所期において、まず上記1から検討することにした。

急性期川崎病患者および健康な対象群から末梢血単核球細胞を分離し、mRNA および miR の microarray を施行した。川崎病患者はすべて免疫グロブリン大量療法（IVIG）に反応した患者とし、治療前、治療開始後 2-4 日および 7-10 日に全血 4ml を採取した。データは GeneSpring GX 11.0 software および Ingenuity Pathways Analysis tools を用いて解析した。

本研究は、すでに富山大学倫理委員会の承認を得ている（受付番号 122）。対象は、文書による同意が得られた患者とする。血液試料は、富山大大学医学部小児科で、試料提供者の氏名が分からないように番号化した上で保存する。また、研究成果を学会・論文等で報告する場合は、試料提供者の氏名が分かるような形での公表は避ける。

C. 研究結果

本研究においては、川崎病急性期にわずかな miR が重要な役割を果たしていることを示唆した。現在知られている 847 種の miRs のうち、わずか 1.06%（9 種）の miRs が、IVIG 治療前に有意に発現亢進あるいは抑制され、特に川崎病に特異性が高いと思われる miR は miR-93、miR-877、miR-92b、miR-182、miR-296-5p の 5 種（0.59%）と考えられた。これらは IVIG 治療後、10 日以内にすべてが正常化した。

これらの miR とネットワークを形成すると報告されている mRNA のうち、12 種は有意に発現亢進あるいは低下し、これらも治療後 10 日以内に正常化した。

D. 考察

冠動脈病変に関連した 10 種の mRNA（*SGCZ, EIF4G3A, PBX3, ANO4, FAM129B, LIMCH1, MAPKBP1, AUTS2, DEPDC1, HIVEP3*）を先に述べた。これまでの報告を元にした IPA の解析によると、*SGCZ (sarcoglycan zeta)* は miR-93 によって、*EIF4G3A (eukaryotic translation initiation factor 4 ganma3A)* は miR-150 によって制御 されることが分かっており、川崎病においてもこれらの miR は有意に変化しており、それぞれの mRNA と miR の発現の変化は強い負の相関関係を示した。

今回明らかになった miR（今回検索した miR の約 0.6%）が川崎病の病勢に相関し、その発現とそれらのコントロールする mRNA のネットワークを変化させ、治療に反応してわずかな間に正常化していくことは、非常に興味深い。すなわちこれは発現に有意差のあった 5 個の miR は関連する mRNA を制御し、急性期症状の発現に関与し、かつ治療への反応性も反映している可能性が考えられた。

特に、IPA は miR-93 と我々がこれまで報告してきた VEGF の中のサブクラスである *VEGFA* の関係を予測した。miR-93 は *VEGFA* の発現を調節し、治療に伴い miR が発現亢進するとターゲットとなる mRNA の発現は低下する強い負の相関を示した。この事実は miR-93 が川崎病冠動脈病変における重要なシグナル伝達系を担っている可能性を示唆した。

E. 結論

川崎病急性期における流血中の人末梢血単核球細胞における miR の変化の同定を試みたところ、川崎病に特異性が高いと考えられる 5 種の miR（miR-93、miR-877、miR-92b、miR-182、 miR-296-5p）が同定された。中でも miR-93 は *VEGFA* の発現を調節し、川崎病冠動脈病変における重要なシグナル伝達系を担っている可能性が考えられた。つまり、今回の研究において、川崎病急性期の単球において、miRNA の発現の亢進および低下を調べることで、川崎病の急性期で、他の急性炎症とは異なる特異な炎症反応の系が亢進しているかを明らかにすることができた。このことにより、川崎病の特異な病態を明らかにすることができ、今後の治療ならびに診断の一助に成り得ると考えられる。

F. 研究発表

1. 論文発表

1) Bowles NE, Arrington CB, Hirono K, Nakamura T, Ngo L, Wee YS, Ichida F, Weis JH. Kawasaki disease patients homozygous for the rs12252-C variant of interferon-induced transmembrane protein-3 are significantly more likely to develop coronary artery lesions. Mol Genet Genomic Med. 2014; 2(4): 356-61.

2) Hirono K, Ichida F. Possible new role of vascular endothelial growth factor-D during the acute phase of Kawasaki disease. Circ J. 2011; 75(6): 1324-5.

2. 学会発表

1) Hideyuki Nakaoka, Keiichi Hirono, Keijiro Ibuki, Sayaka Ozawa, Fukiko Ichida. Increased circulating endothelial microparticles in the acute phase of Kawasaki Disease. 11th International Kawasaki Disease Symposium. 2015.2.3 Hawaii, USA

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

# 多施設共同による川崎病の発症および重症化の遺伝的背景に関する研究

尾内善広

千葉大学大学院医学研究院公衆衛生学

研究要旨

近年，川崎病の病態への関与が示唆されている $Ca^{2+}$/NAFT 経路上の分子，ORAI1 について，川崎病罹患感受性遺伝子の候補としての検討を行った．94 人のゲノム DNA について遺伝子全長を含む 17.4kb の領域を直接シーケンス法で解析，見出されたアレル頻度 5%以上の多型部位 37 ヶ所から 9 つの一塩基多型(SNP)をタグ SNP として選出，関連解析（患者 729 人，対照 1315 人）によるスクリーニングを行った．関連の傾向を示したものについては別の患者－対照セット（患者 1813 人，対照 1097 人）で検証，メタ解析を行った．その結果，エクソン 2 内の非同義 SNP（rs3741596, c.652 A>G, S218G）において統計学的に有意な関連が示された（オッズ比 1.21，P=0.00041）．さらに多型検索において見出された頻度の低い 2 アミノ酸挿入多型（c. 126-127 ins CCACCG）にも有意な関連を観察した（オッズ比 3.91，P=0.010）．rs3741596 のアレル頻度には人種間の差が大きく（アジア人 8.9%~22%，欧米人 0.6%未満），川崎病の罹患率の人種差との関係も示唆される知見であると考える．

## A. 研究目的

川崎病は乳幼児を主に冒す原因不明の全身性血管炎症候群である．冠状動脈の瘤や拡張という合併症(CAL)が無治療で経過した患者の 20%前後に生じ，瘤の破裂や閉塞により死に至ることもある．経静脈免疫グロブリン大量療法(IVIG)と経口アスピリン内服の併用の標準治療により CAL の合併頻度は 5%未満へと大幅に減少したが，IVIG 治療に抵抗性であるケースが 10-15%あり CAL のハイリスク群となる．

川崎病は先進国における小児の後天性心疾患の最大の原因であり，高額かつ未知なる病原体の感染リスクが完全に払拭されていない血液製剤を幼小児に対し大量投与する現行の治療法に対する懸念は根強い．IVIG より安価，安全かつ有効な治療法の開発が望まれているが，その上で重要となる病因の解明は十分ではない．そこで本分担研究では川崎病の病因の全貌解明に資するため，近年川崎病の病態への関与が強く示唆されている $Ca^{2+}$/NFAT 経路上の分子，ORAI1 についてその遺伝子周辺の遺伝子多型と川崎病罹患感受性との関連を検討した．

## B. 研究方法

試料収集：2544 人の川崎病患者 DNA を日本国内の共同研究機関より収集した．大阪御堂筋ロータリークラブ（940 人），ヒューマンサイエンス研究資源バンク（950 人），慶応義塾大学（374 人），日本医科大学（168 人）より健常対照あるいは対照として使用可能な他疾患患者 DNA を得た．試料収集を行った全医療機関において，研究倫理委員会における承認が得られており，全ての試料提供者からは書面による同意意思表明を得た．

多型の検索とタイピング：94 人の川崎病患者 DNA を用い，ORAI1 遺伝子全長を含む 17.4kb の領域(NC_000012.11: 122,062,619 ~ 122,076,990)の多型検索を PCR-直接シークエンス法により実施した．アレル頻度が 5%以上の多型について Haploview 4.2 ソフトウェアによる連鎖不平衡解析を実施，連鎖不平衡係数 $r^2$ 値>0.8 を指標に SNP 群を代表する SNP（タグ SNP）を決定した．SNP のタイピングはインベーダー法により，挿入・欠失多型のタイピングは直接シークエンス法で行った．

統計解析：頻度5%以上のタグSNPについてはピアソンの $\chi^2$ 乗分析にて，頻度が5%未満の多型についてはフィッシャーの正確確率検定により患者群・対照群間での頻度の違いを統計学的に検定した．$\chi^2$ 乗検定はMicrosoft Excelにて，フィッシャーの正確確率検定は統計環境R 2.15.2を用いて行った．

C. 研究結果

94人のシークエンス解析の結果，69ヶ所の多型部位が見出された．そのうちマイナーアレル頻度が5%以上あるものが37あり，それらについて連鎖不平衡解析を行ったところ，互いに強く連鎖した多型からなる9つのグループに分類することが出来た．そこで各グループを代表するタグSNPを1つずつ選択し，患者－対照セット1（患者729人，対照1315人）で関連を検討したところ，9つのうち1つ(rs3741596)のみにP<0.05となる関連の傾向が観察された(表1)．次に患者－対照セット2（患者1813人，対照1097人）で，この関連の傾向を追試したところ，セット1と同様の関連が観察された．Mantel Haenszel法によるメタ解析で，関連は有意性をもって確かめられた（表2）．続いて，頻度が5%未満であった多型部位のうち，ORAI1タンパクのアミノ酸配列に変化をもたらすものについてのみ川崎病との関連を検討したところ，NCBIのデータベースにない新規挿入・欠失多型の挿入型が川崎病群に有意に多く観察された（表3）．

D. 考察

ORAI1はT細胞，B細胞，樹状細胞等で知られるストア作動性カルシウム流入のメカニズムにおいて細胞外からのカルシウムイオンの流入を司るチャネル分子である．ORAI1および小胞体膜上に発現し小胞体内の貯蔵カルシウムの減少を感知するセンサー分子STIM1との相互作用がORAI1のチャネルとしての活性化に必須であり，ORAI1，STIM1ともに常染色体劣性の原発性免疫不全症候群の原因遺伝子として知られている．*ORAI1*遺伝子が存在する12番染色体長腕(12q24)の領域には尾内らが2007年に報告した川崎病の罹患同胞対解析で連鎖の傾向が観察されており，位置的候補遺伝子として着目された．今回川崎病との関連が認められた2つの多型はいずれもORAI1のアミノ酸配列に変化をもたらすが，それぞれが機能的にどのような意義を有するか現時点では不明である．rs3741596は多くの連鎖不平衡にあるSNPの代表として選抜されたが，遺伝子外あるいは非コード領域の他のSNPが機能的意義を有する可能性も残存する．しかし，挿入欠失多型については川崎病と関連する挿入型がrs3741596の低リスクアレルと連鎖しており，かつ他に強く連鎖する多型は認められていないことからrs3741596が代表するSNP群からは独立した機能的意義を有するリスク因子であると考えられる．またrs3741596が代表するSNP群が分布する97.7kbの領域内には*ORAI1*以外には*MORN3*という精子形成への関与以外には機能に関する知見に乏しい遺伝子が存在するのみである．以上を総合的に考察すると今回候補遺伝子とした*ORAI1*以外の近隣の遺伝子が真の罹患感受性遺伝子である可能性は高くないものと思われる．*ITPKC*, *CASP3*に続き，$Ca^{2+}$/NFAT経路上の遺伝子に罹患感受性多型が見出されたことにより，同経路の川崎病の病態における重要性がさらに強く支持する結果であると考える．

rs3741596が代表するSNP群は全てアジア人集団ではアレル頻度が8.9~22%程度あるのに対し、欧米人集団では1%未満と低いため、川崎病の罹患率の人種差との関連も推察される。またこれらは過去に実施されたゲノムワイド関連解析において用いられたDNAチップに非搭載であった。今回の結果は、ありふれた遺伝子多型やこれまで検討されていない稀な遺伝子多型の中にも罹患感受性遺伝子多型がまだ多数存在する可能性を示しており，さらなる検索の継続は不可欠であると思われる．

E. 結論

先に実施した罹患同胞対解析において連鎖がみられた 12 番染色体長腕の位置的候補遺伝子解析により，*ORAI1* 遺伝子内の多型と川崎病との関連を見出した．

F. 研究発表

1. 論文発表

Onouchi Y., Fukazawa R., Yamamura K., Suzuki H., Suenaga T., Takeuchi T., Yoshikawa N., Hamada H., Honda T., Yasukawa K., Terai M., Ebata R., Higashi K., Saji T., Kemmotsu Y., Takatsuki S., Ouchi K., Kishi F., Yoshikawa T., Nagai T., Hamamoto K., Sato Y., Honda A., Kobayashi H., Sato J., Shibuta S., Miyawaki M., Oishi K., Yamaga H., Aoyagi N., Iwahashi S., Miyashita R., Murata Y., Fujino A., Ozaki K., Kawasaki T., Abe J., Seki M., Kobayashi T., Arakawa K., Ogawa S., Hara T., Hata A. and Tanaka T. Variants in *ORAI1* gene associated with Kawasaki Disease. manuscript in preparation

2. 学会発表

Onouchi Y., Fukazawa R., Yamamura K., Suzuki H., Suenaga T., Takeuchi T., Yoshikawa N., Hamada H., Honda T., Yasukawa K., Terai M., Ebata R., Higashi K., Saji T., Kemmotsu Y., Takatsuki S., Ouchi K., Kishi F., Yoshikawa T., Nagai T., Hamamoto K., Sato Y., Honda A., Kobayashi H., Sato J., Shibuta S., Miyawaki M., Oishi K., Yamaga H., Aoyagi N., Iwahashi S., Miyashita R., Murata Y., Fujino A., Ozaki K., Kawasaki T., Abe J., Seki M., Kobayashi T., Arakawa K., Ogawa S., Hara T., Hata A. and Tanaka T. "Variants in *ORAI1* gene associated with Kawasaki Disease." The 11th International Kawasaki Disease Symposium. 2015.2.3, Honolulu.

H. 知的財産権の出願・登録状況

なし

**表 1. *ORAI1* 遺伝子近傍のタグSNPと川崎病との関連**

| dbSNP ID | Chromosomal location[a] | position on NT_022792.17 | Alleles | | Genotype distribution | | | | | | | | | | Statistic analysis[f] | | | | Position in gene |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | KD | | | | | Control | | | | | | | | | |
| | | | 1 | 2 | 11 | 12 | 22 | Total | MAF[b] | 11 | 12 | 22 | Total | MAF[b] | HWEP[c] | OR[d] | 95% C.I.[e] | | P | |
| rs12313273 | 122063010 | 12639540 | C | T | 43 | 291 | 394 | 728 | 0.26 | 75 | 500 | 698 | 1273 | 0.26 | 0.24 | 1.02 | 0.88 - 1.18 | | 0.801 | 5' falnking (-1465base) |
| rs6486789 | 122067972 | 12644502 | T | C | 160 | 369 | 198 | 727 | 0.47 | 312 | 649 | 315 | 1276 | 0.50 | 0.54 | 1.11 | 0.97 - 1.26 | | 0.129 | intron1 (IVS1+3016) |
| rs117324670 | 122070246 | 12646776 | A | T | 4 | 112 | 613 | 729 | 0.082 | 9 | 197 | 1064 | 1270 | 0.085 | 0.97 | 1.03 | 0.82 - 1.30 | | 0.797 | intron1 (IVS1+5290) |
| rs7484839 | 122070961 | 12647491 | T | C | 0 | 109 | 614 | 723 | 0.075 | 0 | 182 | 1076 | 1258 | 0.072 | 0.0057 | 1.05 | 0.82 - 1.34 | | 0.724 | intron1 (IVS1+6005) |
| rs7486943 | 122071163 | 12647693 | T | C | 520 | 193 | 14 | 727 | 0.15 | 919 | 325 | 31 | 1275 | 0.15 | 0.73 | 1.00 | 0.84 - 1.20 | | 0.984 | intron1 (IVS1+6207) |
| rs3741595 | 122079189 | 12655719 | T | C | 32 | 266 | 432 | 730 | 0.23 | 66 | 449 | 794 | 1309 | 0.22 | 0.81 | 1.02 | 0.88 - 1.19 | | 0.763 | exon2 (c. 546 C->T; I182I) |
| rs3741596 | 122079295 | 12655825 | A | G | 444 | 252 | 33 | 729 | 0.22 | 867 | 398 | 50 | 1315 | 0.19 | 0.62 | 1.19 | 1.02 - 1.40 | | 0.028 | exon2 (c. 652 A->G; S218G) |
| rs3825175 | 122079441 | 12655971 | T | C | 101 | 334 | 290 | 725 | 0.37 | 195 | 632 | 491 | 1318 | 0.39 | 0.72 | 1.08 | 0.95 - 1.23 | | 0.256 | exon2 (c. 798 T->C; T266T) |
| rs712853 | 122079668 | 12656198 | A | G | 255 | 363 | 108 | 726 | 0.40 | 469 | 606 | 194 | 1269 | 0.39 | 0.94 | 1.03 | 0.90 - 1.18 | | 0.658 | 3' UTR[g] |

a: Chromosomal locations were based on GRCh37 Patch Release 13 (GRCh37. p13).
b: Minor allele frequency.
c: *P* values for Hardy-Weinberg equilibrium.
d: Odds ratio.
e: Confidence interval.
f: Associations in allelic model were evaluated by using chi-square test.
g: Untranslated region.



**表 2. *ORAI1*遺伝子の一塩基多型（rs3741596）と川崎病との関連の検証**

| | Replication panel[a] | | | | | | | | Combined[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA | AG | GG | Total | MAF[c] | OR[d] | 95% C.I.[e] | P | OR | 95% C.I. | | P |
| KD | 1172 | 577 | 64 | 1813 | 0.19 | 1.22 | 1.06 - 1.4 | 0.0056 | 1.21 | 1.09 | - 1.34 | 0.00041 |
| Control | 760 | 311 | 26 | 1097 | 0.17 | | | | | | | |

a: Association in allelic model was evaluated by using chi-suqre test.
b: A meta-analysis was conducted with Mantel-Haenszel method.
c: Minor allele frequency.
d: Odds ratio.
e: Confidence interval.

**表3. *ORAI1*遺伝子の6塩基挿入多型（c. 126-127 ins CCACCG）と川崎病との関連**

| | wt/wt | wt/ins6 | ins6/isn6 | Total | MAF[a] | OR[b] | 95% C.I. | *P* |
|---|---|---|---|---|---|---|---|---|
| KD | 2304 | 15 | 0 | 2319 | 0.0032 | 3.91 | 1.30 - 11.80 | 0.010 |
| Control | 2410 | 4 | 0 | 2414 | 0.00083 | | | |

Association in allelic model was evaluated by using Fisher's exact test.
a: Minor allele frequency.
b: Odds ratio.
c: Confidence interval.

# Ⅲ. 学会等発表実績

学 会 等 発 表 実 績

委託業務題目「川崎病の病因・病態解明に基づく新規治療・予防法の開発」
機関名　九州大学

１．学会等における口頭・ポスター発表

| 発表した成果（発表題目、口頭・ポスター発表の別） | 発表者氏名 | 発表した場所（学会等名） | 発表した時期 | 国内・外の別 |
|---|---|---|---|---|
| Association between primary immunodeficiency diseases and vasculitis syndrome. | Hara T, Ishimura M, Takada H, Kusuda Y, Nakashima Y, Murata K, Kanno S, Nishio H | 16th Biennial Meeting of the European Society for Immunodeficiencies | 2014 | 国外 |
| Microbe-associated molecular patterns (MAMPs) in the biofilms: A possible cause of Kawasaki disease | Kusuda T, Nakashima Y, Murata K, Hara T | The 34th Annual Meeting of the Japanese Society of Kawasaki Disease | 2014 | 国内 |
| 川崎病患者検体における自然免疫受容体NOD1活性物質の検出とその構造解析 | 楠田 剛, 中島康貴, 西尾壽乗, 原 寿郎 | 第18回川崎病治療懇話会 | 2014 | 国内 |
| Analysis of the mechanisms of intravenous immunoglobulin-resistant Kawasaki disease using iPS cell technology. | Ikeda K, Ameku T, Nomiya Y, Nakamura M, Mae S, Matsui S, Yahata T, Okamoto-Hamaoka A, Suzuki C, Yoshioka A, Kuchitsu Y, Watanabe A, Osafune K, Hamaoka K | Eleventh International Kawasaki Disease Symposium | 2015 | 国外 |
| Analysis of the mechanisms of intravenous immunoglobulin-resistant Kawasaki disease using iPS cell technology. | Ikeda K, Ameku T, Nomiya Y, Nakamura M, Matsui S, Yahata T, Okamoto-Hamaoka A, Suzuki C, Kuchitsu Y, Watanabe A, Osafune K, Hamaoka K | American Heart Association 2014 | 2014 | 国外 |
| iPS細胞由来血管内皮細胞を用いたガンマグロブリン不応川崎病の病態解明 | 池田和幸、天久朝廷、松井敏、八幡倫代、岡本亜希子、鈴木千夏、朽津有紀、渡辺亮、長船健二、濱岡建城 | 第34回日本川崎病学会学術集会 | 2014 | 国内 |
| iPS細胞技術を用いたガンマグロブリン不応川崎病の病態解明 | 池田和幸、天久朝廷、松井敏、八幡倫代、岡本亜希子、鈴木千夏、長船健二、濱岡建城 | 第117回日本小児科学会学術集会 | 2014 | 国内 |
| Midkine, a new functional cytokine, increased after IVIG may protects from vascular injury in acute Kawasaki disease. | Saji T, Takatsuki S, Kenmotsu Y, Naoi K, Ikehara S, Nakayama T, Matsuura H, Kusunoki N, Kawai S | 11th International Kawasaki Disease Symposium | 2015 | 国外 |
| 急性期川崎病におけるミッドカインによる治療反応性の予見 | 高月晋一、楠夏子、直井和之、池原聡、中山智孝、松裏裕行、川合眞一、佐地勉 | 第34回日本川崎病学会 | 2014 | 国内 |

| | | | | |
|---|---|---|---|---|
| Human oral, gut, and blood microbiota in patients with Kawasaki disease. | Abe J, Ebata R, Saito N, Okunushi K, Nakabayashi K, Kuroda M | International Kawasaki Disease Symposium 2015 | 2015 | 国外 |
| 川崎病患者の末梢血、咽頭・直腸スワブのメタゲノム解析 | 阿部淳、中林一彦、江畑亮太、黒田誠 | 第34回日本川崎病学会 | 2014 | 国内 |
| Anti TNF-$\alpha$ drug inhibits initial process of vasculitis in animal model of Kawasaki disease. | Oharaseki T, Yokouchi Y, Katsuzaki J, Yamada H, Ihara F, Miura N, Ohno N, Saji T, Suzuki K, Naoe S, Takahashi K | PAS/ASPR | 2014 | 国外 |
| 川崎病類似マウス血管炎モデルとサイトカイン | 大原関利章、横内幸、榎本泰典、山田仁美、三浦典子、大野尚仁、直江史郎、鈴木和男、髙橋啓 | 第20回MPO研究会 | 2014 | 国内 |
| エタネルセプトによるCAWS誘導マウス動脈炎抑制機序の病理組織学的検討 | 大原関利章、横内幸、榎本泰典、勝碕譲児、山田仁美、伊原文恵、三浦典子、大野尚仁、佐地勉、鈴木和男、直江史郎、髙橋啓 | 第19回血管病理研究会 | 2014 | 国内 |
| Lessons from epidemiologic studies of Kawasaki disease in Japan. | Nakamura Y | The 11th International Symposium of Kawasaki disease | 2015 | 国外 |
| 疫学像からみた川崎病病因論へのアプローチ | 中村好一 | 第34回日本川崎病学会学術集会 | 2014 | 国内 |
| 川崎病急性期治療Pros and Cons | 小林　徹 | 第117回日本小児科学会学術集会教育セミナー | 2014 | 国内 |
| Increased circulating endothelial microparticles in the acute phase of Kawasaki Disease. | Nakaoka H, Hirono K, Ibuki K, Ozawa S, Ichida F | 11th International Kawasaki Disease Symposium | 2015 | 国外 |
| Variants in ORAI1 gene associated with Kawasaki Disease. | Onouchi Y, Fukazawa R, Yamamura K, Suzuki H, Suenaga T, Takeuchi T, Yoshikawa N, Hamada H, Honda T, Yasukawa K, Terai M, Ebata R, Higashi K, Saji T, Kemmotsu Y, Takatsuki S, Ouchi K, Kishi F, Yoshikawa T, Nagai T, Hamamoto K, Sato Y, Honda A, Kobayashi H, Sato J, Shibuta S, Miyawaki M, Oishi K, Yamaga H, Aoyagi N, Iwahashi S, Miyashita R, Murata Y, Fujino A, Ozaki K, Kawasaki T, Abe J, Seki M, Kobayashi T, Arakawa K, Ogawa S, Hara T, Hata A, Tanaka T | The 11th International Kawasaki Disease Symposium | 2015 | 国外 |

２．学会誌・雑誌等における論文掲載

| 掲載した論文（発表題目） | 発表者氏名 | 発表した場所（学会誌・雑誌等名） | 発表した時期 | 国内・外の別 |
|---|---|---|---|---|
| Kawasaki disease-specific molecules in the sera are linked to microbe-associated molecular patterns in the biofilms. | Kusuda T, Nakashima Y, Murata K, Kanno S, Nishio H, Saito M, Tanaka T, Yamamura K, Sakai Y, Takada H, Miyamoto T, Mizuno Y, Ouchi K, Waki K, Hara T | PLoS One | 2014 | 国外 |
| Evaluation of echogenicity of the heart in Kawasaki disease. | Nagata H, Yamamura K, Uike K, Nakashima Y, Hirata Y, Morihana E, Mizuno Y, Ishikawa S, Hara T | Eur J Pediatr | 2014 | 国外 |
| 3-O-sulfo-$\beta$-d-galactose moiety of endogenous sulfoglycolipids is a potential ligand for immunoglobulin-like receptor LMIR5. | Phongsisay V, Iizasa E, Hara H, Yamasaki S | Mol Immunol | 2015 | 国外 |
| Clec12a: quieting the dead. | Yamasaki S | Immunity | 2014 | 国外 |
| Glycerol monomycolate is a novel ligand for the human, but not mouse macrophage inducible C-type lectin, Mincle. | Hattori Y, Morita D, Fujiwara N, Mori D, Nakamura T, Harashima H, Yamasaki S, Sugita M | J Biol Chem | 2014 | 国外 |
| LMIR5 extracellular domain activates myeloid cells through toll-like receptor 4. | Phongsisay V, Iizasa E, Hara H, Yamasaki | Mol Immunol | 2014 | 国外 |
| Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. | Yonekawa A, Saijo S, Hoshino Y, Miyake Y, Ishikawa E, Suzukawa M, Inoue H, Tanaka M, Yoneyama M, Oh-Hora M, Akashi K, Yamasaki S. | Immunity | 2014 | 国外 |
| Involvement of mannose-binding lectin in the pathogenesis of Kawasaki disease-like murine vasculitis. | Nakamura A, Okigaki M, Miura N, Suzuki C, Ohno N, Kametani F, Hamaoka K. | Clin Immunol | 2014 | 国外 |
| The involvement of the vasa vasorum in the development of vasculitis in animal model of Kawasaki disease. | Hamaoka-Okamoto A, Suzuki C, Yahata T, Ikeda K, Nagi-Miura N, Ohno N, Arai Y, Tanaka H, Takamatsu T, Hamaoka K | Pediatr Rheumatol Online J | 2014 | 国外 |
| A survey of the 3-decade outcome for patients with giant aneurysms caused by Kawasaki disease. | Tsuda E, Hamaoka K, Suzuki H, Sakazaki H, Murakami Y, Nakagawa M, Takasugi H, Yoshibayashi M | Am Heart J | 2014 | 国外 |

| | | | | |
|---|---|---|---|---|
| Platelet Activation Dynamics Evaluated Using Platelet-Derived Microparticles in Kawasaki Disease. | Yahata T, Suzuki C, Yoshioka A, Hamaoka A, Ikeda K, Hamaoka K | Circ J | 2014 | 国外 |
| Increased TLR2 and TLR4 expression in peripheral neutrophils isolated from Kawasaki disease. | Mitsui K, Yusa T, Miyazaki S, Ohara A, Saji T: | Pediatr Allergy Immunol Pulmonol | 2014 | 国外 |
| Update on etio and immunopathogenesis of Kawasaki disease. | Takahashi K, Oharaseki T, Yokouchi Y | Curr Opin Rheumatol | 2014 | 国外 |
| The role of TNF-$\alpha$ in a murine model of Kawasaki disease arteritis induced with a Candida albicans cell wall polysaccharide. | Oharaseki T, Yokouchi Y, Yamada H, Mamada H, Muto S, Sadamoto K, Miura N, Ohno N, Saji T, Naoe S, Takahashi K | Mod Rheumatol | 2014 | 国外 |
| Descriptive epidemiology of Kawasaki disease in Japan, 2011-2012: from the results of the nationwide survey. | Makino M, Nakamura Y, Yashiro M, Ae R, Tsuboi S, Aoyama Y, Kojo T, Uehara R, Kotani K, Yanagawa H | J Epidemiol | 2015 | 国外 |
| Tropospheric winds from northeastern China carry the etiologic agent of Kawasaki disease from its source to Japan. | Rodó X, Curcoll R, Robinson M, Ballester J, Burns JC, Cayan DR, Lipkin WI, Williams BL, Couto-Rodriguez M, Nakamura Y, Uehara R, Tanimoto H, Morguí JA | Proc Natl Acad Sci USA | 2014 | 国外 |
| Kawasaki disease patients homozygous for the rs12252-C variant of interferon-induced transmembrane protein-3 are significantly more likely to develop coronary artery lesions. | Bowles NE, Arrington CB, Hirono K, Nakamura T, Ngo L, Wee YS, Ichida F, Weis JH | Mol Genet Genomic Med | 2014 | 国外 |
| Activation of an Innate Immune Receptor, Nod1, Accelerates Atherogenesis in Apoe-/- Mice. | Kanno S, Nishio H, Tanaka T, Motomura Y, Murata K, Ihara K, Onimaru M, Yamasaki S, Kono H, Sueishi K, Hara T | J Immunol | 2014 | 国外 |

# Ⅳ.　研究成果の刊行物・別刷

PLOS ONE

# Kawasaki Disease-Specific Molecules in the Sera Are Linked to Microbe-Associated Molecular Patterns in the Biofilms

**Takeshi Kusuda[1☯], Yasutaka Nakashima[1☯], Kenji Murata[1], Shunsuke Kanno[1], Hisanori Nishio[1], Mitsumasa Saito[2], Tamami Tanaka[1], Kenichiro Yamamura[1], Yasunari Sakai[1], Hidetoshi Takada[1], Tomofumi Miyamoto[3], Yumi Mizuno[4], Kazunobu Ouchi[5], Kenji Waki[6], Toshiro Hara[1]***

**1** Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, **2** Department of Bacteriology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, **3** Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan, **4** Department of Pediatric Infectious Disease, Fukuoka Children's Hospital and Medical Center for Infectious Disease, Fukuoka, Japan, **5** Department of Pediatrics, Kawasaki Medical School Hospital, Okayama, Japan, **6** Department of Pediatrics, Kurashiki Central Hospital, Okayama, Japan

## Abstract

***Background:*** Kawasaki disease (KD) is a systemic vasculitis of unknown etiology. The innate immune system is involved in its pathophysiology at the acute phase. We have recently established a novel murine model of KD coronary arteritis by oral administration of a synthetic microbe-associated molecular pattern (MAMP). On the hypothesis that specific MAMPs exist in KD sera, we have searched them to identify KD-specific molecules and to assess the pathogenesis.

***Methods:*** We performed liquid chromatography-mass spectrometry (LC-MS) analysis of fractionated serum samples from 117 patients with KD and 106 controls. Microbiological and LC-MS evaluation of biofilm samples were also performed.

***Results:*** KD samples elicited proinflammatory cytokine responses from human coronary artery endothelial cells (HCAECs). By LC-MS analysis of KD serum samples collected at 3 different periods, we detected a variety of KD-specific molecules in the lipophilic fractions that showed distinct m/z and MS/MS fragmentation patterns in each cluster. Serum KD-specific molecules showed m/z and MS/MS fragmentation patterns almost identical to those of MAMPs obtained from the biofilms formed *in vitro* (common MAMPs from *Bacillus cereus*, *Yersinia pseudotuberculosis* and *Staphylococcus aureus*) at the 1st study period, and from the biofilms formed *in vivo* (common MAMPs from *Bacillus cereus*, *Bacillus subtilis/Bacillus cereus/Yersinia pseudotuberculosis* and *Staphylococcus aureus*) at the 2nd and 3rd periods. The biofilm extracts from *Bacillus cereus*, *Bacillus subtilis*, *Yersinia pseudotuberculosis* and *Staphylococcus aureus* also induced proinflammatory cytokines by HCAECs. By the experiments with IgG affinity chromatography, some of these serum KD-specific molecules bound to IgG.

***Conclusions:*** We herein conclude that serum KD-specific molecules were mostly derived from biofilms and possessed molecular structures common to MAMPs from *Bacillus cereus, Bacillus subtilis, Yersinia pseudotuberculosis and Staphylococcus aureus*. Discovery of these KD-specific molecules might offer novel insight into the diagnosis and management of KD as well as its pathogenesis.

**Citation:** Kusuda T, Nakashima Y, Murata K, Kanno S, Nishio H, et al. (2014) Kawasaki Disease-Specific Molecules in the Sera Are Linked to Microbe-Associated Molecular Patterns in the Biofilms. PLoS ONE 9(11): e113054. doi:10.1371/journal.pone.0113054

**Editor:** Adam Driks, Loyola University Medical Center, United States of America

**Received** August 4, 2014; **Accepted** October 16, 2014; **Published** November 20, 2014



**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

**Funding:** This work was supported by grants (Grant A for Toshiro Hara [No. 22249043], Grant C for Hisanori Nishio [No. 22591183]) from the Japan Society for the Promotion of Science, the Health and Labour Science Research Grants (for Toshiro Hara) from the Japanese Ministry of Health, Labour and Welfare, and grants (for Takeshi Kusuda and Kenji Murata) from the Japan Therapeutic Study Group for Kawasaki Disease (JSGK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* Email: harat@pediatr.med.kyushu-u.ac.jp

☯ These authors contributed equally to this work.

## Introduction

The etiology of Kawasaki disease (KD) remains unknown, however, KD has long been considered to be caused by an infectious agent, because of its characteristics of the symptoms, age distribution, seasonality, occurrence of community outbreaks and epidemic cycles. On the other hand, no consistently recoverable agents, lack of person-to-person transmission or a common

contagious source, and paucity of case clusters in families, schools or nurseries are supportive of a non-infectious cause for KD [1–3]. Temporal clustering and marked seasonality in KD occurrence in Japan, Hawaii and San Diego also suggest a wind-borne environmental trigger for this disease [4].

KD is also characterized by marked elevations of serum levels of proinflammatory cytokines and chemokines [2] and the activation of the innate immune system [5–7]. We have established a novel murine model of KD coronary arteritis by oral administration of FK565, which functions as a synthetic microbe-associated molecular pattern (MAMP) and a ligand to one of the innate immune receptors, nucleotide-binding oligomerization domain-containing protein (NOD) 1 [8]. In this report, we performed liquid chromatography-mass spectrometry (LC-MS) analysis of KD sera to find out KD-specific molecules and demonstrated that serum KD-specific molecules were closely linked to MAMPs in the biofilms.

## Materials and Methods

### Study subjects

All patients enrolled in this study were admitted to Kyushu University Hospital, Fukuoka Children's Hospital and Medical Center for Infectious Diseases, Kawasaki Medical School Hospital or Kurashiki Central Hospital between June 2010 and March 2014. The study subjects consisted of 117 patients with KD (median age, 21 months; range 3–96 months; male/female, 65/52), 101 controls with other febrile illnesses (DC: median age, 16 months; range 0–121 months; male/female, 61/40), and 5 normal controls (NC: median age, 6 months; range 3–39 months; male/female, 1/4). A diagnosis of KD was made according to the Diagnostic Guidelines of KD [9]. The Ethical Committee of Kyushu University approved the study. Written informed consent was obtained from all guardians. The 1st study subjects (samples were collected mostly between July 2011 and February 2012) consisted of 43 patients with KD, 41 controls with DC (respiratory syncytial virus infection: $n=4$, influenza A virus infection: $n=7$, adenovirus infection: $n=2$, exanthema subitum: $n=5$, varicella: $n=2$, bacteremia: $n=2$, pneumonia: $n=6$, tonsillitis: $n=1$, lymphadenitis: $n=5$, cellulitis: $n=1$, urinary tract infection: $n=1$, gastritis: $n=5$), and 5 NC. The 2nd (mostly between May 2012 and July 2013) and 3rd (mostly between November 2013 and March 2014) study subjects consisted of 41 KD patients and 30 DC controls (respiratory syncytial virus infection: $n=8$, influenza A virus infection: $n=4$, adenovirus infection: $n=2$, exanthema subitum: $n=2$, herpetic stomatitis: $n=1$, pneumonia: $n=5$, bronchitis: $n=2$, upper respiratory infection: $n=1$, tonsillitis: $n=2$, deep neck abscess: $n=1$, acute otitis media: $n=1$, urinary tract infection: $n=1$), and 33 KD patients and 30 DC controls (respiratory syncytial virus infection: $n=8$, influenza A virus infection: $n=1$, adenovirus infection: $n=1$, pneumonia: $n=6$, bronchitis: $n=1$, upper respiratory infection: $n=4$, tonsillitis: $n=2$, lymphadenitis: $n=1$, sinusitis: $n=1$, acute otitis media: $n=1$, urinary tract infection: $n=2$, gastritis: $n=2$), respectively.

### Sample collection

Blood samples were collected at the time of routine examinations before and after high-dose intravenous immunoglobulin (IVIG) therapy, and after resolution of symptoms. The sera were separated by centrifugation and stored at −30°C until the analysis.

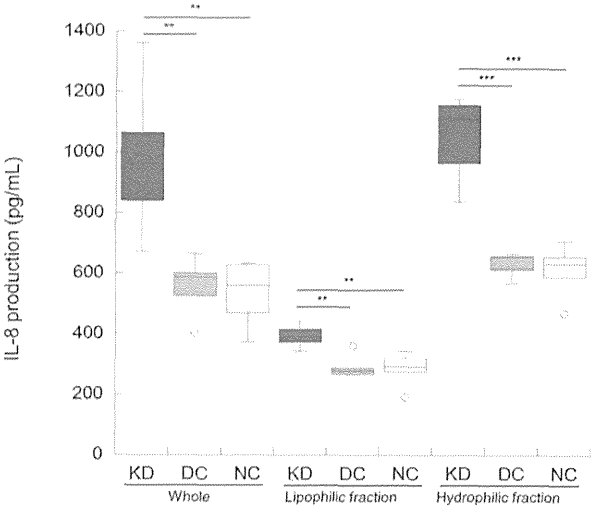


**Figure 1. Whole and fractionated serum samples from KD patients induce cytokine production in HCAECs.** The production of IL-8 by HCAECs was measured in triplicate after 24-hour stimulation with whole sera or lipophilic and hydrophilic fractions from KD patients ($n=6$), DC controls ($n=5$; pneumonia; $n=2$, influenza A virus infection; $n=1$, adenovirus infection; $n=1$ and urinary tract infection; $n=1$), or NC subjects ($n=5$). Lipophilic and hydrophilic fractions were separated by ethyl acetate extraction. The bottom and top edges of the box plot correspond to the 25th and 75th percentiles, respectively. The horizontal line inside the box represents the median of the distribution. The whiskers indicate the 10th and 90th percentiles. $^{**}P<0.01$; $^{***}P<0.001$ (Welch's *t*-test).
doi:10.1371/journal.pone.0113054.g001

Routine bacterial cultures were performed with throat, tongue, nasal and rectal swabs. Biofilms from teeth, tongue, nasal cavity, or rectum (stool) were collected by cotton swabs or interdental brushes (for teeth). These swabs or brushes were suspended in double distilled water ($ddH_2O$) immediately and stored at −30°C until the analysis. Simultaneous collection of biofilm and serum samples was performed at 2nd ($n=12$, mostly October-December, 2012) and 3rd ($n=11$, mostly January-February, 2014) study periods.

### Lipid extraction

Serum samples or other specimens were separated into lipophilic and hydrophilic fractions by Folch method [10] or ethyl acetate extraction [11,12]. As for Folch method [10], 100 μL of serum was acidified to pH5 with acetic acid and mixed with 2:1 chloroform-methanol mixture (v/v) to a final volume 300 μL. The mixture was shaken and centrifuged at 3000 rpm for 10 minutes, and the bottom lipophilic layer and upper hydrophilic layer were collected and evaporated. The lipophilic pellet was dissolved in 5 μL of chloroform, 5 μL of dimethyl sulfoxide (DMSO), and 40 μL of $ddH_2O$ and hydrophilic pellet was in 50 μL of $ddH_2O$. As for ethyl acetate extraction [11,12], 100 μL of serum was mixed with the same volume of ethyl acetate. After centrifugation, the upper lipophilic layer including the interface and the bottom

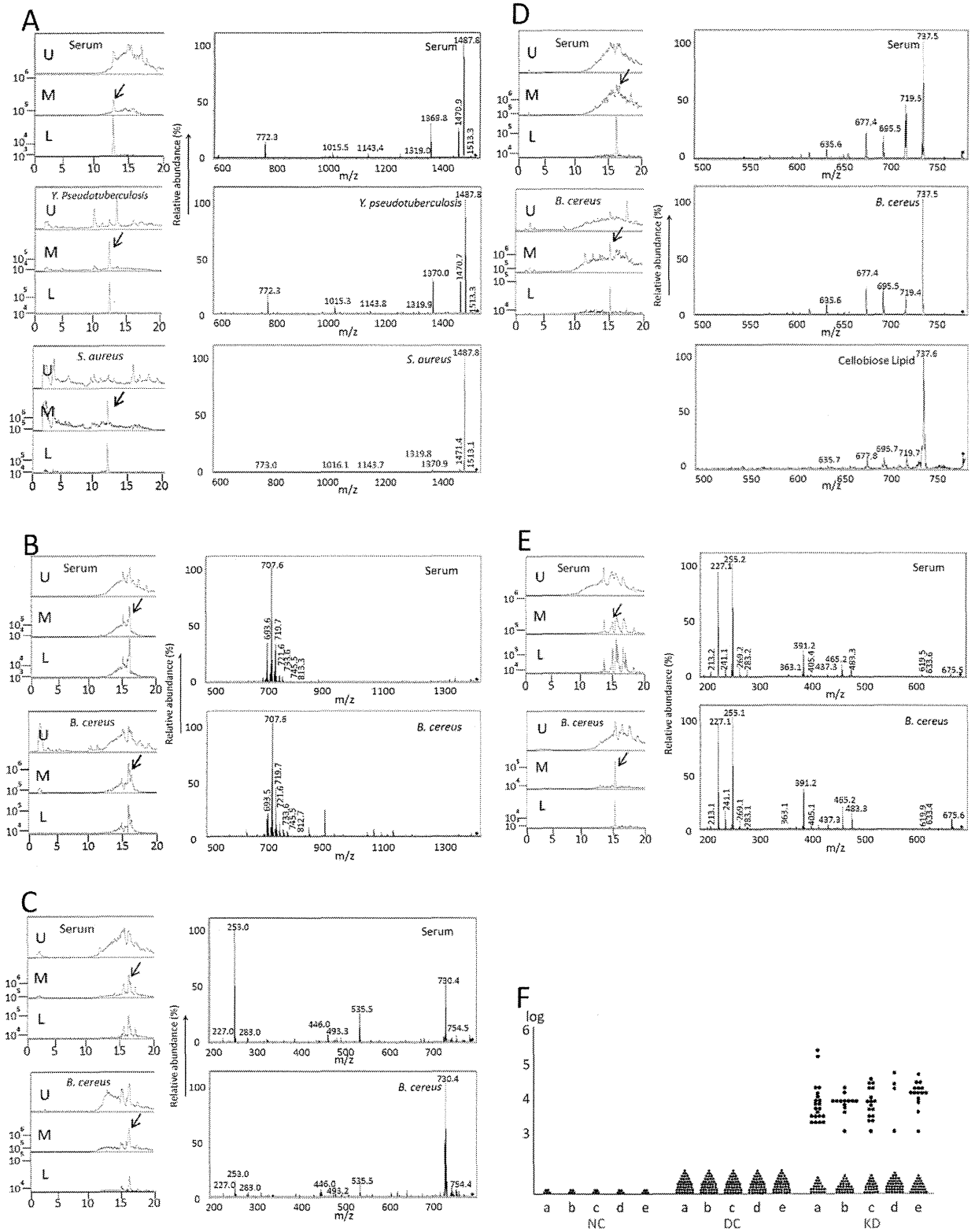
A
Serum
U
M
L
Y. Pseudotuberculosis
S. aureus
Relative abundance (%)
m/z
B
B. cereus
C
D
Cellobiose Lipid
E
F
log
a b c d e
NC
DC
KD

**Figure 2. LC-MS chromatograms and MS/MS fragmentation patterns of serum KD-specific molecules at the 1st study period.** A–E: Each left upper panel: LC-MS chromatograms of KD-specific molecules (A: m/z 1531.8, B: m/z 1414.3, C: m/z 790.9, D: m/z 779.8, and E: m/z 695.0), Each left lower panel: LC-MS chromatograms of biofilm extracts (or initial culture supernatants) from *Y. pseudotuberculosis* and *S. aureus* (A) and *B. cereus* (B–E). U: Total ion current chromatograms, M: Extracted-ion chromatograms at m/z 1500–1600 (A), m/z 1400–1500 (B), m/z 700–800 (C and D), and m/z 600–700 (E), L: Extracted-ion chromatograms at m/z 1531.8 (A), m/z 1414.3 (B), m/z 790.9 (C), m/z 779.8 (D), and m/z 695.0 (E). Arrows indicate peaks of target molecules. Each right upper panel: MS/MS fragmentation patterns of KD-specific molecules (A: m/z 1531.8, B: m/z 1414.3, C: m/z 790.9, D: m/z 779.8, and E: m/z 695.0), Each right lower panel: MS/MS fragmentation patterns of biofilm extracts (or initial culture supernatants) from *Y. pseudotuberculosis* and *S. aureus* (A) and *B. cereus* (B–E). As for the molecule at m/z 779.8, cellobiose lipid shows a MS/MS fragmentation pattern similar to that of KD sera (D, right lowest panel). The intensity is shown by relative abundance. F: The detection rates of each molecule in NC (N = 5), DC (N = 41) or KD (N = 43) sera are shown. Twenty-one (48.8%) of 43 are positive at m/z 1531.8 (a), 13 (30.2%) of 43 at m/z 1414.3 (b), 17 (39.5%) of 43 at m/z 790.9 (c), 4 (9.3%) of 43 at m/z 779.8 (d) and 15 (34.9%) of 43 at m/z 695.0 (e) when the intensity above $1 \times 10^3$ is considered to be significant. The overall detection rate was 76.7% (33 of 43). $P < 0.0001$ (a, b, c and e); $P = 0.0364$ (d) (Fisher's exact test).
doi:10.1371/journal.pone.0113054.g002

hydrophilic layer were transferred, evaporated, and dissolved in 50 μL of 20% methanol (lipophilic layers), and in 100 μL $ddH_2O$ (hydrophilic layers) for cell stimulation, respectively. Since the human coronary artery endothelial cell (HCAEC)-stimulatory activities of KD serum samples were not stable after extraction with Folch method, we used ethyl acetate instead of chloroform. For LC-MS, lipophilic fractions were dissolved in 100% methanol. Other samples were also mixed with the same volumes of ethyl acetate, and centrifuged. Upper lipophilic layers including interfaces were collected, evaporated and dissolved in 100% methanol. To each sample, dibutyl hydroxytoluene was added at a final concentration of 1.0% as an antioxidant [13].

### Cell stimulation

HCAECs (purchased from Lonza and no mycoplasma contamination) were cultured in EBM-2 medium with EGM-2MV (Lonza) in a 5% $CO_2$ incubator at 37°C. These cells, between passages 5 and 7, were suspended and seeded into 75 $cm^2$ flask. After passage, HCAECs were introduced in a 96 well plate ($3 \times 10^3$ cells/well). On the following day, the medium was changed and the supernatants were collected for assay 24 hours after stimulation.

### Cytokine assay

The concentrations of IL-8, IL-6, IL-1β, TNF-α, IL-12p70, and IL-10 in culture supernatants were measured by EC800 cell analyzer (Sony Corporation) with a BD Cytometric Bead Array human inflammation kit (BD Biosciences) [8]. We performed the experiments at least 3 times.

### LC- MS analysis

Samples were analyzed by high performance liquid chromatography (HPLC, Agilent 1200 HPLC instrument, Agilent Technologies) on Dionex Acclaim surfactant column (3 μm, 120Å, 2.1×150 mm, DIONEX) and MS (Esquire 6000 electrospray ionization: ESI, Bruker Daltonics). The mobile phases were $H_2O$ with 0.1% formic acid (eluent A) and acetonitril with 0.1% formic acid (eluent B). They were delivered at a flow rate of 0.2 ml/min and the column was operated at 25°C. The gradient was as follows: 0–3 min. 20% B, 3–12 min. 20–100% B, 12–70 min. 100% B. The injection volume to the system was fixed at 10 μl. The column eluent was connected to MS. The ESI-$MS^n$ spectrum conditions were optimized in the negative-ion mode with the conditions as follows: nebulizer gas, 30.0 psi; drying gas, flow 8 l/min; dry temperature 330°C; high voltage (HV) capillary, 4500 V; HV end plate offset, −500 V; target ion trap, 30000; scan range 100–3000 m/z. The width for targeted precursor ions was set at 4 m/z.

### Biofilm extraction from glass slides

After removing the medium, the culture tube and glass slides were washed once with PBS and vortexed in the presence of ethyl acetate. The ethyl acetate was transferred and evaporated, and the pellet was dissolved in 100% methanol. Details were described in Text S1 in File S1.

### IgG affinity chromatography

Columns used included human polyclonal IgG-conjugated Sepharose 6 Fast (GE Healthcare Life Science), human IgG $F(ab')_2$ fragment-conjugated agarose (ROCKLAND), human IgG Fc fragment full length protein (Abcam)-coupled to cyanogen-bromide (CNBr) Sepharose 4B (GE Healthcare Life Science), mouse monoclonal IgG against a specific antigen (Myc-tag)-conjugated agarose (MBL), rabbit monoclonal IgG against a specific antigen (Phospho-Met (Tyr1234/1235) (D26) XP)-conjugated sepharose (Cell Signaling), and inactivated CNBr Sepharose 4B (GE Healthcare Life Science). Coupling to and inactivation of CNBr Sepharose 4B were performed according to the manufacturer's instructions. Each column was washed once with 10 volumes of PBS with 0.05% Tween20, and twice with 20 volumes of PBS. Biofilms extracts dissolved in PBS with 20% methanol or sera without dilution were applied to a column. After incubation for 30–60 minutes, the mixture was centrifuged and washed twice with PBS. Elution was performed with ethyl acetate. The ethyl acetate elutes were evaporated and the pellets were dissolved in 100% methanol. Inactivated CNBr Sepharose 4B was used as a control column. We performed the experiments at least 3 times.

### Statistics

Data were analyzed by Welch's *t*-test and Fisher's exact test using a statistical software, JMP version 8.0 (SAS Institute), and *P*-values of $<0.05$ were considered to be statistically significant.

## Results

### Activation of HCAECs by KD sera *in vitro*

Since NOD1 ligand directly activates endothelial cells [8] and the expression of endothelial activation antigens was detected in KD skin biopsy specimens [14], HCAECs were employed for the

**Table 1.** The detection rates of serum KD-specific MAMPs at each study.

| | 1st KD study serum MAMPs | | | | | | 2nd KD study serum MAMPs | | | | | 3rd KD study serum MAMPs | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1531.8** | **1414.3** | **790.9** | **779.8** | **695.0** | | **1171.4** | **1169.4** | **906.8** | **695.0** | | **667.4** | **619.4** | **409.3** |
| | **% positive (positive number)** | | | | | | **% positive (positive number)** | | | | | **% positive (positive number)** | | |
| 1st KD study $n=43$ | 48.8 (21) | 30.2 (13) | 39.5 (17) | 9.3 (4) | 34.9 (15) | | 0.0 (0) | 0.0 (0) | 0.0 (0) | 34.9 (15) | | 0.0 (0) | 4.7 (2) | 2.3 (1) |
| | 76.7* (33) | | | | | | 34.9* (15) | | | | | 7.0* (3) | | |
| 2nd KD study n=41 | | | | | | Pre | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | | | | |
| | 0.0 (0) | 0.0 (0) | 5.4 (2) | 0.0 (0) | 16.2 (6) | SBA period | 16.7 (2) | 16.7 (2) | 33.3 (4) | 50.0 (6) | | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| | | | | | | Post | 0.0 (0) | 0.0 (0) | 10.0 (1) | 0.0 (0) | | | | |
| | | | 16.2* (6) | | | | SBA period: 83.3* (10) | | | | | 0.0* (0) | | |
| | | | | | | | Pre & Post: 3.4* (1) | | | | | | | |
| 3rd KD study n=33 | | | | | | | | | | | Pre | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | | 0.0 (0) | 0.0 (0) | 16.1 (5) | 0.0 (0) | SBA period | 63.6 (7) | 45.5 (5) | 81.8 (9) |
| | | | | | | | | | | | Post | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| | 0.0* (0) | | | | | | 16.1* (5) | | | | | SBA period: 90.9* (10) | | |
| | | | | | | | | | | | | Pre & Post: 0.0* (0) | | |

At the 2nd and 3rd studies, both *in vivo* biofilms and serum samples were simultaneously collected. *In vivo* biofilms samples were searched for MAMPs common to those in serum samples by LC-MS and MS/MS analyses. SBA: simultaneous biofilm analysis, *: overall % positive (overall positive numbers). DC samples at the 1st ($n=41$), 2nd ($n=30$) and 3rd ($n=30$) study periods were all negative for all KD MAMPs. The detection rates of KD-specific serum MAMPs between KD samples ($n=43$) and DC samples ($n=41$) at the 1st study showed statistically significant differences at m/z 1531.8, m/z 1414.3, m/z 790.9, m/z 695.0, and overall ($P<0.0001$), but not at m/z 779.8 ($P=0.1164$) by Fisher's exact test. The detection rates between SBA period KD samples ($n=12$) and DC samples ($n=30$) at the 2nd study showed statistically significant differences at m/z 906.8 ($P=0.0044$), m/z 695.0 ($P=0.0002$) and overall ($P<0.0001$), but not at m/z 1171.4 ($P=0.0767$) and m/z 1169.4 ($P=0.0767$). The detection rates between SBA period KD samples ($n=11$) and DC samples ($n=30$) at the 3rd study showed statistically significant differences at all 3 molecules and overall ($P<0.0001$).

doi:10.1371/journal.pone.0113054.t001

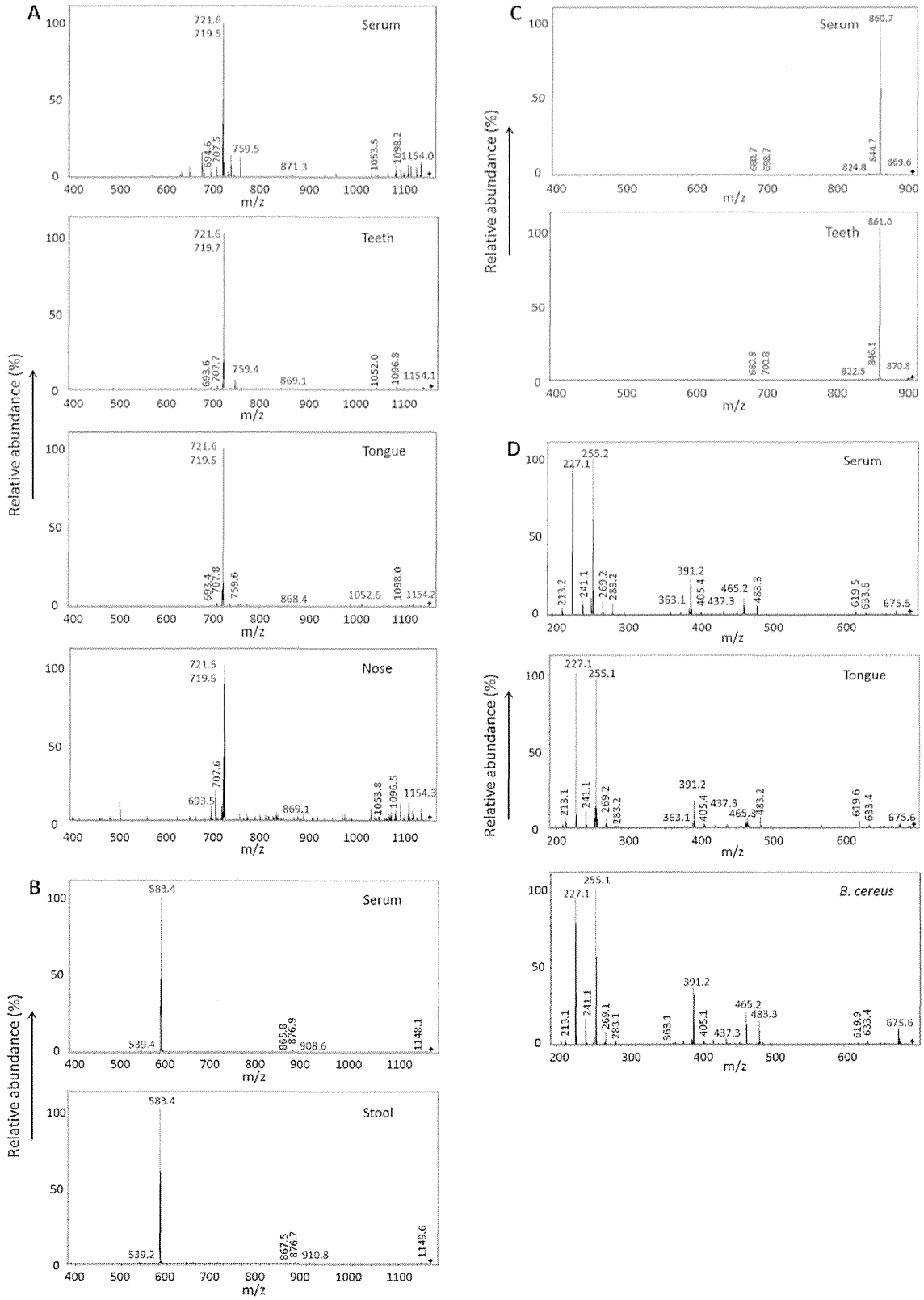
A
Serum
Teeth
Tongue
Nose
B
Serum
Stool
C
Serum
Teeth
D
Serum
Tongue
B. cereus
Relative abundance (%)
m/z

**Figure 3. KD *in vivo* biofilms contain MAMPs common to serum KD-specific molecules (2nd study period).** Extensive search for common molecules in the *in vivo* biofilms and sera from KD patients or DC controls revealed that 4 KD-specific molecules (m/z 1171.4, 1169.4, 906.8, and 695.0) showed similar MS/MS fragmentation patterns between the two in KD patients (Table S3 in File S1). A: The molecule at m/z 1171.4 was common in KD serum and biofilm extracts from teeth, tongue, or nose. B: The molecule at m/z 1169.4 was common in KD serum and stool biofilm extracts. C: The molecule at m/z 906.8 was common in KD serum and teeth biofilm extracts. D: The molecule at m/z 695.0 was common in KD serum and tongue biofilm extracts and *in vitro* biofilm extracts from *B. cereus*.
doi:10.1371/journal.pone.0113054.g003

search of such molecules as MAMPs in KD sera. KD samples induced significantly higher IL-8 production than DC and NC samples in whole sera. After separation into the lipophilic and hydrophilic fractions with ethyl acetate, KD samples elicited higher IL-8 production in each fraction (Figure 1). Similar results were obtained regarding IL-6 production. IL-6 and IL-8 levels in most of the tested sera from KD patients were under detection limits or negligible (data not shown). These results suggested that sera from KD patients contained molecules that stimulated HCAECs to produce IL-8 and IL-6. NOD1-stimulatory activity was also examined in whole and fractionated serum samples from KD, DC and NC, as described in Text S1 in File S1. However, no NOD1 activity was detected in any of these samples (data not shown).

## Serum KD-specific molecules common to MAMPs from the *in vitro* biofilms

We explored serum KD-specific molecules in the lipophilic and hydrophilic fractions by LC-MS analysis, and found numerous KD-specific molecules in the lipophilic fractions in 10 KD patients of the 1st study period (data not shown). It has been reported that *Yersinia (Y.) pseudotuberculosis*-infected children sometimes develop KD [15,16]. Moreover, *Bacillus (B.) cereus* and *B. subtilis* were 2 major spore-forming bacteria isolated from KD patients (Table S1 in File S1), which might work as possible wind-borne environmental triggers for KD [4]. Therefore, to find out the MAMPs identical to serum KD-specific molecules, we initially analyzed culture supernatants (later biofilms) of *Y. pseudotuberculosis*, *B. cereus* and *B. subtilis* from KD patients by LC-MS. Five KD-specific molecules at m/z 1531.8, 1414.3, 790.9, 779.8, and 695.0 showed the m/z and MS/MS fragmentation patterns almost identical to those of the MAMPs from *Y. pseudotuberculosis* and *B. cereus* (Figure 2 and Figure S1 in File S1). The 5 serum KD-specific molecules were detected with 100% specificity and 9.3%–48.8% sensitivity. At least one of the 5 KD-specific molecules was detected in 33 (76.7%) out of 43 patients at the 1st study period (Figure 2, Table 1). All serum KD-specific molecules decreased after IVIG treatment (Figure S1F in File S1). By comparison with 5 authentic microbial glycolipids, only one molecule at m/z 779.8 showed a MS/MS fragmentation pattern similar to that of cellobiose lipid (Figure 2D).

As these microbes ceased production of these MAMPs after 1 or 2 passages, we investigated the optimal culture conditions (medium, temperature, duration, shaking, nutrition and biofilm formation) for the production of these MAMPs. We found that they produced these MAMPs reproducibly in the biofilm-forming conditions in the presence of lipid, especially butter (Figure S2 in File S1). We thus examined the culture supernatants and biofilm extracts from all the spore-forming microbes isolated from KD patients as well as additional microbes by LC-MS and MS/MS analyses. In addition to the 3 bacteria mentioned above, almost all KD-specific molecules were detected not in the culture supernatants but in the biofilm extracts. Although a KD-specific molecule at m/z 1531.8 was detected in biofilm extracts from several bacteria (Table S2 in File S1), *Y. pseudotuberculosis* and *Staphylococcus (S.) aureus* were isolated from KD patients. In addition, *B. cereus*-associated MAMPs were detected in the sera of KD patients from whom *B. cereus* was actually isolated (Figure 2, Figure S1 in File S1 and Table S1 in File S1).

## Serum KD-specific molecules common to MAMPs from the *in vivo* biofilms

Although numerous KD-specific molecules were present in the lipid extracts from KD serum samples of the 2nd study period, the 5 KD-specific MAMPs observed at the 1st study period were no longer detected in the tested 10 samples. As the number of oligosaccharides, and the length, position, degree of saturation and configuration of the hydrophobic moieties in microbial glycolipids are known to change according to the environmental conditions and microbial origins [17,18], we examined lipid extracts from the *in vivo* biofilms in respective KD patients by LC-MS analysis. We detected 4 serum KD-specific molecules with MS/MS fragmentation patterns similar to one (m/z 695.0) of the 5 MAMPs at the 1st study period and 3 additional ones in the biofilms formed *in vivo* (teeth, tongue, nose and stool), respectively, in 10 (83.3%) out of 12 KD patients (Table S3 in File S1, Figure 3, Table 1). By the analysis of 20 microbial biofilm extracts and 5 authentic glycolipids, only one molecule at m/z 695.0 in tongue biofilms showed a MS/MS fragmentation pattern similar to that of a MAMP of *B. cereus* (Table S2 in File S1).

At the 3rd study period, we examined teeth and tongue biofilms and found 3 distinct KD-specific molecules with MS/MS fragmentation patterns similar to those from the *in vivo* biofilms in the respective KD patients by LC-MS and MS/MS analyses (Table S4 in File S1, Figure 4). Two of the 3 serum KD-specific molecules showed the MS/MS fragmentation patterns similar to a MAMP from *S. aureus*, and that from *B. subtilis*, *B. cereus* or *Y. pseudotuberculosis*, respectively. Actually, *B. subtilis* and *S. aureus* were detected from the patients. At least one of the 3 KD-specific MAMPs was detected in 10 (90.9%) out of 11 KD patients. The detection rates of KD-specific serum MAMPs at the 1st, 2nd and 3rd study periods are shown in Table 1. By LC-MS analysis, all the 106 control samples were negative for the 5, 4 and 3 KD-specific MAMPs detected at the 1st, 2nd and 3rd study periods, respectively.

## IgG sepharose binds some serum KD-specific MAMPs

It has been reported that certain microbial glycolipids bound to various species of IgG [19,20]. Therefore, we checked IgG-binding activity of KD-specific MAMPs using various kinds of IgG affinity columns. LC-MS analysis of IgG sepharose-binding