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## Defending the Fort: A Role for Defensin-2 in Limiting *Rickettsia montanensis* Infection of *Dermacentor variabilis*

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### Abstract

The importance of tick defensins is evidenced by their expression in a wide variety of tick tissues and prevalence across many tick genera. To date, the functional and biological significance of defensin-2 as a rickettsiastatic or rickettsiacidal antimicrobial peptide has not been addressed. In a previous study, defensin-2 transcription increased in *Dermacentor variabilis* ticks challenged with *Rickettsia montanensis*. In this study, the hypothesis that defensin-2 is functional as a rickettsiastatic and/or rickettsiacidal antimicrobial peptide is tested. We show that defensin-2 plays a role in reducing burden after acquisition of *Rickettsia montanensis* through capillary feeding. Moreover, defensin-2 associates with *R. montanensis* *in vitro* and *in vivo* causing cytoplasmic leakiness.

### Introduction

Ticks encounter and harbor many foreign microbes during their lifetime. The tick innate immune system has likely contributed significantly to their success as hosts and vectors. The tick immune system is characterized as having both cell-mediated and humoral defense components (Kopacek et al., 2010). Cell-mediated defense involves phagocytosis and encapsulation of foreign microbes that infiltrate the hemocoel (Ceraul et al., 2002; Eggenberger et al., 1990) The humoral arm of the defense response is driven by soluble antimicrobial peptides (AMPs) that are presumably secreted from the hemocytes and fat body into the hemocoel or from the midgut epithelium. AMPs can attack invading microbes through opsonization, agglutination, and/or lysing (Kopacek et al., 2010).

The tick midgut is the first point of contact for rickettsiae imbibed with a bloodmeal from an infected mammal. As the American dog tick, *D. variabilis*, imbibes a rickettsia-infected blood meal, genes encoding a number of AMPs and bacteriostatic factors are activated for

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transcription and translation, including defensin and *D. variabilis* kunitz-type serine protease inhibitor (Ceraul et al., 2011; Ceraul et al., 2007; Ceraul et al., 2008). Defensins are a ubiquitous class of AMPs that are active against Gram-positive and Gram-negative bacteria, fungi, virus, and protozoans (Dugan et al., 2008; Kopacek et al., 2010; Smith and Nemerow, 2008; Tanaka et al., 2012). Tick defensins are 4 – 6.5 kDa cationic and anionic peptides with a secondary structure comprised of alpha helical and beta sheet structures that form a tertiary structure by way of three disulphide bonds (Kopacek et al., 2010; Wang and Zhu, 2011). Tick defensins also possess a  $\gamma$ -core within the C-terminus (Wang and Zhu, 2011). The  $\gamma$ -core is a feature common to defensins regardless of species and is responsible for their antimicrobial activities (Wang and Zhu, 2011; Yount and Yeaman, 2004). The cationic charge, amphipathicity, and  $\gamma$ -core are shown to mediate attraction, attachment, and membrane insertion that facilitate the toroidal pore, barrel-stave or carpet mechanisms of pore-forming action by AMPs like defensin (Brogden, 2005; Wang and Zhu, 2011; Yount and Yeaman, 2004).

Ticks are second only to dipterans in their importance as vectors of pathogens in humans and are the number one vector of pathogens of veterinary importance (Parola et al., 2005). Some of the most detrimental human tick-borne pathogens are members of the spotted fever group rickettsiae (SFG). SFG rickettsiae are obligate intracellular bacteria that replicate in the cytoplasm and nucleus of host cells and are transmitted by ticks. Although experimental studies suggest that there are some lethal effects associated with infection in *D. variabilis* by the etiologic agent of Rocky Mountain spotted fever, *R. rickettsii*, it is successfully acquired, maintained, and transmitted both horizontally and vertically in nature (Niebylski et al., 1999). These observations suggest that even if ticks suffer long-term morbidity as a result of infection, they can control rickettsial infections long enough to transmit the pathogens thereby perpetuating the zoonotic cycle.

Previous studies show that challenge of *D. variabilis* with SFG *R. montanensis* results in activation of defensin-1 and -2 and lysozyme (Ceraul et al., 2007). We hypothesize that defensin-2 is functionally active against *R. montanensis*. Experiments demonstrate that *D. variabilis* defensin-2 associates with and causes cytoplasmic leakage of *R. montanensis*. Our results suggest that defensin-2 function is one factor that limits *R. montanensis* infection of the tick.

## Results

### Defensin limits *R. montanensis* infection *in vitro* and *in vivo*

Purified *R. montanensis* ( $1 \times 10^7$ ) incubated with recombinant defensin, thioredoxin, or PBS were placed on L929 cells, allowed to infect, and collected 24 hours later. Rickettsial burden was assessed with qRT-PCR. There was no statistical difference between the two control treatments. The average burden in the recombinant defensin-2-treated *R. montanensis* decreased 86-percent when compared to PBS-treated rickettsia ( $p < 0.017$ ) (Figure 1A). To lend biological significance to our *in vitro* results, a defensin-2 neutralization experiment was performed. *D. variabilis* ticks were capillary fed anti-defensin-2 IgG at increasing concentrations or preimmune IgG at 2 mg/ml, incubated for 1 hour, and fed  $2.4 \times 10^5$  total *R. montanensis*. Whole tissues were harvested 24 hours post-infection for RNA isolation.

Rickettsial burden rose in direct proportion to the increasing concentrations of anti-defensin-2 IgG. Rickettsial burden in ticks fed 1.0 mg/ml and 2.0 mg/ml anti-defensin-2 IgG increased significantly above that observed in the ticks treated with 2 mg/ml preimmune IgG ( $p=0.004$  and  $p=0.043$ , respectively) (Figure 1B). Collectively, the *in vivo* and *in vitro* burden assays, demonstrate that the control of *R. montanensis* infection seen is specific to the defensin-2 response. Interestingly, the *in vivo* and *in vitro* burden assays, respectively, demonstrate that the growth limiting effects of defensin-2 for *R. montanensis* are specific.

### Incubation of *R. montanensis* with defensin causes cytoplasmic leakage

The most recognized function of defensins is pore formation. To address this, an *in vitro* lysis assay was performed and the data correlated to the live count data from the same experiments. An Imperial Blue stained polyacrylamide gel indicated that the recombinant defensin-2 and thioredoxin proteins were relatively pure (Figure 1C). Purified *R. montanensis* were incubated with defensin-2 or thioredoxin with agitation and the mixtures were separated through high-speed centrifugation into pellet and supernatant fractions. In addition, the lysis assay was performed using a synthetic defensin-2 peptide in increasing concentrations. The number of rickettsia/mm<sup>3</sup> were counted using a small aliquot from each rickettsia sample incubated with the synthetic peptide. The same samples were used to blot for cytoplasmic leakage to correlate lysis with a decrease in live rickettsia and increasing concentrations of synthetic peptide. We theorized that perforations in the cytoplasmic membrane of *R. montanensis* as a result of defensin-2 treatment would cause the leakage of a cytoplasmic protein EF-Ts (Elongation Factor-Thermo stable) into the supernatant.

*R. montanensis* EF-Ts was first expressed in *E. coli* to determine the level of cross-reactivity of our antiserum generated against *R. typhi* cytoplasmic EF-Ts (Figure 2A). A higher weight molecular host protein is known to cross-react with our EF-Ts antiserum. The higher molecular weight band was shown previously to be of host origin (Kaur et al., 2012). To support these previous observations, a lysate sample from uninfected L929 cells was subjected to western blotting to demonstrate that EF-Ts antiserum cross-reacts with a host protein that migrates around 40 kDa (Figure 2B). Thioredoxin-treated *R. montanensis* showed a strong EF-Ts band for the pellet, but not for the supernatant, while recombinant defensin-2-treated *R. montanensis* showed an EF-Ts band in both the pellet and supernatant lanes (Figure 2C). These results are consistent between the two independent experiments performed. Similar results were documented for synthetic peptide-treated *R. montanensis* (Figure 2D). The samples shown in Figure 2D were subjected to longer electrophoretic runs to attain better separation between the host protein and rickettsial EF-Ts, which accounts for EF-Ts migration to approximately 35 kDa.

The number of live rickettsia in the samples used to demonstrate lysis post-treatment with synthetic defensin was estimated using Live/Dead BacLight Viability kit. The BacLight assay allows us to estimate the number of live rickettsia and correlate this number to the appearance of EF-Ts in the supernatant fractions from the lysis assay (cytoplasmic leakage). The numbers of live *R. montanensis* decrease with increasing concentrations of synthetic defensin-2 peptide tested (Figure 2E). We note that defensin-2 made either as a recombinant or synthetic peptide show the same trend. This indicates that our results are not due to a)

contaminants in the recombinant defensin-2 preparation or b) an artifact of the synthetic peptide manufacturing process. Collectively, these results demonstrate that defensin-2 treatment causes leakage of cytoplasmic proteins from *R. montanensis*, which ultimately leads to bacterial death.

### Defensin-2 associates with *R. montanensis* *in vitro* and *in vivo*

*R. montanensis* ( $1 \times 10^7$ ) were incubated with recombinant defensin-2 for 30 minutes and then affixed to nickel grids. Rickettsiae were incubated with rabbit defensin-2 antiserum followed by incubation with 10 nm gold particle-conjugated anti-rabbit IgG. Grids were visualized with TEM to determine if defensin-2 associates with the rickettsia (Figure 3). The immunogold staining observed in the defensin-2 treated, preimmune probed (Figure 3A) control is random and unorganized. Surface adhesion of the gold particles suggests that defensin-2 associates with the outer membrane of *R. montanensis* *in vitro* (Figure 3B).

*In vivo* staining for defensin-2 and *R. montanensis* was performed to determine if staining colocalizes to the same area in an attempt to lend biological significance to our *in vitro* findings. Infected *D. variabilis* midguts were sectioned on a cryo-microtome and incubated with rabbit defensin-2 and mouse *R. montanensis* antisera followed by incubations with Alexa Fluor 488 (defensin) anti-rabbit and Alexa Fluor 594 (rickettsia) anti-mouse sera. Sections were treated with SYTOX Blue nucleic acid stain and visualized on a Zeiss LSM 510 confocal microscope. In order for defensin-2 to be functional it needs to associate with the rickettsia. Defensin-2 staining appears to be localized with *R. montanensis* signal (Figure 4 A). We observe diffuse staining in sections from *R. montanensis*-infected tick midguts probed with normal rabbit and mouse control sera (Figure 4 B). *R. montanensis* staining in control sera probed sections is absent (Figure B).

## Discussion

Tick defensins belong to a class known as ancient invertebrate-type defensins (AITDs) that are found in insects, arachnids, bivalvia and fungi (Wang and Zhu, 2011). Defensins from several genera possess antimicrobial activity for Gram-positive and Gram-negative bacteria, protozoa and fungi (Chrudimska et al., 2011; Lu et al., 2009; Tsuji et al., 2007; Wang and Zhu, 2011). In this study, we demonstrate that defensin-2 associates with *R. montanensis*, causing cytoplasmic leakage. We speculate that these antimicrobial properties of defensin-2 are responsible, in part, for the antimicrobial effect observed *in vitro* and *in vivo*.

Although cationic charge is one property that mediates the association between the bacterium and defensin (Brogden, 2005; Ganz, 2003), defensin-2, a weak anionic peptide (pI = 5.14; [http://web.expasy.org/cgi-bin/compute\\_pi/pi\\_tool](http://web.expasy.org/cgi-bin/compute_pi/pi_tool)) associates with rickettsia. This study does not explore the mechanism underlying defensin-2 association with rickettsia. Our findings present the possibility that defensin-2 interacts with ligands alternative to LPS or that the LPS structure of *R. montanensis* is different from reported Gram-negative bacteria with respect to charge and facilitates the interaction with a weak anionic protein. However, charge does not appear to be the sole factor dictating the functional capacity of AMPs. Both cationic and anionic derivatives of dermcidin-derived peptides from eccrine sweat glands in humans possess broad range antimicrobial activity (Steffen et al., 2006). Interestingly,

Steffen, H et al (2006) demonstrate that the dermcidin derivative, DCL-1 (pI = 5.07) requires 2 hours before *E. coli* viability decreases 100% compared to the positive control peptide LL-37, which attains 100% killing in minutes. In designing experiments for this study, the effects that extended extracellular periods of time have on the viability of an obligate intracellular bacterium were considered. However, it is feasible that anionic peptides function at slower rates and that greater loss in rickettsial viability will be attained when exposed to defensin-2 for a time period exceeding 30 minutes.

After the initial attraction and attachment, AMPs are theorized to assume their folded secondary structure (Manzo et al., 2012; Marcellini et al., 2009; Matsuzaki, 1999; Rahman et al., 2009). A transmembrane pore is thought to form through three mechanisms and appears to be peptide concentration dependent. All mechanisms initiate with a parallel arrangement of the AMP at the surface of the phospholipid membrane. The barrel-stave model dictates that high peptide:lipid ratios encourage insertion of AMP into the membrane (Brogden, 2005). It is thought that the carpet mechanism works through peptide-mediated formation of micelles and that the toroidal pore model is a function of peptide-induced membrane bending followed by pore formation (Brogden, 2005). Clues about the mechanism driving pore formation by defensin-2 (anionic) may come from data for the anionic peptide DCL-1 (net charge of -2). Recent data proposes that DCL-1 folds into an  $\alpha$ -helical structure in the presence of bacterial membranes, self associates in a time-dependent manner, and may form pores through the toroidal pore mechanism (Paulmann et al., 2012). This series of events is also documented for a defensin-like peptide, Longicin from the hard tick, *Haemaphysalis longicornis*. Similar to DCL-1, Longicin remains unstructured in aqueous solution but assumes a secondary structure in membrane mimetic conditions (Rahman et al., 2009).

Our *in vitro* and *in vivo* antimicrobial assays lend support to the biological significance of defensin-2 as a rickettsiastatic or rickettsiacidal protein. We do not address defensin-2 function beyond its ability to limit rickettsial infection. However, the findings have implications for the role of defensin-2 in maintaining homeostatic commensal microflora populations. In *Drosophila*, antimicrobial bacterio-static /-cidal peptides play critical roles in maintaining homeostasis with microflora as well as fighting microbes that may harm the individual (Ryu et al., 2010). Endosymbionts and commensal microflora may be maintained at low levels in specific tissues to avoid detrimental physiological effects to the host (Login et al., 2011; Paredes et al., 2011).

It is unknown if activation of the midgut (local response) translates to a systemic response in the hemocoel or how this may impact colonization of hemocoelic tissues such as the salivary glands and ovaries. In *Drosophila*, peptidoglycan recognition protein-LB (PGRP-LB), is responsible for controlling midgut immune activation and participates in activation of the systemic response after an oral challenge with the Gram-negative bacterium *Erwinia carotovora carotovar-15* (Paredes et al., 2011). Considering this, midgut infection with rickettsia may prime hemocoelic immune effectors for an impending rickettsial invasion. In doing so, AMPs like defensin-2 will limit transmigration of rickettsiae to the hemocoel, ovaries and salivary glands resulting in less infection, which could have implications for transovarial or horizontal maintenance.

While this study does not address the physiochemical mechanism of pore formation for defensin-2, we have correlated loss of rickettsial viability with defensin-2 induced membrane damage that results in the leakage of cytoplasmic contents. Future experiments will address biochemical properties of defensin-2 as it relates to its antimicrobial function.

## Experimental Procedures

### Rickettsia and host cells

Mouse fibroblast cells (L929, ATCC® CCL-1) were cultured in DMEM supplemented with 10% FBS at 5% CO<sub>2</sub> at 34°C. Host cells were inoculated with *R. montanensis* at 80% confluency. Infected host cells were grown for 5 to 6 days. *R. montanensis* were purified from host cells and enumerated as described (Ceraul et al., 2008).

### Synthetic defensin-2 peptide

Six milligrams of synthetic defensin-2 (GenBank accession number: AAO18363.1) peptide was synthesized by Peptide 2.0, Inc. (Chantilly, Va) at > 96% purity. The signal sequence was identified using Signal P (<http://www.cbs.dtu.dk/services/SignalP/>) and excluded from synthesis. The final peptide sequence is NH<sub>2</sub>-TGERSEERSEEARASGCKADACKSYCKSLGSGGGYCDQGTWCVCN-COOH. The peptide was resuspended to 5 mM in acetonitrile:water (1:3) with 1% dimethylformamide (DMF) and 1% dimethylsulfoxide (DMSO). Rabbit anti-defensin-2 polyclonal serum was generated against a synthetic peptide with the sequence NH<sub>2</sub>-EEARASGCKADACK-OH by Biosynthesis, Inc. (Lewisville, TX). Biosynthesis, Inc. also provided the rabbit preimmune serum used in these experiments.

### Expression and purification of recombinant defensin-2

The ORF for tick defensin-2 (AAO18363) was amplified as previously described (Ceraul et al., 2007) using primers AZ3519 (5'-CTTTGCATCTGCCTTGTCTTTCTC-3') and AZ3518 (5'-AATTCCTGTAGCAGGTGCAGG-3') and Hi-Fidelity Polymerase (Life Technologies, Grand Island, NY), ligated to pET32a+ (Novagen Darmstadt, Germany) and transformed into either Origami (Novagen Darmstadt, Germany) or RosettaGami (Novagen) *Escherichia coli* strains. pET32+a was also transformed for expression of thioredoxin as a control protein. Thioredoxin was expressed and purified simultaneously with defensin-2. All procedures were performed according to the respective manufacturers' protocols. Expression of recombinant defensin-2 was induced using either IPTG or auto-induction medium composed of ZY broth supplemented with NPS (20 mM PO<sub>4</sub>, 1.25 mM SO<sub>4</sub>, 2.5 mM NH<sub>4</sub>, 20 mM Na, and 2.5 mM K), 5052 (0.5 g glycerol, 0.5 g glucose, and 0.2 g lactose) and 100 µg/ml ampicillin. Overnight cultures of transformed *E. coli* were diluted 50-fold in LB broth supplemented with 100 µg/ml ampicillin, grown to an OD<sub>600nm</sub> of 0.5 and either supplemented with 1 mM IPTG or diluted five-fold into auto-induction medium. *E. coli* induced with IPTG was grown at 37°C for three to four hours, centrifuged at top speed for 10 minutes at 4°C and the pellet stored at -80°C until lysed for purification. *E. coli* diluted in auto-induction medium was grown at 28–30°C overnight and the pellets collected and stored as described above. *E. coli* pellets were resuspended in 10 ml of NPI-10 (50 mM NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O, 300 mM NaCl, 10 mM imidazole) and lysed by pressure using a French



press (ThermoFisher Scientific, Waltham, MA) or LV1 low volume microfluidizer (Microfluidics, Newton, MA). Recombinant defensin-2 was purified using Ni-NTA agarose magnetic beads or Superflow columns according to the manufacturer's protocol (Qiagen, Valencia, CA). Each sample was electrophoresed on a 4–20% Bis-Tris gel (Life Technologies) and stained with Imperial Blue (ThermoScientific) to assess purity of each sample.

### Antibacterial assay

Separate aliquots of *R. montanensis* ( $1 \times 10^7$ ) designated for the lysis and antibacterial assays respectively were incubated with 5  $\mu\text{g}$  of recombinant defensin-2 or thioredoxin in a 30  $\mu\text{l}$  volume of PBS for 30 minutes at 30°C with shaking. *R. montanensis* was pelleted by centrifugation at top speed for 5 minutes. Thioredoxin and recombinant defensin-2-treated *R. montanensis* were washed with PBS (1X) and placed onto L929 host cells and allowed to incubate at 37°C/5% CO<sub>2</sub> for 24 hours. Infected cells were washed with PBS 3 times and processed for RNA isolation. Rickettsial burden was determined using qRT-PCR by normalizing rickettsial *16s* rRNA to host cell *gapdh* transcript (Ceraul et al., 2008). *16s* rRNA is amplified using AZ4915 (5'-GTTCGGAATTACTGGGCGTA-3') and AZ4916 (5'-AATTAACCGCATGCTCCAC-3'). *gapdh* was amplified using AZ3674 (5'-TCAACGACCCCTTCATTGAC-3') and AZ3675 (5'-ATGCAGGGATGATGTTCTGG-3').

### Lysis Assay

Separate aliquots of *R. montanensis* ( $1 \times 10^7$ ) were prepared as described for the antibacterial assay above. Alternatively, *R. montanensis* was treated with 8  $\mu\text{M}$ –1 mM synthetic defensin-2 or 0.9% NaCl buffer supplemented with the same volume of synthetic defensin diluent buffer (acetonitrile (1:3 ACN:water):1% DMF:1% DMSO) as a negative control buffer. The concentrations (8  $\mu\text{M}$ –1 mM) were obtained using serial dilution. The negative control buffer was diluted equivalently to the highest peptide concentration of 1 mM. *R. montanensis* was incubated with defensin or the negative control buffer for 30 minutes at room temperature. *R. montanensis* were pelleted by centrifugation at 16,000  $\times$  g for 5 minutes. The *R. montanensis* pellet was separated from the supernatant and both were resuspended in LDS (1X) sample buffer with reducing agent (1X). Samples were heated to 70°C for 10 minutes and separated on a 4–20% Bis-Tris gel (Life Technologies) and transferred to a PVDF membrane using an iBlot semi-dry transfer apparatus (Life Technologies). Membranes were probed with preimmune or immune sera directed to *R. typhi* cytoplasmic protein EF-Ts generated by Primm Biotechnologies (Cambridge, MA). To determine if the *R. typhi* anti-EF-Ts serum recognized EF-Ts from *R. montanensis*, the ORF for EF-Ts from *R. montanensis* was cloned into pET101D (Life Technologies) and expressed from BL-21 Star *E. coli* (Life Technologies) using IPTG induction. Pellets from two to four hour cultures were resuspended in LDS buffer, heated to 70°C for 10 minutes and separated on 4–20% Bis-Tris gels and transferred to PVDF membranes. Blots were probed with EF-Ts anti-serum to confirm serum cross-reactivity.

## Live Counts

A 10  $\mu$ l aliquot was removed from rickettsia incubated with 8  $\mu$ M-1 mM synthetic defensin-2. Rickettsia were centrifuged at  $16,000 \times g$  for 5 minutes at 4°C, the supernatant removed and the pellet resuspended in 100  $\mu$ l of 0.9% NaCl. A 200-fold dilution was performed in 0.9% NaCl and the sample was counted on an iN CYTO C-Chip Improved Neubauer hemocytometer (Chungcheongnam-do, Korea) using a Nikon Eclipse E600 (Melville, NY). Each experiment was performed in duplicate and a total of four experiments were completed. Values are reported as the average with the standard deviation. Statistical analysis was performed as described below.

## Defensin-2-*R. montanensis* association assay negative staining electron microscopy of defensin-2 on rickettsia

Purified *R. montanensis* ( $1 \times 10^7$ ) were washed twice with PBS, resuspended in PBS and 5–10  $\mu$ g of recombinant defensin-2 or thioredoxin, and incubated at 30°C for 30 minutes with shaking. After incubation, *R. montanensis* were washed with PBS and fixed with 4F1G fixative (4% formaldehyde, 1% glutaraldehyde in 0.5 M NaH<sub>2</sub> PO<sub>4</sub> Buffer-pH 7.2). After an hour fixation on ice, cells were washed and resuspended in HEPES buffer. Following resuspension, 10  $\mu$ l of *R. montanensis* were spotted on nickel grids and allowed to air dry before an additional 10  $\mu$ l were spotted onto the grids. A total of  $1 \times 10^7$  rickettsia were spotted onto each grid. Grids were blocked with 5% BSA + 0.1% Cold Water Fish Skin gelatin for 15 minutes at room temperature and incubated with the rabbit anti-defensin-2 primary antibody at room temperature (1:20) overnight. Rabbit preimmune serum was used to probe select samples previously incubated with defensin-2 to demonstrate specificity of primary antibody binding. To demonstrate the specificity of defensin-2 association with *R. montanensis*, rabbit anti-defensin-2 antibody was used to probe rickettsia that were incubated with thioredoxin control protein. Grids were washed three times with HEPES, and then incubated with the 10 nm gold particle-labeled anti-rabbit secondary antibody. Grids were fixed with 1% paraformaldehyde, washed with distilled water, and incubated with 1% ammonium molybdate prior to viewing. Samples were viewed on a JEOL 5700 Transmission Electron Microscope (Jeol USA, Inc. Peabody, MA).

## *In vivo* neutralization of defensin-2

IgG was purified from rabbit anti-defensin-2 or preimmune serum using MelonG IgG purification kit (ThermoFisher Scientific) and the concentration estimated using BCA protein assay (ThermoFisher Scientific) or Fluoroprobe protein quantitation kit (Sigma, St. Louis, MO). Five microliters of IgG was provided to four-day part-fed *D. variabilis* in capillaries at concentrations of 0.5, 1 or 2 mg/ml. Preimmune IgG was provided at a 2 mg/ml concentration. To monitor evaporation of fluid from the capillaries, we place a capillary containing five microliters of solution (evaporation control) on a petri dish. The capillary was not attached to the mouthparts of a tick. The fluid in capillaries attached to the ticks' mouthparts empties before the fluid in the “evaporation control” capillary. One hour after the IgG was imbibed, ticks were capillary-fed eight microliters of *R. montanensis* (30,000 bacteria/ $\mu$ l) suspended whole sheep's blood diluted 125 fold with 0.9% NaCl ( $2.4 \times 10^5$  total). Ticks incubated overnight at 25°C and 90–100% humidity. Whole tissues were



collected for total RNA extraction (Qiagen, Valencia, CA). After extraction, burden was estimated using qRT-PCR. Rickettsial *gltA* transcript was normalized with host *actin* transcript (Ceraul et al., 2011). We were unable to use the *16s* rRNA primers to estimate burden *in vivo* due to nonspecific amplification of symbiotic bacteria transcript.

### ***In vivo* IFA of infected tick midgut**

Four-day part-fed *D. variabilis* ticks were capillary-fed *R. montanensis* and incubated for 48 hours as described (Ceraul et al., 2007). Following incubation, midgut tissues from time-matched uninfected and *R. montanensis*-infected ticks was dissected and flash frozen in Tissue-Tek, Optimal Cutting Temperature (OCT) medium (Miles Inc., Elkhart, IN). Samples were stored at  $-80^{\circ}\text{C}$  until used for cryosectioning. Five-micron sections were cryosectioned and air-dried onto glass slides. After fixation, samples were blocked with PBS supplemented with 10% FBS and washed twice with PBS. Samples were then incubated with rabbit anti-defensin-2 serum (1:400) and mouse anti-*R. montanensis* serum (1:100) for one hour. A separate slide was incubated with normal rabbit and normal mouse control sera as a control. Following incubation with the primary antibodies, sections were washed three times with PBS for five minutes each. A 1:500 dilution of anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 594 secondary antibodies were applied for 30 minutes. Sections were washed three more times and counterstained with  $1\ \mu\text{M}$  SYTOX Blue nucleic acid stain in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 for 10 minutes at room temperature. Sections were mounted using Vectashield (Vector Laboratories, inc., Burlingame, CA) and a coverslip. Slides were visualized and images captured at  $400\times$  total magnification on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Thornwood, NY) and analyzed using LSMIX software package (Zeiss LSM Image Examiner Version 3.2.0.70).

### **Statistical Analysis**

Each experiment was repeated at least twice. Individual ticks are designated as biological replicates. Each treatment is represented by at least seven individual biological replicates collected from all experiments performed. An Interquartile range outlier test was performed to exclude outlier data points from statistical analysis. Data from each treatment group was subjected to the Shapiro-Wilk test and log transformed if lacking normality. An F-Test was performed to assess equality of variances between groups. A one-tailed T-Test was performed to compare each treatment to a control. All statistical analyses were performed using Microsoft Excel.

### **Acknowledgments**

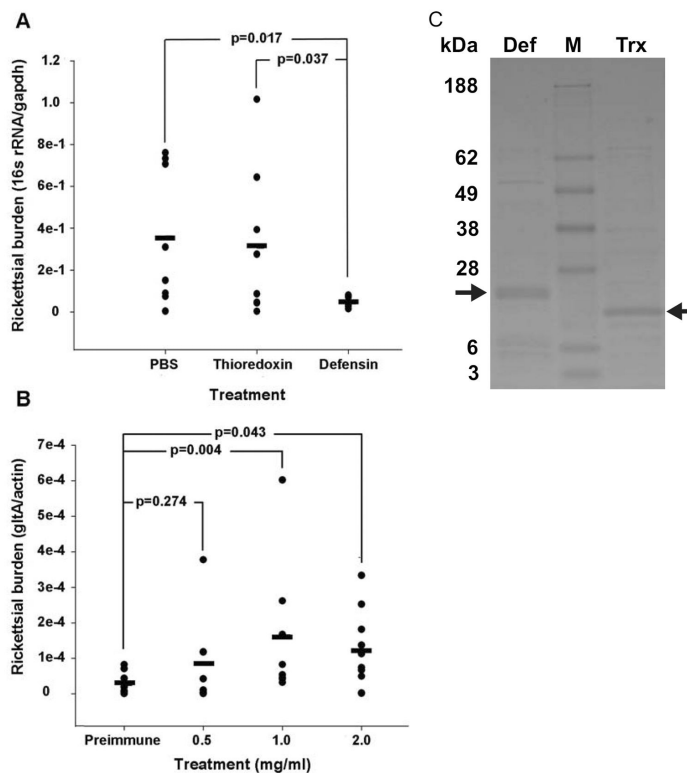
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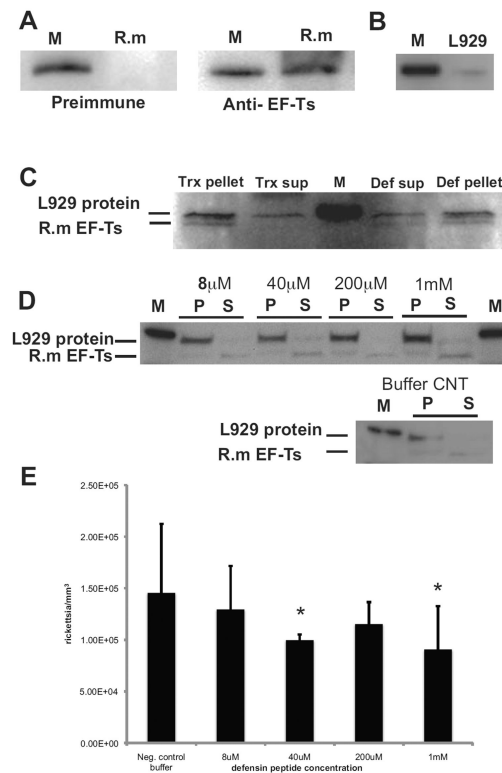
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**Figure 1. Defensin-2 limits *R. montanensis* infection *in vitro* and *in vivo***

(A) *R. montanensis* was incubated with recombinant defensin-2, PBS, or recombinant thioredoxin and then used to infect an L929 monolayer for 24 hours. Rickettsial burden in the infected L929 cells was determined using qRT-PCR. The bar represents the average for each treatment. There are a total of four separate experiments performed in duplicate (two wells per treatment). The number of samples analyzed per treatment are as follows: untreated,  $n = 8$ ; thioredoxin,  $n = 8$ ; defensin-2,  $n = 7$ . (B) Part-fed *D. variabilis* ticks were capillary-fed anti-defensin-2 IgG at increasing concentrations followed by an oral *R. montanensis* challenge. Whole tissue was dissected and rickettsial burden determined using qRT-PCR. Each individual tick is represented by a closed circle. The mean for each treatment is represented by a bar. Values represent the transcript abundance measured in the total number of ticks collected from at least two separate experiments. P-values were determined using the T-Test. Biological replicates are as follows: preimmune,  $n = 10$ ; 0.5 mg/ml,  $n = 8$ ; 1 mg/ml,  $n = 8$ ; 2 mg/ml, 10. (C) Representative Imperial blue stained LDS polyacrylamide gel of recombinant defensin-2 (Def) and thioredoxin (Trx) purification. Defensin-2 migrated to approximately 28 kDa due to the thioredoxin and histidine fusion tags. M, molecular weight marker. Arrows indicate purified recombinant defensin-2 and thioredoxin.

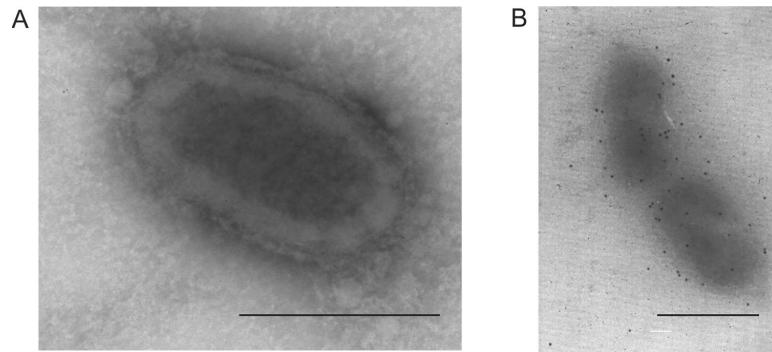


**Figure 2. Defensin-2 causes cytoplasmic leakage in *R. montanensis***

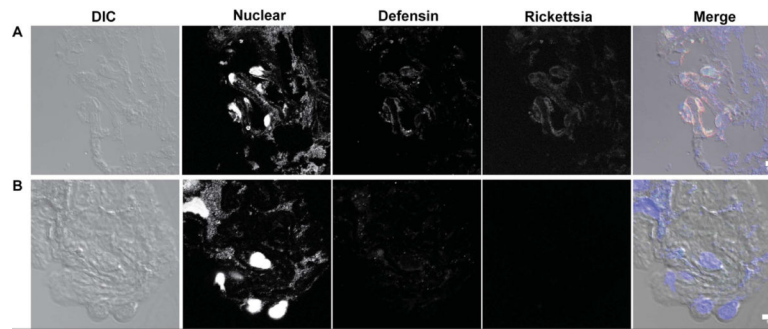
*R. montanensis* was incubated with recombinant defensin-2 or thioredoxin (Trx) followed by centrifugation to separate the pellet and supernatant fractions. Alternatively, *R. montanensis* was treated with synthetic defensin-2 or diluent buffer (buffer control) followed by centrifugation to separate the pellet and supernatant fractions. Each fraction was subjected to LDS-PAGE, blotted to a PVDF membrane, and probed with anti-EF-Ts serum. (A) Lysate from *E. coli* expressing recombinant EF-Ts from *R. montanensis* was detected by EF-Ts antiserum but not rabbit preimmune serum. Recombinant *R. montanensis* EF-Ts migrated to 40 kDa due to the histidine tag. (B) Nonspecific band detected by EF-Ts antiserum in uninfected L929 lysate. (C) *R. montanensis* EF-Ts was detected in the pellet from Trx-treated, as well as in the supernatant and pellet, from defensin-2-treated *R. montanensis*. EF-Ts was not observed in supernatant from Trx-treated *R. montanensis*. The nonspecific L929 and *R. montanensis* EF-Ts (35–40 kDa) proteins are indicated. (D) Pellet and supernatant fractions from *R. montanensis* treated with increasing concentrations of synthetic defensin-2 peptide or diluent buffer (Buffer CNT). *R. montanensis* EF-Ts ran closer to 35 kDa due to an increased running time on the polyacrylamide gels to ensure adequate separation from the nonspecific L929 protein that cross-reacts with EF-Ts antiserum. (E) Decrease in the number of live rickettsia/mm<sup>3</sup> with increasing concentrations of synthetic defensin-2 peptide. Live counts were performed on the same samples used to generate the blots demonstrating lysis to correlate lysis with the decrease in live counts. A T-Test was performed to test for statistical differences between the means for treatment with increasing concentrations of synthetic defensin-2 and the negative control buffer. P-values for each synthetic defensin-2 concentration are 8 µM (p=0.21), 40 µM (p=0.027), 200 µM (p=0.09),

1mM ( $p=0.018$ ). Asterisks indicate significant differences from negative control buffer. M, molecular weight marker migrating to 40 kDa; R.m., *R. montanensis*.





**Figure 3. Defensin-2 associates with *R. montanensis* *in vitro***  
Purified *R. montanensis* was incubated with recombinant defensin-2 and processed for negative staining electron microscopy. Rickettsia specimens incubated with preimmune serum (A) or defensin antiserum (B). Bars equal 5  $\mu$ m



**Figure 4. Defensin-2 associates with *R. montanensis* in vivo**

(A) *R. montanensis*-infected tick midgut incubated with immune sera. (B) *R. montanensis*-infected tick midgut incubated with control sera. *R. montanensis*-infected *D. variabilis* midguts 48 hours post-infection were probed with rabbit defensin and mouse *R. montanensis* antisera or rabbit and mouse preimmune serum. Rabbit and mouse antibodies were detected with anti-rabbit Alexa-488 (defensin) or anti-mouse Alexa-594 (rickettsia), respectively. SYTOX Blue was used to visualize the nucleus. DIC, differential interference contrast. Bars represent 5  $\mu$ m.