

LCC-09, a Novel Salicylanilide Derivative, Exerts Anti-Inflammatory Effect in Vascular Endothelial Cells

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Objective: Endothelial cell (EC) activation facilitates leukocyte adhesion to vascular walls, which is implicated in a variety of cardiovascular diseases and is a target for prevention and treatment. Despite the development of anti-inflammatory medications, cost-effective therapies with significant anti-inflammatory effects and lower organ toxicity remain elusive. The goal of this study is to identify novel synthetic compounds that inhibit EC inflammatory response with minimal organ toxicity.

Methods and Results: In this study, we discovered LCC-09, a salicylanilide derivative consisting of the functional fragment of magnolol, 2,4-difluorophenyl, and paeonol moiety of salicylate, as a novel anti-inflammatory compound in cultured ECs and zebrafish model. LCC-09 was shown to inhibit pro-inflammatory cytokine tumor necrosis factor- α (TNF α)-induced expression of adhesion molecules and inflammatory cytokines, leading to reduced leukocyte adhesion to ECs. Mechanistically, LCC-09 inhibits the phosphorylation of signal transducer and activator of transcription 1 (STAT1), TNF α -induced degradation of NF- κ B Inhibitor- α (I κ B α) and phosphorylation of NF κ B p65, resulting in reduced NF κ B transactivation activity and binding to E-selectin promoter. Additionally, LCC-09 attenuated TNF α -induced generation of reactive oxygen species in ECs. Molecular docking models suggest the binding of LCC-09 to NF κ B essential modulator (NEMO) and Janus tyrosine kinase (JAK) may lead to dual inhibition of NF κ B and STAT1. Furthermore, the anti-inflammatory effect of LCC-09 was validated in the lipopolysaccharides (LPS)-induced inflammation model in zebrafish. Our results demonstrated that LCC-09 significantly reduced the LPS-induced leukocyte recruitment and mortality of zebrafish embryos. Finally, LCC-09 was administered to cultured ECs and zebrafish embryos and showed minimal toxicities.

Conclusion: Our results support that LCC-09 inhibits EC inflammatory response but does not elicit significant toxicity.

Keywords: endothelial cells, inflammation, salicylanilide derivative, tumor necrosis factor- α , lipopolysaccharides, toxicity

Introduction

Vascular endothelial cells (ECs) form a non-adhesive, highly selective physical barrier between blood flow and vessel wall, and regulate vascular tone and remodeling through secretion of multiple vasoactive substances.¹ Upon stimulation with diverse numbers of pro-inflammatory factors, EC activation is induced to mediate leukocyte adhesion, increase vascular permeability and thrombosis, all of which have been recognized as key early events of a variety of cardiovascular diseases.^{2,3} Although current anti-inflammatory therapies including statins and human anti-interleukin-1 β monoclonal

antibody have been convincingly shown to improve disease outcomes, they are costly and are associated with significant side-effects.⁴⁻⁸ Thus, the development of a more cost-effective anti-inflammatory therapeutic strategy with minimal organ toxicity is still necessary for the prevention and treatment of cardiovascular diseases.

In this study, we discovered LCC-09 as a novel synthetic compound with anti-inflammatory effects. LCC-09 is a 5-(2',4'-difluorophenyl)-salicylanilide derivative consisting of the functional fragment of magnolol, 2,4-difluorophenyl, and paeonol moiety of salicylate, with the International Union of Pure and Applied Chemistry (IUPAC) name N-(3-cyanophenyl)-2',4'-difluoro-4-hydroxy[1,1'-biphenyl]-3-carboxamide.^{9,10} LCC-09 was initially designed and synthesized to block multiple signaling pathways to inhibit osteoclastogenesis and growth of glioma stem cells.^{9,10} It was recently observed to relieve disease severity and chronic pain in the mouse rheumatoid arthritis model, indicating it has a potential anti-inflammatory effect.¹¹

To study the role of LCC-09, we utilized a tumor necrosis factor- α (TNF α)-induced EC inflammation model in vitro. TNF α is widely accepted as a major inducer of EC inflammatory response during the pathogenesis of multiple cardiovascular diseases,^{12,13} it binds to membrane receptors to elicit multiple downstream signaling pathways including NF κ B and Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT).¹⁴⁻¹⁶ Additionally, we have examined the effect of LCC-09 on leukocyte recruitment and extravasation in the zebrafish lipopolysaccharide (LPS)-induced inflammation model. To further evaluate the cytotoxic effect of LCC-09, we studied how LCC-09 affected cell viability of cultured ECs and development of zebrafish embryos.

Zebrafish have been widely used for high-throughput drug screening studies for human inflammatory diseases because of the similarity of immune cell lineages to mammals, high fecundity, rapid ex utero development, and optical transparency of embryos and larvae.^{17,18} Our results collectively support that LCC-09 is a novel anti-inflammatory compound with minimal cardiac toxicity which suppresses EC inflammatory response in vitro and in vivo through dual inhibition of NF κ B and STAT1 activation.

Materials and Methods

Cells, Chemicals and Reagents

HUVECs (Lonza) were passaged in endothelial cell basal medium supplemented with EGM-MV SingleQuots (Lonza). In all experiments, HUVECs within five passages were used

and randomized for groups in the experiments. Group sizes are equal by design. THP-1 (ATCC) were grown in RPMI 1640 containing 10% FBS. LCC-09 (Figure 1A) was synthesized as previously described.¹⁰ Stock solution of LCC-09 were dissolved in dimethyl sulfoxide (20 mM) (DMSO; Sigma Aldrich) and stored at -20°C away from light, then diluted in sterile culture medium just before use. Recombinant human TNF α protein was purchased from R&D. Adenoviruses bearing an NF- κ B luciferase reporter were used as previously described¹⁹ and luciferase activity was examined with a luciferase assay kit according to the manufacturer's instructions (Promega). CellroX Green and Hoechst 33342 were obtained from Thermo Fisher Scientific. In-situ staining of FTIC Annexin V and PI (BioLegend) was used to analyze cell apoptosis. Cell viability was analyzed with a CellTiter Aqueous One Solution Cell Proliferation Assay (MTS) from Promega.

Leukocyte Adhesion Assay

HUVECs were pretreated with LCC-09 and then exposed to TNF α for 20h, after which the medium was removed and replaced with fresh medium. Then, HUVECs were co-cultured with THP-1 cells (ATCC) labeled with CellTracker™ Green CMFDA Dye (0.6 μM , Thermo Fisher Scientific) for 30 min. The adhered cells were fixed with 4% paraformaldehyde for 10 minutes and unbound THP-1 cells were removed by washing. The adhered cells were imaged with EVOS Imaging System (Thermo Fisher Scientific) and blindly counted.

Zebrafish Model

Zebrafish were maintained and raised according to the protocol described by Dr. Westerfield.²⁰ Embryos were collected after natural spawning and the unfertilized eggs were removed. Fertilized embryos were cultured at 28.5°C in clean Petri dishes in egg water containing 60 mg/L "Instant Ocean" salt and 2 mg/L methylene blue (Sigma) and randomized to groups. To inhibit skin pigment formation, 0.003% 1-phenyl-2-thiourea (PTU) (Sigma) was added to the egg water after 12 h. All fish experiments have been approved by IACUC at Mayo Clinic. The fish water qualities including pH, conductivity, hardness, and ammonium level were monitored daily. The fish are housed in 2.5L or 6L transparent polycarbonate tanks according to their population densities. The zebrafish housing units are fully integrated with filter systems, germicidal irradiation (UVC) and light, temperature control units, and a recirculating water system which pumps feed

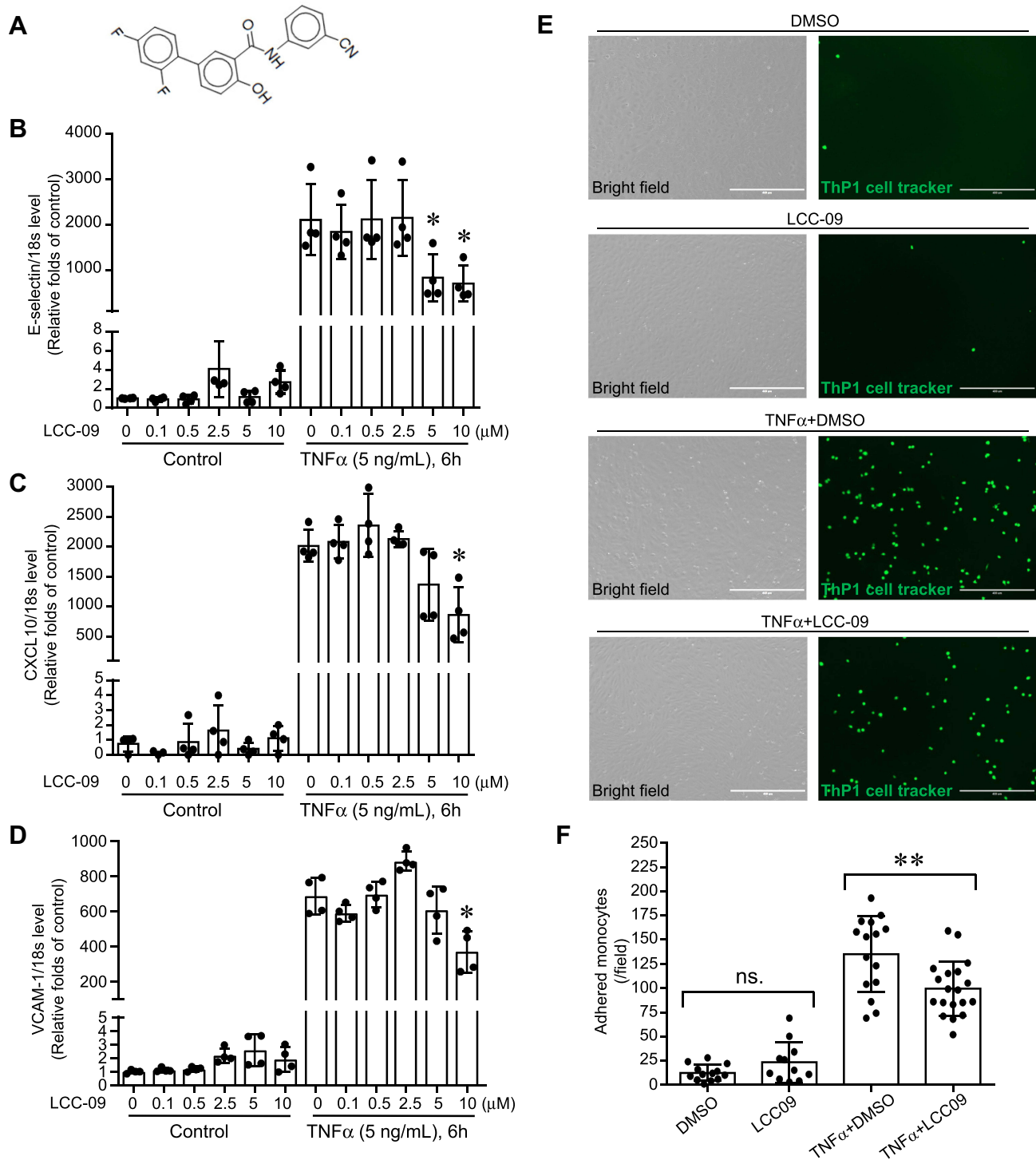


Figure 1 LCC-09 reduces TNF α -induced leukocyte adhesion on endothelial cells. **(A)** Chemical structure of LCC-09. **(B–D)** HUVECs were pre-treated with LCC-09 or control DMSO vehicle control for 30 mins at the indicated concentrations and then stimulated with TNF α (5 ng/mL) for 6 h. qPCR was performed (N=4 per group) and expressed as relative folds of control group, which was normalized to 1. **(E and F)** HUVECs were co-cultured with LCC-09 (5 μ M) or DMSO vehicle control for 30 mins and then stimulated with TNF α (5 ng/mL) for 20 h. Then HUVECs were co-cultured with CMFDA-labeled monocytic THP1 cells for 30 mins and adhered monocytes were imaged **(D)** and counted **(E)**. * $p < 0.05$; ** $p < 0.01$.

water into the tanks. The wastewater is partially purified before being recirculated. Humane endpoints include morphological abnormalities such as swollen abdomen, skin discoloration, frayed fins, etc., and physical abnormalities, such as difficult swimming and reaching food, lack of balance, etc. At the experimental endpoint, fish were euthanized using a chemical overdose of 0.04% tricaine as approved by the Mayo Clinic IACUC.

To examine the role of LCC-09 *in vivo*, we used the transgenic zebrafish myeloperoxidase promoter (MPO):GFP line,²¹ a generous gift from Dr. Stephen C Ekker's laboratory at Mayo Clinic, Rochester, which expresses green fluorescent protein in neutrophils under the control of the myeloperoxidase promoter. At 48 hours post fertilization (hpf), the MPO:GFP zebrafish larvae were anesthetized using 0.015% tricaine and LPS (0.4 mg/mL, 2 nL) was injected either into the yolk or the blood circulation via the caudal vein. The control group was injected with phosphate-buffered saline (PBS). Microinjection (PLI-100, Harvard Apparatus, Inc., Massachusetts, MA, USA) was performed at a volume of 2 nL per larva. The injected larvae were then cultured at 28.5 °C and observed for signs of disease and mortality. Prior to the LPS injection, larvae were treated with 5 µM LCC-09 by static immersion in the egg water for 3 h in a total volume of 1 mL in a 12 well plate. Control larvae were exposed to DMSO in regular egg water. The larvae were observed under the fluorescence microscope to visualize the GFP-labelled neutrophil accumulation at the specified time points. To quantify neutrophil accumulation after LPS injection in yolk sac, the fluorescent intensity was measured. Group sizes are equal by design in all the zebrafish experiments. However, more embryos were included in the LPS injection groups as we expected mortality induced by the LPS, which resulted in unequal group sizes. Additionally, zebrafish embryos which were not properly imaged due to improper orientation were excluded from the data analysis, and we have taken into consideration every fish in each group which was in similar orientation at the time of data acquisition. Residual dark region (Figure 2B, bright field image) and fluorescent signal foci (Figure 2B, fluorescent image) in the zebrafish yolk region were observed after LPS injection, which is likely caused by necrosis of neutrophil and yolk tissues as previously reported,²² but was not quantified in the current study.

In the cardiotoxicity analysis, WT AB zebrafish embryos received from Zebrafish International Resource

Center at 6 hpf were incubated with 10 µM LCC-09 in the water for 48 h and 72 h, respectively. Five zebrafish embryos from each group were randomly selected for visual observation and imaging acquisition of specific phenotype under the microscope (Zeiss Observer Z.1). The occurrence of pericardial edema and abnormal circulation were thereby evaluated by blinded observation. The heart rate was counted for 60 seconds, and heart rhythm was also monitored for any potential arrhythmia, as described previously.²³

Western Blotting

HUVECs were lysed with RIPA lysis buffer supplemented with proteinase inhibitor and phosphatase inhibitor cocktails (Thermo Fisher Scientific). Protein extracts were resolved in 10% SDS-PAGE gels and electroblotted to PVDF membranes (BioRad), which was performed by an investigator who is blinded to the group information. Membranes were blocked for 1 hour at room temperature in TBST containing 5% (weight/vol) non-fat dry milk and incubated overnight with primary antibodies. After TBST washing, membranes were incubated with a Horseradish Peroxidase-conjugated secondary antibody (Santa Cruz) diluted at 1:10000 for 1 hour and scanned using ChemiDoc Image System. To control unwanted source of variation, the intensity of the protein bands was quantified using Image Lab Software (BioRad) and expressed as relative folds of control group which was normalized to 1 for comparison (Figure 3B–D). Antibodies against NFκB p65 (#4764, 1:1000), phosphorylated p65 S536 (#3031, 1:1000), STAT1 (#9172, 1:1000), phosphorylated STAT1 Y701 (#9167, 1:1000), STAT3 (#4904, 1:1000), phosphorylated STAT3 Y705 (#9131, 1:1000), JNK (#9258, 1:1000), phosphorylated JNK T183/Y185 (#4668, 1:1000), ERK (#9102, 1:1000), phosphorylated ERK T202/Y204 (#9101, 1:1000), p38 (#8690, 1:1000) and phosphorylated p38 T180/Y182 (#4511, 1:1000), and IκBα (#2682, 1:1000), were purchased from Cell Signaling Technology. The antibody against β-Actin (A2228; 1:5000) was purchased from Sigma-Aldrich.

qPCR

Total RNA was isolated from HUVECs 6 h post TNFα stimulation with the RNeasy Mini Kit (Qiagen) by an investigator who is blinded to the group information. Previous studies suggest that TNFα-induced E-selectin peaked at 2–4 hours, while VCAM-1 peaked at later

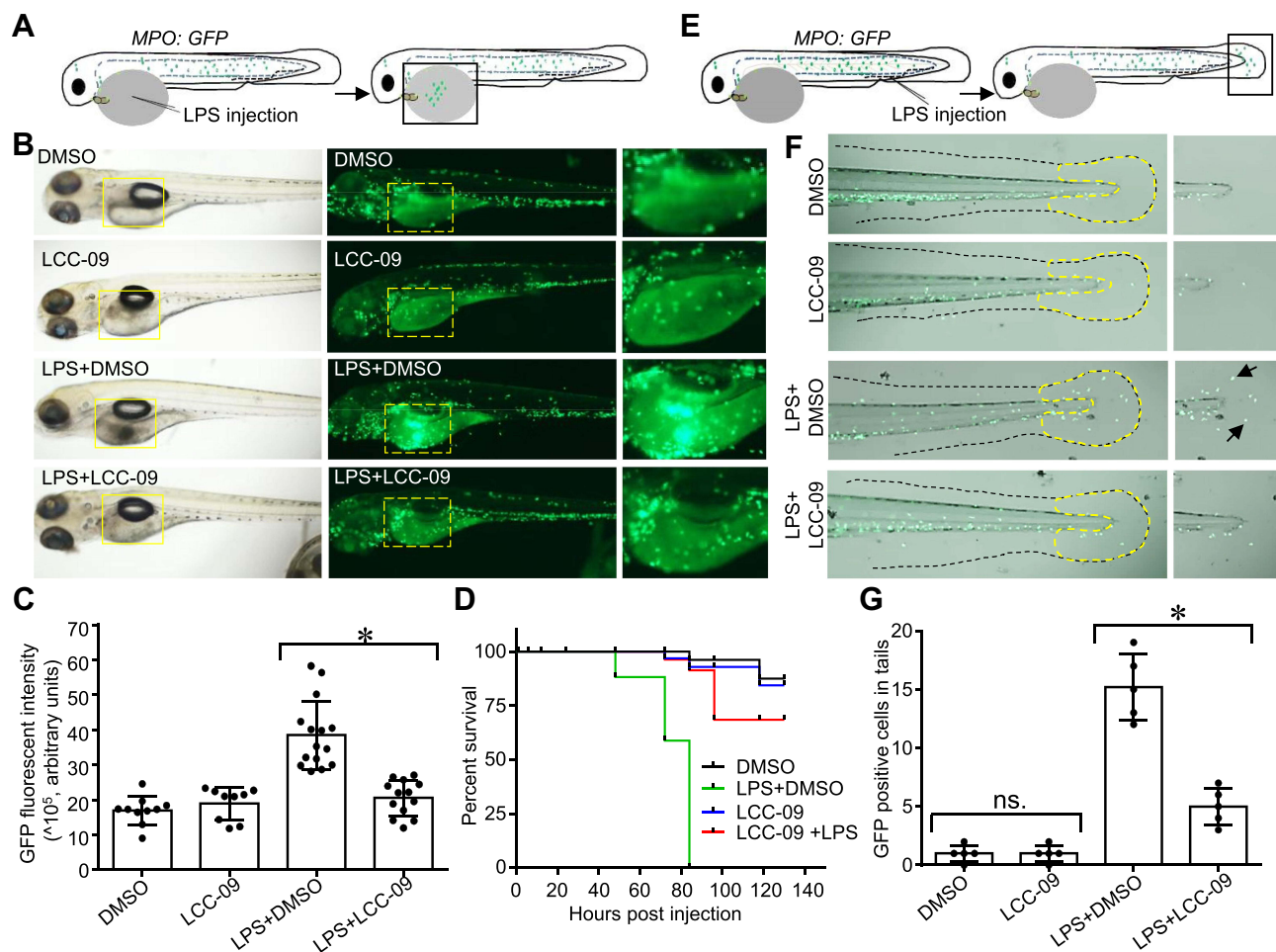


Figure 2 LCC-09 inhibits LPS-induced inflammatory response in zebrafish. (**A–D**) Zebrafish (MPO;GFP) embryos at 48 hpf were treated with LCC-09 (5 μ M) and DMSO as control for 3 h and then injected with LPS (0.4 mg/mL, 2 nL) through yolk sac. Images were collected 12 h after injection (**B**). Local accumulation of neutrophils was quantified using GFP fluorescent intensity and compared (**C**). Zebrafish survival was monitored (N=8 per group) (**D**). (**E–G**) LPS (0.4 mg/mL, 2 nL) was intravenously injected to the caudal vein of zebrafish (MPO;GFP) embryos at 48 hpf. Images were collected 6 h post-injection. Neutrophil accumulation in tail fins (outlined by yellow dotted dash lines) was counted and compared. Arrow denotes infiltration of GFP positive neutrophils induced by LPS injection. * $p < 0.05$.

times (12–24h).²⁴ Thus, 6 h time point has been used in many previous studies to evaluate the expression of adhesion molecules and inflammation cytokines after TNF α stimulation.^{25–27} RNA was reverse-transcribed by using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed with a TaqMan SYBR Green Master Mix (Applied Biosystems). The comparative cycle threshold method was used to calculate the relative abundance of E-selectin mRNA to 18s ribosome RNA expression. E-selectin forward primer: 5'-CTGTGCACTGGAAAGCTTCA-3', reverse primer: 5'-AGCCCAGGTTGAATGCAC-3'; CXCL10 forward primer: 5'-AACCAGAGGGGAGCAAAATC-3', reverse primer: 5'-CTGTGTGGTCCATCCTTGG-3'; VCAM-1 forward primer: 5'-ACT CCG CGG TAT CTG CAT-3', reverse primer: 5'-TTT GTG TCC CAC CTG TGT GT-3'; 18s

forward primer: 5'-GTAACCCGTTGAACCCCAT-3', 18s reverse primer: 5'-CCATCCAATCGGTAGTAGCG-3'. Technical replicates were used in qPCR experiments to ensure reliability of single values, but single values from each experimental sample were presented and analyzed. To control unwanted source of variation, the qPCR data was expressed as relative folds of control group which was normalized to 1 (Figures 1B–D and 3C; Supplemental Figure 1).

ChIP Assay

HUVECs were pretreated with LCC-09 or DMSO as control for 30 mins and then stimulated with TNF α (5 ng/mL) for 2 h. Cells were then cross-linked with 1% formaldehyde, lysed in buffer (Tris-HCl 50 mM, SDS 1%, EDTA 5 mM, pH 7.0), and then sonicated. The sheared chromatin

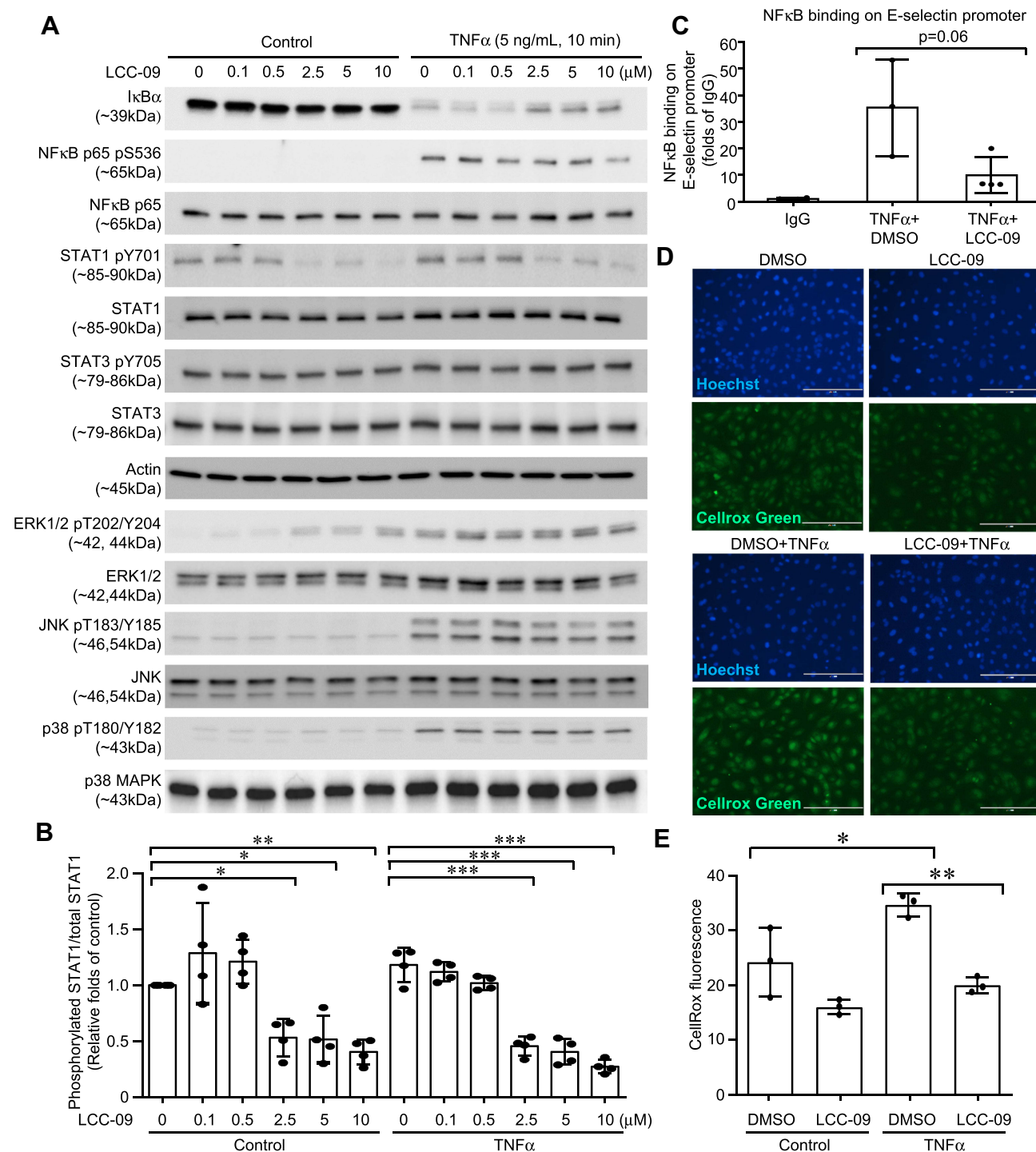


Figure 3 LCC-09 inhibits activation of NFκB and STAT1, and ROS generation in endothelial cells. HUVECs were pre-treated with LCC-09 and control DMSO for 30 mins at the indicated concentrations and then stimulated with TNFα (5 ng/mL). (**A** and **B**) Cell lysates were subjected to Western blotting. (**C**) ChIP was performed to examine the binding of NFκB to E-selectin promoter 2 h after TNFα stimulation. (**D** and **E**) Cells were stained with CellRox Green and imaged (**D**). Fluorescent intensity was measured with a SpectraMax plate reader and compared (**E**) 30 mins after TNFα stimulation. *p<0.05; **p<0.01; ***p<0.001.

was immunoprecipitated with Dynabeads (R&D) conjugated with control IgG or NFκB p65 primary antibodies (#sc-109, Santa Cruz). The eluted immunoprecipitates were incubated at 65 °C for 6 h for reverse formaldehyde

cross-linking. DNA was extracted with a Qiagen PCR purification kit and subjected to qPCR with specific primers which amplify a region in human E-selectin promoter containing a known NFκB binding site required for TNFα-

induced maximum expression of E-selectin.²⁸ Forward primer: 5'-GGCATGGACAAAGGTGAAGT-3', reverse primer: 5'-GGAGGATATTGTCCACATCCAG-3'.

Protein Docking Model

The LCC-09 ligand was prepared by drawing on Avogadro software. The ligand was converted into pdb format using Pymol and converted into pdbqt format using AutoDock Tools 1.5.6. All the protein targets; JAK1 (PDB ID: 6W8I), JAK2 (PDB ID: 6AAJ), JAK3 (PDB ID: 1YVJ), STAT1 (PDB ID: 1BF5) and NEMO (PDB ID: 3BRT) were retrieved from Protein Data Bank as pdb format file, and converted into pdbqt format. The molecular docking experiments were performed by using AutoDock Vina, which is more accurate than Autodock 4.2, has a shorter running time and the ability to process many rotatable bonds. The grid box of 40Å × 40Å × 40Å was generated on defined binding site residues of the protein targets. The protein targets were prepared through deleting water, adjusting hydrogen (polar only) and charges (Kollman charges). All parameters of software were set as default, and all bonds in the ligand are rotated freely, presuming the receptor as rigid. The final visualization of the molecular docking was performed using Pymol.

Statistics

All analyses were performed using GraphPad Prism 5 (GraphPad Software). All the values are expressed as means ± SD. In the figures, the value of each individual sample was represented as a single dot in the scatter graph. Statistical significance was determined using 2-sided Student's *t*-test (2 groups) and one-way ANOVA with Tukey's multiple comparisons test (more than 2 groups). A value of $p < 0.05$ was considered significant.

Results

LCC-09 Reduced TNF α -Induced Leukocyte Adhesion to Endothelial Cells

We first examined the effect of LCC-09 (Figure 1A) on TNF α -induced adhesion molecules and inflammatory cytokines of cultured HUVECs. HUVECs were pretreated with LCC-09 at 0, 0.1, 0.5, 2.5, 5, 10 μ M for 30 mins and then stimulated with TNF α (5 ng/mL) for 6 h. Our results showed that LCC-09 reduced TNF α -induced expression of E-selectin, C-X-C motif chemokine ligand 10 (CXCL10) and vascular cell adhesion molecule 1

(VCAM-1) at 10 μ M (Figure 1B–D). Adhesion molecules and inflammatory cytokines of endothelial cells mediate the recruitment and accumulation of leukocyte to vascular wall,^{29–31} thus we evaluated the effect of LCC-09 on TNF α -induced leukocyte adhesion. Since 5 μ M is the lowest dose of LCC-09 which decreased TNF α -induced adhesion molecules and chemokines, we validated the effect of 5 μ M LCC-09 on leukocyte adhesion. As shown in Figure 1E and F, LCC-09 (5 μ M) did not alter the leukocyte adhesion to control HUVECs, which barely bound any monocytes. However, it significantly reduced the numbers of attached monocytes to HUVECs that were primed with TNF α (135.1±39.3 vs 99.5±28.2, $p < 0.05$). Additionally, LCC-09 (5 μ M) was administered 30 minutes after TNF α stimulation to examine its therapeutic effect. As shown in the Supplemental Figure 1, post-treatment LCC-09 also effectively reduced TNF α -induced expression of CXCL-10 and E-selectin. These results demonstrate LCC-09 attenuates both TNF α -induced upregulation of adhesion molecules and leukocyte adhesion.

LCC-09 Attenuated STAT1 Phosphorylation, and TNF α -Induced Activation of NF κ B and Generation of ROS in Endothelial Cells

To investigate the molecular mechanism through which LCC-09 reduced TNF α -induced inflammatory response in ECs, we examined the activation of downstream-signaling pathways of TNF α . As shown in Figure 3A and Supplemental Figure 2, pre-treatment of LCC-09 for 30 mins did not significantly affect TNF α -induced activation of JNK, ERK1/2 or p38MAPK, but attenuated TNF α -stimulated I κ B α degradation in a dose-dependent manner, which was accompanied with reduced phosphorylation of NF κ B p65. Meanwhile, LCC-09 shows inhibitory effect on phosphorylation of STAT1 both at basal level and upon TNF α stimulation but did not affect phosphorylation of STAT3 (Figure 3A and B). Previous studies have identified one NF κ B binding site in the human E-selectin promoter which is required for its maximum expression induced by TNF α .²⁸ Thus, ChIP assay was performed to assess how LCC-09 affected NF κ B binding on E-selectin gene promoter upon TNF α stimulation. As shown in Figure 3C, LCC-09 significantly reduced the binding of NF κ B p65 on E-selectin promoter. Additionally, we assessed the effect of LCC-09 on intracellular ROS levels, signal transducers that are implicated in endothelial dysfunction.³² Our

results (Figure 3D and E) show that LCC-09 significantly reduced ROS levels after TNF α stimulation but not under basal conditions.

Furthermore, molecular docking was performed to investigate how LCC-09 regulates TNF α -induced NF κ B and STAT1 activation in ECs. In the docking model, LCC-09 was docked as a ligand with several receptors including IKK β and NEMO, JAK1, JAK2, JAK3 and STAT1. The ligand is shown as green stick model and the interaction bonds are illustrated with yellow dash lines in the ligand-receptor interaction model (Figure 4). After visualizing in Pymol, it was found that NEMO is the most promising protein target, which is explained by lowest binding energy of -10.6 kcal/mol with LCC-09 as a ligand, following with JAK1 and JAK3 with binding energy of -10.1 and -10.0 kcal/mol respectively, while IKK β revealed the lowest binding energy of -7.3 kcal/mol (Table 1). Specifically, in the docking model with NEMO, LCC-09 acquired the central pocket of NEMO which leads to several hydrogen bond-mediated interactions between LCC-09 and the amino acid residues of the protein target. The hydrogen in the hydroxyl group and hydrogen in the amine group (in ketone group) shows significant hydrogen bonding of LCC-09 with GLU-89 of NEMO.

LCC-09 Inhibited LPS-Induced Inflammatory Response in vivo

To further define the role of LCC-09 in vivo, we utilized a previously established LPS-induced zebrafish inflammation model, where LPS induces systemic inflammatory response with the release of several inflammatory cytokines, including TNF α , and results in organ dysfunction, multiple organ failure, and death.^{22,33,34} At 48 hpf, transgenic zebrafish Tg (MPO:EGFP), expressing GFP under the neutrophil-specific myeloperoxidase promoter,²¹ were pre-treated with LCC-09 ($5 \mu\text{M}$) for 3 h before receiving a LPS injection (0.4 mg/mL , 2 nL) (Figure 2A). Consistent with previous studies,²² LPS increased the retention of leukocytes at the injection sites, as shown by the enhanced fluorescent intensities (Figure 2B and C), and high mortality 12 h after injection (Figure 2D). LCC-09 did not affect the recruitment of neutrophils under basal conditions but significantly inhibited LPS-induced neutrophil retention in the yolk (Figure 2B and C). Consequently, LCC-09 increased the survival rates from 0% to 68% at 130 h after LPS injection (Figure 2D). Furthermore, we intravenously

injected LPS through caudal veins to validate the effect of LCC-09 on systemic inflammatory response (Figure 2E). Systemic LPS induces vascular inflammatory response, increased vascular permeability and tissue damage, all of which lead to the extravasation of leukocytes into extravascular compartments.³³ As shown in Figure 2F and G, 6 h after injection, the tail fin showed increased neutrophil retention after LPS injection but treatment of LCC-09 significantly inhibited LPS-induced neutrophil accumulation. Taken together, these results collectively support that LCC-09 inhibits inflammatory response in vivo.

LCC-09 Did Not Elicit Significant Toxicity in Cultured Endothelial Cells or Zebrafish Embryos

Organ toxicity is a major limiting factor in drug development and is commonly observed in the applications of small molecule kinase inhibitors.³⁵ Thus, we examined the cytotoxic effect of LCC-09 on cultured endothelial cells. MTS assay was first performed and showed that LCC-09 at $5 \mu\text{M}$ and $10 \mu\text{M}$ decreased the MTS absorbance (Supplemental Figure 3), indicating that LCC-09 reduces the cell proliferation or viability. Annexin V-PI staining was further performed and showed that LCC-09 did not induce cell death (Figure 5A), suggesting LCC-09 does not induce death but inhibits the proliferation of ECs. Furthermore, the effect of LCC-09 on cardiac toxicity was examined in zebrafish models, which have close resemblance of the genetic cascade governing heart development, physiological and functional regulation to that of human heart.³⁶ Wild-type zebrafish embryos (Danio rerio) were incubated with LCC-09 or DMSO as control up to 48 and 72 h, and then heart chamber size and heart rates were examined, respectively, to evaluate the effect of LCC-09 on cardiac morphology and function. As shown in Figure 5B and C, no significant growth delay or developmental defect was observed after the treatment of LCC-09 for 48 and 72 h. Meanwhile, LCC-09 did not significantly affect the heart chamber size or basal-level heart rates (Figure 5D and E). These results collectively support that LCC-09 application has no significant cardiac toxicity in vivo.

Discussion

Leukocyte recruitment to the vessel wall in inflammation is a multistep process which involves tethering, rolling,

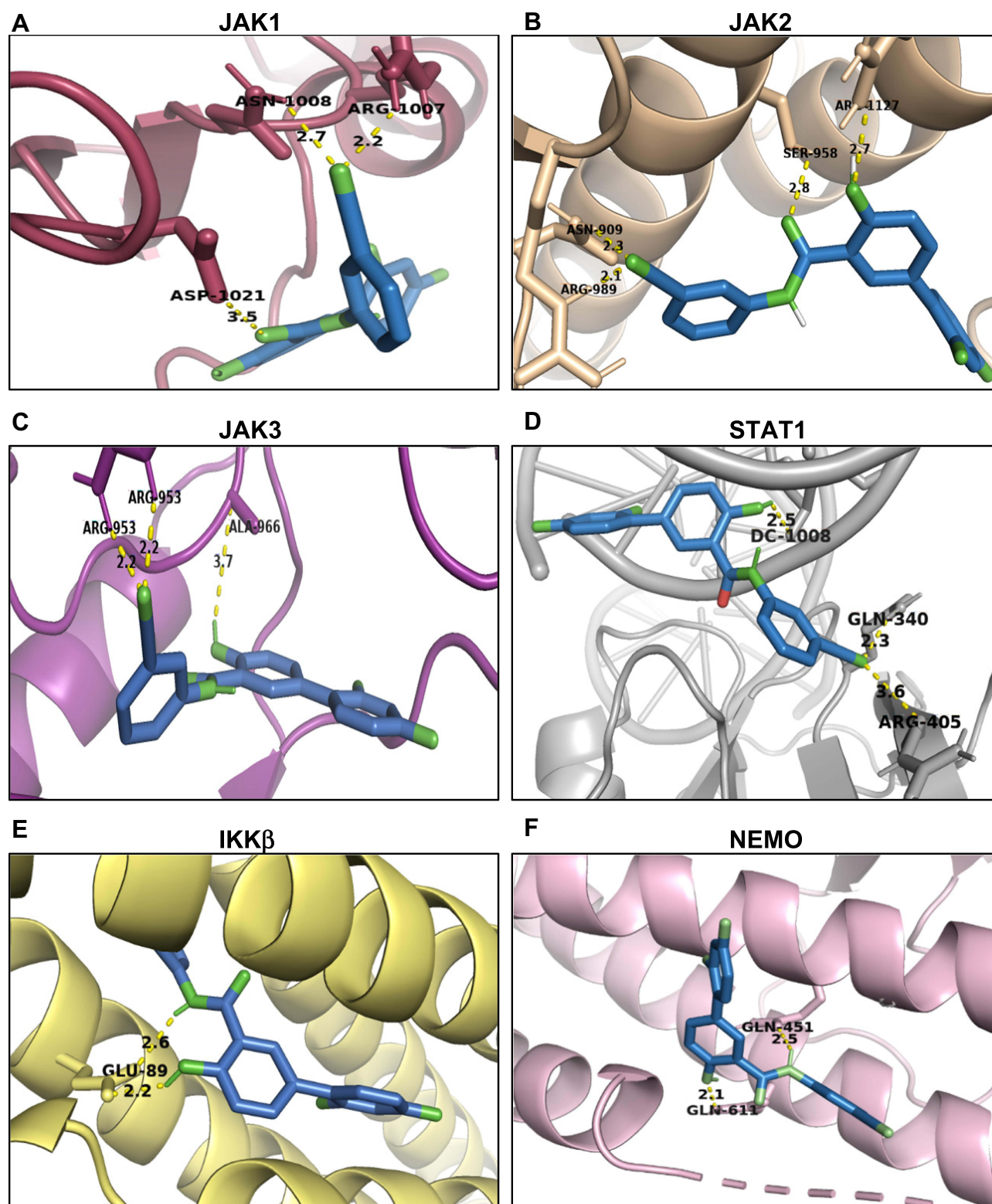


Figure 4 Molecule docking model of LCC-09. LCC-09 (green color sticks) was docked in JAK1 (PDB ID 6W8I) (A), JAK2 (PDB ID 6AAJ) (B), JAK3 (PDB ID 1YVJ) (C), STAT1 (PDB ID 1BF5) (D), IKKβ (PDB ID 4KIK) (E) and NEMO (PDB ID 3BRT) (F).

Table 1 Molecular Docking of LCC-09 (Ligand) and Candidate Proteins (Receptor)

Ligand	Receptor	PDB ID	Binding Affinity	Length of H Bonds	Formed Amino Acid with H-Bonds
LCC-09	JAK1	6W8I	-10.1	2.3 Å 5.8 Å 2.3 Å, 4.3 Å	GLU-966 ASN-1008 ARG-1007
	JAK2	6AAJ	-8.9	2.7 Å 2.8 Å 2.1 Å 2.3 Å	ARG-127 SER-958 ARG-989 ASN-909
	JAK3	1YVJ	-10	2.2 Å 3.7 Å	ARG-953 ALA-966
	STAT1	1BF5	-8.3	3.6 Å 2.3 Å 2.5 Å	ARG-405 GLN-340 DC-1008
	NEMO	3BRT	-10.6	2.4 Å, 2.7 Å	GLU-89
	IKKβ	4KIK	-7.3	2.1 Å 2.6 Å	GLN-611 GLN-451

Note: The molecular docking experiment of LCC09 (ligand) and target proteins (receptor) was performed by using AutoDock Vina.

Abbreviation: PDB, protein data bank.

activation, arrest, spreading, and crawling of leukocytes on the endothelium, and finally transmigration across the endothelium.³⁷ Induction of surface adhesion molecules in ECs is a rate-limiting step to mediate adhesive interactions between leukocytes and ECs.³⁸ Adhesion molecules recognize and bind to ligands expressed by leukocytes such as glycan epitopes and $\alpha 4\beta 1$ -integrin, etc., to mediate efficient leukocyte rolling and transmigration.^{39,40} CXCL10 is a chemokine which facilitates recruitment of monocytes, and it is shown to be involved in sustained monocyte influx upon chronic TNF α stimulation.⁴¹ Our results indicate LCC-09 reduced TNF α -induced expression of E-selectin, CXCL10 (Figure 1), and VCAM-1 (Figure 1), resulting in decreased leukocyte adhesion to cultured ECs and demonstrating that LCC-09 attenuates inflammation through regulation of multiple steps involved in leukocyte recruitment. The effect of E-selectin antibody on leukocyte adhesion has been confirmed by several previous studies,^{39,42–45} in which E-selectin antibody consistently blocked leukocyte adhesion in vitro and in vivo. Interestingly, LCC-09 did not significantly affect the levels of monocyte chemoattractant protein-1 or intercellular adhesion molecule 1 (data not shown). Given that LCC-09 effectively reduced the leukocyte adhesion to endothelial cells (Figure 1E and F), it is thus likely that the reduced expression of E-selectin and VCAM-1 contributes to the decreased leukocyte-endothelial cell adhesion.

The trans-migration of leukocyte determines the levels of leukocyte recruitment to inflamed tissues and is regulated by several molecules including junctional adhesion molecules, clusters of VCAM-1 and ICAM-1, etc.^{46,47} The inhibitive effect of LCC-09 on VCAM-1 expression suggests that it may reduce the transmigration of leukocytes. Furthermore, the trans-migration of leukocytes was examined in zebrafish model in vivo (Figure 2), in which the extravasation and accumulation of neutrophils in yolk sac and tail fin were quantified after local and systemic LPS injection, respectively. Although the molecular details through which LCC-09 controls the trans-migration process require further investigation, our current results support that LCC-09 inhibits the recruitment and extravasation of leukocytes in vivo. The anti-inflammatory effect of LCC-09 is supported by results from cultured endothelial cells in vitro and zebrafish models in vivo. Specifically, endothelial cells pre-treated with LCC-09 exhibited reduced expression of adhesion molecules, inflammatory cytokines, and decreased leukocyte adhesion upon TNF α stimulation. Zebrafish embryos treated with LCC-09 also showed reduced leukocyte extravasation when challenged with LPS. Given that NF κ B and STAT1 pathways, and ROS generation are also required for myeloid cell activation, it is possible that LCC-09 has dual effects on both myeloid cells and endothelial cells, which collectively contributes to the reduced neutrophil retention at the site of LPS injection. In our in vitro experiments, monocytes were co-cultured with HUVECs (Figure 1),

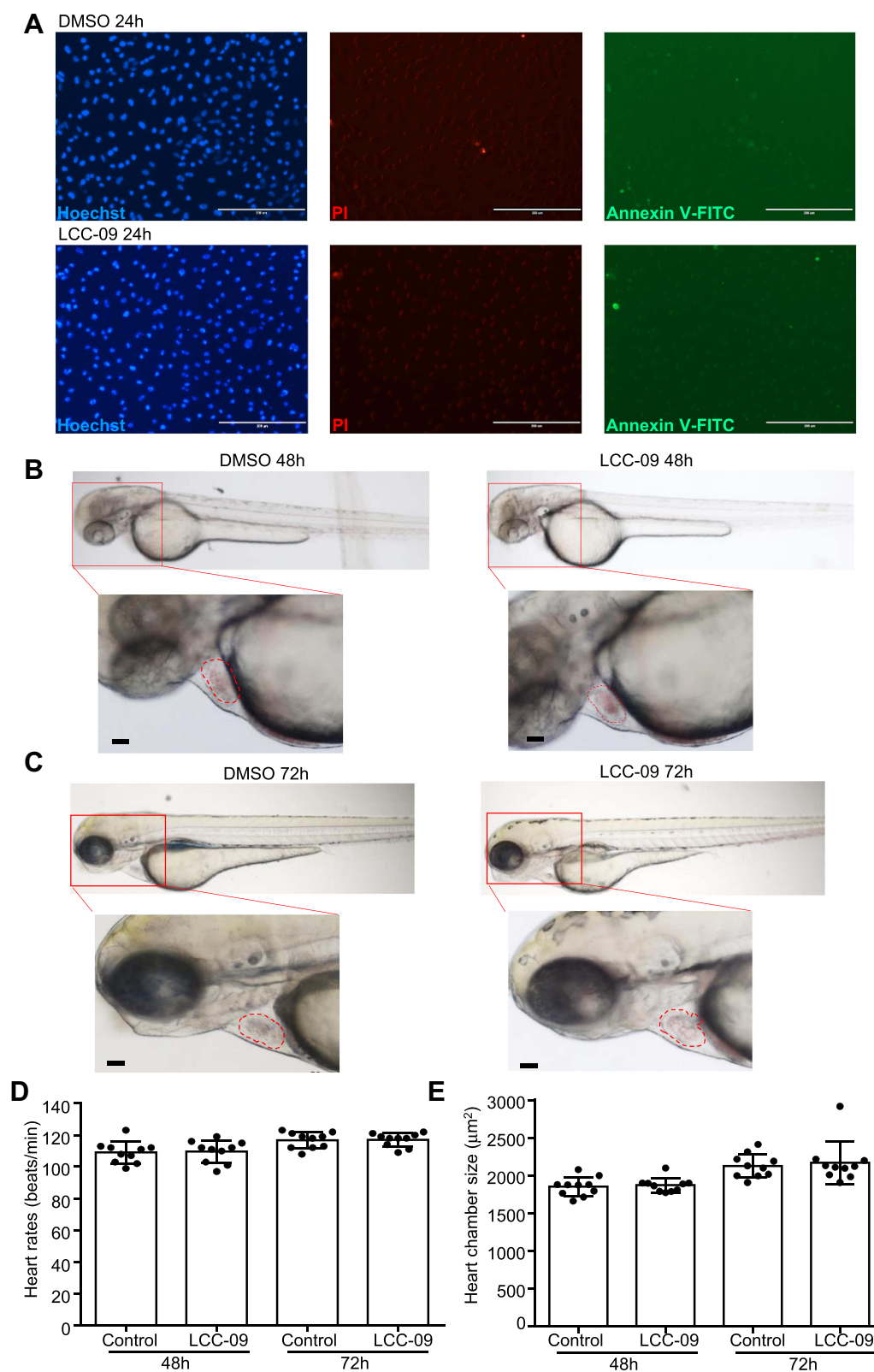


Figure 5 LCC-09 does not induce toxicity in cultured endothelial cells or zebrafish embryos. **(A)** HUVECs were cultured with LCC (10 μ M) or DMSO control for 24 h and then stained with Hoechst, PI and annexin V-FITC. Images are representative of 3 independent experiments. **(B–E)** Zebrafish embryos at 6 hpf were incubated with LCC-09 (10 μ M) or DMSO as control for 48 and 72 h, respectively. Heart rates and chamber sizes were measured and compared. Scale bar: 200 μ M **(A)**, 50 μ M **(B and C)**.

while neutrophil accumulation was examined in the zebrafish model *in vivo* (Figure 2). Although monocytes and neutrophil are distinct subsets of leukocyte populations, the molecular interactions mediating leukocyte-endothelial cell interactions are similar. Thus, reduced expression of adhesion molecules and chemokines in endothelial cells can lead to decreased recruitment of both monocytes and neutrophil. Given that endothelial cells and zebrafish embryos exhibited reduced monocyte adhesion and neutrophil extravasation, respectively, these results collectively indicate that LCC-09 is an anti-inflammatory compound. Interestingly, post-treatment of LCC-09 showed a profound inhibitive effect on the expression of E-selectin and CXCL10 (Supplemental Figure 1). TNF α is known to induce the peak activation of its downstream signaling pathways including NF κ B in a few minutes, however, oscillations of NF κ B activation happens over time^{48,49} and sustained activation of STAT1 is required for continuous expression of inflammatory cytokines.⁵⁰ It is thus likely that the inhibitive effect of LCC-09 on NF κ B and STAT1 contributes to reduced expression of E-selectin and CXCL10 post-TNF α stimulation. Nevertheless, this result suggests that LCC-09 is a promising anti-inflammatory compound for the treatment of inflammatory disease.

We used LPS-induced zebrafish inflammation model to evaluate the anti-inflammatory role of LCC-09 in this study. Zebrafish has been widely used in the studies of inflammatory responses because of their conservation with mammals in immune cell lineages and advantages of rapid development, high fecundity, and optical transparency. Even though the zebrafish TLR4 paralogs do not bind to LPS, they still have a significant inflammatory response after LPS injection.^{33,51} LPS-induced downstream signaling pathways, including induction of TNF α , activation of NF κ B, and enhanced production of ROS, have shown to be conserved between zebrafish and mammals.³³ Indeed, the anti-inflammatory effect of LCC-09 in the zebrafish model is further supported by results from cultured human endothelial cells (Figure 1) and a recent study using murine rheumatoid arthritis models.¹¹ Taken together, our results support that LPS-induced zebrafish inflammation model is an excellent tool for screening new anti-inflammatory drugs.

One recent study has shown that administration of LCC-09 for 4 weeks in a murine glioblastoma model did not elicit any significant toxicity or side effect.⁹ Another study reported that administration of LCC-09 over a 12-week period attenuated synovial inflammation and macrophage infiltration in rheumatoid arthritis mouse models with no observed side effects.¹¹ Taken together with our results in cultured ECs and zebrafish

embryos, these data support that LCC-09 reduces inflammation and has mild toxicity.

Activation of IKK/NF κ B pathway and JAK/STAT is well recognized to play essential roles in TNF α -induced EC inflammation.⁵² The essential roles of NF κ B and STAT1 in TNF α -induced inflammatory response are supported by several previous studies^{52–55} which reported that inhibitors/siRNAs of NF κ B and STAT1 decreased TNF α -induced expression of pro-inflammatory adhesion molecules and leukocyte adhesion. Our results indicate that LCC-09 inhibited TNF α -induced I κ B α degradation and NF κ B p65 phosphorylation, as well as the binding of NF κ B to the human E-selectin promoter, collectively suggests LCC-09 inhibits NF κ B pathway. Interestingly, the molecular docking model suggests that LCC-09 preferentially binds to NEMO but not IKK β , the upstream kinase of I κ B α . NEMO is a noncatalytic component in the activation of NF κ B pathway and exerts its function by recruiting IKK to form the IKK complex.⁵⁶ An extensive-binding interface is present between NEMO amino residues 44–111 and IKK β residues 701–745,⁵⁷ thus, previous studies have developed small peptide inhibitors to prevent the interaction between NEMO and IKK β to inhibit NF κ B activation in inflammation.^{56,58} Our molecular docking experiments suggest that LCC-09 potentially binds to NEMO Glu-89 to reduce the interaction between NEMO and IKK β , leading to decreased I κ B α phosphorylation and degradation.

The JAK-STAT pathway is essential for a wide range of cytokines and growth factors in both development and diseases.^{59,60} STAT1 was originally identified to be a downstream signaling component of TNF receptor (TNFR)-1 and shown to be required for TNF α -induced TNFR2-interferon regulatory factor-1 (IRF1)-interferon- β autocrine loop in monocyte recruitment to endothelial cells,^{41,61} suggesting the essential role of STATs in TNF α -induced inflammation. Although our molecular docking model suggests that LCC-09 potentially binds to JAK1, JAK2 and JAK3 (Figure 4), LCC-09 was shown to selectively inhibit the phosphorylation of STAT1 but not STAT3 (Figure 3). Due to the strict homolog and distinct function between STAT1 and STAT3,⁶² successful development of a specific STAT inhibitor has been challenging. Our results raise the possibility that LCC-09 can be used in conditions when blockage of STAT1 but not STAT3 activation is required. However, how LCC-09 specifically inhibits STAT1 activation remains to be investigated and is out of the scope of the current study. Notably, LCC-09 was shown to inhibit the STAT1 phosphorylation at basal level, suggesting that it interferes with some endogenous mechanism to block STAT1 activation.

It is also possible that some JAK-independent mechanism of STAT1 activation is involved.^{60,63,64} Meanwhile, our current results do not exclude the possibility that other NFκB- and STAT1-independent signaling pathways are involved in the anti-inflammatory effect of LCC-09.

Salicylates have a long history in the clinical practice of treating pain and inflammation. Previous studies have reported that sodium salicylate inhibits EC inflammatory response, TNFα-induced IκBα degradation, and adhesion molecule expression through both cyclooxygenase-dependent and independent mechanisms.^{65–68} However, salsalate, the prodrug dimer of salicylates, did not provide additional benefits in overweight or obese patients with coronary artery diseases receiving statins.⁶⁹ Furthermore, high-dose disalicylate therapy was shown to impair endothelium-dependent vasodilation,⁷⁰ thus raising concerns about the clinical use of salicylates in cardiovascular diseases and highlighting the need to develop novel anti-inflammatory agents. Salicylanilide derivatives have been known for a wide range of biological activities including anti-infection, anti-tumor and anti-oxidation, etc.^{71,72} Our results show that LCC-09, a derivative of salicylanilide consisting of the functional fragment of magnolol, 2,4-difluorophenyl, and paeonol moiety of salicylate, inhibits EC inflammatory response in both cultured ECs and zebrafish models but does not induce significant cardiac toxicity. Additionally, our results show that LCC-09 inhibited expression of adhesion molecules and NFκB activation at the dose of 5 μM, suggesting it is more potent than sodium salicylate and aspirin, which were shown to reduce NFκB activation at 10 mM previously.^{65–67} Previous studies reported that LCC-09 reduced the monocyte accumulation in the rodent rheumatoid arthritis models,¹¹ and our results suggest LCC-09-mediated inhibition of the EC inflammatory response is likely involved in the beneficial role of LCC-09 in this disease model.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, agreed to the submitted journal, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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