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Resistin-like molecule β is a bactericidal protein that promotes spatial segregation of the microbiota and the colonic epithelium

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The mammalian intestine is colonized by trillions of bacteria that perform essential metabolic functions for their hosts. The mutualistic nature of this relationship depends on maintaining spatial segregation between these bacteria and the intestinal epithelial surface. This segregation is achieved in part by the presence of a dense mucus layer at the epithelial surface and by the production of antimicrobial proteins that are secreted by epithelial cells into the mucus layer. Here, we show that resistin-like molecule β (RELMβ) is a bactericidal protein that limits contact between Gram-negative bacteria and the colonic epithelial surface. Mouse and human RELMβ selectively killed Gram-negative bacteria by forming size-selective pores that permeabilized bacterial membranes. In mice lacking RELMβ, Proteobacteria were present in the inner mucus layer and invaded mucosal tissues. Another RELM family member, human resistin, was also bactericidal, suggesting that bactericidal activity is a conserved function of the RELM family. Our findings thus identify the RELM family as a unique family of bactericidal proteins and show that RELMβ promotes host–bacterial mutualism by regulating the spatial segregation between the microbiota and the intestinal epithelium.

antibacterial protein | microbiota | innate immunity | intestinal epithelium

The trillions of microbes that colonize the mammalian gut are in a mutually beneficial nutrient-sharing relationship with their hosts (1). The intestinal epithelial barrier plays an essential role in ensuring that the mutualistic nature of this interaction is maintained. A key element of this barrier is a thick covering of mucus that overlies the epithelial surface and is organized into distinct inner and outer layers (2). Commensal microorganisms are abundant in the outer mucus layer but are largely excluded from the inner layer (3). This ensures that bacteria are spatially segregated from the intestinal epithelial surface, thus limiting their ability to invade host tissues and cause disease.

Antibacterial proteins are essential for enforcing this spatial segregation. For example, RegIIIγ is a bactericidal protein produced by intestinal epithelial cells that maintains physical separation between Gram-positive bacteria and the small intestinal epithelium (4). Other secreted proteins limit access of bacteria to the inner mucus layer of the colon, including Ly6/PLAUR domain containing 8 (Lypd8), which binds to flagellated bacteria (5), and zymogen granulae protein 16 (ZG16), which aggregates Gram-positive bacteria (6). The diversity of antimicrobial mechanisms required to maintain spatial segregation likely reflects the taxonomic complexity of the microbiota. Because of this complexity, we still have an incomplete understanding of the antimicrobial mechanisms that contribute to spatial segregation of microbiota and host.

Resistin-like molecule β (RELMβ) belongs to the RELM protein family, which also includes RELMα, RELMγ, and resistin. RELMβ is produced predominantly by colon goblet cells (7), is induced by the microbiota, and is markedly up-regulated

during intestinal inflammation (8, 9). Initially, both RELMβ and resistin were characterized as hormones that modulate insulin action (10, 11). However, subsequent studies revealed that RELMβ also plays a role in several aspects of host defense, including protection against infection by parasitic nematodes and *Citrobacter rodentium* (7, 12). Although this has been attributed to cytokine-like activities of RELMβ, the mechanistic basis for RELMβ's contributions to host defense remains unclear.

Here, we show that RELMβ kills Gram-negative bacteria. RELMβ binds to bacterial lipids and forms a membrane-permeabilizing pore that lyses the targeted bacterial cells. In mice lacking RELMβ, Proteobacteria are more abundant in the inner mucus layer of the colon, indicating that RELMβ is essential for maintaining spatial segregation of the intestinal microbiota. Human resistin can also disrupt microbial membranes and kill bacteria, suggesting that bactericidal activity is a conserved function of the RELM family. Thus, we identify RELM proteins as a previously unknown family of bactericidal proteins and provide essential insight into the mechanisms that separate the microbiota from the intestinal epithelium.

Significance

The mammalian gastrointestinal tract is home to diverse communities of bacteria that contribute to the metabolic health of their hosts. The epithelial lining of the intestine produces a diverse repertoire of antimicrobial proteins that limit the ability of these microorganisms to enter host tissues and cause disease. We have discovered that resistin-like molecule β (RELMβ) is a previously unknown member of the intestine's antibacterial arsenal. RELMβ is secreted from the intestinal surface and kills Gram-negative bacteria by damaging their membranes, thereby preventing these bacteria from coming into close contact with host tissues. Our findings reveal a new family of endogenous antibiotic proteins and contribute to the understanding of how mammals maintain mutually beneficial relationships with complex communities of intestinal bacteria.

Results

RELMβ Is a Bactericidal Protein That Targets Gram-Negative Bacteria.

We began to consider whether RELMβ might be an antibacterial protein when we noted structural features that are consistent with an antimicrobial function. First, the predicted pI is 8.5 and therefore basic. This is a common characteristic of bactericidal proteins that target bacterial membranes, which are negatively charged (13). Second, the 3D structure of RELMβ (14) features a β-sheet–rich head region containing several aromatic residues that form a hydrophobic surface (Fig. 1A). Similar hydrophobic regions are a feature of several proteins that permeabilize membranes, such as RegIIIα (15), Bcl-2 (16), and *Staphylococcus aureus* α-toxin (17), where the hydrophobic residues are involved in contacting membrane lipids and driving membrane insertion.

To test RELMβ for bactericidal activity, we produced recombinant mouse and human RELMβ in *Escherichia coli* and purified folded, monomeric protein (Fig. S1). We added the purified proteins to a panel of enteric bacteria that included both Gram-positive and Gram-negative species (Fig. 1 B and C). We observed a marked dose-dependent reduction in the viability of the Gram-negative bacteria *Pseudomonas aeruginosa* and *Citrobacter rodentium* (>99% decline in viability after a 2-h exposure to 10 μM RELMβ), but not *Bacteroides thetaiotaomicron*. The viability of the Gram-positive species *Listeria monocytogenes* and *Enterococcus faecalis* also declined, but less markedly (∼50% decline in viability after a 2-h exposure to 10 μM mouse RELMβ) (Fig. 1 B and C). Dimeric RELMβ retained bactericidal efficacy (Fig. S2 A and B), supporting the idea that the dimeric secreted form of RELMβ (8) is also bactericidal. Thus, mouse and human RELMβ have bactericidal activity against Gram-negative

bacteria, and the effective antibacterial concentrations of both proteins are similar to those of other intestinal antimicrobial proteins (18–20).

We used transmission electron microscopy to visualize morphological changes in *P. aeruginosa* cells after exposure to mouse RELMβ. The images showed evidence of cell wall damage and cytoplasmic leakage (Fig. 1D). These findings are similar to those obtained with the antibacterial proteins RegIIIγ (20) and human β-defensin-3 (18), which both kill bacteria by membrane permeabilization, suggesting that RELMβ might kill bacteria through a similar mechanism.

We assessed the capacity of RELMβ to permeabilize bacterial membranes by quantifying bacterial uptake of the membrane impermeant dye propidium iodide (PI). Mouse and human RELMβ increased PI uptake into *C. rodentium* in a dose-dependent manner, (Fig. 1 E and F). Thus, RELMβ permeabilizes the bacterial membrane, suggesting a mechanism for its bactericidal activity.

Interestingly, the bactericidal activity of RELMβ was dependent on the growth phase of the target bacteria, as *C. rodentium* grown to midlogarithmic phase was more readily killed than stationary-phase bacteria (Fig. S3A). Additionally, bactericidal activity and PI uptake required low salt concentrations and an acidic pH (Fig. S3 B and C). This is consistent with the salt and pH sensitivity of other antimicrobial peptides (21) and likely reflects the fact that such antibacterial proteins have evolved to function in the acidic, low-salt environment that is present in the mucus layer (22). The requirement for these specialized conditions might also explain why RELMβ was previously reported to lack antibacterial activity (12).
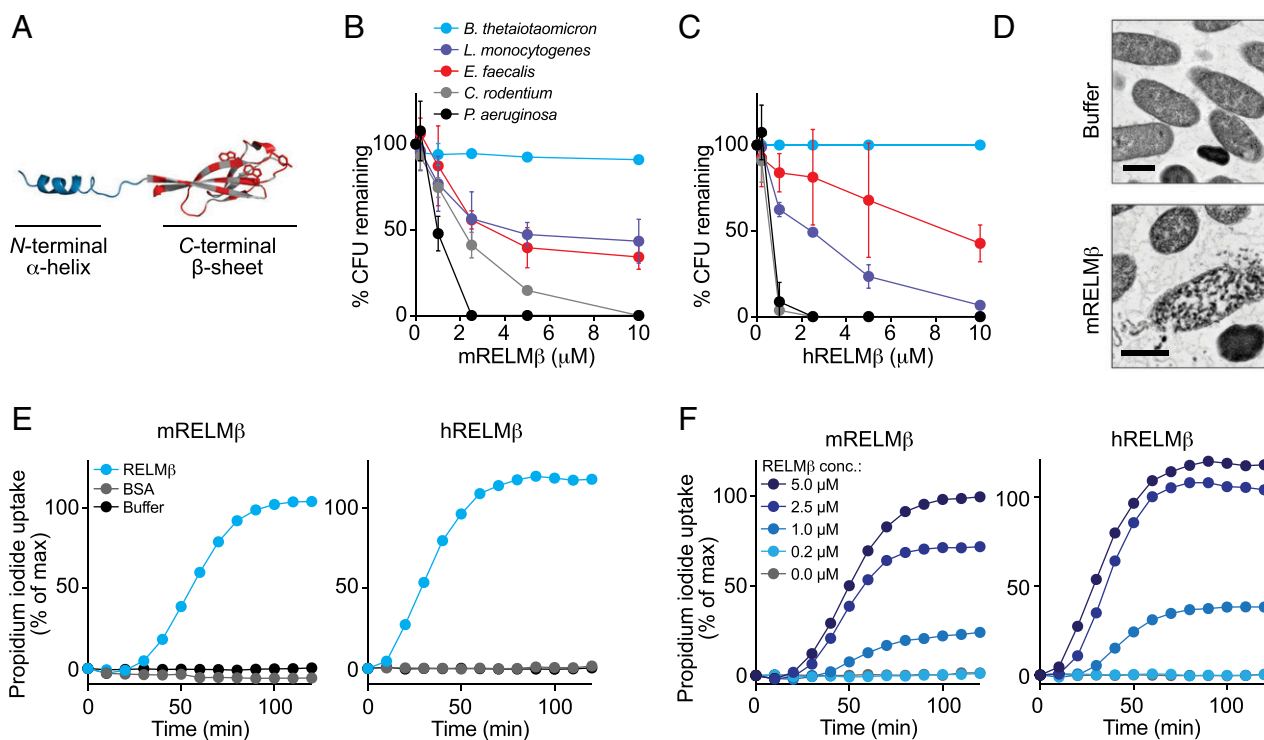

Fig. 1. RELMβ is a bactericidal protein that targets Gram-negative bacteria. (*A*) Crystal structure of mRELMβ (PDB ID code 1RH7) (14) showing the N-terminal α-helix and the C-terminal β-sheet with its aromatic residues. (*B* and *C*) RELMβ bactericidal activity. Purified recombinant mouse RELMβ (mRELMβ) (*B*) or human RELMβ (hRELMβ) (*C*) were added to midlogarithmic phase bacteria for 2 h, and numbers of surviving bacteria were quantified by dilution plating. Means ± SD are plotted. (*D*) Transmission electron microscopy of *P. aeruginosa* following a 2-h exposure to purified recombinant mRELMβ. (Scale bar: 0.5 μm.) (*E*) RELMβ permeabilizes bacterial membranes. *C. rodentium* was treated with 5 μM mRELMβ, hRELMβ, or BSA, and PI uptake was measured over 2 h. (*F*) PI uptake by *C. rodentium* in the presence of increasing concentrations of mRELMβ or hRELMβ. Assays were performed at least twice and repeated in triplicate within each experiment.

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RELMβ Binds to Negatively Charged Lipids and Forms a Multimeric Pore in Membranes. The ability of RELMβ to permeabilize bacterial membranes suggested that it might bind bacterial lipids. We tested this idea by performing an initial screen using membranes displaying various lipids. We found that RELMβ binds to lipids bearing negatively charged lipid head groups, but not to zwitterionic or neutral lipids (Fig. S4A). To determine whether lipid charge is important for RELMβ membrane permeabilization activity, we performed liposome disruption assays on liposomes having varying lipid composition. The liposomes encapsulated carboxyfluorescein (CF), a self-quenching dye that fluoresces upon dilution. RELMβ induced rapid dye efflux from liposomes composed of both phosphatidylcholine (PC), a zwitterionic phospholipid, and phosphatidylserine (PS), an acidic phospholipid (Fig. 2 A and B). The rate of efflux was reduced when PC-only liposomes were used (Fig. 2 A and B), indicating a preference for acidic phospholipids. Liposomes composed of PS alone also yielded a reduced rate of dye efflux, suggesting that charge density is an important factor for RELMβ membrane-disrupting activity, a characteristic shared with other cationic antimicrobial proteins (23, 24). Thus, RELMβ preferentially permeabilizes negatively charged lipid membranes, consistent with the salt sensitivity of RELMβ bactericidal activity (Fig. S3C), and with the acidic lipid content of bacterial membranes (13).

The crystal structure of mRELMβ reveals two distinct domains: an α-helix at the N terminus and a C-terminal β-sheet structure having a cluster of aromatic residues (14) (Fig. 1A). To determine which domain of mRELMβ drives membrane permeabilization, we synthesized a peptide representing the N-terminal α-helix and expressed a recombinant mRELMβ C terminus. When added to PC/PS liposomes, the mRELMβ C terminus yielded a dye efflux rate that exceeded that of full-length mRELMβ, while the mRELMβ N terminus resulted in virtually no dye release (Fig. 2 C and D). This finding was supported by measurements of mRELMβ lipid binding activity in which we measured fluorescence resonance energy transfer (FRET) between mRELMβ tryptophan residues and dansyl-labeled PC/PS liposomes (15). The mRELMβ C terminus produced greater FRET than full-length mRELMβ (Fig. 2E and Fig. S4B), supporting the idea that the C terminus drives mRELMβ–membrane interactions.

We next sought to gain insight into the mechanism by which RELMβ permeabilizes bacterial membranes. The intestinal bactericidal protein RegIIIα is a membrane-permeabilizing protein that forms a hexameric transmembrane pore (15). To determine whether mRELMβ also forms multimers in the presence of membranes, we added the purified monomeric protein to liposomes in the presence of the cross-linking agent bis(sulfosuccinimidyl)suberate. After solubilizing the products in detergent and separating them by size exclusion chromatography, we observed
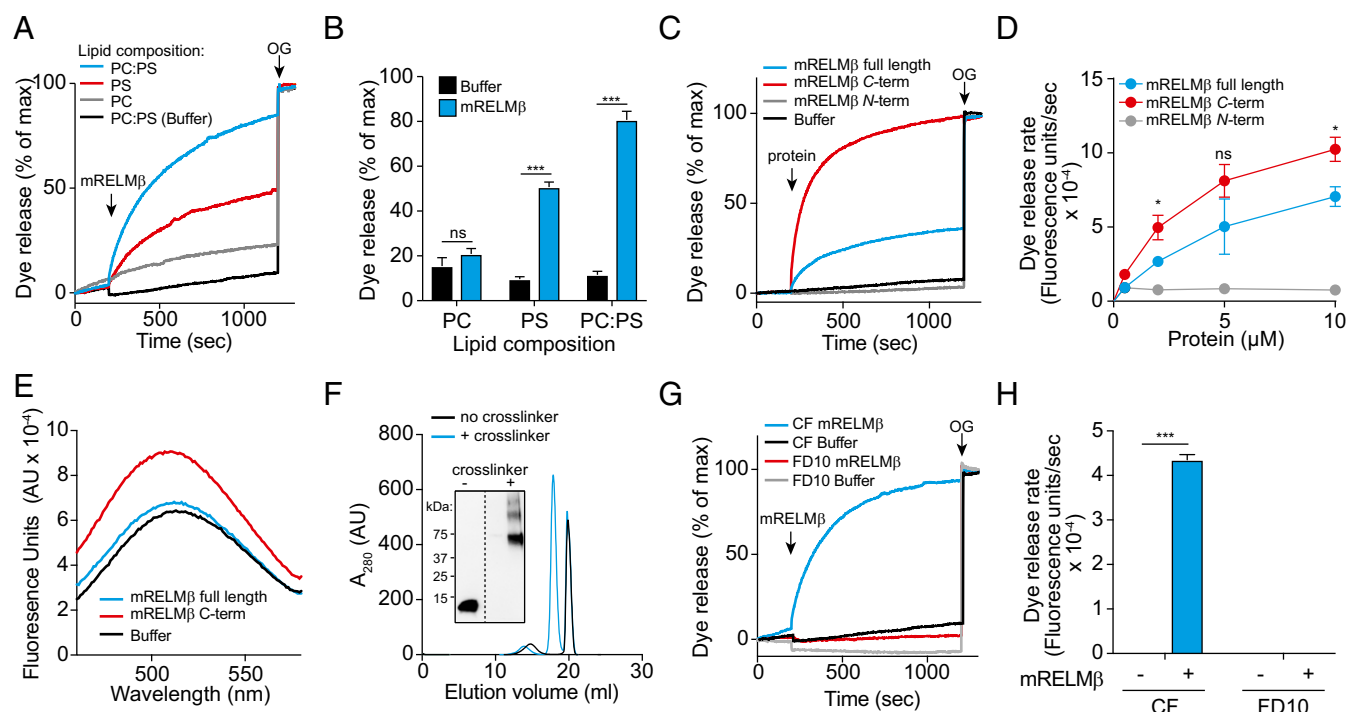
Fig. 2. RELMβ binds to negatively charged lipids and forms a multimeric pore in membranes. (A) mRELMβ disrupts carboxyfluorescein (CF)-loaded unilamellar liposomes containing the negatively charged lipid phosphatidyl serine (PS), but not liposomes composed of the zwitterionic lipid phosphatidylcholine (PC). Liposomes were treated with 5 μM mRELMβ, and dye efflux was monitored over time. The 1.0% octyl glucoside (OG) was added toward the end to disrupt remaining liposomes. Dye efflux is expressed as a percentage of maximal release by OG. (B) Means ± SD from three independent replicates of the experiment shown in A. (C) mRELMβ membrane-disrupting activity is confined to the C terminus. PC:PS liposomes (100 μM) were incubated with 5 μM full-length mRELMβ or the mRELMβ N or C terminus. (D) Initial rate of liposome dye efflux as a function of mRELMβ concentration. Assays were done in triplicate, means ± SD are shown, and statistical significance was calculated relative to the mRELMβ C terminus. (E) The C-terminal portion of mRELMβ binds lipid. The 5 μM full-length mRELMβ or the mRELMβ N or C terminus was added to liposomes incorporating 5% dansyl-PE, and dansyl fluorescence was monitored as measure of binding. (F and G) mRELMβ forms a multimeric complex in the presence of liposomes. Full-length mRELMβ was incubated with 100 mM PC:PS liposomes and cross-linked with bis(sulfosuccinimdyl) suberate. Cross-linked complexes were solubilized in detergent, resolved by size exclusion chromatography (F), and analyzed by Western blotting with anti-RELMβ antibody (F, Inset). mRELMβ forms a complex of ~60–70 kDa, or roughly six to eight protein units. (G) mRELMβ forms size-selective pores in liposomes. The 10 μM full-length mRELMβ was added to 100 μM PC:PS liposomes loaded with carboxyfluorescein (CF) (~10-Å Stokes diameter) or fluorescein isothiocyanate-dextran 10 (FD10) (~44-Å Stokes diameter). (H) Means ± SD from three independent replicates of the experiment shown in G. Statistics were performed with Student's t test; *P < 0.05; ***P < 0.001; ns, not significant.

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a product that migrated at a lower retention volume compared with the non–cross-linked monomer peak (Fig. 2F). The ability to form multimers was retained by the mRELMβ C terminus, supporting the importance of the C terminus in mediating interactions with lipid bilayers (Fig. S4C). Western blotting of the cross-linked protein showed a mobility of ~60–70 kDa (Fig. 2F, Inset). Given the predicted molecular weight of monomeric RELMβ (8.8 kDa), this suggests that the multimeric membrane-associated mRELMβ assembly is composed of six to eight mRELMβ subunits.

To further define the functional properties of membrane-associated RELMβ, we loaded PC/PS liposomes with fluorescent dyes having different Stokes diameters. Both full-length mRELMβ and the mRELMβ C terminus triggered rapid dye efflux in liposomes loaded with CF (~10-Å Stokes diameter), but not liposomes loaded with fluorescein isothiocyanate-dextran 10 (FD10) (~44-Å Stokes diameter) (Fig. 2 G and H and Fig. S4 D and E). This indicates that mRELMβ forms size-selective transmembrane pores.

RELMβ Limits Entry of Gram-Negative Bacteria into the Colon Inner Mucus Layer.
Our finding of a bactericidal function for RELMβ suggested that RELMβ might be involved in regulating microbiota composition and/or restricting host–bacterial contact in vivo. To test this idea, we used CRISPR/Cas9-mediated targeting to generate a frameshift mutation in the mouse Retnlb gene (encoding RELMβ) that produced a premature stop codon within the RELMβ signal sequence (Fig. S5A). We verified that mRELMβ was absent in the colons of Retnlb$^{-/-}$ mice (Fig. S5B) and showed that C. rodentium infection led to higher numbers of tissue-associated bacteria in the absence of RELMβ (Fig. S5C), as previously reported (12).

Other intestinal antibacterial proteins, including RegIIIγ, Lypd8, and ZG16, limit contact between intestinal bacteria and the intestinal epithelial surface, thus enforcing spatial segregation of microbiota and host (4–6). We therefore compared bacterial loads in the intestines of cocaged wild-type and Retnlb$^{-/-}$ mice by quantitative PCR (Q-PCR) determination of total 16S rRNA gene copy number. Bacterial loads in the colonic lumen trended higher in the Retnlb$^{-/-}$ mice, although the difference was not statistically significant. However, there was a significant two-log increase in the numbers of colonic tissue-associated bacteria in Retnlb$^{-/-}$ compared with wild-type mice (Fig. 3A). No significant differences were observed in either total luminal or tissue-associated bacteria in the small intestine (Fig. S6A), consistent with the lower abundance of RELMβ in the small intestine compared with the colon (11). The increase in colonic tissue-associated bacteria was unlikely to result from an altered mucus barrier, as Retnlb$^{-/-}$ mice did not show reduced expression of Muc2, which encodes a key mucus protein (3) (Fig. 3B), and the thickness of the mucus layer was not altered (Fig. 3C). Thus, RELMβ limits the association of bacteria with colonic tissues.

Because RELMβ preferentially kills Gram-negative bacteria, we predicted that Retnlb$^{-/-}$ mice would show an increased abundance of tissue-associated Gram-negative bacteria. We therefore compared the abundance of specific bacterial taxa in cocaged wild-type and Retnlb$^{-/-}$ mice by Q-PCR with 16S rRNA gene primers targeting specific bacterial groups. These included the Gram-positive Firmicutes, the Gram-negative Bacteroidetes, and the Gram-negative γ- and ε-Proteobacteria. While similar numbers of Firmicutes and Bacteroides were associated with colonic tissue, there was a marked increase in the numbers of γ- and ε-Proteobacteria in Retnlb$^{-/-}$ mice (Fig. 3D). These findings were supported by 16S rRNA deep sequencing, which revealed an increase in the abundance of tissue-associated Proteobacteria in Retnlb$^{-/-}$ mice, and minimal alterations in phylum-level abundances among luminal bacteria (Fig. S7 A and B).

We further analyzed specific subgroups of Proteobacteria, finding markedly elevated numbers of Helicobacter (ε-Proteobacteria) associated with Retnlb$^{-/-}$ colonic tissue (Fig. 3F) and in the colon lumen (Fig. S6G). The 16S rRNA Q-PCR analysis was supported by 16S deep sequencing (Fig. S7 C and D) and by visualization of bacteria within the colonic mucus layer of wild-type and Retnlb$^{-/-}$ mice. When we detected Gram-positive bacteria using an anti-lipoteichoic acid (LTA) antibody, the bacteria remained confined to the outer mucus layer in both wild-type and Retnlb$^{-/-}$ mice (Fig. 3E). In contrast, detection with an anti-Helicobacter antibody showed a marked increase in the numbers of bacteria in the inner mucus layer of Retnlb$^{-/-}$ mice, as well as within the epithelial layer (Fig. 3 G and H). Thus, RELMβ limits the numbers of Proteobacteria that associate with colon tissues.

Our in vivo findings are consistent with the potent bactericidal activity of RELMβ for P. aeruginosa and C. rodentium (both Proteobacteria) and diminished mRELMβ bactericidal activity toward B. thetaiotaomicron (belonging to the Bacteroidetes) and E. faecalis (belonging to the Firmicutes) (Fig. 1B). The abundances of lumen- and tissue-associated bacteria in the small intestine remained similar between wild-type and Retnlb$^{-/-}$ mice across all of the taxonomic groups (Figs. S6 B and C and S7), and we did not detect significantly altered numbers of bacteria translocating to mesenteric lymph nodes and spleen (Fig. S6 D and E). Despite there being no difference in the abundances of Firmicutes and Bacteroides associated with colon tissue, we did detect an increase in the abundances of these two groups in the colon lumen of Retnlb$^{-/-}$ mice (Fig. S6F). It is not yet clear how RELMβ deficiency causes these changes in luminal microbiota composition. However, we propose that they may arise as a consequence of the altered tissue-associated communities, which in turn could alter the luminal environment to promote blooms in certain taxonomic groups.

Human Resistin Is a Bactericidal Protein.
The RELM family member resistin (RETN) is produced by adipocytes and has been proposed to be a hormone that functions in metabolic regulation (25). However, human resistin (hRETN) is also expressed in monocytes and epithelial cells (26), suggesting a possible antimicrobial function. hRETN has a high degree of homology with hRELMβ (51% identity overall), particularly in the C terminus (60% amino acid identity), leading us to postulate that hRETN might also have bactericidal activity. Purified recombinant hRETN had potent bactericidal activity for the Gram-negative species P. aeruginosa (>99% decline in viability after a 2-h exposure to 2.5 μM hRETN) (Fig. 4A). The viability of C. rodentium and L. monocytogenes also declined (~90% decline in viability after a 2-h exposure to 10 μM hRETN), while E. faecalis and B. thetaiotaomicron were mostly resistant to hRETN (Fig. 4A). hRETN permeabilized C. rodentium membranes (Fig. 4B) and induced rapid dye release from PC/PS liposomes (Fig. 4 C and D). Indeed, full-length hRETN induced dye release at a faster rate than the mRELMβ C terminus (Fig. 4D). Finally, hRETN formed multimers in association with PC/PS liposomes as revealed by cross-linking experiments (Fig. 4E). Thus, hRETN kills bacteria by forming membrane-permeabilizing pores, suggesting that bactericidal activity is a conserved function of the RELM family.

Discussion
We have identified a bactericidal function for members of the RELM family. RELMβ preferentially kills Gram-negative bacteria through a mechanism involving the formation of multimeric membrane-permeabilizing pores that lyse targeted bacterial cells. In mice, RELMβ restricts entry of Proteobacteria into the colon inner mucus layer and thus limits bacterial contact with the colonic mucosal surface. Human resistin is also bactericidal through the formation of multimeric membrane-permeabilizing pores, suggesting that membrane toxicity and bactericidal activity are conserved functions of the RELM family. Altogether, our findings identify RELM proteins as a previously unknown family of

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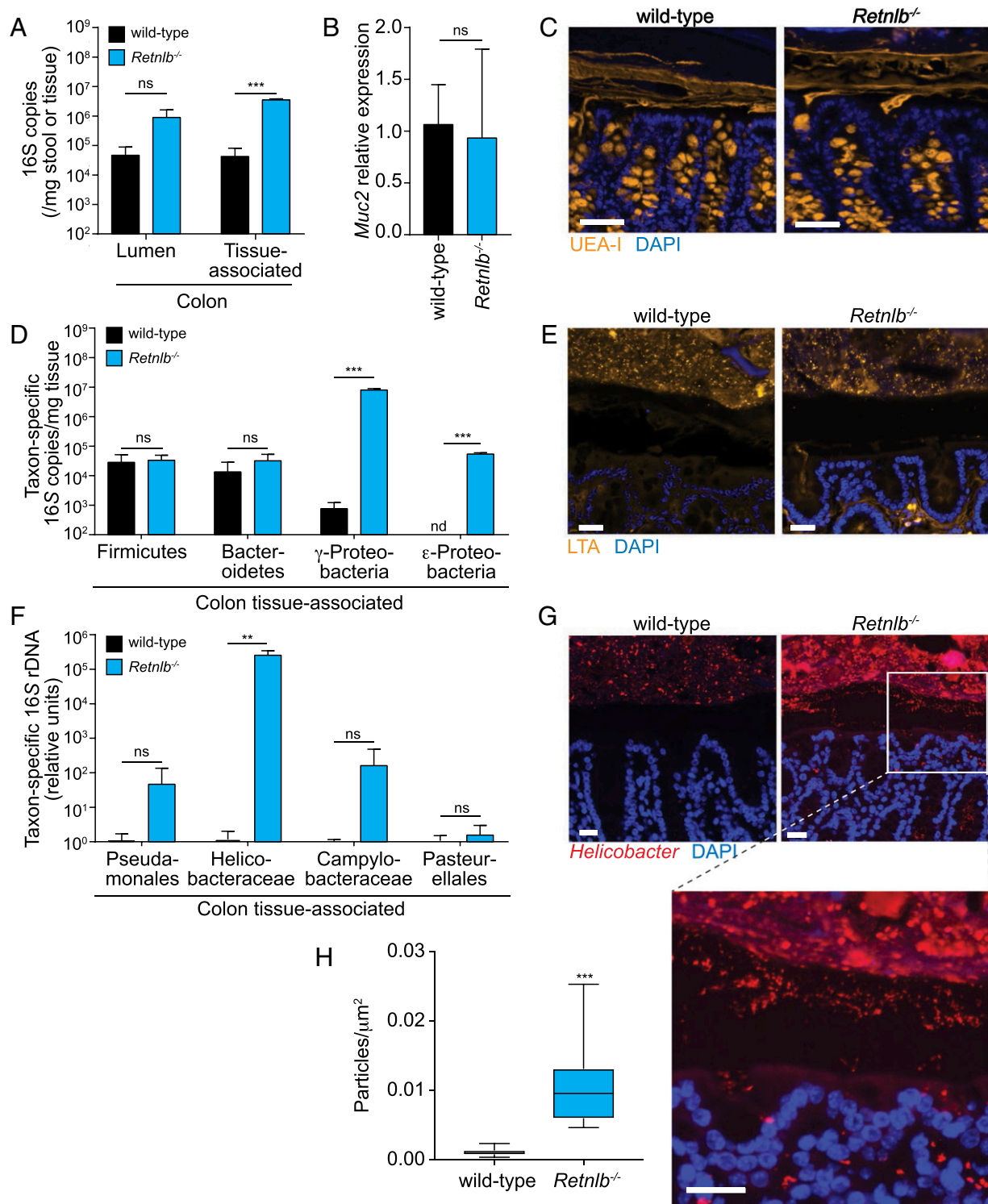
Fig. 3. RELMβ limits entry of Gram-negative bacteria into the colon inner mucus layer. (*A*) Quantification of total colonic luminal and tissue-associated bacteria by Q-PCR determination of 16S rRNA gene copy number in cohoused wild-type and RELMβ-deficient (*Retnlb*$^{-/-}$) mice. (*B* and *C*) MUC2 expression is not altered in RELMβ-deficient mice. (*B*) Q-PCR analysis of colonic *Muc2* transcripts. (*C*) Immunofluorescence detection of the mucus layer in colons of wild-type and *Retnlb*$^{-/-}$ mice with *Ulex europaeus* agglutinin-I (UEA-I), which detects mucus glycans (34). (Scale bars: 50 μm.) (*D*) Q-PCR quantification of 16S gene copy number from specific bacterial groups. Bacteria were recovered from colonic tissue and analyzed using taxon-specific 16S rDNA primers. (*E*) Immunofluorescence detection of lipoteichoic acid (LTA) in colonic tissues indicates that spatial segregation of Gram-positive bacteria is not markedly impacted by RELMβ deficiency. (*F*) Q-PCR quantification of specific bacterial groups at the colonic mucosal surface. Values for each bacterial group are expressed relative to 16S rDNA levels in wild-type mice. (*G*) Immunofluorescence detection of *Helicobacter* species at the colon surface. (*H*) *Helicobacter*$^+$ particles per square micrometer in the colon inner mucus layer. Quantification of particle density was performed using ImageJ from five fluorescent images from three mice of each genotype. For the 16S analyses, four mice per genotype were analyzed for each experiment, and Q-PCR assays were repeated in triplicate within each experiment. Means ± SD are plotted. Statistics were performed with Student's *t* test; **$P < 0.01$; ***$P < 0.001$; ns, not significant. All tissues were counterstained with DAPI (blue), and antibody isotype controls are shown in Fig. S8. (Scale bars: 25 μm.)

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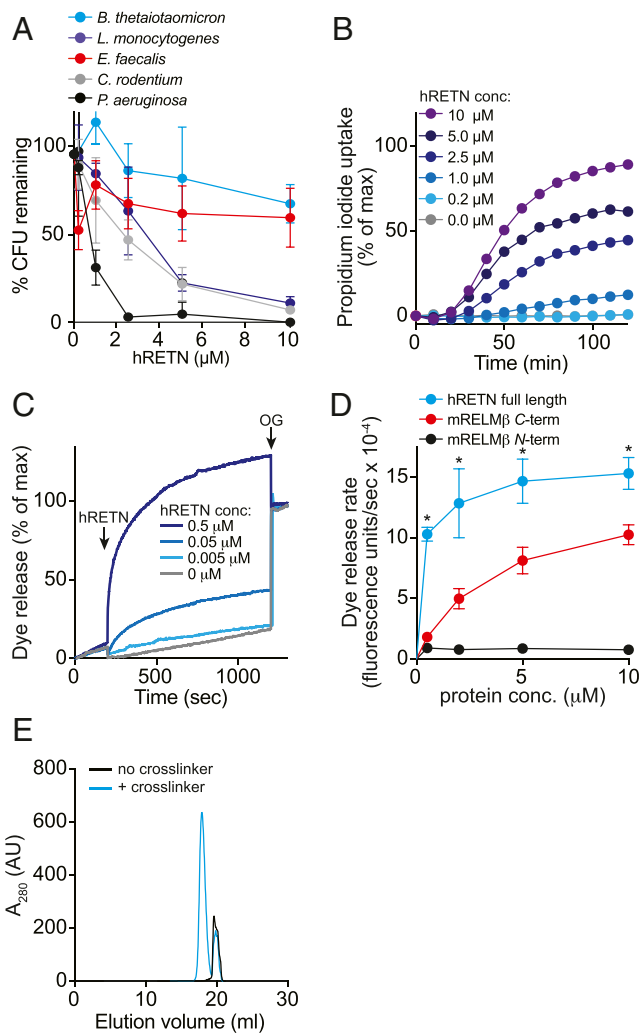
Fig. 4. Human resistin (hRETN) is a bactericidal protein. (*A*) Human resistin (hRETN) bactericidal activity. Purified recombinant hRETN was added to midlogarithmic phase bacteria for 2 h, and numbers of surviving bacteria were quantified by dilution plating. Means ± SD are plotted. (*B*) hRETN permeabilizes bacterial membranes. *C. rodentium* was treated with increasing concentrations of hRETN, and PI uptake was measured over 2 h. The assay was performed twice and was repeated in triplicate within each experiment. (*C*) hRETN disrupts carboxyfluorescein (CF)-loaded PC:PS liposomes. Liposomes were treated with increasing concentrations of hRETN, and dye efflux was monitored over time. The 1.0% octyl glucoside (OG) was added at the end to disrupt remaining liposomes. Dye efflux is expressed as a percentage of maximal release by OG. (*D*) hRETN membrane-disrupting activity is superior to the membrane-disrupting activity of C terminus of mRELMβ. CF-loaded PC:PS liposomes (100 μM) were incubated with varying concentrations of full-length hRETN or the mRELMβ N or C terminus, and initial rates of liposome dye efflux as a function of hRETN concentrations are plotted. Assays were done in triplicate, and means ± SD are shown. (*E*) hRETN forms a multimeric complex in the presence of liposomes. The 10 μM full-length hRETN was incubated with 100 mM PC:PS liposomes and cross-linked with bis(sulfosuccinimdyl) suberate. Cross-linked complexes were solubilized in detergent and resolved by size exclusion chromatography. Statistics were performed with Student's *t* test; *P < 0.05.

bactericidal proteins and enhance our understanding of how bacteria are kept physically separated from the intestinal epithelium.

The complexity of intestinal microbial communities suggests that multiple antimicrobial mechanisms are required to maintain spatial segregation of the intestinal microbiota. Accordingly, several distinct antimicrobial mechanisms have been identified that limit bacterial penetration of the inner mucus layer of the

intestine. RegIIIγ is a bactericidal protein that specifically targets Gram-positive bacteria in the small intestine (4, 15, 20), Lypd8 binds to flagellin and thus reduces the motility of flagellated Gram-negative bacteria in the colon (5), and ZG16 binds and aggregates Gram-positive bacteria in the colon (6). RELMβ is mechanistically unique relative to these antimicrobial factors in that it is a bactericidal protein that selectively targets Gram-negative bacteria, thus reducing their penetration into the colonic inner mucus layer.

An interesting question is whether the bactericidal activity of RELMβ can help to explain the metabolic abnormalities that are observed in RELMβ-deficient mice (27). The composition of intestinal bacterial communities has a marked influence on metabolic outcomes such as susceptibility to obesity, glucose tolerance, and insulin resistance (28). Thus, it seems plausible that alteration of intestinal bacterial communities by RELMβ, particularly those that are most closely associated with host tissues, could have metabolic consequences. In particular, Proteobacteria (targeted by RELMβ) have been shown to contribute to changes in host metabolism (29, 30).

A related question is whether the bactericidal activity of RELMβ could in part account for the various ways in which RELMβ alters intestinal immune function in vivo. For example, RELMβ-deficient mice show enhanced colonic expression of T_H2 cytokines and IL-17 (31), as well as decreased T cell recruitment during intestinal infection with *C. rodentium* and parasitic worms (7, 12). It will be important to determine whether these effects are secondary to RELMβ-dependent alterations in the composition or location of intestinal microbial communities, or arise from cytokine-like activities of RELMβ that may be independent of its bactericidal function.

Finally, our finding that human resistin has bactericidal activity suggests that this member of the RELM family may also function in host defense. Resistin is expressed in monocytes and epithelial cells (26), and thus could also be involved in host defense against pathogenic infections. The bactericidal activity of resistin and its expression in adipose tissue are also consistent with the known role of adipocytes in producing antimicrobial proteins that protect the host from bacterial infection (32).

Methods

Full methods are presented in *SI Methods*.

Mice. *Retnlb*^{−/−} mice were generated using CRISPR/Cas9 genome editing as described in *SI Methods*. Wild-type and *Retnlb*^{−/−} mice were cocaged to ensure a shared microbiota. All mice were housed and bred in the specific pathogen-free facility at University of Texas (UT) Southwestern according to protocols approved by the Institutional Animal Care and Use Committees of UT Southwestern Medical Center.

Cloning, Expression, and Purification of Recombinant RELM Family Proteins. cDNAs encoding mRELMβ, hRELMβ, and hRETN were PCR amplified from codon-optimized genes, using the primers listed in *Table S1* (full details are available in *SI Methods*). The expression and purification of the RELM proteins were based on a previously published protocol (33) and are detailed in *SI Methods*.

Assays for Bactericidal Activity. Bactericidal assays were performed as previously described (20). Briefly, purified proteins were added to logarithmic-phase bacteria and incubated for 2 h at 37 °C. Remaining live bacteria were quantified by dilution plating (*Table S2*). Surviving colonies were counted and calculated as a percentage of the colonies on the control plate.

Dye Uptake Assays. Midlogarithmic phase bacteria were diluted into assay buffer (10 mM Mes, pH 5.5, 25 mM NaCl) containing 5.5 μg/mL PI. Recombinant purified RELM proteins were added and fluorescence output was measured for 2 h using a Spectramax plate reader (Molecular Devices). Dye uptake was measured against the maximum fluorescence output from the positive control [0.05% (wt/vol) SDS].

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Assays for Lipid Binding and Liposome Disruption. Recombinant mRELMβ (1 mg/mL) was incubated with membrane lipid strips (Echelon) overnight at 4 °C, followed by washing and detection with rabbit anti-RELMβ antibody (raised against the purified recombinant mRELMβ). Liposome disruption assays were performed as previously described (15). The mRELMβ N-terminal peptide (QCSFESLVDQRIKEALSRQE) was synthesized by the Protein Chemistry Core at UT Southwestern and purified by HPLC. FRET assays were performed as previously described (15) on liposomes composed of 80% PC, 15% PS, and 5% dansyl-PE.

Real-Time Q-PCR. RNA was isolated from tissue using the RNeasy Midi kit (Qiagen), and cDNA was synthesized using the MMLV kit (Thermo Fisher). Q-PCR analysis was performed using SYBR Green master mix (Thermo Fisher). Primer sequences are listed in Table S3, and gene expression was normalized to 18S rRNA.

16S rRNA Sequencing. Fecal and tissue DNAs were extracted as described (6). Two micrograms of DNA were amplified using primers specific for the 16S rRNA sequence (forward, 5′-AGAGTTTGATCMTGGCTCAG-3′, and reverse, 5′- CGGTTACCTTGTTACGACTT-3′) (6), yielding an amplicon that encompassed the entire 16S rRNA sequence (~1,450 bp). Amplification reactions were carried out with the HotStarTaq polymerase kit (Qiagen) and then diluted 1:10 into H_2O. The diluted DNA samples were then analyzed by Q-PCR using the SYBR Green kit

(Thermo Fisher) and the primers found in Table S4. PCRs were quantified using standard curves generated from template controls for each primer set.

Immunofluorescence Detection and Electron Microscopy. Segments of unflushed colons from each mouse were fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) for at least 4 h at room temperature and further prepared as described in SI Methods. Tissues were detected with *Ulex europaeus* agglutinin I (EY Labs) or antibodies against lipoteichoic acid (Thermo Fisher) and *Helicobacter* species (Abcam) and imaged by fluorescence microscopy. mRELMβ-treated *Pseudomonas aeruginosa* was prepared for transmission electron microscopy as described in SI Methods.

Statistical Analysis. All statistical analyses were performed using two-tailed Student's *t* test. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; and ns, $P > 0.05$.

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