

# Journal Pre-proof

Oral anaphylaxis to peanut in a mouse model is associated with gut permeability but not with *Tlr4* or *Dock8* mutations

Jake A. Gertie, BS, Biyan Zhang, PhD, Elise G. Liu, MD, Laura R. Hoyt, BA, Xiangyun Yin, PhD, Lan Xu, MS, Lauren L. Long, PhD, Arielle Soldatenko, Uthaman Gowthaman, PhD, Adam Williams, PhD, Stephanie C. Eisenbarth, MD, PhD

PII: S0091-6749(21)00820-4

DOI: <https://doi.org/10.1016/j.jaci.2021.05.015>

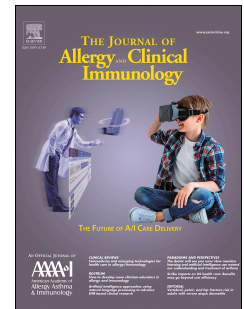
Reference: YMAI 15125

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 8 July 2020

Revised Date: 6 May 2021

Accepted Date: 10 May 2021



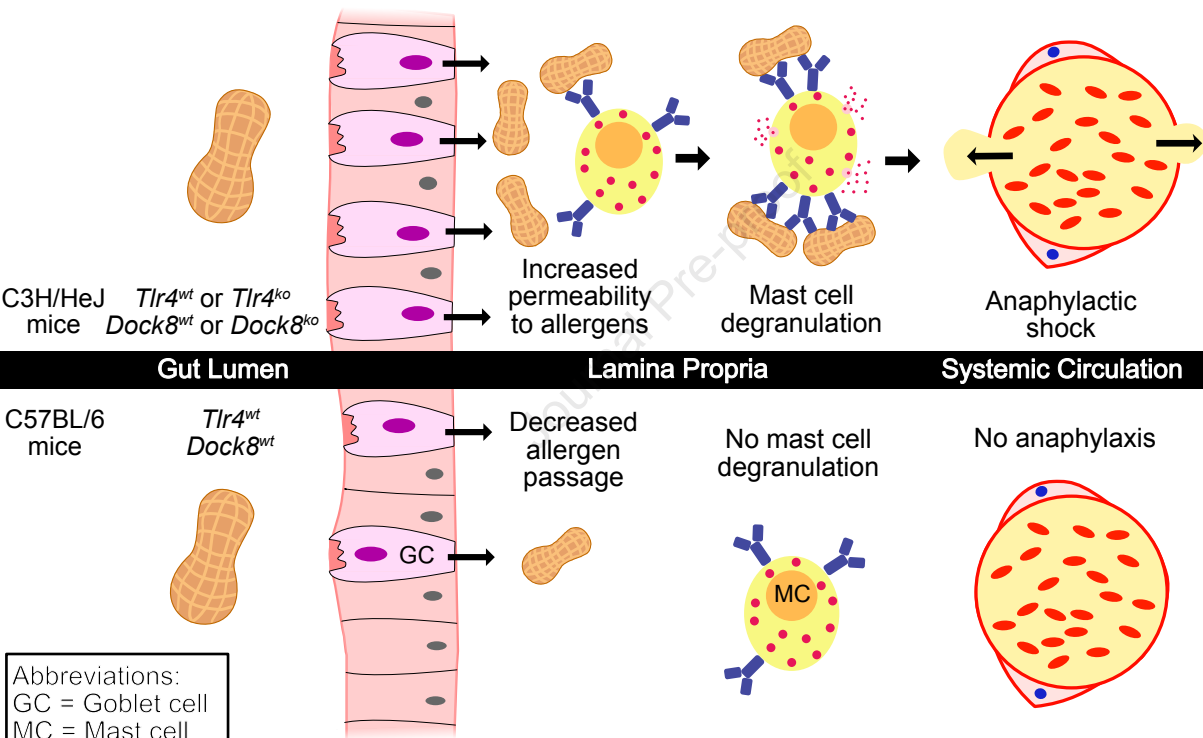
Please cite this article as: Gertie JA, Zhang B, Liu EG, Hoyt LR, Yin X, Xu L, Long LL, Soldatenko A, Gowthaman U, Williams A, Eisenbarth SC, Oral anaphylaxis to peanut in a mouse model is associated with gut permeability but not with *Tlr4* or *Dock8* mutations, *Journal of Allergy and Clinical Immunology* (2021), doi: <https://doi.org/10.1016/j.jaci.2021.05.015>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2021 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology.



associated with gut permeability but not with *Tlr4* or *Dock8* mutations



**Oral anaphylaxis to peanut in a mouse model is associated with gut permeability but not with *Tlr4* or *Dock8* mutations**

Jake A. Gertie<sup>a,b,h</sup>, BS, Biyan Zhang<sup>a,b,d,h</sup>, PhD, Elise G. Liu<sup>a,b,c</sup>, MD, Laura R. Hoyt<sup>a,b</sup>, BA, Xiangyun Yin<sup>a,b</sup>, PhD, Lan Xu<sup>a,b</sup>, MS, Lauren L. Long<sup>e</sup>, PhD, Arielle Soldatenko<sup>a,b</sup>, Uthaman Gowthaman<sup>a,b</sup>, PhD, Adam Williams<sup>e,f\*</sup>, PhD, and Stephanie C. Eisenbarth<sup>a,b,c\*</sup> MD, PhD

<sup>a</sup>Department of Laboratory Medicine, <sup>b</sup>Department of Immunobiology, <sup>c</sup>Section of Rheumatology, Allergy & Immunology, Yale University School of Medicine, New Haven, CT 06520, USA. <sup>d</sup>Singapore Immunology Network (SigN), A\*STAR, 8A Biomedical Grove, Immunos Building, Singapore 138648, Singapore., <sup>e</sup>The Jackson Laboratory for Genomic Medicine, Farmington, CT 06030, USA, <sup>f</sup>The Department of Genetics and Genome Sciences, University of Connecticut Health Center, Farmington, CT 06032, USA. <sup>g</sup>Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01605, USA. <sup>h</sup>These authors contributed equally

\*Correspondence to: Stephanie C. Eisenbarth, Yale School of Medicine, 330 Cedar St. Clinic Building 407, New Haven, CT 06520; Phone: (203) 737-4188; Fax: (203) 785-4636; e-mail: [stephanie.eisenbarth@yale.edu](mailto:stephanie.eisenbarth@yale.edu)

\*Correspondence to: Adam Williams, The Jackson Laboratory for Genomic Medicine, Department of Genetics and Genome Sciences, University of Connecticut Health Center, 10 Discovery Dr., Farmington, CT 06032; Phone: (860) 837-2351; Fax: (860) 837-2398; e-mail: [adam.williams@jax.org](mailto:adam.williams@jax.org)

**Funding**

Funding for this study was provided by Food Allergy Research & Education (FARE), The Ira & Diana Riklis Family Research Award in Food Allergy (SCE), NIAID AI136942 (SCE), a Gift from the Colton Foundation (SCE), 5T32AR007107 (EGL), NCATS Grant UL1TR001863 (EGL), National Science Scholarship from Agency for Science, Technology and Research, Singapore (BZ) and a generous philanthropic gift from Mrs. Francoise Haasch-Jones and Mr. Rhett Jones (AW).

Conflict of Interest: Outside of these grant-related research support the authors declare no other relevant conflicts of interest.

## Abstract

**Background:** The etiology of food allergy is poorly understood; mouse models are powerful systems to discover immunologic pathways driving allergic disease. C3H/HeJ mice are a widely used model for the study of peanut allergy because, unlike C57BL/6 or BALB/c mice, they are highly susceptible to oral anaphylaxis; yet the immunologic mechanism of this strain's susceptibility is not known.

**Objective:** We aimed to determine the mechanism underlying the unique susceptibility to anaphylaxis in C3H/HeJ mice. We tested the role of deleterious toll-like receptor 4 (*Tlr4*) or dedicator of cytokinesis 8 (*Dock8*) mutations in this strain as both genes have been associated with food allergy.

**Methods:** We generated C3H/HeJ mice with corrected *Dock8* or *Tlr4* alleles and sensitized and challenged with peanut. We then characterized the antibody response to sensitization, anaphylaxis response to both oral and systemic peanut challenge, gut microbiome and biomarkers of gut permeability.

**Results:** In contrast to C3H/HeJ mice, C57BL/6 mice were resistant to anaphylaxis following oral peanut challenge; however, both strains undergo anaphylaxis with intraperitoneal challenge. Restoring TLR4 or DOCK8 function in C3H/HeJ mice did not protect from anaphylaxis. Instead, we discovered enhanced gut permeability resulting in ingested allergens in the bloodstream in C3H/HeJ mice when compared with C57BL/6 mice, which correlated with increased number of goblet cells in the small intestine.

**Conclusions:** Our work highlights the potential importance of gut permeability in driving anaphylaxis to ingested food allergens and that genetic loci outside of *Tlr4* and *Dock8* are responsible for the oral anaphylactic susceptibility of C3H/HeJ mice.

## Key Messages

- C3H/HeJ mice are predisposed to oral anaphylaxis to peanut, but not due to mutations in *Tlr4* or *Dock8*.
- Gut permeability is increased in C3H/HeJ mice and is associated with oral anaphylactic susceptibility.
- Future therapy for food allergy may address changes in gut permeability.

## Capsule Summary

C3H/HeJ mice are prone to food allergy and anaphylaxis, and this is associated with increased gut permeability. Future treatment or prophylaxis of food allergy may utilize therapies targeting gut permeability.

## Keywords

C3H/HeJ  
Food allergy  
Anaphylaxis  
Peanut  
TLR4  
DOCK8  
Gut permeability

## Abbreviations used

DOCK8: Deducator of Cytokinesis 8  
TLR4: Toll-like receptor 4

- 78 *Tlr4*<sup>C3H/HeJ</sup>: C3H/HeJ mice with two mutant, non-functional copies of *Tlr4*
- 79 *Tlr4*<sup>Het</sup>: C3H/HeJ mice with one mutant, non-functional and one wild type, functional
- 80 copy of *Tlr4* resulting in mice with the ability to respond through the TLR4 receptor
- 81 *Dock8*<sup>C3H/HeJ</sup>: C3H/HeJ mice with two mutant copies of *Dock8*
- 82 *Dock8*<sup>WT</sup>: C3H/HeJ mice with two wild type copies of *Dock8*
- 83 BMDC: Bone marrow-derived dendritic cell
- 84 IgE: Immunoglobulin E
- 85 mMCP1: Mucosal mast cell protease 1
- 86 PCA: Passive cutaneous anaphylaxis
- 87
- 88

## Introduction

Food allergy and resulting anaphylaxis is a serious and potentially life-threatening condition affecting 5% of adults and 8% of children in the United States, with increasing prevalence(1). Murine models of food allergy are vital to reveal mechanisms of anaphylaxis and allergic sensitization. To model human pathophysiology, it is imperative to identify the genetic, molecular, and cellular mechanisms operational in each model system. While most mouse strains can generate IgE antibodies to food allergens in the presence of adjuvants, including the common C57BL/6 strain, they are resistant to oral anaphylaxis(2). C3H/HeJ mice have become the archetypical strain used for studies of anaphylaxis following oral challenge as it is one of the few to react systemically to oral allergen challenge(3). However, the mechanism of anaphylactic predisposition of C3H/HeJ mice remains unclear.

C3H/HeJ mice are rarely used for immunologic studies because many immunologic tools do not exist on this background. One exception is food allergy models, which have focused on this strain because they demonstrate a heightened anaphylactic propensity after oral administration of food antigens(3). A spontaneous mutation in Toll-like receptor 4 (*Tlr4*) in the C3H/HeJ genome inactivates TLR4 signaling(4). TLR4 is a Toll-like receptor that recognizes lipopolysaccharide (LPS) from gram-negative bacteria. The role of TLR4 in food allergy models is unresolved. One study ascribed microbiota changes to TLR4 inactivation in C3H/HeJ mice and suggested that this enhances peanut-specific IgE production and susceptibility to anaphylaxis(5). Indeed, changes in the microbiota have recently been associated with food allergy development in both mice and humans(6–10). However, a study of TLR4-deficiency in C3H and BALB/c mouse strains did not find a definitive link between TLR4 and food allergy(11). These studies compared the C3H/HeJ strain with other related mouse strains such as C3H/HeOuJ, or with genetically disparate mice on the C57BL/6 or BALB/c background. No study has directly compared the effects of TLR4 sufficiency and deficiency on the C3H/HeJ background.

In addition to harboring a *Tlr4* mutation, we recently discovered a point mutation in *Dock8* (dedicator of cytokinesis 8) in C3H/HeJ mice that does not affect protein production but impairs dendritic cell (DC) migration(12). DOCK8 is an intracellular

protein that regulates multiple aspects of immune cell function including regulation of the actin network and lymphocyte signaling(13,14). Patients with *DOCK8* deficiency suffer a primary immunodeficiency associated with food allergy, though its role in murine models of food allergy is untested(15,16). We therefore hypothesized that the deleterious mutation in *Dock8* on the C3H/HeJ background might drive oral anaphylactic susceptibility in this strain.

In this study, we generated C3H/HeJ mice with wildtype *Tlr4* or *Dock8* sequences and studied the effects on the allergic response. We found that neither *Dock8* nor *Tlr4* rescue in C3H/HeJ mice alters IgE production to peanut or abrogates anaphylaxis following oral challenge. We further confirmed that gut microbial diversity and composition are comparable in *Tlr4*-deficient and -sufficient C3H/HeJ mice. Having discounted two strong genetic candidates for food allergy susceptibility, we compared the phenotype of C3H/HeJ mice to the most common inbred mouse strain, C57BL/6, which are resistant to anaphylaxis following oral peanut challenge, even when both strains are cohoused and IgE levels are comparable. In contrast to oral challenge, both C3H/HeJ and C57BL/6 strains have similar anaphylactic responses to systemic peanut challenge. As bypassing the gut barrier led to anaphylaxis in both strains, we tested the steady-state integrity of the gut barrier. We discovered that C3H/HeJ mice have enhanced gut permeability to both model dextran and protein antigens when compared with C57BL/6 mice. The enhanced gut permeability in C3H/HeJ mice was associated with increased goblet cell numbers in the small intestine when compared with C57BL/6 mice.

The loss of barrier integrity is thought to allow for sensitization through the damaged epithelium, promoting allergy to food(17,18) or other misdirected immune responses such as autoimmune reactions(19). Therefore, our work suggests that aberrant gut permeability may allow for more allergen to pass into the bloodstream, leading to anaphylaxis to ingested food allergens in C3H/HeJ mice but not in other strains with less permeable gut barriers. This finding likely explains why the C3H/HeJ strain is particularly suited as a model of oral anaphylaxis. It also emphasizes that the

integrity of the intestinal barrier may have a central role in protection from food allergy and anaphylaxis.

## Methods

**Mice.** Age- and sex-matched 6-to-12 week old mice were kept on peanut- and egg- free Teklad Global 18% Protein Rodent Diet (2018S, Harlan laboratories). WT CD45.2 C57BL/6N mice were purchased from National Cancer Institute. C3H/HeJ mice were originally purchased from The Jackson Laboratory (JAX) and bred in Yale animal facilities for over 10 generations. C3H/HeJ mice from JAX were crossed with CD45.2 C57BL/6. Resulting F1 mice were then backcrossed over 10 generations with wildtype C3H/HeJ mice to generate C3H/HeJ mice with either two copies of C57BL/6-derived *Dock8* or heterozygous for C3H/HeJ and C57BL/6 *Tlr4*. Genotypes of mice were ascertained by sanger sequencing using primers for *Dock8* (F-5' CTCTTTCCCTCCAGCTGTCA 3') and (R-5' CTCGCCCCTTTCCTGTAGTT 3') and *Tlr4* (F-5' GGA CTG GGT GAG AAA TGA GC 3') and (R-5' CCA ACG GCT CTG AAT AAA GTG 3') . Littermates were used whenever possible. Prior to each experiment, mice were cohoused for at least 2 weeks such that at least one mouse of each genotype was present in each cage. All protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

**Immunization and challenge.** Mice were immunized weekly for 6 weeks by oral gavage with 5mg ground blanched peanut (Western Mixers Produce & Nuts) and 10µg cholera toxin (CT) (List Biologicals) in 200µl of 0.2M sodium bicarbonate buffer per mouse. After 6 weeks of immunization, mice were rested for one week prior to challenge. Mice were fasted for four hours before being challenged orally with 800µl (25mg protein) defatted homemade crude peanut extract(20) or intraperitoneally with 500µg crude peanut extract (Greer) in 200µL PBS. Rectal temperature was measured every 15 minutes for up to 80 minutes, clinical symptoms scores were assessed at 45 minutes (Table E1), and sera were collected for analysis 1 hour post-challenge.

**Serum collection.** Serum was collected prior to sensitization, 7 days after six weeks of peanut sensitization, and after oral challenge. Collected blood was incubated at RT for 1

hour and the resulting clot removed. Samples were centrifuged for 10 minutes at 1500 x g at RT and sera were collected and stored at -80 °C prior to analyses.

*Passive cutaneous anaphylaxis (PCA).* 15µL of sera from sensitized or naïve mice were injected intradermally into the left or right ear of a naïve mouse, respectively. 24 hours following injection, mice were challenged intravenously with 100 µg of crude peanut extract (Greer) in 1% Evans Blue solution in phosphate buffered saline (PBS). 30 minutes following challenge, mice were euthanized and ears collected for analysis. Ears were incubated at 56 °C in 500 µL formamide for 72 hours before being assayed for Evans Blue concentration at 650nm using a spectrophotometer.

*Gut Permeability PCA.* PCA was performed as described earlier with modification(21). Briefly, ~20 µL of pooled serum from peanut- and CT-sensitized C57BL/6 and C3H/HeJ mice (or serum from naïve control mice) were injected into ear pinnae of naïve C57BL/6 or C3H/HeJ recipients. 16 hours later, the recipient mice were deprived of food for 4 hours. They were then injected intravenously with 100µL 2% Evans blue dye and immediately challenged with 800µL of homemade crude peanut extract (25mg protein) through oral gavage. 45 minutes later, mice were euthanized, and ear pinnae were harvested and incubated in formamide before assaying for Evans Blue concentration as above.

*16S rRNA sequence data availability.* All sequence read data have been deposited with links to BioProject accession number PRJNA689615 in the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/>)

*Statistical analysis.* Except for 16S data, all statistical analyses were performed using Prism software (Graphpad Software). Exact tests used are denoted in figure legends. Statistical significance is defined as \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

Complete methods are available in this article's supplementary materials.

## Results

*C3H/HeJ but not C57BL/6 mice are susceptible to anaphylaxis following oral sensitization and challenge.* C57BL/6 mice were cohoused at weaning with C3H/HeJ mice for 3-4 weeks prior to experiments. Following a 6-week sensitization with peanut and CT via gavage, mice were challenged orally with peanut (Fig 1, A). We observed a decrease in temperature in C3H/HeJ mice following oral challenge, indicative of an anaphylactic response (Fig 1, B and E1, A)(22). In contrast, C57BL/6 mice did not have a drop in temperature, indicating a lack of systemic anaphylactic response. This was also reflected in the clinical presentation of the mice: mean anaphylactic score (Table E1) in C3H/HeJ mice was significantly higher than in C57BL/6 mice (Fig 1, C). As mucosal mast cell protease 1 (mMCP1) is a factor released by degranulating mast cells(23,24), it is a proxy for mast cell degranulation during anaphylaxis. Serum mMCP1 was detected in all C3H/HeJ mice following oral challenge, but not in C57BL/6 mice (Fig 1, D). The clinical response difference between these groups was not due to differences in sensitization as peanut (PN)-specific immunoglobulin E (IgE) present in pre-challenge serum samples was similar between C3H/HeJ and C57BL/6 mice (Fig 1, E). Total PN-specific IgG antibodies were moderately but not statistically higher in C3H/HeJ mice, indicating that possible protection afforded by PN-specific IgG in C57BL/6 mice was not a primary explanation for the observed phenotypic difference (Fig E1, B).

*Rescue of TLR4 function does not alter the microbiome.* As defects in TLR4 signaling have previously been suggested to contribute to the susceptibility of C3H/HeJ mice to orally-induced anaphylaxis when compared with similar mouse strains(5), we sought to correct the *Tlr4* gene on the C3H/HeJ background to definitively determine its contribution to the anaphylactic phenotype. We crossed C3H/HeJ mice with C57BL/6 mice, generating mice with a functional *Tlr4*<sup>WT</sup> allele derived from C57BL/6. We subsequently backcrossed mice with a functional *Tlr4* allele onto the C3H/HeJ background for ten generations. This resulted in C3H/HeJ mice which had one functional TLR4 gene (indicated as *Tlr4*<sup>Het</sup>) as well as littermate C3H/HeJ mice that were homozygous for the original non-functional *Tlr4* gene (indicated as *Tlr4*<sup>C3H/HeJ</sup>) (Fig 2, A). To determine that the rescue was successful, we generated bone marrow-derived dendritic cells (BMDCs) from each of these genotypes and stimulated with LPS, which is the ligand for TLR4(4). Following LPS stimulation, *Tlr4*<sup>Het</sup> but not *Tlr4*<sup>C3H/HeJ</sup> BMDCs

upregulated CD86, indicating activation of DCs with a restored WT copy of *Tlr4* (Fig 2, B), confirming intact function of the TLR4 protein in *Tlr4*<sup>Het</sup> mice.

Since TLR4 is the innate sensor for lipopolysaccharide found on gram-negative bacteria and its loss-of-function could alter microbiota composition, we performed 16S sequencing of fecal bacterial DNA from littermate *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice pre- and post- peanut sensitization to compare their bacterial composition. Similar to a previous study(25) in TLR4-deficient and -sufficient C57BL/6 mice, microbial composition was analogous between *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice as shown by the relative abundance of different bacterial classes found in the stool samples from cohoused mice (Fig 2, C and E2, A). This similarity was maintained even after peanut sensitization (Fig 2, C and E2, A). Using both principle coordinate analyses (PCoA) (Fig 2, D) and non-metric multidimensional scaling (nMDS) ordination (Fig E2, B), we further demonstrate that beta diversity of fecal bacteria was not different between *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> littermates. Statistical analysis (PERMANOVA) confirmed no significant differences in community composition by genotype. Lastly, Shannon indices revealed that alpha diversity of fecal bacteria was also similar in *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> littermates (Fig 2, E). Statistical analysis (ANOVA) confirmed that genotype did not significantly impact microbial diversity. Collectively, these data suggest that the loss-of-function mutation in TLR4 did not lead to major alterations in gut microbiota of C3H/HeJ mice at steady-state or post sensitization, allowing us to directly test the role of TLR4 on susceptibility to oral anaphylaxis without confounding effects of disparate microbiomes.

*Rescue of TLR4 function does affect susceptibility to orally-induced anaphylaxis.* We asked whether rescuing TLR4 function in the C3H/HeJ strain impacted allergic antibody production. We quantified serum PN-specific IgG1 and IgE(26) in *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice after six weeks of oral sensitization. We found no difference in PN-specific IgG1 or IgE between the two genotypes (Fig 3, A and B). We next tested the anaphylactic capacity of the IgE generated in each group through passive cutaneous anaphylaxis (PCA) challenge(27–30). PCA challenge revealed similar IgE functionality between *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice (Fig 3, C). Therefore, there was no detectable effect of TLR4 signaling rescue on the oral induction of allergic antibodies in C3H/HeJ mice. We next

challenged cohoused 6-week sensitized *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice orally with peanut. Following challenge, both sensitized groups showed significant drops in temperature when compared to challenged naïve mice, but there was no statistically significant difference between sensitized *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice (Fig 3, D). The two groups also demonstrated similar anaphylactic scores (Fig 3, E) and serum mMCP1 concentrations (Fig 3, F). Thus, there was no detectable effect of TLR4 signaling on anaphylactic susceptibility in C3H/HeJ mice.

*Rescue of DOCK8 function does not impact allergic sensitization to peanut or susceptibility to orally-induced anaphylaxis.* We previously discovered that C3H/HeJ mice harbor a point mutation in *Dock8*(12). Given the allergic predisposition of patients with mutations in *DOCK8*, we tested whether impaired DOCK8 function in C3H/HeJ mice impacted the susceptibility of this strain to food allergy. We generated C3H/HeJ mice with a functional C57BL/6 *Dock8* allele (Fig E3, A). We confirmed that DOCK8 function was restored by immunizing mice homozygous for mutated *Dock8* (*Dock8*<sup>C3H/HeJ</sup>) or homozygous for C57BL/6-derived rescued *Dock8* (*Dock8*<sup>WT</sup>) intranasally with the TLR2 ligand Pam2CysK4. Consistent with our previous work(31), we found that *Dock8*<sup>C3H/HeJ</sup> mice had a selective defect in CD11b<sup>+</sup> but not CD103<sup>+</sup> DC migration to draining lymph nodes (LNs) when compared with mice containing WT *Dock8* sequence (Fig E3, B-D).

We then investigated the effect of *Dock8* rescue on oral allergic sensitization and challenge. In 6-week peanut-sensitized *Dock8*<sup>WT</sup> and *Dock8*<sup>C3H/HeJ</sup> mice we detected similar levels of PN-specific IgG1 and IgE (Fig 4, A and B). PCA also indicated that the IgE was similarly functional (Fig 4, C). Therefore, rescue of DOCK8 function did not alter oral peanut sensitization in C3H/HeJ mice. We then challenged cohoused sensitized mice orally with peanut. Both *Dock8*<sup>WT</sup> and *Dock8*<sup>C3H/HeJ</sup> groups had significant temperature drops relative to naïve mice following challenge (Fig 4, D), but were not statistically different from each other. Further, neither the mean anaphylactic scores (Fig 4, E) nor serum mMCP1 (Fig 4, F) between the two groups were significantly different. Therefore, the rescue of DOCK8 function did not affect anaphylactic susceptibility in C3H/HeJ mice.

Both C3H/HeJ and C57BL/6 mice are susceptible to intraperitoneal challenge. Having demonstrated a difference in oral anaphylactic susceptibility between C3H/HeJ and C57BL/6 mouse strains (Fig 1), we asked if this was due to a gut-specific defect or if it was indicative of a systemic effect. To investigate this, we cohoused C3H/HeJ and C57BL/6 mice to equalize microbiota before sensitizing with peanut and CT. We then challenged these mice orally or interperitoneally with peanut antigen (Fig 5, A). As we had observed before, C3H/HeJ but not C57BL/6 mice demonstrated evidence of anaphylaxis with oral peanut challenge (Fig 5, B). However, when the same mice were challenged interperitoneally (bypassing the gut barrier) we observed temperature drop, positive anaphylactic scores and detectable serum mMCP1 levels in both C3H/HeJ and C57BL/6 strains (Fig 5, B-D and E4, A). Together these data indicate that both sensitized C3H/HeJ and C57BL/6 mice are capable of anaphylactic responses to peanut when antigen is introduced systemically, but only C3H/HeJ mice respond to oral peanut challenge. Therefore, the gut mucosa might block systemic exposure to peanut in C57BL/6 mice.

C3H/HeJ mice demonstrate enhanced translocation of luminal antigens into the circulation compared with C57BL/6 mice. We hypothesized that the difference between susceptibility to oral anaphylaxis in between C3H/HeJ and C57BL/6 mouse strains may be due to differences in gut barrier integrity. To explore this, we directly tested the intestinal permeability at steady-state of cohoused C3H/HeJ and C57BL/6 mice. As the permeability of the gut might increase following sensitization and oral challenge coincident with mMCP1 release(32), we utilized naïve mice. We orally administered FITC-conjugated dextran and screened for FITC fluorescence in the serum after two hours. We found that there was markedly increased FITC fluorescence in the serum of C3H/HeJ mice compared with C57BL/6 mice following challenge with 4kDa FITC-Dextran conjugate (Fig 6, A). 4kDa FITC-dextran is capable of traversing the epithelial barrier via multiple routes including paracellular transport, whereas larger antigens cross epithelia via transcellular pathways in the absence of overt epithelial damage(33). Therefore, the increased gut permeability we observed in C3H/HeJ mice could be due to differences in either transcellular or paracellular transport.

We next tested whether there was a difference in intestinal permeability to a larger 40kDa FITC-Dextran molecule that could only reach the systemic circulation via the transcellular route. Similar to the low molecular weight dextran, we found that only C3H/HeJ but not C57BL/6 mice transported high molecular weight dextran into the blood (Fig 6, B). As these experiments used a model sugar-fluorophore conjugate, we then investigated the gut barrier with the food protein antigen ovalbumin (OVA). We gavaged cohoused C3H/HeJ and C57BL/6 mice with OVA in solution before measuring OVA concentration in the serum 30 minutes later by ELISA. We saw a marked increase in mean OVA serum concentration in C3H/HeJ mice compared with C57BL/6 mice (Fig 6, C), demonstrating that a food protein also passages more efficiently through the gut barrier in C3H/HeJ mice than in C57BL/6 mice.

A positive correlation between the amount of antigen necessary to induce a PCA reaction in the ear and the quantity of antigen present has been previously observed(30). Therefore, we injected serum from immunized mice into the pinnae of both naïve C3H/HeJ and naïve C57BL/6 mice. We then injected Evans Blue dye intravenously into these mice before challenging orally with peanut. C3H/HeJ but not C57BL/6 mice demonstrated a local edematous response in the ear after oral challenge, demonstrating that sufficient antigen could pass through the gut into the serum of C3H/HeJ but not C57BL/6 mice (Fig 6, D). Together, these data indicate greater absorption of both carbohydrate and protein antigens from the gut lumen in C3H/HeJ than C57BL/6 mice. Therefore, the susceptibility of C3H/HeJ mice to anaphylaxis following oral antigen challenge is associated with a more permeable gut than in mice that are resistant to oral challenge such as C57BL/6 mice.

*C3H/HeJ mice have increased jejunal goblet cell numbers compared to C57BL/6 mice.*

One potential explanation for increased antigen entry into the serum from the gut is increased antigen passaging by secretory cells such as goblet cells. As goblet cells are known to form secretory antigen passages (SAPs) that traffic food antigen across the intestinal barrier(34), we performed histological analysis by periodic acid-Schiff staining to quantitate goblet cell numbers in the small intestine. We found that C3H/HeJ mice have increased number of goblet cells as compared with C57BL/6 (Fig 6, E). These

surprising findings suggest a potential cellular mechanism for gut permeability to food allergens in C3H/HeJ mice, thereby predisposing this mouse strain to oral anaphylaxis.

## Discussion

C3H/HeJ mice are a common model of oral food allergy and anaphylaxis(3). In this study, we have demonstrated an association between enhanced gut permeability to oral antigens and susceptibility to anaphylaxis. By challenging both anaphylaxis-prone C3H/HeJ and anaphylaxis-resistant C57BL/6 mice in parallel we demonstrated that sensitized C3H/HeJ and C57BL/6 mice are both susceptible to anaphylaxis following intraperitoneal challenge, but only C3H/HeJ mice are susceptible to anaphylaxis following oral challenge. This is associated with increased gut permeability, which may allow for more antigen to pass into the systemic circulation following challenge, thus triggering tissue mast cells, leading to an anaphylactic phenotype in C3H/HeJ mice when challenged orally. Further, we demonstrated that this phenotype may be due in part to increased goblet cell presence in the gut of C3H/HeJ mice, which warrants further study.

As the anaphylactic susceptibility of C3H/HeJ mice could have been due to a strain differences in microbiome, which have been previously observed in C3H/HeJ mice when compared with other non-cohoused strains and were associated with mutations in *Tlr4*(5), we characterized the gut microbial composition. The microbiome has also been implicated in other mouse and human studies of allergy(6–9,35). In mice, changes in the microbiome have been directly linked to changes in antigen passage through the epithelium during sensitization as well(36). We did not find any significant changes in microbiome composition or subsequent protection between either functional *Tlr4*<sup>Het</sup> or inactive *Tlr4*<sup>C3H/HeJ</sup> genotypes. Changes in gut permeability may instead be due to genes outside of the *Tlr4* loci that regulate the gut barrier, including specialized epithelial cells that transport antigen across from the gut lumen(34).

DOCK8 deficiency is associated with severe immunodeficiency in humans, as well as high rates of food allergy(14). As we have previously found a deleterious

mutation in the *Dock8* gene in the C3H/HeJ strain(12), we sought to determine whether this was responsible for the strain's oral anaphylactic susceptibility. After correcting the *Dock8* gene on the C3H/HeJ background, we did not see demonstrable protection against anaphylaxis. The deleterious mutation in the C3H/HeJ strain is a single point mutation that hampers DOCK8 function, but does not eliminate DOCK8 protein(12), as opposed to a variety of mutations found in human cases of DOCK8 deficiency that often result in complete DOCK8 loss(37). Therefore, although the mutation in *Dock8* in C3H/HeJ mice is not responsible for the strain's susceptibility to anaphylaxis, total loss of DOCK8 function in human patients predisposes an individual to other aspects of food allergy through loss of tolerance and aberrant IgE production (14,16,21,38).

Food allergy is driven by complex interactions of multiple environmental and genetic risk factors(1). Different environments may lead to drastically different results. Indeed, C3H/HeJ mice in some facilities, but not all, demonstrate this unique susceptibility to oral anaphylaxis(5,11,39–43). Recent studies of outbred mice to identify genetic risk factors for anaphylaxis to peanut observed that C3H/HeJ mice did not mount an anaphylactic response, while an outbred strain, CC027, did(10,40). Although the genes underlying this phenotype have not yet been identified, the differences between this study and our own in the same inbred strain of C3H/HeJ mice highlight the impact of environment on susceptibility to food allergy. It also highlights that food allergy is multi-factorial and that multiple different possible perturbations of the immune system can result in disease.

Secretory cells such as goblet cells have been implicated as conduits for passage of food antigens in the gut(34). The observation that C3H/HeJ have more goblet cells and the association between oral anaphylactic susceptibility in C3H/HeJ mice and increased gut permeability to model food antigens such as OVA, invites further study towards mechanistic understanding of gut barrier defects or alterations in luminal antigen sampling. This has the promise of identifying causative genetic pathways as well as cellular mechanistic aspects underlying the risk for food allergy. In addition, other aspects of mucosal immunity that could impact gut permeability such as IgA levels, tight junction integrity and cellular passages remain to be

investigated(18,44). Further studies of both murine models and clinical cases should be mindful of physical alterations in gut barrier integrity that could lead to increased susceptibility to oral anaphylaxis.

## Acknowledgements

We would like to thank Cecilia Berin, Cathryn Nagler, Jennifer S. Chen, Sam Olyha, Kenneth Zhou, and Emily Siniscalco for helpful discussion and critical review of the manuscript. We also would like to thank Zuri Sullivan, William Khoury-Hanold, Joan Goldstein and Mark Firla for technical assistance.

## References

1. Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *Journal of Allergy and Clinical Immunology*. 2014 Feb 1;133:291-307.e5.
2. Smit JJ, Willemsen K, Hassing I, Fiechter D, Storm G, van Bloois L, et al. Contribution of Classic and Alternative Effector Pathways in Peanut-Induced Anaphylactic Responses. *PLoS One* [Internet]. 2011
3. Liu T, Navarro S, Lopata AL. Current advances of murine models for food allergy. *Molecular Immunology*. 2016 Feb 1;70:104–17.
4. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998 Dec 11;282(5396):2085–8.
5. Bashir MEH, Louie S, Shi HN, Nagler-Anderson C. Toll-Like Receptor 4 Signaling by Intestinal Microbes Influences Susceptibility to Food Allergy. *The Journal of Immunology*. 2004 Jun 1;172(11):6978–87.
6. Abdel-Gadir A, Stephen-Victor E, Gerber GK, Noval Rivas M, Wang S, Harb H, et al. Microbiota therapy acts via a regulatory T cell MyD88/ROR $\gamma$ t pathway to suppress food allergy. *Nature Medicine*. 2019 Jul;25(7):1164–74.
7. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, et al. Infant gut microbiota and food sensitization: associations in the first year of life. *Clinical & Experimental Allergy*. 2015;45(3):632–43.
8. Feehley T, Plunkett CH, Bao R, Choi Hong SM, Culleen E, Belda-Ferre P, et al. Healthy infants harbor intestinal bacteria that protect against food allergy. *Nature Medicine*. 2019 Mar;25(3):448–53.

9. Rachid R, Chatila TA. The role of the gut microbiota in food allergy. *Current Opinion in Pediatrics*. 2016 Dec;28(6):748–53.
10. Smeekens JM, Johnson-Weaver BT, Hinton AL, Azcarate-Peril MA, Moran TP, Immormino RM, et al. Fecal IgA, Antigen Absorption, and Gut Microbiome Composition Are Associated With Food Antigen Sensitization in Genetically Susceptible Mice. *Front Immunol* [Internet]. 2021 [cited 2021 Feb 23];11. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.599637/full>
11. Berin MC, Zheng Y, Domaradzki M, Li X-M, Sampson HA. Role of TLR4 in allergic sensitization to food proteins in mice. *Allergy*. 2006 Jan;61(1):64–71.
12. Krishnaswamy JK, Singh A, Gowthaman U, Wu R, Gorrepati P, Sales Nascimento M, et al. Coincidental loss of DOCK8 function in NLRP10-deficient and C3H/HeJ mice results in defective dendritic cell migration. *Proc Natl Acad Sci USA*. 2015 Mar 10;112(10):3056–61.
13. Kearney CJ, Randall KL, Oliaro J. DOCK8 regulates signal transduction events to control immunity. *Cell Mol Immunol*. 2017 May;14(5):406–11.
14. Su HC, Jing H, Angelus P, Freeman AF. Insights into immunity from clinical and basic science studies of DOCK8 immunodeficiency syndrome. *Immunol Rev*. 2019 Jan;287(1):9–19.
15. Engelhardt KR, Gertz EM, Keles S, Schäffer AA, Sigmund EC, Glocker C, et al. The extended clinical phenotype of 64 patients with DOCK8 deficiency. *J Allergy Clin Immunol*. 2015 Aug;136(2):402–12.
16. Boos AC, Hagl B, Schlesinger A, Halm BE, Ballenberger N, Pinarci M, et al. Atopic dermatitis, STAT3- and DOCK8-hyper-IgE syndromes differ in IgE-based sensitization pattern. *Allergy*. 2014;69(7):943–53.
17. Venkataraman D, Soto-Ramírez N, Kurukulaaratchy RJ, Holloway JW, Karmaus W, Ewart SL, et al. Filaggrin loss-of-function mutations are associated with food allergy in childhood and adolescence. *Journal of Allergy and Clinical Immunology*. 2014 Oct 1;134(4):876–882.e4.
18. Wesemann DR, Nagler CR. The Microbiome, Timing, and Barrier Function in the Context of Allergic Disease. *Immunity*. 2016 Apr 19;44(4):728–38.
19. Peters A, Wekerle H. Autoimmune diabetes mellitus and the leaky gut. *PNAS*. 2019 Jul 23;116(30):14788–90.
20. Chen JS, Grassmann JDS, Gowthaman U, Olyha SJ, Simoneau T, Berin MC, et al. Flow cytometric identification of Tfh13 cells in mouse and human. *Journal of Allergy and Clinical Immunology*. 2021 Feb 1;147(2):470–83.
21. Gowthaman U, Chen JS, Zhang B, Flynn WF, Lu Y, Song W, et al. Identification of a T follicular helper cell subset that drives anaphylactic IgE. *Science* [Internet]. 2019 Aug 30 [cited 2020 Jul 1];365(6456). Available from: <https://science.sciencemag.org/content/365/6456/eaaw6433>

- 486 22. Li X-M, Serebrisky D, Lee S-Y, Huang C-K, Bardina L, Schofield BH, et al. A murine model  
487 of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human  
488 responses. *Journal of Allergy and Clinical Immunology*. 2000 Jul 1;106(1):150–8.
- 489 23. Benedé S, Berin MC. Mast cell heterogeneity underlies different manifestations of food  
490 allergy in mice. *PLOS ONE*. 2018 Jan 25;13(1):e0190453.
- 491 24. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. *Nature Reviews*  
492 *Immunology*. 2014 Jul;14(7):478–94.
- 493 25. Sodhi CP, Neal MD, Siggers R, Sho S, Ma C, Branca MF, et al. Intestinal epithelial Toll-like  
494 receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in  
495 mice. *Gastroenterology*. 2012 Sep;143(3):708–718.e5.
- 496 26. Schülke S, Albrecht M. Mouse Models for Food Allergies: Where Do We Stand? *Cells*.  
497 2019 Jun 6;8(6):546.
- 498 27. Xiong H, Dolpady J, Wabl M, Curotto de Lafaille MA, Lafaille JJ. Sequential class switching  
499 is required for the generation of high affinity IgE antibodies. *J Exp Med*. 2012 Feb  
500 13;209(2):353–64.
- 501 28. Finkelman FD, Rothenberg ME, Brandt EB, Morris SC, Strait RT. Molecular mechanisms  
502 of anaphylaxis: Lessons from studies with murine models. *Journal of Allergy and Clinical*  
503 *Immunology*. 2005 Mar 1;115(3):449–57.
- 504 29. Lehrer SB. Role of mouse IgG and IgE homocytotropic antibodies in passive cutaneous  
505 anaphylaxis. *Immunology*. 1977 Apr;32(4):507–11.
- 506 30. Ovary Z. Passive Cutaneous Anaphylaxis in the Mouse. *The Journal of Immunology*. 1958  
507 Oct 1;81(4):355–7.
- 508 31. Krishnaswamy JK, Gowthaman U, Zhang B, Mattsson J, Szeponik L, Liu D, et al. Migratory  
509 CD11b+ conventional dendritic cells induce T follicular helper cell-dependent antibody  
510 responses. *Science Immunology* [Internet]. 2017 Dec 1 [cited 2020 Jul 8];2(18). Available  
511 from: <https://immunology.sciencemag.org/content/2/18/eaam9169>
- 512 32. Gurish MF, Austen KF. Developmental Origin and Functional Specialization of Mast Cell  
513 Subsets. *Immunity*. 2012 Jul 27;37(1):25–33.
- 514 33. Ménard S, Cerf-Bensussan N, Heyman M. Multiple facets of intestinal permeability and  
515 epithelial handling of dietary antigens. *Mucosal Immunol*. 2010 May;3(3):247–59.
- 516 34. Noah TK, Knoop KA, McDonald KG, Gustafsson JK, Waggoner L, Vanoni S, et al. IL-13–  
517 induced intestinal secretory epithelial cell antigen passages are required for IgE-mediated  
518 food-induced anaphylaxis. *Journal of Allergy and Clinical Immunology*. 2019 Oct  
519 1;144(4):1058–1073.e3.
- 520 35. Sicherer SH, Sampson HA. Food allergy: A review and update on epidemiology,  
521 pathogenesis, diagnosis, prevention, and management. *Journal of Allergy and Clinical*  
522 *Immunology*. 2018 Jan 1;141(1):41–58.

36. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, et al. Commensal bacteria protect against food allergen sensitization. *PNAS*. 2014 Sep 9;111(36):13145–50.
37. Su HC, Jing H, Zhang Q. DOCK8 deficiency. *Annals of the New York Academy of Sciences*. 2011;1246(1):26–33.
38. Singh AK, Eken A, Hagin D, Komal K, Bhise G, Shaji A, et al. DOCK8 regulates fitness and function of regulatory T cells through modulation of IL-2 signaling. *JCI Insight* [Internet]. 2017 Oct 5 [cited 2020 Jul 5];2(19). Available from: <https://insight.jci.org/articles/view/94275>
39. López-Expósito I, Chicón R, Belloque J, López-Fandiño R, Berin MC. In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of  $\beta$ -lactoglobulin are immunologically inert. *Journal of Dairy Science*. 2012 Feb 1;95(2):541–8.
40. Orgel K, Smeekens JM, Ye P, Fotsch L, Guo R, Miller DR, et al. Genetic diversity between mouse strains allows identification of the CC027/GeniUnc strain as an orally reactive model of peanut allergy. *Journal of Allergy and Clinical Immunology*. 2019 Mar;143(3):1027-1037.e7.
41. Morafo V, Srivastava K, Huang C-K, Kleiner G, Lee S-Y, Sampson HA, et al. Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *Journal of Allergy and Clinical Immunology*. 2003 May 1;111(5):1122–8.
42. Smarr CB, Hsu C-L, Byrne AJ, Miller SD, Bryce PJ. Antigen-Fixed Leukocytes Tolerize Th2 Responses in Mouse Models of Allergy. *The Journal of Immunology*. 2011 Nov 15;187(10):5090–8.
43. Capobianco F, Butteroni C, Barletta B, Corinti S, Afferni C, Tinghino R, et al. Oral sensitization with shrimp tropomyosin induces in mice allergen-specific IgE, T cell response and systemic anaphylactic reactions. *Int Immunol*. 2008 Aug 1;20(8):1077–86.
44. Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke J-D, Serino M, et al. Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterology*. 2014 Nov 18;14(1):189.

## **Figure Legends**

**Figure 1. C3H/HeJ mice, but not C57BL/6 mice are susceptible to anaphylaxis following oral sensitization and challenge.** Cohoused C3H/HeJ mice and C57BL/6 mice were sensitized with peanut and CT for six weeks before being orally challenged with defatted peanut protein. (A) Sensitization model. Peanuts represent an oral immunization with peanut and CT. (B) Temperature change following oral challenge. (C) Anaphylactic scores 45 minutes following oral challenge. ELISA quantification of (D) Serum-mucosal mast cell protease 1 (mMCP1) one hour following oral challenge and

(E) peanut-specific IgE in the serum following sensitization. For each experiment, each group was assessed for normality via normal quantile plots before being compared using (B) one-way ANOVA or (C,D,E) Student's t-test. Dotted lines represent limits of detection. AU denotes arbitrary units assigned to ELISA standards. Error bars represent SEM for 4-5 mice per group. \* $P < 0.05$ , \*\*\* $P < 0.001$ ; ns, not significant, n.d., not detected. 1 representative experiment out of 3 (B,C,E) or 2 (D) independent experiments.

**Figure 2. Rescue of TLR4 function does not alter the microbiota.** The *Tlr4* point mutation in C3H/HeJ mice was rescued by crossing to C57BL/6 mice with a WT *Tlr4* gene. Mice were selectively crossed back onto the C3H/HeJ background for 10 generations, preserving the functional *Tlr4* gene. (A) Chromatogram of the *Tlr4* point mutation in C3H/HeJ mice (*Tlr4*<sup>C3H/HeJ</sup>) and heterozygous with the WT C57BL/6 *Tlr4* gene (*Tlr4*<sup>Het</sup>). (B) Representative histogram and quantification of surface CD86 expression on LPS-stimulated bone marrow-derived dendritic cells. (C) Relative bacterial abundance in stool between pre-immunization ("Naïve") and post-immunization ("Immunized") *Tlr4* sufficient (*Tlr4*<sup>Het</sup>) and deficient (*Tlr4*<sup>C3H/HeJ</sup>) C3H/HeJ cohoused littermates. Each bar represents pooled stool samples from a single mouse. Samples are color-coded by bacterial composition at class level (D) Principal coordinate analysis (PCoA), of fecal bacteria from *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> cohoused littermates, using the proportionally normalized Bray-Curtis dissimilarity matrix. (E) Shannon diversity indices for measurement of alpha diversity in bacterial fractions isolated from stool samples of cohoused *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> littermates pre- and post-peanut sensitization. Each circle represents pooled stool samples from a single mouse in (D) and (E). *Tlr4*<sup>Het</sup> and *Tlr4*<sup>C3H/HeJ</sup> mice were sensitized and challenged orally with peanut as in (Fig 1). Error bars in (B) represent SEM for 4 mice per group (\*\*\* $P < 0.001$ ). 1 representative experiment out of 2 (B) or pooled data from two independent experiments (C-E).

**Figure 3. Rescue of TLR4 function does not affect susceptibility to orally-induced anaphylaxis.** Representative ELISA quantification of peanut-specific (A) IgG1 or (B) IgE in serum one week after the final immunization with peanut and CT. Data from one representative experiment of three. Dotted lines represent limit of detection. (C) Evans

blue dye extravasation after passive cutaneous anaphylaxis (PCA) assay performed by transferring day seven post-6<sup>th</sup> immunization sera from *Tlr4*<sup>Het</sup> or *Tlr4*<sup>C3H/HeJ</sup> mice into naïve C3H/HeJ mice and challenging intravenously with peanut and 1% Evans blue in PBS. Data from 1 representative experiment out of 2. The dotted line represents reading from a naïve mouse after challenge. (D) Temperature change following oral peanut challenge from 1 representative experiment out of 3 independent experiments. (E) Pooled anaphylactic scores 45 minutes following oral peanut challenge from 3 independent experiments. ELISA quantification of (F) serum-mucosal mast cell protease 1 (mMCP1) one hour post-challenge pooled from 2 independent experiments. For each experiment, groups were assessed for normality via normal quantile plots before being compared with either (D) 2-way ANOVA with Tukey-corrected multiple comparisons by timepoint or (A-C,E,F) Student's t-test. Error bars represent (D) SD or (A-C,E,F). SEM for 4-15 mice per group. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, \*\*\*\**P*<0.0001; *ns*, not significant.

**Figure 4. Rescue of DOCK8 function does not impact allergic sensitization to peanut or susceptibility to orally-induced anaphylaxis.**

The *Dock8* point mutation was corrected to produce C3H/HeJ mice which were homozygous for the WT C57BL/6 *Dock8* allele or homozygous for the deficient C3H/HeJ *Dock8* allele. ELISA quantification of peanut-specific (A) IgG1 or (B) IgE in serum one week after final immunization with peanut and CT. Dotted line represents limit of detection. (C) Evans blue dye extravasation after passive cutaneous anaphylaxis (PCA) assay. The dotted line represents reading from a naïve mouse after challenge. Mice were sensitized then challenged orally with peanut as in (Fig 1). (D) Temperature change following oral peanut challenge of *Dock8*<sup>C3H/HeJ</sup> and *Dock8*<sup>WT</sup> C3H/HeJ mice. (E) Anaphylactic scores from mice 45 minutes post-challenge. (F) Serum-mucosal mast cell protease 1 (mMCP1) levels one hour following challenge. For each experiment, groups were assessed for normality via normal quantile plots before being compared with either (D) 2-way ANOVA with Tukey-corrected multiple comparisons per timepoint, or (A,B,C,E,F) Student's t-test. Error bars represent (D) SD or (A,B,C,E,F) SEM for 4-15 mice per group. \**P*<0.05,; *ns*, not significant. 1 representative experiment of 3 (A, B, D)

or 2 (C) independent experiments. (E, F) Pooled data from 2 of 3 independent experiments.

**Figure 5. C57BL/6 mice are susceptible to intraperitoneally-induced anaphylaxis, but not orally-induced anaphylaxis.**

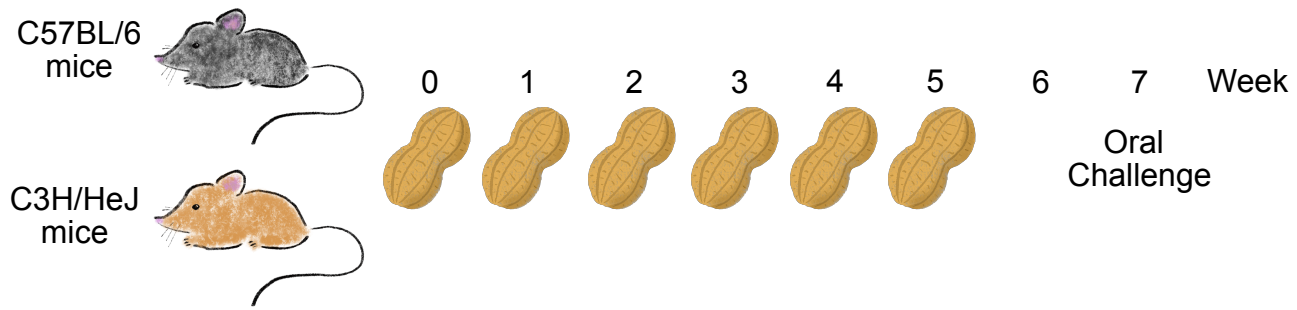
Wildtype C3H/HeJ and C57BL/6 mice were cohoused for 2 weeks prior to experimentation. Mice were orally sensitized for six weeks with peanut and CT. (A) Sensitization and challenge model. Peanuts represent an oral immunization with peanut and CT. (B) Temperature change 45 minutes following either 500µg intraperitoneal (IP) or 25mg oral (PO) peanut challenge of sensitized C3H/HeJ or C57BL/6 mice; temperature change was taken at 15 minutes for mice that succumbed during challenge (marked with an open symbol). (C) Anaphylactic scores from mice 45 minutes post-IP challenge; open symbols are mice that died during challenge. (D) Serum mMCP1 levels one hour following IP challenge were assayed by ELISA. For each experiment, groups were assessed for normality via normal quantile plots before being compared with Student's t-test. Dotted lines represent limits of detection. Error bars represent SEM for 3-7 mice per group.  $**P<0.01$ ,  $***P<0.001$ ; *ns*, not significant. 1 representative experiment of 3 (B and C) or pooled data from two (D) independent experiments of mice that survived challenge.

**Figure 6. Increased oral anaphylaxis susceptibility of C3H/HeJ mice is associated with increased gut permeability.**

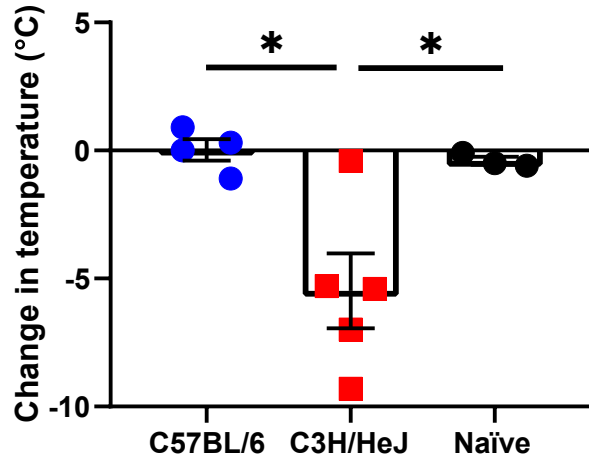
Cohoused naïve C3H/HeJ and C57BL/6 mice were administered FITC-dextran orally. (A) FITC fluorescence detected in serum 2 hours following 4 kDa and (B) 40 kDa FITC-dextran administration to cohoused naïve mice. Dotted line represents the average naïve reading (autofluorescence). (C) ELISA quantification of serum OVA concentration 30 minutes following oral gavage with solubilized OVA. Dotted line represents the reading in naïve mice (no OVA administration), which is at the limit of detection of the assay. (D) Quantitation of Evans blue dye extravasation in ear after passive cutaneous anaphylaxis (PCA) assay in mice orally challenge with 25mg peanut protein. The dotted line represents the post-challenge reading from mice receiving naïve serum (without antigen-specific IgE). (E) Representative image of Periodic acid-Schiff stain for goblet

650 cells (dark purple cells) in C57BL/6 and C3H/HeJ jejunum and quantification. Scale bar  
651 represents 200 $\mu$ m. For each experiment, groups were assessed for normality via  
652 normal quantile plots before being compared with Student's t-test. Error bars = SEM.  
653 \* $P < 0.05$ , \*\* $P < 0.01$ . (A-E) Pooled data from 2 independent experiments with 3-5 mice  
654 per group.

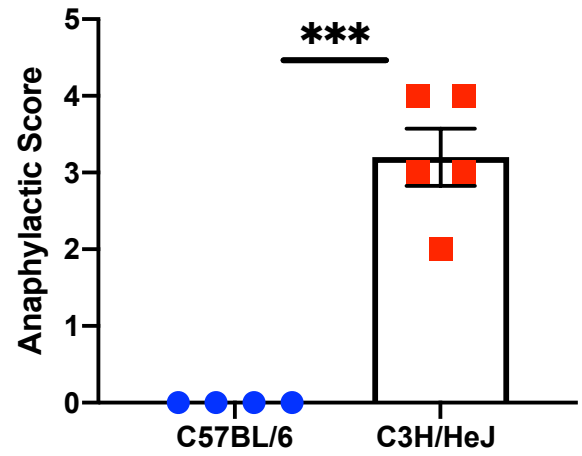
A



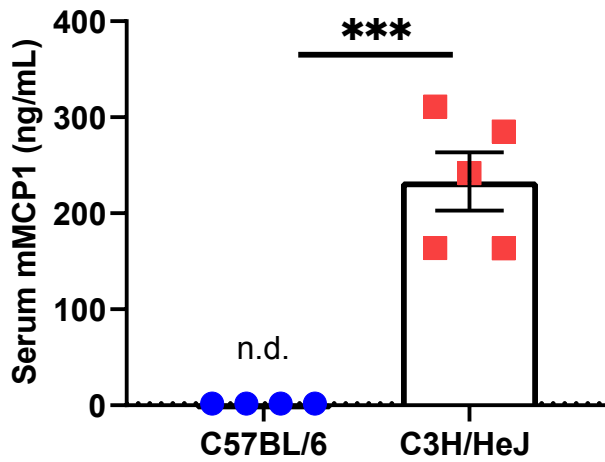
B



C



D



E

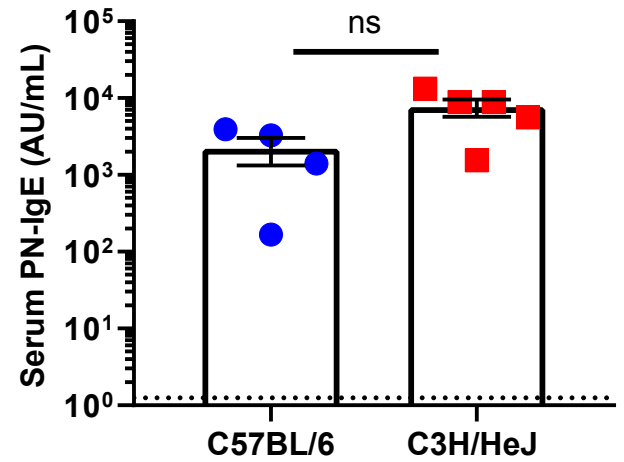


Figure 2

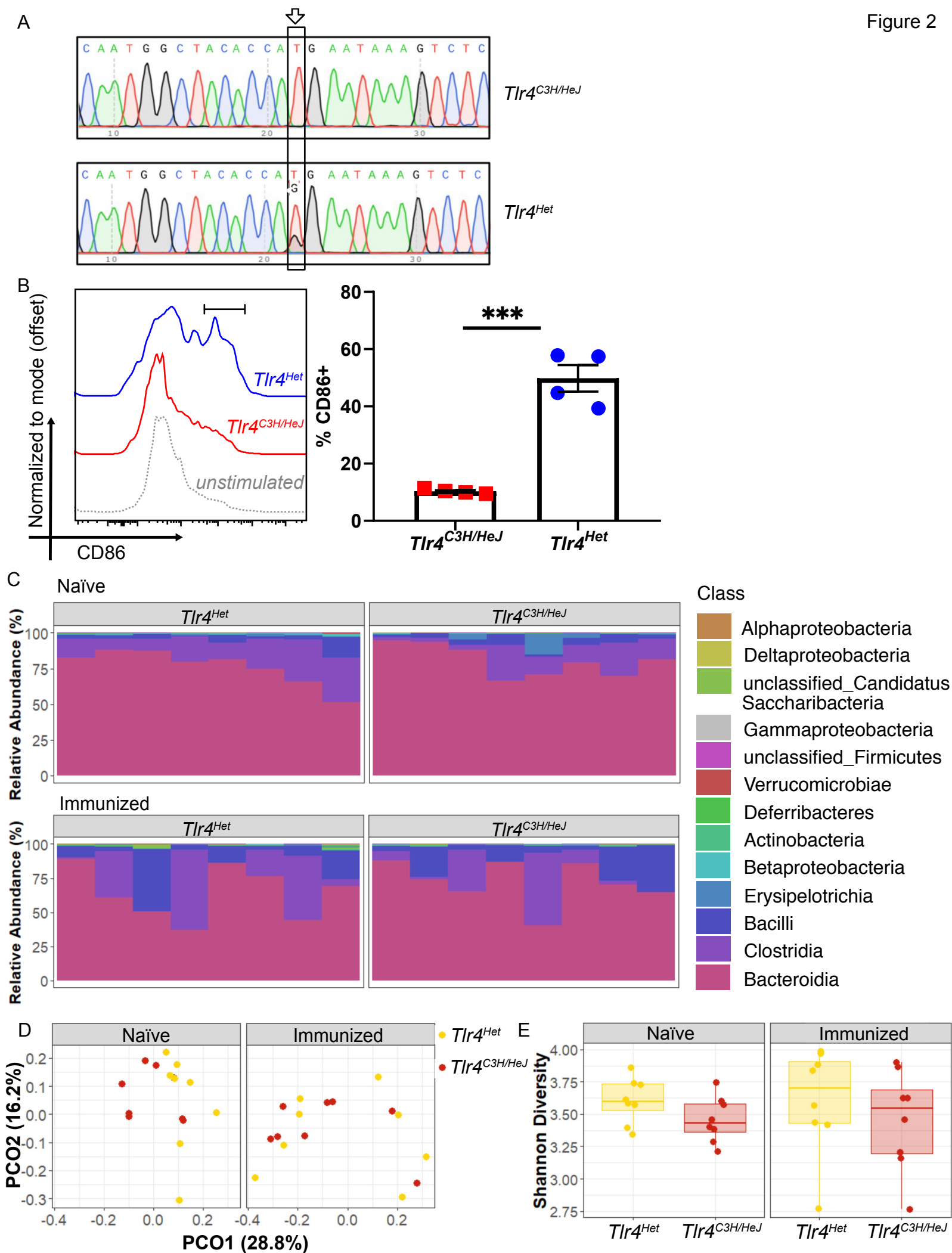
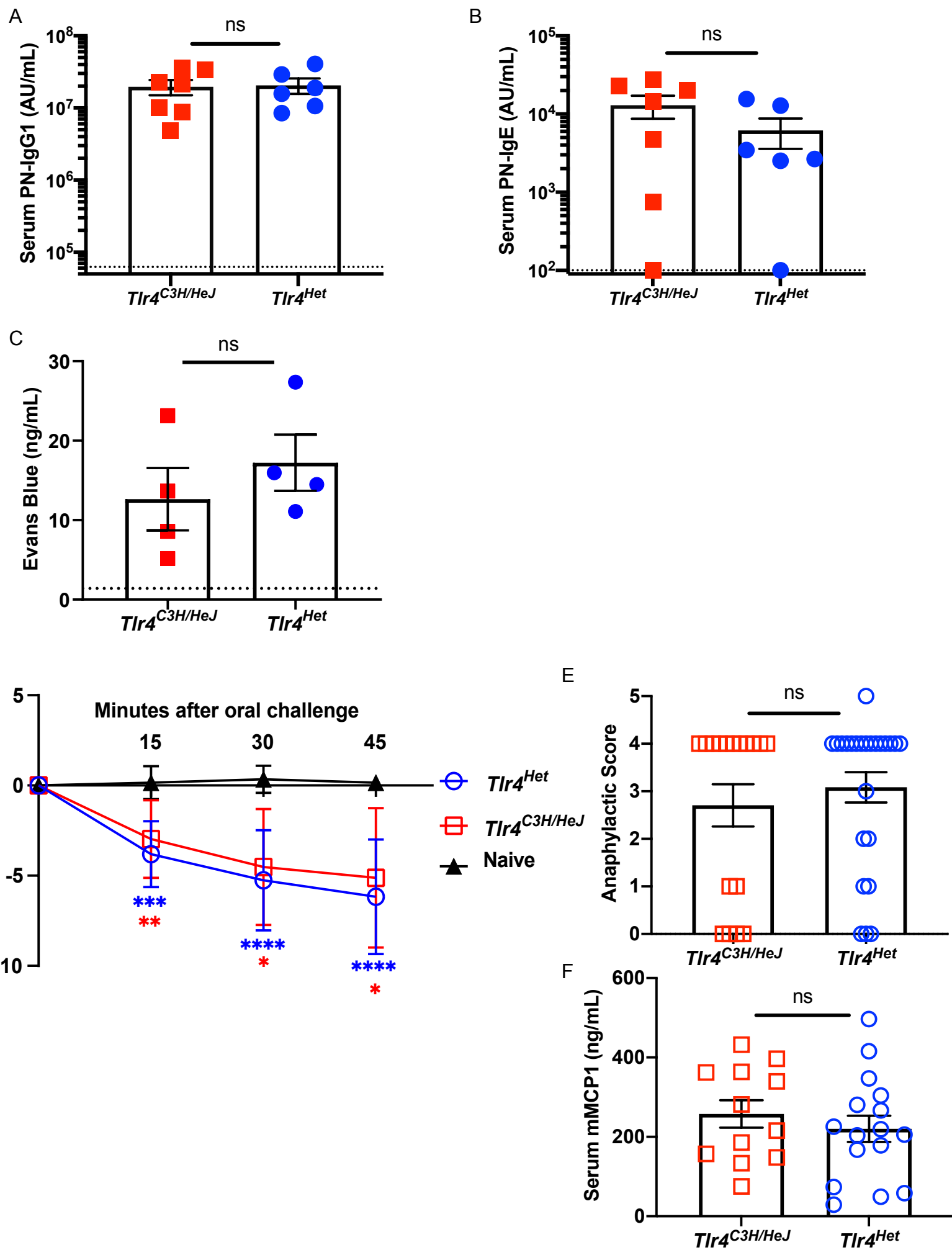


Figure 3



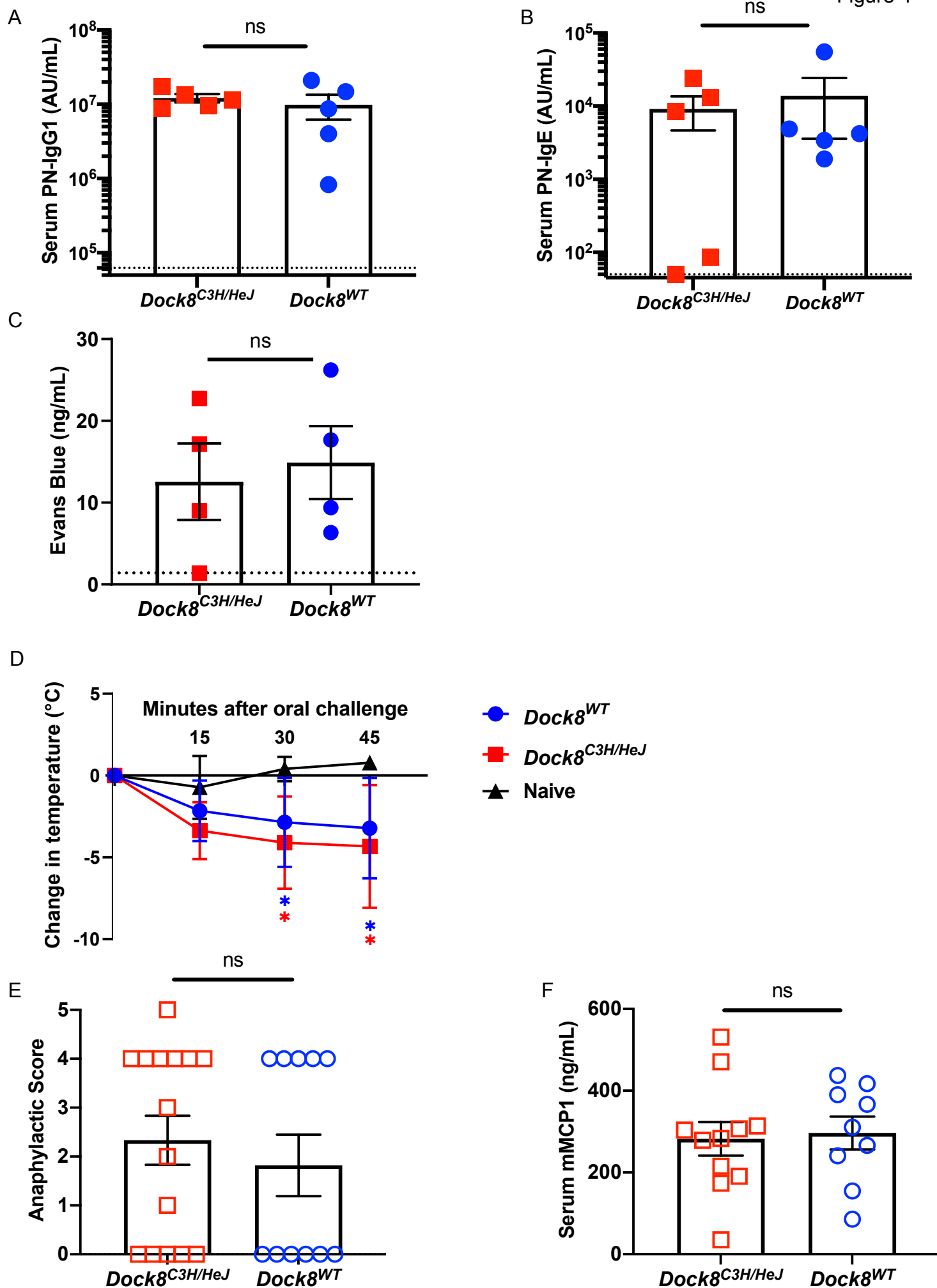


Figure 5

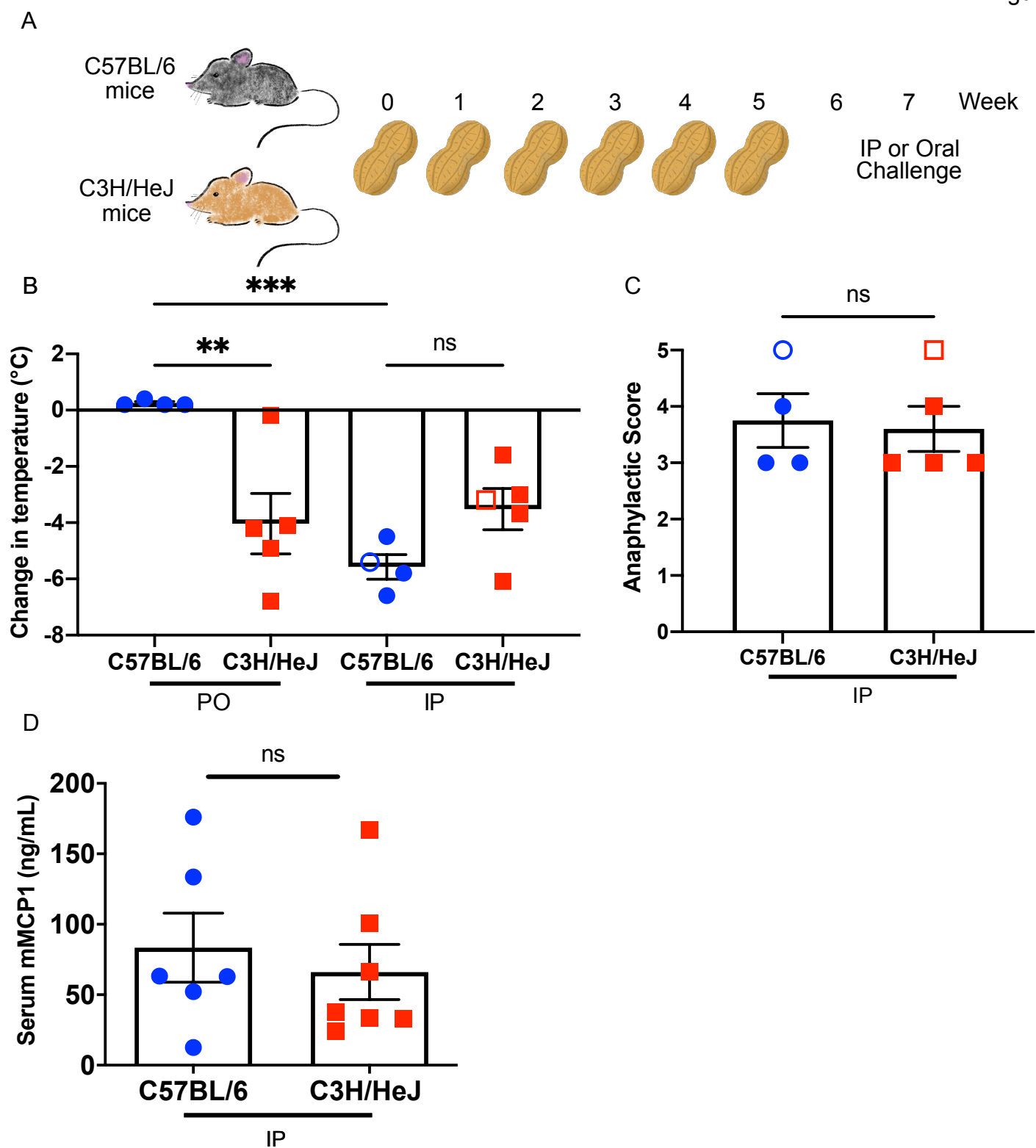


Figure 6

