

Angiogenins: a new class of microbicidal proteins involved in innate immunity

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Although angiogenins have been implicated in tumor-associated angiogenesis, their normal physiologic function remains unclear. We show that a previously uncharacterized angiogenin, Ang4, is produced by mouse Paneth cells, is secreted into the gut lumen and has bactericidal activity against intestinal microbes. Ang4 expression is induced by *Bacteroides thetaiotaomicron*, a predominant member of the gut microflora, revealing a mechanism whereby intestinal commensal bacteria influence gut microbial ecology and shape innate immunity. Furthermore, mouse Ang1 and human angiogenin, circulating proteins induced during inflammation, exhibit microbicidal activity against systemic bacterial and fungal pathogens, suggesting that they contribute to systemic responses to infection. These results establish angiogenins as a family of endogenous antimicrobial proteins.

Human angiogenin (ANG) was first isolated nearly two decades ago based on its ability to stimulate vasculogenesis in the chick chorioallantoic membrane assay¹. Although implicated in the growth of human tumor cells in mice², ANG is also produced under nonpathologic conditions³. Furthermore, the pattern of angiogenin gene expression is not temporally correlated with vascular development⁴, suggesting that its primary function *in vivo* may be related to processes other than vascular growth.

Several observations indicate that angiogenins may function in host defense. Inflammation provokes a rise in ANG mRNA expression in the liver⁵ and ANG protein concentrations increase in serum during the acute phase response⁵. In addition, angiogenins have undergone the type of rapid sequence divergence commonly encountered in genes involved in host defense. Such high rates of mutation are thought to be driven by selective pressures from microorganisms⁶. Orthologous ANG genes have diversified considerably even among primates⁶. Moreover, whereas humans, nonhuman primates and rats harbor a single ANG gene, cattle have two and mice have four⁷.

Mouse Ang genes are clustered together on chromosome 14 and encode proteins with 72–81% sequence identity⁷. Ang1 and Ang3 are angiogenic *in vitro*^{8,9}, whereas angiogenin-related protein (Angrp) lacks such activity⁸. Here, we show that Ang4, a family member whose cellular patterns of expression and biological activities were previously unknown, is a novel, species-selective, bactericidal gut protein regulated by components of the normal intestinal microflora. We also show that mouse Ang1 and human ANG exhibit bactericidal and fungicidal activities against known pathogens, suggesting that these angiogenins are important in systemic innate immunity.

Results

Ang4 identification, and tissue distribution

We previously established a simplified mouse model for characterizing the molecular foundations of commensal host-bacterial interactions in the gut. Germ-free NMRI mice were colonized with *Bacteroides*

thetaiotaomicron, a prominent and genetically manipulatable member of the normal mouse and human distal intestinal microflora¹⁰. High-density oligonucleotide-based microarrays (GeneChips) were then used to define the transcriptional responses of the distal small intestine to colonization with this Gram-negative anaerobe¹¹. One of the most prominent changes was an 11-fold increase in a transcript identified by a GeneChip probe set designed to detect Ang3 mRNA¹¹. However, sequencing of a cDNA clone derived from this transcript revealed that it encoded Ang4, which is 81% identical to Ang3 (see **Supplementary Figs. 1 and 2** online).

Because Ang4 mRNA represented a previously uncharacterized transcript, we first determined its tissue distribution in conventionally raised adult NMRI mice. Real-time quantitative reverse transcriptase PCR (qRT-PCR) assays, using primers designed to distinguish the four mouse Ang mRNAs, showed that Ang4 mRNA is restricted to the intestine (**Fig. 1a**). In contrast, Ang1 is expressed primarily in liver, and to a lesser extent in lung and pancreas. The highest levels of Ang3 mRNA occur in prostate and lung, whereas AngRP was not detected in any of the 15 tissues surveyed.

We next used laser capture microdissection (LCM) to establish the cellular localization of Ang4 expression within the small intestine. LCM allows recovery of specific cell types from tissue cryosections, thus preserving the influence of surrounding cells and the environment on gene expression in the harvested population¹². We used qRT-PCR to compare Ang4 mRNA expression in three microdissected cell populations: Paneth cells (specialized, granule-filled epithelial cells located at the base of small intestinal crypts); the remaining epithelium (including villus epithelium and transit amplifying cells descended from the crypt's multipotent stem cells); and mesenchymal cells captured from the villus core (**Fig. 1b**, inset). The purity of LCM Paneth cell, non-Paneth epithelial cell, and mesenchymal cell preparations was established by measuring the distribution of secretory phospholipase A₂ (*Pla2g2a*; **Fig. 1b**), keratin-8 (*Krt2-8*) and vimentin (*Vim*) mRNAs,

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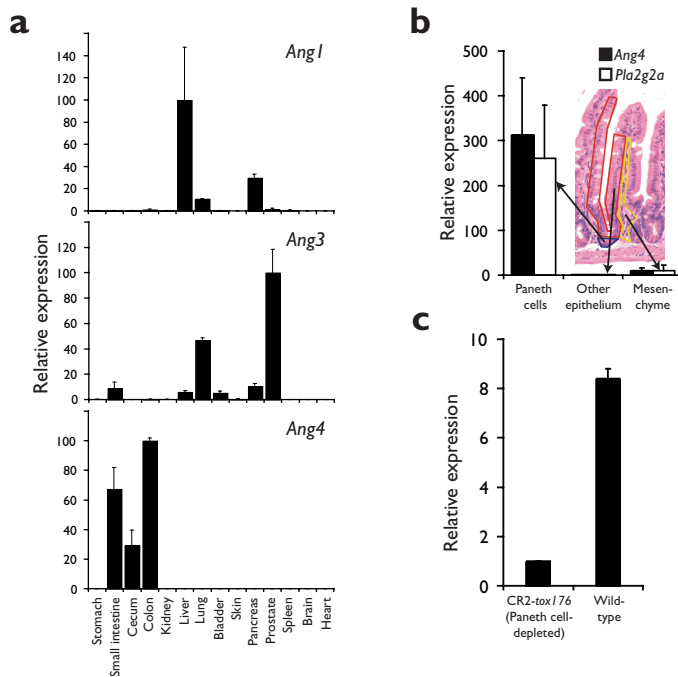


Figure 1. Tissue-specific patterns of expression of mouse angiogenin family members. (a) *Ang* mRNA expression was determined by qRT-PCR analysis of tissue RNAs, using primers that distinguish among *Ang* family members. *Ang* transcripts were normalized to those of glyceraldehyde 3-phosphate dehydrogenase mRNA and are expressed relative to the tissue showing the highest expression, which is set at 100. *Angrp* mRNA was not detected in any tissues tested. Mean \pm s.d. from triplicate determinations are plotted and are representative of results obtained from two adult mice. (b) *Ang4* mRNA expression in laser-capture microdissected small intestinal cell populations. qRT-PCR analysis was carried out on Paneth cells, the remaining crypt and villus epithelium ("other epithelium"), and mesenchyme (inset). Results are plotted as mean \pm s.d. of triplicate experiments, and are expressed relative to mRNA expression in "other epithelium" (arbitrarily set at 1). (c) Epithelial cells were harvested by laser-capture microdissection from the crypt bases from normal FVB/N mice and from age- and gender-matched Paneth cell-depleted CR2-tox176 transgenic mice. *Ang4* mRNA expression was determined by qRT-PCR assays of RNAs from microdissected cells.

respectively (data not shown). *Ang4* transcripts were enriched 310-fold in Paneth cells relative to the other small intestinal epithelial cell types, and 30-fold relative to the mesenchyme. This enrichment matched that of the Paneth-cell-specific *Pla2g2a* mRNA (Fig. 1b). Expression of the other three mouse *Ang* family members were undetectable by qRT-PCR in any of the LCM fractions.

We also compared *Ang4* mRNA expression in epithelial cells harvested by LCM from the bases of small intestinal crypts present in young adult FVB/N mice and in age- and gender-matched CR2-*tox176* transgenic littermates that have an approximately 95% ablation of their Paneth cell lineage due to expression of an attenuated diphtheria toxin A fragment (*tox176*)¹³. qRT-PCR showed that *Ang4* mRNA expression was nine-fold higher in normal epithelium compared with CR2-*tox176* crypt base epithelium (Fig. 1c), providing additional evidence for Paneth-cell-specific expression of *Ang4*.

Ang4 secretion from Paneth cells

Ang4 mRNA encodes a 144-residue protein with a predicted amino-terminal signal peptide, indicating that it is secreted. To gain insight into *Ang4*'s secretory destination, we used immunogold electron microscopy to localize *Ang4* within Paneth cells. Our results revealed that *Ang4* was targeted to dense core secretory granules (Fig. 2a). The degree of gold labeling of these granules was similar to that obtained using antibodies to lysozyme, an established Paneth cell granule protein (data not shown). Pre-treatment of the *Ang* antibody preparation

with purified recombinant *Ang4* blocked its reaction with Paneth cell granules (data not shown). Detection of *Ang4* in these granules is unlikely to represent cross-reaction with other *Ang* family members because neither *Ang1*, *Ang3*, nor *Angrp* mRNAs are detectable by qRT-PCR analysis of microdissected Paneth cells.

The localization of *Ang4* to Paneth cell secretory granules suggested that it is secreted into the intestinal lumen along with other secretory granule contents. Exposure to bacterial lipopolysaccharide (LPS) results in export of Paneth cell proteins into the intestinal lumen¹⁴. To determine whether bacterial LPS promotes secretion of *Ang4*, we used an *ex vivo* system in which pure populations of crypts are isolated by EDTA treatment of the small intestines of conventionally raised mice¹⁴. Immunoblot analysis of secreted proteins revealed that incubation of isolated crypts with bacterial LPS elicited the release of both *Ang4* and lysozyme (Fig. 2b). These results establish that bacterial products stimulate *Ang4* secretion from Paneth cells.

Microbicidal activities of angiogenins

Ang4, like other mouse and human angiogenins, is a member of the RNase superfamily¹⁵. In humans, two members of the RNase family, eosinophil-derived neurotoxin and eosinophil cationic protein, are localized to eosinophil secretory granules and exhibit antibacterial and antiviral activities^{16,17}. The localization of *Ang4* to Paneth cell secretory granules and its export in response to bacterial signals are consistent with a role in Paneth-cell-based innate immunity. To test this hypothe-

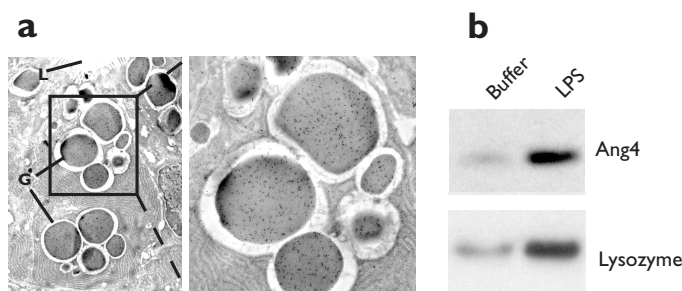
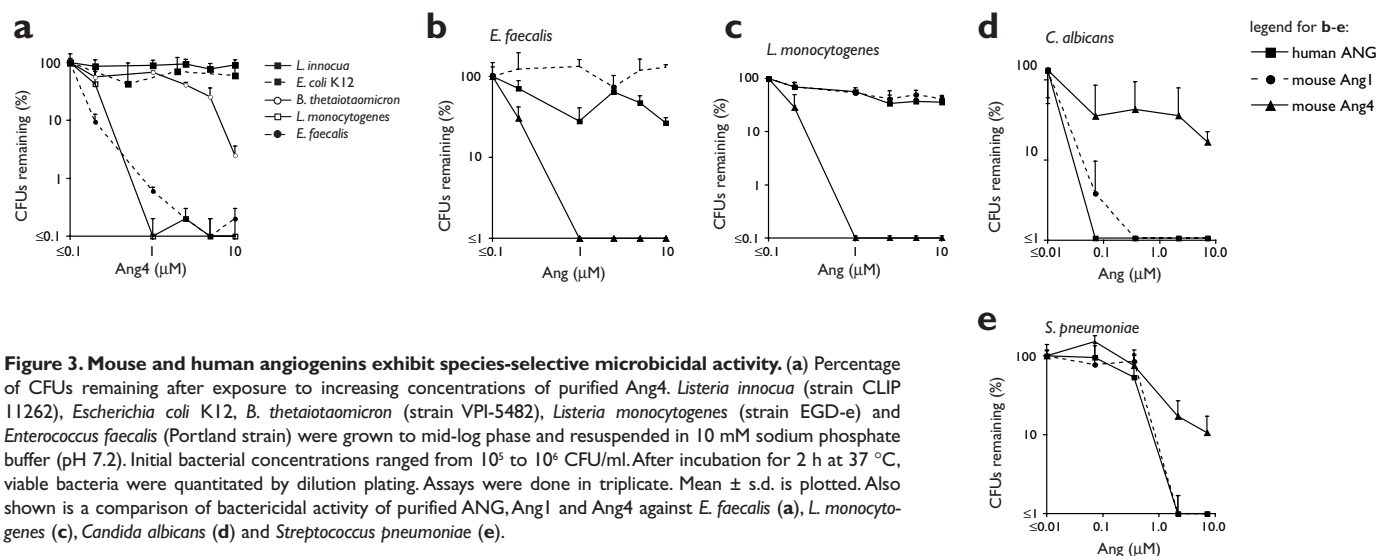


Figure 2. *Ang4* localizes to Paneth cell secretory granules and is secreted in response to LPS. (a) Electron micrograph of mouse Paneth cell granules after immunostaining with rabbit anti-*Ang4* and gold-labeled goat anti-rabbit IgG. G, granule; L, gut lumen. Magnification, $\times 3,300$ and $\times 10,000$ in the left and right panels, respectively. (b) LPS-stimulated secretion of *Ang4* from isolated small intestinal crypts. Approximately 500 crypts were resuspended in isotonic PIPES buffer¹⁴ and incubated for 30 min at 37 °C with purified *Salmonella typhimurium* LPS or with buffer alone. Supernatants were analyzed by immunoblot analysis using antibodies to *Ang* or lysozyme. Results are representative of three independent experiments.



sis, we assayed purified Ang4 for bactericidal activity against several Gram-positive and Gram-negative enteric microbes. The number of colony forming units (CFU) of log-phase *Enterococcus faecalis* or *Listeria monocytogenes* (both Gram-positive pathogens) declined by >99% after a 2 h exposure to 1 μ M of Ang4 (Fig. 3a). The intestinal commensal *B. thetaiotaomicron* was less sensitive: the number of viable organisms was reduced only 30% with 1 μ M of Ang4. Another Gram-negative commensal, *Escherichia coli* K12, was resistant even to 10 μ M Ang4 (Fig. 3a). Despite its close genetic relationship to *L. monocytogenes*¹⁸, *Listeria innocua* was resistant to Ang4 at concentrations of up to 10 μ M, emphasizing that species-specific features mediate susceptibility or resistance to this host-derived, antimicrobial protein. Approximately equivalent amounts of immunoreactive lysozyme and Ang4 were detected in Paneth cell secretory granules (data not shown). Assuming that Ang4 is secreted into the crypt lumen *in vivo* in amounts similar to those of other Paneth cell granule proteins^{14,19}, its estimated concentration in the crypt (>1 mM) would be 1,000 times greater than that required to kill *L. monocytogenes* or *E. faecalis* (Fig. 3a-c).

Because Ang1 and ANG appear in the circulation during the acute phase response to infection⁵, we examined whether these angiogenins function as mediators of systemic innate responses to infection. In

contrast to the potent bactericidal activity exhibited by Ang4 towards *E. faecalis* and *L. monocytogenes*, both bacterial species were resistant to Ang1 and ANG (Fig. 3b,c). However, two organisms that cause systemic infections in humans were sensitive to Ang1 and ANG. *Candida albicans*, an opportunistic fungal pathogen, exhibited a 97% and >99% decline in CFU at 2 h after exposure to 70 nM of Ang1 and ANG, respectively (Fig. 3d)—a concentration comparable to the concentrations of these proteins in serum⁵. Likewise, 2 μ M of Ang1 or ANG produced a >99% decline in the viability of *Streptococcus pneumoniae*, the Gram-positive pathogen that commonly causes pneumonia and sepsis (Fig. 3e). Both pathogens were considerably less sensitive to Ang4. Thus, Ang1 and ANG, which share 77% amino acid sequence identity, likely represent previously unappreciated microbicidal components of systemic innate host defense.

The salt sensitivity of these purified angiogenins is identical to that reported for other well-characterized microbicidal proteins, including human β -defensin²⁰ and α -defensin family members²¹. The antimicrobial activities of ANG, Ang1 and Ang4 were unaffected by NaCl concentrations up to 50 mM, but progressively declined as concentrations were increased from 75 to 150 mM (<1% at 150 mM; data not shown). Serum proteins, such as serpins²² and complement components²³, inhib-

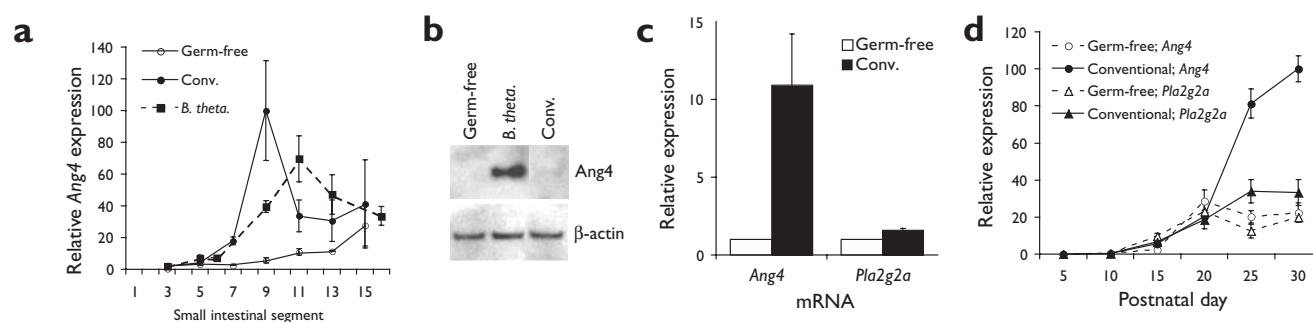


Figure 4. (a) Line graph showing relative Ang4 expression (y-axis, 0-140) across small intestinal segments (x-axis, 1-15) for Germ-free (open circles), Conv. (filled circles), and *B. theta.* (filled squares) conditions. (b) Immunoblot showing Ang4 and β -actin protein levels in Germ-free, *B. theta.*, and Conv. mice. (c) Bar graph showing relative expression of Ang4 and *Pla2g2a* mRNA (y-axis, 0-15) in Germ-free (white bars) and Conv. (black bars) mice. (d) Line graph showing relative expression of Ang4 and *Pla2g2a* mRNA (y-axis, 0-120) over postnatal days (x-axis, 5-30) for Germ-free and Conventional mice, with and without Ang4 or *Pla2g2a* treatment.

it the antibacterial and cytotoxic effects of defensins. Ang1 and ANG were also inhibited by serum (data not shown). Like defensins, ANG is cytotoxic²⁴. These findings suggest that the antimicrobial activities of ANG and Ang1 are modulated by local environmental conditions encountered at sites of infection.

Microbial induction of Ang4 expression

Several observations demonstrate that *Ang4* expression is regulated *in vivo* by components of the intestinal microbiota. A 10-day colonization of adult germ-free NMRI mice with an unfractionated microflora, harvested from the intestines of conventionally raised mice, elicited an increase in *Ang4* mRNA concentrations throughout the small bowel (Fig. 4a). This response was recapitulated by colonization with *B. thetaiotaomicron* alone, indicating that *Ang4* expression is enhanced by at least one normal member of the microflora (Fig. 4a). Immunoblot analysis revealed a corresponding increase in Ang4 protein in *B. thetaiotaomicron*-colonized intestines (Fig. 4b). This increase in Ang4 protein was not detected in the intestine after colonization of germ-free mice with a microflora harvested from conventionally raised animals (defined as "conventionalization"; Fig. 4b). This difference may be due to translational or post-translational regulation of Ang4 expression. Alternatively, conventionalization (but not *B. thetaiotaomicron* monoassociation) may enhance de-granulation of Paneth cells, leading to reduced amounts of stored protein in small intestines from conventionalized animals. We also detected bacterial induction of *Ang4* mRNA in laser-capture microdissected Paneth cells. qRT-PCR analysis revealed an increase in *Ang4* mRNA expression in Paneth cells isolated from conventionalized compared with germ-free mice (Fig. 4c). In contrast, *Pla2g2a* mRNA concentrations were unaffected (Fig. 4c), indicating that the microbe-dependent increase in *Ang4* expression is specific and not merely part of a generalized induction of genes encoding Paneth cell secretory granule proteins.

We obtained further evidence for microbial regulation of *Ang4* expression by comparing the postnatal developmental patterns of *Ang4* expression in mice with and without a microflora. *Ang4* mRNA expression rose in the small intestines of conventionally raised but not germ-free NMRI mice during the weaning period (postnatal days 17–28; Fig. 4d). This suggests that the presence of gut bacteria is responsible for driving higher *Ang4* expression as animals advance to adulthood. This effect is not attributable to a generalized enhancement of Paneth cell secretory granule components in colonized mice, because expression of *Pla2g2a* mRNA increased only slightly in germ-free and conventionally raised mice (Fig. 4d). Weaning is associated with a dramatic change in the composition of the gut microflora, as the abundance of Gram-positive and Gram-negative facultative anaerobes declines and Gram-negative obligate anaerobes become predominant²⁵. Microbial regulation of *Ang4* expression during weaning suggests that this protein may be a host factor that helps shape the composition of the adult gut microflora.

Discussion

We have identified a new class of endogenous microbicidal proteins. Our results indicate that Ang4 is a mediator of epithelial host defense in the intestine. Two other members of this family, Ang1 and ANG, likely constitute previously unappreciated components of systemic defenses. Furthermore, these findings provide an explanation for why mice have evolved four angiogenins. The species-selective microbicidal activities and differential expression patterns of Ang family members suggest that diversification of these genes was driven by selective pressures exerted on the host by different microbes in different host contexts.

The induction of *Ang4* by commensal bacteria is a property that distinguishes it from genes encoding other intestinal microbicidal proteins. The small intestine expresses multiple members of the defensin family, primarily in Paneth cells. Expression of defensin mRNA is similar in the intestines of germ-free and conventionally raised mice²⁶. In addition, our previous DNA microarray analyses revealed no enhancement in intestinal defensin mRNA expression after *B. thetaiotaomicron* colonization of germ-free mice¹¹.

Microbial regulation of a secreted Paneth cell protein with selective bactericidal activity represents a mechanism whereby the intestine's commensal bacteria can shape the composition of their own evolving microbial community during postnatal development, as well as contribute to innate immunity and mucosal barrier function in adults. The intestine exhibits a remarkable ability to accommodate a complex society of indigenous microbes without mounting an inflammatory response²⁷. By inhibiting access of microbes to the gut epithelium, Ang4 should help limit activation of inflammatory responses. This may be particularly important in the crypt, where protecting the stem cell niche is critical. Furthermore, by inducing and sustaining expression of mediators of innate defense, such as Ang4, commensal bacteria may be critical for the development of an adequate mucosal barrier, helping to ensure rapid and efficient deployment of these mediators against enteropathogens. Disrupted or dysregulated host-commensal relationships may thus undermine microbial contributions to mucosal barrier integrity. This may, in turn, lead to enteric infections and contribute to the pathogenesis of disorders such as inflammatory bowel diseases, where there is a loss of tolerance to gut commensals.

Methods

Ang4 cloning. A 438-bp amplicon was generated by RT-PCR using small intestinal cDNA and primers specific for the 3' and 5' ends of the ORF in *Ang3* mRNA (5'-CCTTGGATC CATGGTATGAGCCCCAGGTTCTTTG-3' and 5'-CCTTCTAGACTACGGACTGATAA AAGACTCATCGAAG-3', respectively). The 3' primer incorporated a 5' *Bam*HI site and the 5' primer included a 5' *Xba*I site. The amplicon was subcloned into *Bam*HI/*Xba*I-digested pGEX-KG²⁸. The SmartRACE kit (Clontech, Palo Alto, CA) was used to obtain sequence data from the 5' and 3' regions of *Ang4* mRNA (using primers 5'-GCCAGGGAGACC CTCTTGA-3' and 5'-CTCTGGCTCAGAATGTAAGGTACGA-3', respectively). *Ang3* and *Ang4* cDNA and protein sequences are compared in Supplementary Figs. 1 and 2 online, respectively.

qRT-PCR. SYBR Green-based qRT-PCR was carried out as described¹¹ using gene-specific primers (glyceraldehyde 3-phosphate, 5'-TGGCAAAGTGGAGATTGTTGCC-3' and 5'-AAGATGGTATGGGGCTTCCCG-3'; *Ang1*, 5'-AGCGAATGGAAGCCCTTACA-3' and 5'-CTCATCGAAGTGGACCGCA-3'; *Ang3*, 5'-CTGGCTCAGGATAACTACAGGT ACAT-3' and 5'-GCCTGGGAGACCCTCTTT-3'; *Ang4*, 5'-CTCTGGCTCAGAATG TAAGGTACGA-3' and 5'-GAAATCTTAAAGGCTCGGTACCC-3'; *Pla2g2a*, 5'-CCAAATCACCTGTTCTGCAAAC-3' and 5'-CAITTCAGCGCGGCTTTA-3'; *Vim*, 5'-TGCTTCTGGCAGCTCTTG-3' and 5'-GGACATGCTGTTCGTAATCTG-3'; *Krt2-8*, 5'-GCTGAAGTTCGTGCCAGTAC-3' and 5'-CTTTGTGCGGCGCAGAT-3'). RT-PCR of *Angpr* mRNA used two primer sets: 5'-GATACTGCCAAAGTATGATGGT-3' and 5'-AGACTTGTCTATTCTAAATTTCG-3'; and 5'-AGAAAGGAAGCCCTTATGGACG-3' and 5'-CCAGCCATTCTCACAGCCAATAAT-3'.

Electron microscopic immunohistochemistry. Small intestines from conventionally raised 6-week-old FVB/N mice were flushed with PBS and fixed for 2 h at 4 °C in a solution containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.1 M cacodylate, pH 7.4. Samples were embedded in K4M Lowicryl (Polysciences, Warrington, PA) and 80-nm sections were cut. Sections were placed on formvar-coated nickel grids and stabilized with carbon deposition. Unreacted aldehydes were removed with a 10-min incubation in Tris-buffered saline (TBS; 0.02 M Tris, 0.2 M NaCl, pH 7.6) containing 50 mM glycine. Grids were pretreated for 1 h in blocking buffer (10% normal mouse serum, 0.05% Tween-20 in TBS), followed by an overnight incubation at 4 °C with rabbit anti-Ang4 (generated as described below; diluted 1:500 in blocking buffer). Control sections were incubated with pre-immune serum, with Ang antibodies that had been pre-incubated with purified recombinant mouse Ang4, or with rabbit antibodies to lysozyme (Dako, Carpinteria, CA; diluted 1:200). Goat anti-rabbit IgG conjugated to 18-nm diameter gold particles (Jackson ImmunoResearch, West Grove, PA; 1:30 dilution) was added. Antibodies were cross-linked with 2% glutaraldehyde, sections were stained with uranyl acetate and Reynolds lead citrate, and subsequently viewed with a JEM100C transmission electron microscope (JEOL, Peabody, MA).

Purification of mouse and human angiogenins. ORFs encoding mouse Ang1, mouse Ang4 and human ANG were amplified by RT-PCR using cDNAs from adult mouse liver, adult mouse mid-small intestine (jejunum) and human small intestine, respectively, plus gene-specific primers (*Ang4*, 5'-GGGAATTCATATGCAGAATGAAAGGTAC-GAAAAATTCCTAC-3' and 5'-CCTTGGATCCTACGGACTGATAAAGACTCATCG-3'; *Ang1*, 5'-GGGAATTCATATGCAGGATGACTCCAGGTACACAAAATT-3' and 5'-CCTTGGATCCTATAGACTGAAAAATGACTCATCGAAG-3'; *ANG*, 5'-GGGAATTCATATGCAGGATAACTCCAGGTACACACACTT-3' and 5'-CCTTGGATCCTACGGACTGACGAGAAAATTGACTGA-3'). The resulting amplicons contained a Met codon in place of the signal peptide sequence, and incorporated 5' *Nde*I and 3' *Bam*HI sites. PCR products were digested with *Nde*I and *Bam*HI, cloned into *Nde*I/*Bam*HI-digested pET3a (Invitrogen, Carlsbad, CA) and sequenced. The recombinant plasmids were then introduced into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA). Protein expression was induced in mid-log phase cultures with 0.5 mM isopropylthiogalactoside using a protocol developed by the manufacturer. Cells were subsequently harvested by centrifugation (6,500g), dispersed in 0.1 volume IB buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100) containing 100 µg/ml lysozyme, and disrupted by sonication. Inclusion bodies were collected by centrifugation at 10,000g, washed twice in IB buffer and solubilized in 7 M guanidine-HCl, 0.15 M reduced glutathione, 0.1 M Tris-HCl, pH 8, and 2 mM EDTA. Angiogenins were refolded and then purified by cation-exchange chromatography on SP-Sepharose (Sigma, St. Louis, MO)¹⁵. The purity of each protein preparation was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by N-terminal sequencing. RNase activity²⁹ was measured to determine that proteins had refolded properly. Angiogenins were dialyzed against 10 mM sodium phosphate, pH 7.2, before bactericidal assays. Antibodies to purified Ang4 were generated in rabbits.

Accession number. The genbank accession number for Ang4 cDNA is AY219870.

Note: Supplementary information is available on the Nature Immunology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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