

Pharmacogenetics of asthma in children

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Allergic diseases such as bronchial asthma and atopic dermatitis develop by a combination of genetic and environmental factors. Several candidate causative genes of asthma and atopy have been reported as the genetic factors. The clinical features of patients and causes of diseases vary. Therefore, personalized medicine (tailor-made medicine) is necessary for the improvement of quality of life (QOL) and for asthma cure. Pharmacogenetics is very important for personalized medicine. Here, we present the genetics and pharmacogenetics of asthma in children. Finally, we show the guideline for personalized medicine for asthma, particularly in childhood, including the pharmacogenetics of anti-asthmatic drugs, preliminarily produced by the authors.

Key Words: Pharmacogenetics; asthma; individualized medicine

INTRODUCTION

Allergic diseases such as bronchial asthma and atopic dermatitis develop as a result of a combination of genetic and environmental factors. Several candidate causative genes of asthma and atopy have been reported as the genetic factors.

Recently treatment/management guidelines on bronchial asthma and many other disorders have been published, and they are used in clinical practice. However, the clinical features of patients and causes of diseases vary. Therefore, personalized medicine (tailor-made medicine) is necessary for the improvement of quality of life (QOL) and for asthma cure. Pharmacogenetics is very important for personalized medicine.

Here, we present the genetics and pharmacogenetics of asthma in children.

GENETIC PREDISPOSITION TO DEVELOPMENT OF ASTHMA AND ATOPY

There is sufficient evidence to indicate that asthma is hereditary. A number of studies have shown an increased prevalence of asthma and the phenotype associated with asthma among the offspring of subjects with asthma compared with the offspring of subjects without asthma.

Many studies have shown that there is a genetic accumulation in the development of asthma and allergic disorders. Therefore, the development of asthma and allergic disorders is correlated

with some genes. We consider that multiple causative genes are involved, and not a single gene, because there are multiple pathogenesises of asthma and allergic reactions.

GENES RELATED TO DEVELOPMENT OF ASTHMA AND ATOPY

Many candidate genes related to the development of asthma and atopy have been identified, and different genes may be involved in different ethnic groups.¹ Among more than 100 genes determined from candidate gene association studies, 79 genes are associated with an asthma- or atopy-related phenotype in 2 or more independent study samples.²

Next, we focus on the several genes related to the development of asthma and atopy, in accordance with the various stages of allergic reaction and development of asthma and atopy.

HLA genes and asthma

HLA genes have been reported to be associated with bronchial asthma.³ Moreover, a relationship between the severity of childhood asthma and HLA type has been reported.⁴

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Genetic variation of cytokine signaling in atopy, and enhanced IgE production

IgE production is upregulated by Th2 cytokines, particularly interleukin-4 (IL-4), and is downregulated by Th1 cytokines, particularly interferon- γ (IFN- γ). Interleukin-12 (IL-12) and interleukin-18 (IL-18) are the cytokines that induce IFN- γ and downregulate IgE production.⁵ We review the genetic variation of the cytokine signaling in atopy, and enhanced IgE production.

This item is divided into two parts, (i) "*Genes related to upregulation of IgE production in asthma and atopy*" and (ii) "*Genes related to downregulation of IgE production in asthma and atopy*."

Genes related to upregulation of IgE production in asthma and atopy

Several linkage analyses and mutations of candidate genes of atopy (i.e., enhanced IgE production) have been reported. In 1989, Cookson et al.⁶ reported a linkage between IgE responses underlying asthma and rhinitis and chromosome 11q. Moreover, Shirakawa et al.⁷ reported that a common variant of Fc ϵ RI β on chromosome 11, Ile181Leu within the 4th transmembrane domain, shows significant association with positive IgE responses. Several associations have been noted between atopy and genes on the chromosome 5 cytokine cluster, including IL-4.

An Ile50Val (numbering for mature peptide) variant of human IL-4R α has been identified. In 1998, Mitsuyasu et al.⁸ reported that the Ile50Val variant of the IL-4R α chain upregulates IgE synthesis and is associated with atopic asthma. Ile50 is associated with atopic asthma but not with nonatopic asthma; Ile50 is specifically and significantly associated with increased total serum IgE levels and mite-specific IgE. The association with atopy was particularly strong in children.⁸ The data from both mouse and human cell lines strongly suggest that the Ile50 variant of IL-4R α significantly upregulates receptor response to IL-4, with a resultant increased activation of Stat6, and hence, increased cell proliferation and increased IgE production. Moreover, Shirakawa et al.⁹ noted genetic variants of IL-13.

Genes related to downregulation of IgE production in asthma and atopy

The genetic defects in the downregulation (brake) of IgE production, particularly in terms of IL-12 and IL-18 signalings, are discussed. We found that the reduced IFN- γ production by peripheral blood mononuclear cells (PBMCs) following stimulation with IL-12 or IL-18 is associated with heterozygous IL-12 receptor β 2 (IL-12R β 2) chain gene mutations (2496 del 91, 1577 A to G (Arg 313 Gly), 2799 A to G (His 720 Arg) or IL-18 receptor α (IL-18R α) chain gene mutation (del 950 CAG) in atopic subjects.^{10,11}

We identified a novel heterozygous single-nucleotide substitution 1400 T to C (Leu 467 Pro), in the seventh exon of the IFN- γ receptor 1 (IFN- γ R1) chain gene.¹² This substitution was detected in six of 89 allergic patients (including asthma), but not

in 72 nonallergic subjects. There was a difference in the Leu 467 Pro frequency between allergic and nonallergic subjects ($P < 0.05$). The serum IgE levels of the allergic patients with the Leu 467 Pro substitution were higher than those of the nonallergic subjects ($P < 0.001$). These results suggest that Leu 467 Pro in the IFN- γ R1 chain gene is one of the candidate susceptibility genes for asthma or atopic diseases.

Genetic variation of mediators and other molecules in asthma and atopy

LTC4S and asthma

The locus of leukotriene C4 synthase (LTC4S) is on chromosome 5q35 and has been associated with allergic diseases on the basis of a genomewide search. Cysteinyl leukotrienes (cysLTs) play important roles in asthma and can mediate bronchial smooth muscle constriction and increase mucous secretion, vascular permeability, and cellular infiltration.¹³ LTC4S converts LTA4 to LTC4 by conjugation to reduced glutathione. A single-nucleotide promoter polymorphism (A-444C) in LTC4S has been associated with aspirin-sensitive asthma,^{14,15} although recent studies have found no association between this promoter polymorphism and aspirin-sensitive asthma.¹⁶

Very recently, we have reported that a novel single-nucleotide substitution 10G>A (Glu 4 Lys) in LTC4S is associated with asthma.¹⁷

nNOS and asthma

Nitric oxide (NO) is produced by a group of enzymes referred to as nitric oxide synthase: endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS). The association of some nNOS markers with asthma or related phenotypes has been reported.¹⁸

Genetic defects in target organs in asthma and atopy

ADRB2 and asthma

There was no relationship between β 2-adrenergic receptor (ADRB2) polymorphisms and asthma prevalence, but the Gly-16 variant was apparently associated with a more severe form of asthma.¹⁹ Subsequently, Turki et al.²⁰ found that the Gly-16 allele is more frequent among subjects with nocturnal asthma than among nonnocturnal asthmatics.

ADAM33 and asthma

Van Eerdewegh and Holgate et al.²¹ performed a genomewide scan on 460 Caucasian families and identified a locus on chromosome 20p13 that is linked to asthma (Log_{10} of the likelihood ratio LOD, 2.94) and bronchial hyperresponsiveness (LOD, 3.93). A survey of 135 polymorphisms in 23 genes identified the ADAM33 gene as being significantly associated with asthma using case control, transmission disequilibrium and haplotype analyses ($P = 0.04$ - 0.000003). ADAM proteins are membrane-anchored metalloproteases with diverse functions, which include the shedding of cell-surface proteins such as cytokines

and cytokine receptors. The identification and characterization of ADAM33, a putative asthma susceptibility gene identified by positional cloning in an outbred population, should provide insights into the pathogenesis and natural history of this common disease.

GENETIC CLASSIFICATION OF ATOPY AND ASTHMA

On the basis of many reports and our results, we present a new genetic classification of atopy and asthma in Fig. 1.²² There are four categories of genes that control the expression of allergic disorders, which include (i) antigen recognition, (ii) IgE production (downregulation=brake, and upregulation), (iii) production and release of mediators, and (iv) events on target organs. This genetic classification will facilitate the development of pharmacogenetics and personalized medicine.

PHARMACOGENETICS OF ASTHMA

The response of an individual patient to any given drug depends on several factors such as pathogenesis of disease, compliance, disease severity, and genetic background.²³ The great hope in pharmacogenetics is that it will help predict either treatment response (efficacy) or the risk of adverse drug reactions in the general population and that it will prove cost-effective to genotype individuals before treatment,²³ that is, for personalized medicine (tailor-made medicine). Here, we review the pharmacogenetics of anti-asthmatic drugs.

Pharmacogenetics of β 2-agonists

Beta2-agonists act via binding to the β 2-adrenergic receptor (ADRB2), that is, a cell surface G-protein-coupled receptor. The β 2-adrenergic receptor has several polymorphisms in the coding region, such as Arg 16 Gly, Gln 27 Glu, and Thr 164 Ile. The

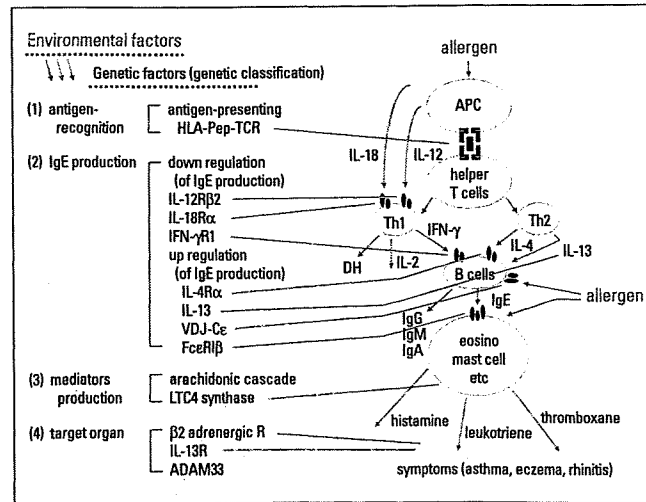


Fig. 1. A new genetic classification of asthma and atopy.

Arg 16 Gly and Gln 27 Glu are functionally important. Patients with homozygous for Arg 16 did worse clinically in terms of the major end points and showed adverse effects, rather than homozygous for Gly 16.²⁴⁻²⁶

Pharmacogenetics of leukotriene antagonists

Leukotrienes are very important mediators in asthma in children as well as in adults. Leukotrienes are released by eosinophils, mast cells, and alveolar macrophages. In the production of leukotrienes, several enzymes such as 5-lipoxygenase (ALOX5) and leukotriene C4 synthase (LTC4S) are important. LTC4S is a membrane-bound glutathione transferase, and synthesizes cysteinyl-leukotrienes, converting LTA4 to LTC4.

In the treatment with zafirlukast (one of the leukotriene receptor antagonists), patients with the homozygous for A-444 showed a lower FEV1 response than those with the A/C or C/C genotype.²⁷

Moreover, treatment with leukotriene receptor antagonists (montelukast or pranlukast) was more effective on adult or pediatric patients with the A/C or C/C genotype at position-444 (LTC4S-444) than on those with the A/A genotype.^{28,29}

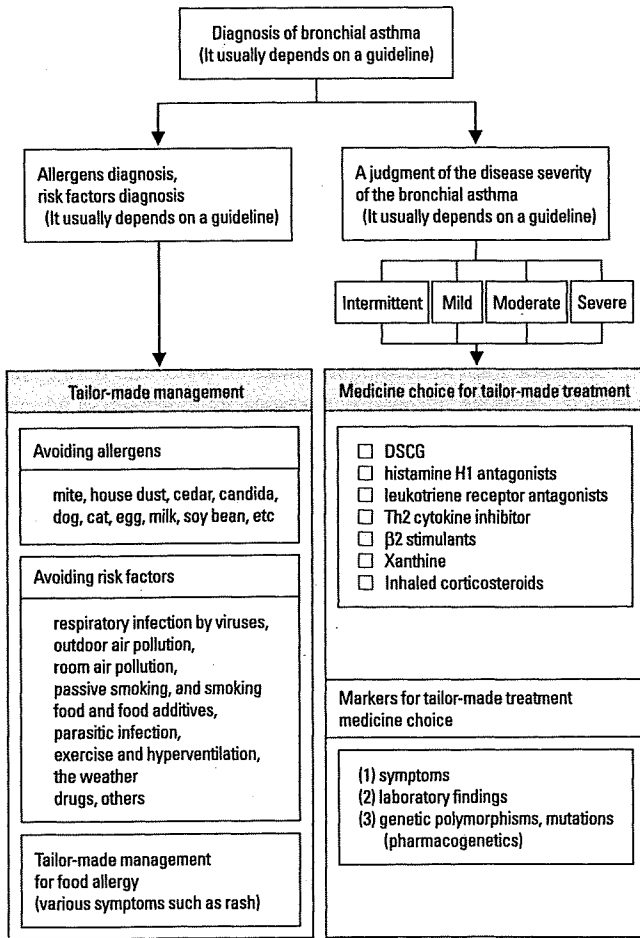


Fig. 2. Management of personalized medicine (tailor-made medicine) for asthma.

Pharmacogenetics of a Th2 cytokine inhibitor

Suplatast tosilate is a Th2 cytokine inhibitor.³⁰ In our recent study, treatment with suplatast tosilate was more effective in children without the -444 A/C polymorphism of the LTC4S gene, and in children without the IL-13 variant Arg 110 Gln. In children who responded well, IFN- γ production was significantly increased after treatment.

Pharmacogenetics of corticosteroids

Inhaled corticosteroids (ICS) are very effective for asthma in children as well as adults. However, there are patients with severe asthma who show no response to treatment with ICS. There are reports concerning the pharmacogenetics of corticosteroids. The IL-4 589T allele (589 C/T SNP) was found to be associated with ICS-resistant asthma.^{31,32} The TBX21 (T-bet) gene

(His 33 Gln) and the corticotropin-releasing hormone receptor 1 (CRHR1) gene are important for the pharmacogenetics of ICS.³³ Particularly in Caucasian children, the heterozygous TBX21 33 His/Gln individuals demonstrated a marked improvement in airway hyperresponsiveness compared with 33 His/His homozygotes on ICS therapy.^{33,34}

PERSONALIZED MEDICINE (TAILOR-MADE MEDICINE) FOR ASTHMA IN CHILDHOOD

The clinical features of patients and causes of diseases vary. Therefore, personalized medicine is necessary for the improvement of QOL and for asthma cure. Pharmacogenetics is very important for personalized medicine. Here, we show the guideline for personalized medicine for asthma, particularly in chil-

	DSCG	H1-antagonists	LTRA	Th2 cytokine inhibitor	long-acting theophylline	β 2 agonists		ICS
	effective	effective	effective	effective	(side effect)	effective	(non-effective)	effective
(1) Symptoms and clinical findings								
viral infection			○					
exercise induced asthma	○		○			○		
aspirin induced asthma	○		○					
complicated with allergic rhinitis			○					
complicated with atopic dermatitis		○						
(2) Laboratory findings								
reduced No. of eosinophil or basophil in peripheral blood				○				
reduced production of IFN- γ				○				
increased LT level in urine			○					
reduced expression of CysLT1R in eosinophil (less than 60%)			○					
(3) Gene polymorphisms and mutations (Pharmacogenetics)								
LTC4S A-444C (AA)				○				
LTC4S A-444C (AC or CC)			○					
IL-13 R110Q (R)				○				
ALOX5 (rs2115819 GG)			○					
MRP1 (rs119774 CT)			○					
CYP1A2 -3594(GT)					○			
ADRB2 R16G (R)							(○)	
TBX21 (T-bet) H33Q (H/Q or Q/Q)								○
CRHR1 (rs1876828)								○

Fig. 3. Medicine choice for personalized medicine based on the symptoms, laboratory findings and pharmacogenetics.

○: positive markers; (○): possible markers.

DSCG, disodium cromoglycate; H1-antagonists, histamine H1-receptor antagonists; LTRA, leukotriene receptor antagonists; ICS, inhaled corticosteroids; LT, leukotriene; LTC4S, leukotriene C4 synthase; ALOX5, 5-lipoxygenase; MRP1, Multidrug resistance-associated protein 1; ADRB2, β 2-adrenergic receptor; CRHR1, corticotropin-receptor 1 releasing hormone; R, Arginine; G, Glycine; H, Histidine; Q, Glutamine.

dren, including the pharmacogenetics of anti-asthmatic drugs, preliminarily produced by the authors (Figs. 2, 3).

Asthma develops by a combination of genetic and environmental factors. There are many environmental factors such as allergens (house dust, mites, foods, and pets so on), viral infections, exercise, passive smoking, and air pollution. The environmental factors are different for each patient. Therefore, it is very important to first eliminate each environmental factor for each patient and to take preventive measures against each environmental factor (Fig. 2), that is, the personalized preventive measures. The preliminary guideline for personalized medicine (tailor-made medicine) for asthma has been published, on the basis of various clinical symptoms, various laboratory findings, as well as pharmacogenetics of anti-asthmatic drugs.

Figure 3 shows the guideline for personalized medicine. For example, as one of the markers of symptoms, for viral-induced asthma, leukotriene receptor antagonists are effective. For exercise induced asthma, disodium cromoglycate (DSCG), leukotriene receptor antagonists, or β_2 -agonists are effective. As one of the laboratory findings, in the patients with poor IFN- γ production, Th2 cytokine inhibitor is effective. As one of the gene markers, in LTC4S-444 A/A type, Th2 cytokine inhibitor is effective, and in LTC4S-444 A/C or C/C type, leukotriene receptor antagonists are effective. In TBX21 (T-bet) 33 His/Gln or Gln/Gln, ICS is effective.

In the near future, the guidelines for the personalized medicine (tailor-made medicine) for asthma will become more available. For personalized medicine, it is necessary for us to obtain both the approval of an ethical committee and informed consent.

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REFERENCES

1. Global Initiative for Asthma (GINA): Global Strategy for Asthma Management and Prevention. 2006. Available from: <http://www.ginasthma.org>
2. Ober C, Hoffman S. Asthma genetics 2006: the long and winding road to gene discovery. *Genes and Immunity* 2006;7:95-100.
3. Juhn YJ, Kita H, Lee LA, Smith RW, Bagniewski SM, Weaver AL, Pankratz VS, Jacobson RM, Poland GA. Childhood asthma and human leukocyte antigen type. *Tissue Antigens* 2007;69:38-46.
4. Juhn YJ, Kita H, Bagniewski SM, Weaver AL, Pankratz VS, Jacobson RM, Poland GA. Severity of childhood asthma and human leukocyte antigens type. *J Asthma* 2007;44:163-8.
5. Kondo N, Matsui E, Kaneko H, Fukao T, Teramoto T, Kato Z, Onishi H, Nishimura A. Genetics of pediatric asthma. In: Pawankar R, Holgate ST, Rosenwasser LJ, editors. *Allergy frontiers volume 1 allergy frontiers: epigenetics, allergens and risk factors*. Heidelberg: Springer; 2009. 189-203.
6. Cookson WO, Sharp PA, Faux JA, Hopkin JM. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. *Lancet* 1989;1:1292-5.
7. Shirakawa T, Li A, Dubowitz M, Dekker JW, Shaw AE, Faux JA, Ra C, Cookson WO, Hopkin JM. Association between atopy and variants of the beta subunit of the high-affinity immunoglobulin E receptor. *Nat Genet* 1994;7:125-30.
8. Mitsuyasu H, Izuhara K, Mao XQ, Gao PS, Arinobu Y, Enomoto T, Kawai M, Sasaki S, Dake Y, Hamasaki N, Shirakawa T, Hopkin JM. Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. *Nat Genet* 1998;19:119-20.
9. Shirakawa T, Deichmann KA, Izuhara I, Mao I, Adra CN, Hopkin JM. Atopy and asthma: genetic variants of IL-4 and IL-13 signaling. *Immunol Today* 2000;21:60-4.
10. Matsui E, Kaneko H, Fukao T, Teramoto T, Inoue R, Watanabe M, Kasahara K, Kondo N. Mutations of the IL-12 receptor β_2 chain gene in atopic subjects. *Biochem Biophys Res Commun* 1999;266:551-5.
11. Watanabe M, Kaneko H, Shikano H, Aoki M, Sakaguchi H, Matsui E, Inoue R, Kato Z, Kasahara K, Fukutomi O, Kondo T, Kondo N. Predominant expression of 950delCAG of IL-18R alpha chain cDNA is associated with reduced IFN-gamma production and high serum IgE levels in atopic Japanese children. *J Allergy Clin Immunol* 2002;109:669-75.
12. Aoki M, Matsui E, Kaneko H, Inoue R, Fukao T, Watanabe M, Teramoto T, Kato Z, Suzuki K, Suzuki Y, Kasahara K, Kondo N. A novel single-nucleotide substitution, Leu 467 Pro, in the interferon-gamma receptor 1 gene associated with allergic diseases. *Int J Mol Med* 2003;12:185-91.
13. Henderson WR Jr. The role of leukotrienes in inflammation. *Ann Intern Med* 1994;121:684-97.
14. Sanak M, Hu S, Szczeklik A. Leukotriene C4 synthase promoter polymorphism and risk of aspirin-induced asthma. *Lancet* 1997;29:1599-600.
15. Kim SH, Hur GY, Choi JH, Park HS. Pharmacogenetics of aspirin-intolerant asthma. *Pharmacogenomics* 2008;9:85-91.
16. Van Sambeek R, Stevenson DD, Baldasaro M, Lam BK, Zhao J, Yoshida S, Yandora C, Drazen JM, Penrose JF. 5'flanking region polymorphism of the gene encoding leukotriene C4 synthase does not correlate with the aspirin-intolerant asthma phenotype in the United States. *J Allergy Clin Immunol* 2000;106:72-6.
17. Yoshikawa K, Matsui E, Kaneko H, Fukao T, Inoue R, Teramoto T, Shinoda S, Fukutomi O, Aoki M, Kasahara K, Kondo N. A novel single-nucleotide substitution, Glu 4 Lys, in the leukotriene C4 synthase gene associated with allergic diseases. *Int J Mol Med* 2005;16:827-31.
18. Gao PS, Kawada H, Kasamatsu T, Mao XQ, Roberts MH, Miyamoto Y, Yoshimura M, Saitoh Y, Yasue H, Nakao K, Adra CN, Kun JE, Morooka S, Inoko H, Ho LP, Shirakawa T, Hopkin JM. Variants of NOS1, NOS2, and NOS3 genes in asthmatics. *Biochem Biophys Res Commun* 2000;267:761-3.
19. Reihnsaus EM, Innis M, MacIntyre N, Liggett SB. Mutations in the gene encoding for the beta 2-adrenergic receptor in normal and asthmatic subjects. *Am J Respir Cell Mol Biol* 1993;8:334-9.
20. Turki J, Pak J, Green SA, Martin RJ, Liggett SB. Genetic polymorphisms of the beta 2-adrenergic receptor in nocturnal and non-nocturnal asthma. Evidence that Gly16 correlates with the nocturnal

- nal phenotype. *J Clin Invest* 1995;95:1635-41.
21. Van Eerdewegh P, Little RD, Dupuis J, Mastro RGD, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, Walsh A, Liu Z, Hayward B, Folz C, Manning SP, Bawa A, Saracino L, Thackston M, Benchekroun Y, Capparell N, Wang M, Adair R, Feng Y, Dubois J, FitzGerald MG, Huang H, Gibson R, Allen KM, Pedan A, Danzig MR, Umland SP, Egan RW, Cuss FM, Rorke S, Clough JB, Holloway JW, Holgate ST and Keith TP. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 2002;418:426-30.
 22. Kondo N, Matsui E, Kaneko H, Kato Z, Fukao T, Teramoto T, Shikano H, Aoki M, Onishi H, Tatebayashi K, Omoya K, Kondo M, Matsukuma E, Kasahara K, Morimoto N. Genetic defects in down-regulation of IgE production and a new genetic classification of atopy. *Allergy International* 2004;53:77-85.
 23. Hall IP. Pharmacogenetics of asthma. *Chest* 2006;130:1873-8.
 24. Israel E, Drazen JM, Liggett SB, Boushey HA, Cherniack RM, Chinchilli VM, Cooper DM, Fahy JV, Fish JE, Ford JG, Kraft M, Kunselman S, Lazarus SC, Lemanske RF, Martin RJ, McLean DE, Peters SP, Silverman EK, Sorkness CA, Szeffler SJ, Weiss ST, Yandava CN. The effect of polymorphisms of the beta(2)-adrenergic receptor on the response to regular use of albuterol in asthma. *Am J Respir Crit Care Med* 2000;162:75-80.
 25. Israel E, Chinchilli VM, Ford JG, Boushey HA, Cherniack R, Craig TJ, Deykin A, Fagan JK, Fahy JV, Fish J, Kraft M, Kunselman SJ, Lazarus SC, Lemanske RF Jr, Liggett SB, Martin RJ, Mitra N, Peters SP, Silverman E, Sorkness CA, Szeffler SJ, Wechsler ME, Weiss ST, Drazen JM; National Heart, Lung, and Blood Institute's Asthma Clinical Research Network. Use of regularly scheduled albuterol treatment in asthma: genotype-stratified, randomised, placebo-controlled cross-over trial. *Lancet* 2004;364:1505-12.
 26. Wechsler ME, Lehman E, Lazarus SC, Lemanske RF Jr, Boushey HA, Deykin A, Fahy JV, Sorkness CA, Chinchilli VM, Craig TJ, DiMango E, Kraft M, Leone F, Martin RJ, Peters SP, Szeffler SJ, Liu W, Israel E; National Heart, Lung, and Blood Institute's Asthma Clinical Research Network. beta-Adrenergic receptor polymorphisms and response to salmeterol. *Am J Respir Crit Care Med* 2006;173:519-26.
 27. Palmer LJ, Silverman ES, Weiss ST, Drazèn JM. Pharmacogenetics of asthma. *Am J Respir Crit Care Med* 2002;165:861-6.
 28. Sampson AP, Siddiqui S, Buchanan D, Howarth PH, Holgate ST, Holloway JW, Sayers I. Variant LTC(4) synthase allele modifies cysteinyl leukotriene synthesis in eosinophils and predicts clinical response to zafirlukast. *Thorax* 2000;55 Suppl 2:S28-31.
 29. Asano K, Shiomi T, Hasegawa N, Nakamura H, Kudo H, Matsuzaki T, Hakuno H, Fukunaga K, Suzuki Y, Kanazawa M, Yamaguchi K. Leukotriene C4 synthase gene A(-444)C polymorphism and clinical response to a CYS-LT(1) antagonist, pranlukast, in Japanese patients with moderate asthma. *Pharmacogenetics* 2002;12:565-70.
 30. Tamaoki J, Kondo M, Sakai N, Aoshiba K, Tagaya E, Nakata J, Isono K, Nagai A. Effect of suplatast tosilate, a Th2 cytokine inhibitor, on steroid-dependent asthma: a double-blind randomised study. *Lancet* 2000;356:273-8.
 31. Leung DY, Bloom JW. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol* 2003;111:3-22.
 32. Tantisira KG, Weiss ST. The pharmacogenetics of asthma: an update. *Curr Opin Mol Ther* 2005;7:209-217.
 33. Szalai C, Ungvári I, Pelyhe L, Tölgyesi G, Falus A. Asthma from a pharmacogenomic point of view. *Br J Pharmacol* 2008;153:1602-14.
 34. Tantisira KG, Hwang ES, Raby BA, Silverman ES, Lake SL, Richter BG, Peng SL, Drazen JM, Glimcher LH, Weiss ST. TBX21: a functional variant predicts improvement in asthma with the use of inhaled corticosteroids. *Proc Natl Acad Sci USA* 2004;101:18099-104.

Structural basis for the multiple interactions of the MyD88 TIR domain in TLR4 signaling

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Myeloid differentiating factor 88 (MyD88) and MyD88 adaptor-like (Mal) are adaptor molecules critically involved in the Toll-like receptor (TLR) 4 signaling pathway. While Mal has been proposed to serve as a membrane-sorting adaptor, MyD88 mediates signal transduction from activated TLR4 to downstream components. The Toll/Interleukin-1 receptor (TIR) domain of MyD88 is responsible for sorting and signaling via direct or indirect TIR–TIR interactions between Mal and TLR4. However, the molecular mechanisms involved in multiple interactions of the TIR domain remain unclear. The present study describes the solution structure of the MyD88 TIR domain. Reporter gene assays revealed that 3 discrete surface sites in the TIR domain of MyD88 are important for TLR4 signaling. Two of these sites were shown to mediate direct binding to the TIR domain of Mal. Interestingly, Mal–TIR, but not MyD88–TIR, directly binds to the cytosolic TIR domain of TLR4. These observations suggested that the heteromeric assembly of TIR domains of the receptor and adaptors constitutes the initial step of TLR4 intracellular signal transduction.

docking simulation | Mal | innate immunity | NMR | protein structure

Myeloid differentiating factor 88 (MyD88) is a cytosolic adaptor protein that plays essential roles in both innate and acquired immune responses by mediating signal transduction pathways that are initiated by Toll-like receptors (TLRs) and IL-1 and IL-18 receptors (IL-1R and IL-18R). MyD88 consists of an N-terminal death domain (DD) (approximately 90 aa residues), a C-terminal Toll/Interleukin-1 receptor (TIR) domain (approximately 150 aa residues), and a short connecting linker (1). In innate immune responses, the TIR domain of MyD88 has pivotal functions in the formation of signal initiation complexes involving the cytosolic domain of TLRs. The best characterized pathway is the TLR4 pathway, in which the cytosolic TIR domain of LPS-stimulated TLR4 interacts with the TIR domain of MyD88 (MyD88–TIR), in cooperation with another TIR-containing adaptor protein, MyD88 adaptor-like (Mal). Subsequently, signal is transmitted to the IL-1 receptor-associated kinase (IRAK) through an interaction between the death domains of MyD88 and IRAK. This eventually activates the transcription factors NF- κ B and activator protein 1 (AP-1) via a phosphorylation cascade (2).

MyD88 has been reported to be involved in signaling pathways initiated by all TLRs thus far reported, with the exception of TLR3 (3). Of the MyD88-dependent pathways involving TLR2, 4, 5, 7, and 9, only the TLR2 and TLR4 pathways require Mal for efficient signal transduction (4). TLR4 also possesses the MyD88-independent signaling pathway, which comprises other TIR-containing cytosolic adaptors, TIR domain-containing adaptor inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α and huntingtin-elongation-A subunit-TOR (HEAT) Armadillo motifs (SARM) (5). Therefore, in general, specific complexes involving more than one TIR-containing adaptor are likely to be required for initiation of each TLR signal transduction pathway.

Recently, Kagan and Medzhitov (6) revealed that MyD88 and Mal have distinctly different roles in TLR4 signaling: MyD88 serves as an essential “signaling adaptor,” which transmits signals from ligand-activated TLRs to downstream factors to initiate kinase-dependent signaling cascades, while Mal functions as a “sorting adaptor,” which recruits MyD88 to the plasma membrane via its PIP2 binding domain to promote interaction between MyD88 and activated TLR4 beneath the membrane. Indeed, Mal was shown to be dispensable for TLR4 signaling when MyD88 is fused to a PIP2 targeting domain. In the TLR4–TRIF pathway, TRAM has been proposed to serve as a sorting adaptor, which delivers TRIF to a specific membrane portion via its myristoylation site (7). These findings suggest that specific combinations of “sorting” and “signaling” TIR-containing adaptors might be involved in TLR signaling pathways.

The specificities of TIR–TIR interactions between adaptors, and between adaptors and TLRs, define the formation of various complexes that initiate TLR signaling pathways. However, little is known about the mechanism of heteromeric interactions between TIR domains. The crystal structures of cytosolic TIR domains of the membranous receptors, TLR1, TLR2, TLR10, and IL-1RAPL have been reported (8–10), and the homomeric TIR interfaces observed in the crystals have been described. However, the functional relevance of these homomeric interactions remains obscure because the formation of a homomeric dimer in these TIR domains has not been observed in solution (9, 10). Based on crystal structures and mutational data, several structural models have been proposed for heteromeric TIR–TIR interactions, which commonly suggest the importance of the so-called BB loop in these interactions (11, 12).

The present study describes the solution structure of MyD88–TIR using NMR spectroscopy. The isolated domain was shown to exist as a monomer in solution state on the basis of size-exclusion chromatography, although full-length MyD88 forms a dimer, which appears to be mediated via homomeric interactions within its death domain. By combining in vitro mutational binding experiments with an NF- κ B reporter system in mammalian cells, 2 surface sites were identified as binding interfaces for the TIR domain of the sorting adaptor Mal, one of which includes a critical residue for MyD88 function, whose mutation causes the pyogenic bacterial infections

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2Z5V).

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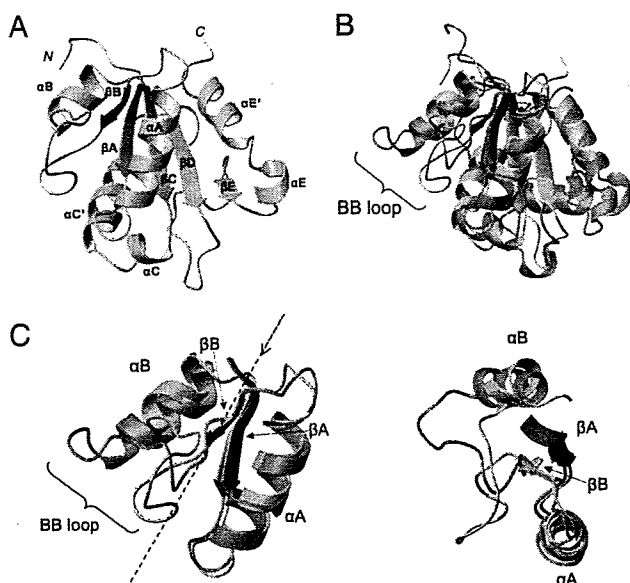


Fig. 1. Solution structure of the TIR domain of human MyD88. (A) A representative ribbon drawing of the NMR structure of MyD88, generated with MOLMOL 2K.2. The notation of the secondary structures (β A– β E, α A– α C, and α E) is based on TLR TIR domains. (B and C) A superimposed representation of MyD88-TIR (sky blue) and the crystal structure of the TIR domain of TLR2 (orange). Although the β -sheet cores are similar (rmsd = 0.90 Å), there are differences in some regions.

(13). Furthermore, the *in vitro* binding experiments demonstrated that MyD88-TIR does not directly bind to the cytosolic TIR domain of TLR4, while Mal-TIR does. The distal location of the Mal binding sites on the MyD88-TIR surface suggests that the TIR domain of MyD88 simultaneously interacts with 2 Mal-TIR molecules, which may provide a highly efficient scaffold for signal transduction.

Results

Structure Determination of the TIR Domain of MyD88. Based on sequence comparison between TIR domains, a region was selected that comprised residues 148–296 of human MyD88 and was used for structure determination by solution NMR spectroscopy (see Fig. S1). In the buffer used for structural studies, MyD88-TIR resided in a monomeric state, as assumed from size-exclusion chromatography. However, the death domain including internal domain (DD+ID) of MyD88 existed in a dimeric state (see Fig. S2). Therefore, the reported MyD88 dimerization was likely mediated by DD+ID but not by the TIR domain (14). The TIR domain structure of human MyD88 (residues 157–296), which presented the lowest overall energies in the 20 final structures generated by calculations, is shown in Fig. 1A. Statistics for the final 20 conformers are summarized in Table S1, which shows that the rmsd for the coordinates of backbone heavy atoms (N, C α , and C') of residues 157–185, 188–194, and 203–295 is 0.45 Å. The N-terminal 9 residues (148–156) displayed random coil propensity, which was characterized by the lack of medium-to-long range NOESY cross-peaks for the region. Hence, these residues were omitted from the figure and statistics for clarity.

Previous studies have indicated that 3 short, sequence motifs, called box 1–3 motifs, which are (F/Y)DA, RDXXPG, and FW, respectively, are conserved between TIR domains (see Fig. S1) (15). Of these, the box 2 motif, which resides in the so-called BB loop region, has been suggested to be important for TIR–TIR interactions and specificities (8, 16). In the calculated conformers of MyD88-TIR, a section of the BB loop, namely residues 194–208,

was not well converged. With the exception of Gly-201, Val-204, and Ser-206, the backbone amide resonances of these residues were not identified in 2D ^1H – ^{15}N hetero-nuclear single quantum coherence (HSQC) spectra, although resonances of some side chain protons were observed and assigned in HCCH-TOCSY and 3D ^{13}C -edited NOESY spectra. This region appeared to not form a single, definite structure, as judged from observations of a relatively few number of long-range NOEs. A $\{^1\text{H}\}$ – ^{15}N heteronuclear NOE experiment showed that some main-chain amide groups in the BB loop region, namely Gly-201, Val-204, and Ser-206, displayed NOE values of 0.67, 0.55, and 0.67, respectively, which were less than the average value for residues 157–297 (0.76 ± 0.09). These results implied that the BB loop was mobile in solution. The presumed conformational flexibility in the BB loop region might exert broadening effects due to chemical exchange, which results in absence of the backbone amide resonances of residues 195–200, 202, and 203. In addition, the turn region comprising residues 185–188, which follows helix α A, was poorly defined as the main chain amide resonances of Thr-185, Asp-186, Tyr-187, and Arg-188 could not be identified in the HSQC spectrum.

During the preparation of this manuscript, Rossi et al. released the solution structure of the TIR domain of human MyD88 in the Protein Data Bank (PDB ID: 2JS7). The overall folding was identical to our findings, despite minor differences. However, a detailed comparison of these 2 structures would not be appropriate, because of substantial differences in solution conditions, such as organic additives and pH. The structure of Rossi et al. was determined in a buffer containing 5% acetonitrile at pH 5.0, while the present structure determination was performed in a buffer at pH 6, with no organic solvent.

Structural Description of the TIR Domain of MyD88, and Comparison with Other TIR Domains. The MyD88 TIR domain structure (residues 157–296) comprised a central 5-stranded parallel β -sheet (β A– β E) surrounded by 4 α -helices (α A– α C and α E) (Fig. 1A). As predicted, the global fold was similar to what was observed in previously determined crystal structures of TIR domains of receptors TLR1, TLR2, TLR10, and IL-1RAPL (PDB codes: 1FYV, 1FYW, 2J67, and 1T3G, respectively). Of the known structures, the MyD88 TIR domain exhibited highest sequence similarity to TLR2. Fig. 1B and C show superimposed representations of the TIR domain structures from MyD88 and TLR2. While the β -sheet cores displayed high structural similarity, as indicated by an rmsd value for backbone N, C α , and C' of 0.90 Å, several regions displayed notable conformational differences. The largest structural discrepancy was observed in the region from the BB loop (Ser-194–Ala-208 of the MyD88 TIR domain) to α B (Fig. 1C). The BB loop was exposed to solvent in MyD88 and TLR2, but the direction in which the loops orient was markedly different. This was mainly due to a structural difference in the C-terminal region of the BB loop (residues 205–208), which precedes α B of MyD88. These residues adopted an extended conformation in MyD88, whereas corresponding residues are involved in an α -helix (α B) in TLR2. Therefore, α B of MyD88 was much shorter than TLR2. Another major conformational difference was the lack of an α -helix in the region between strands β D and β E (residues 257–273) of the MyD88 TIR domain (see Fig. S1). This region adopted an extended and a short helical coil conformation in MyD88, but the corresponding residues formed an α -helix (α D) in TLR2 (positions 266–270 in MyD88 numbering).

Cell-Based Functional Assays of the MyD88 TIR Domain in TLR4 Signaling Pathways. To explore residues that are important for function of the MyD88 TIR domain, we performed mutational analysis of the domain, using dominant negative effects of ectopically expressed isolated TIR domain (17). For the assay, a luciferase reporter system for NF- κ B activation was constructed in HEK293 cells, where MyD88-TIR or mutants harboring single amino acid substitutions of surface residues, was ectopically expressed. Expression of

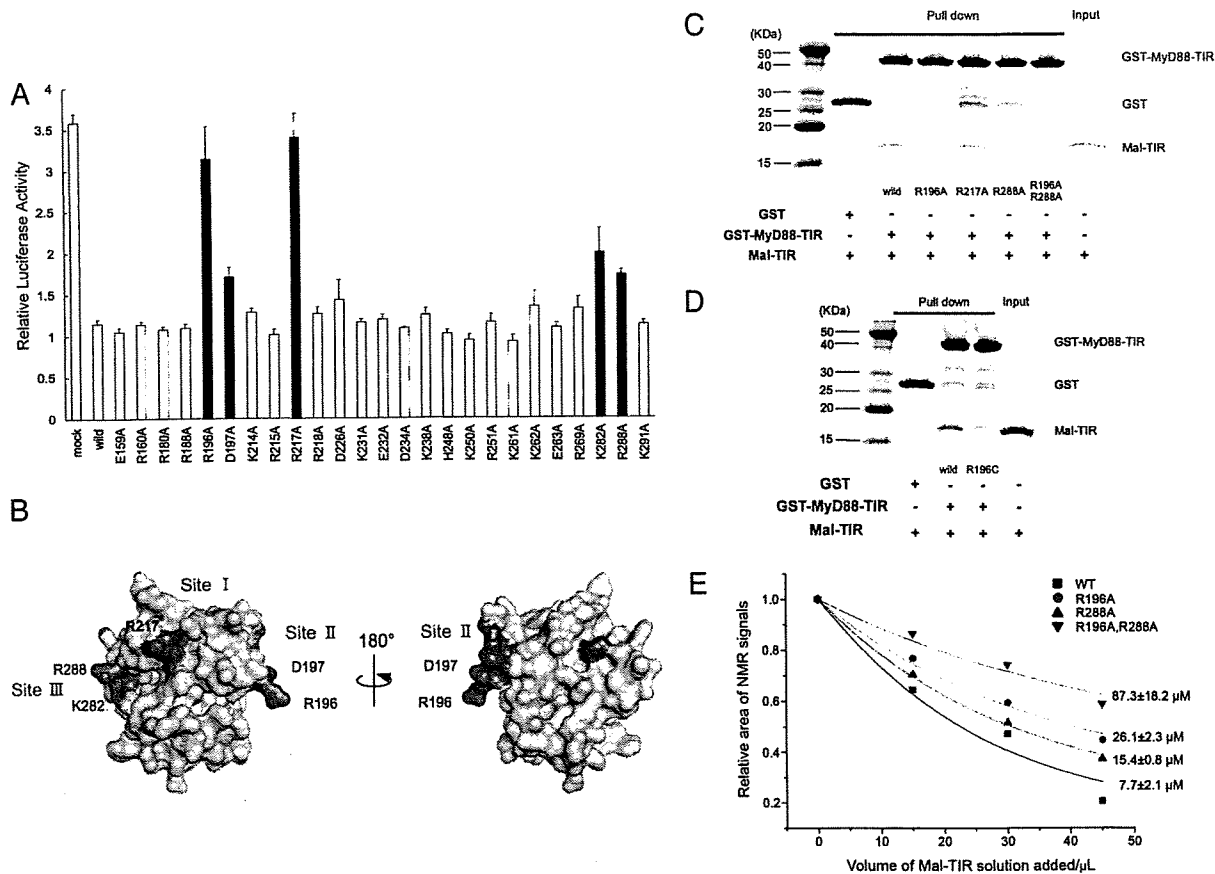


Fig. 2. Functional sites for signaling and binding sites for Mal. (A) The NF- κ B reporter gene assay of MD2 cotransfected with LPS-induced (1.0 μ g/ml) 293-hTLR4A-HA cells. In these graphs, each column indicates relative luciferase activity of stimulated cells over nonstimulated cells. Color code: black bars indicate a significant increased NF- κ B activity, compared with the wild-type group. The statistical significance of differences in luciferase activities between wild type and mutants was analyzed using the Dunnett's multiple comparison test. Statistical significance was assumed to be $P < 0.05$. (B) Results of the functional assays of LPS/TLR4 signaling presented on the 3D structure of the TIR domain of MyD88. The results of the functional assays are mapped onto the molecular surface of the MyD88 TIR domain. The amino acid residues judged to be significant by the luciferase assay are shown in red, while nonsignificant residues are shown in light brown. The conserved motifs of boxes 1–3 (FDA of box1, VLPG of box2, FW of box3) are shown in blue. (C) Assay of binding of Mal TIR domain and MyD88 TIR domain wild type or mutants. Alanine substitutions in Site II (R196A) or Site III (R288A) in MyD88 resulted in reduced interaction with Mal. The double alanine substituted mutant of Site II and Site III caused complete abolition of the interaction with Mal. (D) Cysteine substitution in R196 also caused reduced interaction with Mal. (E) Plots of relative integrated area of NMR signals, which were derived from NMR titration data, in a function of added volume of Mal-TIR into 15 N MyD88-TIR sample. The best-fit lines to the data, assuming a simple 1:1 complex model, are also shown. Apparent dissociation constants calculated are also indicated with standard errors (see Fig. S3). The black box, red circle, blue triangle, and green triangle indicate the dissociation curves of wild-type, R196A, R288A, and R196A-R288A, respectively.

MyD88-TIR, which lacked the N-terminal death domain and thus was supposedly unable to transmit signals, suppressed LPS-induced luciferase expression. This effect was presumably due to inhibition of signaling pathways by competitive binding of the isolated TIR domain to signaling components that interact with endogenous MyD88. When a TIR mutant harboring a substitution of a functionally important residue is expressed, such suppression is alleviated, which leads to higher LPS-induced luciferase activity than observed with a TIR domain harboring the wild-type sequence. It should be noted that the dominant negative effect of ectopically expressed TIR domain has been used for functional analysis of some key residues of MyD88-TIR in IL-1 signaling (11).

Results from luciferase assays of mutant-expressing cells upon LPS stimulation are shown in Fig. 2A. LPS addition to HEK293 cells expressing MD2 and TLR4 resulted in an approximately 3-fold increase in reporter activity, which was consistent with a previous report (18). Alanine substitution of 5 residues, Arg-196, Asp-197, Arg-217, Lys-282, or Arg-288 resulted in significantly reduced inhibitory effects in LPS-induced luciferase activity. Interestingly,

these 5 residues are closely associated with the box 1, 2, and 3 motifs, which are highly conserved across TIR domains (see Fig. S1). Arg-196 and Asp-197 are located within the box 2 motif. The side chains of Lys-282 and Arg-288 form a continuous protein surface with box 3 forming residues, and Arg-217 is located distant in the sequence, but proximal in space to the box 1 motif (Fig. 2B). Hence, we designated the sites that these residues form as Site II, Site III, and Site I, respectively.

Binding Sites of MyD88-TIR for Mal. Because Site I, Site II, and Site III of the MyD88 TIR domain are important for TLR4-mediated cellular responses following LPS stimulation, the involvement of these sites was examined in direct binding to the TIR domain of Mal (Mal-TIR), the sorting adaptor in MyD88-dependent TLR4 pathways (19). The effect of alanine substitution of Arg-196, Arg-217, or Arg-288 (which forms Site II, Site I, and Site III of the MyD88 TIR domain, respectively) on interactions with Mal-TIR was analyzed by GST pull-down assay. Mal-TIR was pulled down by wild-type MyD88-TIR. However, substitution of either Arg-196 or

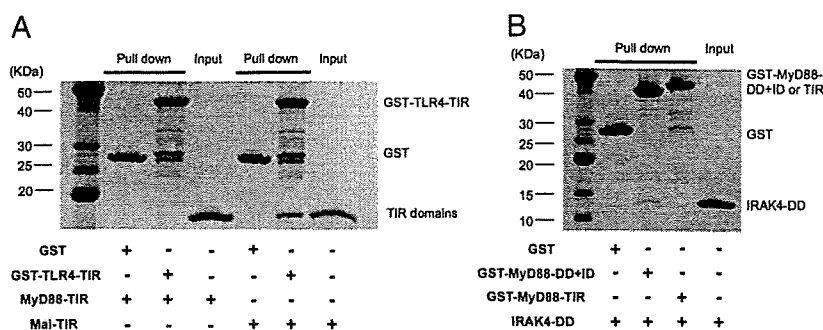


Fig. 3. Direct interactions of MyD88, Mal, TLR4, and IRAK4. (A) Binding assay of the TLR4 TIR domain with the MyD88 TIR domain or Mal TIR domain. GST-TLR4-TIR binds Mal-TIR but not MyD88-TIR. (B) Binding assay of the IRAK4 death domain with the MyD88 TIR domain or death domain. IRAK4-DD binds the MyD88-DD+ID but not MyD88-TIR.

Arg-288 resulted in moderate, but significant, decreases in MyD88-TIR affinity for Mal-TIR. The MyD88-TIR affinity of the Arg-196 and Arg-288 double-substituted mutant for Mal-TIR was completely abolished (Fig. 2C). In contrast, the Arg-217 mutation had no significant effect. The results, therefore, suggested that Site II and Site III, but not Site I, contributed to the interface with Mal-TIR. In addition, Arg-196 substitution by cysteine, which was detected in MyD88 deficiency patients (13), also caused reduced interaction with Mal-TIR (Fig. 2D). The effect of alanine substitution of those arginine residues on interactions with Mal-TIR was also examined by observing 2D ^1H - ^{15}N correlation NMR spectra of ^{15}N -labeled MyD88-TIR and its derivative in the absence or presence of various concentrations of nonlabeled Mal-TIR. The ^{15}N -labeled MyD88-TIR signals uniformly decreased upon titration of Mal-TIR (see Fig. S3). Signal attenuation was presumably due to increased apparent molecular weight upon complexation or chemical exchange (Fig. 2E). A MyD88-TIR mutation of either Arg-196 or Arg-288 caused moderate effects. However, double mutations of these residues resulted in large effects on signal attenuation, suggesting that signal attenuation was due to interactions between TIR domains of MyD88 and Mal. The apparent dissociation constants were estimated from the signal attenuation of wild-type and mutant MyD88 as previously described (Fig. 2E) (20). These results indicated that contributions from Site II and Site III to the interaction were comparable to each other. It should be noted that the effect of tested alanine substitution on the structure of the TIR domain was minor and only limited to the region close to the mutational sites, as judged from the 2D spectra of those mutants. Thus, these substitutions do not affect the opposite functional surface. Therefore, allosteric effect was neglected in interpreting the data.

The Function of Site I in TLR4 Signaling. Site I could serve as an interaction site with cytoplasmic TIR domain of TLR4 (TLR4-TIR), as previously suggested (6). However, an interaction between TIR domains of MyD88 and TLR4 was not detected (Fig. 3A). The GST pull-down experiment in the present study further demonstrated that the DD of IRAK4 exhibited no detectable binding activity to MyD88-TIR, but rather bound to MyD88 that lacked the TIR domain (Fig. 3B). This indicated that Site I was not involved in interactions with the downstream effector IRAK4, although results from a previous study showed that a small region of TIR, which included box 1 motif, ID, and DD of MyD88, interacts with IRAK4 (21).

Discussion

The TIR domain is typically composed of 135–160 aa residues, with sequence conservation ranging from 20 to 30%. While the hydrophobic core residues are conserved, the surface exposed residues vary greatly between TIR domains. Consequently, the distribution

of surface electrostatic potential differs significantly between TIR domains (22), possibly underlying the differences in binding specificity. For TIR domains of membranous receptors, 4 structures from TLR1, TLR2, TLR10, and IL-1RAPL have been reported (8–10). When comparing these, the BB loop of MyD88 displayed the largest structural difference (Fig. 1C). The BB loop region has been proposed to be important for interactions between TIR domains. Thus, structural deviation of the BB loop, and differences in surface electrostatic potential, might reflect specificities of TIR–TIR interactions. It should be noted that the isolated MyD88-TIR domain existed as a monomer in solution state, while some TIR domains have been reported to form a dimer in crystal structure (9, 10). On the other hand, full-length MyD88 is known to form a dimer, which seems to be mediated via homomeric interactions between the death domains (see Fig. S2).

The present study identified 3 functional surface sites (Sites I–III) of MyD88-TIR that were important for the LPS-activated TLR4-signaling pathway. Two of these sites, Sites II and III, served as binding sites for Mal-TIR (Fig. 2C and E). Results from the GST pull-down and NMR titration experiments suggested that these 2 sites equally contributed to interactions between MyD88-TIR and Mal-TIR. The Site II-forming residues Arg-196 and Asp-197 were located in the BB loop, and were highly conserved across TIR domains (see Fig. S1). Another Mal binding site, Site III, which was formed by 2 basic residues, Lys-282 and Arg-288, flanks the box 3 comprised of FW motif, creating a positively charged surface patch. Because basic amino acids were conserved at positions 282 and 288 (see Fig. S1), this positively charged patch appeared to be common in TIR domains. The present data revealed that Arg-217 in Site I played a crucial role in the TLR4-mediated cellular response to LPS stimulation but was not involved in direct binding to Mal-TIR (Fig. 2C). In the GST pull-down experiments, direct interaction of MyD88-TIR with either the TIR domain of TLR4 or the death domain of IRAK4 was not observed (Fig. 3). This was consistent with previous observations that MyD88 does not directly bind to the cytosolic domain of TLR4 (23). Therefore, Site I is unlikely involved in MyD88 interaction with any of the known possible binding partners, such as Mal, TLR4, and IRAK4, but might serve as a contact surface with a yet unidentified MyD88 binding protein or specific membrane portion. The functional role of Site I in TLR4 signaling remains to be clarified in further studies.

The 2 Mal binding sites of MyD88-TIR, Sites II and III, are distantly located from each other and are on opposite molecular surfaces. Thus, it is impossible to assume that one Mal-TIR can make simultaneous contact with both MyD88-TIR sites. In addition, contributions from Site II and Site III to the interaction were shown to be comparable to each other. Thus, assuming 2 Mal-TIR molecules would bind to one MyD88-TIR molecule, we constructed a complex model between MyD88-TIR and Mal-TIR using a molecular docking method similar to previous studies (12, 24). The

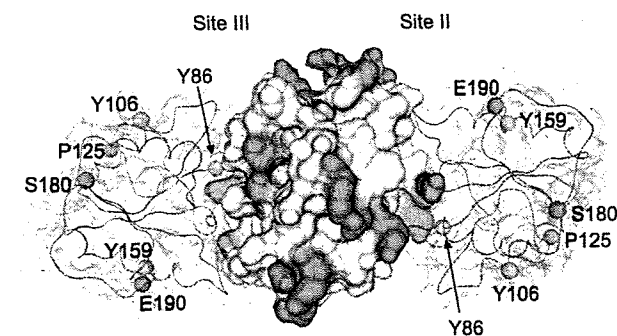


Fig. 4. Structural model of signaling complex formed by MyD88 and Mal. The Mal binding sites, Sites II and III residues, are shown in red, and noncritical residues for signaling and Site I residue are shown in light blue. The positions of the previously reported functional residues (P125, S180, and E190) are shown as orange spheres, and the phosphorylation sites in Mal for signaling, Tyr-86, Tyr 109, and Tyr-159, are shown as yellow spheres.

model indicated that Sites II and III residues are well situated at the interface centers of each Mal-TIR. In addition, all noncritical residues, including Arg-217 (Site I), avoided the interfaces, which supported validity of this model (Fig. 4). Previous reports have shown that P125H mutation or S180L polymorphism of Mal causes decreased interactions between Mal and TLR4 or TLR2, respectively, but has no effect on interactions between Mal and MyD88 (25, 26). Moreover, the TRAF-6 binding site on Mal was shown to include Glu-190 (27). These 3 Mal residues were not included in the Mal-MyD88 complex model interfaces (Fig. 4). Therefore, the model suggested that MyD88-TIR binding might not interfere with interactions between Mal-TIR and TLR4, TLR2, or TRAF-6. Recently, Tyr-86 phosphorylation of Mal was shown to negatively regulate interactions with MyD88 (28). In addition, Tyr-86 mutation, not Tyr-106 or Tyr-159, significantly altered affinity of Mal to MyD88. These observations were consistent with the present complex model, in which Tyr-86, not others, was at the molecular interface (Fig. 4). Moreover, the model predicted that Tyr-86 phosphorylation might perturb MyD88 interface steric complementarity of Mal and result in an electrostatic repulsion to the acidic surface of MyD88-TIR that Mal binds to.

Recently, one of the Site II residues, Arg-196, has just been found to be mutated to cysteine in the new primary immuno-deficiency (MyD88 deficiency) patients (13). This mutation did not cause destabilization of the MyD88 protein, but showed a significant decrease of the direct binding ability between MyD88-TIR and Mal-TIR (Fig. 2D). Patients with the MyD88 deficiency were highly susceptible to Gram-positive bacteria, while they showed normal resistance to other kinds of pathogens, such as Gram-negative bacteria and viruses. The phenotypes suggest that TLR2 signaling is more critical than other self-defense systems in early life (3) because the other innate immune signaling pathways have a kind of redundancy, acting as alternative signaling pathways; i.e., TLR2 and TLR4 signaling needs MyD88 and Mal to signal, but TLR4 has other MyD88-independent pathways with TRIF (5). It suggests that the Gram-positive bacterial recognition system is much more dependent on TLR2/MyD88/Mal signaling. The loss of interaction between MyD88 and Mal caused by the mutation would be a critical molecular mechanism for MyD88 deficiency patients.

It is of special interest that the TIR domain of Mal, and not MyD88, directly interacted with the TIR domain of TLR4 (Fig. 3A) as described in the previous predicted docking model (29). This observation raises the possibility that Mal-TIR might simultaneously bind to the TIR domains of TLR4 and MyD88, and thereby mediate association as previously predicted (5). Because Mal has been shown to be dispensable for TLR4 signaling when MyD88 is artificially fused to a PIP2 targeting domain (6) there is the

possibility that weak interactions between TIR domains of MyD88 and TLR4 mediate signal transduction. Alternatively, an unidentified alternate Mal-independent pathway could contribute to signaling as previously discussed (30). Previous mutation analysis suggests that Mal Pro-125 contributes a binding interface with TLR4 (26). This residue was located distal to the putative MyD88 interface in the present MyD88:Mal complex model and was therefore consistent with the hypothesis that Mal-TIR mediates TIR-TIR interactions between TLR4 and MyD88.

Conclusion

Structure determination combined with functional assays of human MyD88-TIR revealed that 3 sites, which are related to conserved boxes 1–3 of the domain, were important for the LPS/TLR4 pathway. Two of these sites were located at opposite surfaces of the molecule and were shown to mediate direct interaction with Mal-TIR. Thus, the 2 independent binding sites served by MyD88-TIR might contribute to formation of higher order TIR-TIR complexes, which may result in amplification of TLR signal activation. Identification of the key residue in MyD88, which is a direct interacting residue for Mal, is of the clinical significance because one of these residues was shown to be critical for the primary immunodeficiency syndrome. Distribution of the 3 functional sites dispersed on the molecular surface of MyD88-TIR suggested that MyD88 provided multiple interaction surfaces to protein factors that form the signal initiation complex at the cytosolic TLR4 domain. Knowledge of the sites revealed in this study will facilitate further identification of factors and mechanisms used in TLR signaling pathways.

Materials and Methods

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 (GE Healthcare). This vector was transformed into *Escherichia coli* BL-21 (DE3) (Novagen). The TIR domain of MyD88, which was expressed as a GST (GST) fusion protein, was first purified by glutathione Sepharose 4B FF (GE Healthcare) affinity chromatography, and the GST-tag was removed by digestion with Factor Xa (GE Healthcare). 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). Using the purification protocol, ^{15}N -labeled, and ^{13}C , ^{15}N -doubly-labeled monomeric TIR domain of MyD88 wild-type proteins were prepared. The protein sample buffer was replaced by 20 mM potassium phosphate buffer (pH 6.0) containing 0.1 mM EDTA and 10 mM DTT. The final protein sample concentration for typical NMR experiments was approximately 0.3 mM.

NMR Spectroscopy. All NMR spectra were recorded at 25 °C on a Bruker DRX500 or DRX800 spectrometer equipped with a cryogenic probe. For assignment of backbone and side chain ^1H , ^{13}C , and ^{15}N resonances, a series of triple-resonance experiments were conducted (31). Distance restraints for structure calculations were obtained from 3D ^{15}N -edited NOESY and 3D ^{13}C -edited NOESY experiments, with a mixing time of 150 msec. NMR spectra were processed with NMRPipe software (32) and analyzed using Sparky (33). The pulse sequence used to obtain 2D (^1H)- ^{15}N steady-state NOE spectra has been previously described (34). The (^1H)- ^{15}N NOE values were determined from ratios of peak intensities with or without a 3 sec ^1H -saturation applied before each scan: $\text{NOE} = I_{\text{sat}}/I_{\text{unsat}}$.

Structure Calculation. Automated NOESY cross-peak assignment and iterative structure calculation were performed using CYANA version 2.1 (35). The obtained assignment of NOESY cross-peaks was manually validated, and the final structure calculation was performed using CNS version 1.1 (36). Surface electrostatic potentials were calculated using MOLMOL 2K.2 (37).

Cell Culture. Human embryonic kidney (HEK) 293-hTLR4A-HA cells were purchased from InvivoGen. These cells were cultured in Dulbecco's modified Eagle's medium (high glucose-containing DMEM, Invitrogen) supplemented with 10% heat-inactivated FBS (Sigma), penicillin (100 U/ml), and streptomycin (100 pg/ml). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 .

Vector Preparations. A cDNA encoding the TIR domain (amino acid residues 148–296) that was tagged at the N terminus with a myc-epitope was cloned into

the plasmid vector pcDNA3.1+ (Invitrogen). Mutants of the MyD88 TIR domain were generated using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). Mutants of each of 25 charged polar amino acid residues (Asp, Glu, Arg, Lys, and His) substituted by alanine were generated. Mutants with poor expression were not included to avoid possible misinterpretation of the loss of dominant negative inhibitory effect. The MD2 construct was also cloned into pcDNA3.1+. A pGL3-Basic Vector (Promega) containing 4 kb binding sites, which was used in the NF- κ B luciferase reporter assay, and a Renilla luciferase reporter vector used as an internal control in the assay were gifts from Drs. Sewon Ki and Tetsuro Kokubo (Yokohama City University, Yokohama, Japan).

NF- κ B Reporter Gene Activity. 293-hTLR4A-HA cells were transfected with pcDNA3.1+ control vector or pcDNA3.1+ myc-MyD88 TIR domain (wild type or mutant), pcDNA3.1+ MD2, NF- κ B luciferase reporter vector, and Renilla luciferase reporter vector, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were stimulated with LPS O127 (1.0 μ g/ml, Sigma) and incubated for 6 hours. Luciferase reporter gene activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega). The inhibitory effect of each TIR mutant expression was assessed in at least 3 independent experiments. The statistical significance of differences in luciferase activities between wild type and mutants in the NF- κ B reporter assays was analyzed using Dunnett's multiple comparison test. Statistical significance was assumed to be $P < 0.05$.

GST Pull-Down Assay. The TIR domain of MyD88 wild type and mutants (R196A, R196C, R217A, R288A, and R196A-R288A) was purified as GST-fusion proteins. These expression vectors were generated by subcloning the pcDNA3.1+ myc-tagged MyD88 TIR domain into pGEX 5X-1 (GE Healthcare). The DD+ID of MyD88 (amino acid residues 18–141) and TLR4-TIR were also purified as GST-fusion proteins. The GST-fusion proteins were purified by glutathione Sepharose 4B FF (GE Healthcare) affinity chromatography. The TIR domain of human Mal, as well

as the DD of IRAK4, was purified using a modified previously reported method (22, 38). These purified proteins were incubated with Glutathione Sepharose 4B (GE Healthcare) for 3 hours. After 4 wash steps with wash buffer (20 mM potassium phosphate buffer (pH 6.0), 100 mM KCl, 0.1 mM EDTA, 10 mM DTT, and 0.5% Triton X-100), the resin was analyzed by SDS polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

NMR Titration. One 15- μ l aliquot of 100 μ M nonlabeled Mal-TIR was added to 150 μ l of 20 μ M 15 N-labeled MyD88-TIR or its alanine substituted mutants up to 1.5 molar equivalent of 15 N MyD88-TIR. At each titration point, 1D 1 H- 15 N and 2D 1 H- 15 N SOFAST-HMQC spectra were measured. Quantification of NMR signal attenuation in the titration experiments, and evaluation of apparent dissociation constant (K_d^{app}) for the interaction, are described in *SI Materials and Methods*.

Docking Studies Between MyD88 and Mal. Structure modeling of the TIR domain of Mal was performed using the MyD88-TIR structure as a template on molecular operating environment (MOE) software (39, 40). The docking simulation was performed on AutoDock without any specific restraints between the molecules as previously reported (24, 41, 42). (See detailed method of the docking study in *SI Materials and Methods*.)

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- Bonnert TP, et al. (1997) The cloning and characterization of human MyD88: A member of an IL-1 receptor related family. *FEBS Lett* 402:81–84.
- Kawai T, Akira S (2007) Signaling to NF- κ B by Toll-like receptors. *Trends Mol Med* 13:460–469.
- Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124:783–801.
- Hornig T, Barton GM, Flavell RA, Medzhitov R (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 420:329–333.
- O'Neill LA, Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7:353–364.
- Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125:943–955.
- Rowe DC, et al. (2006) The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. *Proc Natl Acad Sci USA* 103:6299–6304.
- Xu Y, et al. (2000) Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature* 408:111–115.
- Khan JA, Brint EK, O'Neill LA, Tong L (2004) Crystal structure of the Toll/interleukin-1 receptor domain of human IL-1RAPL. *J Biol Chem* 279:31664–31670.
- Nyman T, et al. (2008) The crystal structure of the human Toll-like receptor 10 cytoplasmic domain reveals a putative signaling. *J Biol Chem* 283:11861–11865.
- Li C, Zienkiewicz J, Hawiger J (2005) Interactive sites in the MyD88 Toll/interleukin (IL) 1 receptor domain responsible for coupling to the IL1 β signaling pathway. *J Biol Chem* 280:26152–26159.
- Jiang Z, et al. (2006) Details of Toll-like receptor:adaptor interaction revealed by germ-line mutagenesis. *Proc Natl Acad Sci USA* 103:10961–10966.
- von Bernuth H, et al. (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321:691–696.
- Burns K, et al. (1998) MyD88, an adapter protein involved in interleukin-1 signaling. *J Biol Chem* 273:12203–12209.
- Slack JL, et al. (2000) Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. *J Biol Chem* 275:4670–4678.
- Poltorak A, et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science* 282:2085–2088.
- Adachi O, et al. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9:143–150.
- Shimazu R, et al. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 189:1777–1782.
- Kagan JC, et al. (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon- β . *Nat Immunol* 9:361–368.
- Lu J, Chen M, Dekoster GT, Cistola DP, Li E (2008) The RXRALpha C-terminus T462 is a NMR sensor for coactivator peptide binding. *Biochem Biophys Res Commun* 366:932–937.
- Burns K, et al. (2003) Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *J Exp Med* 197:263–268.
- Dunne A, Ejdeback M, Ludidi PL, O'Neill LA, Gay NJ (2003) Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. *J Biol Chem* 278:41443–41451.
- Brown V, Brown RA, Ozinsky A, Hesselberth JR, Fields S (2006) Binding specificity of Toll-like receptor cytoplasmic domains. *Eur J Immunol* 36:742–753.
- Kato Z, et al. (2008) Positioning of autoimmune TCR-Ob. 2F3 and TCR-Ob. 3D1 on the MBP85–99/HLA-DR2 complex. *Proc Natl Acad Sci USA* 105:15523–15528.
- Khor CC, et al. (2007) A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. *Nat Genet* 39:523–528.
- Hornig T, Barton GM, Medzhitov R (2001) TIRAP: An adapter molecule in the Toll signaling pathway. *Nat Immunol* 2:835–841.
- Mansell A, Brint E, Gould JA, O'Neill LA, Hertzog PJ (2004) Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF- κ B activation by toll-like receptor (TLR)-2 and TLR4. *J Biol Chem* 279:37227–37230.
- Piao W, et al. (2008) Tyrosine phosphorylation of MyD88 adapter-like (Mal) is critical for signal transduction and blocked in endotoxin tolerance. *J Biol Chem* 283:3109–3119.
- Nunez Miguel R, et al. (2007) A dimer of the Toll-like receptor 4 cytoplasmic domain provides a specific scaffold for the recruitment of signalling adaptor proteins. *PLoS ONE* 2:e788.
- Monie TP, Moncrieffe MC, Gay NJ (2009) Structure and regulation of cytoplasmic adapter proteins involved in innate immune signaling. *Immunol Rev* 227:161–175.
- John C, Wayne JF, Arthur GIP, Rance M, Nicholas JS (2007) *Protein NMR Spectroscopy: Principles And Practice* (Academic, San Diego), 2nd Ed.
- Delaglio F, et al. (1995) NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6:277–293.
- Goddard TD, Kneller DG (1999) SPARKY 3 (University of California, San Francisco).
- Farrow NA, et al. (1994) Backbone dynamics of a free and a phosphopeptide-complexed Src homology-2 domain studied by N-15 Nmr relaxation. *Biochemistry* 33:5984–6003.
- Güntert P (2004) Automated NMR structure calculation with CYANA. *Methods Mol Biol* 278:353–378.
- Brunker AT, et al. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D* 54:905–921.
- Koradi R, Billeter M, Wuthrich K (1996) MOLMOL: A program for display and analysis of macromolecular structures. *J Mol Graphics* 14:51–55:29–32.
- Lasker MV, Gajjar MM, Nair SK (2005) Cutting edge: Molecular structure of the IL-1R-associated kinase-4 death domain and its implications for TLR signaling. *J Immunol* 175:4175–4179.
- Levitt M (1992) Accurate modeling of protein conformation by automatic segment matching. *J Mol Biol* 226:507–533.
- Fechter T, Dengler U, Schomburg D (1995) Prediction of protein three-dimensional structures in insertion and deletion regions: A procedure for searching data bases of representative protein fragments using geometric scoring criteria. *J Mol Biol* 253:114–131.
- Stoddard BL, Koshland DE, Jr (1992) Prediction of the structure of a receptor-protein complex using a binary docking method. *Nature* 358:774–776.
- Morris GM, Goodsell DS, Huey R, Olson AJ (1996) Distributed automated docking of flexible ligands to proteins: Parallel applications of AutoDock 2.4. *J Comput Aided Mol Des* 10:293–304.

Theophylline-associated status epilepticus in an infant: pharmacokinetics and the risk of suppository use

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Background: Theophylline has been widely used to treat asthma, but recent studies have revealed that the possible risks for seizure may result in the revision of the therapeutic guidelines.

Methods: An 8-month-old boy who had been treated with oral sustained-release theophylline and additional aminophylline suppository was hospitalized. A combination of diazepam, lidocaine and thiopental was required to stop his convulsion.

Results: The pharmacokinetic study indicated that the usage of a sustained-release formula should not usually be over 15 mg/ml, but the additional use of an aminophylline suppository elevated the concentration to over 20 mg/ml and resulted in the severe adverse effects.

Conclusion: The parents of children and also physicians should be educated to ensure the proper use of the suppository formula.

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Key words: convulsion;
pharmacokinetics;
suppository;
theophylline

Introduction

Adverse neurological symptoms such as seizure during theophylline therapy are associated with the serum level of theophylline, a significant factor for increased morbidity.^[1,2] We present a case of

intoxication caused by add-on use of an aminophylline suppository during daily use of a sustained-release formulation. The pharmacokinetic study of this case suggests that the use of suppositories should be revised to avoid the possible adverse effects.

Case report

An 8-month-old boy who had been neurologically normal prior to this episode was transferred to our hospital due to status epilepticus. Before admission he was diagnosed as having bronchitis without fever and was treated with oral sustained-release theophylline (40 mg/day, 5.7 mg/kg per day) but without any antihistamines or antiallergic drugs at a local clinic. On the second day of illness, his mother gave him an aminophylline suppository (50 mg, 7 mg/kg), which had been previously provided by another clinic as a "cough stopper" to attenuate his coughing.

On admission, he was drowsy and his eyes were deviated and fixed to the left side, and he would respond only to painful stimuli. He had tachycardia (180/min) and tachypnea (50/min) without fever. He was diagnosed with a tonic convulsion, but not in a non-convulsive status epilepticus, and the initial dose of diazepam injection (2.5 mg, 0.35 mg/kg) slightly improved his tonic state. However, he developed a massive tonic seizure on the right arm and leg, which required three times of injections of diazepam (2.5 mg, 0.35 mg/kg), lidocaine (20 mg, 3.0 mg/kg) and thiopental (25 mg, 3.5 mg/kg) to cease the seizures. He showed right-sided paresis after the seizure, but the paresis diminished after several hours, suggesting Todd's paresis. No paroxysmal discharge was shown on electroencephalography, but there were periodic high voltage delta waves predominantly on the left hemisphere. Head computed tomography showed no abnormal lesion and he was discharged after three days of hospitalization without any neurological sequelae.

Laboratory tests showed the elevation of muscle related enzymes (aspartate amino transferase 66 mg/ml, lactate dehydrogenase 614 mg/ml, and creatine kinase 410 mg/ml) and abnormalities in a blood gas analysis

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(pH 7.113 and base excess -9.0), which would have been due to the status epilepticus. Routine examination of his cerebrospinal fluid (CSF) was normal. The theophylline concentrations of serum and CSF were 18.2 mg/ml (at 1 hour after the last dose) and 5.2 mg/ml (at 8 hours after last dose), respectively.

Discussion

The theophylline concentration in this case was analyzed according to a pharmacokinetic study of the suppository and sustained-release formulae.^[3,4] The results showed that the use of a sustained-release formula should not usually be over 15 mg/ml, but the additional use of an aminophylline suppository elevated the concentration to over 20 mg/ml and resulted in the severe adverse effects (Fig.).

Theophylline associated seizures have a tendency to have more severe outcomes than those without theophylline, possibly due to its pharmacological impairments on the energy metabolisms in cells.^[1,2] We previously reported a 2-year-old girl with severe theophylline associated seizure that resulted in quadriplegia, who now at 15 years of age remains a bed-ridden life without any ability for meaningful communication.^[5] Our studies of that case and the present case clearly indicate the risk of suppository use of theophylline especially for infants. Our other study about unbound free theophylline in serum also showed a potent risk for neurological complications in infants due to their significant higher ratios of unbound theophylline compared to adults (65%-80% in neonates or early infants; 45%-50% in adults).^[6] Moreover, our study showed that the ratio could easily change depending on several intrinsic factors such as albumin concentration.^[7]

As suggested by the previous studies, theophylline

associated seizures seem to be intractable and it is difficult to stop it by standard treatments with diazepam.^[8] Theophylline is known to antagonize the effect of benzodiazepines, and this may explain why drugs such as diazepam are relatively ineffective in treating theophylline-associated seizures. The prompt use of barbiturates is recommended when diazepam is not effective as seen in our case, in which a combination of diazepam, lidocaine, and thiopental was required to stop his convulsion. In addition, cautions also should be taken in using histamine H1 antagonists in young infants because such drugs could potentially disturb the anticonvulsive central histaminergic system.^[9]

Aminophylline suppositories are commercially available and widely used for treatment of coughing and wheezing in children, although such use is not recommended mainly because of the unreliable absorption.^[10] Pharmacokinetic analysis in the present case showed that the serum theophylline concentration increased more rapidly and to a much greater extent than the concentration predicted by the use of the same dose of an oral sustained-release formulation^[5] (Fig.). According to the results and previously described data,^[3-7] suppository dosing at home is equivalent to drip infusion without an adequate medical supervision and assessment, thus exposing the user to an increased risk of intoxication.

We previously recommended that aminophylline suppositories should be used only for short-term management with just a single dosing, and not given as maintenance therapy.^[5] However, the recommendation should be revised with a caution issued for its additional use with daily sustained-release theophylline treatment. The parents of pediatric patients and also physicians should be educated to ensure the proper use of the formula, not as a "cough stopper".

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Competing interest: None declared.

Contributors: Kato Z wrote the first draft of this paper. Kato Z and Nakamura M performed the pharmacokinetic analysis. All the authors contributed to the intellectual content and approved the final version. Kondo N is the guarantor.

References

- 1 Dunn DW, Parekh HU. Theophylline and status epilepticus in children. *Neuropediatrics* 1991;22:24-26.
- 2 Nolke AC. Severe toxic effects from aminophylline and theophylline suppositories in children. *JAMA* 1956;161:693-697.
- 3 Ooniwa T, Kuwahata K, Matsumoto T, Watanabe K, et al. The Clinical Report 1985;19:444-454.

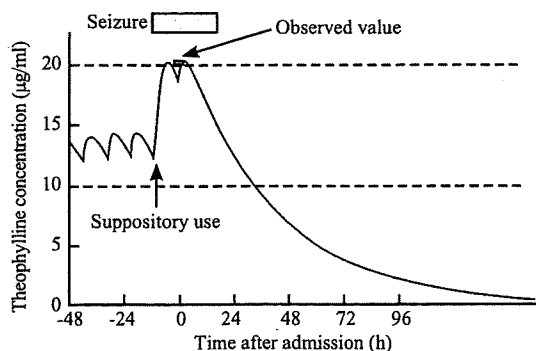


Fig. Time course of serum theophylline concentration predicted by a one-compartment model for sustained-release or suppository formulation.^[4,5]

- 4 Kato Z, Fukutomi O, Yamazaki M, Kondo N, Imaeda N, Orii T. Prediction of steady-state serum theophylline concentration in children by first-order and zero-order absorption models. *Int J Clin Pharmacol Ther* 1994;32:231-234.
- 5 Kato Z, Yasuda K, Shimozawa N, Kondo N, Horiuchi T. Theophylline-associated status epilepticus resulting in quadriplegia. *Ann Allergy Asthma Immunol* 1997;78:332.
- 6 Kato Z, Fukutomi O, Kondo N. Developmental changes of unbound theophylline. *Ann Allergy Asthma Immunol* 1998;80:517.
- 7 Kato Z, Fukutomi O, Kondo N. Intrinsic factors for unbound theophylline. *J Investig Allergol Clin Immunol* 2002;12:217-219.
- 8 Yoshikawa H. First-line therapy for theophylline-associated seizures. *Acta Neurol Scand Suppl* 2007;186:57-61.
- 9 Haruyama W, Fuchigami T, Noguchi Y, Endo A, Hashimoto K, Inamo Y, et al. The relationship between drug treatment and the clinical characteristics of febrile seizures. *World J Pediatr* 2008;4:202-205.
- 10 Drug Information for the Health Care Professional: USP DI, 15th ed, 1995.

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床小児気管支喘息患児における
トシル酸プラタストの有用性の検討まつ い えい こ *1
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近 藤 直 実
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折 居 建 治
はな おか やす ひこ *4
花 岡 康 彦かね こ ひで お *1
金 子 英 雄
さ さ き せい *2
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別表参照

Key Words

小児気管支喘息

トシル酸プラタスト

QOL (quality of life) 調査

要 旨

ロイコトリエン受容体拮抗薬を投与していても、十分なコントロールが得られない小児気管支喘息患児に対し、トシル酸プラタストを12週間投与し、その有用性を検討した。また、QOL (quality of life) 調査票を用いて、QOLの変化もあわせて検討した。トシル酸プラタスト投与により気管支喘息症状発現日数は有意に減少し、全般改善度は62.9%であった。臨床的に問題となる副作用は認められなかった。また、QOL調査項目の多くで改善が認められた。

はじめに

小児気管支喘息に対して、「小児気管支喘息治療・管理ガイドライン」の普及により長期管理薬が適切に投与されるようになってきている。その治療においては、気道炎症を抑制し、無発作状態をできる限り長期に持続させることが重要である。

代表的な気管支喘息の長期管理薬として、吸入ステロイド薬および抗アレルギー薬などがあげられるが、抗アレルギー薬には効果発現までに一定の時間を要すること、薬剤の有効例、無効例が存在することや、投与前に予知するマーカーが明確でないといった問題がある。効果に差がみられるのは、個々の患者での病態が種々であることなどに起因すると考えられる。

気管支喘息の適応をもつ抗アレルギー薬の一つであるトシル酸プラタストは、ヘルパーT細胞からのIL-4およびIL-5の産生抑制に基づく好酸球浸潤抑制作用、IgE抗体産生抑制作用^{1)~6)}などにより抗アレルギー作用が発揮されるTh2サイトカイン阻害薬である。成人のガイドラインにおいては、重症喘息患者において吸入ステロイド薬と併用する長期管理薬の一つとして位置づけられている。

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今回、個々の患者での病態が種々であることを考慮して、ロイコトリエン受容体拮抗薬を投与していても十分なコントロールが得られない間欠型および軽症持続型の小児気管支喘息患児に対して、トシル酸スプラタストドライシロップへ切替え、もしくは追加投与した際の有用性について検討した。

対象および方法

1. 対 象

本調査では、日本小児アレルギー学会「小児気管支喘息治療・管理ガイドライン（2005）」の重症度によって間欠型、軽症持続型と判定され、ロイコトリエン受容体拮抗薬を主体とする治療を4週間以上実施され、主治医が効果不十分と判断した15歳未満の患児を対象にした。

2. 除外基準

本調査登録前3カ月以内にトシル酸スプラタストによる治療歴のある患児、および皮膚外用薬以外の副腎皮質ステロイド薬を長期管理薬として使用中の患児は除外した。

3. 薬剤投与期間および併用薬剤

本調査は多施設共同調査であり、2006年7月～2007年6月までの12カ月間、全国202施設において施行した特定使用成績調査である。調査期間は、観察期2週間、治療期（トシル酸スプラタストドライシロップを1回3 mg/kg 1日2回）12週間とした。なお、観察期に投与されていたロイコトリエン受容体拮抗薬は継続投与、もしくは中止とした。併用薬剤に関しては、観察期、治療期を通じて、中等度以上の急性発作対応のために静注、経口または吸入にてステロイド薬を短期併用することは制限しなかった。

4. 評価項目および判定方法

1) 有効性の評価

①気管支喘息症状発現日数評価

投与開始前、投与4週後、8週後、12週後の各時期における過去2週間の気管支喘息症状発

現日数〔夜に咳や喘鳴に気がついた日数、夜に咳や喘鳴で目覚めた日数、昼に咳や喘鳴、息切れに気がついた日数、喘息症状によって活動が制限された日数、発作止めの薬（ β_2 刺激薬吸入など）を使用した日数、喘息症状によって予定外に医療機関を受診した日数〕を、日誌および問診にて調査した。担当医師は、診察時に喘息日誌の記載内容を確認し、臨床症状について2週間ごとにまとめた。

②QOL調査票による評価

4歳未満には15問、4歳以上には20問の「小児気管支喘息患児と親または保護者のQOL調査票改訂版2001」⁷⁾を用い、投与開始前と12週後（12週以前に投与終了する場合は終了時点とした）に患児の保護者に調査した。QOL調査票は身体的領域と精神的領域からなり、いずれの項目ともに喘息により最大の支障をきたした場合を1点、喘息による支障がない場合を5点とし、5段階評価を行った。QOL点数はそれぞれに属する因子スコアの合計とし、QOL合計点数は全合計点数として評価した。

③全般改善度

臨床経過を総合的に判断し、投与前と比較して5段階（著明改善、改善、軽度改善、不変、悪化）で主治医が評価した。また、アトピー性皮膚炎あるいはアレルギー性鼻炎を合併している患児に対しては、さらに3段階（有効、やや有効、無効）でアトピー性皮膚炎あるいはアレルギー性鼻炎に対する効果を評価した。

④検査値の推移

観察期、治療終了時に血液検査を施行することに同意の得られた症例では、血清IgE、末梢血好酸球数の測定を行った。

2) 安全性の評価

本剤投与中または投与後に新たに発現した臨床的に問題となる有害事象（自他覚所見、合併症、偶発症、臨床検査値異常など）を、患児あるいはその保護者への問診などをふまえ調査し

た。また有害事象のうち、担当医によりトシル酸スプラストドライシロップとの因果関係が否定できないと判断した場合を副作用とした。

5. 解析方法

気管支喘息症状の発現日数に関する投与前後の比較では、観察時期が複数であるため経時観察データとして線形混合モデルを用い、症状ごとに固定効果に対する検定を行った。検定の結果が有意であった場合には、探索的に時期別に対応のある t 検定を行った。また、QOL調査項目および領域別合計点数に関する投与前後の比較では、Wilcoxonの符号付き順位和検定をそれぞれ行った。検定は投与前後のデータがある症例を対象にして、統計解析パッケージソフトSAS® 9.1 (SAS Institute Japan株式会社)を用い、有意水準5% (両側) で実施した。

結 果

1. 症例内訳および背景

2006年7月～2007年6月までに202施設から754例登録された。そのうち、前治療薬としてのロイコトリエン受容体拮抗薬の投薬期間が4週間未満であった56例、吸入ステロイドを本剤投与初日から併用していた47例、初回投与後来院がない14例および投与前に喘息症状がない26例などの計123例を除いた631例で安全性を評価した。有効性は投与後の喘息症状が未記載であった54例を除外した577例で、さらにQOL評価は投与前後のQOL評価がない11例を除外した566例で実施した。

安全性評価症例631例の患児背景を表1に示す。3歳未満が141例 (1歳未満：12例，1歳：48例，2歳：81例)，平均年齢は4.4歳，間欠型が256例，軽症持続型が375例であった。

2. 有効性の評価

1) 気管支喘息症状発現日数評価

気管支喘息症状発現日数推移一覧を表2に示す。いずれの項目ともに、トシル酸スプラタス

表1 患児背景

因 子	区 分	例 数
性 別	男	388
	女	243
入院 外来区分	入院	3
	外来	607
	入院+外来	21
年 齢	3歳未満	141
	3歳以上5歳未満	223
	5歳以上11歳未満	241
	11歳以上	26
発作型	間欠型	256
	軽症持続型	375
病 型	アトピー型	451
	非アトピー型	177
	不明	3
合併症	無	326
	有	305
	アトピー性皮膚炎	163
	アレルギー性鼻炎	180
併用薬 (重複あり)	クロモグリク酸ナトリウム (吸入)	137
	プロピオン酸ベクロメタゾン (吸入)	12
	テオフィリン (経口)	193
	ツロブテロール (外用)	269
1日投与量	4 mg/kg未満	30
	4 mg/kg以上6 mg/kg未満	281
	6 mg/kg以上8 mg/kg未満	290
	8 mg/kg以上	30
総投与日数 (週)	2週未満	16
	2週以上4週未満	31
	4週以上6週未満	45
	6週以上8週未満	20
	8週以上10週未満	16
	10週以上12週未満	21
	12週以上	482

表2 気管支喘息症状の発現日数推移

気管支喘息の症状 (2週)		時 期				P value *
		投与前	4週後	8週後	12週後	
夜に咳や喘鳴に 気がついた日数	平均値±標準誤差	4.4 ± 0.2	2.2 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	$p < 0.001$
	例 数	554	531	465	477	
夜に咳や喘鳴で 目覚めた日数	平均値±標準誤差	2.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	$p < 0.001$
	例 数	545	525	461	469	
昼に咳や喘鳴, 息切れに 気がついた日数	平均値±標準誤差	3.6 ± 0.2	1.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	$p < 0.001$
	例 数	546	524	461	471	
喘息症状によって活動が 制限された日数	平均値±標準誤差	1.8 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	$p < 0.001$
	例 数	541	519	457	469	
発作止めの薬 (β_2 刺激薬吸入 など) を使用した日数	平均値±標準誤差	3.3 ± 0.2	1.6 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	$p < 0.001$
	例 数	544	523	461	472	
喘息症状によって予定外に 医療機関を受診した日数	平均値±標準誤差	1.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	$p < 0.001$
	例 数	544	523	461	471	

* : 線型混合モデルによる固定効果の検定

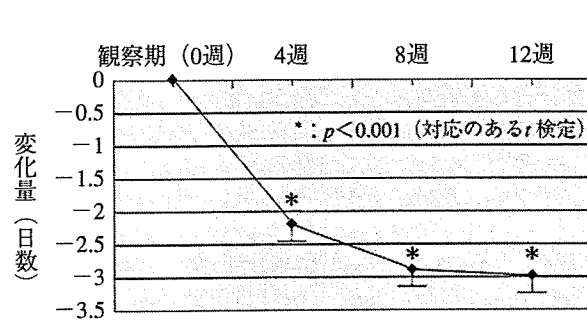


図1 夜に咳や喘鳴に気がついた日数

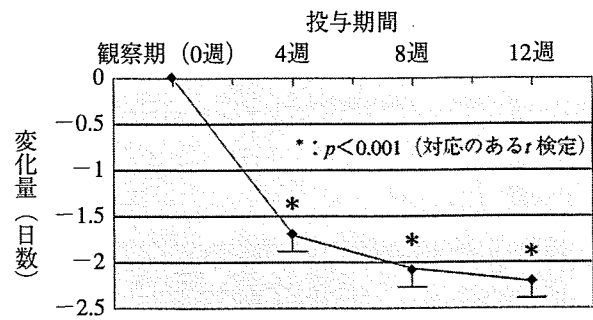


図2 発作止めの薬 (β_2 刺激薬吸入など) を使用した日数

トドライシロップ投与後の症状発現日数は有意 ($p < 0.001$) に減少した。夜間の咳および喘鳴日数は、治療4週目から有意に減少していた (図1)。発作コントロールのために発作止めの薬 (β_2 刺激薬吸入など) の吸入を使用する頻度も、治療4週目から有意に減少していた (図2)。

2) QOL調査票による評価

トシル酸スプラスタ投与前後の各調査項目の平均値レーダーチャートを図3に、領域別の合計点を表3に示した。4歳未満 (図3-A) ではQ3, Q4, Q14, 4歳以上 (図3-B) ではQ4を除いた各項目に対し、投与前に比し有意に改善し

ていた。つまり、身体的領域、精神的領域ともに改善を認めたが、とくに、4歳未満における身体的領域の改善が著明であった (図3-A, Q1, Q2, Q15)。

3) 全般改善度

中等度改善以上は357例 (著明改善111例, 中等度改善246例) であり, 全般改善率は62.9% (357/568例) であった (有効性評価例577例から評価不能と判定された9例を除く)。患児背景別の改善度を表4に示す。

ロイコトリエン受容体拮抗薬の投与を中止し切替えた場合 (60.7% : 204/336例) とそのまま

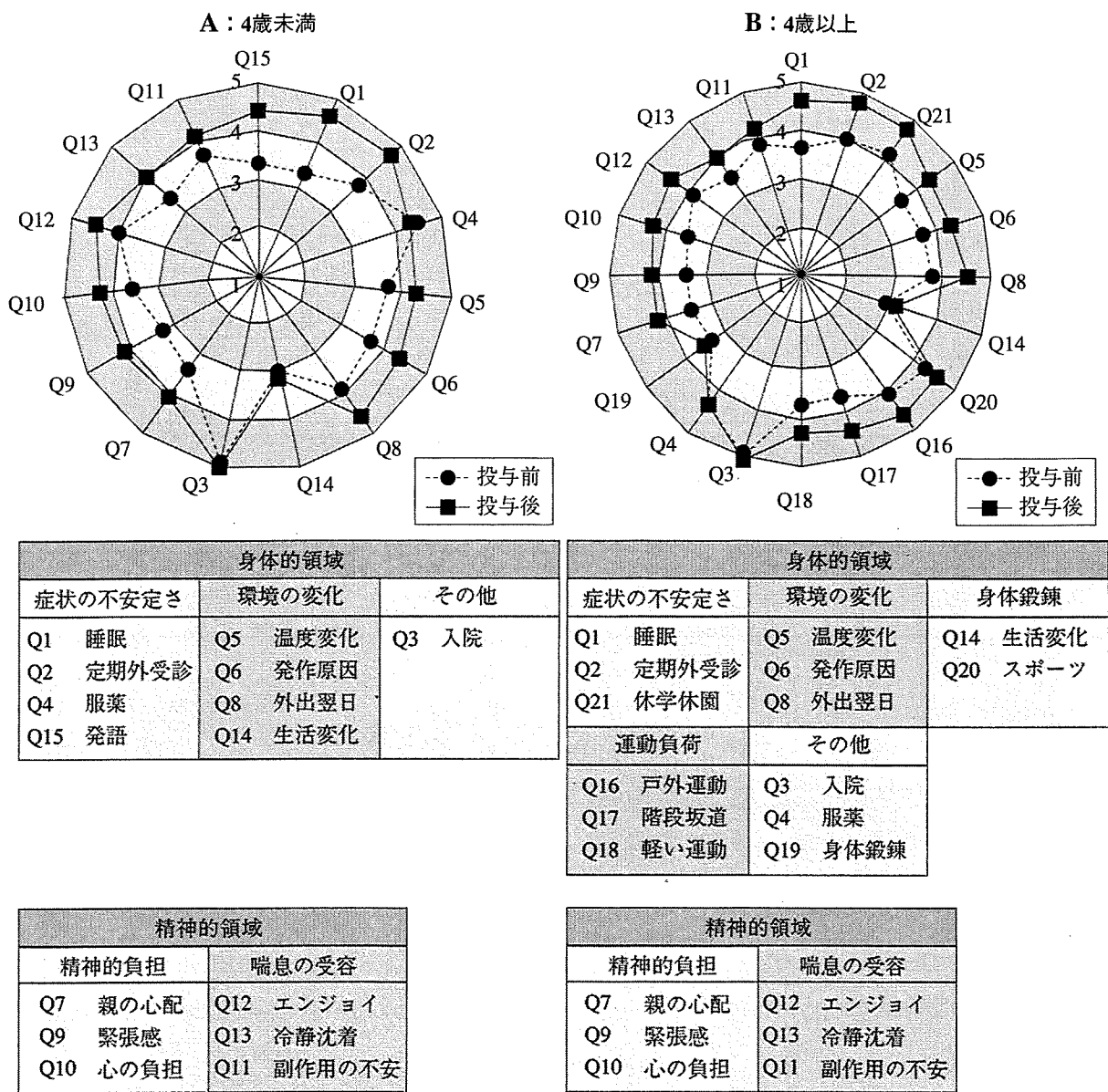


図3 QOL変化

表3 領域別QOL変化

		4歳未満			4歳以上		
		症例数	スコア	P値*	症例数	スコア	P値*
QOL合計	投与前	189	55.0	$p < 0.001$	293	75.3	$p < 0.001$
	12週後	189	64.3		293	85.5	
身体的領域	投与前	194	34.0	$p < 0.001$	298	53.9	$p < 0.001$
	12週後	194	39.4		298	60.8	
精神的領域	投与前	203	21.0	$p < 0.001$	303	21.5	$p < 0.001$
	12週後	203	24.9		303	24.7	

* : Wilcoxonの符号付き順位和検定