学 位 論 文

G0S2 regulates innate immunity of Kawasaki disease via lncRNA RP1-28O 10.1

（G0S2 は lncRNA RP1-28O 10.1 を介して川崎病における自然免疫を制御する）

富山大学大学院

医学薬学教育部（博士課程）生命・臨床医学専攻

小児科学教室

氏名　岡部　真子

Table of contents

Abstract

Objective

Kawasaki disease (KD) is a systemic vasculitis that is the most common cause of acquired heart disease in children. However, the etiology of KD remains unknown. Long-cording RNAs (lncRNA) contribute to the pathophysiology of various diseases, including myocardial infraction and cardiomyopathy. There have been few studies reporting a role for lncRNAs in KD-associated inflammation and we conducted this study to investigate whether there is a role for lncRNAs.

Methods

We enrolled 50 patients with KD and 45 controls. lncRNA expression in monocytes were determined by Cap analysis gene expression sequencing (CAGE-seq) and validated by quantitative real-time PCR. Differentially-expressed lncRNAs were further investigated to determine whether they were directly regulated by immune responses.

Result

Twenty-one candidate lncRNA transcripts were identified by CAGE-seq as being differentially expressed during the acute phase of KD. Of the twenty-one lncRNAs, the expression level of RP1-28O10.1 was up-regulated and rapidly decreased in acute phase of KD, confirming that the expression level of RP1-28O10.1 was regulated by TLR ligand.

Moreover, G0S2 was co-expressed with RP1-28O10.1 and G0S2 regulate inflammatory cytokines via RP1-28O 10.1.

Conclusion

RP1-28O10.1 and G0S2 interact with each other and play a pivotal role of innate immunity and inflammation during acute stage of KD.

Introduction

Kawasaki disease (KD) is an acute inflammatory syndrome in the form of systemic vasculitis that occurs in children(1,2). KD is the most common cause of acquired cardiac disease in children in developed countries(3). Its vasculitis sometimes results in the disruption of the coronary artery structure, which leads to coronary artery aneurysms. However, administering high-doses of intravenous immunoglobulin (IVIG) during the acute stage of KD is the standard initial treatment for KD and can reduce the incidence of coronary artery lesions (CAL). However, coronary artery aneurysms are still likely to occur, especially in IVIG non-responders (4,5).

Several studies suggest that infectious agents and the genetic makeup of the patient are involved in the pathogenesis of KD, however, the detailed etiology and pathology are not fully understood.

Non-coding RNAs are non-protein-coding transcripts, and long non-coding RNNAs (lncRNA) are classified as those longer than 200 nucleotides(6). In recent years, lncRNAs have come under significant attention for their potential role in disease pathogenesis and progression, especially because lncRNAs are involved in many biological processes through the regulation of gene transcription. Several studies have suggested that dysregulated expression of lncRNAs play a role in multiple human diseases, including myocardial infraction and cardiomyopathy, and that they may be therapeutic targets for these conditions(7,8).

However, there have been few studies on the relationship between KD and lncRNAs. Therefore, we hypothesized that lncRNAs have an important role in inflammation during the acute of KD.

Materials and methods

Enrollment of patients.

Fifty patients with KD (aged 4 months to 4 years, 29 males, 21 females), and 50 controls (25 non-KD febrile patients and 25 healthy children) were enrolled from August 2016 to March 2018 at the Toyama University Hospital and affiliated hospitals (Table 1). The study was approved by the Ethics Committee of the University of Toyama and performed

in accordance with the Declaration of Helsinki. Patients and controls were enrolled after informed consent. Patients with KD who responded to IVIG treatment and whose fever decreased within 48 hours of treatment were designated as responders and patients with KD who did not respond to IVIG were designated as non responder patients. Blood samples were collected at two time points; acute stage, at the time of diagnosis before IVIG treatment; convalescent stage, 3-7 days after IVIG infusion.

Separation of monocytes and confirmation of accuracy by FACS (fluorescence-activated cell sorting)

Peripheral blood monocular cells were separated by centrifugation of whole blood through Histopark. Monocytes were separated and collected using magnetic beads carrying antibodies against CD14 magnetic beads, with a purity of 98% after FACS.

RNA isolation

Total RNA was extracted from the human monocyte samples using RNeasy Kits (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions, and quantitated using a NanoVue (GE imagination at work). The extracted RNA samples were stored at −150 °C until further processing.

CAGEseq

The sequenced read data were submitted to the DDBJ Read Archive (accession number DRAXXXXXX). CAGE library preparation, sequencing, mapping, gene expression, and motif discovery analysis were performed. Total RNA quality was assessed using a Bioanalyzer (Agilent) to ensure that RIN (RNA integrity number) was over 7.0. cDNAs were synthesized from total RNA using random primers. The ribose diols in the 5' cap structures of RNAs were oxidized, and then biotinylated. The biotinylated RNA/cDNAs were captured using streptavidin beads (cap-trapping). After RNA digestion using RNaseONE/H, and adaptor ligation to both ends of the cDNA, double-stranded cDNA libraries (CAGE libraries) were constructed. The CAGE libraries were sequenced using single end reads of 75nt on a NextSeq 500 instrument (Illumina).

Differentially expressed gene analysis

After removing reads derived from ribosomal RNA and reads containing non-A/T/G/C base, the remained reads were mapped to the human reference genome (hg19) using BWA (ver. 0.7) (9) and HISAT (ver.2.0.5) (10). For tag clustering, CAGE-tag 5'

coordinates were entered for the RECLU clustering pipeline (11) with the following parameters: Maximum irreproducible discovery rate (IDR) of 0.1; Minimum count per million (CPM) value, 0.1; Gene annotation, GENCODE human gene annotation release 19. The gene-level tag count outputs of the RECLU clustering were subjected to differentially expressed gene using R package edgeR (ver. 3.26) (12). The genes with an FDR adjusted p-value of less than 0.05, and an absolute log2 fold change value of 1 or more were defined as differentially expressed genes (DEGs). The heatmap of the gene expression level of DEGs was drawn using R with the following parameters: Clustering method, Ward; Distance measure, Euclidean.

Enrichment analysis

The differentially expressed genes were subjected to enrichment analysis using R package goseq (ver. 1.3) (13). GO terms with p-values less than 0.05 were defined as significantly enriched GO terms. In addition, significantly enriched KEGG pathways were determined using the same criteria as GO enrichment analysis.

DEGs were subjected to enrichment analysis using R package goseq (ver. 1.3) (X6). GO terms in biological processing having p-values < 0.05 were defined as significantly

enriched. In addition, significantly enriched KEGG pathways were defined using the same criteria as GO enrichment analysis.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For real-time PCR (RT-PCR) analysis, RNA samples were reverse-transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Japan), according to the manufacturer's protocol. RT-PCR was conducted using a THUNDERBIRD SYBR qPCR Mix (Toyobo) on Thermal Cycler Dice Real Time System II (Takara Bio). The quantification was performed using ΔΔCt method with *GAPDH* expression used as a reference gene.

Cell culture

The human monocyte cell line, THP-1, was purchased from the JCRB Cell Bank (National Institute of Biomedical Innovation, Japan). Cells were maintained in RPMI media (Manufacturer) with 10% calf serum and antibiotics. To induce inflammation, 1.0 x 10^5 THP-1 cells were plated in 24 well plates containing 1ml RPMI media and after 24 hours LPS (1μM/ml: Manufacturer) or Pam (500nM/ml: Manufacturer) were added to the media. RNA samples were collected in 24 hours after the stimulation of LPS or Pam.

Knockdown of G0S2 in THP1

The siRNA delivery system (Dharmacon) was used to design and develop reagents to knockdown the expression of candidate lncRNAs. Control Pools (Nippongene), containing non-targeting siRNAs with no homology to known human, mouse, or rat genes, was used as a control in all siRNA transfection experiments. The THP1 cells plated in 24 well plates, cultured with growth media (RPMI medium supplemented with 10% FBS) for 24 hours and then transfected with siRNAs using the Lipofectamine® RNAiMAX(Thermo Fisher Scientific), per the manufacturer's protocol: a10 μM siRNA solution was prepared by mixing the siRNA stock solution with Opti-MEM (Thermo Fisher Scientific) for 5 minutes, and then 50μl was added to the well containing 1 ml of media. The cells were cultured at 37 °C with 5% CO2 for 48 hours and then treated with LPS or Pam, as described above.

Statistical analysis

To determine statistical significance, standard errors of the mean were calculated, and two-tailed tests were employed. Significant differences in all figures are indicated with single, double or triple asterisks when p value is <0.05, <0.01 or <0.005, respectively.

Graphs were drawn using GraphPad Prism 6.04 (GraphPad Software, Inc., La Jolla, CA). Quantified data are presented as mean ± SD.

Results

Clinical characteristics

In demographic data, KD patients had higher / longer regarding fever duration, levels of CRP, white blood cell count, and platelet count than in the febrile patient group. Other items were not significantly different between the patients with KD and the febrile patients (Table 1).

	Kawasaki Disease (n=50)		Non-KD Febrile (n=25)	Healthy (n=25)	P-value (KD vs Febrile control)
	Responder (n=45)	Nonresponder (n=5)			
Age at onset, month, mean (range)	18.2 (4-42)	27(12-55)	14 (4-48)	26.1 (12-72)	0.061
Male sex, n (%)	25 (55)	4(80)	11 (55.0)	15 (60.0)	–
Fever duration, days, mean±SD *	5.5(3-9)	9.3(8-11)	3.5(2-5)	–	<0.001
CRP, mg/dl, mean±SD *	6.8±5.7*	4.6±1.1	2.8±1.0	0.2±0.29	0.006
White blood cell count, /mm^3,mean±SD *	14,735±3777	13300±3874	10,835±1521	8,220±836	<0.001
Neutrophils, %, mean±SD	65.8±14.4	61.7±19.8	65.9±6.9	42.6±5.5	0.871
Platelet count, x10^4/mm^3, mean±SD *	37.5±10.5	48.7±16	26.2±5.3	27.7±4.7	<0.001
Na, mmol/l, mean±SD	134.6±2.4	135±1.5	136.7±3.0	137±3.2	0.023
AST, IU/L, mean±SD	90(23-580)	203(23-549)	33.6±11.9	36.3±8.4	0.095
Maximum CA RCA(#1), mm, mean (range)	1.92(1.4-2.8)	2.5(1.8-3.4)	–	–	–
Maximum CA LCA(#5), mm, mean (range)	2.1(1.7-2.8)	2.5(2.2-4.0)	–	–	–

Table 1. Demographic and clinical characteristics of enrolled patients. *P < 0.05.

CAGEseq of all transcripts from human samples of patients with acute KD and controls.

To identify globally affected expression profiles during the acute stage of KD, we performed CAGE-seq analysis of monocyte RNA (Fig.1A). We compared the KD and healthy control groups for the expression of the transcription start site of RNA obtained from CAGE. (Figure 1B). Using a combination of expression level and statistical filters that satisfy both an expression change of more than two-fold (log2 value 1.00 or more)

and an FDR of less than 0.05, we identified 21 candidate lncRNA transcripts significantly expressed during the acute stage of KD**;** ten were elevated, 11 were reduced (Table 2).

Figure.1

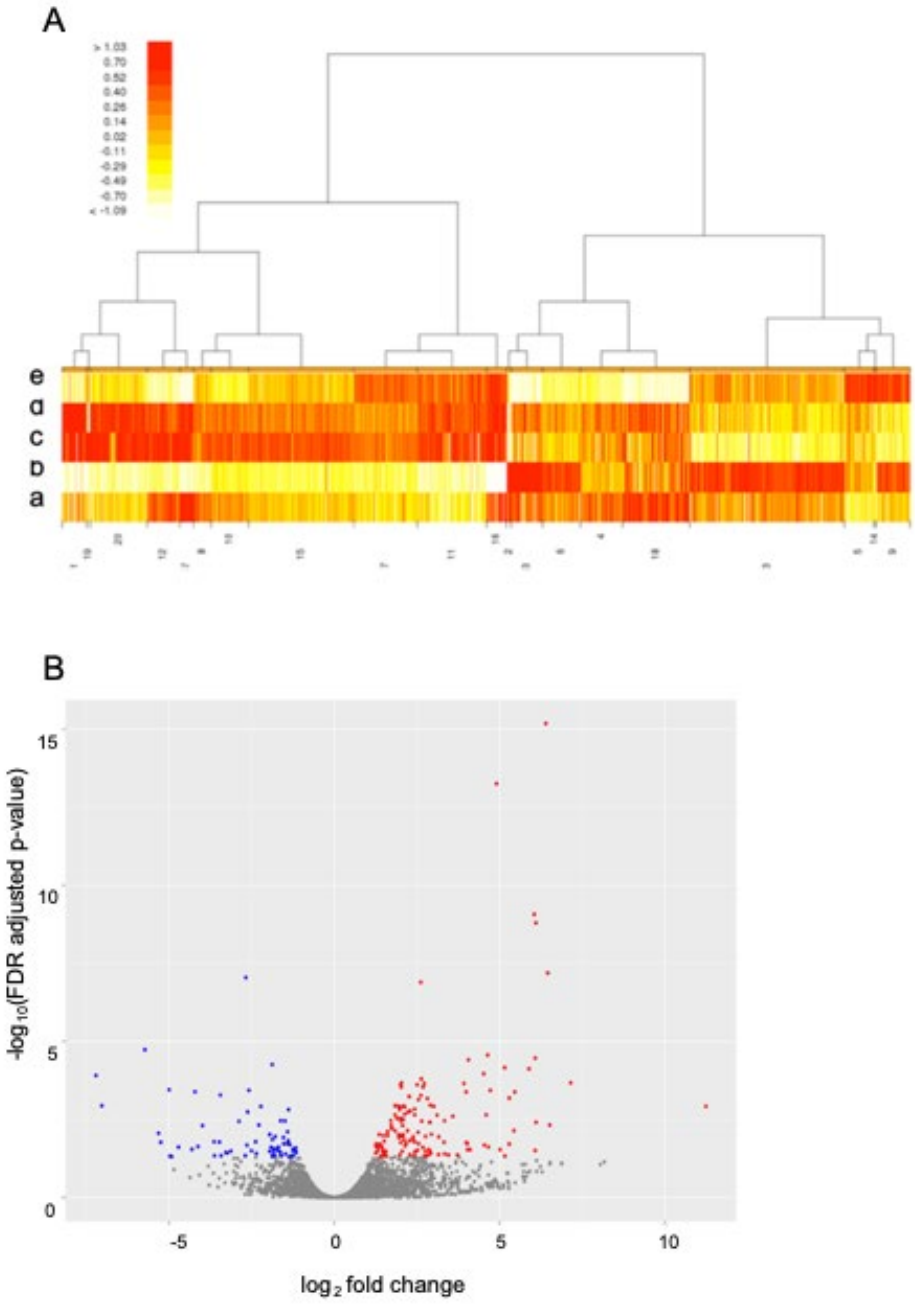

Figure1. CAGE-seq revealed the differentially expressed genes in Kawasaki disease

A: Heat map of the expression levels of all transcription start points by CAGE-seq. The lines a, b, c, d, e, represent the average expression level of each transcript in monocytes from KD patients before treatment with intravenous immunoglobulin (IVIG) who respond, KD patients after treatment with IVIG who respond, KD patients before treatment with IVIG who are refractory, KD patients after treatment with IVIG who are refractory, and Healthy controls, respectively.

B: Volcano plot comparing the gene expression of all KD patients, before IVIG treatment, with Healthy controls. The genes increased in KD are depicted in red and the genes decreased in KD are depicted in blue.

Gene	Gene type	Log2 fold change	Log2 CPM	FDR adjusted p-value
SNORD3C	lincRNA	-2.68	4.78	9.03E-08
7SK	lincRNA,misc_RNA,antisense	-5.73	6.26	1.85E-05
AC009950.2	antisense	-4.99	-1.32	3.56E-04
SNORD3B-1	lincRNA	-3.44	4.46	5.27E-04
XIST	lincRNA	11.24	3.46	1.20E-03
RP1-28O10.1	antisense	3.33	4.10	3.50E-03
RP11-47I22.2	lincRNA	2.90	2.84	3.88E-03
RP11-96C21.1	lincRNA	6.10	0.25	3.91E-03
RP11-764K9.1	lincRNA	-5.31	0.92	8.82E-03
RP11-689B22.2	antisense	2.43	1.97	1.66E-02
RP11-6N17.9	processed_transcript	-3.47	-1.42	1.68E-02
CTC-378H22.1	antisense	-1.53	3.23	1.90E-02
AC017002.2	lincRNA	-2.64	3.03	2.12E-02
MIR4435-1HG	lincRNA	-2.50	3.06	3.21E-02
RP11-624L4.1	antisense	2.76	2.96	3.28E-02
HLA-AS1	antisense	2.05	3.93	3.31E-02
RP3-322G13.5	antisense	2.90	1.29	3.34E-02
XXbac-BPG252P9.10	antisense	2.44	2.81	3.46E-02
FAM157A	lincRNA	3.15	0.04	4.22E-02
AP003774.6	lincRNA	-1.86	1.99	4.38E-02
RP11-598F7.3	antisense	-1.62	3.77	4.61E-02

Table 2. The ranking of gene expression of 21 candidate lncRNA trancropts that were significantly expressed in the acute stage of KD.

lncRNA RP1-28O 10.1 was upregulated in KD and stimulated by LPS and Pam3CSK4

Twenty-one differentially expressed lncRNA transcripts were validated by quantitative

real-time-PCR. Comparing the acute with the convalescent stages, only the RP1-28O10.1 transcript was significantly higher in the acute stage (Fig. 2A) (acute KD vs convalescent KD: $p < 0.004$, acute KD vs fever control: $p<0.0001$, acute KD vs healthy control: $p<0.0001$, respectively). The AC009950.2 transcript was significantly lower in the KD patients compared to healthy control samples ($p< 0.05$) (Fig. 2B). To investigate whether RP1-28O 10.1 could be associated with KD, THP1 monocytes were stimulated with lipopolysaccharide (LPS) and Pam3CSK. RP1-28O 10.1 was elevated significantly one hour after LPS stimulation by 1.7fold ($p=0.02$) and Pam3CSK4 stimulation by 1.8fold ($p=0.01$) (Fig. 2C). The expression level of interleukin (IL)-6 and tumor necrosis factor (TNF)-α, also increased by stimulation with LPS and Pam3CSK **(**Fig. 2D, E).

Figure.2

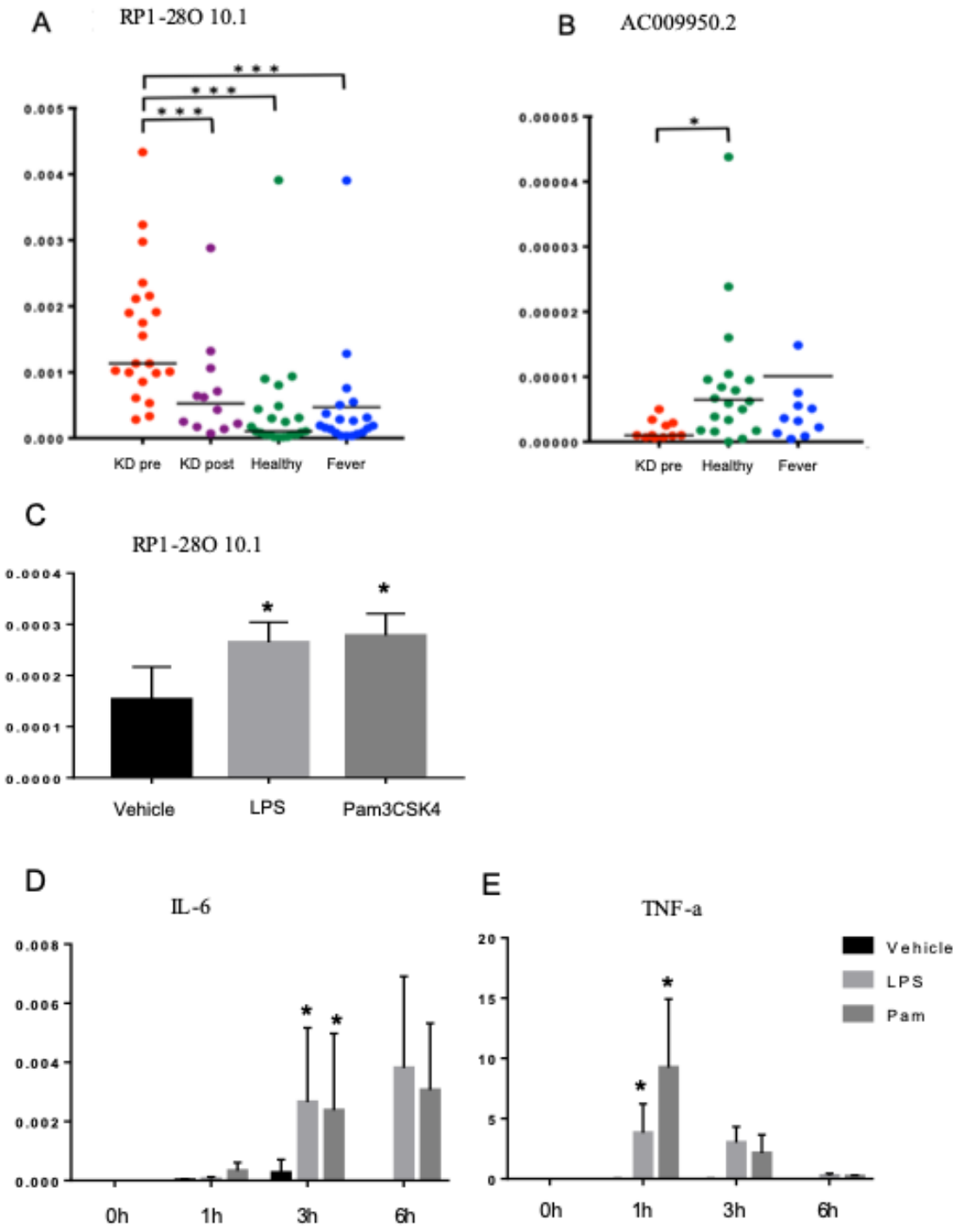

Figure2. RP1-28O10.1 was increased in monocytes in KD patients.

A, B: Relative gene expression of RP1-28O10.1 and AC009950.2 in human monocytes were compared between KD and controls.

C: Relative gene expression of RP1-28O10.1 in THP-1 monocytes was measured following 1hour stimulation with LPS or Pam3CSK4.

D, E: Inflammatory cytokines in THP-1 monocytes were measured during 6 hours of stimulation with LPS or Pam3CSK4.

G0S2 was associated with RP1-28O 10.1 and regulated by innate immunity.

We sought to identify genes associated with RP1-28O 10.1 and which behave as same as RP1-28O 10.1 during acute stage of KD. The absolute value of Pearson correlation showed that G0S2 strongly correlated with RP1-28O 10.1 (Table 3, Fig. 3A). Similar to RP1-28O 10.1, G0S2 was higher during the acute stage than the recovery stage, as well as for samples from fever and healthy controls ($p=0.03$, $p=0.0019$ respectively) (Fig. 3B).

We hypothesized that the RP1-28O10.1 and G0S2 were regulated together and related to the pathophysiology of KD. Therefore, we assessed how they affected the inflammatory response in monocytes using the in vitro model of THP-1 monocytes. G0S2 expression rose 3-6 hour after LPS and Pam3CSK4 stimulation. ($p<0.05$) (Fig. 3C)

Since it has been established that markers of inflammation of in THP-1 monocytes (such as TNF-α and IL-6) are induced by stimulation with LPS or Pam, we knocked down expression of G0S2 using siRNA and then stimulated the cells with these TLR ligands. After treatment with the siRNA the expression of G0S2 was significantly downregulated ($p = 0.04$) (Fig.3D), as was the expression of RP1-28O10.1 (Fig. 3E). TNF expression following LPS stimulation was also significantly decreased in cells in which G0S2 was knocked down (Fig. 3F). These data suggest that the expression of RP1-28O10.1 and G0S2 are tightly controlled and that they regulate the inflammatory response in monocytes from KD patients via an innate immunity pathway, in particular through the TLR4 receptor.

Gene 1	Gene 2	Pearson's correlation
RP1-28O10.1	GPR97	0.950
RP1-28O10.1	G0S2	0.942
RP1-28O10.1	IL8	0.935
RP1-28O10.1	PRKCH	0.924
RP1-28O10.1	CD82	0.921
RP1-28O10.1	ADAM9	0.920
RP1-28O10.1	ALPL	0.920
RP1-28O10.1	BCL2A1	0.920

RP1-28O10.1	CTD-3214H19.16	0.911
RP1-28O10.1	RP11-96C21.1	0.908
RP1-28O10.1	MAP3K8	0.907
RP1-28O10.1	LIN7A	0.904
RP1-28O10.1	RP11-689B22.2	0.904
RP1-28O10.1	ANXA3	0.901
RP1-28O10.1	LRRC70	0.895
RP1-28O10.1	PDE4D	0.895
RP1-28O10.1	SBNO2	0.895
RP1-28O10.1	SLC25A37	0.894
RP1-28O10.1	CXCL16	0.894
RP1-28O10.1	ACTA2	0.893
RP1-28O10.1	GRAP2	0.892
RP1-28O10.1	CST7	0.888
RP1-28O10.1	GZF1	0.886
RP1-28O10.1	PPP1R3B	0.885
RP1-28O10.1	CXCR1	0.882
RP1-28O10.1	ICAM1	0.882
RP1-28O10.1	NAMPT	0.880
RP1-28O10.1	PPARG	0.880
RP1-28O10.1	RNF122	0.877
RP1-28O10.1	LIMK2	0.876
RP1-28O10.1	MCTP2	0.874
RP1-28O10.1	MYL9	0.874
RP1-28O10.1	RP11-47I22.2	0.869
RP1-28O10.1	KIAA0226L	0.868
RP1-28O10.1	MERTK	0.864
RP1-28O10.1	ABCA1	0.859
RP1-28O10.1	SOD2	0.853
RP1-28O10.1	HIF1A	0.853
RP1-28O10.1	PPBP	0.851
RP1-28O10.1	RP3-322G13.5	0.851
RP1-28O10.1	PGS1	0.843
RP1-28O10.1	SDCBP2	0.842
RP1-28O10.1	RP11-624L4.1	0.841
RP1-28O10.1	ARHGAP11B	0.841

RP1-28O10.1	LITAF	0.834
RP1-28O10.1	C10orf54	0.832
RP1-28O10.1	WDR81	0.831
RP1-28O10.1	CLU	0.829
RP1-28O10.1	AQP9	0.829
RP1-28O10.1	AP001055.1	0.826
RP1-28O10.1	CR1	0.825
RP1-28O10.1	HAMP	0.819
RP1-28O10.1	SLC39A8	0.815
RP1-28O10.1	NAMPTL	0.812
RP1-28O10.1	SLC22A16	0.810
RP1-28O10.1	SLCO4A1	0.809
RP1-28O10.1	FGF13	0.808
RP1-28O10.1	TIMP1	0.808

Gene 1	Gene 2	Pearson's correlation
RP1-28O10.1	TRGV7	-0.930
RP1-28O10.1	RASSF4	-0.924
RP1-28O10.1	POLR1B	-0.918
RP1-28O10.1	MRPS33	-0.914
RP1-28O10.1	XYLB	-0.905
RP1-28O10.1	GHRL	-0.881
RP1-28O10.1	MRPS18B	-0.876
RP1-28O10.1	TMA16	-0.868
RP1-28O10.1	C11orf21	-0.862
RP1-28O10.1	RABGAP1L	-0.834
RP1-28O10.1	CTNND1	-0.833
RP1-28O10.1	MRPS25	-0.821
RP1-28O10.1	ARHGAP22	-0.813
RP1-28O10.1	HDAC9	-0.806
RP1-28O10.1	NAT10	-0.801

Table 3. Ranking of correlated mRNAs with RP1-28O10.1 during acute phase of KD

Figure.3

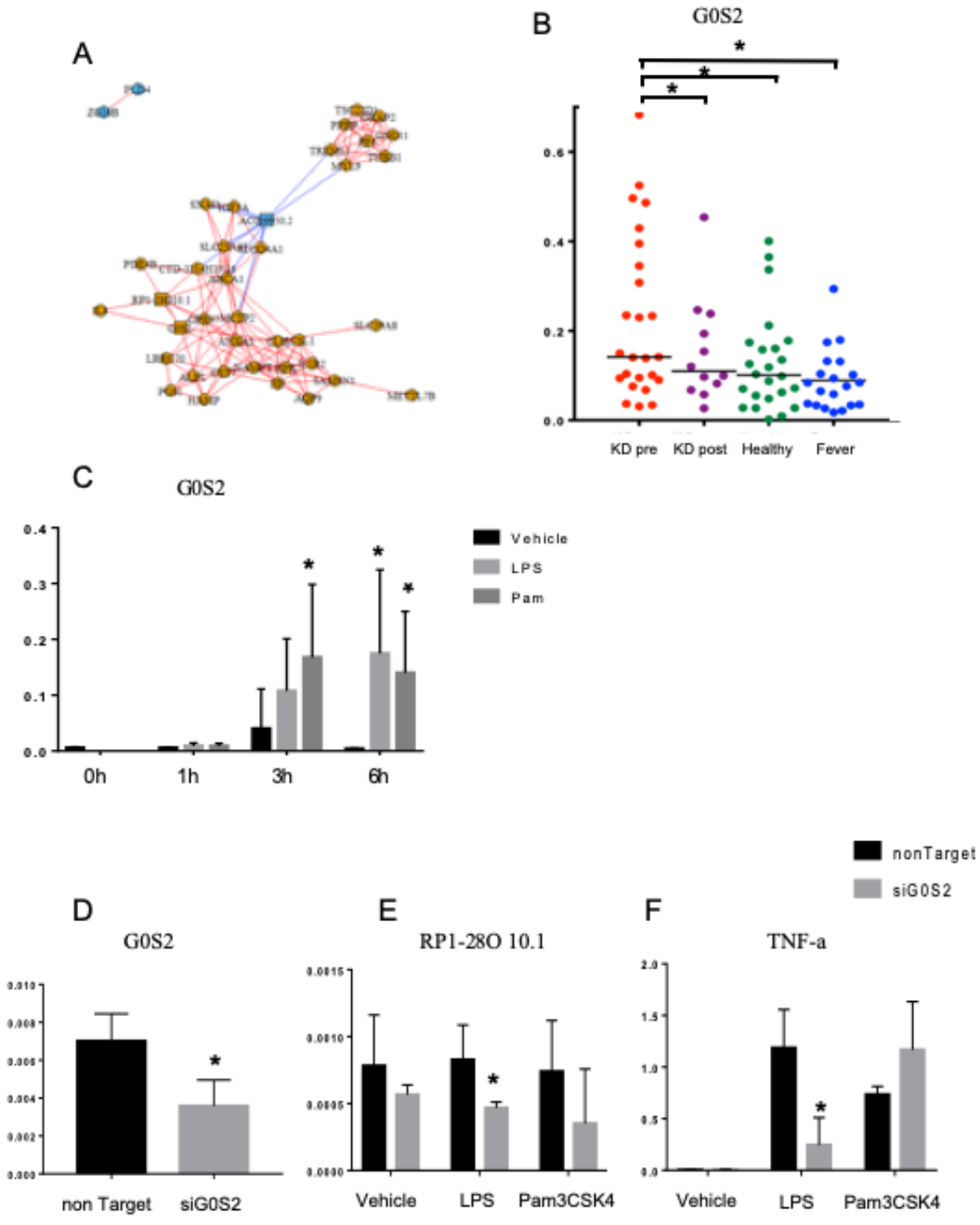

Figure3. G0S2 were highly expressed in KD patients and correlated with RP1-28O10.1.

A: The correlation network between DEGs are depicted based on Pearson's correlation

B: Relative gene expression of G0S2 in human monocytes were compared in acute stage of KD and controls.

C: G0S2 in THP-1 monocytes were estimated along time course until 6 hours after the stimulation of LPS or Pam3CSK4 (n=4).

D: Relative gene expression of G0S2 in THP-1 monocytes treated with siG0S2 (n=3)

E, F: Relative gene expression in THP-1 monocytes treated with siG0S2 after the stimulation of LPS or Pam3CSK4 (n=3).

Gene Ontology analysis

Gene ontology (GO) analysis (Fig.4) identified potential pathways involved in KD. Biological Process revealed that genes involved in the immune system process (-log p = 13.5) and acute inflammatory response (-log p = 12) (Fig4.A). The expression of receptor ligand and receptor regulatory activities are increased in molecular function analysis (Fig4. B). In the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, there were many genes involved in cytokine receptor interaction (-log p = 5) (Fig4.C).

Figure.4

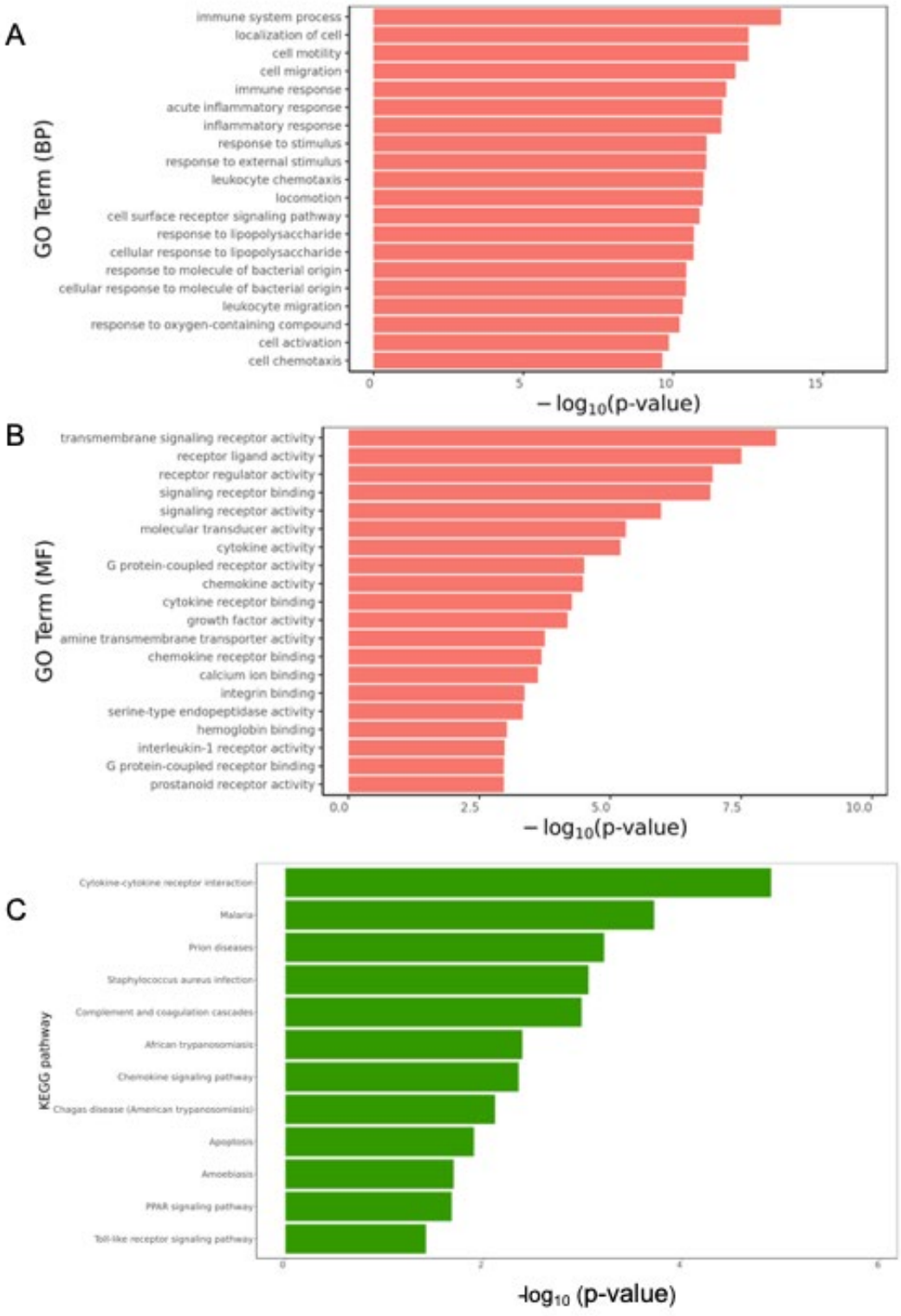

Figure 4. Gene ontology analysis and KEGG pathway analysis represent potential

pathways involved in KD.

The analysis of the Biological Process (A), molecular function (B), and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways with respect to genes differentially expressed in KD (n=6).

Discussion

Our data revealed that there are lncRNAs specifically expressed during the acute stage of KD as determined by CAGE-seq analysis. The expression of lncRNA RP1-28O10.1 was upregulated in acute stage and then rapidly decreased after initial treatment in monocytes from patients with KD. This lncRNA is directly regulated by G0S2 via TLR pathways. Our results suggest that RP1-28O 10.1 and G0S2 contribute to the inflammatory response in KD.

Two previous articles have identified relationships between lncRNAs and KD. Li et al. reported that lnc1992 regulates TNF-α expression, and that its expression was upregulated in KD patients(14). Ko et al. reported that XLOC_00627 is associated with CAA in KD patients(15).

Innate immunity is thought to play an important role in the pathogenesis of KD(16). Monocytes, which play an important role in innate immunity, increase and change

morphologically in peripheral blood during acute stage of KD(17,18). It has been observed that the majority of cells infiltrating the coronary arteries are macrophages (19,20). Further, the levels of damage-associated molecular patterns (DAMPs), such as S100 proteins are elevated in the serum of the patients of KD in the acute stage (21-23). KD patients have higher recurrence within 1 year after the first episode. (24). Therefore, we focused on monocyte analysis. This is the first study showed comprehensive expression profiling of monocytes with the acute KD patients.

Recently, several studies have been reported an association between KD and non-coding RNAs, using RNA-seq and microarray analyses(25,26). CAGE-seq is an improved method of RNA expression analysis(27,28). Since the cap structure at the 5 'end of RNA is specifically biotinylated and collected (cap trap method), the full-length cDNA is collected. In addition to the expression level of mRNA and lncRNA, the transcription start sites are precisely detected(29). Moreover, CAGE-seq is more quantitative than other methods by lacking the PCR amplification during the library production process. It enables one RNA to be detected with 99.99% accuracy among 2 million RNA molecules. In this study it resulted in the detection RP1-28O 10.1 as being differentially expressed.

Our results show the expression of lncRNA28O was increased during the acute stage of KD and co-expressed with the mRNA G0S2 in monocytes. The expression of G0S2 is suppressed by TNF-α (30,31), and G0S2 has been identified as a regulator of ATP production under cardiac ischemia(32). Kamikubo et al. showed that G0S2 increases the energy production efficiency under hypoxia via ATP synthase(33). Jakobsson et al. reported that monocytes/macrophages mediate inflammatory via ATP signaling(34). These studies support our data that G0S2 is involved in the inflammatory response of acute KD.

There have been several studies reporting the importance of the toll like receptor (TLR) pathway in KD. Recent studies showed that the expression of TLR2 increased in monocytes and that the TLR4 pathway induces S100A8, A100A9, and S100A12 proteins in KD (35)(36). The expression of both RP1-28O10.1 and G0S2 were found to be upregulated by stimulation with Pam3CSK4 and LPS, which are ligands for TLR1/2 and 4, respectively, suggesting that RP1-28O10.1 and G0S2 are regulated by TLR TLR1/2 and 4.

Further the expression of RP1-28O 10.1 was not stimulated by LPS or Pam3CSK4 when G0S2 was knocked down using siRNA. Moreover, the level of TNF-α was suppressed even under LPS stimulation. These data suggest that RP1-28O10.1 is regulated by G0S2

and that these molecules induce inflammation via TLR pathways. A relationship between G0S2 and RP1-28O 10.1 has not been reported previously. However, they are encoded close to each other in the genome and 28O is an antisense RNA which is likely to regulate the expression of a nearby gene, such as G0S2. Therefore, we propose that G0S2 plays a central role in innate immunity in KD.

Limitation

The number of the samples for CAGE-seq was small and it was possible that our study was lacking the power to detect candidate lncRNAs comprehensively. However, we believe these data are satisfyingly reliable because we were able to detect an lncRNA previously implicated in KD and coding genes already known to participate in the inflammatory response to appropriate stimuli. Although we demonstrated the involvement of RP1-28O10.1 and G0S2, how they involved in the inflammation are unknown and future studies are warranted. None of these patients developed CAL and, thus, no tissue samples from lesions could not be examined. The gene involved in the pathogenesis of KD may differ by race, and the further studies including all races are required.

Conclusion

RP1-28O10.1 is regulated by G0S2 and these molecules induce inflammation via TLR pathways in monocytes during the acute stage of KD. Our study shows that lncRNA may be involved in the initial stages of inflammation in KD and may have crucial roles in innate immune responses. Further studies of the roles of lncRNAs in KD are warranted and may prove to be an attractive therapeutic target of KD.

Source of Fundings : Grants

This study was supported by Grant-in-Aid for Scientific Research 00831041 (to M.O.) from The Ministry of Education, Culture, Sports, Science and Technology,

Grant-in-Aid from Japanese Kawasaki Disease Research Center (to M.O.), and a grant for Kawasaki Disease Research from Japan Blood Products Organization (to M.O.).

Disclosures

None.

.

References

1. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. Circulation 2004;110:2747-71.
2. Kawasaki T. [Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children]. Arerugi 1967;16:178-222.
3. McCrindle BW, Rowley AH, Newburger JW, Burns JC, Bolger AF, Gewitz M, et al. Diagnosis, Treatment, and Long-Term Management of Kawasaki Disease: A Scientific Statement for Health Professionals From the American Heart Association. Circulation 2017;135:e927-e99.
4. Dusser P, Kone-Paut I. IL-1 Inhibition May Have an Important Role in Treating Refractory Kawasaki Disease. Front Pharmacol 2017;8:163.
5. Dajani AS, Taubert KA, Gerber MA, Shulman ST, Ferrieri P, Freed M, et al. Diagnosis and therapy of Kawasaki disease in children. Circulation 1993;87:1776-80.

6. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009;458:223-7.

7. Zangrando J, Zhang L, Vausort M, Maskali F, Marie PY, Wagner DR, et al. Identification of candidate long non-coding RNAs in response to myocardial infarction. BMC Genomics 2014;15:460.

8. Li X, Zhou J, Huang K. Inhibition of the lncRNA Mirt1 Attenuates Acute Myocardial Infarction by Suppressing NF-kappaB Activation. Cell Physiol Biochem 2017;42:1153-64.

9. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754-60.

10. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods 2015;12:357-60.

11. Ohmiya H, Vitezic M, Frith MC, Itoh M, Carninci P, Forrest AR, et al. RECLU: a pipeline to discover reproducible transcriptional start sites and their alternative regulation using capped analysis of gene expression (CAGE). BMC Genomics 2014;15:269.

12. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for

differential expression analysis of digital gene expression data. Bioinformatics 2010;26:139-40.

13. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 2010;11:R14.

14. Li Z, Chao TC, Chang KY, Lin N, Patil VS, Shimizu C, et al. The long noncoding RNA THRIL regulates TNFalpha expression through its interaction with hnRNPL. Proc Natl Acad Sci U S A 2014;111:1002-7.

15. Ko TM, Chang JS, Chen SP, Liu YM, Chang CJ, Tsai FJ, et al. Genome-wide transcriptome analysis to further understand neutrophil activation and lncRNA transcript profiles in Kawasaki disease. Sci Rep 2019;9:328.

16. Hara T, Nakashima Y, Sakai Y, Nishio H, Motomura Y, Yamasaki S. Kawasaki disease: a matter of innate immunity. Clin Exp Immunol 2016;186:134-43.

17. Furukawa S, Matsubara T, Yabuta K. Mononuclear cell subsets and coronary artery lesions in Kawasaki disease. Arch Dis Child 1992;67:706-8.

18. Luo Y, Yang J, Zhang C, Jin Y, Pan H, Liu L, et al. Up-regulation of miR-27a promotes monocyte-mediated inflammatory responses in Kawasaki disease by inhibiting function of B10 cells. J Leukoc Biol 2019.

19. Takahashi K, Oharaseki T, Yokouchi Y, Hiruta N, Naoe S. Kawasaki disease as a

systemic vasculitis in childhood. Ann Vasc Dis 2010;3:173-81.

20. Sato N, Sagawa K, Sasaguri Y, Inoue O, Kato H. Immunopathology and cytokine detection in the skin lesions of patients with Kawasaki disease. J Pediatr 1993;122:198-203.

21. Foell D, Ichida F, Vogl T, Yu X, Chen R, Miyawaki T, et al. S100A12 (EN-RAGE) in monitoring Kawasaki disease. Lancet 2003;361:1270-2.

22. Ye F, Foell D, Hirono KI, Vogl T, Rui C, Yu X, et al. Neutrophil-derived S100A12 is profoundly upregulated in the early stage of acute Kawasaki disease. Am J Cardiol 2004;94:840-4.

23. Ebihara T, Endo R, Kikuta H, Ishiguro N, Ma X, Shimazu M, et al. Differential gene expression of S100 protein family in leukocytes from patients with Kawasaki disease. Eur J Pediatr 2005;164:427-31.

24. Hirata S, Nakamura Y, Yanagawa H. Incidence rate of recurrent Kawasaki disease and related risk factors: from the results of nationwide surveys of Kawasaki disease in Japan. Acta Paediatr 2001;90:40-4.

25. Saito K, Nakaoka H, Takasaki I, Hirono K, Yamamoto S, Kinoshita K, et al. MicroRNA-93 may control vascular endothelial growth factor A in circulating peripheral blood mononuclear cells in acute Kawasaki disease. Pediatr Res

2016;80:425-32.

26. Nakaoka H, Hirono K, Yamamoto S, Takasaki I, Takahashi K, Kinoshita K, et al. MicroRNA-145-5p and microRNA-320a encapsulated in endothelial microparticles contribute to the progression of vasculitis in acute Kawasaki Disease. Sci Rep 2018;8:1016.

27. Arner E, Daub CO, Vitting-Seerup K, Andersson R, Lilje B, Drablos F, et al. Transcribed enhancers lead waves of coordinated transcription in transitioning mammalian cells. Science 2015;347:1010-4.

28. Hon CC, Ramilowski JA, Harshbarger J, Bertin N, Rackham OJ, Gough J, et al. An atlas of human long non-coding RNAs with accurate 5' ends. Nature 2017;543:199-204.

29. Liu S, Wang Z, Chen D, Zhang B, Tian RR, Wu J, et al. Annotation and cluster analysis of spatiotemporal- and sex-related lncRNA expression in rhesus macaque brain. Genome Res 2017;27:1608-20.

30. Sun J, Yang Z, Shi XC, Ji H, Du ZY, Chen LQ. G0S2a1 (G0/G1 switch gene 2a1) is downregulated by TNF-alpha in grass carp (Ctenopharyngodon idellus) hepatocytes through PPARalpha inhibition. Gene 2018;641:1-7.

31. Jin D, Sun J, Huang J, He Y, Yu A, Yu X, et al. TNF-alpha reduces g0s2 expression

and stimulates lipolysis through PPAR-gamma inhibition in 3T3-L1 adipocytes. Cytokine 2014;69:196-205.

32. Kioka H, Kato H, Fujikawa M, Tsukamoto O, Suzuki T, Imamura H, et al. Evaluation of intramitochondrial ATP levels identifies G0/G1 switch gene 2 as a positive regulator of oxidative phosphorylation. Proc Natl Acad Sci U S A 2014;111:273-8.

33. Kamikubo K, Kato H, Kioka H, Yamazaki S, Tsukamoto O, Nishida Y, et al. A molecular triage process mediated by RING finger protein 126 and BCL2-associated athanogene 6 regulates degradation of G0/G1 switch gene 2. J Biol Chem 2019;294:14562-73.

34. Jakobsson PJ. Pain: how macrophages mediate inflammatory pain via ATP signaling. Nat Rev Rheumatol 2010;6:679-81.

35. Lin IC, Kuo HC, Lin YJ, Wang FS, Wang L, Huang SC, et al. Augmented TLR2 expression on monocytes in both human Kawasaki disease and a mouse model of coronary arteritis. PLoS One 2012;7:e38635.

36. Armaroli G, Verweyen E, Pretzer C, Kessel K, Hirono K, Ichida F, et al. Monocyte-Derived Interleukin-1beta As the Driver of S100A12-Induced Sterile Inflammatory Activation of Human Coronary Artery Endothelial Cells:

Implications for the Pathogenesis of Kawasaki Disease. Arthritis Rheumatol 2019;71:792-804.

Acknowledgements

We are grateful to Professor Yuichi Adachi.

We thank our colleagues and collaborating hospitals for always supporting us: Hitoshi Moriuchi, Haruna Hirai, Eriko Masuda, Miho Arai, Syokei Murakami, Shintaro Terashita, Yu Saito, Haruka Ushio, Taisuke Kato, Nao Sakata, Asami Takasaki, Osamu Higuchi, Tomoko Sakuma, Yoshie Okabe, Junko Yamaoto, Tatsuya Fuchizawa, Keiichiro Uese, Shinichi Tsubata, Itaru Yamaguchi, Yujiro Takegami, Ichiro Takasaki, Kesuke Okabe, and Koshi Kinoshita.

We thank Neil Bowles for help editing this manuscript.