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特集　最先端医療の進歩－臓器移植・再生医療・遺伝子治療

Ⅱ．再生医療の進歩

# 総論：再生医療の進歩

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要旨　再生医療の基盤となる細胞は幹細胞であり，この細胞は自己複製能とさまざまな細胞への分化能を併せもった細胞として知られている．現在わが国で行われている再生医療は体性幹細胞を利用したものであるが，ヒトES細胞を用いた再生医療が2010年に米国で開始された．受精卵を滅失して作成するES細胞に比し倫理的問題の少ないiPS細胞の再生医療への応用も，近い将来開始されようとしている．

Key words　再生医療，体性幹細胞，ES細胞，iPS細胞

## はじめに

従来の医療は，臓器障害をできるだけ早期に発見し，その原因の除去および生体防御反応の修飾により，障害を受けた臓器の自然回復を待つものであった．そのための薬物療法や外科的手法を用いた患部の除去などの医療が行われてきた．しかしながら，臓器障害も一定の限度を超えると不可逆的となり，臓器の機能回復は困難となる．このような患者に対して障害を受けた細胞，組織，さらには臓器を体内で再生し（*in vivo*法），あるいは人為的に再生させた細胞や組織などを移植したり，臓器としての機能を有するようになった再生組織で置換すること（*ex vivo*法）で，治療しようというのが再生医療である．このための基礎研究が盛んに行われ，その成果が続々と臨床の場に持ち込まれようとしている．再生医療の基盤となる細胞は幹細胞であり，この細胞は自己複製能とさまざまな細胞への分化能を併せもった細胞として知られている．現在行われている再生医療の多くはわれわれの身体の中に存在する体性幹細胞を利用したものであるが，ほぼ無限の自己複製能をもつ胚性幹細胞（embryonic stem cell，以下ES細胞と略す）を用いた再生医療が2010年に米国で開始され，人工多能性幹細胞（induced pluripotent stem cell，以下iPS細胞と略す）の再生医療への応用も近い将来開始されようとしている．

## 幹細胞

再生医療の基盤となる幹細胞には，大きく分けると多能性（pluripotent）幹細胞であるES細胞，iPS細胞と，分化の方向性が限定された（multipotent）体性幹細胞（組織幹細胞）がある（図1）．

幹細胞には階層性（hierarchy）があり，より未分化な幹細胞は自己複製能が高く，非常に広範な細胞系列に分化できる多分化能を有するが，幹細胞も下位になるに従い自己複製能は低下し，分化できる細胞系列も限定されてくる．後述するES細胞やiPS細胞はほぼ無限の自己複製能をもち，未分化な状態でいくらでも増やすことができ，分

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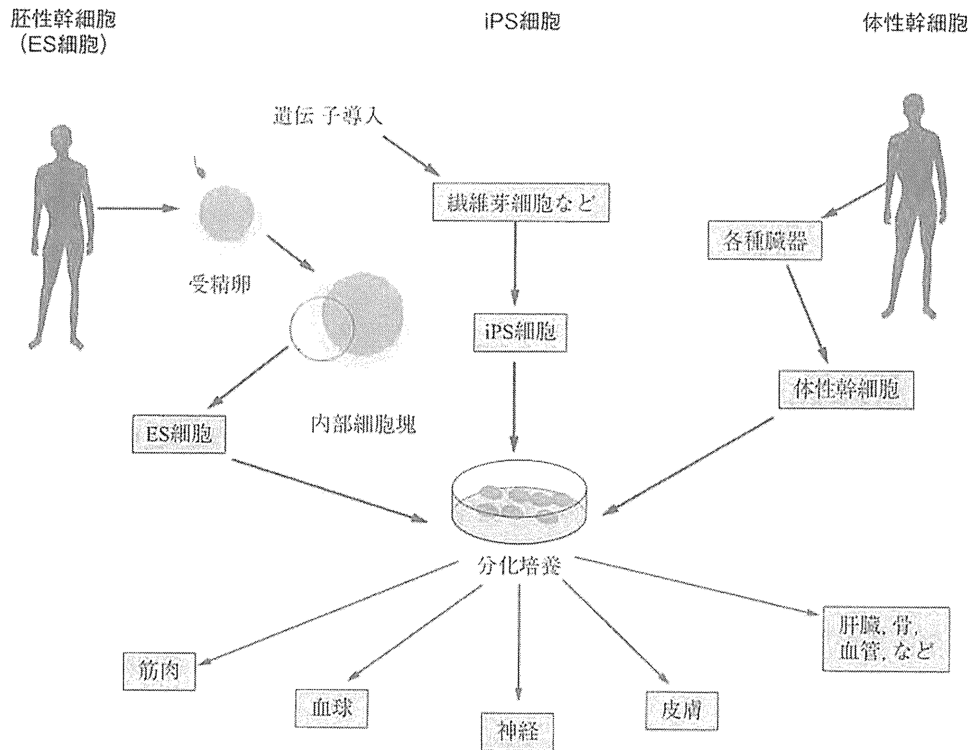


図1 幹細胞の種類

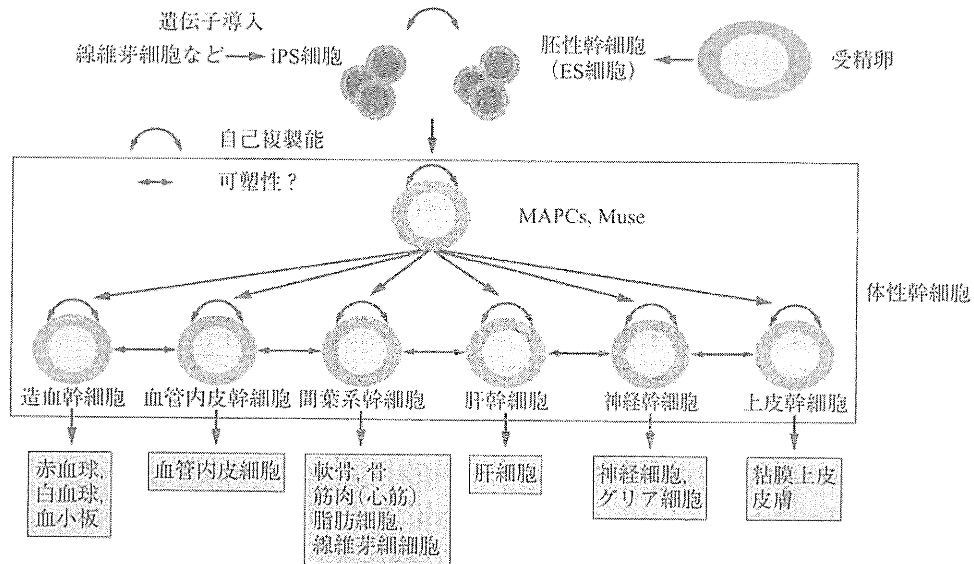


図2 幹細胞のhierarchy(階層性)

化させる培養系に転換すれば多くの細胞系列へ分化できる多分化能を有することから，hierarchyの上位に位置していると考えられる．一方，多くの体性幹細胞は限定された自己複製能と分化能をもつことから，下位に位置する幹細胞と考えられている(図2)．

**1．体性幹細胞**

われわれの多くの臓器には，組織特異的な体性

幹細胞が存在し，臓器が障害を受けた際にはその修復を担っていると考えられている．体性幹細胞は固有の系列への分化能をもつとともに，分裂した際，自分と同じ能力をもった細胞を再生（自己複製）することにより，それぞれの組織を維持していると考えられている．造血幹細胞はもっとも研究の進んでいる体性幹細胞であり，すべての血液細胞の母細胞である．すでに世界的に骨髄，末梢血，臍帯血中の造血幹細胞を用いた移植が盛んに行われ，さまざまな難治性疾患に対する根治を目指す治療法としての地位が築かれている．造血幹細胞移植が成功すれば，骨髄に生着したドナーの造血幹細胞から一生にわたり各種血球が供給されることとなる．このような造血幹細胞移植はまさに再生医療の先駆けと位置づけることができるし，さらに造血幹細胞を体外で増幅する研究が盛んに行われ，増幅した細胞を用いた実際の臨床応用も開始されている．その他，血管内皮，皮膚，腸上皮クリプトの基底細胞，生殖器などにそれぞれ固有な体性幹細胞が存在することが知られ，最近では，肝臓，腎臓，網膜のような三次元構造をもった組織や成人の神経組織においても幹細胞が存在することが報告されている．骨髄や脂肪組織などに存在し，骨，軟骨，脂肪細胞に分化できる間葉系幹細胞（mesenchymal stem cell：MSC）の中に，通常の体性幹細胞よりさらに未分化な性質をもつ幹細胞の存在も示唆されている[1]．このようなさまざまな幹細胞を用いて再生医学を目指す研究が盛んに行われている．欧米では死亡胎児の脳組織中の神経幹細胞を用いたParkinson病などに対する再生医療が行われているが，わが国では死亡胎児（中絶胎児）を用いた医療についての社会的合意は得られていない．現在，わが国で実際の患者に対して行われている再生医療は，すべて体性幹細胞を利用したものである．

2．胚性幹細胞（ES細胞）

1981年，Evansらは，マウス胚盤胞の将来胎仔を構成する内部細胞塊（inner cell mass：ICM）の細胞を取りだして*in vitro*で培養することにより，多能性幹細胞の樹立に成功した[2]．この細胞は生殖細胞を含むすべての組織・細胞に分化しうる能力をもつことが明らかにされた．初期胚の胚盤胞の内部細胞塊へES細胞を注入し，それを子宮に戻せば，発生への寄与が可能になり，キメラマウスが誕生し，生殖細胞に分化したES細胞はキメラマウスの交配により，その遺伝情報が次世代個体に伝達されることが確認された．このことによりDNA相同組換えによる遺伝子破壊や外来遺伝子が導入されたES細胞クローンを選択し，個体に戻すことで生体における未知の新規遺伝子の機能解析が可能となり，ES細胞は分子生物学の発展に大きく寄与することとなった．

マウスES細胞は，適切な条件下で培養すればさまざまな細胞に分化していく様子を観察することができる．われわれは血液，心筋，骨格筋，各種神経細胞やグリア細胞などへの分化を報告してきた[3][4]．

1998年，米国ウイスコンシン大学のThomsonらにより，はじめてヒト胚盤胞からのES細胞の樹立が報告された．彼らは不妊治療で不用となり廃棄される前のヒト胚盤胞を使ってES細胞株の樹立に成功した[5]．今では多数の国からヒトES細胞の樹立が報告されている．

ヒト受精卵を用いたES細胞の樹立は社会的，倫理的に多くの問題を抱えているが，一方，いったん樹立されるとES細胞はほぼ無限に増やせることから，これらの細胞から特定の細胞や特定の組織幹細胞だけに分化させる培養系を確立することができれば，幅広い再生医療への応用が可能になると期待されている（図3）．しかし，ヒトES細胞が再生医療や生物学の発展に大きく貢献する可能性がある反面，人間の生命の萌芽であるヒト胚を使用することなどの生命倫理上の問題点が存在する．とくに，ES細胞由来の細胞を用いた再生医療を考えた場合には，当然，ヒト組織適合白血球抗原（human histocompatibility leukocyte antigen,

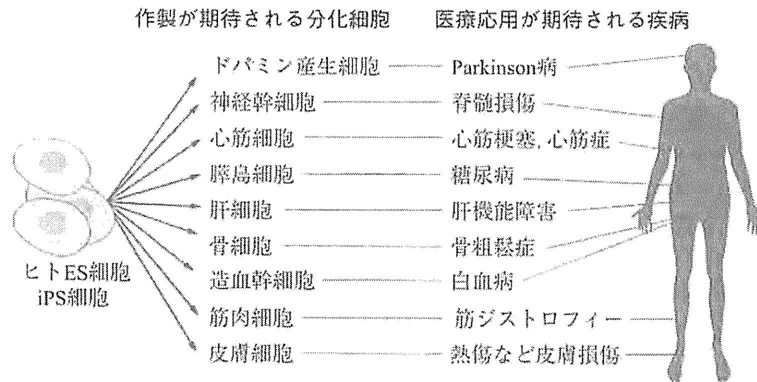


図3　ES/iPS細胞による再生医療

以下HLAと略す）の合致した細胞を移植に用いる必要がある．そのためには膨大な数の受精卵を滅失してHLAの異なるES細胞株を樹立せねばならず，二重の生命倫理的問題が生じてしまう．一方，受精卵の核を除き代りに患者体細胞の核を移植する方法で患者に合致したHLAをもつES細胞の樹立も試みられたが成功していない．また，このような方向での医療は，膨大な数の受精卵を必要とすることから，とても社会的に受け入れられるものではない．

わが国ではヒトES細胞の樹立および使用に関して，文部科学省により「ヒトES細胞の樹立および使用に関する指針」が公表されている．すでにこの指針を遵守したヒトES細胞が京都大学の末盛，中辻らにより樹立され，広く使われるようになった．最近，わが国における2番目のヒトES細胞樹立機関として，国立成育医療研究センターでヒトES細胞が樹立された．

筆者らは平成15年よりヒトES細胞を用いた研究を行ってきた．ヒトES細胞は培養条件を変化させると，神経細胞，筋肉細胞（とくに心筋細胞），血管内皮細胞，軟骨や骨の細胞，膵島細胞，肝細胞，各種血液細胞などさまざまな細胞に*in vitro*で分化可能である．ヒトES細胞からの血球系の細胞分化の過程を検討すると，ほぼ*in vivo*における発生過程を模倣する形で血球が作られてくることがわかった[6)]．

ES細胞の再生医療への応用を考えた場合，ES細胞の未分化性を維持した細胞が一つでも残っていると移植後腫瘍を形成してしまうし，目的とする細胞系列以外の細胞への分化能を残したままでは医療への応用はむずかしいことから，特定の細胞系列だけに分化させる系の確立が急がれている．

現在のわが国の指針ではヒトES細胞の使用は研究に限られており，臨床に使用することはできない．

2010年10月，米国食品医薬品局（Food and Drug Administration，以下FDAと略す）の認可を受け，米国ジェロン社が脊髄損傷に対するヒトES細胞由来オリゴデンドロサイトを用いた移植試験を多施設で開始した．同年11月にはAdvanced Cell Technology（ACT）社がFDAからヒトES細胞由来網膜色素上皮細胞を用いた家族性黄斑萎縮症治療の臨床試験申請の承認が得られたと発表した．前述したようにES細胞を用いた再生医療は倫理的な問題を多く抱えているものの，これらの発表は再生医療を待つ患者さんにとっては記念碑的な出来事といえよう．

わが国では「ヒト幹細胞を用いる臨床研究に関する指針」の改正が2010年11月1日に行われた．後述するヒトiPS細胞はこの指針に盛り込まれたものの，ES細胞については，ヒト胚の臨床利用に関する基準が定められるまではヒトES細胞を

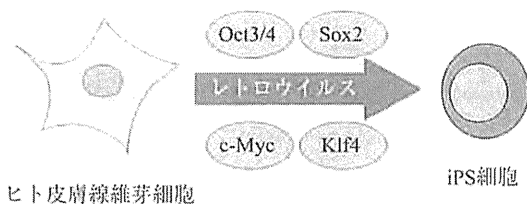


図4 ヒトiPS細胞の作製成功（文献9）より引用）

用いる臨床研究は実施しないこととするとされている．現在，ヒトES細胞を含む新たな指針改正に向けた議論が始まっている．

### 3．人工多能性幹細胞（iPS細胞）

2006年，京都大学の山中教授らはマウス体細胞にたった四つの転写因子（*OCT3/4*，*Sox2*，*c-Myc*，*Klf4*）遺伝子をレトロウイルスベクターで導入することにより，旺盛な自己複製能と多分化能をもった人工多能性幹細胞（iPS細胞）の樹立に成功し[7]，世界中に大きな衝撃を与えた．

iPS細胞はES細胞特異的なマーカーを発現するようになり，免疫不全マウスに移植すると三胚葉系を含む奇形腫を形成することができ，このiPS細胞は受精卵の胚盤胞に戻すと，生殖細胞を含む全身の細胞に分化し，次の世代では全身がiPS細胞に由来するマウスも正常に誕生したことから，iPS細胞のもつ多能性はES細胞と比べても遜色がないことが示された．iPS細胞を分化に適した条件で培養すると，ES細胞同様，心筋，神経細胞，グリア細胞，各種血液細胞，骨格筋細胞，血管内皮細胞などさまざまな細胞への分化がみられた．筋ジストロフィーへの再生医療の開発を目的に，正常マウスiPS細胞から分化してきた骨格筋を分離し，その中に含まれる骨格筋幹細胞（サテライト細胞）をFACSで分取し，筋ジストロフィーのモデルマウスであるジストロフィンを欠損したmdxマウスに移植すると，多数の正常骨格筋の再生がみられた[8]．

マウスiPS細胞が報告された翌年，山中らはヒト皮膚線維芽細胞にマウスと同様の四つの遺伝子を導入することによりヒトiPS細胞の樹立に成功した（図4）[9]．

ヒトiPS細胞は，受精卵を滅失することなく作成できることから，ES細胞のもつ社会的，倫理的な多くの問題を回避することができる．iPS細胞はES細胞と同様に未分化な状態のままほぼ無限に増やせること，培養条件を変化させると，神経細胞，心筋，骨格筋，血管内皮細胞，軟骨や骨の細胞，膵島細胞，肝細胞，各種血液細胞などさまざまな細胞に*in vitro*で分化可能であることから，幅広い再生医療への応用が期待されている（図3）．現在，臨床応用可能な，より安全性の高いiPS細胞を樹立するためさまざまな検討が行われている．

#### 1）iPS細胞樹立の材料

当初，皮膚線維芽細胞から樹立されていたiPS細胞はその後，骨髄細胞，臍帯血，末梢血，毛根細胞，歯髄細胞，脂肪組織など，さまざまな組織から樹立可能となっている．どの組織を材料にして作製したiPS細胞がもっとも優れているか，さまざまな角度から検討が行われている．

#### 2）最適な誘導法は何か

当初*OCT3/4*，*Sox2*，*Klf4*，*c-Myc*の四つの転写因子遺伝子を導入してiPS細胞の樹立が行われていたが，がん遺伝子でもある*c-Myc*の代りに*L-Myc*が用いられるようになった[10]．遺伝子導入にはレトロウイルスベクターが用いられていたが，染色体に組み込まれないエピゾーマルプラスミドベクターを中心に検討が進んでいる[11]．

#### 3）最適な評価法

どのようなiPS細胞をもっともよい標準的なiPS細胞とするかについても世界的な検討が始まり，目的の細胞にきちんと分化できるか．分化抵抗性をもった細胞が残存しないか，導入遺伝子はしっかりサイレンシングされているか，などの問題が検討されている．

2010年11月1日に改正された「ヒト幹細胞を用いる臨床研究に関する指針」では，ヒトiPS細胞を用いた再生医療も指針の中に含まれている．

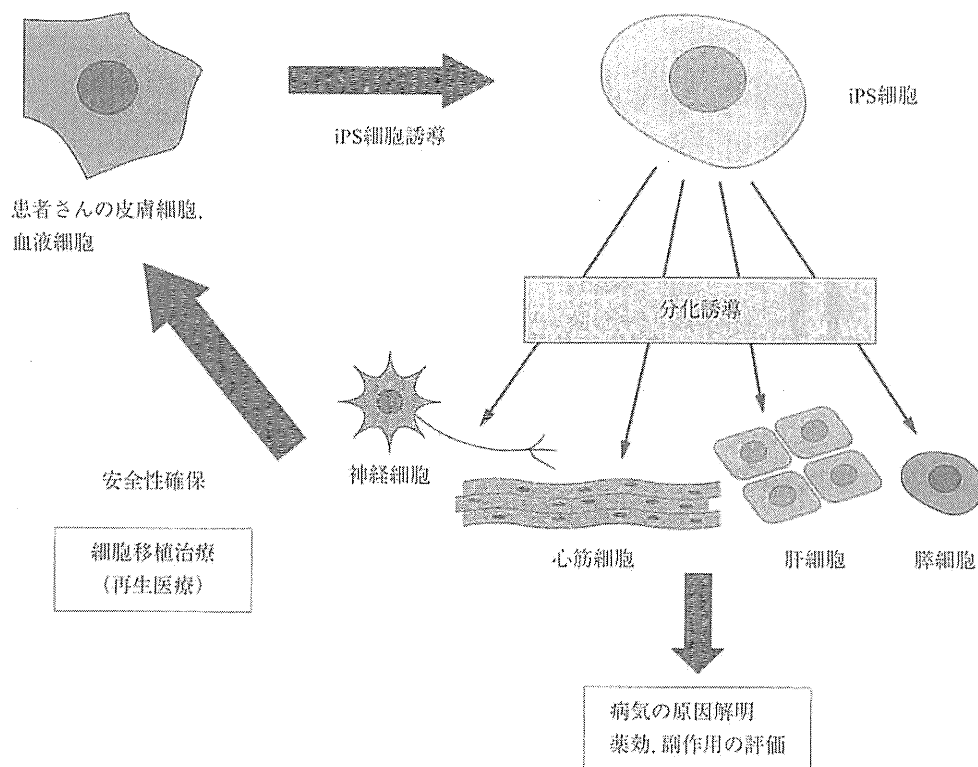


図5　疾患特異的iPS細胞の医療応用への可能性

表　再生医療の対象となる疾患

| | |
|---|---|
| 血液，腫瘍疾患 | 白血病，再生不良性貧血，小児がんなどに対する造血幹細胞移植，間葉系幹細胞（MSC）の移植への応用，人工血液 |
| 神経疾患 | Perkinson病，Alzheimer，脳性小児麻痺，脳室周囲白質軟化症（PVL），神経変性疾患，脊髄損傷，末梢神経障害，など |
| 循環器疾患 | 心筋梗塞（川崎病），拡張型心筋症，慢性閉塞性動脈硬化症（ASO），Buerger病，など |
| 筋疾患 | 各種筋ジストロフィー |
| 肝疾患 | 劇症肝炎，肝硬変，など |
| 内分泌疾患 | I型糖尿病，など |
| 骨疾患 | 骨系統疾患，骨欠損，など |
| 皮膚疾患 | 重症熱傷，など |
| その他 | 先天性難聴，網膜色素変性症，未熟児網膜症，など |

MSC：mesenchymal stem cell，PVL：periventricular leukomalacia，ASO：arteriosclerosis obliterans

iPS細胞を用いた再生医療はそう遠くない将来わが国でも始まり，さまざまな疾患が対象となるであろう（表）．

## おわりに

造血幹細胞や間葉系幹細胞などの体性幹細胞を用いた再生医療で始まったわが国においても，ヒトES細胞やiPS細胞を用いた再生医療が始まろうとしている．とくにiPS細胞は患者自身の皮膚や血液から樹立可能である．この疾患特異的iPS細胞は，疾患の病因，病態の解明や新規薬剤の開発に有用であるとともに，遺伝子治療と組み合せた再生医療への発展が期待されている（図5）．この分野の研究の発展を期待したい．

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# Clinical and Host Genetic Characteristics of Mendelian Susceptibility to Mycobacterial Diseases in Japan

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**Abstract**
*Purpose* The aim of this study is to investigate clinical characteristics and genetic backgrounds of Mendelian susceptibility to mycobacterial diseases (MSMD) in Japan.
*Methods* Forty-six patients diagnosed as having MSMD were enrolled in this study. All patients were analyzed for the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* gene mutations known to be associated with MSMD.
*Results* Six patients and one patient were diagnosed as having partial interferon-γ receptor 1 deficiency and nuclear factor-κB-essential modulator deficiency, respectively. Six of the seven patients had recurrent disseminated mycobacterial infections, while 93% of the patients without these mutations had only one episode of infection.
*Conclusions* The patients with a genetic mutation were more susceptible to developing recurrent disseminated mycobacterial infections. Recurrent disseminated mycobacterial infections occurred in a small number of patients even without these mutations, suggesting the presence of as yet undetermined genetic factors underlying the development and progression of this disease.

**Keywords** Disseminated mycobacterial infection · IFN-γR1 deficiency · NEMO deficiency · flow cytometric analysis

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## Introduction

Although the outcome of mycobacterial infection is influenced by many factors, including the virulence of the pathogen and the environment of the host, it has been demonstrated that host genetic factors play important roles in the defense against mycobacteria [1]. Mendelian susceptibility to mycobacterial diseases (MSMD, MIM 209950) is a rare primary immunodeficiency syndrome characterized by a predisposition to develop infections caused by weakly virulent mycobacteria, such as *Mycobacterium bovis* bacille Calmette-Guerin (BCG) and environmental non-tuberculous mycobacteria (NTM) [2–4]. These patients are vulnerable to systemic salmonellosis and infections with *Mycobacterium tuberculosis*, the virulent mycobacterial species, to a lesser extent [5, 6]. Diseases caused by other intracellular pathogens, such as *Nocardia*, *Listeria*, *Paracoccidioides*, *Histoplasma*, and *Leishmania*, and some viruses, such as human herpes virus-8, have only rarely been reported, mostly in single patients [7–12].

To date, interferon (IFN)-γ receptor 1 (*IFNGR1*) [13–15], IFN-γ receptor 2 (*IFNGR2*) [16], interleukin (IL)-12 p40 subunit (*IL12B*) [17], IL-12 receptor β subunit (*IL12RB1*) [18–20], signal transducer and activator of transcription-1 (*STAT1*) [21], and nuclear factor-κB-essential modulator (*NEMO*) [22] mutations were identified as the causes of this primary immunodeficiency. On the other hand, no genetic etiology has yet been reported to be identified for about half of all patients with MSMD [3]. In addition, there have been no precise reports on the clinical characteristics and genetic backgrounds of MSMD in Asian countries, including Japan, which has a high prevalence of tuberculosis.

In this study, we analyzed patients who had a recurrent or disseminated infection with intracellular pathogens to clarify the clinical manifestations and host genetic backgrounds of MSMD in Japan.

## Materials and Methods

### Subjects

We studied 46 patients (30 males and 16 females) diagnosed as having MSMD because of recurrent infections, or blood-borne infections such as osteomyelitis/arthritis, and multiple infections at different anatomic sites by intracellular bacteria including BCG, NTM, *Salmonella* species, *Listeria monocytogenes*, or *M. tuberculosis* in 34 hospitals in Japan from 1999 to 2009. There was no consanguinity in these families. The clinical information on each patient was collected using a standardized case report form. Informed consent was obtained from the parents of the subjects before the study. This study was approved by the Ethics Committee of Kyushu University.

### Flow Cytometric Analysis

Two-color flow cytometric analysis was performed to investigate IFN-γ receptor 1 (IFN-γR1) expression levels on the patients' monocytes by using an EPICS XL instrument (Beckman Coulter, Miami, FL, USA). Peripheral blood mononuclear cells (PBMCs) were stained with mouse anti-IFN-γR1 monoclonal antibody (MAb) (Genzyme, Cambridge, MA, USA), followed by rat phycoerythrin anti-mouse immunoglobulin antibody (BD Bioscience Pharmingen, San Diego, CA, USA). Cells were washed twice and stained with a phycoerythrin 5.1 (PC5)-anti-CD14 MAb (Beckman Coulter). IFN-γR1 expression was analyzed on monocytes determined by their side scatter and CD14 positivity.

### Genomic DNA and cDNA Sequence Analysis

The *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes were analyzed for coding exons and flanking intronic sequences. These genes were amplified by polymerase chain reaction (PCR) after whole genome amplification with a GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, UK). The PCR products were treated with an Exo-SAP-IT kit (GE Healthcare, Amersham, UK) and then were analyzed by direct sequencing with an ABI 3130 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). Detected mutations were confirmed by sequencing the PCR product using cDNA as a template.

### Statistical Analysis

Comparisons of the proportions were analyzed by the $\chi^2$ test. The Mann–Whitney $U$ test was used to compare differences between quantitative variables. A $P$ value less than 0.05 was considered to be statistically significant.

## Results

The median age of the patients was 8 years (range, 6 months–41 years), and the median age at the onset of infection was 1 year and 4 months (range, 4 months–6 years). The male to female ratio was 1.9:1. Only one patient had not received a BCG vaccination. There were 59 episodes of disseminated mycobacterial infections in the 46 patients. Nine (19%) of 46 patients had two or more episodes of these infections. Two of the patients had three episodes, and one had four episodes of these infections. In all episodes, BCG was the most common pathogen (82.6%, Table I). The *Mycobacterium avium* complex (MAC) was isolated during eight episodes of these infections. *M. tuberculosis* was also confirmed in two episodes of infection. No severe *Salmonella* species, *L. monocytogenes*, or viral infections were observed.

The common clinical manifestations were osteomyelitis/arthritis, lymphadenitis, and subcutaneous abscess/dermatitis (Table I and Fig. 1a). Only one patient was diagnosed as having arthritis, and the lesion spread to the adjacent bone. Two patients showed hepatosplenomegaly during the BCG infection, and two patients with the MAC infection developed pulmonary abscess. Among the BCG infections, the median intervals of time between BCG vaccination and the development of primary BCG infection were 3 (1–10 months), 4 (2–36 months), and 11 months (5–46 months) for the subcutaneous abscess/dermatitis, lymphadenitis, and osteomyelitis/arthritis, respectively (Fig. 1b).

We performed the genetic analysis on these patients for the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes. Six patients (five families) and one patient had mutations in the *IFNGR1* and *NEMO* genes, respectively (Table II). Five of the seven patients who had a mutation in the *IFNGR1* gene were the patients that we

**Table I** The clinical manifestations of the patients with MSMD

| | Patients with genetic mutation, *n* (%) | Patients without a genetic mutation, *n* (%) | Total *n* (%) |
|---|---|---|---|
| Causative pathogen[a] | | | |
| BCG | 3 (42.9) | 35 (89.7) | 38 (82.6) |
| *M. avium* complex | 1 (14.3) | 3 (10.2) | 4 (8.7) |
| BCG+*M. avium* complex | 2 (28.5) | 0 (0) | 2 (4.3) |
| *M. avium* complex+*M. tuberculosis* | 1 (14.3) | 1 (2.6) | 2 (4.3) |
| Sites of infection[b] | | | |
| Osteomyelitis/arthritis | 7 (43.8) | 24 (55.8) | 31 (52.5) |
| Lymphadenitis | 8 (50.0) | 8 (18.6) | 16 (27.1) |
| Dermatitis/subcutaneous | 3 (18.8) | 11 (25.6) | 14 (23.7) |
| Pulmonary abscess | 0 (0) | 2 (4.7) | 2 (3.4) |

The total number exceeds 59 because some patients had multiple lesions at the same time

[a] $n=7$ for patients with a genetic mutation and $n=39$ for patients without a genetic mutation

[b] $n=16$ for patients with a genetic mutation and $n=43$ for patients without a genetic mutation

reported previously [14, 15], and the other two patients were newly identified. All of the IFN-γR1-deficient patients were heterozygotes, and the mutation was in the transmembrane domain in one patient (774del4: patient 5) and in the intracellular domain in five patients (811del4: patient 1, 818del4: patients 2–4, and 832 G>T, E278X: patient 6), which led to the expression of a truncated protein with a dominant negative effect on the IFN-γR1 signaling (Table II and Fig. 2a). The IFN-γR1 expression levels were significantly increased in all six patients with IFN-γR1 deficiency (Fig. 2b). Patient 7 had a missense mutation in *NEMO* (943 G>C, E315Q). The CD14-positive cells from this patient produced a lower level of TNF in response to LPS stimulation (data not shown), which was consistent with the defect in NF-κB signaling.

The proportions of the patients with recurrent mycobacterial infection or multiple osteomyelitis/arthritis were

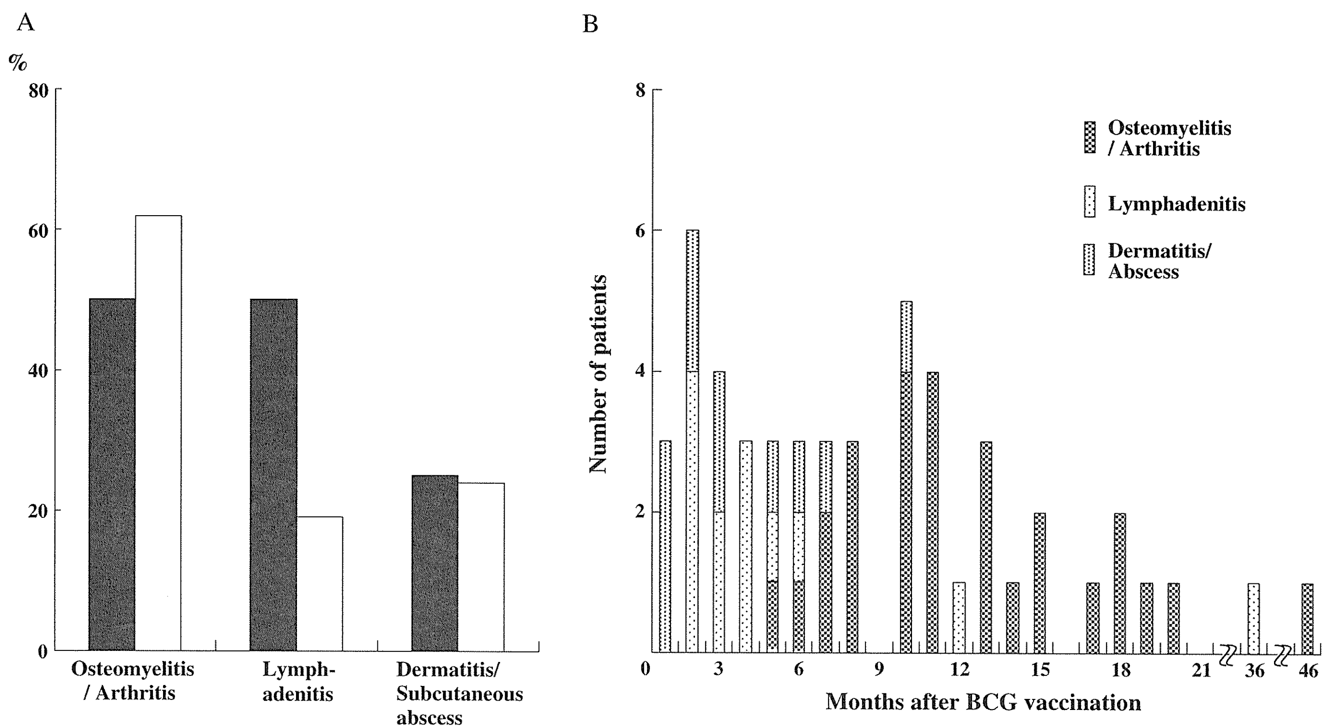


**Fig. 1** The clinical features of the patients with BCG infection. The distribution of the sites of infections (**a**) and the intervals between BCG vaccination and the first onset of BCG infection (**b**) are shown. The *black bar* and the *white bar* represent the proportion of the patients with and without genetic mutations, respectively

**Table II** Characteristics of the patients with a genetic mutation

| Patient no. | Sex | Age | Age of onset | Episodes of infections prior to detection of the genetic mutation | Genetic mutation |
|---|---|---|---|---|---|
| 1[a] [14] | F | 1 year 7 months | 10 months | BCG lymphadenitis and dermatitis<br>Multiple BCG osteomyelitis | *IFNGR1* 811del4 |
| 2[a] [14] | M | 1 year 9 months | 8 months | BCG lymphadenitis, hepatomegaly<br>Multiple BCG osteomyelitis | *IFNGR1* 818del4 |
| 3[a] [14] | M | 2 years | 2 years | Multiple BCG osteomyelitis | *IFNGR1* 818del4 |
| 4[a] [14] | M | 41 years | 3 years | *M. tuberculosis* lymphadenitis (twice)<br>Multiple MAC octeomyelitis | *IFNGR1* 818del4 |
| 5[a] [15] | F | 12 years | 6 months | BCG lymphadenitis<br>Multiple MAN osteomyelitis | *IFNGR1* 774del4 |
| 6 | M | 19 years | 4 months | BCG lymphadenitis and dermatitis<br>Multiple BCG osteomyelitis<br>MAC subcutaneous abscess<br>Multiple MAC osteomyelitis | *IFNGR1* E278X |
| 7 | M | 10 years | 10 months | *M. tuberculosis* lymphadenitis<br>Multiple MAC lymphadenitis<br>Sepsis, bacterial pneumonia (four times) | *NEMO* E315Q |

Patient 4 is the father of patient 2

*MAC Mycobacterium avium* complex

[a] These patients were reported previously

significantly higher in those with the genetic mutations (Table III). There were no significant differences in the age at the onset of mycobacterial infection, or in the interval of time between BCG vaccination and the first onset of BCG infection between the patients with and without genetic mutations. One patient diagnosed with BCG dermatitis died of persistent diarrhea of unknown etiology, while the others are still alive.

## Discussion

In the present study, we investigated the clinical characteristics and the genetic backgrounds of the patients diagnosed as having MSMD in Japan. We observed that the patients with the genetic mutation were susceptible to developing recurrent mycobacterial infections and multiple osteomyelitis/arthritis, and IFN-γR1 deficiency was the most

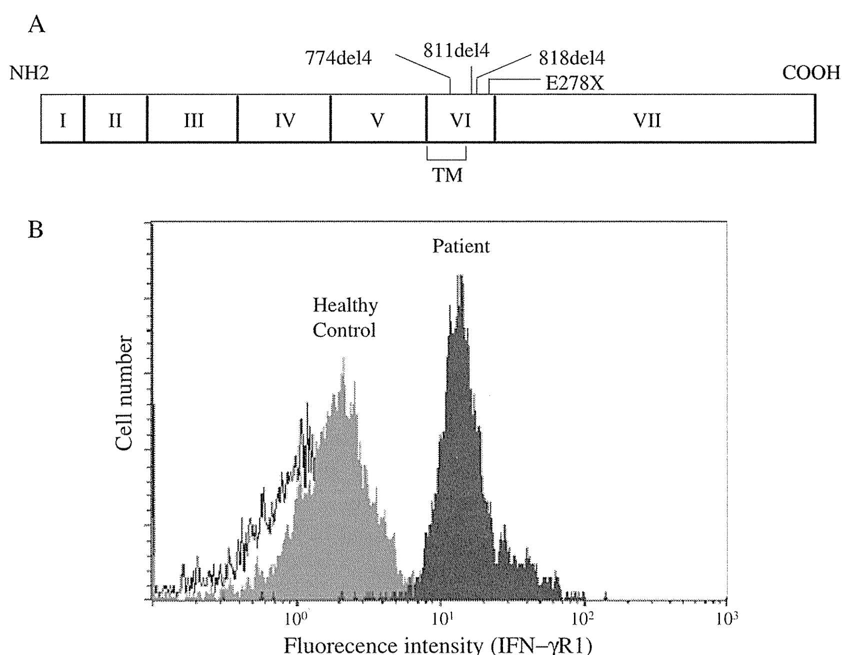


**Fig. 2** *IFNGR1* gene mutations and the analysis of IFN-γR1 expression on monocytes. The sites of *IFNGR1* gene mutations in the six IFN-γR1-deficient patients (**a**) and the increased IFN-γR1 expression level on monocytes in patient 2 are shown (**b**)

**Table III** Comparison of the patients with and without a genetic mutation

| | Patients with a genetic mutation ($n$=7) | Patients without a genetic mutation ($n$=39) |
|---|---|---|
| Age of onset (months) | 10 (4–36) | 14 (4–75) |
| Male to female ratio | 2.5:1 | 1.8:1 |
| Familial history ($n$) | 2 | 0 |
| Median interval between BCG vaccination and the first onset of BCG infection (months) | 9.5 (7–15, $n$=4) | 10 (1–46, $n$=35) |
| Recurrent cases (%) | 85.7* | 7.7 |
| Patients with multiple osteomyelitis/arthritis (%) | 100* ($n$=6) | 4.2 ($n$=24) |

*$p<0.0001$

frequent genetic defect identified in these patients. The prevalence of MSMD is estimated to be at least 0.59 cases per million births, and the disease does not seem to be confined to any ethnic group or geographic region, according to a national retrospective study of idiopathic disseminated BCG infection in France [23, 24]. This is the first epidemiological study associated with MSMD in Japan which showed the difference in the clinical manifestation and the genetic background between Japan and Western countries.

The *IFNGR1* mutations identified in this study were in exon IV, within the transmembrane domain, or the intracellular domain of the *IFNGR1* gene (Fig. 2a), which led to a truncated protein lacking signaling motifs [25]. The truncated protein also lacks the recycling motif, which leads to the overexpression of the mutant protein (Fig. 2b) [25]. These mutations are located in important hot spots in the patients diagnosed with dominant partial IFN-γR1 deficiency [13], and the flow cytometric analysis of IFN-γR1 expression levels may be a useful method for the screening for this disease [15]. The *NEMO* mutation found in patient 7 was in exon VIII within the leucine zipper domain of the *NEMO* gene. A previous study reported that a mutation in this region disrupted a common salt bridge in the leucine zipper domain and impaired T-cell-dependent IL-12 production [22].

The patients with the genetic mutations were susceptible to recurrent mycobacterial infections and multiple osteomyelitis/arthritis as described previously [3], but no fatal mycobacterial infection was observed in this study. Unlike complete IFN-γR1 and IFN-γR2 deficiencies, which often cause fatal mycobacterial infections [13, 16], the patients with dominant partial IFN-γR1 and NEMO deficiencies have been reported to have a relatively mild disease and a better prognosis [13, 22]. These factors might have contributed to the good outcome of the patients in this study. In addition, the low virulence of BCG might contribute to the characteristics of BCG infection in Japan, because the BCG Tokyo 172 strain that is used in Japan for vaccination is the least virulent BCG substrain.

The *IL12RB1* mutation has been reported to be the most common cause of MSMD [4]. However, none of the patients in this study was diagnosed as having an IL-12 receptor β1 deficiency. In Japan, this disease was reported in only one patient with disseminated lymphadenitis caused by *M. avium* complex [18]. It has been suggested that most complete IL-12 receptor β1-deficient individuals may be asymptomatic, and only those that also have a second mutation in another gene may be more prone to infections [26, 27]. These symptomatic IL-12 receptor β1-deficient patients are mainly found in families with consanguineous parents [19, 27]. Consanguineous marriages are uncommon in Japan, and there were no consanguineous families in this study. This might be the reason why no IL-12 receptor β1-deficient patients were observed. Alternatively, it is possible that the causative gene mutations associated with MSMD are different among races, because the number of patients with IL-12 receptor β1 deficiency was also lower than those with IFN-γR1 deficiency in Taiwan [28].

Although another patient had multiple osteomyelitis, and three patients had recurrent disseminated mycobacterial infections in these studies, they did not have mutations in any of the six genes. It was previously reported that no genetic etiology has yet been identified in about half of patients with disseminated and recurrent mycobacterial infections [3, 4]. This suggests the presence of as yet undetermined genetic factors in the development of this disease.

In the present study, the number of patients with genetic mutations might be too small to conclusively indicate the differences in the clinical manifestations and the host genetic backgrounds of MSMD between Japan and Western countries. However, in terms of the genetic etiology and the prognosis, it remains possible that the features of the patients diagnosed as having MSMD in the present study are different from those in previous reports [3]. Further investigations of a large number of patients are therefore warranted to more precisely evaluate the clinical manifestations and the host genetic background of MSMD in Japan.

## Conclusions

We found that the patients diagnosed as having MSMD in Japan seem to have different genetic features, as well as

different clinical manifestations, compared with those in Western countries. A few patients with recurrent mycobacterial infections without mutations in the six known genes might suggest a contribution of other genetic, as well as environmental, factors in the susceptibility to recurrent infections.

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# Nod1 Ligands Induce Site-Specific Vascular Inflammation

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***Objective***—The goal of this study was to investigate the effects of stimulants for a nucleotide-binding domain, leucine-rich repeat-containing (NLR) protein family on human artery endothelial cells and murine arteries.

***Methods and Results***—Human coronary artery endothelial cells were challenged in vitro with microbial components that stimulate NLRs or Toll-like receptors. We found stimulatory effects of NLR and Toll-like receptor ligands on the adhesion molecule expression and cytokine secretion by human coronary artery endothelial cells. On the basis of these results, we examined the in vivo effects of these ligands in mice. Among them, FK565, 1 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 libitum drinking of FK565. The degree of the 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.

***Conclusion***—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. ***(Arterioscler Thromb Vasc Biol.* 2011;31:1093-1099.)**

**Key Words:** coronary artery disease ■ immune system ■ Kawasaki disease ■ pathology ■ coronary arteritis ■ inflammation

Germ-line encoded pattern-recognition receptors of the innate immune system sense exogenous microbial components and endogenous danger signals to protect the host.[1–4] The pattern-recognition receptors include Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors, the leucine-rich repeat-containing (NLR) protein family, and as-yet-unidentified pattern-recognition receptors that recognize double-stranded DNA.[1,3] The TLR, RIG-I-like receptor, 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 among the structural cells to sense microbial components through pattern-recognition receptors. 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, nucleotide-binding oligomerization domain 1 (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]

Because innate immunity has been suggested to be involved in the pathogenesis or pathophysiology of cardiovascular diseases in adults,[7] as well as vasculitis in Kawasaki disease (KD) in children,[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 a Leica M500 ophthalmology microscope. Cryostat sections were used for the correct detection of 3 aortic valve cusps in these studies.

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Severity of coronary arteritis was assessed by the cross-section with 3 aortic valve cusps as described,[12] defined as follows: − indicates no inflammatory infiltration in the whole layer (from intima to adventitia) of nearest coronary arteries from aorta or in the aorta; + indicates that less than one third of the circumference showed inflammatory infiltration in the whole layer; 2+, between one third and 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 Intercellular Adhesion Molecule-1 Expression and Cytokine Production by Human Coronary Artery Endothelial Cells In Vitro

To investigate the direct effects of innate immune stimulants on the endothelial cells, human coronary artery 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 intercellular adhesion molecule-1 (ICAM-1, CD54) expression and cytokine secretion by HCAEC on 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 a TLR2 ligand, peptidoglycan from *Escherichia coli* K12 in HCAEC (Figure 1). Because 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 cytokine production were observed by combined stimulation with pure synthetic iE-DAP plus MDP or iE-DAP plus lipid A in HCAEC. No release of IL-1β, IL-10, tumor necrosis factor-α, or IL-12p70 was 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 on the day 3 experiment, we performed experiments on day 1 as well. Similar additive effects were observed between NOD1 and TLR4 ligands on day 1 (Supplemental Figure IA). In addition, NOD1 small interfering RNA completely inhibited additive effects of NOD1 and TLR4 ligands on day 1 experiments (Supplemental Figure 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, peptidoglycan from *E. coli* K12, and another bacterial com-

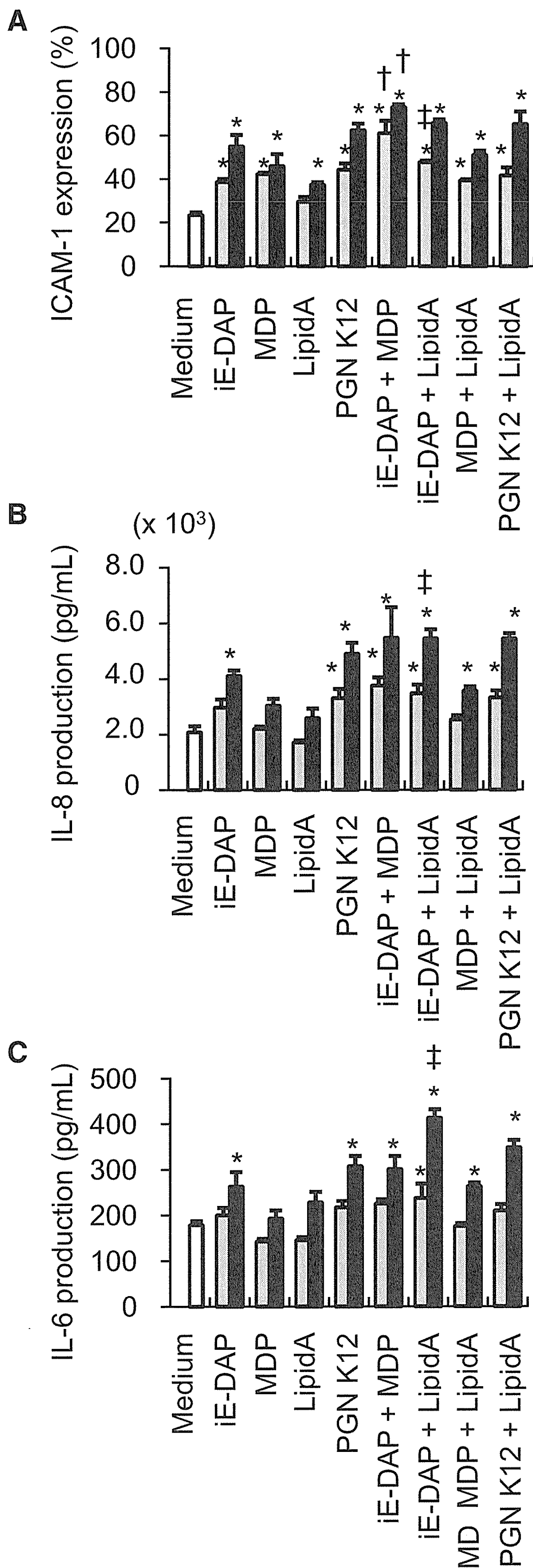


**Figure 1.** Effects of innate immune stimulants on HCAEC. HCAEC ($1\times10^4$ cells) were incubated with NOD1, NOD2, TLR, and other stimulants in various combinations. ICAM-1 expression (A) and IL-8 (B)/IL-6 (C) production in the culture supernatants were investigated in triplicate on day 3. The concentrations of stimulants are as follows: iE-DAP, MDP, and peptidoglycan from *E. coli* K12 (PGN K12), 1 (gray bars) or 10 (black bars) μg/mL; lipid A 10 (gray bars) or 100 (black bars) ng/mL. Data are presented as mean±SD. *$P<0.01$ 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 test).

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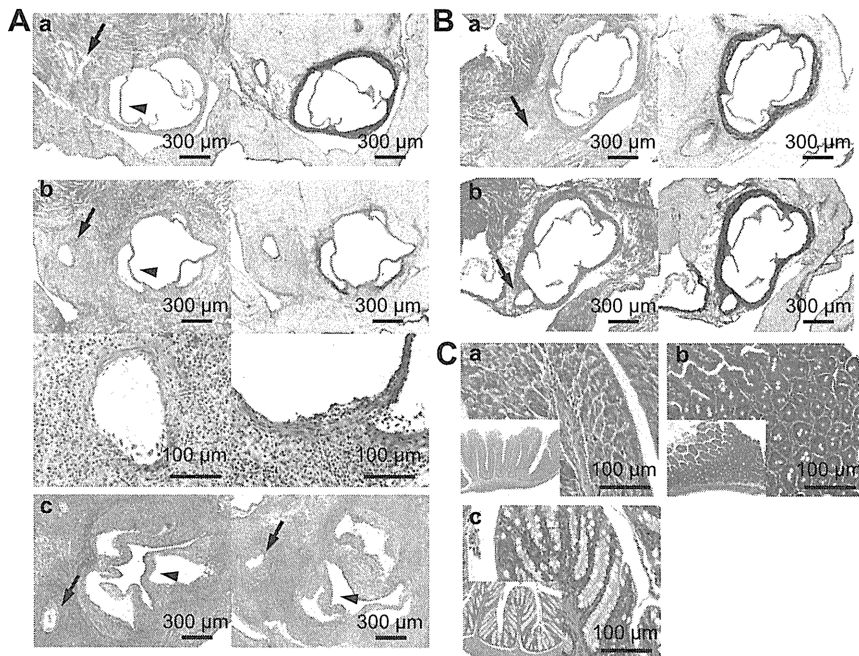


**Figure 2.** Histopathologic changes after administration of Nod1 ligand in BALB/c, SCID, and Nod1 knockout mice. A, a, Cross sections with 3 aortic valve cusps of a BALB/c mouse treated with 100 $\mu$g of LPS IP 4 times (Supplemental Table IA). Severity of coronary arteritis: grade 0. Left, hematoxylin and eosin stain; right, elastica Van Gieson stain. b, Grade 3+ coronary arteritis/valvulitis of the mouse subcutaneously challenged by FK565 (500 $\mu$g) with LPS priming weekly 4 times. Upper panels, hematoxylin-eosin stain (left) and elastica Van Gieson stain (right); magnification, ×40. Lower panels, coronary artery (left) and aortic valve (right) in hematoxylin-eosin stain; magnification, ×200. c, Grade 3+ coronary arteritis and valvulitis of FK565–orally administrated mouse without LPS priming (left, 100 $\mu$g once a day, 6 days/week, 4 weeks; right: tap water ad libitum, 120 $\mu$g/day, 4 weeks, hematoxylin-eosin stain; magnification, ×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 libitum 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 with FK565 with LPS priming, as shown in Supplemental Table IB. Grade 3+ coronary arteritis/valvulitis and no coronary arteritis/valvulitis are shown in SCID mice and Nod1$^{-/-}$ mice, respectively. Shown are hematoxylin-eosin stain (left) and elastica Van Gieson stain (right). Coronary artery is indicated by arrows. C, Oral administration of FK565 (100 $\mu$g for 6 consecutive days) after LPS priming induces no inflammation in the gut mucosa. a, Stomach (magnification: large panel, ×200; small panel, ×40). b, Small intestine (magnification: large panel, ×200; small panel, ×100). c, Colon (magnification: large panel, ×200; small panel, ×100).

ponent 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, because FK565 is generally most effective among Nod1 ligands and showed stronger effects on HCAEC (Supplemental Figure II). ICAM-1 expression and cytokine production by HCAEC were enhanced by the combined addition of LPS (Figure 1), and priming with LPS upregulated 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 (Supplemental Table IA). Subcutaneous injection of MDP, peptidoglycan from *E. coli* K12, zymosan, or OK432 with LPS priming or LPS priming alone did not induce any cellular infiltration in the arteries (Supplemental Table IA and Figure 2Aa). On the other hand, when mice were subcutaneously injected by FK565 (500 $\mu$g) with LPS priming, diffuse cellular infiltration was observed in the aortic root, including aortic valves and the origin of coronary arteries in all mice (Figure 2Ab). The histopathologic features of this coronary arteritis model were characterized by panarteritis with dense inflammatory infiltrate consisting mainly of neutrophils and macrophages (Supplemental Figure III) and not associated with fibrinoid necrosis, similar to those in the 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 did not show coronary aneurysm, but the rupture of elastic fiber in coronary artery was observed, just as in KD. Formation of neither thrombus nor granuloma was recognized in aortic and coronary lesions of all experimental mice. MDP, by itself, did not induce coronary arteritis at 500 $\mu$g, but the enhancement of the effect of Nod1 agonist by MDP became apparent when MDP was added to suboptimal doses of FK565 (200 and 100 $\mu$g). Vascular inflammation was not observed in pulmonary, celiac, renal, or other arteries, whereas mild cellular infiltration was observed in other parts of aorta and in common carotid and subclavian arteries (data not shown). Subcutaneous injection of iE-DAP or FK156 induced a slight inflammatory reaction (data not shown), whereas that of FK565 induced 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, whereas no arteritis was observed in Nod1-knockout mice (Supplemental Table IB and Figure 2B).

As FK565 is highly stable and effective by parenteral and oral routes,[16] FK565 was orally administered (Supplemental Table IC). All BALB/c mice showed coronary arteritis/valvulitis after oral administration of 6 days (100 $\mu$g/day) per 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 of 5 mice developed severe

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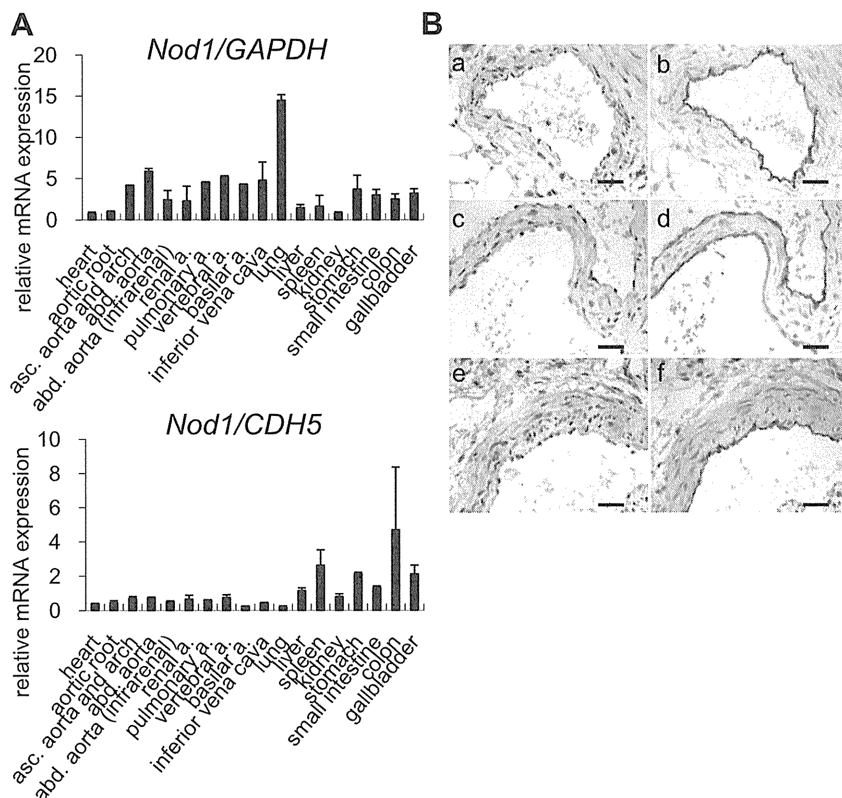


**Figure 3.** Nod1 expression in various tissues and organs of normal mice. A, *Nod1* expression levels in various tissues and organs of normal mice were determined by quantitative real-time reverse transcription–polymerase chain reaction with *GAPDH* (top) and *CDH5* (bottom) as internal controls. a. indicates artery; asc. ascending; abd., abdominal. Similar expression levels of *Nod1* were observed in the vascular system when *CDH5* was used as an internal control. B, Immunohistochemical stainings with Nod1-specific (left) and CD31-specific (right) antibodies in normal mice. a and b, coronary artery; c and d, aortic valve; e and 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 bars=20 $\mu$m.

coronary arteritis/valvulitis after 4 weeks (Figure 2Ac). When mice were given FK565-containing tap water (estimated daily doses of 120 $\mu$g/day [n=3] and 180 $\mu$g/day [n=3]) ad libitum for 4 weeks in the absence of LPS priming, all 6 developed severe (grade 3+) coronary arteritis/valvulitis (Figure 2Ac). On the other hand, oral administration of FK565 induced no inflammation in gut mucosa (Figure 2C) or in many arteries and organs (data not shown).

## Site-Specific Vascular Inflammation In Vivo Is Associated With High Expression of Chemokine/Cytokine and Metallopeptidase Genes in Each Tissue

First, *Nod1* expression levels in normal mice were quantified by real-time reverse transcription–polymerase chain reaction 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 (Figure 3A). Consistent with 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 immunohistochemical staining (Figure 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), uninflamed tissue (pulmonary artery), and much less inflamed tissue (ascending to abdominal aorta) from the vascular system and in 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 0 were continuously elevated more than 5-fold in microarray data from FK565-stimulated pulmonary artery, aorta and spleen, respectively (Supplemental 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, 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 (Supplemental Figure IV, Supplemental 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) and IL-6, of which genes were highly expressed in in vivo FK565-stimulated aortic root (Supplemental Table II), was studied with ex vivo organ culture of aortic root, pulmonary artery, aortic arch, and abdominal

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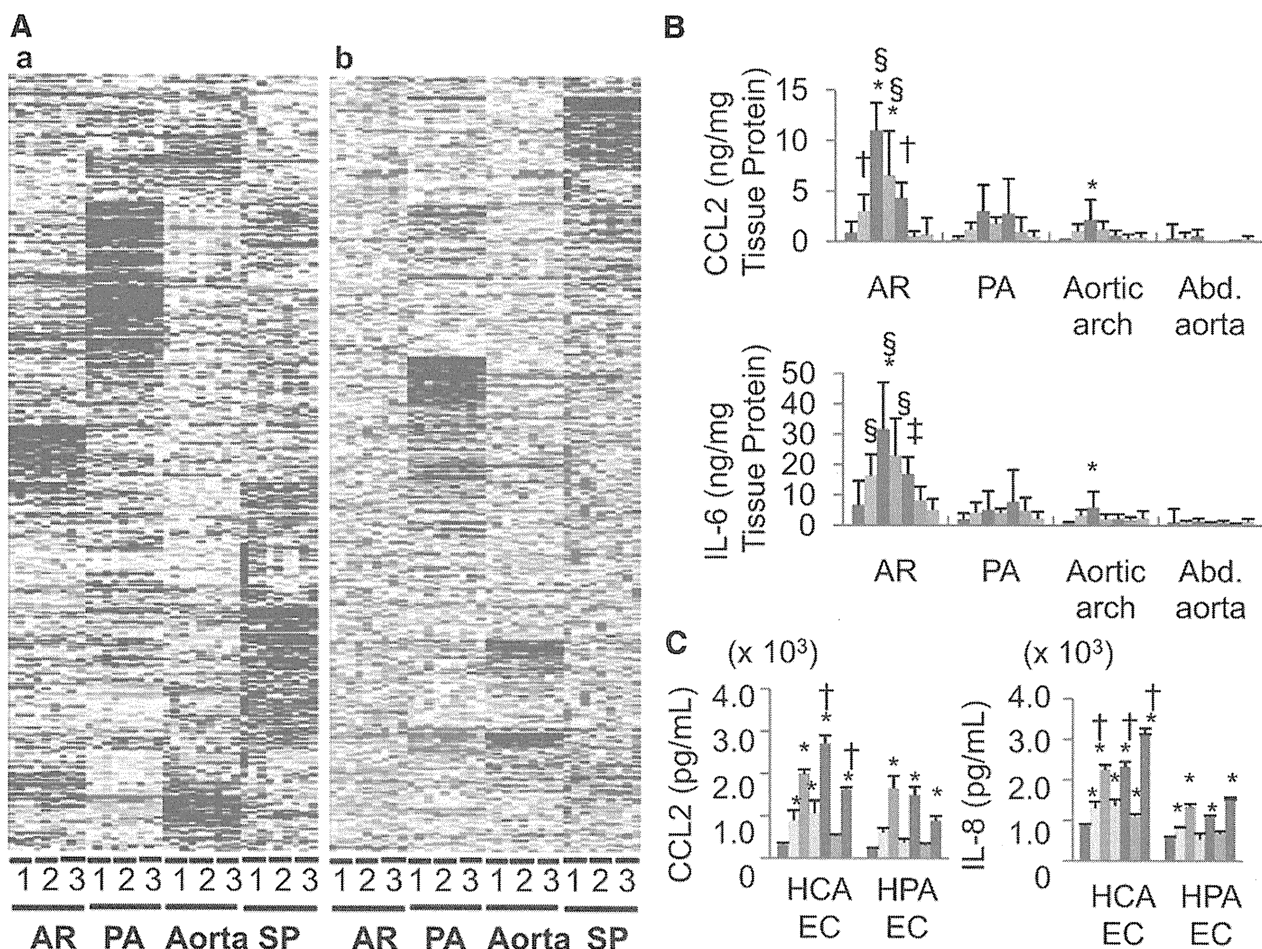


**Figure 4.** The in vivo gene expression patterns of vascular and immune tissues of mice treated with LPS, FK565, or LPS plus FK565 (A) and the production of chemokine/cytokine by vascular tissues ex vivo (B) and endothelial cells in vitro (C) treated with innate immune ligands. A, The gene expression 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 are shown. a, 44 170 genes; b, 13 546 genes of which expression levels were more than 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 (less than half) to high (more than 16-fold) compared with those with no stimulation (yellow indicates no change after stimulation). B, Supernatants collected from each tissue after 24 hours of culture with each reagent were assayed for CCL2 and IL-6 (n=6). The reagents were as follows: darker blue, none; green, 10 μg/mL iE-DAP; purple, 10 μg/mL FK565; medium blue, 10 μg/mL MDP; red, 100 ng/mL lipid A; yellow, none in NOD1$^{-/-}$ mice; lighter blue, 10 μg/mL FK565 in Nod1$^{-/-}$ mice. *$P<0.01$ vs none (Dunnett 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 honestly significant differences 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 or 0.57±0.25 ng/mg tissue protein in aortic root), proving that the arteries were properly prepared and viable. C, HCAEC and human pulmonary artery endothelial cells (HPA EC) ($4\times10^4$ 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 HPA EC stimulated with Nod1 or Nod2 ligand. The concentrations of stimulants are as follows: darker blue, none; very light blue, 1 μg/mL iE-DAP; green, 10 μg/mL iE-DAP; lighter purple, 1 μg/mL FK565; darker purple, 10 μg/mL FK565; medium blue, 10 μg/mL MDP; red, 100 ng/mL lipid A. *$P<0.01$ vs none (Dunnett test), †$P<0.01$ vs HPA EC (Student *t* test).

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 stimulation with FK565 in normal mice, whereas no production of CCL2/IL-6 in response to FK565 was observed in any vascular tissue from Nod1$^{-/-}$ mice (Figure 4B). In addition, the production of CCL2 and IL-8 by FK565 was also higher in HCAEC than in human pulmonary artery endothelial cells (Figure 4C), suggesting that site-specific vascular inflammation is ascribed to the intrinsic nature of vascular cells, including endothelial cells.[18]

## Discussion

The present study has demonstrated that pure selective Nod1 ligands (iE-DAP, a dipeptide with a molecular mass of 319.3 Da; FK565, an acyltripeptide with a molecular mass of 502.6 Da; and FK156, a synthetic tetrapeptide originally isolated from culture filtrates of *Streptomyces* strains, with a molecular mass of 519.5 Da) and a Nod2 ligand (MDP) induced

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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 the 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 in acute-phase KD, which showed edema and a dominant infiltration of neutrophils with some macrophages and lymphocytes at early stages (until 9 days after KD onset).[20] No animal models of coronary arteritis have been reported with a pure or synthetic reagent.

On the basis of 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 libitum drinking of FK565-containing tap water.

Nod1 stimulants include *meso*-DAP and *meso*-L-anthionine, amino acids specific to bacterial peptidoglycans, iE-DAP, L-Ala-γ-D-Glu-*meso*-DAP (TriDAP), FK156, FK565, GlcNAc-(β1 to 4)-(anhydro)MurNAc-L-Ala-γ-D-Glu-meso-DAP, bacterial extracts (*Bacillus* species, *Bacillus anthracis* spores, *Legionella pneumophila, Salmonella 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 (*E. coli* breaks down nearly 50% of its peptidoglycan every generation).[27] As peptidoglycan is constantly turned more than[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, because 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 receptor interacting protein-2, mitogen-activated protein kinases, and nuclear factor-κB, and their inhibitors or activators.[1,3,4] Among them, A20 (tumor necrosis factor-α-induced protein 3) is a candidate because it is a negative regulator of TLR and NLR signaling via nuclear factor-κB[31] and is 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 or 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 noninflammatory tissues and the use of knockout or transgenic mice.

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

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## Disclosures

None.

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