TABLE 3.— Comparisons of patients' characteristics and VAS scores before treatment

N	Effective 22	Ineffective 14	Median (range)
Age (y)	37.5 (21.0–77.0)	39.5 (26.0–71.0)	N.S.
IgE (IU/ml)	105.2 (3.0–1051.0)	112.6 (5.6–691.9)	N.S.
Eosinophils in blood (/μl)	133 (0–403)	131 (0–572)	N.S.
%FVC	106.6 (80.1–132.4)	99.1 (83–133.1)	N.S.
FEV1/FVC (%)	84.7 (70.0–93.9)	79.25 (63.1–114)	N.S.
%V25/HT	70.6 (29.1–122.4)	49.2 (5.7–121.8)	N.S.
Log PC20	3.57 (1.83–4.30)	3.54 (3.20–4.30)	N.S.
Cough (VAS)	5.5 (3.0–10.0)	6.0 (4.0–10.0)	N.S.
Daily life (VAS)	5.0 (0.0–10.0)	4.5 (0.0–10.0)	N.S.
Sleep (VAS)	3.0 (0.0–10.0)	0.5 (0.0–10.0)	N.S.

of the degree of cough improved further after the subsequent 2-week treatment period (Figures 3A–C).

In the ineffective group, the VAS scores of the degree of cough, effect on daily life, and effect on sleep did not change significantly after the initial 2-week treatment with montelukast. However, they improved substantially after 2 weeks of FP inhalation therapy; the improvements in the VAS scores were similar to those seen after 2 weeks of montelukast treatment in the effective group (Figures 3D-F).

Double Staining of the Bronchial Mucosa Biopsy Specimens with the Anti-CD63 Antibody and the Anti-Tryptase Antibody

A luminous spot with a nucleus or intense brightness on staining with anti-human tryptase antibody was regarded as a tryptase-positive mast cell (Figures 4A, C, E); tryptase-positive mast cells were considered to be CD63-positive when the mast cell was also stained by the anti-CD63 antibody (Figures 4B, D, F). Both in CVA patients and in control subjects, there were very few CD63-positive cells that were not stained by anti-human tryptase antibody. The number of mast cells in the bronchial mucosa was not significantly different among control subjects, the effective group, and the ineffective group (Figure 5A). However, the ratio of the number of CD63-positive cells to the number of tryptase-positive mast cells was significantly higher in the effective group than in the ineffective group or the control group (Figure 5B).

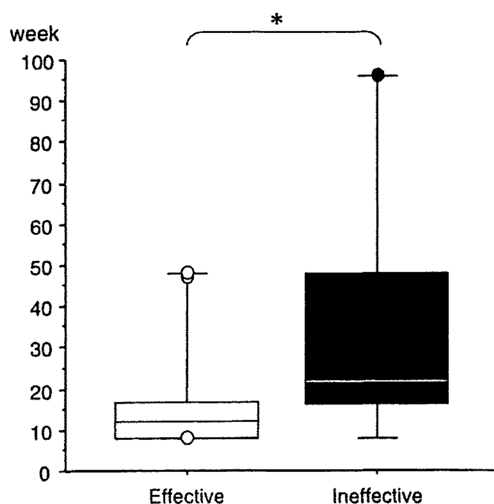


FIGURE 2.—The time interval from the onset of cough to the initiation of montelukast treatment. *$p < 0.03$, Mann-Whitney test.

The Number of Eosinophils in the Bronchial Mucosa Biopsy Specimens

There were more eosinophils in the bronchial mucosa obtained from CVA patients in the effective group than in the ineffective group; however, the difference was not statistically significant (Figure 5C).

Ratio of CD63-Positive Cells to CD203c-Positive Cells in the Peripheral Blood

The ratio of the number of CD63-positive cells to the number of CD203c-positive cells present in the peripheral blood (CD63/CD203c) was not significantly different among the control group, the effective group, and the ineffective group (Figure 5D).

Relationships Between Atopy in the CVA Patients and the Clinical Effects of Montelukast and the Bronchial Mucosa Immunohistochemical Analysis

The relationships between atopy in the CVA patients and the clinical effects of montelukast and the bronchial mucosa immunohistochemical analysis were analyzed. As shown in Table 1, atopy was defined as the presence of a high total serum IgE level (> 150 IU/mL) and/or the presence of serum IgE specific to any one of 26 common environmental antigens. There was no significant relationship between the clinical effect of montelukast and the presence of atopy (among the patients in whom montelukast was effective: 13 of 18 (72.2%) with atopy compared to 9 of 18 (50.0%) without atopy: $p = 0.3051$). The ratio of the number of CD63-positive mast cells to the number of tryptase-positive cells present in the bronchial mucosa was not significantly related to atopy; the ratio was 38.5% (0-100) in patients with atopy compared to 33.0% (0–71.0) in patients without atopy ($p = 0.9417$).

The CD63/CD203c ratio in the peripheral blood was also not related to atopy; the ratio was 45.9% (0–77.4) in patients with atopy compared to 34.5% (12.8-78.8) in patients without atopy ($p = 0.7285$).

DISCUSSION

It has been reported that the number of mast cells in the subepithelial bronchial mucosa does not differ between normal subjects and CVA patients (17) or mild asthma patients in whom significantly more mast cells are found in the smooth muscle layer (2). In the present study, few of the biopsy specimens included the smooth muscle layer; thus, the mast cell profiles around the smooth muscle layers could not be investigated. However, the present study is unique in that immunohistochemical analysis using anti-CD63 antibody showed that airway mast cells, even in the area of the subepithelial mucosa, had specific characteristics that were related to whether montelukast treatment was effective.

CD63 is one of the granular membrane proteins that belongs to the tetraspan family; it is known to be expressed on the cell surface of activated basophils (18). Thus, CD63 is

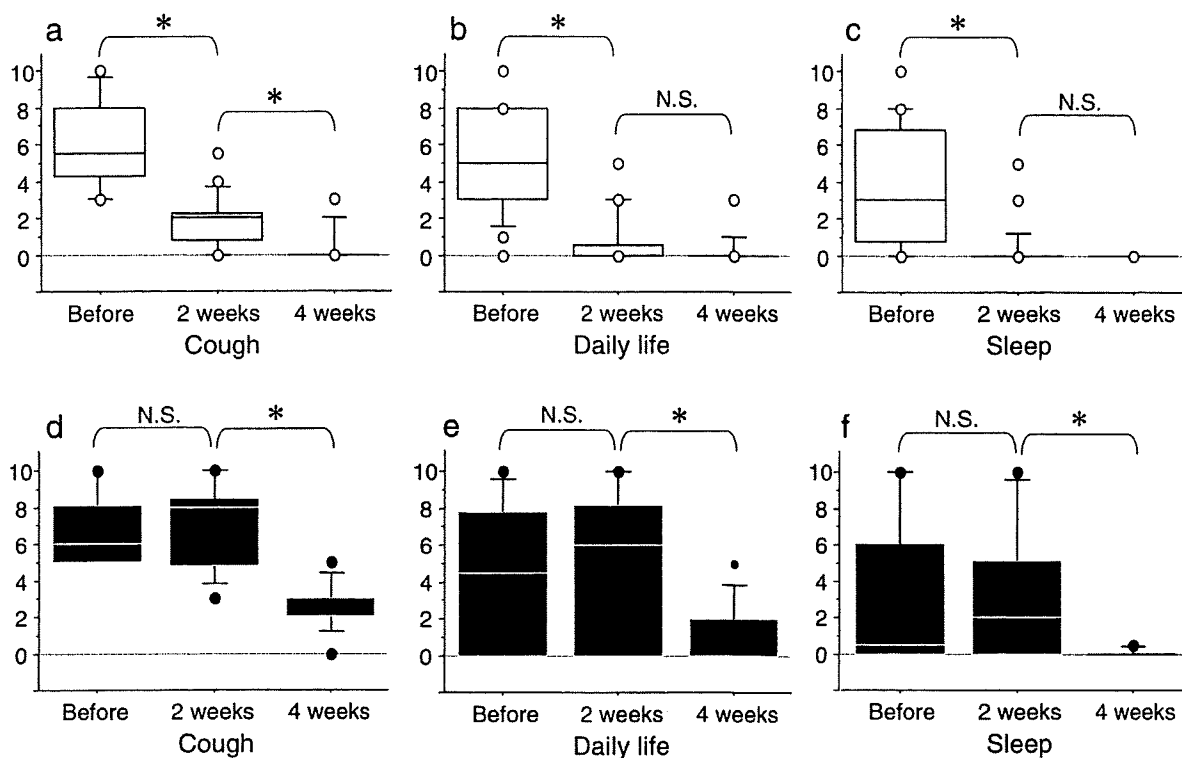


FIGURE 3.—VAS scores of the degree of cough, interference with daily life, and interference with sleep. (A—C)in the effective group; (D—F) in the ineffective group. The ordinate represents the VAS score. $*p < 0.03$, Wilcoxon signed-rank test.

Q2

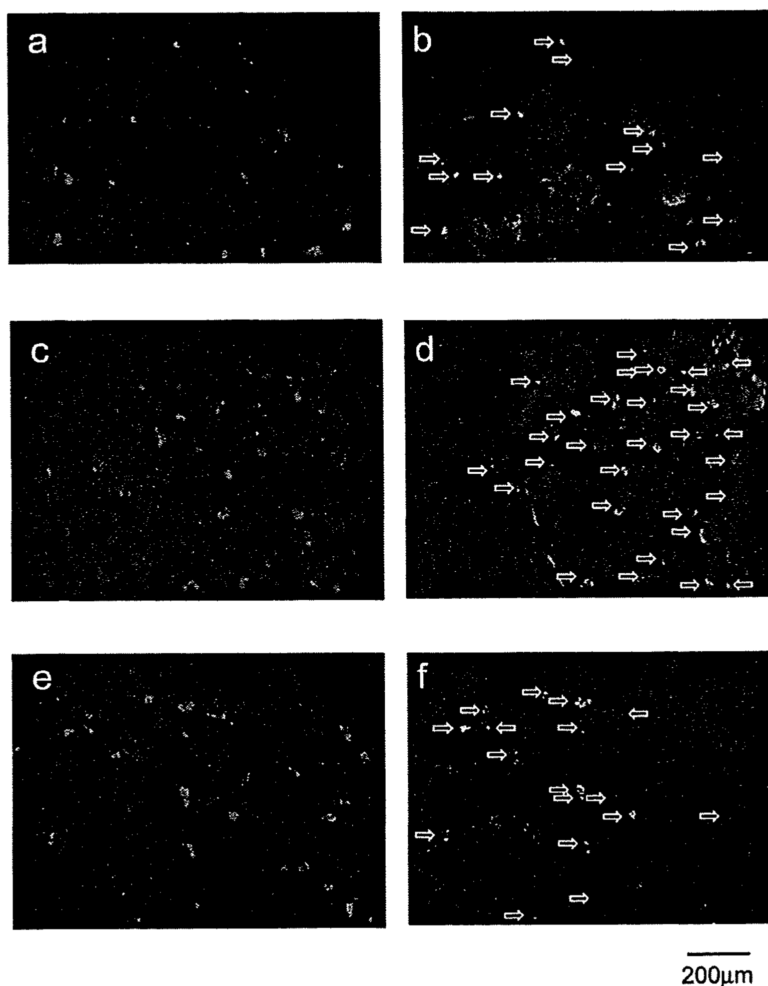
regarded as a marker of their activation. In mast cells, CD63 has been reported to exist in the plasma membrane (19) and in intracellular structures (20). Furthermore, the increased expression of CD63 and its translocation from the intracellular compartments to the plasma membrane are reported to be related to the activation of mast cells (20–22). In unstimulated mast cells, CD63 is considered to participate in adhesion or to be involved in intracellular signal transduction [20]. In the present study, the immunohistochemical analysis using thin sections of the biopsy specimens could not determine whether immunostained CD63 was present on the cell surface or in the cytoplasm. However, the effective group had a higher ratio of CD63/tryptase, which reflects an increased expression of CD63. This suggests that the submucosal airway mast cells may be either activated to a greater degree or be more ready to be activated in CVA patients in whom montelukast was effective than in CVA patients in whom it was ineffective.

Kraft et al. (23)reported that anti-CD63 antibody inhibited the adhesion of mast cells to extracellular matrix (ECM) proteins and also inhibited FcεRI-induced degranulation in mast cells adherent to ECM proteins. Antibodies recognizing integrins have also been reported to suppress mast cell degranulation (24). However, the mechanisms responsible for the activation of airway mast cells in CVA patients seem to be independent of IgE; in the present study, neither the clinical efficacy of montelukast nor the ratio of CD63/tryptase of the airway mast cells was significantly different between patients who were atopic and those who were not.

It is important to consider how LT may be involved in the coughing that characterizes CVA. LTRA is reported to lower the eosinophil levels in the sputum, peripheral blood, and bronchial mucosa of patients with classic asthma (25, 26). In the present study, the degree of eosinophil infiltration in the airways appeared to be higher in the effective group than in the ineffective group. Thus, LT may cause and/or may originate from the allergic inflammation present in CVA patients' airways, and the inflammation could stimulate cough receptors in the airways.

Neural excitement after cough receptors are stimulated is known to be transmitted to the cough center through two nerve fibers: the medullated A δ fiber and the non-medullated C-fiber (27). The C-fiber may be more crucial to the pathophysiology of CVA, since, in CVA patients, the density of the substance P-immunoreactive nerve fibers has been reported to be significantly higher than in patients with classic asthma (28). LT is known to stimulate the C-fiber directly (29, 30). This could result in the release of the neuropeptide, which elicits activation of mast cells (31, 32) as well as transmission of the excitation to the cough center, airway contractions, and/or the accentuation of vascular permeability through an axon reflex (30, 33).

Thus, the present results might be explained, at least in part, by the following hypothesis: excitation of the two cough-related nerve fibers by several kinds of inflammatory stimulation, including LTs originating from various allergic inflammation processes, may bring about further degranulation



4C/Art

FIGURE 4.—Double staining of bronchial mucosa biopsy specimens with anti-CD63 and anti-human tryptase antibodies. Tryptase-positive cells are stained from orange to red (A, C, E), and CD63-positive cells (arrows) are stained green (B, D, F). (A, B) A control subject. (C, D) A CVA patient in whom montelukast was effective. (E, F) A CVA patient in whom montelukast was not effective.

of chemical mediators, such as LTs, by a positive feedback mechanism on mast cells that are adherent to ECM by the expression of CD63.

The results of the flow cytometric analysis of the peripheral blood suggest that the behavior of the mast cell in the bronchial mucosa is more important in the pathophysiology of CVA than the level of peripheral blood basophil activation.

In the present study, the time interval from the onset of cough to the initiation of the treatment was significantly shorter in the effective group than in the ineffective group. This finding indicates that the pathophysiology of CVA includes an initial stage during which the patient is sensitive to montelukast, and that this sensitivity may change during the clinical course of CVA. The cells possibly related to such a changed pathophysiology during the clinical course would have to be neither airway mast cells nor eosinophils since the ineffective group had fewer CD63-positive mast cells and eosinophils than the effective group. Furthermore, the responsible cells appear to be sensitive to corticosteroids since FP relieved cough in the ineffective group. T-lymphocytes could be a possible candidate, although further investigations are necessary.

The finding regarding the relationship between the time interval from the onset of cough and the effectiveness of montelukast raises two interesting issues: (1) whether montelukast would still have been effective if it had been started at a different time in the effective group; and (2) whether, in the effective group, montelukast treatment would continue to be effective for longer than the 4 weeks of treatment of the present study. If montelukast improves the fundamental pathophysiology of CVA, then montelukast treatment in the early phase after onset would have a greater long-term clinical efficacy and might prevent the development of classic asthma. The clinical course of CVA in patients who receive early intervention and are given long-term LTRA needs to be investigated.

The limitations of the present study may be that it was an open label study and that the sample size was relatively

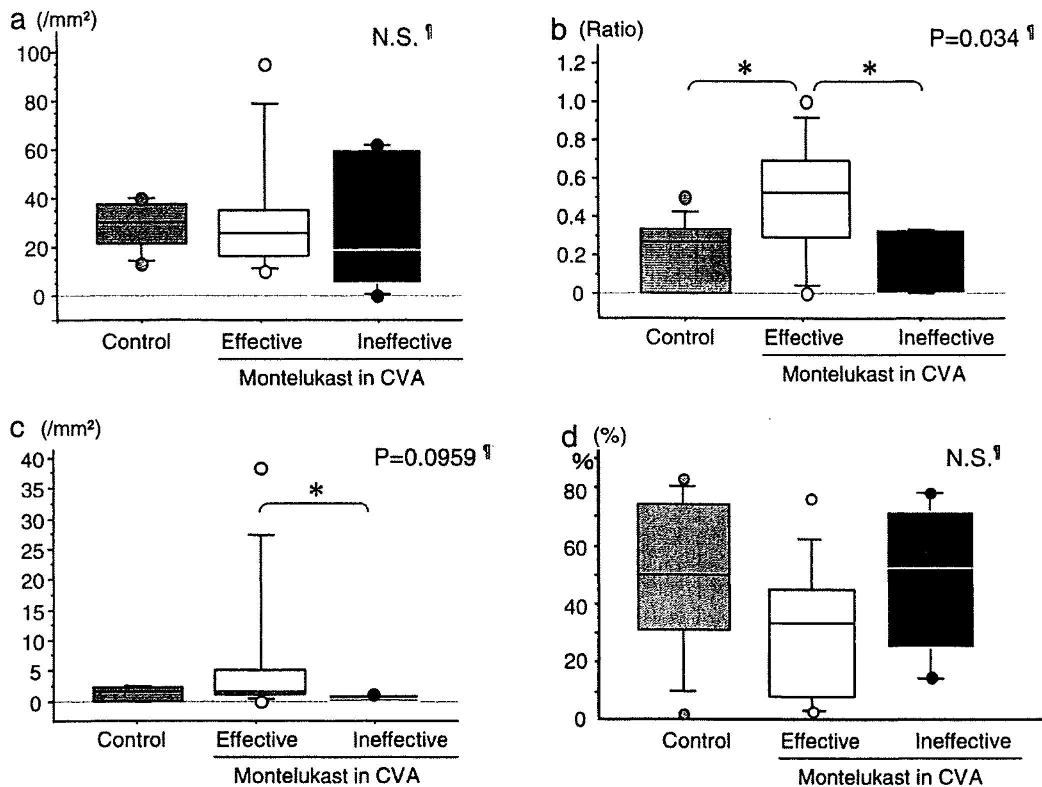


FIGURE 5.—Parameters in the bronchial mucosa (A-C) or in the peripheral blood (D) before treatment. (A)The number of mast cells; (B) the ratio of the number of CD63-positive cells to the number of tryptase-positive cells, and (C)the number of eosinophils in the control group ($n = 11$), the effective group ($n = 8$), and the ineffective group ($n = 5$), respectively. (D) The ratio of the number of CD63-positive cells to the number of CD203c-positive basophils in the control group ($n = 10$), the effective group ($n = 11$), and the ineffective group ($n = 7$). ¶Kruskal-Wallis test. *$p < 0.05$, Mann-Whitney test.

small. However, the central aim of this study was to clarify the hypothesis that leukotriene was involved in the pathophysiology of CVA. It was found that cysteinyl-leukotrienes probably originate from activated airway mast cells and may be related to the pathogenesis of chronic cough, especially during the early phase of CVA. Thus, given this study's encouraging results, a blinded, controlled study to evaluate the clinical usefulness of montelukast for the treatment of CVA is warranted.

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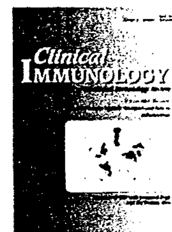

Toll-like receptor signaling is impaired in dendritic cells from patients with X-linked agammaglobulinemia

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KEYWORDS
Bruton's tyrosine kinase;
Dendritic cells;
Toll-like receptor;
TNF-α;
X-linked agammaglobulinemia

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.
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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.

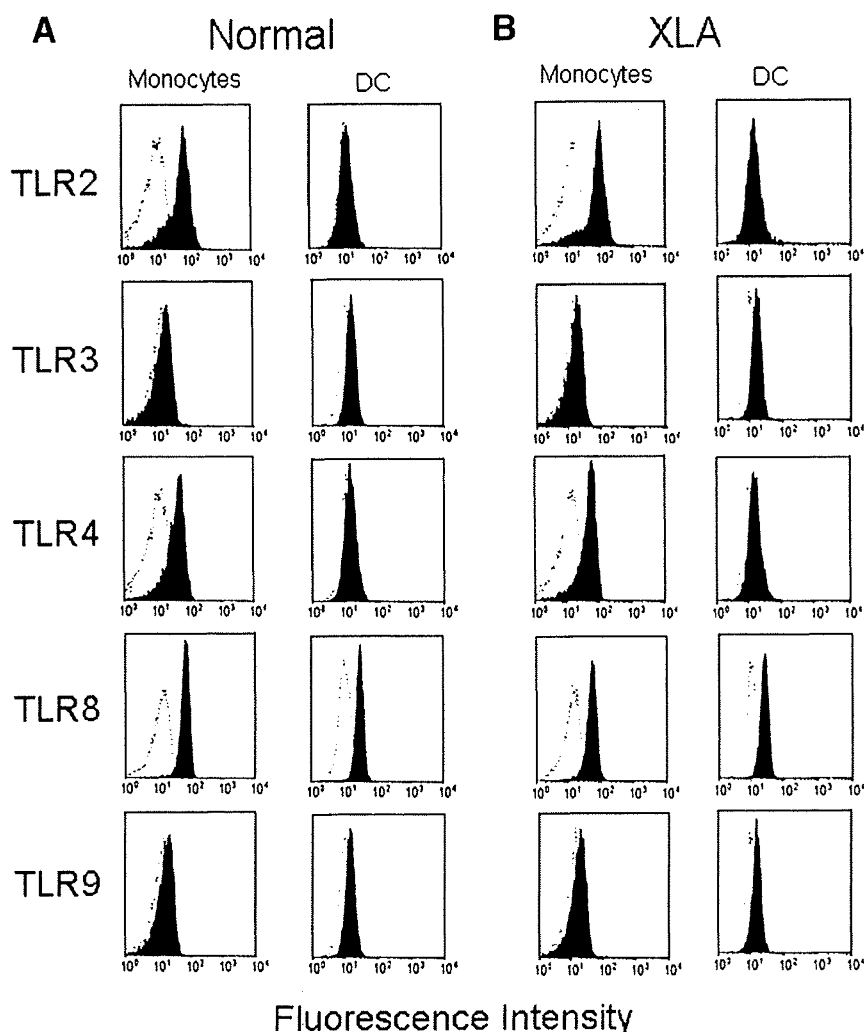


Figure 1 TLR expression in monocytes and DCs. The expression of TLR2, TLR3, TLR4, TLR, and TLR9 was examined in monocytes and monocyte-derived DCs. To detect TLR3, TLR8, and TLR9, the cells were fixed and permeabilized in 0.5% saponin prior to staining. These cells were incubated with PE-labeled mAbs 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.

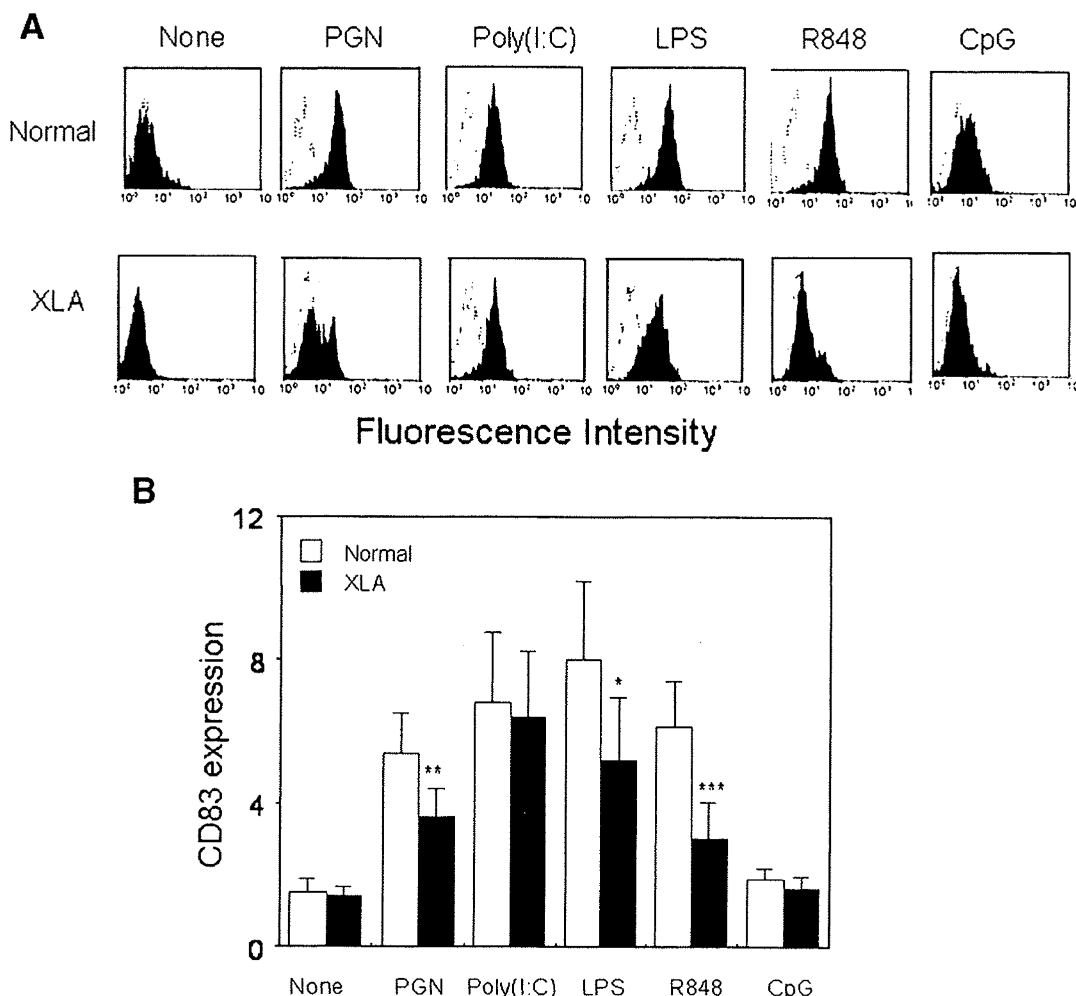
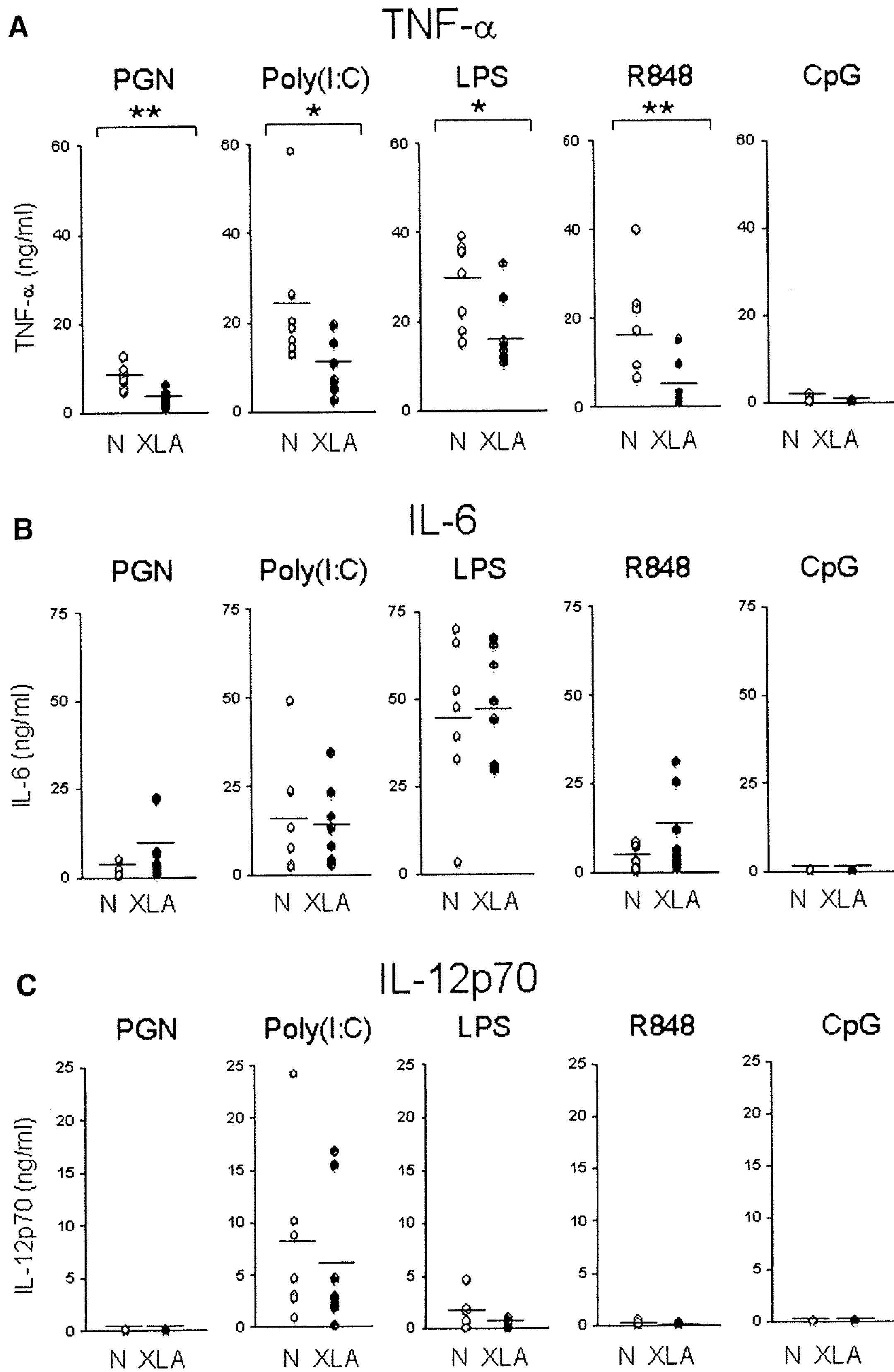


Figure 2 Induction of CD83 expression on DCs by stimulation with TLRs. Monocyte-derived DCs were cultured with various TLR ligands including PGN, Poly(I:C), LPS, R848, and CpG. CD83 expression on DCs after stimulation with TLR ligands was assessed by a flow cytometry. (A) Representative histograms obtained 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)
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TNF-α (ng/ml)
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PGN
Poly(I:C)
LPS
R848
CpG
IL-6 (ng/ml)
N XLA
C
IL-12p70
PGN
Poly(I:C)
LPS
R848
CpG
IL-12p70 (ng/ml)
N XLA

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.

Acknowledgments

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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.

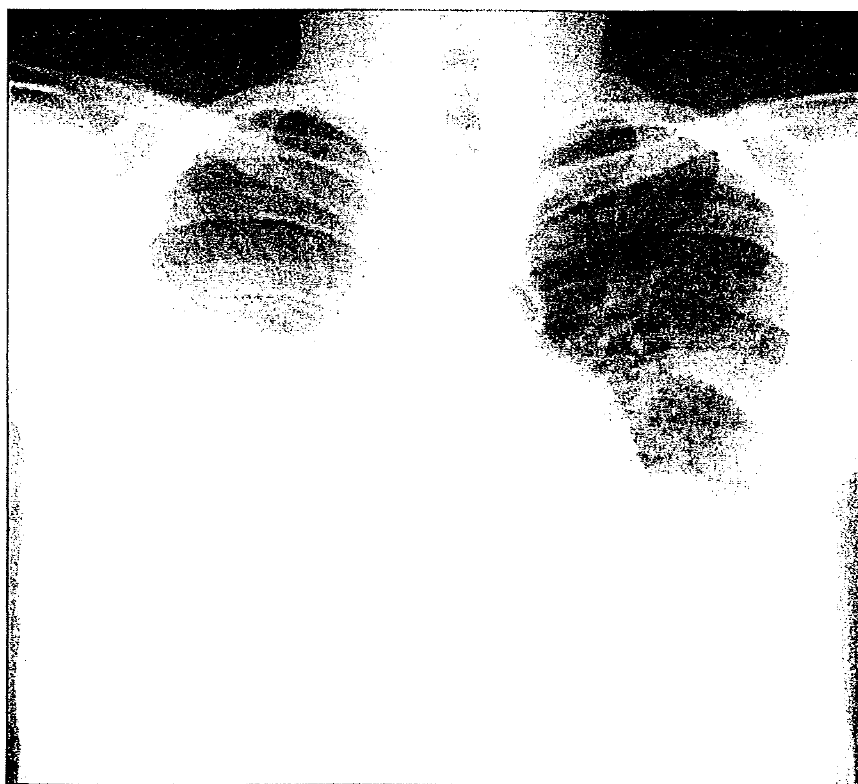
CASE REPORT

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.

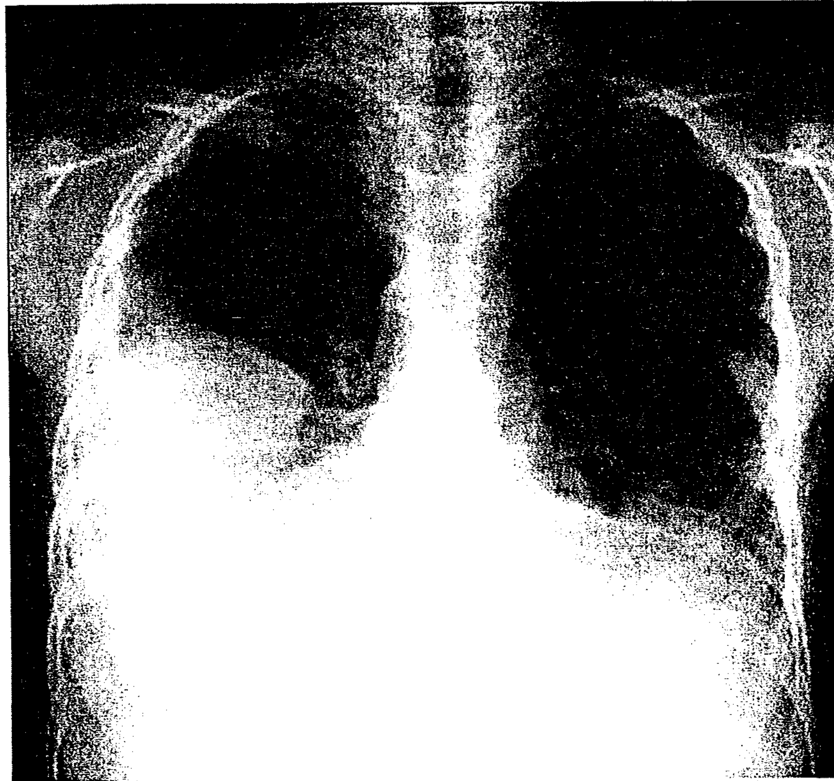
He was admitted again with complaints of sudden dyspnea after 2 years. A chest radiograph showed an increase of the right pleural effusion

(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)

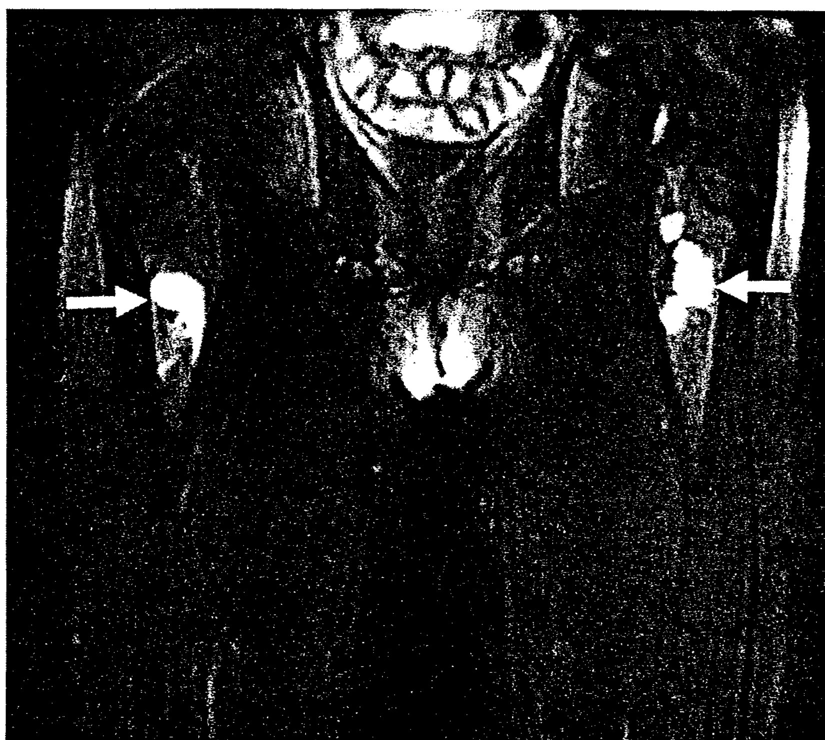
FIGURE 1 (Continued)

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.

DISCUSSION

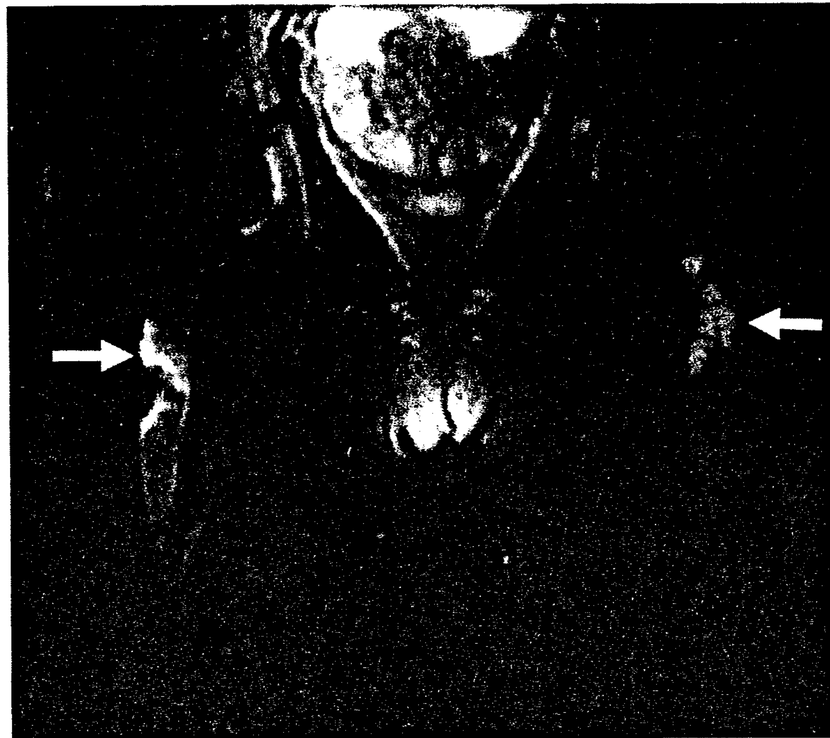
We have presented a case with a rare, progressive, diffuse lymphangiomatosis treated with pegylated IFN alfa-2b. This agent markedly regressed pulmonary effusion caused by thoracic lesion and also regressed multiple bone lesions.

The theoretical concept for using IFN alfa-2b to treat this disease was based on laboratory and clinical findings that have evolved over the past decade. IFN alfa is a cytokine with multiphasic cellular functions, including antiviral, antiproliferative, immunomodulatory, and antiangiogenic activities [6]. The delivery of IFN alfa represents a significant challenge and has potential implication in multiple therapeutic indications, including patients with hepatitis B and C infections [7], malignant melanoma [8], renal cell carcinoma [9], and chronic myelogenous leukemia [10]. Laboratory findings demonstrated that IFN alfa had antiangiogenic effects in 1980. It was discovered that interferon inhibits endothelial cell motility in vitro and that both IFN alfa and IFN beta can inhibit tumor-induced angiogenesis in murine animal models [11]. This led to the first successful application



(a)

FIGURE 2 Femoral MRI showed a multiple high-intensity areas on T2-weighted images as indicated by arrows (a). These lesions were apparently reduced by IFN treatment for 3 months (b). *(Continued)*



(b)

FIGURE 2 (Continued)

of IFN alfa-2b, based on its antiangiogenic activity in a 7-year-old boy with diffuse pulmonary hemangiomatosis; this was the first demonstration of antiangiogenic therapy in humans [12]. There have been several reports documenting successful therapy with IFN alfa-2b for complicated hemangiomas [13]. IFN alfa has also been used with effective results in the treatment of lymphangiomatosis. In our review of the literature (Table 1) [14–21], patients aged between 1 and 15 years (10 males and 4 females) who had recurrent, intractable, progressive lymphangiomatosis and received IFN alfa were treated with IFN alfa. All but patient 8 [19] had pulmonary involvement. Patient 8 was affected in bone, liver, and spleen. The clinical symptoms were coughing, wheezing, dyspnea, and chest pain. Bone lesions exisited in 64.2% (9/14) of the cases. Almost all of them experienced clinical improvement of disease to a greater or lesser extent and several continued that therapy. The most commonly reported side effects in these patients were fever, neutropenia, thrombocytopenia, and nausea. Two cases were unable to tolerate these side effects, leading to the discontinuation of IFN alfa therapy.

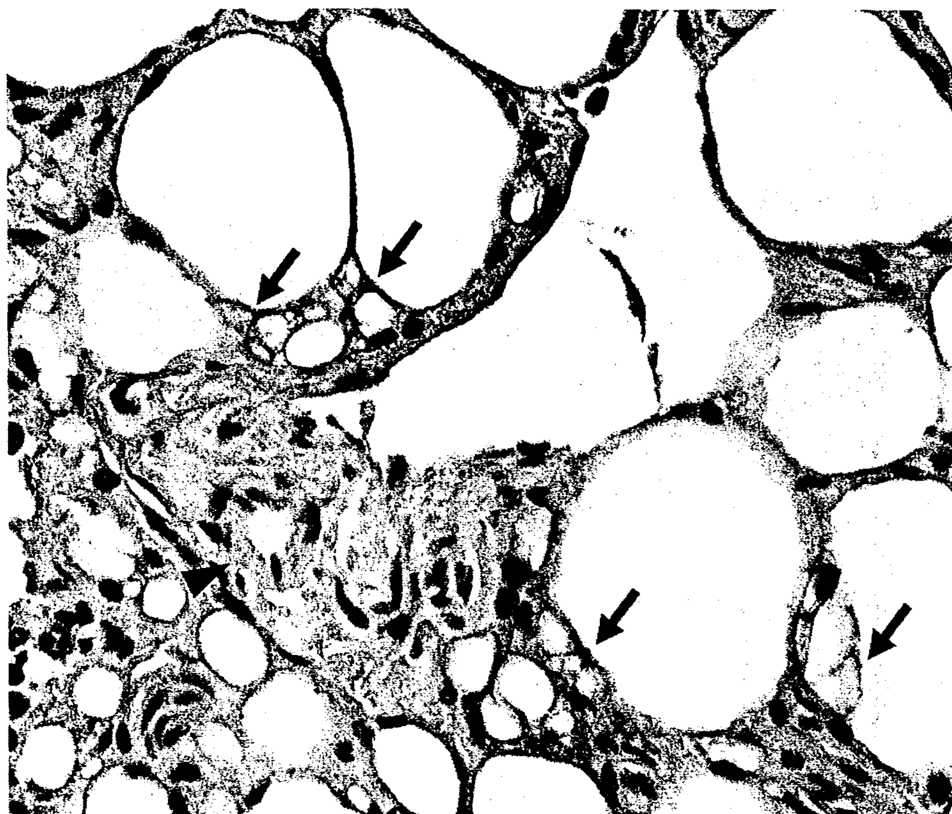


FIGURE 3 Specimen of bone biopsy from left upper forelimb. Dilated endothelial cells are strongly labeled by D2-40 (arrows). In contrast, those of adjacent small blood vessels and capillaries are D2-40 negative (an arrowhead).

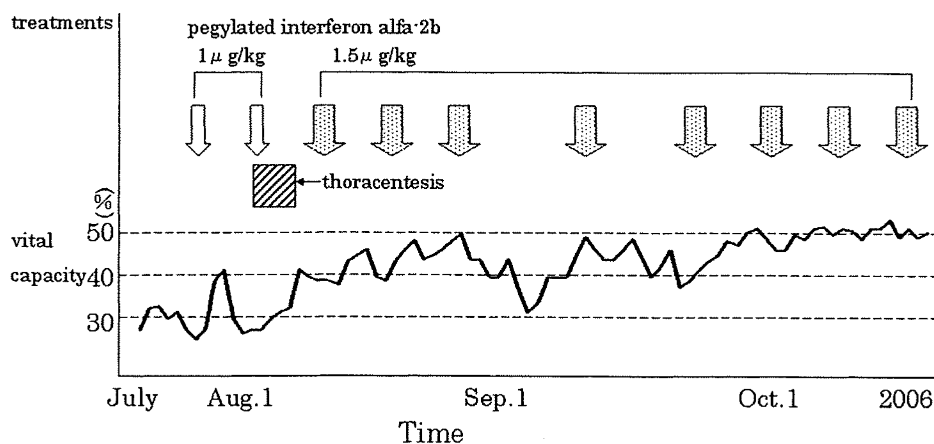


FIGURE 4 Treatments and patient's vital capacity levels during the course of pegylated interferon alfa-2b therapy. For our patient, a compact electronic spirometer was used for measuring vital capacity every day.

TABLE 1 Cases of Lymphangiomatosis with Interferon Alfa Therapy

No.	Reference	Age/sex	Involvement	Symptom	Therapy	Response to IFN therapy	Reason for stopping IFN	Outcome, follow up
1	Kelso et al. 1991	3 Y/M	Lung, Pe	Cough, dyspnea, hemoptysis	$3\frac{1}{2}$ weeks	Regression of symptoms	Thrombocytopenia	Died of hemoptysis 4 weeks after discontinuation of IFN
2	Tazelaar et al. 1993	7 Y/M	Lung, Pe	Unknown	20 months	Clinical improvement	NA	Alive 7 years later
3	Margraf et al. 1996	10 Y/M	Lung, Pe	Chest pain, nausea, dyspnea	Short term	Abate the impending respiratory failure without success	Died	Died of disease
4	Reinhardt et al. 1997	14 Y/M	Thorax, Pe, abdomen	Dyspnea, decrease exercise tolerance	12 months	Improvement, no further thoracetesis, no hospitalization	NA	Alive
5		12 Y/M	Thorax, Pe, bone, spleen	Dyspnea, chest pain	40 months	Slight improvement, then stabilization of effusion	Not reported	Alive
6	Patton et al. 1998	1 Y/M	Lung, pericardium, spleen	Wheezing, fatigue, easy bruising	Several months	Not reported	Responded to splenic embolization	Alive
7	Maki et al. 1999	12 Y/F	Bone, liver, spleen	leg and back pain	About 5 years	clinical improvement, but necessary use of analgesics	NA	Alive
8		12 Y/M	Pe, bone, abdomen	Dyspnea, limitation of motion	$3\frac{1}{2}$ years	Clinical and radiological improvement	The dose was reduced due to anemia, hepatic abnormality	Alive, he remains asymptomatic

(Continued)