DOI: 10.1111/all.13757

# Differential food protein-induced inflammatory responses in swine lines selected for reactivity to soy antigens

To the Editor,

Food protein-induced enterocolitis, commonly triggered by milk and soy protein, is on the rise, but immunological mechanisms of the disease are poorly understood.[1] Most animal models of food allergy utilize mice which have significant limitations in obtaining translatable information.[2] Here, we report a novel porcine model of soy-induced enteritis mimicking Food Protein-Induced Enterocolitis Syndrome (FPIES) that mainly affects neonates and young children.[3-6] An advantage of using a swine model is their relative longer growing period during which induction and assessment of food allergy responses can be studied.[7] Moreover, higher similarities in anatomy, immunology, and diet are also useful characteristics. Our model utilizes two related pig lines (L1 and L2), created by selective breeding for 8 generations based on their low (L1) and high (L2) responses to soy proteins injected in the hypodermis.[8] L2 animals develop eosinophilic enteritis similar to human FPIES upon sensitization and subsequent oral challenges with soy proteins, while L1 animals develop moderate neutrophilia in the small intestine but do not develop clinically overt inflammatory responses. Enhanced responses of soy-reactive IL-4-producing $CD4^+$ T and non-T cells were detected in the intestine of L2, whereas low levels of Th2 but normal levels of Th1 cells were detected in L1 animals.

To induce food allergy responses, L1 and L2 animals were sensitized 3 times with a soy extract and cholera toxin (i.p.), and then orally challenged with soy-containing diet (Figure 1A). L1 and L2 had different levels of inflammation in the jejunum. While both L1 and L2 developed enteritis based on leukocyte infiltration in the jejunum, L2 developed a significantly higher inflammatory response, indicated by low villus heights and high mucosal layer destruction (Figure S1A, B; Figure 1B, C), which is reminiscent of the small intestinal lesions of certain FPIES patients.[4-6] Histological examination of the inflamed jejunum tissues revealed eosinophilic infiltration (some marked by black arrows), particularly in the lamina propria area of L2 animals (Figure 1B). In contrast, mononuclear phagocytes and neutrophils (green arrows) with small numbers of eosinophils infiltrated the jejunum of soy-challenged L1 animals.

To more quantitatively examine leukocytes, we determined the frequency of the infiltrating eosinophils and neutrophils in the soy-challenged animals by flow cytometry. $SWC1^+SIRP1\alpha^+$ cells represent neutrophils, whereas $SWC1^-$ $SIRP1\alpha^+$ cells represent eosinophils in pigs.[9] The frequency of eosinophils was greatly increased in the blood and jejunum of soy-challenged L2 animals (Figur S2A; Figure 2A). In contrast, the frequency of neutrophils was increased in the jejunum of L1 animals upon soy challenge (Figure S2B).

GATA3 is a major transcription factor expressed by Th2 cells and innate type 2 lymphoid cells (ILC2). CCL11 is a chemoattractant for eosinophils. IL18 is also called interferon-gamma inducing factor and associated with Th1 responses. In line with the eosinophil response, *GATA3* and *CCL11* were highly up-regulated in the jejunum of L2, but *IL18* expression was up-regulated in the jejunum of L1 following soy challenge (Figure S3A). In addition, L2 had lower expression of *IL17A* compared to L1 (Figure S3B).

Next, we examined the levels of Th1 and Th2 effector cells. L1 has higher steady-state levels of Th1 cells in the blood. Soy challenge decreased them in the blood but slightly increased them in the jejunum (Figure S3C, D). Th2 numbers were decreased in the blood of both lines following soy challenge but were considerably increased in the MLN and the jejunum of L2 animals only (Figure S3C; Figure 2B). Overall, the Th2/Th1 ratio was high in the blood of unchallenged and in the gut tissues of challenged L2 animals (Figure S3E). Soy challenge appears to shift effector T cells, particularly Th2 cells, from the blood to gut tissues.

We also detected soy-responsive $CD4^+$ T cells and non-$CD4^+$ cells in the blood of L2 animals challenged with soy diet (Figure S4A). Only L2, but not L1, $CD4^+$, and $CD4^-$ cells underwent proliferation ex vivo in the presence of soy antigens (Figure S4A; Figure 2C). These cells expressed IL-4, but not IFN-γ, at increased levels (Figure S4B). These results confirm that L2 animals have increased numbers of soy protein-reactive Th2 cells. Non-T cells, such as innate lymphoid cells (ILCs), can also produce the Th1/2 cytokines. IL-4-, but not IFN-γ−, expressing $CD3^-$ non-T cells were also increased in the jejunum of L2 (Figure S5A, B). L2 had higher frequencies of $FoxP3^+$ T cells than L1 animals upon soy challenges (Figure S5C, D). Thus, Tregs were not quantitatively suppressed in the L2 animals.

Importantly, soy-fed L2 animals displayed retarded growth during the 20-day feeding period (Figure 2D). Flow cytometry examination of intestinal tissues revealed increased frequencies of Th2, Th1, and $FoxP3^+$ T cells in the jejunum of soy-fed L2 pigs (not shown). These results indicate that natural soy exposure through the oral route can cause adverse immune responses in the intestine of L2 animals, leading to decreased growth performance.

We have established a swine model of food allergy. This model will be particularly useful in studying food protein-induced allergy responses in the intestine. This model is unique in that it employs two swine lines with a ~12% genetic relatedness among individual animals. Therefore, this model better mimics the genetically heterogeneous human populations. The two lines were different in

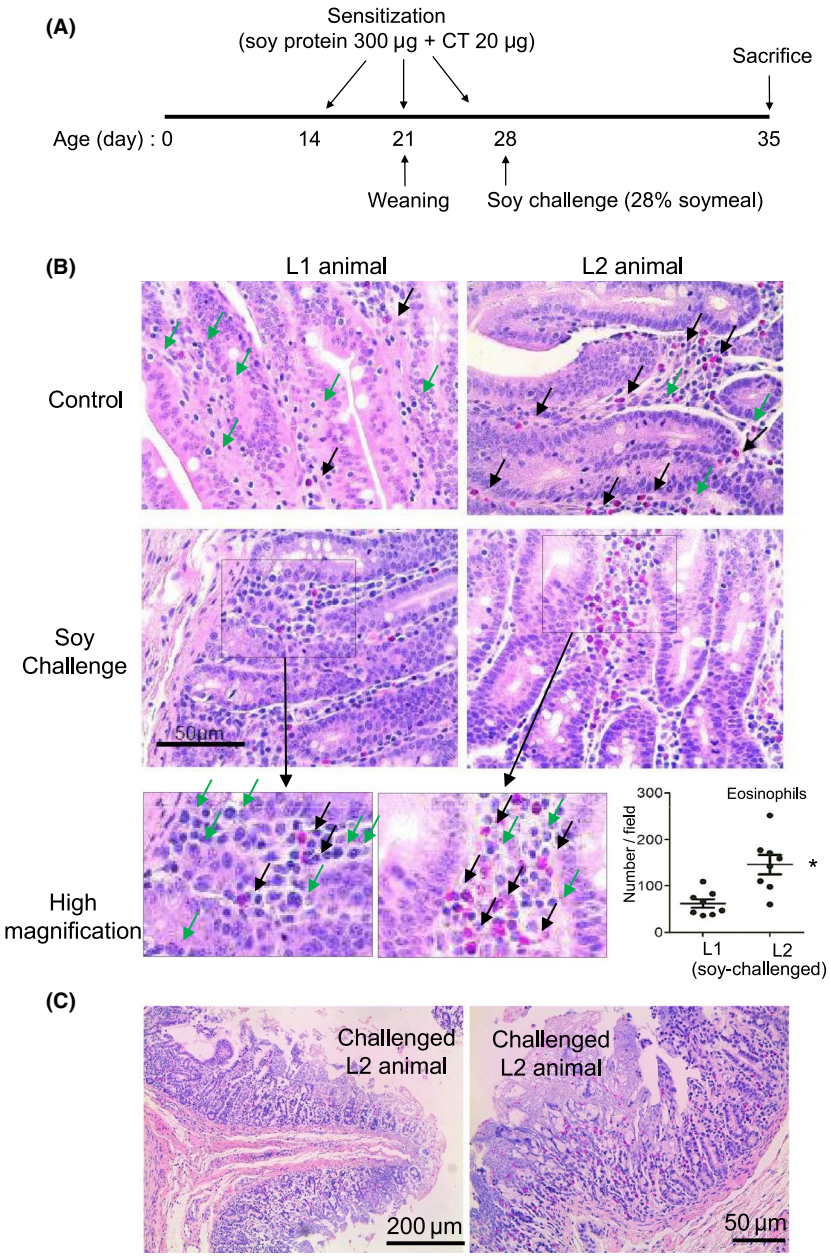


**FIGURE 1** Differential soy-induced inflammatory responses in two pig lines. A, The soy challenge group was sensitized with immunization i.p. with soy protein extract (300 µg) and cholera toxin (CT, 20 µg) and then challenged with 28% soy meal. Control groups received CT only without soy proteins and were not challenged with soy. B, Representative histological images of jejunum of L1 and L2 pigs with eosinophil counts in challenged animals. Representative eosinophils (black) and mononuclear cells/neutrophils (green) are highlighted with arrows. C, Severe cases of intestinal inflammation in L2 animals. *Significant differences ($P < 0.05$; n = 8 per group)

immune responses to soy proteins in terms of Th2 cells, eosinophils, and non-T cell IL-4 producers, which could be ILC2. Thus, the two lines represent individuals with high and low susceptibility to food protein-induced inflammatory responses. Especially, the L2 animals have heavy infiltration with eosinophils and Th2 cells in the small intestine, thus similar to the eosinophil type FPIES.[4-6] We demonstrated that the increased sensitivity to soy antigens can deteriorate animal health evidenced by retarded growth. This model will be highly useful for developing pharmaceuticals for prevention or treatments of food allergy responses. It can also serve as a testing model for developing hypo-allergenic foods including baby formulas and animal feeds. Future work includes generation of stable lines for in-depth immunological and genetic studies to understand underlying mechanisms.

## ACKNOWLEDGMENTS

We thank the Purdue animal science graduate students and staff for their helpful assistance with animal experiments and tissue preparation.

## FUNDING INFORMATION

This study was supported, in part, from grants from the Purdue College of Veterinary Medicine, University of Michgan, USDA NIFA (Grant no. 2015-67017-23140), and NIH (1R01AI121302, 1R01DK076616, and R01AI080769). CK is the Kenneth and Judy Betz Endowed Professor at the Mary H. Weiser Food Allergy Center at University of Michigan.

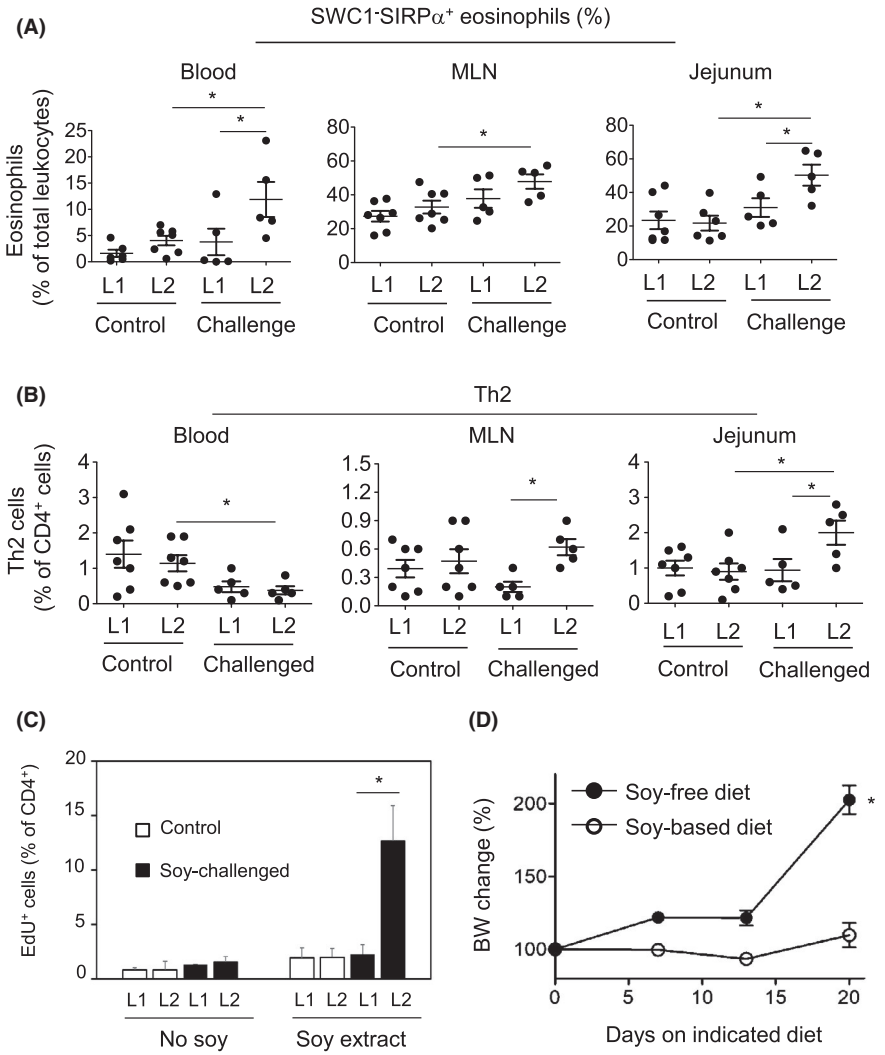


**FIGURE 2** Elevated levels of eosinophils and Th2 cells and soy diet-induced growth retardation. Frequencies of eosinophils (A) and Th2 cells (B) in L2 animals. (C) Ex vivo proliferation of peripheral blood $CD4^+$ T cells in response to soy proteins. (D) Growth rates of L2 animals on soy diet. For panels A-C, the data from animals challenged again on day 41 and euthanized on day 42 were similar to those challenged once in Figure 1A, and therefore the data were combined. For panel D, weaned L2 pigs were placed on soy-free diet for 7 days and then on soy-free or 18% soy diet for the next 21 days. *Significant differences ($P < 0.05$; n = 4-9 per group)

## CONFLICT OF INTEREST

The authors declare no financial or commercial conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DOI: 10.1111/all.13772

# The presence of virus significantly associates with chronic rhinosinusitis disease severity

To the Editor,

Chronic rhinosinusitis (CRS) is an inflammatory disorder of the paranasal sinuses occurring with and without nasal polyps (CRSsNP and CRSwNP). Although not objectively demonstrated, an initial viral insult is commonly described by patients prior to the development of CRS. If viruses were demonstrated to play a role in CRS, novel prophylactic and/or therapeutic targets might be uncovered.

Findings in previous studies investigating CRS and viruses are variable.[1-4] Possible reasons include small sample sizes, unvalidated collection methods, seasonal limitation, heterogenous CRS cohorts, and limited viral species screening. No studies to date have investigated disease severity in relation to viral presence.

We aimed to investigate the sinonasal virome of patients with CRS in relation to disease phenotype, to compare it to healthy controls, and to explore any association between more severe disease and viral presence. Cytobrush samples were taken from the sinonasal passages, and DNA/RNA extracts underwent PCR for a number of viral species and strains. The *Herpesviridae* were excluded due to their near-ubiquity in adult sinuses. Methodology details appear in the Data S1.

A total of 288 patients were recruited: 71 controls, 133 CRSsNP, and 84 CRSwNP (Table S1). Of the 288, 45 patients were virus-positive: 5 control, 27 CRSsNP, and 13 CRSwNP (Figure 1). The rate of viral positivity was significantly higher in the CRSsNP group ($P < 0.05$).

Objective disease severity scores (Lund-Mackay [LMS] and Lund-Kennedy [LKS]) revealed significantly worse disease in the CRSsNP virus-positive cohort compared with the CRSsNP virus-negative cohort ($P < 0.05$, Figure 2). No significant differences were observed in the control or CRSwNP cohorts. Subjective scores (Sino-Nasal Outcome Test 22 [SNOT-22] and Adelaide Disease Severity Score [ADSS]) revealed no difference between patients with or without virus in any of the groups (Figure 2). PCR cycle thresholds also revealed no difference between virus-positive or virus-negative individuals (Table S2). Viral species detected did not vary significantly from the previously published studies; these were largely rhinovirus and coronavirus (Tables S2 and S3 and Figure S1). Peak viral detection occurred in spring and winter; there was no significant difference in detection when analyzed by season (Figure S2).

This study identified common respiratory viruses as more prevalent in patients with CRSsNP than in controls. It is the first study to demonstrate their significant association with more severe radiological and endoscopic disease in *virus-positive* CRSsNP patients but not *virus-positive* patients with CRSwNP.

The lack of any significant difference in subjective symptom scores in any of the groups is not unexpected. The absence of correlation between subjective and objective measures of disease severity has been well documented.[5] Although the inclusion of nonrhinologic questions in the SNOT-22 score is a possible explanation, no difference was observed when using the more specific ADSS. Another possible explanation may be the timing of sampling. As most viruses tested in the assay are shed from the nasopharynx up to 3 weeks after symptom resolution, it is possible that sampling occurred either during this time or early in the infection prior to symptom development.

The viruses identified largely were consistent with those seen in previous CRS studies, with the exception that this study did not identify metapneumovirus. The main viruses observed across all cohorts were rhinovirus and coronavirus, with influenza featuring strongly in the CRS group. However, it seems likely there is no one