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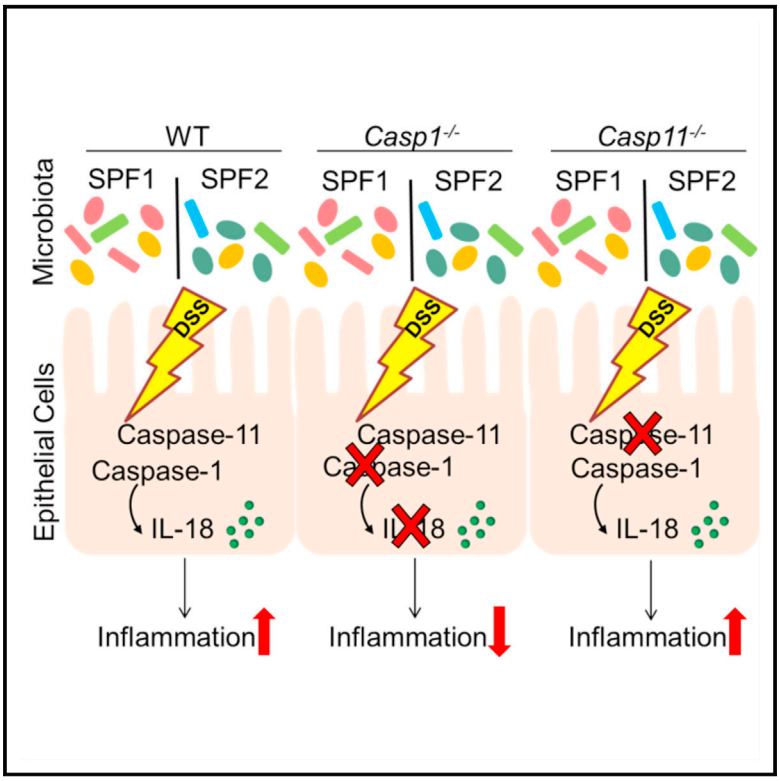
Microbiota Normalization Reveals that Canonical Caspase-1 Activation Exacerbates Chemically Induced Intestinal Inflammation

Graphical Abstract



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In Brief

The discovery of a passenger mutation and colitogenic alterations in the microbiota of Casp1$^{-/-}$Casp11$^{129mt/129mt}$ mice has called into question the role of caspase-1 in DSS colitis. Błażejewski et al. show using Casp1$^{-/-}$ mice with a standardized gut microbiota that caspase-1, but not caspase-11, exacerbates DSS-induced colitis.

Highlights

- Microbiota normalization enables the generation of isobiotic mouse lines
- Caspase-1, but not caspase-11, exacerbates inflammation during DSS-induced colitis
- Caspase-1-driven colitis is independent of the intestinal microbiota composition
- Caspase-1 expressed in intestinal epithelial cells exacerbates DSS colitis

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Microbiota Normalization Reveals that Canonical Caspase-1 Activation Exacerbates Chemically Induced Intestinal Inflammation

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SUMMARY

Inflammasomes play a central role in regulating intestinal barrier function and immunity during steady state and disease. Because the discoveries of a passenger mutation and a colitogenic microbiota in the widely used caspase-1-deficient mouse strain have cast doubt on previously identified direct functions of caspase-1, we reassessed the role of caspase-1 in the intestine. To this end, we generated *Casp1*$^{-/-}$ and *Casp11*$^{-/-}$ mice and rederived them into an enhanced barrier facility to standardize the microbiota. We found that caspase-11 does not influence caspase-1-dependent processing of IL-18 in homeostasis and during DSS colitis. Deficiency of caspase-1, but not caspase-11, ameliorated the severity of DSS colitis independent of microbiota composition. Ablation of caspase-1 in intestinal epithelial cells was sufficient to protect mice against DSS colitis. Moreover, *Casp1*$^{-/-}$ mice developed fewer inflammation-induced intestinal tumors than control mice. These data show that canonical inflammasome activation controls caspase-1 activity, contributing to exacerbation of chemical-induced colitis.

INTRODUCTION

The mammalian gastrointestinal tract is inhabited by a diverse microbial ecosystem, referred to as intestinal microbiota, which contributes to the physiology of the host in health and disease (Lozupone et al., 2012). Specifically, in diseases such as metabolic syndrome and inflammatory bowel diseases (IBDs), the microbiota is now considered a disease-modulating entity (Jostins et al., 2012; Kau et al., 2011). Large-scale studies of IBD patients identified numerous mutations in immune-related genes, including ones that affect the functionality of the innate immune system (Liu et al., 2015). In line with these findings, it is now accepted that the innate immune system contributes to the prevention of recurring inflammatory reactions to commensal microbes, while preserving the ability to defend against pathogens (Thaiss et al., 2016). Inflammasomes are part of the innate immune system, recognizing both pathogenic and beneficial microbial constituents (Henao-Mejia et al., 2012). They are multiprotein complexes composed of receptor and adaptor molecules, which control the activity of caspase-1. Active caspase-1 is responsible for processing of the cytokines pro-IL-1β and pro-IL-18 into their active forms as well as initiating a unique form of cell death called pyroptosis (Henao-Mejia et al., 2012; Strowig et al., 2012).

The roles of inflammasomes and caspase-1 have been studied extensively in different animal models of human IBD, including chemical-induced colitis (Siegmund et al., 2001), T cell transfer colitis (Harrison et al., 2015), and IL-10-deficient mice (Zhang et al., 2014). Notably, conflicting results regarding the role of inflammasome in the dextran sulfate sodium (DSS) colitis model have been reported (Siegmund, 2010). Specifically, mice deficient in caspase-1 or NLRP3 were found to feature decreased disease severity (Bauer et al., 2010; Siegmund et al., 2001), while others reported that deficiency in caspase-1 or NLRP3 exacerbates the disease (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). These disagreements potentially stem from two recently discovered complications. First, it was identified that the *Casp1*$^{-/-}$ mice (Kuida et al., 1995; Li et al., 1995) used in all previous studies were deficient in caspase-11, a closely related inflammatory caspase, as a consequence of an inactivating mutation in the *Casp4* gene encoding caspase-11 (i.e., they are *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ mice) (Kayagaki et al., 2011). Importantly, caspase-11 has, in addition to its initially described caspase-1-dependent functions, also inflammasome-independent functions, including serving as a receptor for intracellular lipopolysaccharide (LPS) as well as the regulation of cell death and cytokine release (Broz et al., 2012; Kayagaki et al., 2011; Shi et al., 2014). Any interpretation of the role of caspase-1 during DSS colitis using *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ is complicated by the

influence of caspase-11 on the severity of disease in this model (Demon et al., 2014; Oficjalska et al., 2015; Williams et al., 2015). Moreover, non-canonical activation of caspase-1 in intestinal epithelial cells by caspase-11 during oral infection with *Salmonella* Typhimurium suggests that caspase-11-dependent and caspase-11-independent processes may operate in the intestine (Knodler et al., 2014). The second complication stems from our observation that *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ mice feature a colitogenic microbiota that actively enhances severity of DSS colitis (Elinav et al., 2011). Moreover, subsequent studies by us and other groups demonstrated that alterations in the microbiota of these mice also actively modulate other complex in vivo disease models (Henao-Mejia et al., 2012; Hu et al., 2013; Lukens et al., 2014). Consequently, in order to characterize the intrinsic functions of caspase-1 in vivo, novel experimental systems involving a standardized genetic background and carefully controlled environmental conditions are required.

To this end, we generated *Casp1*$^{-/-}$ and *Casp11*$^{-/-}$ mice on a pure C57BL/6N genetic background. Moreover, we rederived both lines into a barrier mouse facility to maintain them with a defined non-dysbiotic microbiota, which was confirmed by 16S rRNA sequencing of the intestinal microbiota. We found that *Casp1*$^{-/-}$ mice, but not *Casp11*$^{-/-}$ mice, are characterized by decreased disease severity compared with wild-type (WT) animals in the DSS colitis model, suggesting that canonical caspase-1 activation is responsible for exacerbating the disease in this model. Using cell type-specific ablation of caspase-1, we demonstrate that expression of caspase-1 in intestinal epithelial cells is responsible for this phenotype and that absence of IL-18 in *Casp1*$^{-/-}$ mice correlates with a protective effect in our setting. In summary, our results clarify the function of caspase-1 in chemical-induced intestinal inflammation and highlight the need to use carefully controlled animal models for complex in vivo models of human diseases.

RESULTS

Caspase-11-Independent Activation of Caspase-1 in the Intestine in the Steady State

Caspase-1 controls numerous immune-related processes in the intestine during steady state and inflammation (Strowig et al., 2012). In order to precisely analyze the functions of both capase-1 and caspase-11, we have generated two mouse lines lacking either caspase-1 or caspase-11 on a on pure C57BL/6N genetic background (Figures S1A and S1B) (Case et al., 2013). We validated the mouse lines using previously identified bacterial triggers of canonical and non-canonical caspase-1 activation, namely, *Salmonella enterica* serovar Typhimurium (a trigger of the canonical inflammasome activation) and *Escherichia coli* (a trigger of the non-canonical inflammasome activation) (Kayagaki et al., 2011). Bone marrow-derived macrophages (BMDMs) deficient in caspase-1 (*Casp1*$^{-/-}$) failed to release IL-1β and to undergo pyroptotic cell death after infection with *S.* Typhimurium, comparable with previously described *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ BMDCs, while *Casp11*$^{-/-}$ BMDMs behaved like WT BMDMs (Figures S1C–S1E). Upon infection with *E. coli*, both *Casp1*$^{-/-}$ and *Casp11*$^{-/-}$ BMDMs displayed decreased IL-1β secretion, while only *Casp11*$^{-/-}$ BMDMs failed to induce cell death as expected (Figures S1C–S1E). This confirmed previous findings obtained using transgenic complementation of caspase-11 into *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice and demonstrating the utility of these mouse lines (Kayagaki et al., 2011).

Next, we assessed caspase-1 and caspase-11 function in vivo and chose to focus on the intestine, as previous reports identified a role for caspase-1 in regulating the composition of the intestinal microbiota (Elinav et al., 2011) as well as the severity of intestinal inflammation and tissue repair (Allen et al., 2010; Bauer et al., 2010; Dupaul-Chicoine et al., 2010; Siegmund et al., 2001; Zaki et al., 2010a). Caspase-1 and caspase-11 were readily detectable by western blot (WB) in colonic tissue of WT mice in homeostatic conditions, and they were absent in the respective gene-deficient mice (Figure 1A). We aimed to generate WT and gene-deficient mice with a comparable microbiota to avoid secondary effects on in vivo models due to alterations in the microbiota (Elinav et al., 2011; Levy et al., 2015). Recent studies demonstrated long-lasting effects of the microbiota on the host already during fetal and neonatal development (Cahenzli et al., 2013; Gomez de Agüero et al., 2016). Hence, instead of classic cohousing, we decided to pursue another approach, namely, rederivation by embryo transfer. Specifically, we rederived the mouse lines in an enhanced barrier facility by embryo transfer using foster mothers with a standardized microbiota devoid of any pathogens (SPF1). After rederivation, we analyzed the fecal microbiota composition in WT (n = 21), *Casp1*$^{-/-}$ (n = 26), and *Casp11*$^{-/-}$ (n = 18) mice using 16S rRNA analysis. We observed a similar abundance of all bacterial families present in the intestinal community of mice housed in the same room of our vivarium and a similar α diversity between genotypes (Figures 1B and S1F). Furthermore, clustering of mice during analysis of β diversity was independent of the genotype (analysis of similarities [ANOSIM]: R = 0.091, p = 0.004) (Figure 1C).

Production and release of IL-18 in the intestine is regulated both on the transcriptional and post-translational level by microbial products (Levy et al., 2015; Singh et al., 2014). Analysis of *Il18* expression revealed comparable *Il18* mRNA levels (Figure 1D). Because caspase-11 serves as sensor for intracellular LPS (Shi et al., 2014), we considered the possibility that LPS derived from intestinal bacteria may activate caspase-11 and contribute to caspase-1 processing in the steady state. However, comparable levels of active caspase-1 (p10) were detected by WB in the colon of WT and *Casp11*$^{-/-}$ mice (Figure 1A). Moreover, only caspase-1 but not caspase-11 deficiency resulted in decreased release of IL-18 in the colon in these non-inflammatory conditions (Figure 1E). Notably, the release of active IL-18 triggered by the production of taurine in the microbiota during the steady state has been shown to regulate expression of the antimicrobial peptides (AMPs) *Ang4* and *Retnlb1* (Levy et al., 2015). But under the tested environmental conditions in this study, equal expression of *Ang4* and *Retnlb1* was found in all mouse lines (Figures 1F and 1G). In summary, these results demonstrate that caspase-1 activation in the intestine is regulated independent of the non-canonical activation pathway in the steady state. Importantly, under these restrictive environmental conditions, the composition of the fecal microbiota is independent of the genotype, allowing in vivo experiments focusing on the host genotype rather than the microbiota.

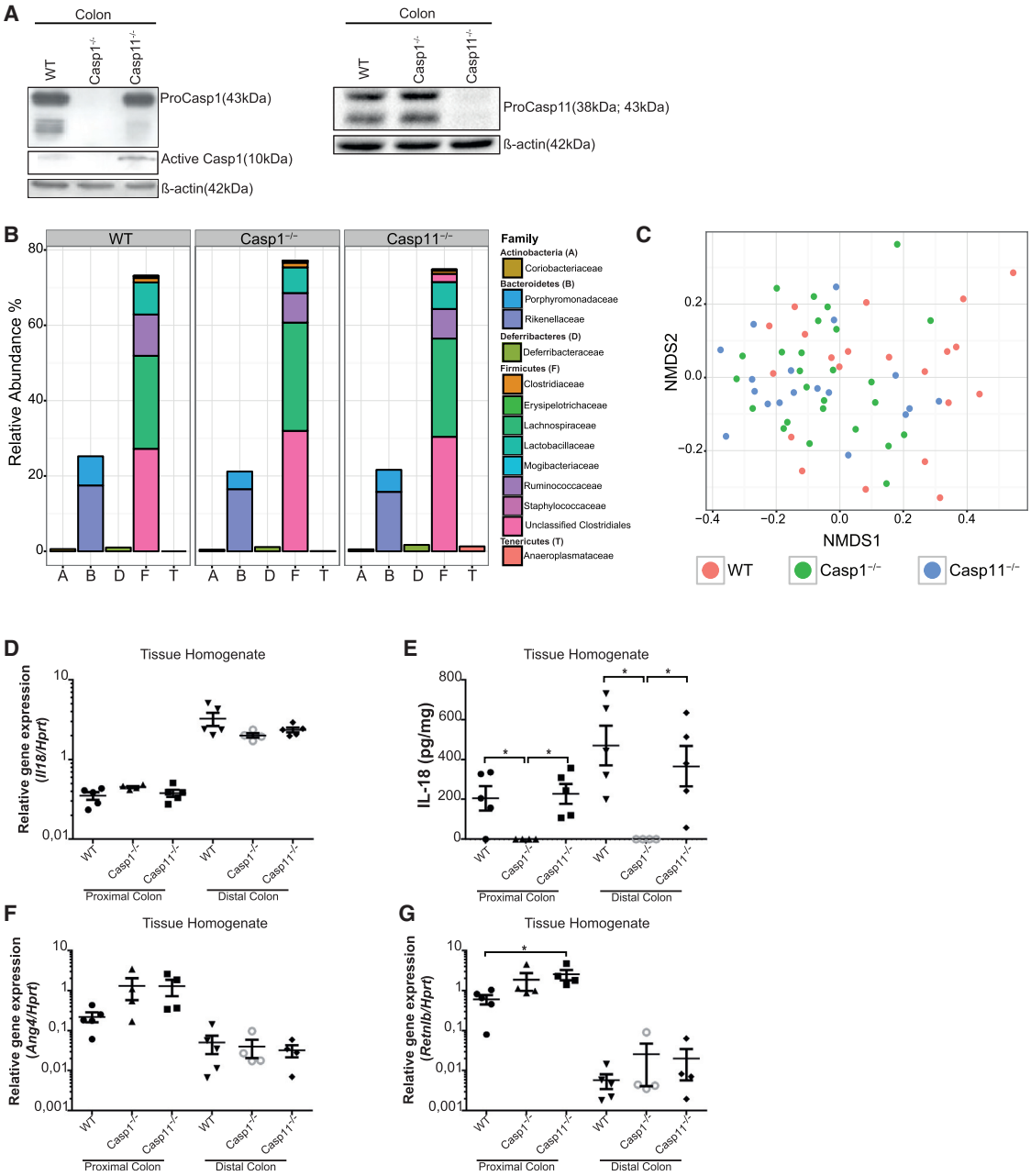

Figure 1. Caspase-11-Independent Activation of Caspase-1 in the Intestine in the Steady State

Characterization of microbiota and inflammasome activation in WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ male and female mice in the steady state.

(A) Caspase-1 and caspase-11 expression in proximal colon of indicated mice by western blot.

(B and C) Microbiota composition analysis using 16S rRNA sequencing of WT (n = 21), *Casp1*$^{-/-}$ (n = 26), and *Casp11*$^{-/-}$ (n = 18) mice. Relative abundances of bacterial families are shown grouped by phylum (B). β diversity analysis of fecal microbiota using NMDS (C). Data are from two independent experiments.

(D and E) Analysis of *Il18* gene expression (D) and production of active IL-18 (E) from indicated sites.

(F and G) Analysis of gene expression of *Ang4* (F) and *Retnlb* (G) from indicated sites. Representative data are from one out of three experiments.

Data shown as mean ± SEM. *$p < 0.05$ (performed with nonparametric Kruskal-Wallis test with multiple comparison).

Caspase-1 Deficiency Protects Mice from DSS-Induced Colitis

In order to investigate the functions of caspase-1 as well as the importance of non-canonical inflammasome activation during inflammation in the colon, we induced DSS colitis by providing DSS containing water (2.5% w/v). Colitis severity was evaluated by monitoring weight loss and survival of WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice. We included both male and female mice to

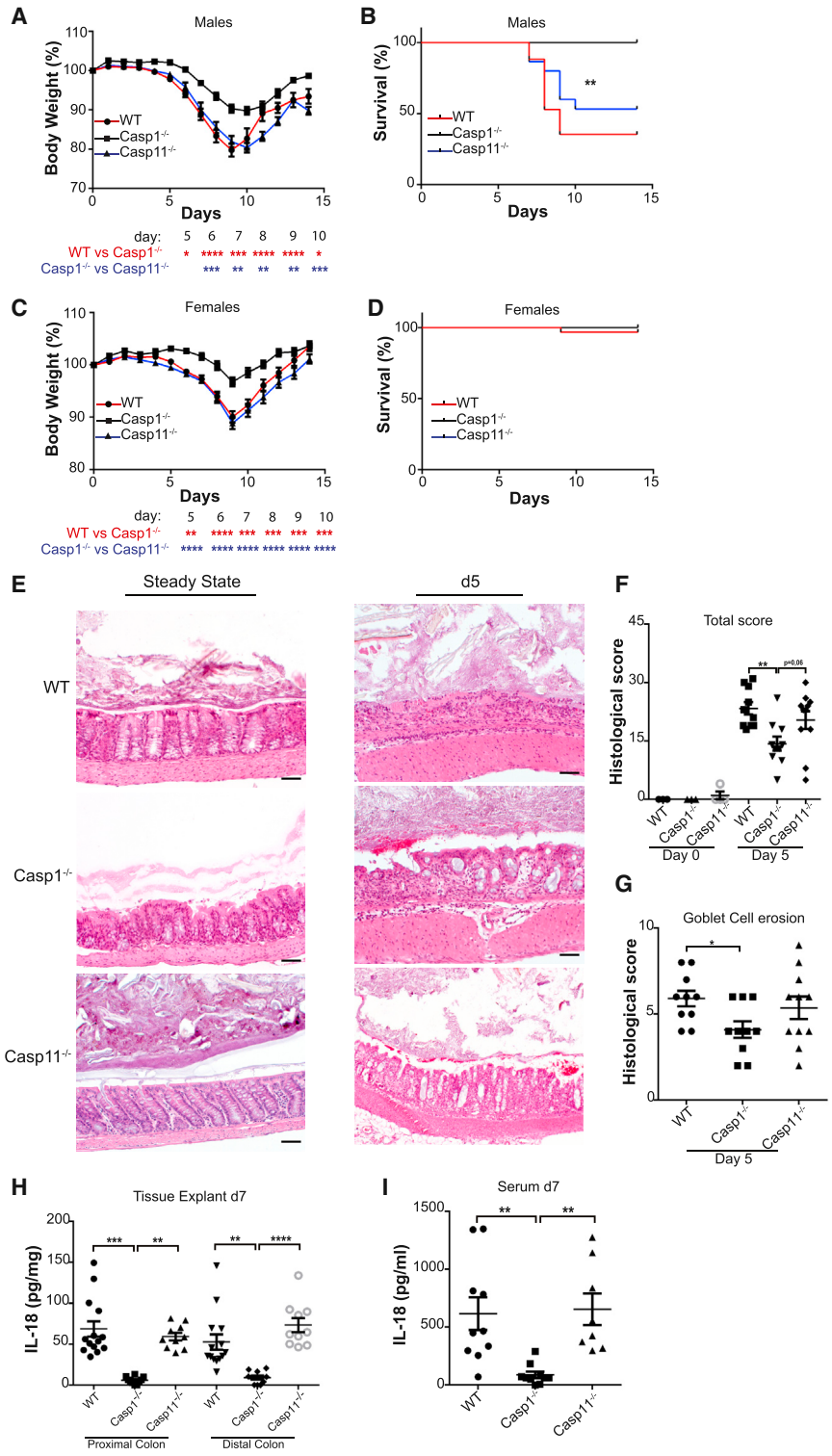

Figure 2. Caspase-1 Deficiency Protects Mice from DSS-Induced Colitis

DSS colitis was induced in WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice using 2.5% DSS (w/v) for 7 days.

(A and B) Weight loss (A) and survival (B) of WT (n = 17), *Casp1*$^{-/-}$ (n = 17), and *Casp11*$^{-/-}$ (n = 15) male mice. Data are pooled from two independent experiments.

(C and D) Weight loss (C) and survival (D) of WT (n = 32), *Casp1*$^{-/-}$ (n = 34), and *Casp11*$^{-/-}$ (n = 31) female mice. Data are pooled from three independent experiments.

(E–G) Histological analysis of WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ male and female mice were performed at indicated time points. Representative pictures of H&E-stained colon sections. The bar represents approximately 50 μm (E). Quantification of the total histological colitis severity score (F) and goblet cell erosion (G). Data are pooled from two independent experiments.

(H and I) Production of active IL-18 from WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice at day 7 of DSS colitis in colon (H) and serum (I). Data are from two independent experiments.

Data shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (performed with nonparametric Kruskal-Wallis test with multiple comparison).

investigate whether gender differences may contribute to the conflicting results previously reported (Bauer et al., 2010; Dupaul-Chicoine et al., 2010; Siegmund et al., 2001; Zaki et al., 2010a). Independent of the gender, WT mice lost significant more body weight compared with *Casp1*$^{-/-}$ mice (Figures 2A and 2C), and male WT mice displayed enhanced mortality (Figures 2B and 2D). In our experimental conditions, *Casp11*$^{-/-}$ mice exhibited comparable disease severity with WT mice (Figures 2A–2D), which is in contrast to other studies that had found enhanced diseases severity (Demon et al., 2014; Oficjalska et al., 2015; Williams et al., 2015). In order to corroborate the phenotype of *Casp1*$^{-/-}$ mice, we performed histological characterization of WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice before (steady state) and after induction of DSS colitis (d5) (Figure 2E). Prior to induction of DSS colitis, no histological changes were observed in all mouse lines, whereas during colitis, significantly higher colitis score was detected in sections of WT mice compared with *Casp1*$^{-/-}$ mice; in contrast, *Casp11*$^{-/-}$ mice showed similar colitis score to WT animals (Figure 2F). It was recently reported that IL-18 release during intestinal inflammation enhances goblet cell loss (Nowarski et al., 2015). We concomitantly observed reduced goblet cell loss in *Casp1*$^{-/-}$ mice (Figure 2G). In addition, the histological findings were further supported by colonoscopy. Namely, *Casp1*$^{-/-}$ mice displayed a lower endoscopic colitis score than WT controls (Figures S2A and S2B). In

parallel, we characterized cytokine production in the tissue and serum 7 days after induction of DSS colitis. Caspase-1 deficiency prohibited processing and activation of IL-18 in both proximal and distal colon as well as serum (Figures 2H, 2I, and S2C). Previous studies reported conflicting findings regarding the involvement of caspase-11 in processing IL-18 during DSS colitis (Demon et al., 2014; Oficjalska et al., 2015; Williams et al., 2015). In our experimental setting, IL-18 levels were unaffected in *Casp11*$^{-/-}$ mice during colitis, corroborating that non-canonical inflammasome activation is not involved in caspase-1 activation under these conditions (Figures 2H and 2I). Finally, IL-1β was barely detectable in WT mice under these conditions, but a significant reduction was observed in the proximal colon of *Casp1*$^{-/-}$ mice (Figure S2D). Higher severity of disease correlated with increased production of IL-6 in the colon of WT mice compared with *Casp1*$^{-/-}$ mice (Figure S2F). However, no significant differences were observed in the serum of WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice regarding IL-6 (Figure S2F) and other tested inflammatory cytokines, including IL-1β, TNF-α (Figures S2H and S2I), IL-4, IL-5, IL-1, IL-12p70, IL-17A, IL-17F, IL-22, IL-23, and IL-27 (data not shown). These results strongly suggest that caspase-1 promotes the severity of DSS-induced colitis in a caspase-11-independent manner via controlling the release of IL-18.

Promotion of DSS Colitis by Caspase-1 Is Independent of Microbiota Composition

Several recent studies have identified specific interactions between distinct intestinal microbes or microbial products and corresponding immune pathways that regulate the outcome of intestinal inflammation (Levy et al., 2015; Round et al., 2011; Seo et al., 2015; Smith et al., 2013). In order to determine whether the phenotype was a result of the specific microbiota community structure in our animal facility, we introduced a different microbiota into the mice prior to the start of the experiments. Therefore, WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice bred in our vivarium (SPF1) were cohoused for 4 weeks with WT mice from a commercial vendor (SPF2) containing a community with members from several bacterial families not represented in our animal facility (e.g., S24-7, Desulfovibrionaceae, and Verrucomicrobiaceae), including ones with potentially pro-inflammatory properties (i.e., Prevotellaceae, Enterobacteriaceae) (Figure 3A) (Elinav et al., 2011; Seo et al., 2015). Microbiota analysis showed that cohousing resulted in equal transfer of the microbiota into WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice as demonstrated by similar α diversity and the lack of cluster formation according to genotype in β diversity analysis (ANOSIM: $R = 0.018$, $p = 0.001$) (Figures 3A, 3B, and S3A). Next, we evaluated whether this altered community influenced the gene expression and release of IL-18 in the steady state, respectively. Gene expression of *Il18* was not different among WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice with SPF1 and SPF2 microbiota (Figures 1D and 3C). However, an enhanced amount of active IL-18 was detected in the proximal, but not distal, colon of SPF2 WT mice compared with SPF1 conditions (Figures 3D and 3E). Similar to SPF1 conditions, IL-18 processing was strictly dependent on caspase-1 but not caspase-11 (Figures 3D and 3E). Despite the differences in active IL-18 between genotypes and similar to SPF1 conditions no differences in *Ang4* and *Retnlb1* were detected between WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice (Figures 3F and 3G). In order to test whether the SPF2 microbiota influences the phenotype of *Casp1*$^{-/-}$ and *Casp11*$^{-/-}$ mice differently than the SPF1 microbiota, we induced DSS colitis after 4 weeks of cohousing. Compared with WT mice, *Casp1*$^{-/-}$ mice displayed ameliorated disease after induction of DSS colitis, as characterized by reduced weight loss (Figures 3H and 3I). In line with our findings in SPF1 conditions, WT and *Casp11*$^{-/-}$ mice were characterized by comparable weight loss (Figures 3H and 3I). Similar to SPF1 conditions, IL-18 processing during DSS colitis was independent of caspase-11 (Figure 3J). In addition, we assessed the DSS colitis phenotype in *Casp1*$^{-/-}$ mice raised in conventional housing conditions and containing a microbial ecosystem similar to the one described in dysbiotic *Nlrp6*$^{-/-}$ mice (Figures S3B and S3C) (Elinav et al., 2011). Again, *Casp1*$^{-/-}$ mice were protected relative to cohoused WT mice (Figures S3D–S3F). These results corroborate that the phenotype of *Casp1*$^{-/-}$ mice is independent of the composition of the microbiota, at least in the microbial environments studied here, and independent of caspase-11 demonstrating that canonical inflammasome activation exacerbates DSS colitis in presence of different intestinal microbial communities.

Caspase-1 Exacerbates Inflammation-Induced Intestinal Tumorigenesis

Human IBD patients have an increased risk to develop colorectal cancers as a consequence of repeated mucosal damage and repair during bouts of disease (Malik, 2015). Several studies have implicated inflammasomes in intestinal tumorigenesis showing that *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ mice, have enhanced tumorigensis during colitis-associated cancer development (Allen et al., 2010; Hu et al., 2010; Zaki et al., 2010b). The interpretation of these results is complicated by studies showing that alterations in the microbiota composition influence tumorigenesis in the AOM/DSS colitis model (Hu et al., 2013; Man et al., 2015). Hence, we investigated the influence of caspase-1 in this model using WT and *Casp1*$^{-/-}$ mice with standardized microbiota. WT and *Casp1*$^{-/-}$ mice displayed differences in the weight loss similar to the acute DSS colitis model (Figure 4A). Endoscopy revealed larger numbers of tumors and higher total tumor scores in WT mice compared with *Casp1*$^{-/-}$ mice (Figures 4B and 4C). Histopathological investigation further corroborated decreased number and size of adenomas in *Casp1*$^{-/-}$ compared with WT mice (Figure 4D). Moreover, adenomas in *Casp1*$^{-/-}$ mice were less likely to develop malignant characteristics such as undifferentiated foci and submucosal herniation (Figure 4E). These results show that in isobiotic SPF conditions, reduced inflammation and tissue damage as a consequence of caspase-1 deficiency ameliorates intestinal tumorigenesis.

Deletion of Epithelial Caspase-1 Protects against DSS Colitis

Caspase-1 is expressed in hematopoietic and non-hematopoietic cell types, and its expression is furthermore modulated by inflammatory signals (Strowig et al., 2012). In the intestine, epithelial cells and resident myeloid cells express caspase-1 in non-inflammatory conditions (Figure 5A), and it is additionally

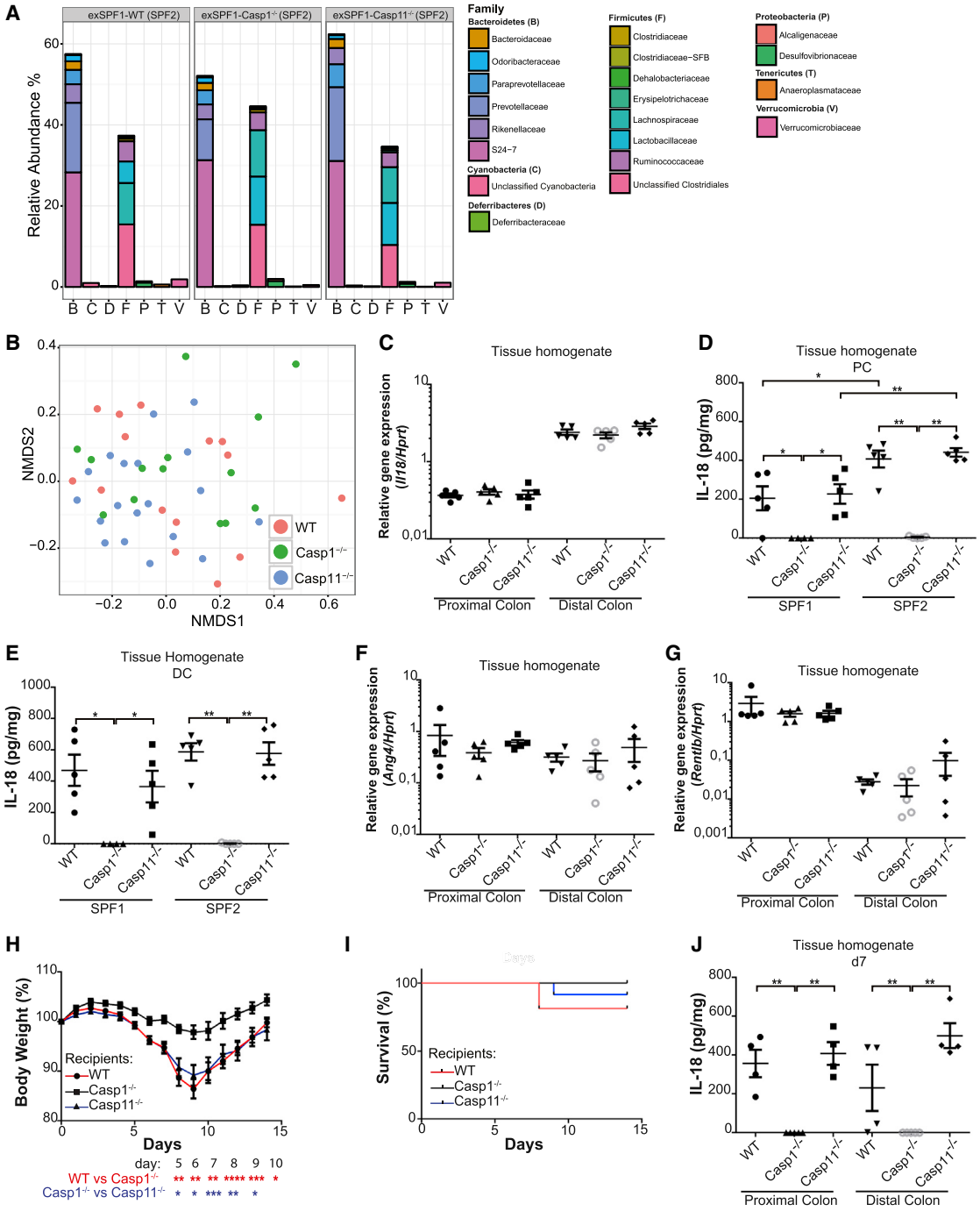

Figure 3. Promotion of DSS Colitis by Caspase-1 Is Independent of Microbiota Composition

WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ recipient female mice (SPF1) were cohoused with WT donor female mice (SPF2) for 4 weeks. After cohousing, characterization of microbiota and inflammasome activation in control and gene-deficient mice in the steady state was performed.

(A and B) Microbiota composition analysis using 16S rRNA sequencing of WT (n = 14), *Casp1*$^{-/-}$ (n = 14), and *Casp11*$^{-/-}$ (n = 18) recipient mice. Relative abundances of bacterial families are shown grouped by phylum (A). β diversity analysis of fecal microbiota using NMDS (B). Data are from two independent experiments.

(C–E) Analysis of *Il18* gene expression (C) and production of active IL-18 (D and E) from indicated sites (SPF1; see also Figure 1E).

(F and G) Analysis of gene expression of *Ang4* (F) and *Retnlb* (G) from indicated sites. Representative data are from one out of three experiments.

(H–J) After 4 weeks of cohousing, DSS colitis was induced using 2% DSS (w/v) in recipient (SPF2) WT (n = 16), *Casp1*$^{-/-}$ (n = 17), and *Casp11*$^{-/-}$ (n = 12) female mice. Data are pooled from two independent experiments. Weight loss (H) and survival (I) of recipient mice, as well as production of active IL-18 at day 7 of DSS colitis in colon (J).

Data shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (performed with nonparametric Kruskal-Wallis test with multiple comparison).

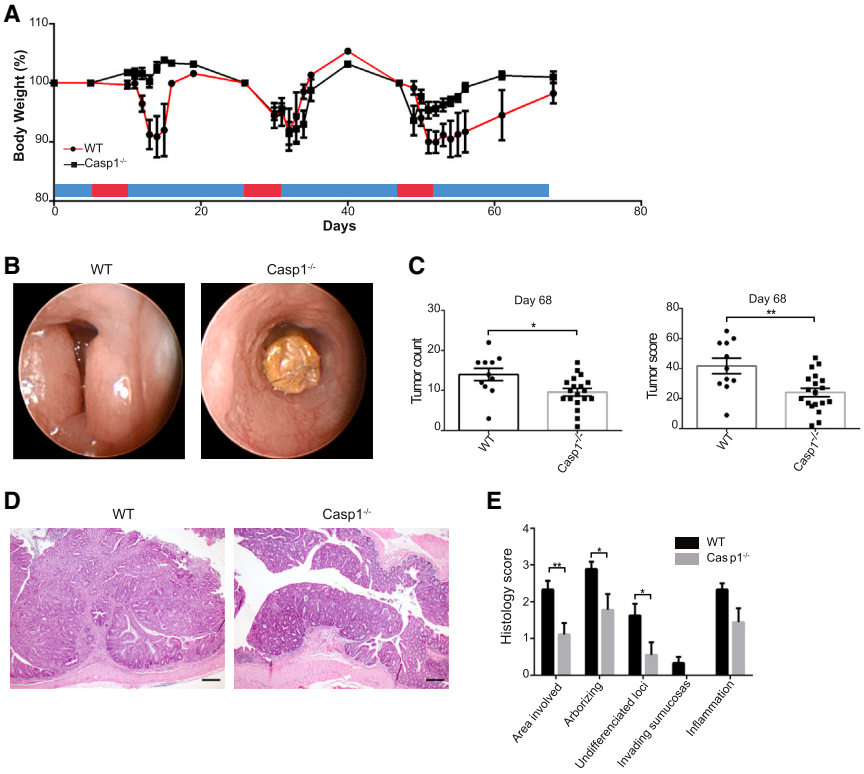

Figure 4. Caspase-1 Exacerbates Inflammation-Induced Intestinal Tumorigenesis

Colon tumorigenesis was induced in WT and *Casp1*$^{-/-}$ male mice by administration of AOM followed by three cycles of DSS colitis.

(A) Weight loss of WT (n = 8) and *Casp1*$^{-/-}$ (n = 10) mice during course of experiment.

(B and C) Quantification of intestinal tumorigenesis using endoscopy at day 68. Representative appearance of colon of AOM/DSS-treated mice (B). Tumor number and total tumor score of WT (n = 11) and *Casp1*$^{-/-}$ (n = 19) male mice (C). Data are pooled from two independent experiments.

(D and E) Representative H&E-stained sections of colon (the bar represents approximately 200 μm) (D) and scoring of AOM/DSS-induced colon cancer in WT (n = 9) and *Casp1*$^{-/-}$ (n = 9) male mice on day 68 (E). Representative data are from one out of two independent experiments.

Data shown as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ (performed with a nonparametric Mann-Whitney test).

expressed in infiltrating monocytes and granulocytes during inflammation (Seo et al., 2015). In order to investigate the relative contribution of caspase-1 in different cell types to the regulation of disease severity during DSS colitis, we generated mice conditionally deficient in caspase-1 (Figure S1). We first assessed the relative contribution of intestinal epithelial cells to caspase-1 expression in the intestine using Casp1$^{fl/fl}$/Vilcre^{+} mice (*Casp1*$^{\Delta IEC}$) and littermate control mice in SPF1 conditions. Analysis of tissue lysates by WB demonstrated that caspase-1 in the intestine is largely expressed in epithelial cells, as shown by the almost complete absence of pro-caspase-1 in Casp1$^{\Delta IEC}$ mice (i.e., less than 5% of pro-caspase-1 remaining) prior to induction of DSS colitis; moreover, we observed no differences in caspase-11 expression in in Casp1$^{\Delta IEC}$ mice (Figures 5B, 5C, and S4A). Reduced levels of active caspase-1 as revealed by WB (p10) and ELISA for IL-18 demonstrated that caspase-1 activity in the steady state was largely limited to epithelial cells in the colon (Figures 5B and 5D). Characterization of microbiota composition by 16S rRNA gene analysis showed no clustering and similar α diversity of Casp1$^{\Delta IEC}$ and littermate control mice according to genotypes (ANOSIM: R = 0.29, p = 0.005) (Figures 5E, 5F, and S4B). Upon induction of DSS colitis, Casp1$^{\Delta IEC}$ mice were characterized by decreased disease severity as demonstrated by reduced weight loss compared with littermate controls (Figure 5G). Histological characterization before and after induction of DSS colitis (d5) showed that prior to induction of DSS colitis, no histological changes as well as no disturbances in mucus integrity were observed. In contrast, during colitis, significantly higher colitis score was detected in sections of Casp1^{fl} mice compared with *Casp1*$^{\Delta IEC}$ mice; furthermore, we observed reduced goblet cell loss in *Casp1*$^{\Delta IEC}$ mice (Figures S4C–S4E). Similar to *Casp1*$^{-/-}$ mice, IL-18 was absent in tissue lysates of Casp1$^{\Delta IEC}$ mice during DSS colitis under these environmental conditions (Figure 5H). We did not detect IL-1β in Casp1$^{\Delta IEC}$ and littermate control mice during DSS colitis. Moreover, no significant differences were observed in other tested inflammatory cytokines including IL-6, IL-1α (Figures S4F–S4I), TNF-α, IL-12p70, IL-17A, IL-17F, IL-22, IL-23, and IL-27 (data not shown). In order to assess whether caspase-1 expressed in myeloid cells contributes to the phenotype, we similarly performed DSS colitis using mice deficient in capase-1 in myeloid cells, Casp1$^{\Delta Lyz}$. No significant difference in weight loss was observed between Casp1$^{\Delta Lyz}$ and littermate control mice (Figure S4J); also, no changes in IL-18 levels during DSS were noted (Figure S4K). These data show that intestinal epithelial cells are the main cell type for activation of caspase-1 and are the major source of IL-18 during acute DSS colitis, exacerbating the severity of disease.

DISCUSSION

Inflammasomes are multiprotein platforms that control the activity of caspase-1. Since their generation, caspase-1-deficient mice have been used to study the functions of inflammasomes in homeostasis and pathophysiological conditions. However, the discoveries of a passenger mutation and disease-modifying alterations in the microbiota of caspase-1-deficient mice have called into question some of the physiological functions of Caspase-1 (Elinav et al., 2011; Kayagaki et al., 2011; Siegmund, 2010). Similar to many other gene-deficient mouse lines, caspase-1-deficient strains were generated using embryonic stem cells (ESCs) from 129 mouse lines (Kuida et al., 1995; Li et al., 1995). Comparative genomic analysis of the C57BL/6J and 129

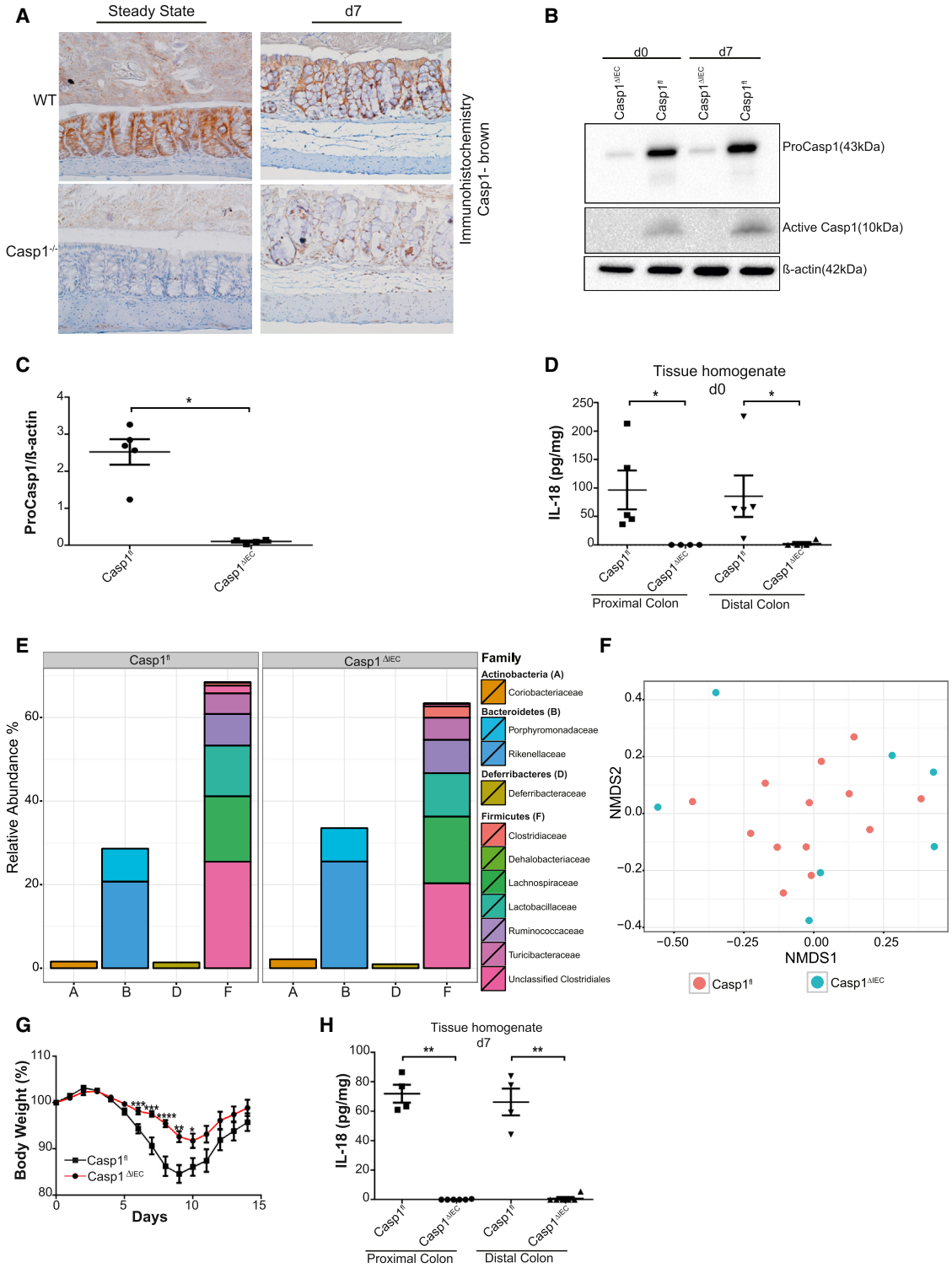

Figure 5. Deletion of Caspase-1 in Intestinal Epithelial Cells Protects against DSS Colitis

Characterization of microbiota composition, inflammasome activation, and DSS colitis severity in male and female mice with caspase-1 cell type-specific deletion.

(A) Detection of caspase-1 expression in the colon of WT and *Casp1*$^{-/-}$ female mice using immunohistochemistry (Casp1: brown staining) on day 0 and day 7 of DSS treatment. The bar represents approximately 50 μm.

(B) Analysis of caspase-1 expression in the proximal colon of *Casp1*$^{fl/fl}$ and *Casp1*$^{\Delta IEC}$ male mice using WB.

(legend continued on next page)

strains has recently identified genetic changes in more than 1,000 genes between these lines (Vanden Berghe et al., 2015). Specifically, the missense mutation in the *Casp11* gene of the 129 strain (*Casp11*$^{129mt/129mt}$) is estimated to be present in up to 86 other mouse lines in addition to the *Casp1*$^{-/-}$ *Casp11*$^{129mt/129mt}$ mice (Vanden Berghe et al., 2015). Importantly, the use of littermate mice does not correct for this issue but rather requires isogenic mouse lines (i.e., the de novo gene targeting on backgrounds suitable for the relevant experiment models).

Beyond the importance of precise genetic models, the impact of the microbiota as a disease-modulating entity on experimental animal models has been identified in the past years (Belkaid and Hand, 2014; Honda and Littman, 2016; Thaiss et al., 2016). Differences in microbiota composition as well as specific interactions between the immune system and intestinal bacteria have been proposed to contribute to the phenotypic discrepancies that have been observed in experimental animals on the same genetic background but maintained in different facilities (Macpherson and McCoy, 2015). However, the exact extent of how much microbiota composition compared with other experimental variables, such as genetic background, influences disease models is currently not fully understood.

The situation has been similarly controversial in the case of inflammasomes and specifically caspase-1 activation (Allen et al., 2010; Bauer et al., 2010; Dupaul-Chicoine et al., 2010; Siegmund et al., 2001; Zaki et al., 2010a) (Table S1). Yet, besides our own work (Elinav et al., 2011), studies characterizing the role of caspase-1 in DSS colitis have not formally considered the influence of the microbiota on the severity of disease, potentially contributing to opposing results and great confusion in the field (Siegmund, 2010). Some of these studies used WT mice that were purchased from vendors, and others obtained control mice from separately maintained lines at the same facility, which both could potentially harbor distinct microbial communities compared with the gene-deficient mouse lines (Ubeda et al., 2012) (Table S1). In contrast, we rederived both WT and gene-deficient mice through embryo transfer into an enhanced barrier facility with a standardized microbiota introduced through the foster mothers. Analysis of the microbiota composition by 16S rRNA sequencing demonstrated that under these controlled conditions, no significant differences in composition were detectable in these mouse lines. The observed lack of clustering by genotype under these conditions does not exclude that caspase-1 influences the microbiota composition per se, as the microbial community in our facility lacks representatives of many of the bacterial groups that had altered abundances in previous studies (i.e., the family Prevotellaceae and the phylum Proteobacteria) (Elinav et al., 2011).

Notably, IL-18 was recently shown to regulate gene expression of the AMPs *Ang4* and *Retnlb*, regulating thereby the interplay of host and microbiota (Levy et al., 2015). In our tested environmental conditions, no differences could be detected in the expression of *Ang4* and *Retnlb* between WT and *Casp1*$^{-/-}$. This suggests that IL-18 modulates expression of these AMPs only under specific environmental conditions (i.e., in the presence of distinct commensals that were absent in the two communities we tested). These findings support the notion that normalization and state-of-the-art documentation of microbiota composition are essential to disentangle the relative contributions of microbiota and specific genes in animal models.

In order to characterize the role of caspase-1 in intestinal inflammation, we used the above-described precise genetic models and normalization of the microbiota. Comparison of severity of DSS colitis in WT, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ mice demonstrated that caspase-1 exacerbates disease severity. This phenotype was confirmed in three different microbiota settings. Moreover, we concluded that canonical (i.e., caspase-11-independent) inflammasome activation was responsible for inducing inflammation during DSS colitis. Notably, a similar conclusion had been made by Siegmund et al. (2001) despite using *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ mice. But the results reported here contradict the findings of other studies (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a), which we hypothesize could have been influenced by differences in microbiota composition in combination with the described passenger mutation. Although in this study, caspase-11-deficient mice showed a similar phenotype during DSS-induced colitis as WT mice, other studies had observed that *Casp11*$^{-/-}$ mice were more susceptible to disease (Demon et al., 2014; Oficjalska et al., 2015; Williams et al., 2015). Differences in the mouse strains used, genetic backgrounds, and experimental variables again complicate the comparison among individual studies (Table S1). Two of these studies used *Casp11*$^{-/-}$ mice generated on a C57BL/6J background, comparing them to WT C57BL/6J mice (Demon et al., 2014; Williams et al., 2015), while the other used *Casp11*$^{-/-}$ mice generated on a 129 background, comparing them with littermate control mice (Oficjalska et al., 2015). As discussed above, the use of WT mice bred separately from the gene-deficient mice without careful normalization of the microbiota may result in microbiota-modulated and genotype-independent effects of the course of disease. Conspicuously, it was noted by Demon et al. (2014) that *Casp11*$^{-/-}$ mice harbored an altered microbiota compared with WT mice, as indicated by reduced abundance of the family Prevotellaceae as measured by qPCR. Upon cohousing for 4 weeks with WT mice, the phenotype did not change, but recently identified effects of the microbiota on the development of the immune system in utero or in the

(C) Quantification of pro-caspse-1 protein expression in proximal colon.
(D) Quantification of active IL-18 at indicated sites. Representative data are from one experiment.
(E and F) Microbiota composition analysis using 16S rRNA sequencing of *Casp1*$^{fl/fl}$ (n = 7) and *Casp1*$^{\Delta IEC}$ (n = 13) female mice. Relative abundances of bacterial families are shown grouped by phylum (E). β diversity analysis of fecal microbiota using NMDS (F). Representative data are from one experiment.
(G and H) Colitis was induced in *Casp1*$^{\Delta IEC}$ male and female mice and their littermate control mice using 2.5% DSS. Weight loss of *Casp1*$^{fl/fl}$ (n = 12) and *Casp1*$^{\Delta IEC}$ (n = 16) mice (G). Data are from two independent experiments. Active IL-18 production in *Casp1*$^{fl/fl}$ (n = 4) and *Casp1*$^{\Delta IEC}$ (n = 6) from indicated sites (H). Representative data are from one of two independent experiments.
Data shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (performed with a nonparametric Mann-Whitney test).

postnatal period cannot be excluded (Cahenzli et al., 2013; Gomez de Agüero et al., 2016). The use of littermates by Oficjalska et al. (2015) likely normalized the microbiota composition, but as discussed above, additional unwanted passenger mutations may have contributed to the observed phenotype. Furthermore, as a potential mechanism how caspase-11 influences the severity of DSS colitis, two studies reported that caspase-11 deficiency resulted in decreased IL-18 processing, which they linked to exacerbation of disease. In contrast, in addition to our study, another study did not observe any influence of caspase-11 on the processing of IL-18 (Demon et al., 2014). We hypothesize that differences in IL-18 processing observed in the two studies are likely linked to the presence of specific caspase-11-activating microbes or reflects the different degrees of inflammation between the mouse lines. But we cannot exclude that the role of caspase-11 is also partly influenced by genetic changes between C57BL/6J and C57BL/6N backgrounds. Despite remaining uncertainties about the contribution of caspase-11, our results demonstrate that caspase-1 exacerbates DSS colitis independent of its potential influence on microbiota composition.

Repeated bouts of inflammation and tissue destruction followed by tissue repair during IBD have been associated with the development of colon cancer in humans (Malik, 2015). *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ mice were previously found to feature enhanced intestinal tumorigenesis in the AOM-DSS colitis model (Allen et al., 2010; Zaki et al., 2010b), presumably as a consequence of increased microbiota induced inflammation during DSS colitis. In contrast, we observed decreased intestinal tumorigenesis in *Casp1*$^{-/-}$ mice, as demonstrated by endoscopy and histology. Interestingly, we observed similar weight loss between WT and *Casp1*$^{-/-}$ mice during the second cycle but not other cycles of AOM/DSS treatment, indicating a possible mechanical immunoregulatory differences between acute and chronic colitis models (Bento et al., 2012). Alterations in the microbiota, which have been found to alter susceptibility of intestinal tumorigenesis (Hu et al., 2013), or the passenger mutations in caspase-11 may have influenced previously reported contradicting results. Notably, Williams et al. (2015) did not find evidence of a role of caspase-11 in the AOM/DSS colitis model (Williams et al., 2015), suggesting that other genetically linked passenger mutations in *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ mice could have contributed to the enhanced tumorigenesis.

Active caspase-1 cleaves the proinflammatory cytokines pro-IL-1β and pro-IL-18 into their active forms, resulting in their release (Strowig et al., 2012). As expected we observed a complete reduction of active IL-18 in *Casp1*$^{-/-}$ mice compared with WT mice during DSS colitis, which correlated with lower disease severity. This finding is in line with a recent study, which used precise genetic models and microbiota normalization, to show that IL-18 exacerbates severity of DSS colitis (Nowarski et al., 2015). In contrast, a number of other studies had suggested predominantly protective properties for IL-18 contributing to tissue repair based on the observation of increased tissue damage and inflammation in *Casp1*$^{-/-}$*Casp11*$^{129mt/129mt}$ and *Il18*$^{-/-}$ mice (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Salcedo et al., 2010; Zaki et al., 2010b). Beyond IL-18, IL-1β has been associated with enhanced colitis severity in rabbit models of colitis, as its neutralization decreases the degree of inflammation (Cominelli et al., 1992). However, contradicting results regarding the role of IL-1β have been reported with *Il1β*$^{-/-}$ mice displaying exacerbated disease (Bersudsky et al., 2014). The conflicting observations regarding the role of IL-18 and IL-1β in DSS colitis may reflect the lack of microbiota normalization and effects of unwanted passenger mutations. But it has also been recently demonstrated that IL-1β processed by the Nlrp3 inflammasome in inflammatory monocytes contributes to the exacerbation of DSS colitis, specifically after recognition of *Proteus mirabilis* isolated from the mouse intestine (Seo et al., 2015). Because numerous different cell types express caspase-1 in the intestine, we used cell type-specific ablation of caspase-1 to identify the cell type responsible for its role in DSS colitis in our experimental conditions. Deletion of caspase-1 in intestinal epithelial cells abrogated IL-18 processing and lowered disease severity to similar levels what had been observed in *Casp1*$^{-/-}$ mice, suggesting that this cell type is largely responsible for the activity of caspase-1 in the intestine. This is in line with observations by Nowarski et al. (2015) that identified the intestinal epithelium as source and target of IL-18 during DSS colitis, resulting in exacerbation of disease. Additional studies combining cell type-specific ablation with gnotobiotic conditions will be required to advance our understanding of the interactions between intestinal bacteria and inflammasomes during intestinal inflammation.

In summary, using these gene-deficient mouse lines and normalization of the microbiota, we identify that canonical activation of caspase-1 in intestinal epithelial cells is responsible for exacerbation of DSS colitis in three different microbiota settings. However, the role of inflammasomes and their substrates in intestinal inflammation is very likely more complex than currently understood. Yet the need to harmonize the microbiota between cohorts of gene-deficient and control mice has emerged as necessity in many mouse models of complex diseases to distinguish the effect of host genes from the effect of microbial communities. Variation of microbiota conditions as well as detailed characterization of microbiota composition will be subsequently required to evaluate whether observed phenotypes are general principles or reflect specific interactions between distinct commensal and corresponding host factors.

EXPERIMENTAL PROCEDURES

Mouse Lines and Microbiota Manipulation

Generation of *Casp1*$^{-/-}$ (Casp1$^{tm2.1Flv}$) and Casp1$^{fl/fl}$ (Casp1^{tm2Flv}) mice has been described (Case et al., 2013). Casp1$^{\Delta IEC}$ mice and littermate control mice were obtained from crossing Casp1$^{fl/fl}$ with Casp1$^{fl/fl}$-Tg(Vil1-cre)^{997Gum} mice. *Casp11*$^{-/-}$ mice (Casp4$^{tm1a(KOMP)Wtsi}$) were generated from *Casp11*$^{-/-}$ ESCs in which exons 3 and 4 are flanked by loxP sites obtained from the KOMP Repository (project ID: CSD47499). After germline transmission, mice were crossed to mice expressing Cre recombinase under control of the E2A promoter, leading to deletion of the floxed region. Mice were crossed to C57BL/6N mice to segregate Cre recombinase from the deleted allele, and we subsequently intercrossed *Casp11*$^{+/-}$ to generate *Casp11*$^{-/-}$ mice. All mice were further backcrossed to C57BL/6N mice obtained from Charles River to generate mice of comparable background. WT obtained from Charles River and gene-deficient mouse lines were rederived to SPF1 microbiota conditions by embryo transfer and bred at the animal facilities of the Helmholtz Centre for Infection Research (HZI) under enhanced specific pathogen-free (SPF) conditions (Stehr et al., 2009) (Table S2). C57BL/6N SPF2 mice were purchased

from Janvier (Barrier 10C). For cohousing experiments, donor and recipient mice were housed at a ratio of 1:2 for at least 4 weeks together before experiments. All mice were provided with sterilized food and water ad libitum. Mice were kept under strict 12 hr light cycle and housed in groups of up to six mice per cage. We used age- and sex-matched mice between 10 and 14 weeks of age. Both female and male mice were used in experiments. All animal experiments were performed in agreement with the local government of Lower Saxony, Germany or the Yale Institutional Animal Care and Use Committee protocols.

Induction of DSS Colitis

Colitis was induced by administration of DSS (molecular weight [MW] = 36,000–50,000 Da; MP Biomedicals).

Mice were treated with 2.5% DSS (or as indicated otherwise) dissolved in drinking water for 7 days, followed by 7 days of regular access to water. Weight loss and mortality were followed daily until day 14. Experimental samples were collected at days 0, 5, and 7 of DSS. Colonoscopy was performed on day 7. Mice were sacrificed for histological analysis on days 5 and 7.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data are expressed as mean ± SEM. Differences were analyzed by nonparametric Mann-Whitney test and nonparametric Kruskal-Wallis test with multiple comparisons, depending on the number of groups. A p value ≤ 0.05 was considered to indicate statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.058.

AUTHOR CONTRIBUTIONS

A.J.B. and T.S. designed the experiments and wrote the manuscript with input from co-authors. A.J.B., S.T., U.R., and A.S. performed and analyzed experiments. M.C.P. and U.H. performed histological evaluation and analysis. E.J.C.G. supported the analysis of 16S rRNA sequencing data. M.R.Z. and T.S. generated the gene-deficient mice. R.A.F. contributed essential reagents. T.S. and R.A.F. supervised the study.

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