Methodology; Writing-review and editing Mary Dinauer: Supervision; Methodology; Writing-review and editing Tamara Doering: Supervision; Methodology; Writing-review and editing Renee Tsolis: Supervision; Methodology; Writing-review and editing Jonathan Dworkin: Conceptualization; Writing-review and editing Christina Stallings: Supervision; Methodology; Writing-review and editing Gaya Amarasinghe: Conceptualization; Supervision; Methodology; Writing-review and editing Craig Micchelli: Conceptualization; Supervision; Project administration; Writing-review and editing

Funding:
Crohn's and Colitis Foundation (Crohn's & Colitis Foundation); Genetics Initiative #274415; National Science Foundation (NSF); DGE-1143954; National Science Foundation (NSF); DGE-1143954; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); T32AI007163; HHS | NIH | National Institute of General Medical Sciences (NIGMS); T32GM007067; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); T32AI007172; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); F32AI108089; HHS | NIH | National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK); R01DK100644; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007172; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007163; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007172; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007163; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007172; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007163; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007172; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007163; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); R01AI007172; HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID); U19AI007975; Arnold and Mabel Beckman Foundation; Young Investigator Award; Burroughs Wellcome Fund (BWF); Investigators in the Pathogenesis of Infectious Disease Award; WUSTL | Washington University School of Medicine in St. Louis.; Children's Discovery Institute

Reviewer Instructions
LysMD3 is a type II membrane protein without an \textit{in vivo} role in the response to a range of pathogens

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Germline-encoded receptors recognizing common pathogen-associated molecular patterns are a central element of the innate immune system and play an important role in shaping the host response to infection. Many of the innate immune molecules central to these signaling pathways are evolutionarily conserved. LysMD3 is a novel molecule containing a putative peptidoglycan-binding domain that has orthologs in humans, mice, zebrafish, flies, and worms. We found that the lysin motif (LysM) of LysMD3 forms a folded structure and is likely related to a previously described peptidoglycan-binding LysM found in bacteria. Mouse LysMD3 is a type II integral membrane protein that co-localizes with GM130+ structures, consistent with localization to the Golgi apparatus. We describe here two lines of mLysMD3-deficient mice for \textit{in vivo} characterization of mLysMD3 function. We found that mLysMD3-deficient mice were born at Mendelian ratios and had no obvious pathologic abnormalities. They also exhibited no obvious immune response deficiencies in a number of models of infection and inflammation. mLysMD3-deficient mice exhibited no signs of intestinal dysbiosis by 16S analysis or alterations in intestinal gene expression by RNA-Seq. We conclude that mLysMD3 contains a LysM motif with cytoplasmic orientation, but we were unable to define a physiologic role for the molecule \textit{in vivo}.

\textbf{INTRODUCTION}

The innate immune response to infection relies heavily on signals transduced by germline-encoded receptors that recognize common pathogen-associated molecular patterns (PAMPs) such as bacterial and viral proteins, glycoproteins, and microbe-specific nucleic acids \cite{1}. These pattern recognition receptors tend to recognize microbial products that are absent in the host, thereby preventing self-reactivity, are often evolutionarily conserved, and may be members of a protein family that recognize similar but distinct microbial products.

Therefore, we were interested in characterizing the evolutionarily-conserved molecule, LysMD3, which has homologs in humans, mice, zebrafish, flies, and worms. LysMD3 is named for its N-terminal lysin motif (LysM) and, in mice and humans, is a member of a protein family that also includes LysMD1,
LysMD2, and LysMD4. Recent studies of bacterial and plant LysMs suggest that LysMs bind the glycan backbone of peptidoglycan, or the related molecule chitin (2-6). While peptidoglycan is a ubiquitous bacterial component, relatively little is known about its interactions with the mammalian immune system. The intracellular receptors NOD1 and NOD2 have been shown to bind the peptidoglycan fragments Tri-DAP and muramyl-dipeptide, respectively (7), but it is unknown if the mammalian immune system is capable of recognizing the ubiquitous polymeric glycan backbone of peptidoglycan. Furthermore, a recent study suggested that chitin oligomers may modulate mammalian angiogenesis through interactions with a yet unknown receptor (8). We hypothesized that the LysMD family may represent a novel family of pattern recognition receptors, potentially capable of recognizing important microbial ligands such as peptidoglycan or chitin.

Results from this study revealed that the LysM of LysMD3 is evolutionarily conserved, with orthologs in human, mice, zebrafish, flies, and worms. In mammalian cells, we found that LysMD3 was a type II integral membrane protein that co-localizes with the Golgi marker GM130 with a predicted cytoplasmic location of the LysM. To explore the function of LysMD3 in the immune response, we generated two lines of LysMD3-deficient mice and evaluated the role of LysMD3 in a number of infection and inflammation models. We found no evidence for a role for LysMD3 during the mammalian immune response in the models that we tested, nor did we find a role for LysMD3 in the control of the intestinal microbiota or intestinal gene expression. Additional studies will be required to determine a functional role for this evolutionarily-conserved molecule.

RESULTS

LysMD3 is a predicted transmembrane protein containing an evolutionarily conserved LysM. Mouse LysMD3 (mLysMD3) is a 305 amino acid protein with an annotated N-terminal LysM and C-terminal transmembrane domain (Figure 1A). Phylogenetic analysis of the minimal LysM sequence from multiple model organisms (Figure 1B and Figure 1C) revealed that mLysMD1, mLysMD2, mLysMD3, and mLysMD4 each clustered with like sequences from other organisms, including H. sapiens, X. tropicalis, and D. rerio. Further, groups containing LysMD3 and LysMD4 sequences diverged from clusters containing LysMD1 and LysMD2 LysM sequences. Interestingly, the LysM from C. elegans protein F43G9 clustered with LysMD3 and LysMD4 sequences, and apart from LysMD1 and LysMD2. Furthermore, while the exact relationship between the LysM of mLysMD3 and D. melanogaster CG17985 is somewhat less defined, it is apparent that the D. melanogaster CG17985 sequence groups clusters with LysMs from the mLysMD3/4 cluster, apart from the mLysMD1/2 clusters, and apart from other murine LysM-containing proteins Neoc7 and Oxr1, which are more similar to D. melanogaster protein Mtd (9).

Although there was sequence diversity within LysMs across species, alignment of the deduced proteins demonstrated that several residues were remarkably conserved between prokaryotes and higher order organisms (Figure 1B). In particular, an asparagine residue N31 is conserved in all the species analyzed. In addition, amino acids 11-13, with only a few exceptions, were conserved across LysMs from all species analyzed, including several bacterial proteins. A neighboring sequence of amino acids 16-19 had only conservative substitutions across all species.

The mLysMD3 LysM sequence was submitted to the Phyre2 Protein Fold Recognition Server (10) for protein structure prediction. The top hit from this analysis, in which 96% of the sequence was modeled with a confidence of 99.6%, was structure 2dj, a structure of the LysM of human LysMD1 (data not shown). A model of the mLysMD3 LysM could also be generated based on the structure 2mkx, the LysM from E. faecalis protein AtlA, in which 85% of the sequence could be modeled with a confidence of 99.4% (Figure 1D). Together these data suggest that the LysM of mLysMD3, as well as those of mLysMD1, mLysMD2, and mLysMD4 are conserved across multiple divergent species.

LysMD3 colocalizes with GM130+ structures. We next sought to define the subcellular localization of LysMD3. Immunofluorescence staining of endogenous human LYSMD3 (hLYSMD3) in HeLa cells suggested that hLYSMD3 co-localizes
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with GM130+ structures, consistent with localization to the Golgi (Figure 2A; (11)). Importantly, this staining and co-localization is not observed in HeLa cell lines in which hLYSMD3 expression has been eliminated through the use of CRISPR/Cas9 targeted genome editing. These data are further supported by similar staining patterns using a second commercially-available antibody specific to hLYSMD3 as well as transfection of HeLa cells with a mLysMD3-GFP expression construct (Figure 2C) and subsequent staining for GFP (Figure S1A and B). Additionally, mouse embryonic fibroblast cells (MEFs) were stained for mLysMD3 expression using an affinity-purified polyclonal anti-mLysMD3 antibody raised against amino acids 1-205 of mLysMD3 (Figure S1C). Although this antibody appears to non-specifically stain the nucleus, LysMD3-specific signals were revealed by the absence of staining in MEFs deficient for mLysMD3 due to the insertion of a gene-trap cassette downstream of mLysMD3 exon 1 (Figure S2A). GM130 co-localization was also observed for mLysMD3 in MEFs using this antibody (Figure S1C). Taken together, these data suggest that human and mouse LysMD3 molecules are located on GM130+ structures, consistent with localization to the Golgi.

Pfam analysis of the mLysMD3 sequence identified a putative transmembrane domain between amino acids 217 and 237 (Figure 1A), suggesting that mLysMD3 is a single pass transmembrane molecule. To test this hypothesis, we first evaluated the cellular compartment with which mLysMD3 is associated. Differential detergent fractionation followed by immunoblotting suggested that mLysMD3 is associated with the membrane compartment of primary bone marrow macrophages (BMMo), and not the nucleus or cytoplasm (Figure 2B). These data are consistent with the hypothesis that mLysMD3 is a transmembrane protein.

We next assessed mLysMD3 membrane topology in a protease protection assay using a recombinant mLysMD3 molecule with N-terminal Flag and C-terminal HA tags (Flag-LysMD3-HA, diagrammed in Figure 2C). This construct was expressed in HeLa cells, the plasma membrane was selectively permeabilized using digitonin (12), and exposed epitopes were subjected to Proteinase K digestion. Immunoblot analysis of the N-terminal Flag epitope indicated that the Flag tag was degraded upon permeabilization of the plasma membrane and addition of increasing concentrations of Proteinase K, as shown by decreased detection of the Flag-tagged full-length protein (Figure 2D). In contrast, with increasing concentration of Proteinase K, the HA tag was detected on a molecular species that shifted from a molecular weight of about 60kD to about 15kD, indicating that the C-terminal HA tag was within an intracellular vesicle not permeabilized by digitonin and therefore protected from Proteinase K digestion.

This conclusion is further supported by immunofluorescence analysis using GFP-tagged mLysMD3 molecules (diagrammed in Figure 2C). Transfection of HeLa cells with GFP-tagged molecules and anti-GFP staining demonstrated that a mLysMD3 construct with a C-terminal GFP tag could be immunostained at the cell surface without prior membrane permeabilization (Figure 2E). This anti-GFP signal is not observed for a mLysMD3 construct with an N-terminal GFP tag without prior membrane permeabilization.

Overall, these data suggest that mLysMD3 is a type II integral membrane protein located in the Golgi, with the N-terminal LysM located in the cytoplasm.

Generation of LysMD3 deficient mice. To evaluate the role of mLysMD3 during the immune response in vivo, we generated a mouse line with gene-trap (GT) mediated disruption of mLysMD3 using commercially available ES cells. This 129P2Ola/Hsd background cell line contained a β-galactosidase/neomycin resistance GT inserted between exons 1 and 2 of the mLysMD3 gene, upstream of the protein-coding region (Figure S2A). The location of the GT cassette within mLysMD3 intron 1 was confirmed by PCR and Sanger sequencing across the cassette and its boundaries (data not shown). The mLysMD3 GT allele was backcrossed to a C57BL6/J (B6, WT) background with the assistance of microsatellite-based speed congenic analysis at each generation. mLysMD3GT/GT mice were viable and fertile and crosses of mLysMD3WT/GT mice generate offspring with genotypes at the expected Mendelian frequencies on both the 129P2Ola/Hsd and B6 backgrounds.
In knockout (KO) mice generated through gene trap mutagenesis, it is possible that splicing over the gene trap may occur resulting in expression of the endogenous protein, although often at hypomorphic levels (13). Additionally, it is possible that backcrossing the mLysMD3 gene-trap allele from one background to another could select for a linked gene with differential immune responses between 129P2Ola/Hsd and B6.

We therefore also generated a KO mouse in which exon 2 of mLysMD3, containing the coding region for the LysM, was targeted using CRISPR/Cas9 genome editing in B6 embryos (Figure S2B). Sequencing of the edited mLysMD3 allele revealed a deletion of approximately 2kB, corresponding to the genomic sequence between the guide gRNA sites (Figure S2C). As observed for mLysMD3 GT mice, mLysMD3EN/EN mice are viable, fertile, and crosses of heterozygous parents yield offspring at the expected Mendelian frequencies.

To evaluate expression of mLysMD3 in our two mouse lines, we used a qRT-PCR assay with primers spanning the exon 2-exon 3 junction of mLysMD3. This exon-exon junction was abolished in the LysMD3 EN mouse line and was downstream of the gene-trap cassette in the LysMD3 GT mouse line. In this assay, mLysMD3 transcripts are undetectable in littermates from both lines of mLysMD3-deficient mice (Figure 3A) and are reduced in heterozygous mice, compared to WT.

Genomic alterations have the potential to affect neighboring gene expression (14), therefore, we evaluated the expression of selected mLysMD3 neighboring genes. We found that expression of Polr3g was unchanged in both LysMD3 GT and LysMD3 EN mice (Figure S2D), while expression of Adgrv1 was unchanged in LysMD3 EN mice and was marginally altered in LysMD3 GT mice in a gene-dose dependent manner (Figure S2D). Finally, given that LysMD3 is a member of a protein family, we evaluated the expression of LysMD4 in the absence of LysMD3. We found that LysMD4 expression was unaltered in both lines of LysMD3-deficient mice in the tissues evaluated (Figure 3B).

These data suggest that alteration of mLysMD3 has minimal effect on expression of surrounding gene or LysMD4 expression, and that we have established two lines of mLysMD3-deficient mice, referred to as LysMD3 GT and LysMD3 EN hereinafter.

Lack of a role for mLysMD3 in the response to C. rodentium and S. Typhimurium infection. As our expression data suggested that mLysMD3 is expressed in the intestine, we first evaluated the response of mLysMD3-deficient mice to oral infection with the gram-negative bacterium Citrobacter rodentium. In B6 background mice, oral C. rodentium infection causes the formation of lesions similar to those caused by the human enteric pathogen enteropathogenic Escherichia coli (15, 16). Over the course of infection with C. rodentium, minimal morbidity was observed in mLysMD3 WT or mLysMD3 KO littermate mice (Figure 4A). Furthermore, no difference in C. rodentium fecal shedding was observed over 8 days of infection (Figure 4B). Finally, no difference in colonic inflammation was observed 8 days post infection (DPI) in the absence of mLysMD3 (Figure 4C and Figure 4D).

We next evaluated the response of mLysMD3-deficient mice to oral infection with the gram-negative bacterium Salmonella enterica serotype Typhimurium (S. Typhimurium). B6 mice are highly susceptible to S. Typhimurium infection due to a mutation in Nramp (17), but can be used to model intestinal inflammation during enteric salmonellosis (18). We found that mLysMD3 deficiency did not affect intestinal inflammation after S. Typhimurium infection in either the colon or cecum, as evaluated by clinical scoring of histological sections at 2 DPI (Figure 4E, Figure 4F and data not shown).

Lack of a role for mLysMD3 in the response to intracellular bacteria. Given the cytoplasmic orientation of the LysMD3 LysM, we evaluated the role for LysMD3 in the sensing of intracellular bacteria. We evaluated the response of mice to infection with Listeria monocytogenes, an intracellular, cytoplasmic gram-positive bacterium that is a major human pathogen (19). Mice were infected with L. monocytogenes and monitored for lethality. We observed no difference in lethality between mLysMD3 WT and mLysMD3 KO mice over the course of the experiment, with approximately 50% of each genotype succumbing to infection (Figure 5A). Furthermore, we found
no role for mLysMD3 in the ability of bone marrow macrophages (BMMo) to activate T cells in response to \textit{L. monocytogenes} infection \textit{in vitro} (Figure S3A).

We next evaluated the ability of mLysMD3-deficient BMMo to respond to infection by a gram-negative intracellular and cytoplasmic bacterium, \textit{Franciscella novicida}, a close relative of the human pathogen \textit{F. tularensis}, the causative agent of tularemia (20). Two strains of \textit{F. novicida} were used for these experiments: U112, a WT strain of \textit{F. novicida}, and isogenic mutant \textit{AFPI}, which is incapable of escaping the macrophage phagosome to enter the cytoplasm (21). We observed no difference in the ability of either \textit{F. novicida} strain to grow intracellularly (Figure 5B), or stimulate cell death (Figure S3B) or type I IFN production (Figure S3C) in the absence of mLysMD3. Similarly, no difference in cell death or type I IFN production was seen at a high multiplicity of infection (MOI) of 100 (data not shown).

We next evaluated the ability of mLysMD3-deficient mice to control \textit{Mycobacterium tuberculosis} (\textit{Mtb}) infection \textit{in vivo} (22-25). \textit{Mtb} is an intracellular pathogen, classically thought to reside in an early endosomal compartment, however recent studies have suggested that it may also grow in the cytoplasm (26). We found that there was no overall difference in \textit{Mtb} titers in the lungs of B6, \textit{LysMD3\textit{EN/EN}}, or \textit{LysMD3\textit{GT/GT}} mice at three or 13 weeks post infection (WPI, Figure 5C-D). Although there was a slight reduction in bacterial titers in the spleens of \textit{LysMD3\textit{GT/GT}} mice at three WPI compared to B6 controls (Figure S3D), this reduction was not observed in \textit{LysMD3\textit{EN/EN}} mice, and was not seen at 13 WPI (Figure S3E). Additionally, we did not observe differences in immune cell recruitment to the lung, spleen, or mediastinal lymph nodes of mLysMD3-deficient mice at either three WPI or 13 WPI (data not shown).

We next evaluated the ability of mLysMD3-deficient mice to control \textit{Brucella abortus} infection \textit{in vivo}. \textit{Brucella} species cause chronic granulomatous infection in both domestic animals and humans (27, 28). Littermate-matched mice were infected with \textit{B. abortus} intraperitoneally (IP) and bacterial titers were determined at 3 DPI. We observed no difference in bacterial titers in the spleen or liver or serum IL6 levels in either LysMD3 deficient IL6 levels in either LysMD3 deficient mouse line (Figure 5E-G and Figure S3F-H).

We next evaluated the ability of mLysMD3-deficient BMMo to respond to infection with \textit{Legionella pneumophila}, the causative agent of Legionnaire’s disease (29). BMMo were infected with \textit{L. pneumophila} strain LP02 or isogenic mutants \textit{ΔAflaA} or \textit{ΔdotA}. \textit{AflaA} mutants are non-flagellated, nonmotile, and grow to high titers in BMMo, though growth can be restricted by IFN\textgamma{} priming of BMMo (30). Conversely, \textit{ΔdotA} mutants are lack the type IV secretion apparatus that is strictly required for intracellular growth (31). We found no difference in the ability of these bacterial strains to grow intracellularly or to induce cell death in the absence of mLysMD3 (Figure 5H and Figure S3I).

We next evaluated a possible role for mLysMD3 in the pathogenesis of urinary tract infections caused by the gram negative bacterium uropathogenic \textit{E. coli} (UPEC), which occupies, luminal, intracellular cytoplasmic, and subcellular compartments during infection (32). Mice were transurethrally infected with the clinical UTI isolate, UTI89 and infection as well as the inflammatory response were evaluated 24 hours later. We found no difference in urine bacterial titers or urine IL1\textbeta{} production at 24 hours post infection (HPI) in the absence of mLysMD3 (Figure 5I and Figure 5J). Similarly, there was no difference in serum IL1\textbeta{} production at 24 HPI (Figure 5K). Histological evaluation of bladders from UPEC-infected mice demonstrated no role for mLysMD3 in the severity of inflammation at 24 HPI (Figure S3J). We also evaluated the ability of mLysMD3-deficient BMMo to respond to UPEC infection \textit{in vitro}. We found there was no difference in IL1\textbeta{} production by mLysMD3-deficient BMMo at either 6 or 24 HPI (Figure S3K).

Finally, we evaluated the ability of mLysMD3 deficient MEFs to respond to infection by clinically relevant intracellular bacteria species \textit{Chlamydia trachomatis} and \textit{Shigella flexneri}. We saw no difference between LysMD3 WT and KO cell lines in production of IFN\textbeta{} in response to \textit{C. trachomatis} (Figure 5L) or \textit{S. flexneri} (Figure 5M). Similarly no difference was seen in the...
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production of IL6 in response to infection (Figure S3L-M).

Lack of a role for mLysMD3 in models of inflammation. Datasets from the Immunological Genome Project (Immmgen) (33) suggest that mLysMD3 transcripts are expressed in cells of the immune system, as well as in intestinal tissues that could likely come into contact with pathogens. Therefore, we next evaluated a number of additional models of inflammation and infection.

Immmgen datasets suggest that LysMD3 transcripts are highly expressed in neutrophils (33). Therefore, we hypothesized that mLysMD3 could regulate inflammation in the neutrophil-dependent KRN model of serum-induced arthritis (34, 35). Mice were intravenously injected with arthritogenic serum from KxB/N mice on day 0 and monitored for disease progression. In littermate-matched male mice, we found that there was no difference in arthritis induction, as assessed by weight loss (Figure 6A), ankle swelling (Figure 6B), and clinical scoring (data not shown) up to 7 days post serum transfer. Further, there were no observable histologic differences in the affected joints at that time point (data not shown). Separate experiments with non-littermate-matched mice suggested that resolution of swelling after arthritis induction was similarly unaffected by mLysMD3 deficiency (data not shown).

We next tested whether mLysMD3 played a role in the inflammatory response to bacterial products. To address this, we challenged mice with lipopolysaccharide (LPS) and monitored them for mortality (Figure 6C). We found there was no difference in LPS-induced death in the absence of mLysMD3. Similarly, we found that peritoneal exudate cells (PECs) from littermate-matched mLysMD3-deficient mice produced similar levels of pro-inflammatory cytokines after ex vivo stimulation with LPS (Figure 6D).

Lack of a role for mLysMD3 in response to viral infection. Given that LysM domains have been found capable of binding to chitin (5, 38), we evaluated the role of mLysMD3 in the host response to fungal infection. Mice were infected with C. neoformans strain K99α and monitored for lethality (Figure 8A). In one of three experiments, LysMD3 KO mice were significantly more resistant to C. neoformans infection than their WT littermates. However, this finding was not replicated in two subsequent experiments. We also found no role for mLysMD3 in the response to Aspergillus fumigatus infection in vivo. Mice were infected with A. fumigatus IN and monitored for morbidity. We observed no difference in weight loss over the course of the experiment, and saw no difference in cell recruitment to the lung at 4 DPI (Figure 8B and C). Evaluation of lung tissue from A. fumigatus infected mice revealed no difference in lung inflammation or fungal hyphae growth in the absence of mLysMD3 (data not shown).

Lack of a role for mLysMD3 in controlling intestinal bacterial populations or intestinal gene expression. Interactions between host and pathogen are likely to be distinct from interactions between host and commensal organisms. Given that publicly available datasets (39) and our own analysis (Figure 3A) suggest that LysMD3 was expressed in the intestine, we assessed whether mLysMD3 regulated host interactions with the commensal microbiota. To test this hypothesis, we co-housed littermate WT and mLysMD3-deficient mice for two weeks to homogenize the microbiota between mice and correct for variability due to differences in breeding cohorts. Mice were then singly housed for two weeks on a regimen of
broad spectrum antibiotics in Kool Aid or Kool Aid alone. Fecal pellets were collected for 16S analysis and ileal tissue samples were prepared for RNaseq. We found no difference between WT and mLysMD3-deficient mice in bacterial richness or diversity within treatment groups in either mLysMD3 deficient mouse line (Figure 9A and Figure 9B). We did observe a significant drop in richness and diversity in the antibiotic-treated groups, as expected (Figure 9A and Figure 9B). We also applied principal component analysis to these datasets and found that while there was a clear difference between Kool Aid-treated and antibiotics-treated mice, there was not a distinct separation of mice by genotype (Figure 9C). Finally, RNaseq analysis of ileum samples from both the mLysMD3 GT and LysMD3 EN lines demonstrated no commonly significantly differentially expressed genes between WT and mLysMD3-deficient mice with or without antibiotic treatment (Figure 9D).

**Lack of a role for mLysMD3 in the innate immune response to C. rodentium infection.** Finally, we tested whether mLysMD3 played a role in the innate immune response to infection, which may have been obscured by the presence of the adaptive immune system in previous experiments. To address this possibility, we crossed the LysMD3 EN mouse line to the RAG1 KO background (40). Littermate-matched LysMD3 WT and KO mice on the RAG1 KO background were infected with *C. rodentium* and monitored for lethality. We found no difference in lethality (Figure 10A) or stool titers (Figure 10B) over the course of *C. rodentium* infection.

**DISCUSSION**

The annotated LysM domain of mLysMD3 is evolutionarily related to similar domains in human, frog, and zebrafish with orthologs in flies and worms. We found that mouse and human LysMD3 proteins co-localize with GM130+ structures, consistent with localization to the Golgi, and is a type II integral membrane protein, with the LysM domain with a cytoplasmic orientation. In extensive studies of two independent lines of mLysMD3-deficient mice, we observed no evidence for a physiologic role for this molecule in a range of pathogen infections, models of inflammation, or interactions with commensal bacterial. One explanation for these *in vivo* findings is that, while evolutionarily conserved, mLysMD3 has no physiological role. Alternatively, we simply failed to select the right model in which to detect a phenotype for mLysMD3 deficiency or there is redundancy that obscures the function of this conserved molecule. The latter hypothesis is supported by the lack of an observable developmental phenotype in our mLysMD3-deficient mice.

**Significance of mLysMD3 localization and immune functions of ER and Golgi proteins.** We originally hypothesized that mLysMD3 was a candidate pattern recognition receptor due to homology between the LysMs in flies and plants. Furthermore, publicly available databases (39, 41, 42), as well as our own data, suggest that mLysMD3 is expressed in tissues where it could feasibly be involved in the sensing of invading pathogens or interactions with the commensal microbiota. Interestingly, however, we found that mLysMD3 co-localizes with GM130+ structures in human and mouse cells, consistent with localization to the Golgi (11), and that, furthermore, mLysMD3 is a type II integral membrane protein with LysM in the cytoplasm. While traditional paradigms for PAMP-sensing focus on localization of pattern recognition receptors at the cell surface, it is now appreciated that intracellular organelles such as mitochondria, the ER, and the Golgi apparatus can serve innate immune signaling platforms (43). For instance, in primary cells isolated from *Drosophila* infected with *Wolbachia pipiensis* bacteria, *Wolbachia* can be identified in a GM130+ cellular compartment, or an immediately adjacent compartment (44). In mammals, S. Typhimurium establishes the *Salmonella*-containing-vacuole near the Golgi apparatus (45). Furthermore, *S. enterica, B. abortus, C. trachomatis,* and *L. pneumophila* have all been shown to co-opt intracellular trafficking and redirect Golgi-derived vesicles during infection (46). However, we were unable to define a role for mLysMD3 in models of these infections and multiple others. Furthermore we found that mLysMD3 deficient mice have no evidence of dysbiosis or altered intestinal gene expression. It remains possible that mLysMD3 interacts specifically with a particular microbial product or
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Evolutionary conservation. Despite the fact that mLysMD3 appears to be evolutionarily conserved with proteins found in flies, zebrafish, and worms, it is possible that its function is not conserved. Although Toll and TLR family members are central to immune responses in flies and mammals (47, 48), the evolutionarily-conserved peptidoglycan recognition proteins (PGRP, PGLYRP), while essential for the Drosophila immune response, to date have been found to play a relatively minor role in mammalian immunity (49, 50). It is possible that LysMD3 is conserved for reasons completely unrelated to a role in immunity, despite its defining peptidoglycan-binding lysin motif, such as a role during organismal development. Indeed, the Drosophila molecule Toll plays an important role in dorsal-ventral polarity during embryogenesis (51), with secondary function as an important element of the immune response in adult flies (48, 52). Furthermore, LysMD family member expression in zebrafish has been shown to be upregulated during embryogenesis, with localization to the central nervous system, but without alterations in expression in response to bacterial challenge (53).

mLysMD3 redundancy. LysMD3 is a member of a protein family and LysMD4 is predicted to also contain LysM and transmembrane domains. It is possible that mLysMD4 may compensate for mLysMD3 deficiency in our mouse lines. However, we saw no evidence of mLysMD4 transcript upregulation in mLysMD3 deficient mice, though it is possible that compensation may occur at the level of protein regulation or in the context of physiological levels of expression. It is also possible that the more distantly related family members mLysMD1 and mLysMD2 could compensate for mLysMD3 deficiency. However, obscuring of mLysMD3 function by mLysMD1 or mLysMD2 redundancy seems unlikely, given that the evolutionary distance between these molecules is significant (Figure 1C), and mLysMD1 and mLysMD2 are not predicted to be membrane-bound proteins. Mice deficient for multiple family members will be required to evaluate a role for the LysMD protein family in mammalian biology.

MATERIALS AND METHODS

Mice. The embryonic stem cell line Lysmd3Gt(E201G10)Wrst (clone E201G10, 129P2Ola/Hsd background) was purchased from the German Gene Trap Consortium. A single male chimeric founder was bred to B6 mice and backcrossed to >99% B6J background with high-density (~10cM) microsatellite-marker-based speed congenic analysis at each generation with the assistance of the Speed Congenics Facility of the Rheumatic Diseases Core Center at Washington University School of Medicine. LysMD3 gene trap mutant mice were genotyped using primers listed in Table 1, with GT mutant alleles identified by primers A+C and WT alleles identified by primers B+C. To generate CRISPR/Cas9 modified mLysMD3-deficient mice, B6 embryos were microinjected with mLysMD3 EN gRNA 1 and 2 (Table 1) and Cas9 mRNA and transferred to pseudopregnant recipient female mice, as previously described (54). Mice were screened for loss of exon 2 by Southern blot and targeted alleles verified by Sanger sequencing. A single male founder was crossed to a B6 female and F1 pups were intercrossed to generate subsequent breeding pairs and experimental animals. Mice were genotyped using primers listed in Table 1, with WT alleles identified by primers A+B and EN mutant alleles identified by primers B+C. All mice were bred and housed in an enhanced barrier specific-pathogen-free facility at Washington University in St. Louis. All experiments were performed using age- and sex-matched mice between 8 and 12 weeks of age generated by intercrossing heterozygous mice (littermate mice), unless otherwise noted. B. abortus experiments were performed at the University of California Davis using age- and sex-matched littermate mice between 7 and 17 weeks of age. Experiments conducted at Washington University were approved by the Institutional Animal Care and Use Committee of Washington University. B. abortus experiments conducted at the University of California Davis were approved by the UC Davis Institutional Animal Care and Use Committee.

Cell culture. MEFs were established from embryos derived from progeny of a LysMD3 GT heterozygous mouse cross, genotyped as above,
and used before passage six. Unless otherwise stated, MEFs derived from WT littermates were used as controls. MEFs were grown in DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. All experiments were performed before cell line passage 6. All HeLa cell lines were maintained in DMEM with 10% FBS. hLysMD3 HeLa KO cells (ΔLYSMD3) were generated using CRISPR/Cas9 genome editing at the Genome Engineering and iPSC Center at Washington University School of Medicine (St. Louis, MO), as previously described (55). Briefly, hLysMD3 exon 2 was targeted using hLysMD3 gRNA (Table 1) and single cell clones were sequenced to confirm the complete absence of WT alleles and disruption of the open reading frame.

Plasmids. To generate an N-terminal Flag and C-terminal HA expression vector, the Gateway cloning vector pTAg-attR-C1 (56), containing three N-terminal Flag/CBP tags was modified to encode three in-frame HA tags downstream of the Gateway cloning site. To generate an insert tagged with an N-terminal EGFP, the Flag/CBP motif in pTAg-attR-C1 was replaced with EGFP. The cDNA IMAGE clone 3156298 containing mLysMD3 was purchased from ATCC. The mLysMD3 protein coding region was PCR amplified and cloned into the Gateway destination vectors described above. mLysMD3 was also cloned into pEGFP-N1 (Clontech) using Gibson assembly master mix (NEB). All expression plasmids were sequence verified.

Antibodies. Rabbit polyclonal anti-LysMD3 antibody was raised against amino acids 1-205 of mouse LysMD3 (Cocalico). mLysMD3 1-205 was conjugated to AffiGel15 (BioRad) and used to affinity purify the resulting immune serum. Commercially available antibodies were: anti-β Actin (Sigma A5316), anti-ERK1/2 (Cell Signaling 4695), anti-Flag M2 (Sigma F1804), anti-GFP (Abcam ab6556), anti-GM130 (BD Pharmingen 610822), anti-H2AX (Millipore 07-627), anti-HA (Sigma H9658), anti-hLysMD3 (Sigma HPA018024 and Proteintech 24313-1-AP), and anti-Transferrin Receptor (Life Technologies 13-6800). Secondary antibodies were: donkey anti-rabbit AF488 (Invitrogen A21206), goat anti-mouse AF555 (Invitrogen A21425), goat anti-mouse AF633 (Invitrogen A21052), donkey anti-rabbit AF647 (Invitrogen A-31573), goat anti-mouse HRP (Jackson Immunoresearch 115-035-146), and goat anti-rabbit HRP (Jackson Immunoresearch 111-035-003).

Phylogenetic analysis. Phylogenetic analysis was performed using the maximum likelihood method in the MEGA6 package (57) with 500 bootstrap replicates. The tree with the highest log likelihood is shown. Phylogenetic trees were visualized using TreeView (58). Sequences used for the phylogenetic analysis were: C. briggsae CBG12503: XP_002640031, C. elegans F43G9: NP_001122475, D. melanogaster CG12207: NP_650352, D. melanoaster CG15471: NP_572187, D. melanogaster CG17985: NP_610305, D. melanogaster Mtd: NP_652017, D. rerio LysMD1: NP_001070218, D. rerio LysMD2: NP_001003507, D. rerio LysMD3: NP_001002104, D. rerio LysMD4: NP_957144, D. simulans GD10233: XP_002080462, E. coli MltD-1: NP_414747, E. coli MltD-2: NP_414747, E. faecalis AtA: NP_814543, H. sapiens LysMD1: NP_997716, H. sapiens LysMD2: NP_699205, H. sapiens LysMD3: NP_938014, H. sapiens LysMD4: AAH84545, M. musculus LysMD1: NP_694761, M. musculus LysMD2: NP_081585, M. musculus LysMD3: NP_084533, M. musculus LysMD4: NP_780424, M. musculus Ncoa7: NP_766083, M. musculus Oxr1: NP_001123635, X. tropicalis LysMD1: NP_001096341, X. tropicalis LysMD2: NP_001037868, X. tropicalis LysMD3: NP_001017308, X. tropicalis LysMD4: XP_012815036.

Cell fractionation and immunoblot. WT bone marrow macrophages (BMMo) were generated as previously described (59). Briefly, bone marrow cells were cultured in 10% FBS, 10% CMG14-12 supernatant fluids containing M-CSF (60). At day 7, cells were subjected to differential detergent fractionation using a QProteome kit (Qiagen), according to manufacturer’s instructions. Fractions were subjected to Western blot analysis with antibodies to the indicated proteins. All Western blotting experiments were repeated twice and representative images are shown.

Protease protection assay. TransIT-LT1 (Mirus) was used to transfect an expression construct
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coding for Flag-LysMD3-HA into HeLa cells. 24h post-transfection, cells were treated with 80µM digitonin to permeabilize the plasma membrane. Samples were treated with the indicated concentration of proteinase K on ice and protease activity was inhibited by the addition of PMSF prior to Western blot analysis with the indicated antibodies. Experiments were repeated twice and representative blots are shown.

Immunoefluorescence. MEFs or HeLa cells were seeded on coverslips, fixed in 4% methanol-free paraformaldehyde, permeabilized using 0.5% TritonX100, and blocked in 1% BSA and 10% serum corresponding to the secondary antibody species. Cells were stained using the indicated primary and secondary antibodies and Phalloidin AF594 or AF647 (Invitrogen). Coverslips were mounted using Prolong Gold Antifade reagent (Invitrogen). For determination of membrane orientation by immunoefluorescence staining, TransIT-LT1 (Mirus) was used to transfect HeLa cells with the indicated LysMD3 constructs. Cells were fixed, blocked, and stained with an anti-GFP antibody prior to permeabilization using 0.5% Triton-X100, re-blocking, and re-staining for GFP where appropriate. Phalloidin-Alexa594 (Invitrogen) was used to visualize actin. Immunofluorescence images were acquired using a Zeiss LSM510 confocal microscope or a Zeiss LSM880 confocal laser scanning microscope. Unless otherwise noted, 3 separate experiments were performed and representative images are shown.

qRT-PCR RNA was extracted from tissues and qRT-PCR was performed as described (61). RNA was extracted from tissues with TRIzol (Life Technologies, Carsbad, CA) or Tri Reagent (Sigma, St. Louis, MO) according to the manufacturer’s protocol. One µg of RNA was used as a template for random-primed cDNA synthesis with ImPromII reverse transcriptase (Promega, Madison, WI). Transcripts were detected using predesigned Taqman assays (IDT, Coralville, IA) listed in Table 2 and the absolute number of transcript copies was determined by comparison to target-sequence containing gBlocks (IDT) and normalized to actin (Table 1).

Citrobacter rodentium. Kanamycin-resistant C. rodentium strain DBS120 (62, 63) cultures in log-phase growth were pelleted and resuspended in PBS with 3% bicarbonate. Mice were infected with approximately 1e9 CFU intragastrically (i.g.). Mice were subsequently weighed. Bacterial titers per stool pellet determined by homogenizing a single stool pellet in 1mL PBS with 0.05% Triton-X100 using 1mm silica beads (Biospec) on a mini-beadbeater 24 (Biospec) and plating serial dilutions on LB agar plates.

Salmonella enterica serotype Typhimurium. S. enterica serotype Typhimurium SL1344 was used to inoculate streptomycin-pretreated mice, as previously described (18). Mice fasted and pretreated with 200µg streptomycin 24 hours before infection. On the day of infection, mice were fasted for 4 hours then gavaged with 1e8 CFU of SL1344 in PBS, harvested in log-phase growth.

Histological preparation of tissues and colitis scoring. Colons and ceca were harvested and flushed with 10% neutral buffered formalin. Tissues were cut open, pinned flat, allowed to fix overnight at 4ºC, washed in 70% ethanol, and embedded in agar for processing. Sections were H&E stained and scored for colitis severity using the following scoring system: 0, no acute inflammation; 1, acute inflammation in surface epithelium, lamina propria, or cryptitis; 2, crypt abscess; 3, acute inflammation present at muscularis mucosa or beyond; 4, ulcer or transmural inflammation. The extent of involved colon was also evaluated as an estimated percentage.

Listeria monocytogenes. L. monocytogenes strain EGD from a frozen glycerol stock was used to infect mice IP as described(64). L. monocytogenes experiments used B6 mice as controls and LysMD3-deficient mice were generated by intercrossing KO mice.

Francisella novicida. BMMo were differentiated in DMEM with 10% FBS and 20% MCSF and infected with F. novicida U112 and ΔFPI as previous described (65, 66). Cytotoxicity was measured using LDH release (Promega, Madison,
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**Mycobacterium tuberculosis.** *M. tuberculosis* Erdman strain bacteria were used to aerosolically infect mice as previously described (64, 68). Approximately 100 CFU of *Mtbc* was inoculated using an Inhalation Exposure System (Glass-Col, Terre Haute, IN). At 0 or 24 HPI, 1-2 mice were sacrificed and the number of CFU delivered per mouse were quantitated to determine infection efficiency. Tissue bacterial titers were determined by plating dilutions of lung and spleen homogenate on 7H10 agar plates.

**Brucella abortus.** Littermate-matched mice between the ages of 7-17 weeks, were bred and housed at Washington University and shipped to UC Davis, where they were housed in microisolator cages with sterile bedding and irradiated feed in a biosafety level 3 laboratory. Mice were infected IP with 1e6 CFU of *B. abortus* 2308. Spleens and livers were collected aseptically. Spleens and livers were homogenized, and serial dilutions of the homogenate were plated on tryptic soy agar for enumeration of CFUs.

**Legionella pneumophila.** Primary BMMo were infected with *L. pneumophila*, as previously described (30, 69). LDH release was quantitated using the Cytotox assay (Promega, Madison, WI) as previously described (30).

**Uropathogenic E. coli.** Anesthetized female mice were transurethrally inoculated with 1e7 CFU UPEC strain UTI89. At 24 HPI, urine was collected for CFU enumeration and cytokine analysis, serum was collected for cytokine analysis, and bladder tissues were removed for inflammation scoring (70, 71). IL1β concentrations in the urine and serum were determined by ELISA (R&D systems, Minneapolis, MN). A semiquantitative scoring system, with a scale of 0 (normal) to 5 (necrosis with full-thickness inflammatory infiltration) was used to evaluate bladder inflammation at 24 HPI (71). UTI89 infection of BMMo was performed as described (72). Briefly, macrophages were differentiated from whole bone marrow in DMEM containing 15% FBS and 30% L929 conditioned media. Cells were re-plated on day 8 and challenged with UTI89 on day 9 at an MOI of 0.1. Supernatant fluids were collected at the indicated time points and IL1β concentration was determined as above.

**Chlamydia trachomatis and Shigella flexneri.** Infections of BMMo with *C. trachomatis* serovar L2 434/Bu at an MOI of 10 or *S. flexneri* serovar 2a WT strain 2457T at an MOI of 1 were performed in triplicate as described (73, 74) and supernatant fluids were collected for cytokine analysis using L929-ISRE fibroblasts to measure IFNβ levels (73). Sandwich ELISA was used to derive IL6 levels (BD Biosciences, San Jose, CA).

**Serum induced arthritis.** Serum was isolated from K/BxN mice as previously described (34). Serum was injected intravenously (i.v.) into littermate-matched male mice and morbidity and ankle swelling were determined at the indicated times post serum transfer (75).

**LPS treatments.** Mice were injected IP with 20mg/kg LPS purified by phenol extraction (Sigma L-2880). PECs were isolated by flushing the peritoneal cavity with 10mL of ice cold DMEM containing 10% FBS. Cells were counted, plated on 96-well tissue culture-treated plates, and incubated for 3 days. Cells were stimulated with 100ng/mL LPS purified by ion exchange chromatography (Sigma L4524) ± 16h IFNγ pretreatment. Supernatant fluids were collected 6h post-stimulation and TNFα was quantitated by ELISA (BD Biosciences, San Jose, CA).

**Influenza.** 8-12 week old mice were anesthetized and inoculated with 50 TCID50 H1N1 influenza virus strain PR8 IN, as described (76). Mice losing more than 30% of their initial body weight were sacrificed.

**MHV68 ex vivo limiting dilution assay for reactivation from latency.** MHV68 WUMS (ATCC VR1465) was used and reactivation from latency was measured as described (36, 37, 77). Mice were infected with 1e6 PFU MHV68 IP and after 16 days, PECs or spleens were pooled from 3-5 mice and serial dilutions of cells were plated on a MEF monolayer. After 3 weeks, wells were scored for cytopathic effect to detect reactivation.
Data points represent the mean and SEM for three replicate experiments using three mice per genotype per experiment. MHV68 experiments used B6 mice as controls and LysMD3-deficient mice were generated by intercrossing KO mice.

Cryptococcus neoformans. Cryptococcus neoformans strain KN99α was recovered from 15% glycerol stocks stored at −80°C and maintained on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, and 2% Bacto agar). A single colony was inoculated into YPD broth and grown for 16h at 30°C with shaking, collected by centrifugation, thrice washed with sterile PBS, and counted on a Cellometer Auto M10 (Nexcelom Bioscience, Lawrence, MA). Female WT or LysMD3 KO littermate mice from the LysMD3 GT line were anesthetized by IP injection (of 150 µl of 2 mg/ml xylazine (VEDCO) and 10 mg/ml of ketaset (Fort Dodge Animal Health)) and IN inoculated with 5e4 CFU (verified by quantitative culture on YPD agar) in 50 µl of sterile PBS. Mice were euthanized when body weight fell below 80% of peak weight.

Aspergillus fumigatus A. fumigatus CEA10 (CBS 144.89) was grown and conidia harvested similar to as described (78). Mice were infected IN with 1e7 conidia. Weight was monitored daily and mice were sacrificed at 72 HPI. Lungs were lavaged with 1mL of PBS with EDTA and fetal bovine serum. RBCs in the BAL were lysed and cell counts were performed. Cytospins preparations analyzed by light microscopy to determine differential counts. Lungs were fixed, paraffin-embedded and stained with hematoxylin and eosin for assessment of pathological changes. Hyphae were stained using Grocott methamine silver.

Antibiotic treatment of mice for 16S analysis. Female WT and KO mice were co-housed for 2 weeks, at a ratio of 2 WT and 2 KO mice per cage. After 2 weeks, mice were individually housed and were administered grape Kool-Aid ± a broad-spectrum antibiotic cocktail in their drinking water (79) for another 2 weeks. The antibiotic cocktail consisted of 1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin, 0.5 g/L vancomycin (Sigma, St. Louis, MO)) in 20 mg/mL grape Kool-Aid (Kraft Foods, Northfield, IL). After antibiotic treatment, stool pellets were collected for 16S analysis (79).

16S rRNA Illumina sequencing and analysis. Preparation of fecal pellets for 16S analysis was as previously described (79, 80). Briefly, DNA was phenol:chloroform-extracted and amplified in triplicate with Golay-barcoded primers specific for the V4 region of the 16S rRNA. Amplicons were pooled and purified with 0.6x Agencourt Ampure XP beads (Beckman-Coulter) prior to sequencing at the Center for Genome Sciences, Washington University School of Medicine bythe 2x250bp protocol on the Illumina MiSeq platform. 16S rRNA gene sequences were resolved using dada2 (81). Taxonomy was assigned to resolved sequences according to the Greengenes database (v.13.8) (82). All subsequent analysis were performed using PhyloSeq (v1.16.2) (83) to calculate richness, per sample Shannon diversity and both weighted and unweighted UniFrac distances amongst samples (84). Differential abundance of bacterial taxa between experimental groups was determined using the PhyloSeq DESeq2 extension using the Wald significance test and a local fit type (v.1.6.3) (83, 85).

RNA sequencing RNA from the distal ileum was purified, an Illumina sequencing library was generated, and libraries sequenced (HiSeq platform), as described (37). Five to six mice were included in each group. DESeq2 was used to identify differentially expressed genes (85).

Sequencing data RNA-seq and 16S sequencing data were deposited to the European nucleotide archive under the accession numbers PRJEB23707 and PRJEB25196, respectively.

Antigen presentation. Antigen presentation by BMMo was assessed as described (86, 87). BMMo were generated in 10% FBS and 10% CMG14-12 supernatant fluids (60) and infected with L. monocytogenes strain 10403S or pulsed with LLO peptide ± IFNγ priming. At one HPI, infected BMMo were washed with Dubecco’s PBS and Gentamycin was added. Splenic CD4 T cells were isolated from LLO56tg mice (86), bearing a TCR specific for LLO190-225, were isolated using a CD4 negative selection kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and added to the macrophage cultures. After 24 hours, cells were stained for CD4 (eFluor450, Clone RM4-5,
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eBioscience) and CD69 (PECy7, Clone H1.2F3, BioLegend) to evaluate activation status.

Acknowledgements We thank Darren Kreamalmeyer for assistance with animal husbandry, Andrea Bredemeyer, Barry Sleckman, and W. Tim Schaiff for technical assistance, and Broc T. McCune, Thomas Burke, and Daniel Portnoy for providing reagents. Experimental support was provided by the Genome Engineering Center of the Alvin J. Siteman Cancer Center, the Speed Congenics Facility and the Microinjection Core of the Rheumatic Diseases Core Center, and the Molecular Microbiology Imaging Facility at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, MO. The Siteman Cancer Center is supported by grant P30CA091842. The Rheumatic Diseases Core Center is supported by grant P30AR048335. This work was supported by the Children’s Discovery Institute of Washington University and St. Louis Children’s Hospital (MCD), grant #274415 from the CCFA Genetics Initiative (HWV), NSF fellowships DGE-1143954 (JPH) and DGE-1143954 (JMK), and NIH grants T32AI007163 (CCY), T32GM007067 (JMK), T32AI007172 (CRH), F32AI108089 (KMS), R01DK100644 (IUM), R01AI24157 (PMA), R01AI095396 (DDM), R01AI102882 (TLD), and U19AI109725 (HWV). C.L.S. is supported by a Beckman Young Investigator Award from the Arnold and Mabel Beckman Foundation and a Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Disease award.

Conflict of interest The authors have no conflicts of interest with the contents of this article.

Author contributions Conceptualization, C.C.Y., M.T.B., D.W.L., J.E.D., G.K.A., C.A.M., H.W.V; Methodology and investigation, C.C.Y., D.W.L., T.C.L., V.E.D.O., J.P.H., J.M.K., E.L.S., C.R.H., R.A.I., S.P., K.M.S., C.W., A.V.M., E.C., T.K.; Formal analysis, G.Z., C.D., S.A.H., Writing, original draft, C.C.Y.; Writing, review, and editing, all authors, with primary responsibility falling to C.C.Y. and H.W.V.; Supervision M.T.B., D.W.L., M.N.S., I.U.M., P.M.A., D.M.M., M.C.D., T.L.D., R.M.T., C.L.S., G.K.A., C.A.M., H.W.V.; Funding acquisition, H.W.V.

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Table 1. Primers and gRNA

| Target                                  | Primer sequence                                                                 |
|-----------------------------------------|---------------------------------------------------------------------------------|
| mLysMD3 GT genotyping                   |                                                                                |
| A                                       | GAGCCCCCAAATGAAAGAC                                                             |
| B                                       | GTCTAGACCGGGTGATGAA                                                             |
| C                                       | TCTTCGCGCATCTGTCTCTA                                                            |
| mLysMD3 EN genotyping                   |                                                                                |
| A                                       | TACAACAGGGTCTCAACCACG                                                           |
| B                                       | GCTGGCAGCAAGAACCTTTTG                                                           |
| C                                       | AGGTCACCAGTAAAGGAATTTC                                                         |
| Mouse actin qPCR                        |                                                                                |
| F                                       | GCTCCTTCGTTGCCCGGTCCA                                                          |
| R                                       | TTGCACATGCGGAGGCGGTT                                                            |
| Mouse actin qPCR probe                  |                                                                                |
|                                           | 6-JOEN-CACCAGTTT/GCCATGGATGACGA-IABkFQ                                          |
| mLysMD3 EN gRNA 1                       |                                                                                |
|                                           | GCCAGAGACACCCCGATACCTGG                                                        |
| mLysMD3 EN gRNA 2                       |                                                                                |
|                                           | GCTTTGTAACCTGCCCAGATAGGG                                                       |
| hLysMD3 gRNA                            |                                                                                |
|                                           | TGACCACCTTGACTGAATCCNGG                                                        |
Table 2. Predesigned qPCR assays.

| Target       | Assay               |
|--------------|---------------------|
| mLysMD3      | Mm.PT.58.10100260   |
| mLysMD4      | Mm.PT.58.12179173   |
| Adgrv1       | Mm.PT.58.43743756   |
| Polr3g       | Mm.PT.58.10070464   |
Characterization of the novel molecule LysMD3

Figure 1. Phylogenetic analysis of LysMs. (A) Schematic of predicted domains of mouse LysMD3. Transmembrane, TM. (B) Multiple sequence alignment of LysMs from the indicated organisms. Positions with a single, fully conserved residue are marked with an *. Positions with conservation of residues with strongly similar properties (> 0.5 in the Gonnet PAM 250 matrix) or weakly similar properties (≤ 0.5 in the Gonnet PAM 250 matrix) are marked with a "=" or "~", respectively. Percent conservation of residues is indicated at the bottom. (C) Phylogenetic tree of LysMs based on the multiple sequence alignment in B. Where known, common protein names are listed. For E. coli protein MltD, two LysMs in the protein were
evaluated (MltD-1 and MltD-2). (D) Phyre2 alignment of mLysMD3 with protein 2kmx, a LysM from *E. faecalis* protein AtlA. Known secondary structures within protein 2kmx are indicated at the top. T, hydrogen bonded turn.
Figure 2. LysMD3 is a type II integral membrane protein that co-localizes with GM130+ structures. (A) WT and hLYSMD3-deficient ΔLYSMD3 HeLa cells were stained for LysMD3 using a polyclonal anti-hLYSMD3 antibody. Cells were co-stained for GM130 and Phalloidin (blue) was used to visualize F-actin. (B) WT BMMo were subjected to differential detergent fraction and immunoblot analysis. Control molecules were ERK1/2 (cytoplasmic fraction), transferrin receptor (TIR, membrane fraction) and Histone H2AX (nuclear fraction). (C) Diagram of mLysMD3 constructs used in D and E. (D) HeLa cells were transfected with a construct expressing Flag-LysMD3-HA, then permeabilized with Triton X-100, digested with increasing doses of Proteinase K, as indicated, and subjected to immunoblot analysis for Flag, HA, and actin. (E) HeLa cells were transfected with the indicated constructs. Cells were fixed and stained for GFP ± prior permeabilization with Triton-X100, as indicated. Following GFP staining, all samples were permeabilized and Phalloidin-594 was used to visualize F-actin.
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Figure 3. LysMD3 deficient mice lack LysMD3 RNA expression. qRT-PCR analysis of (A) LysMD3 and (B) LysMD4 expression in the indicated tissues from LysMD3 GT and LysMD3 EN mouse lines. All experimental mice were littermate-matched.
Figure 4. Lack of a role for LysMD3 in the control of *C. rodentium* and *S. Typhimurium* infection. Littermate-matched male mice were infected with 1e9 CFU *C. rodentium* on day 0 and morbidity (A) and fecal shedding (B) were evaluated. At 8 DPI, colons were evaluated for inflammation (C and D). Each dot represents the histology score for one mouse. (E and F) Mice were infected with 1e8 CFU *S. Typhimurium*. At 2 DPI, colons were harvested and evaluated for inflammation. All experiments were performed in duplicate using littermate-matched male mice from the LysMD3 EN mouse line.
Figure 5. Lack of a role for LysMD3 in the control of multiple bacterial infections. (A) Age-matched mice from the LysMD3 GT mouse line were infected with 5e5 CFU L. monocytogenes strain EGD IP and monitored for lethality. Data are pooled from 3 experiments using male mice and 1 experiment using female mice. Non-littermate B6 mice were used as controls. (B) BMMo from the LysMD3 GT mouse line were infected with F. novicida strain U112 or isogenic mutant FPI at an MOI of 10 and intracellular growth was evaluated. (C-D) Mice were aerosoly infected with M. tuberculosis strain Erdman and titers in the lungs were determined at 3 (C) and 13 (D) WPI. (E-F) Mice from the LysMD3 EN mouse line were infected with 1e6 CFU B. abortus IP and titers in the spleen (E) and liver (F) and serum IL6 levels (G) were determined at 3 DPI. Data are combined from 2 experimental replicates. (H) BMMo from the LysMD3 EN mouse line were infected with L. pneumophila strains Lp02, ΔdotA, or ΔflaA ± IFNγ and intracellular growth was evaluated. Data are combined from two experimental replicates. (I-L) Female littermate mice from the LysMD3 GT mouse line were inoculated transurethrally with 1e7 CFU and UPEC strain UTI89. At 24 HPI, urine was collected for CFU enumeration (I) and cytokine analysis (J) and serum was collected for cytokine analysis (K). (L) MEFs from the LysMD3 GT mouse line were infected with C. trachomatis at an MOI of 10 and levels of secreted IFNb were evaluated by ELISA. (M) MEFs from the LysMD3 GT mouse line were infected with S. flexneri at an MOI of 1 and levels of secreted IFNb were evaluated by ELISA.
Figure 6. Lack of a role for LysMD3 in mouse models of inflammation. Mice were i.v. injected with KBN serum at day 0 and monitored for weight loss (A) and ankle swelling (B). Data are pooled from two experimental replicates using littermate male mice from the LysMD3 EN mouse line. (C) Mice were injected IP with 20mg/kg LPS and monitored for death every 12 hours. Data are pooled from 3 experimental replicates using littermate matched female mice from the LysMD3 GT mouse line on a mixed B6/129P2Ola/Hsd background. (D) PECs from littermate-matched LysMD3 EN mouse line were stimulated with 10ng/mL LPS ± IFNγ pretreatment. At 6 hours post stimulation, TNFα levels were measured in the supernatant fluids by ELISA. Data are pooled from two separate experimental replicates.
Figure 7. Lack of a role for mLysMD3 in the control of influenza and gHV68 viral infection. (A) Mice were anesthetized and infected IN with H1N1 strain PR8 and monitored for weight loss. Data are pooled from two experiments using littermate matched female mice from the LysMD3 EN line. (B-C) Mice were infected with MHV68 IP. At 16 DPI, PECs (D) and splenocytes (C) were harvested, serially diluted, and plated on a monolayer of MEFs. At 3 weeks post-harvest, MEF monolayers were scored for cytopathic effect, indicating the present of latent virions. Data are pooled from 3 experiments using non-littermate B6 mice as a control and the LysMD3 GT mouse line.
Figure 8. Lack of a role for mLysMD3 in the response to *C. neoformans* and *A. fumigatus* infection. (A) Mice were infected IN with 5e4 CFU of *C. neoformans* and monitored for lethality. Aggregated results from three independent experiments are shown. Mice were sacrificed if body weight dropped below 80% of initial starting weight. (B, C) Mice were infected IN with 1e7 *A. fumigatus* conidia and monitored for weight loss (B). At 4 DPI, lungs were lavaged and BAL cell counts were determined (C).
Figure 9. No microbiota or gene expression alterations in intestines of LysMD3 deficient mice. Littermate matched WT and LysMD3-deficient (KO) mice from both mouse lines were co-housed for two weeks, then subjected to two weeks of oral broad spectrum antibiotics in Kool Aid, or Kool Aid alone. Post-treatment fecal samples were subjected to 16S rRNA sequencing and ileal RNA was subjected to RNA-Seq analysis. Based on 16S sequencing, bacterial species richness (A) and alpha diversity (B) were evaluated. (C) Principal component analysis was applied to weighted UniFrac distances based on 16S rRNA sequences. (D) Average expression values were calculated for each gene across replicates and plotted on a log10 scale by genotype. Genes with average expression values <10 were omitted. Genes with at least two fold change in expression with adjusted p values<0.1 appear as black, with the remainder in gray. There were no genes with adjusted p values<0.01.
Figure 10. Lack of a role for LysMD3 in the innate immune response to \textit{C. rodentium} infection. The LysMD3 EN mouse line was crossed onto the RAG1 KO background. Littermate mice were infected with $1\times10^9$ CFU \textit{C. rodentium} i.g. Lethality (A) and \textit{C. rodentium} fecal shedding (B) were monitored.
LysMD3 is a type II membrane protein without an in vivo role in the response to a range of pathogens

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J. Biol. Chem. published online March 1, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001246

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