Genetic diversity between mouse strains allows identification of the CC027/GeniUnc strain as an orally reactive model of peanut allergy

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Background: Improved animal models are needed to understand the genetic and environmental factors that contribute to food allergy.
Objective: We sought to assess food allergy phenotypes in a genetically diverse collection of mice.
Methods: We selected 16 Collaborative Cross (CC) mouse strains, as well as the classic inbred C57BL/6J, C3H/HeJ, and BALB/cJ strains, for screening. Female mice were sensitized to peanut intragastrically with or without cholera toxin and then challenged with peanut by means of oral gavage or intraperitoneal injection and assessed for anaphylaxis. Peanut-specific immunoglobulins, T-cell cytokines, regulatory T cells, mast cells, and basophils were quantified.
Results: Eleven of the 16 CC strains had allergic reactions to intraperitoneal peanut challenge, whereas only CC027/GeniUnc mice reproducibly experienced severe symptoms after oral food challenge (OFC). CC027/GeniUnc, C3H/HeJ, and C57BL/6J mice all mounted a T_H2 response against peanut, leading to production of IL-4 and IgE, but only the CC027/GeniUnc mice reacted to OFC. Orally induced anaphylaxis in CC027/GeniUnc mice was correlated with serum levels of Ara h 2 in circulation but not with allergen-specific IgE or mucosal mast cell protease 1 levels, indicating systemic allergen absorption is important for anaphylaxis through the gastrointestinal tract. Furthermore, CC027/GeniUnc, but not C3H/HeJ or BALB/cJ, mice can be sensitized in the absence of cholera toxin and react on OFC to peanut.
Conclusions: We have identified and characterized CC027/GeniUnc mice as a strain that is genetically susceptible to peanut allergy and prone to severe reactions after OFC. More broadly, these findings demonstrate the untapped potential of the CC population in developing novel models for allergy research. (J Allergy Clin Immunol 2019;143:1027-37.)

Key words: *Collaborative Cross, food allergy, peanut allergy, anaphylaxis, mouse model, Ara h 2*

Food allergy is a potentially life-threatening disease characterized by IgE-mediated degranulation of mast cells and basophils on allergen ingestion. Affecting 6% of children and 4% of the general population, food allergy is a growing public health concern, with peanut allergy present in at least 1% of the US population.[1-3] Although many food allergies are outgrown before adulthood, peanut and tree nut allergies persist in roughly 80% to 90% of the affected population.[4] Significant progress in food allergy research has occurred over the last 10 years, including the development of potential therapies,[5-10] identification of improved diagnostic approaches,[3] and discovery of underlying immunologic mechanisms driving food allergies.[11,12] However, critical knowledge gaps exist surrounding the cause of peanut allergy, including genetic, microbial, and environmental influences.

The laboratory mouse has been the premier model organism for understanding complex human diseases and developing therapies for a variety of diseases. Despite concerns about the translation of data from specific mouse strains to larger human health responses,[13] there has been a growing appreciation for the role that genetic diversity between inbred mouse strains has in different outcomes within experimental models of human disease.[14,15] A number of mouse genetic reference panels, including the BxD panel[16] and the subsequently generated inbred Collaborative Cross (CC)[17] and outbred Diversity Outbred,[18] have been developed to better leverage and identify the causal genetic variants driving such disease differences. These resources, panels of diverse mice with well-characterized genetics, have been used to (1) characterize the breadth of disease phenotypes that can be attributed to genetic variation, (2) define new models of disease phenotypes not found in the small pool of classic mouse strains used in standard studies, and (3) identify those polymorphic genes driving

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Abbreviations used

CC:	Collaborative Cross
FoxP3:	Forkhead box P3
HRP:	Horseradish peroxidase
MMCP-1:	Mucosal mast cell protease 1
MSD:	Meso Scale Discovery
OFC:	Oral food challenge
OIT:	Oral immunotherapy
PNsIgE:	Peanut-specific IgE
$PNsIgG_1$:	Peanut-specific IgG_1
$PNsIgG_{2a/2c}$:	Peanut-specific $IgG_{2a/2c}$
sIgE:	Specific IgE
Treg:	Regulatory T
UNC:	University of North Carolina

differential disease responses. Critically, such systems improve on the utility and rigor of experimental models, ultimately making them more relevant for modeling diverse human disease responses.

Because peanut allergy within the human population is a heritable (ie, genetically influenced) trait,[19,20] we sought to use the high levels of genome-wide genetic diversity present in the CC mice to improve our understanding of peanut allergy and its contributing factors. Numerous murine models are currently in use by our group and many others to study the mechanisms and treatments of peanut allergy.[21-23] However, these models often require powerful T_H2-skewing adjuvants (eg, cholera toxin,[24] staphylococcal enterotoxin B,[25] or aluminum hydroxide[26]) to sensitize animals, intraperitoneal challenge to elicit a reaction,[24,27] or complex modifications, such as humanization.[28-30]

In a model commonly used by our group and others, C3H/HeJ mice are sensitized by means of weekly oral gavage of peanut extract and cholera toxin and challenged by means of intraperitoneal injection with peanut extract.[24] Importantly, although some reports demonstrate reactions on oral challenge in the C3H/HeJ model described above,[23] other groups, including our own, have not been able to successfully reproduce these findings.[29,31] A model that can both be sensitized and reproducibly react orally would allow for the study of therapies that alter the immune system in the gastrointestinal tract, such as oral immunotherapy (OIT), as well as genetic and environmental factors driving these severe allergic reactions in a more physiologically relevant model of human disease. Here we report our screen of CC strains to identify orally induced peanut-induced anaphylaxis, characterization of peanut-specific immunologic responses, and novel insights into anaphylactic reactions in mice through the gastrointestinal tract. In concordance with prior assessment in the human population, we identified strong genetic control of the propensity to experience anaphylaxis after sensitization. We also identified the CC strain CC027/GeniUnc as a mouse strain that had anaphylaxis after oral sensitization and challenge, even in the absence of the T_H2-skewing cholera toxin.

METHODS

Mice

CC mice were purchased from the University of North Carolina (UNC) Systems Genetics Core.[32] C57BL/6J, C3H/HeJ, and BALB/cJ mice were obtained from colonies maintained for less than 5 generations by the Pardo-Manuel de Villena laboratory from mice purchased from the Jackson Laboratory (Bar Harbor, Me). All mice were bred at UNC, raised on standard mouse chow free of any peanut-containing ingredients, kept on a 12:12 light/dark cycle, and transferred for sensitization at 4 to 6 weeks of age. Female mice were weaned into cages at a common cage density (between 3-5 mice per cage depending on the experiment) but with a diverse set of strains within each cage. In this way effects of cage density and other cage-specific effects were removed from these studies. Throughout these studies, where possible, experimenters were blinded to the mouse strains being studied. All mouse work was conducted in compliance with UNC Institutional Animal Care and Use Committee protocols 16-045 and 17-286.

Reagents

Peanut extract was created by mixing peanut flour (12% fat light roast and 50% protein; Golden Peanut, Alpharetta, Ga) in a 1:5 (wt/vol) ratio of PBS with 1 mol/L NaCl, and the soluble fraction was filter sterilized, as described previously.[27] Protein concentrations were determined by using a bicinchoninic acid assay (Pierce, Rockford, Ill). Peanut extract was run on a NuPage gel to identify and compare relative quantities of peanut allergens before use.

Sensitization with peanut plus cholera toxin and challenge

Four- to 6-week-old female mice underwent weekly sensitization with 2 mg of peanut extract and 10 μg of cholera toxin (List Biological laboratories, Campbell, Calif) in a 200-μL volume for 3 weeks by means of oral gavage, followed by 1 week of 5 mg of peanut extract and 10 μg of cholera toxin administered by means of oral gavage. One week after this final sensitization, mice were bled by means of submandibular bleed to collect serum for immunoglobulin quantification. The following day, mice undergoing an oral challenge underwent gavage with 9 mg of peanut extract, whereas mice undergoing intraperitoneal challenge received 200 μg of peanut extract. Core body temperatures were monitored every 15 minutes with a rectal thermometer (Physitemp, Clifton, NJ).

For serum mucosal mast cell protease 1 (MMCP-1), histamine, and Ara h 1, 2, and 3 measurements, blood was collected 60 minutes after oral challenge. Serum levels of MMCP-1 (eBioscience, San Diego, Calif), histamine (Beckman Coulter, Brea, Calif), and Ara h 1, 2, and 3 (Indoor Biotechnologies, Charlottesville, Va) were measured by means of ELISA, according to the manufacturer's instructions.

Sensitization with peanut in the absence of cholera toxin and challenge

Four- to 6-week-old female mice underwent sensitization for 3 weeks with either PBS once per week, 2 mg of peanut extract once per week, 2 mg of peanut extract 3 times per week, or 2 mg of peanut extract plus 10 μg of cholera toxin once per week. All peanut groups received 1 final week of 5 mg of peanut extract with or without cholera toxin at the frequency described. Bleeding and oral challenges were consistent with the procedure described above.

Immunoglobulin quantification

For peanut-specific IgE (PNsIgE), peanut-specific IgG_1 ($PNsIgG_1$), and peanut-specific $IgG_{2a/2c}$ ($PNsIgG_{2a/2c}$) quantification, plates were coated with 20 μg/mL peanut extract diluted in carbonate-bicarbonate buffer (Sigma-Aldrich, St Louis, Mo). Samples were assayed on plates at 1:100, 1:20,000, and 1:1,250, respectively. Ara h 1–specific IgE (sIgE), Ara h 2 sIgE, and Ara h 3 sIgE plates were coated with 5 μg/mL of the appropriate purified peanut component diluted in carbonate-bicarbonate buffer. Samples were plated at a 1:20 dilution. IgE plates were all detected by using the following antibodies in sequence: sheep anti-mouse IgE (0.5 μg/mL; Binding Site, Birmingham, United Kingdom), biotinylated donkey anti-sheep IgG (0.5 μg/mL; Accurate Chemical, Westbury, NY), and neutravidin-horseradish peroxidase (HRP; 0.2 μg/mL; Pierce). IgG_1 and $IgG2_{a/2c}$ ELISAs were detected with HRP-conjugated goat anti-mouse IgG_1 (SouthernBiotech,

Birmingham, Ala) or HRP-conjugated goat anti-mouse IgG_{2a} (SouthernBiotech) and HRP-conjugated goat anti-mouse IgG_{2c} (SouthernBiotech), respectively. Sure Blue TMB Microwell Peroxidase Substrate and Stop Solution (KPL, Gaithersburg, Md) were applied to all plates. Plates were read on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, Vt). Total IgE was analyzed by means of ELISA (Affymetrix, Santa Clara, Calif) and run according to the manufacturer's instructions. All ELISA data were analyzed by using Gen5 software.

mRNA and cytokine protein quantification

Spleens were collected from both naive and peanut-sensitized mice 1 week after oral challenge. mRNA abundance levels were quantified by using real-time PCR and SYBR Green methodology, as previously described.[33] Briefly, total RNA was extracted with RNA kits (Qiagen, Germantown, Md). Reverse transcription was performed by using random decamers as primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif). The abundance of resultant mRNA-derived cDNA was determined by using quantitative RT-PCR analysis with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Calif), primers specific for genes of interest (see Table E1 in this article's Online Repository at www.jacionline.org for sequences), and a StepOne Plus cycler (Applied Biosystem, Foster City, Calif). Each set of primers spans at least 2 introns so that contaminating genomic DNA either cannot be amplified because of large product size or can be easily identified based on its size on agarose gel. Primers for 18S rRNA were obtained from Ambion (Austin, Tex). The specificity of each real-time PCR target was confirmed by using melt temperature analysis and agarose gel fragmentation of amplicons.

A standard curve for each target mRNA, as well as for 18S rRNA, was generated from serial dilutions of cDNAs derived from a pooled intestinal cDNA library to quantify mRNA abundance. The relative abundance of mRNA of interest in each sample was determined based on its corresponding standard curve and normalized against the abundance of 18S rRNA. For protein analysis, splenocytes were isolated and cultured for 96 hours in the presence of 200 μg/mL peanut extract. Supernatants were collected and run on Meso Scale Discovery (MSD) plates to determine levels of IL-4, IL-5, IL-13, TNF-α, IFN-γ, IL-12p40, and IL-10, according to the manufacturer's instructions (MSD, Rockville, Md).

Flow cytometry

For analysis of regulatory T (Treg) cells, splenocytes were collected 1 week after oral challenge. Splenocytes were stimulated with 200 μg/mL peanut extract for 7 days. Treg cells were then labeled with fluorescein isothiocyanate rat anti-mouse CD4 clone RM4-5 (BD Biosciences, San Diego, Calif), phycoerythrin anti-mouse/rat/human forkhead box P3 (FoxP3) clone 150D (BioLegend, San Diego, Calif), and allophycocyanin rat anti-mouse CD25 clone PC61 (BD Biosciences). For determination of basophil levels, whole blood was collected by means of submandibular bleed 1 week after sensitization. Cells were stained with anti-mouse IgE fluorescein isothiocyanate clone 23G3 (eBioscience, San Diego, Calif), peridinin-chlorophyll-protein complex/Cy5.5 anti-mouse CD49b clone DX5 (BioLegend), and phycoerythrin anti-mouse CD200R clone OX-110 (BioLegend). Flow cytometry was performed on a Beckman Coulter CyAn ADP (Beckman Coulter, Fullerton, Calif) and analyzed with FlowJo software (v10; TreeStar, Ashland, Ore). Basophils were gated as IgE^+CD49b^+ cells and expressed as a percentage of lymphocytes. Treg cells were gated as $CD4^+CD25^+FoxP3^+$ and expressed as a percentage of $CD4^+$ lymphocytes.

Histology

Proximal jejunum was harvested from peanut-sensitized mice 1 week after challenge, fixed with cold 4% paraformaldehyde in PBS overnight, and paraffin embedded. Cross-cut sections (at a thickness of 7 μm) were subjected to immunostaining with an antibody specific for mast cell tryptase (1:180; ab151757; Abcam, Cambridge, United Kingdom) or CD117 (c-kit; 1:150, PA5-16770; Thermo Fisher, Waltham, Mass). Antibody-antigen complexes were detected with an ABC kit (Vector Laboratories, Burlingame, Calif) and visualized by means of incubation with DAB (Vector Laboratories or Sigma-Aldrich).

For mast cell tryptase–positive cell quantification, immunostained sections were then subjected to counterstaining for cell nuclei with 0.1% methylene blue in acetic acid. Two to 4 villi and the crypts under the villi were randomly selected and mast cell tryptase–positive cells with clear nuclei within delineated villi and crypts were counted to estimate the number of mast cell tryptase–positive cells in mucosa. The total number of cells was determined by counting methylene blue–stained cell nuclei, and the percentage of mast cell tryptase–positive cells was calculated. For each mouse, 850 to 1640 cells were counted.

Statistical analysis

GraphPad Prism software (version 7.02; GraphPad Software, La Jolla, Calif) was used to analyze all data. Mann-Whitney *U*, Spearman correlation, and unpaired *t* tests were performed, and a *P* value of less than .05 was considered significant. For cytokine protein measurement, values at or less than the lower limit of detection were assigned half of the value of the lower limit of detection for that particular MSD assay.

RESULTS

CC027/GeniUnc female mice react severely to both oral and intraperitoneal challenge with peanut extract

Female mice from 16 CC strains were screened by using an established sensitization model (Fig 1) to assess the role that genetic variation plays in controlling anaphylaxis after sensitization with peanut allergen.[27] These CC strains were chosen based on the fact that their well-characterized genetic makeup is representative of the larger CC population,[34] as well as prior reports[14,35] of aberrant disease present in specific strains. All mice underwent the same 4-week sensitization regimen, followed by half of the mice in each strain receiving a 200-μg peanut extract challenge by means of intraperitoneal injection and the other half receiving a 9-mg peanut extract challenge by means of oral gavage.

After either oral food challenge (OFC) or intraperitoneal challenge, CC strains were grouped into 3 types of reactors: strains that do not react regardless of challenge route (Fig 2, *A*: OFC; Fig 2, *D*: intraperitoneal challenge), strains that reacted mildly (mean body temperature decreases between 1.5°C and 3°C; Fig 2, *B*: OFC; Fig 2, *E*: intraperitoneal challenge), and strains that reacted severely (mean body temperature decreases, >3°C; Fig 2, *C*: OFC; Fig 2, *F*: intraperitoneal challenge). As expected, the screen identified many more mild and severe reactors after intraperitoneal challenge than after oral challenge; however, responses were highly concordant across routes of challenge (ie, if a strain was a nonreactor in the intraperitoneal cohort, it was also a nonreactor in the OFC cohort). Two strains, CC027/GeniUnc (referred to as CC027 in figures) and CC012/GeniUnc, were classified as strong reactors after OFC and were also classified as strong reactors to intraperitoneal challenge with peanut extract, suggesting that they could be potential models for severe anaphylaxis after peanut sensitization.

To validate the findings of this initial screen, we conducted a second experiment with the OFC severe responders CC027/GeniUnc and CC012/GeniUnc, as well as the nonresponders CC028/GeniUnc and the intraperitoneal-only responders CC011/Unc. Although the results of this experiment were largely concordant with the initial screen (Fig 2, *G-J*), only CC027/GeniUnc mice exhibited a severe reaction after OFC. Therefore we concluded that the CC027/GeniUnc strain represents a robust OFC reaction model derived from our screen of the CC.

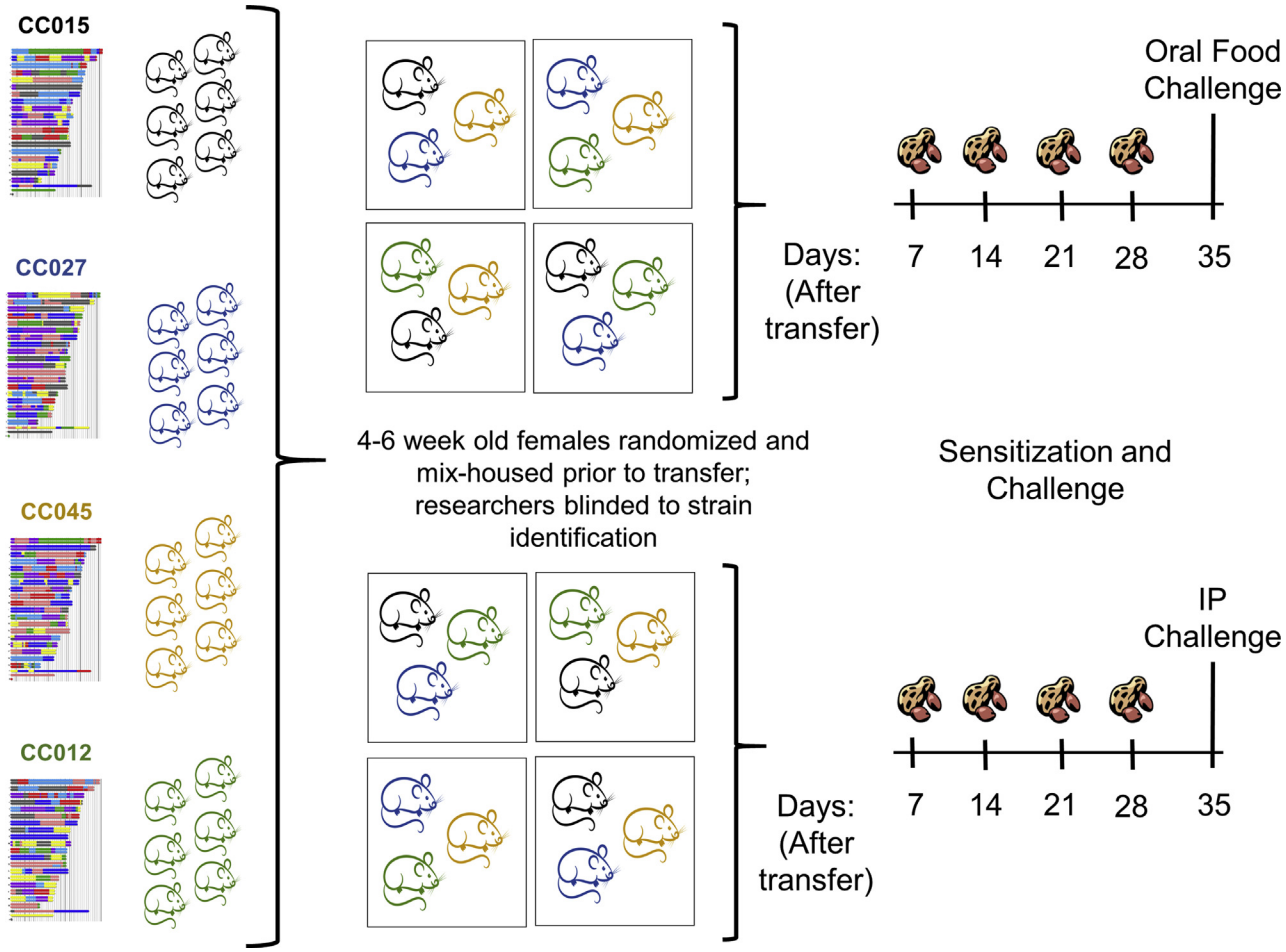

FIG 1. CC screening approach. Schematic showing 4 representative strains of the 16 strains screened. Six female mice between the ages of 4 and 6 weeks from each strain were mixed so that each cage contained 3 to 5 mice from different strains. Mice were then transferred from the UNC Systems Genetics Core to the UNC Food Allergy Initiative, where researchers were blind to the identification of each strain. Mice were sensitized intragastrically with peanut extract and cholera toxin for 4 weeks before undergoing either an OFC (n = 3 per strain) or intraperitoneal *(IP)* challenge (n = 3 per strain) with peanut extract.

CC027/GeniUnc, but not C3H/HeJ, C57BL/6J, or BALB/cJ, mice react on oral challenge, despite all making IgE to peanut allergens

Immune responses of sensitized CC027/GeniUnc female mice were compared with those of female mice from the classical inbred C3H/HeJ, C57BL/6J, and BALB/cJ strains. All 4 strains were sensitized by using the previously described 4-week sensitization schedule and then underwent an OFC with 9 mg of peanut extract. Consistent with our previous experiments, CC027/GeniUnc mice experienced severe systemic reactions with body temperatures decreasing over the course of 60 minutes after OFC (Fig 3, *A*), whereas C3H/HeJ, C57BL/6J, and BALB/cJ mice had essentially no change in body temperature after OFC. These results confirm the utility of the CC027/GeniUnc strain as an orally reacting allergy model. Despite only CC027/GeniUnc mice reacting on OFC, C3H/HeJ and C57BL/6J mice make PNsIgE, $PNsIgG_1$, and $PNsIgG_{2a/2c}$, as well as IgE to the major peanut components Ara h 1, Ara h 2, and Ara h 3 (Fig 3, *B-G*). After sensitization, CC027/GeniUnc mice make significantly more PNsIgE than C3H/HeJ ($P < .05$) but not C57BL/6J (Fig 3, *B*) or BALB/cJ (see Fig E1 in this article's Online Repository at www.jacionline.org) mice. $PNsIgG_1$ levels are not different between CC027/GeniUnc, C3H/HeJ, and C57BL/6J mice (Fig 3, *C*), whereas $PNsIgG_{2a/2c}$ levels are greater in CC027/GeniUnc than C57BL/6J mice but not different from those in C3H/HeJ mice ($P < .01$; Fig 2, *D*). CC027/GeniUnc mice also had significantly more total IgE than C3H/HeJ mice ($P < .01$) but not significantly different total IgE levels from C57BL/6J mice after sensitization (Fig 3, *H*).

Given that PNsIgE and $PNsIgG_1$ levels were different between CC027/GeniUnc mice and at least one of the 2 classical inbred strains, we assessed whether PNsIgE or $PNsIgG_1$ levels within CC027/GeniUnc mice were correlated with anaphylaxis reaction severity. We found that PNsIgE and $PNsIgG_1$ levels did not correlate with reaction severity in CC027/GeniUnc mice and thus do not explain the increased reactivity of these mice (see Fig E2 in this article's Online Repository at www.jacionline.org). Together, these data show that CC027/GeniUnc mice make immunoglobulins to peanut and peanut components but that the strain-specific production of immunoglobulins alone does not distinguish CC027/GeniUnc mice from C3H/HeJ or C57BL/6J mice.

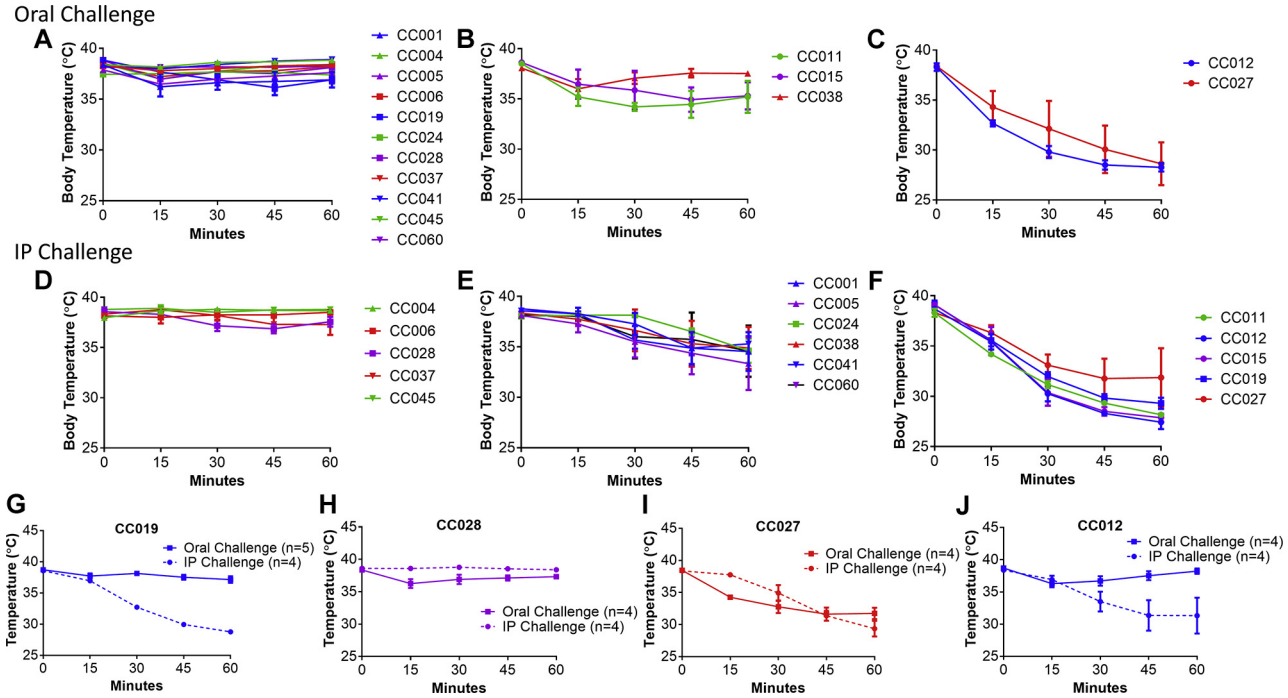

FIG 2. Anaphylaxis in peanut-sensitized CC strains after peanut challenge. **A-F,** Oral and intraperitoneal *(IP)* challenges revealed CC strains that are nonreactors (Fig 2, *A* and *D*), mild reactors (Fig 2, *B* and *E*), and severe reactors (Fig 2, *C* and *F*), as measured based on decreased body temperature. **G-J,** Challenges were repeated with an IP reactor control (Fig 2, *G*), a nonreactor control (Fig 2, *H*), and oral reactors (Fig 2, *I* and *J*).

CC027/GeniUnc mice mount a T_H2 cellular response with little T_H1 or regulatory cytokine response to peanut

Secreted cytokines from peanut-stimulated splenocytes were quantified for the CC027/GeniUnc, C3H/HeJ, and C57BL/6J strains to determine T-cell phenotypes. All 3 strains produce IL-4, and levels are not significantly different across the strains (Fig 4, *A*). IL-12p40 levels were significantly increased in C3H/HeJ mice relative to both C57BL/6J and CC027/GeniUnc mice (Fig 4, *F*). For the remaining 5 cytokines (IL-5, IL-13, TNF-α, IFN-γ, and IL-10), we found that CC027/GeniUnc mice had significantly lower levels than either C57BL/6J or C3H/HeJ mice (Fig 4, *B-E* and *H*). The ratio of IL-4 to IFN-γ, which is indicative of a T_H2-skewed response,[23-25] was greater in CC027/GeniUnc mice compared with C3H/HeJ or C57BL/6J mice (Fig 4, *G*). These secreted cytokine data demonstrate that CC027/GeniUnc mice mount T_H2 responses to peanut antigen with limited production of IFN-γ, IL-12p40, TNF-α, and IL-10.

Concurrent with the finding that CC027/GeniUnc mice show a T_H2 response to peanut, we found that CC027/GeniUnc mice have greater levels of *Gata3* mRNA than C3H/HeJ mice ($P < .05$), although with similar levels to C57BL/6J mice (Fig 4, *I*). CC027/GeniUnc mice also had a lower Treg cell response, as indicated by reduced $CD4^+CD25^+FoxP3^+$ Treg cell counts (Fig 4, *K*) and decreased IL-10 protein production than the classical inbred strains ($P < .01$; Fig 4, *H*).

Sensitization-induced changes in mRNA expression were also analyzed for a few selected genes. Interestingly, CC027/GeniUnc mice have lower expression levels of *Il10* and *Il12* after sensitization than they do at baseline ($P < .05$, see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that sensitizing these animals results in decreased production of Treg and T_H1-type cytokines. However, sensitization did not change *Il10* or *Il12* mRNA levels in either C3H/HeJ or C57BL/6J mice (see Fig E3). Other reports have shown that *Ox40l* expression increases in dendritic cells after sensitization.[36] We found *Ox40l* mRNA levels in the small intestine to be increased in CC027/GeniUnc mice compared with those in C3H/HeJ ($P = .0592$) and C57BL/6J ($P < .05$) mice, showing an additional effect of sensitization in these mice (Fig 4, *J*). Overall, T-cell responses in CC027/GeniUnc mice appear to favor proallergic responses to peanut, with the presence of T_H2 cytokines and limited T_H1 cytokine production, lower numbers of Treg cells, and less regulatory cytokine IL-10.

Effector cells are more prevalent in CC027/GeniUnc mice than classic inbred strains

Basophils and mast cells are the 2 main effector cells implicated in allergic reactions to foods.[37] We quantified basophil frequency in blood after sensitization by using flow cytometry. CC027/GeniUnc mice had an increased percentage of IgE^+CD49b^+ basophils circulating after sensitization compared with C3H/HeJ ($P < .05$) and C57BL/6J ($P < .01$) mice (Fig 5, *A*). Furthermore, basophils from CC027/GeniUnc mice also had less of the inhibitory receptor CD200R1 than the other 2 strains ($P < .001$; Fig 5, *B*).[38] Tissue samples of the small intestine were stained for tryptase-positive mast cells. CC027/GeniUnc mice had an increased percentage of tryptase-positive cells, suggesting increased mast cell presence in the tissue (Fig 5, *C* and *D*). Similar findings were noted with toluidine blue staining of mast cells in tissue (see Fig E4 in this article's Online Repository at

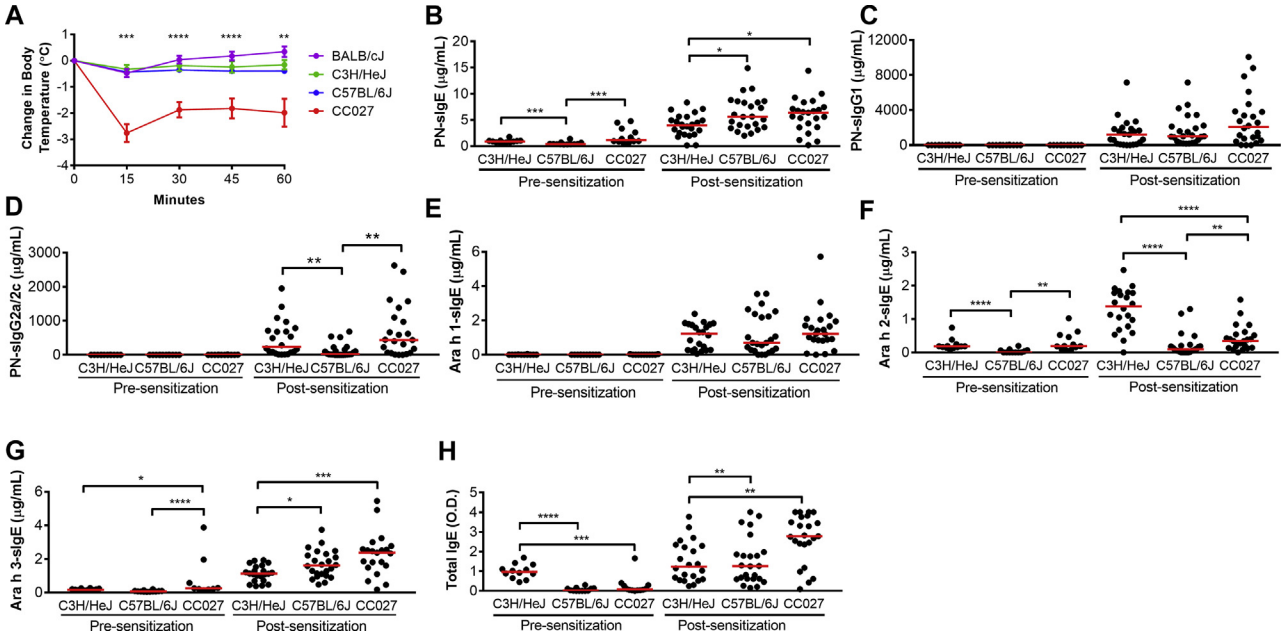

FIG 3. Immune response of CC027/GeniUnc mice to peanut extract relative to that of C3H/HeJ, C57BL/6J, and BALB/cJ mice. CC027/GeniUnc mice are represented as *CC027* in figures. **A,** Body temperatures after oral challenge with peanut extract (n ≥ 12 per strain; representative of 3 independent experiments). **B-H,** Serum levels of immunoglobulins after 4 weeks of sensitization. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, Mann-Whitney *U* test. Statistical significance represents comparisons of C3H/HeJ, C57BL/6J, and BALB/cJ mice relative to CC027/GeniUnc mice (Fig 3, *A*).

www.jacionline.org). Taken together, CC027/GeniUnc mice have an increased number of basophils in circulation that can lack negative feedback mechanisms driven by CD200R and also an excess of mast cells in the gastrointestinal tract.

Reaction severity in CC027/GeniUnc mice correlates with serum levels of Ara h 2 but not MMCP-1 during oral challenge

Blood was collected from mice 60 minutes after OFC to further characterize the severe reaction observed in CC027/GeniUnc mice. Serum levels of MMCP-1, a mediator released by degranulated mast cells in the gastrointestinal tract, was measured to verify that mast cell degranulation could be detected in the reacting animals. Serum MMCP-1 was detectable in both C3H/HeJ and CC027/GeniUnc mice but not C57BL/6J mice (Fig 6, *A*), although only CC027/GeniUnc mice showed signs of a systemic reaction. Histamine levels were also quantified after OFC and were not different between strains (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). Within CC027/GeniUnc mice, serum levels of MMCP-1 were not correlated with reaction severity (Spearman $r = 0.2196$, $P = .4109$; Fig 6, *B*); histamine levels were also not correlated with reaction severity (see Fig E5, *B*). Histamine levels in serum 5 minutes after OFC were also not different between strains and did not correlate with reaction severity (data not shown). Concurrently, serum levels of the major peanut allergen Ara h 2 were measured 60 minutes after challenge by means of ELISA to determine the amount of allergen being absorbed into the bloodstream. CC027/GeniUnc mice had significantly greater levels of Ara h 2 in serum compared with C57BL/6J ($P < .05$) and C3H/HeJ ($P < .0001$) mice (Fig 6, *C*). Interestingly, Ara h 2 quantity positively correlates with reaction severity in CC027/GeniUnc mice (Spearman $r = 0.69$, $P = .0028$; Fig 6, *D*). Two other major peanut allergens, Ara h 1 and Ara h 3, were not detectable in serum after challenge.

CC027/GeniUnc mice can be sensitized in the absence of cholera toxin and react on oral challenge with peanut extract

Given the oral reactivity of the CC027/GeniUnc female mice, we then tested whether they could be sensitized with peanut extract alone (ie, without cholera toxin or any additional adjuvant) in comparison with C3H/HeJ and BALB/cJ mice. After oral challenge with peanut, all groups of C3H/HeJ and BALB/cJ mice did not experience decreased body temperatures (Fig 7, *A* and *B*). However, CC027/GeniUnc mice sensitized with peanut alone, 1 or 3 times per week, experienced, on average, a greater than 3°C and 4°C decrease, respectively, that is comparable with that seen in mice sensitized with peanut plus cholera toxin (Fig 7, *C*). All strains had increased PNsIgE levels after sensitization with peanut plus cholera toxin. CC027/GeniUnc mice sensitized with peanut 3 times per week had greater levels of PNsIgE, $PNsIgG_1$, and $PNsIgG_{2a}$ after sensitization compared with C3H/HeJ mice ($P < .05$) but not BALB/cJ mice (Fig 7, *D*, and see Fig E6 in this article's Online Repository at www.jacionline.org). CC027/GeniUnc mice sensitized 3 times a week with peanut had increased PNsIgE levels compared with the group sensitized with PBS ($P < .01$), whereas mice sensitized with peanut plus cholera toxin had higher PNsIgE levels ($P < .0001$). There was no difference in PNsIgE levels between CC027/GeniUnc mice sensitized with peanut alone 3 times or once per week. Overall, these data demonstrate that CC027/GeniUnc mice can be sensitized with peanut in the absence of cholera toxin, as evidenced by their decreased body temperature after OFC and increased levels of PNsIgE.

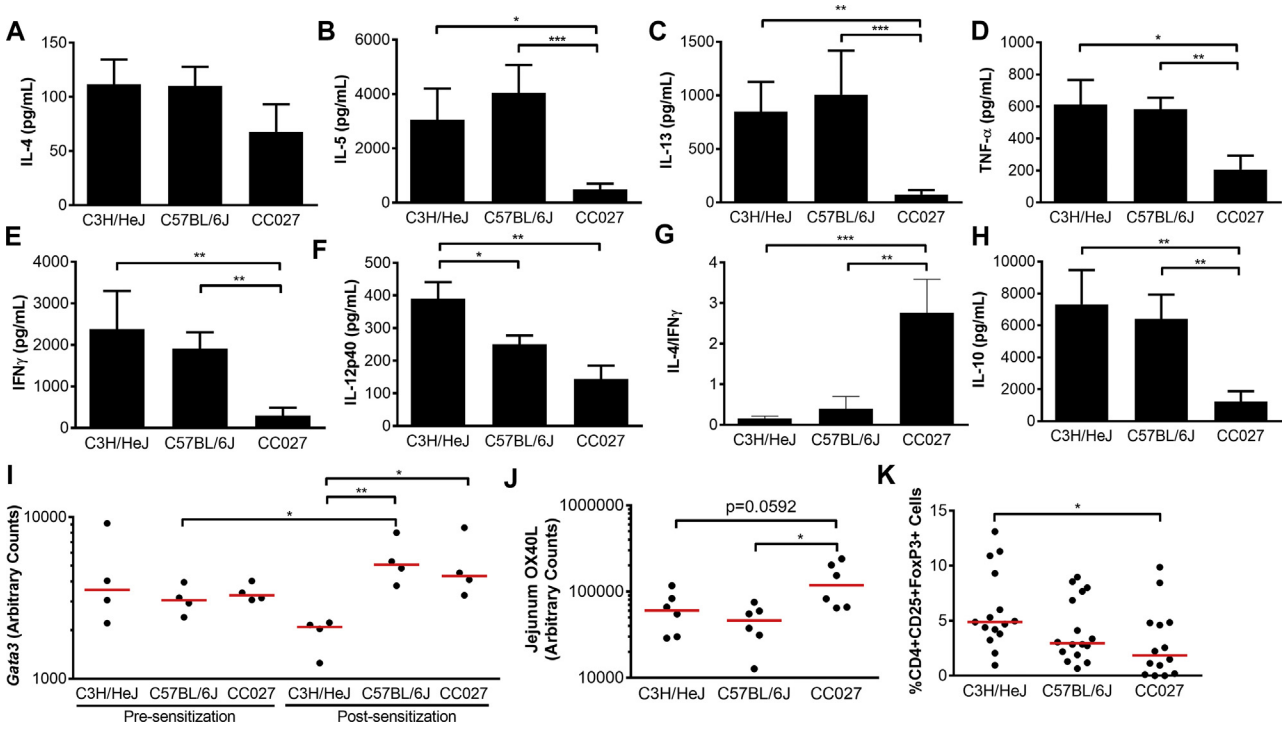

FIG 4. Cellular responses in CC027/GeniUnc, C3H/HeJ, and C57BL/6J mice. Splenic cytokines 96 hours after peanut stimulation (n = 10 per strain; **A-H**), mRNA expression (**I** and **J**), and $CD4^+CD25^+FoxP3^+$ Treg cells (**K**) 1 week after oral challenge. $*P < .05$, $**P < .01$, and $***P < .001$, Mann-Whitney *U* test (Fig 4, *A-H* and *K*). $*P < .05$ and $**P < .01$, unpaired *t* test (Fig 4, *I* and *J*).

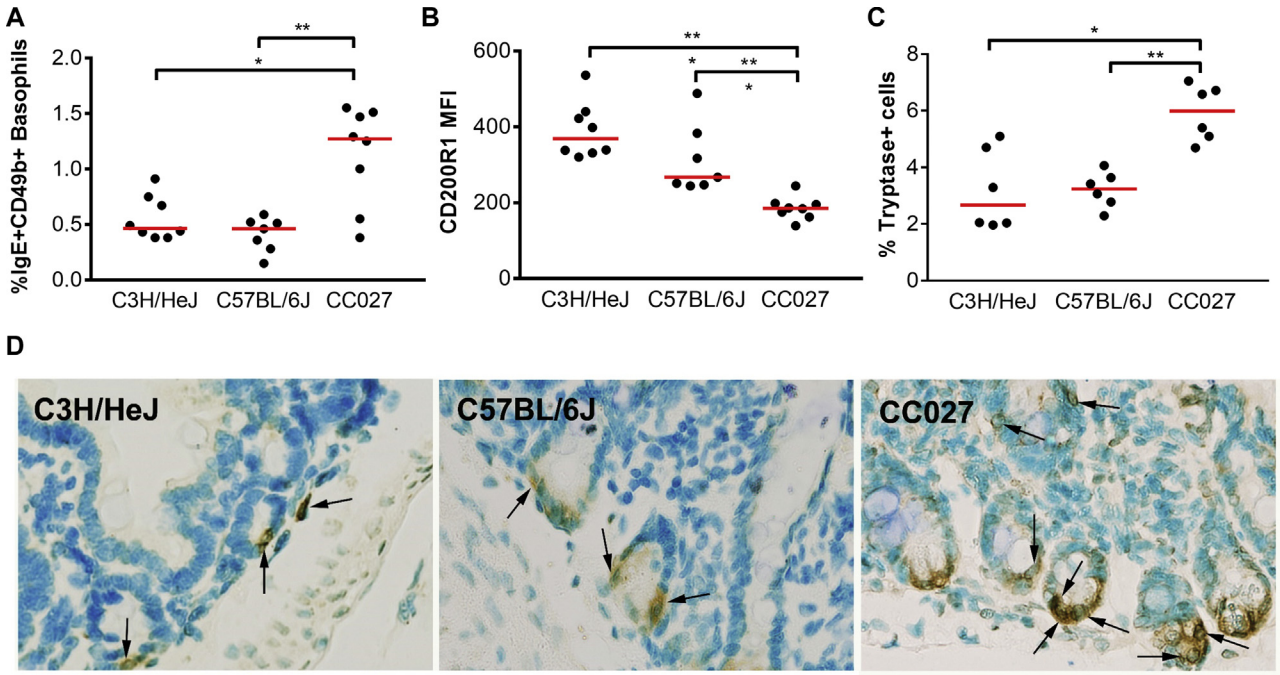

FIG 5. Enumeration of effector cells in CC027/GeniUnc, C3H/HeJ, and C57BL/6J mice. **A** and **B,** Percentage of IgE^+CD49b^+ basophils (Fig 5, *A*) and the basophil inhibitory receptor CD200R1 in whole blood (Fig 5, *B*). **C** and **D,** Jejunal tryptase-positive mast cells were quantified 1 to 3 weeks after challenge (Fig 5, *C*), and representative staining images are shown, with *arrows* indicating tryptase-positive cells (Fig 5, *D*). $*P < .05$ and $**P < .01$, Mann-Whitney *U* test.

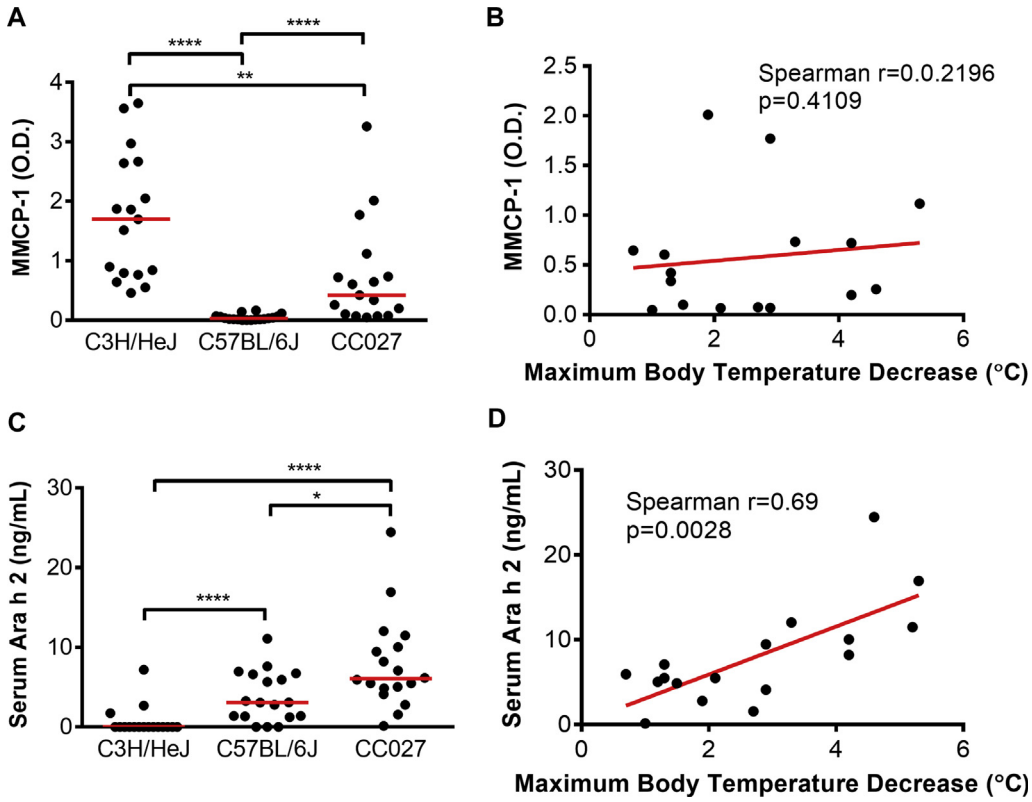

FIG 6. Post-OFC serum levels of mast cell degranulation marker and the major peanut allergen Ara h 2. **A** and **C,** Serum levels of MMCP-1 (Fig 6, *A*) and Ara h 2 (Fig 6, *C*) 60 minutes after oral challenge. **P* < .05, ***P* < .01, and *****P* < .0001, Mann-Whitney *U* test. **B** and **D,** Correlations between MMCP-1 (Fig 6, *B*) or Ara h 2 (Fig 6, *D*) levels and maximum body temperature decrease after oral challenge in CC027/GeniUnc mice.

DISCUSSION

An accurate translation between small-animal models and human health outcomes requires that models accurately recapitulate key aspects of the human disease. Previously, we used a mouse model of food allergy that requires intraperitoneal challenge with peanut extract to elicit an anaphylactic response after sensitization with peanut and a T_H2-skewing adjuvant.[24,27] However, a mouse that reacts on oral challenge would provide a more physiologically relevant platform to study both the cause of the disease and potential treatments. Within the human population, increasing evidence has shown that host genetic variation affects allergic responses. A twin study[19] estimated the heritability (proportion of genetic contribution) to peanut allergy at approximately 0.8. However, identification of genetic variants contributing to peanut allergy responses and outcomes has been limited to associations with the MHC locus and others associated with asthma and eczema.[20,39,40] Undoubtedly, there are additional genetically variable factors driving the propensity for and severity of allergic responses to peanut. Therefore we sought to determine whether genetic variation between mouse strains could explain variation in food allergy disease severity and whether we could develop a more relevant oral challenge model by assessing genetically diverse inbred mouse strains.

Here we described the use of 16 strains from the CC genetic reference panels to screen for an orally reacting animal model of peanut allergy. The CC strains are a set of reproducible inbred strains with high genetic diversity throughout the genome,[34] and the CC has been used to identify genetic factors driving aberrant disease outcomes[41-43] and has enabled the development of more relevant models of human disease responses.[14,35,44] We identified a single strain, CC027/GeniUnc, as a promising model of food allergy. CC027/GeniUnc mice experience a severe systemic reaction, as evidenced by decreased body temperature after OFC with peanut extract, whereas the other 15 CC strains (as well as the well-studied inbred strains C3H/HeJ, C57BL/6J, and BALB/cJ) did not react accordingly. CC027/GeniUnc mice produce detectable levels of IL-4 protein and produce PNsIgE, Ara h 1 sIgE, Ara h 2 sIgE, and Ara h 3 sIgE similar to the immune responses seen in peanut allergy in human subjects.[45] Quantities of IL-4 and *Il4ra* mRNA in the jejunum were similar among strains, although protein levels of IL-4 and IL-4 receptor α might be different. CC027/GeniUnc mice have increased levels of the T_H2-promoting transcription factor *Gata3* mRNA relative to C3H/HeJ mice, which do not react on oral challenge. Notably, our experimental design measured secreted cytokine and gene transcript levels 1 week after oral challenge; it would be interesting to investigate changes in these levels at several time points before and after challenge in future studies to better understand the dynamic changes in activation and responses to allergen across different time points. Also, similar to human disease, PNsIgE levels do not correlate with disease severity in these mice. Furthermore, CC027/Geni/Unc mice have lower numbers of Treg cells based on flow cytometric data, as well as lower levels of the important regulatory cytokine IL-10 at protein and mRNA

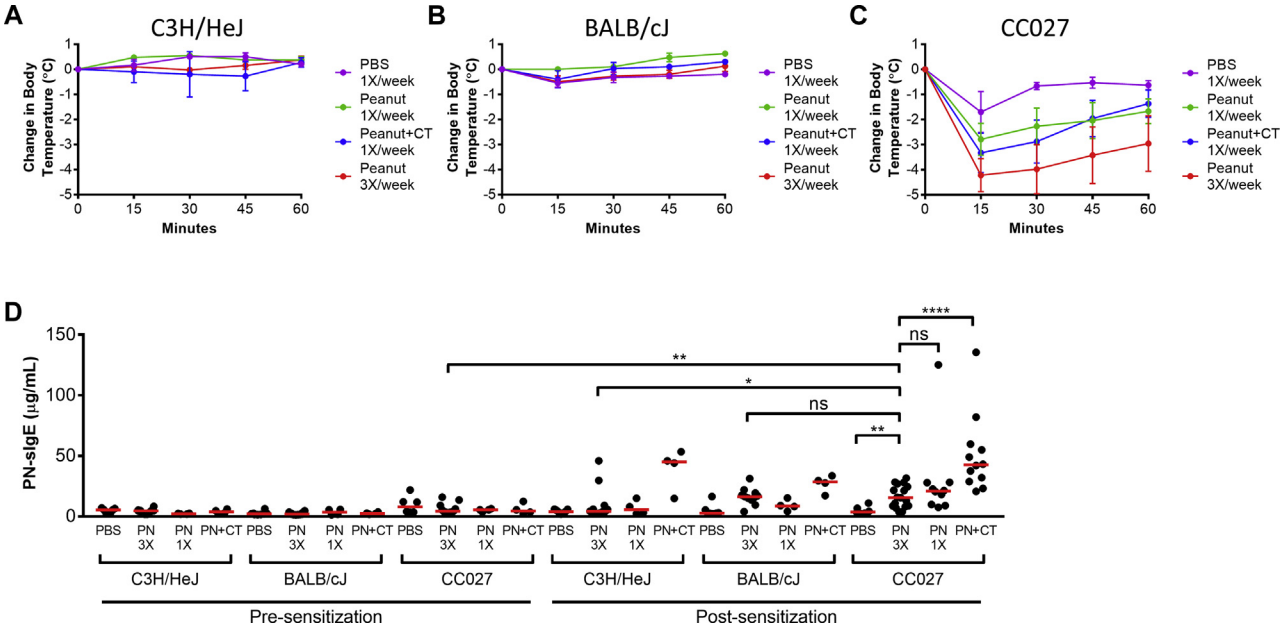

FIG 7. Anaphylaxis in peanut-sensitized mice after oral peanut challenge. **A-C,** Body temperatures after oral challenge with peanut extract in C3H/HeJ (Fig 7, *A*), BALB/cJ (Fig 7, *B*), and CC027/GeniUnc (Fig 7, *C*) mice sensitized with PBS, peanut extract once per week *(PN 1X)*, peanut extract plus cholera toxin once per week *(PN+CT)*, or peanut extract 3 times per week *(PN 3X)*. **D,** PNsIgE quantities in mice before and after sensitization. $*P < .05$, $**P < .01$, and $****P < .0001$, Mann-Whitney *U* test. *ns*, Not significant.

levels. Together, these results reveal a model of peanut allergy that, like other models,[22] produces IL-4 in response to the allergen and a decreased regulatory response but also demonstrate signs of a severe systemic reaction on oral challenge with the allergen, making it a highly relevant model recapitulating key features of peanut allergy in human subjects.

As in human food allergy, the exact mechanistic causes of the increased reactivity of CC027/GeniUnc mice need to be studied further. Our findings suggest many potential contributing factors likely driven by the underlying genetic differences in these mice. As already stated, PNsIgE levels do not correlate with reaction severity, signifying that differences beyond IgE levels must be important for the severe oral reactions observed. In addition to hallmarks of acquired immune differences in CC027/GeniUnc mice, this strain has a greater quantity of basophils and mast cells, the effector cells responsible for the manifestations of allergic symptoms. Recent reports suggest an important interplay between activating and inhibitory signals from the surfaces of mast cells on allergic disease.[46] Although we did not assess mast cell activation, we found that the increased numbers of basophils possess less of the inhibitory receptor CD200R1 in CC027/GeniUnc mice than in C3H/HeJ or C57BL/6J mice, similar to what has been reported for subjects with birch pollen allergy.[47] Thus CC027/GeniUnc mice might have a larger number of more easily activated effector cells than the other less reactive strains. Although histamine levels are similar between strains, the possibility remains that CC027/GeniUnc mice might have greater sensitivity to histamine, as has been shown previously in other mouse strains.[48]

Furthermore, we demonstrated that CC027/GeniUnc mice absorbed higher levels of Ara h 2 protein into their bloodstream during OFC than either C3H/HeJ or C57BL/6J mice, and these levels of serum Ara h 2 in CC027/GeniUnc mice correlated with reaction severity. Ara h 1 and Ara h 3 were not detectable in any strain, which could be due to the time frame we bled the mice after OFC because these proteins have lower stability compared with Ara h 2. C57BL/6J mice had detectable levels of serum Ara h 2 protein after OFC but did not exhibit any signs of a systemic reaction or any detectable serum MMCP-1 after OFC. Taken together, these findings suggest mast cells in C57BL/6J mice are difficult to degranulate compared with mast cells in CC027/GeniUnc mice. However, C3H/HeJ mice had high levels of MMCP-1 after OFC but no serum Ara h 2 or symptoms of anaphylaxis. It is possible that only local mast cells in the mucosa degranulate in C3H/HeJ mice after oral challenge, whereas CC027/GeniUnc mice experienced both local and systemic degranulation. The positive correlation observed between serum Ara h 2 levels and reaction severity in CC027/GeniUnc mice suggests that CC027/GeniUnc mice experience more severe reactions because of increased allergen absorption into their bloodstream, which can trigger anaphylaxis by degranulation of mast cells, basophils, and/or neutrophils. These findings suggest that both Ara h 2 absorption into systemic circulation along with readily degranulating effector cells is required for anaphylaxis on OFC. Increased Ara h 2 absorption could be due to increased gut permeability in CC027/GeniUnc mice. Although a role for intestinal permeability in food allergy has been suggested,[49,50] attempts by our own group and others[51] to measure Ara h 2 levels in human serum after ingestion has proved difficult and inconclusive. Thus CC027/GeniUnc mice offer insight into a potential disease mechanism that is currently difficult to investigate in human subjects. Future investigation of the uptake of Ara h 2 through the gastrointestinal tract is needed.

By also investigating whether CC027/GeniUnc mice could be sensitized with peanut extract alone, we have further validated the utility of this strain in food allergy research. The model in CC027/

GeniUnc mice has addressed the 2 main weaknesses of current food allergy animal models: sensitization requiring an adjuvant, such as cholera toxin or staphylococcal enterotoxin B, and anaphylactic reactions on intraperitoneal challenge but not OFC. CC027/GeniUnc mice can be orally sensitized with peanut extract alone and react orally to peanut, which more closely mimics our current understanding of the human disease. The ability of CC027/GeniUnc mice to be sensitized in the absence of cholera toxin was further suggested by increased levels of PNsIgE measured at baseline. Further studies are needed to understand this IgE production in the absence of a known antigen exposure in a subset of mice and the underlying mechanisms leading to failure of oral tolerance in CC027/GeniUnc mice.

CC027/GeniUnc mice represent a highly relevant model of peanut allergy to the field at large. This small-animal model should allow for more robust evaluation of therapeutic treatments in a preclinical setting before transition into clinical trials. Leading investigational treatments in the field include various routes of peanut immunotherapy, including OIT, sublingual immunotherapy, and epicutaneous immunotherapy. Despite promising results from OIT, sublingual immunotherapy, and epicutaneous immunotherapy studies,[7,9,10,52] these therapies have limitations, including daily dosing, side effects, and difficulty in achieving long-term tolerance after stopping therapy. Therefore new therapies that induce immunologic tolerance are needed. CC027/GeniUnc mice provide a preclinical model to develop these therapies and study the effects on the development of oral tolerance. Furthermore, genetic dissection of the repressive and enhancing phenotypes observed across mouse strains can lead to identification of novel genes and pathways that might be critical in promoting peanut allergy within the human population. More broadly, our results highlight the utility of integrating the experimental robustness of inbred small-animal models of disease with defined and broad genetic diversity in attempting to better understand and address human disease needs.

We thank Soheila Maleki for providing purified Ara h 1, 2, and 3.

Key messages

- **Screening of 16 genetically diverse CC strains was used to identify 1 strain, CC027/GeniUnc, that reacts severely after oral sensitization and oral challenge with peanut extract.**
- **The CC provides a platform to study the immunology and contributing factors, such as genetics and environment, in the development of food allergy.**

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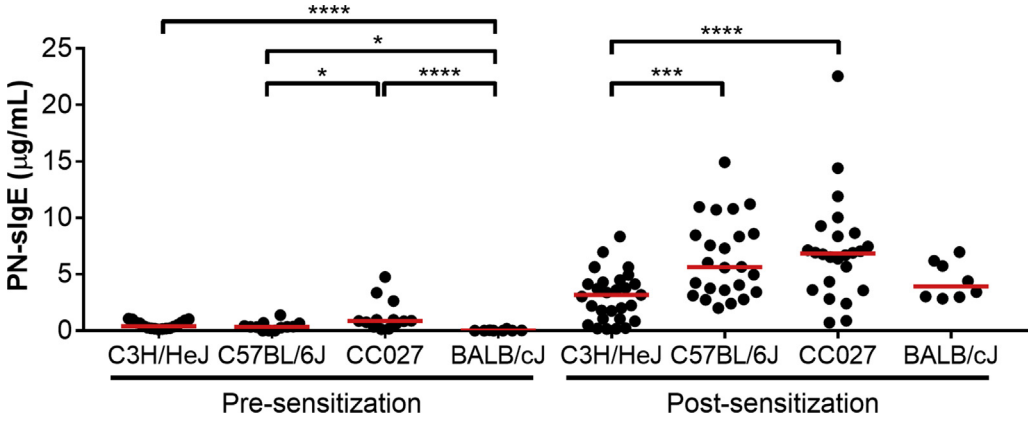

FIG E1. Presensitization and postsensitization PNsIgE levels. Mice from 4 strains underwent sensitization with peanut and cholera toxin, and serum was collected before and after sensitization to quantify PNsIgE levels. $*P < .05$, $***P < .001$, and $****P < .0001$.

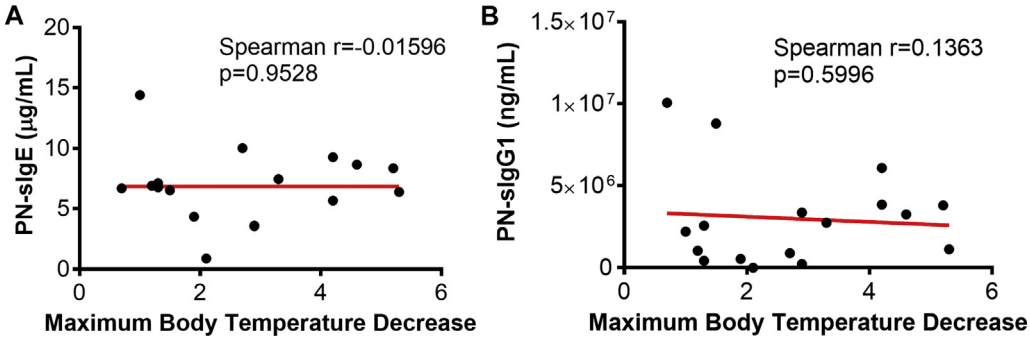

FIG E2. Postsensitization peanut-specific immunoglobulins and reaction severity correlation. Correlations between serum levels of PNsIgE **(A)** and PNsIgG$_1$ **(B)** and maximum body temperature decrease after oral challenge are shown (Spearman correlation).

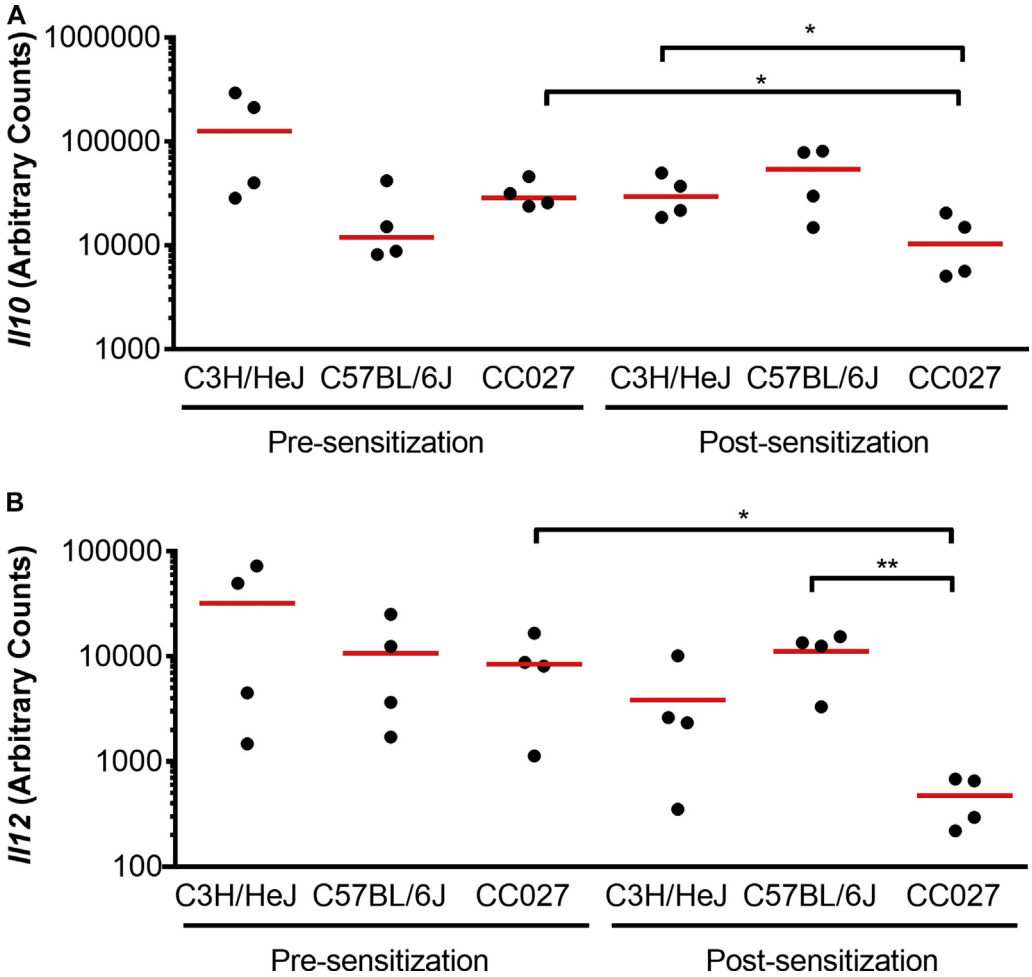

FIG E3. Presensitization and postsensitization mRNA levels. *Il10* **(A)** and *Il12* **(B)** mRNA levels at baseline and after sensitization for C3H/HeJ, C57BL/6J, and CC027 mice are shown. $*P < .05$ and $**P < .01$, unpaired *t* test.

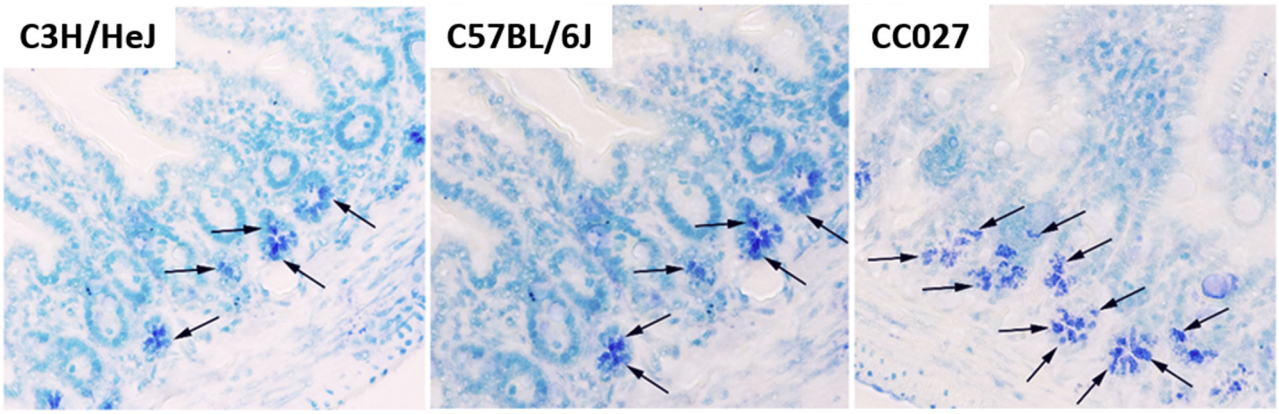

FIG E4. Mast cells in jejunum after peanut sensitization. Toluidine blue staining was performed on jejunal tissue sections, with *arrows* indicating mast cells.

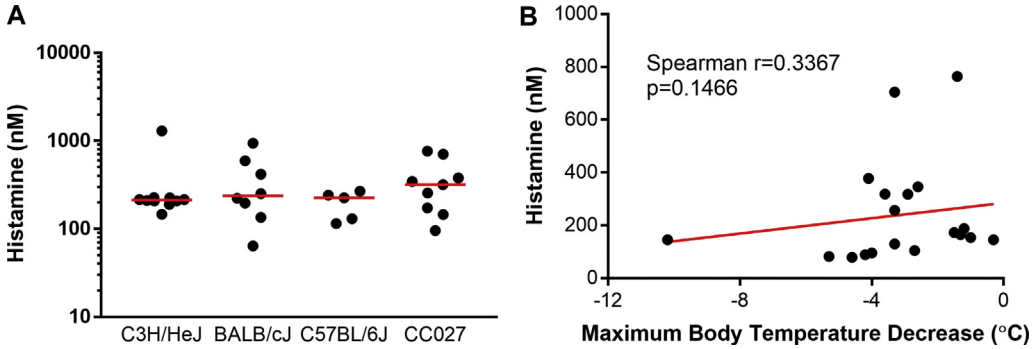

FIG E5. Histamine levels after OFC. **A,** Mice from 4 strains underwent OFC with peanut and then were bled 60 minutes after OFC, and histamine was quantified. **B,** Histamine levels were not correlated with maximum body temperature decrease in CC027/GeniUnc mice.

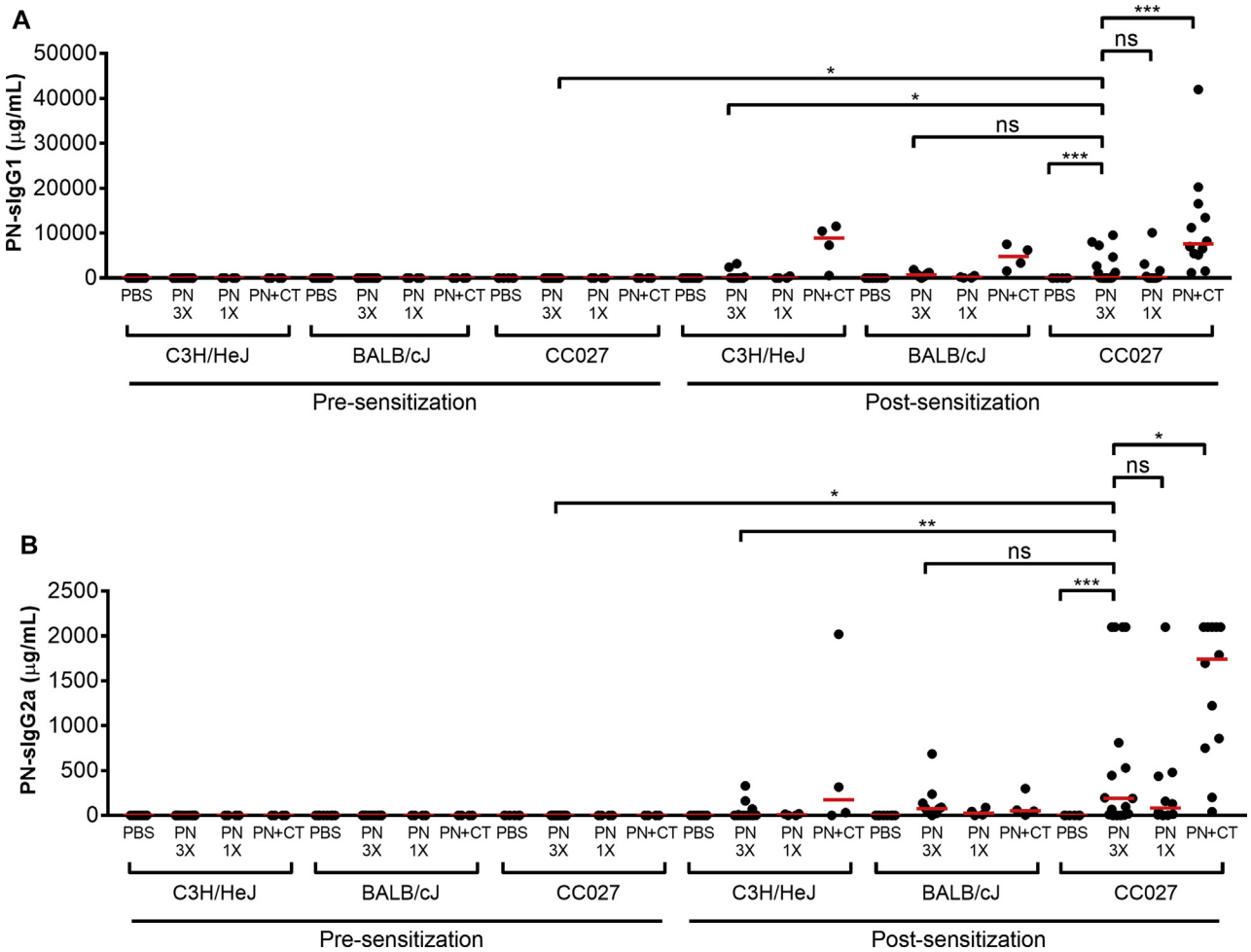

FIG E6. Peanut-specific immunoglobulin quantities before and after sensitization with PBS, peanut extract 3 times per week, peanut extract once per week, or peanut extract plus cholera toxin once per week. PNsIgG$_1$ **(A)** and PNsIgG$_{2a}$ **(B)** levels in C3H/HeJ, BALB/cJ and CC027/GeniUnc mice are shown. Values greater than the limit of detection were assigned the upper limit of detection for that assay. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$. *ns*, Not significant.

TABLE E1. Quantitative real-time PCR primers

	Forward	Reverse	Size	Accession no.
Gata3	5′-AAGGAGAGCAGGGACATCCT-3′	5′-TTTCGGGTCTGGATGCCTTC-3′	165	NM_008091
Il10	5′-GGTTGCCAAGCCTTATCGGA-3′	5′-AATCGATGACAGCGCCTCAG-3′	152	NM_010548
Il12	5′-TCTTCTCACCGTGCACATCC-3′	5′-TGGCCAAACTGAGGTGGTTT-3′	162	NM_001159424
Ox40l	5′-TCCTCTCCGGCAAAGGACC-3′	5′-GCCCATCGCACTTGATGACA-3′	148	NM_009542

Primer sequences for *Gata3*, *Il10*, *Il12*, and *Ox40l* mRNA quantification are shown.