TA cloning kit (Invitrogen) and sequenced as described above.

Reporter Assay for Detecting a Mutant NEMO Function: NEMO-NF-κB Luciferase Reporter Assay

NEMO cDNAs from a healthy volunteer and our patient were subcloned into the p3xFLAG-CMV14 vector (Sigma), respectively. NEMO null rat fibroblast cells (kindly provided by Dr. S. Yamaoka) were plated at a density of 3×10^4 cells/well in a 24-well culture dish and were transfected with 200 ng of plasmid, containing 40 ng of NF-κB reporter plasmid (pNF-κB-Luc; BD Biosciences Clontech, USA), 2 ng of a *NEMO* mutant expression construct, 148 ng internal control for normalization of transfection efficiency (pRL-TK; Toyo Ink, Japan), and the corresponding mock vector, using the FuGENE® HD Transfection Reagent (TOYO-B-Net, Japan) according to the manufacturer's protocol. At 12 h after transfection, the cells were stimulated with 15 ng/mL LPS for 4 h and the NF-κB activity was measured using the PicaGene® Dual SeaPansy assay kit (TOYO-B-NET) according to the manufacturer's protocol. Experiments were performed in triplicate and firefly luciferase activity was normalized to Renilla luciferase activity.

Vβ and Vα Analysis of T Cells

T cell receptor (TCR) β and α chain variable region (Vβ and Vα) repertoires were analyzed by a reverse transcription polymerase chain reaction (RT-PCR) method as described [16]. Briefly, each Vβ fragment (from Vβ1 to Vβ20) or Vα fragment (from Vα1 to Vα18, Vα21, and Vα24) was prepared from a series of HBVT/HBVP or HAVT/HAVP plasmids originating from thymus or peripheral T cells [17] and was dotted on filters. PCR products obtained from the patient by RT-PCR were labeled by α-^{32}P-dCTP and hybridized to the filters. Using densitometry, a semiquantitative assessment of V gene usage was made from the amounts of hybridized products.

Flow Cytometry

Peripheral blood samples were analyzed by three-color flow cytometry. Cells were stained with monoclonal antibodies to the following cell surface markers: CD3, CD4, CD8, CD19 (Becton-Dickinson), and CD14 (eBioscience, USA). Flow cytometry analysis of intracellular NEMO protein was performed as described previously [18]. Flow cytometric data from the stained cells were collected by FACScalibur and analyzed with CellQuest software (Becton-Dickinson).

Intracellular Cytokine Staining

Whole blood samples from our X-EDA-ID patient and healthy donors were stimulated with 1-μg/mL ionomycin (Sigma-Aldrich) and 25-ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) in the presence of 10-μg/mL brefeldin A (Sigma-Aldrich) for 4 h. Cultured cells were stained with monoclonal antibodies against CD4 and CD8 for 30 min at room temperature. Stained cells were fixed and permeabilized with BD Lysing solution (Becton-Dickinson) and incubated with anti-TNFα monoclonal antibody or IgG1 isotypic control (Becton-Dickinson). Cells were analyzed by flow cytometry as described above. Analysis of intracellular TNFα in CD14+ cells was performed after stimulation with LPS (1 μg/mL) at 37°C for 4 h.

Endoscopy and Immunohistochemical Staining

Endoscopy was performed with the consent of legal guardians. Colon biopsies were obtained at regions of visual abnormalities. Formalin-fixed paraffin-embedded tissues blocks were cut into 2-μm sections and stained with hematoxylin and eosin. Subsequently, immunohistochemical analysis using the following primary antibodies with optimized experimental protocols was performed: CD3ε (DAKO, Denmark, rabbit, polyclonal, diluted 1:100, incubated for 24 h at 4°C after microwave heat-induced antigen retrieval for 40 min in pH 6.0 citrate buffer), CD79a (DAKO, mouse, monoclonal, 1:100, microwave for 40 min, pH 6.0), CD68 (DAKO, mouse, monoclonal, 1:50, proteinase K (DAKO) for 10 min at room temperature), CD4 (Novocastra, USA, 1:100, microwave for 40 min, pH 9.0 (NICHIREI BIOSCIENCES, Japan)), CD8 (DAKO, mouse, monoclonal, 1:100, microwave for 40 min, pH 9.0), and TNFα (Santa Cruz Biotechnology, USA, goat, polyclonal, 1:200, microwave for 40 min, pH 6.0). An Envision-HRP Detection kit (DAKO) was used for visualization, except for anti-TNFα, which was visualized using donkey biotin conjugated anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, USA) and LASB2-System/HRP kit (DAKO).

Infliximab Treatment

Infliximab treatment for our X-EDA-ID patient was approved by the medical ethics committee of the University of Miyazaki. We obtained written consent concerning treatment from both the patient and his guardian. Before initiating infliximab, we confirmed that he had no severe infection including tuberculosis according to laboratory data, mycobacterium culture test, skin tuberculin test, and chest computed tomography. Cardiac dysfunction was excluded by echocardiography and electrocardiogram.

Infliximab was given intravenously over 2 h at a dose of 5 mg/kg on 0, 2, and 6 weeks, with follow-up treatments every 7–8 weeks depending on clinical symptoms. The patient was monitored regularly throughout the infliximab treatment.

Results

Case

The patient was born to unrelated Japanese parents after an uncomplicated pregnancy of 41 weeks. There was no history of any first-degree relative diagnosed with incontinentia pigmenti. On the first day after birth, he presented high fever with a markedly increased white blood cell count ($40\times10^3/\mu L$) and was treated successfully with antibiotics. He has had a history of recurrent, severe infections including varicella at 3 months of age, penicillin-resistant *Streptococcus pneumoniae* meningitis at 6 months of age, and zoster at 8 months of age. Persistent diarrhea was also observed.

He was introduced to our hospital at 8 months of age for examination of his immunological status. On admission, he showed a marked increase in both white blood cells ($31.9\times10^3/\mu L$) and platelets ($872\times10^3/\mu L$). Peripheral blood T cell count was decreased (CD3-positive cells, 25.8%), and B cell count was highly increased (CD20-positive cells,: 69.2%). PHA induced a normal proliferation response of T cells, and concentrations of immunoglobulins were within the normal range except IgD (less than 0.2 mg/dL). Natural killer cell activity was markedly impaired. Superoxide-generating ability from neutrophils was intact. LPS-induced TNFα production from patient's PBMC was impaired (Fig. 1a). Interferon (IFN) γ-producing lymphocytes were also reduced apparently at 8 months of age (Table I). All the genes involving in the IL-12 signal pathway, including *IL12RB1*, *IL12RB2*, *JAK2*, and *STAT4* were sequenced, but no mutations were found (data not shown). Surprisingly, both IFNγ-producing T cells and natural killer cells had expanded significantly by 11 months of age (Table I). In addition, we observed that he had ectodermal dysplasia including anhidrosis and conical teeth (Supplementary Fig. 1). A skin biopsy revealed the absence of eccrine sweat glands. When he was 3 years old, a G505C (A169P) missense mutation in his *IKBKG* gene was confirmed and diagnosed as X-EDA-ID. His mother was a carrier. An expression of mutant NEMO protein was not markedly

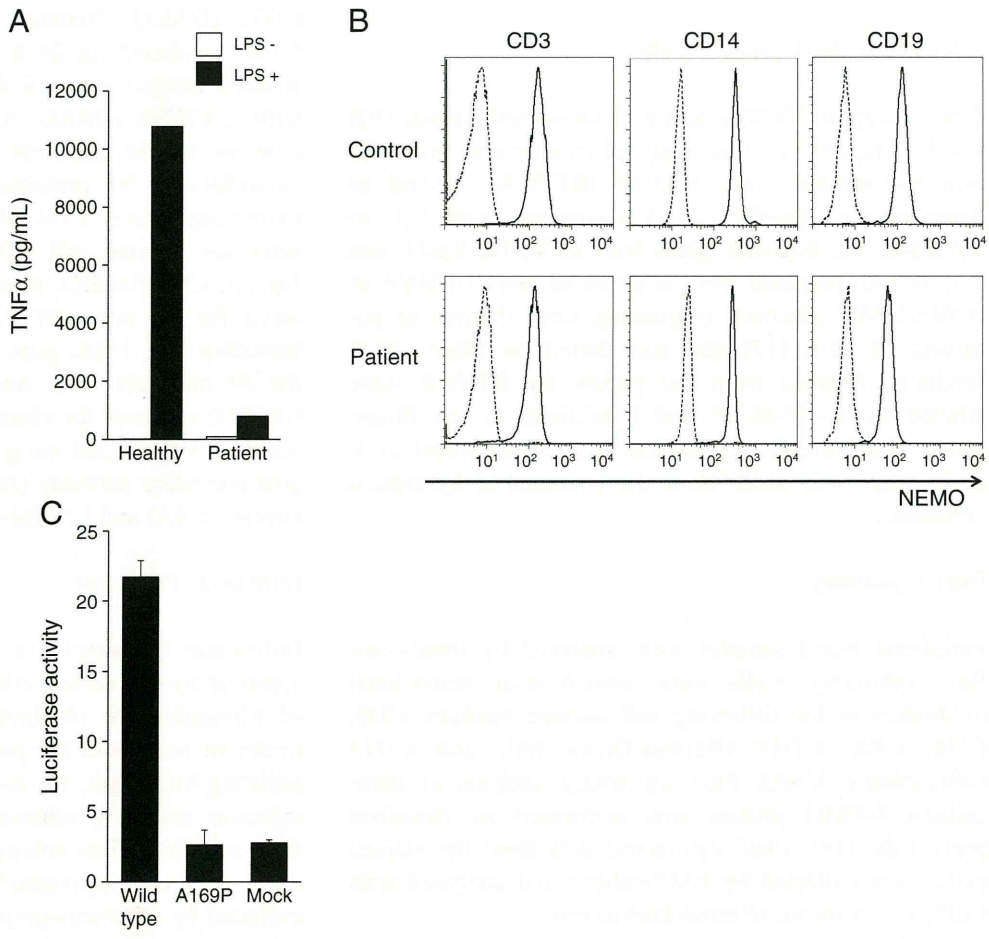


Fig. 1 Analysis of mutant NEMO protein. **a** Reduced production of TNFα from LPS-stimulated PBMCs. PBMCs from our patient and healthy volunteer were stimulated with LPS (1 μg/mL). **b** Analysis of NEMO protein expression using flow cytometry. Intracellular NEMO protein in PBMCs from the patient was not reduced markedly. **c** The result of NEMO-NF-κB luciferase reporter assay. The activity of mutant NEMO in the patient was almost defective. Mock vectors and wild-type NEMO were used as controls. *Error bars* indicate SD

Table I Proportion of IFNγ-expressing T and NK cells in the patient

Age	$IFN\gamma^+/IL\text{-}4^-$		
	CD4 (%)	CD8 (%)	CD56 (%)
8 months	1.14	8.83	2.00
11 months	3.18	70.40	66.29
3 years 11 months	11.89	65.48	82.79
Healthy control	15	60–80	80–90

reduced by flow cytometer (Fig. 1b), but the activity of mutant NEMO was almost defective which was confirmed by a mutant NEMO-NF-κB luciferase reporter assay (Fig. 1c). He has been prescribed prophylactic cotrimoxazole before and after the diagnosis.

He presented with chest pain, erythema, polyarthritis, continuous high fever refractory to antibiotics, and marked elevation of C-reactive protein (7.4 mg/dL) at 4 years of age. Autoantibodies such as anti-centromere antibody were detected transiently. Chest computed tomography revealed multiple nodular shadows resembling bronchiolitis obliterans organizing pneumonia. The repertoire of T cell receptor showed high expression of limited Vβ subsets (Supplementary Fig. 2). Combination therapy using corticosteroids, cyclosporine A, and methotrexate was effective and was continued to control his symptoms.

Severe abdominal pain and intractable frequent diarrhea recurred when the corticosteroid dose was reduced, and he presented perianal fistula at 8 years of age. A mild elevation was observed in both erythrocyte sedimentation rate and C-reactive protein under the preceding immunosuppressive treatments (Table II). No significant pathogen was detected by stool culture and the use of antibiotics and antifungal drugs resulted in no improvement in clinical symptoms.

Endoscopic and Microscopic Findings of the Colon

Colonic endoscopy revealed many polyp-like lesions with mucosal redness and edema at the sigmoid/descending junction (Fig. 2). A longitudinal ulcerative lesion found in the sigmoid colon was suggestive of Crohn's disease. Passing the endoscope beyond these obstructive clusters of polyps was difficult; therefore, we could not observe the upper part of the colon. Neither stenosis nor ulcer formation was observed by intestinal radiocontrast analysis.

Histopathological examination of the colonic biopsied specimens showed diffuse lymphoplasmacytic infiltration, superficial edema, and hyperemia in lamina propria. Foamy cells and some eosinophils were also seen (Fig. 3a, b). No definite neutrophilic infiltration, crypt abscesses, or granulomatous lesions were observed. Cultures from biopsied specimens yielded neither bacterial nor fungal growth.

Immunohistochemical staining revealed predominant infiltration of CD79a-positive, plasma cells in the lamina propria. Infiltration of CD68-positive macrophages and CD3-positive T cells was also observed (Fig. 3c–g).

Detection of TNFα-Producing Cells in the Lamina Propria and Peripheral Blood

To investigate the possibility that TNFα blockade therapy can ameliorate inflammatory colitis as well as NEMO-deficient mice as suggested by previous analysis [15], we analyzed TNFα-producing mononuclear cells in the lamina propria in the colon of our patient. Immunohistochemical staining showed abundant TNFα in infiltrated mononuclear cells in the lamina propria (Fig. 3h) which would be associated with progression of inflammatory colitis.

We also analyzed TNFα-producing T cells and monocytes in the peripheral blood (Fig. 4a). The majority (72.49%) of CD4-positive T cells in our patient expressed intracellular TNFα, while 40% to 70% of CD4-positive T cells expressed TNFα in adults with IBD in our study. Forty-eight percent of CD8-positive T cells in our patient expressed TNFα. CD14-positive monocytes from our patient expressed small amounts of intracellular TNFα after LPS stimulation, while similarly treated CD14-positive cells from healthy subjects expressed abundant TNFα (Fig. 4b).

Reversion Analysis

Nishikomori et al. reported that in an X-EDA-ID patient, the mutation had been reverted to the normal state in IFNγ-

Table II Laboratory data on admission (8 years old)

WBC	13,600/μL	CD3	76.2%	IgG	790 mg/dL
Neutrophils	10,200/μL	CD4	22.2%	IgA	666 mg/dL
Lymphocytes	1,632/μL	CD8	58.3%	IgM	71 mg/dL
Monocytes	952/μL	CD19	4.8%	IgD	<0.6 mg/dL
Hemoglobin	12.0 g/dL	CD20	3.8%	C3	134 mg/dL
Platelets	84.7×10^4/μL	CD16	0.5%	C4	46 mg/dL
		CD56	33.6%	CH50	56 U/mL
		HLA-DR	26.5%	ESR	43 mm/h

WBC white blood cell, *CH50* total complement activity, *ESR* erythrocyte sedimentation rate

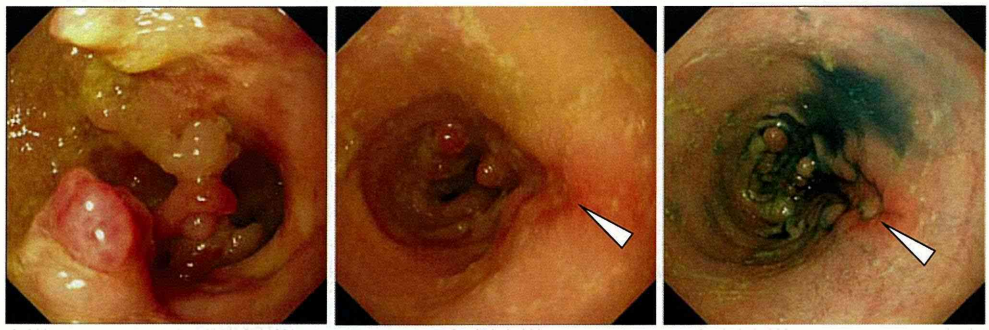


Fig. 2 Findings of colonoscopy performed before initial treatment with infliximab. Colonoscopy revealed polyp-like lesions with mucosal redness and edema at the sigmoid/descending junction (*left panel*). A longitudinal ulcer (*arrowhead*) was found in the sigmoid colon (*center panel*). Same segment as in the *center panel* after indigo carmine dye (*right panel*)

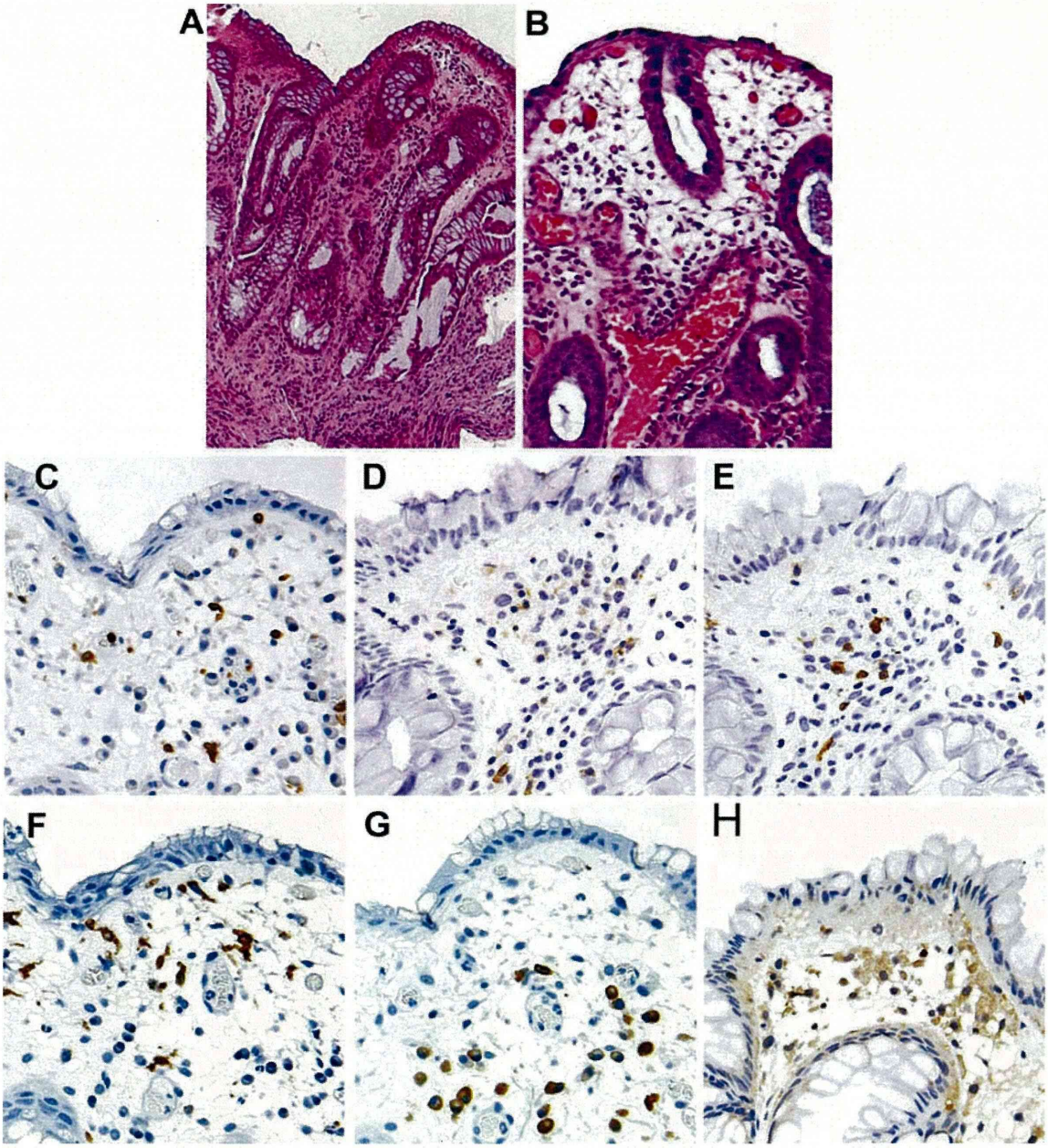


Fig. 3 Microscopic findings of affected colonic specimens. **a**, **b** Hematoxylin and eosin staining. **a** and **b** are low-power field and high-power field views, respectively. **c**–**h** Staining profiles of cellular surface antigens: **c** CD3ε, **d** CD4, **e** CD8, **f** CD68, and **g** CD79a. **h** Staining with anti-human TNFα antibody

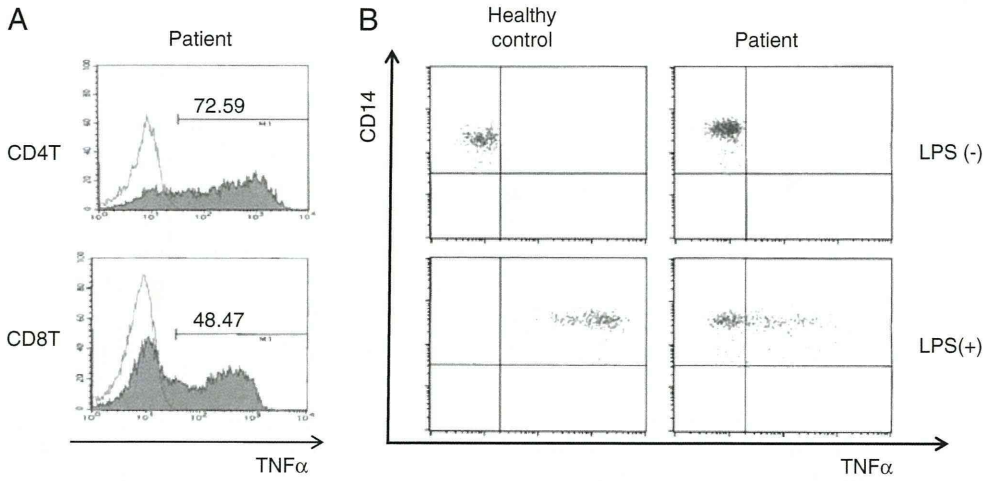


Fig. 4 Analysis of TNFα-producing mononuclear cells in peripheral blood. **a** TNFα-expressing T cells increased markedly before infliximab treatment. PBMCs were stimulated with ionomycin and PMA for 4 h in the presence of brefeldin A then stained for intracellular TNFα. For FACS analysis, gates were set on lymphocytes according to forward and side scatter properties. Representative histograms of TNFα expression in stimulated (*solid histograms*) or unstimulated (*black line histograms*) T cells. The proportion of TNFα-positive CD4-positive T cells in adult IBD patients is 40–70%. **b** The percentage of TNFα-positive monocytes was examined. Cells from our patient and healthy volunteer were incubated with or without LPS for 4 h in the presence of brefeldin A. Monocytes were identified by CD14. Approximately 50% of stimulated monocytes produced a small amount of TNFα

expressing T cells [18]. Our patient showed expansion of IFNγ-expressing T cells during infancy and an increase in TNFα-producing T cells at that time. We hypothesized that the A169P mutation in the *IKBKG* gene had been reverted to wild type and that the reverted T cells had expanded in our patient. Indeed, before initiating infliximab treatments, reversion mutation was detected in 23/67 (34%) from non-stimulated PBMCs (Table III). At 24 months after the initiation, reversion mutation was detected in both messenger RNA (mRNA) and genomic DNA from lymphocytes stimulated with PHA and IL-2 for 10 days, whereas only mutated mRNA was identified from non-stimulated lymphocytes (Fig. 5). Reverted mRNA was observed in CD3-positive T cells. Sex chromosome analysis with fluorescent in situ hybridization revealed no maternal cells and therefore graft-versus-host disease secondary to maternal–fetal transfusion was unlikely. These findings suggest that reverted T cells activated NF-κB in response to growth signals and had a growth advantage over mutant cells.

Table III Frequency of reverted clones before and after initiation of infliximab treatments

	Before	After 12 months	After 24 months
PBMCs	23/67 (34%)	nd	2/6 (33%)[a]
CD3	nd	3/16 (19%)	nd
CD14	nd	0/19 (0%)	nd
CD19	nd	0/47 (0%)	nd

nd not done

[a] A result using stimulated mononuclear cells

Reverted T cells decreased with repeated administrations of anti-TNFα monoclonal antibody. In contrast, CD14-positive monocytes and GM-CSF-induced monocyte-derived dendritic cells had no reversion (Table III).

Anti-TNFα Treatment Improved NEMO Colitis

We initially treated NEMO colitis with high dose corticosteroid therapy (2 mg/kg prednisolone, daily) (Fig. 6). However, steroid therapy did not improve clinical symptoms and resulted in compression fracture in the thoracic spine from corticosteroid-induced osteoporosis.

The increase in TNFα-producing T cells suggested the possibility that TNFα blockade therapy would be an effective treatment for the intractable NEMO colitis. After confirming the absence of severe bacterial or mycobacterial infection, we initiated administration of the chimeric anti-TNFα monoclonal antibody, infliximab, to our patient.

Soon after the first infusion of infliximab, abdominal pain disappeared and his appetite recovered. Frequency of diarrhea decreased as administrations of infliximab were repeated (Fig. 6). Colonoscopy after his third administration showed mild improvement of both mucosal redness and edema (Fig. 7a). These mucosal inflammatory findings had almost disappeared after 1-year treatment with infliximab, although polyp-like lesions remained (Fig. 7b).

The proportion of TNFα-producing cells in CD4-positive and CD8-positive T cells markedly decreased by his third infliximab infusion (from 72.6% to 26.7% in CD4-positive T cells and from 48.5% to 23.1% in CD8-positive T cells), and reduction of TNFα-producing cells was

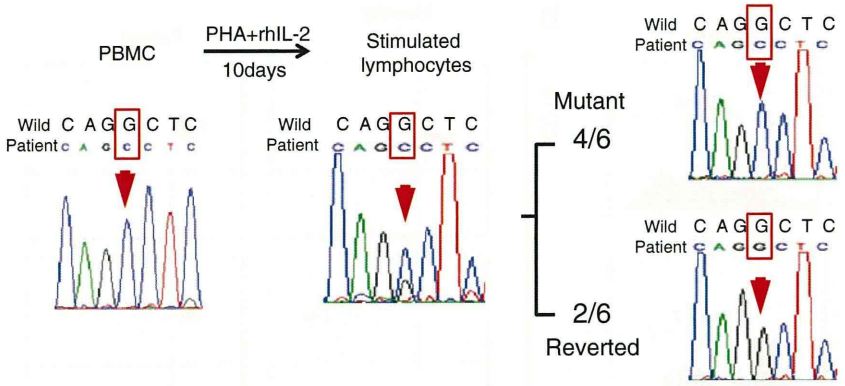


Fig. 5 Reversion analysis of cDNA of gene encoding NEMO isolated from mononuloclear cells after 24 months of infliximab treatment. PBMC was obtained from our patient and incubated with PHA and IL-2 for 10 days. Direct sequence for mRNA encoding NEMO was performed using PBMC and the stimulated mononuclear cells. Before stimulation, no reverted mononuclear cells were detected. After PHA and IL-2 stimulation, reverted mononuclear cells apparently increased. Subcloning of cDNA from stimulated cells showed that two of six cells had reversion of mutation in the gene

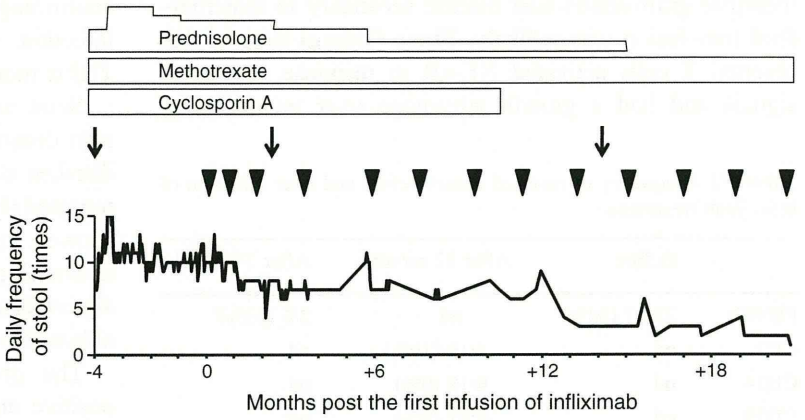
associated with improvement of clinical symptoms (Fig. 6). Administration of cyclosporine A was discontinued by the eighth infliximab treatment, and corticosteroid was reduced and then discontinued by the tenth infliximab infusion.

Our patient had one serious adverse event, pneumonia, after his fourth administration of infliximab. *Campylobacter jejuni* was isolated from his blood culture at that time. He was successfully treated with antibiotics and infliximab administration was resumed after confirming resolution of pneumonia. He has been treated safely for more than 2 years with regular administrations of infliximab (once every 7–8 weeks). Neither mycobacterial infections nor severe infusion reactions have been observed.

Discussion

Our patient showed immunodeficiency with very low IFNγ-production during his younger years as shown in Table I and suffered from many opportunistic infections (Zoster virus infection, penicillin-resistant *Streptococcus pneumoniae* meningitis, and other undetermined infections). A novel missense mutation, A169P, in the first coiled-coil domain resulted in defective NEMO function (Fig. 1) and was responsible for recurrent severe infections. However, he later suffered from autoimmune diseases at 4 years of age (bronchiolitis obliterans organizing pneumonia, severe arthritis, and vasculitis) and severe chronic inflammatory colitis at 8 years. Based on the facts that, in the mice model, TNFα played a major role in the pathogenesis of NEMO colitis [15], and that, in our patient, TNFα-producing mononuclear cells in the peripheral blood were markedly increased (Fig. 4a), infliximab was employed for the patient's treatment. This treatment led to improvement in his symptoms and colonoscopic findings for 2 years.

Increases in TNFα-producing cells similar to that seen in other IBD [19] were detected (Fig. 4). We confirmed G/C



TNF+/CD4T(%)	72.6	26.7	38.0	45.7	52.4	32.7
TNF+/CD8T(%)	48.5	23.1	27.5	37.7	44.4	N.D.

Fig. 6 Clinical course of NEMO colitis after infliximab treatment in our patient. Colonoscopy (*arrows*), administrations of infliximab (*arrowheads*), and other immunosuppressive drugs (*bars*) are indicated. Daily frequency of stools (times) is graphed at the *center*. Changes in the proportion of TNFα-producing T cells in CD4-positive and CD8-positive cells are also indicated at the *bottom*

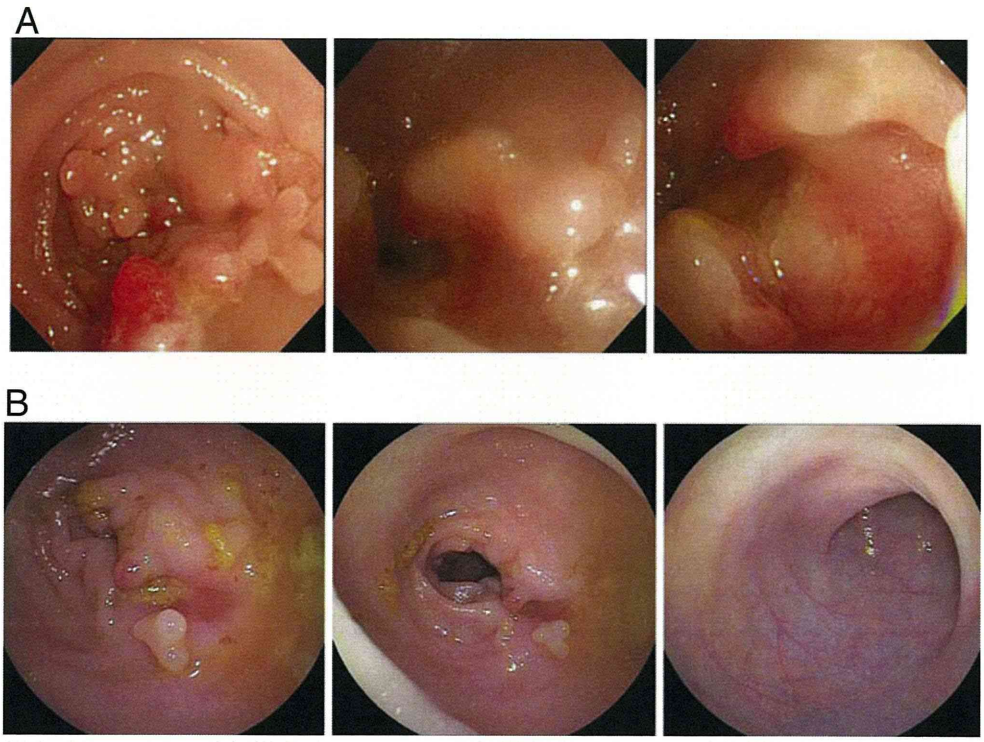


Fig. 7 Findings of colonoscopy after infliximab treatment. **a** Colonoscopy performed after the third infliximab treatment. Mild improvement was observed. Both mucosal redness and edema decreased. However, polyp-like lesions remained. At this point, the patient showed neither abdominal pain nor watery diarrhea. **b** Colonoscopy after 1-year treatment. Almost no mucosal redness or edema. A clear vascular pattern was also observed. Inflammatory polyps could still be found

reversion in T cells after co-stimulation with PHA and IL-2 before and even after infliximab therapy (Table III and Fig. 5). Reversion mosaicism has been reported in primary immunodeficiencies such as X-linked severe combined immunodeficiency [20, 21], adenosine deaminase deficiency [22], *RAG1* deficiency [23], and Wiskott–Aldrich syndrome [24]. Most of these patients reduced the frequency of severe infections and showed survival for longer periods. Our patient also had very few episodes of severe infection after expansion of IFNγ-producing peripheral blood mononuclear cells, contrary to increased susceptibility to diverse pathogens in X-EDA-ID [5, 25]. However, none of the patients with reversion mosaicism involving reverted T cells developed IBD other than X-EDA-ID. Our patient and patients with Omenn's syndrome [21, 23] developed systemic inflammatory conditions and exhibited a restricted TCR repertoire. In our patient, oligoclonal expansion of reverted T cells caused impairment of immune regulation.

According to the report by Nenci et al. in a murine model of intestinal epithelium-specific NEMO deficiency, intestinal epithelial cells exhibit increased sensitivity to TNFα-induced apoptosis and cause disruption of the epithelial barrier if mucosal immune cells have normal immune functions and produce proinflammatory cytokines [15]. They also showed that an additional TNF receptor-1 knockout ameliorated this intestinal inflammation [15]. The pathogenesis of severe colitis in the mouse model seems to be similar to that of our patient. Specifically, NEMO-deficient intestinal epithelium was damaged by TNFα produced from both T cells and macrophages in the lamina propria (shown in Fig. 3c–e, h), and anti-TNFα antibody suppressed progression of intestinal inflammation. Although reversion in peripheral blood monocytes was not confirmed after culture with GM-CSF and analysis of TNFα expression after LPS stimulation, submucosal and peripheral macrophages produced a fair amount of TNFα detectable by immunohistochemistry and flow cytometry (Figs. 3 and 4). Production of TNFα from lamina propria macrophages may be augmented by IFNγ released from reverted T cells.

In addition to the amelioration of clinical symptoms and colonic mucosal inflammation, in our patient, TNF blockade therapy restored his dry skin with thick epidermis to moderately moist skin of normal thickness. Nenci et al. described in another paper using the epidermis-specific NEMO-deficient mice that mice showed severe skin inflammation with thick epidermis and predominant infiltration of inflammatory cells and showed further that an additional knockout of TNFR1 suppressed the inflammatory condition [26]. We postulate that TNFα is also a key cytokine in the pathogenesis of inflammation in diverse epithelial tissues and that infliximab treatment suppresses the TNFα-mediated inflammatory response by inducing apoptosis of TNFα-producing cells [27]. In fact, the patient's peripheral blood TNFα-producing cells reduced along with the improvement of clinical symptoms, and this reduction provided an available marker to assess inflammatory status. Reverted cells in peripheral blood also decreased after repeated anti-TNFα antibody administrations. Unfortunately, we could not obtain consent for re-biopsy so we could not

confirm a vulnerability for apoptosis of intestinal epithelium and lamina propria after the treatment.

Since patients with X-EDA-ID were well known to have increased susceptibility to mycobacterium, and in addition, anti-TNFα monoclonal antibody indeed caused infection-related deaths in a few patients with inflammatory colitis associated with primary immunodeficiencies [28–30], the side effects of anti-TNFα monoclonal antibody treatment should be paid attention to, especially, mycobacterial infections. Before infliximab treatment, we confirmed the absence of active mycobacterial infections by culture tests for mycobacterium including atypical mycobacteria, laboratory examinations, and chest radiographs. He also has no history of Bacillus Calmette-Guérin immunization. Although the patient experienced bacterial pneumonia after his third infliximab infusion, he has not suffered from severe infections for several years. This may be because of the patient's mosaicism of mutated and reverted cells. The risks concerning severe infections and oncogenic effects [31–33] should be considered before employing infliximab for NEMO colitis.

Conclusion

Reversion of mutation in T cells contributes to the pathogenesis of mucosal immunity in NEMO-deficient patients. Moreover, treatment with anti-TNFα monoclonal antibody therapy can improve the symptoms of the disease by both preventing exposure of the mucosa to TNFα and reducing the number of T cells carrying the reverted gene. Anti-TNFα monoclonal antibody therapy provides a promising treatment for intractable NEMO colitis.

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Conflict of Interests The authors declare no competing financial interests.

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LETTERS TO THE EDITOR

Interleukin 12 and myeloperoxidase (MPO) in Vietnamese children with acute respiratory distress syndrome due to Avian influenza (H5N1) infection*

Dear Editor,

We read with interest the paper by HO and colleagues concerning prognostic factors for fatal adult influenza.[1] A previous study in Vietnam in 2004 also focused on severe disease in H5N1 infected individuals.[2] Leukopenia and thrombocytopenia were common findings in all studies; these are prognostic indicators for acute respiratory distress syndrome (ARDS) and death.[2,3] When the influenza A virus infects epithelial cells, it replicates and infects other cells; macrophages and leukocytes respond to the infection by producing proinflammatory chemokines and other immunoregulatory cytokines.[4] High levels of oxidized phospholipids injure the air space of lungs in H5N1 virus-infected patients.[5]

We examined the levels of cytokines, chemokines and MPO activity in plasma and nasopharyngeal aspirate (NPA) of patients with ARDS (age >1 month) in 3 patients with H5N1 influenza infection compared with 31 patients with non-H5N1 influenza infection who were admitted to the NHP from January 2008 to December 2009 (Table 1). In addition, we determined the correlation of these data with clinical parameters.

Plasma levels of IL-12p40 and TNF-R2 in the H5N1-positive group were significantly higher in the present study (Table 1). Moreover, levels of TNF-α, IL-6, IL-12p70 and sIL-6R in the H5N1-positive group were slightly increased. Our results suggest that IL-12p40, TNF-R2 and other inflammatory mediators may be released from alveolar epithelial or endothelial cells to circulate in the blood. MCP-1 slightly increased in the H5N1-positive group and IL-8 strongly

Table 1 Levels of cytokines, chemokines and MPO activity in plasma and NPA of pediatric patients with ARDS infected with H5N1 influenza.

	Plasma			NPA			NPA	
Cytokines	H5N1-negative (n = 31)	H5N1-positive (n = 3)	P-value	H5N1-negative (n = 31)	H5N1-positive (n = 3)	P-value	H1N1-positive (n = 9)	P-value
Chemokines	Mean value ± SD (pg/ml)	Mean value ± SD (pg/ml)		Mean value ± SD (pg/ml)	Mean value ± SD (pg/ml)		Mean value ± SD (pg/ml)	
TNF-α	56.4 ± 237.6	81.8 ± 171.3	0.858	5.9 ± 19.1	0.0 ± 0.0	0.668	240.4 ± 479.6	0.01
IL-1β	10.8 ± 23.5	8.0 ± 17.0	0.842	56.4 ± 161.6	16.3 ± 0.4	0.732	349.7 ± 669.4	0.03
IL-6	48.4 ± 66.1	117.9 ± 18.1	0.097	34.2 ± 79.1	33.9 ± 23.3	0.996	291.4 ± 393.9	0
MCP-1	125.3 ± 362.9	303.1 ± 524.9	0.440	134.6 ± 412.3	73.2 ± 103.5	0.837	116.4 ± 170.9	0.9
IFN-γ	1.5 ± 8.3	ND	–	3.34 ± 14.6	ND	–	ND	–
IL-8	122.1 ± 390.8	3.6 ± 6.3	0.608	1201.5 ± 1556.0	244.1 ± 191.7	0.398	807.8 ± 1130.2	0.48
IL-12p40	813.1 ± 786.2	2416.9 ± 1080.6	0.002	210.7 ± 175.1	252.1 ± 28.9	0.744	ND	–
IL-12p70	91.5 ± 117.8	181.7 ± 100.3	0.211	15.6 ± 15.5	18.6 ± 10.7	0.794	ND	–
TNF-R2	9813.8 ± 2983.7	21796.7 ± 285.6	0.001	83.8 ± 92.3	149.6 ± 68.1	0.332	863.7 ± 1408.3	0
sIL-6R	26728.8 ± 7945.1	28520.6 ± 903.7	0.709	52.8 ± 88.2	345.8 ± 217.3	0.0002	103.8 ± 135.2	0.19
MPO	units/min/ml 6.6 ± 5.8	units/min/ml 24.1 ± 17.4	0	units/min/ml 3.0 ± 3.2	units/min/ml 2.8 ± 1.0	0.708	units/min/ml 3.6 ± 6.3	0.042

Cytokines and chemokines were measured by an enzyme-linked immunosorbent assay (ELISA) and MPO by the TMB (3,3′, 5,5′-tetramethylbenzidine) method (modified from Analytical Biochemistry 1983; 132:345–52.); this method was introduced to the National Hospital of Pediatrics (NHP) in Hanoi.
ND: Not detectable.

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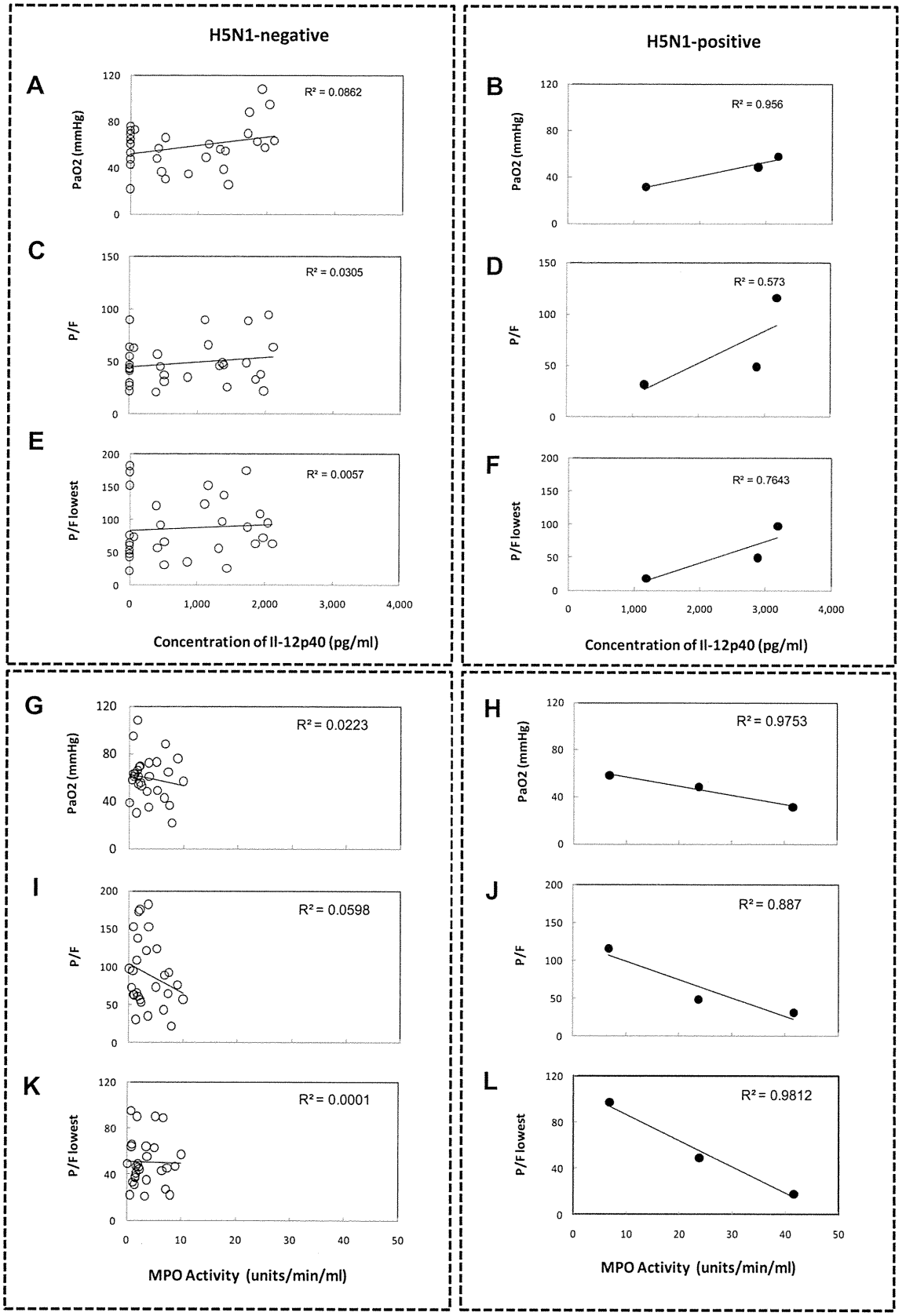

Figure 1 Significant correlation of IL-12p40 level and MPO activity in plasma with clinical parameters of H5N1-positive and H5N1-negative ARDS. Correlation of the IL-12p40 level in plasma with clinical parameters in H5N1-positive (black dot) and H5N1-negative groups (white dot): $PaCO_2$ (A, B), P/F (C. D) and P/F lowest (E, F). Correlation of MPO activity in plasma with clinical parameters in H5N1-positive (black dot) and H5N1-negative groups (white dot): $PaCO_2$ (G, H), P/F (I. J) and P/F lowest (K, L).

increased in the H5N1-negative group, suggesting the contribution of macrophages to lung injury in the initial stage of H5N1 infection. This association of MCP-1 may be particularly relevant to the development of ARDS.[6] Furthermore, a slight increase in IFN-γ in the plasma of H5N1-negative patients, undetected in the H5N1-positive group, may be due to sampling at the early stage of infection.

On the other hand, in NPA, the level of sIL-6R, but not TNF-α, in the H5N1-positive group was significantly higher in the present study. However, in nasal lavage fluid, levels not only of IL-6 but also of TNF-α increase with influenza A virus infection.[6] Our results suggest that sIL-6R in particular may contribute to the nasal space associated with lung injury. In addition, NPA concentrations of the chemokines MCP-1 and IL-8 in the H5N1-negative group were slightly increased, confirming IL-8 in adult ARDS[7] and neutrophil predominance in influenza infection.[1] Morbidity and mortality of influenza infection might have been overlooked in the immunocompetent population.[1]

Furthermore, MPO activity increased much more in plasma than in NPA of patients in both groups, but MPO activity in plasma of H5N1-positive patients was significantly higher in our study. A significant increase in serum MPO levels has been reported in patients with ARDS, including those with no myonecrosis evident at initial presentation.[8] MPO in neutrophils kills influenza virus *in vitro*[9] and, released from neutrophils which are activated by the H5N1 virus in the epithelial cells in lung, it could then damage lung tissue.[5]

Furthermore, we determined that there were positive relationships between IL-12p40 and MPO levels in plasma and PaO_2, P/F and P/F lowest in ARDS patients in the H5N1-positive group (Fig. 1). Multivariate analysis assigns a PaO_2/FiO_2 ratio value of 150 as a marker for mortality, which may be more useful in clinical analysis. H5N1 infection results in a cytokine storm in fatalities among young healthy adults.[10] These levels of inflammatory mediators may be responsible in part for the sepsis syndrome, ARDS, and multiorgan failure.[6] The positive correlations shown in the present study strongly suggest that IL-12p40 and MPO may play key roles in acute lung injury, resulting in respiratory dysfunction in H5N1 infection.

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