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ORIGINAL CONTRIBUTION

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Expression of Leucine-rich Repeat-containing Protein 32 Following Lymphocyte Stimulation in Patients with Non-IgE-mediated Gastrointestinal Food Allergies

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The lymphocyte stimulation test (LST) facilitates the diagnosis of non-IgE-mediated gastrointestinal food allergies (non-IgE-GI-FAs). However, LSTs require large volumes of blood and prolonged culture durations. Recently, we found that *IL2RA* mRNA expression in peripheral blood mononuclear cells (PBMCs) of patients with non-IgE-GI-FAs increased after a 24 h stimulation with milk proteins. We designated this gene expression test as the instant peripheral blood allergen stimulation test (iPAST). In this study, we investigated whether other activated T cell-associated genes are superior to *IL2RA* in the iPAST for the supplementary diagnosis of non-IgE-GI-FAs. After incubating PBMCs with milk proteins for 24 h, the mRNA levels of three genes, *LRRC32*, *TNFRSF4*, and *CD69*, were assessed using quantitative RT-PCR. The diagnostic significance of the mRNA expression was evaluated by analyzing the receiver operating characteristic (ROC) curve. Upon stimulation with α-casein, κ-casein, α-lactalbumin, or a mixture of four milk protein components (Pmix), *LRRC32* expression in the PBMCs of 16 patients with non-IgE-GI-FAs was found to be higher than that in their 17 control counterparts, whereas *TNFRSF4* and *CD69* levels remained unaltered. Except for β-lactoglobulin and cow's milk (CM), the area under the ROC curve (AUC) for *LRRC32* mRNA expression upon stimulation was >0.7, which validated the diagnostic ability of this test. Notably, α-casein and Pmix had higher AUC scores of 0.820 and 0.842, respectively, than other antigens. iPAST assessed by *LRRC32* as well as *IL2RA* may be useful for the supplementary diagnosis of non-IgE-GI-FAs as an alternative to LSTs and provide insight into the pathogenesis of non-IgE-GI-FAs.

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Abbreviations: non-IgE-GI-FAs, non-IgE-mediated gastrointestinal food allergy; CM, cow's milk; FPIES, food protein-induced enterocolitis syndrome; FPIAP, food protein-induced allergic proctocolitis; FPE, food protein-induced enteropathy; GI, gastrointestinal; OFC, oral food challenge; LST, lymphocyte stimulation test; IL2RA, interleukin-2 receptor-α; iPAST, instant peripheral blood allergen stimulation test; PBMCs, peripheral blood mononuclear cells; LRRC32, leucine-rich repeat-containing protein 32; TNFRSF4, tumor necrosis factor receptor superfamily, member 4; qRT-PCR, quantitative reverse transcription polymerase chain reaction; BrdU, 5-bromo-2'-deoxyuridine; SI, stimulation index; ROC, receiver operating characteristic; AUC, area under the curve; GWAS, genome-wide association analysis.

Keywords: non-IgE-GI-FAs, LSTs, LRRC32, IL2RA, diagnosis, pathogenesis

INTRODUCTION

Non-IgE-mediated gastrointestinal food allergies (non-IgE-GI-FAs) are a type of food allergies that are primarily caused by the ingestion of cow's milk (CM) and is especially prevalent among neonates and infants. Non-IgE-GI-FAs include food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), and food protein-induced enteropathy (FPE) [1-3]. The number of neonates and infants with non-IgE-GI-FAs has been increasing rapidly in Japan since the late 1990s [4,5]. The global prevalence of FPIES is 0.015–0.7% [6-9], while the prevalence of non-IgE-GI-FAs has been reported to be 0.21% in Japan [10] and 0.13-0.72% in Europe [11]. As cases of non-IgE-GI-FAs show nonspecific symptoms, including fever, poor weight gain, and minor gastrointestinal (GI) troubles, the diagnosis of non-IgE-GI-FAs is sometimes challenging. Additionally, approximately 10% of patients develop severe complications, including mechanical ileus and delayed growth. Therefore, highly accurate diagnosis and prompt treatment are urgently needed for this disease. Oral food challenge (OFC) is necessary for definitive diagnosis. However, OFC can have risks, as it can induce the symptoms of non-IgE-GI-FAs, including severe symptoms, such as shock. Therefore, OFC cannot be performed easily as the patients comprise neonates and infants. Furthermore, OFC should not be performed until the patients are sufficiently healthy, which can subsequently delay diagnosis.

The lymphocyte stimulation test (LST), which is used to determine the reactivity of T-lymphocytes to specific allergens *in vitro* [12-14], aids in the diagnosis of non-IgE-GI-FAs. However, LSTs require substantial amounts of fresh peripheral blood and prolonged culture times of 5-7 days. Therefore, alternative tests that require small amounts of blood and shorter experimental times are needed on an urgent basis. Following activation by antigen recognition and co-stimulation, the T-cells express inflammatory cytokines, such as IL2, within a few hours. This is followed by the activation of certain genes, including *CD69* and *CD25* (interleukin-2 receptor-α (IL-2RA)). A few days later, cell proliferation is observed by LSTs [15]. We hypothesized that the early transcriptional response of the allergen-stimulated lymphocytes of patients with non-IgE-GI-FAs replaces LSTs as an auxiliary *in vitro* test for assessing the antigen response. We designated this method as the instant peripheral blood allergen stimulation test (iPAST). In the iPAST, lymphocytes are stimulated with a specific allergen for 24 h, following which the mRNA levels are analyzed. In this study, we observed that *IL2RA* mRNA expression increased in the peripheral blood mononuclear cells (PBMCs) of patients with non-IgE-GI-FAs after stimulation with the allergens in CM for 24 h *in vitro* [16].

In the present study, we evaluated the mRNA levels of known T-cell-related genes, namely, leucine-rich repeat-containing protein 32 (*LRRC32*) [17], tumor necrosis factor receptor superfamily, member 4 (*TNFRSF4*) (OX40) [18], and *CD69,* upon stimulation with the allergens in CM for 24 h, for identifying superior biomarkers for the diagnosis of non-IgE-GI-FAs.

MATERIALS AND METHODS

Patients

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 consents from parents or guardians for this study. The control group comprised individuals who did not have non-IgE-GI-FAs or other immune diseases. Non-IgE-GI-FAs were diagnosed on the basis of the criteria proposed by the Japanese Pediatric Guidelines for Food Allergy, and modified by Powell: (i) development of GI symptoms after the ingestion of causative foods; (ii) disappearance of the symptoms after discontinuation of the causative foods; (iii) exclusion of other disorders that can cause GI symptoms, including infections and surgical problems; and (iv) recurrence of GI symptoms during OFC or after re-administration of the causative foods. This study was conducted after obtaining approval from the Ethics Committee of Gunma University (approval number: 1507).

The Instant Peripheral Blood Allergen Stimulation Test (iPAST)

Samples of peripheral blood were obtained during the onset of the symptoms or before OFC. The PBMCs, collected from the patients and their control counterparts, were incubated with α-casein, κ-casein, α-lactalbumin, β-lactoglobulin, a mixture of all the four components (Pmix), or CM for 24 h, as previously described [16]. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed as previously described [16], with TaqMan Gene Expression Assays for *LRRC32* (Assay ID: Hs00194136_m1; Thermo Fisher Scientific), *TNFRSF4* (Assay ID: Hs00937194_g1; Thermo Fisher Scientific), *CD69* (Assay ID: Hs00934033_m1; Thermo Fisher Scientific), and 18S rRNA (Assay ID: Hs99999901_s1; Thermo Fisher Scientific), which was used as the control. The gene expression was determined by dividing the gene expression in the stimulated samples with that of the non-stimulated samples, after normalizing the expression to that of 18S rRNA.

Lymphocyte Stimulation Test

The lymphocyte stimulation test was conducted with

Table 1. mRNA levels and ROC curve analysis for the patients and controls.

			Fold induction of mRNA†			ROC curve	
		n	Patients with non-IgE-GI-FAs	Controls	*p* value*	AUC	*p* value
LRRC32	α-casein	32	1.394 (0.655–2.862)	0.486 (0.152–0.776)	**<0.01**	0.8203	**<0.01**
	κ-casein	32	1.028 (0.451–3.147)	0.696 (0.238–1.402)	**<0.05**	0.7294	**<0.05**
	α-lactalbumin	33	1.638 (1.125–2.817)	1.252 (0.958–1.529)	**<0.05**	0.7132	**<0.05**
	β-lactoglobulin	33	1.968 (1.593–2.637)	1.637 (1.157–2.047)	>0.1	0.6654	>0.1
	Pmix	33	2.224 (1.270–4.400)	0.730 (0.350–1.156)	**<0.001**	0.8419	**<0.001**
	CM [a]	30	2.016 (1.084–2.363)	1.049 (0.706–2.172)	>0.05	0.6964	>0.05
TNFRSF4	α-casein	33	0.929 (0.714–1.218)	0.740 (0.482–0.997)	>0.1	0.6397	>0.1
	κ-casein	33	0.798 (0.502–0.997)	0.599 (0.373–0.792)	>0.05	0.6985	>0.05
	α-lactalbumin	33	1.631 (1.422–2.381)	1.372 (0.960–1.937)	>0.1	0.6507	>0.1
	β-lactoglobulin	33	1.781 (1.596–2.236)	1.700 (1.021–2.198)	>0.1	0.6066	>0.1
	Pmix	33	1.696 (1.372–2.401)	1.186 (0.905–1.830)	>0.05	0.6985	>0.05
	CM [a]	30	1.285 (0.886–2.222)	1.296 (0.797–1.607)	>0.1	0.5625	>0.1
CD69	α-casein	32	1.076 (0.823–1.255)	0.894 (0.739–1.005)	>0.05	0.6980	>0.05
	κ-casein	32	0.952 (0.744–1.125)	0.877 (0.494–1.034)	>0.1	0.6314	>0.1
	α-lactalbumin	32	1.370 (1.097–1.969)	1.086 (0.799–1.747)	>0.1	0.6510	>0.1
	β-lactoglobulin	32	1.314 (0.934–1.535)	1.061 (0.776–1.558)	>0.1	0.5784	>0.1
	Pmix	32	1.330 (0.990–2.006)	1.067 (0.860–1.582)	>0.1	0.5961	>0.1
	CM [a]	30	1.232 (0.880–1.381)	1.025 (0.813–1.261)	>0.1	0.6161	>0.1

[a] CM, Cow's milk. Data are expressed as the median (interquartile ranges). *Mann–Whitney U test.
† Fold induction of *LRRC32*, *TNFRSF4*, and *CD69* mRNAs was calculated by dividing the expression in the stimulated samples with that of the non-stimulated samples, following normalization to that of 18S rRNA.
p values < 0.05 are indicated by bold text.

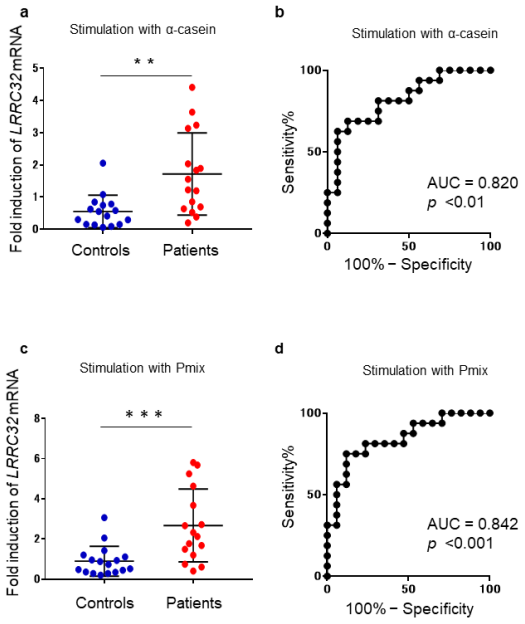


Figure 1. **Comparison of the expression levels of *LRRC32* mRNA between the patients and controls**. **a**. The fold induction of *LRRC32* mRNA was calculated by dividing the expression of *LRRC32* mRNA in α-casein-stimulated samples with that of the non-stimulated samples, following normalization to that of 18S rRNA. Each blue dot represents the data obtained from a single control and each red dot represents the data obtained from a single patient. The bars indicate the median values. Mann–Whitney U tests were performed for comparisons. **b**. ROC curves for the expression levels of *LRRC32* mRNA, in the PMBCs of the patients and controls stimulated with α-casein. **c**. The fold induction of *LRRC32* mRNA was calculated by dividing the expression in the Pmix-stimulated samples with that of the non-stimulated samples, following normalization to that of 18S rRNA. Each blue dot represents the data obtained from a single control and each red dot represents the data obtained from a single patient. The bars indicate the median values. Mann–Whitney U tests were used for comparisons. **d**. The ROC curves for *LRRC32* mRNA expression in the PMBCs of patients and controls stimulated with Pmix. **Refers to comparisons between patients and controls, $p < 0.01$. ***Refers to comparisons between patients and controls, $p < 0.001$.

a few modifications to previously described implementations [19,20]. Briefly, PBMCs from each patient were incubated separately with CM and four different milk protein components (α-lactalbumin, β-lactoglobulin, α-casein, and κ-casein) for 5 days. Subsequently, proliferating cells were labeled by further incubation with 100 μM of 5-bromo-2-deoxyuridine (BrdU) for 6 h, and the incorporated BrdU contents were determined by an enzyme-linked immune-solvent assay. The results were described as stimulation index (SI), which was calculated as the ratio of BrdU-contents in a stimulated to unstimulated sample.

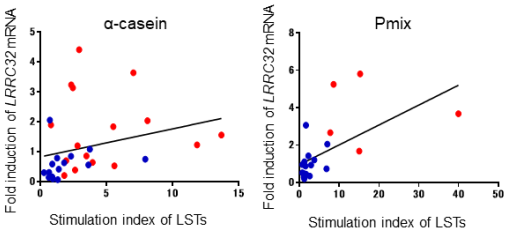
Statistical Analysis

The mRNA levels were statistically evaluated by Mann–Whitney U test, and diagnostic accuracy was assessed by analyzing the receiver operating characteristic (ROC) curve. Furthermore, the correlation between the expression levels of mRNA and the SI of LSTs by Spearman correlation coefficients. All analyses were performed using GraphPad Prism version 7 for Windows (GraphPad Inc., San Diego, CA, USA). The results were considered statistically significant if $p < 0.05$.

RESULTS

Sixteen patients (8 boys and 8 girls) with non-IgE-GI-FAs participated in this study. Seventeen individuals (11 boys and 6 girls) who did not have non-IgE-GI-FAs or other immune diseases comprised the control group in this study. 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 ($p = 0.0016$, Mann–Whitney U test). Among the 16 patients, eight met the criteria for acute FPIES proposed in the 2017 International Consensus Guidelines [21]. For the other eight patients, three had FPIAP, one had chronic FPIES or FPE clinical features, and one had reproducible characteristic features of acute FPIES but did not meet the diagnostic criteria. The remaining three cases mainly showed bloody stools without vomiting, as well as poor general conditions. These three cases were categorized as unclassified. We assessed the mRNA expression levels and evaluated the diagnostic significance of *LRRC32*, *TNFRSF4*, and *CD69* genes. Compared with the control group, the PBMCs obtained from the patients showed higher mRNA expression of *LRRC32* after stimulation with α-casein, κ-casein, α-lactalbumin, or Pmix, but not β-lactoglobulin or CM (Table 1, Figure 1a and c). The area under the ROC curve (AUC) for *LRRC32* mRNA expression after 24 h of stimulation with the aforementioned components, was greater than 0.7, with the exception of β-lactoglobulin and CM, and thus validated the diagnostic value of this analysis. Notably, the AUC scores of α-casein (AUC: 0.8203; $p = 0.002$) and Pmix (AUC: 0.8419; $p = 0.0008$) were higher than other antigens (Table 1, Figure 1b and d). As the two disease-related samples of PMBs were obtained during the onset of symptoms or before OFC, we examined whether testing during symptom onset and before OFC would give different results. There was no significant difference in the induced levels of *LRRC32* mRNA expression between samples collected in the two different situations (Appendix A: Supplementary Table 1). Furthermore,

a



b

	n	r	*p* value
α-casein	32	0.5048	**<0.01**
κ-casein	29	0.3562	>0.05
α-lactalbumin	33	0.2199	>0.1
β-lactoglobulin	33	−0.0160	>0.1
Pmix	22	0.6702	**<0.001**
Cow milk	29	0.0739	>0.1

Figure 2. Comparison of the fold induction of *LRRC32* mRNA using the stimulation index determined by conventional LSTs following stimulation with milk proteins. a. Spearman correlation analysis of the levels of *LRRC32* mRNA and stimulation indexes obtained from LSTs after α-casein and Pmix stimulation. Levels of *LRRC32* mRNA were described by the fold induction of *LRRC32* mRNA expression, upon stimulation with the indicated milk proteins, relative to that of the unstimulated cells. Each blue dot represents the data obtained from a single control and each red dot represents the data obtained from a single patient. **b**. Summary of Spearman correlations. *p* values < 0.05 are indicated by bold text.

because the control and patient groups had significantly different ages, there were no significant correlation between the *LRRC32* mRNA levels and age for all antigens in all participants including in controls. In contrast, when the control group was exclusively analyzed, *LRRC32* mRNA was significantly increased with age following α-casein, κ-casein, Pmix, and CM stimulation (see Appendix A: Supplementary Figure 1). The mRNA levels of *TNFRSF4* and *CD69* did not differ between the patients and the controls after 24 h of stimulation with any of the allergens (Table 1). Furthermore, subpopulation analysis after stimulation with milk allergens revealed no difference in *LRRC32* mRNA expression between patients who fulfilled the diagnostic criteria for FPIES and those who did not or between the FPIES group and FPIAP group (Appendix A: Supplementary Table 2 and 3).

We subsequently assessed the correlation between *LRRC32* mRNA levels and the results obtained by conventional LSTs. The median (interquartile ranges) SI of LSTs of the patients and controls were 3.222 (2.377–6.686; α-casein), 15.04 (8.17–27.6; Pmix), and 0.959 (0.699–2.071; α-casein), 1.663 (1.169–2.795; Pmix), respectively. Using Spearman correlation, we observed that the correlations obtained for α-casein (r = 0.5048; p = 0.0032) and Pmix (r = 0.6702; p = 0.0006) were moderate and significant; however, this did not hold true for the other allergens (Figure 2a and b). These results indicated that α-casein- and Pmix-stimulated *LRRC32* mRNA expression levels correlated with the SI obtained by LSTs.

DISCUSSION

In this study, we found that among *LRRC32*, *TNFRSF4*, and *CD69*, the expression of *LRRC32* mRNA could be used to discriminate the patients from the control group, particularly after stimulation with α-casein and Pmix. The levels of *LRRC32* mRNA stimulated by α-casein and Pmix correlated with those obtained from conventional LSTs. These results indicated that *LRRC32* plays an important role in non-IgE-GI-FAs, and further suggested that α-casein was a major allergen in non-IgE-GI-FAs.

The *LRRC32* gene is located at the 11q13 region, which is associated with a wide range of allergic diseases, including asthma [22], atopic dermatitis [23], allergic rhinitis [24], and food allergies [25]. LRRC32 is a surface marker of activated FoxP3$^+$ regulatory T-cells [17,26]. It directly binds to the TGF-β latency-associated peptide complex and suppresses the secretion of TGF-β [17]. Pathological conditions in FPIES may involve the decreased expression of the TGF-β1 receptor in GI tissues, with consequent impairment of GI barrier function resulting from the decreased activity of TGF-β [27]. Moreover, children with FPIES to CM have deficient T cell-mediated TGF-β responses to casein [28]. Taken together, a specific increase in *LRRC32* expression was observed in this study, which suggested its important role in non-IgE-GI-FAs. The AUC of *IL2RA* mRNA expression following stimulation with α-casein in ROC curve analysis in our previous report was 0.9596 (p < 0.0001) [16]. This is higher than the AUC of *LRRC32* mRNA (0.8203, p < 0.01) in the current study, indicating that IL2RA is a better marker for diagnosing non-IgE-GI-FAs.

Some of the major limitations of this study include the small number of patients in the group and the significant age difference between the non-IgE-GI-FAs patients and the control group. However, it is important to note that as non-IgE-GI-FAs mostly develop in young children, it is difficult to obtain samples from age-matched controls owing to ethical constraints and difficulties in obtaining consent. However, in analysis of the control group alone, α-casein-, κ-casein-, Pmix-, and CM-stimulated *LRRC32* mRNA expression levels significantly increased with increasing age; *LRRC32* expression in younger patients was higher after stimulation with milk

allergens compared to in controls. This result indicates that *LRCC32* levels were affected by the disease, which can be used as a diagnostic index. Additionally, we did not investigate which cells expressed the *LRRC32*, *TNFRSF4*, and *CD69* mRNAs. The LST assay is useful for diagnosing non-IgE-GI-FA, but it is unclear which types of cells were proliferating after 5 days of stimulation with the milk allergen. In this study, the expression level of *LRRC32* mRNA was correlated with conventional LST. Although an antigen-specific initial increase in *LRRC32* mRNA levels may reflect an antigen-specific lymphocyte proliferation response, the corresponding mechanism is unknown. However, one of the strengths of this study is that the diagnosis of non-IgE-GI-FAs was based on strict diagnostic criteria and included an OFC or re-administration for confirming the diagnosis.

In conclusion, we have presented evidence that, in addition to IL2RA iPAST, *LRRC32* has a good diagnostic value and its increase may provide insights into the pathogenesis of non-IgE-GI-FAs.

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Author Contributions: HY performed the experiments and statistical analysis and prepared the manuscript. TT designed the study, reviewed the collected data, and supervised the study. YY and HA analyzed the results and suggested revisions. All the authors, except YY, contributed to data collection. All the authors read and approved the final manuscript.

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Appendix A

Supplementary Table 1. Comparison of the expression levels of *LRRC32* mRNA between patients during the onset and before OFC.

	Fold induction of *LRRC32* mRNA†				
	Patients with onset		Patients with OFC [b]		
	n		n		*p* value*
α-Casein	12	1.379 (0.655–2.826)	4	1.633 (0.598–3.238)	>0.1
κ-Casein	12	0.962 (0.5205–3.028)	3	1.677 (0.328–3.867)	>0.1
α-Lactalbumin	12	2.012 (1.311–3.371)	4	1.207 (0.855–1.977)	>0.1
β-Lactoglobulin	12	1.968 (1.593–2.171)	4	2.240 (1.488–2.900)	>0.1
Pmix	12	2.495 (1.270–5.098)	4	1.901 (0.721–3.290)	>0.1
CM [a]	10	2.033 (1.159–3.378)	4	1.555 (0.945–2.395)	>0.1

[a] CM, Cow milk. [b] OFC, Oral food challenge

Data are expressed as the median (interquartile ranges). *Mann–Whitney U test.

† Fold induction of *LRRC32* mRNAs was calculated by dividing the expression in the stimulated samples with that of the non-stimulated samples, following normalization to that of 18S rRNA.

Supplementary Table 2. mRNA levels in patients with FPIES and patients without FPIES.

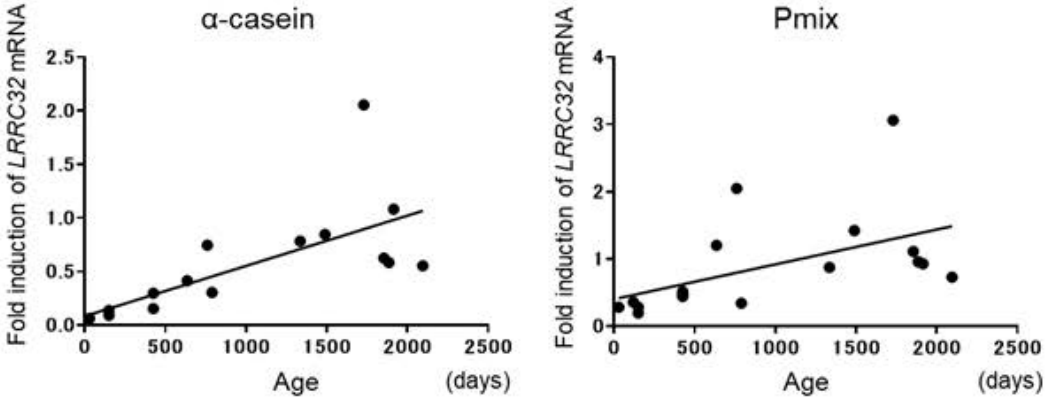
		Fold induction of mRNA†		
		Patients with FPIES [b]	Patients without FPIES	*p* value*
LRRC32	α-casein	1.865 (1.207–2.937)	0.777 (0.556–2.742)	>0.1
	κ-casein	2.020 (0.895–3.867)	0.590 (0.4153–2.617)	>0.05
	α-lactalbumin	1.763 (0.984–2.817)	1.638 (1.311–3.205)	>0.1
	β-lactoglobulin	1.987 (1.551–2.900)	1.851 (1.593–2.171)	>0.1
	Pmix	2.394 (1.536–3.438)	2.047 (0.856–5.575)	>0.1
	CM [a]	2.093 (1.373–2.455)	1.404 (0.993–3.289)	>0.1
TNFRSF4	α-casein	0.959 (0.677–1.218)	0.929 (0.714–1.279)	>0.1
	κ-casein	0.862 (0.517–0.997)	0.748 (0.502–1.024)	>0.1
	α-lactalbumin	1.678 (1.108–2.521)	1.532 (1.422–2.381)	>0.1
	β-lactoglobulin	1.851 (1.426–4.130)	1.761 (1.596–2.177)	>0.1
	Pmix	1.735 (1.072–2.658)	1.652 (1.435–2.219)	>0.1
	CM [a]	1.808 (0.896–2.229)	1.065 (0.886–1.840)	>0.1
CD69	α-casein	1.060 (0.784–1.290)	1.164 (0.850–1.255)	>0.1
	κ-casein	0.925 (0.771–1.137)	1.074 (0.703–1.125)	>0.1
	α-lactalbumin	1.328 (0.936–1.463)	1.370 (1.164–2.223)	>0.1
	β-lactoglobulin	1.353 (0.652–1.466)	1.322 (1.062–1.647)	>0.1
	Pmix	1.289 (0.873–1.394)	1.444 (1.278–2.399)	>0.1
	CM [a]	1.140 (0.920–1.332)	1.341 (0.775–1.505)	>0.1

Supplementary Table 3. mRNA levels in the patients with FPIES and patients with FPIAP.

		Fold induction of mRNA†		
		Patients with FPIES [b]	Patients with FPIAP [c]	*p* value*
LRRC32	α-casein	1.865 (1.207–2.937)	0.697 (0.641–1.558)	>0.1
	κ-casein	2.020 (0.895–3.867)	0.729 (0.434–1.028)	>0.05
	α-lactalbumin	1.763 (0.984–2.817)	2.303 (1.266–3.505)	>0.1
	β-lactoglobulin	1.987 (1.551–2.900)	1.556 (1.187–2.226)	>0.1
	Pmix	2.394 (1.536–3.438)	2.325 (1.197–5.812)	>0.1
	CM [a]	2.093 (1.373–2.455)	1.649 (1.100–2.198)	>0.1
TNFRSF4	α-casein	0.959 (0.677–1.218)	0.862 (0.683–1.015)	>0.1
	κ-casein	0.862 (0.517–0.997)	0.759 (0.410–0.888)	>0.1
	α-lactalbumin	1.678 (1.108–2.521)	1.587 (1.351–2.357)	>0.1
	β-lactoglobulin	1.851 (1.426–4.130)	1.874 (1.541–2.274)	>0.1
	Pmix	1.735 (1.072–2.658)	1.762 (1.478–2.095)	>0.1
	CM [a]	1.808 (0.896–2.229)	1.190 (0.970–1.410)	>0.1
CD69	α-casein	1.060 (0.784–1.290)	1.053 (0.850–1.255)	>0.1
	κ-casein	0.925 (0.771–1.137)	0.925 (0.750–1.099)	>0.1
	α-lactalbumin	1.328 (0.936–1.463)	1.969 (1.370–3.153)	>0.1
	β-lactoglobulin	1.353 (0.652–1.466)	2.027 (1.201–2.853)	>0.1
	Pmix	1.289 (0.873–1.394)	2.116 (1.330–2.902)	>0.1
	CM [a]	1.140 (0.920–1.332)	1.246 (0.805–1.686)	>0.1

[a] CM, Cow milk. [b] FPIES, Food protein-induced enterocolitis syndrome. [c] FPIAP, food protein-induced allergic proctocolitis. Data are expressed as the median (interquartile ranges). *Mann–Whitney U test. † Fold induction of *LRRC32*, *TNFRSF4*, and *CD69* mRNAs was calculated by dividing the expression in the stimulated samples with that of the non-stimulated samples, following normalization to that of 18S rRNA.

a



b

	n	r	*p* value
α-casein	16	0.8133	**<0.001**
κ-casein	17	0.8039	**<0.001**
α-lactalbumin	17	-0.2053	>0.1
β-lactoglobulin	17	-0.1684	>0.1
Pmix	17	0.6550	**<0.005**
Cow milk	16	0.5221	**<0.05**

Supplementary Fig. 1 Spearman correlation analysis of *LRRC32* mRNA levels and age in controls after α-casein and Pmix stimulation.

A, Spearman correlation analysis of LRRC32 levels and age after α-casein and Pmix stimulation.

B, Summary of Spearman correlations.