3) 現在までフローボリュームカーブの測定を行なっている9歳以上の小児気管支喘息患者約250名の唾液採取、幼児期の生活環境等についてのアンケートを回収している。一部の患者についてはβ2刺激薬吸入前後でのフローボリュームカーブを測定した。気道閉塞の程度や部位、寛解の有無と遺伝子解析、環境因子の関連について解析を行なっている。

D. 考 察

小児気管支喘息の予後にはダニ感作レベルの程度や変化が大きく関与することが示唆された。症例数が少なく、また症例・対照研究であることから、今後はより大規模な前方視的研究が必要と考えられるが、本研究からダニ特異IgE値を決定する因子の解析は喘息の予後を予測し、さらに寛解を誘導する方略を考える上で極めて重要であることが示された。本研究からは、調節性サイトカインであるIL-10とTh2サイトカインのバランスなどの獲得免疫機能がそのひとつであることが示唆された。IL-10は喘息患者においては低値であるとする報告と高値であるとする報告があるが、Th2サイトカイン産生に対するカウンターレギュレーションと考えれば、IL-10/Th2サイトカインバランスがよい指標となると思われる。

MMPは、細胞外マトリックスの分解、細胞表面のタンパク質の分解、サイトカインを含む生理活性物質のプロセシング、など多岐にわたる作用を有する。本研究では、MMP9が血清総IgEやダニ特異IgE値に関与することが明らかとなった。アレルゲン特異IgE低下にMMP9活性を制御する薬物が有用である可能性がある。

CD14-159C/T多型とIgE値の多寡については相反する報告が出されている。近年、これらの異なる結果は遺伝子・環境因子の相互作用から説明されている。我が国において本研究で得られた結果と同様の報告は現在までなく、極めて重要な知見である。海外の結果と異なり、我々の検討ではIgE値との関連はCD14-159C/T多型よりもCD14-550C/T多型との関連が強く、これは人種の違い等によるものかもしれない。いずれにせよ、喘息をはじめとするアレルギー疾患の発症や経過には遺伝因子のみならず環境因子の関わりが大きく、それらが単独でなく相互に関連することは極めて重要な意義を有する。ダニ特異IgEの制御には単に環境調整をすれば良いのではなく、免疫関連分子の遺伝子多型も考慮しての対応が必要であることが示唆される。

以上をまとめると、本研究から、ダニ特異IgE値には、獲得免疫のみならず自然免疫・炎症に関与する遺伝子の多型、さらに乳幼児期の生活環境等が関与することが示唆された。今後、思春期以降の寛解、非寛解例調査患者数を増やして呼吸機能の点からも予後に関連する因子を明らかにしたい。

E. 結 論

小児気管支喘息の予後に関連するいくつかの因子を明らかとした。

F. 健康危険情報

なし

G. 研究発表

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なし

2. 学会発表

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（免疫アレルギー疾患等予防・治療研究事業）
分担研究報告書
アレルギーのテーラーメイド治療管理ガイドラインの確立と実用化

分担課題名：遺伝子情報の網羅的解析、および小児喘息におけるロイコトリエン受容体拮抗薬の治療反応性予測遺伝子因子とテーラーメイド治療開発

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研究要旨

気管支喘息、アトピー性皮膚炎などアレルギー疾患の増加が大きな社会問題になっている。本研究の目的は、アレルギー疾患の病因・病態および治療反応性予測因子を系統的に検出できる遺伝子診断キットを中心とする診断システムを開発すること、および、その診断をもとにして各病因・病態に合致したテーラーメイド治療法を確立することである。今回は、多数報告のあるアレルギー病因遺伝子群のなかで、TGF-β1遺伝子多型とアレルギー発症との関連解析を行うとともに、トシル酸スプラタストの使用とQOLとの関連解析を行った。

A．研究目的

近年、気管支喘息、アトピー性皮膚炎などアレルギー疾患の増加が大きな社会問題になっている。本研究の目的は、アレルギー疾患の病因・病態および治療反応性予測因子を系統的に検出できる遺伝子診断キットを中心とする診断システムを開発することおよび、その診断をもとにして各病因・病態に応じたテーラーメイド治療管理ガイドラインを確立し、実用化をはかることである。

B．研究方法

①アレルギーの病因遺伝子群の系統的、多角的な解明に基づいて、アレルギーの系統的遺伝子診断キットをインベーダーアッセイ法を用いて開発した。さらに、新規SNPとの関連を検討する目的で、ジーンチップ（500K Mapping assay）を利用し、薬剤反応性と関連しているSNPの検索を行った。

②気管支喘息の発症とTGF-β1遺伝子多型との関連解析を行った。

③抗アレルギー薬のうち、トシル酸スプラタストの使用前後における患者および保護者のQOLに関する調査を行い、薬剤による小児気管支喘息患者およびその保護者のQOLの変化についての検討を行った。

（倫理面への配慮）

研究対象者には本研究の内容、方法および予想される結果について充分に説明し充分な理解（インフォームドコンセント）を得た上で採血が行なわれた。また、倫理面でも、結果による不利益は全く生じないか、または配慮が充分になされることから問題がないと判断された。

C．研究結果

①アレルギーの系統的遺伝子診断キットをインベーダーアッセイ法を利用して構築した。ジーンチップ（500K Mapping assay）によるロイコトリエン受容体拮抗薬（LTRA）の効果とSNPの関連解析では、GATA3などの免疫関連遺伝子領域に存在する22種類の多型、IL-8, IL-19, TNFなどのサイトカイン関連遺伝子領域に存在する20種類の多型が、有効性との関連（$p<0.001$）が認められた。

②気管支喘息の発症とTGF-β1遺伝子多型との関連解析を行った。

図1

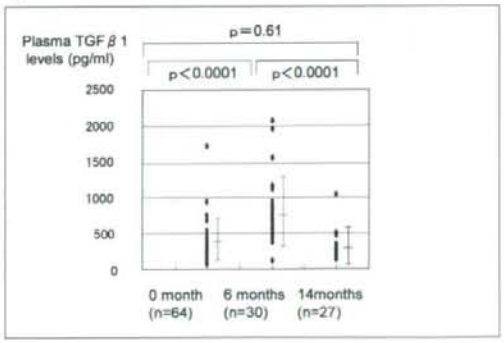


図１に示すように、生後６ヶ月の血漿中TGF-β1濃度は、出生時、生後14ヶ月時に比較して高値であった。

また、TGF-β1遺伝子プロモーター領域に存在する遺伝子多型（C-509T）の検索を行ったところ、生後14ヶ月までに気管支喘息を発症した３例はいずれもTTgenotypeであった（表1）。

表１

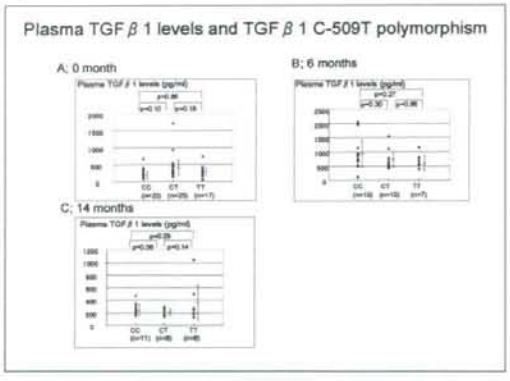
Genotype frequency of C-509T *TGF-β1* promoter SNP in subjects with/without bronchial asthma at 14months

	CC	CT	TT	Total
bronchial asthma -	22	25	14	61(96%)
bronchial asthma +	0	0	3	3(4%)

(P=0.016)

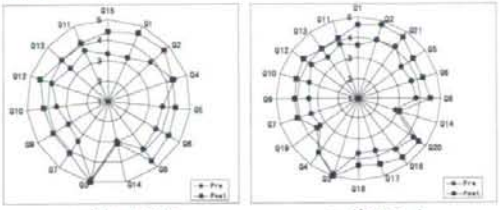
さらに、TGF-β1遺伝子C-509T多型と、血漿中TGF-β1濃度を検討したところ、出生時、生後６ヶ月では、一定の傾向を認めなかったが、生後14ヶ月では、TTgenotypeで、血漿中TGF-β1濃度が高い傾向がみられた（図2）。

図２



③抗アレルギー薬のうち、トシル酸スプラタストの使用前後における患者および保護者のQOLに関する調査を行い、薬剤による小児気管支喘息患者およびその保護者のQOLの変化についての検討を行った。

図３



４歳未満　　４歳以上

表２

		<4 years of age			≥4 years of age		
		No of patient	Score	P value*	No of patient	Score	P value
Overall QOL score	Pre	188	69.0	p< 0.001	293	75.3	p< 0.00
	Post		84.3			85.5	
Physical domain	Pre	194	34.0	p< 0.001	288	53.8	p< 0.00
	Post		39.4			60.8	
Emotional domain	Pre	203	21.0	p< 0.001	303	21.5	p< 0.00
	Post		24.9			24.7	

*Wilcoxon signed-ranks test

図3、表２に示すように、全体としては、４歳未満、４歳以上ともに薬剤投与前後を比較すると、QOLが改善していた。

しかし、症例によっては、症状の改善およびQOLの改善がみられないことが明らかになった。そこで、現在、QOLの改善度と遺伝子多型との関連について検討を加えている。

D．考　察

増加を続けるアレルギー疾患患者の個々の病態を遺伝子レベルで整理し、分類することは、テーラーメイド医療を考える上で、非常に重要なことであると考えられる。多数報告のみられるアレルギー関連遺伝子多型情報を、臨床レベルで活用するためには、多くのサンプルを迅速に処理する必要があり、これを実現するために、遺伝子検出キットは、非常に有用であると考えられた。今後、診断、治療に着目した感受性、特異性の向上、薬理遺伝学的見地からの検討を十分に行い、テーラーメイド医療に活用していきたいと考える。

E．結　論

アレルギー疾患の遺伝子診断キットを開発し、臨床へ応用するため、診断、治療に着目した感受性、特異性の向上、薬理遺伝学的見地からのさらなる検討が必要である。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況（予定を含む）

1．特許取得

近藤直実，松井永子，金子英雄，青木美奈子，近藤 應：遺伝子多型を利用した抗アレルギー薬の感受性予測方法（特許出願中）：平成 17 年度

2．実用新案登録

なし

3．その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（平成 20 年度）

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Ⅳ. 研究成果の刊行物・別冊

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Toll-like receptor signaling is impaired in dendritic cells from patients with X-linked agammaglobulinemia

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Abstract Bruton's tyrosine kinase (BTK), which is defective in patients with X-linked agammaglobulinemia (XLA), is expressed not only in B cells but also in monocytes and dendritic cells (DCs). DCs play a crucial role in the innate immune response against infections by sensing pathogens through Toll-like receptors (TLRs). However, it is not known whether BTK deficiency in XLA might impair TLR-mediated signaling in DCs, which are susceptible to various infections. The phenotypic maturation and cytokine production mediated by TLRs were examined in monocyte-derived DC from XLA patients and normal controls. The TLR expression in DCs was analyzed by flow cytometry. TLR-mediated signaling in DCs was evaluated for the phenotypic maturation based on CD83 expression and production of cytokines, such as TNF-α, IL-6 and IL-12p70. TLR levels in DCs were similar between XLA and controls. TLR2, TLR4 and TLR7/8 ligands elicited less phenotypic maturation of DCs from XLA patients than normal controls based on CD83 expression. Stimulation with TLR2, TLR4 and TLR7/8 ligands, as well as TLR3 ligand, resulted in significantly lower production of TNF-α, but neither IL-6 nor IL-12p70, by DCs from XLA patients in comparison to normal controls. These findings suggest that BTK may thus be required for TLR signaling in DCs. The impaired TLR signaling in DCs may therefore be partly responsible for the occurrence of severe infections with bacteria and some viruses in XLA patients.

Abbreviations: BTK, Bruton's tyrosine kinase; DCs, dendritic cells; IRAK, IL-1 receptor-associated kinase; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; mAb, monoclonal antibodies; Mal, MyD88 adaptor-like protein; MAP, mitogen-activated protein; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; MyD88, myeloid differentiation primary-response protein 88; NF-κB, nuclear factor-κB; PAMPs, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline solution; PE, phycoerythrin; PGN, peptidoglycan; ss, single stranded; TIR, Toll/IL-1 receptor; TLRs, Toll-like receptors; XLA, X-linked agammaglobulinemia.

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Introduction

X-linked agammaglobulinemia (XLA) is the prototypical antibody deficiency syndrome, clinically characterized by recurrent bacterial infections from early childhood, hypogammaglobulinemia, and markedly reduced numbers of circulating B cells [1,2]. In 1993, the causative gene was identified as an intracytoplasmic tyrosine kinase, *Bruton's tyrosine kinase* (*BTK*) [3,4]. BTK plays a crucial role in early B cell development; however, it is expressed not only in B cells but also in monocytes and dendritic cells (DCs) [5,6]. The function of BTK in monocytes and DCs remains to be precisely determined. XLA patients are sometimes associated with enteroviral infections, which often lead to meningoencephalitis, and severe or fatal infections of the central nervous system [1,7,8]. The reason for this susceptibility to enterovirus infection may be caused by a diminished neutralization of these viruses in the patients, but the precise mechanism remains unknown. The associations of enteroviral infections might be caused by the impaired innate immunity in XLA patients. It has been demonstrated that lipopolysaccharide (LPS)-induced TNF-α production is reduced in monocytes from XLA patients [9]. These data suggest that BTK could play a functional role in the innate immune system.

The immune response can be divided into the innate and adaptive immunity, and the innate immunity is the first line of defense against pathogens [10]. Toll-like receptors (TLRs) are widely distributed on cells of the immune systems and play a crucial role in the early host defense against invading pathogens. The TLRs consist of 10 family members in humans. They function to sensor the pathogen-associated molecular patterns (PAMPs), derived from viruses, bacteria, fungi and parasites. TLRs possess intracytoplasmic regions, which are known as Toll/IL-1 receptor homologous (TIR) domains, based on their structural similarity among TLR and IL-1R family members. These regions play a critical role in signaling, which thus leads to the activation of a variety of transcription factors such as nuclear factor-κB (NF-κB) or mitogen-activated protein (MAP) kinases. A cytoplasmic adapter, myeloid differentiation primary-response protein 88 (MyD88), associates with TIR domains and is essential for most function of TLRs. In addition to MyD88, four adapters with TIR domains, including MyD88 adapter-like protein (Mal), TIR-domain-containing adaptor protein inducing interferon-β, and TRIF-related adaptor molecule, play roles as adapters in TLR signaling. Several findings suggested that BTK is a critical molecule for TLR signaling [11,12]. BTK has been shown to be involved in the signaling by LPS to activate NF-κB via TLR4 and interact with MyD88, Mal, and IL-1 receptor-associated kinase (IRAK)-1 [11]. However, the production of cytokines by LPS-treated DCs from XLA patients was unaffected [6]. The role of BTK in innate immunity, especially in human, has remained unclear.

To address the biological role of BTK in TLR-stimulated DC, the present study has been intended to investigate TLR-induced responses in monocyte-derived DCs from XLA patients. The results show impaired TLR function in DC from XLA patients and discuss its involvement in susceptibility to bacteria and some viruses.

Materials and methods

Reagents and antibodies

Recombinant human GM-CSF and IL-4 were obtained from R&D Systems, Inc. (Minneapolis, MN). Peptidoglycan from *Staphylococcus aureus* (PGN; TLR2 ligand), Poly(I:C) (TLR3 ligand), lipopolysaccharide (LPS; TLR4 ligand), and ODN2006 (CpG oligonucleotide type B; TLR9 ligand) were purchased from InvivoGen (San Diego, CA), and R-848 (Resiquimod; TLR7/8 ligand) was obtained from 3M Pharmaceuticals (St. Paul, MN). Phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) against TLR2, TLR3, TLR4, and TLR9 were purchased from eBioscience (San Diego, CA), and PE-conjugated anti-human TLR8 mAb was obtained from IMGENEX (San Diego, CA).

Subjects

Eight XLA patients and seven age-matched healthy controls were included in this study. The clinical and genetic data for the XLA patients are described in Table 1 [13,14]. All the patients were doing well under intravenous immunoglobulin treatment. The diagnosis of XLA was made based on a flow cytometric analysis combined with direct sequencing of the *BTK* gene. Written informed consent for the study was obtained from either all patients or their parents. Ethical approval was obtained from the ethics committee of the University of Toyama.

Generation of DCs

Venous blood was obtained from XLA patients and healthy normal controls. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on Histopaque®-1077 (Sigma-Aldrich, Inc., St. Louis, MO). Monocytes were obtained from PBMCs by positive selection using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for magnetic separation according to the manufacturer's instructions. The purity of monocytes was >98% as assessed by flow cytometry with PE-conjugated anti CD14 mAb (Dako Japan, Kyoto, Japan). The monocytes were cultured at 3×10^5/ml in RPMI 1640 medium (Sigma-Aldrich, Inc.) supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate,

Table 1 Clinical and genetic data on XLA patients

Patient	Age at diagnosis	Age at sampling	BTK mutation
P1	13 m	10 y	Exon 6 skip
P2	3 y	9 y	Exons 16–18 skip
P3	7 y	26 y	S554P
P4	4 m	5 y	787delG (V219delX228)
P5	5 y	31 y	L111R
P6	6 m	10 y	Exons 16–18 skip
P7	10 m	12 y	1176ins (60nt)
P8	3 y	16 y	1176ins (60nt)

Abbreviations: m, months; y, years; del, deletion; ins, insertion; nt, nucleotides.
P7 and P8 were cousins.

penicillin G, and streptomycin, in the presence of 50 ng/ml recombinant human GM-CSF and 20 ng/ml recombinant human IL-4 for 6–7 days. The purity of generated DCs was assessed by a flow cytometry analysis with fluorescein isothiocyanate (FITC)-labeled anti-CD1a (Dako Japan, Kyoto, Japan) [15]. For stimulation with TLR ligands, immature DCs were cultured at a concentration of 3×10^5 cells/ml with various TLR ligands, such as 5 μg/ml PGN, 25 μg/ml Poly(I:C), 100 ng/ml LPS, 5 μM R-848, and 5 μg/ml CpG for 24 h. Thereafter, the culture supernatants were collected to assess the levels of cytokines, such TNF-α, IL-6, and IL-12p70 as below. The maturation of DCs by TLR ligand stimulation was evaluated by flow cytometric staining with FITC-conjugated anti-CD83 (Immunotech, Marseille, France) mAb, which might be the most indicative marker of mature DCs, although CD80, CD86, or MHC class II were also markers of mature DCs [15].

Flow cytometric analysis of DCs

To examine the TLR expression on DCs, TLRs were detected by flow cytometry using anti-human TLR mAbs. While TLR2 and TLR4 are expressed on the cell surface, TLR3, TLR8, and TLR9 are principally expressed in the cytoplasm [16]. For TLR3, TLR8, and TLR9 staining, DCs were first fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and then permeabilized in 0.5% saponin (Sigma-Aldrich, Inc.) for 15 min on ice. Untreated and permeabilized DCs were incubated with PE-labeled mAbs against TLR2 and TLR4, and TLR3, TLR8 and TLR9, respectively, for 20 min on ice and then washed in a staining buffer. The stained cells were analyzed with a flow cytometer. TLR7 expression was not evaluated because mAb against TLR7 was not available.

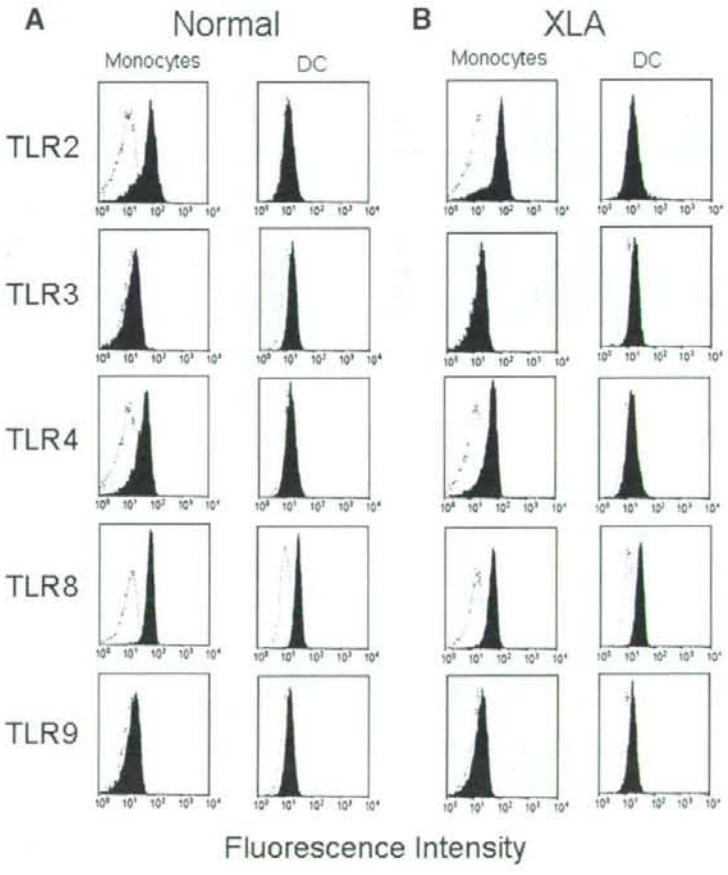


Figure 1 TLR expression in monocytes and DCs. The expression of TLR2, TLR3, TLR4, TLR, and TLR9 was examined in monocytes and monocyte-derived DCs. To detect TLR3, TLR8, and TLR9, the cells were fixed and permeabilized in 0.5% saponin prior to staining. These cells were incubated with PE-labeled mAbs against respective TLRs, and the stained cells were analyzed by a flow cytometry. Histograms are representative results obtained from normal controls (A) and an XLA patient (P4) (B). The *closed area* and *dashed area* indicate the staining with anti-TLR and control mAbs.

Cytokine measurement

The levels of cytokines in the culture supernatants from the DCs stimulated with TLR ligands were measured using ELISA kits (TNF-α: BioSource International, Camarillo, CA; IL-6: R&D Systems; and IL-12p70: BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis

The statistical significance of differences was determined with unpaired Student's *t*-test. A value of $P<0.05$ was considered to be statically significant.

Results and discussion

DCs from XLA patients and normal controls were generated by the cultures of purified monocytes in the presence of GM-CSF and IL-4. The recovery of homogenous DCs in both subjects was confirmed based on the expression of high levels of CD1a and the down-regulation of CD14 as evaluated by flow cytometry (data not shown). This implies that the generation of immature DC does not require BTK.

The generated DCs were investigated for TLR expression in a comparison with monocytes. TLR expression in monocytes and DCs might be regulated differently [16]. A flow cytometry analysis shows that monocytes from XLA patients as well as normal controls express high levels of TLR2, TLR4, and TLR8, but little TLR3 and TLR9 (Fig. 1). It was noted that DCs from both subjects showed less expression of TLR2 and TLR4 and greater expression of TLR3 and TLR9 than monocytes. The expression of TLR8 was comparable between monocytes and differentiated DCs and its expression was normal in XLA patient-derived monocytes and DCs. These results indicate that the expression of all TLRs examined are normal in the XLA patient-derived monocytes and DCs.

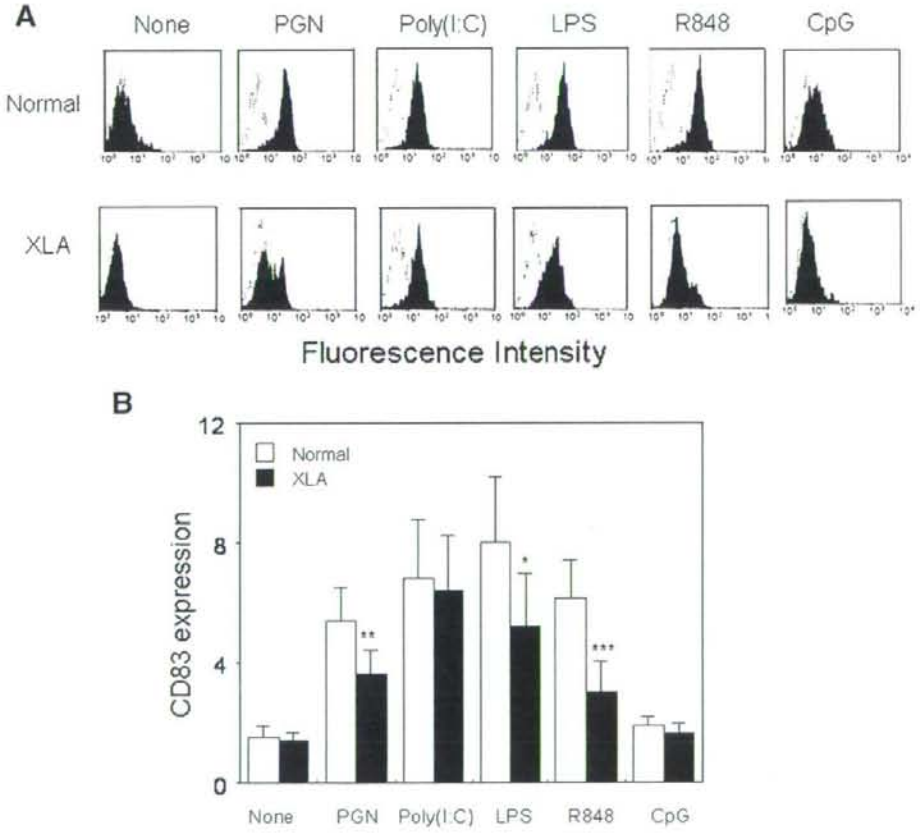
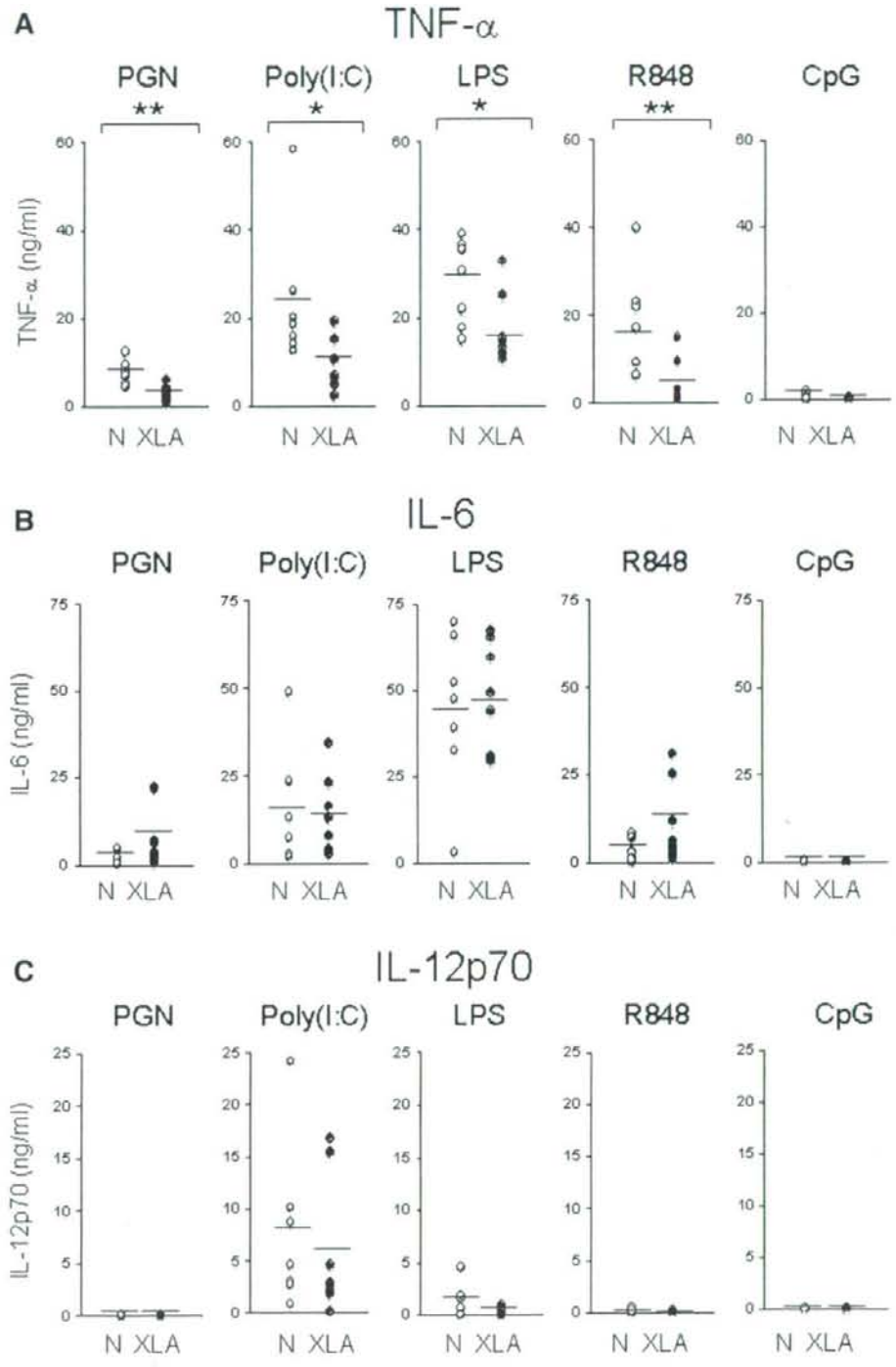


Figure 2 Induction of CD83 expression on DCs by stimulation with TLRs. Monocyte-derived DCs were cultured with various TLR ligands including PGN, Poly(I:C), LPS, R848, and CpG. CD83 expression on DCs after stimulation with TLR ligands was assessed by a flow cytometry. (A) Representative histograms obtained from a normal control and an XLA patients (P8). The closed area and dashed area indicate the staining with anti-CD83 and control mAbs. (B) CD83 expression on XLA and normal DCs. The levels of CD83 expression were expressed as a ratio of the mean fluorescence intensity (MFI) with FITC-labeled anti-CD83 mAb to MFI with FITC-labeled control antibody. Data indicate the means ± SD of 8 XLA patients and 7 normal controls. $^{*}P<0.02$, $^{**}P<0.005$, and $^{***}P<0.0002$ *versus* normal controls.


A
TNF-α
PGN
Poly(I:C)
LPS
R848
CpG
**
*
*
**
TNF-α (ng/ml)
60
40
20
0
N XLA
B
IL-6
IL-6 (ng/ml)
75
50
25
C
IL-12p70
IL-12p70 (ng/ml)
25
20
15
10
5

The responses of DCs against a variety of TLR ligands were next analyzed. First, the phenotypic maturation of DCs was evaluated based on the mean fluorescence intensity (MFI) of CD83 expression [15]. Although the surface expression of TLR2 and TLR4 is fundamentally low, PGN (TLR2 ligand) and LPS (TLR4 ligand) could appreciably induce the CD83 mature phenotype in normal DCs (Fig. 2A). Likewise, Poly(I:C) (TLR3 ligand) and R848 (TLR7/8 ligand), but not CpG (TLR9 ligand), promoted a marked CD83 expression in normal DCs, thus reflecting the expression of respective TLRs. In contrast, regardless of a similar TLR expression, it seemed that CD83 induction by TLRs in XLA-DCs was lower than that in normal DCs (Fig. 2A). Intriguingly, the MFI of CD83 expression induced by PGN, LPS, and R848 was significantly weaker in XLA DCs than control DCs (Fig. 2B). This suggested that the DC maturation induced by TLR signaling, especially TLR2, TLR4, and TLR7/8 signaling, was defective in XLA patients. Other studies have shown that DCs from XLA patients and normal controls led to an upregulation of surface molecules including CD80, CD83, CD86, and MHC class II at similar levels [6,17]. However, partially defective DC maturation was found in XLA patients in this study.

The next question was whether the different phenotypic maturation by TLR ligands between XLA-DCs and normal DCs as above might be associated with TLR ligand signaling in terms of cytokine production. Therefore, cytokine production of TNF-α, IL-6, and IL-12p70 by XLA-DCs and normal DCs after stimulation with TLR ligands was examined. DCs from both XLA patients and normal controls produced substantially TNF-α and IL-6 after stimulation with PGN, Poly(I:C), LPS, and R848, although cytokine levels varied among donors and TLR ligands (Fig. 3). On the other hand, only Poly(I:C) was observed to efficiently stimulate IL-12p70 induction. However, CpG could not induce TNF-α or IL-6 even in normal DCs, which might be related to its failure to induce the phenotypic maturation in DCs. IL-6 and IL-12p70 were similarly produced by TLR-stimulated DCs from XLA patients and normal controls. Consistent with these findings, Gagliardi et al. [6] demonstrated that the production of IL-12 and IL-10 by DCs was not affected by BTK deficiency. In another study, the phosphorylation state of MAP kinases including Erk1/Erk2, JNK, and p38 in monocytes from XLA patients and normal controls was also evaluated, and the phosphorylation increment of MAP kinases in response to LPS was totally similar [18]. Erk1/Erk2 and JNK activation after B-cell antigen receptor engagement is impaired in Btk-deficient B cells [19]. BTK may play a different role in the MAP kinase activation in response to either B-cell antigen receptor or TLR4. In contrast to the current results, the production of IL-12 is greater in macrophages from *xid* mice than those from wild-type mice, and such production is also associated with the reduced production of nitric oxide [20]. In Btk-deficient mice, macrophages secrete decreased levels of IL-10 and increased levels of IL-6 in response to TLR ligands, and these actions correlated with the Btk-dependent induction of NF-κB and the AP-1 DNA binding activity [21]. The difference in cykokine production between humans and mice remained unclear. The function of BTK might differ among species, and it must be clarified in the future.

Fig. 3 demonstrates that the amounts of TNF-α produced in response to TLR ligands including PGN, Poly(I:C), LPS, and R-848 were significantly lower in XLA-DCs than normal DCs. Although the MFI of CD83 expression induced by Poly(I:C) was not significantly different between XLA DCs and control DCs, TNF-α production levels in response to PGN, LPS, and R-848 seemingly correspond to the induction of CD83. However, no significant correlation was observed between the TNF-α level and the CD83 expression in this study. Such a difference between XLA-DCs and normal DCs was not observed in the induction of IL-6 and IL-12p70. It was shown that, in response to LPS, monocytes from XLA patients induce early MAP kinase activation and TNF-α and IL-6 production in a comparable level to those from normal donors, indicating that BTK is not essential for early LPS signaling in human monocytes [18]. In a previous study, however, TNF-α and IL-6 production was determined by intracellular flow cytometry, which might be less sensitive than ELISA. Consistent with these results, BTK would be required for TLR2- and TLR4-induced production of TNF-α and IL-1β, but not the production of IL-6, IL-8, and IL-10, by stabilizing mRNAs [22]. In another study, a profound defect in IL-6 and TNF-α production by XLA-DCs in response to single stranded (ss)RNA which was used as TLR8 ligand was demonstrated, although DC function in XLA was not shown to be impaired by the TLR4 ligand [17].

The present results implied that BTK might be involved in the TLR signaling required for production of TNF-α, but not IL-6 nor IL-12p70. TNF-α production following stimulation with R-848 was mostly diminished in XLA patients. R-848 as well as ssRNA viruses are ligands for TLR7 and TLR8 [23–26]. In XLA patients, the production of TNF-α might be impaired during the infection of enteroviruses which are ssRNA viruses. Consistent with these data, a severe impairment of TNF-α production in response to TLR8 ligand was observed [17]. TNF-α is a proinflammatory cytokine, which induces the production of other inflammatory cytokines. In addition, TNF-α has anti-viral activity, while also playing a pivotal role in the pathogenesis of inflammation. A reduced production of TNF-α might be associated with severe infections of enterovirus in XLA patients. Type I interferons also play an important role in viral infection and this should be investigated in future studies.

Taken together, the present study indicates that BTK may play an important role in TLR signaling although the precise mechanism remained to be elucidated. These results suggest that susceptibility to infections in XLA patients may be caused not only by hypogammaglobulinemia but also by impaired TLR signaling.

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Figure 3 Cytokines produced by DCs stimulated with TLR ligands. Monocyte-derived DCs from normal control (open circle) and XLA patients (closed circle) were cultured with TLR ligands, including PGN, Poly(I:C), LPS, R848, and CpG, for 24 h. The concentrations of TNF-α (A), IL-6 (B), and IL-12p70 (C) in the culture supernatants were determined by ELISA. *$P<0.05$ and **$P<0.01$ *versus* normal controls.

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Case Report

CLINICAL IMPROVEMENT OF DIFFUSE LYMPHANGIOMATOSIS WITH PEGYLATED INTERFERON ALFA-2B THERAPY: Case Report and Review of the Literature

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□ *Diffuse lymphangiomatosis is a very rare congenital disease, characterized by diffuse or multifocal lymphangioma in the skeletal tissue, spleen, liver, mediastinum, and/or lung. The prognosis is usually poor, especially for children with thoracic lesion, and treatments for the disease are controversial. The authors report a 9-year-old boy with diffuse lymphangiomatosis involving the thorax with pleural effusions, the spleen, and systemic bone. The patient was treated with pegylated interferon alfa-2b, and achieved good clinical and radiological improvement.*

Keywords D2-40, diffuse lymphangiomatosis, pegylated interferon alfa-2b, pleural effusion, vascular endothelial growth factor

Diffuse lymphangiomatosis is an uncommon lymphatic disorder occurring mostly in children and young adults. The etiology of the disease is unknown, but it might be the result of an abnormal development of the lymphatic system. This lymphangiomatosis involves the skeletal system and thoracic and abdominal organs, but does not affect the central nervous system due to its lack of lymphatic vessels. Patients present with symptoms corresponding to their site of infiltration. Thoracic involvement results in pleural effusions, pericardial effusions, and severe respiratory infections, which lead to a serious prognosis. Ofelia et al. reviewed 53 cases of thoracic lymphangiomatosis from the literature [1]. Thirty-two cases (60.3%) were

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children (<16 years old), 26 cases of which (81.2%) had lung or pleural involvement. There was no mortality among adult patients, but 12 of 32 children (39%) died of the disease. All of them had pleural effusion and/or pulmonary infiltration. Therefore, it is important for children with thoracic lesions to be treated appropriately.

Treatments for thoracic lymphangiomatosis have not yet been determined. Current therapeutic modalities include operative treatment (pleurocentesis, pleurodesis or pleurodectomy, ligation of the thoracic duct, and resection of lymphangioma), radiation therapy [2], corticosteroids, tamoxifen, vincristine, OK-432 [3], interferon (IFN) alfa, and so on. The pleiotropic cytokine IFN alfa has shown beneficial effects in the treatment of a diverse array of malignant tumors. Several reports described effectiveness of IFN alfa treatment of diffuse lymphangiomatosis [4]. However, there are no reports on pegylated IFN alfa-2b therapy for diffuse lymphangiomatosis in the literature thus far.

We present here the case of a 9-year-old boy with diffuse lymphangiomatosis involving the thorax and bones with massive pleural effusions and he has been treated successfully with systemic administration of pegylated IFN alfa-2b without severe side effects.

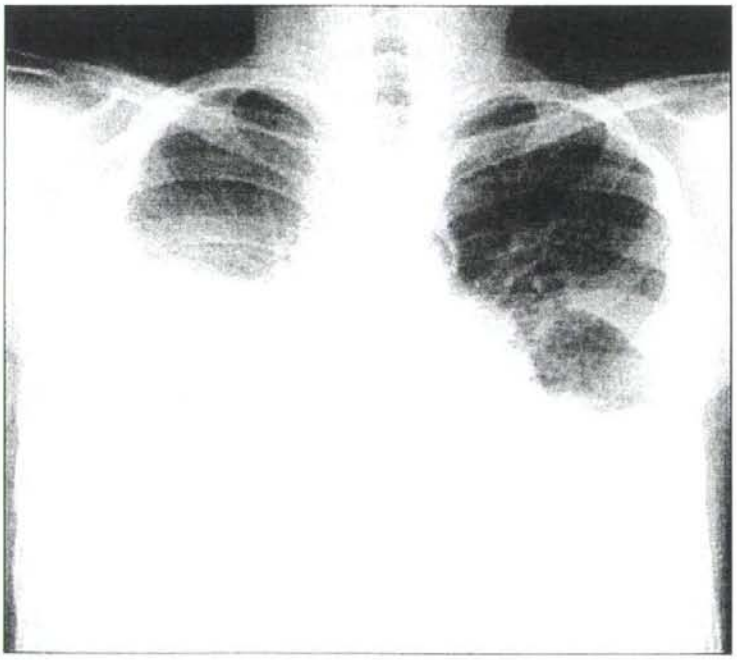
CASE REPORT

A 5-year-old boy developed a severe back pain and lumbago. A radiograph of the spine showed compressed fracture of the 10th thoracic spine and a T2-weighted imaging on MRI showed a high-intensity lesion around the 10th thoracic spine. He had no history of trauma, illness, or surgery. On physical examination, he had no fever, body weight loss, or fatigue, and laboratory tests including tumor markers were within normal limits. Open biopsy of the affected thoracic spine was done. The specimen contained no tumor cells and a small number of histiocytes. This finding was compatible with the diagnosis of Langerhans cell histiocytosis. However, these cells were negative for S-100 protein by immunohistochemical analysis and Birbeck's granules were not found by electron microscopic examination. He was tentatively diagnosed with Langerhans cell histiocytosis and received multiagent chemotherapy (vincristine, ara-C, and predonisone) despite a lack of histological evidence. His pathologic lesions on MRI had not changed by the chemotherapy and he was discharged from the hospital and followed-up by outpatient visits.

One year later, a chest radiograph showed a right pleural effusion during routine examination and he was admitted to our hospital. Chylothorax was confirmed by thoracentesis. He was successfully treated by total parenteral nutrition at that time.

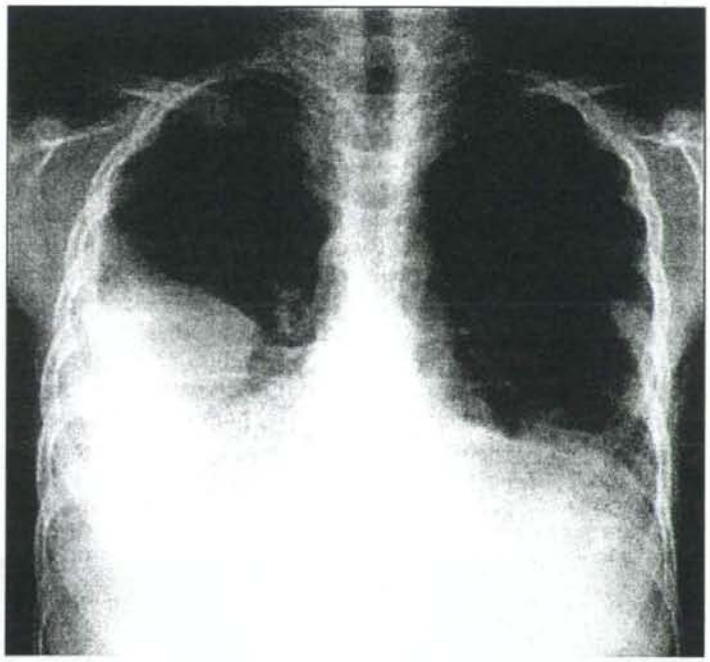
He was admitted again with complaints of sudden dyspnea after 2 years. A chest radiograph showed an increase of the right pleural effusion

(Figure 1a). Systemic MRI showed a multiple high-intensity areas on T2-weighted and proton-density images in skull, bilateral humeri, thoracic vertebra, pelvis, caput femoris, and spleen (Figure 2a). These lesions were not enhanced by an imaging agent. Pulmonary function tests revealed a severe restrictive pattern characterized by a vital capacity (VC) of 0.69 L (36.3% of predicted value), and an $FEV_{1.0}$/VC ratio of 92.1%. He was treated by total parenteral nutrition and thoracentesis. The biopsy specimen at the affected lesion of the left upper forelimb bone was a delicate structure, and showed nothing but fluid, blood, and scattered tissues. Histologically, vascular connective tissue was dominant. Immunohistochemically, CD31 and CD34, markers for vascular endothelial cells, were positive. Some of endothelia were positive for the monoclonal antibody D2-40 (Signet Laboratory, Dedham, MA, USA), a novel monoclonal antibody to a MW 40,000 *O*-linked sialoglycoprotein that reacts with a fixation-resistant epitope in lymphatic endothelium (Figure 3). Then, the diagnosis of diffuse lymphangiomatosis was defined.



(a)

FIGURE 1 Chest radiograph: (a) at the time of admission to our hospital with extensive pleural effusion; (b) 3 months after IFN treatment showing decreased pleural effusion. *(Continued)*



(b)

FIGURE 1 (Continued)

Administration of pegylated IFN alfa-2b was started at a dose of 1.0 μg/kg once a week initially. After the second administration, drainage of the thoracic cavity (about 1.8 L) was performed transiently due to increasing pleural effusion. The dosage was then increased to 1.5 μg/kg from the third administration. During treatment, his exercise tolerance improved, a chest radiograph showed gradual regression of the pleural effusion, and VC was increased. Moderate side effects of pegylated IFN alfa-2b, including elevated body temperature, a mild headache, and general fatigue, were observed in the first months of treatment. His general status was improved in a few weeks and he was discharged from hospital. Three months after the initiation of IFN treatment, he was evaluated. No further progression of pleural effusion was observed (Figure 1b) and pulmonary function tests indicated clear improvement (Figure 4); a vital capacity (VC) of 0.96 L (51.3% of predicted value), an $FEV_{1.0}$/VC ratio of 94.9% and multiple bone involvements have also regressed radiologically (Figure 2b). He has been on the interferon therapy for 9 months without further complications.