Environmental Factors Modify the Severity of Acute DSS Colitis in Caspase-11-Deficient Mice

Ting-Jia Fan, MS,^{*,†} Sandrine Y. Tchaptchet, PhD,[†] Diana Arsene, MD,^{†,‡} Yoshiyuki Mishima, MD, PhD,^{†,§} Bo Liu, PhD,[†] R. Balfour Sartor, MD,^{*,†,‡} Ian M. Carroll, PhD,[†] Edward A. Miao, MD, PhD,^{*} Anthony A. Fodor, PhD,[¶] and Jonathan J. Hansen, MD, PhD^{†,‡}

Background: Human and mouse studies implicate the inflammasome in the pathogenesis of inflammatory bowel diseases, though the effects in mice are variable. The noncanonical inflammasome activator caspase-11 (*Casp11*) reportedly attenuates acute dextran sodium sulfate (DSS) colitis in mice. However, the effects of *Casp11* on chronic experimental colitis and factors that influence the impact of *Casp11* on acute DSS colitis are unknown.

Methods: We studied the role of *Casp11* in *Il10*^{-/-} mice and acute and chronic DSS colitis mouse models. We quantified colonic *Casp11* mRNA using quantitative polymerase chain reaction and colitis using weight loss, blinded histological scoring, IL-12/23p40 secretion by colonic explants, and fecal lipocalin-2. We determined fecal microbial composition using 16S amplicon sequencing.

Results: We detected increased colonic *Casp11* mRNA in *Il10*^{-/-} mice with chronic colitis, but not in mice with DSS colitis. The presence of *Casp11* did not alter the severity of chronic colitis in DSS-treated or *Il10*^{-/-} mice. Contrary to prior reports, we initially observed that *Casp11* exacerbates acute DSS colitis. Subsequent experiments in the same animal facility revealed no effect of *Casp11* on acute DSS colitis. There were pronounced stochastic changes in the fecal microbiome over this time. The majority of bacterial taxa that changed over time in wild-type vs *Casp11*^{-/-} mice belong to the Clostridiales.

Conclusions: *Casp11* does not impact chronic experimental colitis, and its effects on acute DSS colitis vary with environmental factors including the microbiota, particularly Clostridiales. Stochastic drifts in intestinal microbiota composition, even in mice in the same housing facility, should be considered when interpreting studies of acute DSS colitis models.

Key Words: caspase-11, colitis, microbiota, DSS, IL-10

INTRODUCTION

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel diseases (IBDs), afflict approximately 1.6 million people in the United States and incur between \$12 and \$21 billion in annual direct medical costs alone.¹ Inflammatory bowel diseases are characterized by chronic

T-cell-mediated intestinal inflammation and are thought to be due to maladaptive innate and adaptive immune responses to resident intestinal microbiota.² Although medical cures for IBDs are currently elusive, the most effective therapeutic strategies to induce and maintain remission rely on immunosuppressive agents, many of which have significant potential toxicities and risks. Until we better understand the pathogenesis of IBDs, we will not be able to develop new treatments that function in a targeted manner to treat the diseases without causing significant side effects.

Genome-wide association studies can assist in determining the causes of disease and have revealed more than 200 genetic polymorphisms that are associated with IBDs—including polymorphisms in genes of the innate and adaptive immune systems.^{3,4} Polymorphisms in the NLR family member NOD2, an innate immune sensor, are the strongest known genetic risk factors for Crohn's disease.^{5,6} Also, polymorphisms in the NLRP3 component of the inflammasome of the innate immune system and in IL-1Ra and IL-18, effector pathways downstream of inflammasome activation, are significantly associated with Crohn's disease.⁷⁻¹⁰ These data, along with others demonstrating increased caspase-1 activity, IL-1 β levels, and IL-18 levels in immune cells from Crohn's patients, indicate that inflammasome activity of the innate immune system likely plays a role in the development of Crohn's disease.¹¹⁻¹⁵ However,

Received for publications July 20, 2017; Editorial Decision June 16, 2018.

From the *Department of Microbiology and Immunology, †Center for Gastrointestinal Biology and Disease, and ‡Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; §Department of Internal Medicine II, Shimane University Faculty of Medicine, Izumo, Shimane, Japan; ¶Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, North Carolina

Conflicts of interest: The authors have no conflicts of interest to declare.

Supported by: This work was supported by the following funding sources: National Institutes of Health (NIH) P30 DK034987 (R.B.S., J.J.H.); NIH P40 OD010995 (R.B.S.); Crohn's and Colitis Foundation 381227 (J.J.H.).

Address correspondence to: Jonathan J. Hansen, MD, PhD, Internal Medicine, Center for Gastrointestinal Biology and Disease, University of North Carolina at Chapel Hill, 7341 MBRB, CB 7032, Chapel Hill, NC 27599-7032 (jjhansen@med.unc.edu).

© 2018 Crohn's & Colitis Foundation. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

doi: 10.1093/ibd/izy244
Published online 26 July 2018

the functional consequences of these polymorphisms in inflammasome genes and downstream pathways are unknown.

Although animal models of IBDs are valuable tools to help decipher the functional role of the inflammasome in chronic intestinal inflammation, such studies have produced conflicting results.¹⁶ In short-term dextran sodium sulfate (DSS) colitis that recapitulates the acute rather than the chronic colitis observed in IBDs, blockade of caspase-1, IL-1 β , or IL-18 reduces colitis. In *Il10*^{-/-} mice that do not have colitis when raised in a germ-free state but develop spontaneous chronic immune-mediated colitis characteristic of IBDs when exposed to resident intestinal bacteria, a caspase-1 inhibitor attenuates colitis.¹⁷ These studies suggest a pro-inflammatory role of the inflammasome during colitis. On the other hand, deficiency of the AIM2, NLRP3, or NLRP6 components of the inflammasome worsens acute DSS colitis, indicating an anti-inflammatory role of the inflammasome in this model.^{18–21} Importantly, studies of mice lacking other components of the inflammasome have produced variable results in acute DSS colitis, suggesting that environmental factors such as the intestinal microbiota that differ between labs may interact with the inflammasome to profoundly affect the colitis phenotype of these mice.¹⁶

In addition to the canonical inflammasome, activation of the caspase-11-containing noncanonical inflammasome influences the severity of acute DSS experimental colitis. Others have previously shown that *Casp11* protein expression is increased in colon tissue from mice with acute DSS colitis and that *Casp11*^{-/-} mice are more sensitive to acute DSS colitis, suggesting that caspase-11 limits inflammation in this model.^{22–25} More recently, another group has reported that caspase-11 does not affect the severity of acute DSS colitis.²⁶ The reasons for these discrepant results are not clear. In addition, the effects of caspase-11 on more IBD-relevant chronic immune-mediated experimental colitis are unknown.

In the current work, we demonstrate that *Casp11* mRNA is increased in colon tissue during chronic colitis in *Il10*^{-/-} mice but is decreased in colon tissue during acute and chronic DSS colitis. Despite this, caspase-11 does not affect the severity of chronic colitis in either model. Interestingly, during the course of these experiments, we noted that *Casp11*^{-/-} mice had attenuated acute DSS colitis in our facility, a result opposite to that previously published. This pro-inflammatory role of caspase-11 in acute DSS colitis varies with time and is associated with alterations in intestinal microbiota in our facility. These data suggest that although the caspase-11-containing noncanonical inflammasome does not affect chronic colitis in IBD-relevant models, it does variably influence the sensitivity of mice to acute DSS colitis in a manner that may be dependent on the state of the microbial community.

METHODS

Animals

Wild-type C57BL/6 and *Il10*^{-/-} mice on the C57BL/6 background were originally purchased from Jackson Laboratories and

maintained in vivarium facilities at UNC Chapel Hill. *Casp11*^{-/-} mice on the C57BL/6 background were originally obtained from Dr. Vishva Dixit (Genentech, Inc.). *Il10*^{-/-}; *Casp11*^{-/-} mice were generated by crossing *Il10*^{-/-} with *Casp11*^{-/-} mice. All mice were housed in autoclaved cages and provided with autoclaved food and drinking water ad libitum. In some experiments, germ-free WT and *Il10*^{-/-} mice on the C57BL/6 background were obtained from the National Gnotobiotic Rodent Resource Center (UNC Chapel Hill) and then colonized with specific pathogen-free microbiota for the indicated time periods. Mice for littermate experiments were produced from 7 heterozygous (*Casp11*^{+/-}) mating pairs/litters and then combined as needed to house 4–5 mice per cage until 8 weeks of age, at which point DSS was added to the drinking water. Animal protocols were performed in accordance with American Association for Laboratory Animal Care standards and were approved by the UNC Institutional Animal Care and Use Committee.

DSS Colitis

For colonic *Casp11* mRNA expression experiments during acute DSS colitis, groups of 5–6 C57BL/6 mice age 8–10 weeks raised in specific pathogen-free facilities were treated for up to 5 days with 2% DSS (TdB Consultancy AB, Uppsala Sweden) in their drinking water, followed by regular water for up to 6 days. These mice were born and raised at a time point after the 18-month acute DSS colitis experiment described in the Results section. For other DSS colitis experiments, age- and sex-matched *Casp11*^{-/-} and WT mice were co-housed (2–3 mice of each genotype in each cage) beginning at the time of weaning (3–4 weeks), with the exception of mice for the 3-month time point, which were not co-housed. At 8–12 weeks of age, fecal specimens were collected for subsequent 16S amplicon sequencing, and then mice were given either regular water or water containing 2% DSS for 5 days followed by 4 days of regular water. For chronic DSS colitis, mice were given 3 cycles of 2% DSS for 5 days followed by regular water for 7 days. Mice were weighed daily.

Quantitative Polymerase Chain Reaction

Total RNA was isolated from whole colon tissue using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using 500 ng of total RNA, 125 U mMLV reverse transcriptase (Invitrogen, Carlsbad, CA), 250 ng random primers (Invitrogen, Carlsbad, CA, USA), 25 U RNasin (Promega, Madison, WI, USA), and 500 μ M dNTPs (Invitrogen, Carlsbad, CA, USA) in 25 μ L volume. Reaction was incubated at 39°C for 60 minutes followed by 93°C for 7 minutes and 4°C until ready to use. Two μ L of cDNA reaction was added to 8 μ L of iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 0.4 μ M of the following forward and reverse primers: B-ActinF 5'-ATGGAGGGGAATACAGCCC-3' and B-ActinR 5'-TTCTTTGCAGCTCCTTCGTT-3' or *Casp11*F 5'-ACAATGCTGAACGCAGTGAC-3' and *Casp11*R

5'-CTGGTTCCTCCATTTCAG-3'; this was placed in the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) running universal conditions. Data were analyzed using the ΔCt method with reference to Beta-actin.

Histological Analysis

In *Il10*^{-/-} mice, inflammation in formalin-fixed, hematoxylin and eosin-stained (H&E-stained) sections of cecum, proximal colon, midcolon, and distal colon was quantified in a blinded fashion and summed together as described previously.²⁷ In DSS experiments, inflammation in formalin-fixed, H&E-stained sections of cecum, proximal, mid, and distal colon was quantified in a blinded fashion as described previously.²⁸

TUNEL Staining

Formalin-fixed, paraffin-embedded sections of colon were deparaffinized in xylenes, rehydrated in ethanol, and then tested for apoptotic cells using the Click-iT TUNEL assay per the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Numbers of TUNEL-positive epithelial cell nuclei per colon crypt were enumerated by using fluorescence microscopy to examine approximately 200–300 crypts per mouse.

Enzyme-Linked Immunosorbent Assays

Segments of proximal colon (2–3 cm long) were washed, weighed, minced, and cultured for 22 hours as described previously, after which culture supernatants were stored at -80°C .²⁷ Mouse IL-12/23p40 in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) as described previously.²⁷ Mouse fecal Lcn-2 was measured by homogenizing feces in PBS containing 0.1% Tween 20, removing debris by centrifugation, and assaying Lcn-2 in diluted supernatants using the mouse lipocalin-2/NGAL DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Fecal DNA Isolation

Fecal DNA was isolated by incubating feces in 500 μL lysis buffer (200 mM NaCl, 100 mM Tris pH 8.0, 20 mM EDTA, 20 mg/mL lysozyme) at 37°C for 30 minutes, then adding 30 μL of 20% SDS and 30 μL proteinase K (20 mg/mL) and incubating at 60°C for 30 minutes; 300 μL of 0.1-mm glass beads (Biospec) and 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) were added, and samples were lysed in the TeSeE Precess48 bead beater (Bertin Technologies) for 3 rounds of 90 seconds at 6200 rpm. After centrifugation, the aqueous layer was extracted with another 500 μL of phenol:chloroform:isoamyl alcohol followed by 500 μL chloroform. The DNA in the aqueous phase was precipitated with ethanol, resuspended in Tris-EDTA buffer, and then further purified using DNEasy minicolumns according to the manufacturer's instructions (Qiagen).

Fecal Microbiome Analysis

Fecal bacterial composition was analyzed by amplification of the V4 variable region of the 16S rRNA gene by polymerase chain reaction (PCR), as described previously²⁹; 16S rRNA amplicon libraries of each fecal DNA sample were generated in 2 PCR steps: (1) amplification using 200 ng of fecal DNA as the DNA template, a mix of the 6 forward and 6 reverse modified 16S V4 primers (Supplementary Table 1), and the KAPA2G Robust PCR kit (Kapa Biosystems, Wilmington, MA, USA); (2) amplification using 5 μL of purified amplicons from the first amplification as template, forward and reverse primers containing 12-bases Golay barcode and Illumina adapter sequences (Supplementary Table 1), and the KAPA HiFi HotStart ReadyMix PCR kit. Amplicons were purified after each amplification using the HighPrep PCR clean-up kit (MagBio, Lausanne, Switzerland) and sequenced on the Illumina MiSeq platform (250 paired-end) at the UNC High Throughput Sequencing Facility. Sequencing data were processed by the Quantitative Insights Into Microbial Ecology (QIIME) pipeline³⁰ with quality control. We aligned reads (average of 125,087 reads/sample) and assigned them to operational taxonomic units (OTUs; total 101,318 OTUs) by a QIIME de novo OTU picking pipeline. Mixed linear and univariate models were created using the lme function from the nlme package in R. Scripts used to generate figures and tables in this paper are available at <https://github.com/afodor/casp11>.

RESULTS

Caspase-11 mRNA Levels Are Altered During Chronic Experimental Colitis

Others have previously shown that caspase-11 attenuates the severity of acute DSS colitis and that this protection is mediated by cells in both the hematopoietic and nonhematopoietic compartments.^{23–25} In addition, others have detected increased *Casp11* protein in colon tissue during acute DSS colitis.^{23,25} However, the roles of caspase-11 in chronic experimental colitis, a condition that is more representative of human IBDs than acute DSS colitis, have not been elucidated. We hypothesized that as *Casp11* protein is more abundant in colon tissue from mice with acute DSS colitis, *Casp11* mRNA would similarly be increased in colon tissue from mice with chronic experimental colitis. To test this hypothesis, we induced acute DSS colitis by feeding WT mice a 5-day cycle of DSS, and we induced chronic DSS colitis by feeding mice 3 cycles of DSS and then measured *Casp11* mRNA in midcolon tissue. Contrary to prior reports of increased *Casp11* protein expression in the colons of mice with acute DSS colitis, we observed modest decreases in *Casp11* mRNA in mice with acute DSS colitis at day 9 compared with healthy controls (Fig. 1A).^{23, 25} Likewise, *Casp11* mRNA levels are slightly decreased in colon tissues from mice with chronic DSS colitis as compared with

FIGURE 1. Colonic *Casp11* mRNA levels are altered during experimental colitis. **A**, *Casp11* mRNA in midcolon tissue from WT mice with acute and chronic DSS colitis at the indicated day postinitiation of DSS administration was measured by real-time PCR. Results were normalized to β -actin and are presented as mean $\pm$ SEM fold-change relative to day 0 ($n = 5$ –8 mice/group, $*P < 0.05$ relative to time 0, Student t test). **B**, *Casp11* mRNA in colon tissue from germ-free WT and *Il10*^{-/-} mice colonized for the indicated times with specific pathogen-free mouse feces. Results are normalized to β -actin and presented as mean $\pm$ SEM fold-change relative to day 0 ($n = 3$ to 10 mice/group, $*P < 0.05$ relative to WT, Student t test).

healthy controls (Fig. 1A; Supplementary Fig. 1). To confirm these findings in another model of chronic experimental colitis, we colonized germ-free WT and *Il10*^{-/-} mice with specific pathogen-free microbiota for up to 6 weeks, a time point at which *Il10*^{-/-} mice develop moderate chronic T-cell-mediated colitis,³¹ and measured *Casp11* mRNA in colon tissue. In contrast to what we observed during acute and chronic DSS colitis, we detected significantly increased levels of *Casp11* mRNA in the colon tissue of *Il10*^{-/-} vs WT mice at 6 weeks postcolonization, but not at 0 or 2 weeks (Fig. 1B; Supplementary Fig. 1). Together, these findings indicate that *Casp11* mRNA levels vary in colon tissue during experimental colitis and that the levels may depend upon the specific colitis model under investigation. The factors that induce or repress *Casp11* mRNA transcription

or stability during chronic colitis and the cell types in which *Casp11* is predominantly expressed remain to be determined.

Caspase-11 Does Not Affect the Severity of Chronic Experimental Colitis in *Il10*^{-/-} Mice

As we found altered *Casp11* mRNA in colon tissue during chronic experimental colitis, we hypothesized that caspase-11 may play a role in the pathogenesis of chronic intestinal inflammation. In particular, as others have previously shown that caspase-11 attenuates the severity of acute DSS colitis, we hypothesized that caspase-11 would similarly decrease the severity of chronic experimental colitis. To test this hypothesis, we generated *Il10*^{-/-}; *Casp11*^{-/-} double knockout mice (DKO) and monitored them for signs of colitis in a specific pathogen-free environment over the course of 6 months. Interestingly, we found no differences in histological inflammation in colon pieces, spontaneous secretion of IL-12/23p40 by colonic explant cultures, or levels of lipocalin-2 (Lcn-2) in feces from DKO mice compared with *Il10*^{-/-} mice (Fig. 2). In addition, we detected no differences in stool consistency or body weight between the 2 groups of mice (24.2 g in *Il10*^{-/-} mice vs 25.3 g in DKO mice; $P = 0.54$). These data suggest that, contrary to our hypothesis, caspase-11 does not affect the severity of colitis in the *Il10*^{-/-} mouse model of chronic colitis.

Caspase-11 Does Not Affect the Severity of Chronic Experimental DSS Colitis

As caspase-11 did not affect severity of colonic inflammation in *Il10*^{-/-} mice, we predicted that caspase-11 would similarly not impact the severity of chronic DSS colitis, even though others have previously shown that caspase-11 attenuates acute DSS colitis.^{23–25} We treated WT and *Casp11*^{-/-} mice with 3 rounds of DSS to induce chronic DSS colitis. After the third cycle of DSS administration, we observed no significant difference in weight loss, histologic colon inflammation, or spontaneous secretion of IL-12/23p40 by colonic explants in WT vs *Casp11*^{-/-} mice (Fig. 3). As others have reported different frequencies of apoptosis in colonic epithelial cells in *Casp11*^{-/-} vs WT mice with acute DSS colitis (1 report of increased and 1 report of decreased frequency in *Casp11*^{-/-} vs WT mice),^{23,25} we examined the effect of caspase-11 on colonic epithelial cell apoptosis during chronic DSS colitis. We detected low and similar levels of apoptosis in both WT and *Casp11*^{-/-} mice with chronic DSS colitis (Fig. 3E and F). We conclude that caspase-11 does not impact the severity of inflammation or colonic epithelial cell apoptosis in chronic DSS colitis.

Severity of Acute DSS Experimental Colitis Varies in *Casp11*^{-/-} Mice

During the chronic DSS colitis experiments, we noticed that *Casp11*^{-/-} mice consistently lost significantly less weight than WT mice during the acute phase of DSS colitis (ie, after the first round of DSS administration), suggesting that, contrary to previously

FIGURE 2. *Casp11* does not affect severity of chronic immune-mediated colitis in *Il10*^{-/-} mice. A, Composite blinded colon histological inflammation scores. B, Spontaneous IL-12/23 p40 secretion by colonic explant cultures. C, Lipocalin-2 (mLcn-2) levels from mouse feces. D, Representative photomicrographs of H&E-stained colon sections from 6-month-old WT, *Casp11*^{-/-}, *Il10*^{-/-}, and *Casp11*^{-/-}; *Il10*^{-/-} (DKO) mice housed in a specific pathogen-free environment. Data are presented as mean $\pm$ SEM, n = 6 to 8 mice/group. Abbreviation: N.S. = no statistically significant difference.

published reports, caspase-11 potentiates the severity of acute DSS colitis (Fig. 3A). Indeed, in repeated acute DSS colitis experiments in a total of 80 animals in 5 replicate experiments over a period of 18 months, we initially observed significantly increased weight loss in WT vs *Casp11*^{-/-} mice, but this difference disappeared after the second set of experiments (Fig. 4). In general, the severity of acute DSS colitis in *Casp11*^{-/-} mice worsened in repeated experiments over the 18-month time period. After the 18-month time period, we repeated the acute DSS colitis experiment in WT and *Casp11*^{-/-} littermates as opposed to co-housed mice and still detected no difference in colitis severity (Supplementary Fig. 2). Therefore, the method of microbiota transfer between mice of different genotypes (ie, co-housed from time of weaning vs littermates) did not appear to alter the acute DSS colitis phenotype after the 18-month time period.

Pronounced Stochastic Shifts in the Intestinal Microbiota Occur During the Time of Variable Acute DSS Colitis Severity in *Casp11*^{-/-} Mice

We next aimed to determine factors that might account for the variable acute DSS colitis phenotype that we observed

in our facility. As the DSS vendor, mouse colony, housing facility, water source, and chow type remained constant during this time period, we hypothesized that changes in fecal bacterial composition in the mice in our housing facility associate with changes in acute DSS colitis phenotype. To determine if the different experimental results at different time points correlate with changes in the fecal microbial composition, we performed 16S bacterial rRNA amplicon sequencing of fecal DNA from all 80 animals. Multidimensional scaling (MDS) ordination was performed using Bray-Curtis dissimilarity to describe the relative similarity of the entire microbial community in the 80 mice (Fig. 5). We noted that each time point clustered separately, but, surprisingly, the beginning (time point 0) and end (time point 18) showed the most similarity. Comparing statistical models with time as a factor (unordered) or numeric (ordered) variables (Supplementary Fig. 3) confirmed that the fecal microbiome appeared to be on a “random walk” moving from state to state but not moving consistently in any 1 direction over time.

Each cohort of mice was housed within distinct cages with 4–6 animals per cage (except for 1 cage having 2 animals). Within our MDS plot, some clustering of cages within each time point

FIGURE 3. *Casp11* does not affect severity of inflammation or epithelial cell apoptosis during chronic DSS colitis. WT and *Casp11*^{-/-} mice were treated with 3 cycles of 2% DSS in their drinking water for 5 days, followed by 7 days of regular water. A, Percent change of initial body weight. B, Composite blinded colon histological scores. C, Spontaneous IL-12/23 p40 secretion by colon explant cultures. D, Representative photomicrographs of H&E-stained cecal sections from WT and *Casp11*^{-/-} mice killed at 35 days. E and F, Frequency of TUNEL-positive intestinal epithelial cells per crypt (arrowheads) in mice with chronic DSS colitis as in (A–D). Data are presented as mean $\pm$ SEM, $n = 5$ to 9 mice/group. * $P < 0.05$ based on Student t test. Abbreviation: N.S. = not statistically different.

could be observed (Supplementary Fig. 4), but nonetheless distinct cages of cohorts clustered together by time point (Fig. 5). We therefore concluded that the impact of changes to the microbial community in different cohorts over time was not just a function of the previously demonstrated tendency³² of animals within similar cages to share similar microbial communities.

To explicitly model the effects of genotype on changes to the microbiota, we next considered the following mixed linear model:

$$Y = \text{genotype} + \text{time} + \text{genotype}:\text{time} + 1/\text{cage},$$

where genotype is a categorical variable with 2 possible values (*Casp11*^{-/-} vs WT), time is a factor (with possible values of 0, 3, 4, 12, and 18 months), and cage is a categorical variable within the mixed linear model.³² In each model, the Y variable is 1 of the first 10 MDS axes or the log-normalized relative abundance of each taxa at the genus level. We also included the genotype:time interaction terms in the model as the microbiota composition in mice of each genotype may change differently over time. We found that MDS axes 2, 3, and 4 were highly associated with changes over time, as evidenced by highly significant adjusted P values (blue line in Fig. 6A). One MDS axis (MDS2) had a significant term for the time:genotype interaction (black line in Fig. 6A). There were no other significant associations for the models of the first 10 MDS axes at a 5% false discovery rate

(FDR) threshold. Together, these data indicate that stochastic changes over time, rather than changes according to genotype alone, are a dominant signal within the fecal microbiome of these mice and may explain some of the variation in acute DSS colitis phenotype that we observed.

Within mixed models, categorical random variables (such as cage in our model) were modeled as a single off-diagonal parameter in the variance–covariance matrix. Mixed models therefore imposed additional parametric assumptions over canonical linear models in the form of an assumption that a single parameter is sufficient to account for covariation in each cage. To demonstrate that this assumption was not driving our conclusions, we constructed a series of univariate models in which genotype or time was directly compared with each MDS axis as the only parameter in the model without attempting to correct for cage effects. As was the case for our full mixed linear model, the results of univariate modeling showed strong effects for time and modest effects for genotype (Supplementary Fig. 5A). The concordance of the results of models with and without explicit terms for cage is consistent with our conclusion that there are stochastic changes to the microbial community over time within our animal facility and that these changes are unlikely to be explained only by cage effects.

To determine changes in the microbial community with higher resolution, we applied our full mixed linear model to the log-normalized relative abundance of each of 68 nonrare

FIGURE 4. The severity of acute DSS-induced colitis in *Casp11*^{-/-} mice in individual experiments worsens over time. A–E, Percent change of initial body weight in WT and *Casp11*^{-/-} mice treated with 2% DSS for 5 days followed by 4 days of regular water in repeated experiments over an 18-month time period. F, Sum of blinded histological inflammation scores of 4 segments of colon from mice in (B). G, Representative photomicrographs of colon tissue scored in (F). H, Sum of blinded histological inflammation scores of 4 segments of colon from mice in (E). I, Representative photomicrographs of colon tissue scored in (H). Data are presented as mean $\pm$ SEM, $n = 5$ to 10 mice/group. * $P < 0.05$ relative to WT based on Student t test. Abbreviation: N.S. = not statistically significant.

taxa that occurred in at least 25% of all of our samples. We corrected for multiple hypothesis testing with Benjamini-Hochberg correction and used a 5% FDR cutoff. As we would expect from our MDS analyses, changes over time remained

the dominant signal in our data set (Fig. 6B). For many taxa, there were time points in which the taxa were entirely absent, even though they were unambiguously present in other time points (Supplementary File 1). In general, the significance of

FIGURE 5. Fecal microbiota in WT and *Casp11*^{-/-} mice varies substantially over time. Fecal microbial composition, represented by MDS axes 2 and 3, in each of the 80 mice in acute DSS colitis experiments represented in Fig. 4. Each dot represents an individual WT or *Casp11*^{-/-} mouse, and the colors distinguish mice from different experimental time points. The data file used to generate this graph is available at https://github.com/afodor/casp11/blob/master/pcoa_rerunPlusMetadata_L6.txt.

the association with time is not well correlated to the abundance of each taxa (Fig. 6C), demonstrating that the changes to the microbial community are not limited to low-abundance taxa. As was the case for our PCA axes, univariate models lacking a term for cage replicated strong time effects and modest genotype effects (Supplementary Fig. 5B), confirming that our conclusions are not dependent on the assumptions of mixed linear models.

There were a number of taxa with significant terms for genotype and time:genotype interactions (Fig. 6B; Supplementary Tables 2 and 3; Supplementary File 1). Of the 9 taxa that had a significant time:genotype interaction at a 5% FDR threshold, 7 were in the order Clostridiales (Table 1). These data suggest that the abundances of these Clostridia fluctuated differently between WT and *Casp11*^{-/-} mice during the time period of our experiments and may have influenced the acute DSS

colitis phenotype. The effect sizes are modest, however, and this hypothesis will need to be confirmed in future work.

DISCUSSION

Others have previously shown that the transcripts of human orthologs to murine *Casp11* are increased in colon tissue from patients with active vs inactive ulcerative colitis²⁴ and that *Casp11* protein is elevated in colon tissue from mice with acute DSS colitis.²⁵ It has also been shown in acute DSS colitis that type II interferons are required for *Casp11* protein induction in the colon and that pro-*Casp11* protein is transiently upregulated in intestinal epithelial cells.²⁵ In cultured macrophages infected with a murine intestinal bacterial pathogen, reactive oxygen species also enhance *Casp11* protein expression.³³ In the current study, we show that *Casp11* transcripts are increased in whole colon tissue from *Il10*^{-/-} mice with spontaneous chronic immune-mediated colitis but are decreased in colon tissue in mice with acute and chronic DSS colitis. The difference in our finding of decreased *Casp11* mRNA and the previously published finding of increased *Casp11* protein expression during acute DSS colitis may be due to effects of acute DSS colitis on *Casp11* protein translation or mRNA stability. The reasons why colon *Casp11* mRNA is slightly decreased during chronic DSS colitis but markedly increased during chronic colitis in *Il10*^{-/-} mice are not clear but probably reflect the inherent differences in the disease mechanisms in these colitis models. Although others have identified that *Casp11* expression in both hematopoietic and nonhematopoietic cells affects acute DSS colitis and that reactive oxygen species induce *Casp11* expression in macrophages during acute bacterial infection,^{23–25, 33} the relative contributions of *Casp11*-expressing cell types to chronic intestinal inflammation and reactive oxygen species to *Casp11* transcription during chronic experimental colitis are unknown.

Despite significant alteration of *Casp11* transcription in colon tissue during acute and chronic colitis, our results suggest that *Casp11* deficiency does not affect severity of chronic

FIGURE 6. Fecal microbiota in mice from the acute DSS experiments varies stochastically with time and genotype + time. Results of a full mixed linear model with terms for genotype, time, and the genotype:time interaction. A, For the first 10 MDS axes. B, For each nonrare taxa present in at least 25% of all samples. C, Correlation between abundance of nonrare taxa and *P* value for time association. *P* values were corrected for false discovery rate with the Benjamini and Hochberg FDR procedure for (A) and (B), but uncorrected for (C). The dashed line indicates a 5% FDR.

TABLE 1: Taxa With Significant Terms for Time:Genotype Interaction in Mixed Linear Model

Bacterial Taxa	Adjusted <i>P</i> Values for Time:Genotype Interaction
k_Bacteria.p__Proteobacteria.c__Deltaproteobacteria.o__Desulfovibrionales.f__Desulfovibrionaceae.g__Bilophila	0.000678
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia	0.000708
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Peptostreptococcaceae.g__	0.000708
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Christensenellaceae.g__Christensenella	0.000982
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__SMB53	0.000982
k_Bacteria.p__Proteobacteria.c__Deltaproteobacteria.o__Desulfovibrionales.f__Desulfovibrionaceae.g__	0.009638
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__	0.014329
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Peptococcaceae.g__	0.017025
k_Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.Other.Other	0.043698

experimental colitis. This stands in contrast to results noted by others in acute DSS colitis models in which *Casp11* deficiency has been shown to exacerbate acute DSS colitis.^{23–25} The reasons why caspase-11 affects severity of acute, but not chronic, colitis are unknown. However, we speculate that mechanisms of inflammation that dominate acute DSS colitis such as intestinal epithelial cell damage and innate immune cell infiltration may be influenced more strongly by disruptions in the noncanonical inflammasome compared with mechanisms that dominate in chronic colitis such as intestinal CD4⁺ T-cell activation and infiltration. This reasoning is consistent with other data indicating *Casp11* expression in intestinal epithelial cells and macrophages and caspase-11 mediated inflammasome activation and pyroptosis in macrophages.^{24, 25, 34} Whether caspase-11 directly affects the function of CD4⁺ T cells, an important cell type in IBDs and chronic experimental colitis, is currently unknown.

In our facility, the presence of *Casp11* either worsens or has no effect on severity of acute DSS colitis. This is in contrast to previous reports in which caspase-11 protects against acute DSS colitis^{23–25} but partially consistent with a more recent report that caspase-11 does not affect acute DSS colitis severity.²⁶ The reasons for these discrepancies are not clear. The *Casp11*^{−/−} mice used in our studies are from the same source as in 2 of the prior studies.^{23, 24} Genetic drift in the *Casp11*^{−/−} colonies at the institutions or differences in vendor source of DSS, mouse chow, and mouse housing practices could explain the contrasting results. However, the fact that the severity of acute DSS colitis in our *Casp11*^{−/−} mice varied over time, during which all other previously mentioned factors presumably remained constant, suggests that differences in the environmental microbiota may contribute to the variation in phenotype.

The microbiota has been shown to alter the acute DSS colitis phenotype in mice deficient in other inflammasome components. For instance, Prevotellaceae are associated with increased susceptibility to DSS colitis in NLRP6^{−/−} mice.^{18, 35, 36} NLRP3^{−/−} mice have attenuated acute DSS colitis compared with WT mice, but co-housing WT and NLRP3^{−/−} mice restored

the acute DSS colitis phenotype of NLRP3^{−/−} mice to that of WT mice, presumably due to microbiota transfer between the 2 strains.³⁷ Casp3/11 double knockout mice also have less severe acute DSS colitis than WT mice, but this difference disappears when Casp3/11 double knockout and WT mice are co-housed.³⁸

Therefore, we propose that the severity of acute DSS colitis in *Casp11*^{−/−} mice is affected by changes in the intestinal microbiota. It is important to note that the retrospective nature of our study of changes in acute DSS colitis severity over time limits our ability to control for potential factors besides the microbiota that may also explain the observed changes. For example, unidentified changes in mouse genetic composition, animal husbandry practices, mouse chow, and/or DSS composition might also account for our results. It should also be noted that some of the observed variation in microbiota composition in our experiments could be due to technical variation in DNA isolation or sequencing depth, as the fecal DNA was isolated in batches for each time point and there was slight variation in sequencing depth at the different time points (Supplementary Table 4). However, the slight variation in sequencing depth does not likely account for all of the variation in microbiota composition over time as such variation occurs even for high-abundance taxa that are less sensitive to measurement error (Fig. 6C).

The majority of operational taxonomic units that demonstrate a time–genotype interaction in our model are in the Clostridiales order (Table 1). Some Clostridia are positively associated with improved intestinal epithelial cell barrier function, increased luminal concentrations of short chain fatty acids, and increased numbers and function of regulatory T cells, all of which can decrease intestinal inflammation.^{39–42} Although other unidentified environmental differences may explain the phenotype variability we observed, we hypothesize that stochastic changes in Clostridia in our study interact with genotype in complex ways, impacting the reproducibility of acute DSS colitis severity. Also, as caspase-11 is important in sepsis and macrophage responses to cytosolic lipopolysaccharide (LPS),^{43, 44} one could hypothesize that the microbiota

from early time points in our studies, at which acute DSS colitis is more severe in WT vs *Casp11*^{-/-} mice, may harbor bacterial species that are more prone to delivering LPS to the cytosol. Testing these hypotheses would require repeating the acute DSS colitis experiments in germ-free *Casp11*^{-/-} mice selectively colonized with specific Clostridia, specific species of cytosol-invasive bacteria, or with microbiota from the various facilities used by investigators in previously published studies.

In summary, we show that despite changes in *Casp11* mRNA in colon tissue during both acute and chronic experimental colitis, the presence of *Casp11* variably affects disease severity in these models. *Casp11* does not impact the severity of chronic experimental colitis, and its effect on acute DSS colitis severity varies over time, presumably depending on environmental factors. Profound stochastic changes in the microbiome that interact differently with WT and *Casp11*^{-/-} phenotypes, including members of the Clostridiales order, represent one such changing environmental factor. These findings reinforce the need for investigators to thoroughly consider environmental influences such as the microbiota when studying models of acute DSS colitis.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

REFERENCES

- Kappelman MD, Rifas-Shiman SL, Porter CQ, et al. Direct health care costs of Crohn's disease and ulcerative colitis in US children and adults. *Gastroenterology*. 2008;135:1907–1913.
- Sartor RB, Wu GD. Roles for intestinal bacteria, viruses, and fungi in pathogenesis of inflammatory bowel diseases and therapeutic approaches. *Gastroenterology*. 2017;152:327–339.e4.
- Jostins L, Ripke S, Weersma RK, et al; International IBD Genetics Consortium (IIBDGC). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012;491:119–124.
- Liu JZ, van Sommeren S, Huang H, et al; International Multiple Sclerosis Genetics Consortium; International IBD Genetics Consortium. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet*. 2015;47:979–986.
- Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001;411:603–606.
- Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001;411:599–603.
- Villani AC, Lemire M, Fortin G, et al. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet*. 2009;41:71–76.
- Schultz I, Verma D, Halfvarsson J, et al. Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. *Am J Gastroenterol*. 2009;104:1180–1188.
- Yamamoto-Furusho JK, Santiago-Hernández JJ, Pérez-Hernández N, et al. Interleukin 1 β (IL-1 β) and IL-1 antagonist receptor (IL-1RN) gene polymorphisms are associated with the genetic susceptibility and steroid dependence in patients with ulcerative colitis. *J Clin Gastroenterol*. 2011;45:531–535.
- Tountas NA, Casini-Raggi V, Yang H, et al. Functional and ethnic association of allele 2 of the interleukin-1 receptor antagonist gene in ulcerative colitis. *Gastroenterology*. 1999;117:806–813.
- Mahida YR, Wu K, Jewell DP. Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. *Gut*. 1989;30:835–838.
- Casini-Raggi V, Kam L, Chong YJ, et al. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *J Immunol*. 1995;154:2434–2440.
- Monteleone G, Trapasso F, Parrello T, et al. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol*. 1999;163:143–147.
- Pizarro TT, Michie MH, Bentz M, et al. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol*. 1999;162:6829–6835.
- Ligumsky M, Simon PL, Karmeli F, et al. Role of interleukin 1 in inflammatory bowel disease—enhanced production during active disease. *Gut*. 1990;31:686–689.
- Opipari A, Franchi L. Role of inflammasomes in intestinal inflammation and Crohn's disease. *Inflamm Bowel Dis*. 2015;21:173–181.
- Zhang J, Fu S, Sun S, et al. Inflammasome activation has an important role in the development of spontaneous colitis. *Mucosal Immunol*. 2014;7:1139–1150.
- Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 2011;145:745–757.
- Zaki MH, Boyd KL, Vogel P, et al. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity*. 2010;32:379–391.
- Allen IC, TeKippe EM, Woodford RM, et al. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med*. 2010;207:1045–1056.
- Wilson JE, Petrucci AS, Chen L, et al. Inflammasome-independent role of AIM2 in suppressing colon tumorigenesis via DNA-PK and Akt. *Nat Med*. 2015;21:906–913.
- Namba T, Tanaka K, Ito Y, et al. Positive role of CCAAT/enhancer-binding protein homologous protein, a transcription factor involved in the endoplasmic reticulum stress response in the development of colitis. *Am J Pathol*. 2009;174:1786–1798.
- Demon D, Kuchmyi A, Fossoul A, et al. Caspase-11 is expressed in the colonic mucosa and protects against dextran sodium sulfate-induced colitis. *Mucosal Immunol*. 2014;7:1480–1491.
- Williams TM, Leeth RA, Rothschild DE, et al. Caspase-11 attenuates gastrointestinal inflammation and experimental colitis pathogenesis. *Am J Physiol Gastrointest Liver Physiol*. 2015;308:G139–G150.
- Oficialska K, Raverdeau M, Aviello G, et al. Protective role for caspase-11 during acute experimental murine colitis. *J Immunol*. 2015;194:1252–1260.
- Blażewski AJ, Thiemann S, Schenk A, et al. Microbiota normalization reveals that canonical Caspase-1 activation exacerbates chemically induced intestinal inflammation. *Cell Rep*. 2017;19:2319–2330.
- Patwa LG, Fan TJ, Tchaptchet S, et al. Chronic intestinal inflammation induces stress-response genes in commensal *Escherichia coli*. *Gastroenterology*. 2011;141:1842–51.e1.
- Hansen JJ, Holt L, Sartor RB. Gene expression patterns in experimental colitis in IL-10-deficient mice. *Inflamm Bowel Dis*. 2009;15:890–899.
- Kleiman SC, Bulik-Sullivan EC, Glenney EM, et al. The gut-brain axis in healthy females: lack of significant association between microbial composition and diversity with psychiatric measures. *PLoS One*. 2017;12:e0170208.
- Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010;7:335–336.
- Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun*. 1998;66:5224–5231.
- McCafferty J, Mühlbauer M, Gharibeh RZ, et al. Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. *ISME J*. 2013;7:2116–2125.
- Lupfer CR, Anand PK, Liu Z, et al. Reactive oxygen species regulate caspase-11 expression and activation of the non-canonical NLRP3 inflammasome during enteric pathogen infection. *PLoS Pathog*. 2014;10:e1004410.
- Kayagaki N, Warming S, Lamkanfi M, et al. Non-canonical inflammasome activation targets caspase-11. *Nature*. 2011;479:117–121.
- Levy M, Thaiss CA, Zeevi D, et al. Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell*. 2015;163:1428–1443.
- Wlodarska M, Thaiss CA, Nowarski R, et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell*. 2014;156:1045–1059.
- Bauer C, Duesell P, Lehr HA, et al. Protective and aggravating effects of nlrp3 inflammasome activation in IBD models: influence of genetic and environmental factors. *Dig Dis*. 2012;30(Suppl 1):82–90.
- Brinkman BM, Becker A, Ayis RB, et al. Gut microbiota affects sensitivity to acute DSS-induced colitis independently of host genotype. *Inflamm Bowel Dis*. 2013;19:2560–2567.
- Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*. 2011;331:337–341.
- Atarashi K, Tanoue T, Oshima K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*. 2013;500:232–236.
- Furusawa Y, Obata Y, Fukuda S, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*. 2013;504:446–450.
- Peng L, Li ZR, Green RS, et al. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J Nutr*. 2009;139:1619–1625.
- Hagar JA, Powell DA, Aachoui Y, et al. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxemic shock. *Science*. 2013;341:1250–1253.
- Kayagaki N, Wong MT, Stowe IB, et al. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science*. 2013;341:1246–1249.