The Peritrophic Matrix Mediates Differential Infection Outcomes in the Tsetse Fly Gut following Challenge with Commensal, Pathogenic, and Parasitic Microbes

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Metazoans serve as hosts to a wide variety of pathogenic, commensal, and beneficial microbes, the majority of which are housed in the gut. As such, the gut tissue must be able to differentiate between these microorganisms and respond to perceived threats. The gut of insects and vertebrates share developmental, structural, and functional characteristics (1). Thus, the insect gut is being used with increased frequency as a model tissue to study the basic cellular and molecular mechanisms that underlie immune homeostasis (2, 3). Acquiring a comprehensive understanding of insect gut immunity is also of paramount importance from a medical perspective, because zoonotic pathogens must surmount this hostile environment within their respective vector before being transmitted to a human host.

The insect gut is lined by a protective, chitinous peritrophic matrix (PM) that separates immunoreactive epithelial cells from microbes present within the luminal contents. Tsetse flies (Glossina spp.) imbibe vertebrate blood exclusively and can be exposed to foreign microorganisms during the feeding process. We used RNA interference–based reverse genetics to inhibit the production of a structurally robust PM and then observed how this procedure impacted infection outcomes after per os challenge with exogenous bacteria (Enterobacter sp. and Serratia marcescens strain Db11) and parasitic African trypanosomes. Enterobacter and Serratia proliferation was impeded in tsetse that lacked an intact PM because these flies expressed the antimicrobial peptide gene, attacin, earlier in the infection process than did their counterparts that housed a fully developed PM. After challenge with trypanosomes, attacin expression was latent in tsetse that lacked an intact PM, and these flies were thus highly susceptible to parasite infection. Our results suggest that immunodeficiency signaling pathway effectors, as opposed to reactive oxygen intermediates, serve as the first line of defense in tsetse’s gut after the ingestion of exogenous microorganisms. Furthermore, tsetse’s PM is not a physical impediment to infection establishment, but instead serves as a barrier that regulates the fly’s ability to immunologically detect and respond to the presence of these microbes. Collectively, our findings indicate that effective insect antimicrobial responses depend largely upon the coordination of multiple host and microbe-specific developmental factors. The Journal of Immunology, 2014, 193: 773–782.

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Abbreviations used in this article: AMP, antimicrobial peptide; BSF, bloodstream form; CS, chitin synthase; dpc, d postchallenge; DUOX, dual oxidase; ECS, ectoperitrophic space; EP, epithelial cell; Gmn<sup>++</sup>Δ<sup>-4</sup>, wild-type teneral Glossina morsitans morsitans; hpc, h postchallenge; IMD, immunodeficiency; iNOS, inducible NO synthase; LC, peptidoglycan recognition protein LC; MAMP, microbe-associated morsitans; hpc, h postchallenge; IMD, immunodeficiency; iNOS, inducible NO synthase; LC, peptidoglycan recognition protein LC; MAMP, microbe-associated molecule; MG, midgut; P1, peritrophin pro 1; P2, peritrophin pro 2; PF, procyclic form; PGRP-LB, peptidoglycan recognition protein LB; PM, peritrophic matrix; qPCR, quantitative real-time PCR; RNAi, RNA interference; ROI, reactive oxygen intermediate; Serratia<sup>++</sup>Δ<sup>-4</sup>, heat-killed Serratia; SG, salivary gland.
(16, 17), and tsetse EP protein (18). Currently, only circumstantial evidence suggests that tsetse’s PM influences microbial infection outcomes in the fly’s gut (19, 20).

In this study, we characterized the functional relationship between tsetse’s PM and microbial infection outcomes in the gut. To do so, we used a robust RNA interference (RNAi)-based gene knockdown strategy that inhibited production of prominent structural components of tsetse’s PM. Subsequently, flies with intact and structurally compromised PMs were challenged per os with three taxonomically and phenotypically distinct exogenous microbes, including bacteria that present commensal and pathogenic phenotypes in other insect hosts, as well as parasitic trypanosomes. We determined that tsetse’s PM indirectly regulates infection outcomes after challenge with foreign microorganisms by mediating the presentation of epithelial immune responses within the fly gut. We discuss our findings in the context of physiological processes that regulate innate epithelial immune responses in the gut, and microbial adaptations that promote differential infection outcomes in this niche.

Materials and Methods

Ethical consideration

This work was carried out in strict accordance with the recommendations in the Office of Laboratory Animal Welfare at the National Institutes of Health and the Yale University Institutional Animal Care and Use Committee. The experimental protocol was reviewed and approved by the Yale University Institutional Animal Care and Use Committee (Protocol No. 2011-07260).

Tsetse, trypanosomes, and bacteria

Glossina morsitans morsitans were maintained in Yale’s insectary at 24°C with 50–55% relative humidity. Throughout this article, flies referred to as “teneral” were unfed adults recently eclosed (<24 h) from their pupal case, whereas those referred to as “mature” were fed three times. All flies received defibrinated bovine blood every 48 h through an artificial membrane feeding system (21).

In vivo Trypanosoma brucei brucei (RUMP 503) was expanded in rats, and harvested from infected blood at peak parasitemia. Blood was aliquoted and cryopreserved for subsequent tsetse challenge experiments. T. b. brucei completes development in tsetse and was used to initiate midgut (MG) and salivary gland (SG) infections. Enterobacter sp. (Zygaena) isolated from the gut of the mosquito, Anopheles gambiae (22) and Serratia marcescens strain DB1 (isolated from a moribund Drosophila sp.) (23) were grown in Luria-Bertani media at 37°C and 30°C, respectively. Serratia were heat killed by incubating log-phase cells at 80°C for 1.5 h.

RNAi-mediated knockdown of gene expression

GFP (GFP), pro1 (P1), pro2 (P2), chitin synthase (CS), and pgpt-lc (peptidoglycan recognition protein LC [LC]) dsRNA were prepared according to the manufacturer’s protocol (Ambion, Austin, TX). Gene-specific PCR primers, each encoding a 5’ T7 RNA polymerase binding site, are listed in Supplemental Table 1. All dsRNA PCR primer sequences are listed in Supplemental Table 1. All dsRNA PCR primer sequences were BLASTed against a tsetse RNASeq library and a complete set of tsetse ESTs (both available at the VectorBase Web site: www.vectorbase.org), to eliminate the possibility that they could amplify any sequence other than their target. In addition, all PCR products were sequenced before the synthesis of dsRNA to confirm that they encoded only target genes.

Newly emerged adult male tsetse were divided into treatment groups (dsP1/2, received P1 and P2 dsRNA; dsCS, received CS dsRNA; dsCS/P, received P1, P2, and CS dsRNA; dsCS/LC, received CS and LC dsRNA) and one control group (dsGFP, received GFP dsRNA). Teneral individuals of each group were provided a blood meal containing 3 μg dsRNA/20 μl blood (the approximate volume a tsetse fly imbibes each time it feeds). The following day, all flies received an intrathoracic microinjection containing a total of 5 μg of the same dsRNAs they had previously received per os. All flies received the same total quantity of dsRNA regardless of their group assignment. As such, additional GFP dsRNA was administered to treatment groups dsP1/2 dsCS and dsCS/P, to equilibrate the total quantity of dsRNA received by each fly. Sample sizes for all experiments are indicated in the corresponding figure legend.

Tissue sectioning and staining

We sectioned and stained MG tissues from dsRNA treatment (with the exception of dsCS/LC) and control tsetse flies in an effort to visually confirm whether our RNAi-based gene knockdown strategy effectively compromised PM integrity. To do so, we collected guts (inclusive of the bacteriome through posterior MG) from all dsRNA-treated tsetse 10 d after dsRNA injection and immediately fixed the tissues in Carnoy’s solution (60% EtOH, 30% chloroform, 10% glacial acetic acid). Tissues were embedded in agar (1.5%), dehydrated and cleared through a xylene and ethanol series, and embedded in paraffin. Serial 5-μM tissue sections were cut with a rotary microtome and mounted on poly-l-lysine-coated glass slides (Richard-Allan Scientific). Before staining, slide-mounted samples were counterstained with additional xylene and ethanol series. Tissues were then stained with H&E according to the manufacturer’s protocol (Poly Scientific) and hard-mounted using Permount mounting solution containing toluene. Finally, samples were visualized under differential interference contrast optics using a Zeiss Axio Observer Z1 inverted microscope equipped with a Hamamatsu camera.

Dextran feeding assay

Dextran feeding assays were performed by using a modified version of previously described protocols (8, 24). In brief, 500 kDa FITC-labeled dextran molecules (Sigma) were dissolved in a 2.5% sucrose solution and filtered using PD MiniTrap Sephadex G10 columns (GE Healthcare). Tsetse (n = 10 individuals of each dsRNA-treated group, with the exception of dsCS/LC) were inoculated with dextran by feeding flies a 2.5% sucrose solution containing 10% bovine blood and 10% filtered dextran molecules (1 mg/ml). Six hours postfeeding, MGs from dsGFP, dsP1/2, dsCS, dsCS/LC, and dsCS-P-treated animals (n = 3 per group) were dissected and dsRNA injected. Forty days after trypanosome challenge, all surviving flies were dissected and stained with Hoechst and FITC signal observed using a fluorescent dissecting microscope (Zeiss Discovery) equipped with a digital camera (Zeiss AxioCam MRC 5).

Microbial infection assays

Per os bacterial challenge of dsRNA-treated tsetse flies was performed by feeding (in their second blood meal) all individuals a blood meal inoculated with 1 × 106 CFUs Enterobacter or Serratia per milliliter of the total culture. This bacterial density was used because it was the minimum number of bacterial cells that are capable of successfully colonizing tsetse’s gut. The vertebrate host complement system was heat inactivated (56°C for 1 h) before inoculating blood meals with bacterial cells. All bacteria-challenged flies were maintained on heat-inactivated blood throughout the 14-d experimental time course. At 3 and 14 d postchallenge (dpc), a previously developed bacterial quantification assay (25) was used to determine the number of bacteria present within the guts of dsRNA-treated flies. The same assay was used to quantify Serratia density in tsetse hemolymph at 3 and 6 dpc.

For trypanosome infection experiments, dsRNA treatment and control flies were challenged with 1 × 105/ml bloodstream form (BSF) T. b. brucei (RUMP 503) parasites in their second blood meal (administered 2 d after dsRNA injection). Forty days after trypanosome challenge, all surviving flies were dissected and stained with Hoechst and SGs microscopically examined for the presence of parasite infections.

dCS/P flies were coinfected with 1 × 106/ml BSF T. b. brucei (RUMP 503) parasites and 1 × 105 heat-killed Serratia (Serratia) in their second blood meal (administered 2 d after dsRNA injection) to evaluate the impact of heightened IMD pathway activity on trypanosome infection outcomes in tsetse’s gut. Wild-type teneral control flies (wild-type teneral G. m. morsitans [GmmYT]) that house an underdeveloped PM and mature flies with an intact PM (dsGFP) received the same challenge. Trypanosome MG infection prevalence was determined 14 dpc. Sample size for all trypanosome infection experiments is indicated in the corresponding figure.

Tissue dissections and quantitative analysis of gene expression

Quantitative real-time PCR (qPCR) was used to validate the effectiveness of our RNAi procedure and to analyze the expression of immunity-related genes after bacterial and parasitic challenge. Dissected tissues were immediately placed into TRIzol reagent, after which RNA was extracted according to the manufacturer’s protocol (Invitrogen). Contaminating DNA was removed from all samples via treatment with DNaseI, and cDNAs were generated using an iScript cDNA synthesis kit (Bio-Rad). qPCR analysis was performed using SYBR Green supermix and a Bio-Rad C1000 thermal cycler. Amplification primers are listed in Supplemental Table 2. Samples sizes are indicated by individual dots, bars indicate the median fold change for each gene assayed. Quantitative measurements were performed in duplicate for all samples. Gene expression levels were below the limit of detection of our assay for samples that lack values.
Flies were fed blood meals supplemented with 500 kDa FITC-labeled dextran molecules. Six hours postinoculation, MGs (the dextran is present in a diffuse pattern in contact with the EP. Scale bar, 500 μm). The ECS, which contains no fluorescent dextran, is visible between the PM and MG EP in these panels. In molecules are contained within the sleevelike PM (which resembles wrinkled cellophane) of dsRNA-treated group (the other two replicates from each dsRNA-treated group exhibited similar patterns of dextran diffusion as did the one shown). Dextran molecules are contained within the sleevelike PM (which resembles wrinkled cellophane) of dsGFP- and dsP1/2-treated individuals. In addition, a dark ECS, which contains no fluorescent dextran, is visible between the PM and MG EP in these panels. In dsCS- and dsCS/P-treated tsetse, no PM is visible and the dextran is present in a diffuse pattern in contact with the EP. Scale bar, 500 μm.

To confirm gene knockdown, we dissected cardia tissues from dsP1/2, dsCS and dsCS/P treatment, and dsGFP control flies, immediately before challenge with Enterobacter, Serratia, or T. brucei, which was 2 d after thoracic microinjection of dsRNA. To confirm gene knockdown in dsCS/CS/P treatment flies, we dissected MGs (including the cardia) 12 h after challenge with Enterobacter or Serratia. qPCR results were normalized relative to tsetse’s constitutively expressed β-tubulin gene (determined for each corresponding sample).

To analyze the expression of immunity-related genes, we dissected MGs from dsRNA-treated flies 12 h postchallenge (hpc) with Enterobacter and Serratia. For trypanosome infection experiments, MGs were dissected 24 h after parasite challenge (treatment group 1) and after SerratiaHK and T. brucei challenge (treatment group 2, and control groups 1 and 2). For comparative purposes, tissues were dissected from a subset of age-matched, dsRNA-treated flies that were not exposed to an infective blood meal. All fold-change values are represented as a fraction of the average normalized gene expression levels from age-matched, microbe-challenged versus -unchallenged flies.

**Statistical analyses**

Statistical significance between various treatments, and treatments and controls, is indicated in the corresponding figure legends. Survival curves are presented as Kaplan–Meier plots, and statistical analyses were performed by log-rank analysis using GraphPad Prism software (v.6). Statistical analysis of bacterial colonization in tsetse’s gut was performed by Mann–Whitney U test (GraphPad Prism software, v.6), qPCR data by Student t test (Microsoft Excel), and trypanosome infection outcomes by Fisher exact test (JMP software, v.9).

**Results**

Knockdown of PM-associated genes compromises the structure’s integrity

In an effort to better understand how the PM mediates host–microbe interactions, we artificially compromised the integrity of this structure through the use of an RNAi-based gene knockdown approach. We did so by generating dsRNAs that specifically target the expression of the peritrophins pro1 (P1) and pro2 (P2) and CS. These genes were chosen because they are an integral component of PM formation processes in other insects (CS) (26, 27). In addition, P1 and P2 are highly expressed in tsetse’s cardia, and Pro1 and Pro2 are proteinaceous components of the fly’s PM (28, 29).

In an effort to achieve potent knockdown of target gene expression, we used a robust RNAi-based protocol that included feeding and injecting target dsRNAs into distinct groups of tsetse. We fed four groups of tsetse with 2 μg of either P1/2, CS, CS/P, or GFP dsRNA (these flies are hereafter referred to as dsP1/2, dsCS, dsCS/P, and dsGFP, respectively) in their first blood meal posteclosion. One day later, all flies received an additional treatment with 5 μg of the same dsRNAs, this time via intrathoracic microinjection. This consecutive knockdown approach reduced the expression of target genes by 85–91% in treatment flies (dsP1/2, dsCS, and dsCS/P) compared with dsGFP controls (Supplemental Fig. 1A).

Histological analysis of gut sections from treatment and control individuals revealed that the PM of dsP1/2 and dsGFP flies was intact, although the structure was severely compromised, or entirely absent, from dsCS and dsCS/P individuals (Fig. 1A). To further validate the effectiveness of our experimental procedure, we fed dsRNA-treated tsetse a modified blood meal supplemented with FITC-labeled dextran molecules (500 kDa). The presence of fluorescent dextran in tsetse’s gut allowed us to visualize structural integrity of the fly’s PM. Six hours postfeeding, we observed that dextran molecules were surrounded by an intact PM in dsGFP and dsP1/2 fly groups. Under these circumstances, the ECS was visible and contained no dextran molecules. In contrast, diffuse fluorescence was observed immediately adjacent to surrounding intestinal epithelial tissues within MGs of dsCS and dsCS/P individuals, and no ECS was visible (Fig. 1B), further confirming that these flies lack a robust PM.
Our results confirm that we were able to artificially alter the structural integrity of the PM through the use of an RNAi-based gene knockdown approach. Furthermore, tsetse chitin, as opposed to P1 and P2, appears to play a prominent role in the formation of a structurally robust PM.

The PM modulates infection outcomes with different bacteria

We next set out to determine whether tsetse’s PM contributes to fly survival after challenge with exogenous bacteria. First, we orally challenged wild-type tsetse (GmmWT) with either Enterobacter sp. (Esp_Z) or Serratia marcescens (strain Db11). In Anopheles gambiae, Enterobacter benignly colonizes the host gut (22), whereas Serratia causes a lethal infection in Drosophila (23). We found that 85% of GmmWT individuals were able to survive the 14-d experimental period after oral challenge with 1 \times 10^3 CFU Enterobacter (Fig. 2A). In contrast, when GmmWT flies were orally challenged with the same dose of Serratia, only 13% of flies survived their infection (Fig. 2A).

To determine whether the PM modulates bacterial infection outcomes in tsetse, we orally challenged dsP1/2, dsCS, dsCS/P, and dsGFP individuals (confirmation of target gene knockdown is shown in Supplemental Fig. 1A) with 1 \times 10^3 CFU of either Enterobacter or Serratia and monitored host survival over the course of 14 d. After challenge with Enterobacter, we found no significant difference in host survival in any of the dsRNA-treated groups (Fig. 2B). However, after challenge with Serratia, 75% of dsCS flies and 83% of dsCS/P flies survived the 14-d experimental period. In contrast, significantly fewer dsP1/2 (11%) and dsGFP (12%) individuals survived (Fig. 2C). Thus, in the absence of a robust PM, tsetse were protected from this otherwise virulent microbe.

The PM modulates bacterial colonization dynamics in the fly gut

We discovered that Enterobacter presents a benign phenotype regardless of PM status, whereas Serratia is pathogenic in hosts that house an intact PM. We thus investigated whether the PM

FIGURE 3. The PM regulates bacterial colonization dynamics in tsetse’s gut. (A) Enterobacter and (B) Serratia densities (CFU/gut) within the gut of dsRNA-treated tsetse at 3 and 14 dpc. At both time points, significantly higher densities of both bacteria were present in dsGFP and dsP1/2 versus dsCS and dsCS/P tsetse guts (p < 0.001). (B) Values shown in gray represent what became lethal infections. (A and B) Dotted lines are indicative of the initial inoculate. (C) Serratia quantification in the hemocoel of GmmWT flies at 3 and 6 dpc. Symbols on all graphs represent one fly gut, and bars indicate the median bacterial density per time point.
mediates infection outcomes by regulating bacterial colonization dynamics in tsetse’s gut. We monitored Enterobacter and Serratia densities in the gut of dsRNA-treated fly groups at 3 and 14 dpc, and found that dsGFP and dsPI/2 individuals harbored significantly more Enterobacter 3 dpc ($1.6 \times 10^7 \pm 5.0 \times 10^7$ and $2.0 \times 10^7 \pm 6.7 \times 10^7$, respectively) than did their dsCS or dsCS/P counterparts ($108 \pm 67$ and $2.5 \times 10^7 \pm 2.3 \times 10^7$, respectively; Fig. 3A). More so, Enterobacter densities in each dsRNA-treated fly group did not change significantly over the remainder of the 14-d experiment (Fig. 3A). With respect to Serratia infections, we found that susceptible dsGFP and dsPI/2 flies harbored significantly more Serratia at 3 dpc ($2.1 \times 10^7 \pm 5.4 \times 10^6$ and $1.4 \times 10^7 \pm 4.5 \times 10^6$, respectively) than did resistant dsCS and dsCS/P individuals ($4.1 \times 10^6 \pm 1.5 \times 10^6$ and $4.5 \times 10^6 \pm 2.1 \times 10^6$, Fig. 3B). By 14 dpc, Serratia density in resistant dsCS and dsCS/P flies had returned to levels less than that present in the initial inoculum ($141 \pm 60$ and $928 \pm 334$, respectively; Fig. 3B). In contrast, susceptible dsGFP and dsPI/2 flies harbored high Serratia densities at this same time point ($6.9 \times 10^5 \pm 3.4 \times 10^5$ and $3.3 \times 10^5 \pm 1.6 \times 10^5$, respectively), indicating they were unable to control bacterial growth (Fig. 3B). Thus, in the absence of a robust PM, proliferation of both Enterobacter and Serratia is impeded early in the infection process.

In Drosophila, entomopathogenic Serratia translocates from the fly’s gut into its hemocoel (23). To determine whether a similar phenomenon occurs in tsetse, we repeated our initial Serratia infection experiment in wild-type tsetse (Fig. 1A) and then collected and plated hemolymph from flies 3 and 6 dpc. By 3 dpc, we could readily detect Serratia in tsetse’s hemocoel, and by 6 dpc the bacterium was present at a density of $3.6 \times 10^6$ CFU/μl hemolymph (Fig. 3C). This result suggests that Serratia invades the tsetse’s hemocoel, and that after entering this niche, the bacterium likely replicates to intolerable densities that result in host death.

Thus, with respect to Serratia, the presence of an intact PM creates a protective niche where bacteria can proliferate in the gut lumen and subsequently cause disease.

**The PM modulates host immune detection of exogenous bacteria**

We determined that PM structural integrity influences infection dynamics after per os challenge with Enterobacter and Serratia. To gain functional insight into this phenomenon, we surveyed the expression pattern of immunity-related genes in normal and PM-compromised tsetse 12 hpc with these two bacteria. We monitored attacin (an AMP product of the IMD pathway), as well as dual oxidase (DUOX) and inducible NO synthase (iNOS), which are signaling molecules involved in the activation of humoral immune responses and the production of antimicrobial ROIs (30, 31). After challenge with Enterobacter, we observed that attacin expression increased 126- and 59-fold in dsCS- and dsCS/P-treated tsetse, respectively. Similarly, after Serratia challenge, attacin expression increased 99-fold and 203-fold in dsCS- and dsCS/P-treated tsetse, respectively. In contrast, challenge with these bacteria resulted in no significant change in iNOS or DUOX expression in either of the PM-compromised fly groups (Fig. 4A).

Our results suggested that IMD pathway activation could eliminate Serratia from the gut of PM-compromised flies before bacterial cells were able to translocate into the fly’s hemocoel. We thus reasoned that PM-compromised, Serratia-resistant tsetse would be rendered susceptible to this bacterium in the absence of a competent IMD pathway. We used our previously described RNAi-based approach to simultaneously knock down the expression of both tsetse CS and pgrp-lc (these treatment flies are designated dsCS/LC). The latter gene encodes the IMD pathway receptor, and knocking down its expression reduces production of downstream AMPs, including Attacin (16). This procedure sig-

**FIGURE 4.** The PM modulates IMD pathway-mediated detection of exogenous bacteria in tsetse’s gut. (A) Fold change in the expression of genes that encode Attacin, iNOS, and DUOX in dsRNA-treated tsetse 12 hpc with $10^7$ CFU of either Enterobacter (top panels) or Serratia (bottom panels). Genes shown in gray were significantly upregulated in challenged versus unchallenged individuals ($p < 0.05$). (B) Survival of dsCS/LC tsetse (these flies present a structurally compromised PM and dysfunctional IMD pathway) after challenge with $10^7$ CFU Enterobacter or Serratia. Gray curves depict lethal treatments. (C) Enterobacter and Serratia densities (CFU/gut) within the gut of dsCS/LC versus dsGFP tsetse. Symbols represent one fly gut, and bars indicate the median bacterial density per time point. Values shown in gray represent what become lethal infections.
significantly reduced the expression of CS and pgp-lc in dsRNA-treated tsetse (Supplemental Fig. 1B). In addition, upon microbial challenge, attacin expression was significantly reduced in dsCS/LC versus dsGFP individuals (Supplemental Fig. 1B). As demonstrated previously, we found that all tsetse survived challenge with Enterobacter. However, when dsCS/LC tsetse were challenged with Serratia, 68% of flies perished within 14 d (Fig. 4B). In addition, we found that Enterobacter and Serratia densities in guts of dsCS/LC tsetse were not significantly different from those in guts of dsGFP control flies (Fig. 4C). Thus, effectors that prohibit Serratia and Enterobacter proliferation in PM-compromised guts appear to be products of the IMD signaling pathway as opposed to ROIs (summarized in Supplemental Fig. 2).

The PM modulates trypanosome infection outcomes

We next used our RNAi-based gene knockdown approach to determine whether the PM also modulates infection outcomes after challenge with trypanosomes (confirmation of target gene knockdown is shown in Supplemental Fig. 1C). Forty dpc, parasite infection status in MG and SG tissues was determined in flies with and without intact PM structures. We observed that significantly more dsP1/2, dsCS, and dsCS/P flies harbored both MG and SG infections than did dsGFP controls (Fig. 5A). Furthermore, dsCS and dsCS/P exhibited a statistically significant increase in trypanosome infection prevalence compared with dsP1/2 flies (Fig. 5A). Our results suggest that the presence of an intact PM reduces the ability of parasitic trypanosomes to establish infections in tsetse’s gut.

The IMD pathway is critical for host resistance to trypanosome establishment

A higher percentage of PM compromised adult tsetse harbor trypanosome MG infections than do flies with intact PMs. This finding indicates that PM structural integrity at the time of exposure to trypanosomes correlates with parasite survival. To determine the functional basis of PM-mediated infection outcomes, we developed an assay that allowed us to investigate the relation between PM status and activity of distinct host immune pathways after challenge with trypanosomes (Fig. 5B). We treated a group of tsetse (treatment group 1) with CS/P dsRNA (effectiveness of this dsRNA treatment is shown in Supplemental Fig. 1D), challenged the flies per os with trypanosomes, and then monitored expression patterns of gut-associated attacin, iNOS, and DUOX 24 h later. Furthermore, we determined parasite MG infection prevalence 2 wk postchallenge. After trypanosome challenge, we observed that attacin expression in the gut of dsCS/P individuals remained unchanged, whereas iNOS and DUOX were significantly upregulated. Under such conditions, 51% of these flies eventually harbored
MG trypanosome infections 2 wk postchallenge (Fig. 5C, first graph) despite the initial significant increase in iNOS and DUOX expression detected upon parasite acquisition.

IMD pathway–associated AMPs and ROIs are produced by tsetse’s cardia and MG in response to parasite challenge (13, 15). Thus, latent attacin expression in dsCS/P tsetse after parasite challenge was surprising. In an effort to induce expression of this trypanocidal effector in tsetse’s gut at the time of parasite acquisition, we coinoculated dsCS/P individuals with Serratia $^{HK}$ and trypanosomes (treatment group 2). This challenge induced a 283-fold increase in attacin expression, and 0% of dsCS/P flies harbored MG trypanosome infections (Fig. 5C, second graph). We also administered the same cochallenge to 2-d-old wild-type adults (33). Forty-one percent of WT/T; control group 1), which housed an immature PM structure (Fig. 5). As such, trypanosome differentiation and replication processes proceed unobstructed in tsetse’s gut. By the time adults by 8 dpc, bacterial cells have entered into the ECS where they come into contact with the gut EPs. These bacteria, along with commensal trypanosomes (from immune-competent epithelial cells. (A) At 0 hpc, Enterobacter are replicating in the fly’s gut lumen (endoperitrophic space). By 3 and 8 dpc, bacterial cells have entered into the ECS where they come into contact with the gut EPs. These bacteria, along with commensal Sodalis, persist in the ECS. This phenomenon may occur because these bacteria form protective biofilms that shield them from exposure to lytic host AMPs. Serratia is lethal to wild-type tsetse. Early in the infection process, Serratia replicates undetected in tsetse’s gut lumen. By 3 dpc, Serratia cells have invaded the ECS where they induce a host immune response. Despite this outcome Serratia translocates into the fly’s hemocoel where it replicates to intolerable densities. (B) Upon ingestion of a blood meal containing infectious trypanosomes, slender BSF parasites perish while stumpy BSFs quickly differentiate to PFs. These PF parasites express a surface coat composed of GPEET procyclin molecules. By 3 dpc, GPEET PFs begin to proliferate tsetse’s endoperitrophic space. These parasites encounter trypanolytic effectors in this environment and undergo a significant process of attrition that leads to parasite clearance in the majority of adults by 8 dpc. Teneral tsetse, which present a structurally similar PM to that of dsCS and dsCS/P adults, are significantly more susceptible to infection than trypanosomes with mature flies. Trypanosome susceptibility exhibited by teneral tsetse may be due to their underdeveloped PM structure and/or immunologically immature gut EPs (Fig. 5). As such, trypanosome differentiation and replication processes proceed unobstructed in tsetse’s gut. By the time tsetse gut EPs have developed immuno-competence (~5–7 dpc), most PF parasites have likely switched their GPEET procyclin coat to an EP form that resists trypanolytic activity of host AMPs. FG, foregut; C, cardia; B, bacteriome; HL, hemocoel; HG, hindgut; PT, peritrophin.

FIGURE 6. Models depicting the relationship between tsetse’s PM and infection outcomes following challenge with exogenous microbes. The presence of an intact PM in tsetse’s gut creates endo and ectoperitrophic “microenvironments” that separate luminal contents (including exogenous bacteria and trypanosomes) from immune-competent epithelial cells. 

Discussion

The insect gut can be exposed to commensal microbes that are benign or beneficial to their host, as well as potentially harmful opportunistic pathogens and parasites. As such, the maintenance of immunological homeostasis in the insect gut likely reflects a delicate balance between host and microbial adaptations that ultimately favor host fitness. Specifically, insects must be able to tolerate the presence of beneficial gut bacteria, whereas selectively recognizing and mounting an efficacious immune response against potentially virulent microbes. Reciprocally, commensal bacteria must evade or tolerate local immune responses and persist without reducing host fitness, whereas pathogens must disseminate to new hosts. The relation between insect vectors and the mammalian...
parasites they transmit represents a different situation in that these organisms have likely coevolved over millions of years. This type of association may have resulted in the presentations of unique adaptations in both insect vector hosts and parasites that permit coexistence with as little harm as possible to both members of the relationship. Thus, the success of microbial survival in insects relies on different physiological mechanisms commensurate with the level of functional integration that the association represents.

Results we acquired using the tsetse model system indicate that both commensal and pathogenic bacteria elicit an immune response by gut EPs. However, the timing of this response is influenced by the structural integrity of the PM barrier, which initially separates microbes present in the gut lumen from immune-responsive EPs. While in tsetse’s gut lumen, Enterobacter and Serratia remain invisible to the fly’s immunogenic gut EP and thus proliferate unimpeded. After reaching high densities in this niche, both bacteria reposition into, and subsequently colonize, tsetse’s ECS. At this stage of infection, Enterobacter exhibits a benign phenotype, whereas Serratia translocates into the fly’s hemocoel and causes systemic disease. As such, tsetse’s PM barrier promotes Serratia pathogenicity. From the perspective of challenge with trypanosomes, the PM serves tsetse by severely restricting the establishment of fecundity-reducing parasite infections (34). However, coevolutionary processes may have resulted in host–parasite adaptations that facilitate successful parasite infection establishment, and thus transmission of the parasite to a subsequent mammalian host. The trypanosome-refractory phenotype exhibited by tsetse hosts suggests that parasite infections exert a more pronounced fitness cost on the fly than does the production of a highly effective PM. We summarize our data in models presented in Fig. 6, which depict the dynamics of gut colonization after challenge with bacteria and parasites.

The PM has been proposed to serve as a barrier that physically prevents Plasmodium and Leishmania from establishing infections in the gut of mosquitoes and sandflies, respectively (8, 35–38). More recent experimental evidence using the Drosophila model system indicates that the PM also mediates infection outcomes in this environment by regulating the induction of antimicrobial immune mechanisms (9). Our findings presented in this article support that tsetse’s PM does not function as a physical barrier that prevents exogenous microorganisms from establishing an infection in the fly’s gut. Instead, this structure mediates infection outcomes by temporally regulating immunological detection of microbe-associated molecular patterns (MAMPs) by host gut EP. This phenomenon may account for the differential infection outcomes we observed after oral challenge of tsetse with Enterobacter and Serratia. Specifically, proliferation of these bacteria in the gut was restrained when gut EPs detected the presence of foreign cells early in the infection process and responded via the production of IMD pathway–associated AMPs. Under these circumstances, normally pathogenic Serratia was maintained at tolerable densities within tsetse’s MG. A robust PM may further facilitate Enterobacter and Serratia colonization of tsetse’s ECS by serving as an acellular substrate on which to form immune-evasive biofilms. In fact, Vibrio cholerae forms defensive biofilms on Drosophila’s PM during its normal colonization program within the fly’s gut (39). Many additional enteric bacteria, including tsetse’s commensal symbiont Sodalis glossinidius, evade lethal gut-associated immune responses by hiding within protective biofilms (25, 40–42). As such, when tsetse lacks an intact PM, Enterobacter and Serratia may be unable to form biofilms and are thus exposed to host antimicrobial immune responses. Under these conditions, both bacteria are prohibited from proliferating.

The structural integrity of tsetse’s PM, and the fly’s refractoriness to trypanosome infection, increase as a function of adulthood postpupal eclosion (19). The higher parasite susceptibility presented by young adults has been largely attributed to the absence of a robust PM at this stage of host development (32). However, in this study, we also observed that teneral tsetse did not present an effective epithelial immune response after exposure to parasites. This outcome, which is similar to that observed in a previous study (20), suggests that the absence of an intact PM in teneral flies is likely not the sole factor for their high susceptibility to parasites. Instead, the teneral gut may also be immunologically immature, and thus unable to respond to exogenous microbes. Also of interest was the observation that PM-compromised adults conspicuously failed to express trypanocidal attacin after exposure to parasites. Assuming that the absence of an intact PM significantly decreases the time between trypanosome acquisition and recognition, the immunoreactive gut epithelial cells of PM-compromised flies would be exposed to MAMPs present on the surface of BSF parasites. During the natural course of infection, slender BSF parasites acquired in the blood meal are quickly eliminated and insect-stage-adapted stumpy BSF cells differentiate into procyclic forms (PFs) (43). Although PF trypanosomes initially circumvent the PM (via an unknown mechanism) and colonize the ECS, these infections are subsequently eliminated in the majority of tsetse at ~3 dpc (44). Our results suggest that this trypanolytic host response is mediated by IMD pathway effectors as opposed to ROIs. However, the tsetse–trypanosome interaction may be more complex, because parasites that can survive initial host responses eventually proliferate in tsetse’s gut despite the presence of host AMP expression (13). Trypanosomes that compose established infections may be either resistant to tsetse effectors or host immune responses maintain these parasites at densities that do not cause a harmful fitness reduction to infected flies. In support of the former theory, during the course of infection in tsetse’s gut PF, parasites switch from producing a surface coat composed of GPEET procyclins to one composed predominantly of immune-evasive EP molecules (45, 46). It remains to be seen whether host AMPs are differentially toxic to PF parasites that present different procyclin coats. Given that trypanosome infections persist in the MG of infected flies, presentation of a less antigenic surface coat may represent an adaptation that enables parasite survival in close proximity to immunocompetent host gut EPs (Fig. 6B). This adaptation may reflect long-term host–parasite coevolutionary processes (47), because tsetse is the obligate host through which African trypanosomes must pass to complete their life cycle. Thus, tsetse may exhibit resistance to infection with trypanosomes because trypanocidal molecules, including tsetse EP protein and PGRP-LB, are present in the ECS when parasites enter this niche (17, 18). Furthermore, differential expression of parasite MAMPs may regulate trypanolytic immune responses by the fly’s MG EPs.

The mucosal EP that comprises the vertebrate gut is separated from luminal contents by a mucous lining composed of mucin glycoproteins (48). Several lines of evidence suggest that the insect PM is structurally and functionally analogous to the vertebrate mucous layer. First, numerous insect peritrophins exhibit mucin-like structural properties in that they are highly glycosylated and encode chitin binding domains that are similar to those from their vertebrate counterparts (6). From a functional perspective, the mucous layer also plays a critical role in determining bacterial infection outcomes in the vertebrate gut. AMPs that are constitutively expressed by epithelial cells as a result of continuous exposure to resident commensal microbes are found within the mucous layer, or concentrated between it and the gut surface (49–
51). These immune molecules function by killing pathogenic bacteria that become trapped within, or manage to circumvent, the agglutinative mucous layer (52). In the vertebrate gut, the presence of a compromised mucous layer induces aberrant production of innate defense peptides upon exposure to microbial elicitors (53). We observed a similar phenomenon in tsetse in that flies presenting a structurally compromised PM expressed AMPs earlier after microbial challenge than did individuals in which the structure was intact. Interestingly, abnormal expression of immune genes in the vertebrate gut alters microbiota composition in a way that promotes colonization of the environment by pro-inflammatory bacteria that can induce disease (54). In contrast, results from this study suggest that a compromised PM may benefit tsetse when the fly is faced with a challenge by pathogenic bacteria. Specifically, in the absence of an intact matrix, we observed a significant increase in AMP expression that likely controlled proliferation of highly virulent *Serratia* in tsetse’s gut. Our results further the notion that the mucous layer and the PM are functionally similar structures that play a prominent role in regulating infection outcomes in the vertebrate and insect gut, respectively. The insect gut and PM may serve as an efficient alternative model system for deciphering the basic physiological mechanisms that mediate host–pathogen interaction at the mucosal surface of the vertebrate gut.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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Supplemental Data

Figures

Figure S1. dsRNA-mediated knockdown of genes characterized in this study.

Relative expression of target genes in control (GFP dsRNA) versus treatment (PM dsRNA) tsetse flies challenged with (A) *Enterobacter* or *Serratia*, (B) *Enterobacter* or *Serratia* following RNAi-mediated inhibition of tsetse’s IMD pathway, and (C) BSF *T. brucei*. (D) Relative *chitin synthase* expression in tsetse treated with either GFP or CS/P dsRNAs. These flies were subsequently challenged with either *T. brucei* alone, or co-challenged with *T. brucei* and *Serratia*\(^{\text{HK}}\) (see Figure 5B for experimental details). Gene expression in all cases is normalized relative to constitutively-expressed tsetse \(\beta\)-tubulin.
All quantitative measurements were performed in duplicate. In (A-C) target gene expression was significantly lower control versus treatment flies ($p<0.05$). In (D) *chitin synthase* expression is significantly lower in GFP dsRNA versus CS/P dsRNA treated tsetse ($p<0.05$). Four dsRNA treatment and control flies were sacrificed per experiment to determine (via qPCR) the efficiency of gene knockdown.
**Figure S2. PM-mediated antibacterial immunity in tsetse’s gut.** Tsetse’s PM mediates infection outcomes following challenge with commensal *Enterobacter* and entomopathogenic *Serratia*. When tsetse houses an intact PM (dsGFP and dsP1/2 treated individuals), the fly’s gut epithelium fails to immunologically detect and respond to the presence of these microbes. Under these circumstances both bacteria are able to rapidly multiply. Conversely, when tsetse presents a structurally compromised PM (dsCS and dsCS/P treated individuals), bacterial cells swiftly induce IMD pathway
activity that curtails microbial proliferation. In the absence of both a robust PM and functional IMD pathway (dsCS/LC treated individuals), *Enterobacter* and *Serratia* proliferate uncontrolled. FG, foregut; C, cardia; B, bacteriome; HL, hemocoel; HG, hindgut; PT, peritrophin; PM, peritrophic matrix.
Figure S3. PM status and tsetse’s susceptibility to trypanosomes. (A)
Trypanosome susceptibility of teneral tsetse. Wild-type teneral tsetse (Gmm\textsuperscript{WT/T}), which house an immature PM, are equally susceptible to infection with trypanosomes regardless of whether or not they were administered an infectious blood meal that also contains \textit{Serratia}\textsuperscript{HK}. (B) PM-mediated trypanocidal immunity in tsetse’s gut. The PM is functionally correlated with tsetse’s immune response following challenge with pathogenic trypanosomes. BSF trypanosomes promptly contact tsetse midgut cells in flies that lack an intact PM (treatment group 1). Interestingly, this association induces the production of ROI exclusively, but fails to prohibit the establishment of trypanosome infections in tsetse’s midgut. Conversely, in the absence of a PM, co-challenge with
trypanosomes and Serratia\textsuperscript{HK} induces potent attacin expression, and tsetse exhibits a parasite-refractory phenotype (treatment group 2). Teneral tsetse are highly susceptible to trypanosome infection even in the presence of Serratia\textsuperscript{HK} (Figure 5). This outcome indicates that Serratia extracts do not contain trypanocidal factors. Additionally, Gmm\textsuperscript{WT/T} flies likely results from the fact that they present an immature immune system that is incapable of producing a trypanocidal response (control group 1). Conversely, the same treatment of mature tsetse induces attacin, iNOS and DUOX expression, and a parasite-refractory phenotype (control group 2). FG, foregut; C, cardia; B, bacteriome; HL, hemocoel; HG, hindgut; PT, peritrophin; PM, peritrophic matrix.
## Supplemental Data

### Table

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