Interactions between mutualist Wigglesworthia and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission

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Tsetse flies, the sole vectors of African trypanosomes, have co-evolved with mutualistic endosymbiont Wigglesworthia glossinidiae. Elimination of Wigglesworthia renders tsetse sterile and increases their trypanosome infection susceptibility. We show that a tsetse peptidoglycan recognition protein (PGRP-LB) is crucial for symbiotic tolerance and trypanosome infection processes. Tsetse pgrp-lb is expressed in the Wigglesworthia-harboring organ (bacteriome) in the midgut, and its level of expression correlates with symbiont numbers. Adult tsetse cured of Wigglesworthia infections have significantly lower pgrp-lb levels than corresponding normal adults. RNA interference (RNAi)-mediated depletion of pgrp-lb results in the activation of the immune deficiency (IMD) signaling pathway and leads to the synthesis of antimicrobial peptides (AMPs), which decrease Wigglesworthia density. Depletion of pgrp-lb also increases the host's susceptibility to trypanosome infections. Finally, parasitized adults have significantly lower pgrp-lb levels than flies, which have successfully eliminated trypanosome infections. When both PGRP-LB and IMD immunity pathway functions are blocked, flies become unusually susceptible to parasitism. Based on the presence of conserved amidase domains, tsetse PGRP-LB may scavenge the peptidoglycan (PGN) released by Wigglesworthia and prevent the activation of symbiont-damaging host immune responses. In addition, tsetse PGRP-LB may have an anti-protozoal activity that confers parasite resistance. The symbiotic adaptations and the limited exposure of tsetse to foreign microbes may have led to the considerable differences in pgrp-lb expression and regulation noted in tsetse from that of closely related Drosophila. A dynamic interplay between Wigglesworthia and host immunity apparently is influential in tsetse's ability to transmit trypanosomes.

Beneficial symbiosis with maternally transmitted obligate and facultative mutualistic bacteria is common in insects from many different taxonomic groups. Obligate symbioses are often ancient in origin and indispensable for host physiological processes including fecundity. Although facultative mutualists are more recently associated with their hosts, they also confer important traits such as tolerance to environmental stress (1) and protection from natural enemies (2). Mechanisms that result in host tolerance to symbionts and that regulate symbiont density and invasion processes without reducing host fitness remain largely unknown. Also unknown is the influence of the symbionts on host immune physiology, which regulates the outcome of other parasitic infections. We used the tsetse fly, which harbors 3 symbionts and transmits the parasite African trypanosomes, as a model system to investigate the interactive dynamics of mutualism and parasitism.

Adult tsetse flies feed exclusively on vertebrate blood, which is largely free of microbes. Additionally, tsetse’s unique viviparous reproductive strategy where offspring develop in utero and acquire nutrients from mother’s milk, restricts the exposure of immature stages to a broad range of environmental microbes during a crucial period of immune system development. This is in contrast to closely related insect Drosophila, which reproduces on and consumes organic material undergoing decomposition by a wide variety of bacteria. To supplement their nutritionally restricted diet, tsetse harbors the obligate endosymbiont Wigglesworthia glossinidiae. Wigglesworthia resides within differentiated midgut cells that form an organ called the ‘bacteriome’ (3). Tsetse also harbors the commensal mutualist Sodalis glossinidius, which proliferates in the midgut and hemolymph (4). Both symbionts are vertically transmitted to the developing progeny via their mother’s milk secretions (5, 6). The densities of Sodalis and Wigglesworthia are tightly regulated during immature and adult development. One exception to this phenomenon occurs in young adults immediately posteclosion when both symbionts are allowed to undergo unregulated proliferation for a short duration (7). Young adults also show a high susceptibility to infective trypanosomes in contrast to older flies that exhibit high resistance (8). Recently we have been able to maintain tsetse cured of Wigglesworthia infections (GmmWig−) (5). In contrast to normal flies (GmmWT), older GmmWig− are unusually susceptible to parasitism (5). Although a role for host nutrient provisioning had been proposed for Wigglesworthia, its role in host immunity was previously unknown.

We focused on a family of conserved proteins, the peptidoglycan recognition proteins (PGRPs), which function in diverse processes by binding pathogen specific peptidoglycan (PGN) molecules. In Drosophila, upon microbial recognition some PGRPs (PGRP-SA, -SD, and -SC1) activate the Toll signal transduction pathway, whereas others (PGRP-LC and –LE) trigger the IMD pathway (9). Other PGRPs, such as Drosophila PGRP-SC1, -LB, and -SB1 and 1 of the 4 human PGRPs (PGLYRPs) have catalytic N-acetylmuramoyl-l-alanine amidase activities to scavenge free PGN. In doing so, the catalytic PGRPs prevent full-blown activation of host immune responses after exposure to environmental microbes (10, 11). In tsetse, immune induction has been shown to be costly and reduce fecundity (12). Still other PGRPs including the 3 human proteins and PGRP-SB1 from Drosophila and zebrafish embryos have been shown to have bactericidal activity (13, 14). Although symbionts also have PGN, the functions of host PGRPs in the context of symbiosis are unknown. In the weevil, Sitophilus zeamais, which also has mutualistic symbionts, pgrp-lb expression was detected in the bacteriome organ and was found to be up-regulated in the nymphal phase during a time when the symbionts are released from host cells (15, 16). In Drosophila, which lacks evolutionarily coevolved symbiotic partners, pgrp-lb is expressed in the fat body tissue, and typically immune challenge activates pgrp-lb expression via the NF-κB pathway (10).

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We investigated the role of PGRP-LB in symbiont density regulation and symbiotic tolerance and trypanosome infection processes in tsetse. Our data support the role for PGRP-LB in symbiont density regulation. Our data also suggest an additional role for PGRP-LB as an immune effector that enhances host fitness by eliminating costly parasite infections and by protecting tsetse’s indispensable mutualistic symbiosis with Wigglesworthia.

Results

PGRP-LB Is Expressed in the Bacteriome in Response to Wigglesworthia’s Proliferation. The putative tsetse PGRP-LB (GmmPGRP-LB) is a 24-kDa secreted protein with 70% identity to Drosophila LB, whereas PGRP-LC (GmmPGRP-LC) is 46 kDa with 61% identity to Drosophila LCx. Phylogenetic analysis of PGRP-LB proteins from tsetse, Drosophila, mosquitoes, and sand flies shows a distinct lineage, signifying their close ancestral relationship among Diptera (Fig. S1). Tsetse PGRPs have conserved N-terminal cytoplasmic and C-terminal ectodomains involved in PGN binding. Tsetse PGRP-LB has retained residues that function as zinc-ligands for N-acetylmuramoyl-t-alanine amidase activity associated with the catalytic activity of PGRPs (Fig. S2).

To understand the role of PGRPs in tsetse’s symbiotic homeostasis, we evaluated the spatial and temporal expression of pgrp-lb and pgrp-lc (Fig. 1 and Fig. S3). Spatial expression analysis indicated abundant and preferential synthesis of pgrp-lb in the bacteriome organ and in the fat body fraction of older females (Fig. 1 A and B). Because separating fat bodies from milk gland tubules during the dissection process can be difficult, pgrp-lb expression noted in the older female fat body tissues may originate from the female milk gland organ. The milk gland organ has also been shown to harbor free-living Wigglesworthia, which is transmitted to the intratuterine larva in mother’s milk (5). In contrast, transcripts for pgrp-lc, which encode the IMD receptor, are preferentially detected in the fat bodies of both male and female adults (Fig. S3). Temporal expression analysis indicated that pgrp-lb levels increase in females over the first 2 weeks post eclosion (Fig. 1C). The increasing pgrp-lb levels we noted parallel the increase observed during the same time-span in Wigglesworthia densities (7). To validate our laboratory-observed correlation between the Wigglesworthia densities and host pgrp-lb levels, we measured the symbiont genome copy number and pgrp-lb levels from dissected bacteriomes of a natural population of G. f. fuscipes (P = 0.019; Fig. 1D), indicating that the positive correlation observed in cultured lines is similar to the dynamics present in natural populations. We also showed that the bacteriomes of young and old GmmWig females, which have been artificially cured of their Wigglesworthia infections, do not display the increasing temporal profile and express significantly less pgrp-lb levels compared with normal controls (Fig. 1E). Based on spatial and temporal analysis, there appears to be a tight correlation between the host pgrp-lb levels and the presence and abundance of Wigglesworthia.

PGRP-LB Maintains Symbiotic Homeostasis in Tsetse. The host-symbiont interplay, which regulates symbiont densities for optimal host fitness outcomes, is largely unknown. The preferential expression of pgrp-lb in symbiont harboring tissues, the bacteriome and the milk gland, and the known amidase and antibacterial activities associated with PGRP-LB proteins suggested that tsetse PGRP-LB might play a role in symbiotic homeostasis. We analyzed the functional roles of PGRPs in vivo using a gene-specific double stranded (ds) RNA-based RNAi silencing methodology. We measured Wigglesworthia and Sodalis genome numbers 20 days after reduction of host pgrp-lc, pgrp-lb, and pgrp-lb/lc levels following treatment with corresponding dsRNAs (Fig. 2 A and B). No difference in the density of either symbiont was observed when pgrp-lc levels (IMD immune pathway functions) were reduced. In contrast, Wigglesworthia densities decreased significantly when pgrp-lb expression was inhibited. Given the putative role of PGRP-LB as the negative regulator of IMD in Drosophila, we speculated that Wigglesworthia density reduction in the absence of PGRP-LB might result from host immune activation. To validate host immune induction, we measured levels of the antimicrobial
peptide (AMP) attacin, synthesized downstream of tsetse’s IMD pathway (17). As predicted, we found significantly higher attacin levels in dsLB treated flies compared with normal controls (Fig. 2C). When both pgrp-lb and pgrp-lc were silenced, Wigglesworthia densities remained the same as those from the control groups. It appears that the decrease in Wigglesworthia numbers observed in the absence of PGRP-LB results from the bacteriocidal actions of the host AMPs. During homeostasis, tsetse PGRP-LB may contribute to a fine-tuned symbiotic density regulation process. It may directly function to regulate optimal Wigglesworthia densities via its putative bacteriocidal activity. PGRP-LB can also prevent the activation of damaging host innate immune responses by scavenging Wigglesworthia PGN, a byproduct of symbiont turnover.

Densities of the commensal symbiont Sodalis, which lies free in the gut milieu and hemolymph, do not appear to be regulated by PGRP-LB. Interestingly whereas tsetse’s immune effectors can harm Wigglesworthia, they do not impact Sodalis in vivo—a finding also supported by our previous studies where Sodalis was found to be highly resistant to the actions of tsetse’s AMPs Attacin (18) and Diptericin (19) in vitro.

Role of PGRP-LB in Innate Immune Activation. We next investigated the interplay between PGRP-LB and host innate immune activation following microbial challenge. We again used attacin expression as a measure of IMD pathway induction (Fig. 3). The levels of gene-specific silencing via dsRNA treatments were ≈87% for pgrp-lb and ≈80% for pgrp-lc (Fig. 3 B and C, respectively). As expected, silencing of pgrp-lc (IMD functions) prevented the downstream attacin expression upon E. coli challenge. Interestingly, pgrp-lb silencing led to significantly greater induction of attacin in challenged flies; a 17-fold increase was noted following E. coli challenge (Fig. 3D) and a 35-fold increase was noted after trypanosome acquisition (Fig. 3D). Our data indicate that loss of PGRP-LB renders the host immune system hypersensitive to pathogens, thus resulting in super activation of host immune functions in response to both bacteria and eukaryotic parasites. It appears that PGRP-LB in tsetse acts early in the infection process and clears pathogen-associated molecules, which typically induce the major host immune pathways.

Regulation of pgrp-lb Expression. In Drosophila, microbial challenge induces pgrp-lb expression in the fat body tissue (10). Furthermore, silencing of the NF-κB family transcription factor relish prevents pgrp-lb induction upon microbial challenge. In tsetse, we did not detect significant induction of pgrp-lb in the bacteriome upon E. coli challenge (Fig. 4A), although attacin expression in the fat body was induced (Fig. 4B). In addition, E. coli challenge of relish-silenced flies did not result in a decrease in bacteriome pgrp-lb levels, although attacin expression was reduced in the fat body tissue. The molecular signals that lead to increasing levels of pgrp-lb transcription in bacteriocytes in response to Wigglesworthia densities remain unknown. However, it appears that unlike Drosophila, pgrp-lb in the bacteriome is not under the regulation of the transcription factor Relish or the IMD pathway in tsetse.

Role of PGRP-LB in Parasitism. The lack of Wigglesworthia in Gmm/W1 results in an unusually high parasite infection prevalence, especially when infections are initiated in older adults; 80% midgut infection prevalence in Gmm/W1 compared with only 5% in age-matched Gmm/W2 (5). We showed that the pgrp-lb levels in the bacteriomes of young and old Gmm/W1 adults were significantly less than the Gmm/W2 counterparts, especially in older adults (Fig. 1E). The high susceptibility of Gmm/W1 to parasite infections may arise from low pgrp-lb levels. It is, however, not possible to rule out a role for other aspects of host physiology that may be perturbed in the absence of the highly integrated mutualistic symbiosis in Gmm/W1. We have been able to partially rescue the parasite resistance phenomenon in Gmm/W1 by provisioning flies PGN-supplemented blood meals before the parasite infection process. Such Gmm/W1 flies have higher pgrp-lb levels at the time of parasite acquisition and display greater parasite resistance than Gmm/W2 maintained on normal blood meals (Fig. S4). We had previously observed an increased parasite susceptibility in tsetse in the absence of IMD pathway functions (17). It is interesting that IMD pathway functions do not confer resistance to trypanosome infections in Gmm/W1. It is possible that the AMP effectors, activated by accumulating parasite immunogenic molecules, play a secondary role in infected flies in the parasite density regulation process. Given the role of pgrp-lb in tsetse’s IMD pathway induction and the high parasitism associated with lack of Wigglesworthia, we investigated the role of pgrp-lb in midgut trypanosome infections.

We provided a group of newly eclosed Gmm/W1 trypanosome-containing blood meal, and subsequently identified susceptible (parasitized) and resistant (self-cured) individuals. We then measured the expression of pgrp-lb from the bacteriomae of both groups. The resistant group had significantly higher pgrp-lb levels than their susceptible counterparts (Fig. 5A). In fact, the pgrp-lb levels observed between susceptible and resistant Gmm/W2 were similar to those observed between Gmm/W1 and Gmm/W2 adults. Our results suggest that pgrp-lb expression may influence trypanosome infection outcomes in tsetse, with the host parasitism resistance phenomenon correlating with increased pgrp-lb levels.

Finally, we evaluated the independent roles of PGRP-LB and IMD pathway constituents in the trypanosome transmission process. Midgut parasite infections were significantly more prevalent in flies when either the IMD pathway or PGRP-LB functions were reduced (Fig. 5B). Interestingly, infection outcome was dependant on the developmental stage of the parasite provided for infection establishment. While provisioning either procyclic or bloodstream form parasites gave rise to equally high midgut infection prevalence in the absence of pgrp-lb, bloodstream form parasites resulted in higher midgut infection prevalence in the absence of pgrp-lc. Our previous observation that infections with bloodstream form parasites induced a more robust host immune response supports our
current data that demonstrate tsetse’s immune system can differentially recognize distinct parasite developmental stages (19). Given that dsRNA treatments can only result in gene expression reduction (by ~80% in this case), the high infection prevalence we observed indicates that both IMD pathway effectors and PGRP-LB provide protection to Wigglesworthia in turn benefit host fitness by preventing trypanosome infections, that can activate host immune responses, which result in loss of fecundity and damage the mutualistic symbiosis.

Intracellular bacteriome-dwelling Wigglesworthia are required for tsetse to remain fertile, whereas extracellular forms present in the female milk glands are transmitted to the intrauterine progeny (5, 6). The host bacteriome is packed with as many as 108 bacterial cells (3). Interestingly, the streamlined genome of Wigglesworthia, which is only ~700 kb in size, encodes enzymes involved in LPS and PGN biosynthesis—products integral to the Gram-negative cell wall structure (20). The retention of membrane capabilities may confer the symbiont protection against host defenses, especially during transmission to the intrauterine progeny. Symbiont LPS and PGN can also, however, stimulate host immune responses leading to the synthesis of antimicrobial effectors. How can Wigglesworthia escape the bacteriocidal actions of its host immune responses and at the same time how does tsetse host regulate symbiont densities for optimal fitness outcomes? We show that pgrp-lb levels in the bacteriome are closely related to the number of Wigglesworthia harbored, a correlation observed both in the laboratory and in natural fly populations. When Wigglesworthia infections are artificially cured, significantly lower levels of pgrp-lb are detected in such bacteriomatomes. The high levels of pgrp-lb expression apparently provide protection to Wigglesworthia from hostile immune responses of its host. Loss of PGRP-LB functions reduce Wigglesworthia fitness as a consequence of the activation of the major host immune pathways and subsequent expression of symbiont damaging antibacterial effectors. On the basis of the conserved structure of the active site, tsetse PGRP-LB is predicted to have catalytic
Fig. 5. pgrp-lb levels and parasite infection prevalence. (A) pgrp-lb levels from dissected bacteriomes of normal (N), trypanosome infected (IF) and trypanosome resistant (RE) flies. Error bars indicate standard error (n = 5). (B) % parasite infection prevalence in flies treated with dsL and dsLB, respectively. (C) % parasite infection prevalence in dsLC/LB treated flies. P values indicate the level of significance between treatments.

zinc-dependent peptidoglycan-lytic amidase activity, which has been demonstrated biochemically for several other PGRP-LB homologs (10, 21). Digestion of pathogen released PGN with the amidase activity can reduce or eliminate the ability of polymeric PGN to stimulate insect immune pathways. Thus, protective function of PGRP-LB in the bacterioocytes can result from its ability to scavenge Wigglesworthia PGN released during cell division processes. In fact, loss of PGRP-LB scavenging activity would be undesirable for both partners as activation of the host immune responses by accumulating Wigglesworthia PGN can lead to significant reductions in host fecundity in the absence of Wigglesworthia. A study with mammalian TLRs also indicated their recognition of microbial ligands produced by intestinal commensal microflora (22). Thus, preventing full-blown activation of immunity in response to symbiont molecules may be part of a general requirement in animals for maintaining symbiotic homeostasis. Several PGRPs-LBs have been shown to have direct antimicrobial activities as well. Because Wigglesworthia lies free in the cytoplasm of the bacterioocytes that express pgrp-lb, a fine-tuned regulatory mechanism that relies on both the direct effector and PGN scavenging functions of PGRP-LB may regulate Wigglesworthia proliferation. It will be of interest now to show through biochemical experiments whether tsetse PGRP-LB protein exhibits an antibacterial activity.

Both laboratory lines and natural populations of tsetse demonstrate high resistance to trypanosome infections (8). Tsetse’s IMD pathway has been shown to play an important role in resistance to parasites. When levels of attacin, cecropin, and the IMD pathway regulating transcription factor relish were reduced by corresponding dsRNAs, tsetse had significantly higher midgut parasite infection prevalence (17, 23). In addition, bacteriocidal peptides, such as Dipterocin (19) and the recombinant Attacin, synthesized by the IMD pathway displayed trypanocidal activity both in vitro and in vivo in tsetse midgut (18). It was thus surprising that reduction of pgrp-lb, which increased IMD pathway activity, could increase the susceptibility of flies to parasite infections. Our study provides several lines of evidence that incriminate PGRP-LB’s role in parasite transmission. First, although reduction of pgrp-lb renders innate immune responses such as the IMD pathway hypersensitive to the presence of microbes (Fig. 3), such flies remain highly susceptible to trypanosome infections. In fact, parasite infection prevalence in pgrp-lb silenced flies, which expressed high levels of host AMPs, was comparable to those with suppressed AMP expression. Second, Wigglesworthia-free Gmm\textsuperscript{Wg\textsuperscript{-}} adults, which naturally express significantly less pgrp-lb, are highly susceptible to infections with trypanosomes (5). The age-dependent resistance to parasite infections typically observed in tsetse was not present in Gmm\textsuperscript{Wg\textsuperscript{-}}. Third, parasitized adults have significantly less pgrp-lb levels than resistant flies that have successfully cleared parasite infections. Finally, when both effectors, PGRP-LB and AMPs are reduced, flies display higher susceptibility to parasite infections, pointing to a synergistic impact of multiple effectors. Glycosylphosphatidylinositol (GPI) anchors are abundant molecules in trypanosome membranes and the biosynthesis of GPI is initiated by transfer of N-acetylgalcosamine (GlcNAc), a component of PGN. The amidase functions of PGRP-LB may also contribute to scavenging of trypanosome molecules released by division or dying parasites in the midgut milieu. In fact, absence of this scavenging activity apparently results in hyper stimulation of the host innate immune system. In addition we suggest that PGRP-LB secreted into the midgut milieu may have an antiparasitic activity effective against eukaryotic protozoa. Studies in humans have demonstrated antibacterial roles for all PGRPs, and in Drosophila bacteriocidal activity against limited microbes such as Bacillus megaterium has been shown for PGRP-SB1 (13). The observed variable susceptibility of natural flies to trypanosome infections may arise from varying Wigglesworthia numbers, which in turn result in different PGRP-LB levels present in the anterior midgut at the time of parasite acquisition. The age-dependent increase observed in PGRP-LB levels may also provide one explanation for the higher parasite susceptibility noted in newly eclosed flies whereas older adults are resistant. It is also possible, however, that expression of host pgrp-lb levels may be suppressed in parasite infected flies by a trypanosome factor. It is difficult to test retrospectively whether the parasitized flies had lower Wigglesworthia densities and pgrp-lb levels at the time of parasite acquisition. When we measured endogenous levels of pgrp-lb from groups of young and old adults, the percentage of flies that had low and high pgrp-lb levels were similar to the percentage of parasitized and resistant flies we typically obtain in each age cohort. This observation further supports our hypothesis that it is the level of the host PRGP-LB protein at the time of parasite acquisition that determines the infection outcome. It has been shown that the mammalian immune receptors TLR2 and TLR4 can be activated by trypanosome GPI anchors, and that TLR9 can recognize genomic DNA and subsequently trigger host immune responses (24). Thus, trypanosome GPI may also be involved in triggering the invertebrate host innate immune responses. In the presence of high parasitemia, both the scavenging and trypanolytic capacity of the PGRP-LB protein may be overwhelmed, thus provoking the activation of the major host immune pathways. It is likely that both the levels of the endogenous PGRP-LB regulated by Wigglesworthia density and the number of parasites ingested in the infectious blood meal act as influential determinants of infection outcome in tsetse. It is possible that the host antimicrobial functions may have a role in parasite density regulation once infections are established as our previous studies had indicated higher parasite intensity when the IMD pathway functions were abolished (17).

Our data demonstrate that PGRPs and host innate immune responses interact to regulate a fine-tuned balance for tolerance to indispensable mutualistic bacteria and resistance to pathogenic microbes. In this regulatory process, PGRP-LB appears to play multiple roles, first by scavenging pathogen or symbiont...
PGN to prevent full-blown activation of the host’s innate immune system that can be costly, and second by acting as a defense molecule in preventing trypanosome infection establishment and third, possibly by contributing to symbiotic density regulation. Further, chemical studies will provide insight into the mode of trypanolytic and antibacterial actions of tsetse PGRP-LB. Having exposure to a restricted microbial fauna as a result of strict hematophagous and viviparous physiology, tsetse flies provide a unique model to investigate mechanisms that enable tolerance to symbionts and resistance to foreign microbes.

**Materials and Methods**

**Insects and Microbes.** *G. m. morsitans* colony and the apomictic symbiont *GmmWG* line are maintained as described (5). *T. b. rhodesiense* (strain YTat1.1) bloodstream parasites (BsF) were obtained from a rat infection and stored as frozen stabiles for fly infections. *T. b. rhodesiense* procyclic cells (PcF) were maintained in SDM-70 medium (25). Natural populations of *G. f. fuscipes* were collected in 2008 spring in Tororo, Uganda.

**Real-time Quantitative PCR (qPCR).** For *pgrp-lb* expression, RNA was prepared from bacteriomes, midguts and fat bodies of normal flies at designated time points. RNA was also prepared from dissected bacteriomes of parasitized and self-cured *GmmWG* and *GmmWG* Tsetse *β-tubulin* was used for expression normalization. qPCR was performed with an iCycler IQ real time PCR detection system (Bio-Rad). Primer sequences are shown in Table S1. The normality of sample means from each treatment was determined by Shapiro-Wilk test before *t* test analysis. Values are represented as the mean (± SEM), and statistical significance was determined using a Student’s *t* test and Microsoft Excel software.

**Gene Silencing by dsRNA Treatments.** PCR amplimers tagged with T7 promoter sequences were used to synthesize dsRNAs as described (17). The cDNA clones *gmmpgrp-lb*, *gmmppgr-lb*, *gmmrelisch* (GenBank Accession No. DQ307161, DQ307160, and DQ177419), and plasmid eGFP (BD Biosciences) served as templates for amplification using gene specific primers (Table S1). The dsRNAs (8 μg/μL) were injected into 3-day-old adults as described (17).

**Symbiont Density Analysis.** Female flies were analyzed 20 days after dsRNA treatments. Total RNA and DNA were prepared using TRIzol reagent (Invitrogen) and analyzed by qPCR. Symbiont genome numbers were quantified by qPCR using *thiC* and *chi* primer sets for *Wigglesworthia* and *Sodalis*, respectively, and data were normalized to host *β-tubulin* (Table S1). For IMD path-

** IMD Pathway Regulation.** Four days after dsRNA treatment, males received an intrathoracic injection of 10⁷ *E. coli* DH5α cells in PBS or a blood meal supplemented with 4 × 10⁶/mL bloodstream stage *T. b. rhodesiense*. AMP attacin abundance was measured by northern analysis 2 days after bacteria challenge and 3 days after parasite challenge, respectively. Total whole fly RNAs were pooled from 3 flies for each analysis. For hybridizations, tsetse *β-tubulin* cDNA fragment was labeled and used as hybridization probe as described (17). Northern data were confirmed by 3 independent experiments and 1 representative dataset is presented.

**Trypanosome Infection Prevalence.** Fourteen days post infectious blood meal, flies were dissected and midgut parasite infection status was microscopically determined. Bloodstream or procyclic form parasites (4 × 10⁶/mL) were given to flies 4 days post single dsRNA (8 μg of dsRNA) injections. Bloodstream form parasites (1 × 10⁶/mL) were given to flies that received double dsRNA (4 μg of dsRNA each). Results from 5 independent experiments with high-dose parasite infections and 3 replicates in low-dose infections are shown. After conducting an arc sine transformation on the proportional infection data, a single-factor ANOVA was performed for each group. The analysis revealed no significant differences in infection prevalence between replicates (F < 0.05, P > 0.05). This affirmed the reliability of the experimental procedures and allowed for the replicates to be pooled. Differences were evaluated by Chi² analysis and were considered significant if P value < 0.05.

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Fig. S1.  Phylogenetic analysis of the PGRPs using T7 N-acetylmuramoyl-L-alanine amidase (T7 lysozyme) (P00806) as outroot. *G. m. morsitans* (GmmPGRP-LC: DQ307161 and GmmPGRP-LB: DQ307160), *D. melanogaster* (DmPGRP-LCx: NM_168324; DmPGRP-LCa:NM_140041; DmPGRP-LCy:NM_206308; DmPGRP-LE:AF313391; DmPGRP-SA:AF207541; DmPGRP-SC1b:AF207542 and DmPGRP-LB:NM_169393), *Apis mellifera* (AmPGRP-LC:XM_392452), *Homo sapiens* (HsPGRP4:AK292203), *Anopheles gambiae* (AgPGRP-LB:EAA01800) and *S. zeamais* (SzPGRP-LB:CN612423), and *Lutzomyia longipalpis* (LIPGRP:EU124614). Tree generation was done by MEGA3.1, and bootstrap analysis was performed for 5,000 replicates. PGRP-LC and PGRP-LB clusters are indicated in blue and red, respectively.
Fig. S2. Alignment of conserved PGRP domains from G. m. morsitans (GmmPGRP-LC: DQ307161, and GmmPGRP-LB: DQ307160) and PGRPs from Fig. S1 including D. melanogaster (DmPGRP-LCx; DmPGRP-LCa; DmPGRP-LCy; DmPGRP-LE; DmPGRP-SA; DmPGRP-SC1b; and DmPGRP-LB), Apis mellifera (AmPGRP-LC), Homo sapiens (HsPGRP43), An. gambiae (AgPGRP-LB), and S. zeamais (SzPGRP-LB). Three conserved PGRP domains are boxed in black and numbered. The highly conserved residues among all PGRP proteins are shown in black, conserved residues present in the recognition PGRPs and catalytic PGRPs are shown in light gray and dark gray shadow, respectively. Residues interacting with PGN are boxed in red. Residues required for amidase activity are indicated by a star.
Fig. S3. Tissue specific expression of pgrp-lc. pgrp-lc is preferentially expressed in the fat body fraction of both male and female adults. In females, pgrp-lc levels are significantly higher in the bacteriome (B) than in midgut (MG). pgrp-lc expression level was normalized by host β-tubulin.
Fig. S4. The effect of PGN supplementation of the blood meal diet on bacteriome pgpr-lb levels (A) and on parasite infection prevalence (B) in aposymbiotic Gmm\textsuperscript{Wig-} flies. (A) Groups of newly enclosed Gmm\textsuperscript{Wig-} were given either normal blood meals or blood meals supplemented with PGN (50 \mu g/mL) (Sigma). RNA was prepared from the dissected bacteriomes of 8-day-old Gmm\textsuperscript{Wig-} and corresponding normal blood receiving Gmm\textsuperscript{WT} adults. PGN provisioning resulted in a significant increase in the pgpr-lb levels of Gmm\textsuperscript{Wig-} flies than normal bloodmeal receiving Gmm\textsuperscript{Wig-} flies. The pgpr-lb levels were normalized by host tubulin and presented as fold change relative to wild type flies. Error bars, standard error (n = 5). (B) Groups of flies that received the same treatments as described above (with and without PGN supplementation) were given 1 infectious blood meal containing T. b. rhodesiense (2 \times 10^6/mL) on day 8. Flies were dissected and midguts were microscopically examined for parasite infections 14 days after infection acquisition. P values indicate the level of significance between treatments. Results indicate that Gmm\textsuperscript{Wig-} flies, which had higher pgpr-lb levels at the time of parasite acquisition as a result of PGN supplementation, are significantly more resistant to parasite infections that their Gmm\textsuperscript{Wig-} counterparts maintained on normal bloodmeal diets.
Table S1. Primer sets used for dsRNA preparation and qRT-PCR reactions

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer pair sequence</th>
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<tr>
<td>F, Forward</td>
<td>R, Reverse</td>
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<tr>
<td>dsLC</td>
<td>F: 5' TAATACGACTCACTATAGGGACTTATGCGCAACATGAACA 3'R: 5' TAATACGACTCACTATAGGGAACTTCTCCAGCTACTCTTCG 3'</td>
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<tr>
<td>dsLB</td>
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<tr>
<td>dsGFP</td>
<td>F: 5' TAATACGACTCACTATAGGGTCAGGAGGGTGGAAG 3'R: 5' TAATACGACTCACTATAGGCTAGTTGAACGGATCCATC 3'</td>
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<td>qPGRP-LC*</td>
<td>F: 5' CCAAGAGGAACCCCAATAAT 3'R: 5' CCAAGAGGAACCCCAATAAT 3'</td>
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<tr>
<td>qPGRP-LB*</td>
<td>F: 5' TCAATGATGGGGATGATAA 3'R: 5' GAACGATCACAAACACGAGA 3'</td>
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<td>qGmmattA</td>
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<tr>
<td>qGmmtub-β†</td>
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<tr>
<td>qPGRP-LC-2†</td>
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<tr>
<td>qGffPGRP-LB‡</td>
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<td>qthiC</td>
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<td>qchi</td>
<td>F: 5' TGGGAAGCTGCGTGAGGGAAG 3'R: 5' TCATAGGCGGGATGATAATTGAG 3'</td>
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<td>Wigglesworthia§</td>
<td>F: 5' ATGCCACCTTCAACGAC 3'R: 5' ATGCCACCTTCAACGAC 3'</td>
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<tr>
<td>Sodalis</td>
<td>F: 5' ATGCCACCTTCAACGAC 3'R: 5' ATGCCACCTTCAACGAC 3'</td>
</tr>
</tbody>
</table>

*Primer set used for qRT-PCR analysis in dsRNA treated flies.
†Primer set used to test gene silencing efficacy of dsRNA treatment.
‡Primer set used for qRT-PCR analysis of G. f. fuscipes.
§The same primer pair is used for Wigglesworthia density analysis in G. m. morsitans and G. f. fuscipes.