Calcineurin inhibitors exacerbate coronary arteritis via the MyD88 signalling pathway in a murine model of Kawasaki disease

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TITLE PAGE

Original Article

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Short title

CNIs exacerbate coronary arteritis in a murine model

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Key words
Calcineurin inhibitors; Kawasaki disease; Coronary arteritis; MyD88; Mouse model

Abbreviations

KD	Kawasaki disease
IVIG	intravenous immunoglobulin
CN	calcineurin
CNIs	calcineurin inhibitors
CsA	cyclosporine A
Tac	tacrolimus
ITPKC	inositol 1,4,5-trisphosphate 3 kinase C
NFAT	nuclear factor of activated T cells
SCID	severe combined immunodeficiency

54	Nod1	nucleotide-binding oligomerization domain-containing protein 1
55	CAL	coronary artery lesions
56	TLR	toll-like receptor
57	MyD88	myeloid differentiation primary response gene 88
58	CARD9	caspase-associated recruitment domain 9
59	Cnb1	calcineurin subunit B1
60	MDP	muramyl dipeptide
61	LPS	lipopolysaccharide
62	DMSO	dimethyl sulfoxide
63	HE	hematoxylin and eosin
64	LC	liquid chromatography
65	MS	mass spectrometry
66	HPLC	high performance liquid chromatography
67	BMDMs	bone marrow-derived macrophages
68	FBS	fetal bovine serum
69	BMDCs	bone marrow-derived dendritic cells
70	HCAECs	human coronary artery endothelial cells
71	HCASMCs	human coronary artery smooth muscle cells
72	DMEM	Dulbecco's modified Eagle's medium
73	MHECs	mouse heart endothelial cells
74	FITC	fluorescein isothiocyanate
75	ICAM-1	intercellular adhesion molecule-1
76	PE	phycoerythrin
77	VCAM-1	vascular cell adhesion molecule-1
78	qRT-PCR	quantitative real time polymerase chain reaction
79	TDM	trehalose-6,6’-dimycolate
80	WT	wild type

p.o.	per os
i.p.	intraperitoneal injection
s.c.	subcutaneous injection

Summary

Calcineurin inhibitors (CNIs) have been used off-label for the treatment of refractory Kawasaki disease (KD). However, it remains unknown whether CNIs show protective effects against the development of coronary artery lesions in KD patients. To investigate the effects of CNIs on coronary arteries and the mechanisms of their actions on coronary arteritis in a mouse model of KD, we performed experiments with FK565, a ligand of nucleotide-binding oligomerization domain-containing protein 1 (NOD1) in wild-type, severe combined immunodeficiency (SCID), caspase-associated recruitment domain 9 $(CARD9)^{-/-}$, and myeloid differentiation primary response gene 88 $(MyD88)^{-/-}$ mice. We also performed *in-vitro* studies with vascular and monocytic cells, and vascular tissues. A histopathological analysis showed that both cyclosporin A and tacrolimus exacerbated the NOD1-mediated coronary arteritis in a dose-dependent manner. Cyclosporin A induced the exacerbation of coronary arteritis in mice only in high doses, while tacrolimus exacerbated it within the therapeutic range in humans. Similar effects were obtained in SCID and $CARD9^{-/-}$ mice but not in $MyD88^{-/-}$ mice. CNIs enhanced the expression of adhesion molecules by endothelial cells and the cytokine secretion by monocytic cells in our KD model. These data indicated that both vascular and monocytic cells were involved in the exacerbation of coronary arteritis. Activation of MyD88-dependent inflammatory signals in both vascular cells and macrophages appears to contribute to their adverse effects. Particular attention should be paid to the development of coronary artery lesions when using CNIs to treat refractory KD.

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Introduction

Kawasaki disease (KD) is an acute febrile illness of unknown etiology characterized by a systemic vasculitis of small and medium-sized arteries, particularly coronary arteries (1). The standard therapy for KD consists of aspirin and high-dose intravenous immunoglobulin (IVIG). Approximately 10-30% of KD patients are resistant to IVIG (2-5), and some develop coronary artery lesions (CALs) such as coronary aneurysms, a clinically important problem. Although therapeutic options for KD patients refractory to high-dose IVIG include high-dose corticosteroid, infliximab, and plasma exchange, no definite treatments for such patients have been established.

Calcineurin inhibitors (CNIs), such as cyclosporine A (CsA) and tacrolimus (Tac), have been used as off-label drugs to treat KD (6-10). *Inositol 1,4,5-trisphosphate 3 kinase C (ITPKC)*, a susceptibility gene of KD, encodes a kinase that negatively regulates the intracellular Ca^{2+} level and inhibits calcineurin (CN)-dependent activation of nuclear factor of activated T cells (NFAT) by phosphorylating inositol trisphosphate. Given the inhibitory effects on the CN/NFAT pathway, CNIs are expected to be useful as an alternative treatment for KD patients. In several observational studies (7, 8), the anti-pyretic and anti-inflammatory effects of CsA have been described in refractory KD patients; however, it remains to be determined whether or not CNIs exert any effects on coronary arteries.

We recently established a mouse model of KD by the administration of FK565, a synthetic ligand of nucleotide-binding oligomerization domain-containing protein 1 (NOD1) (11). The histopathologic features of FK565-induced coronary arteritis in mice are similar to those of CALs in acute-phase KD (11, 12). We also reported that vascular cells and cardiac $CD11c^{+}$ macrophages play a pivotal role in the pathogenesis of acute coronary

arteritis (13).

Using a NOD1-mediated KD animal model, we investigated the effects of CNIs on coronary arteries and examined the mechanism of CNI-induced exacerbation of coronary arteritis.

Materials and Methods

Animals

C57BL6/N [wild type (WT)] and CB-17 SCID (severe combined immunodeficiency) mice were purchased from KBT Oriental (Charles River Grade, Saga, Japan). Myeloid differentiation primary response gene 88 (MyD88)-knockout (*MyD88*$^{-/-}$) mice in a C57BL/6 background were purchased from Oriental Yeast (Tokyo, Japan). Caspase-associated recruitment domain 9 (CARD9) knockout (*CARD9*$^{-/-}$) mice were generated as described (14). *Calcineurin subunit B1* (*Cnb1*)$^{flox/flox}$ and *Cd11c-Cre* mice in a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were 5-7 weeks old, and were kept under specific-pathogen-free conditions. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Kyushu University (Protocol number: A-27-154), and followed the Guideline for Proper Conduct of Animal Experiments, Science Council of Japan.

Administration of innate immune ligands and CNIs

FK565, a synthetic NOD1 ligand, was supplied initially by Astellas Pharma (Tokyo, Japan) and prepared later by Shimoyama and Fukase (in the Supporting information). CsA and Tac were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Enzo Life Sciences (Lausen, Switzerland), respectively. Muramyl dipeptide (MDP; Nod2 ligand) and lipopolysaccharide [LPS; Toll-like receptor (TLR)-4 ligand] were purchased from Lonza (Basel, Switzerland) and InvivoGen (Toulouse, France), respectively.

CsA and Tac were dissolved in endotoxin-free dimethyl sulfoxide (DMSO) (Hybri-Max^{TM}; Sigma-Aldrich, St. Louis, MO, USA), and we confirmed that these solutions were endotoxin-free with a ToxinSensor^{TM} Chromogenic LAL endotoxin

assay kit (GenScript, Piscataway, NJ, USA).

The protocol to induce arteritis in mice was as follows: mice were administered 100 μg of FK565 orally, which was dissolved in sterile distilled H_2O, once daily for 5 consecutive days, or administered 10, 100, or 500 μg of FK565 subcutaneously on Days 0 and 3. Simultaneously, CsA (4, 12, 40, or 120 mg/kg body weight per dose) or Tac (0.2, 0.6, 2, or 6 mg/kg body weight per dose), dissolved in 10% DMSO, was administered intraperitoneally for 5 consecutive days. MDP (500 μg), dissolved in sterile distilled H_2O, was administered intraperitoneally on Days 0 and 3. LPS (10 μg), dissolved in sterile distilled H_2O, was administered intraperitoneally twice (Days 0 and 3) or once (Day -1 or 0).

Histological analyses

After mice were euthanized, the hearts were dissected on Day 5. They were 4% paraformaldehyde-fixed and paraffin-embedded for histological analyses. Cross-sections of the aortic roots were prepared, with the three aortic valve cusps and coronary arteritis assessed by hematoxylin and eosin (HE) staining as described previously (11). For scoring inflammatory cell infiltration, the lesion area and cell infiltration around the coronary arteries were measured in one section per heart using the Image J software program (National Institutes of Health, Bethesda, MD, USA) (13).

Blood concentrations of CsA and Tac

CsA (4, 12, 40, or 120 mg/kg body weight per dose) or Tac (0.2, 0.6, 2, or 6 mg/kg body weight per dose) was administered intraperitoneally to 5-weeks-old mice once daily for 5 consecutive days. Blood samples were collected from mice 0 (trough), 2, and 4 h after drug administration on Day 5. Blood concentrations of CsA and Tac were measured by liquid chromatography (LC)–tandem mass

199 spectrometry (MS)/MS, as described previously (15) with minor modifications.
200 Briefly, all whole blood samples (150 µL) were transferred to glass tubes and
201 spiked with 25 µL of ascomycin (20 µg/mL; Sigma-Aldrich), which served as the
202 internal standard. Then, 600 µL of water and 2 mL of extraction solution
203 (methyl-t-butyl ether/cyclohexane, 1:3 v/v) were added to the glass tubes. Each
204 tube was capped securely, mixed on a horizontal shaker for 15 min, and
205 centrifuged at 3,000 rpm for 15 min. The organic layer was transferred to a new
206 tube and evaporated using an Automatic Environmental Speed Vac® System
207 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each sample was
208 reconstituted with 200 µL of 50% methanol solution and then mixed with a vortex
209 mixer for 1 min. A 20-µL aliquot of each sample was injected into the LC-MS/MS
210 system. Each sample was analyzed by high-performance liquid chromatography
211 (HPLC, Eksigent® ekspert™ ultra LC SYSTEM100XL; AB Sciex, Framingham,
212 MA, USA) on an analytical column (Inertsil-ODS3, 150 × 2.1mm i.d.; GL
213 Sciences, Inc., Tokyo, Japan) and an MS/MS detector (QTRAP® 4500; AB
214 Sciex). The mobile phase consisted of a multiple gradient of solvent A (1mM
215 ammonium acetate) and solvent B (methanol/1mM ammonium acetate). The
216 flow rate was set at 250 µL/min, the column was operated at 60 °C, and the
217 eluent was introduced directly into the electro-spray ion source of the mass
218 spectrometer. Selected reaction monitoring transitions in the positive ion mode
219 were m/z 1220 → m/z 1203 for CsA, m/z 821 → m/z 768 for Tac, and m/z 809 →
220 m/z 756 for ascomycin. CsA, Tac and their metabolites were detected as
221 ammonium adducts (m+ NH_4). Peak areas were linear from 0.5 to 30 ng/mL for
222 CsA, Tac, and their metabolites.
223
224 *Organ culture and protein determination*
225 The aortic roots sterilely isolated from C57CL/6 mice were cultured for 24 h in a

96-well plate with endothelial basal medium (EBM)-2 ~~medium~~ with EGM-2MV (Lonza) in a CO_2 (5%) incubator at 37°C as described previously (11). The protein concentrations of aortic root tissues were measured by a Bio-Rad protein assay (BioRad, Hercules, CA, USA) after homogenization with phosphate-buffered saline containing Cell Culture Lysis Reagent (Promega, Madison, WI, USA) and Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). Whole protein contents of aortic root tissue were measured to calculate the chemokine (C-C motif) ligand 2 (CCL2)/interleukin (IL)-6 levels per tissue protein content.

Cells

Murine bone marrow cells were harvested from the femur and tibia. Bone marrow-derived macrophages (BMDMs) were prepared from bone marrow cells cultured in RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Santa Ana, CA, USA) and 10% L929 culture supernatant (source of macrophage colony-stimulating factor) for 7-10 days (13). Bone marrow-derived dendritic cells (BMDCs) were prepared from bone marrow cells cultured in RPMI-1640 supplemented with 10% FBS and 10% MGM-5 culture supernatant (source of granulocyte-macrophage colony-stimulating factor) for 7-10 days (16). BMDCs were stained with phycoerythrin-conjugated anti-CD11c antibody (BD Biosciences, San Jose, CA, USA) and analyzed using an EC800 Analyzer (Sony Biotechnology, Tokyo, Japan).

Human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (HCASMCs) derived from healthy donors were purchased from Lonza. Murine monocyte/macrophage cell line RAW264.7 (RCB0535) was obtained from Riken Cell Bank (Tsukuba, Japan). HCAECs and

HCASMCs were cultured in EBM-2 medium with EGM-2MV and smooth muscle basal medium (SmBM) with SmGM-2 (Lonza), respectively. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) with 10% FBS (MP Biomedicals). These cells were incubated in a 5% CO_2 incubator at 37 °C.

Mouse heart endothelial cells (MHECs) were isolated from murine heart using CD31 microbeads (Miltenyi Biotec, Auburn, CA, USA) and cultured as described previously (13). Briefly, diced heart tissues were treated with collagenase II (Worthington Biochemical, Freehold, NJ, USA) for 1 h at 37 °C. Cell suspensions were incubated with rat anti-mouse CD31, and CD31-positive cells were purified using MACS (Miltenyi). CD31-positive cells were cultured in DMEM containing endothelial cell growth supplement (Sigma-Aldrich) for 7 days and used for subsequent analyses.

Flow cytometric analyses

HCAECs stimulated with FK565 (10 μg/ml) in the presence or absence of CsA (10 μM = 12.03 μg/mL) or Tac (10 μM = 8.22 μg/mL) for 24 h were collected and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD54 (intercellular adhesion molecule-1; ICAM-1) monoclonal antibody or IgG1 isotype control (Beckman Coulter, Miami, FL, USA), and phycoerythrin (PE)-conjugated anti-CD106 (vascular cell adhesion molecule-1; VCAM-1) monoclonal antibody or IgG1 isotype control (BD). The expression of ICAM-1 and VCAM-1 was analyzed using an EPICS XL flow cytometer (Beckman Coulter). The data were analyzed with the Kaluza software program, ver. 1.5 (Beckman Coulter).

278

279 *Quantitative real time polymerase chain reaction*

280 HCAECs stimulated with FK565 (10 μg/ml) in the presence or absence of CsA
281 (10 μM ~~= 12.03 μg/mL~~) or Tac (10 μM ~~= 8.22 μg/mL~~) for 6 h were collected, and
282 the total RNA was extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany).
283 Complementary DNA was synthesized using a High-Capacity RNA to cDNA Kit
284 (Life Technologies, Gaithersburg, MD, USA) in accordance with the
285 manufacturer's protocol. A quantitative real time polymerase chain reaction
286 (qRT-PCR) was performed using Fast SYBR Green Master Mix and
287 StepOnePlus (Life Technologies). Human β-actin (ACTB) was used as an
288 internal control gene. The sequences of the gene-specific primers were as
289 follows:

290 (F: forward, R: reverse primers): ICAM-1: 5'
291 -CGGCCAGCTTATACACAAGAAC- 3' (F) and 5'
292 -AATTTTCTGGCCACGTCCAG- 3' (R), VCAM-1: 5'
293 -AAGGCAGAGTACGCAAACAC- 3' (F) and 5'
294 -ATTTTCGGAGCAGGAAAGCC- 3' (R), E-selectin: 5'
295 -TGAGGAAGGCTTCATGTTGC- 3' (F) and 5'
296 -TGTGCACTGGAAAGCTTCAC- 3' (R), and ACTB: 5'
297 -CACCCTGAAGTACCCCATCG- 3' (F) and 5'
298 -TGCCAGATTTTCTCCATGTCG- 3' (R)

299 To evaluated the expression of NOD1 in the murine vascular tissue, aorta
300 tissues (from arch to abdominal aorta) isolated from wild type C57BL/6 mice
301 were stimulated with DMSO (control), CsA (0.1, 1 or 10 μM), or Tac (0.1, 1 or 10
302 μM) for 48 h and collected. Then, total RNA was extracted using RNeasy Mini Kit
303 (Qiagen), and complementary DNA was synthesized. A qRT-PCR was
304 performed using predesigned PrimeTime® qPCR Assay (Integrated DNA

Technologies, Coralville, IA, USA) for mouse Nod1, Tlr4 and Actb.

The PCR conditions were 95 °C (20 seconds), 40 cycles of 95 °C (3 seconds), and 60 °C (30 seconds). The relative gene expression was calculated by the ddCt method and presented as $2^{[Ct_{(ACTB)} - CT_{(gene)}]}$ (17).

Cell stimulation and cytokine assay

BMDMs/BMDCs/RAW264.7 (1.0×10^5 cells/well) or HCAEC/HCASMC (3200 cells/well) were seeded into 96-well plates. The next day, the medium was changed, and the cells were stimulated with each of the following reagents: PolyI:C (TLR3 ligand; Invivogen), LPS, or plate-coated trehalose-6,6'-dimycolate (TDM; C-type lectin Mincle ligand; Sigma-Aldrich) as positive controls. The supernatants were collected for assay 24 h after stimulation.

The concentrations of tumour necrosis factor (TNF), IL-6, IL-8 and CCL2 were measured using a BD™ Cytometric Bead Array Flex Set System, Mouse Inflammation Kit, and Human Inflammatory Cytokine kit (BD Biosciences). The cytokines were analyzed using an EC800 Analyzer (Sony Biotechnology).

Statistical analysis

The data were analyzed using Student's *t*-test, Dunnett's test, or Tukey-Kramer test using the statistical software program JMP® version 9.0 (SAS Institute, Cary, NC, USA). Values of $p < 0.05$ were considered statistically significant.

Results

CNIs Exacerbate Nod1-mediated Coronary Arteritis

To investigate whether or not CNIs exert anti-inflammatory effects on coronary arteritis in a NOD1-mediated KD animal model (11), we administered CsA or Tac to mice in combination with FK565 (Fig. 1a). In the preliminary experiments, we determined the dose and duration of FK565 administration to induce coronary arteritis in mice (Supporting information, Fig. S1A). Unexpectedly, CNIs exacerbated NOD1-mediated coronary arteritis in a dose-dependent manner (Fig. 1b). In the quantitative evaluation of inflammatory lesions by number and infiltration area of inflammatory cells, coronary arteritis was exacerbated significantly by the administration of CsA 120 mg/kg or Tac 2 and 6 mg/kg (Fig. 1c, d). No coronary arteritis was induced by CNIs alone (Supporting information, Fig. S2).

To determine the doses at which CNIs exacerbated coronary arteritis, we measured the blood concentrations of CNIs before and after 5-day administration. When mice were administered 120 or 40 mg/kg of CsA, the trough levels were over the toxic level of CsA in humans (300 ng/mL) (18). The trough levels after the administration of 6 mg/kg of Tac did not reach the toxic level in humans (20 ng/mL) (19) (Fig. 1e). The blood levels after administration of 12 mg/kg of CsA and 0.6 mg/kg of Tac in mice were close to those after administration of standard therapeutic doses of 5.26 mg/kg of CsA (18) and 0.16 mg/kg of Tac (20) in humans, respectively, as shown in Fig. 1e. These results indicated that coronary arteritis might be exacerbated by high concentrations of CsA and at therapeutic concentrations of Tac in humans.

Non-T, Non-B Cells Are Associated with Exacerbation of Coronary Arteritis by CNIs

To examine which cells were associated with the exacerbation of coronary arteritis by CNIs, we repeated the same experiments as above using SCID mice. Exacerbation of coronary arteritis by CNIs was also found in the SCID mice, suggesting that T and B cells were not essential for the exacerbation of arteritis (Fig. 2a-c).

Effects of CNIs on Vascular and Monocytic Cells

We reported that monocytic and vascular cells play an essential role in the pathogenesis of NOD1-mediated coronary arteritis (13). First, to examine the effects of CNIs on murine vascular cells *in vitro*, we stimulated MHECs with CNIs and FK565 and assayed the production of cytokines. When a high concentration of Tac (10 μM) was added, the production of IL-6 and CCL2 was enhanced in the absence of FK565 stimulation but not in the presence of FK565 (Fig. 3a). In contrast, the production of these cytokines was suppressed with high concentrations (1 and 10 μM) of CsA in the presence or absence of FK565 (Fig. 3a).

To investigate the effects of CNIs on human vascular cells, we stimulated HCAECs and HCASMCs with CNIs in the presence or absence of FK565 and examined the cytokine production. The stimulation of HCAECs and HCASMCs with Tac did not increase cytokine production in the presence or absence of FK565 (Figure 3b). The production of IL-6 and IL-8 was increased in the presence of 10 μM of CsA with FK565 in HCAECs but was somewhat suppressed in HCASMCs under these conditions. Taken together, these data showed that the co-administration of CNIs with FK565 resulted in variable responses of endothelial cells and smooth muscle cells in the production of

proinflammatory cytokines.

We next performed experiments focusing on monocytes/macrophages. To investigate the effect of CNIs on monocytes/macrophages *in vitro,* we stimulated BMDMs and RAW264.7 cells with CNIs in the presence or absence of FK565 and examined the production of cytokines. We found that CNIs enhanced the spontaneous and FK565-induced production of TNF and IL-6 in a concentration-dependent manner, and the effect of Tac was stronger than that of CsA in BMDMs and RAW264.7 cells (Fig. 3c, d).

Effects of CNIs on the Cytokine Production in Vascular Tissues

We previously demonstrated that NOD1 ligands enhanced the production of inflammatory cytokines from aortic roots *ex vivo* (11). To examine the direct effect of CNIs on the aortic roots, we cultured the aortic root tissues isolated from C57BL/6 mice with FK565 and CNIs and measured the IL-6 and CCL2 levels in the culture supernatant. In the presence of FK565, the production of IL-6 and CCL2 was increased in the presence of high concentrations of Tac (Fig. 3e). In contrast, 10 μM CsA did not enhance the IL-6 and CCL2 production from aortic tissues. These data recapitulated the differential effects of CsA and Tac on FK565-associated coronary arteritis (Fig. 1) and confirmed that Tac exaggerated the cytokine releases from aortic roots.

CNIs Increase the Expression of Adhesion Molecules on Endothelial Cells.

As CNIs did not necessarily enhance the production of cytokines in endothelial cells, we examined the effects of CNIs on the adhesion of leukocytes to endothelial cells in the presence of FK565. First, we performed an endothelial cell-leukocyte adhesion assay using HCAECs and U937 cells (human monocyte-like cell line) (21). We found that CNIs did not enhance the adhesion

between endothelial cells and leukocytes significantly under stimulation with FK565 (data not shown).

Next, to examine whether or not CNIs increase the expression of adhesion molecules on endothelial cells, we stimulated HCAECs with CNIs in the presence or absence of FK565 and analyzed the expression of ICAM-1, VCAM-1 and E-selectin by flow cytometry and qRT-PCR. We found that CsA and Tac enhanced FK565-induced ICAM-1 expression on HCAECs (Fig. 4a and Supporting information, Fig. S3). In the quantitative real-time PCR, CsA increased ICAM-1 and E-selectin expression in the absence of FK565 (Fig. 4b). Although there were some discrepancies between the expression of *ICAM1* mRNA and the cell-surface ICAM-1 protein, we interpreted the dissociated results as a sequential time-course of the whole-cell expression of *ICAM1* mRNA in HCAECs, followed by the post-transcriptional regulation and post-translational modification of the ICAM1 protein towards its cell-surface expression. Taken together, these results suggested that CNIs increased the expression of adhesion molecules on vascular endothelial cells, which may lead to the migration of inflammatory cells, such as macrophages, and induce the progression of coronary arteritis.

The Role of CN in the CNI-induced Cytokine Production of Monocytic Cells

To examine the functional role of CN in the CNI-induced cytokine production of monocytic cells, we employed the *Cre-loxP* system to obtain CN-deficient cells, as CN-knockout was lethal to mouse embryos (22, 23). CD11c-positive macrophages play a critical role in NOD1-mediated coronary arteritis (13); therefore, we used CD11c-positive cell-specific Cnb1 subunit conditional-knockout mice (*Cd11c-Cre*$^{+}$*Cnb1*$^{flox/flox}$; Cnb1 CKO) (24). First, we ensured that Tac increased TNF production, regardless of the absence or

presence of FK565 in WT BMDCs, the majority of which expressed CD11c (Fig. 5a).

To investigate a possible role of CN in the effects of CNIs, we performed stimulation experiments using BMDCs from Cnb1 CKO and *Cnb1*$^{flox/flox}$ mice (Cnb1 WT). We found that the production of TNF in Cnb1 CKO mice induced by Tac and FK565 was significantly higher than that in Cnb1 WT mice (Fig. 5b upper). In addition, the TNF production of CD11c-positive macrophages from Cnb1 CKO mice after stimulation with LPS (TLR-4 ligand) and PolyI:C (TLR-3 ligand) was slightly higher than that in control mice. In contrast, no increase in cytokine production was observed after stimulation with TDM (ligand of Mincle). These results were consistent with the previous finding that CN regulates TLR-mediated activation pathways negatively (25).

The Exacerbation of Coronary Arteritis by CNIs Depends on MyD88

KD is characterized by the activation of the innate immune system (26-29). A previous report showed that CNIs activated the TLR-4-MyD88 signal pathway, an innate immune pathway, and increased the production of proinflammatory cytokines, such as TNF, from macrophages (25). MyD88 is the adapter molecule downstream of TLRs. In contrast, CARD9 is a signal transducer of the non-TLR innate immune pathway. We therefore investigated whether or not a deficiency of MyD88 or CARD9 affected the exacerbation of NOD1-mediated coronary arteritis by CNIs.

To create the experimental conditions to induce mild coronary arteritis, we optimized the treatment doses of FK565 at 100 μg for WT and CARD9$^{-/-}$ mice and 500 μg for MyD88$^{-/-}$ mice (Fig. 6a and Supporting information, Fig. S1B). In WT and CARD9$^{-/-}$ mice, 0.6 mg/kg of Tac exacerbated NOD1-mediated coronary arteritis similarly (Figure 6b-d). In contrast, Tac was unable to exacerbate

461 coronary arteritis in MyD88$^{-/-}$ mice (Figure 6e-g). As the NOD2 ligand MDP,
462 exacerbated NOD1-mediated coronary arteritis in WT mice (11), we
463 administered MDP to MyD88$^{-/-}$ mice as a positive control, and the coronary
464 arteritis was exacerbated by MDP as expected (Fig. 6e-g). Therefore, we
465 concluded that CNIs exacerbated NOD1-mediated coronary arteritis by
466 activating the MyD88-dependent inflammatory signals, whereas CARD9 was not
467 essential in the exacerbation process.
468 We examined further whether or not a MyD88 deficiency might reduce the
469 macrophage activation by CNIs *in vitro*. We stimulated BMDMs from MyD88$^{-/-}$
470 and WT mice with CNIs in the absence or presence of FK565 (Fig. 6h). We
471 confirmed that the TNF production in BMDMs from MyD88$^{-/-}$ mice was not
472 enhanced by LPS but was enhanced by polyI:C (Fig. 6h, right). These data were
473 consistent with the fact that LPS is a stimulator of the TLR-4-MyD88 signaling
474 pathway, whereas polyI:C is a stimulator of the MyD88-independent TLR-3
475 signaling pathway (30). TNF production by Tac and FK565 was suppressed by
476 56% in MyD88$^{-/-}$ BMDMs compared with WT BMDMs ($p < 0.01$), suggesting that
477 the activation of macrophages by CNIs was partially MyD88-dependent (Fig. 6h,
478 left).
479
480 *CsA Exacerbates Coronary Arteritis in the Presence of LPS.*
481 When BMDMs from WT mice were stimulated with various doses of CsA in the
482 absence or presence of LPS *in vitro*, the TNF production by BMDMs was
483 increased in a dose-dependent manner (Fig. 7a). These data prompted us to
484 explore *in-vivo* evidence for the additive actions of LPS and CsA on coronary
485 arteritis, as LPS was shown previously to enhance NOD1-mediated coronary
486 arteritis (11). When we administered 10 μg of FK565 and 10 μg of LPS to induce
487 mild coronary arteritis, 4 mg/kg of CsA, which is equivalent to the therapeutic

488 dose in humans (Fig. 1e), enhanced NOD1-mediated coronary arteritis (Fig.
489 7b-d). These results therefore suggested that doses of CsA similar to those
490 usually used in humans might enhance coronary arteritis in the presence of
491 innate immune pathogen-associated molecular patterns such as LPS.
492

Discussion

In this report, we first demonstrated that both CsA and Tac exacerbated NOD1-mediated coronary arteritis in a mouse model of KD (11). CsA induced the exacerbation of coronary arteritis in mice only in high doses, the trough levels of which exceeded the toxic level in humans. In contrast, Tac exacerbated it even when administered at a dose within the therapeutic range in humans.

CsA and Tac bind to CN and inhibit its kinase activity, and thus regulate the activation of the downstream transcription factor, NFAT (31-34). Although CNIs are known as potent immunosuppressants, they affect not only T cells but also many other cells, including B cells, neutrophils, macrophages, vascular endothelial cells, and smooth muscle cells (35-41).

In our KD model, vascular cells and cardiac $CD11c^+$ macrophages play a critical role in the pathogenesis of acute coronary arteritis (13). In this study, we also found that the exacerbation of coronary arteritis by CsA and Tac involved both vascular and monocytic cells, but not T cells/B cells by the experiments using SCID mice. Tac was sufficient to increase the cytokine secretion by vascular tissues *ex vivo.* Furthermore, both CsA and Tac enhanced the surface expression of adhesion molecules on endothelial cells but not the cytokine secretion from endothelial cells or smooth muscle cells. As NOD1 expression of vascular tissue was not elevated by CNIs (Supporting information, Fig. S4), we speculate that the exacerbation of coronary arteritis due to CNI was not related directly to the change in the expression levels of NOD1.

Further experiments revealed that the cytokine secretion by monocytic cells (macrophages, RAW264.7 cells and dendritic cells) was enhanced by CsA and Tac in a dose-dependent manner. Controversies exist concerning the effects of CsA and Tac, especially on monocytic cells. Some reports have argued that CsA and Tac inhibit TNF production from monocytic cells (35, 40, 41), and others

insist that CsA and Tac enhance cytokine production, such as TNF from macrophages (35, 40, 41). Kang *et al.* (25) reported that CN regulated the TLR-mediated activation pathways of macrophages negatively. Their data appear to support ours, as CNIs (CsA and Tac) activated the TLR-MyD88 and nuclear factor (NF)-κB-associated pathways, thereby enhancing the TNF production by macrophages. With regard to other cell types, several studies have demonstrated experimental evidence for the enhanced production of inflammatory cytokines by CNIs using fibroblasts (36), aortic endothelial cells (42) and smooth muscle cells (39). Thus, CsA and Tac show either immunosuppressive or immunostimulatory effects, depending on the dose, cell type and interaction with endothelial cells (21, 43, 44).

A recent report showed that CNIs (CsA and Tac) induced endothelial adhesion molecule synthesis and *ex-vivo* vascular inflammation by activation of NF-kB / p65, oxidative stress and reactive oxygen species (ROS) in vascular tissue through TLR-4/MyD88 signaling pathway (42). We also demonstrated that the exacerbation of coronary arteritis by CNI (Tac) in mice was MyD88-dependent and CARD9-independent, using *MyD88*$^{-/-}$ and *Card9*$^{-/-}$ mice. Although our findings have partly overlapped with those in the previous report by Rodrigues-Diez et al. (42) , the fundamental distinction between our and their reports is that our conclusion was based on not only *ex-vivo* and *in-vitro* data, but also *in-vivo* experiments. It was useful because we could evaluate the effect of CNIs directly by the analysis of severity of vasculitis, which is accompanied with marked cellular infiltration around the coronary artery. Thus, to our knowledge, our report has first shown the histological exacerbation of coronary arteritis caused by CNIs.

Furthermore, one of the important additional findings is that the combination of CsA and LPS exacerbated coronary arteritis further (Fig. 7). As

547 LPS priming was as effective as LPS-CsA simultaneous administration in
548 exacerbation of the coronary arteritis (Supporting information, Fig. S5), it was
549 speculated that there might be a higher inflammatory alert status of the cells in
550 terms of the additive effect of LPS on CNI. However, it remains to be determined
551 whether the priming effect was due to the utilization of the same receptor or a
552 general activation of the cells.

553 The effect of CNI (Tac) on TNF production by dendritic cells was enhanced
554 in the absence of CN, consistent with the negative regulatory effect of CN on the
555 TLR-mediated activation pathways (25). Another report showed that the
556 proinflammatory activity of CNIs in endothelial cells was partially dependent
557 upon CN (42), whereas other reports showed that CNIs (CsA and Tac) also have
558 CN-independent effects and do not reproduce the phenotype obtained upon CN
559 gene deletion (45-47). Thus, it is likely that the CN-independence of the
560 proinflammatory activity of CNIs varies, depending on the cell type.

561 CsA exerts anti-pyretic and anti-inflammatory effects in most refractory KD
562 patients. However, clinical studies have suggested that CsA at the therapeutic
563 concentration has no strong protective or promotive effect on the development of
564 coronary aneurysms. Suzuki *et al.* reported that CsA was not able to suppress
565 the progression of CAL in two cases with CAL among 28 IVIG-refractory cases,
566 and two of 26 cases without CAL developed CAL during CsA treatment (7).
567 Hamada *et al*. reported that, among 19 IVIG refractory cases who were treated
568 by CsA, one of 14 CsA responders (afebrile within 5 days) and two of five CsA
569 non-responders developed CAL (9). Tremoulet *et al*. reported that among 10
570 IVIG-resistant KD cases, three of nine CsA-treated cases and the one
571 Tac-treated case developed coronary aneurysms (8). Currently, a phase III
572 multi-center, randomized, open-label, blinded end-point trial is in progress to
573 evaluate the efficacy and safety of immunoglobulin plus CsA in patients with

574 severe KD (KAICA Trial) (48). With the primary end-point ~~at~~ set as the
575 occurrence of CAL, this prospective study will clarify the favorable or unfavorable
576 effects of the CsA on the CAL development in KD patients.
577 The present study was based mainly on animal and cell experiments,
578 whereas these data might not necessarily reflect the exacerbating effect of CNIs
579 on coronary artery lesions in humans. Taking the above reports (7-9) into
580 account, we speculate that the results of this study may be related partly to the
581 mechanism of exacerbation of human coronary artery lesions. As even a small
582 amount of CsA can exacerbate coronary arteritis in the presence of other innate
583 immune stimulants such as LPS in mice, it would be safer that the use of CNIs is
584 monitored carefully, especially for severe forms (KD shock syndrome, etc.) of KD
585 patients, who might also be receiving certain innate immune stimulants.
586 In summary, the present study revealed the exacerbation of coronary
587 arteritis by CNIs in a NOD1-mediated KD murine model. CNIs appear to exert
588 their effects through the activation of vascular cells and macrophages via the
589 MyD88 pathway (Figure 8). CsA may still be used in the treatment of refractory
590 KD provided that particular care is taken with respect to the coronary arteries.
591

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Disclosures

The authors have no conflicts of interest.

Author contributions

S. Y. and T. H. designed the study; K. M., Y. M., T. T., S. K., T. Y., M. O. A. S. and H. N. performed animal and laboratory experiments; H. T., S. M., K. F., M. O. and H. H. analyzed the results and interpreted the data; K. M., H. N. Y. S. and T. H. wrote the manuscript; S. O. and T. H. supervised the study and scrutinized the manuscript.

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771 patients with severe Kawasaki disease (KAICA Trial). BMJ Open.
772 2015;5(12):e009562.
773

774 **Figure legends**

775 **Figure 1. Exacerbation of coronary arteritis by calcineurin inhibitors**
776 **(CNIs).**

777 **A,** The experimental protocol. p.o.: per os, i.p.: intraperitoneal injection

778 **B-D,** The histological evaluation of coronary arteritis (B) induced by cyclosporine
779 A (CsA) or tacrolimus (Tac) and 100 μg of FK565 in wild type mice (scale bar,
780 100 μm). The numbers (C) and infiltration areas (D) of inflammatory cells are
781 shown. The data are presented as the mean ± standard deviation (s.d.) (n = 3).
782 *: $p < 0.05$ (Dunnett's test).

783 **E,** After CsA (4, 12, 40, or 120 mg/kg body weight per dose) or Tac (0.2, 0.6, 2,
784 or 6 mg/kg body weight per dose) was administered i.p. once daily for 5
785 consecutive days, blood concentrations of CsA and Tac at 0 (trough), 2 and 4 h
786 after drug administration on day 5. The data are presented as the mean ± s.d. (n
787 = 3). CsA (120 mg/kg) and 6 mg/kg of Tac (blue squares), 40 mg/kg of CsA and 2
788 mg/kg of Tac (red diamonds), 12 mg/kg of CsA and 0.6 mg/kg of Tac (green
789 circles), and 4 mg/kg of CsA and 0.2 mg/kg of Tac (purple triangles). Gray
790 dashed lines represent the blood concentrations of human therapeutic doses
791 [5.26 mg/kg of CsA and 0.16 mg/kg of Tac from Refs (18, 20)].

792

793 **Figure 2. Exacerbation of coronary arteritis by calcineurin inhibitors (CNIs)**
794 **in CB-17 severe combined immunodeficiency (SCID) mice.**

795 CB-17 mice were administered 40 mg/kg of cyclosporin A (CsA), 2 mg/kg of
796 tacrolimus (Tac), or a solvent control [10% dimethylsulphoxide (DMSO)]
797 intraperitoneally with 100 μg of FK565 [per os (p.o.)] once daily for 5 consecutive
798 days. A histological evaluation of coronary arteritis (**A**) induced by CsA or Tac
799 and 100 μg of FK565 in CB-17 SCID mice (scale bar, 100 μm). The numbers (**B**)
800 and infiltration areas (**C**) of inflammatory cells are shown. The data are

presented as the mean ± standard deviation (s.d.) (n = 3). * $p < 0.05$ (Dunnett's test).

Figure 3. The effects of calcineurin inhibitors (CNIs) on vascular and monocytic cells.

A and B, The culture supernatants of mouse heart endothelial cells (MHECs) (**A**), human coronary artery endothelial cells (HCAECs) and human coronary artery smooth muscle cells (HCASMCs) (**B**) stimulated with cycloporin A (CsA) or tacrolimus (Tac) in the presence of FK565 were assayed for interleukin (IL)-6, chemokine (C-C motif) ligand 2 (CCL2) and IL-8.

C, The culture supernatants of bone marrow-derived macrophages (BMDMs) from wild type mice stimulated with CsA or Tac in the presence or absence of FK565 were assayed for tumour necrosis factor (TNF) and IL-6.

D, The culture supernatants of RAW264.7 monocytic cells stimulated with CsA or Tac in the presence or absence of FK565 were assayed for TNF.

E, The culture supernatants of aortic root tissues stimulated with CsA or Tac and 10 μg/ml FK565 were assayed for IL-6 and CCL2.

The data are presented as the mean ± standard deviation (s.d.) (n = 4). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ ~~as~~ compared to controls (0 μM CsA or Tac) of each group (Dunnett's test).

Figure 4. Effect of calcineurin inhibitors (CNIs) and FK565 on adhesion molecule expression on human coronary artery endothelial cells (HCAECs).

A. HCAECs were stimulated with cyclosporine A (CsA) or tacrolimus (Tac) and FK565. The frequencies of intercellular adhesion molecule-1 (ICAM-1)- and vascular cell adhesion molecule-1 (VCAM-1)-positive cells on HCAECs were

828 measured by fluorescence activated cell sorter (FACS). The data are presented
829 as the mean ± standard deviation (s.d.) (n = 6).
830 **B.** HCAECs were stimulated with CsA or Tac and FK565. The mRNA expression
831 of ICAM-1, VCAM-1, and E-selectin in HCAECs was measured by a quantitative
832 real-time-polymerase chain reaction (PCR) analysis, and were normalized to
833 those of β-actin. The data are presented as the mean ± standard deviation (s.d.)
834 (n = 3).
835 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Dunnett's test).
836
837 **Figure 5. Tumour necrosis factor (TNF) production enhanced by**
838 **calcineurin inhibitors (CNIs) was calcineurin-independent.**
839 **A,** The culture supernatants of bone marrow-derived dendritic cells (BMDCs)
840 from wild-type (WT) mice stimulated with cyclosporine A (CsA) or tacrolimus
841 (Tac) in the presence or absence of FK565 were assayed for TNF production.
842 The data are presented as the mean ± standard deviation (s.d.) (n = 4). *: p <
843 0.05, **: $p < 0.001$ ~~as~~ compared to the controls (0 μM CsA or Tac) of each group
844 (Dunnett's test).
845 **B,** The culture supernatants of BMDCs from *Cnb1*$^{flox/flox}$ (Cnb1 WT) and
846 *Cd11c-Cre*$^{+}$*Cnb1*$^{flox/flox}$ (Cnb1 CKO) mice stimulated with CsA or Tac and FK565,
847 and with plate-coated trehalose-6,6'-dimycolate (TDM), lipopolysaccharide
848 (LPS) or PolyI:C as positive controls were assayed for TNF. The data are
849 presented as the mean ± standard deviation (s.d.) (n = 4). *: $p < 0.05$, **: $p <$
850 0.001 (Student's *t*-test).
851

852 **Figure 6. The coronary arteritis exacerbation and macrophage activation**
853 **by calcineurin inhibitors (CNIs) were myeloid differentiation primary**
854 **response gene 88 (MyD88)-dependent.**
855 **A,** The experimental protocol; subcutaneous injection (s.c.), intraperitoneal
856 injection (i.p.).
857 **B-D,** A histological evaluation of coronary arteritis (**B**) induced by tacrolimus
858 (Tac) and 100 μg of FK565 in caspase-associated recruitment domain 9
859 $(CARD9)^{-/-}$ mice (scale bar, 100 μm). The numbers (**C**) and infiltration areas (**D**)
860 of inflammatory cells are shown. The data are presented as the mean ± standard
861 deviation (s.d.) (n = 5). *: $p < 0.05$ (Dunnett's test).
862 **E-G,** A histological evaluation of coronary arteritis (**E**) induced by Tac or muramyl
863 dipeptide (MDP) and 500 μg of FK565 in $MyD88^{-/-}$ mice (scale bar, 100 μm). The
864 numbers (**F**) and infiltration areas (**G**) of inflammatory cells are shown. The data
865 are presented as the mean ± s.d. (control or Tac: n = 3, MDP; n = 1).
866 **H,** The culture supernatants of bone marrow-derived macrophages (BMDMs)
867 from wild-type (WT) and $MyD88^{-/-}$ mice stimulated with CsA or Tac in the
868 presence or absence of FK565, and with PolyI:C and lipopolysaccharide (LPS)
869 as positive controls were assayed for tumour necrosis factor (TNF). The data are
870 presented as the mean ± s.d. (n = 4). *: $p < 0.01$, **: $p < 0.001$ (Student's *t*-test).
871
872 **Figure 7. The combination of cyclosporine A (CsA) and lipopolysaccharide**
873 **(LPS) further activated macrophages and exacerbated coronary arteritis.**
874 **A.** The culture supernatants of bone marrow-derived macrophages (BMDMs)
875 from wild-type (WT) mice stimulated with CsA in the presence or absence of LPS
876 were assayed for TNF. The data are presented as the mean ± standard deviation
877 (s.d.) (n = 4). *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$ ~~as~~ compared to controls (0
878 μM CsA) of each group (Dunnett's test).

879 **B.** The experimental protocol. We set the treatment protocol as 10 μg of FK565
880 for 2 days with or without 4 mg/kg of CsA for 5 consecutive days. This protocol
881 induced only mild coronary arteritis in the WT mice.
882 **C and D.** Injections of LPS (10 μg) for 2 days were added to this mild coronary
883 arteritis-inducing protocol. The numbers (**C**) and infiltration areas (**D**) of
884 inflammatory cells are shown. The data are presented as the mean ± s.d. (n =
885 3-4). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (Tukey-Kramer test).
886
887 **Figure 8. Graphical abstract**
888 A nucleotide-binding oligomerization domain-containing protein 1 (NOD1) ligand,
889 FK565, activates vascular cells, which produce large amounts of chemokines.
890 Chemokine receptor-expressing monocytes in the peripheral blood are recruited
891 to FK565-activated vascular cells. This process subsequently induces the
892 differentiation of the monocytes into cardiac macrophages, which play a pivotal
893 role in the pathogenesis of acute coronary arteritis. Calcineurin inhibitors
894 exacerbate NOD1-mediated coronary arteritis in a dose-dependent manner in a
895 murine model of Kawasaki disease through the activation of vascular cells and
896 macrophages via the myeloid differentiation primary response gene 88 (MyD88)
897 pathway.

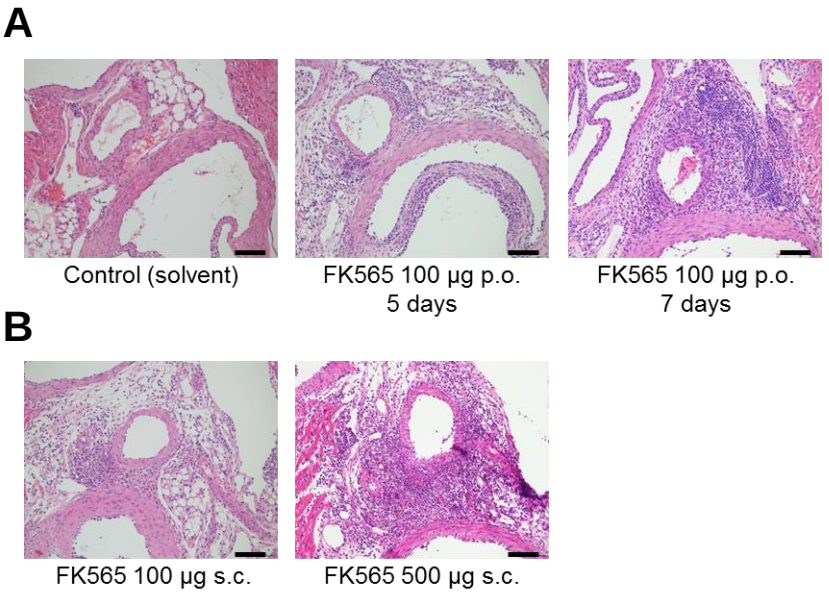

Figure S1. FK565-induced coronary arteritis after oral or subcutaneous administration

A, Histological evaluation of coronary artery on the following day after oral administration of 100 µg of FK565 or distilled H_2O (solvent of FK565) for 5 or 7 days (scale bar, 100 µm).

B, Histological evaluation of coronary artery on the following day after subcutaneous administration of 100 or 500 µg of FK565 twice (Days 0 and 3) (scale bar, 100 µm).

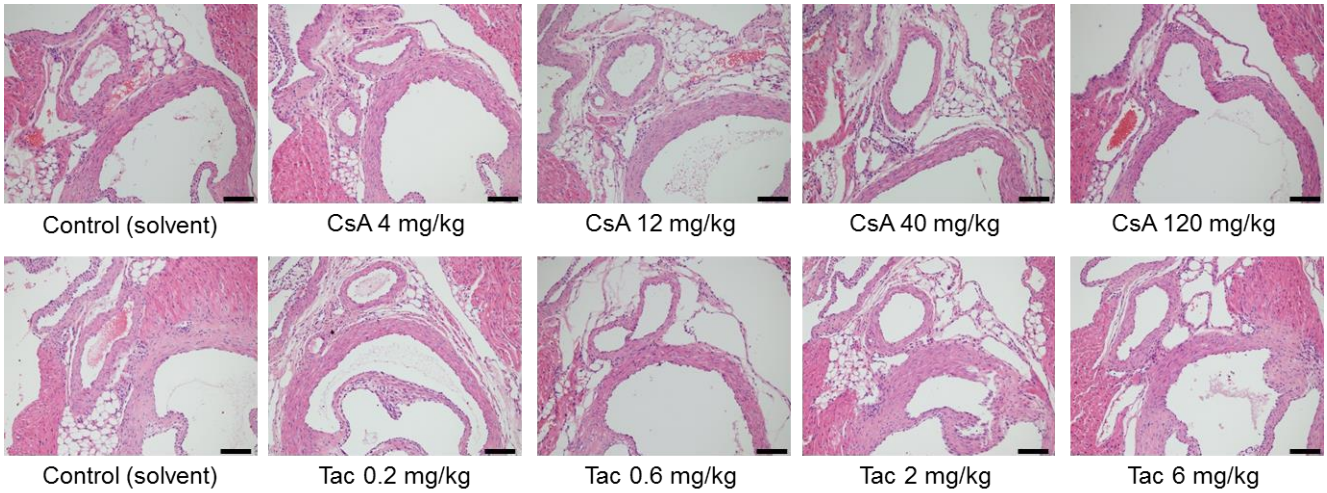

Figure S2. No coronary arteritis was induced only by calcineurin inhibitors (CNIs).

A histological evaluation of the coronary artery after 5 days' administration of cyclosporin A (CsA), tacrolimus (Tac) and distilled H_2O (solvent of FK565) (scale bar, 100 μm).

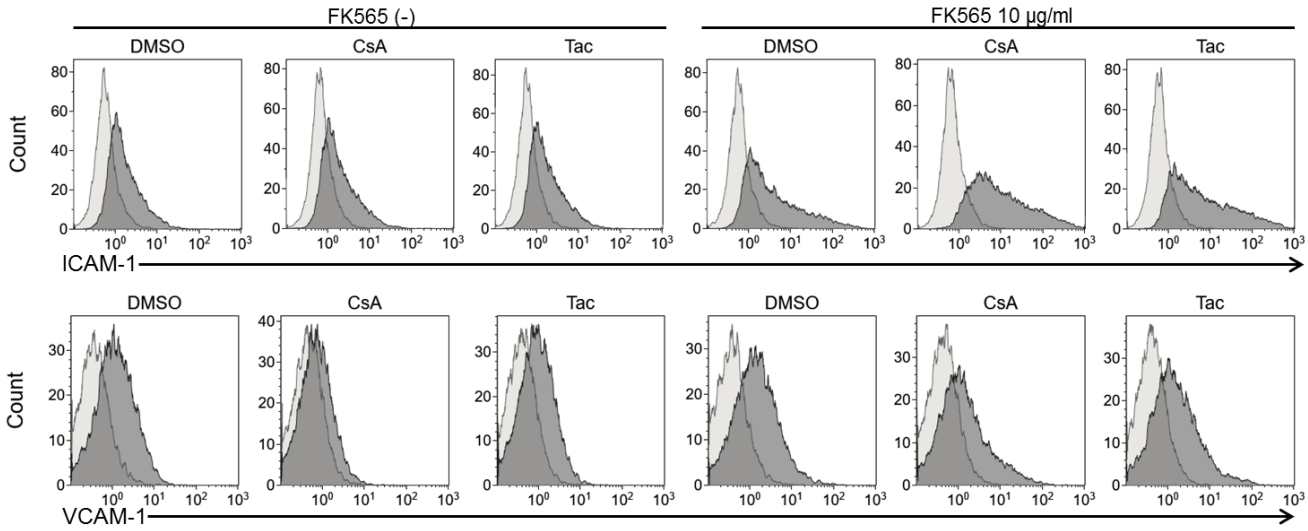

Figure S3. Histogram of intercellular adhesion molecule-1 (ICAM)-1 and vascular adhesion molecule-1 (VCAM-1) expression in human coronary artery endothelial cells (HCAECs)

Histograms show flow cytometric analysis of ICAM-1 and VCAM-1 expression in HCAECs after stimulation of FK565 with cyclosporin A (CsA) or tacrolimus (Tac). Respective isotype controls are shown as light gray area.

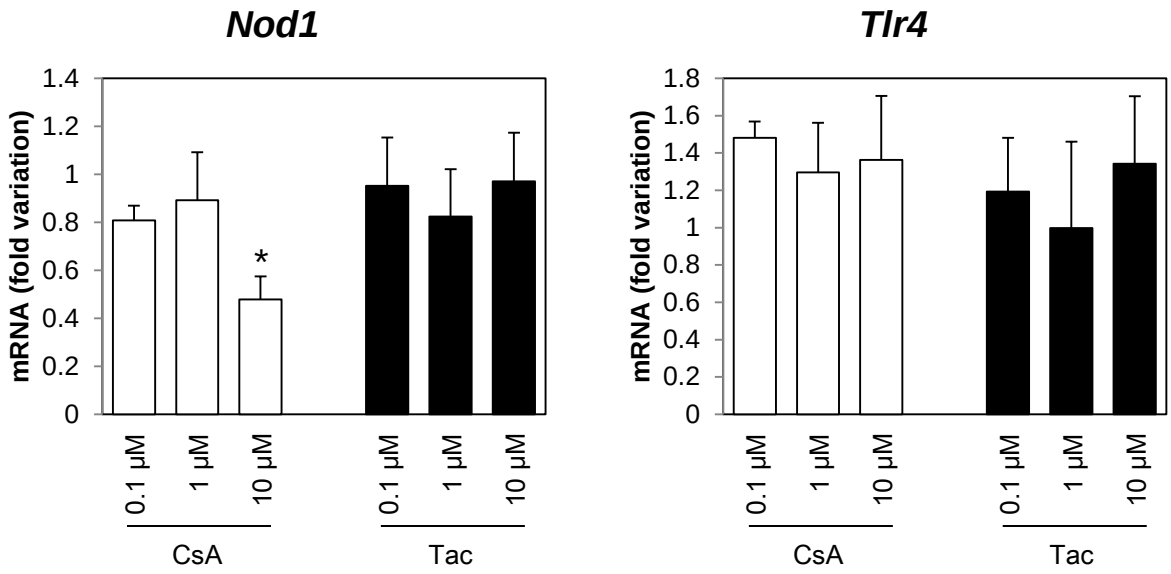

Figure S4. Effect of calcineurin inhibitors (CNIs) on nucleotide-binding oligomerization domain-containing protein 1 (*NOD1*) expression in human coronary artery endothelial cells (HCAECs).

HCAECs were stimulated with cyclosporin A (CsA) or tacrolimus (Tac). *NOD1* and *Tlr4* mRNA expression levels in HCAECs were measured by a quantitative real-time PCR analysis, normalized to those of β-actin, and compared to controls (relative levels in untreated HCAECs). The data are presented as the mean ± standard deviation (s.d.) (n = 3). * $p < 0.01$ (Dunnett's test).

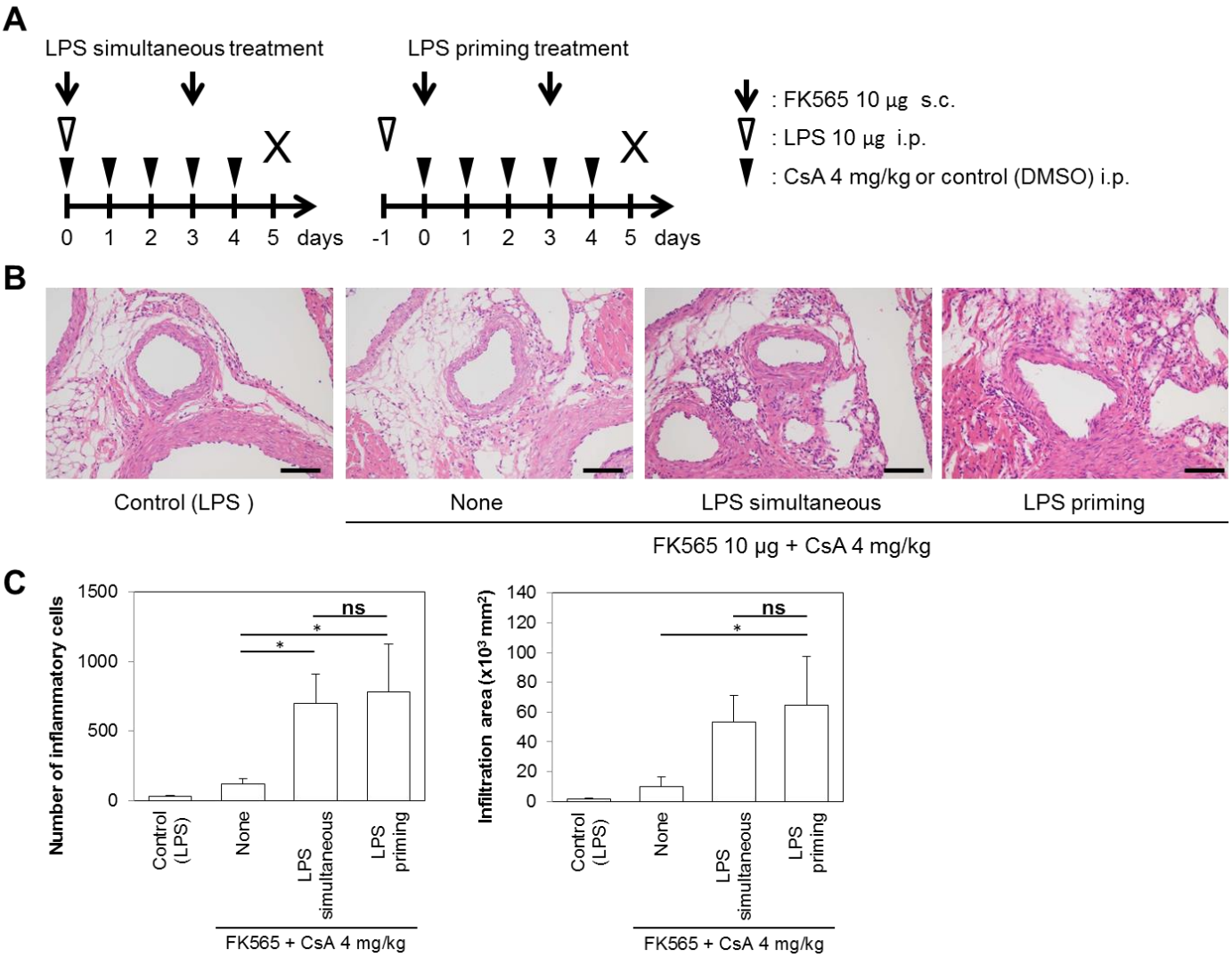

Figure S5. Comparison of coronary arteritis exacerbation between simultaneous administration and priming of lipopolysaccharide (LPS)

A, The experimental protocol. We set the treatment protocol as 10 μg of FK565 for 2 days and 4 mg/kg of cyclosporin A (CsA) for 5 consecutive days with or without single dose of LPS (10 μg). LPS was administrated simultaneously with calcineurin inhibitor (CNI)/FK565 or primed 1 day prior to CNI/FK565.

B-D, The histological evaluation of coronary arteritis (B) induced by CsA and FK565 with LPS (simultaneous or priming treatment) in wild-type (WT) mice (scale bar, 100 μm). The numbers (C) and infiltration areas (D) of inflammatory cells are shown. The data are presented as the mean ± standard deviation (s.d.) (n = 3). *: $p < 0.05$, ns: not significant (Tukey-Kramer test).

Supporting information

Calcineurin inhibitors exacerbate coronary arteritis via the MyD88 signaling pathway in a murine model of Kawasaki disease

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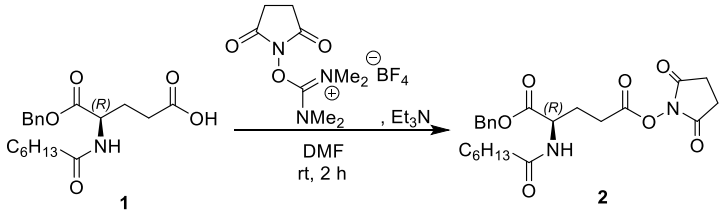
Preparation of FK565

Succinimidyl esterification of compound (1)

To a solution of **1** (387 mg, 1.11 mmol) (1) and *N,N,N'N'*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (367 mg, 1.22 mmol) in anhydrous *N,N*-dimethylformamide (DMF) (5.0 mL) was added triethylamine (337 mg, 3.33 mmol) at room temperature under Ar atmosphere. After stirring for 2 h, the mixture was concentrated *in vacuo* to give crude product **2** as yellow oil, which was used for the subsequent coupling without further purification.



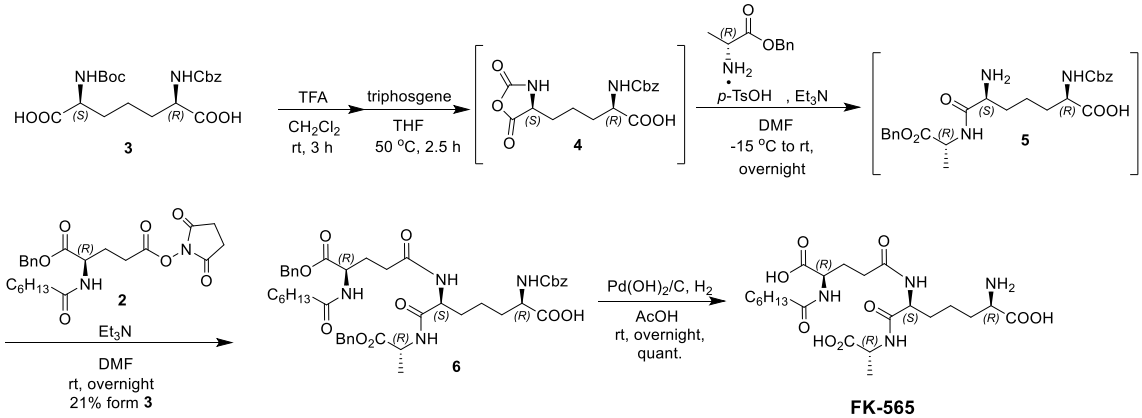
Synthesis of FK-565

To a solution of **3** (235 mg, 554 μmol) (2) in CH_2Cl_2 (300 μL) was added trifluoroacetic acid (100 μL) in CH_2Cl_2 (100 μL). The reaction mixture was stirred for 3 h and concentrated *in vacuo*. The residual trifluoroacetic acid was removed by co-evaporation with toluene. To the residue was added 1 M hydrogen chloride diethylether solution to give Boc-deprotected intermediate as a white solid, which was lyophilized in 1,4-dioxane. The crude residue was dissolved in dry tetrahydrofuran (THF) (5.0 mL) at 50 °C, followed by the addition of triphosgene (82 mg, 277 μmol) in dry THF (5.0 mL). The solution was stirred at 50 °C for 2.5 h. After cooling to room temperature, the reaction mixture was concentrated *in vacuo* and the residue was washed by dry *n*-hexane. Without purification, the next step was carried out immediately. To the solution of the crude compound **4** and triethylamine (280 mg, 2.77 mmol) in dry DMF (3.0 mL) was added *p*-toluenesulfonic acid·H-D-Ala-OBn (253 mg, 720 μmol) in dry DMF (2.0 mL) at -15 °C. The mixture was stirred at -15 °C for 0.5 h, and then warmed to room temperature. The reaction mixture was stirred overnight and concentrated *in vacuo*. The residual DMF was removed by co-evaporation with toluene. After removing DMF, the next step was carried out directly. To a solution of crude compound **5** and

triethylamine (280 mg, 2.77 mmol) in dry DMF (3.0 mL) was added compound **2** (1.11 mmol) in DMF (2.0 mL) dropwise. After being stirred overnight, the reaction was diluted with EtOAc and the mixture was washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4, and filtered. After being concentrated *in vacuo*, the residue was purified with gel permeation chromatography ($CHCl_3$/MeOH = 1/1) and silica-gel flash column chromatography ($CHCl_3$/MeOH =30/1 to 10/1 to 3/1) to give **6** as a white solid (95 mg, 21% from **3**). To a solution of **6** (30 mg, 36.7 μmol) in acetic acid (2.0 mL) was added $Pd(OH)_2$/C (20% [w/w] on carbon) (20 mg). The mixture was stirred under 10 kg/cm^2 of hydrogen at room temperature overnight. The Pd catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The residual acetic acid was removed by co-evaporation with toluene. The residue was lyophilized from water to give **FK-565** as a white solid (18.0 mg, quant.).

HRMS (ESI-TOF, positive) Calcd. for $C_{22}H_{40}N_4O_9$ $[M+H]^+$: 503.2712, Found 503.2723.

^{1}H NMR (500 MHz, CD_3OD) δ: 4.32-4.28 (m, 3H, DAP-αH, Gln-αH, Ala-αH), 3.86 (t, *J* = 6.2 Hz, DAP-εH), 2.29 (t, *J* = 7.2 Hz, 2H, Glu-γH), 2.18-2.11 (m, 3H, Glu-βH, α-CH_2 of heptanoyl chain), 1.96-1.74 (m, 4H, DAP-βH, δH Glu-βH), 1.67-1.42 (m, 5H, DAP-βH, γH, β-CH_2 of heptanoyl chain) 1.31 (d, 3H, *J* = 7.3 Hz, Ala-αH), 1.28-1.19 (m, 6H, CH_2 of heptanoyl chain), 0.81 (t, *J* = 6.8 Hz, 3H, -CH_2-$C\underline{H_3}$ of heptanoyl chain).



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