LysMD3 is a type II membrane protein without an 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 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 in vivo characterization of mLysMD3 function. We found that mLysMD3-deficient mice were born at Mendelian ratios and had no obvious pathological 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 sequencing. We conclude that mLysMD3 contains a LysM with cytoplasmic orientation, but we were unable to define a physiological role for the molecule in vivo.

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 (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). Although peptidoglycan is a ubiquitous bacterial component, relatively little is known about its interactions with the mammalian immune sys-

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This article contains Figs. S1–S3.

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The abbreviations used are: PAMP, pathogen-associated molecular pattern; qRT, quantitative RT; CFU, colony-forming unit; CBP, calmodulin-binding peptide; DPI, days post-infection; MOI, multiplicity of infection; WPI, weeks post-infection; IFN, interferon; HPI, hours post-infection; MEF, mouse embryonic fibroblast; LPS, lipopolysaccharide; UPEC, uropathogenic E. coli; PEC, peritoneal exudate cell; TNF, tumor necrosis factor; KO, knock-out; RNASeq, RNA sequencing; EGFP, enhanced green fluorescent protein; i.n., intranasally; i.g., intragastrically; i.p., intraperitoneally; LysM, lysin motif; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; BMMo, bone marrow macrophage; UPEC, uropathogenic E. coli; IAV, influenza virus; h, human; GT, gene trap; UTI, urinary tract infection.
Characterization of the novel molecule LysMD3

Mouse LysMD3 (mLysMD3) is a 305-amino acid protein with an annotated N-terminal LysM and C-terminal transmembrane domain (Fig. 1A). Phylogenetic analysis of the minimal LysM sequence from multiple model organisms (Fig. 1, B and C) revealed that mLysMD1, mLysMD2, mLysMD3, and mLysMD4 clustered with like sequences from other organisms, including Homo sapiens, Xenopus tropicalis, and Danio rerio. Furthermore, groups containing LysMD3 and LysMD4 sequences diverged from clusters containing LysMD1 and LysMD2 LysM sequences. Interestingly, the LysM from Caenorhabditis elegans protein F43G9 clustered with LysMD3 and LysMD4 sequences and apart from LysMD1 and LysMD2. Furthermore, whereas the exact relationship between the LysM of mLysMD3 and Drosophila melanogaster CG17985 is somewhat less defined, it is apparent that the D. melanogaster CG17985 clusters with sequences including mLysMD3/4 and not with mLysMD1/2. The other murine LysM-containing proteins Ncoa7 and Oxr1 cluster separately from CG17985 and the mLysMD family members and are more similar to the mLysMD4 sequences and apart from LysMD1 and LysMD2.

Furthermore, groups containing LysMD3 and LysMD4 sequences diverged from clusters containing LysMD1 and LysMD2 LysM sequences. Interestingly, the LysM from Caenorhabditis elegans protein F43G9 clustered with LysMD3 and LysMD4 sequences and apart from LysMD1 and LysMD2. Furthermore, whereas the exact relationship between the LysM of mLysMD3 and Drosophila melanogaster CG17985 is somewhat less defined, it is apparent that the D. melanogaster CG17985 clusters with sequences including mLysMD3/4 and not with mLysMD1/2. The other murine LysM-containing proteins Ncoa7 and Oxr1 cluster separately from CG17985 and the mLysMD family members and are more similar to the 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 (Fig. 1B). In particular, an asparagine residue at position 31 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 2dip, 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 2mks, the LysM from Enterococcus faecalis protein AtlA, in which 85% of the sequence could be modeled with a confidence of 99.4% (Fig. 1D). Together, these data suggest that the LysM of mLysMD3 and those of mLysMD1, mLysMD2, and mLysMD4 are conserved across multiple divergent species.

**LysMD3 co-localizes 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 with GM130+ structures, consistent with localization to the Golgi (Fig. 2A) (11). Importantly, this staining and the co-localization are 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 anti-hLYSMD3 antibody as well as transfection of HeLa cells with an mLysMD3-GFP expression construct (Fig. 2C) and subsequent staining for GFP (Fig. S1, A 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 (Fig. S1C). Although this antibody appears to nonspecifically 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 (Fig. S2A). GM130 co-localization was also observed for mLysMD3 in MEFs using this antibody (Fig. 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 amino acid sequence identified a putative transmembrane domain between amino acids 217 and 237 (Fig. 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 (Fig. 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 Fig. 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 (Fig. 2D). In contrast, with increasing concentration of proteinase K, the HA tag was detected on a molecular species that shifted from a molecular mass of about 60 kDa to about 15 kDa, 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 Fig. 2C). Transfection of HeLa cells with GFP-tagged molecules and anti-GFP staining demonstrated that an mLysMD3 construct with a C-terminal GFP tag could be immunostained at the cell surface without prior membrane permeabilization (Fig. 2E). This anti-GFP signal is not observed for an 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 con-
Characterization of the novel molecule LysMD3

Figure 3. LysMD3-deficient mice lack LysMD3 RNA expression. A and B, qRT-PCR analysis of LysMD3 (A) and LysMD4 (B) expression in the indicated tissues from LysMD3 GT and LysMD3 EN mouse lines. All experimental mice were littermate-matched.

Additionally, 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 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 backgrounds.

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 (Fig. S2B). Sequencing of the edited mLysMD3 allele revealed a deletion of ~2 kb, corresponding to the genomic sequence between the guide gRNA sites (Fig. S2C). As observed for the mLysMD3 GT mouse line, mLysMD3^EN/EN mice are viable and fertile, and crosses of mLysMD3~GT/GT mice generate offspring with genotypes at the expected Mendelian frequencies on both the 129P2Ola/Hsd and B6 backgrounds.

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 C. 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 (Fig. 4A). Furthermore, no difference in C. rodentium fecal shedding was observed over 8 days of infection (Fig. 4B). Finally, no difference in colonic inflammation was observed 8 days post-infection (DPI) in the absence of mLysMD3 (Fig. 4, C and D).

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 they 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 (Fig. 4, E and F 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 (Fig. 5A). Furthermore, we found no role for mLysMD3 in the ability of BMMo to activate T cells in response to *L. monocytogenes* infection in vitro (Fig. S3A).

We next evaluated the ability of mLysMD3-deficient BMMo to respond to infection by a Gram-negative intracellular and cytoplasmic bacterium, *Francisella novicida*, a close relative of the human pathogen *Francisella tularensis*, the causative agent of tularemia (20). Two strains of *F. novicida* were used for these experiments: U112, a WT strain of *F. novicida*, and isogenic mutant ΔFPI, which is incapable of escaping the macrophage phagosome to enter the cytoplasm (21). We observed no difference in the ability of either *F. novicida* strain to grow intracellularly (Fig. 5B) or to stimulate cell death (Fig. S3B) or type I IFN production (Fig. 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 *Mycobacterium tuberculosis* infection in vivo. *M. tuberculosis* 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 *M. tuberculosis* titers in the lungs of B6, mLysMD3<sup>EN/EN</sup>, or mLysMD3<sup>GT/GT</sup> mice at 3 or 13 weeks post-infection (WPI) (Fig. 5, C and D). Although there was a slight reduction in bacterial titers in the spleens of mLysMD3<sup>GT/GT</sup> mice at three WPI compared with B6 controls (Fig. S3D), this reduction was not observed in mLysMD3<sup>EN/EN</sup> mice and was not seen at 13 WPI (Fig. 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 3 or 13 WPI (data not shown).

We next evaluated the ability of mLysMD3-deficient mice to control *Brucella abortus* infection in vivo. *Brucella* species cause chronic granulomatous infection in both domestic animals and humans (27, 28). Littermate-matched mice were infected with *B. abortus* intraperitoneally (i.p.), 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 mLysMD3-deficient mouse line (Fig. 5, E–G, and Fig. S3, F–H).

We next evaluated the ability of mLysMD3-deficient BMMo to respond to infection with *Legionella pneumophila*, the causative agent of Legionnaire’s disease (29). BMMo were infected with *L. pneumophila* strain LP02 or isogenic mutants ΔflaA or ΔdotA. ΔflaA mutants are nonflagellated, nonmotile, and grow to high titers in BMMo, although growth can be restricted by IFNγ priming of BMMo (30). Conversely, ΔdotA mutants 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 (Fig. 5H and Fig. S3I).
We next evaluated a possible role for mLysMD3 in the pathogenesis of urinary tract infections (UTIs) caused by the Gram-negative bacterium uropathogenic \textit{E. coli} (UPEC), which occupies the 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 h later. We found no difference in urine bacterial titers or urine IL1/\(\beta\) production at 24 h post-infection (HPI) in the absence of mLysMD3 (Fig. 5, I and J). Similarly, there was no difference in serum IL6 levels (G) determined at 3 DPI. Data were combined from two experimental replicates. H. BMMo from the LysMD3 EN mouse line were infected with \textit{L. pneumophila} strains Lp02, \textit{ΔdotA}, or \textit{ΔflaA ΔIFN-γ}, and intracellular growth was evaluated. Data were combined from two experimental replicates. I-L, female littermate mice from the LysMD3 GT mouse line were inoculated transurethrally with 1\(\times\)106 CFU of \textit{B. abortus} i.p., and titers in the spleen (E), liver (F) and serum IL6 levels (G) were determined at 3 DPI. Data were pooled from three experiments using male mice and one experiment using female mice. Nonlittermate B6 mice were used as controls. B, BMMo from the LysMD3 GT mouse line were infected with \textit{F. novicida} strain U112 or isogenic mutant FPI at an m.o.i. of 10, and intracellular growth was evaluated. C and D, mice were infected by aerosol method with \textit{M. tuberculosis} strain Erdman, and titers in the lungs were determined at 3 (C) and 13 (D) WPI. E-G, mice from the LysMD3 EN mouse line were infected with 1\(\times\)106 CFU \textit{B. abortus} i.p., and titers in the spleen (E) and liver (F) and serum IL6 levels (G) were determined at 3 DPI. Data were combined from two experimental replicates. H. BMMo from the LysMD3 GT mouse line were infected with \textit{L. pneumophila} strains Lp02, \textit{ΔdotA}, or \textit{ΔflaA ΔIFN-γ}, and intracellular growth was evaluated. Data were combined from two experimental replicates. I-L, female littermate mice from the LysMD3 EN mouse line were infected with \textit{C. trachomatis} at an m.o.i. of 1, and levels of secreted IFNβ were evaluated by ELISA. M, MEFs from the LysMD3 GT mouse line were infected with \textit{S. flexneri} at an m.o.i. of 1, and levels of secreted IFNβ were evaluated by ELISA.

Lack of a role for mLysMD3 in models of inflammation

Datasets from the Immunological Genome Project (Immgen) (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.

Immgen 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 (Fig. 6A), ankle swelling (Fig. 6B), and clinical scoring (data not shown) up to 7 days post-serum transfer. Furthermore, there

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characterization of the novel molecule lysmd3

lack of a role for mllysmd3 in response to fungal infection

Given that lysm domains have been found capable of binding to chitin (5, 38), we evaluated the role of mllysmd3 in the host response to fungal infection. Mice were infected with cryptococcus neoformans strain kn99α and monitored for lethality (fig. 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 mllysmd3 in the response to aspergillus fumigatus infection in vivo. Mice were infected with a. fumigatus i.n. and monitored for moribidity. We observed no difference in weight loss over the course of the experiment, and we saw no difference in cell recruitment to the lung at 4 dpi (fig. 8b, c). Evaluation of lung tissue from a. fumigatus-infected mice revealed no difference in lung inflammation or fungal hyphae growth in the absence of mllysmd3 (data not shown).

lack of a role for mllysmd3 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 (fig. 3a) suggest that lysmd3 was expressed in the intestine, we assessed whether mllysmd3 regulated host interactions with the commensal microbiota. To test this hypothesis, we co-housed littermate wt and mllysmd3-deficient mice for 2 weeks to homogenize the microbiota between mice and to correct for variability due to differences in breeding cohorts. Mice were then singly housed for 2 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 rnasseq. We found no difference between wt and mllysmd3-deficient mice in bacterial richness or diversity within treatment groups in either mllysmd3-deficient mouse line (fig. 9, a and b). We did observe a significant drop in richness and diversity in the antibiotic-treated groups, as expected (fig. 9a and b). We also applied principal component analysis to these datasets and found that although there was a clear difference between kool aid-treated and antibiotic-treated mice, there was not a distinct separation of mice by genotype (fig. 9c). Finally, rnasseq analysis of ileum samples from both the mllysmd3 gt and lysmd3 en lines demonstrated no differentially expressed genes between wt and mllysmd3-deficient mice with or without antibiotic treatment that reached significance in both mouse lines (fig. 9d).

lack of a role for mllysmd3 in the innate immune response to c. rodentium infection

Finally, we tested whether mllysmd3 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

were no observable histological differences in the affected joints at that time point (data not shown). Separate experiments with nonlittermate-matched mice suggested that resolution of swelling after arthritis induction was similarly unaffected by mllysmd3 deficiency (data not shown).

We next tested whether mllysmd3 played a role in the inflammatory response to bacterial products. To address this, we challenged mice with lipopolysaccharide (lps) and monitored them for moribidity (fig. 6c). We found there was no difference in lps-induced death in the absence of mllysmd3. Similarly, we found that peritoneal exudate cells (pecs) from littermate-matched mllysmd3-deficient mice produced similar levels of pro-inflammatory cytokines after ex vivo stimulation with lps (fig. 6d).

lack of a role for mllysmd3 in response to viral infection

Given that mllysmd3 is highly expressed in the lung (fig. 3a), we tested whether mllysmd3 played a role during respiratory viral infection. We first evaluated the ability of mllysmd3 to control influenza virus (iav) infection in vivo. Mice were infected with mouse-adapted influenza virus h1n1 strain pr8 intranasally (i.n.) and monitored for moribidity and mortality. We found no difference in the susceptibility of mllysmd3-deficient mice to infection with iav pr8 over the course of the experiment (fig. 7a).

We also evaluated the role of mllysmd3 in the control of herpesvirus reactivation from latency. Mice were latently infected with the γ-herpesvirus mHV68 and the ability of mHV68 to reactivate from pecs was evaluated (36, 37). We found no difference in viral reactivation from latently infected lysmd3 wt or lysmd3-deficient pecs (fig. 7b and c).

lack of a role for mllysmd3 in response to fungal infection

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 were pooled from two experimental replicates using littermate male mice from the lysmd3 en mouse line. C, mice were injected i.p. with 20 mg/kg LPS and monitored for death every 12 h. Data were pooled from three experimental replicates using littermate-matched female mice from the lysmd3 gt mouse line on a mixed B6/129P2Ola/Hsd background. D, PECs from the littermate-matched lysmd3 en mouse line were stimulated with 10 ng/ml LPS ± IFNγ pretreatment. At 6 h post-stimulation, TNFα levels were measured in the supernatant fluids by ELISA. Data were pooled from two separate experimental replicates.

KO background were infected with *C. rodentium* and monitored for lethality. We found no difference in lethality (Fig. 10 A) or stool titers (Fig. 10 B) 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/H11001 structures and that they are type II integral membrane proteins, 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 physiological 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 endoplasmic reticulum 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 and datasets (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 furthermore, mLysMD3 is a type II integral membrane protein with LysM in the cytoplasm. Although 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 endoplasmic reticulum, 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, *Salmonella 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 ligand that we did not evaluate in our studies.

**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
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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 2 weeks, then subjected to 2 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 α 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 of <10 were omitted. Genes with at least 2-fold change in expression with adjusted p values of <0.1 appear as black with the remainder in gray. There were no genes with adjusted p values of <0.01.

Figure 10. Lack of a role for LysMD3 in the innate immune response to C. rodentium infection. The LysMD3 EN mouse line was crossed onto the Rag1 KO background. Littermate mice were infected with 1e9 CFU C. rodentium i.g. Lethality (A) and C. rodentium fecal shedding (B) were monitored.

Toll-like receptor family members are central to immune responses in flies and mammals (47, 48), the evolutionarily-conserved peptidoglycan recognition proteins (PGRP and 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 up-regulated 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 a LysM and a transmembrane domain. It is possible that mLysMD4 may compensate for mLysMD3 deficiency in our mouse lines. However, we saw no evidence of mLysMD4 transcript up-regulation in mLysMD3-deficient mice, although 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 (Fig. 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 Lysmd3Gtt(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% B6 background with high-density (~10 centimorgans) microsatellite–marker-based speed congenic analysis at each generation with the assistance of the Speed Congenics Facility at 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 listed in Table 1, with WT alleles identified by breeding pairs and experimental animals. Mice were genotyped female, and F1 pups were intercrossed to generate subsequent

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 (New England Biolabs). 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 (Bio-Rad) and used to affinity-purify the resulting immune serum. Commercially available antibodies were as follows: anti-β-actin (Sigma A5316); anti-ERK1/2 (Cell Signaling 4695); anti-FLAG M2 (Sigma F1804); anti-GFP (Abcam ab6556); anti-GM130 (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, Inc., 13-6800). Secondary antibodies were as follows: 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 115-035-003).

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 as follows: Caenorhabditis briggsae CBG12503, XP_002640031; C. elegans F43G9, NP_001122475; D. melanogaster CG12207, NP_650352; D. melanogaster CG15471, NP_572187; D. melanogaster CG17985, NP_610305; D. melanogaster Mtd, NP_652017; D. rerio LysMD1, NP_001070218; D. rerio

Table 1

<table>
<thead>
<tr>
<th>Primers and gRNA</th>
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<td>mLysMD3 GT genotyping B</td>
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<tr>
<td>hLysMD3 gRNA</td>
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Cell culture

MEFs were established from embryos derived from progeny of an 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 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 described previously (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 ORF.
LysMD2, NP_001003507; D. rerio LysMD3, NP_001002104; D. rerio LysMD4, NP_957144; Drosophila simulans GD10233, XP_002080462; E. coli MltD-1, NP_414747; E. coli MltD-2, NP_414747; E. faecalis AtlA, 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; and X. tropicalis LysMD4, XP_012815036.

Cell fractionation and immunoblot

WT bone marrow macrophages were generated as described previously (59). Briefly, bone marrow cells were cultured in 10% FBS, 10% CMG14-12 supernatant fluids containing macrophage colony-stimulating factor (60). At day 7, cells were subjected to differential detergent fractionation using a QProteome kit (Qiagen), according to the manufacturer’s instructions. Fractions were subjected to Western blot analysis with the indicated antibodies. 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 coding for FLAG-LysMD3-HA into HeLa cells. 24 h post-transfection, cells were treated with 80 μM 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 phenylmethylsulfonyl fluoride prior to Western blot analysis with the indicated antibodies. Experiments were repeated twice, and representative blots are shown.

Immunofluorescence

MEFs or HeLa cells were seeded on coverslips, fixed in 4% methanol-free paraformaldehyde, permeabilized using 0.5% Triton X-100, 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). Immunofluorescence images were acquired using a Zeiss LSM510 confocal microscope or a Zeiss LSM880 confocal laser-scanning microscope. Unless otherwise noted, three separate experiments were performed, and representative images are shown.

Characterization of the novel molecule LysMD3

RNA was extracted from tissues, and qRT-PCR was performed as described (61). RNA was extracted from tissues with TRIzol (Life Technologies, Inc.) or TRI Reagent (Sigma) 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). 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 with target sequence-containing gBlocks (IDT) and normalized to actin (Table 1).

C. rodentium

Kanamycin-resistant C. rodentium strain DBS1220 (62, 63) cultures in log-phase growth were pelleted and resuspended in PBS with 3% bicarbonate. Mice were infected with ~1e9 CFU intragastrically. Mice were subsequently weighed. Bacterial titers per stool pellet were determined by homogenizing a single stool pellet in 1 ml of PBS with 0.05% Triton X-100 using 1-mm silica beads (Biospec) on a mini-beadbeater 24 (Biospec) and plating serial dilutions on LB agar plates.

S. enterica serotype Typhimurium

S. enterica serotype Typhimurium SL1344 was used to inoculate streptomycin-pretreated mice, as described previously (18). Mice were fasted and pretreated with 200 μg of streptomycin 24 h before infection. On the day of infection, mice were fasted for 4 h, then gavaged with 1e8 CFU of SL1344 in PBS, and 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 4 °C, 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 i.p. as described (64). L. monocytogenes experiments used B6 mice as controls and LysMD3-deficient mice were generated by intercrossing KO mice.

F. novicida

BMMo were differentiated in DMEM with 10% FBS and 20% macrophage colony-stimulating factor and infected with
Characterization of the novel molecule LysMD3

F. novicida U112 and ΔFPI as described previously (65, 66). Cytotoxicity was measured using lactate dehydrogenase release (Promega), and type I IFN levels were measured using ISRE-L929 reporter cells (67).

M. tuberculosis

M. tuberculosis Erdman strain bacteria were used to infect mice by the aerosol route, as described previously (64, 68). Approximately 100 CFU of M. tuberculosis 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.

B. abortus

Littermate-matched mice between the ages of 7 and 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 intraperitoneally (i.p.) 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.

L. pneumophila

Primary BMMo were infected with L. pneumophila, as described previously (30, 69). Lactate dehydrogenase release was quantitated using the Cytotox assay (Promega) as described previously (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). 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 condition 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.

C. trachomatis and S. 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 determine IL6 levels (BD Biosciences).

Serum-induced arthritis

Serum was isolated from K/BxN mice as described previously (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 i.p. with 20 mg/kg LPS purified by phenol extraction (Sigma L-2880). PECs were isolated by flushing the peritoneal cavity with 10 ml 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 100 ng/ml LPS purified by ion-exchange chromatography (Sigma L4524) ± 16 h IFNγ pretreatment. Supernatant fluids were collected 6 h post-stimulation, and TNFα was quantitated by ELISA (BD Biosciences).

Influenza

8–12-Week-old mice were anesthetized and inoculated with 50 TCID 50 H1N1 influenza virus strain PR8 i.n., 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 i.p., and after 16 days, PECs or spleens were pooled from 3 to 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 standard error for three replicate experiments using three mice per genotype per experiment. MHV68 experiments used C57BL/6 mice as controls, and LysMD3-deficient mice were generated by intercrossing KO mice.

C. neoformans

C. 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 16 h at 30 °C with shaking, collected by centrifugation, washed three times 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 i.p. injection of (150 μl of 2 mg/ml xylazine (Vedco) and 10 mg/ml of ketaset (Fort Dodge Animal Health)) and i.n. 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.

A. fumigatus

A. fumigatus CEA10 (CBS 144.89) was grown, and conidia were harvested similar to Ref. 78. Mice were infected i.n. with 1e7 conidia. Weight was monitored daily, and mice were sacrificed at 72 HPI. Lungs were lavaged with 1 ml of PBS with EDTA and fetal bovine serum. RBCs in the BAL were lysed, and
cell counts were performed. Cytospin preparations were 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, as described previously (37). Five to six mice were pooled and purified with 0.6× Agencourt Ampure XP beads (Beckman-Coulter) prior to sequencing at the Center for Genome Sciences, Washington University School of Medicine, with the 2× 250-bp 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 (version 13.8) (82). All subsequent analyses were performed using PhyloSeq (version 1.6.2) (83) to calculate richness, per sample Shannon diversity, and both weighted and unweighted UniFrac distances among 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 (version 1.6.3) (83, 85).

**RNA-Seq**

RNA from the distal ileum was purified; an Illumina sequencing library was generated, and libraries were sequenced (HiSeq platform), as described previously (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 1 HPI, infected BMMo were washed with Dulbecco’s PBS, and gentamycin was added. Splenic CD4 T cells isolated from LLO56tg mice (86), bearing a T cell receptor 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 h, cells were stained for CD4 (eFlour450, Clone RM4-5, eBioscience) and CD69 (PECy7, Clone H1.2F3, BioLegend) to evaluate activation status.

**References**

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LysMD3 is a type II membrane protein without an in vivo role in the response to a range of pathogens


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