In-depth proteomic profiles prior to symptom development in food protein-induced enterocolitis

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Short title: Proteomic profiles of pre-symptom onset in FPIES

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Conflict of interest

The authors declare that this research was conducted without commercial or financial relationships that could be construed as potential conflicts of interest.

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Abstract

Background: The innate immune system is activated at the onset of food protein-induced enterocolitis syndrome (FPIES) symptoms. However, the precise mechanism through which this immune response is initiated remains unclear.

Keywords

Food protein-induced enterocolitis syndrome, Neddylation, Neutrophil, Proteome, Proteasome

Abbreviations

DIA: data-independent acquisition

FPIES: food protein-induced enterocolitis syndrome

MS: mass spectrometry

SDS: sodium dodecyl sulfate

ACN: acetonitrile

TFA: trifluoroacetic acid

AGC: auto gain control

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1. Introduction

Food protein-induced enterocolitis syndrome (FPIES) is a non-IgE-dependent food allergy that causes gastrointestinal symptoms, such as vomiting, diarrhea, and blood loss, following ingestion of food allergens[1,2]. The introduction of allergenic foods early in life can prevent

the development of IgE-dependent food allergies[3], and the prevalence of IgE-dependent food allergies is decreasing. However, the increase in FPIES cases despite the early introduction of such foods has become problematic[4,5]. The incidence of egg yolk-related FPIES has increased in recent years, especially in Japan[4], contributing to a reduced quality of life. Therefore, there is an urgent need to elucidate the pathogenesis of FPIES and establish effective prevention and treatment strategies.

Since FPIES is a non-IgE-dependent allergy, the responses of antigen-specific T cells to the food allergen have been extensively studied[6]. However, although FPIES was associated with a significant increase in serum IL-17 family levels[7]—which is mainly secreted by Th17 cells and associated with neutrophil activation—cytometric analysis of peripheral blood has shown activation not of antigen-specific T cells but of systemic innate immune responses, such as increased neutrophil counts and activation of neutrophils, eosinophils, monocytes, and natural killer cells[8]. Similarly, transcriptome analysis of the whole peripheral blood showed high expression of genes related to innate immune responses in FPIES[9].

To advance the pathogenetic analysis of FPIES, further research is needed on how innate immune overactivation occurs in an antigen-specific manner. However, few studies have focused on the time point before abnormal activation of innate immunity. This may be due to the difficulty in evaluating immunological status using classical immunological analyses such as flow cytometry or ELISA.

In this study, we aimed to elucidate the pathogenic mechanisms underlying FPIES symptom development by examining the serum and salivary proteomic profiles of individuals with FPIES before symptom onset. This study could provide empirical evidence of the potential causes of FPIES.

2. Methods

2.1. Study design

This multicenter observational study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Ethical Review Board of Chiba University (Chiba, Japan; approval number: M10335). Written informed consent was obtained from each participant and/or their guardian.

The exclusion criteria were a) complications of atopic dermatitis or bronchial asthma; b) history of or current treatment for underlying medical conditions other than allergic diseases, such as heart, liver, or renal disease; and c) [?]3.5 kUA/L specific IgE against egg yolk.

2.2. Sampling of clinical specimen

Whole blood was collected at 1 and 2 h after egg yolk ingestion and, if possible, at the onset of acute FPIES symptoms from nine participants consenting to both serum and saliva collection; eight participants consented only to saliva collection. Serum was separated from the blood, divided into aliquots, cryopreserved, and stored frozen at < -20 °C until analysis.

2.3. Proteomic analysis

The Kazusa DNA Research Institute, a collaborating institution, performed proteomic analysis of cryopreserved serum and saliva.

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2.3.1. Sample preparation for proteome analysis

Σαμπλε πρεπαρατιον ανδ προτεομε αναλψσις ωερε περφορμεδ ας δεσςριβεδ πρειουσλψ[11-13] Βριεφλψ, 51 σαλια σαμπλες (17 σαμπλες βεφορε ΟΦ˜, 17 σαμπλες 1 η αφτερ ΟΦ˜, ανδ 17 σαμπλες 2 η αφτερ ΟΦ˜) ωερε δισσολεδ ιν 100 μM Τρισ-Η˜λ (πΗ 8.0) ςονταινινγ 4% σοδιυμ δοδεςψλ συλφατε (ΣΔΣ), 20 μM Να˜λ, ανδ 10% ασετονιτριλε (Α˜Ν) υσινγ α Βιορυπτορ ΒΡ-ΙΙ (ΣΟΝΙ˜ ΒΙΟ, Καναγαωα, Θαπαν). Τηε εξτραςτεδ προτεινς (40 μγ) ωερε χυαντιφιεδ υσινγ α Πιερςε Β˜Α Προτειν Ασσαψ Κιτ (Τηερμο Φισηερ Σςιεντιφις, Ωαλτηαμ, ΜΑ, ΥΣΑ) ατ 200 νγ/μΛ. Τηε προτειν εξτραςτς ωερε ρεδυςεδ ωιτη 20 μM τρισ(2-ςαρβοξψετηψλ)πηοσπηινε φορ 10 μιν ατ 80 °˜, φολλοωεδ βψ αλκψλατιον ωιτη 35 μM ιοδοαςεταμιδε φορ 30 μιν ιν τηε δαρκ. Προτειν πυριφιςατιον ανδ διγεστιον ωερε περφορμεδ υσινγ τηε ΣΠ3 μετηοδ[11]. Τρψπτις διγεστιον ωας περφορμεδ υσινγ 500 νγ/μΛ Τρψπσιν πλατινυμ (Προμεγα, Μαδισον, ΩΙ, ΥΣΑ) οερνιγητ ατ 37 °˜. Τηε διγεστς ωερε πυριφιεδ υσινγ ΓΛ-Τιπ ΣΔΒ (ΓΛ Σςιενςες, Τοκψο, Θαπαν) αςςορδινγ το τηε μανυφαςτυρερ′ς προτοςολ. Τηε πεπτιδες ωερε ρεδισσολεδ ιν δεςψλ μαλτοσε νεοπεντψλ γλψςολ (ΔΜΝΓ) ςονταινινγ 0.1% τριφλυοροαςετις αςιδ (ΤΦΑ)[12] ανδ χυαντιφιεδ υσινγ α Β˜Α ασσαψ ατ 200 νγ/μΛ.

To remove high-abundance proteins, 32 serum samples (9 samples before OFC, 9 samples 1 h after OFC, 9 samples 2 h after OFC, and 5 samples at symptom onset) were treated using Top14 Abundant Protein Depletion Mini Spin Columns (Thermo Fisher Scientific) following the manufacturer's instructions. The filtrates were dissolved in 100 mM Tris-HCl (pH 8.0) containing 4% SDS, 20 mM NaCl, and 10% ACN using the Bioruptor BR-II. The reduction and alkylation of proteins and the SP3 method were performed as previously described. Peptides were dissolved in 0.01% DMNG containing 0.1% TFA.

2.3.2. Liquid chromatography with tandem mass spectrometry (LC–MS/MS)

Λ˜ ωας περφορμεδ ωιτη διγεστεδ πεπτιδες λοαδεδ διρεςτλψ ιντο α 75 μμ × 30 ςμ νανοΛ˜ νανοςαπιλλαρψ ςολυμν (ὅΑνν Τεςηνολογιες, Ριςηλανδ, ΩΑ, ΥΣΑ) ατ 50 °˜ ανδ τηεν σεπαρατεδ υσινγ α 100-μιν γραδιεντ (μοβιλε πηασε Α = 0.1% ΦΑ ιν ωατερ, Β = 0.1% ΦΑ ιν 80% Α˜Ν) ςονσιστινγ οφ 0 μιν 7% Β, 86 μιν 37% Β, 93 μιν 70% Β, ανδ 100 μιν 70% Β ατ α φλοω ρατε οφ 150 νΛ/μιν ον αν ΥλτιΜατε 3000 ΡΣΛ˜νανο Λ˜ σψστεμ (Τηερμο Φισηερ Σςιεντιφις). ΜΣ/ΜΣ οφ τηε ελυτεδ πεπτιδες ωας περφορμεδ υσινγ α χυαδρυπολε Ορβιτραπ Εξπλορις 480 ηψβριδ μασς σπεςτρομετερ (Τηερμο Φισηερ Σςιεντιφις) ωιτη α νορμαλ ΔΙΑ ωινδοω. Τηε ΜΣ1 σςαν ρανγε ωας σετ το α φυλλ σςαν οφ μ/ζ 495–745 ατ μασς ρεσολυτιον οφ 60,000, αυτο γαιν ςοντρολ (ΑΓ˜) ταργετ οφ 3 × 10^6, ανδ μαξιμυμ ινθεςτιον τιμε οφ 'Αυτο.' ΜΣ2 ωας περφορμεδ ατ μ/ζ 200–1,800, ρεσολυτιον οφ 45,000, αν ΑΓ˜ ταργετ οφ 3 × 10^6 (μαξιμυμ ινθεςτιον τιμε οφ 'Αυτο'), ανδ φιξεδ νορμαλιζεδ ςολλισιον ενεργψ οφ 26%. Τηε ισολατιον ωιδτη φορ ΜΣ2 ωας σετ το 4 Τη. Φορ τηε 500–740 μ/ζ ωινδοω παττερν, αν οπτιμιζεδ ωινδοω αρρανγεμεντ ωας υσεδ ιν Σςαφφολδ ΔΙΑ (Προτεομε Σοφτωαρε, Πορτλανδ, ΟΡ, ΥΣΑ).

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2.3.3. Data processing

The raw data were searched against an in silico predicted spectral library using DIA-NN (version 1.8.1, https://github.com/vdemichev/DiaNN). The in silico predicted spectral library was generated from the human protein sequence database (UniProt id UP000005640, reviewed, canonical, 20,591 entries, March 7, 2023, download). The spectral library was generated using the following parameters: digestion enzyme, trypsin; missed cleavage, 1; peptide length, 7–45; precursor charge, 2–4; precursor m/z, 495–745; fragment ion m/z, 200–1800. Additional-

ly, "FASTA digest for library-free search/library generation," "Deep learning-based spectra, RTs, and IM prediction," "n-term M excision," and "C carbamidomethylation" were enabled. For the DIA-NN search, the following parameters were applied: mass accuracy, 10 ppm; MS1 accuracy, 10 ppm; protein inference based on genes; utilization of neural network classifiers in single-pass mode; quantification strategy using robust LC (high precision); cross-run normalization set to "RT-dependent." Additionally, "unrelated runs," "use isotopologues," "heuristic protein inference," and "no shared spectra" were enabled. The protein identification threshold was <1% for both peptide and protein false discovery rates.

2.3.4. Statistics and bioinformatics

Protein expression was analyzed using Perseus software 1.6.15.0 (https://maxquant.net/perseus/). Protein quantification data were log2-transformed and filtered to ensure that at least one group contained a minimum of 70% valid values for each protein. The remaining missing values were imputed using random numbers drawn from a normal distribution (width = 0.3, downshift = 1.8). A two-tailed Welch's t-test was used to compare significant differences between the groups. The$p$-values were corrected post hoc using the Benjamin and Hochberg procedure for multiple comparison tests. Statistical significance was set at $p < 0.05$.

For functional annotation and pathway enrichment analysis of differentially expressed proteins, we utilized Enricher-KG[14], a knowledge graph and web server application applying gene set libraries from Enrichr[15]. Gene Ontology (GO) biological process terms (https://geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) 2021 human pathways (https://www.genome.jp/kegg/pathway.html) were used for the analysis. Statistical significance was set at $p < 0.05$. Metascape[16] (https://geneontology.org) was used for cluster analysis of enriched ontologies of the top 150-rankeddifferentially expressed proteins and their protein–protein interactions. The molecular complex detection (MCODE) algorithm[17] was applied to identify densely connected network components.

2.4. ELISA and statistical analysis

Serum proteasome levels were measured using a 20S/26S Proteasome ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA). Serum levels of NEDD8 were measured using a Human NEDD8 ELISA Kit (MyBioSource, San Diego, CA, USA). Statistical analysis of the ELISA data was performed using JMP Pro® 15.2.1 software. The Mann–Whitney U test was used to compare groups (two-sided), with $p < 0.05$ considered significant.

3. Results

4. Discussion

We aimed to clarify the pathogenesis of FPIES symptom development based on the serum and saliva proteomic profiles before symptom onset. We observed a transient increase in serum proteasome- and neddylation-related protein levels before the onset of FPIES symptoms, possibly triggering the activation of various innate immune cell types at symptom onset. Regardless of the onset of symptoms, high expression of proteins related to neutrophil activation was found in the serum and saliva before symptom onset, suggesting that initial neutrophil activation may not be necessary to initiate FPIES symptom development. However, it might be prolonged and worsen the symptoms when they occur.

The novelty of this study lies in its use of proteomics to explore factors related to FPIES pathogenesis up to symptom onset. FPIES is considered as triggered by specific antigens. After symptom onset, there is an increase in IL-17 levels[7], indicating Th17 cell activation. However, the onset of FPIES symptoms within 1–4 h suggests that the initial response of FPIES may be

due to an innate rather than a cellular immune response. Moreover, early ingestion of allergenic foods to prevent the development of IgE-mediated food allergies by inducing oral immune tolerance (induction of antigen-specific regulatory T cells) may conversely be related to a higher incidence of FPIES[4,5]. Further, the relatively early acquisition of tolerance[18,19] compared with that in IgE-dependent allergy suggests that FPIES pathogenesis may be associated with abnormal innate immunity activation, while the T cell-mediated immune response may be a secondary event.

In our in-depth proteomic analysis, the levels of proteasome subunit- and neddylation-related proteins were significantly increased in the sera of the OFC-positive group before FPIES symptom onset, suggesting that pathways involving these proteins participate in symptom development. Both are involved in protein degradation and regulation of inflammation, suggesting that key pathway activation events occur early after exposure to the trigger food in FPIES patients.

The ubiquitin-proteasome system[20], which labels unnecessary or defective proteins polyubiquitin for subsequent degradation to peptides via the proteasome, is responsible for selective non-lysosomal protein degradation. The biologically functional proteasome complex has been detected in normal human blood plasma/serum (known as the circulating proteasome) and is highly expressed in various diseases, including malignancies, autoimmune disorders, sepsis, and other conditions[21]. Notably, this is the first study to report the high expression of proteasomes in allergic disease.

Neddylation is a post-translational modification that occurs when NEDD8, a ubiquitin-like protein, is covalently bound to a target protein[22]. Neddylation is catalyzed by Cullin family and non-Cullin proteins. NEDD8 and neddylation-related proteins are often upregulated in various diseases, such as cardiac, metabolic, chronic liver, neurodegenerative, and immune-related diseases[22]. As with proteasomes, no reports exist on the high expression of neddylation-related proteins in allergic diseases; however, they play a regulatory role in inflammatory cytokine and interferon production during innate immune responses in various infectious diseases. Neddylation-related proteins may also be involved in the innate immune response to trigger food in FPIES.

Proteins related to neutrophil activation were upregulated in the serum and saliva after ingestion of the trigger food. In the OFC-positive group, we observed high expression of proteins related to neutrophil extracellular trap formation[23] at symptom onset, which may trigger the previously reported increase in peripheral blood neutrophil count and activation after symptom onset. However, the high expression of proteins associated with neutrophil activation was also observed in blood and saliva samples of the OFC-negative group, suggesting that neutrophil activation may be inconsequential in the development of FPIES symptoms. Regardless, this is the first evidence in a proteomic study of neutrophil activation before the onset of symptoms.

The present study has some limitations. First, we compared positive and negative groups in the OFC using a specific quantity of trigger food (cooked egg yolk) rather than comparing healthy subjects and patients experiencing egg yolk-induced FPIES. This approach was ethically justified because it avoided oral challenge tests in healthy children and collecting blood and saliva samples. Although it was not feasible to examine protein variation in FPIES relative to healthy subjects, in-depth proteomic analysis allowed for the examination of a vast number of proteins, both in serum and saliva, revealing factors related to symptom induction. Second, MS-based proteomic analysis could not detect proteins of all molecular weights, such as cytokines and chemokines, owing to its limited measurement range, which precluded evaluation of the IL-17 inflammatory signature. Integrated analysis of the proteome using multiple advanced approaches for molecule and pathway analysis is needed to elucidate FPIES pathogenesis. Finally, this study was designed with a small sample size because only a few patients consented to collect both blood and saliva samples, as it is relatively invasive to take multiple blood samples from infants during OFC, when symptom induction can occur. However, as proteomic studies can be performed with high precision even with a small number of samples, we considered that our serum data do reveal the proteomic profiles of FPIES. Nevertheless, analysis of a larger sample is warranted to obtain detailed proteomic profiles and corroborate the results of the current study.

Taken together, our findings demonstrate that proteasome- and neddylation-related proteins were highly expressed before the onset of FPIES symptoms, which has not been previously reported. The presence or degree of this response to the trigger food may be related to FPIES onset, though further detailed investigation of their involvement in pathogenesis is warranted.

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Tables

Table 1. Characteristics of study participants

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	All participants(N = 17)	O
		P
Participants consenting to both serum and saliva collection, n	9	4
Participants consenting to saliva but not serum collection, n	8	2
Female, %	58.8	5
Age (months), median (IQR)	15 (13–17)	1
Height (cm), median (IQR)	76.0 (75.1–78.3)	7
Weight (kg), median (IQR)	9.3 (8.7–10.1)	9.
Peripheral blood eosinophils (cells/μL), median (IQR)	290 (146–475)	3
Serum total IgE (IU/mL)	4.0 (0.0–14.0)	1
Egg yolk-specific IgE (kUA/L), median (IQR)	0.0 (0.0–0.33)	0.
Egg white-specific IgE (kUA/L), median (IQR)	0.0 (0.0–1.21)	0.
Duration between last symptom of FPIES due to egg yolk and OFC (weeks), median (IQR)	29 (25–37)	2
Time to symptom onset at OFC (minutes), median (IQR)		2
IgE-dependent symptoms at OFC, %	0	0

OR, odds ratio; CI, confidence interval; IQR, interquartile range. Odds ratios with 95% CI not >1 and $p< 0.5$ are written in bold.

Figure legends

Figure 1. Serum proteins differentially expressed 1 and 2 h after OFC and at symptom onset relative to before OFC

Colored areas in the volcano plots show differentially expressed proteins upregulated and downregulated (A) 1 h after OFC, (B) 2 h after OFC, (C) at symptom onset relative to before OFC in the OFC-positive group

and (D) 1 h after OFC and (E) 2 h relative to before OFC in the OFC-negative group. Proteome analysis of serum samples was performed using four biological replicates for the OFC-positive group and five biological replicates for the OFC-negative group.

Figure 2. Comparison of enriched ontology clusters among top 150-ranked proteins upregulated 2 h after OFC between OFC-positive and OFC-negative group

We performed enrichment analysis using the top 150-ranked proteins upregulated 2 h after OFC. We selected the term with the lowest$p$ -value within each cluster as the representative term in the dendrogram. The heatmap cells are colored according to their$p$ -values, and grey cells indicate a lack of enrichment for that term in the corresponding gene list.

Figure 3. Protein–protein interaction network and MCODEs of merged proteins upregulated 2 h after OFC in the OFC-positive and OFC-negative group

Densely connected protein–protein networks were identified using the Molecular Complex Detection (MCODE) algorithm in Metascape. Blue circles: proteins in Serum-UpPOS2. Red circles: proteins in Serum-UpNEG2. The biological interpretation of each MCODE is presented in Table S1.

Figure 4. Enrichment analysis of proteins upregulated at symptom onset

Most relevant GO terms (pink bars) and KEGG pathways (gray bars) related to 73 proteins in serum-UpS, but not in Serum-UpPOS2 or Serum-UpNEG2. Bar length represents the significance of the specific gene set or term in the enrichment analysis using Enricher-KG.

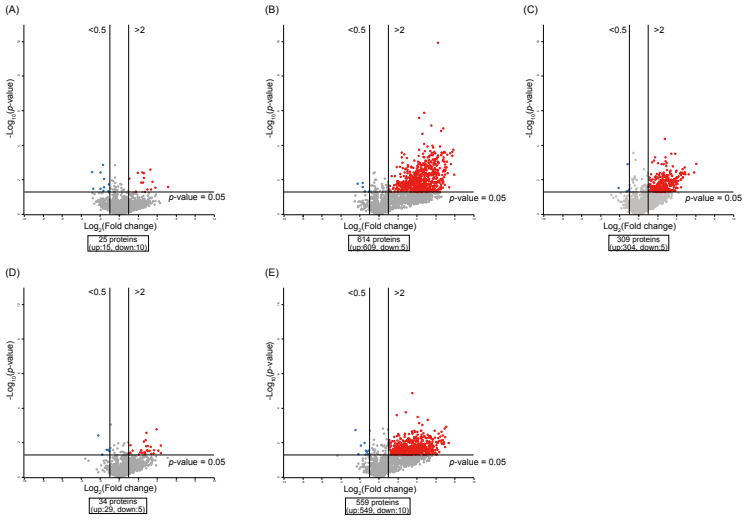

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Figure 1_Inoue et al.

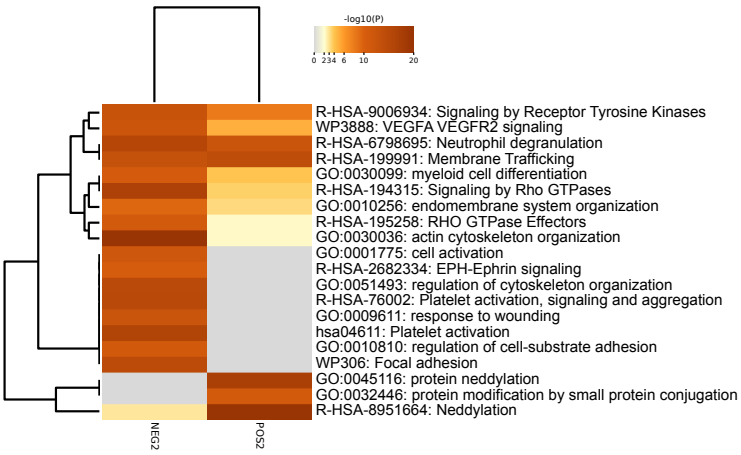

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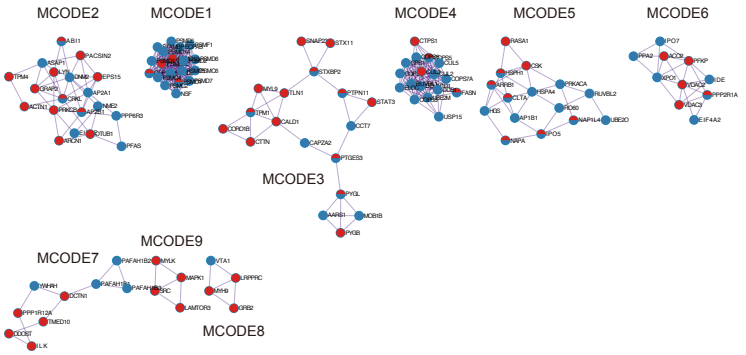
Figure 2_Inoue et al.



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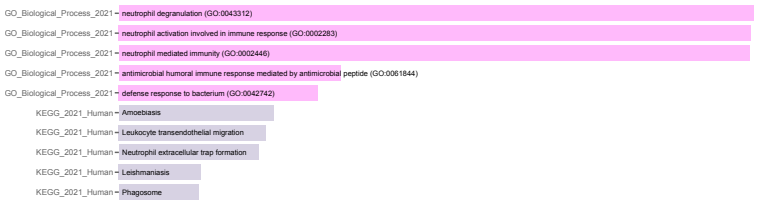

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Figure 4_Inoue et al.

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