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INTRODUCTION

Apart from environmental factors, there are also genetic factors responsible for asthma. To identify the asthma-related gene, the single nucleotide polymorphism (SNP) of the total genome has been used. Asthma is a Th2-dominant disease, and IL-4/IL-13 and the genetic polymorphism of the associated receptors are involved in atopy and asthma[1)-4)]. IL-4/IL-13 also act on tissue-forming cells such as the epithelial cells of the airways and smooth muscle (SM) cells. Both these cytokines are in the asthmatic pathology (polyblennia, hypersensitivity and remodeling of the airways). IL-4/IL-13 are also involved in the modification of the expression of the various types of chemical mediator receptor.

1. CHEMICAL MEDIATOR RECEPTORS

Table 1 shows the expression loci and action of the various types of chemical mediator receptor, the modifying factors for the asthmatic pathology. Table 2 presents the genetic polymorphism of these receptors.

1) Histamine Receptors

Four types of histamine receptor (HR) have been identified: H1R - H4R. H1R is involved in histamine-caused reactions (histamine-induced contraction of airways, mucus secretion, and vascular permeability). The airway SMs express H1R, H2R and H4R. The expression of these histamine receptors is not subject to the effects of IL-4/IL-13 stimulation. Histamine will promote the Th1 reaction via H1R activation while H2R activation will suppress both the Th1 and the Th2 reactions[5)]. The H1R and H2R genes are located at 3p25 and 5q35, respectively, and polymorphism of H1R receptor can occur[6)] as −17C/T and 1045G/A in the Japanese. H2R receptor can also occur in polymorphic SNP variants. These two polymorphic variants are not asthma-related. The 3p25 chromosome position is one of the markers of a link to atopic factors in the Japanese[7)]. Genetic H1R and H2R receptor polymorphism is related to atopy rather than asthma. The H4R gene (18q11) is not involved in asthma or atopy.

2) Cysteinyl Leukotriene Receptor

Two cysteinyl leukotriene receptors (CysLTR) are CysLT1R and CysLT2R. CysLTs as LTC_4 and LTD_4 are responsible for airway contraction, polyblennia and high vascular permeability, mediated through LT1R. Airway SM express CysLT1R and CysLT2R. IL-4/IL-13 selectively enhance CysLT1R expression. IL-13 reportedly promotes SM cell proliferation[8)]. CysLT1R and CysLT2R are important lipid mediator receptors involved in the modification of asthma and the genetic polymorphism of the receptors has been studied[9)-11)]. The CysLT1R and CYsLT2R receptor genes are found at Xq13-q21 and 13q14-q21 and there are various SNPs. CysLT2R receptor may be connected to asthma.

3) Thromboxane A_2 Receptor

Thromboxane A_2 receptor (TXA_2R) is involved in the contraction of airway SM and blood vessels. The TXA_2R gene is located at 19p13. The polymorphic variant 924T/C has a link with asthma in the Japanese[12)]. 924T/C polymorphism may also be involved in infantile asthma and a correlation with decreases in $FEV_{1.0}$ and FVC is possible[13)].

表1　ケミカルメディエーター受容体の発現部位・作用
Table 1　Expression sites and action of chemical mediator receptors

リガンド Ligand	受容体 Receptor	発現部位 Expression site	作用 Action
ヒスタミン Histamine	H1R	気道平滑筋，血管内皮細胞，粘液分泌細胞，T細胞，単球，マクロファージ，好酸球，好塩基球，肥満細胞，樹状細胞 Smooth muscles of airways, hemangioendothelial cells, mucus-secreting cells, T-cells, monocytes, macrophages, eosinophils, basophils, mast cells, dendritic cells	気道収縮作用，血管透過性亢進作用，粘液分泌亢進作用，Th1反応の促進 Contraction of smooth muscles, enhancement of vascular permeability, increased mucus secretion, promotion of Th1 reaction
	H2R	気道平滑筋，粘液分泌細胞，T細胞，単球，マクロファージ，肥満細胞，好塩基球，樹状細胞 Smooth muscles of airways, mucus-secreting cells, T-cells, monocytes, macrophages, mast cells, basophils, dendritic cells	粘液分泌亢進作用，Th1・Th2反応の抑制，樹状細胞におけるサイトカイン産生調節，マクロファージにおける好中球・単球遊走因子産生促進 Increased mucus secretion, suppression of the Th1 and Th2 reaction, regulation of cytokine production in the dendritic cells, promotion of production of eosinophil/monocyte emigration factors
	H4R	気道平滑筋，線維芽細胞，気道上皮細胞，血管内皮細胞，T細胞，B細胞，好酸球，肥満細胞，樹状細胞 Smooth muscles of airways, fibroblasts, epithelial cells of the airways, hemangioendothelial cells, T-cells, B-cells, eosinophils, mast cells, dendritic cells	$CD8^+$細胞におけるIL-16産生調節作用，肥満細胞・好酸球遊走作用 Regulation of IL-16 production in $CD8^+$ cells, emigration of mast cells/eosinophils
システイニルロイコトリエン Cysteinyl leukotrienes	CysLT1R	気道平滑筋，$CD34^+$細胞，B細胞，単球，マクロファージ，好酸球，好塩基球，肥満細胞 Smooth muscles of airways, $CD34^+$ cells, B-cells, monocytes, macrophages, eosinophils, basophils, mast cells	気道収縮作用，血管透過性亢進作用，粘液分泌亢進作用，$CD34^+$細胞遊走作用，細胞分化・増殖作用，好酸球浸潤誘導作用 Contraction of smooth muscles, enhancement of vascular permeability, increased mucus secretion, emigration of $CD34^+$ cells, cell differentiation/proliferation, induction of eosinophil infiltration
	CysLT2R	気道平滑筋，肺静脈，単球，マクロファージ，好酸球 Smooth muscles of airways, pulmonary veins, monocytes, macrophages, eosinophils	血管収縮作用 Vascular contraction
トロンボキサン A_2 Thromboxane A_2	TXA_2R	気道平滑筋，気道上皮細胞，血管内皮細胞，粘液分泌細胞，単球，マクロファージ，肥満細胞 Smooth muscles of airways, epithelial cells of the airways, hemangioendothelial cells, monocytes, macrophages, mast cells	気道収縮作用，血管収縮作用，粘液分泌亢進作用 Contraction of smooth muscles of airways, vascular contraction, enhancement of mucus secretion
アセチルコリン Acetylcholine	M1R	副交感神経節 Parasympathetic nerve synapses	迷走神経伝導の促進 Stimulation of vagus nerve transmission
	M2R	節後神経終末 気道平滑筋 Postsynaptic nerve terminal bud Smooth muscles of airways	アセチルコリン放出の抑制 アデニル酸シクラーゼの阻害，Ca^{2+}チャネルの抑制 Suppression of Ach release Adenylate cyclase, Ca^{2+} channel suppression
	M3R	気道平滑筋 粘膜下腺，杯細胞 Smooth muscles of airways Submucosal gland, goblet cells	気道収縮作用 粘液分泌促進作用 Contraction of smooth muscles of airways, enhancement of mucus secretion

表2 ケミカルメディエーター受容体の遺伝子多型
Table 2 Genetic polymorphism of chemical mediator receptors

受容体 Receptor	染色体座位 Chromosome site	遺伝子多型 Genetic polymorphism	喘息との関連 Relation with asthma	アトピーとの関連 Relation with atopy
H1R	3p25	-17C/T, 1045G/A	−*	+
H2R	5q35	543G/A, 826C/T	−*	?
H4R	18q11	?	?	?
CysLT1R	Xq13-q21	927T/C	−	?
CysLT2R	13q14-q21	His57Gly	+	?
TXA_2R	19p13	924T/C	+	+
M1R	11q13	Cys417Arg	?	?
M2R	7q31-q35	1696T/A	?	?
M3R	1q41-q44	$(CTTT)_{12-20}$-$(GT)_{6-19}$ ハプロタイプ haplo-type	−	+

*：アレル頻度の不均衡を考慮する必要性がある.
*: Signifies that allelism should be taken into consideration.

4) Muscarinic Receptor

Bioactivation of acetylcholine (Ach) is mediated via the muscarinic receptor (MR). There are 5 sub-types of MR with different functions: M1R - M5R, M1R, M2R and M3R occur in the lung. Airway SM contraction and increased mucus secretion in asthma are triggered via the M3R receptor. SM stimulation by IL-4/IL-13 enhances M3R expression and increases intracellular Ca^{2+}. The M3R genes are located at 1q41-q44, close to the airway hypersensitivity area in Australians[14),15)]. In Maltese asthmatics no SNP was detected in the M3R gene translation and in the 3' untranslation areas (3'-UTR)[16)]. In Caucasians and African Americans, four SNPs are present in the promoter region of the M3R gene. They have no connection with asthma[17)]. Haplo-type short tandem repeat polymorphism (STRPs) in this region is not directly related to asthma.

M2R suppresses the release of Ach at the postsynaptic terminal. Cyclic AMP decreases due to inhibition of adenylate cyclase in the SM causing muscle contraction. The M2R gene is located at 7q31-q35, with 2 silent SNPs in the translation area and one SNP in the 3'-UTR[16)].

M1Rs are present in the autonomous nerve synapses and promote vagus nerve transmission. In the translation region of the M1R gene (at 11q13) Cys417Arg mutates across animal boundary lines[18)]. There is no clear relation to asthma. Binding affinity for ligands and antagonists is altered in rats by mutation.

II. ARGINASE

Arginase expression is enhanced in a IL-4/IL-13-dependent manner. This enzyme has a strong involvement in asthma[19)-21)]. Arginase catalyzes the hydrolytic cleavage of arginine to regenerate ornithine and yield urea. Importantly, arginase catalyzes the NO synthesis reaction and inhibits stromal competition (Fig. 1). NO exhibits diverse effects on the airways. In the guinea pig asthma model, for example, enhanced arginase activation suppresses NO with enhanced airway sensitivity[22)]. Arginase also plays an important role in murine pulmonary fibrosis due to Puromycin[23)]. In asthma patients, high arginase expression occurs in the airway epithelial cells and the submucosal inflammatory cells[21)].

Arginase I deficient patients have two mutation sites associated with the loss of enzyme activity[24)].

CONCLUSION

The chemical mediator receptors and arginase are factors modifying the asthmatic pathology. The regulating effect of IL-4/IL-13 on the expression of these chemical mediator receptors and arginase and the polymorphism of the receptor genes are relevant to the pathology of asthma.

に関与する重要な脂質メディエーター受容体であるので，これらの遺伝子多型については今までに多くの解析が行われた[9)-11)]．CysLT1R と CysLT2R の遺伝子はそれぞれ Xq13-q21と13q14-q21に存在し，いずれの遺伝子にもいくつかの SNPs が同定されている．CysLT1R の遺伝子多型と喘息との関連を積極的に支持する報告はないが，CysLT2R は喘息関連候補遺伝子の1つであると考えられている．

3) トロンボキサン A_2受容体

トロンボキサン A_2受容体(TXA_2R)は気道や血管の平滑筋収縮作用に関与している．TXA_2R 遺伝子は19p13に存在し，日本人では924T/C 多型が成人喘息と有意な関連を示すことが報告されている[12)]．この多型はサイレント SNP であるので，転写制御あるいは病態修飾にかかわる遺伝子と連鎖不平衡にある可能性が示唆される．また，中国人を対象とした解析結果によれば，924T/C 多型は小児喘息とも関連し，$FEV_{1.0}$(1秒量)や FVC (努力肺活量)の低下などとの相関が示されている[13)]．さらに，ネコ抗原特異的 IgE 値との相関も認められるので，924T/C 多型はアトピーとも連鎖していると考えられる．

4) ムスカリン受容体

アセチルコリン(Ach)の生物活性はムスカリン受容体(MR)を介して発現され，また MR には M1R-M5R の5つのサブタイプが同定されている．これらのうち，肺には M1R，M2R および M3R が発現されている．各サブタイプは異なった機能をもっており，喘息反応においては副交感神経終末から放出された Ach が気道平滑筋や粘液下腺に発現されている M3R を介して気道収縮や粘液分泌亢進を引き起こす．気道平滑筋細胞を IL-4/IL-13で刺激すると，M3R の発現が増強され，また Ach による細胞内 Ca^{2+}濃度のさらなる増加が認められる(山本ら，投稿準備中)．このように，M3R の発現は IL-4/IL-13によって正の調節を受ける．M3R 遺伝子は染色体1q41-q44に存在し，またこの部位はオーストラリア人の喘息患者において気道過敏性と関連していることが示されている領域に約20cM 近接している[14),15)]．M3R の遺伝子多型に関しては，マルタ島の喘息患者とランダムコントロールを対象とした解析結果によれば，M3R 遺伝子の翻訳領域と3'-非翻訳領域(3'-UTR)には SNP は検出されていない[16)]．しかし，ヨーロッパ系とアフリカ系のアメリカ人においては M3R 遺伝子のプロモーター領域に4つの SNPs が同定されたが，いずれの SNP も喘息との関連は認められなかった[17)]．一方，この領域において同定された短縦列繰り返し配列多型(STRPs)ハプロタイプは，アトピー患者を対象とした伝達不平衡テストによる解析でプリックテストにおけるゴキブリ抗原陽性率と相関していた．すなわち，STRPs ハプロタイプは喘息や気道過敏性との直接的な関連を示さないが，アトピー感受性遺伝子マーカーとは連鎖不平衡にある可能性が示唆される．

M2R は節後神経終末では，ネガティブフィードバック機構を介して Ach の放出を抑制する．また，気道平滑筋ではアデニル酸シクラーゼの活性化を阻害することによってサイクリック AMP レベルを低下させ，その結果収縮的に作用する．M2R 遺伝子は7q31-q35に存在し，翻訳領域には2つのサイレント SNPs，また3'-UTR には1つの SNP が同定されている[16)]．これら3つの SNPs と喘息との関連については今後の検討が待たれる．

M1R は自律神経節に存在し，迷走神経伝導を促進する．M1R 遺伝子(11q13)の翻訳領域には Cys417Arg 変異が同定されているが[18)]，喘息との関連はまだ検討されていない．この変異領域は動物種を超えて保存されており，たとえばラットに変異を誘導すると，リガンドや拮抗薬に対する結合能は有意に変化することが知られている．

Ⅱ．アルギナーゼ

アルギナーゼは IL-4/IL-13依存的に発現が増強され，また喘息への関与が強く示唆されている酵素である[19)-21)]．アルギナーゼはアルギニンの加水分解を触媒し，オルニチンと尿素を生成する酵素であり，2種類のアイソフォームが存在する．このうち，タイプⅠは特に尿素サイクルに関与し，肝臓に多く認められる．タイプⅡに関しては，前立腺や腎臓に高発現し，細胞増殖やコラーゲン産生に関わるプロリンとポリアミンを合成する．アルギナーゼの注目すべき活性は，NO (一酸化窒素)合成酵素と基質競合阻害することである(**図1**)．NO は気道系に多彩な作用を示し，たとえばモルモット喘息モデルではアルギナーゼ活性の増強が NO 産生を抑制し，その結果気道過敏性が亢進する[22)]．また，アルギナーゼはブレオマイシンによるマウス肺線維症にも重要な役割を果たしている[23)]．喘息患

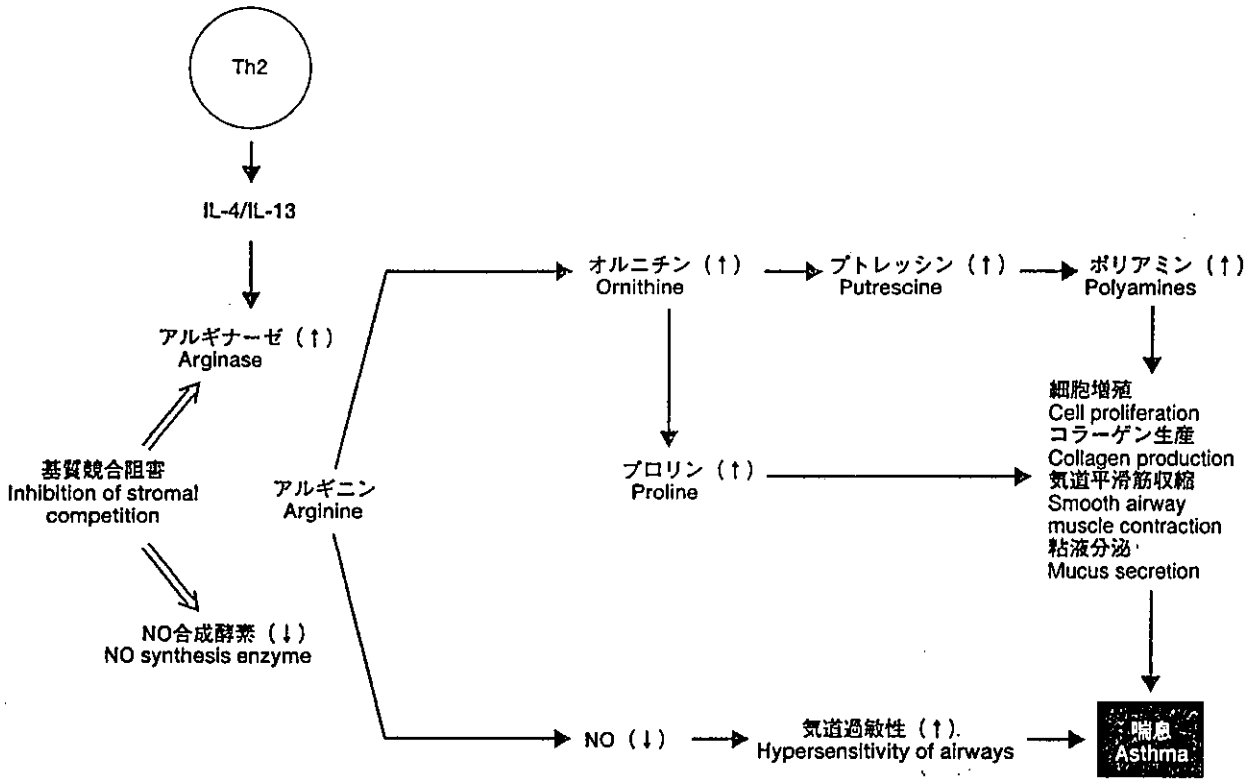

図1　喘息とアルギニン代謝経路
Fig. 1　Asthma and Arginine metabolic pathway

（文献20より引用改変）
(based on literature reference 20)

者では気道上皮や粘膜下炎症細胞にアルギナーゼの高発現が認められるので[21]，アルギニン代謝経路は喘息の病態形成に深く関与していると考えられる．

タイプⅠアルギナーゼ遺伝子は6q23上に存在し，またアルギナーゼⅠ欠損症患者では酵素活性の失活に関わる変異が2ヶ所同定されている[24]．アルギナーゼ活性の調節に関与する多型部位と喘息との関連については，今後の解析結果を待ちたい．

おわりに

以上，喘息の病態修飾因子であるケミカルメディエーター受容体やアルギナーゼについて，IL-4/IL-13による発現調節作用および受容体遺伝子の多型と喘息との関連を中心に概説した．喘息関連遺伝子の同定や遺伝子多型間の相互作用の解析は，創薬開発を含めたポストゲノム戦略として有用であるので，今後の進展を期待したい．

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The structure and binding mode of interleukin-18

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Interleukin-18 (IL-18), a cytokine formerly known as interferon-γ- (IFN-γ-) inducing factor, has pleiotropic immunoregulatory functions, including augmentation of IFN-γ production, Fas-mediated cytotoxicity and developmental regulation of T-lymphocyte helper type I. We determined the solution structure of IL-18 as a first step toward understanding its receptor activation mechanism. It folds into a β-trefoil structure that resembles that of IL-1. Extensive mutagenesis revealed the presence of three sites that are important for receptor activation: two serve as binding sites for IL-18 receptor α (IL-18Rα), located at positions similar to those of IL-1 for IL-1 receptor type I (IL-1RI), whereas the third site may be involved in IL-18 receptor β (IL-18Rβ) binding. The structure and mutagenesis data provide a basis for understanding the IL-18-induced heterodimerization of receptor subunits, which is necessary for receptor activation.

IL-18, which is functionally similar to IL-12 in terms of IFN-γ production, is associated with severe inflammatory diseases. The aberrant expression of IL-18 has been inferred to be associated with severe inflammatory conditions, such as autoimmune diseases, allergies or neurological disorders[1–4]. Successful therapeutic approaches such as tumor suppression in mice using IL-18 have been reported[5]. The *in vivo* functions of IL-18 are closely related to those of IL-12; thus, IL-18-deficient mice exhibit a phenotype similar to that of those deficient in IL-12. However, IL-18 and its receptors show no structural similarity with IL-12 and its receptors, respectively[2,3]. In contrast, despite the absence of apparent functional resemblance in terms of IFN-γ production, IL-1β and IL-18 show a moderate sequence similarity (17% identity). In addition, the polypeptide processing schemes for IL-18 and IL-1 share a common feature; that is, caspase-1 cleavage of both precursors is essential for maturation[2,3]. Receptors for both IL-1β and IL-18 belong to the IL-1 receptor family; thus, the intracellular signaling pathways for responding to these cytokines share the same downstream mediators, such as TRAF6 and NF-κB[2,3]. For activation of these receptors, heterodimerization of two structurally related but distinct immunoglobulin (Ig)-like chains are required: IL-18Rα (formerly called IL-1Rrp) and IL-18Rβ (formerly called IL-1AcPL) for the initiation of the IL-18 pathway, and IL-1RI and IL-1 accessory protein (IL-1AcP) for the initiation of the IL-1 pathway[2,3]. The interaction between IL-1 and IL-1RI has been revealed by crystal structures of their complex[6,7]. However, the recognition of IL-1AcP by IL-1 and hence the activation mechanism of the IL-1 pathway remain unclear[2,3].

As a first step toward understanding the receptor activation by IL-18, we have determined the solution structure of human IL-18 using NMR spectroscopy. The structure shows that the β-trefoil fold of IL-18 is similar to those of IL-1 family members, such as IL-1β, but the surface residues are totally dissimilar. We also constructed point mutants for the 50 surface-exposed residues of IL-18. Receptor binding and cellular response analyses using these mutants revealed the presence of three sites: two of these are important for IL-18Rα binding, and the third site is involved in the cellular response but not in IL-18Rα binding. Comparing the structure and receptor binding sites of IL-18 to those of IL-1 family members, we propose a model for the interaction between IL-18 and IL-18Rα.

RESULTS

Structure of IL-18

The overall structure of IL-18 is well defined except the segment between residues 34 and 43. IL-18 consists of 12 strands (S1–S12), which form three twisted four-stranded β-sheets, with one short α-helix (H1) and one 3_{10}-helix (H2). The three β-sheets are packed against each other to adopt a β-trefoil fold (**Fig. 1**). The loop, residues 34–42, spreads out from the body of the protein and seems to be flexible, as characterized by small heteronuclear NOE values (0.49 ± 0.025) and by the lack of long-range NOE values.

Despite the moderate sequence identity (17%), the overall architecture of IL-18 shows marked similarity with that of such IL-1 family members as IL-1β (**Fig. 2a**), as previously predicted on the basis of the conservation of hydrophobic residues[8]. The r.m.s. deviation of 1.60 Å between IL-18 and IL-1β for 70 Cα atoms in secondary structure elements indicates that they are related proteins belonging to the same structure class. However, IL-18 and IL-1 differ substantially in the

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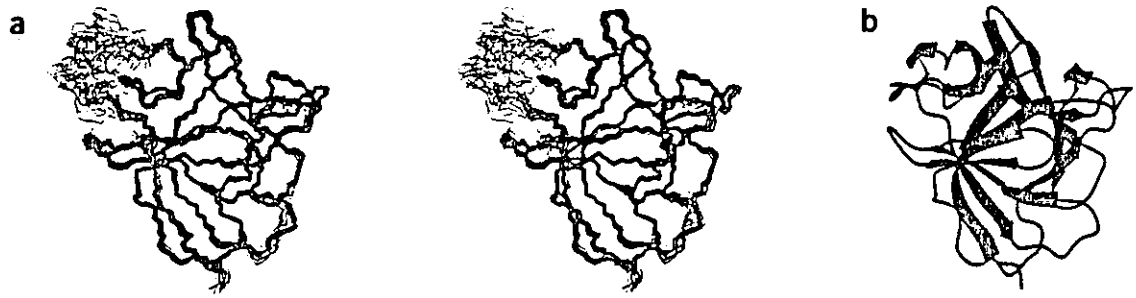

Figure 1 Solution structure of human IL-18. (a) Stereo view of the best-fit backbone superposition of the 20 final structures. The backbone atoms of residues 1–157 are superimposed. (b) Schematic ribbon drawing of the NMR structure of IL-18, drawn with MolMol[30].

length and conformation of several segments between the strands, S3–S4, S4–S5, S7–S8 and S11–S12.

Contact sites for IL-18Rα

To identify residues important for the IL-18 function, we generated a series of mutants and analyzed their abilities to induce IFN-γ production and to bind to IL-18Rα (Table 1). We chose 50 residues that covered most of the surface-exposed residues of IL-18. For each mutation that caused a decreased activity, we checked the effect of the overall native fold and protein stability by ^{1}H-^{15}N HSQC and circular dichroism spectra (see Methods). Of the 50 mutant proteins, 14 showed a substantially decreased ability to induce IFN-γ production without any apparent disruption of the native fold and stability (data not shown). Of the 14 mutants that showed decreased ability to induce IFN-γ production, 11 also showed a marked reduction in receptor binding activity (Table 1). These residues form two distinct surface patches, designated as sites I and II (Fig. 2b,c). Site I, formed by five residues (Arg13, Asp17, Met33, Asp35 and Asp132), is located on one side of the core barrel of the β-trefoil fold, whereas site II, formed by six residues (Lys4, Leu5, Lys8, Arg58, Met60 and Arg104), is located at the top of the β-barrel. It has recently been shown that the charge-reversal mutation of Glu6 or Lys53 alters the biological activity of IL-18 (ref. 9). These two residues are located on the same surface as site II, suggesting the importance of the site II–forming residues for receptor binding.

Table 1 Biological activity and dissociation constant of the mutants

	Biological activity (% wild type)[a]	Dissociation constant (nM)[b]
Wild type	100.0 ± 17.3	3.4 ± 0.3
K4A	7.0 ± 2.2	11.1 ± 0.5
L5A	1.0 ± 0.3	34.5 ± 3.5
K8A	9.5 ± 3.6	10.4 ± 2.1
R13A	8.0 ± 5.3	7.4 ± 1.2
D17A	20.1 ± 7.5	8.5 ± 1.7
M33A	0.6 ± 0.2	142.9 ± 49.0
D35A	0.5 ± 0.2	418.8 ± 181.6
R58A	1.9 ± 1.9	60.8 ± 2.6
M60A	2.1 ± 0.0	200.0 ± 55.0
K79A	9.6 ± 1.4	3.3 ± 0.3
K84A	25.7 ± 13.8	3.6 ± 0.3
D98A	16.7 ± 1.7	4.5 ± 0.3
R104A	7.5 ± 1.3	8.0 ± 0.5
D132A	14.2 ± 6.5	8.0 ± 0.6

[a]Mean values of triplicated interferon-γ induction assays are shown with standard deviation. [b]Dissociation constants for the interaction of IL-18 and IL-18Rα determined from Scatchard analysis of the equilibrium bound values are shown with standard error.

The structure of the complex of IL-1β and IL-1RI and mutational data have shown that IL-1β has two interfacial sites, sites A and B, for the IL-1RI (Fig. 2d,e). Site A makes contacts with the two N-terminal Ig domains of the extracellular portion of the receptor, and site B with the third Ig domain (Fig. 3a). Comparison of the tertiary and primary structures of IL-18 and IL-1β revealed that sites I and II of IL-18 correspond to sites A and B of IL-1β, respectively, because these sites are positioned equivalently in terms of sequences and tertiary structures.

These observations allowed us to construct a model for the complex of IL-18 and IL-18Rα (Fig. 3b), using the coordinates of the IL-1β–IL-1RI complex (PDB entry 1ITB) and of IL-18. The sequence identity (22%) and conservation of characteristic amino acids, such as cysteine, between the extracellular domains of IL-1RI and IL-18Rα, made the modeling of the tertiary structure of IL-18Rα relatively straightforward. The model obtained for the IL-18–IL-18Rα complex resembles closely the crystal structure of IL-1β–IL-1R1 and is consistent with the present mutational data for IL-18.

Electrostatic complementarity may account for the specificity of receptor binding of IL-18 and IL-1β. The molecular surface at and around site I of IL-18 is negatively charged, whereas the molecular surface of IL-18Rα that makes contact with this site in the model is highly positively charged (Fig. 2b,c). Mutational study has shown that the charges on IL-18 are important for receptor binding: alanine substitution of Asp17, Asp35 or Asp132 of IL-18 markedly abolished the receptor binding. In contrast, site A of IL-1β is positively charged (Fig. 2e), and the interface of IL-1RI is negatively charged. The electrostatic status of the receptor-binding site of the cytokines may contribute to receptor discrimination.

Possible binding site for IL-18Rβ

The mutagenesis studies also revealed that the residues Lys79, Lys84 and Asp98 are functionally important but not relevant to binding to IL-18Rα. Mutants of each of these residues are capable of binding to IL-18Rα but have lost the capacity for IFN-γ induction (Table 1). These residues are clustered at the bottom of the barrel, located opposite to site II, and are designated as site III (Fig. 2b,c). In the model of the IL-18–IL-18Rα complex, site III of IL-18 is exposed to the solvent, facing opposite to IL-18Rα (Fig. 3b). Thus, the observation that alanine substitutions of the site III–forming residues exert little effect on IL-18Rα binding is consistent with the model (Fig. 3b and Table 1).

What, then, is the function of the site III–forming residues? We assume that these residues are important for binding to IL-18Rβ, whose association with IL-18Rα is essential for initiation of IL-18 receptor signaling[2,3]. It is thought that IL-18 induces the association of the extracellular domains of IL-18Rα and IL-18Rβ and facilitates the homotypic interaction of their cytoplasmic domains to initiate the

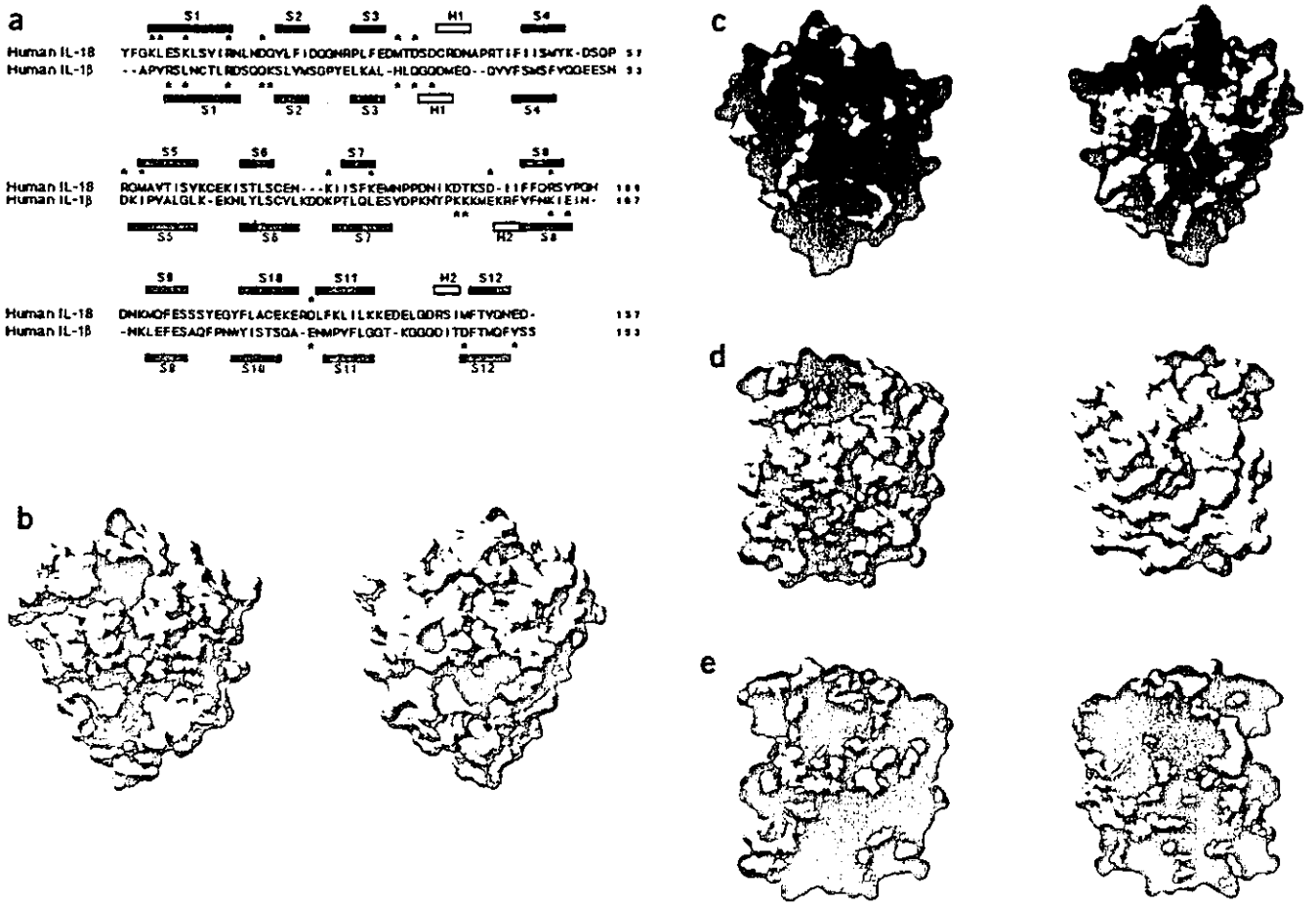

Figure 2 Mutational analysis of IL-18. (a) Sequence alignment of human IL-18 and IL-1β. IL-18 and IL-1β residues, the mutations of which resulted in a substantial reduction in biological activities, are indicated by asterisks. The secondary structure elements of IL-18 and IL-1β (PDB entry 2I1B) are indicated at the top and bottom, respectively. Conserved residues are boxed in yellow. (b) Surface representation of IL-18 residues, the mutations of which resulted in a substantial reduction in the activity. Residues in sites I, II and III are red, orange and blue, respectively (see text). (c) Distribution of the electrostatic potential on the solvent-accessible surface. Blue corresponds to positive potential and red to negative potential. In **b** and **c**, the molecule is shown in the same orientation as for **Figure 1** (left) and rotated by 180° about the vertical axis (right). (d) Structure of IL-1β. Surface representation of IL-1β residues, the mutations of which resulted in a substantial reduction in the activity. Residues in sites A and B are red and orange, respectively (see text). (e) Distribution of the electrostatic potential on the solvent-accessible surface. In **d** and **e**, the molecule is shown in the same orientation as for IL-18 (left) and rotated by 180° about the vertical axis (right).

signal transduction process[3]. A truncated form of IL-18Rβ lacking the cytoplasmic domain inhibits IL-18 signaling[10].

To determine whether site III is important for the association between IL-18Rα and IL-18Rβ, we conducted surface plasmon resonance measurements. IL-18Rβ cannot bind on its own to either IL-18 or IL-18Rα (Fig. 3c,d). However, in the presence of IL-18, IL-18Rβ is capable of binding to IL-18Rα immobilized on the sensor chip, showing that IL-18Rβ associates with the IL-18–IL-18Rα complex to form a ternary complex (Fig. 3d). We then examined the effects of mutations of the site III–forming residues on the formation of the ternary complex of IL-18, IL-18Rα and IL-18Rβ. The mutation of Lys79, Lys84 or Asp98 causes a large reduction in the degree of association between IL-18Rβ and the IL-18–IL-18Rα complex, suggesting that the residues at site III are important for the ternary complex formation (Fig. 3d). These IL-18 residues have charged side chains that form characteristic positively and negatively charged surface patches (Fig. 2b,c).

DISCUSSION

Initially, the structural basis of the receptor activation by cytokine was addressed for the growth hormone (GH) system[11]. The crystal structure of the GH–receptor complex revealed that monomeric GH binds to the boundary region between two fibronectin domains of the receptor 1 in a phenomenon known as elbow recognition, then simultaneously facilitates the formation of a ternary complex including another receptor, receptor 2, through interactions between GH and receptor 2, and between receptor 1 and receptor 2. It has been suggested that formation of this ternary complex allows cross-phosphorylation of the cytoplasmic domains of receptors 1 and 2, a reaction that is essential for GH-induced receptor activation. Such an activation mechanism involving monomeric ligand and receptor homo- or heterodimers having fibronectin domains seems to be widely employed in many of the signaling systems that include hematopoietic helical cytokines[12–14].

In contrast to hematopoietic helical cytokines, those adopting β-trefoil folds, such as fibroblast growth factors (FGFs) and IL-1 (refs. 6,7,15), activate their receptors in different ways. The structure of the signaling complex of FGF revealed that the two Ig domains of each receptor molecule interact mainly with the top of the β-barrel of the cytokine, similarly to elbow recognition, forming a dimeric 2:2:2 FGF–FGFR–heparin ternary complex[15]. In contrast, IL-1β exists as a

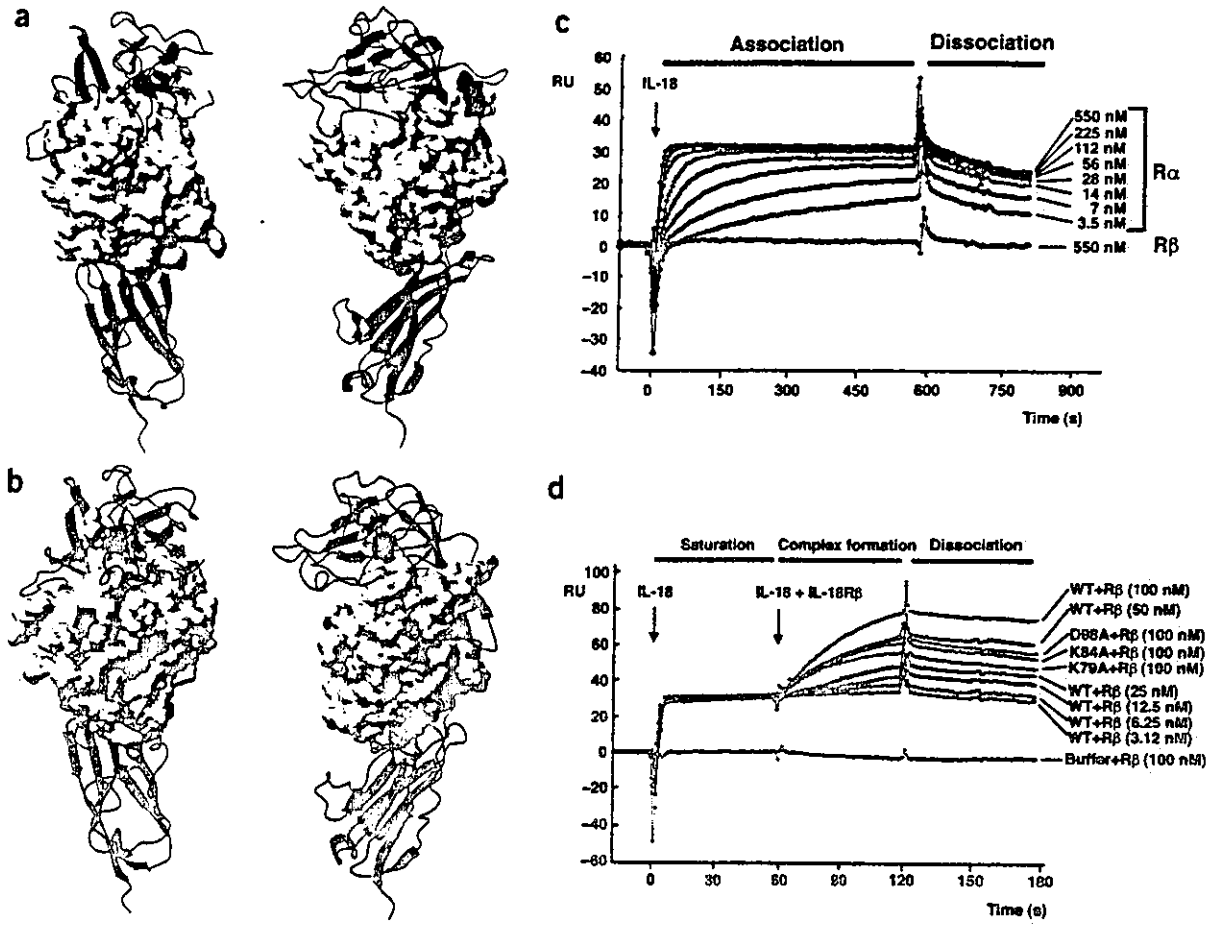

Figure 3 Models for the complex of IL-18 and IL-18Rα, and interactions among IL-18, IL-18Rα and IL-18Rβ. (**a**) Crystal structure of the complex of IL-1β and IL-1RI (PDB entry 1ITB). IL-1β residues in sites A and B are red and orange, respectively. (**b**) Modeled structure of the complex of IL-18 and IL-18Rα. IL-18 residues in sites I, II and III are red, orange and blue, respectively. In **a** and **b**, the molecule on the right is viewed as rotated by 90° about the vertical axis relative to the image on the left. (**c**) Dose-response curves of surface plasmon resonance for wild-type IL-18 to immobilized IL-18Rα or to immobilized IL-18Rβ. Concentration of wild-type IL-18 is also indicated. (**d**) Response curves of surface plasmon resonance for IL-18Rβ for to the complex of immobilized IL-18Rα with wild-type IL-18 or the site III mutant proteins.

monomer in solution, and the ligand–receptor stoichiometry for the active IL-1β signaling complex is unclear. Thus, although the mechanism of receptor activation by this cytokine remains to be characterized, the structure of the IL-1β–IL-1Rα binary complex shows that the three Ig domains of IL-1Rα embrace the ligand monomer interacting with the top, the side and the bottom of the β-barrel of IL-1β (refs. 7,16).

Our structural determination and mutagenesis of IL-18 suggest that the mode of interaction between IL-18 and IL-18Rα is similar to that seen in the IL-1β–IL-1Rα binary complex, and that residues Lys79, Lys84 and Asp98 of IL-18 are important for binding of IL-18Rβ to the binary complex. Although we cannot completely exclude the possibility, it seems improbable that these residues are directly involved in homodimerization of IL-18 to form the 2:1:1 complex of IL-18, IL-18Rα and IL-18Rβ, because we did not observe any indications of homodimerization of IL-18 by itself (such as significant line broadening and intermolecular NOEs) in the NMR spectra of IL-18 at a high concentration (1–2 mM). Therefore, we assume that these residues are involved in direct binding to IL-18Rβ and thus that the active ternary complex consists of a single IL-18 molecule. Future study needs to be directed at establishing the role of site III–forming residues in the formation of the active complex.

The molecular architecture of IL-18 and its receptors shows marked similarities to other IL-1 molecules and their receptors[17,18]. The residues of IL-1β (Lys77, Leu82 and Glu96), which correspond to the site III–forming residues of IL-18, may analogously form an interfacial surface with IL-1AcP and thus mediate its association with the IL-1β–IL-1RI complex. This raises the possibility that mutation of these residues of IL-18 or IL-1β might lead to the generation of a functional antagonist for these signaling systems, because the mutant can block one of the receptor molecules without activating it. Therefore, the determination of IL-18 residues important for receptor activation not only provides a molecular basis of receptor activations by the members of the IL-1 cytokine family[19,20], but also furnishes information that can contribute to therapeutic approaches for IL-1 family–related diseases[3,21].

METHODS

Sample preparation. The ^{15}N-labeled and ^{15}N-^{13}C-labeled wild-type IL-18 proteins were expressed as a glutathione *S*-transferase (GST) fusion protein. After purification by affinity chromatography, the GST tag was removed by digestion with Factor Xa. Samples for NMR measurements typically consisted of 1–2 mM protein in 50 mM Tris-HCl buffer (pH 6.0), 150 mM KCl and 10 mM DTT in H_2O/D_2O (90%:10%) or D_2O.

Table 2 Summary of restraints and structural statistics

NOE distance restraints	
Total	2,289
$\|i-j\|=0$	646
$\|i-j\|=1$	603
$2\le\|i-j\|<5$	239
$\|i-j\|\ge5$	801
Dihedral angle restraints	
ϕ	111
ψ	89
$\chi1$	10
Hydrogen bonds[a]	50
Energies (kcal mol^{-1})[b]	
E_{total}	93.9 ± 0.85
E_{LJ}[c]	−590 ± 17
Maximum violation	
Distance (Å)	0.24
Angle (°)	0.94
R.m.s. deviation from ideal geometry	
Bonds (Å)	0.0011 ± 0.00006
Angles (°)	0.2831 ± 0.0023
Improper (°)	0.1193 ± 0.0054
Coordinate precision (residues 1–157)	
Backbone (Å)	0.940
Heavy (Å)	1.442
Coordinate precision (residues 2–33, 43–157)	
Backbone (Å)	0.570
Heavy (Å)	1.147
Ramachandran statistics (residues 1–157)	
Most favored (%)	78.5
Additionally (%)	18.9
Generously (%)	1.9
Disallowed (%)	0.7

Statistics are calculated for 20 of the lowest-energy structures of 100 calculated.

[a]Two distance restraints per hydrogen bond were used, providing a total of 100 restraints. [b]The default parameters and force constants of protein-allhdg.param and anneal.inp in CNS 1.1 were used for calculation. [c]The Lennard-Jones van der Waals energy was not used in the structure calculation.

A total of 50 mutant IL-18 proteins were prepared in the same manner as that of wild-type protein, in which each amino acid was replaced by alanine or glycine. Residues to be mutated were selected considering sequence alignments with IL-1 and IL-18 family members, comparison of the structures of IL-18, IL-1β (PDB entry 2I1B) and the IL-1β–IL-1RI complex (PDB entry 1ITB), and previously reported mutagenesis data for IL-1 molecules[22].

NMR spectroscopy. All NMR spectra were acquired at 303 K on a Bruker DMX500, DRX500 or DRX800 NMR spectrometer. For assignment of the ^{1}H, ^{13}C and ^{15}N resonances of the backbone and the side chains, a series of three-dimensional experiments were conducted[23]. The stereospecific assignment of methyl groups of the valine and leucine residues was carried out as described[24]. Distance restraints were obtained from ^{15}N-^{15}N-, ^{15}N-^{13}C- or ^{13}C-^{13}C-resolved 4D NOESY experiments with a mixing time of 100 ms (ref. 23).

Structure calculation. The obtained main chain resonance assignment indicated the presence of a minor conformation at or around the segment between strands S3 and S4, possibly as a result of *cis-trans* isomerization of the Ala42–Pro43 peptide bond. Differences of ^{1}H and ^{15}N chemical shifts between the major and minor conformations are relatively small. We obtained structure restraints for only the major conformation. Initially, structure calculation and NOE peak assignment were done in an iterative and manual manner using DYANA (version 1.5)[25]. Backbone torsion angle restraints were derived from $^{3}J_{HNH}\alpha$ of HNHA[23] and TALOS[26]. The torsion angles $\chi1$ of Phe30, Phe47, Phe52, Phe83, Phe101, Phe102, Phe115, Phe124, Phe151, Tyr52 and His109 were estimated from $^{3}J_{C'C}$ and $^{3}J_{NC}$ coupling constants[27]. After determining the global fold manually, the CANDID algorithm was used to assign the remaining NOE peaks[28], yielding 2,289 meaningful NOE upper distance restraints. With these restraints, final structures were calculated using CNS (version 1.1)[29]. At this stage, hydrogen bond restraints from the slowly exchanging backbone amides were added as distance restraints of 2.8–3.4 Å for N–O and 1.8–2.4 Å for HN–O atom pairs, respectively. Non-stereospecifically assigned protons were treated as floating chirality and $\sum (r^{-6})^{-1/6}$ sum. A total of 100 structures were refined, and the 20 lowest-energy structures were analyzed using MOLMOL[30], AQUA and PROCHECK-NMR software[31] (Table 2).

IFN-γ induction and receptor binding assay. We prepared the following mutants of IL-18: Y1G, F2A, K4A, L5A, E6A, K8A, R13A, L15G, N16A, D17A, D23A, R27A, E31A, M33A, D35A, R39A, D40A, M51G, K53A, D54A, R58A, M60A, K67A, E69A, K70A, E77A, K79A, K84A, E85A, D90A, K93A, D94A, K96A, D98A, R104A, D110A, K112A, E121A, E128A, E130A, D132A, F134G, K135A, K139A, K140A, E141A, E143A, R147A, V153G and D157A. The wild-type and mutant IL-18 proteins were examined for their ability to induce IFN-γ production at 37 °C as described[32]. For each of the mutants that showed substantial reduction in activity, ^{15}N-labeled proteins were prepared and the ^{1}H-^{15}N-correlation spectrum at 30 °C was compared with that of the wild-type IL-18. All these mutants showed no evidence of mutation-induced disruptions of the native fold. The stability of the wild-type and mutant proteins, F2A, M33A, D35A, M60A, K79A and D98A, was further examined by measuring their circular dichroism spectra at 37 °C. Results suggest that the structural stability of these mutant proteins, except that of F2A, is similar to that of the wild-type protein at this temperature. Mutant F2A was omitted from the additional studies because of the possible destabilization of the structure.

In vitro affinities of the wild-type and mutant IL-18 for IL-18 receptors were measured at 25 °C by surface plasmon resonance experiments using BIACORE3000 (Pharmacia Biosensor AB). A specific binding surface was prepared by coupling the anti-human IgG Fc antibody (Rockland) to a CM5 sensor chip by the amine coupling method. Then, the recombinant IL-18Rα–Fc or IL-18Rβ–Fc chimera proteins (R&D Systems) were immobilized on chips. The coupling density was limited to 200 resonance units (RU). Samples of IL-18 were diluted in HBS–EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, 0.005% (v/v) surfactant P-20). For the dissociation constant analysis of the wild-type and mutant IL-18 proteins to IL-18Rα, different amounts of the samples were injected over the sensor chip at 2 μl min^{-1} until the equilibrium phases were obtained. The sensor surface was regenerated by two 60-s pulses of 0.2 M glycine-HCl, pH 1.5. Dissociation constants were determined by Scatchard plot analysis. For evaluation of the trimeric complex–forming abilities, the IL-18 proteins were first passed over the sensor chips until the IL-18Rα binding sites were saturated, and then the same amount of the IL-18 proteins mixed with the IL-18Rβ protein (3.12–100 nM) was injected.

Modeling. Modeling of the IL-18 and IL-18Rα complex is based on the structure of IL-18 and the crystal structure of the IL-1β–IL-1RI complex (PDB entry 1ITB). The MOE (http://www.chemcomp.com) was used for model construction and structure verification. The coordinates of the NMR structure of IL-18 and the modeled structure of IL-18Rα were superimposed on the IL-1β–IL-1RI complex. The IL-18–IL-18Rα complex model was refined by the molecular dynamics calculation of 70 ps at 300 K with the AMBER 5.0 package (http://www.amber.ucsf.edu) using the all-atom force field. The final structure was additionally energy minimized.

Coordinates. Coordinates have been deposited in the Protein Data Bank (accession code 1J0S).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Original Article

(27) Interleukin-18 is associated with the severity of atopic dermatitis

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Abstract

Background: Interleukin (IL)-18 acts as both a Th1 and Th2 cytokine, but its association with allergic diseases remains unclear. The aim of the present study was to measured plasma IL-18 and serum IgE levels in atopic children to evaluate how IL-18 is associated with allergic diseases.

Methods: The plasma IL-18 and serum IgE levels in 51 atopic children, 28 healthy control children and 14 healthy control adults were measured by enzyme-linked immunosorbent assay (ELISA). The 5′ end of the IL-18 gene of 48 atopic children and 20 healthy control children was sequenced.

Results: The plasma IL-18 level was significantly elevated in children with bronchial asthma and/or atopic dermatitis. Plasma IL-18 levels in the moderate or severe atopic dermatitis group were significantly higher than those in either the control group or the mild atopic dermatitis group. There was a positive correlation between plasma IL-18 and serum IgE levels. Three allelic combinations of polymorphisms in the IL-18 gene promoter region were observed. There was no significant difference in the plasma IL-18 levels between groups carrying these genotypes. However, bronchial asthma patients had significantly higher frequencies of the –137 G/G genotype than did control children.

Conclusions: The plasma IL-18 level was elevated, particularly in patients with atopic dermatitis. As the clinical severity of atopic dermatitis increased, the plasma IL-18 level also tended to increase. These findings suggest that IL-18 may be associated with the severity of atopic dermatitis.

Key words: atopic dermatitis, bronchial asthma, IgE, interferon-γ, interleukin-18, interleukin-18 promoter.

Introduction

Interleukin (IL)-18, originally known as an interferon (IFN)-γ-inducing factor (IGIF), is a recently cloned cytokine secreted by Kupffer cells of the liver and activated macrophages.[1] Interleukin-18 strongly enhances IFN-γ production by T cells, natural killer cell cytotoxicity and T cell proliferation. Moreover, recent studies have demonstrated that IL-12, which is also known as an IFN-γ-inducing factor, and IL-18 exert a synergistic effect on IFN-γ production by T cells.[2,3]

Interleukin-18 is a proinflammatory cytokine. Its increased production was observed in the acute phase of experimental autoimmune encephalomyelitis (EAE)[4] and antibodies to IL-18 could prevent EAE in Lewis rats.[5] In humans, IL-18 expression has been observed in Th1-mediated chronic inflammatory diseases, such as Crohn's disease,[6] rheumatoid arthritis[7] and acute infectious mononucleosis.[8] Increased serum or plasma IL-18 levels have been observed in severe melioidosis,[9] hemophagocytic lymphohistiocytosis[10] and multiple sclerosis.[11] Increased IL-18 levels in cerebral spinal fluid have been observed in bacterial meningitis[12] and multiple sclerosis.[11]

However, how IL-18 is associated with allergic diseases remains unclear. We have reported previously that the serum IgE level was negatively correlated with IFN-γ production by phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC).[13] The IFN-γ

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production by IL-12-stimulated PBMC in the atopic group was lower than that in the control group and the serum IgE level was negatively correlated with IFN-γ production by IL-12-stimulated PBMC.[14] One of the reasons for this was that reduced IFN-γ production following IL-12 stimulation was associated with heterozygous IL-12Rβ2 mutations in atopic patients.[15] The presence of abnormalities not only in the IL-12, but also in the IL-18, signal transduction pathway could be assumed in atopic patients. However, recently there have been some reports that IL-18 is also associated with Th2 reactions.[16–19] Nakanishi *et al.* reviewed the dual regulatory roles of IL-18 in the immune system; that is, IL-18 regulates not only the Th1 pathway, but also the Th2 pathway.[20]

In the present study, we found a correlation between the plasma IL-18 level and the severity of atopic dermatitis in children.

METHODS

Patients and control subjects

All patients and healthy volunteers were recruited from the Department of Pediatrics, Gifu University School of Medicine, and informed consent was obtained from all subjects or their parents. Fifty-one children with allergic diseases, particularly atopic dermatitis and bronchial asthma, 28 healthy control children and 14 healthy control adults were studied. Seventeen patients had bronchial asthma only (eight patients were in the no asthma attack phase, nine patients were in the asthma attack phase), 18 patients had atopic dermatitis only and 16 patients had both bronchial asthma and atopic dermatitis. The diagnosis of atopic dermatitis was made according to the criteria of Hanifin,[21] whereas that of bronchial asthma was made according to the criteria of the American Thoracic Society.[22] The severity of atopic dermatitis was evaluated based on the criteria of Rajka and Langeland.[23] The serum IgE levels, age and sex of the study subjects are listed in Table 1. The healthy controls did not have a history of atopic diseases and their serum IgE levels were within normal limits for their age. They were healthy and free of acute infection at the time of testing.

Plasma and cell preparation

Plasma and leukocytes were separated from the heparinized blood of control donors and patients. All plasma samples were stored at –30°C until assay.

Interleukin-18 assay

Plasma IL-18 levels were measured with a human IL-18 enzyme-linked immunosorbent assay (ELISA) kit (Medical & Biological Laboratories, Nagoya, Japan); the lower detection limit was 12.5 pg/mL.

IgE assay

Plasma or serum IgE levels were determined by chemiluminescent enzyme immunoassay. Plasma IgE values were regarded as serum IgE levels. Specific IgE antibodies for house dust, *Dermatophagoides*, hen egg and cows' milk were measured with a fluoroenzyme immunoassay by means of a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden). Scores of 3+ to 6+ were considered positive.

Sequencing of the 5' end of the IL-18 gene

Genomic DNA was extracted from leukocytes using a SepaGene (Sanko Junyaku, Tokyo, Japan). A 1492 bp fragment of the IL-18 gene was amplified by polymerase chain reaction (PCR) using primers 5'-TTGATCCCACTTCGTGCTTTCA-3' and 5'-CCTTTCCTCTTCCCGAAGCTGT-3'. Conditions for the PCR were 40 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 2 min. DNA was purified using geneclean II (BIO 101, Carlsbad, CA, USA) and used for big dye terminator bidirectional sequencing (Applied Biosystems, Foster

Table 1 Subject characteristics

	Control		Bronchial asthma	Atopic dermatitis	Atopic dermatitis + bronchial asthma
	Adults	Children			
No. subjects	14	28	17	18	16
Age (years)	33.9 ± 9.4	4.8 ± 4.4	8.1 ± 4.1	4.9 ± 4.6	5.1 ± 3.8
Sex (males/females)	5/9	16/12	14/3	13/5	10/6
IgE (IU/mL)	8–140	5–110	24–2400	11–11 000	18–5400

City, CA, USA). Primers 5'-CCCTTCCTAGCAAAG-TAATAC-3', 3'-GAATAATCAGTCCTATTGGGG-5', 5'-CCAATAGGACTGATTATTCCGCA-3' and 3'-AGGAG-GGCAAAATGCACTGG-5 were used for sequencing an approximate 700 bp fragment upstream of the known IL-18 cDNA sequence. For the position –607 specific PCR, a common reverse primer 5'-TAACCTCATTCAG-GACTTCC-3' and two sequence-specific forward primers 5'-GTTGCAGAAAGTGTAAAAATTATTAC-3' and 5'-GTTGCAGAAAGTGTAAAAATTATTAA-3' were used. For the position –137 specific PCR, a common reverse primer 5'-AGGAGGGCAAAATGCACTGG-3' and two sequence-specific forward primers 5'-CCCCAACTTT-TACGGAAGAAAAG-3' and 5'-CCCCAACTTTTACG-GAAGAAAAC-3' were used. Each PCR was performed with rTaq (Takara Shuzo, Shiga, Japan) and the conditions for PCR were 40 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min. Products separated by 2% agarose gel electrophoresis were visualized by staining with ethidium bromide.

Statistical analyses

The significance of differences between two groups was analyzed by the Mann–Whitney *U*-test. The significance of differences between multiple groups was evaluated by the Kruskal–Wallis test and further analysis was performed by the Bonferroni/Dunn test. Spearman's correlation coefficient (*R*) was used to calculate the correlation between two variables. The frequencies of alleles were compared through the use of χ^2 statistics. Statistical significance was assumed for $P < 0.05$.

RESULTS

Mean (± SD) plasma IL-18 levels in patients with allergic diseases (394 ± 220 pg/mL), healthy control adults (201 ± 79 pg/mL) and control children (174 ± 87 pg/mL) are shown in Fig. 1a. We found significantly higher levels of plasma IL-18 in patients with allergic diseases ($P < 0.0001$) than in healthy control children. There was no significant difference in plasma IL-18 levels between healthy control adults and healthy control children.

In the present study, we particularly investiaged bronchial asthma and atopic dermatitis as representative allergic diseases in children. Mean (± SD) plasma IL-18 levels in patients with bronchial asthma (324 ± 122 pg/mL), atopic dermatitis (508 ± 238 pg/mL) and atopic dermatitis and bronchial asthma (338 ± 236 pg/mL)

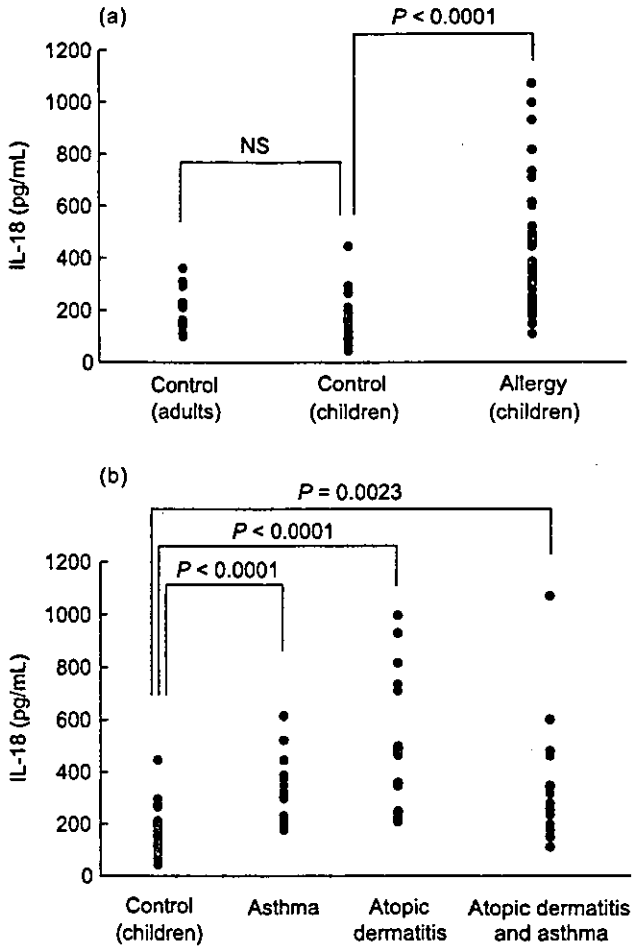

Fig. 1 (a) Comparison of plasma interleukin (IL)-18 levels in control adults, control children and children with allergic disease. Plasma IL-18 levels in children with allergic disease were significantly higher than those in healthy control children. (b) Comparison of plasma IL-18 levels in control children and children with bronchial asthma, atopic dermatitis and atopic dermatitis plus bronchial asthma. Plasma IL-18 levels in children with bronchial asthma, atopic dermatitis and atopic dermatitis plus bronchial asthma were significantly higher than those of healthy control children.

are shown in Fig. 1b. We found significantly higher levels of plasma IL-18 in the bronchial asthma group ($P < 0.0001$), the atopic dermatitis group ($P < 0.0001$) and the atopic dermatitis and bronchial asthma group ($P = 0.0023$) compared with healthy control children. Furthermore, the plasma IL-18 level of the atopic dermatitis group was particularly high. The atopic dermatitis group has significantly higher levels of plasma IL-18 than the bronchial asthma group ($P = 0.01$). There was no significant difference in plasma IL-18 levels between the

acute phase (323 ± 153 pg/mL) and non-acute phase of asthma (325 ± 85 pg/mL).

Figure 2a,b shows the plasma IL-18 and serum IgE levels of each group classified according to the severity of atopic dermatitis with or without bronchial asthma.[23] Using the Kruskal–Wallis test, we found significant differences in levels of plasma IL-18 ($P < 0.0001$) and serum IgE ($P < 0.0001$) between groups of patients classified according to the severity of atopic dermatitis. Further analysis using the Bonferroni/Dunn test was performed for comparisons among groups. Plasma IL-18 levels in the moderate or severe atopic dermatitis group were significantly higher than those in either the control group or the mild atopic dermatitis group. Serum IgE levels in the severe atopic dermatitis group were significantly higher than those in the other groups and serum IgE levels in the mild and moderate atopic dermatitis groups were significantly higher than those in the control group. Figure 2c shows the relationship between plasma IL-18 and serum IgE levels in healthy controls and patients. It was reported that serum IL-18 levels in Nc/Nga mice tended to be negatively correlated with serum IgE levels[24] but, in the present study, there was a positive correlation between plasma IL-18 and serum IgE levels ($R = 0.472$; $P < 0.0001$). Three patients (P1, P2, P3) had higher plasma IL-18 levels more than the control group + 2SD, but their serum IgE levels were within normal limits for their age. These three patients were also negative for specific IgE.

We analyzed the 5′ end of the IL-18 gene sequence in 48 allergic patients and 20 healthy control children. Five single-nucleotide polymorphisms were detected. Two are located in the 5′-untranslated region of the IL-18 gene and three are located in the promoter region. Three allelic combinations of the polymorphisms observed in the Japanese populations were the same as those found in the Swedish population[25] (Table 2). Figure 3 shows the

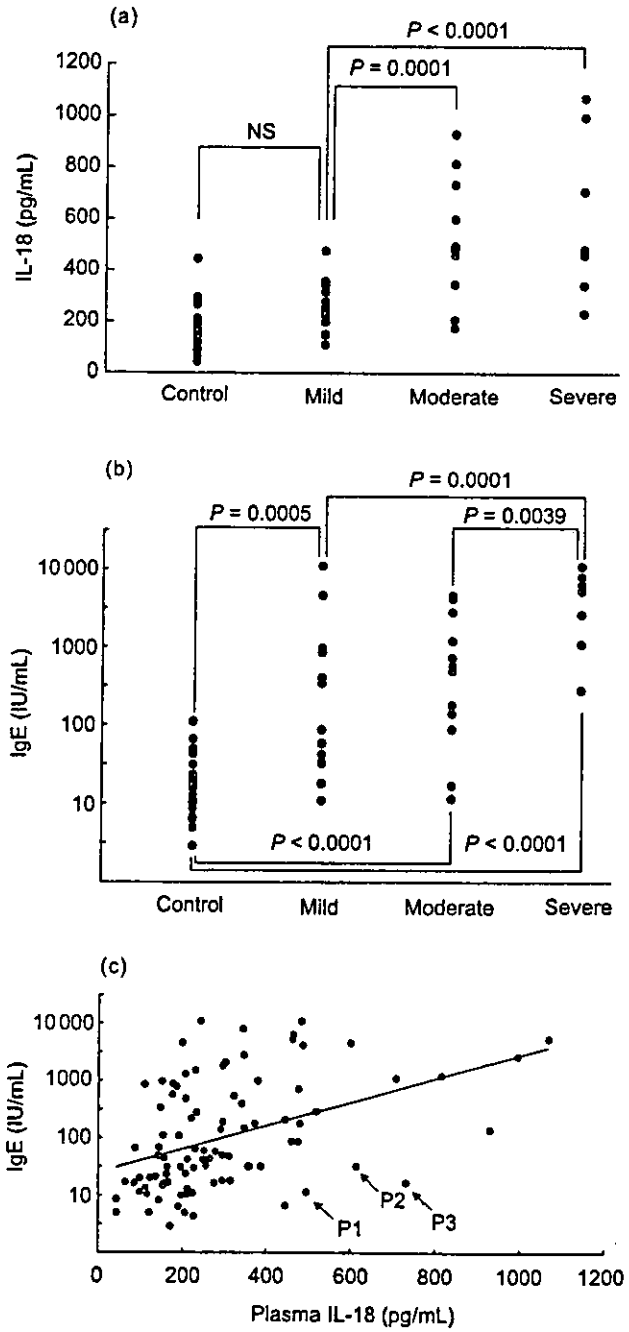

Fig. 2 (a) Relationship between plasma interleukin (IL)-18 levels and severity of atopic dermatitis. Plasma IL-18 levels in the moderate or severe atopic dermatitis group were significantly higher than those in the control group or mild atopic dermatitis group (mild ($n = 14$) 257 ± 95.9 pg/mL; moderate ($n = 12$) 549 ± 226 pg/mL; severe ($n = 8$) 595 ± 302 pg/mL). (b) Relationship between serum IgE levels and the severity of atopic dermatitis. Serum IgE levels in the severe atopic dermatitis group were significantly higher than those in the other groups and serum IgE levels in the mild and moderate atopic dermatitis groups were significantly higher than those in the control group (mild ($n = 14$) 1320 ± 3030 IU/mL; moderate ($n = 12$) 1250 ± 1660 IU/mL; severe ($n = 8$) 5020 ± 3600 IU/mL). (c) Relationship between plasma IL-18 levels and serum IgE levels in healthy controls and patients. Plasma IL-18 levels were significantly and positively correlated with serum IgE levels ($R = 0.472$; $P < 0.0001$). Arrows indicate patients with high plasma IL-18 levels, but low serum total IgE levels; they were also negative for specific IgE (patient 1, patient 2, patient 3).

Table 2 Ploymorphisms at the 5′ end of the interleukin-18 gene

	Position				
	−656	−607	−137	+113	+127
Allele 1	G	C	G	T	C
Allele 2	T	A	C	G	T
Allele 3	T	A	G	T	C

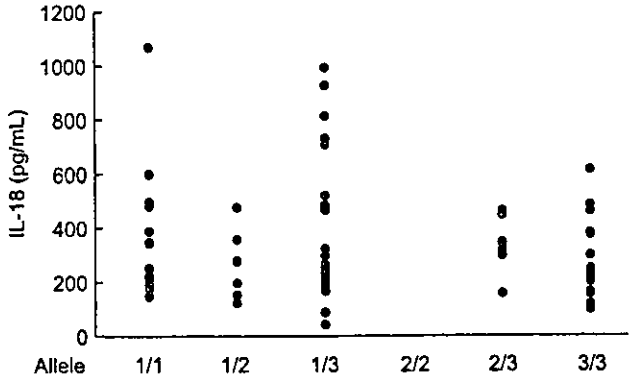

Fig. 3 Relationship between levels of plasma interleukin (IL)-18 and genotypes of the 5′ end of IL-18 gene polymorphisms in atopic patients and healthy control children. There was no significant difference ($P = 0.3596$) between these groups (1/1 ($n = 14$) 365 ± 246 pg/mL; 1/2 ($n = 8$) 252 ± 121 pg/mL; 1/3 ($n = 21$) 408 ± 282 pg/mL; 2/2 ($n = 0$); 2/3 ($n = 7$) 267 ± 143 pg/mL; 3/3 ($n = 18$) 336 ± 217 pg/mL).

Table 3 Genotype frequencies in 48 allergic patients and 20 healthy control children

Genotype	Control	Allergic patients
1/1	2	12
1/2	4	4
1/3	7	14
2/2	0	0
2/3	3	4
3/3	4	14
P		0.3559

plasma IL-18 levels of children with different combinations of alleles. Children with the genotype 1/1 and 1/3 showed a relatively higher mean level of plasma IL-18 (1/1, 365 ± 246 pg/mL; 1/3, 408 ± 282 pg/mL). However, there was no significant difference between these groups ($P = 0.3596$). Table 3 shows genotype frequencies of the IL-18 promoter region in allergic patients and healthy control children. There were no significant differences. Further analysis was performed for comparisons among allergic patients and healthy control children at position −137 of the IL-18 promoter region. Table 4 shows the frequencies of single nucleotide polymorphism −137 G/C of the IL-18 promoter region in allergic patients and healthy control children. Bronchial asthma patients had significantly higher frequencies of the −137 G/G genotype than did control children.

Discussion

Interleukin-18 was initially identified as a strong inducer of a Th1-mediated cytokine, IFN-γ, and this function is synergistically performed with IL-12.[26] Moreover, IgE suppression had been thought as the result of IFN-γ induction by IL-18 because the serum IgE level was negatively correlated with the amount of IFN-γ secreted by the PBMC of atopic patients.[13] However, it was reported that IL-18 levels in the sera of adult patients and in atopic dermatitis model mice (Nc/Nga) were elevated.[24] In the present study, we showed that the plasma IL-18 levels increased significantly in children with allergic diseases, particularly atopic dermatitis. Tanaka *et al.* reported that the serum IL-18 level may reflect asthma disease activity,[27] but we found no significant difference in plasma IL-18 levels between the acute and non-acute phases of asthma in the present study. In contrast with patients with bronchial asthma, the plasma IL-18 levels of patients with atopic dermatitis tended to increase as the severity of the disease increased.

Table 4 Frequencies of single nucleotide polymorphism −137 G/C of the interleukin-18 promoter region in patients and healthy control children

Genotype	Control ($n = 20$)	Allergy ($n = 48$)	Bronchial asthma ($n = 14$)	Atopic dermatitis ($n = 18$)	Atopic dermatitis + bronchial asthma ($n = 16$)
G/G	13	40	14	15	11
G/C	7	8	0	3	5
C/C	0	0	0	0	0
P		0.0967	0.0130	0.2000	0.8125

Why is the IL-18 level elevated particularly in patients with bronchial asthma and severe and moderate atopic dermatitis? One of the possible reasons is the association with lipopolysaccharide (LPS) or endotoxin. Jorgensen *et al.* reported that, in leukocytes from patients with atopic dermatitis, bacteria and endotoxin induce the release of basophil histamine.[28] Recently, it was reported that endotoxemia was associated with elevations in the plasma IL-18 level in infections after acute lung injury.[29] Seki *et al.* reported that IL-18 secretion is mediated by activation of endogenous caspase-1 without *de novo* protein synthesis after stimulation with LPS.[30] El-Mezzein *et al.* reported an increase in the secretion level of IL-18 by LPS-stimulated PBMC of patients with bronchial asthma or atopic dermatitis.[31] Therefore, LPS and endotoxin could be associated with caspase-1 activity and IL-18 secretion in atopic dermatitis.

The second possible reason is differences in IL-18 promoter activity among IL-18-producing cells in the patients. Vilmantas *et al.* reported that alleles 1 and 3 of IL-18 promoter region polymorphisms showed a higher transcriptional activity than allele 2, as determined by luciferase assay using transfected human HeLa 229 cells that were stimulated with phorbol myristate acetate and ionomicin.[25] A change from C to A at position –607 disrupts a potential cAMP-responsive element binding protein binding site. A change from G to C at position –137 changes the H4TF-1 nuclear factor binding site to a binding site for an unknown factor found in the granulocyte–macrophage colony stimulating factor promoter.[25] In the present study, children with the genotype 1/1 and 1/3 showed a relatively higher average level of plasma IL-18, but the association of plasma IL-18 level with polymorphisms of IL-18 promoter alleles and their frequencies was not significant. However, the –137 G/G genotype had a significantly higher frequency in bronchial asthma patients than in control children. Therefore although the number of subjects is too small to conclusively determine the association between plasma IL-18 and these polymorphisms, we can speculate that alleles 1 and 3, which had G at position –137 of the IL-18 promoter region, may affect the allergy state through IL-18.

Another possibility is the existence of abnormalities in the signal transduction pathway of IL-18. Matsui *et al.* reported that IL-12Rβ2 mutations resulted in a decrease of IFN-γ production.[15] Similarly, Shikano *et al.* reported that, in several atopic patients; IFN-γ production was not induced sufficiently by IL-18, but it was induced sufficiently by phytohemagglutinin or IL-12.[32] In our recent study on the IL-18 receptor, *950delCAG* in the IL-18Rα chain cDNA was found to be associated with reduced IFN-γ production and a high serum IgE level in atopic patients.[33] Mühl *et al.* reported that IFN-γ upregulated the expression of IL-18 binding protein, which is an inhibitor of IL-18 activity, suggesting a negative feedback mechanism between IL-18 and IFN-γ.[34] Therefore, abnormalities of IL-18 signal transduction to IFN-γ production and the impairment of the negative feedback mechanism may induce the increase in plasma IL-18 levels.

How is IL-18 associated with exacerbation of atopic dermatitis? Atopic dermatitis is a chronic inflammatory skin disease, with remissions and exacerbations. It is generally known that serum IgE levels are elevated in 80% of patients with atopic dermatitis[35,36] and the severity of atopic dermatitis highly correlates with the levels of serum IgE.[35] In the present study, as the clinical severity of atopic dermatitis increased, the serum IgE and plasma IL-18 levels tended to increase. Moreover, the plasma IL-18 levels were significantly correlated with serum IgE levels. Yoshimoto *et al.* showed that, in the presence of IL-3, IL-18 induces basophils and mast cells to release large amounts of Th2 cytokines both *in vitro* and *in vivo*.[18] Moreover, IL-18 alone has the capacity to induce IgE accumulation *in vivo*.[18,19] Therefore, IL-18 may be associated with allergic diseases, particularly atopic dermatitis exacerbation through IgE production.

It is interesting that three patients (P1, P2, P3) had high plasma IL-18 levels, but low serum total IgE levels; they were also negative for specific IgE. In fact, many allergic patients have low serum total IgE levels and specific IgE levels. It has been reported that 20% of atopic dermatitis patients with typical eczema have normal serum IgE levels.[21,37] Yoshimoto *et al.* reported that IL-18 could directly stimulate histamine release by basophils.[18] Recently, Tsutsui *et al.* also reported that keratinocyte-caspase-1 transgenic/signal transducers and activators of transcription (STAT) 6-deficient mice developed atopic dermatitis without IgE production and their serum IL-18 levels were high.[38] Moreover, Yagi *et al.* recently reported that STAT6-deficient NC/Nga mice showed development of atopic dermatitis, although these mice failed to produce IgE and IL-18 had been highly expressed at the skin lesions.[39] Interleukin-18 may participate in atopic dermatitis exacerbation by histamine production from basophils or mast cells without IgE production.

In the present study, we found that the plasma IL-18 level was elevated in children with bronchial asthma and atopic dermatitis. The plasma IL-18 level showed a

positive correlation with serum IgE levels. Furthermore, we showed that the plasma IL-18 level correlated with the clinical severity of atopic dermatitis. Interleukin-18 has dual roles in the Th1/Th2 system depending on conditions, such as the existence of antigens or other cytokines, including IL-10.[40] Further studies of cytokine profiles in allergic diseases and analysis of gene polymorphisms of IL-18 and IL-18-related molecules (IL-18Rα, IL-18Rβ, MyD88, IL-1 receptor-associated kinase (IRAK), TNF receptor-associated factor (TRAF) 6) are important in order to clarify the association of IL-18 with atopic diseases.[41–43]

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