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Letter to the Editor

Interleukin 2 receptor- α expression after lymphocyte stimulation for non-IgE-mediated gastrointestinal food allergies

Dear Editor,

Non-IgE-mediated gastrointestinal food allergies (non-IgE-GI-FAs) are often found in neonates and infants and are mainly caused by cow's milk (CM). Some cases show nonspecific symptoms, such as fever, poor sucking, and poor weight gain, but with minor gastrointestinal (GI) symptoms. Additionally, approximately 10% of patients develop severe complications, such as mechanical ileus, perforation of the GI wall, shock, and developmental retardation, owing to delayed start of treatment.¹ Oral food challenge (OFC) is necessary for definitive diagnosis, but may induce severe symptoms; accordingly, OFC should not be performed until patients are healthy enough, resulting in delayed diagnosis.

The pathology of non-IgE-GI-FAs involves antigen-presenting cells, allergen-specific lymphocytes, and eosinophils, although the specific mechanisms are still unclear. The lymphocyte stimulation test (LST), which examines the reactivity of T lymphocytes to specific allergens *in vitro*, facilitates the diagnosis of non-IgE-GI-FAs^{2,3} because LSTs can be performed irrespective of the patient's condition, thereby permitting early diagnosis. However, in patients with drug allergies, LSTs should not be performed until 4 weeks after an allergic reaction to prevent false-negative results.⁴

LSTs require substantial amounts of fresh peripheral blood and lengthy culture times (5–7 days). Alternative tests that require small amounts of blood and shorter experimental times are urgently needed. In LSTs for non-IgE-dependent drug allergies, T cells increase the expression of CD25 (interleukin-2 receptor- α [IL2RA]) on their surface within several hours after activation by antigen recognition and costimulation.⁵ Subsequently, the cells proliferate and differentiate into effector and memory cells. IL2RA expression has been reported in *in vitro* and *in vivo* testing and is upregulated on natural killer (NK) cells after a positive food challenge in food protein-induced enterocolitis syndrome.⁶ Thus, we hypothesized that IL2RA mRNA may be increased early in allergen-stimulated lymphocytes from patients with non-IgE-GI-FAs and that monitoring of such an increase may be an alternative to conventional LSTs for evaluation of cell proliferation.

Patients with non-IgE-GI-FAs to CM who were treated at our hospital from June 1, 2011 to July 31, 2018 were recruited after obtaining informed consent from parents or guardians. Controls who did not have non-IgE-GI-FAs or other immune diseases were also recruited. Non-IgE-GI-FAs were diagnosed based on the criteria proposed by the Japanese Pediatric Guidelines for Food Allergy,⁷ modified from those proposed by Powell.⁸ Blood

specimens were collected at onset or before OFC. Peripheral blood mononuclear cells (PBMCs) from patients and controls were incubated with α -casein, κ -casein, α -lactalbumin, β -lactoglobulin, a mixture of all four components (Pmix), or CM for 24 h, and IL2RA expression was assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The same sets of samples were also subjected to conventional LSTs (Supplementary Methods).

The median age (interquartile ranges) of 33 participants (19 boys and 14 girls) was 330 days (97.5–1275 days). Sixteen patients had non-IgE-GI-FAs (Supplementary Table 1), and 17 patients were controls. The median ages (interquartile ranges) of the patients and controls were 112.5 (61.3–292.5) days and 760 (287.5–1793) days, respectively. We first addressed the diagnostic significance of early IL2RA mRNA expression. All antigens significantly increased IL2RA mRNA expression in patients' PBMCs compared with that in controls' (Table 1, Fig. 1A). The area under the curve (AUC) of the receiver operating characteristic (ROC) curve of IL2RA mRNA expression after 24-h stimulation with all antigens was above 0.7, supporting the diagnostic value of this analysis. Notably, α -casein showed the largest AUC (0.9596; $p < 0.0001$; Table 1, Fig. 1B). These AUCs were comparable to those of LSTs (AUC: 0.846, $p = 0.0007$, α -casein; Supplementary Table 2). Additionally, after stimulation with a control antigen, ovomucoid, no difference was found between non-IgE-GI-FAs and disease controls, indicating the antigen specificity of the results (four non-IgE-GI-FAs to CM, six disease controls, $p = 0.3524$, Supplementary Fig. 1). Since the small amount of LPS that is present in milk proteins affected the results of LST,⁹ we examined the influence of LPS on IL2RA mRNA expression. We found that stimulation with LPS (1 μ g/mL and 100 pg/mL) did not affect IL2RA mRNA expression (data not shown). Furthermore, subpopulation analysis after stimulation with milk allergens showed no difference in IL2RA mRNA expression between the patients who fulfilled diagnostic criteria for food protein-induced enterocolitis syndrome (FPIES)¹⁰ and those who did not (Supplementary Fig. 2).

Next, we assessed the correlation of IL2RA mRNA expression to the results of conventional LSTs. We found weak but significant correlations for all antigens, particularly α casein ($r = 0.5144$; $p = 0.0022$), κ -casein ($r = 0.5791$; $p = 0.0008$), and Pmix ($r = 0.5833$; $p = 0.0044$) using Spearman correlations (Supplementary Fig. 3). AUC showed better prediction for IL2RA mRNA assay of α -casein (0.8444, $p = 0.0008$) and Pmix (0.9294, $p = 0.0042$) to LST assays compared with the other antigens (Supplementary Fig. 4). These results indicated that IL2RA mRNA expression levels were correlated with conventional LSTs.

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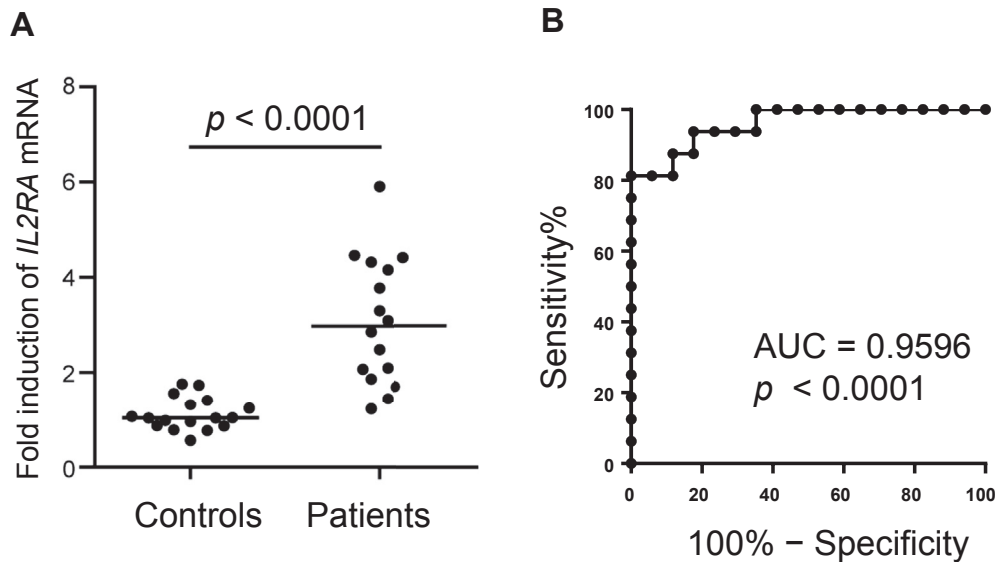
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Table 1
IL2RA mRNA levels and ROC curve analysis in patients and controls.

	n	Fold induction of <i>IL2RA</i> mRNA [†]			ROC curve				
		Patients with non-IgE-GI-FAs	Controls	<i>p</i> value*	AUC	<i>p</i> value	Cut off	Sensitivity (%)	Specificity (%)
α -Casein	33	3.061 (1.969–4.403)	1.066 (0.896–1.404)	<0.0001	0.9596	<0.0001	1.68	87.5	88.2
κ -Casein	33	2.640 (1.300–4.131)	1.218 (0.947–1.405)	0.0003	0.8493	0.0006	1.46	75.0	82.4
α -Lactalbumin	33	4.015 (2.973–5.808)	2.770 (1.787–3.704)	0.0061	0.7757	0.0069	3.21	75.0	70.6
β -Lactoglobulin	33	5.675 (4.645–7.005)	3.223 (2.312–3.725)	0.0009	0.8272	0.0013	3.97	87.5	82.4
Pmix	33	6.401 (4.360–10.29)	3.081 (1.769–5.573)	0.0042	0.7868	0.0050	4.32	81.3	70.6
Cow's milk	30	4.411 (3.225–7.796)	3.243 (1.575–3.875)	0.0177	0.7522	0.0188	3.60	71.4	68.8

Data are expressed as medians (interquartile ranges).

* Mann–Whitney test.

[†] Fold induction of *IL2RA* mRNA was calculated by dividing stimulated samples with nonstimulated samples after normalization to 18S rRNA.**Fig. 1.** Comparison of *IL2RA* mRNA levels between patients and controls. **A**, Fold induction of *IL2RA* mRNA was calculated by dividing α -casein-stimulated samples with nonstimulated samples after normalization to 18S rRNA. Each dot represents data from a single participant. Bars indicate median values. Mann–Whitney tests were used for comparisons. **B**, ROC curves for *IL2RA* mRNA expression levels from patients with non-IgE-GI-FAs and controls.

Our results showed that *IL2RA* mRNA expression was increased after 24-h stimulation of PBMCs in a disease-specific manner, consistent with the results of conventional LSTs using the same set of samples. Thus, an antigen-specific early increase in the levels of *IL2RA* mRNA could reflect antigen-specific lymphocyte proliferative responses. The test of *IL2RA* expression does not require a lengthy culture time, whereas LSTs usually require 5–7 days. Furthermore, using our current test, after stimulation, cells do not have to be kept alive because the test uses mRNA. Thus, our method could be a useful, rapid complementary test for non-IgE-GI-FAs. We designated this method the instant peripheral blood allergen stimulation test (iPAST); the iPAST includes 24-h stimulation of lymphocytes with a specific allergen and analysis of mRNA levels. Further studies are needed to determine whether other genes or a combination of genes may be superior to *IL2RA* in this assay. We are currently using genome-wide analyses to identify alternative gene candidates.

Notably, α -casein showed the best diagnostic accuracy in this study. Caseins consisting of four types of proteins (α S1-, α S2-, β -, and κ -) account for approximately 80% of milk proteins. Caseins are major allergens that cause immediate-type milk allergies, and casein-specific IgE is found in most patients; hence, these proteins are considered highly immunogenic. The results of our study

suggested that casein may be a major allergen in non-IgE-GI-FAs as well.

There were some limitations to this study. First, the study included a small number of patients, and the age differed between non-IgE-GI-FAs patients and controls. Notably, because non-IgE-GI-FAs develop mostly in young children, it is difficult to obtain samples from age-matched controls owing to ethical constraints and difficulties in obtaining consent. Additionally, we did not investigate which cells expressed *IL2RA* mRNA. Thus, we examined the presence of NK cells and neutrophils in PBMCs based on a recent study showing *IL2RA* expression in these cells in non-IgE-GI-FAs patients.⁶ We evaluated the expression of an NK cell-specific gene, natural cytotoxicity receptor 1 (*NCR1*), and a neutrophil-specific gene, cathepsin G (*CTSG*) in PBMCs (Supplementary Fig. 5). *NCR1* mRNA was detected in PBMCs from controls and patients (Supplementary Fig. 5A), suggesting that NK cells may promote *IL2RA* expression, as previously shown.⁶ In contrast, *CTSG* was barely detected in PBMCs from controls but faintly detected in that from patients (Supplementary Fig. 5B). This suggested contamination of neutrophils in patient-derived PBMCs, which could be attributable to a decrease in neutrophil density caused by inflammation. Accordingly, neutrophils may be a source of *IL2RA* expression in patients, as described previously.⁶ Further

studies are needed to elucidate the detailed mechanisms underlying the *in vitro* expression of *IL2RA* mRNA.

In conclusion, iPAST may be useful for the supplementary diagnosis of non-IgE-GI-FAs as an alternative to LSTs owing to its high diagnostic value, rapid analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.11.003>.

Conflict of interest

The authors have no conflict of interest to declare.

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SUPPLEMENTARY MATERIALS

Supplementary Methods

Diagnosis

Non-IgE-GI-FAs were diagnosed based on the following criteria proposed by the Japanese Pediatric Guidelines for Food Allergy,¹ modified from those proposed by Powell:² (i) development of GI symptoms after ingestion of causative foods; (ii) disappearance of symptoms after discontinuation of causative foods; (iii) exclusion of other disorders that could cause GI symptoms, such as infection or surgical problems; and (iv) recurrence of GI symptoms during OFC or re-administration. FPIES was diagnosed based on the criteria proposed by the 2017 International Consensus Guidelines.³

Isolation of PBMCs

PBMCs were isolated from heparinized peripheral blood using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA). Heparinized blood (3 mL) was diluted with 12 mL phosphate-buffered saline, and 5 mL each of diluted blood sample was layered over 4 mL of Ficoll-Paque. After centrifugation at $2300 \times g$ for 20 min at 20°C, the top layer containing plasma was removed, and the PBMC layer was transferred to a sterile tube. PBMCs collected from 2 mL whole blood were used for conventional LSTs, and 1 mL was used for iPAST. The procedures yielded approximately 65–80% PBMCs. The iPAST required 1×10^5 PBMCs for a single stimulation; 1 mL of peripheral whole blood yielded 19–24 assays given that the average number of lymphocytes (e.g., 6000/ μ L).

Quantification of mRNA levels

PBMCs (1×10^5) were incubated in AIM-V Medium with CM (100 $\mu\text{g/mL}$; Morinaga Milk, Tokyo, Japan), four different milk protein components (α -casein, κ -casein, α -lactalbumin, or β -lactoglobulin 100 $\mu\text{g/mL}$ each; Sigma-Aldrich, St. Louis, MO, USA), a mixture of these four proteins (Pmix; 100 $\mu\text{g/mL}$), phosphate-buffered saline, OVM (100 $\mu\text{g/mL}$; Sigma-Aldrich) or LPS (1 $\mu\text{g/mL}$, 100 pg/mL ; Sigma-Aldrich), at 37°C for 24 h. RNA was extracted from PMBCs using Ambion's ToTALLY RNA or TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized from 150 μg total RNA in a final volume of 20 μL using SuperScript VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer's protocol. qPCR using Taq Man primers was performed in a 20- μL reaction volume containing 1 μL cDNA, 7.5 μL RNase-free water, 10 μL Applied Biosystems TaqMan Gene Expression Master Mix, and 1 μL Taq Man primers according to the protocol for TaqMan Gene Expression Master mix (PN 4371135; Thermo Fisher Scientific). After incubation for 2 min at 50°C and 10 min enzyme activation at 95°C, 45 cycles of amplification were run at 95°C. Each cycle included denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. *IL2RA* (Assay ID: Hs00907779_m1; Thermo Fisher Scientific), *CTSG* (Assay ID: Hs00175195_m1; Thermo Fisher Scientific), and 18S rRNA (Assay ID: Hs99999901_s1; Thermo Fisher Scientific) as a control were analyzed using TaqMan Gene Expression Assays. For *NCR1* expression, qPCR was performed in a 20- μL reaction volume containing 0.5 μL cDNA, 0.5 μL Tris/EDTA, 7.4 μL RNase-free water, 10 μL KAPA Sybr Fast qPCR Master Mix (2 $\times$) (Kapa Biosystems, Wilmington MA USA), and 0.8 μL each of a 20 μM forward primer and a reverse primer. The reaction was performed according to the protocol for KAPA Sybr Fast qPCR Master Mix (2 $\times$): 3 min enzyme activation at 95°C, 40 cycles of amplification were run at 95°C. Each cycle included denaturation at 95°C for 10 s

and annealing/extension at 60°C for 1 min. The specific primers were used as follows, *NCRI*: 5'-TCTAGACGGCAGTAGAAGGTC-3' and 5'-CTTGCTGGATCTGGTGGTAA-3'; 18S rRNA: 5'-GATATGCTCATGTGGTGTG-3' and 5'-AATCTTCTTCAGTCGCTCCA-3'. Expression levels of mRNA for each gene were normalized to those of 18S rRNA expression levels. For *IL2RA* expression, fold induction of 18S rRNA-normalized *IL2RA* in antigen-stimulated samples to that in nonstimulated samples was determined and indicated as fold induction of *IL2RA* expression.

LST

LSTs were conducted as previously described,⁴ with some modifications. Briefly, PBMCs from each patient were incubated separately with CM, each of four different milk protein components (α -casein, κ -casein, α -lactalbumin, and β -lactoglobulin), or Pmix (100 μ g/mL) for 5 days. Proliferating cells were then labeled by further incubation with 100 μ M of 5-bromo-2'-deoxyuridine (BrdU) for 6 h, and incorporated BrdU contents were determined by enzyme-linked immunosorbent assays. The results were described as the stimulation index (SI), which was calculated as BrdU contents in a stimulated sample divided by those in an unstimulated sample.

Statistical analysis

Correlations were tested by Spearman correlation coefficients, and levels of *IL2RA* mRNA were evaluated by Mann-Whitney tests. Cut-off levels and diagnostic accuracy were assessed with ROC curve analysis using GraphPad Prism version 7 for Windows (GraphPad Inc., San Diego, CA, USA) The results were statistically significant if *p* values were less than 0.05.

Ethics

This study was conducted after receiving approval from the Ethics Committee of Gunma University (1507).

Supplementary Tables

Supplementary Table 1: Demographic characteristics and laboratory findings

	FPIES								Non-FPIES							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Age (days)	65	105	150	1215	2035	330	180	120	90	66	45	37	15	120	60	371
Sex	M	M	F	F	M	M	F	F	F	F	M	M	M	F	M	F
Gestational age (weeks, days)	41, 1	38, 0	41, 3	37, 1	36, 6	37, 6	38, 3	37, 5	39, 0	N.D	38, 0	37, 1	39, 1	41, 0	41, 1	36, 4
Birth weight (g)	3484	2334	2860	2710	2726	3800	3136	2466	2718	2468	2130	2398	3286	2628	3474	2350
Age at onset (days)	30	8	129	2	1	6	120	0	60	30	10	33	4	18	30	40
Symptom at onset	V,B	V, D, PWG	V, B, PWG	V, D, Fe, PWG	V, D, Fe, PWG	V, B, D, Fe, PWG	V, B, D	V, B, D, PWG	V	B, D	B	B	V, D	B, D, PWG	B, PWG	B, D, PWG
WBC (/μL) at onset	11200	15000	12760	15100	20500	8100	8400	18100	11500	10600	9100	22000	9900	10400	7310	27300
Neutrophil (/μL) at onset	1534	5910	1590	7079	8220	2646	3192	9919	2040	3074	3203	3508	5148	1560	986	12558
Eosinophil (/μL) at onset	1209	540	110	30	2460	153	336	887	800	318	373	3175	198	1560	161	819
CRP (mg/dL) at onset	0.1>	0.23	0.23	1.7	0.1	0.44	0.8	0.1>	0.18	0.1>	0.1>	0.1>	0.13	0.25	0.1>	1.8
Total IgE (IU/mL) at onset	5.0	22	3.0>	3.0>	3.0>	3.0>	3.0>	55	10	3.0>	3.0>	3.0>	3.0>	3.0>	3.0>	169.2
Milk specific IgE (IU/mL) at onset	0.35>	1.35	0.61	0.35>	0.35>	0.35>	0.35>	1.9	1.04	0.35>	0.35>	0.35>	0.35>	0.35>	0.35>	0.35>

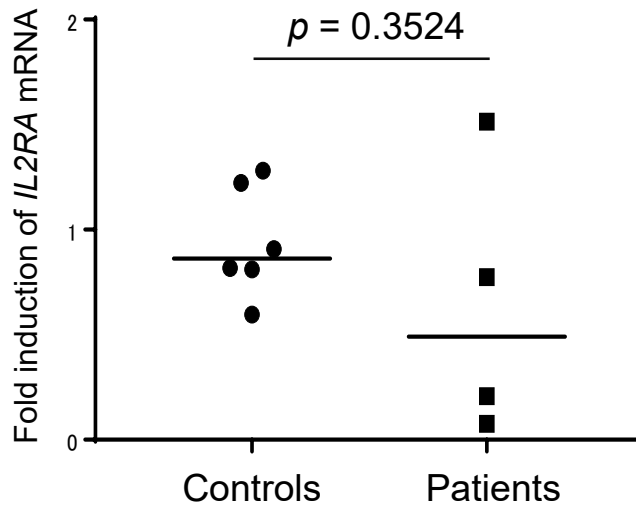
FPIES, food protein-induced enterocolitis syndrome; M, male; F, female; V, vomiting; B, bloody stool; D, diarrhea; Fe, fever; PWG, poor weight gain; WBC, white blood cell; CRP, C-reactive protein.

Poor weight gain was defined as a decrease of -0.5 standard deviations of weight.

Supplementary Table 2: ROC curve analysis of LSTs for patients with non-IgE-GI-FAs (n = 16) and controls (n = 17)

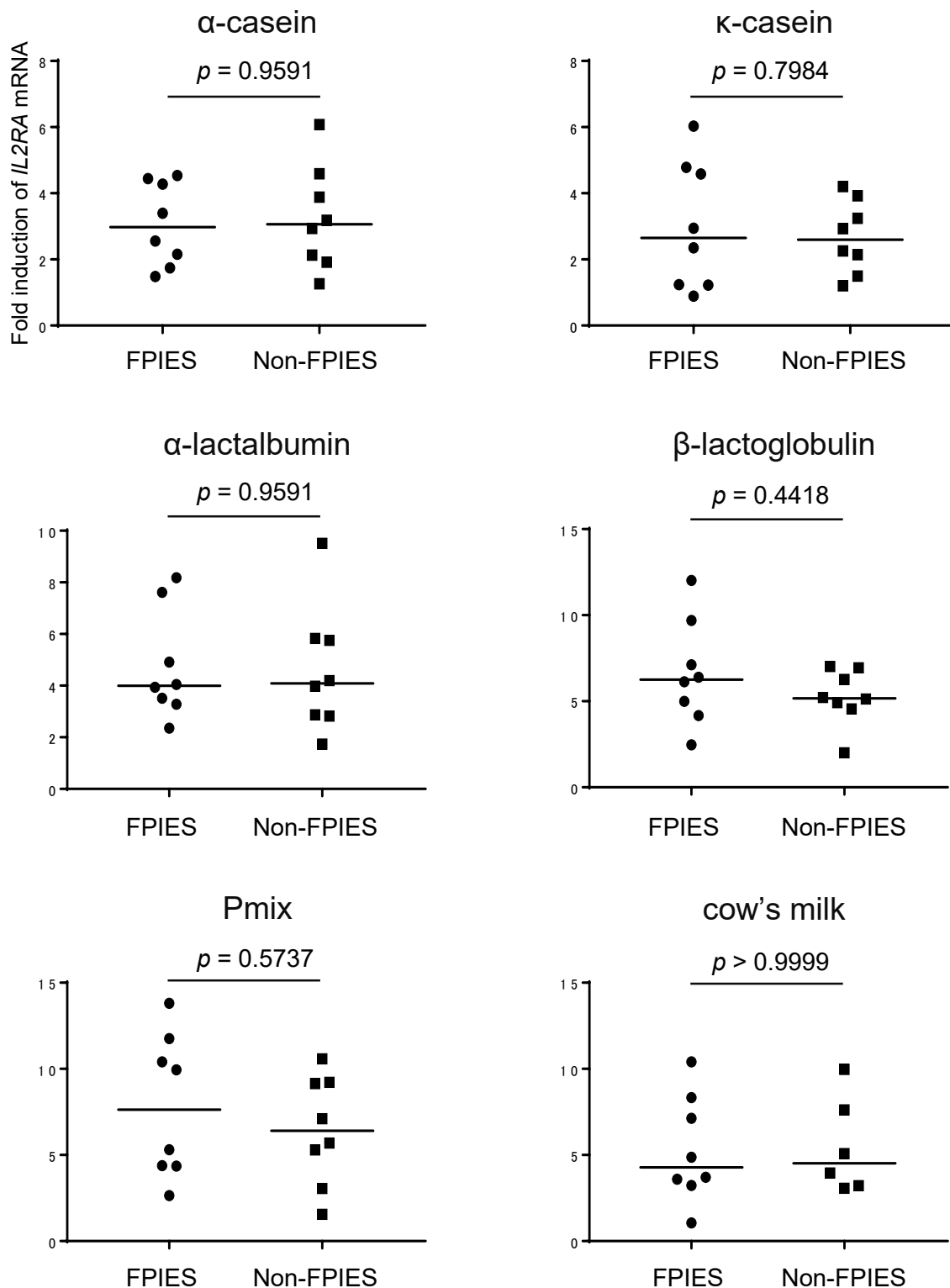
	ROC curve		
	AUC	<i>p</i> value	SI (Cut off)
α -Casein	0.846	0.0007	1.82
κ -Casein	0.857	0.0007	1.16
α -Lactalbumin	0.717	0.0336	1.60
β -Lactoglobulin	0.706	0.0437	1.99
Pmix	1.000	0.0009	7.38
Cow's milk	0.812	0.0027	5.65

SI, stimulation index



Supplementary Fig. 1 Comparison of *IL2RA* mRNA levels after stimulation with ovomucoid (OVM) between patients and controls.

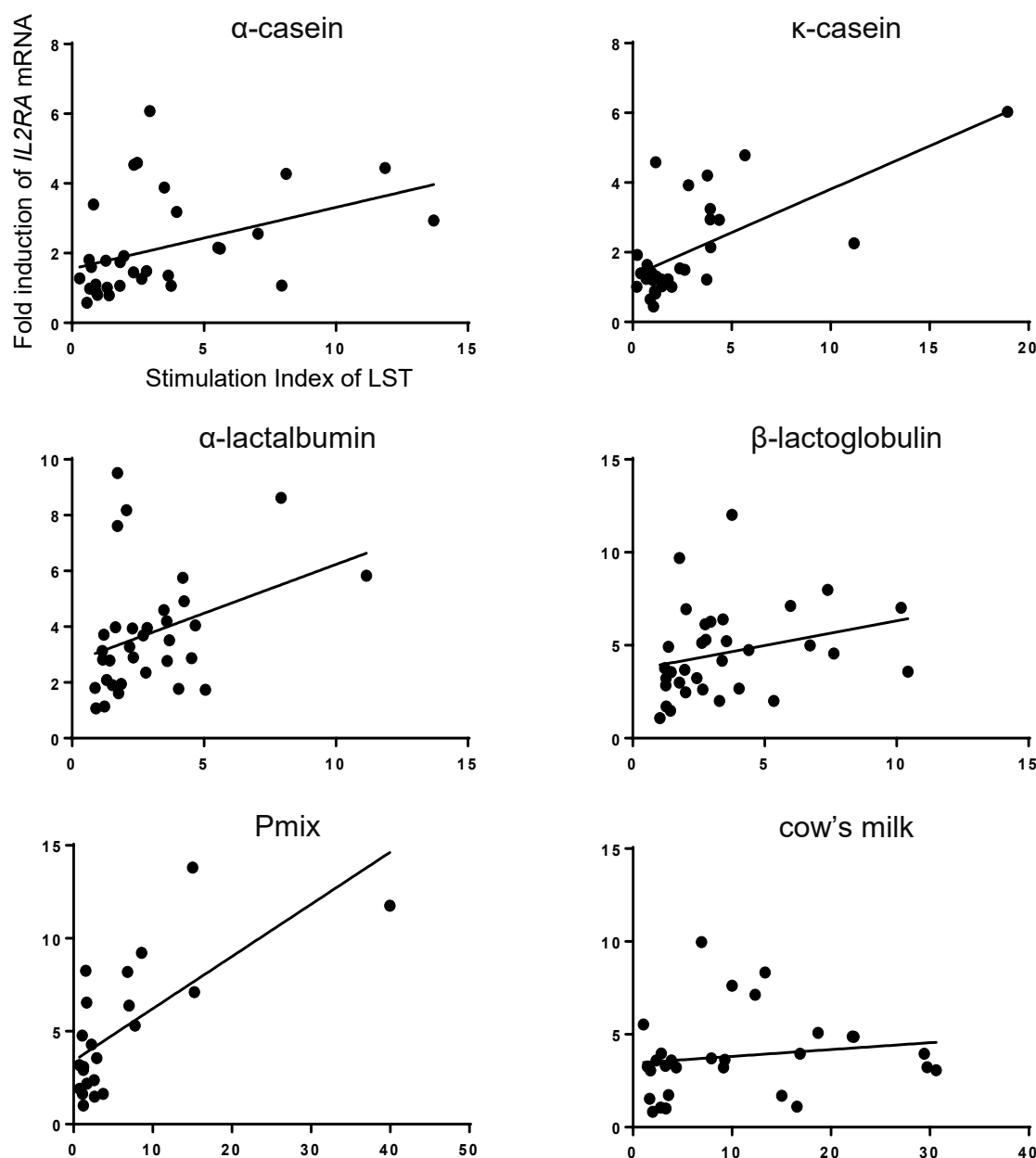
PBMCs were incubated with 100 $\mu\text{g/mL}$ OVM for 24 h, and *IL2RA* mRNA was determined with RT-PCR.



Supplementary Fig. 2 Comparison of *IL2RA* mRNA levels between PBMCs derived from patients with food protein-induced enterocolitis syndrome (FPIES) and patients without FPIES.

Each dot represents data from a single participant. Bars indicate median values. Mann-Whitney tests were used for comparisons.

A



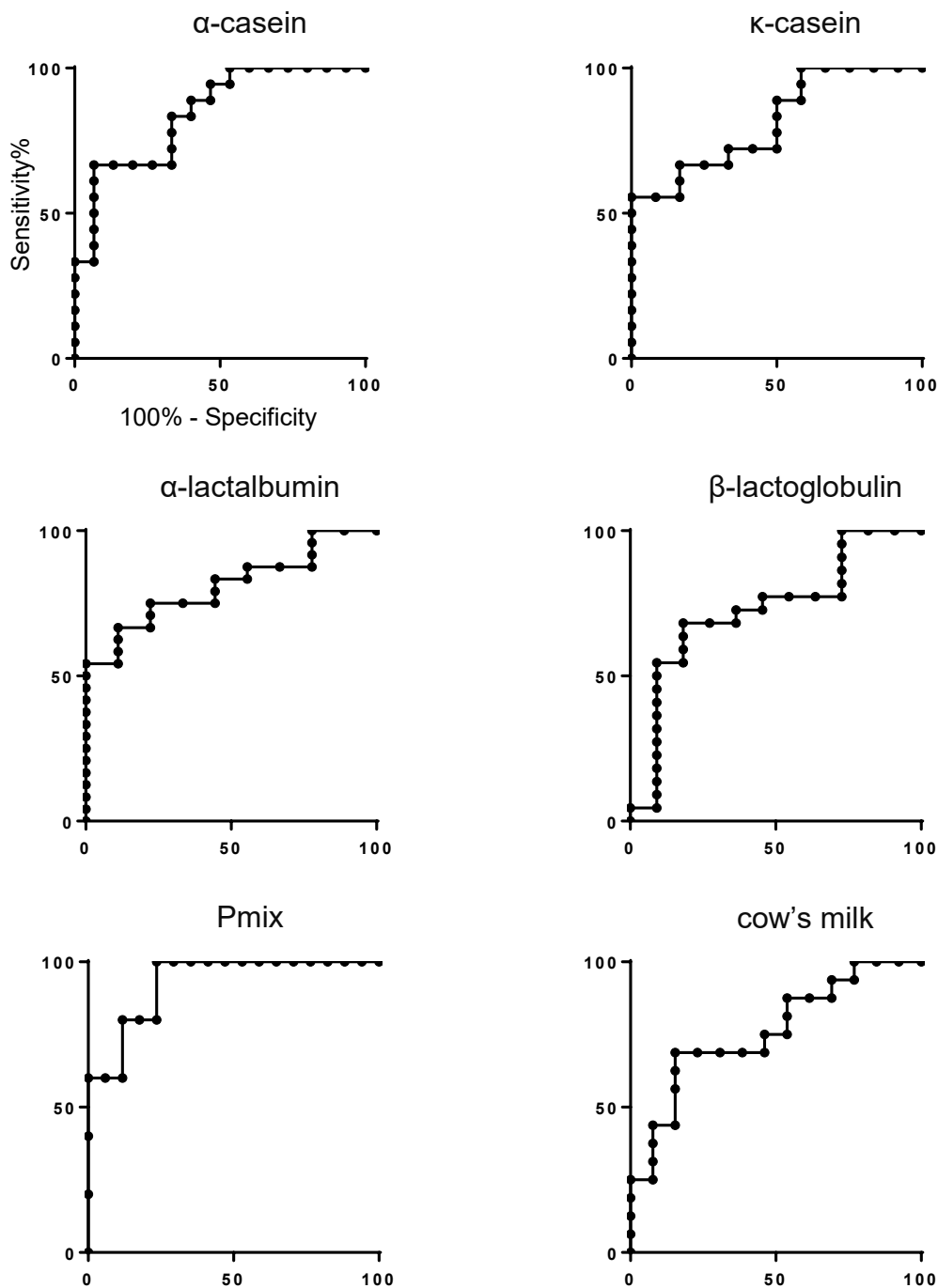
B

	n	r	p value
α-casein	33	0.5144	0.0022
κ-casein	30	0.5791	0.0008
α-lactalbumin	33	0.3777	0.0302
β-lactoglobulin	33	0.4248	0.0137
Pmix	22	0.5833	0.0044
cow's milk	29	0.2902	0.1268

Supplementary Fig. 3 Comparison of fold induction of *IL2RA* mRNA with stimulation index of conventional LSTs after milk proteins stimulation.

A, Spearman correlation analysis of *IL2RA* levels and stimulation indexes for LSTs with milk proteins. The results of *IL2RA* expression are described as fold induction of *IL2RA* mRNA expression by stimulation with the indicated milk proteins relative to that in unstimulated cells.

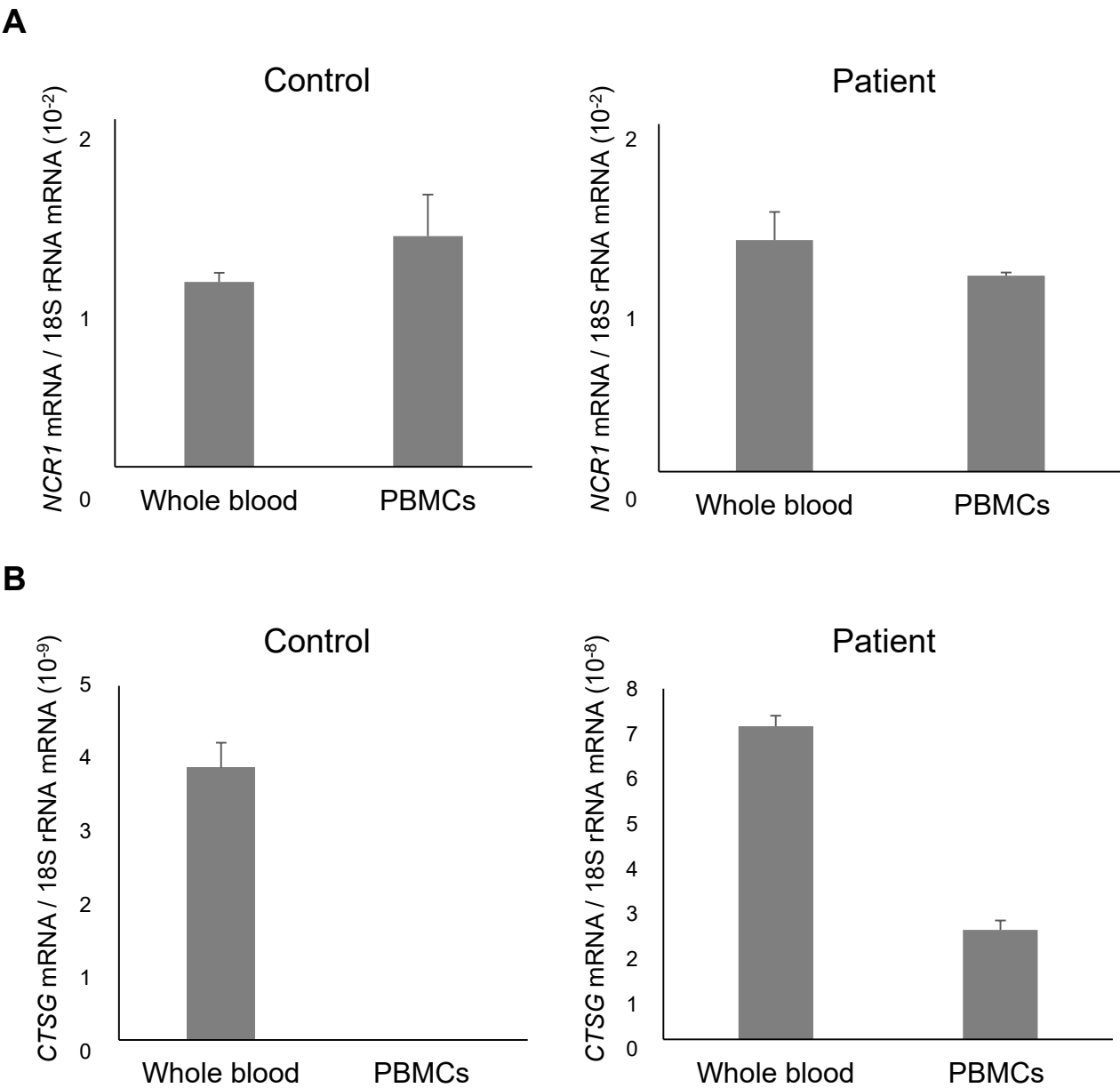
B, Summary of Spearman correlations.

A**B**

	n	AUC	<i>p</i> value
α -casein	33	0.8444	0.0008
κ -casein	30	0.8148	0.0040
α -lactalbumin	33	0.8102	0.0068
β -lactoglobulin	33	0.7273	0.0357
Pmix	22	0.9294	0.0042
cow's milk	29	0.7596	0.0179

Supplementary Fig. 4 ROC curve analysis of *IL2RA* expression in LSTs with milk proteins.

A, ROC curve analysis of *IL2RA* mRNA levels in LSTs after stimulation with the indicated milk proteins. **B**, ROC curve of *IL2RA* expression in LSTs using milk proteins.



Supplementary Fig. 5 *NCR1* and *CTSG* mRNA expression levels in whole blood and PBMCs.

Expression levels of an NK cell-specific gene, natural cytotoxicity receptor 1 (*NCR1*) (**A**), and a neutrophil-specific gene, cathepsin G (*CTSG*) (**B**), in a representative control and patient were determined with qRT-PCR.