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### Authors

Shimizu, Chisato

Kim, Jihoon

He, Ming

et al.

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ORIGINAL RESEARCH

# RNA Sequencing Reveals Beneficial Effects of Atorvastatin on Endothelial Cells in Acute Kawasaki Disease

Chisato Shimizu , MD\*; Jihoon Kim, MS\*; Ming He , MD\*; Adriana H. Tremoulet , MD, MAS; Hal M. Hoffman, MD; John Y-J. Shyy , PhD; Jane C. Burns , MD

**BACKGROUND:** Damage to the coronary arteries during the acute phase of Kawasaki disease (KD) is linked to inflammatory cell infiltration, myointimal proliferation, and endothelial cell (EC) dysfunction. To understand the response of ECs to KD treatment, we studied the genome-wide transcriptional changes in cultured ECs incubated with KD sera before and after treatment with or without atorvastatin.

**METHODS AND RESULTS:** RNA sequencing of human umbilical vein ECs incubated with pooled sera from patients with acute KD before or after treatment with intravenous immunoglobulin and infliximab revealed differentially expressed genes in interleukin-1, tumor necrosis factor- $\alpha$ , and inflammatory cell recruitment pathways. Subacute sera pooled from patients treated with intravenous immunoglobulin, infliximab, and atorvastatin uniquely induced expression of *NOS3*, Kruppel like factor (*KLF2*, and *KLF4* (promotes EC homeostasis and angiogenesis) and ZFP36 ring finger protein (*ZFP36*) and suppressor of cytokine signaling 3 (*SOCS3*) (suppresses inflammation), and suppressed expression of *TGFB2* and *DKK1* (induces endothelial-mesenchymal transition) and sphingosine kinase 1 (*SPHK1*) and C-X-C motif chemokine ligand 8 (*CXCL8*) (induces inflammation).

**CONCLUSIONS:** These results suggest that atorvastatin treatment of patients with acute KD may improve EC health, reduce mediators of inflammation produced by ECs, and block KD-induced myofibroblast proliferation.

**Key Words:** atorvastatin ■ endothelial cell ■ infliximab ■ Kawasaki disease ■ transcriptome

Acute systemic inflammation associated with Kawasaki disease (KD) induces coronary artery (CA) aneurysms in a subset of genetically susceptible children. Early in the acute phase of this vasculitis, neutrophils adhere to activated endothelial cells (ECs) and enter the arterial wall.<sup>1</sup> Cytotoxic T cells follow and mediate further arterial damage, resulting in focal weakening and aneurysm formation.<sup>2</sup> Endothelial-to-mesenchymal transition (EndMT), a process that transforms ECs into myofibroblasts, can result in remodeling of the arterial wall with myointimal proliferation and stenosis of the arterial lumen.<sup>3,4</sup> Stenoses commonly occur at the inlet or outlet of the

aneurysm and can contribute to the risk of thrombosis, leading to myocardial infarction and death.<sup>5</sup>

The standard treatment of acute KD is intravenous immunoglobulin (IVIG) plus aspirin. At the time of KD diagnosis, 25% to 30% of patients have CA Z scores  $\geq 2.5$  (CA internal diameter normalized for body surface area) and are at risk for progression to aneurysms.<sup>6</sup> Elevated levels of the proinflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are associated with the acute phase of KD and are highest among patients developing CA lesions.<sup>7,8</sup> Intensification of initial therapy with infliximab, a monoclonal antibody against TNF- $\alpha$ , is one strategy to mitigate the arterial wall inflammation in

Correspondence to: Chisato Shimizu, MD, Department of Pediatrics, University of California, San Diego, School of Medicine, 9500 Gilman Dr, La Jolla, CA 92093-0641. Email: [c1shimizu@health.ucsd.edu](mailto:c1shimizu@health.ucsd.edu)

\*C. Shimizu, J. Kim, and M. He contributed equally.

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## CLINICAL PERSPECTIVE

### What Is New?

- Analyzing the gene expression profile of cultured endothelial cells incubated with sera from patients who received different treatments provides a window into the vascular effects of those treatments.
- Endothelial cells incubated with serum from patients with acute Kawasaki disease treated with standard therapy plus atorvastatin showed gene expression profiles with improved endothelial cell health and decreased inflammation compared with controls.

### What Are the Clinical Implications?

- Although the American Heart Association guidelines encourage adjunctive therapy for patients with acute Kawasaki disease with early echocardiographic signs of coronary artery damage, there are currently no clinical trial data to guide best practice to stop the progression of arterial wall damage and prevent coronary artery aneurysm formation during the acute phase of Kawasaki disease.
- We demonstrated deleterious changes in endothelial cell transcriptional profiles that were mitigated by exposure to sera from patients treated with atorvastatin. The anti-inflammatory and endothelial-protective effects of atorvastatin may yield benefit in blocking coronary artery aneurysm progression in patients with Kawasaki disease when added to standard therapy.

## Nonstandard Abbreviations and Acronyms

<b>CA</b>	coronary artery
<b>DEG</b>	differentially expressed gene
<b>EC</b>	endothelial cell
<b>EndMT</b>	endothelial-to-mesenchymal transition
<b>HUVEC</b>	human umbilical vein endothelial cell
<b>KD</b>	Kawasaki disease
<b>NOS</b>	NO synthase
<b>RT-PCR</b>	reverse transcriptase–polymerase chain reaction
<b>TGF-<math>\beta</math></b>	transforming growth factor- $\beta$

patients with KD having elevated CA Z scores.<sup>9</sup> Despite intensification of initial therapy with steroids, infliximab, or cyclosporine, a subset of patients develop progression of CA aneurysms, resulting in permanent destruction of the coronary wall architecture.<sup>10–12</sup>

Statins are well documented to reduce vascular wall inflammation, reduce transforming growth factor- $\beta$  (TGF- $\beta$ )–induced EndMT, and induce NO synthase (NOS), all of which actions could have potential benefits in acute KD.<sup>13–15</sup> The addition of atorvastatin to IVIG and infliximab was studied in a phase 1/2a trial in children with KD with early dilation of the coronary arteries on echocardiography.<sup>16</sup> In this dose escalation trial, 34 patients with KD were treated with atorvastatin, 0.125 to 0.75 mg/kg, for 6 weeks in addition to acute treatment with IVIG, aspirin, and infliximab. Although atorvastatin appeared to be safe and well tolerated, with no dose-limiting toxicities, it is unlikely that an adequately powered phase 3 clinical trial will ever be performed given the small number of eligible patients. In the absence of clinical trial data, we developed an ex vivo experimental system using patient sera and cultured vascular ECs to explore the effects of acute KD sera on the endothelium.<sup>17</sup> Using this experimental approach, we elucidated the effects of different anti-inflammatory treatments and the addition of atorvastatin for intensification of initial therapy in patients with KD with early signs of CA inflammation. To better understand the statin effects in patients with acute KD, we examined the genome-wide transcriptional changes in ECs incubated with sera from patients with KD treated with or without atorvastatin. We also evaluated differentially expressed genes (DEGs) in whole blood RNA from these same patients and learned that the primary benefit of atorvastatin is on the ECs. These studies revealed a marked beneficial effect of atorvastatin on EC homeostasis and reduction in markers of inflammation.

## METHODS

### Patients and Samples

The demographic and clinical characteristics of study patients are presented in the Table. All patients were diagnosed by 1 of 2 KD clinicians (J.C.B. and A.H.T.) at Rady Children's Hospital San Diego and met the American Heart Association criteria for complete KD.<sup>18</sup> All patients were followed up longitudinally in the dedicated KD Clinic until the age of 15 years. The internal dimensions of the right and left anterior descending coronary arteries were expressed as SD units from the mean normalized for body surface area (Z score). All patients who received atorvastatin were enrolled in a phase 1/2a dose-escalating clinical trial of atorvastatin in patients with KD with CA Z score  $\geq 2.5$ .<sup>16</sup> The clinical samples in these experiments were collected from patients who received atorvastatin, 0.75 mg/kg per day, in addition to IVIG (2 g/kg) plus infliximab (5–10 mg/kg)<sup>16</sup> (Table and Table S1A and S1B). Patients with KD who received IVIG and infliximab or IVIG alone were matched 1:1 by age  $\pm 4$  years, illness day  $\pm 5$  days

**Table. Demographic and Clinical Characteristics of the Patients With KD Treated With IVIG and Infliximab With or Without Atorvastatin**

Variable	IVIG+infliximab		IVIG+infliximab+atorvastatin		P value*
	(n=5)		(n=5)		
Age, y <sup>†</sup>	4.8 (2.6–5.8)		4.7 (2.9–5.1)		0.936
Female sex, n (%)	5 (100)		1 (20)		0.048
Race or ethnicity, n (%)					0.753
Asian	1 (20)		1 (20)		
White	1 (20)		1 (20)		
Hispanic	2 (40)		3 (60)		
>2 Races or ethnicities	1 (20)		0		
Maximum Z score, median (IQR, range) <sup>‡</sup>	1.14 (0.6–1.72, 0.5–1.8)		3.2 (2.8–3.2, 2.8–11.3)		0.008
	Pre-IVIG	Subacute	Pre-IVIG	Subacute	
Illness day of sample collection <sup>§</sup>	5 (5–7)	21 (19–23)	5 (5–7)	22 (20–23)	0.846
Laboratory data <sup>¶</sup>					
WBC count, 10 <sup>9</sup> /μL	11.7 (11.6–13.1)	8.0 (7.8–8.1)	10.7 (10.5–11.5)	6.5 (5–7.3)	0.218
ANC, /μL	7254 (6240–9490)	3270 (2604–4212)	7351 (6625–7665)	3120 (2024–3796)	0.894
ZHgb	0.2 (–2.1 to 0.2)	–1.0 (–2.0 to 0.2)	–1.9 (–2.2 to –1.1)	–0.9 (–1.0 to –0.6)	0.346
Platelet count, 10 <sup>9</sup> /mm <sup>3</sup>	287 (267–447)	359 (353–407)	322 (279–352)	312 (280–618)	0.523
ESR, mm/h	62 (31–64)	31 (30–32)	68 (67–83)	63 (38–64)	0.82
CRP, mg/dL <sup>  </sup>	7.9 (3.4–8.3)	0.5 (0.5–0.5)	8.2 (5.6–13.9)	0.5 (0.5–0.6)	0.581

ANC indicates absolute neutrophil count; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IQR, interquartile range; IVIG, intravenous immunoglobulin; KD, Kawasaki disease; WBC, white blood cell; and ZHgb, hemoglobin concentration normalized for age.

\*P values were calculated by Mann-Whitney test for continuous variables between 2 groups (IVIG+infliximab vs IVIG+infliximab+statin) and Fisher exact test for categorical variables. Laboratory test P values were calculated between 2 groups using acute or subacute data.

<sup>†</sup>Data are given as median (IQR).

<sup>‡</sup>Maximum Z score (internal diameter normalized for body surface area) for the right and left anterior descending coronary arteries.

<sup>§</sup>Illness day 1=first day of fever.

<sup>||</sup>Subacute CRP levels were measured in 3 (IVIG+infliximab) and 2 (IVIG+infliximab+atorvastatin) patients.

(first day of fever=illness day 1) (Table and Table S1). All patients received aspirin (30–50 mg/kg per day). Whole blood RNA in PAXgene tubes, EDTA plasma, and serum samples was collected before treatment (illness day 2–8) and again on illness day 15 to 25, as previously described<sup>17</sup> (Table S1). For statin-treated patients, subacute blood was also collected 5 to 32 hours after oral administration of atorvastatin (Table S1). Late convalescent sera (illness day 440–990) from healthy children with a remote history of KD and with always normal coronary arteries by echocardiography served as healthy control sera. The Human Research Protection Program of the University of California San Diego approved this research protocol, and written informed consent and assent were obtained from the parents and patients as appropriate.

### Cell Culture

Detailed methods were as previously described.<sup>17</sup> In the experiments involving patients' sera, M199 was supplemented with 10% patient serum (pooled or individual) and 2% fetal bovine serum (100 μL patient

serum, 20 μL fetal bovine serum, and 880 μL medium per well of a 6-well plate). For RNA sequencing (RNA-seq), human umbilical vein ECs (HUVECs) were incubated with pooled, pretreatment (illness day 5–7) or posttreatment (illness day 19–23) sera from patients with KD treated with or without atorvastatin for 24 hours (Table S1). For experiments to validate the RNA-seq by reverse transcriptase–polymerase chain reaction (RT-PCR), HUVECs were incubated with sera from individual patients with KD treated with or without atorvastatin either before treatment (illness day 5–7) or after treatment (illness day 19–23) for 24 hours (Table S1). All cell culture experiments were repeated 3 times.

### RNA Extraction, RNA-Seq, and RT-PCR

The data that support the findings of this study are available from the corresponding author on reasonable request. Total RNA from HUVECs was isolated using miRvana (ThermoFisher) for RNA-seq or Trizol reagent (Invitrogen) for RT-PCR. To extract RNA from whole blood, PAXgene tubes were processed following the manufacturer's instruction (Qiagen). For

RNA-seq analysis, total RNA was assessed for quality using an Agilent TapeStation 4200, and samples with an RNA integrity number >8.0 were used to generate RNA-seq libraries using the TruSeq Stranded mRNA Sample Prep Kit with TruSeq Unique Dual Indexes (Illumina, San Diego, CA). Samples were processed following manufacturer's instructions, modifying RNA shear time to 5 minutes. Resulting libraries were multiplexed and sequenced with 100–base pair paired end reads (PE100) to a depth of  $\approx$ 50 million reads per sample on an Illumina NovaSeq 6000. Samples were demultiplexed using bcl2fastq v2.20 Conversion Software (Illumina). TaqMan RT-PCR was performed for *NOS3*, *KLF4*, *DKK1*, *TGFB2*, *CXCL8*, and *ZFP36* (Life Technologies), as previously described.<sup>17</sup> *YWHAZ* for HUVECs<sup>19</sup> was used as a housekeeping gene control to normalize expression in the RT-PCR assays. RNA-seq data have been made publicly available at FigShare (<https://figshare.com/>) and can be accessed (10.6084/m9.figshare.19164383).

### Differential Expression Analysis

Because of an imbalance of female patients between the 2 treatment groups ( $P=0.048$ ), we excluded genes on chromosome X and Y from the whole blood RNA-seq data analysis. The RNA-seq analysis pipeline consisted of the following steps: quality control using fastp<sup>20</sup> and MultiQC,<sup>21</sup> quantification using salmon,<sup>22</sup> and differential expression analysis using DESeq2.<sup>23</sup> R version 4.3.4 and Python3 version 3.8.5 were used in data analysis, file management, and visualization. To eliminate genes with low transcript abundance, we mapped the distribution of all transcript copy numbers and eliminated the lower 10%. The cutoff value of the adjusted  $P$  value for multiple testing was predefined as 0.0001. The minimum required fold change was set as 2. All computation was conducted in Amazon Elastic Computing instances, with the virtual servers in the cloud computing environment. A heat map was generated after applying hierarchical clustering on the normalized gene expression values with Euclidean distance and complete linkage.

### ELISA

Human DKK1 was quantified in culture medium using the Quantikine ELISA Kit (R&D Systems), following manufacturer's instructions. Intracoefficient and intercoefficient of variations were <13% and 11%, respectively.

### Statistical Analysis

We used different statistical tests for different types of data. For RNA-seq count data with a 2-group comparison, we used DESeq2 to deal with dispersion using an

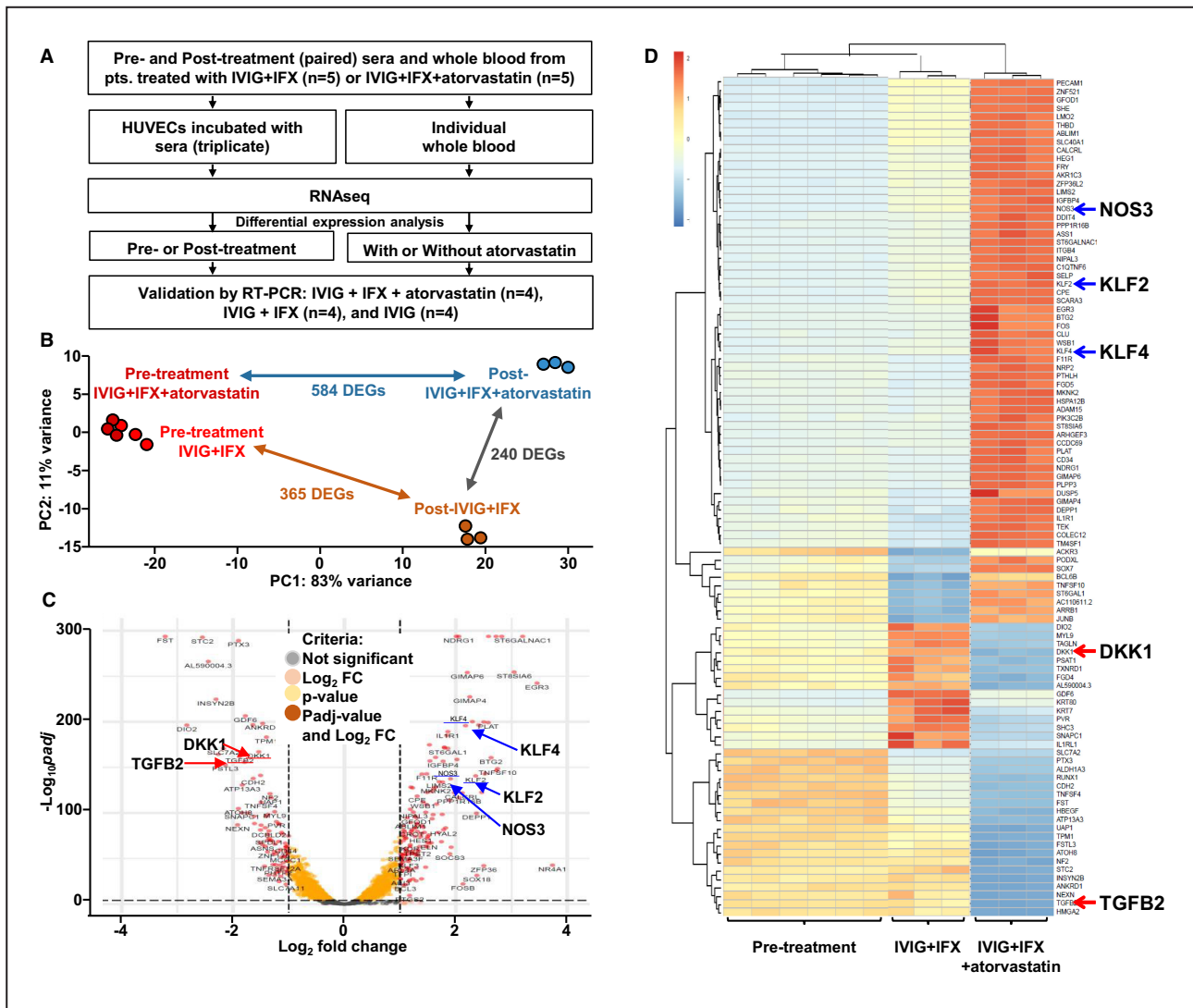
adjusted  $P$ -value threshold of 0.0001. For RT-PCR numeric data with a 3-group comparison, as a first step, we used Kruskal-Wallis test to test for equivalence across the 3 groups. As a second step, if Kruskal-Wallis test was significant, then we applied Mann-Whitney test for paired comparisons to test for equivalence of 2 groups. For clinical variables, we applied a similar approach as for the RT-PCR numeric data.  $P$  values were calculated by Mann-Whitney test for continuous variables between 2 groups (IVIG+infliximab versus IVIG+infliximab+statin) and Fisher exact test for categorical variables with an unadjusted  $P$ -value cutoff of 0.05.

## RESULTS

### Effects of Patient Treatment With IVIG and Infliximab on Cultured ECs

Clinical samples were collected from patients with KD treated with either IVIG+infliximab or IVIG+infliximab+atorvastatin (Table). Although the CA Z scores differed between the 2 groups, there was no statistically significant difference in proinflammatory cytokine levels, such as TNF- $\alpha$  and interleukin-6 (Figure S1). We performed RNA-seq on cultured ECs incubated with pooled sera (5 patients/group) from these 2 groups to compare the transcriptomic profiles. We also sequenced whole blood RNA from these same 5 patients with KD treated with or without atorvastatin (Figure 1A). Principal component analysis of cultured EC RNA-seq performed in triplicate showed agreement among all 6 samples using sera collected from patients before any treatment. However, RNA expression profiles were significantly different when compared with those from ECs incubated with post-treatment sera (Figure 1B). Analysis of triplicate experiments of ECs incubated with pretreatment and posttreatment sera from 5 patients treated with IVIG and infliximab revealed 365 DEGs, with 114 upregulated and 251 downregulated (adjusted  $P<0.0001$  and absolute fold change >2) (Table S2, Figure 1B, and Figure S2). The top 100 DEGs were displayed in a heat map (Figure S3). Among these DEGs, 81 were downregulated after treatment with IVIG and infliximab, which included chemokine genes (*CCL2*, *CCL20*, *CXCL8*, *CXCL1*, and *CXCL6*), adhesion molecule genes (*ICAM1*, *VCAM1*, and *SELE*), cytokine genes (interleukin-1 and TNF- $\alpha$ ), and genes encoding molecules involved in the nuclear factor- $\kappa$ B pathway (eg, *TIFA* and *NFKB2*). The upregulated genes after treatment included *NOS3*, platelet-related genes (thrombomodulin [*THBD*] and platelet-EC adhesion molecule 1 [*PECAM1*]), and angiogenesis-related genes (integrin subunit  $\beta$  4 [*ITGB4*] and endomucin [*EMCN*]).





**Figure 1. Study workflow and differentially expressed genes (DEGs).**

**A**, Endothelial cells (ECs) were incubated with pretreatment or posttreatment (illness day 17–25) pooled sera from patients with Kawasaki disease (KD) treated with intravenous immunoglobulin (IVIG), infliximab (IFX), and atorvastatin (n=5) or age- and illness day–matched patients with KD treated with IVIG and IFX but without atorvastatin (n=5). Cell culture experiments were performed in triplicate, and RNA sequencing (RNA-seq) was performed on cell lysates to profile transcriptomic changes. Differential expression analysis between pretreatment and posttreatment or posttreatment with or without atorvastatin treatment was performed. The results were validated using individual sera from independent patients with KD incubated with ECs, and the cell lysates were analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) on targeted molecules. We also performed whole blood RNA-seq followed by differential expression analysis on the same patients used in the EC experiments. **B**, Principal component (PC) analysis using RNA-seq data from cell lysates from ECs incubated with KD sera. Transcript levels of ECs incubated with pretreatment sera from patients subsequently treated with either IVIG and IFX (blue circles) or IVIG, IFX, and atorvastatin (green circles) were compared with posttreatment transcript levels without (blue triangles) or with (green squares) atorvastatin treatment, respectively. Differential expression analysis was also performed on cell lysates from ECs incubated with posttreatment sera from patients treated with (green squares) or without (blue triangles) atorvastatin. **C**, Volcano plot for posttreatment gene expression with or without atorvastatin. *NOS3*, *KLF2*, *KLF4*, *DKK1*, and *TGFB2* are shown with arrows (downregulated genes after treatment with atorvastatin are shown in red, and upregulated genes after atorvastatin treatment are shown in blue). **D**, Heat map using top 100 DEGs. Expression levels of *NOS3*, *KLF2*, *KLF4*, *DKK1*, and *TGFB2* are shown with the same color codes described in **(C)**. FC indicates fold change; HUVEC, human umbilical vein EC; Padj, adjusted *P* value; and pts., patients.

### Effects of Patient Treatment With Atorvastatin on Cultured ECs

Analysis of triplicate experiments of ECs incubated with pretreatment and posttreatment sera from 5 patients

treated with IVIG, infliximab, and atorvastatin revealed 584 DEGs, with 246 upregulated and 338 downregulated DEGs (adjusted *P*<0.0001 and absolute fold change >2) (Table S3, Figure 1B, and Figure S2). Of these 584 DEGs, 259 genes were the same as those

detected in ECs incubated with sera from patients treated with IVIG and infliximab alone (Table S3). We performed pathway analysis (Enrichr, <https://maayanlab.cloud/Enrichr/>) on these 259 common DEGs. The top pathways were TNF- $\alpha$  effects on cytokine activity, cell motility, and apoptosis (adjusted  $P=3.1\times 10^{-27}$ ) (Table S4). Unsupervised clustering was performed on the top 100 DEGs between pretreatment and posttreatment with IVIG, infliximab, and atorvastatin (Figure S4). There were 48 unique DEGs following atorvastatin treatment, which included the downregulation of *TGFB2*, a key molecular marker for EndMT.

Next, we compared DEGs from ECs incubated with subacute sera from patients treated with or without atorvastatin (Figure 1). With atorvastatin, there were 152 upregulated and 88 downregulated genes (adjusted  $P<0.0001$ , and an absolute fold change  $>2$ ) (Table S5). Unsupervised clustering of the top 100 DEGs selected by a volcano plot is shown in Figure 1C and 1D. The top 100 DEGs from ECs incubated with sera from atorvastatin-treated patients included upregulated *NOS3*, *KLF2*, and *KLF4*, which are essential to maintain EC homeostasis (Figure 1D, blue arrows). The downregulated genes included *TGFB2* and Dickkopf WNT signaling pathway inhibitor 1 (*DKK1*), which are associated with EndMT (Figure 1D, red arrows). To understand the effect of atorvastatin treatment on ECs, we performed pathway analysis using the top 100 DEGs. The top ranked pathway was TGF- $\beta$  regulation of extracellular matrix (adjusted  $P=9.7\times 10^{-9}$ ) that included *TGFB2* and *DKK1* (Table S4). To confirm these findings, we measured *DKK1* levels in the conditioned media of ECs incubated with sera from patients with KD treated with or without atorvastatin. *DKK1* levels were lower in the media from ECs incubated with sera from atorvastatin-treated patients compared with pretreatment sera or posttreatment sera without atorvastatin ( $P=0.003$  for pretreatment versus poststatin treatment, and  $P=0.03$  for posttreatment with versus without statin) (Figure S5). Thus, transcript levels in HUVEC lysates were consistent with protein levels of secreted *DKK1* in the media.

### Atorvastatin Decreases EC Inflammation

Atorvastatin has well-described effects on EC homeostasis, EndMT, and inflammation. We selected genes that regulate those pathways (Figure 2A and 2F) and graphed transcript levels in EC lysates using RNA-seq data. To compare the effect of healthy control sera on cultured ECs, we performed independent experiments using sera from individual pretreatment patients with KD ( $n=6$ ), individual healthy controls ( $n=3$ ), and no serum controls in triplicate (Figure 2). As seen in the heat map (Figure 1D), *NOS3* and *KLF4* transcripts were increased, whereas those of *TGFB2* and

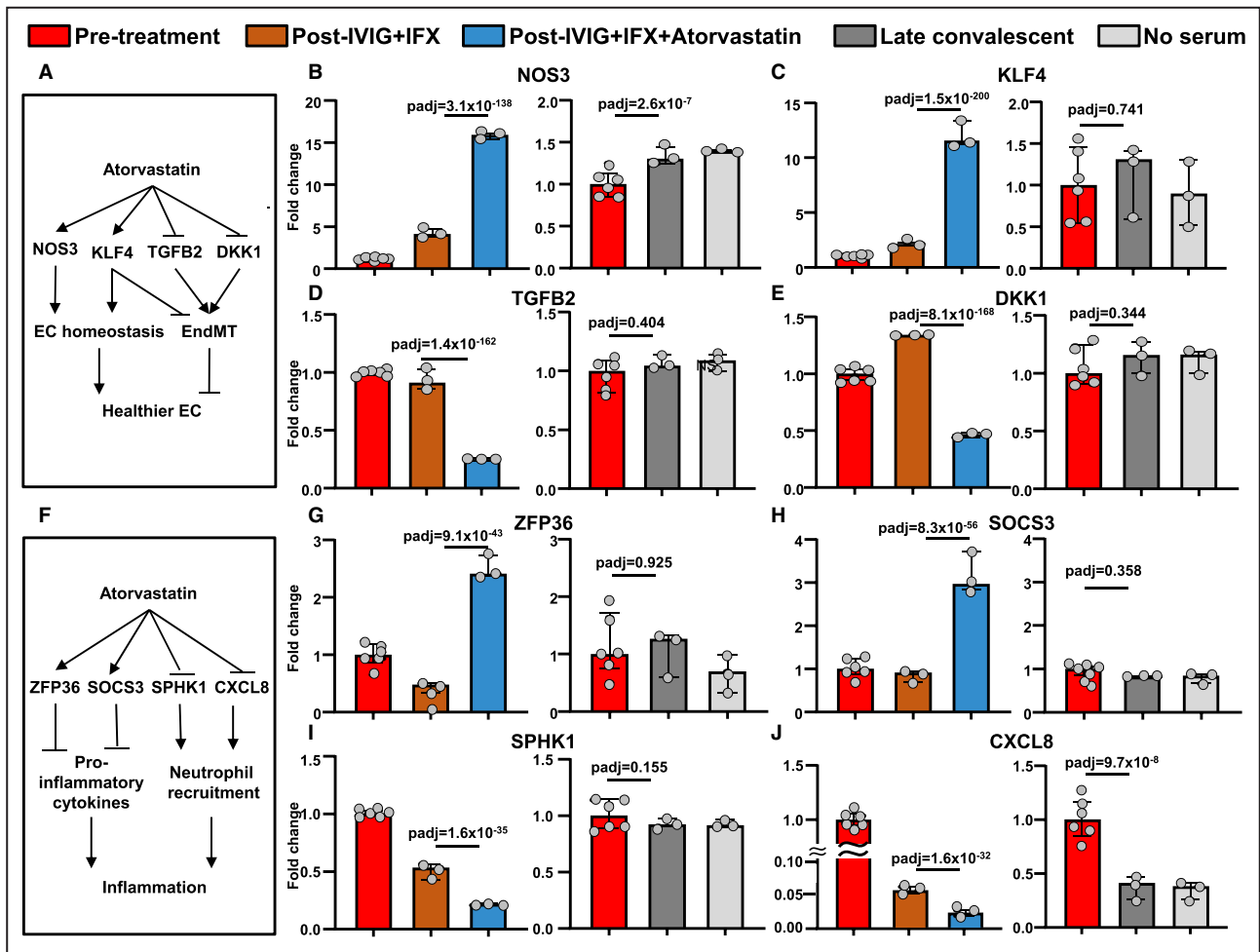
*DKK1* were decreased in atorvastatin-treated patients ( $P=3.1\times 10^{-138}$  to  $1.5\times 10^{-200}$ ). Transcripts for *ZFP36* and *SOCS3* that encode for proteins that inhibit proinflammatory cytokines were increased, whereas transcripts for *SPHK1* that stimulates proinflammatory cytokines and *CXCL8* that recruits neutrophils were decreased in ECs incubated with sera from atorvastatin-treated patients ( $P=1.6\times 10^{-32}$  to  $8.3\times 10^{-56}$ ). Levels of *NOS3* were significantly decreased, and levels of *CXCL8* were significantly increased, in HUVECs incubated with acute, pretreatment sera compared with late convalescent healthy control sera or no sera.

### DEGs in Whole Blood From Patients Treated With or Without Atorvastatin

To determine whether the dramatic changes seen in ECs incubated with sera from patients were also evident in the circulation, we analyzed the whole blood transcriptome from the same 10 individuals whose sera were used for the EC experiments. Principal component analysis of RNA-seq data showed a trend toward grouping of all the subacute data regardless of atorvastatin treatment (pretreatment versus subacute [Figure S6]). Unsupervised clustering of the top 100 DEGs revealed a separation trend of the acute and subacute samples regardless of atorvastatin treatment. The DEGs included genes related to neutrophil activation (*CD177* and *S100A9* [*S100 calcium-binding protein A9*]) and other inflammation-associated genes (*hexokinase 3* [*HK3*], *NLR family CARD domain containing 4* [*NLRC4*], *Toll-like receptor 5* [*TLR5*], and *IL1B*). All DEGs were lower at the subacute time point, except for 1 outlier patient, whose acute DEG pattern clustered with the subacute samples on the heat map. At the subacute time point, no genes were differentially expressed comparing subacute whole blood RNA from patients treated with IVIG plus infliximab with or without atorvastatin (adjusted  $P<0.05$  and absolute fold change  $>2$ ).

### Validation of DEGs

We validated results from RNA-seq analysis in independent sera. Pharmacokinetic analysis from the statin clinical trial showed that levels of atorvastatin and its metabolite, orthohydroxyatorvastatin, reached a peak 1 to 2 hours after an oral dose and levels rapidly declined to  $<10\%$  by 12 hours.<sup>16</sup> Therefore, we incubated ECs with sera from patients with KD who had taken atorvastatin 5, 6, 16, and 32 hours before phlebotomy. As controls, we chose age- and illness day-matched patients with KD treated with IVIG alone or IVIG and infliximab without atorvastatin (Table S1). As anticipated, transcript levels for *NOS3*, *KLF4*, and *ZFP36* were increased (pretreatment versus poststatin treatment:



**Figure 2. RNA-sequencing (RNA-seq) transcript levels from selected genes.**

**A and F,** Predicted effects of atorvastatin treatment are shown in flowcharts. Transcript levels of each endothelial cell (EC) experiment are shown for *NOS3* (**B**), *KLF4* (**C**), *TGFB2* (**D**), and *DKK1* (**E**) (genes in flowchart **A**) and *ZFP36* (**G**), *SOCS3* (**H**), *SPHK1* (**I**), and *CXCL8* (**J**) (genes in flowchart **F**). RNA-seq transcript levels of ECs treated with pooled pretreatment sera and posttreatment without or with atorvastatin are shown in the left panel for each gene. Transcript levels of ECs treated with individual patient pretreatment sera, late convalescent (healthy control) sera, and no serum controls in triplicate are shown in the right panel for each gene. Transcript levels are expressed as fold change normalized to pretreatment median values for each experiment. Adjusted *P* values (Padj) are shown comparing posttreatment transcript levels with or without atorvastatin (left panels) or comparing pretreatment transcript levels with healthy controls (right panels). EndMT indicates endothelial-to-mesenchymal transition; IFX, infliximab; and IVIG, intravenous immunoglobulin.

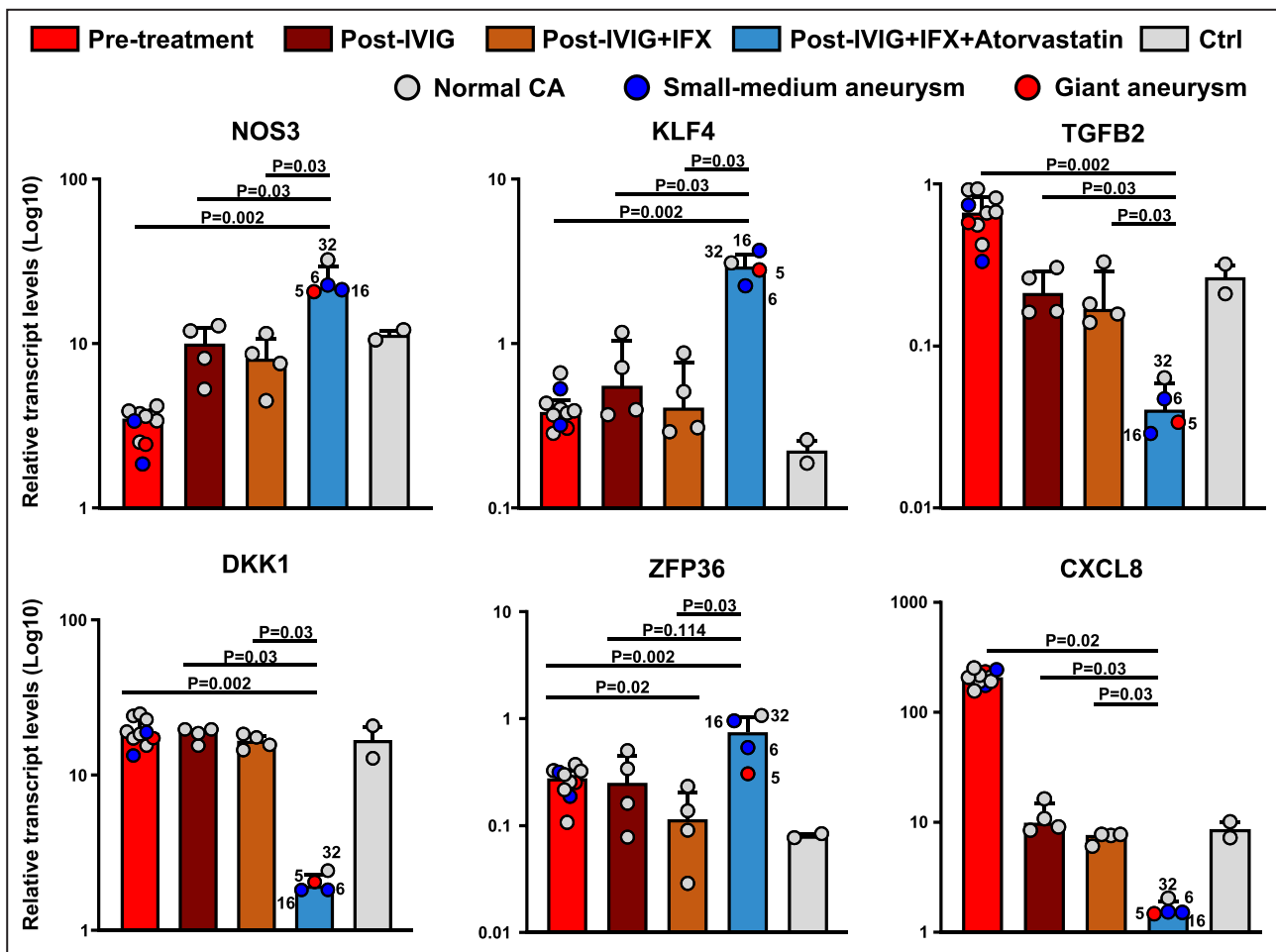
$P=0.002$  for *NOS3* and *KLF4*, and  $P=0.02$  for *ZFP36*; posttreatment IVIG and infliximab versus IVIG, infliximab, and atorvastatin:  $P=0.03$  for all transcripts) (Figure 3). Interestingly, the transcript levels of *DKK1*, *TGFB2*, and *CXCL8* were reduced by statin treatment, compared with treatment without statin (pretreatment versus poststatin treatment:  $P=0.002$ ; posttreatment with versus without atorvastatin:  $P=0.03$  for all molecules) (Figure 3). Although sera from patients treated with IVIG and infliximab without atorvastatin increased gene expression for *NOS3* ( $P=0.002$ ) and decreased gene expression for *TGFB2* ( $P=0.002$ ) compared with pretreatment, the differences were smaller than those with atorvastatin (Figure 3). There was no difference in transcript levels among patients who took atorvastatin

between 5 and 32 hours before phlebotomy (Figure 3). A patient with giant aneurysms had lower *ZFP36* transcript levels after treatment, but no other differences were noted between patients with and without aneurysms (Figure 3).

## DISCUSSION

The experiments reported herein demonstrate that atorvastatin treatment of patients during the acute phase of KD blocks the deleterious effect of pretreatment sera on cultured ECs, characterized by the reduced expression of *NOS3* and the increased expression of *CXCL8*. These findings may have





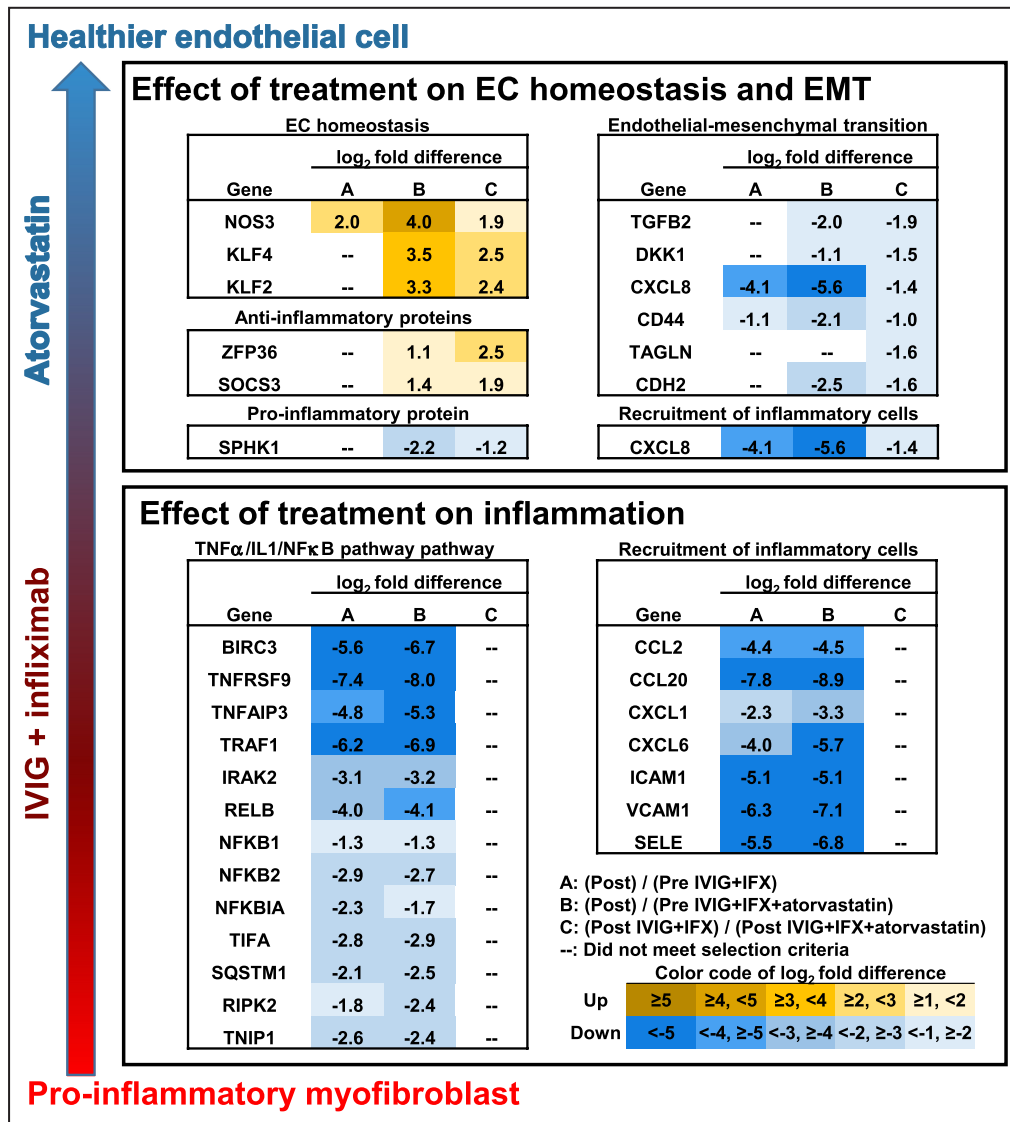
**Figure 3.** Reverse transcriptase–polymerase chain reaction (RT-PCR) validation in independent patient sera analyzed individually.

*NOS3*, *KLF4*, *TGFB2*, *DKK1*, *ZFP36*, and *CXCL8* for RT-PCR. Red dot indicates patient with giant aneurysm with Z score=11, blue dots indicate patients with small-medium coronary artery (CA) aneurysms, and gray dots indicate normal CA. Numbers next to individual dots show the hours between atorvastatin administration and phlebotomy. Mann-Whitney tests were used to compare between 2 groups if Kruskal-Wallis test was significant across groups. Ctrl indicates control; IFX, infliximab; and IVIG, intravenous immunoglobulin.

implications for the use of atorvastatin to preserve EC homeostasis in children with acute vascular inflammation attributable to KD. Although a beneficial transition in gene expression patterns was seen with IVIG alone or IVIG with infliximab, the modification of the serum by atorvastatin treatment had a more profound effect on EC expression of genes associated with restoring EC homeostasis.

KD is an acute vasculitis associated with activated ECs that express adhesion molecules and recruit inflammatory cells to the vessel wall. The data presented herein demonstrate that pretreatment sera from patients with acute KD cause ECs to upregulate transcription of proinflammatory molecules, which was suppressed 2 weeks after patient treatment with IVIG and infliximab. A similar level of suppression was seen in experiments using sera after atorvastatin treatment, but additional

effects promoting EC homeostasis were also noted (Figure 4). One beneficial effect of atorvastatin was the suppression of genes in the TGF- $\beta$  pathway, with downregulation of *TGFB2* among the top 100 DEGs in the comparison of pretreatment versus posttreatment (IVIG+infliximab+atorvastatin) and posttreatment IVIG+infliximab versus IVIG+infliximab+atorvastatin. *TGFB2*, which encodes 1 of the 3 isoforms of TGF- $\beta$ , has been previously implicated in KD susceptibility and aneurysm formation.<sup>24</sup> Although TGF- $\beta$ 2 transcript levels were not increased in cultured ECs treated with pretreatment sera, immunohistochemical staining of the coronary arteries from autopsy cases with acute KD (illness day 12–38) demonstrated highly expressed TGF- $\beta$ 2 in inflammatory cells and spindle-shaped cells in the intima and adventitia.<sup>4</sup> TGF- $\beta$  receptor 2 and phosphorylated (activated) SMAD family member 3



**Figure 4. Effect of atorvastatin treatment added to intravenous immunoglobulin (IVIG) and infliximab (IFX) on endothelial cells (ECs).**

Representative genes with cutoff value ( $P < 0.0001$  and absolute fold change  $> 2$ ) were chosen on the basis of their biologic importance in pathways highlighted in the Enricher analysis. EMT indicates epithelial-to-mesenchymal transition; IL-1, interleukin-1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; and TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

(SMAD3) were also expressed in the spindle-shaped cells in the intima and adventitia, which suggested that the TGF- $\beta$  pathway was activated in myofibroblasts in the arterial wall. Because TGF- $\beta$  is a potent regulator of EndMT, the lower *TGFB2* transcript levels in ECs associated with atorvastatin treatment may reduce EndMT. In contrast, efforts to reduce TGF- $\beta$  signaling with angiotensin receptor blocking agents in KD mouse models produced conflicting results.<sup>25,26</sup> This may be attributable, in part, to the pleiotropic effects of TGF- $\beta$  on different cell types. For example, TGF- $\beta$  induces the differentiation of naïve T cells to a regulatory phenotype, which likely plays an important role in reducing inflammation in acute KD. Transcriptome analysis

using whole blood RNA showed no difference in TGF- $\beta$  expression between pretreatment versus posttreatment (IVIG+infliximab+atorvastatin) or posttreatment IVIG+infliximab versus IVIG+infliximab+atorvastatin. Therefore, downregulation of TGF- $\beta$  signaling by atorvastatin may be restricted to ECs, and not manifested in whole blood gene expression.

A second potentially important effect of atorvastatin was the upregulation of *NOS3*, which encodes a key molecule in EC homeostasis. *NOS3* is the principle enzyme that produces NO in ECs and has important roles in vascular homeostasis, angiogenesis, and reduction of leukocyte adhesion and platelet aggregation.<sup>27</sup> The transcription factors *KLF4* and *KLF2* regulate

expression of NOS3.<sup>28</sup> NOS3, KLF4, and KLF2 were in top 100 DEGs in the comparison between post-treatment with or without atorvastatin. We previously reported that ECs incubated with sera from patients with KD treated with atorvastatin led to increased levels of KLF4 transcripts and protein.<sup>17</sup> Thus, the findings in the present study are consistent with these previous observations that atorvastatin treatment during acute KD promotes EC homeostasis through KLF2/4-NOS3 pathways.

DKK1, a secretory molecule and inhibitor of the WNT signaling pathway, was represented in the upregulated TGF- $\beta$  pathway. Atorvastatin is known to reduce the levels of DKK1 transcript and protein in HUVECs in a dose-dependent manner.<sup>29</sup> It has been estimated that ~27% of protein levels that are influenced by atorvastatin in HUVECs may be mediated through DKK1 reduction. Therefore, DKK1 has emerged as a key molecule regulated by atorvastatin. Although DKK1 is a WNT inhibitor, the WNT pathway also upregulates DKK1 transcription,<sup>30</sup> suggesting that DKK1 is important for fine-tuning the WNT pathway. Because the WNT pathway together with the TGF- $\beta$  pathway are important for EndMT and fibrosis, regulation of DKK1 by atorvastatin might reduce CA fibrosis that occurs in the patients with KD with aneurysms.<sup>3,5</sup>

A third potential effect mediated by atorvastatin was reduction of transcripts associated with inflammation. Inflammation-related genes were differentially expressed compared with posttreatment with and without atorvastatin treatment (Figure 2). The expression of the anti-inflammatory genes, ZFP36 and SOCS3, was significantly increased with atorvastatin, whereas expression of the proinflammatory genes, SPHK1 and CXCL8, was significantly suppressed.

We recognize several limitations to these experiments. First, a direct clinical effect of atorvastatin treatment on CA outcome was difficult to demonstrate in vivo given the small number of patients available for study. In the absence of robust clinical trial data, this ex vivo experimental system allowed us to probe changes in EC homeostasis specifically linked to exposure to sera from matched patients treated with or without atorvastatin. We cannot exclude the possibility that the beneficial effects of sera from atorvastatin-treated patients on cultured ECs were a direct result of low concentrations of atorvastatin in the serum versus the changes in mediators of inflammation and EndMT that resulted from atorvastatin treatment. Atorvastatin enhances proliferation, migration, and tube formation of ECs at low concentrations (0.005–0.01  $\mu\text{mol/L}$ ). On the basis of our pharmacokinetic study, we predicted the concentration of atorvastatin in sera used in our experiments to be between 0.002 and 0.035  $\mu\text{mol/L}$  because we collected sera from patients with KD 5 to

32 hours after atorvastatin administration. As another limitation, we recognize the sex imbalance between the treatment groups whose sera were used in these experiments, and we cannot exclude the possibility that these differences also contributed to the difference in gene transcription profiles. In addition, although the endothelium is the point of entry of inflammatory cells into the arterial wall and is clearly impacted in acute KD, we did not study the effect of atorvastatin on vascular smooth muscle cells or cardiomyocytes. Finally, the use of HUVECs instead of arterial ECs might be considered an experimental limitation. However, newborn ECs may in fact more appropriately model the pediatric ECs relevant for this patient population, and the flow pattern in the umbilical vein simulates arterial flow.<sup>31</sup> In addition, a recent article demonstrated that HUVECs and arterial ECs respond to environmental stimuli with similar changes in transcriptional profiles.<sup>32</sup>

Although the American Heart Association guidelines encourage adjunctive therapy for patients with KD with early signs of CA damage, there are currently no clinical trial data to guide best practice to stop the progression of arterial wall damage and prevent CA aneurysm formation during the acute phase of KD.<sup>18</sup> We demonstrated deleterious changes in EC transcription that were mitigated by exposure to sera from patients treated with atorvastatin. The anti-inflammatory and endothelial-protective effects of atorvastatin may yield benefit in blocking CA aneurysm progression in patients with KD when added to standard therapy.

## ARTICLE INFORMATION

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### Affiliations

Department of Pediatrics (C.S., A.H.T., H.M.H., J.C.B.), Department of Biomedical Informatics (J.K.), and Department of Medicine (M.H., J.Y.-J.S.), University of California, San Diego, La Jolla, CA; and Rady Children's Hospital, San Diego, CA (A.H.T., H.M.H., J.C.B.).

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### Disclosures

None.

### Supplemental Material

Table S1–S5

Figures S1–S6

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## **SUPPLEMENTAL MATERIAL**



**Table S1a. Characteristics of atorvastatin treated patients and matched control KD patients who received only IVIG and IFX or IVIG alone.**

**Table S1b. Summary of demographic and clinical characteristics of patients with KD whose whole blood RNA was used in the validation RT-PCR assays.**

**Table S2. Comparison of transcripts in HUVEC lysates after incubation with pre- and post-treatment sera from patients treated with IVIG and IFX.**

**Table S3. Comparison of transcripts in HUVEC lysates after incubation with pre- and post-treatment sera from patients treated with IVIG, IFX and atorvastatin.**

**Table S4. Top pathway of DEGs.**

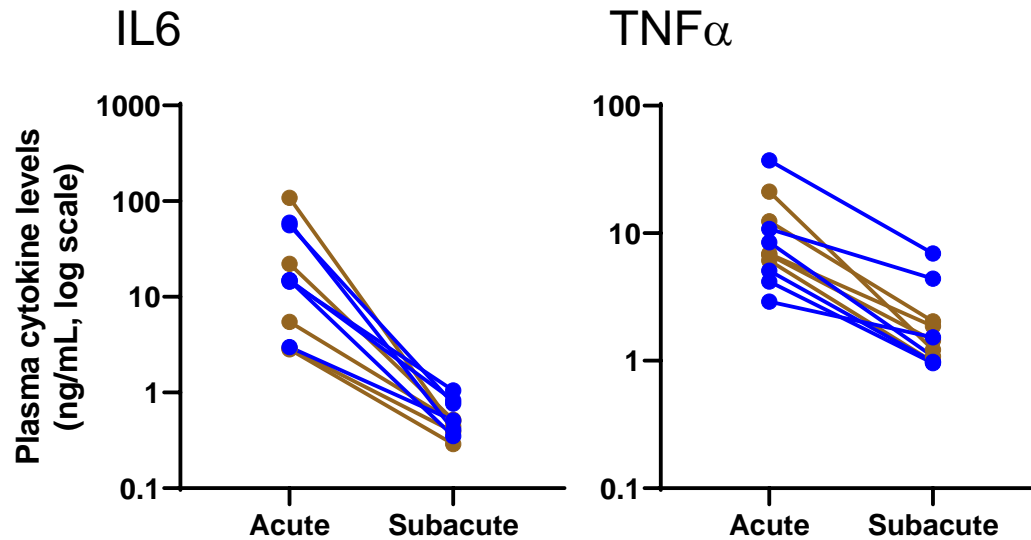
**Table S5. Comparison of transcripts in HUVEC lysates after incubation with sera from patients treated with or without atorvastatin.**

**Figure S1. Plasma TNF $\alpha$  and IL6 levels in the patients studied by RNAseq and RT-PCR.**

TNF $\alpha$  and IL6 levels were measured using mesoscale discovery (MSD) (V-PLEX Human Proinflammatory Panel I) on six of nine patients treated with IVIG+IFX+atorvastatin (3 RNAseq and 3 validation) and five of nine patients treated with IVIG+IFX (4 RNAseq and 1 validation).

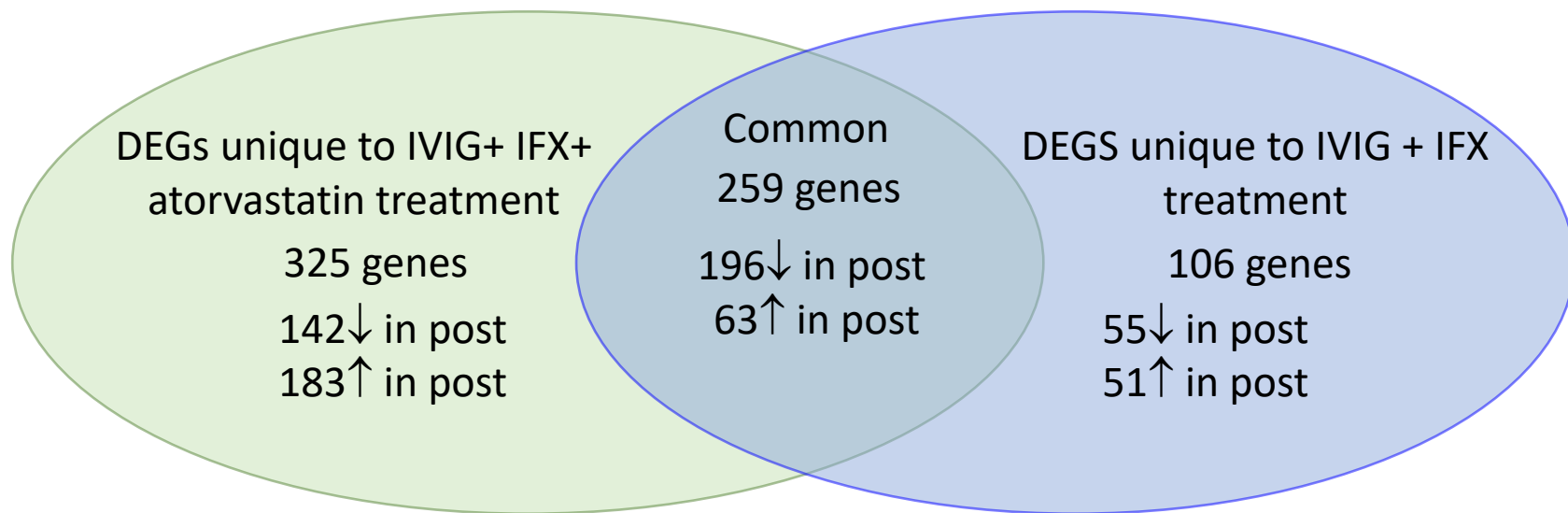
IL-6 levels: no statistically significant difference between IVIG+IFX+atorvastatin vs IVIG+IFX (Acute p=0.54, Subacute p=0.25)

THF $\alpha$  levels: no statistically significant difference between IVIG+IFX+atorvastatin vs IVIG+IFX (Acute p=0.93, Subacute p=0.97)



**Figure S2. Venn diagram of differentially expressed genes (DEGs) between pre-treatment and post-treatment.**

Venn diagram shows number of DEGs between pre-treatment and post-treatment (with IVIG and IFX or with IVIG, IFX and atorvastatin) with adjusted p-value < 0.0001, absolute fold change > 2 and base mean counts >2433.22, Chr.1-22 (excluded X and Y). The overall count is the mean of normalized counts across all samples and is calculated per gene.



DEGs: Differentially expressed genes







**Figure S5. DKK1 protein levels in EC culture medium.**

Secreted DKK1 protein levels measured in culture media. Red dot indicates patient with giant aneurysm with Z score=11, blue dots indicate patients with small-medium coronary aneurysms and gray dots indicate normal CA. Numbers next to individual dots show the hours between atorvastatin administration and phlebotomy. Mann-Whitney tests were used to compare between two groups if Kruskal-Wallis test was significant across groups.

