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ARTICLE

^{1}H, ^{13}C, and ^{15}N resonance assignment of the TIR domain of human MyD88

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Abstract Myeloid differentiating factor 88 (MyD88) is one of a critical adaptor molecule in the Toll-like receptor (TLR) signaling pathway. The TIR domain of MyD88 serves as a protein–protein interaction module and interacts with other TIR-containing proteins such as Mal (MyD88 adaptor-like) and Toll-like receptor 4 to form signal initiation complexes. Here we report the ^{15}N, ^{13}C, and ^{1}H chemical shift assignments of the TIR domain of MyD88. The resonance assignments obtained in this work will contribute to the study of heteromeric TIR–TIR interactions between MyD88 and TIR-containing receptors or adaptors.

Keywords Innate immunity · Toll-like receptor · NMR · MyD88 · TIR domain

Biological context

MyD88 is an essential and universal cytosolic adaptor protein in the signal transduction pathways mediated by members of the IL-1 family, including IL-18, and the Toll-like receptors (TLRs). It consists of an N-terminal death domain, a C-terminal Toll/Interleukin-1 receptor (TIR) domain (~150 amino acid residues), and a small linker segment between them. In innate immune responses, MyD88 plays pivotal roles in bridging between membraneous TLR and downstream kinases, such as the IL-1 receptor-associated kinases (IRAKs), in the cytosol (O'Neill and Bowie 2007). For example, in the TLR4 pathway, the cytosolic TIR domain of lipopolysaccharide (LPS)-stimulated TLR4 interacts with the TIR domain of MyD88 (MyD88-TIR). Concurrently, the death domain of MyD88 interacts with the death domain of IRAK4, activating the kinase. This initiates a phosphorylation cascade that eventually activates the transcription factors NF-κB (nuclear factor κB) and AP-1 (activator protein 1) (Akira et al. 2006).

The TIR domains serve as protein–protein interaction modules, which are conserved in the intracellular region of TLRs and also in cytosolic adaptor proteins such as MyD88, Mal (MyD88 adaptor-like), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule), and SARM (sterile α and HEAT Armadillo motifs). The crystal structures of TIR domains of some mammalian membranous receptors (TLR1, TLR2, TLR10, IL-1RAPL) and a bacterial protein (*Paracoccus denitrificans* TIR (PdTIR)) have been reported (Chan et al. 2009; Khan et al. 2004; Nyman et al. 2008; Tao et al. 2002; Xu et al. 2000), in some of which homomeric TIR interfaces were observed. However, the functional relevance of such interactions remains obscure, as the formation of these homo-dimers of TIR domains had not been observed in solution (Khan et al. 2004; Nyman et al. 2008).

In our previous study, the isolated MyD88-TIR was shown to exist as a monomer, while full-length MyD88 forms a dimer in solution. The dimerization appears to be mediated via homomeric interactions within its death

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domain (Ohnishi et al. 2009). In that study, we performed NMR titration experiments using ^{15}N MyD88-TIR and characterized a functionally relevant heteromeric TIR–TIR interaction with another TIR domain derived from Mal, which had been shown to enhance LPS-stimulated TLR4 signaling. Interestingly, no interaction has been observed between TLR4-TIR and MyD88-TIR, while Mal has been shown to directly bind TLR4-TIR. Thus, Mal seems to bridge between TLR4 and MyD88. These results exemplified the binding specificity among TIRs and its importance in forming correct signal initiation complexes (Ohnishi et al. 2009).

Here, we describe the NMR assignments of the TIR domain of MyD88. In IL-1R and TLR signaling, the heteromeric TIR interactions of MyD88 play a central role in the above-mentioned formation of the TLR signalosome. The resonance assignments form a substantial contribution to the study of the heteromeric TIR–TIR interactions between MyD88 and TIR containing receptors or adaptors.

Methods and experiments

Sample preparation

The portion of the human MyD88 gene encoding the TIR domain (amino acid residues 148–296) was cloned into the vector pGEX-5X-3 digested with restriction enzymes, *ECO*RI and *Not*I (GE Healthcare). This vector was transformed into *E. coli* BL-21 (DE3) (Novagen). The transformed bacterial cells were cultured at 37°C until the OD_{600} reached approximately 0.5. The cells were then induced with 1.0 mM IPTG and cultured for 16 h at 25°C. For ^{15}N- or ^{13}C, ^{15}N-double-labeling, rich growth media containing stable isotope-enriched nutrients (Silantes) were used. Harvested bacterial cells were once frozen and thawed before the next lysis step. The cells were resuspended in lysis buffer (20 mM Tris, pH 8.0, 400 mM KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol and protease inhibitors) and disrupted by sonication on ice. The supernatant was loaded onto a glutathione Sepharose 4B FF (GE Healthcare) affinity chromatography column. Lysis buffer at 30× the bed volume of this resin was used for the washing step. GST fused MyD88-TIR protein was eluted with elution buffer (50 mM Tris, pH 8.0, and 10 mM 2-mercaptoethanol, and 10 mM reduced glutathione). The GST-tag was cleaved by digestion with Factor Xa (1% of total protein volume) (Haematologic Technologies Inc.). Subsequently, the TIR domain was purified by gel filtration (Sephacryl S-100 HR 26/60 column, GE Healthcare) and cation-exchange chromatography (Mono S column, GE Healthcare). The gel filtration running buffer was 20 mM potassium phosphate buffer (pH 6.0) containing 100 mM KCl, 0.1 mM EDTA and 10 mM DTT. Using this purification protocol, ^{15}N-labeled or ^{13}C, ^{15}N-doubly-labeled TIR domain of MyD88 wild-type proteins was prepared. The yields of these proteins were approximately 1.0 mg from 1 L of rich growth media containing stable isotope-enriched nutrients. For preparing samples where selected amino acids were not labeled, proteins were over-expressed by the same method described above with a slight modification. The bacterial cells were first cultured in LB media and collected 30 min before IPTG induction by centrifugation. The cells were resuspended and cultured in ^{15}N-enriched M9 media containing unlabelled Gly, Ser and Cys, or Arg alone to a final composition of 100 mg/L for protein expression (The details of this protocol will be published elsewhere by Dr. H. Hiroaki). To improve the sample solubility and stability, the sample buffer was replaced by 20 mM potassium phosphate buffer (pH 6.0) containing 0.1 mM EDTA, 10 mM DTT, 50 mM deuterated L-arginine, and 50 mM deuterated L-glutamic acid (Golovanov et al. 2004). The final concentrations of the protein samples for typical NMR experiments were about 0.3 mM.

NMR spectroscopy

All NMR spectra were recorded at 25°C on a Bruker DRX500 or Avance 800 spectrometer equipped with a cryogenic probe. For assignment of backbone ^{1}H, ^{13}C, and ^{15}N resonances, HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH and 3D ^{1}H-^{15}N NOESY-HSQC spectra were recorded. For side chain resonance assignment, 2D constant-time ^{1}H-^{13}C HSQC, ^{1}H-^{13}C NOESY-HSQC, HCCH-TOCSY, CC(CO)NNH and HCC(CO)NNH were recorded. All NMR data were processed using NMRPipe (Delaglio et al. 1995), and analyzed using Sparky (Goddard and Kneller 1999).

Assignments and data deposition

Employment of 50 mM L-arginine and L-glutamic acid in the sample buffer significantly improved the solubility of the sample by approximately threefold, resulting in significantly enhanced NMR signal intensities. Following a standard sequential assignment procedure, 89.2% of the $^{1}H^{N}$, ^{15}N resonances of backbone amide groups (124 out of the 139 non-Pro residues) were assigned (Fig. 1). In addition, 91.9% of H^{α} (137 out of 149 residues), 93.3% (139 out of 149 residues) of $^{13}C^{\alpha}$, and 93.2% (136 out of 146 residues) of $^{13}C^{\beta}$ resonances were assigned. The sequential correlations of the backbone resonances of 195–200, 202, and 203 were missing. Those residues are involved in the BB-loop region in the structure of MyD88-TIR. Relatively lower {^{1}H}-^{15}N hetero NOE values of amide groups at the neighboring residues suggested that the region did not adopt a fixed conformation, but had some flexibility (Ohnishi et al. 2009). This might

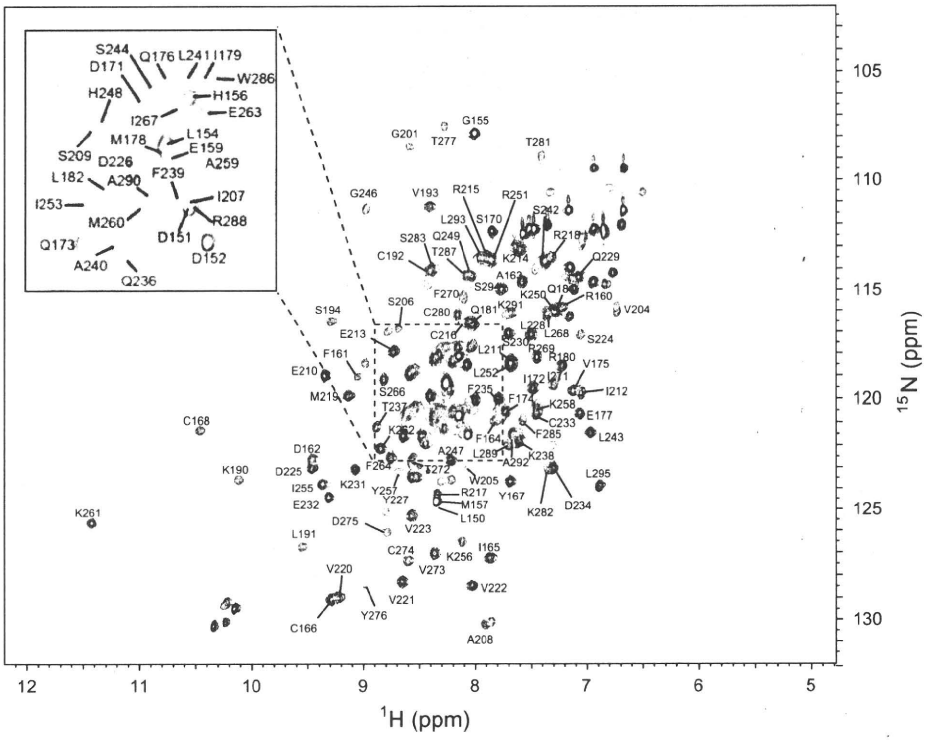

Fig. 1 ^{1}H, ^{15}N HSQC spectrum of uniformly ^{15}N-labeled TIR domain of human MyD88 in 20 mM potassium phosphate buffer (pH 6.0) containing 0.1 mM EDTA, 10 mM DTT, 50 mM deuterated L-arginine, and 50 mM deuterated L-glutamic acid recorded at 25°C

have caused NMR signal broadening at the BB-loop residues. Besides those above-mentioned residues, Thr-185, Asp-186, Tyr-187, and Arg-188 in the turn region were also missing, which could also be due to NMR signal broadening caused by local motions. Nevertheless, substantial numbers of side chain ^{1}H and ^{13}C atoms of those residues have been assigned on the basis of ^{13}C-edited NOESY and HCCH-TOCSY spectra. In total, 81% (859 out of 1,060) of assignable side chain resonances were assigned. The assigned chemical shifts of the TIR domain of MyD88 have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 11078.

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Genetic variations in MyD88 adaptor-like are associated with atopic dermatitis

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Abstract. Toll-like receptors (TLRs) are important pathogen-associated molecular pattern recognition receptors involved in initiating immune responses. The adaptor protein MyD88 adaptor-like (Mal), involved in signaling downstream of TLRs, plays a crucial role in mediating NF-κB activation. The association of Mal polymorphisms with allergic diseases has not previously been defined. The objective of this study was to detect polymorphisms in the Mal gene and to investigate their association with allergic diseases. Mal gene polymorphisms were genotyped in 310 subjects. The functional effects of Mal variants were analyzed *in vitro*. One Mal polymorphism, c.303 G>A (Q101Q), was found at a significantly lower frequency in atopic dermatitis patients (p=0.016). Q101Q is in linkage disequilibrium with -103 A>G (rs1893352) and c.539 C>T (S180L) (rs8177374) in the HapMap database. The A allele of -103 A>G showed significantly reduced transcription of Mal compared with the G allele. In addition, three rare variants were identified in this study, c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D), and were shown to lead to loss-of-function of Mal. It is possible that gene polymorphisms in Mal could affect atopic dermatitis by influencing the innate immune system. We show that Q101Q, which is in linkage disequilibrium with -103 A>G and S180L, may play a protective role against atopic dermatitis. Furthermore, we propose that loss-of-function variants of Mal could predispose individuals to atopic dermatitis or other immunological disorders.

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Abbreviations: Mal, MyD88 adaptor-like; TIR, Toll/interleukin-1 receptor; TIRAP, Toll/interleukin-1 receptor domain-containing adaptor protein; TLR, Toll-like receptor; PAMPs, pathogen associated molecular patterns; MyD88, myeloid differentiation primary response protein 88; TRIF, TIR domain-containing adaptor protein including IFNβ; TRAM, TRIF-related adaptor molecule; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; NF-κB, nuclear factor-κB; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; SNPs, single nucleotide polymorphisms; NCBI, National Center for Biotechnology Information; LD, linkage disequilibrium; HEK, human embryonic kidney; SD, standard deviation; AD, atopic dermatitis

Key words: atopic dermatitis, innate immunity, Mal/TIRAP, polymorphism, Toll-like receptor

Introduction

The toll-like receptor (TLR) protein family plays a central role in the activation of the innate immune system. In humans, CD14 and TLRs are involved in extracellular recognition of pathogen associated molecular patterns (PAMPs), which activate the initial host defense system. Downstream of the TLRs, five Toll/interleukin-1 receptor (TIR) domain containing adaptor proteins have been identified: myeloid differentiation primary response protein 88 (MyD88), MyD88 adaptor-like (Mal) [also known as Toll/interleukin-1 domain-containing adaptor protein (TIRAP)], TIR domain-containing adaptor protein including IFNβ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and heat-armadillo motifs. These adaptor proteins function in mediating the intracellular signaling pathways that lead to inflammatory gene expression (1). One of these adaptor proteins, Mal/TIRAP (hereafter referred to as Mal), is involved in the MyD88-dependent signaling pathway downstream of TLR2 and TLR4 (2,3). The Mal/MyD88-dependent signaling pathway induces the sequential activation of interleukin-1 receptor-associated kinase (IRAK)4, IRAK1 and tumor necrosis factor receptor-associated factor-6 (TRAF6) (1). Mal plays specific roles in interactions with various TIR domain-containing adaptors; Mal mediates the interaction between TLR4 and MyD88 (4,5), and directly interacts with the downstream component TRAF6 (6).

Allergic diseases are caused by inappropriate immunological responses to harmless antigens, driven by T helper type 2 (Th2)-mediated immune responses. Insufficient stimulation of TLRs by PAMPs results in decreased production of T helper type 1 (Th1) cytokines such as, IL-12 and IFNs, which can attenuate the activity of down-regulators of Th2 responses, in turn leading to allergic disease (7). It has been

Table I. Clinical information on patients with allergic diseases and control subjects.

Characteristics	Patients with allergic diseases (n=207)		Control subjects (n=103)
	AD (n=99)	Other allergic diseases (n=108)	
Gender			
Male	62	72	56
Female	37	36	47
Serum total IgE (IU/ml)			
Mean	2491.63	950.17	38.11
Median	461	390	21
SD	4835.59	1882.37	39.36
Min	2.36	1.91	4.34
Max	24838	16000	152

AD, atopic dermatitis; SD, standard deviation.

reported that endotoxin can induce MyD88-deficient dendritic cells to support Th2 cell differentiation (8). Therefore, the Mal/MyD88-dependent signaling pathway may be implicated in allergic diseases caused by overactive Th2 responses.

Genetic variations leading to alterations in proteins involved in PAMP recognition or downstream signaling may alter the balance between Th1 and Th2 immune responses and may therefore influence the individual's susceptibility to developing allergic diseases. Previous studies have found single nucleotide polymorphisms (SNPs) in TLRs and TLR-related proteins to be associated with allergic diseases. For example, the polymorphism R753Q in the TLR2 gene increases the frequency of atopic dermatitis (9,10); the A-16934T promoter polymorphism of the TLR2 gene is associated with severe atopic dermatitis (11); polymorphism D299G of the TLR4 gene is associated with a high prevalence of asthma and reduced IL-12 responses (12); and polymorphisms in IRAK4 and promoter polymorphisms of CD14 are associated with total serum IgE levels (13,14).

In contrast to these associations with allergic disease, the Mal polymorphism S180L (rs8177374) has been associated with protection against some diseases (15). However, there is conflicting evidence for the role of S180L in some ethnic populations (16,17). Recently, the rare mutation D96N (rs8177400) was described as a loss-of-function variant of Mal that leads to reduced TLR2/TLR4 signaling activity (18,19). These recent results suggest that the effects of SNPs in Mal are important in some immunological diseases.

In this study, we detected SNPs in the Mal gene in Japanese population samples with or without allergic diseases and explored whether we could detect associations of SNPs with allergic diseases. Further functional analyses were performed to identify the functional effects of the variants of Mal.

Subjects and methods

Subjects. The association between Mal gene polymorphisms and the presence of atopic dermatitis (AD) (n=99), other allergic diseases (n=108) and non-allergic control subjects (n=103) was analyzed in Japanese subjects. Non-allergic unrelated control subjects had no history of atopic dermatitis or other allergic diseases including bronchial asthma, food allergies, allergic rhinitis or allergic conjunctivitis. Detailed clinical information on the subjects is presented in Table I. The diagnosis of atopic dermatitis was made according to the criteria of Hanifin (20). All subjects provided informed consent to participate in the study.

Polymorphism detection and linkage disequilibrium (LD) analysis. Genomic DNA was extracted from neutrophils using a SepaGene kit (Sanko Junyaku, Tokyo, Japan). Three primer sets (Table II) were designed based on the coding region of the Mal gene sequence available from the National Center for Biotechnology Information (NCBI Reference Sequence: NC_000011.9) to amplify the two coding exons. Genotyping was performed using the ABI 3100 DNA auto-sequencer program (Applied Biosystems). Japanese HapMap data were obtained from http://www.hapmap.org/ (21). Pairwise LD was calculated as the r^2-values by using the Haploview 4.1 program.

Vector preparation and in vitro mutagenesis. The wild-type coding region of the Mal gene (235-aa isoform; Accession number: NM_148910) was cloned into pFLAG-CMV6a (Sigma-Aldrich). This plasmid was used for generating substituted types of Mal using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI). Primers containing the following substituted sites in Mal were designed for generating the six different variations of Mal (S55N, Q101Q, E132K, R143Q, S180L and E190D). Fragments (7500-7808 bp of NC_000011.9) contained in the promoter region of Mal and containing the Mal intron 3 replicated from the genome of individuals with or without the -103G variation, were cloned into pGL4.11 (luc2P) (Promega). These were used to analyze the transcriptional activities of Mal/-103A and Mal/-103G.

Cell culture. Human embryonic kidney (HEK) 293T cells and 293-hTLR4/MD2-CD14 cells (InvivoGen, CA, USA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen,

Table II. Primer details for amplifying exon 4 and 5 in the Mal gene by PCR.

Exon	Primer	Primer sequence	Length of amplified DNA	Annealing temp (°C)
Exon 4	S4 (F)	5'GTCTGGCCCTAATCTCATGA3'		
Exon 4	A4 (R)	5'CACTCTCCACAAAGCATCCAG3'	479 bp	60
Exon 5	S5 (F)	5'GAGAATAAGATGTTTCCCAGTGC3'		
Exon 5	A5 (R)	5'GCAGCATCTGGTACTTGCACCA3'	557 bp	62
Exon 5	S6 (F)	5'GGTCTCCTACTTGGAAGGCA3'		
Exon 5	A6 (R)	5'CAATGGAAACCTGTTGGTCAG3'	569 bp	60

F, forward sense; R, reverse antisense; temp, temperature.

Carlsbad, CA) supplemented with 10% FCS, 100 U/ml penicillin and 100 pg/ml streptomycin. THP-1 cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, 0.01 M HEPES, 100 U/ml penicillin and 100 pg/ml streptomycin. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Luciferase assay. HEK 293T cells ($2x10^4$ cells/well in 96-well plates) were transfected with 100 ng pFLAG-CMV6a empty vector or pFLAG-CMV6a-Mal wild-type or variants (S55N, Q101Q, E132K, R143Q, S180L and E190D) using Lipofectamine 2000 (Invitrogen). NF-κB luciferase reporter vector (50 ng) [pGL4.32 (luc2P/NF-κB-RE/Hygro)] and 50 ng *renilla* luciferase reporter control vector (pGL4-hRluc-TK) were co-transfected into the appropriate wells. At 48 h after transfection, NF-κB luciferase reporter gene activity was analyzed using the Dual-Luciferase Reporter assay system (Promega). For dose-dependent assays, 293-hTLR4/MD2-CD14 cells were transfected with 10, 40 and 80 ng plasmids DNA of Mal variants (E132K, R143Q and E190D), respectively. The DNA content was adjusted to a total volume of 80 ng with mock plasmid DNA. Cells were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for 6 h and NF-κB luciferase reporter gene activity was then analyzed. THP-1 cells are derived from the human monocytic cell line, which expresses TLRs and TLR adaptor proteins. Therefore, we used THP-1 cells to perform transcription activity assays and cytokine production experiments. THP-1 cells ($1x10^6$ cells/ml) were transfected using Lipofectamine LTX (Invitrogen) with 800 ng pGL4.11 (luc2P) control vector, pGL4.11 (luc2P)-Mal promoter (-103A) or pGL4.11 (luc2P)-Mal promoter (-103G). pGL4-hRluc-TK (50 ng) was co-transfected into all wells as an internal control. Transcriptional enhancer activities were measured at 24 h after transfection. Each of the luciferase assays was performed at least three times.

Measurement of TNF-α and IL-12. THP-1 cells ($1x10^6$ cells/ml) were transfected with Mal variant plasmids as described above or with the pSV-β-galactosidase control vector as an internal control using Nucleofector (Amaxa, Program V-001). After 24 h of transfection, cells were cultured with or without 100 ng/ml LPS for a further 24 h. Supernatants were collected at 48 h after transfection and the concentrations of TNF-α and IL-12 in the supernatants were determined using human TNF-α or human IL-12 ELISA kits (BioSource International, Carlsbad, CA).

Western blotting. HEK 293T cells ($8x10^5$ cells/well for 6-well plates) were transfected as described above. After 48 h, cells were lysed in 200 µl lysis buffer (10 mM Tris HCl, pH 7.5, 10 mM NaCl, 2 mM EDTA, 10% glycerol and 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The concentration of each expressed protein was measured by the Lowry method and the quantity of each expressed protein was adjusted to load equal protein quantities for Western blots analysis. Samples were analyzed by Western blotting using an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO).

Statistical analysis. Genotype frequencies were analyzed using a chi-square (χ^2) test. A p-value of <0.05 was considered statistically significant. The differences in luciferase activities and cytokine values between wild-type Mal and the Mal variants were analyzed using the Dunnett's test. The difference between the transcriptional activity of -103A and -103G was analyzed by the Bonferroni test. Each assay was performed with at least three samples.

Protein structure modeling. Structure modeling of the TIR domain of Mal was performed using the MyD88-TIR structure (PDB code: 2z5v) as a template on MOE software according to our previously described method (5). The figure of this structure was made using PyMOL (22).

Results

Characterization of Mal polymorphisms in Japanese subjects. Eight polymorphisms were discovered in the 235-aa coding region of Mal (Fig. 1A). Four of these polymorphisms were already described in the NCBI, while the other four polymorphisms were novel [c.297 C>T (A99A), c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D)]. The three non-synonymous polymorphisms in the Mal gene [c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D)] are relatively rare missense variants. Two of three were only discovered in AD subjects (Table III). These three rare missense variants were found as heterozygotes. On the other hand, one of the synonymous SNPs, c.303 G>A (Q101Q)

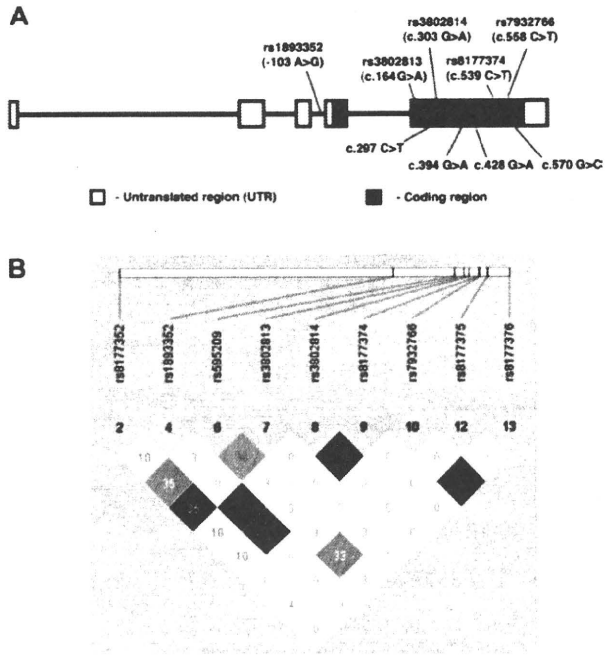

Figure 1. (A) Exon-intron structure of the Mal gene and locations of genotyped SNPs. c.297 C>T (A99A), c.394 G>A (E132K), c.428 G>A (R143Q) and c.570 G>C (E190D) are novel variants. The fragment of the genome containing the novel variants was sequenced for at least three times under different conditions to confirm that these novel variants are not PCR errors. (B) Linkage disequilibrium (LD) plot surrounding the Mal SNPs. The LD plot was generated by Haploview version 4.1 using the HapMap JPT database. The colors of the squares illustrate the magnitude of the pairwise r^2 values on a black and white scale, where black indicates a perfect LD (r^2 = 1.00).

(rs3802814), was found at a significantly different frequency in the three categorized groups (p=0.038, Table III). When we statistically tested the difference between AD and control groups, we found this SNP at a significantly lower frequency in AD subjects than in control subjects (p=0.016, data not shown). We did not find a significant difference between the other allergic disease groups and the control group.

Functional analyses of synonymous and non-synonymous SNPs of Mal. Overexpression of wild-type Mal led to a strong induction of NF-κB reporter gene activity, and three of the studied polymorphisms (S55N, Q101Q and S180L) were comparable to wild-type Mal in their ability to activate NF-κB. Interestingly, the variants E132K, R143Q and E190D were severely incompetent in NF-κB activation (Fig. 2A). The expression of E132K, R143Q and E190D variants in HEK 293T cells was confirmed by Western blotting (Fig. 2B).

The variants S55N, Q101Q and S180L produced comparable levels of TNF-α and IL-12 to the levels produced by wild-type Mal. However, E132K, R143Q and E190D did not produce TNF-α or IL-12 in the presence or absence of LPS (Fig. 2C). These analyses suggest that these three non-synonymous SNPs of Mal are loss-of-function variants.

Dose-dependent assays were performed to classify the mechanism of behavior of the variants E132K, R143Q and E190D. As shown in Fig. 4A, E132K showed a dominant negative inhibitory effect in LPS/TLR4 dependent activation of NF-κB, while R143Q and E190D did not show a significant dominant negative effect.

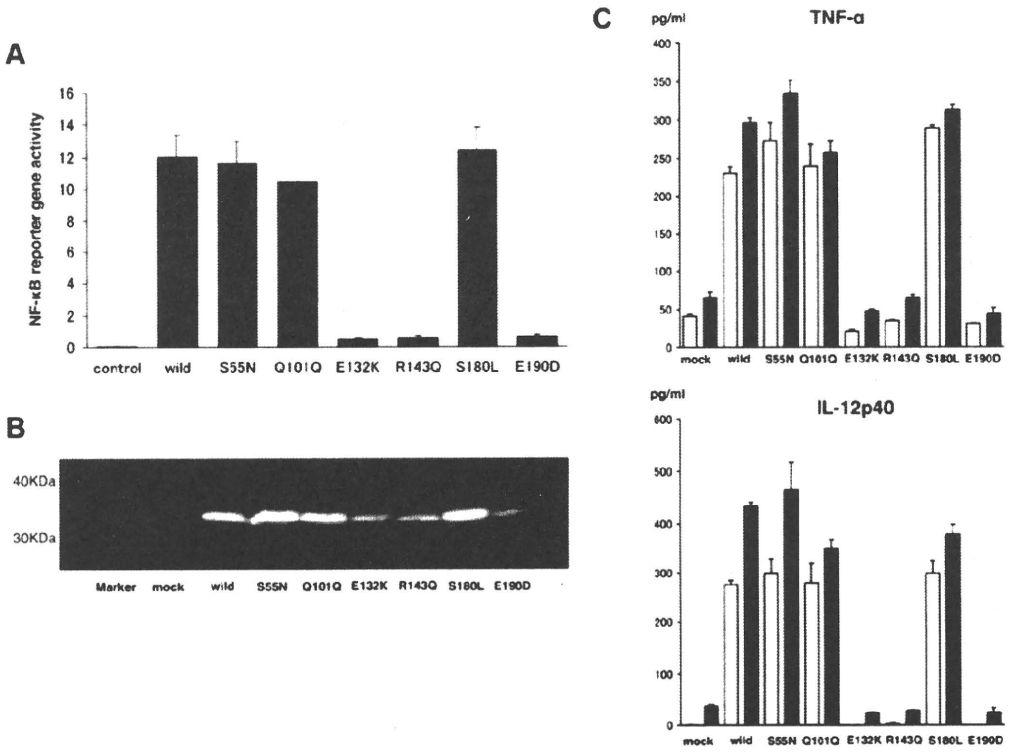

Figure 2. (A) Mal signaling induces NF-κB-driven reporter gene activity. HEK 293T cells were equally transfected with 100 ng vector DNA (pFLAG Mal wild-type or variants of Mal). (B) Protein expression levels of Mal variants. (C) TNF-α and IL-12 production assay. White bars show the cytokine production levels from THP-1 cells without LPS stimulation, black bars show cytokine production levels from LPS stimulated THP-1 cells. These results are shown as the mean of triplicate determinations ± SD.

Table III. Mal single nucleotide polymorphism (SNP) frequencies in the AD (n=99), other allergic diseases (n=108) and control (n=103) groups.

SNP (amino acid change)	dbSNP ID	Genotype	AD (%)	Other allergic diseases (%)	Controls (%)	P-value
c.164 G>A	rs3802813	GG	66 (66.7)	77 (71.3)	64 (62.1)	
(S55N)		GA	32 (32.3)	29 (26.85)	39 (37.9)	0.34
		AA	1 (1.0)	2 (1.85)	0	
c.297 C>T		CC	99 (100)	107 (99.1)	103 (100)	
(A99A)		CT	0	1 (0.9)	0	0.39
		TT	0	0	0	
c.303 G>A	rs3802814	GG	91 (91.9)	94 (87.0)	82 (79.6)	
(Q101Q)		GA	8 (8.1)	14 (13.0)	21 (20.4)	0.038[a]
		AA	0	0	0	
c.394 G>A		GG	97 (98.0)	108 (100)	103 (100)	
(E132K)		GA	2 (2.0)	0	0	0.11
		AA	0	0	0	
c.428 G>A		GG	98 (99.0)	108 (100)	100 (97.1)	
(R143Q)		GA	1 (1.0)	0	3 (2.9)	0.17
		AA	0	0	0	
c.539 C>T	rs8177374	CC	94 (94.9)	104 (96.3)	97 (94.2)	
(S180L)		CT	5 (5.1)	4 (3.7)	6 (5.8)	0.77
		TT	0	0	0	
c.558 C>T	rs7932766	CC	89 (89.9)	102 (94.4)	96 (93.2)	
(A186A)		CT	10 (10.1)	5 (4.6)	7 (6.8)	0.38
		TT	0	1 (1.0)	0	
c.570 G>C		GG	98 (99.0)	108 (100)	103 (100)	
(E190D)		GC	1 (1.0)	0	0	0.34
		CC	0	0	0	

[a]Statistically significant; AD, atopic dermatitis.

-103 A>G and Q101Q are in LD. As displayed in Table III, the polymorphism Q101Q was found at a significantly lower frequency in the AD group. However, this polymorphism did not show a significantly higher ability than wild-type Mal in stimulating NF-κB luciferase activity and producing inflammatory cytokines (Fig. 2). We searched the LD surrounding the Mal gene using the HapMap JPT database to detect any SNPs acting in LD with Q101Q. We found that the SNP -103 A>G (rs1893352) shows complete LD (r^2=1.00) with Q101Q (rs3802814) (Fig. 1B). A luciferase reporter gene assay was performed to assess the functional effect of this SNP on the transcriptional ability of the Mal promoter. As shown in Fig. 3, the -103A allele significantly reduced the transcriptional activity of Mal (p=0.009), showing a 2.6-fold lower activity than the -103G allele. Additionally, we found another SNP, S180L (rs8177374), to be in LD with Q101Q.

Discussion

Many SNPs in the Mal gene have been described, including A9P, R13W, S55N, D96N, Q101Q, D102H, S180L, A186A and V197I (23). Other polymorphisms have been found specifically in some ethnic groups, such as A100T, S131S and R143W in the coding region of Mal (24). With the SNPs discovered in our study (E132K, R143Q and E190D) added to this list, Mal can be considered a relatively polymorphic gene among TIR domain-containing adaptors (25).

In this study, we found Q101Q to be present at a significantly lower frequency in the AD group compared with control subjects. Though we could not explain the exact protective function of Q101Q, -103 A>G was found to be in LD with Q101Q and S180L, and the latter has been previously described as protective factor against bacterial infection (15). Thus, we speculate that Q101Q plays a protective role against the onset of atopic dermatitis via increasing transcriptional activity of Mal.

We searched for transcription factor binding sites in the promoter region of Mal by using Genomatix (http://www.genomatix.de/) to detect whether the polymorphism -103 A>G acts to influence transcription factor. We found that one transcription factor associated with the Mal promoter, named CTCF, precisely binds to the nucleotide sequence 5'tctctct ACCCtctgtaggatggctgc3', in which the capital letters denote the core sequence used by MatInspector (Genomatix: Matrix Library information). The capital A in the core sequence is the A allele of -103 A>G. It has been reported that CTCF

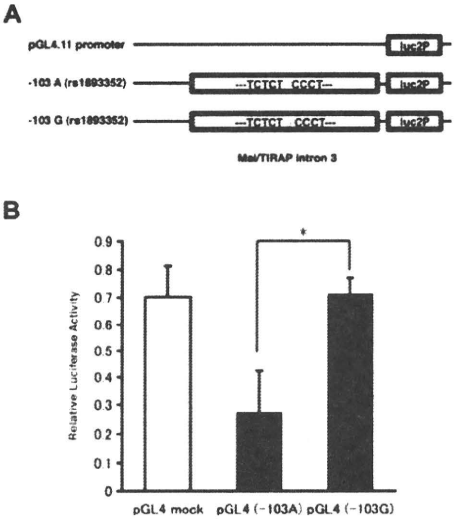

Figure 3. (A) pGL4 plasmid constructs used for transfection. (B) Transcriptional activities of the promoter of Mal with -103 A>G (rs1893352). THP-1 cells were transfected with the pGL4 vector as a control and the pGL4-Mal/-103A and pGL4-Mal/-103G variants. Transcriptional activities were measured by the luciferase reporter assay. Values of relative luciferase activity are shown as the means ± SD. *P=0.009, Bonferroni test.

is a vertebrate insulator protein that blocks the interaction between enhancers and upstream promoters, thereby regulating imprinted expression (26). Therefore, we hypothesize that the variant G allele alters the CTCF binding site sequence and thus inhibits the binding of the insulator protein CTCF to the Mal promoter, thereby enhancing Mal transcriptional activity. This is consistent with the result of our transcriptional activity assay showing that the A allele of -103 has reduced activity compared with the G allele of -103 (Fig. 3).

S180L was the first identified functional SNP in Mal, as described by Khor *et al* (15). L180 led to a failure of Mal to interact with TLR2 but not MyD88. In contrast, Ferwerda *et al* showed increased production of proinflammatory cytokines (TNF, IL-6 and IFN-γ) in response to endotoxin in the S180L heterozygous individuals but not in S180 homozygous individuals (27). Although the overexpression assays of the S180L variants in this study and previously reported studies did not show differences in the activity between the wild-type Mal and S180L (18,19), the linkage of S180L with Q101Q may be another modification factor for Q101Q.

The novel variants identified in this study (E132K, R143Q and E190D) all showed a significantly lower ability to activate NF-κB and induce TNF-α and IL-12 (Fig. 2). IL-12 is an essential signal for the differentiation of naive Th cells into Th1 cells, which produce IFN-γ and thereby inhibit Th2 differentiation and IgE production (28,29). A failure to produce IL-12 can lead to allergic diseases. Because MyD88-dependent pathways are essential for the development of protective IL-12-mediated Th1 responses (30), a loss-of-function or a dominant-negative inhibitory effect of Mal may also lead to polarized Th2 responses, which could increase susceptibility to AD.

E132K is predicted to be located in the BB loop in the TIR domain of Mal (Fig. 4B). One of the important mutations of Mal, P125H, which is also located in the BB loop, has been found to fail to co-immunoprecipitate with TLR4 and to strongly inhibit activation of NF-κB (2,3). Similarly, E132K is considered a critical residue located in the interaction region of the TIR domain. Interestingly, the E132K variant also showed a dominant-negative inhibitory effect in TLR4 signaling (2) (Fig. 4A). Moreover, this variant was found in two AD patients in this study, but was not found in the control subjects (Table III). These results suggest that the E132K variant may increase the risk of developing AD.

Our study found that the Mal variants R143Q and E190D also lead to loss-of-function (Figs. 2 and 4A). R143Q was found in both AD and control subjects, and we did not find a clear role for R143, but we hypothesize that R143Q affects the stability of the Mal protein, since R143 lies in a core position of the Mal TIR domain (Fig. 4B). The loss of the positively charged arginine in this polymorphism may affect the stability of Mal. On the other hand, in a novel feature that distinguishes Mal from MyD88, Mal has been found to possess a TRAF6 interaction motif, P-P-E-L-R-F, at amino acid position 188-193. This motif is essential for the signaling function of Mal. Mansell *et al* experimentally mutated the glutamic acid at position 190 to alanine and found that the mutant failed to induce TLR2- and TLR4-mediated activation of the NF-κB, JNK and MAP kinase pathways (6). These data suggest that E190D can inhibit NF-κB activation and thereby may be associated with susceptibility to developing immunological diseases.

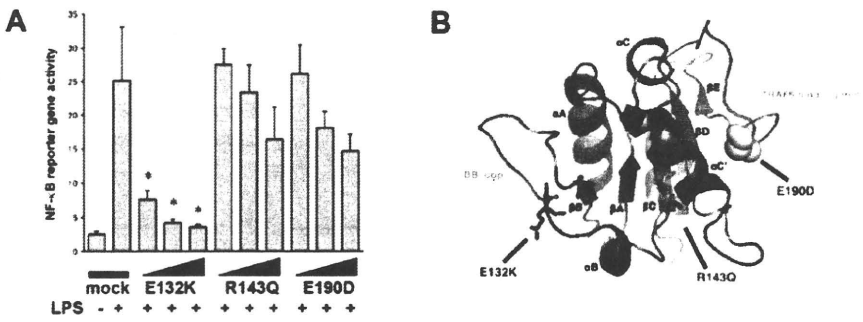

Figure 4. (A) The dose-dependent NF-κB reporter gene assay of Mal variants. Mal variants were cotransfected (10 ng, 40 ng, or 80 ng) in 293-hTLR4/MD2-CD14 cells. E132K showed significant inhibition of LPS/TLR4-mediated NF-κB activation in a dose-dependent manner. *P<0.01. (B) Model structure of the Mal TIR domain. The illustrated structure was created from the protein structure of MyD88-TIR as a template. This structure comprised a central five-stranded parallel β-sheet (βA-βE) surrounded by four α-helices (αA-αC and αE). The orientations of the side chain of E132 and R143 are shown with red or magenta color, respectively. The TRAF6 binding motif on Mal is highlighted with green and E190 is shown with yellow spheres.

The TLR signaling pathways are crucial for the innate immune system to act in efficient host defense. Deletion mutations, compound heterozygous or homozygous missense mutations in MyD88 or IRAK4 have all been associated with immunodeficiency (31,32). Recently, two MyD88 non-synonymous SNPs were reported as loss-of-function variants (33). In addition, Mal knock-out mice have been shown to be susceptible to *Klebsiella pneumonia* (34). Therefore, it is likely that homozygosity or compound heterozygosity of the loss-of-function variants described in this study, which are located in TIR domain of Mal, could lead to immunodeficiency disorder due to an impaired innate immune response.

Efficient expression and appropriate function of Mal is necessary for the induction of sufficient proinflammatory cytokines and in initiating a timely Th1 immune response, thereby helping to balance Th2 and Th1 immune responses and defend against allergic diseases. The polymorphism -103 A>G, linked with Q101Q and/or S180L, may play a key protective role against atopic dermatitis. In contrast, dominant negative or loss-of-function variants in TIR domain of Mal could impair Mal-mediated signaling and lead to deficient production of proinflammatory cytokines, which may lead to AD or the other immunological disorders. The new identified polymorphisms and the functional characteristics described in our study may facilitate genetic diagnosis and lead to new therapies for atopic dermatitis.

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Key words: CCR4, cutaneous lymphocyte-associated antigen, IL-17, IL-22, psoriasiform and pustular eruption, Th17, tumour necrosis factor-α inhibitor

Conflicts of interest: none declared.

Interleukin-17 expression in the urticarial rash of familial cold autoinflammatory syndrome: a case report

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MADAM, Familial cold autoinflammatory syndrome (FCAS) is an autosomal dominant disease characterized by recurrent episodes of fever, urticarial rash, arthralgia and conjunctivitis associated with exposure to cold temperatures.[1] FCAS is an autoinflammatory disorder related to the innate immune system. This syndrome is caused by mutations of NLRP3 (NOD-like receptor family, pyrin domain containing 3).[2,3] The urticarial rash in FCAS is considered to be different from the typical urticaria induced by histamine, but the pathophysiology underlying the urticarial rash in FCAS remains poorly understood. We hypothesized that certain cytokines induced by interleukin (IL)-1β may attract neutrophils and mononuclear cells in this disease. Therefore, we performed immunohistochemical detection of IL-17A in the urticarial lesions of FCAS.

A 34-year-old Japanese man presented with a recurrent, urticarial rash, which developed following physical exhaustion or cold exposure and resolved after rest and warming. The patient had experienced recurrent episodes of the rash since infancy, but the condition had not been previously diagnosed. There was no history of aseptic meningitis, renal amyloidosis or perceptive deafness. The patient's 3-month-old daughter had the same symptoms (H. Ohnishi, T. Teramoto, H. Iwata, Z. Kato, T. Kimura, K. Kubota, R. Nishikomori, H. Kaneko, M. Seishima, N. Kondo, unpublished data).

On examination, a mildly itchy, urticarial rash was distributed over his whole body (Fig. 1). In addition, bilateral conjunctivitis was observed. Histopathological examination of a biopsy specimen from the lower back revealed mild dermal perivascular inflammation with prominent neutrophils and mononuclear cells (Fig. 2a). Blood tests showed an elevated total white blood cell count of 13 120 μL^{-1} with 74·5% neutrophils and an elevated C-reactive protein of 3·05 mg dL^{-1}. A heterozygous missense mutation (Y563N) at exon 3 of NLRP3 was detected in the DNA from the father and daughter by direct sequencing. These findings confirmed the diagnosis of FCAS.

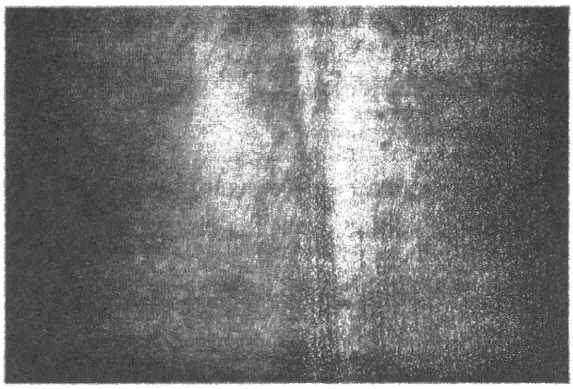

Fig 1. Clinical appearance of the urticarial rash on the back of the patient.

An immunohistochemical analysis of IL-17A expression was performed with an antihuman IL-17A monoclonal antibody (eBioscience, San Diego, CA, U.S.A.), using the Dako EnVision method (Dako, Glostrup, Denmark). Sections (5 μm) were cut and deparaffinized using two xylene exchanges followed by rehydration through an ethanol gradient. Antigen retrieval was performed by boiling the slides in buffer containing 6·5 mmol L^{-1} sodium citrate (pH 6·0). The slides were immersed in absolute methanol containing 0·75% hydrogen peroxide for 30 min to neutralize endogenous peroxidase activity, followed by a 20-min incubation in SuperBlock Blocking Buffer (Thermo Scientific, Rockford, IL, U.S.A.) to block nonspecific binding. Slides were incubated with the IL-17A antibody at 1 : 100 dilution for 45 min at room temperature. The slides were washed three times for 10 min with phosphate buffered saline, and incubated with Dako EnVision for 30 min. Staining was visualized using 3,3′-diaminobenzidine tetrahydrochloride substrate and counterstained with haematoxylin. IL-17A predominantly stains T helper (Th) 17 cells. Immunohistochemistry of the paraffin sections of the biopsy showed positivity for IL-17A in some of the dermal perivascular infiltrate (Fig. 2b); stained cells had multilobulated nuclei suggestive of neutrophils (Fig. 2c). Replacement of the primary antibody with mouse cocktail isotype immunoglobulin (Dako) and omission of the primary antibody resulted in negative staining.

FCAS belongs to the group of cryopyrin-associated periodic syndromes (CAPS), which has been mapped to the gene NLRP3, encoding the NLRP3 (cryopyrin) protein.[2] CAPS is a

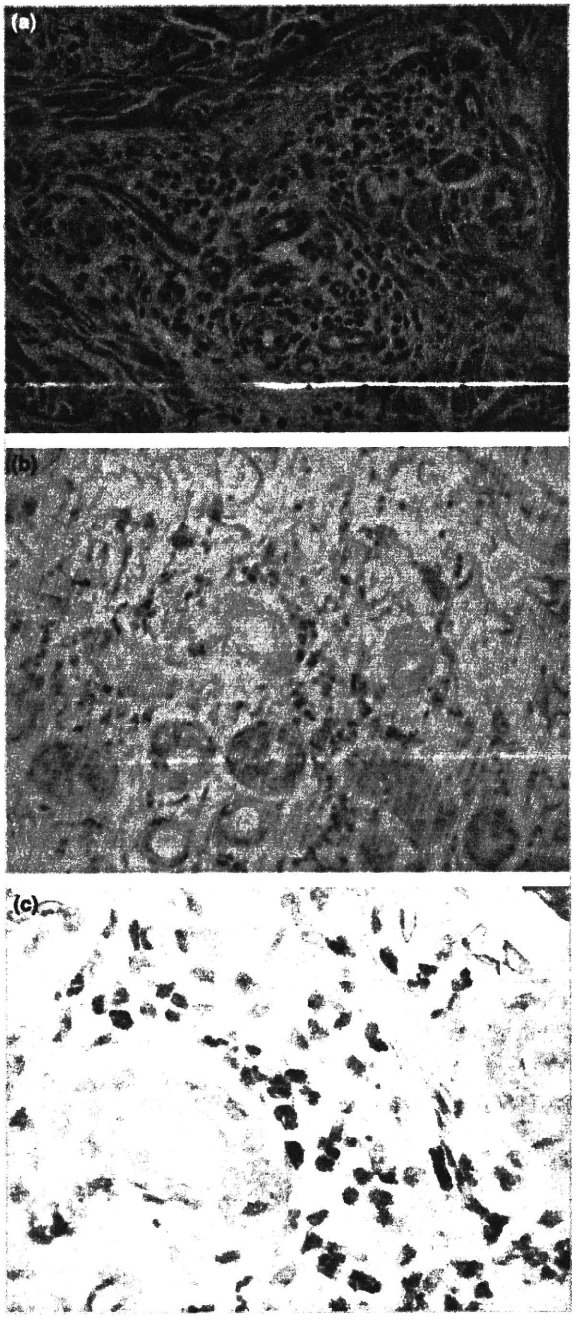

Fig 2. (a) A mild dermal perivascular inflammation with prominent neutrophils and mononuclear cells (haematoxylin and eosin stain; original magnification × 400). (b) Immunohistochemical analysis of interleukin-17A expression in the dermal infiltrate. The brown coloration indicates positive staining (original magnification × 400). (c) Positive-stained cells have multilobulated nuclei suggestive of neutrophils (original magnification × 1000).

spectrum of rare, autoinflammatory disorders which also include Muckle–Wells syndrome and chronic infantile neurological, cutaneous, articular (CINCA) syndrome.[4] CINCA syndrome is the most severe syndrome, while FCAS is the least severe. In some patients with CAPS, the severity of the phenotype correlates with the genotype.[5] However, additional genetic and environmental factors might be influential in some cases.[5]

NLRP3 plays a critical role in innate immunity, as it responds to intracellular pathogens and some danger signals. NLRP3 is composed of three domains: a pyrin domain, a nucleotide oligomerization domain (NOD) and a leucine-rich repeat domain.[2] All mutations have been identified in NLRP3 and most of them are found in exon 3 encoding NOD. In the present case of the father and his daughter, a heterozygous mutation (Y563N) of NLRP3 was detected. This mutation has previously been reported in two cases of FCAS.[5]

NLRP3 participates in the formation of a large protein complex called the inflammasome.[6] NLRP3 is maintained in an inactive state in the cell cytoplasm. The NLRP3 inflammasome is activated by bacterial toxins, bacterial or viral RNA and uric acid crystals.[7] Activated NLRP3 leads to activation of caspase-1, which subsequently activates pro-IL-1β and pro-IL-18 to become active IL-1β and IL-18, respectively. Theses cytokines participate in systemic and local responses to infection or injury. A mutation in NLRP3 might constitutively activate NLRP3, resulting in increased IL-1β secretion.[8] Until recently, nonsteroidal anti-inflammatory drugs and oral corticosteroids have been the only treatment options for CAPS. Their efficacy was unremarkable. With identification of the underlying molecular pathophysiology, treatments targeting the IL-1 pathway are being developed. Anakinra, an IL-1 receptor antagonist, has shown promising results in the treatment of patients with CAPS.[4]

The histological features of the urticarial rash in patients with CAPS show dermal perivascular infiltration with neutrophils.[9] The mode of neutrophil recruitment in the urticarial rash of CAPS is still unclear. In 2009, Nakamura *et al.*[9] reported that mast cells constitutively produce IL-1β in the skin of patients with CAPS. NLRP3 mutants promoted the production of IL-1β and neutrophil recruitment in mice.[9] Patients with FCAS respond markedly to therapy with IL-1β receptor antagonists,[10] suggesting that the urticarial rash in FCAS is mediated predominantly by IL-1β and not histamine. IL-1β is essential for the differentiation of Th17 cells,[11] whose major interleukin, IL-17, induces a variety of neutrophil-activating cytokines such as IL-1, IL-6, tumour necrosis factor-α, CXCL8 and granulocyte colony-stimulating factor.[12] In contrast, Li *et al.*[13] reported that neutrophils are the predominant source of IL-17A, and IL-17A from neutrophils regulates neutrophil infiltration in the kidney. Meng *et al.*[14] reported that a mutation in NLRP3 potentiates Th17 cell-dominant immune responses. NLRP3 mutant mice showed spontaneous skin inflammation in which the inflammatory cell infiltrate was mainly neutrophils. In addition, expression of IL-17A was increased in the inflamed skin as demonstrated by real-time polymerase chain reaction analysis. Blockage of IL-17A decreased skin inflammation and IL-17A expression in NLRP3 mutant mice. On the basis of these results, IL-17 might induce neutrophils into skin and cause the urticarial rash of CAPS. In the present case, we

showed that the dermis in an urticarial lesion of a patient with FCAS was infiltrated with IL-17-positive cells, which appeared to be neutrophils. This is the first report of IL-17 expression in an urticarial lesion of a patient with FCAS.

In conclusion, we hypothesize that the urticarial rash in FCAS is induced by IL-1β as a result of NLRP3 activation; IL-1β activates Th17 cells leading to IL-17-associated neutrophil recruitment into the dermis. In addition, the neutrophil infiltrate might secrete more IL-17, and cause further inflammation. The blockage of IL-1β by anakinra provides therapeutic benefit for patients with CAPS; however, long-term benefit and safety data are needed. We speculate that IL-17 might be a potential therapeutic target.

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Key words: *familial cold autoinflammatory syndrome, interleukin-17, NLRP3,* urticaria

Conflicts of interest: none declared.

Hair casts are a dermoscopic clue for the diagnosis of traction alopecia

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MADAM, Prolonged hair traction may cause cicatricial alopecia, which is more commonly localized at hair margins behind the frontal hairline or along the temporoparietal margin (marginal alopecia) but may involve any scalp area depending on the cause of traction. Traction alopecia is clinically characterized by loss and thinning of hair in the affected area. Traction alopecia due to traumatic hair care practice is a very common problem in African-American women and children. Hairstyling associated with considerable traction includes braids, twists, dreadlocks, braiding and weaving with extensions, and the use of overnight rollers. According to a large epidemiological study, traction alopecia is more common when traction is applied to relaxed hair and in subjects using combined hairstyles.[1,2]

However, marginal traction alopecia due to hairstyle can also occur in straight-haired individuals, particularly in girls wearing a tight ponytail. Occipital loss has been reported in women wearing a chignon or a bun, in nurses who used to fix their cap with a pin and recently in African-American girls wearing sculptured hairstyles requiring multiple pins.[3]

Hair casts are small freely movable cylindrical structures that envelop the proximal hair shaft and may clinically be misdiagnosed as nits.[4,5]

The formation of hair casts is a well-known sign of persistent hair traction. Traction-induced hair casts encircle a single hair shaft, consist of the inner and/or outer root sheath and are not characterized by prominent parakeratosis.[6] The presence of hair casts can give the clinician a clue in the differential diagnosis of alopecias affecting the scalp margin.

In the last 6 months we detected hair casts in five patients with traction alopecia including one Caucasian woman with patchy alopecia that had been previously misdiagnosed as alo-

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Original article

Distinct cytokine profiles of systemic-onset juvenile idiopathic arthritis-associated macrophage activation syndrome with particular emphasis on the role of interleukin-18 in its pathogenesis

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Abstract

Objectives. To compare the pro-inflammatory cytokine profiles and the cytokine kinetics in patients with secondary macrophage activation syndrome (MAS) due to systemic-onset juvenile idiopathic arthritis (s-JIA) and in both active and inactive disease states of s-JIA (but no MAS), with those demonstrated in EBV-induced haemophagocytic lymphohistiocytosis (HLH) and Kawasaki disease (KD), and to investigate the significance of IL-18 in the pathogenesis of s-JIA.

Methods. Five patients with MAS complicating s-JIA (MAS/s-JIA), 10 with HLH due to EBV infection (EBV-HLH), 22 with KD and 28 healthy controls were analysed. Cytokine concentrations (IL-18, IL-6, neopterin and TNF-γ receptor Types I and II) were quantified in serum by ELISA. Results were compared with clinical features of MAS/s-JIA, including ferritin concentrations.

Results. Serum IL-18 concentrations in MAS/s-JIA patients were significantly higher than those in EBV-HLH or KD patients ($P < 0.05$). Serum IL-6 concentrations in KD patients were significantly higher than those in EBV-HLH or MAS/s-JIA patients. Serum neopterin concentrations in EBV-HLH patients were significantly higher than those in MAS/s-JIA or KD patients. Serum IL-18 correlated positively with the following measurements of disease activity: CRP, ferritin, lactate dehydrogenase and other cytokines ($P < 0.05$). Serum concentrations of IL-18 in s-JIA patients remained elevated in the inactive phase of disease, whereas clinical parameters and other cytokines normalized.

Conclusions. IL-18 may be an important mediator in s-JIA. Although serum IL-18 concentrations correlated with markers of the disease activity, IL-18 concentrations remained elevated even when other markers of disease activity normalized. Serum IL-18 concentration may be a promising indicator of the disease activity. The cytokine release pattern in MAS/HLH is different among patients with different aetiologies. Monitoring the cytokine profile, including IL-18, may be useful for differentiation of MAS/HLH and evaluation of disease activity in s-JIA.

Key words: Macrophage activation syndrome, Systemic juvenile idiopathic arthritis, Interleukin-18.

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Introduction

Macrophage activation syndrome (MAS) is a severe, potentially life-threatening complication of childhood systemic inflammatory disorders [1, 2]. It is clinically characterized by fever, hepatosplenomegaly, lymphadenopathy, profound depression of all the three blood cell lines, deranged liver function, intravascular coagulation and CNS dysfunction. Among paediatric rheumatic

diseases, MAS occurs most often in children with systemic-onset juvenile idiopathic arthritis (s-JIA) and is less common in those with other rheumatic diseases, including polyarticular JIA, SLE and Kawasaki disease (KD) [3, 4]. MAS accounts for much of the significant morbidity and mortality observed with s-JIA. A variety of triggers have been implicated in the pathogenesis of MAS associated with s-JIA, including viral infections, NSAID therapy, MTX and etanercept [1, 3, 5]. The hallmark of this syndrome is excessive activation and proliferation of T lymphocytes and macrophages [6]. MAS bears a close resemblance to a group of haemophagocytic lymphohistiocytosis (HLH) syndromes and should be considered among the secondary causes of HLH [7, 8]. Massive hypercytokinaemia is strongly associated with the pathogenesis of MAS/HLH [3]; however, the kinetics of cytokine release in patients with MAS/HLH is still unclear.

IL-18 was originally described as an INF-γ-inducing factor mainly produced by the activated macrophage lineage cells [9, 10]. IL-18 stimulates a variety of inflammatory responses, enhances proliferation and activity of T cells and NK cells and shifts Th-cell balance towards the Th1 response [11, 12]. IL-18 has been reported to enhance the production of Th2 cytokines and IgE and recruitment of eosinophils, suggesting that IL-18 can also regulate allergic inflammation [13]. Some reports have recently shown that serum concentrations of IL-18 are highly elevated in patients with s-JIA [14–16].

To assess the kinetics of cytokine release during MAS/HLH, we measured the concentrations of serum cytokines, including IL-18, IL-6, neopterin and TNF-γ receptor Types I (sTNF-RI) and II (sTNF-RII) in patients with MAS/s-JIA. We compared them with the concentrations in patients with HLH due to EBV infection (EBV-HLH) and KD, which are both characterized by prominent and systemic inflammation in children. We determined the correlation between the concentrations of such markers of cytokine release with measures of disease activity and severity in order to clarify the importance of IL-18 in the pathogenesis of not only MAS but also s-JIA.

Materials and methods

Patients and samples

Serum samples were obtained from 5 patients with MAS as a complication of s-JIA, 10 with EBV-HLH, 22 with KD and 28 age- and sex-matched healthy controls (HCs) {age [MAS/s-JIA: 5.8 (6.8) years; control: 8.8 (7.3) years]}. Samples from MAS/s-JIA patients were also obtained during both the active and inactive phases of the s-JIA disease, but where MAS was not present. Diagnosis of s-JIA was based on the ILAR criteria [17]. MAS was diagnosed based on the combination of cytopenias affecting at least two cell lines, coagulopathy and liver dysfunction (Table 1), according to the guidelines proposed by Ravelli *et al.* [18]. The criteria for the active phase of s-JIA was defined as follows: active arthritis, fever, rash, hepatosplenomegaly, generalized lymphadenopathy, active uveitis and serositis, as well as increased ESR and CRP concentrations, but where criteria noted in the guidelines for MAS proposed by Ravelli *et al.* [18] were not fulfilled. The criteria for the inactive phase of s-JIA on medication were as follows: no clinical symptoms that can be seen in active phase as well as normal ESR and CRP concentrations. All patients of EBV-HLH fulfilled the diagnostic criteria for EBV-HLH [19], positivity for the EBV genome in the blood and bone marrow and other tissues (determined by PCR, Southern blot and/or *in situ* hybridization for EBV encoded RNA) and positive anti-viral capsid antigen-specific-IgG. Diagnosis of KD was based on the classic clinical criteria [20]. Serum was separated from cells, divided into aliquots, frozen and stored at −80°C until use. This study was approved by the Institutional Review Board at Kanazawa University and all specimens were used after the receipt of informed consent.

TABLE 1 Clinical characteristics of patients with s-JIA during the acute phase of MAS

Case	1	2	3	4	5
Age, years	11	1	15	0	2
Sex	F	M	F	M	M
Disease duration, months	10	1	2	0	1
Fever	+	+	+	+	+
Systemic JIA rash	+	+	+	−	+
Arthritis	+	−	+	+	−
Hepatosplenomegaly	+	+	+	+	+
Lymphoadenopathy	+	+	+	−	+
WBC × 1000/μl	2.4	25.3	15.95	5.4	80
Haemoglobin, g/dl	13.4	9.1	13.8	7.8	9.3
Platelets × 1000/μl	5.6	19.2	11.7	10.7	12.9
CRP, mg/dl	17.9	10.3	2.6	0.79	4.69
ALT IU/l	26	145	2437	444	195
AST IU/l	62	241	1382	581	296
LDH IU/l	331	988	2925	1528	1318
Ferritin, ng/ml	13639	1912	7000	19600	729.6

F: female; M: male; WBC: white blood cells; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase.

Quantification of serum cytokines

Serum concentrations of IL-18, IL-6, neopterin, sTNF-RI and sTNF-RII were evaluated by commercial ELISA according to the manufacturer's instructions (IL-18: MBL, Nagoya, Japan; IL-6, sTNF-RI and sTNF-RII: R&D Systems, Minneapolis, MN, USA; neoptrin: IBL, Hamburg, Germany).

Statistical analysis

Within-group comparisons were analysed by the Mann–Whitney test. Correlations were expressed using the Spearman's rank correlation coefficient. For the analysed measures, $P < 0.05$ was considered significant.

Results

Cytokine release in MAS patients

We determined serum concentrations of cytokines, including IL-6, IL-18, sTNF-RI and sTNF-RII, in patients

with MAS complicated with s-JIA (MAS/s-JIA) and compared them with the concentrations in patients with EBV-HLH or KD. It is noteworthy that the magnitude of the difference in serum IL-18 concentrations between patients with MAS/s-JIA and the other patient groups was overwhelming in comparison with that of other cytokine concentrations. As shown in Fig. 1, serum IL-18 concentrations in patients with MAS/s-JIA (median 122 500, range 101 000–830 000 pg/ml) and active phase of s-JIA (median 130 000, range 56 500–203 000 pg/ml) were significantly higher than those in patients with EBV-HLH (median 3825, range 1720–14 800 pg/ml), KD (median 279.5, range 180–560 pg/ml) and HCs (median 140.5, range 76–255 pg/ml) ($P < 0.05$). Serum IL-18 concentrations in patients with s-JIA were markedly elevated even in the inactive phase of s-JIA (median 6025, range 3730–12 000 pg/ml). Other cytokines that were elevated during active disease in the MAS/s-JIA group normalized when patients were in clinical remission. Serum IL-18 concentrations were significantly higher in active s-JIA patients compared with the elevated concentrations also seen in patients with active EBV-HLH ($P < 0.05$). These findings indicate that abnormal production of IL-18 appears to be highly specific for s-JIA.

Serum neopterin concentrations in patients with EBV-HLH (median 68, range 46–135 nmol/l) were higher than those in patients with MAS/s-JIA (median 46, range 10.5–122 nmol/l), KD (median 13.5, range 7–50 nmol/l) and HCs (median 4.35, range 1.8–9.5 nmol/l). Serum IL-6 concentrations in patients with KD (median 57, range 22–310 pg/ml) were higher than those in patients with MAS/s-JIA (median 8.7, range 5–22 pg/ml), EBV-HLH (median 14.3, range 0.5–106 pg/ml) and HCs (< 3.0 pg/ml). Interestingly, serum neopterin and sTNF-RII concentrations in patients with MAS/s-JIA were significantly higher than those in patients with active phase of s-JIA. Because many inflammatory cytokines are associated with the pathogenesis of MAS/HLH, we believe that monitoring the cytokine profile in combination with these cytokines might be more useful for evaluating disease activity. Consequently, we tried to represent the cytokine profile with a radar chart (Fig. 2). The pattern of the cytokine profile was characteristic in each background (Fig. 2).

Markedly elevated concentrations of serum IL-18 in patients with the active phase of s-JIA and MAS

To investigate the relevance of IL-18 to the pathogenesis of s-JIA, serum concentrations of IL-18 were serially monitored in all five cases of s-JIA (Fig. 3A–E). The concentration of serum IL-18 both rapidly and markedly rose with the development of the complication of MAS, but gradually reduced after this manifestation resolved with immunosuppressive therapy including corticosteroid and ciclosporin. However, even a few weeks after normalization of other indicators of the inflammatory reaction such as LDH, IL-18 concentrations were still well above the value of HC. In Case 1, MAS was frequently complicated in this phase with high concentrations of serum IL-18 (Fig. 3A). Since serial monitoring of serum concentrations of IL-18 was started, the patient suffered three relapses but could be treated before MAS was complicated (Fig. 3A). The pattern of cytokine profile of MAS/s-JIA is similar in all cases. Serum concentrations of IL-18 in patients with s-JIA were markedly elevated even in the inactive phase. The other cytokines were detected at significant concentrations in patients with MAS/s-JIA but was undetectable during remission (Fig. 4).

Correlation between serum IL-18 concentrations and measures of disease activity in clinical course of five cases of s-JIA

Since the concentrations of serum ferritin, LDH, aspartate aminotransferase and CRP are clinically used as indicators for disease activity of s-JIA, their concentrations were compared with those of IL-18. The concentrations of serum IL-18 correlated positively with each of these indicators ($P < 0.0001$; Fig. 5A–D). However, even during the clinically inactive phase after remission from MAS, concentrations of serum IL-18 remained extremely elevated, although other clinical parameters were normalized.

Correlation between serum IL-18 and other cytokines in the clinical course of five cases of s-JIA

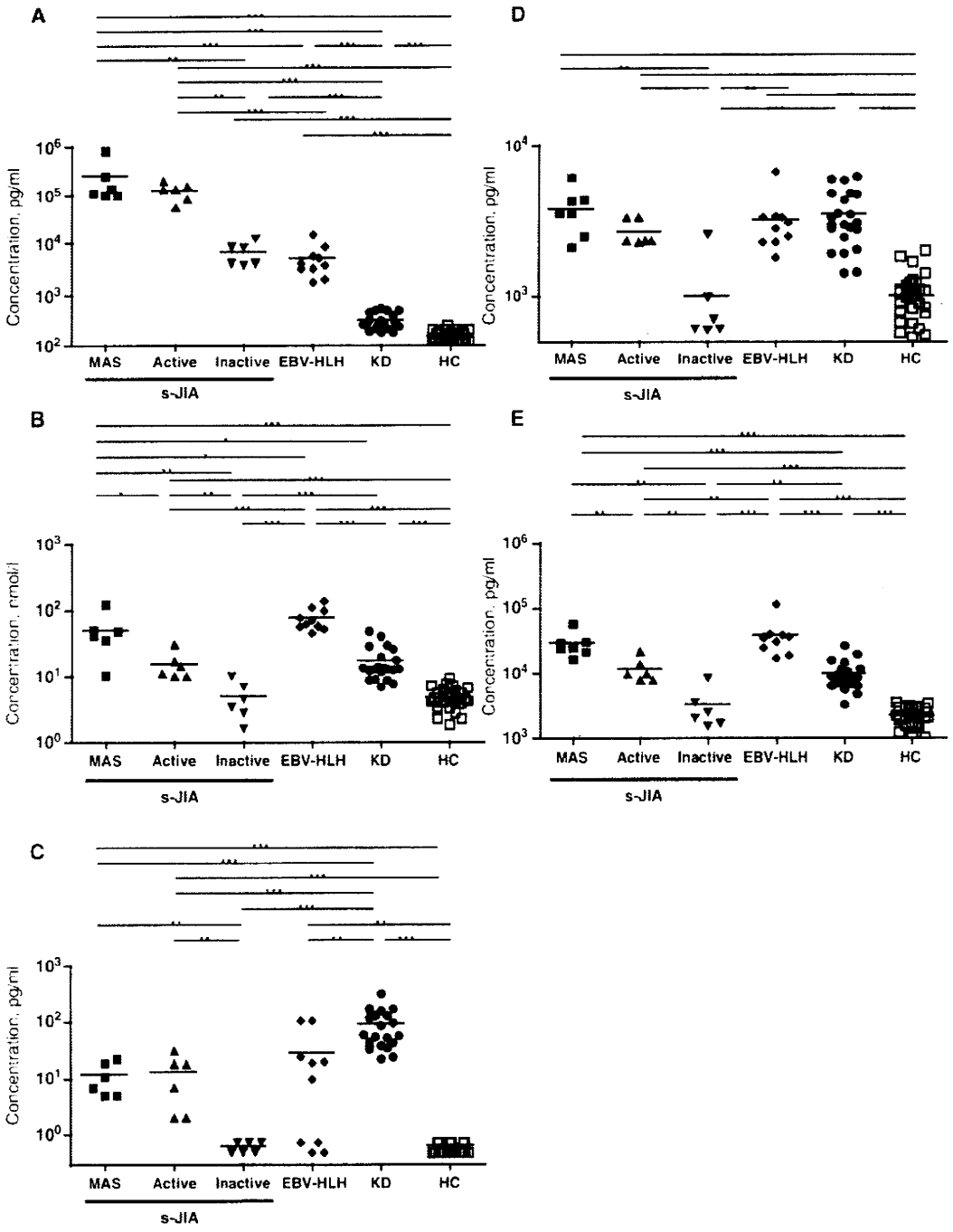
The concentrations of serum IL-18 correlated positively with the concentrations of serum neopterin, IL-6, sTNF-RI and sTNF-RII ($P < 0.0001$; Fig. 5E–H). Although the concentrations of serum IL-6 and neopterin were normalized in the inactive phase, concentrations of serum IL-18 remained significantly elevated.

Discussion

MAS is a severe, potentially life-threatening complication characterized by excessive activation of well-differentiated macrophages, resulting in fever, hepatosplenomegaly, lymphadenopathy, severe cytopenia, serious liver disease, intravascular coagulation and neurological involvement [1, 2]. MAS bears close resemblance to a histiocytic disorder, namely secondary HLH. HLH is a better defined entity observed in a heterogeneous group of diseases, including infections, neoplasms, haematological conditions and autoimmune disorders. It has been suggested that MAS should be replaced with the term autoimmune disease-associated reactive HLH [7, 8]. The hallmark of this syndrome is excessive activation and proliferation of T lymphocytes and macrophages [6]. Massive hypercytokinaemia produced by activated inflammatory cells is strongly associated with the pathogenesis of MAS/HLH; however, the kinetics of cytokine release in patients with MAS/HLH have not been analysed completely.

In the present study, the concentrations of serum IL-18 in patients with s-JIA were markedly increased. In contrast, the concentrations of serum neopterin and IL-6 were significantly increased in patients with EBV-HLH and KD, respectively. These findings show that the pattern

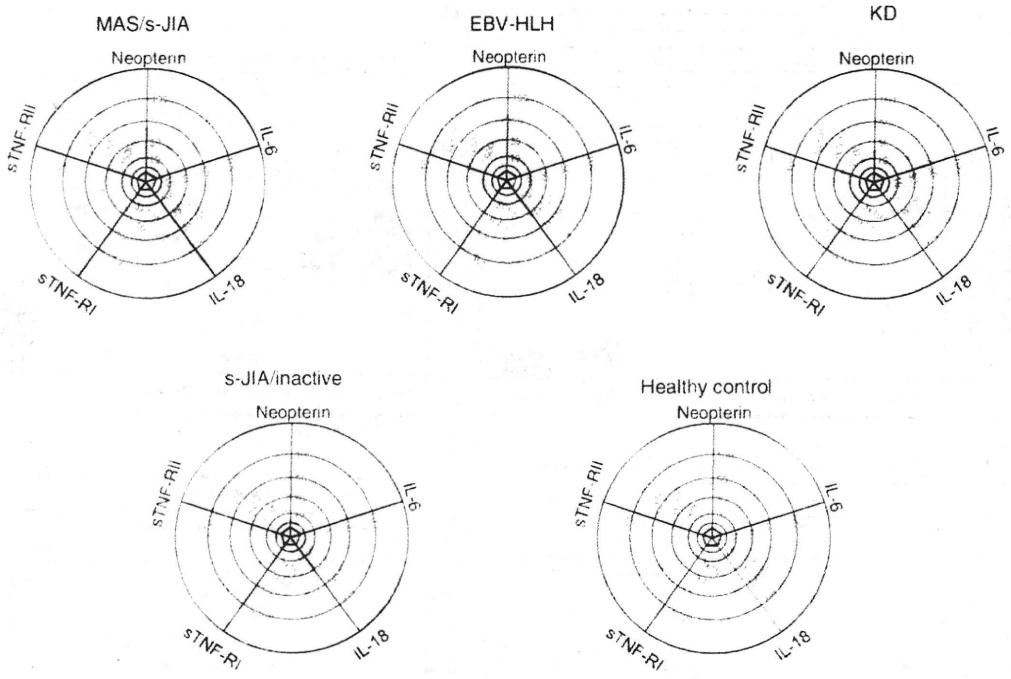
FIG. 1 Serum cytokine concentrations in different patient groups. Serum concentrations of (**A**) IL-18, (**B**) neopterin, (**C**) IL-6, (**D**) sTNF-RI and (**E**) sTNF-RII from the different patient groups are shown. Bars represent median values. Statistically significant differences between each patient group are shown as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.



of cytokine release is different among patients with different conditions, although clinical characteristics bear a close resemblance.

Because many inflammatory cytokines are associated with the pathogenesis of MAS/HLH, we proposed that monitoring the cytokine profile in combination with the individual cytokines might be more useful for evaluating the disease activity. Consequently, we tried to represent the cytokine profile with a radar chart (Fig. 2). The pattern of the cytokine profile was characteristic for each disease

FIG. 2 Cytokine profiles with radar charts in the different patient groups. Representative profiles of serum cytokines including, neopterin, IL-6, IL-18, sTNF-RI and sTNF-RII are shown for different patient groups. Overlaid inner green pentagons show the mean values of HCs.



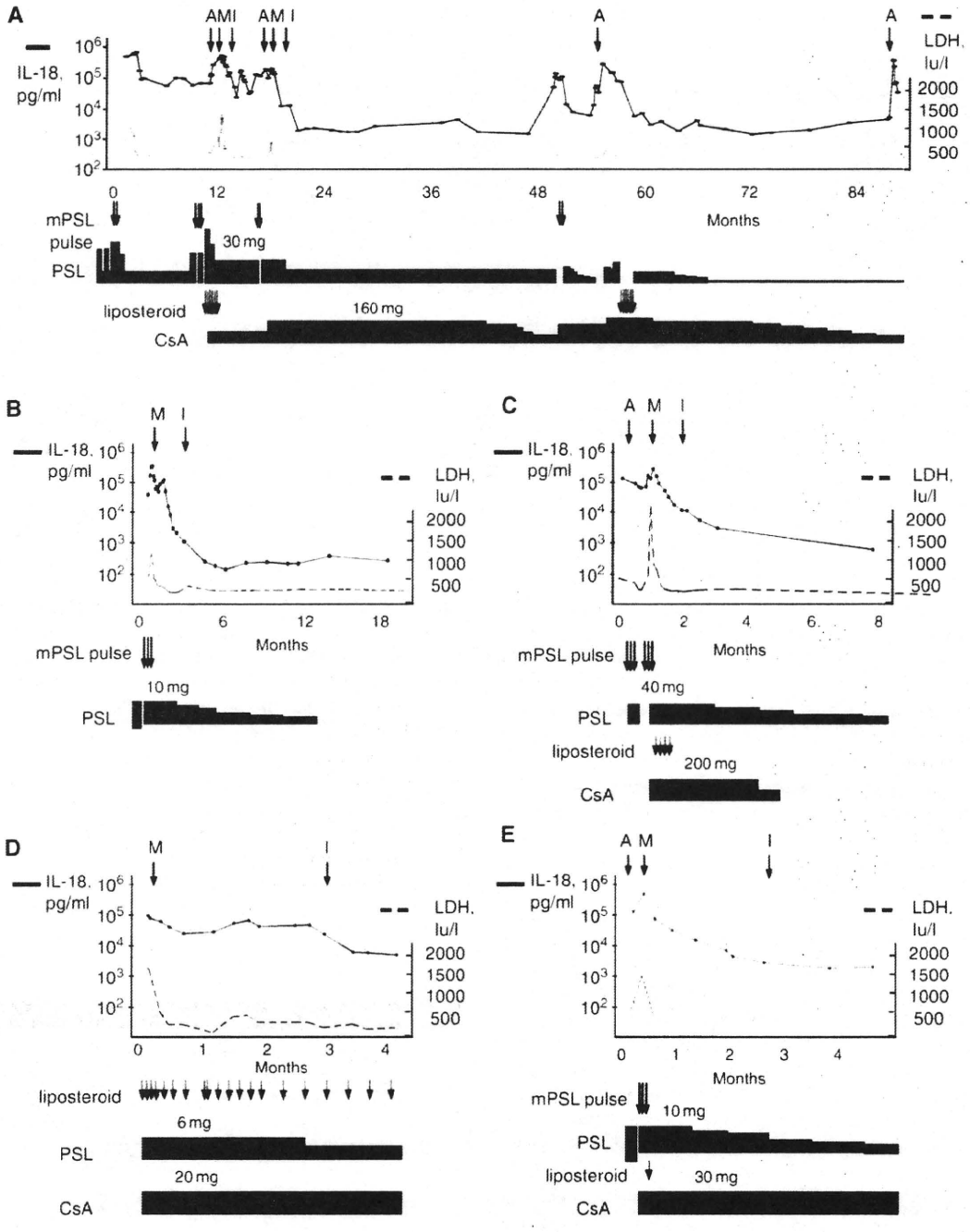
(Fig. 2) and similar in all cases of s-JIA/MAS (Fig. 4). In the acute phase of MAS/HLH, it is often difficult to differentiate the patients' primary disease, but monitoring the cytokine profile might be very useful in achieving early diagnosis and therapeutic decision making.

In the present study, it is noteworthy that the magnitude of the difference in serum IL-18 concentrations between patients with MAS/s-JIA and all other patients was significantly elevated in comparison with that of all the other cytokine concentrations. Serum IL-18 concentrations in patients with MAS/s-JIA and during active disease flares of s-JIA were significantly higher than those in patients with EBV-HLH and KD; these concentrations positively correlated with the measures of disease activity as well as other cytokines. Interestingly, the concentrations of serum IL-18 in patients with s-JIA dropped in the inactive phase of the disease but remained elevated compared with controls and patients with resolved EBV-HLH. Based on serial monitoring of serum IL-18 concentrations in Case 1, relapses of acute flares of s-JIA and the complication of MAS occurred in this phase, indicating that careful monitoring was needed for withdrawal of immunosuppressive drugs until the concentration of serum IL-18 normalized. The concentrations of serum IL-18 increased before other clinical indicators for disease activity of s-JIA including ferritin, LDH, ASP and CRP start to increase. These findings show that serum IL-18 may be a useful biomarker to predict impending disease flares. Interestingly, the concentrations of neoptetrin and sTNF-RII were significantly higher in MAS/s-JIA phase than in the acute-phase s-JIA alone. These concentrations were also extremely high in EBV-HLH, which shows these might be a useful marker to predict the transition to MAS/s-JIA from the acute phase of s-JIA.

Our data indicate that abnormal production of IL-18 appears to be highly specific for s-JIA. However, it is still unknown what causes the induction of extremely high IL-18 concentrations in the serum of patients with s-JIA. IL-18 is the most effective at regulating NK cell activity [21, 22] and it has been reported that decreased NK cell function is found in s-JIA [23–25]. Recently, it was reported that the mechanism of the impaired NK cell function in s-JIA involves a defect in IL-18 receptor β phosphorylation [26]. Further study will be required, but non-functional IL-18/NK cell axis might be associated with the pathogenesis of s-JIA.

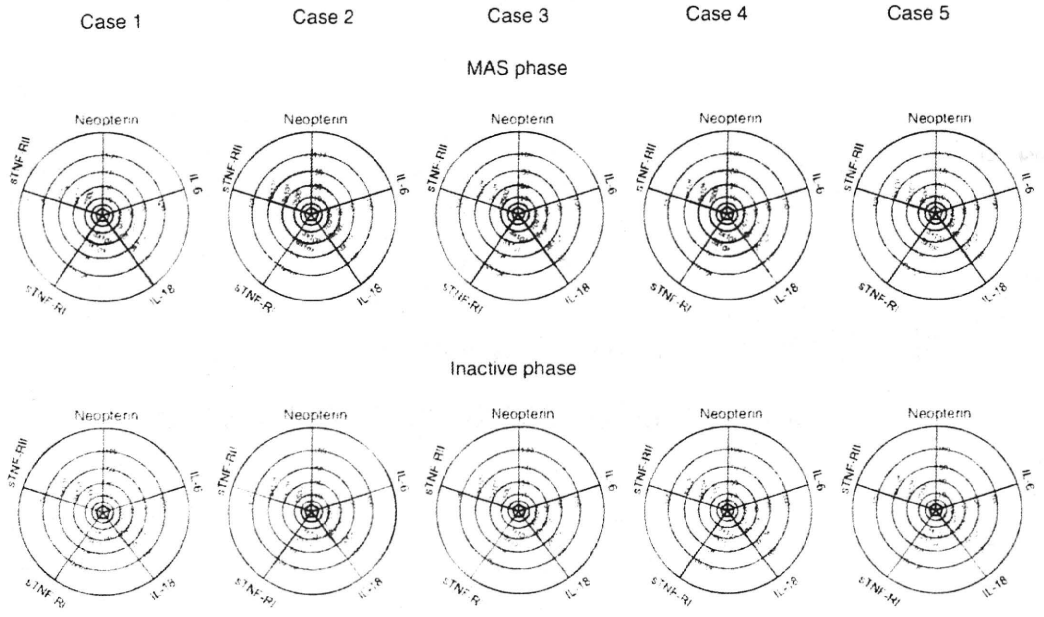
Some reports have recently shown that serum concentrations of IL-18 are also highly elevated in patients with adult-onset Still's disease (AOSD) [27–29]. It is still controversial whether s-JIA and AOSD are identical. It has been reported that no significant difference in clinical features such as systemic manifestations or joint lesions, or in prognosis exists between these two diseases [30]. In addition to these observations, our findings in the present study would be consistent with these two diseases being pathogenically identical.

FIG. 3 Longitudinal follow-up of serum IL-18 in five cases with MAS/s-JIA. (**A–E**) shows the longitudinal follow-up of Cases 1–5, respectively, of five patients with MAS/s-JIA. Changes in serum IL-18 (solid lines) and LDH (dotted lines) concentrations are shown in upper panels and details of therapeutic interventions are shown in the lower panels. Time points at which the blood is drawn are shown with arrows: M: MAS; A: active phase; I: inactive; PSL: prednisolone; mPSL, methylprednisolone.



During the clinically inactive phase after remission from MAS, the concentrations of serum IL-6 and neopterin normalized. However, interestingly, the concentrations of sTNF-RI and sTNF-RII increased in the inactive phase. These findings suggest that TNF-α is also associated with the pathogenesis of the inflammatory process in s-JIA, not only during exacerbation but also during the clinically silent phase of this disease.

FIG. 4 Cytokine profiles at different s-JIA disease phases. Cytokine profiles were examined by radar charts in five cases of s-JIA at MAS (upper panels) and inactive (lower panels) phases. Overlaid inner green pentagons show the mean values of HCs.



Tocilizumab (TCZ) is a humanized mAb recently developed against the human IL-6 receptor [31]. Although the introduction of TCZ has brought about a paradigm shift in the treatment of s-JIA [31], it has been reported that some cases were complicated with MAS during treatment with TCZ (Dr Syuji Takei, personal communication). This finding indicates that IL-6 blocking cannot prevent the onset of MAS. It is important to analyse the kinetics of cytokine release during MAS in these cases, and the results of such analysis may give us findings that are useful in understanding the role of each cytokines in the pathogenesis of MAS and s-JIA.

In the majority of patients with active s-JIA, coagulation abnormalities and greatly elevated serum ferritin concentrations are observed. Some rheumatologists suggest that MAS and s-JIA are included in the same spectrum [32]. Our findings suggest that MAS and s-JIA are at different ends of the same spectrum, which is based on the significant production of IL-18 by activated macrophages [14]. The clinical course at later stages of s-JIA is highly variable. Systemic features such as fever, rash and polyserositis tend to subside during the initial months and up to years of the disease. About half of the children with s-JIA recover almost completely, but the other half continue to show progressive involvement of additional joints. To address whether these two subpopulations have the same spectrum or not, further analysis of the kinetics of cytokine release may be useful.

In spite of the limitations, our results suggest that IL-18 plays a key role in the complex network involved in the inflammation of s-JIA and that serum IL-18 concentration is a promising indicator of the disease activity. The pattern of cytokine release in MAS/HLH is different among patients with different backgrounds. Monitoring of the cytokine profile may be useful for differentiation of the primary underlying disease in patients with MAS/HLH and evaluation of disease activity in s-JIA. Some other potentially useful markers to predict MAS in s-JIA, including soluble CD163 and soluble IL-2 receptor, have been reported [33]. Inclusion of some of these markers in cytokine profiling may improve the quality of the analysis. Further studies are needed to assess what combination of markers are the most useful for the monitoring of MAS/HLH.

Rheumatology key messages

- IL-18 is an important mediator in s-JIA.
- The cytokine release pattern is different among patients with different aetiologies of their in MAS/HLH.
- Monitoring the cytokine profile may be useful for the evaluation of disease activity in s-JIA.

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