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Nishio et al. Nod1 related to vascular inflammation

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Abstract

Objective:

To investigate the effects of stimulants for nucleotide-binding domain, leucine-rich repeat-containing (NLRs) protein family on human artery endothelial cells and murine arteries.

Methods and Results:

Human coronary artery endothelial cells (HCAEC) were challenged *in vitro* with microbial components that stimulate NLRs or Toll-like receptors (TLRs). We found the stimulatory effects of NLR and TLR ligands on the adhesion molecule expression and cytokine secretion by HCAEC. Based on these results, we examined the *in vivo* effects of these ligands in mice. Among them, FK565, one of the nucleotide-binding oligomerization domain (Nod)1 ligands, induced strong site-specific inflammation in the aortic root. Furthermore, coronary arteritis/valvulitis developed after direct oral administration or ad lib drinking of FK565. The degree of respective vascular inflammation was associated with persistent high expression of proinflammatory chemokine/cytokine and matrix metallopeptidase (*Mmp*) genes in each tissue *in vivo* by microarray analysis.

Conclusions:

This is the first coronary arteritis animal model induced by oral administration of a pure synthetic Nod1 ligand. The present study has demonstrated an unexpected role of Nod1 in the development of site-specific vascular inflammation, especially coronary arteritis. These findings might lead to the clarification of the pathogenesis and pathophysiology of coronary artery disease in humans.

Key Words: Nod1; NLR; coronary arteritis; valvulitis; innate immunity

Introduction

Germ-line encoded pattern-recognition receptors (PRRs) of the innate immune system sense exogenous microbial components and endogenous danger signals to protect the host[1-4]. The PRRs include TLRs, RIG-I like receptors (RLRs), NLRs protein family and as-yet-unidentified PRRs that recognize double-stranded DNA[1,3]. The TLR, RLR, and NLR families consist of 10 (human), 3, and more than 20 members, respectively[1,3,4].

In the cardiovascular system, endothelial cells are usually the first ones among the structural cells to sense microbial components through PRRs. Human endothelial cells express functional innate immune receptors such as TLRs and NLRs[5,6]. There is a line of evidence that activation of TLRs, especially TLR4 and TLR2, contributes to the development and progression of cardiovascular diseases including atherosclerosis, cardiac dysfunction in sepsis, and congestive heart failure[7]. With respect to NLRs, only a limited number of studies have shown that human endothelial cells express functional NLRs, NOD1 and NOD2. *Chlamydophila pneumoniae* and *Listeria monocytogenes* elicited NOD1-dependent interleukin (IL)-8 production in endothelial cells[8.9]. A selective NOD1 ligand, FK565, but not a selective NOD2 ligand, muramyl dipeptide (MDP), induced nitric oxide synthase-II protein/activity and vascular hyporeactivity *ex vivo* and

shock *in vivo*[10].

Since innate immunity has been suggested to be involved in the pathogenesis and/or pathophysiology of cardiovascular diseases in adults[7] as well as vasculitis in Kawasaki disease in children (KD)[11], we have investigated the effects of stimulants for innate immune receptors, especially TLRs and NODs, on human artery endothelial cells *in vitro*, and murine arteries *in vivo.* We found the stimulatory effects of pure NOD1 and NOD2 ligands on coronary artery endothelial cells *in vitro*, and the induction of coronary arteritis by oral or parenteral administration of a pure selective NOD1 ligand with or without a microbial component in mice *in vivo*. This evidence indicates a possible linkage between an innate immune receptor, NOD1, and cardiovascular disorders.

METHODS

Histological evaluation. All organs were isolated using Leica M500 ophthalmology microscope. Cryostat sections were used for the correct detection of three aortic valve cusps in these studies. Severity of coronary arteritis was assessed by the cross section with three aortic valve cusps as described[12] and defined as follows: (-) indicates no inflammatory infiltration in the whole layer (from intima to adventitia) of nearest coronary arteries from aorta and/or aorta; (+) indicates that less than one third of the

circumference showed inflammatory infiltration in the whole layer; (2+), between one third to two thirds; (3+), more than two thirds.

For a detailed description of methods, please see the supplemental materials, available online at http://atvb.ahajournals.org.

Results

NOD ligands enhance ICAM-1 expression and cytokine production by HCAEC *in vitro*

To investigate the direct effects of innate immune stimulants on the endothelial cells, HCAEC were challenged *in vitro* with microbial cell wall components that stimulate TLRs and NLRs. After preliminary time course studies (data not shown), we analyzed the effects of each reagent on the intercellular adhesion molecule-1 (ICAM-1, CD54) expression and cytokine secretion by HCAEC at day 3. Significant ICAM-1 expression and IL-8 secretion were induced by a pure synthetic Nod1 ligand, γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP), a pure synthetic Nod2 ligand, MDP, a pure synthetic TLR4 ligand, lipopolysaccharide (LPS) lipid A, and TLR2 ligand, peptidoglycan from *E.coli K12* (PGN K12) in HCAEC (Fig. 1). Since synergistic effects of NLR and TLR ligands were observed in human cells[13], we analyzed the effects of

various components in combination on HCAEC. Enhanced ICAM-1 expression and/or cytokine production were observed by the combined stimulation with pure synthetic iE-DAP plus MDP or iE-DAP plus Lipid A in HCAEC. No releases of IL-1β, IL-10, tumor necrosis factor (TNF)-α and IL-12p70 were observed by any combination in HCAEC (data not shown). These results clearly demonstrate that pure synthetic Nod1 and Nod2 ligands and TLR ligands activate human artery endothelial cells *in vitro*.

To rule out possible secondary effects of NOD stimulation at day 3 experiment, we performed experiments at day 1 as well. Similar additive effects were observed between NOD1 and TLR4 ligands at day 1 (Supplementary Fig. IA). In addition, NOD1 siRNA completely inhibited additive effects of NOD1 and TLR4 ligands at day 1 experiments (Supplementary Fig. IB). Thus, the additive effect between NOD1 and TLR4 ligands appeared to be not secondary but primary.

Nod1 ligands induce site-specific inflammation *in vivo* in mice

Based on these *in vitro* results, we examined the *in vivo* effects of a pure synthetic Nod1 ligand (FK565), MDP, LPS, PGN K12, and other bacterial component or bacteria (zymosan, OK432) on the artery endothelial cells in mice. As a Nod1 ligand, FK565 was mainly used for *in vivo* studies instead of iE-DAP or FK156, since FK565 is generally most effective among Nod1 ligands and showed stronger effects on HCAEC

(Supplementary Fig. II). ICAM-1 expression and cytokine production by HCAEC were enhanced by the combined addition of LPS (Fig. 1) and priming with LPS up-regulated the expression levels of *TLR* gene, resulting in the enhancement of innate immune response to peptidoglycan in mice[14]. Therefore, BALB/c mice were intraperitoneally primed with or without LPS, and 24 hours later, each reagent was injected 4 times at an interval of 1 week (Supplementary Table IA). Subcutaneous injection of MDP, PGN K12, zymosan, or OK432 with LPS priming or LPS priming alone did not induce any cellular infiltration in the arteries (Supplementary Table IA, Fig. 2Aa). On the other hand, when mice were subcutaneously injected by FK565 (500 μg) with LPS priming, diffuse cellular infiltration was observed in the aortic root including aortic valves and the origin of coronary arteries in all mice (Fig. 2Ab). The histopathological features of this coronary arteritis model were characterized by panarteritis with dense inflammatory infiltrate mainly consisting of neutrohophils and macrophages (Supplemantary Fig. III), and not associated with fibrinoid necrosis, similar to those at acute-phase of KD[15], which is an acute febrile illness of childhood characterized by the occurrence of vasculitis, especially coronary arteritis and valvulitis. This model didn't show coronary aneurysm, but the rupture of elastic fiber in coronary artery was observed just like Kawasaki disease. Neither formation of thrombus nor granuloma was recognized in aortic and

coronary lesions of all experimental mice. MDP, by itself, did not induce coronary arteritis at 500 μg but the enhancement of the effect of Nod1 agonist by MDP became apparent when MDP was added to suboptimal doses of FK565 (200 μg, 100 μg). Vascular inflammation was not observed in pulmonary, celiac, renal, and other arteries, while mild cellular infiltration was observed in other parts of aorta, and common carotid and subclavian arteries (data not shown). Subcutaneous injection of iE-DAP or FK156 induced slight inflammatory reaction (data not shown), while that of FK565 did highly reproducible and remarkable inflammation in the aortic root, indicating that the effects of Nod1 agonists vary greatly *in vivo*, depending on the chemical structure of Nod1 agonist used. FK565 administration induced arteritis of the similar severities and frequencies in other strains such as C57B/6, DBA/2, CD1, CBA/J and CH3 (data not shown). Severe combined immunodeficiency (SCID) mice developed weaker but significant arteritis, suggesting a partial involvement of acquired immunity in the inflammation induced by a pure Nod1 ligand, while no arteritis was observed in Nod1-knockout mice (Supplementary Table IB, Fig. 2B).

As FK565 is highly stable and effective by parenteral and oral routes[16], FK565 was orally administered (Supplementary Table IC). All BALB/c mice showed coronary arteritis/valvulitis after oral administration of 6 days (100 μg/day) /week of FK565 with

LPS priming, and the severity of coronary arteritis/valvulitis increased with the duration of the administration. Even in the absence of LPS priming, 4 out of 5 mice developed severe coronary arteritis/valvulitis after 4 weeks (Fig. 2Ac). When mice were given FK565-containing tap water (estimated daily doses of 120 μg/day, N=3 and 180 μg/day, N=3) ad lib for 4 weeks in the absence of LPS priming, all 6 developed severe (grade 3+) coronary arteritis/valvulitis (Fig. 2Ac). On the other hand, oral administration of FK565 induced no inflammation in gut mucosa (Fig. 2C) as well as in many arteries and organs (data not shown).

Site-specific vascular inflammation *in vivo* is associated with high expression of chemokine/cytokine and *Mmp* genes in each tissue

First, *Nod1* expression levels in normal mice were quantified by real-time RT-PCR with glyceraldehyde-3-phospate dehydrogenase (*GAPDH*) and Cadherin 5[17] (*CDH5*, a constitutive endothelial marker) genes as internal controls. Relative *Nod1/GAPDH* and *Nod1/CDH5* ratios in normal aortic root were not higher than those in other arteries (Fig. 3A). Consistent to the above results, Nod1-positive cells were detected with no great differences among normal vascular cells of coronary artery, aortic valve and pulmonary artery by the immunohistochemical staining (Fig. 3B).

To explore the molecular mechanisms of site-specific inflammation induced by Nod1

ligand, microarray analysis was performed in the most inflamed tissue (aortic root), no inflamed tissue (pulmonary artery) and much less inflamed tissue (ascending to abdominal aorta) from the vascular system, and the immune system (spleen). As shown in Figure 4A, tissue-specific gene expression patterns were observed by the *in vivo* stimulation with LPS, FK565, or LPS plus FK565. Among 10 chemokine/cytokine genes highly expressed in FK565- and LPS plus FK565-stimulated aortic root, only 2, 4 and none of which were continuously elevated over 5-fold in microarray data from FK565-stimulated pulmonary artery, aorta and spleen, respectively (Supplementary Table II). Several *Mmp* genes were also highly expressed in FK565-stimulated aortic root, indicating that the degree of inflammation was associated with persistent high expression of chemokine/cytokine and *Mmp* genes in each tissue *in vivo*. Marked inflammatory responses in aortic root by Nod1 ligand and TLR4 agonist were associated with the synergistic induction of chemokine (Ccl2, Cxcl13, Ccl8, Ccl7, Cxcl2), cytokine (Il6), Mmp (Mmp3, Mmp19, Mmp8), and Cam(Icam1, Selp and Jam2) mRNA levels.

Vascular tissue/cell-specific responses to Nod1 ligand *ex vivo* or *in vitro*

Comparison of the gene expression levels of 3 murine vascular tissues (aortic root, pulmonary artery, and arch portion of aorta) *ex vivo* as well as of 2 human endothelial

cells *in vitro* in the presence or absence of FK565 showed vascular tissue/cell-specific responses to FK565 (Supplementary Fig. IV, Supplementary Table III).

To further investigate the mechanisms of site-specific inflammation induced by Nod1 ligand, the production of chemokine (C-C motif) ligand 2 (CCL2) (monocyte chemoattractant protein-1; MCP-1) and IL-6, of which genes were highly expressed in *in vivo* FK565-stimulated aortic root (Supplementary Table II), was studied with *ex vivo* organ culture of aortic root, pulmonary artery, aortic arch and abdominal aorta from the vascular system in the presence or absence of lipid A, Nod1 ligands (iE-DAP, FK565), or MDP. The production of CCL2 and IL-6 was significantly higher in aortic root than in pulmonary artery, aortic arch, and abdominal aorta by the stimulation with FK565 in normal mice, while no production of CCL2/IL-6 in response to FK565 was observed in any vascular tissue from Nod1$^{-/-}$ mice (Fig. 4B). In addition, the production of CCL2 and IL-8 by FK565 was also higher in HCAEC than in human pulmonary artery endothelial cells (HPAEC) (Fig. 4C), suggesting that site-specific vascular inflammation is ascribed to an intrinsic nature of the vascular cells including endothelial cells[18].

Discussion

The present study has demonstrated that pure selective Nod1 ligands, iE-DAP, a

dipeptide with a M.W. of 319.3 daltons, FK565, an acyltripeptide with a M.W. of 502.6 daltons, and FK156, a synthetic tetrapeptide originally isolated from culture filtrates of Stretomyces strains with a M.W. of 519.5 daltons, and a Nod2 ligand, MDP, induced ICAM-1 expression and CCL2/IL-8 production in HCAEC, suggesting a possible role of these bacterial components in the pathogenesis of vasculitis.

In addition, coronary arteritis was induced *in vivo* in mice by selective Nod1 ligands, FK565, FK156 and iE-DAP. Nod2 ligand showed a significant effect on the development of coronary arteritis when FK565 dose was suboptimal. The induction of coronary arteritis by Nod1 ligand was enhanced by various microbial components such as TLR4 ligand (LPS). These findings can be explained by synergistic effects of Nod1 ligands with TLR agonists to produce inflammatory cytokines[19] .

The pathological findings of Nod1 ligand-induced coronary arteritis in mice were consistent with those of human coronary artery lesions at acute-phase KD, which showed edema and a dominant infiltration of neutrophils with some macrophages and lymphocytes at early stage (unitl 9 days after KD onset)[20]. No animal models of coronary arteritis have been reported with a pure or synthetic reagent.

Based on the fact that a Nod1 agonist, FK565, is very stable against temperature and acid[16], coronary arteritis was successfully induced by oral administration of FK565.

This is the first coronary arteritis animal model induced by oral administration of a pure synthetic Nod1 ligand. Absorption site of FK565 is not clear, but it is possible that gut mucosa is a major site because there were no great differences in the efficiencies of the induction of coronary arteritis between direct oral administration of FK565 solution and ad lib drinking of FK565-containing tap water.

Nod1 stimulants include *meso*-DAP and *meso*-Lanthionine, amino acids specific to bacterial peptidoglycans, iE-DAP, L-Ala-gamma-D-Glu-*meso*-DAP (TriDAP), FK156, FK565, GlcNAc-(β1–4)-(anhydro)MurNAc-L-Ala-γ-D-Glu-meso-DAP, bacterial extracts (*Bacillus* species, *Bacillus anthracis* spores, *L. pneumophila, S. typhimurium, Mycobacterium tuberculosis*) and live microbes (*S. flexneri, Helicobacter pylori,* Enteroinvasive *E. coli, Pseudomonas* species*, Chlamydia* species*, L. monocytogenes*)[4,21-24]. Nod1 agonists are considered to be derived from peptidoglycans of most gram-negative and some gram-positive bacteria[25] and *Chlamydia*[4,21,22], although major natural Nod1 stimulants produced by bacteria remain unknown[26]. Biologically active peptidoglycan fragments are released during growth by Gram-negative bacteria (*Escherichia coli* breaks down nearly 50% of its peptidoglycan every generation)[27]. As peptidoglycan is constantly turned over[27] and partly translocated across the gut mucosa into the circulation[23], NOD1 agonists in water-soluble or

water-insoluble (lipophilic) forms[26], together with various microbial components, may be released from normal or pathological microbiota, which contains 10^{14} microbes, which are estimated to weigh 1 kg in an adult human, in the gastrointestinal tract, airways, genitourinary tract, ducts of exocrine glands and skin[28-30].

Site-specific vascular inflammation was not related to Nod1 expression levels but appeared to be due to a site-specific production of chemokine/cytokine by respective vascular structures, since *ex vivo* organ culture in the presence of FK565 showed a site-dominant production of CCL2 and IL-6, as shown in Figure 4. It is likely that higher expression levels of chemokine and *Mmp* genes in *in vivo* FK565-treated aortic root than in *ex vivo* FK565-treated one by microarray analysis suggested an amplification of inflammation by the migration of inflammatory cells in a site-specific manner.

The site-specific nature of arterial inflammation in response to Nod1 ligand might be explained by a difference in the expression levels of certain molecules involved in Nod1 signaling pathway such as RIP2, MAP kinases and NFκB, and the inhibitors or activators[1,3,4]. Among them, A20 (tumor necrosis factor, alpha-induced protein 3) is one of the candidates because it is a negative regulator of TLR and NLR signaling via NF-kB[31], and significantly related to intestinal innate immunity including LPS-tolerance[32,33]. Considering that oral administration of Nod1 ligand does not induce

inflammation in gut mucosa as well as in many arteries such as pulmonary artery, it is possible that a certain Nod1-specific regulatory mechanism such as A20 is responsible for the inhibition of Nod1 signaling[31,34]. Further study is going on to identify which molecule in Nod1 signaling pathway is responsible for the site-specific effect of Nod1 ligand by the extensive comparison of inflammatory and non-inflammatory tissues and the use of knockout or transgenic mice.

The present study has first demonstrated an unexpected role of Nod1 in the development of site-specific vascular inflammation, especially coronary arteritis and valvulitis. These findings might lead to the clarification of the pathogenesis and pathophysiology of coronary artery and valvular lesions in KD in children as well as of coronary artery disease in adults.

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Disclosures

None.

Figure legends

Figure 1. Effects of innate immune stimulants on HCAEC.

HCAEC (1 x 10^4 cells) were incubated with NOD1, NOD2, TLR and other stimulants in various combinations. ICAM-1 expression (a) and IL-8 (b) /IL-6 (c) production in the culture supernatants were investigated in triplicate at day 3. The concentrations of stimulants are as follows: iE-DAP, MDP and PGN K12, 1 (■) or 10 (■) μg/mL; lipid A 10 (■) or 100 (■) ng/mL. Data are presented as mean ± s.d. *: P < 0.01 compared with medium, †: P < 0.01 compared with either iE-DAP or MDP, ‡: P < 0.01 compared with either iE-DAP or Lipid A (Dunnett's test).

Figure 2. Histopathologic changes after administration of Nod1 ligand in BALB/c, SCID, and Nod1 knockout mice.

A. Panel (a) shows cross sections with three aortic valve cusps of a BALB/c mouse treated with LPS 100 μg ip 4 times (Supplementary Table IA). The severity of coronary arteritis: grade 0. Left: hematoxylin and eosin (H&E) stain, right: Elastica Van Gieson (EVG) stain. Panel (b) shows grade 3+ coronary arteritis/valvulitis of the mouse subcutaneously challenged by FK565 (500 μg) with LPS priming weekly 4 times. Upper panel: H&E stain (left) and EVG stain (right), x 40, Lower panel: coronary artery (left) and aortic valve (right) in H&E stain, x 200. Panel (c) shows grade 3+ coronary arteritis and valvulitis of FK565-orally administrated mouse without LPS priming (left: 100μg once a day, 6 days/week, 4 weeks; right: tap water ad lib 120 μg/day, 4 weeks, H&E stain, x 40). Coronary arteritis and aortitis including valvulitis were histopathologically characterized by panarteritis with dense inflammatory infiltrate. Neither aneurysmal dilatation nor thrombus was associated. Control mice did not show any vasculitis by either tap water ad lib or oral administration of water alone. Coronary artery and aortic valves are indicated by arrows and arrow heads, respectively.

B. SCID mice (a) and Nod1 $^{-/-}$ mice (b) were treated by FK565 with LPS priming, as shown in Supplementary Table IB. Grade 3+ coronary arteritis/valvulitis and no coronary

arteritis/valvulitis are shown in SCID mice and Nod1 $^{-/-}$ mice, respectively. H&E stain (left) and EVG stain (right). Coronary artery is indicated by an arrow.

C. Oral administration of FK565 (100 μg for 6 consecutive days) after LPS priming induces no inflammation in the gut mucosa. (a): stomach (large panel: x 200, small panel: x 40); (b): small intestine (large panel: x 200, small panel: x 100); (c): colon (large panel: x 200, small panel: x 100).

Figure 3. Nod1 expression in various tissues and organs of normal mice

A. *Nod1* expression levels in various tissues and organs of normal mice were determined by quantitative real-time RT-PCR with *GAPDH* (upper) and *CDH5* (lower) as internal controls. artery (a.), ascending (asc.), abdominal (abd.). The similar expression levels of *Nod1* were observed among the vascular system when *CDH5* was used as an internal control. **B.** Immunohistochemical stainings with Nod1-specific (left) and CD31-specific antibodies (right) in normal mice. (a), (b): coronary artery, (c), (d): aortic valve, (e), (f): pulmonary artery. Nod1-positive cells were immunohistochemically detected diffusely in normal endothelial cells and focally in smooth muscle cells of coronary and pulmonary arteries, and valvular fibroblasts. Scale bar = 20 μm.

Figure 4. The *in vivo* gene expression patterns of vascular and immune tissues of mice treated with LPS, FK565 or LPS plus FK565 (A), and the production of chemokine/cytokine by vascular tissues *ex vivo* (B) and endothelial cells *in vitro* (C) treated with innate immune ligands.

A. The gene expression patterns of aortic root (AR), pulmonary artery (PA), ascending to abdominal aorta and spleen (SP) of mice stimulated by LPS priming (1), FK565 po (2) or LPS priming+ FK565 po (3) on days 2, 4 and 7 (from left to right lane) are shown. (a): 44,170 genes, (b): 13,546 genes of which expression levels were over 2-fold enhanced in AR after oral administration of FK565 with or without LPS priming, compared with those without administration. Blue-to-red scale indicates expression levels from low (under half) to high (over 16-fold), compared with those with no stimulation (no change after stimulation: yellow).

B. Supernatants collected from each tissue after 24 hours culture with each reagent were assayed for CCL2 and IL-6 (N=6). The reagents are as follow: none (■); iE-DAP 10μg/mL (■); FK565 10μg/mL (■); MDP 10μg/mL (■); lipid A 100ng/mL (■); none in NOD1$^{-/-}$ mice (■); FK565 10μg/mL in Nod1$^{-/-}$ mice (■). *: P <0.01, vs. none (Dunnett's test); †: P < 0.01, vs. abdominal (abd.) aorta; ‡: P < 0.01, vs. aortic arch and abdominal aorta; §: P < 0.01, vs. PA, aortic arch, and abdominal aorta (Tukey-Kramer HSD test).

Both pulmonary artery and aortic root produced similar levels of CCL5 in response to FK565 stimulation (no stimulation: 0.01 ± 0.01 ng/mg tissue protein in pulmonary artery and aortic root; FK565-stimulation: 0.57± 0.40 ng/mg tissue protein in pulmonary artery; 0.57 ± 0.25 ng/mg tissue protein in aortic root), proving that the arteries were properly prepared and viable.

C: HCAEC and HPAEC (4 x 10^4 cells) were cultured in the presence or absence of a reagent for 24 hours, and supernatants were assayed in triplicate for each CCL2/IL-8 level. IL-8 is shown instead of IL-6 because there were no significant differences in the IL-6 production between HCAEC and HPAEC stimulated with Nod1 or Nod2 ligand. The concentrations of stimulants are as follows: none (■), iE-DAP 1 (■) or 10 (■) μg/mL; FK565 1 (■) or 10 (■) μg/mL; MDP 10 μg/mL (■); lipid A 100 ng/mL (■). *: P < 0.01, vs. none (Dunnett's test). †: P < 0.01, vs. HPAEC (Student's t-test).

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Supplemental Material.

METHODS

Ligands. γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) was synthesized by Fujimoto Y. and Fukase K. FK565 and FK156 were supplied by Astellas Pharmaceutical. OK-432 (Picibanil), a viable but static *Streptococcus pyogenes* after penicillin-treatment, was supplied by Chugai Pharmaceutical. LPS from *Escherichia coli* O111:B4 (a ligand for TLR4 and other receptors), and zymosan (a ligand for TLR2 and dectin 1)[1] were purchased from Sigma. *E. coli*-type synthetic LPS lipid A (a ligand for TLR4) was purchased from Peptide Institute. *E.coli*-type synthetic lipid A is the lipid portion of bacterial LPS, the bioactive center of LPS toxicity[2]. Peptidoglycan from *E.coli K12* (PGN K12), and synthetic MDP (a ligand for NOD2) were purchased from InvivoGen. Synthetic NOD ligands (iE-DAP, FK565, FK156, and MDP) showed no endotoxin contamination (less than 0.05 EU/mL by Toxinometer ET-5000, Wako).

Cell stimulation experiments. HCAEC and HPAEC were cultured in EBM-2 medium with EGM-2MV and EGM-2 (Lonza), respectively, in a CO_2 (5%) incubator at 37°C[3]. These cells, between passages 6 and 10, were suspended and seeded into 6- or 12-well plates (3-5 day culture: 1×10^4 cells/well and 1 day culture: 1, 1.6 or 4×10^4 cells/well). One day later, the medium was changed and cells were stimulated with each

reagent or combined reagents at various concentrations for 1, 3 or 5 days. We performed these experiments four times independently.

Flow cytometric analyses. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD54 mAb (Beckman Coulter). The expression levels of ICAM-1 were analyzed by an EPICS XL flow cytometer (Beckman Coulter)[4,5]. Culture supernatants were harvested and the concentrations of IL-8, IL-6, IL-1β, TNF-α, IL-12p70, Interferon (IFN) γ, IL-10 and CCL2 were measured by BD™ Cytometric Bead Array human and mouse inflammation kits and human chemokine kit (BD Biosciences).

RNA interference (RNAi). HCAEC (4×10^4 cells/well) were transfected by NOD1 Stealth RNAi small interfering RNA (siRNA) (HSS115906 or HSS115908, Invitrogen) and Stealth RNAi siRNA Negative Control Med GC (Invitrogen) using Lipofectamin RNAiMAX (Invitrogen) for knockdown of endogenous NOD1 and negative control, respectively. Thirty pmol siRNA per well was used for transfection with Lipofectamin RNAiMAX according to manufacturer's recommendation (Invitrogen) and preliminary experiments. The viability of the cells was over 95% without morphological change at day 1. After transfection for 22hr, HCAEC were stimulated by each reagent or combined reagents for 1 day. We performed these experiments twice independently.

Animal experiments. BALB/c, C57BL/6, DBA/2, CD-1, CBA/J, C3H and SCID

(C57BL/6 background) mice were purchased from KBT Oriental, Charles River Grade. Nod1 $^{-/-}$ mice in C57BL/6 background were a gift from Tak Mak, University Health Network. All mice were 5- to 9-week-old female, and were housed in a specific pathogen-free environment. Experiments were performed three times independently under barrier conditions at the animal facility of the biosafety level P1A. These animal experiments were performed according to the protocol approved by the Kyushu University Institutional Animal Care and Use Committee.

Immunohistochemistry. All sections for immunohistochemistry were 4% paraformaldehyde-fixed and paraffin-embedded. Primary antibodies used were Nod1-specific antibody (1:1000, IMGENEX)[6], F4/80 (macrophage)-specific antibody (1:100, Abcam), NIMP-R14 (neutrophil)-specific antibody (1:1500, Abcam), CD3 (T cell) -specific antibody, (1:400, Abcam), alpha smooth muscle actin-specific antibody (1:100, Dako), and CD31-specific antibody (Platelet endothelial cell adhesion molecule 1) (1:50, Abcam) used for the detection of endothelial cells. Deparaffinized sections with or without antigen retrieval (citrate buffer pH6.0 or trypsin) were incubated with 3% nonfat milk to eliminate nonspecific binding and with peroxidase-labeled secondary antibody (Dako and Nichirei) following the primary antibodies. Horseradish peroxidase activity was visualized with Peroxidase Stain DAB Kit (Nacalai Tesque) to give the reaction

product a brown color, and then the sections were counterstained with hematoxylin.

Quantitative real-time RT-PCR. Total RNA was extracted from HCAEC and murine tissues using RNeasy Micro kit and RNeasy Fibrous Tissue (Qiagen), respectively, followed by cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems). Human NOD1 and mouse Nod1 mRNA expression levels were analyzed by TaqMan® Gene Expression Assay Hs00196075_m1 and Mm00805062_m1 (Applied Biosystems), respectively, and TaqMan Gene Expression Master Mix (Applied Biosystems). Human GAPDH (Pre-Developed TaqMan Asssay Reagents GAPDH Control Reagents, Applied Biosystems), mouse GAPDH (primers: CCTGGAGAAACCTGCCAAGTAT, TTGAAGTCGCAGGAGACAACCT; TaqMan probe: VIC-TGCCTGCTTCACCACCTTCTTGATGT-TAMRA) and Cadherin 5 (CDH5, Mm00486938_m1, Applied Biosystems) were used as internal controls. Calibration curve was generated with serial 5-fold dilutions. The mRNA expression levels of the targeted genes were quantified by an ABI PRISM 7700 or Applied Biosystems StepOnePlus sequence detector (Applied Biosystems), as described[4].

Organ Culture. Each tissue of aortic root, pulmonary artery, aortic arch, and abdominal aorta (between roots of right and left renal arteries) sterilely isolated from BALB/c or Nod1$^{-/-}$ mice was cultured for 24 hours in a 96-well plate with EBM-2 medium and

EGM-2 (Lonza) in the presence or absence of an indicated reagent in a CO_2 (5%) incubator at 37°C. We performed these experiments four times independently.

Protein determination. Protein concentrations of each tissue were measured by Bio-Rad protein assay (BioRad) after homogenization with phosphate buffered saline containing Cell Culture Lysis Reagent (Promega) and Protease Inhibitor Cocktail (Nacalai Tesque). Whole protein contents of each tissue were measured to calculate CCL2/IL-6 levels per tissue protein contents.

Microarray analysis. Microarray analysis was performed with *in vivo*-treated and *ex vivo*-cultured organs isolated from 7 mice and *in vitro*-cultured cells (1×10^4 cells). Total RNA was extracted from murine tissues with an RNeasy Fibrous Tissue and from human cell lines with RNeasy Micro Kit (Qiagen). Total RNA was then amplified using Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion). Briefly, double-standard complementary DNA (cDNA) was synthesized from total RNA and *in vitro* transcription was performed to produce multiple copies of amino allyl-labelled complementary RNA (cRNA). Amino allyl-labelled cRNA was purified, reacted with N-hydroxy succinimide esters of Cy3 (GE Healthcare) using Nimblegen's protocol and hybridized for 19h at 42°C to the mouse and human Nimblegen Gene Expression arrays (090901_MM9_EXP_HX12, and 090828_HG18_opt_expr_HX12, Roche NimbleGen)

that contain approximately 40,000 genes. The arrays were scanned on Gene Pix 4000B (Molecular Devices Corporation). The averages of triplicate spot intensity data were extracted using NimbleScan v2.5 (Roche NimbleGen) and processed using robust multiarray analysis method[7]. The scaled gene expression values were imported into GeneSpring 11.0 software (Agilent Technologies) for preprocessing and data analysis[4]. The expression value of each gene was normalized to the 75 percentile shift expression of all genes in each chip. Probe sets were deleted from subsequent analysis if they were displayed an absolute value below 30 in all experiments. The normalized data were first $\log_2$ transformed. For each gene, $\log_2$ intensities from stimulated samples were compared by non-stimulated samples. Microarray data were deposited in Gene Expression Omnibus under accession numbers, GSE20929 (*in vitro* gene expression) and GSE20930 (*in vivo* and *ex vivo* gene expression). Microarray experiments of in vivo organs from 7 mice were done once but on 3 different days 2, 4 and 7 after treatment with consistent results.

Statistical Analysis. Data were analyzed by Student's t-test, Dunnett's test or Tukey-Kramer honestly significant difference (HSD) test using a statistical software, JMP version 8.0 (SAS Institute).

Supplementary Figure I: Primary augmenting effects between NOD1 and TLR ligands in HCAEC

A. Effects of innate immune stimulants on HCAEC at day 1.

HCAEC (1 x 10^4 cells for ICAM-expression; 1.6 x 10^4 cells for cytokine production) were incubated with NOD1, NOD2, TLR and other stimulants in various combinations for 24 h. ICAM-1 expression (a) and IL-8 (b) /IL-6 (c) production in the culture supernatants were investigated in triplicate at day 1. The concentrations of stimulants are as follows: iE-DAP, MDP and PGN K12, 1 (■) or 10 (■) μg/mL; lipid A 10 (■) or 100 (■) ng/mL. Data are presented as mean ± s.d. *: P < 0.01 compared with medium, †: P < 0.01 compared with either iE-DAP or Lipid A, ‡: P < 0.01 compared with either PGN K12 or Lipid A (Dunnett's test).

B. NOD1 knockdown suppresses the stimulatory effects of NOD1 ligand in the absence or presence of TLR4 ligand in HCAEC.

HCAEC (4 x 10^4 cells) were transfected with either siRNA against NOD1 (#1: HSS115908 or #2: HSS115906) or a non-targeted control siRNA. NOD1 siRNA (#1) and (#2) reduced the expression levels of endogenous NOD1 in HCAEC to 15.9 ± 1.9% and 37.0 ± 7.5% of those treated with control siRNA, respectively, as confirmed by quantitative RT-PCR (data not shown). Secretion levels of IL-8 (a) and IL-6 (b) were

investigated in triplicate at 24 hr after stimulation. The concentrations of stimulants are as follows: iE-DAP and MDP, 10 μg/mL; lipid A 100 ng/mL. Data are presented as mean ± s.d. The effects of NOD1 ligand, iE-DAP, were almost completely inhibited, while those of TLR4 ligand (LipidA) remained uninhibited by NOD1 siRNA (#1). *: $P < 0.01$ compared with those treated with a control siRNA. The additive effect of NOD1 and TLR4 was abolished after NOD1 siRNA (#1) treatment. †: $P < 0.01$ compared with either iE-DAP or Lipid A (Dunnett's test).

Supplementary Figure II. Chemical structures of NOD1 ligands and comparison of the effects on HCAEC.

A. Chemical structures of diaminopimelic acid (DAP)–type peptidoglycan and synthetic NOD1 ligands. iE-DAP: γ-D-Glu-DAP, a synthetic dipeptide with a molecular weight (M.W.) of 319.3 daltons, FK565: heptanolyl-γ-Glu-*meso*-DAP-D-Ala, a synthetic acyltripeptide with a M.W. of 502.6 daltons, FK156: D-lactyl-L-Ala-γ-Glu-*meso*- DAP-Gly, with a M.W. of 519.5 daltons, a synthetic tetrapeptide, originally isolated from culture filtrates of *Streptomyces* strains.

B. HCAEC (4×10^4 cells) were incubated with one of Nod1 stimulants, FK565, FK156 or iE-DAP. ICAM-1 expression (a) and CCL2 (b) /IL-8 (c) production in the culture

supernatants were examined at day 3. The concentrations are 1 (■) or 10 (■) μg/mL. Data are presented as mean ± s.d. (N=4). *: P <0.01, vs medium; †: P < 0.01, vs. FK 156; ‡: P < 0.01, vs. iE-DAP (Dunnett's test).

Supplementary Figure III. Immunohistochemial studies of coronary arteritis induced by oral administration of FK565.

All the sections were serial ones of aortic root from coronary arteritis model which was orally administrated by FK565 100μg for 6 consecutive days after priming of LPS 20μg i.p. Inflammatory cells infiltrating in the both coronary and aorta including valve consisted of many NIMP-R14-positive neutrophils, some F4/80-positive macrophages, and few CD3-T lymophocytes. Endothelial cells, smooth muscle cells and fibroblasts/myofibroblasts in addition to infiltrating inflammatory cells were apparently positive for NOD1. H&E stain (W: aortic root, x 40 (A: aortic valve (AV), B: coronary artery (CA)); A1: AV, x 400; B1: CA, x 400) and immunohistochemical stainings with Nod1- (A2: AV, B2: CA, x400), F4/80 (macrophage)- (A3: AV, B3: CA, x400), NIMP-R14 (neutrophil)- (A4: AV; B4: CA, x 400), CD3- (A5: AV; B5: CA, x 400), CD31- (B6: CA, x 400), and alpha smooth muscle actin- (B7: CA, x 400) specific antibodies.

Supplementary Figure IV. Microarray analysis of the gene expression of vascular tissues from BALB/c mice and human endothelial cells cultured with or without an innate immune stimulant.

A. The expression patterns of 44,170 genes in aortic root (AR), pulmonary artery (PA), and arch portion of aorta (aorta) *ex vivo* cultured for 24 hours with FK565 (10 μg/mL) by microarray analysis. B. The expression patterns of 44,931 genes in HCAEC and HPAEC *in vitro* cultured for 24 hours with FK565 (10 μg/mL) or lipid A (100 ng/mL) by microarray analysis. Blue-to-red scale indicates expression levels from low (under half) to high (A: over 4-fold, B: over 8-fold), compared with those with no stimulation (no change after stimulation: yellow).

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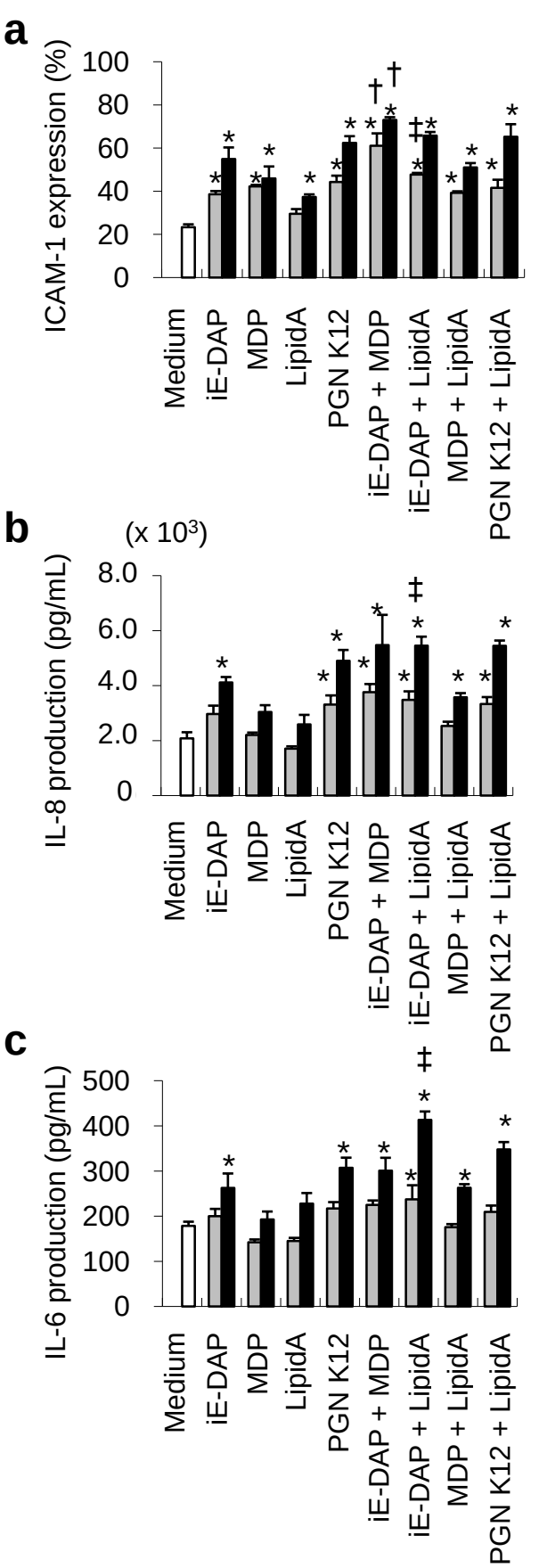
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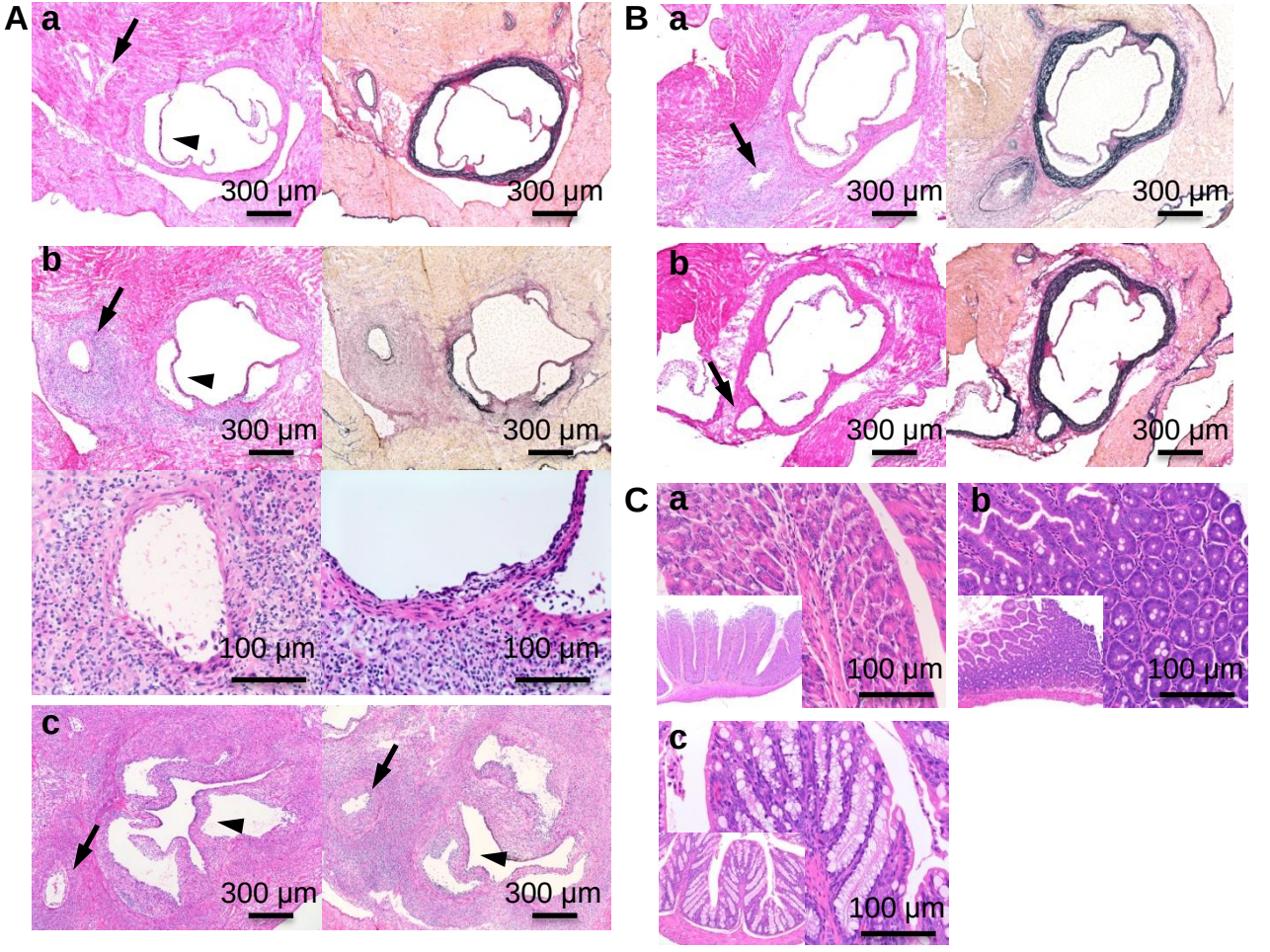
ICAM-1 expression (%)
100
80
60
40
20
0
Medium
iE-DAP
MDP
LipidA
PGN K12
iE-DAP + MDP
iE-DAP + LipidA
MDP + LipidA
PGN K12 + LipidA
b
(x 10³)
IL-8 production (pg/mL)
8.0
6.0
4.0
2.0
0
c
IL-6 production (pg/mL)
500
400
300
200
100
0

Figure 1

Figure 2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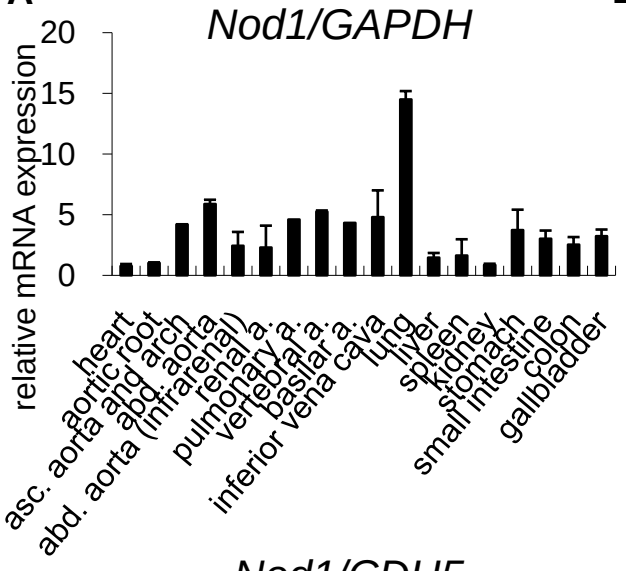
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15
10
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heart
aortic root
asc. aorta and arch
abd. aorta
abd. aorta (infrarenal)
renal a.
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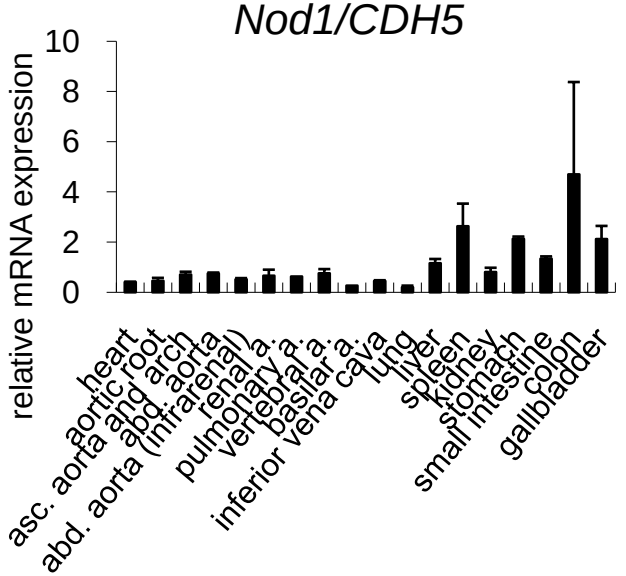
inferior vena cava
lung
liver
spleen
kidney
stomach
small intestine
colon
gallbladder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aortic root
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abd. aorta
abd. aorta (infrarenal)
renal a.
pulmonary a.
vertebral a.
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inferior vena cava
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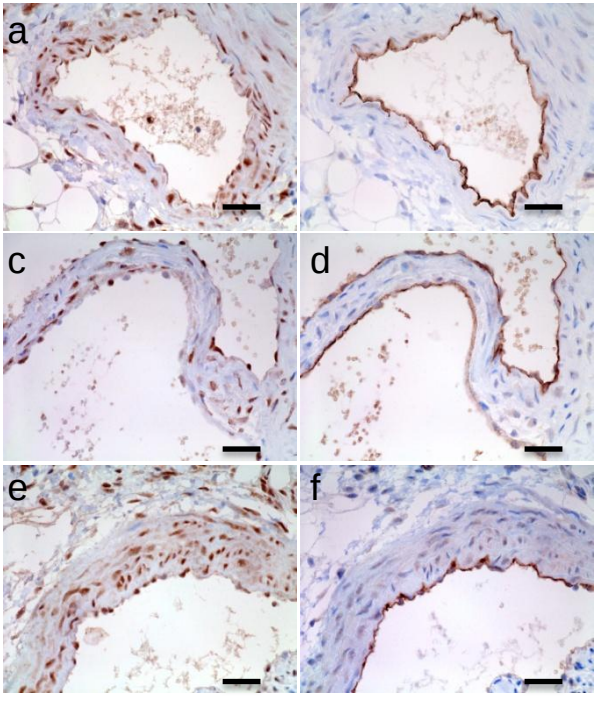
liver
spleen
kidney
stomach
small intestine
colon
gallbladder

B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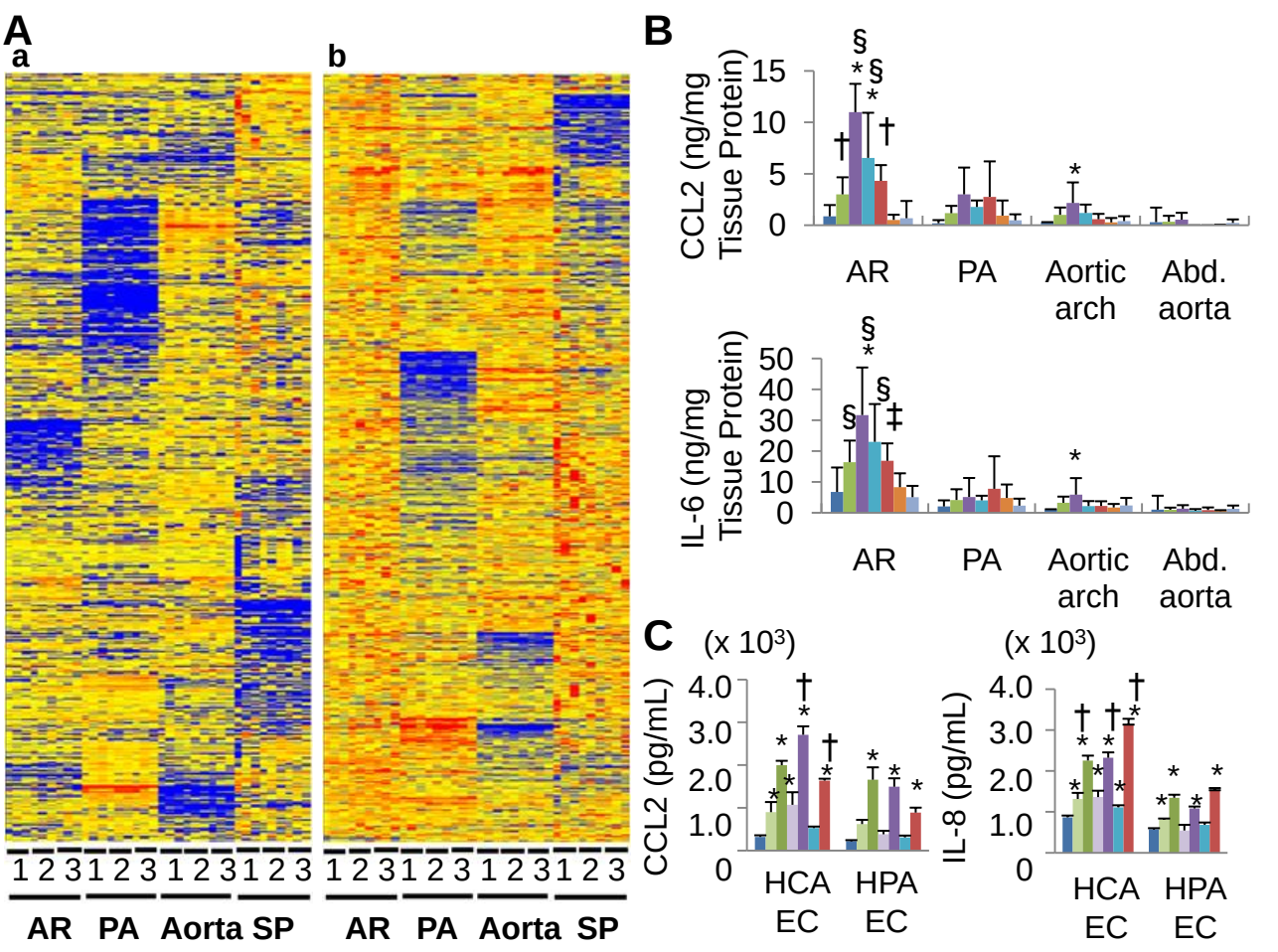
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IL-6 (ng/mg Tissue Protein)
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40
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AR
PA
Aortic arch
Abd. aorta
C
(x 10³)
CCL2 (pg/mL)
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HCA EC
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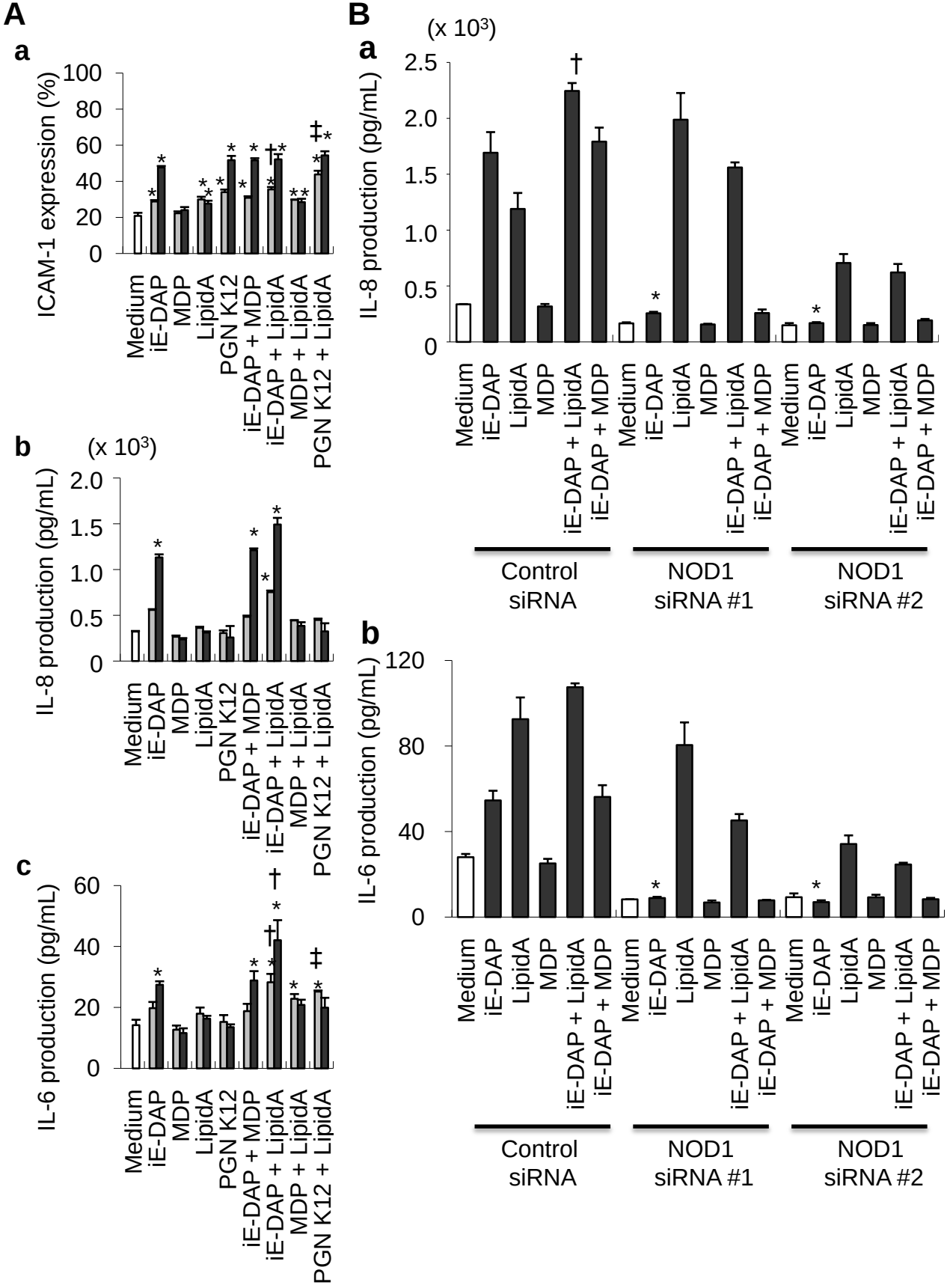
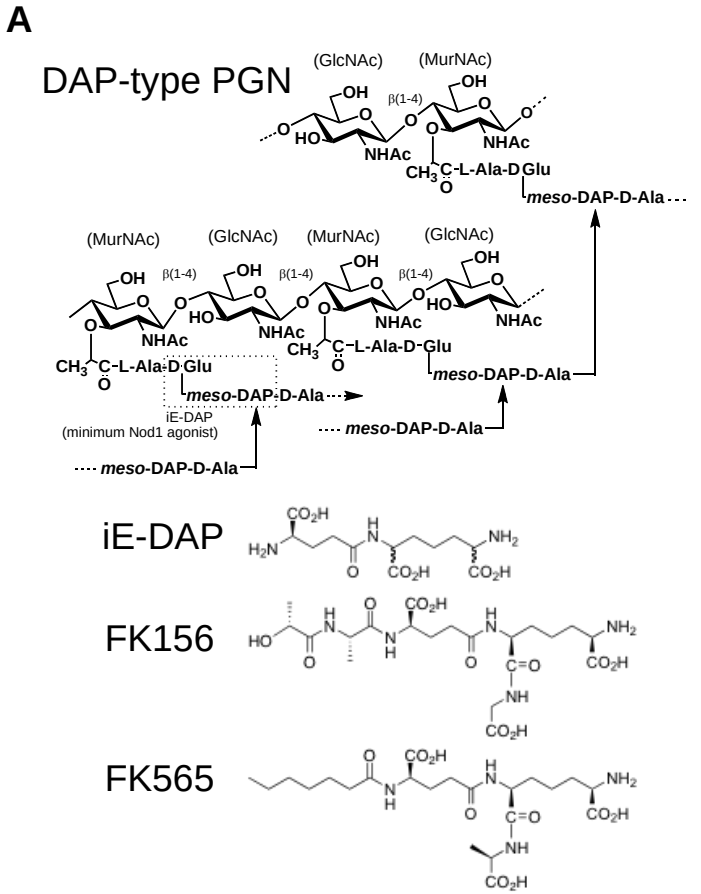
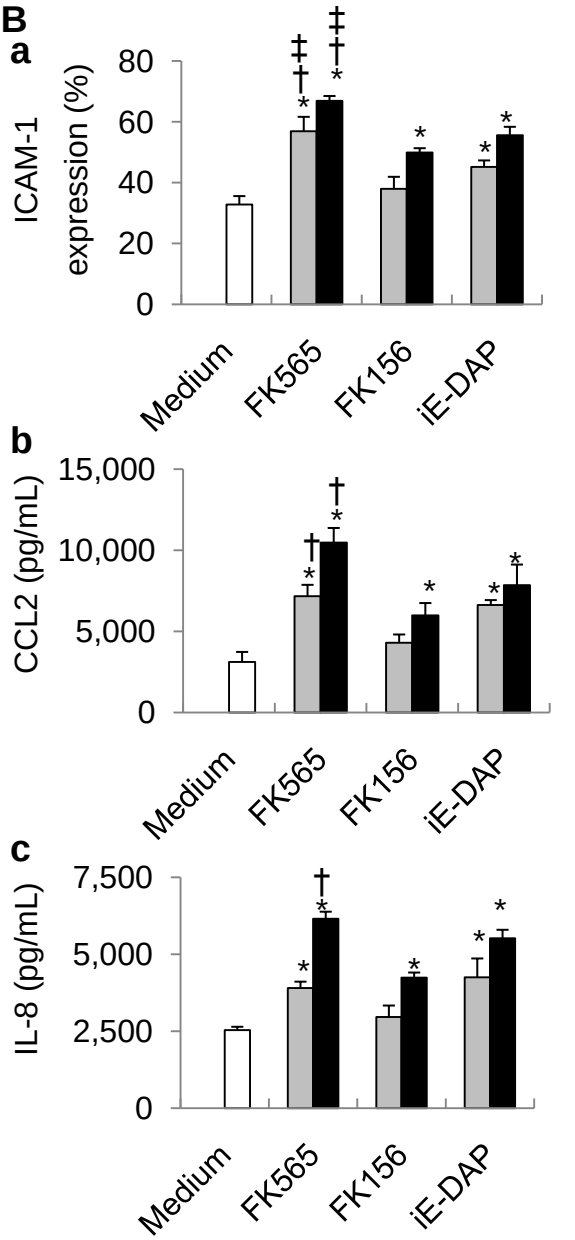
HCA EC
HPA EC

Figure 4

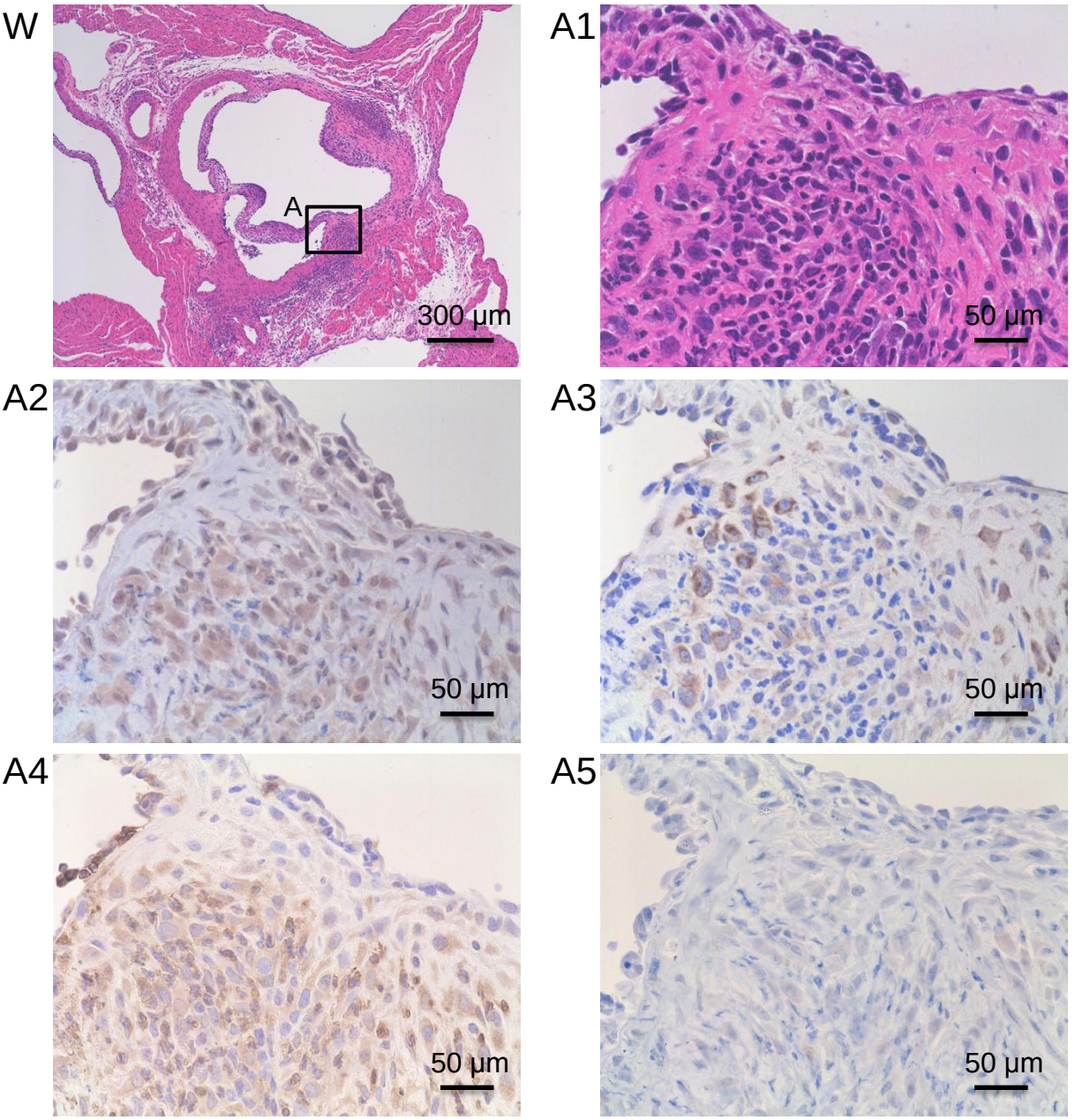


Supplementary Figure I





Supplementary Figure II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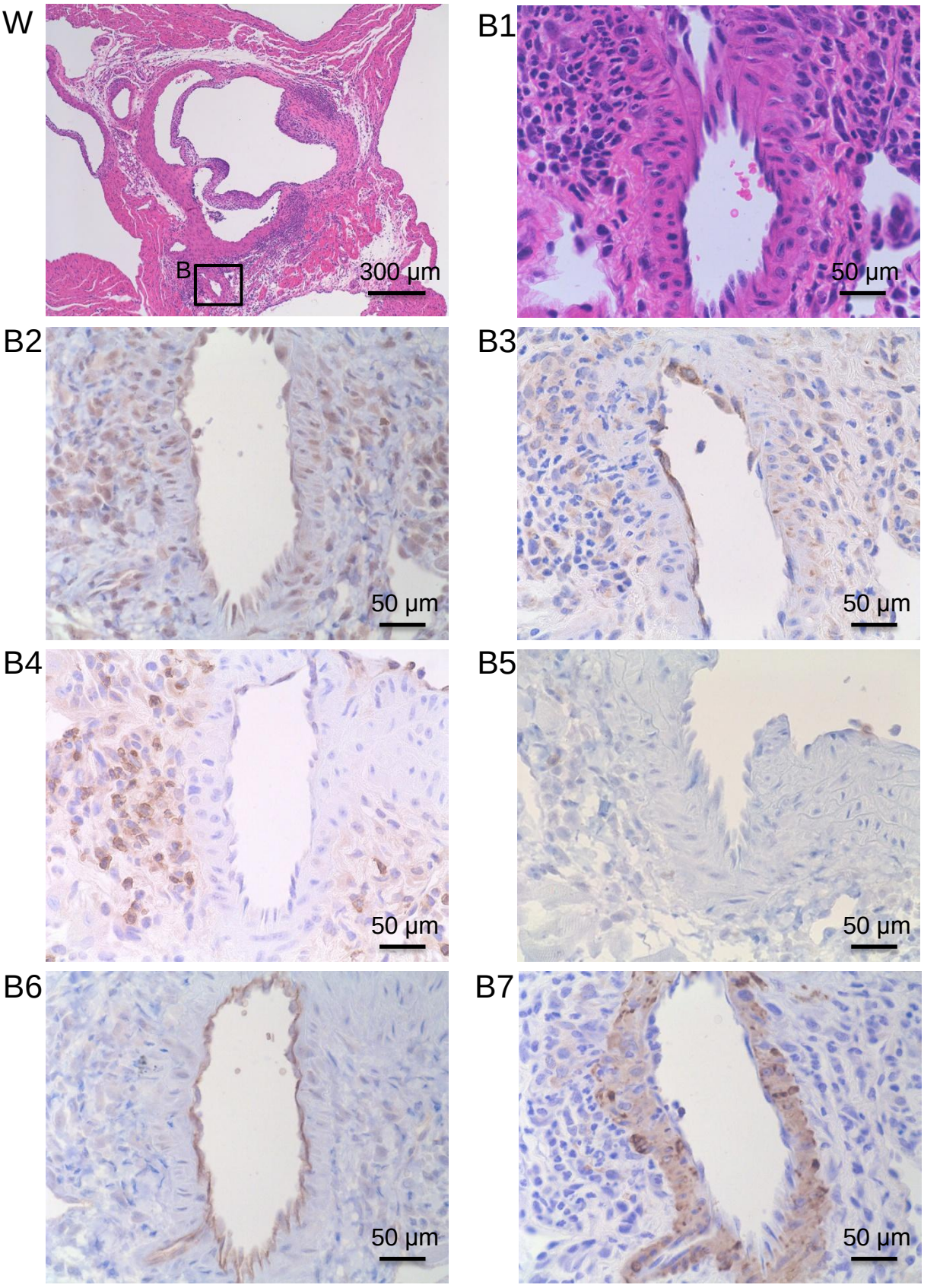
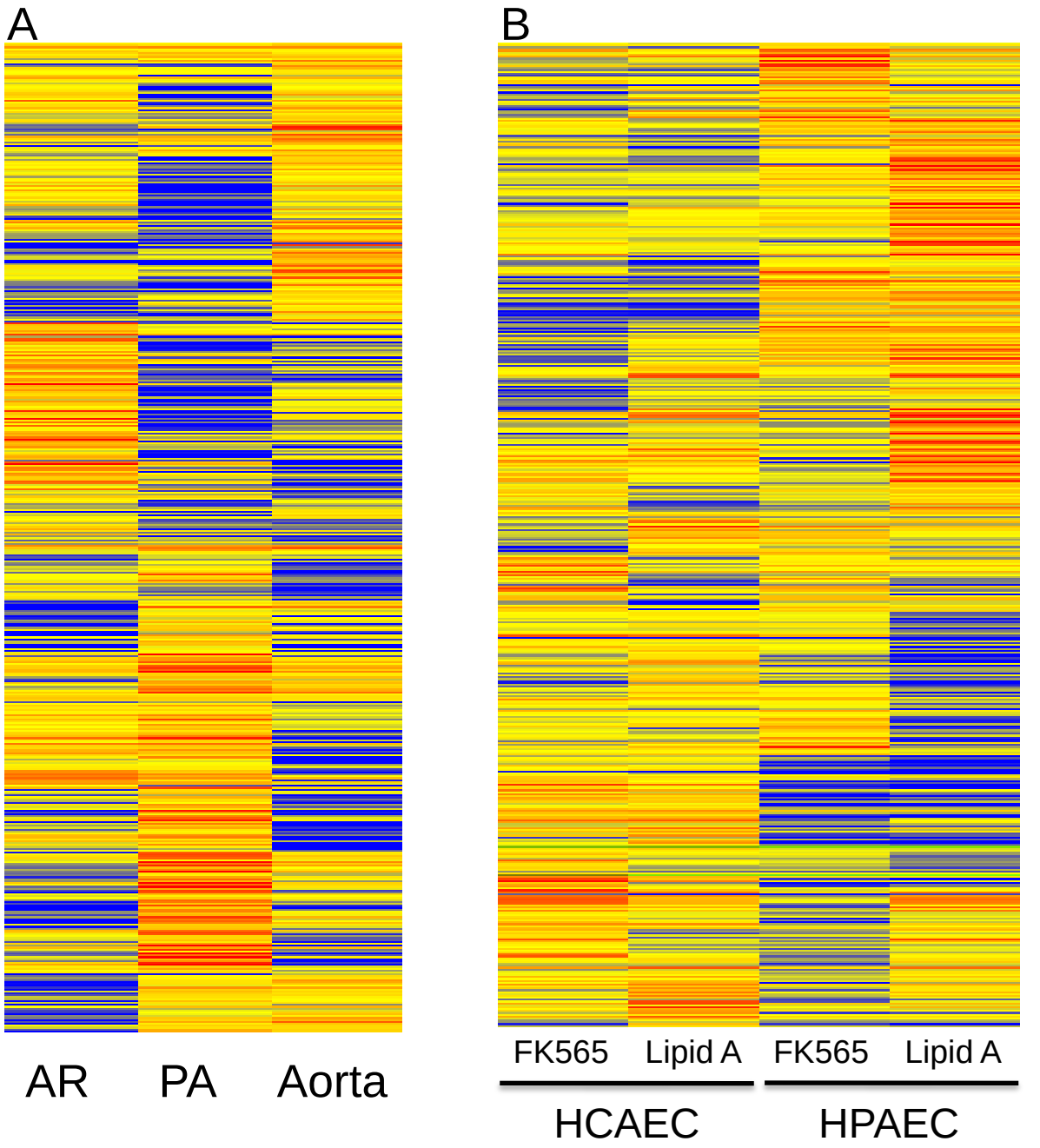


Supplementary Figure III



Supplementary Figure IV

Supplementary Table I. Induction of coronary arteritis by microbial reagents.

A

Reagents	Dose (/mouse)	Administration route	Priming (LPS ip)	Number of Administration	Severity of CA	Incidence of CA+No./	SI score
None	-	-	20 or 50μg/w	4 times	-, -, -	0/3	0
None	-	-	100μg/w	4 times	-, -, -	0/3	0
Zymosan	500μg/w	sc	20μg	4 times	-, -, -	0/3	0
OK432	1KE/w	sc	20μg	4 times	-, -, -	0/3	0
PGN K12	500μg/w	sc	20μg	4 times	-, -, -	0/3	0
MDP	500μg/w	sc	20μg	4 times	-, -, -	0/3	0
FK565	500μg/w	sc	20μg	4 times	3+, 3+, 3+	3/3	9
FK565	500μg/w	sc	-	4 times	+, -, -	1/3	1
FK565	200μg/w	sc	20μg	4 times	+, -, -	1/3	1
FK565	100μg/w	sc	20μg	4 times	-, -, -	0/3	0
FK565+MDP	500μg, 500μg/w	sc	20μg	4 times	3+, 3+, 3+	3/3	9
FK565+MDP	200μg, 200μg/w	sc	20μg	4 times	3+, 2+, -	2/3	5
FK565+MDP	100μg, 100μg/w	sc	20μg	4 times	+, +, -	2/3	2

B

Mouse	Reagent/ Dose (/mouse)	Administration route	Priming (LPS ip)	Number of Administration	Severity of CA	Incidence of CA+No./	SI score
SCID	-	-	+	4 times	-, -, -	0/3	0
SCID	FK565/ 500μg/w	sc	-	4 times	2+, +, - , -, -	2/5	3
SCID	FK565/ 500μg/w	sc	+	4 times	3+, 2+, +, +, +	5/5	8
Nod1 $^{-/-}$	-	-	+	4 times	-, -, -	0/3	0
Nod1 $^{-/-}$	FK565/ 500μg/w	sc	+	4 times	-, -, -, -, -	0/5	0

C

Reagents	Dose (/mouse)	Administration route	Priming (LPS ip)	Duration of Administration	Severity of CA	Incidence of CA+No./	SI score
-	-	-	+	4 w	-, -, -, -, -	0/5	0
FK565	25μg x 6 times/w	po	+	1 w	+, +, -, -, -	2/5	2
FK565	50μg x 6 times/w	po	+	1 w	-, -, -, -, -	0/5	0
FK565	100μg x 6 times/w	po	+	1 w	2+, 2+, +, +, +	5/5	7
FK565	100μg x 6 times/w	po	+	2 w	3+, 3+, 2+, 2+, +	5/5	11
FK565	100μg x 6 times/w	po	+	3 w	3+, 3+, 2+, 2+, 2+	5/5	12
FK565	100μg x 6 times/w	po	+	4 w	3+, 3+, 3+, 3+, 2+	5/5	14
FK565	100μg x 6 times/w	po	-	4 w	3+, 3+, 3+, 2+,-	4/5	11

Panel A shows the incidence and severities of coronary arteritis (CA) in response to various microbial reagents. BALB/c mice were primed with or without LPS, and 1 day later the mice were subcutaneously challenged with each reagent. Injections were repeated weekly, and mice were sacrificed 1 week after the last administration. Panel B shows the incidence and severities of CA in SCID or Nod1$^{-/-}$ mice. Each mouse was primed with or without LPS ip priming (10μg: dose reduction due to high sensitivity), and 1 day later challenged with or without sc FK565 (500μg). Injections were repeated weekly, and mice were sacrificed 1 week after the last administration. Panel C shows the induction of CA by various doses and durations of oral administration of FK565 with or without LPS ip priming (20μg). BALB/c mice were primed with or without LPS and 1 day later challenged with oral administration of FK565 for 6 consecutive days (1 week course). Administration was repeated for 1 to 4 weeks. Mice were sacrificed 1day after last administration. No vasculitis was observed in NOD1$^{-/-}$ mice after oral administration of FK565. ip = intraperitoneal; sc = subcutaneous; po = per os; w: week, KE = Klinische Einheit units; OK432 = penicillin-killed streptococcus pyogenes; PGN = peptidoglycan. SI score is calculated by the summation of severity scores of all mice in each experiment.

Supplementary Table II. Top 10 genes expressed in aortic root after oral administration of FK565 with or without LPS priming *in vivo*.

			Aortic root									Pulmonary artery									Aorta									Spleen								
			LPS			FK565			LPS+FK565			LPS			FK565			LPS+FK565			LPS			FK565			LPS+FK565			LPS			FK565			LPS+FK565		
Category	Symbol	Rank	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7	day2	day4	day7
All	Ccl5	1	5.1	4.5	1.5	**86.2**	**123.9**	**56.1**	108.7	62.9	34.0	0.8	0.8	0.3	3.5	**6.4**	3.0	3.2	3.7	2.1	17.2	9.5	3.7	**42.7**	**85.6**	**44.9**	78.7	87.0	47.9	0.4	1.5	1.1	0.9	3.3	2.8	1.1	2.5	1.3
	Arg1	2	8.9	4.4	2.5	**64.4**	**9.3**	4.6	192.8	27.5	7.1	0.5	0.5	0.4	2.7	0.7	0.5	17.7	6.0	0.8	9.9	9.0	2.6	**16.1**	**6.0**	3.7	48.4	14.9	4.6	3.6	1.2	2.1	**7.9**	1.8	0.6	19.1	4.3	3.1
	Ccl2	3	2.0	2.2	0.7	**34.4**	**36.7**	**15.2**	122.5	47.9	16.2	1.4	1.4	1.0	**10.1**	**11.6**	**8.2**	73.1	11.7	6.4	2.7	2.2	0.9	**20.3**	**26.2**	**12.7**	99.9	53.8	11.5	0.6	2.0	1.1	2.6	3.1	1.3	7.0	1.6	1.7
	Cxcl13	4	15.7	6.2	3.3	**32.1**	**8.2**	**17.0**	149.6	33.2	16.5	0.8	0.5	0.1	0.7	2.8	0.3	23.8	1.1	2.0	15.2	2.2	3.4	4.7	**8.0**	**6.2**	89.2	8.3	4.7	0.1	0.5	0.3	1.8	1.2	3.4	1.8	1.1	1.4
	Ccl8	5	4.3	4.2	2.3	**8.1**	**27.1**	**48.0**	4.1	92.5	36.1	5.4	6.4	2.0	**7.9**	**16.5**	**21.4**	3.7	12.4	13.5	2.9	3.4	0.9	2.5	**12.7**	**21.0**	1.6	8.2	7.2	1.5	2.2	2.3	0.9	3.8	0.8	1.0	1.6	0.8
	Il6	6	1.7	0.9	1.4	2.8	**12.9**	**15.3**	20.5	55.6	106.5	0.4	0.5	0.5	0.8	0.9	0.5	1.8	0.6	0.7	1.9	1.6	1.2	1.3	**7.7**	4.2	5.4	9.1	2.8	0.5	1.0	0.7	0.5	0.6	0.4	0.5	0.7	0.6
	Serpina3n	7	8.9	2.2	2.1	**25.4**	**17.6**	**14.5**	80.7	19.6	16.8	14.7	4.1	3.9	**42.1**	**6.7**	**9.9**	109.1	8.8	9.7	5.8	1.2	0.9	4.3	3.3	4.0	28.7	3.3	1.9	0.3	0.5	4.6	3.6	**5.9**	2.1	6.4	0.5	0.6
	Saa3	9	31.1	12.4	1.0	**35.8**	**25.5**	**20.6**	41.5	21.2	17.4	29.7	16.2	2.1	**24.0**	**11.4**	**16.1**	32.7	35.1	27.2	46.8	34.2	5.3	**28.9**	**25.1**	**42.2**	56.3	33.3	19.7	30.8	12.0	2.8	**21.3**	**15.7**	**9.8**	305.2	35.2	18.9
	Cfb	10	19.8	7.2	2.4	**22.4**	**24.2**	**21.8**	58.2	22.3	12.5	7.4	3.9	1.2	**7.4**	**7.8**	**8.5**	20.0	6.1	4.2	3.4	1.7	0.7	1.9	2.4	2.3	9.5	2.5	2.2	0.4	1.7	2.4	1.5	0.9	2.2	2.6	3.2	1.1
	Lcn2	11	24.9	1.9	0.5	**15.1**	**9.5**	**8.2**	82.3	15.4	17.9	34.5	3.7	1.5	**22.3**	**14.6**	**12.7**	89.1	8.0	1.5	3.8	0.5	0.1	0.9	0.8	0.6	9.5	2.1	0.5	0.2	0.4	2.4	0.9	1.2	0.8	5.1	2.6	2.1
Chemokine/cytokine	Ccl5	1	5.1	4.5	1.5	**86.2**	**123.9**	**56.1**	108.7	62.9	34.0	0.8	0.8	0.3	3.5	**6.4**	3.0	3.2	3.7	2.1	17.2	9.5	3.7	**42.7**	**85.6**	**44.9**	78.7	87.0	47.9	0.4	1.5	1.1	0.9	3.3	2.8	1.1	2.5	1.3
	Ccl2	3	2.0	2.2	0.7	**34.4**	**36.7**	**15.2**	122.5	47.9	16.2	1.4	1.4	1.0	**10.1**	**11.6**	**8.2**	73.1	11.7	6.4	2.7	2.2	0.9	**20.3**	**26.2**	**12.7**	99.9	53.8	11.5	0.6	2.0	1.1	2.6	3.1	1.3	7.0	1.6	1.7
	Cxcl13	4	15.7	6.2	3.3	**32.1**	**8.2**	**17.0**	149.6	33.2	16.5	0.8	0.5	0.1	0.7	2.8	0.3	23.8	1.1	2.0	15.2	2.2	3.4	4.7	**8.0**	**6.2**	89.2	8.3	4.7	0.1	0.5	0.3	1.8	1.2	3.4	1.8	1.1	1.4
	Ccl8	5	4.3	4.2	2.3	**8.1**	**27.1**	**48.0**	4.1	92.5	36.1	5.4	6.4	2.0	**7.9**	**16.5**	**21.4**	3.7	12.4	13.5	2.9	3.4	0.9	2.5	**12.7**	**21.0**	1.6	8.2	7.2	1.5	2.2	2.3	0.9	3.8	0.8	1.0	1.6	0.8
	Il6	6	1.7	0.9	1.4	2.8	**12.9**	**15.3**	20.5	55.6	106.5	0.4	0.5	0.5	0.8	0.9	0.5	1.8	0.6	0.7	1.9	1.6	1.2	1.3	**7.7**	4.2	5.4	9.1	2.8	0.5	1.0	0.7	0.5	0.6	0.4	0.5	0.7	0.6
	Ccl7	16	1.4	1.1	1.0	**12.0**	**12.7**	**13.8**	29.6	22.6	12.5	1.3	1.6	1.2	**6.7**	4.8	1.9	21.9	4.0	1.2	2.3	1.5	0.8	2.1	**12.1**	4.6	14.8	15.6	2.4	1.2	3.3	1.2	1.7	2.4	1.8	10.1	1.8	6.9
	Cxcl9	21	2.3	4.0	2.8	**10.4**	**25.2**	**17.4**	17.0	18.9	5.8	0.4	0.4	0.2	1.1	1.4	0.6	2.9	0.8	0.8	10.4	6.7	6.4	**19.7**	**13.3**	**8.5**	46.7	16.4	9.0	2.1	3.4	0.6	1.8	1.7	2.4	0.9	1.8	2.3
	Cxcl10	22	1.6	1.8	3.0	**17.2**	**18.8**	**11.4**	22.0	10.1	6.1	0.6	1.2	2.7	**7.6**	**6.5**	2.6	10.6	4.8	2.2	6.4	6.9	3.1	**45.1**	**42.7**	**17.5**	89.4	28.0	9.8	0.2	0.3	0.3	3.0	1.6	2.6	1.8	0.9	0.8
	Cxcl2	23	1.6	0.7	1.6	**6.3**	**8.4**	**9.4**	29.0	12.3	17.0	1.5	1.9	1.2	1.8	1.7	2.9	8.1	5.3	1.2	0.7	0.8	0.5	2.5	3.3	3.0	10.5	6.6	3.1	1.7	0.9	4.7	0.7	1.0	1.2	0.9	0.7	0.9
	Ccl19	30	1.5	1.1	1.1	**14.5**	**15.7**	**6.2**	15.4	12.9	6.8	0.4	0.3	0.4	0.8	2.3	0.4	0.5	0.8	0.3	3.4	0.9	1.5	3.7	**7.9**	2.3	3.7	3.5	1.7	0.6	0.4	1.2	0.8	1.1	1.0	0.4	0.7	0.8
Mmp	Mmp3	29	1.2	1.0	0.6	4.6	**14.2**	**10.4**	15.9	12.3	17.0	1.1	0.6	0.6	2.3	1.4	0.7	1.2	1.2	1.1	2.5	1.6	1.4	4.1	4.5	3.4	4.4	6.2	2.5	1.4	0.8	4.4	3.0	**5.3**	2.8	3.6	1.0	6.0
	Mmp12	39	8.0	3.1	2.3	**9.6**	**15.6**	**5.9**	9.8	10.2	13.3	0.7	0.4	0.5	1.0	1.8	1.8	1.2	2.0	2.1	1.2	2.6	1.6	2.0	4.6	3.2	1.0	5.2	4.8	2.2	0.6	0.6	1.7	0.7	2.0	0.5	0.9	2.0
	Mmp19	501	2.8	3.1	1.9	1.9	2.2	**5.2**	6.1	2.3	4.2	0.4	1.2	1.1	0.6	0.6	0.8	1.1	0.6	0.5	0.5	0.9	0.9	0.6	1.2	1.4	2.0	1.0	0.6	8.5	1.4	8.7	4.5	4.5	1.2	3.2	1.3	1.5
	Mmp9	516	3.3	2.3	2.6	2.4	4.3	2.8	4.9	4.7	2.7	4.0	4.2	4.9	4.9	3.5	5.4	14.5	5.6	3.0	1.0	0.5	0.9	0.7	0.5	0.7	2.1	1.1	0.8	0.3	0.3	0.9	1.0	1.2	2.0	2.6	2.1	1.2
	Mmp8	2617	0.8	1.2	0.6	1.0	1.0	0.7	7.3	1.7	1.4	0.7	0.7	0.7	0.8	0.6	1.2	5.7	1.3	0.9	1.5	0.9	0.7	0.7	1.3	2.2	21.3	2.9	0.6	0.2	0.6	0.9	0.4	0.7	0.5	0.5	0.6	0.2
	Mmp20	3942	0.7	0.9	1.5	1.0	1.5	4.4	1.0	1.8	1.7	0.5	1.2	1.2	0.6	0.7	1.1	0.7	1.0	0.6	0.6	0.7	1.0	0.5	1.1	0.8	0.5	0.7	0.6	1.9	2.7	3.0	1.4	1.7	1.2	1.4	2.4	3.9
	Mmp14	4046	1.3	1.7	1.3	1.2	1.5	2.1	2.4	1.7	2.4	1.6	2.2	1.3	1.5	0.9	1.7	1.2	1.0	2.4	1.4	2.2	2.0	1.1	1.8	2.5	1.9	2.7	2.2	2.6	1.2	2.8	0.6	1.8	0.6	1.4	1.6	1.1
	Mmp1a	4838	0.8	0.7	1.3	**5.3**	1.0	1.1	0.8	0.9	1.4	0.6	0.8	1.0	1.0	0.7	0.6	0.9	0.9	0.7	1.5	2.2	2.6	1.9	1.6	1.4	1.3	1.2	1.6	1.1	0.9	0.3	0.3	0.5	0.5	0.3	0.2	1.7
	Mmp27	6457	1.3	1.4	0.9	1.3	1.0	1.2	1.6	2.8	1.9	0.6	1.1	1.0	1.0	1.2	4.6	1.1	1.0	0.7	0.8	3.3	0.7	0.5	1.0	0.8	0.9	0.8	0.6	0.6	1.0	0.6	1.4	**7.5**	0.4	0.6	0.9	0.7
	Mmp7	6484	1.9	0.8	1.9	0.9	0.7	2.7	1.9	2.5	1.1	0.7	0.6	0.6	0.4	0.6	0.5	0.4	0.6	0.5	0.7	1.0	0.8	0.6	0.6	1.0	0.4	0.8	0.9	0.9	0.7	0.7	0.7	0.9	0.8	0.6	1.1	0.9
Cam	Vcam1	40	2.5	1.3	0.9	**15.1**	**9.9**	**6.2**	14.8	10.6	5.7	2.4	1.4	3.3	**23.4**	**5.4**	**6.1**	12.3	11.2	10.0	1.6	1.4	1.0	**18.7**	**6.2**	3.6	12.0	15.1	4.1	0.1	0.4	1.1	1.0	1.5	0.8	1.4	0.9	0.6
	Icam1	258	1.7	1.4	1.1	**5.0**	4.1	3.5	10.1	3.4	2.0	1.4	1.3	2.1	4.8	2.7	3.0	6.0	4.1	2.7	1.4	1.0	1.0	3.3	2.8	2.4	6.2	2.7	1.2	0.4	0.7	1.0	1.3	2.0	2.1	2.4	1.5	1.1
	Selp	311	1.8	1.2	0.7	3.3	3.4	3.7	10.0	2.9	2.6	3.9	1.7	3.3	**5.1**	1.0	2.5	4.9	4.7	23.9	10.4	5.4	8.1	**13.1**	**15.0**	**14.9**	27.4	18.1	13.7	3.4	1.2	6.9	0.9	0.9	0.9	1.1	1.4	2.2
	Madcam1	401	2.0	1.5	1.4	4.6	**5.6**	2.0	7.2	2.6	1.7	1.1	1.0	0.6	1.5	3.0	1.2	2.8	1.0	1.0	2.2	0.8	0.5	2.5	2.7	1.7	5.4	2.2	2.0	0.6	0.6	1.6	0.8	1.6	1.4	0.6	1.4	1.6
	Jam2	2360	1.3	1.3	1.5	2.0	1.1	1.8	1.1	6.2	1.1	2.4	1.2	1.0	0.7	0.4	1.7	3.3	0.5	2.4	1.3	4.5	2.1	1.9	1.5	1.3	1.3	1.4	2.0	1.7	1.1	1.7	1.0	1.3	2.3	1.8	1.1	6.6
	Cd34	3999	1.6	1.6	1.3	1.0	2.1	2.5	2.2	0.9	2.7	1.7	1.1	0.5	1.1	0.9	1.4	1.5	1.6	3.3	1.0	0.9	1.1	0.8	0.9	0.7	1.1	0.5	0.7	1.2	1.8	1.2	0.7	1.7	1.0	1.3	1.6	0.8
	Jam3	7317	1.2	3.5	4.2	1.3	1.8	2.4	1.4	1.3	1.4	1.3	0.6	0.8	1.0	1.2	0.9	1.3	1.6	1.2	1.9	3.2	1.7	1.9	1.0	0.9	1.4	2.0	1.7	3.6	2.3	0.6	0.9	0.5	1.4	0.5	0.7	0.9
	Sele	12984	1.0	0.9	0.9	1.1	1.1	1.2	2.2	1.2	1.0	0.7	0.7	0.5	1.8	0.7	0.9	1.4	0.9	1.1	1.2	1.0	1.8	2.9	1.7	2.6	9.9	2.3	1.2	0.9	2.0	3.1	0.8	1.5	0.7	0.8	0.9	1.4
	Pvrl2	13254	2.2	0.8	1.2	2.9	1.3	1.4	0.8	0.5	0.7	1.4	0.8	1.0	1.2	1.6	1.3	1.3	0.8	1.1	0.9	0.9	1.3	0.6	1.5	1.4	0.9	1.2	1.3	0.5	0.7	0.7	0.3	0.8	0.4	0.5	0.4	1.7
	Icam2	15175	1.0	1.0	1.2	1.1	1.5	1.4	1.3	0.8	1.3	1.3	1.0	1.5	1.1	1.0	1.3	1.0	1.8	2.3	1.3	0.9	1.3	1.9	1.1	1.3	1.3	0.6	1.2	0.6	1.3	0.6	1.7	1.5	4.3	3.6	0.9	1.6

Top 10 genes in the all, Chemokine/cytokine, Mmp and Cell adhesion molecule (Cam) categories are listed among the genes of which expression levels were enhanced in aortic root after oral administration of FK565 with or without LPS priming at days 2, 4 and 7, compared with those without administration. We prepared each 10 mice in the same condition, 7 of which were used for in vivo microarray analysis and the remaining 3 were sacrificed for the histopathology study of heart. At day 2, the sections showed mild cellular infiltration in aortic valve. At day4, the cellular infiltration with edema was enhanced in aortic valve. Then, at day7, marked coronary arteritis and valvulitis were observed. Numbers in rank refer to the order of the gene expression levels in all categories. Data are expressed as fold changes of the respective gene expression levels after stimulation. Numbers in bold in FK565-stimulated vascular tissues and spleen refer to those over 5-fold increases. The genes in the category “Chemokine/cytokine” were selected from those listed in Cytokine- cytokine receptor interaction (map04060), and the genes in the category “Cam” were from those listed in the Endothelial cell, Leukocyte transendothelial migration in Cell adhesion molecules (map04514) in the KEGG database (http://www.genome.jp/kegg/pathway.html). Gene full names are as follows. Ccl5, chemokine (C-C motif) ligand 5; Arg1, arginase, liver; Ccl2, chemokine (C-C motif) ligand 2; Cxcl13, chemokine (C-X-C motif) ligand 13; Ccl8, chemokine (C-C motif) ligand 8; Il6, interleukin 6; Serpina3n, serine (or cysteine) peptidase inhibitor, clade A, member 3N; Saa3, serum amyloid A 3; Cfb, complement factor B; Lcn2, lipocalin 2; Ccl7, chemokine (C-C motif) ligand 7; Cxcl9, chemokine (C-X-C motif) ligand 9; Cxcl10, chemokine (C-X-C motif) ligand 10; Cxcl2, chemokine (C-X-C motif) ligand 2; Cxcl19, chemokine (C-X-C motif) ligand 19; Mmp, matrix metallopeptidase; Vcam1, vascular cell adhesion molecule 1; Icam1, intercellular adhesion molecule 1; Selp, selectin, platelet; Madcam1, mucosal vascular addressin cell adhesion molecule 1 ; Jam2, junction adhesion molecule 2; Cd34, CD 34 antigen; Jam3, junction adhesion molecule 3; Sele, selectin, endothelial cell; Pvrl2, poliovirus receptor-related 2; Icam2, intercellular adhesion molecule 2. When 2 or more genes were picked up by different probes, only one with the highest rank is included in the list (Rank 8 is Il6). Microarray analysis data are shown in Fig. 4Ab.

Supplementary Table III. Top 10 genes expressed in aortic root *ex vivo* and HCAEC *in vitro* stimulated with FK565.

A

Category	Gene Symbol	Rank	AR	PA	Aorta
All	Gm4022	1	28.3	0.7	1
	Gm3727	2	26.7	1.2	1.1
	Ccl5	3	25.8	32.7	22.3
	Krt42	4	20.3	0.8	0.6
	Gm7225	5	20.2	1.0	1.0
	Gm4477	6	18.7	1.3	0.9
	Prlr	7	17.9	2.1	1.0
	Gm15761	8	17.3	1.0	4.8
	Gm7732	9	16.7	1.1	1.0
	Gm7877	10	16.5	0.4	0.6
Chemokine/ cytokine	Ccl5	3	25.8	32.7	22.3
	Ccl22	45	11.0	1.0	0.7
	Kitl	86	9.3	1.9	1.2
	Osm	113	8.7	3.6	0.9
	Il1b	392	5.6	0.9	0.7
	Il6	417	5.5	1.9	1.5
	Cxcl10	638	4.7	0.8	0.4
	Ccl2	880	4.1	4.5	2.1
	Il12b	1227	3.6	3.1	0.7
	Cxcl16	1451	3.3	2.2	4.6
Mmp	Mmp3	1322	3.5	2.2	2.0
	Mmp27	5397	1.9	1.6	5.0
	Mmp7	5453	1.9	1.0	2.6
	Mmp12	7387	1.6	2.0	1.2
	Mmp10	8276	1.5	0.7	0.9
	Mmp19	10649	1.4	0.4	2.0
	Mmp15	12572	1.3	0.7	2.5
	Mmp11	13380	1.3	2.1	0.6
	Mmp24	17089	1.1	1.2	0.9
	Mmp20	17299	1.1	1.2	1.4
Cam	Vcam1	1331	3.5	6.1	8.7
	Pecam1	2742	2.5	0.7	1.5
	Icam1	3917	2.2	3.1	2.6
	Selp	6266	1.8	3.3	1.5
	F11r	8175	1.6	1.4	1.2
	Pvrl2	10882	1.4	0.7	0.4
	Jam3	10972	1.4	0.6	1.2
	Jam2	13969	1.2	1.3	1.2
	Icam2	15417	1.2	1.8	1.0
	Cd34	16883	1.1	1.8	1.9

B

Category	Gene Symbol	Rank	HCAEC		HPAEC	
			FK565	Lipid A	FK565	Lipid A
All	UBD	1	70.3	6.4	43.9	0.7
	PRMT8	3	31.5	1.8	1.1	0.5
	PRRX1	4	20.8	2.4	0.2	0.5
	SH3GL2	5	14.3	1.0	0.8	1.6
	CES1	6	14.3	0.9	1.4	2.3
	PRM1	7	13.2	2.3	1.0	0.9
	C1orf81	8	12.3	1.0	0.7	1.3
	CCL2	9	10.5	3.1	5.3	2.9
	PAMR1	10	10.5	0.8	0.5	0.6
	GNPTAB	11	9.6	2.8	2.2	1.0
Chemokine/ cytokine	CCL2	9	10.5	3.1	5.3	2.9
	CCL5	41	6.0	6.3	4.5	2.9
	CSF2	43	5.8	2.1	0.8	3.9
	LTB	58	5.4	0.8	6.8	1.9
	IL29	124	4.2	1.3	0.3	0.3
	IL5	252	3.3	1.7	8.0	1.5
	CCL20	427	2.9	2.0	1.4	1.6
	CXCL3	446	2.9	2.7	1.5	1.2
	IL6ST	453	2.9	1.8	1.7	0.5
	CXCL9	476	2.9	1.0	1.2	1.0
MMP	MMP9	30	6.7	1.1	0.9	1.5
	MMP3	600	2.7	2.5	1.5	0.8
	MMP10	1675	2.0	1.6	2.4	1.6
	MMP13	1862	2.0	1.9	2.8	0.6
	MMP28	2800	1.8	1.2	0.7	1.1
	MMP23B	4671	1.5	1.1	0.8	0.9
	MMP25	5844	1.5	0.5	1.8	1.5
	MMP12	7211	1.4	1.2	2.5	1.3
	MMP16	7467	1.4	0.8	1.0	1.2
	MMP27	11675	1.2	1.6	1.2	1.1
CAM	ICAM1	53	5.6	2.0	4.4	1.5
	MADCAM1	921	2.4	1.2	1.0	1.4
	VCAM1	1323	2.2	2.0	0.9	1.2
	CD34	1996	2.0	1.8	1.7	3.6
	CD58	4737	1.5	1.3	1.0	0.8
	F11R	12407	1.2	0.9	1.0	0.6
	JAM3	20960	1.0	1.2	0.9	1.2
	ICAM3	24950	1.0	1.0	1.0	1.0
	ICAM2	25062	1.0	0.9	1.0	1.0
	JAM2	27109	0.9	0.9	1.3	0.8

A. Top 10 genes in the all, Chemokine/cytokine, Mmp and Cam categories are listed among the genes of which expression levels were enhanced in aortic root *ex vivo* cultured for 24 hours with FK565 (10μg/mL) compared with those without stimulation by microarray analysis. Numbers in rank refer to the order of the gene expression levels in all categories. Data are expressed as fold changes of the respective gene expression levels after FK565 stimulation. Selection of the genes in each category is described in SupplementaryTable II. Gene full names are as follows. Gm4022, predicted gene 4022; Gm3727, predicted gene 3727; Krt42, keratin 42; Gm7225, predicted gene 7225; Gm4477, predicted gene 4477; Prlr, prolactin receptor; Gm15761, predicted gene 15761; Gm7732, predicted gene 7732; Gm7877, predicted gene 7877; Ccl22, chemokine (C-C motif) ligand 22; Kitl, kit ligand; Osm, oncostatin m; Il1b, interleukin 1 beta; Il12b, interleukin 12b; Cxcl16, chemokine (C-X-C motif) ligand 16; Pecam1, platelet/endothelial cell adhesion molecule 1; F11r, F11 receptor; When 2 or more genes were picked up by different probes, only one with the highest rank is included in the list. AR: aortic root, PA: pulmonary artery, Aorta: arch portion of aorta. Microarray analysis data are shown in Supplementary Fig. IVA.

B. Top 10 genes in the all, Chemokine/cytokine, human matrix metallopeptidase (MMP) and human cell adhesion molecule (CAM) categories are listed among genes of which expression levels were enhanced in HCAEC after 24 hours stimulation with FK565 (10μg/mL) *in vitro* compared with those without any reagent by microarray analysis. Numbers in rank refer to the order of the gene expression levels in all categories. Data are expressed as fold changes of the respective gene expression levels after stimulation. Selection of the genes in each category is described in Supplementary Table II. Gene full names are as follows. UBD, ubiquitin D; PRMT8, protein arginine methyltransferase 8; PRRX1, paired related homeobox 1; SH3GL2, SH3-domain GRB2-like 2; CES1, carboxylesterase 1 (monocyte/macrophage serine esterase 1); PRM1, protamine 1; C1orf81, hypothetical protein LOC647215; CCL2, chemokine (C-C motif) ligand 2; PAMR1, regeneration associated muscle protease; GNPTAB, N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits; CCL5, chemokine (C-C motif) ligand 5; CSF2, colony stimulating factor 2 (granulocyte-macrophage); LTB, lymphotoxin beta (TNF superfamily, member 3); IL29, interleukin 29 (interferon, lambda 1); IL5, interleukin 5 (colony-stimulating factor, eosinophil); CCL20, chemokine (C-C motif) ligand 20; CXCL3, chemokine (C-X-C motif) ligand 3; IL6ST, interleukin 6 signal transducer (gp130, oncostatin M receptor); CXCL9, chemokine (C-X-C motif) ligand 9; ICAM1, intercellular adhesion molecule 1; MADCAM1, mucosal vascular addressin cell adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1; CD34, CD34 molecule ; CD58, CD58 molecule; F11R, F11 receptor; JAM3, junction adhesion molecule 3; ICAM3, intercellular adhesion molecule 3; ICAM2, intercellular adhesion molecule 2; JAM2, junction adhesion molecule 2. When 2 or more genes were picked up by different probes, only one with the highest rank is included in the list (Rank 2 is UBD). Microarray analysis data are shown in Supplementary Fig. IVB.