Environmental factors regulate Paneth cell phenotype and host susceptibility to intestinal inflammation in Irgm1-deficient mice

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ABSTRACT

Crohn’s disease (CD) represents a chronic inflammatory disorder of the intestinal tract. Several susceptibility genes have been linked to CD, though their precise role in the pathogenesis of this disorder remains unclear. Immunity-Related GTPase M (IRGM) is an established CD risk allele. We have shown previously that conventionally-raised (CV) mice lacking the IRGM ortholog, Irgm1, exhibit abnormal Paneth cells (PCs) and increased susceptibility to intestinal injury. In the present study, we sought to utilize this model system to determine if environmental conditions impact these phenotypes, as is thought to be the case in human CD. To accomplish this, wild-type and Irgm1⁻/⁻ mice were re-derived into specific pathogen-free (SPF) and germ-free (GF) conditions. We next assessed how these differential housing environments influenced intestinal injury patterns and epithelial cell morphology and function in wild-type and Irgm1⁻/⁻ mice. Remarkably, in contrast to CV mice, SPF Irgm1⁻/⁻ mice showed only a slight increase in susceptibility to dextran sodium sulfate-induced inflammation. SPF Irgm1⁻/⁻ mice also displayed minimal abnormalities in PC number, morphology, and antimicrobial peptide expression. Goblet cell numbers and epithelial proliferation were also unaffected by Irgm1 in SPF conditions. No microbial differences were observed between wild-type and Irgm1⁻/⁻ mice, but gut bacterial communities differed profoundly between CV and SPF mice. Specifically, Helicobacter sequences were significantly increased in CV mice; however, inoculating SPF Irgm1⁻/⁻ mice with H. hepaticus was not sufficient to transmit a pro-inflammatory phenotype. In summary, our findings suggest the impact of Irgm1-deficiency on susceptibility to intestinal inflammation and epithelial function is critically dependent on environmental influences. This work establishes the importance of Irgm1⁻/⁻ mice as a model to elucidate host-environment interactions that regulate mucosal homeostasis and intestinal inflammatory responses. Defining such interactions will be essential for developing novel preventative and therapeutic strategies for human CD.
INTRODUCTION

Crohn’s disease is a chronic inflammatory disorder of the gastrointestinal tract, which is thought to occur as a result of dysregulated host immune responses to the enteric microbiota within a genetically susceptible host (Abraham and Cho, 2009, Leone et al., 2013). Genome wide association studies have identified a multitude of genes associated with development of Crohn’s disease, many of which encode factors involved in the regulation of the autophagy pathway (Baskaran et al., 2014, Cleynen et al., 2013, Hoefkens et al., 2013, Li et al., 2014, Liu et al., 2015, Khor et al., 2011). Among these is the molecule Immunity-Related GTPase M (IRGM), which has recently been shown to play an integral role in the initiation of autophagy during microbial clearance (Chauhan et al., 2015).

We previously reported that mice deficient in Irgm1, a murine homologue of IRGM, exhibited several phenotypic differences within the gastrointestinal tract, as compared to wild-type (WT) mice (Liu et al., 2013). First, \textit{Irgm1}⁻/⁻ mice showed an overall increased susceptibility to dextran sodium sulfate (DSS)-induced colonic injury. Clinically, these knockout (KO) mice demonstrated greater weight loss, increased fecal blood, and worsened stool consistency than WT mice treated with DSS. \textit{Irgm1}⁻/⁻ mice also exhibited increased inflammation in the ileum in response to DSS, which is not typically observed in this injury model (Chassaing et al., 2014). Second, we reported that \textit{Irgm}⁻/⁻ mice expressed an aberrant Paneth cell (PC) phenotype. PCs are specialized epithelial cells located in the small intestine, which play a major role in innate immunity and regulation of the intestinal microbiota via secretion of antimicrobial peptides (AMPs). \textit{Irgm1}⁻/⁻ mice possessed an increased number of PCs per crypt, decreased PC granule size, and an increased number of ectopically placed PCs. Functionally, the PCs of Irgm1-deficient mice expressed decreased transcript levels of the AMPs \textit{α-defensin 20 (Defa20)} and \textit{lysozyme (Lyz)} (Liu et al., 2013).

It is important to note that mice used in this previous study were born and raised in conventional (CV) conditions that did not exclude, for instance, murine norovirus or \textit{Helicobacter} species. Because previous work has demonstrated that environmental factors, including microbial influences, can affect the penetrance and severity of intestinal inflammation in murine models of inflammatory bowel disease (IBD) (Foltz et
al., 1998, Maggio-Price et al., 2006, Ward et al., 1996, Peloquin and Nguyen, 2013, Cadwell et al., 2010), we sought to determine if environmental conditions impact the inflammatory and PC phenotypes in Irgm1-deficient mice. Specifically, we hypothesized that Irgm1^−/− mice re-derived into “clean” housing conditions would have an attenuated phenotype in regards to DSS susceptibility and PC dysmorphology.

To test our hypothesis, we established colonies of littermate Irgm1^−/− and WT mice in specific pathogen-free (SPF) and germ-free (GF) facilities. In this study, we demonstrate that environmental conditions remarkably attenuate the increased susceptibility of Irgm1^−/− mice to DSS-mediated intestinal injury, as well as the dysmorphic PC phenotype observed in these animals. This interplay between host genetics and the environment parallels similar interactions in human Crohn’s disease, thereby enhancing the value of this murine model in the investigation of the gene-environment interactions within the context of intestinal inflammatory disorders.

RESULTS
Increased susceptibility to DSS-induced intestinal injury is attenuated in Irgm1-deficient mice housed in SPF conditions

To determine whether Irgm1^−/− mice housed in SPF conditions exhibit the increased susceptibility to intestinal inflammation previously observed in CV KO animals, SPF KO and WT littermate mice were subject to acute DSS injury as described (Liu et al., 2013). In contrast to our previous findings in CV housing conditions, in which DSS-treated Irgm1 KO mice lost more weight than their WT counterparts, no differences in weight loss were detected between WT and KO groups when mice were housed in an SPF environment (Fig. 1A). Similarly, no differences between Irgm1 KO and WT mice were observed when fecal samples were scored for consistency (Fig. 1B). At 7 days post-DSS, the Hemoccult index was slightly higher in KO mice versus their WT counterparts, but no differences in stool blood were observed at earlier time points (Fig. 1C).

To assess colonic inflammation, we first measured colon lengths at the time of necropsy (after 7 days of DSS treatment). There were no baseline differences between SPF WT and KO mice that received water control; however, both groups demonstrated
shortened colons relative to controls when treated with DSS (Fig. 2A). This colon shortening was more pronounced in KO mice receiving DSS ($P < 0.01$), though the absolute difference between DSS-treated WT and KO mice was relatively small. Histologically, the vehicle-treated controls of both the KO and WT mice lacked any discernable histologic inflammation or ulceration of the colon. Within the DSS-treated groups (Fig. 2B,C), mild inflammation occurred in the proximal colon of both KO and WT mice; however, there were no differences in severity between these two groups. Similarly, the ceca of both KO and WT DSS-treated groups showed moderate inflammation, but no discernable effect of genotype. In the middle colon, both KO and WT groups treated with DSS exhibited an equally severe inflammatory response. The only differences in histologic inflammatory scores noted in the SPF mice were in the distal colon, where KO animals did display increased inflammation relative to their WT counterparts. Notably, this starkly contrasts with our previous finding in CV-raised mice, in which genotype contributed to differences in inflammation throughout the middle colon, distal colon, and cecum (Liu et al., 2013).

In the ileum, we found no significant differences in inflammation between DSS-treated WT and KO mice raised in SPF conditions (Fig. 2D,E). Ileal tissues were also assessed for expression of TNF$\alpha$ mRNA; however, neither $lrgm1$ KO nor WT mice housed in SPF conditions yielded detectable levels of TNF$\alpha$ mRNA as compared to positive controls (data not shown). Again, this contrasts our previous findings in CV KO mice, which showed increased ileal inflammation and TNF$\alpha$ mRNA expression in response to DSS, as compared to their WT littermates (Liu et al., 2013). In summary, the increased susceptibility to ileal and colonic inflammation in response to DSS that is displayed by CV $lrgm1$-deficient mice is markedly attenuated in SPF housing conditions.

**Environmental conditions influence Paneth cell phenotype in $lrgm1$-deficient mice**

In addition to enteric inflammatory responses to DSS, SPF $lrgm1^{\sim}$ mice were also evaluated for the PC anomalies previously observed in CV-raised KO animals. Similar to CV $lrgm1^{\sim}$ mice, SPF KO mice exhibited an increased number of PCs per histologic crypt-villus unit as compared to their WT controls (Fig. 3A,B). We also observed a trend towards increased ectopic PCs in SPF KO mice relative to their WT
counterparts (Fig. 3C), though this did not reach statistical significance as we previously reported in CV KO mice (Liu et al., 2013). Notably, Irgm1−/− mice housed in SPF conditions failed to show differences in PC granule size compared to WT animals (Fig. 3D), a difference that was apparent in CV mice. Finally, we also measured the expression of genes related to PC function (including the antimicrobial peptides Lyz and Defa20), which were previously shown to be decreased in CV Irgm1−/− mice. Again, SPF Irgm1−/− mice showed no differences in expression of either of these genes compared to WT littermates (Fig. 3E,F). In summary, the PC abnormalities originally observed in CV Irgm1−/− mice are attenuated in SPF conditions.

Irgm1-deficient and wild-type littermate mice display no differences in goblet cell numbers or epithelial proliferation in SPF conditions

In addition to its role in PC biology, autophagy has also been implicated in the regulation of goblet cell function (Patel et al., 2013) and cellular proliferation (Sbrana et al., 2016). Therefore, we assessed goblet cell numbers and epithelial cell proliferation in the ileum of Irgm1−/− and WT littermates, using periodic acid-Schiff (PAS) and Ki67 staining respectively. As shown in Fig. 4A,C, goblet cell numbers were similar in WT and Irgm1−/− mice; however, we did observe an increased intensity of PAS staining in the goblet cells of KO animals. This was consistent across multiple sections from numerous mice. In regards to epithelial proliferation, we found no differences in the Ki67-positive zones of WT and Irgm1−/− mice (Fig. 4B,D). Importantly, there were also no ectopic foci of proliferation that would suggest aberrantly located crypt formation in the KO animals.

Environment, but not genotype, drives microbial composition and diversity in Irgm1-deficient and wild-type littermate mice

Given the differences in the inflammatory and PC phenotypes between populations of mice raised in different housing facilities, we next sought to determine if the intestinal microbiota varied between groups. Deep sequencing of the V6 region of the 16S rRNA gene was performed on stool pellets and ileal tissue collected from Irgm1 KO and WT mice from both housing conditions. Interestingly, PCoA using Bray-Curtis dissimilarity showed no clustering of microbial communities based on Irgm1 status; however, distinct differences between microbial communities from SPF and CV mice
were observed in both stool and ileal tissue compartments (Fig. 5A,D). This is depicted statistically in Table 1. As indicated, \textit{Irgm1} genotype did not significantly impact microbial composition in stool or ileal samples for the first 3 PCoA axes, based on their FDR-corrected \(P\)-values (Table 1A). In contrast, housing conditions did significantly influence microbial composition for the first 2 PCoA axes, in both stool and ileal compartments (Table 1B). Notably, similar results were found through a parallel analysis using the QIIME platform (Fig. S1A).

In addition to its effects on PCoA clustering (\(\beta\)-diversity), the housing environment also significantly impacted the richness of the ileal microbiota, as measured by observed OTU numbers (Fig. 5B,E) and the Chao1 index (Fig. 5C,F). Specifically, CV mice had a decreased richness of their ileal microbiota (FDR = 0.024) when compared to SPF mice. The richness of the fecal microbiota between these 2 groups was similar (FDR > 0.66). Again, \textit{Irgm1} gene status did not appear to influence the richness of the ileal (FDR = 0.817) or fecal (FDR = 0.375) microbiota. Similar results were obtained using the QIIME platform (Fig. S1B,C). In total, these findings suggest that environmental factors, as opposed to \textit{Irgm1} expression, are the primary drivers of gut microbial composition and diversity in these animals.

**Microbial influences modulate Paneth cell morphology in \textit{Irgm1}-deficient mice**

Given our finding that SPF and CV housing facilities support markedly distinct enteric bacterial communities in both KO and WT mice, we speculated that a microbial driver might be responsible for the differences in PC phenotype we observed in these two environmental conditions. To test this hypothesis, we re-derived our \textit{Irgm1} colony into GF conditions and assessed their PCs for both morphology and AMP expression. Similar to the SPF colony (but in contrast to CV-raised mice), there were no significant differences in PC numbers, location, and granule size between WT and KO mice (Fig. 6A-D). Intriguingly, however, we found that GF KO mice showed increased mRNA expression of \textit{Lyz} and \textit{Defa20} compared to WT mice (Fig. 6E,F). These findings suggest that the gut microbiota influences PC morphology in \textit{Irgm1}-deficient mice, though there appears be an effect of \textit{Irgm1} itself on AMP gene expression.
**Helicobacter hepaticus** is not sufficient to reconstitute the inflammatory phenotype of conventional Irgm1-deficient mice

The observed differences in gut bacterial communities in CV versus SPF mice also allowed us to generate hypotheses regarding the impact of specific bacterial groups on DSS susceptibility in Irgm1-deficient animals. Specifically, we were able to determine which bacterial groups were increased in relative abundance in CV versus SPF housing conditions. A complete list of these organisms can be found in Table S1. One bacterial group of particular interest that was increased in the CV versus SPF samples was the *Helicobacter* genus (Fig. 7A). Because various *Helicobacter* spp. have been shown to enhance intestinal inflammation in numerous murine models of colitis (Chichlowski and Hale, 2009), we sought to determine if this organism could be a driver of the inflammatory phenotype observed in our CV *Irgm1* KO mice.

Quantitative PCR testing for specific *Helicobacter* groups identified *H. hepaticus* as the most abundant species in the stool samples of CV mice. Therefore, we next assessed whether *H. hepaticus* colonization of SPF Irgm1 KO mice would enhance their inflammatory response to treatment with DSS. To accomplish this, we colonized SPF Irgm1 KO and WT mice with *H. hepaticus*, administered DSS, and then assessed for intestinal inflammation. In these studies, we found no differences in weight loss between DSS-treated Irgm1 KO and WT mice that had been pre-infected with *H. hepaticus* (Fig. 7B). Moreover, all experimental groups developed similar histologic inflammation in both the ileum and the colon (Fig. 7C,D). Intriguingly, contrary to our original hypothesis, less inflammation was seen in the cecum of KO mice receiving *H. hepaticus* + DSS, as compared to similarly treated WT animals. Regardless, the introduction of *H. hepaticus* was not sufficient to recrudesce the inflammatory phenotype previously shown in CV KO mice.

**DISCUSSION**

To date, genome-wide association studies (GWAS) have linked 206 genetic loci to the development of IBD (Ellinghaus et al., 2016). However, the cumulative disease risk described by these studies is approximately 25% (Denson et al., 2013), a relatively
small fraction of overall IBD heritability. Moreover, the concordance rates for IBD in homozygous twins is approximately 50% for CD and 20% for ulcerative colitis (Halfvarson et al., 2003). These findings suggest that environmental influences play a key role in dictating the penetrance of IBD phenotypes. In the present study, we demonstrate that housing conditions modulate the susceptibility of Irgm1-deficient mice to intestinal injury. Housing environment also interacts with Irgm1 to regulate PC morphology and function. These findings are essential knowledge for investigators using the Irgm1−/− mouse model and set the stage for further understanding the cross talk between the environment and Irgm1 to regulate mucosal homeostasis.

Although numerous IBD susceptibility genes have been identified, the precise mechanisms by which these molecules enhance disease risk remain unclear. In vitro studies have shown that human IRGM regulates key molecular processes, including mitochondrial fission (Singh et al., 2010), autophagy (Chauhan et al., 2015), and the clearance of intracellular pathogens (Kim et al., 2012). Limited studies of murine Irgm1 demonstrate similar functions of this mouse homologue. Specifically, mouse Irgm1 localizes to mitochondria and regulates fission (Henry et al., 2014). Irgm1 is also a mediator of IFNγ-induced autophagy, and promotes the clearance of intracellular bacteria in cultured cells. Despite this in vitro knowledge, however, our understanding of how IRGM (and Irgm1) regulate intestinal inflammation in vivo is unclear.

We previously reported that Irgm1-deficient mice raised in CV housing conditions develop extensive intestinal injury after DSS exposure, as compared to WT mice (Liu et al., 2013). Specifically, DSS-treated CV KO mice develop more severe disease in the ileum, middle colon, distal colon, and cecum relative to WT animals. In contrast, the present study demonstrates that “cleaner”, DSS-treated SPF KO mice only incur more significant inflammation in the distal colon. Importantly, there is no increased susceptibility to ileitis in SPF KO mice treated with DSS. These data suggest that an environmental component modulates DSS responses in Irgm1-deficient animals.

A potential mechanism by which the environment modulates DSS susceptibility is via the enteric microbiota. It is well established that environmental influences can affect the composition and function of the gut microbiota (Ivanov et al., 2008, Thoene-Reineke et al., 2014). Moreover, variations in the enteric microbiota can influence the severity of
DSS colitis (Brinkman et al., 2013, Nagalingam et al., 2011). Therefore, we characterized gut bacterial communities in WT and KO mice in both CV and SPF housing conditions. Remarkably, we found no impact of Irgm1 itself on global enteric microbial composition in either CV or SPF environments. This is intriguing in light of previous work suggesting IRGM can promote the clearance of intracellular bacteria, such as Mycobacterium tuberculosis (Singh et al., 2006). That said, it is unclear how defects in intracellular bacterial killing affect the composition of the luminal/mucosal commensal microbiota, which is the focus of the present study. Such defects may be more relevant to pathogenic organisms, such as Listeria monocytogenes (Collazo et al., 2001) and Salmonella typhimurium (Henry et al., 2007), which do result in increased disease and bacterial burdens in Irgm1⁻/⁻ mice. Additionally, Irgm1 may be primarily involved in mediating inflammatory responses to enteric bacteria, rather than shaping the composition of those bacteria. Despite the lack of a genotype effect, our microbiota analyses did demonstrate the gut microbiota of mice reared in CV conditions differed profoundly from those in SPF housing. This supports the possibility that the enteric microbiota may influence the variable inflammatory phenotypes exhibited between CV and SPF KO mice in response to DSS.

A complete list of gut bacterial taxa exhibiting differential abundances in CV versus SPF mice is shown in Table S1. Of interest is the Helicobacter genus, which is increased in CV mice compared to SPF counterparts. Numerous Helicobacter species have been shown to induce intestinal inflammation in susceptible mice, the most common of which include H. hepaticus and H. bilis (Chichlowski and Hale, 2009). Using species-specific, targeted qPCR, we identified H. hepaticus as the primary Helicobacter species present in our CV housing facility. This organism is a known trigger of intestinal inflammation in murine IBD models, such as the IL10-deficient mouse (Kuhn et al., 1993, Kullberg et al., 1998). Based on these findings, we postulated that H. hepaticus infection would be able to recapitulate an inflammation-susceptible phenotype in our SPF Irgm1 KO animals.

In contrast to our hypothesis, however, we found that H. hepaticus inoculation was not sufficient to increase the susceptibility of SPF Irgm1 KO mice to DSS-induced intestinal injury over their WT counterparts. There are numerous putative explanations...
for this finding. First, it is possible that *H. hepaticus* is not the sole organism responsible for the increased susceptibility of CV *Irgm1* KO mice to DSS injury. Additional microbes, perhaps in concert with *H. hepaticus*, may be required to drive the inflammatory phenotype of CV KO animals. Indeed, previous work has shown that the composition of the commensal microbiota can regulate host inflammatory pathways that drive intestinal disease in *H. hepaticus* models of colitis (Nagalingam et al., 2013). A second possible explanation for these findings is that the strain of *H. hepaticus* used in our study may not be identical to that found in the CV animals. Ample evidence exists supporting the concept that different bacterial strains within a given species differ in their ability to induce colitis in animal models of IBD (Moran et al., 2009). However, it should be noted that the specific *H. hepaticus* strain used in this study (MU-94) has been shown to induce intestinal inflammation in susceptible mouse strains (Cook et al., 2014, Livingston et al., 2004). Finally, it also possible that the increased susceptibility to intestinal inflammation observed in CV *Irgm1* KO mice is not due to a transmissible microbial factor. Numerous environmental factors have been implicated in IBD pathogenesis (i.e. smoking, nonsteroidal anti-inflammatory drugs, appendectomy, diet, stress), and it is not clear if these exert an impact on intestinal inflammation via the gut microbiota (Frolkis et al., 2013). Similarly, our housing facilities varied in regards to food, water, bedding and caging (Table S2). While it is likely that these differences may contribute to microbial variations between the colonies, it is also possible that they may impact host inflammatory responses through a microbial-independent mechanism.

In addition to its impact on inflammation susceptibility, housing environment also appears to interact with *Irgm1* to influence PC morphology and AMP transcription. We have shown that, in CV housing, *Irgm1*-deficient mice display multiple PC abnormalities including increased PC numbers, ectopic PCs, decreased PC granule size, and decreased transcript levels of specific AMPs (*Lyz* and *Defa20*) (Liu et al., 2013). In the present study, *Irgm1* KO mice housed in SPF conditions continued to demonstrate increased PC numbers per crypt and a trend towards increased ectopic PCs. However, their granule morphology was identical to WT mice. Moreover, differences in *Lyz* and *Defa20* mRNA expression between WT and KO mice were abrogated in SPF conditions. Given the differences in bacterial communities between our CV and SPF
facilities, these data suggest the gut microbiota may modulate PC function in \textit{lrgm1}-deficient mice.

In order to dissect the relative contributions of genotype (\textit{lrgm1} status) and the microbiota to PC regulation in this model, we next re-derived \textit{lrgm1}-deficient mice and WT littermates into GF housing conditions. Elimination of the microbiota resulted in complete loss of the morphological PC differences between WT and KO mice. Specifically, GF WT and KO mice displayed similar PC numbers, location, and granule morphology. Intriguingly, transcript expression of \textit{Lyz} and \textit{Defa20} were increased in GF KO mice compared to WT counterparts. Historically, these AMPs have not been thought to be transcriptionally induced (Bevins and Salzman, 2011). For the \(\alpha\)-defensins, however, elegant work by Wehkamp and colleagues has demonstrated transcriptional regulation of these molecules through Wnt signaling [via Tcf-1 (Beisner et al., 2014) and Tcf-4 (Wehkamp et al., 2007)]. Indeed, polymorphisms of the TCF-4 promoter have been associated with ileal Crohn’s disease (Koslowski et al., 2009). It is possible that the Wnt/\(\beta\)-catenin/Tcf pathway is up-regulated in \textit{lrgm1}-deficient mice, and future experiments are planned to explore this possibility. Presently, however, we can surmise that these findings contrast with the decreased \textit{Defa20} and \textit{Lyz} mRNA expression observed in CV KO mice, and the equivalent AMP expression shown between SPF KO mice and their WT counterparts. This suggests the gut microbiota influences the manner in which \textit{lrgm1} regulates AMP expression.

To date, the precise microbial constituents that regulate PC phenotype in the \textit{lrgm1}-deficient mouse model remain unclear. Previous work studying the autophagy-related CD risk allele, ATG16L1, demonstrated that Atg16l1 hypomorph mice have a similar PC phenotype to our CV \textit{lrgm1} KO animals. Specifically, Atg16l1\textsuperscript{HM} mice possess dysmorphic PCs with reduced numbers of lysozyme granules (Cadwell et al., 2008, Cadwell et al., 2009). Remarkably, this abnormal PC phenotype is dependent on a distinct murine norovirus strain (MNV-CR6) (Cadwell et al., 2010). Notably, murine norovirus is excluded from our SPF mouse colony, and hence SPF \textit{lrgm1} KO mice are not exposed to this microbe (Table S3). Future studies will attempt to recapitulate a dysmorphic PC phenotype in \textit{lrgm1} KO mice by colonizing with the MNV-CR6 strain. Intriguingly, we did observe subtle differences in the ileal goblet cells of SPF \textit{lrgm1}\textsuperscript{-/-}
mice. Although goblet cell numbers were similar between WT and KO mice, the increased intensity of PAS staining in KO animals could indicate differences in the mucin composition of these cells. This will be also be explored further in future studies.

The findings presented in this study demonstrate that environmental conditions interact with \( \text{lrgm1} \), putatively through the enteric microbiota, to direct PC function and host-susceptibility to intestinal injury. This may be a critical consequence of the role \( \text{lrgm1} \) plays in regulating autophagy. An established function of autophagy is to assist in the clearance of certain intracellular organisms (Levine et al., 2011). This antimicrobial process, known as xenophagy, can be triggered by pathogen-associated molecular patterns (Deretic et al., 2015). As such, a mechanism is required to transmit microbial inputs to the initiation of xenophagic processes. Recently, human IRGM has been shown to directly interact with the pattern recognition receptor NOD2, forming a core complex that can regulate autophagy in response to microbial products (Chauhan et al., 2015). The interaction between the environment and \( \text{lrgm1} \) demonstrated in the present study may support a similar role for this murine homologue. It is possible that \( \text{lrgm1} \) may serve to integrate environmental inputs, such as the microbiota, into signals that can regulate autophagy within host cells. While a comprehensive evaluation of the impact of environment on autophagy in \( \text{lrgm1} \)-deficient mice is beyond the scope of the present study, future work will be directed at dissecting the mechanistic aspects of this putative \( \text{lrgm1} \) function.

Overall, the findings described in this study highlight the importance of the \( \text{lrgm1} \)-deficient mouse model for the study of human CD. The pathogenesis of CD is multifactorial, involving both environmental and host factors that interact to drive disease in a susceptible individual. \( \text{lrgm1} \) KO mice appear to display a similar phenotype, in which environmental influences are able to modulate the impact of \( \text{lrgm1} \) deficiency on PC morphology and inflammation susceptibility. The findings presented also parallel work in human CD, which has demonstrated that abnormal PC morphology in ileal biopsies may predict a more rapid time to recurrence in patients with CD (VanDussen et al., 2014). Although direct causality between dysmorphic PCs and susceptibility to DSS has not been shown in the \( \text{lrgm1} \) KO model, a similar phenomenon is observed in these mice: CV KO mice with abnormal PCs are more
susceptible to DSS-mediated injury, while SPF KO mice have relatively normal PCs and less severe responses to DSS.

In summary, the present study characterizes novel aspects of the Irgm1−/− mouse model that are important to the scientific community, and highly relevant to the understanding of human CD. In particular, environmental conditions strongly impact the influence of Irgm1 on PC and inflammatory phenotypes in the mouse small intestine. In contrast, Irgm1 itself does not appear to regulate gut microbial composition. Future work using this model may now focus on how, mechanistically, abnormal PCs drive this increased susceptibility to intestinal injury. Importantly, the role of environmental factors in this process may also be elucidated. Characterizing these gene-environment interactions will enhance our understanding of the role of IRGM in human CD, and ultimately promote the development of novel preventative and therapeutic approaches for these patients.

MATERIALS AND METHODS

Mice

All animals used in this study were Mus musculus, C57BL/6, sex-matched, adult (2-4 month old) mice. SPF and GF Irgm1 heterozygous mice were re-derived from mice housed in CV conditions (Liu et al., 2013, Collazo et al., 2001), by implantation of two-cell stage embryos into WT SPF or GF pseudopregnant females. Heterozygous mice were then mated to produce Irgm1 KO and WT littermate animals for experimental use. Husbandry details for each facility are provided in Table S2. Importantly, our SPF facility excludes Helicobacter spp. and murine norovirus. A complete list of organisms excluded in our CV and SPF facilities is shown in Table S3. All mice were housed under AAALAC accredited facilities and IACUC approved protocols at the Durham VA Medical Center, Duke University Medical Center, and the University of North Carolina Medical Center, respectively.

Dextran Sodium Sulfate-Induced Intestinal Injury

Acute intestinal injury was induced as previously described (Liu et al., 2013). Briefly, male mice received 3% (w/v) DSS (MP Biomedicals, #160110) in their drinking water for 7 days. Control mice received drinking water without DSS. Mice were
weighed and feces collected on days 0, 3, 5, 6, and 7. Fecal blood and stool consistency were assessed at each time point as previously described (Liu et al., 2013). Mice were sacrificed on day 7, and their intestinal tissues collected for analysis. Colons were removed and length measured prior to being opened longitudinally and content removed. Colons were then rolled in a Swiss-roll fashion. Ceca were also opened longitudinally, their content removed, and rolled for histology. Ileal tissue was opened longitudinally, gross contents were removed and the remaining tissue fixed flat prior to paraffin embedding. Tissues bound for histologic analysis were fixed in 10% formalin solution for 48 hours prior to paraffin embedding. Sections were cut (5 μm) and stained with hematoxylin and eosin (H&E). Histologic inflammation was scored in a blinded fashion using validated systems for ileum (Moran et al., 2009) and DSS-induced colitis (Erben et al., 2014).

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed for Ki67 and Lyz using 5 μm, formalin-fixed, paraffin-embedded ileal tissue sections as described above. Deparaffinization was accomplished using fresh xylene, followed by re-hydration via a series of ethanol dilutions. Heat induced epitope retrieval was subsequently utilized for antigen retrieval (ThermoFisher, #TA-135-HBL). This was followed by treatment with 3% hydrogen peroxide to quench endogenous peroxidase activity. Blocking was performed with 10% normal goat serum. After sections were prepared in this fashion, they were incubated overnight at 4°C with either anti-Ki67 (1:250, Vector Laboratories, #VP-RM04) or anti-Lyz (1:100, Diagnostic Biosystems, #RP028) rabbit primary antibodies. Sections were next incubated for 1 hour at room temperature with a biotinylated goat anti-rabbit IgG secondary antibody (1:500, Jackson ImmunoResearch, #111-065-144). Signal amplification was accomplished using the Vectastain Elite ABC-HRP Kit per manufacturer’s instructions (1:50, Vector Laboratories, #PK-6100). Finally, staining was visualized with DAB chromoreagent (ThermoFisher, #TA-125-QHDX).

**Epithelial cell analyses**

Epithelial cell subtypes were enumerated in a blinded fashion and reported as number of cells per crypt-villus unit. To be included in the analysis, a crypt-villus unit was required to contain a properly oriented crypt (full crypt with lumen visible in cross
section) and visibly adjacent villi. At least 10 crypt-villus units were evaluated for each mouse. Paneth cells (PCs) were enumerated by counting the number of Lyz IHC positive cells within a histologic crypt-villus unit. Ectopic PCs were recorded as the number of PCs identified outside their normal position within the crypt base. PC granule sizes were measured on H&E stained sections, using the ellipse tool in ImageJ version 1.48 software (NIH). At least 70 granules from varying regions were measured for each mouse. Goblet cells were enumerated per crypt-villus unit using periodic-acid Schiff (PAS) staining (ThermoFisher, #SS32-500), which readily identifies deep purple cells with classic goblet cell morphology. Finally, proliferating epithelial cells were assessed by counting the number of Ki67 IHC positive cells per crypt-villus unit.

**Quantitative Reverse-Transcriptase Polymerase Chain Reaction**

Ileal tissues were collected into RNA/later solution (Qiagen, #76104) for subsequent RNA isolation. Total RNA was extracted from flushed ileal tissue using an RNeasy Mini Kit per manufacturer’s instructions (Qiagen, #74104). Complementary DNA was generated using SuperScript II Reverse Transcriptase (Invitrogen, #18064014). Quantitative RT-PCR was conducted for *Lyz* (Applied Biosystems, Mm00657323_m1) and *Defa20* (Applied Biosystems, Mm00842045_g1). These specific AMPs were chosen because they have previously been shown to differ between *Irgm1* KO and WT mice (Liu et al., 2013). Each sample was run in triplicate via quantitative RT-PCR using TaqMan Gene Expression Master Mix and the appropriate primer/probe set for the gene of interest (ThermoFisher Scientific, #4369016). Results were analyzed using the comparative threshold cycle (ΔΔCt) method by normalizing to β-actin (Applied Biosystems, Mm02619580_g1) and comparing to the baseline WT group.

**Deep Sequencing of Gut Bacterial Communities**

Bacterial DNA was extracted from 100 mg of frozen tissue or feces as previously described (Shanahan et al., 2013). Briefly, samples were incubated in a sterile lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.0], 20 mM EDTA, 20 mg/ml lysozyme [Sigma-Aldrich, #12671-19-1]) for 30 min at 37°C. Lysis was completed by adding 40 μl of proteinase K (20 mg/ml) and 85 μl of 10% SDS to the mixture, and incubating for an additional 30 min at 65°C. Samples were next homogenized by bead beating for 2 min using 300 mg/sample of 0.1 mm zirconium beads (BioSpec Products, #11079101Z).
DNA was extracted from the resultant supernatants using phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with absolute ethanol for 1 hr at -20°C. Precipitated DNA was purified using a DNeasy Blood and Tissue extraction kit (Qiagen, #69504) per manufacturer’s instructions.

The purified DNA samples were used to construct an Illumina V6 16S rRNA library for deep sequencing, as previously described (Arthur et al., 2012). Briefly, an initial PCR was performed using barcoded universal V6 primers (Table S3). This allowed for amplification of the V6 region, with simultaneous addition of unique barcodes for multiplex analyses. 15 μl of this PCR product was then used in a second PCR reaction using PCRF1/PCRR1 primers (Table S4), to add Illumina flow cell adaptor sequences to the final product. The final PCR amplicons were purified using a Qiagen PCR Purification Kit and visualized on a 1.5% agarose gel to confirm size and purity. Finally, equal amounts of DNA from each sample were pooled (final concentration 21 ng/μl) and subject to 100 bp paired-end read sequencing, using the Illumina HiSeq2000 platform.

Analysis of 16S rRNA Sequences

A total of 88,015,334 paired-end reads were generated and processed as described previously (Arthur et al., 2014). Forward and reverse reads were merged using a minimum of 70 continuous matching bases between them, which resulted in 48,999,102 merged sequences for the current study representing 78 samples. We used AbundantOTU+ v.0.93b (http://omics.informatics.indiana.edu/AbundantOTU/out+.php) with the “-abundantonly” option to cluster these sequences into 1,468 Operational Taxonomic Units (OTUs), incorporating 99.86% of the input sequences. UCHIME (http://www.drive5.com/uchime/) (Edgar et al., 2011) and the Gold reference database were used to screen for the presence of chimeras in our OTU sequences, and a total of 8 OTUs were removed. The remaining 1,460 OTUs were used for downstream analysis. Taxonomic assignments were done using uclust through QIIME assign_taxonomy.py (Caporaso et al., 2010). A parallel analysis using QIIME v.1.9.1 was also conducted, utilizing the close-reference OTU picking approach (at 97% similarity level using the Greengenes 97% reference dataset, release 13_8). We excluded OTUs that had ≤
0.005% of the total number of sequences according to Bokulich and colleagues (Bokulich et al., 2013).

PCoA plots were generated from both Bray-Curtis dissimilarity statistic on the normalized and \(\log_{10}\) transformed reads (Arthur et al., 2014), and UniFrac after rarefying the counts to the minimum number of reads found in all samples (32,698 for Ileum and 78,957 for stool samples). Alpha diversity (observed OTU estimate and Chao1 diversity index) was calculated after rarefying the raw counts to a depth of the minimum count in all samples (32,698 for Ileum and 78,957 for stool samples). We used a linear mixed-effects model utilizing the package nlme_v. 3.1-128 in R (v. 3.3.1) (https://www.R-project.org/) to analyze the data and account for possible contributions that may arise from co-housing the mice in the same cage. In our model, cage was modeled as a random effect and both \textit{Irgm1} status and housing condition as fixed effects (McCafferty et al., 2013). \textit{P}-values are reported from the linear mixed-effects model using \textit{F}-test. We controlled for false discovery rate (FDR) by correcting the \textit{P}-values using Benjamini and Hochberg (BH) approach (Benjamini et al., 2001).

\textbf{Helicobacter Studies}

Testing of fecal matter for \textit{Helicobacter} colonization was conducted via commercial PCR assays utilizing \textit{Helicobacter} genus-level primers, followed by speciation using species-specific primers (IDEXX BioResearch, Columbia, Missouri). For \textit{H. hepaticus} colonization experiments, the MU-94 strain was obtained as a kind gift from Dr. Robert Livingstone (Idexx BioResearch, Columbia, Missouri). SPF mice received \(1 \times 10^8\) colony forming units (CFU) of \textit{H. hepaticus} suspended in 200 μl of \textit{Brucella} broth, which was gavaged on three separate occasions spaced 3-4 days apart (Livingston et al., 2004). After each gavage dosing, an additional 1 mL of \textit{H. hepaticus}-infected broth was added to the floor of each cage. \textit{Helicobacter} colonization using this protocol was confirmed by PCR. Mice were held for six weeks before being exposed to a 7-day DSS treatment, followed by sacrifice as described above.

\textbf{Statistical analysis}

All statistical analyses, other than sequencing analysis described above, were performed using Graphpad Prism version 6 (Graphpad software Inc., San Diego, CA). Results are expressed as standard error of the mean (SEM). Clinical data were
analyzed using two-way analysis of variance (ANOVA) with other comparisons analyzed using the Kruskal-Wallis and Mann-Whitney tests. P<0.05 was considered significant.

**ACKNOWLEDGEMENTS:** We are grateful to Dr. Robert Livingstone for providing the *H. hepaticus* used in our colonization experiments. We also appreciate the assistance of the UNC Center for Gastrointestinal Biology and Disease gnotobiotic (Maureen Bower) and histology (Carolyn Suitt) cores. Immunohistochemistry services were provided by the Histology Research Core Facility in the Department of Cell Biology and Physiology at the University of North Carolina, Chapel Hill NC (Ashley Ezzell).

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**Figure 1.** Clinical response of *Irgm1*-deficient mice receiving DSS. Male SPF WT and *Irgm1* KO mice were administered 3% DSS in drinking water or drinking water alone (control) for 7 days. A: Weight loss shown as a percentage of initial weight prior to
treatment. B: Average stool consistency score. C: Hemoccult score measuring occult fecal blood. Values are means +/- SEM; data are combined from 4 separate experiments. Combined cohort sizes are as follows: n = 22 WT DSS, 21 KO DSS, 11 WT control, 10 KO control. *P < 0.05 (KO DSS vs. WT DSS).
**Figure 2.** *Intestinal inflammation in SPF *lrgm1*-deficient mice receiving DSS.* Male SPF WT and *lrgm1* KO mice were administered 3% DSS in drinking water or drinking water alone (control) for 7 days. A: Gross colon length. B: Representative histological tissue sections from the colon of WT and KO mice treated with DSS (H&E stain, 4x magnification). C: Average histologic inflammation scores from indicated segments of colon. D: Representative histological tissue sections from the ileum of WT and KO mice treated with DSS (H&E stain, 20x magnification). E: Average histologic inflammation scores in ileum. Results from water controls were omitted from B-D, as there was no inflammation present in these samples. Values are means +/- SEM; data are combined from 2 separate experiments. Combined cohort sizes were as follows: n = 14 WT DSS, 9 KO DSS, 8 WT control, 9 KO control. *P < 0.01.*
Figure 3. Histologic and functional Paneth cell (PC) measurements of SPF *Irgm1*−/− (KO) and wild-type (WT) mice. A: Representative ileal tissue sections from SPF WT and KO mice (Lyz IHC, 20x magnification). B: Number of Lyz+ cells per histologic crypt/villus unit. C: Number of ectopic Lyz+ cells existing outside the base of the crypt per crypt/villus unit. D: Average individual PC granule size. E-F: Results of quantitative RT-PCR measurement of ileal tissue transcript levels of the antimicrobial peptides *Lyz* and *Defa20* normalized to β-actin. N = 6-12 mice/group, **P < 0.008.
Figure 4. *Goblet cells and intestinal epithelial proliferation in SPF Irgm1−/− (KO) and wild-type (WT) mice.* Male SPF WT and Irgm1 KO mice were administered 3% DSS in drinking water or drinking water alone (control) for 7 days. A: Representative ileal tissue sections from DSS-treated SPF WT and KO mice (PAS, 20x magnification). B: Representative ileal tissue sections from DSS-treated SPF WT and KO mice (Ki67 IHC,
20x magnification). C: Number of goblet cells per crypt/villus unit, based on PAS staining. D: Number of proliferating epithelial cells per crypt/villus unit, based on Ki67 staining. N = 4-5 mice/group.
Figure 5. Gut microbial composition and diversity differs between housing condition, but not genotype. (A,D) Principal coordinates analysis of stool and ileal microbial composition of wild-type (WT) and Irgm1<sup>−/−</sup> (KO) mice in conventional (CV) and specific pathogen-free (SPF) conditions based on Bray Curtis dissimilarity statistic. Richness measured using observed OTUs (B,E), and Chao1 index (C,F). N = 11 CV WT, 11 CV KO, 7 SPF WT, 10 SPF KO. *FDR = 0.024.
Figure 6. Histologic and functional Paneth cell (PC) measurements of germ-free (GF) lrgm1⁻/⁻ (KO) and wild-type (WT) mice. A: Representative ileal tissue sections from GF WT and KO mice (Lyz IHC, 20x magnification). B: Number of Lyz⁺ cells per histologic crypt/villus unit. C: Number of ectopic Lyz⁺ cells existing outside the base of the crypt per crypt/villus unit. D: Average individual PC granule size. E-F: Results of quantitative RT-PCR measurement of ileal tissue transcript levels of the antimicrobial peptides Lyz and Defa20 normalized to β-actin. N = 6-12 mice/group, **P < 0.005.
Figure 7. Clinical and pathologic response of Helicobacter hepaticus (HH) infected Irgm1^-^- (KO) mice to dextran sodium sulfate (DSS) treatment. A: Relative abundance of Helicobacter species in stool and ileal samples of untreated conventional (CV) and specific pathogen-free (SPF) mice. *FDR < 0.009 (ileum); FDR = 0.125 (stool). N = 11 CV wild-type (WT), 11 CV KO, 7 SPF WT, 10 SPF KO. For B-D, male SPF WT and KO mice were infected with H. hepaticus (HH) for 6 weeks, followed by administration of 3% DSS in drinking water for 7 days. B: Weight loss shown as a percentage of initial weight prior to DSS treatment, P > 0.1 (2-way ANOVA). C-D: Average histologic inflammation scores in the ileum and large intestine. For B-D, values are means +/- SE, combined from 2 separate studies. Cohort sizes ranged between 7-14 mice/group, *P < 0.05.
### A. Genotype Effects

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**Table 1.** Effects of Irgm1 genotype and housing conditions on gut bacterial composition. Standard and false discovery rate (FDR)-corrected P-values are shown for the first 3 axes of principal coordinates analysis of stool and ileal microbial composition of (A) wild-type versus *Irgm1*-deficient mice, and (B) conventional versus specific pathogen free mice, based on Bray Curtis dissimilarity statistic. N = 11 CV WT, 11 CV KO, 7 SPF WT, 10 SPF KO.
**Fig. S1.** QIIME analysis confirms gut microbial composition and diversity differ between housing conditions, but not genotype. 

A: Principal coordinates analysis of stool and ileal microbial composition of WT and Irgm1 KO mice in conventional (CV) and specific pathogen free (SPF) conditions based on QIIME UniFrac distances. Stool CV versus SPF, FDR < 6.4x10^{-10}; ileum CV versus SPF, FDR < 0.007. For WT versus KO mice in both tissue compartments, FDR > 0.09. 

B: Observed OTUs calculated from QIIME close reference
pipeline. C: Chao1 α-diversity index calculated from QIIME close reference pipeline. N = 11 CV WT, 11 CV KO, 7 SPF WT, 10 SPF KO. *FDR = 0.021.
## Supplemental Tables

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**Table S1.** *Bacterial taxa differentially abundant in conventional (CV) versus specific pathogen free (SPF) facilities.* Genus-level bacterial taxa that are significantly different in CV versus SPF facilities are shown. *P*-values are reported from the mixed linear model using *F*-test, which accounts for the contribution of cage. We controlled for false discovery rate (FDR) by correcting the *P*-values using Benjamini and Hochberg (BH) approach.
Table S2. *Husbandry details for individual mouse facilities.* Specific food, water, bedding, enrichment, and caging sources are indicated for conventional (CV), specific pathogen free (SPF), and germ-free (GF) housing facilities. Sterilization techniques are also shown when utilized.

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**Table S3.** Colony health surveillance results from conventional (CV) and specific pathogen free (SPF) facilities. Results of dirty bedding sentinel testing of either exposure (serology) or presence (PCR) of murine pathogens in the CV and SPF colonies during the time course of the study.

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<td>Enzootic diarrhea of mice</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Theiler’s murine encephalomyelitis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Bacterial Agents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycoplasma pulmonis</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Helicobacter spp.</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Pasturella pneumotropica</em></td>
<td>Not tested</td>
<td>-</td>
</tr>
</tbody>
</table>
Table S4. Primers used to create V6 16S rRNA library for sequencing. V6F1-12 and V6R1-12 were used to initial PCR. PCRF1/PCRR1 were used for second stage PCR.