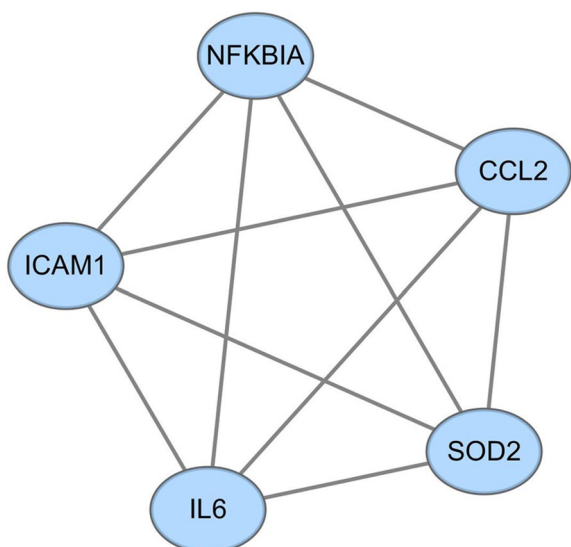


Fig. 4 A module from protein–protein interaction (PPI) network. The module analysis was conducted by the MCODE plug-in of Cytoscape. The MCODE score of the module is 5.0

On the other hand, in the present study, several genes involved in the negative regulation of NF κ B signaling pathway, including NFKBIA and TNFAIP3, were also differentially upregulated. NFKBIA (as referred in I κ B α) inhibits NF κ B activation by sequestering it in the cytosol (Baeuerle and Baltimore 1996) and exerts a proapoptotic activity in most cells (Luo et al. 2005). TNFAIP3 is a potent anti-inflammatory protein that inhibits NF κ B signaling. In smooth muscle cells, TNFAIP3 was found to exert anti-inflammatory and anti-proliferative effects, leading to prevention of neointimal hyperplasia (Patel et al. 2006). Furthermore, in the present study, C1QTNF1 also downregulated the expression of the gene required to sustain inflammatory responses, namely CCL4 [as referred in Macrophage inflammatory protein-1 beta (MIP- β)], which is otherwise upregulated in vulnerable atherosclerotic plaques (Reape and Groot 1999). CCL4 induces cell adhesion to endothelial cells through production of reactive oxygen species (Tatara et al. 2009). Thus, the differential regulation of inflammation-related genes appears to exert pro-inflammatory or anti-inflammatory effects, and thus, their effects may be dependent on the stage of the inflammatory diseases.

In this study, we analyzed genes regulated by C1QTNF1 and their PPI network using integration of microarray with

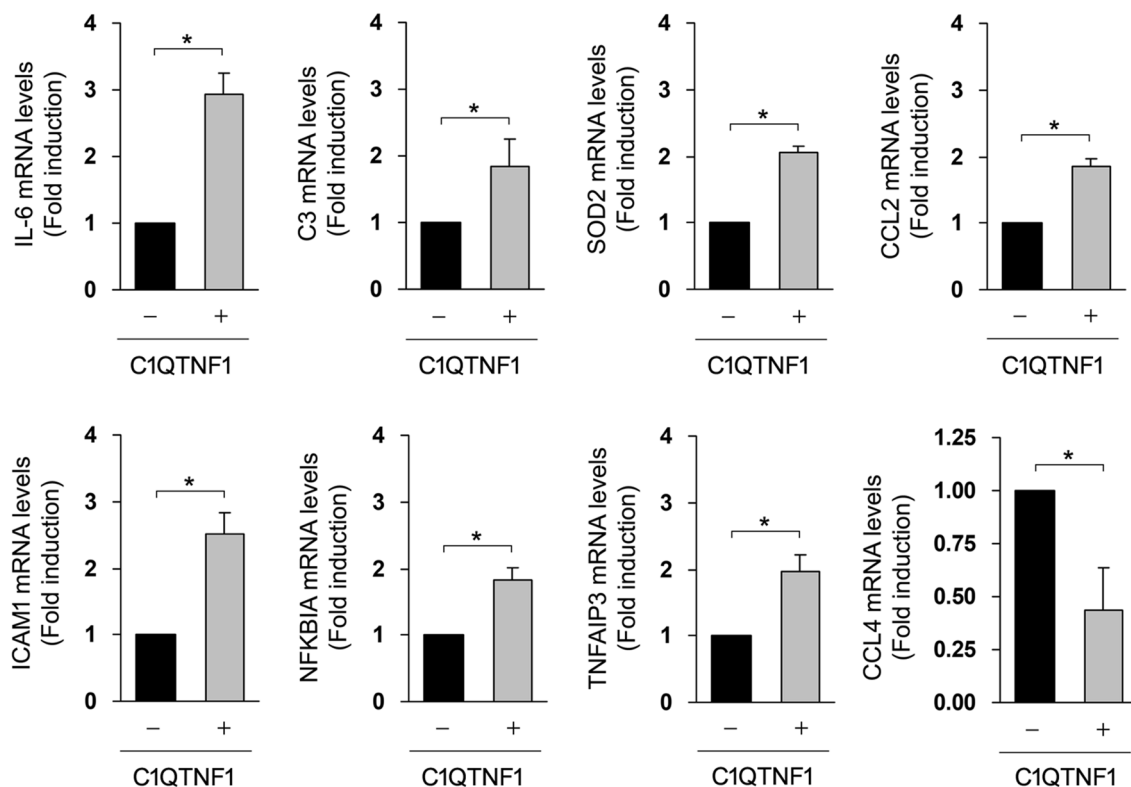


Fig. 5 Quantitative determination of transcript levels by real-time PCR. Human vascular smooth muscle cells were incubated with 5 μ g/ml recombinant C1QTNF1 for 16 h. Expression levels of eight genes

(IL-6, C3, SOD2, CCL2, ICAM1, NFKBIA, TNFAIP3, and CCL4) were examined by quantitative real-time PCR. Results represent the means \pm s.d. (n=4). Mann–Whitney *U* test: **P*<0.05

Table 4 Verification of microarray data by quantitative real-time PCR

Symbol	Description	Fold (genechip)	Fold (qPCR)
IL6	Interleukin-6	2.08	2.93 ± 0.31
C3	Complement component 3	1.82	1.84 ± 0.41
SOD2	Superoxide dismutase 2	1.77	2.05 ± 0.10
CCL2	Chemokine (C–C motif) ligand 2	1.72	1.86 ± 0.11
ICAM1	Intercellular adhesion molecule 1	1.69	2.52 ± 0.31
NFKBIA	Nuclear factor of kappa B inhibitor, alpha	1.63	1.82 ± 0.18
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	1.52	1.66 ± 0.30
CCL4	Chemokine (C–C motif) ligand 4	–2.41	–2.28 ± 1.05

bioinformatics tools and identified important genes for C1QTNF1-mediated cellular responses. However, PPI network did not coverage all genes represented by the array. Furthermore, DEGs from microarray contained many unannotated genes and RNAs. This could result in the loss of information. Thus, characterization of these unannotated genes may be necessary to acquire more information.

The present study provides insights on the effects of C1QTNF1 on gene expression of smooth muscle cells. GO enrichment, KEGG pathway, and PPI network analysis suggest that IL6, CCL2, and ICAM1 are key genes regulated by C1QTNF1. Furthermore, we believe our findings will help to elucidate the molecular mechanisms regarding the effects of C1QTNF1 on smooth muscle cells in coronary artery diseases.

Author contributions Conceptualization: S-YP. Methodology: DK, S-YP. Formal analysis: DK, S-YP. Data curation: DK, S-YP. Investigation: DK, S-YP. Writing-original draft: S-YP. Wright-review and editing: S-YP. Approval of final manuscript: all authors.

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Compliance with ethical standards

Conflict of interest The authors have no potential conflicts of interest to disclose.

References

- Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM (1999) Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 19:1518–1525
- Akiyama H, Otani M, Sato S, Toyosawa S, Furukawa S, Wakisaka S, Maeda T (2013) A novel adipokine C1q/TNF-related protein 1 (CTRP1) regulates chondrocyte proliferation and maturation through the ERK1/2 signaling pathway. *Mol Cell Endocrinol* 369:63–71. <https://doi.org/10.1016/j.mce.2013.01.002>
- Bauerle PA, Baltimore D (1996) NF-kappa B: ten years after. *Cell* 87:13–20
- Braun M, Pietsch P, Schror K, Baumann G, Felix SB (1999) Cellular adhesion molecules on vascular smooth muscle cells. *Cardiovasc Res* 41:395–401
- Cheng X, Wang Y, Chen H, Xu Y, Xiong W, Wang T (2017) Claudin-1 regulates pulmonary artery smooth muscle cell proliferation through the activation of ERK1/2. *Biomed Pharmacother* 89:983–990. <https://doi.org/10.1016/j.biopha.2017.02.063>
- Falk E (2006) Pathogenesis of atherosclerosis. *J Am Coll Cardiol* 47:C7–C12. <https://doi.org/10.1016/j.jacc.2005.09.068>
- Ikeda U, Ikeda M, Seino Y, Takahashi M, Kano S, Shimada K (1992) Interleukin 6 gene transcripts are expressed in atherosclerotic lesions of genetically hyperlipidemic rabbits. *Atherosclerosis* 92:213–218
- Inoue S et al (2002) Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 106:2700–2706
- Jeon JH et al (2008) A novel adipokine CTRP1 stimulates aldosterone production. *FASEB J* 22:1502–1511. <https://doi.org/10.1096/fj.07-9412com>
- Kanemura N et al (2017) C1q/TNF-related protein 1 prevents neointimal formation after arterial injury. *Atherosclerosis* 257:138–145. <https://doi.org/10.1016/j.atherosclerosis.2017.01.014>
- Lasser G et al (2006) C1q/TNF-related protein-1 (CTRP-1): a vascular wall protein that inhibits collagen-induced platelet aggregation by blocking VWF binding to collagen. *Blood* 107:423–430. <https://doi.org/10.1182/blood-2005-04-1425>
- Lee GL, Chang YW, Wu JY, Wu ML, Wu KK, Yet SF, Kuo CC (2012) TLR 2 induces vascular smooth muscle cell migration through cAMP response element-binding protein-mediated interleukin-6 production. *Arterioscler Thromb Vasc Biol* 32:2751–2760. <https://doi.org/10.1161/ATVBAHA.112.300302>
- Liu ZH et al (2017) C1q/TNF-related protein 1 promotes endothelial barrier dysfunction under disturbed flow. *Biochem Biophys Res Commun* 490:580–586. <https://doi.org/10.1016/j.bbrc.2017.06.081>
- Lu L et al (2016) C1q/TNF-related protein-1: an adipokine marking and promoting atherosclerosis. *Eur Heart J* 37:1762–1771. <https://doi.org/10.1093/eurheartj/ehv649>
- Luo JL, Kamata H, Karin M (2005) The anti-death machinery in IKK/NF-kappaB signaling. *J Clin Immunol* 25:541–550. <https://doi.org/10.1007/s10875-005-8217-6>
- Morimoto S et al (1991) Interleukin-6 stimulates proliferation of cultured vascular smooth muscle cells independently of interleukin-1 beta. *J Cardiovasc Pharmacol* 17(Suppl 2):S117–S118
- Ni W et al (2001) New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 103:2096–2101
- Park SY, Choi GH, Choi HI, Ryu J, Jung CY, Lee W (2005) Depletion of mitochondrial DNA causes impaired glucose utilization

- and insulin resistance in L6 GLUT4myc myocytes. *J Biol Chem* 280:9855–9864
- Patel VI et al (2006) A20, a modulator of smooth muscle cell proliferation and apoptosis, prevents and induces regression of neointimal hyperplasia. *FASEB J* 20:1418–1430. <https://doi.org/10.1096/fj.05-4981.com>
- Peterson JM, Aja S, Wei Z, Wong GW (2012) CTRP1 protein enhances fatty acid oxidation via AMP-activated protein kinase (AMPK) activation and acetyl-CoA carboxylase (ACC) inhibition. *J Biol Chem* 287:1576–1587. <https://doi.org/10.1074/jbc.M111.278333>
- Raines EW, Ferri N (2005) Thematic review series: the immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. *J Lipid Res* 46:1081–1092. <https://doi.org/10.1194/jlr.R500004-JLR200>
- Reape TJ, Groot PH (1999) Chemokines and atherosclerosis. *Atherosclerosis* 147:213–225
- Rollins BJ (1991) JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. *Cancer Cells* 3:517–524
- Rong JX, Shapiro M, Trogan E, Fisher EA (2003) Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proc Natl Acad Sci USA* 100:13531–13536. <https://doi.org/10.1073/pnas.1735526100>
- Shannon P et al (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13:2498–2504. <https://doi.org/10.1101/gr.1239303>
- Szklarczyk D et al (2015) STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acid Res* 43:D447–452 <https://doi.org/10.1093/nar/gku1003>
- Tang JN et al (2015) Plasma levels of C1q/TNF-related protein 1 and interleukin 6 in patients with acute coronary syndrome or stable angina pectoris. *Am J Med Sci* 349:130–136. <https://doi.org/10.1097/MAJ.0000000000000378>
- Tatara Y et al (2009) Macrophage inflammatory protein-1 β induced cell adhesion with increased intracellular reactive oxygen species. *J Mol Cell Cardiol* 47:104–111. <https://doi.org/10.1016/j.yjmcc.2009.03.012>
- Wang X, Feuerstein GZ, Clark RK, Yue TL (1994) Enhanced leucocyte adhesion to interleukin-1 β stimulated vascular smooth muscle cells is mainly through intercellular adhesion molecule-1. *Cardiovasc Res* 28:1808–1814
- Wang H et al (2007) Inhibition of terminal complement components in presensitized transplant recipients prevents antibody-mediated rejection leading to long-term graft survival and accommodation. *J Immunol* 179:4451–4463
- Wang H, Wang R, Du D, Li F, Li Y (2016a) Serum levels of C1q/TNF-related protein-1 (CTRP-1) are closely associated with coronary artery disease. *BMC Cardiovasc Disord* 16:92. <https://doi.org/10.1186/s12872-016-0266-7>
- Wang XQ et al (2016b) C1q/TNF-related protein 1 links macrophage lipid metabolism to inflammation and atherosclerosis. *Atherosclerosis* 250:38–45. <https://doi.org/10.1016/j.atherosclerosis.2016.04.024>
- Wong GW, Krawczyk SA, Kitidis-Mitrokostas C, Revett T, Gimeno R, Lodish HF (2008) Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions. *Biochem J* 416:161–177. <https://doi.org/10.1042/BJ20081240>
- Yuasa D et al (2014) Association of circulating C1q/TNF-related protein 1 levels with coronary artery disease in men. *PLoS ONE* 9:e99846. <https://doi.org/10.1371/journal.pone.0099846>
- Yuasa D et al (2016) C1q/TNF-related protein-1 functions to protect against acute ischemic injury in the heart. *FASEB J* 30:1065–1075. <https://doi.org/10.1096/fj.15-279885>