NIH Public Access

Author Manuscript

J Immunol. Author manuscript; available in PMC 2013 April 1.

Published in final edited form as:

J Immunol. 2012 April 1; 188(7): 3395–3403. doi:10.4049/jimmunol.1103691.

Obligate symbionts activate immune system development in the tsetse fly

Brian L. Weiss*, **Michele Maltz**, and **Serap Aksoy**

Department of Epidemiology and Public Health, Division of Epidemiology of Microbial Diseases, Yale University School of Medicine, LEPH 606, 60 College Street, New Haven, Connecticut 06520, USA

Abstract

Many insects rely on the presence of symbiotic bacteria for proper immune system function. However, the molecular mechanisms that underlie this phenomenon are poorly understood. Adult tsetse flies (*Glossina* spp.) house 3 symbiotic bacteria that are vertically transmitted from mother to offspring during this insect's unique viviparous mode of reproduction. Larval tsetse that undergo intrauterine development in the absence of their obligate mutualist, *Wigglesworthia*, exhibit a compromised immune system during adulthood. In this study we characterize the immune phenotype of tsetse that develop in the absence of all of their endogenous symbiotic microbes. Aposymbiotic tsetse (*Gmm*^{Apo}) present a severely compromised immune system that is characterized by the absence of phagocytic hemocytes and atypical expression of immunity-related genes. Correspondingly, these flies quickly succumb to infection with normally non-pathogenic *E. coli*. The susceptible phenotype exhibited by *Gmm*^{Apo} adults can be reversed when they receive hemocytes transplanted from wild-type donor flies prior to infection. Furthermore, the process of immune system development can be restored in intrauterine *Gmm*^{Apo} larvae when their moms are fed a diet supplemented with *Wigglesworthia* cell extracts. Our finding that molecular components of *Wigglesworthia* exhibit immunostimulatory activity within tsetse is representative of a novel evolutionary adaptation that steadfastly links an obligate symbiont with it's host.

INTRODUCTION

All metazoan life forms interact with prokaryotic organisms on a perpetual basis. These associations often result in a fitness advantage for one or both partners involved (1, 2). Insects represent a group of higher eukaryotes that harbor a well-defined bacterial microbiota. Unlike their mammalian counterparts, insects house less complex bacterial communities, are relatively inexpensive to maintain and produce large numbers of offspring in a short period of time. Several studies have demonstrated the importance of symbiotic bacteria as they relate to the proper function of their insect host's immune system. For example, *Drosophila* naturally infected with *Wolbachia* are protected (through an unknown mechanism) from several otherwise harmful RNA viruses (3). The malaria vector, *Anopheles gambiae*, is unusually susceptible to infection with *Plasmodium* parasites when they lack their commensal microbiota. In this case, symbiotic bacteria appear to mediate anti-*Plasmodium* immunity by activating basal expression of AMPs, inducing the production of phagocytic granulocytes, and directly generating anti-malarial reactive oxygen species (4, 5, 6).

*Corresponding author: Phone: (203) 737-4134; Fax: (203) 785-4782; brian.weiss@yale.edu.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Tsetse flies (*Glossina* spp.) harbor 3 symbiotic bacteria that regulate important aspects of their host's physiology. Two of these microbes, obligate *Wigglesworthia* and commensal *Sodalis*, are transferred to developing intrauterine progeny via maternal milk gland secretions (7). Tsetse's third symbiont, *Wolbachia*, is transferred via the germline (8). Tsetse that undergo intrauterine larval development in the absence of *Wigglesworthia* are immuno-compromised during adulthood. This phenotype is characterized by a significantly reduced population of phagocytic sessile and circulating hemocytes, and an unusual susceptibility to infection with pathogenic trypanosomes and normally non-pathogenic *E. coli* K12 (9, 10, 11). Further studies on the tsetse/*Wigglesworthia* symbiosis as it relates to host immunity have been obstructed by our inability to reconstitute symbiont-free flies with this bacterium.

In the present study we investigated the intimate relationship between immunity and symbiosis in tsetse by producing flies that underwent larval development in the absence of all endogenous microbes. We analyzed the immune system phenotype of aposymbiotic tsetse (Gmm^{Apo}) following microbial challenge, and investigated whether loss of immunity in Gmm^{Apo} flies could be rescued through either transfer of immune cells from healthy individuals or symbiont provisioning. We obtain results that reinforce the obligate nature of tsetse's relationship with *Wigglesworthia*, and provide further insights into the basic molecular mechanisms that underlie symbiont-induced maturation of host immunity.

MATERIALS AND METHODS

Tsetse and bacteria

G. morsitans morsitans were maintained in Yale's insectary at 24°C with 50–55% relative humidity. These flies received defibrinated bovine blood (Hemostat Laboratories) every 48 hours through an artificial membrane feeding system (12). Designations of all tsetse cohorts used in this study, the composition of their symbiont populations, and the treatments they received are described in Table 1.

Luciferase-expressing *E. coli* K12 (rec*E. coli*$_{pIL}$) were produced via transformation with construct pIL, which encodes the firefly luciferase gene under transcriptional control of *Sodalis' insulinase* promoter (13). The assay used to quantify rec*E. coli*$_{pIL}$ cells in vivo was performed as described previously (13). GFP-expressing *E. coli* K12 (*recE. coli*$_{GFP}$) were produced via electroporation with pGFP-UV plasmid DNA (Clontech). *Sodalis* were isolated from surface-sterilized *G. m. morsitans* pupae and cultured on *Aedes albopictus* C6/36 cells as described previously (14). *Sodalis*, which has a doubling time of approximately 24 h, were subsequently maintained cell-free *in vitro* at 25°C in Mitsuhashi-Maramorosch medium (1 mM CaCl2, 0.2 mM MgCl2, 2.7 mM KCl, 120 mM NaCl, 1.4 mM NaHCO3, 1.3 mM NaH2PO4, 22 mM D (+) glucose, 6.5 g/L lactalbumin hydrolysate and 5.0 g/L yeast extract) supplemented with 5% heat-inactivated fetal bovine serum (14).

Tsetse infections

Systemic challenge of tsetse was achieved by anesthetizing flies with CO_2 and subsequently injecting individuals with live bacterial cells using glass needles and a Narashige IM300 micro-injector. *Per os* bacterial challenges were performed by adding 500 colony forming units (CFU) of *E. coli* per 20 μl (the approximate amount consumed by a fly) of the total blood meal. The vertebrate host compliment system was heat-inactivated (56°C for 1 hr) prior to inoculating blood meals with bacterial cells. The number of bacterial cells injected or fed, control group designations, and sample size for all infection experiments are indicated in the corresponding figures and their legends.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Hemolymph collection and hemocyte quantification

Hemolymph collection from *Gmm*^{WT} and *Gmm*^{Apo} flies was performed using the high injection/recovery method as described previously (15). Subsequent determination of circulating hemocyte abundance was performed using a Bright-Line hemocytomter (11). Sessile hemocyte abundance was quantified by subjecting *Gmm*^{WT} and *Gmm*^{Apo} flies (n=3) to hemocoelic injection with blue fluorescent microspheres. 12 hr post-injection, flies were dissected to reveal tsetse's dorsal vessel (DV). Exposed tissue was rinsed 3 times with PBS to remove contaminating circulating hemocytes or any beads not engulfed by sessile hemocytes. The left-most panel is a Brightfield image of the 3 chambers that make up the DV (scale bar = 400 μm). The anterior-most chamber is indicated within a white circle, and the 2 remaining panels are the anterior chamber at higher magnification (scale bar = 80 μm). Engulfed beads were visualized microscopically by excitation with UV light (365/415 nm). Relative fluorescence, which was quantified using ImageJ software, represents the average amount of light emitted from 3 *Gmm*^{WT} and *Gmm*^{Apo} individuals.

Quantitative analysis of immunity-related gene expression

For quantitative real-time PCR (qPCR) analysis of immunity-related gene expression, whole flies were homogenized in liquid nitrogen and total RNA was extract using Trizol reagent (Invitrogen). Randomly-primed cDNAs were generated with Superscript II reverse transcriptase (Invitrogen), and qPCR analysis was performed using SYBR Green supermix and a Bio-Rad C1000 thermal cycler. Amplification primers are listed in Supplemental Table 1. Quantitative measurements were performed on 3 biological samples in duplicate and results were normalized relative to tsetse's constitutively expressed β-tubulin gene (determined from each corresponding sample). Fold-change data are represented as a fraction of average normalized gene expression levels in bacteria-infected flies relative to expression levels in corresponding uninfected controls. Values are represented as the mean (±SEM).

Hemolymph transplantation

Undiluted hemolymph was collected by removing one front fly leg at the joint nearest the thorax and then applying gentle pressure to the distal tip of the abdomen. Hemolymph exuding from the wound was collected using a glass micro-pipette and placed into a microfuge tube on ice. Four cohorts of newly emerged aposymbiotic recipient flies were used, 2 of which were designated *Gmm*$^{Apo/WT}$ or *Gmm*$^{Apo/Apo}$ based on whether they received hemolymph transplanted from WT or aposymbiotic donors, respectively. *Gmm*$^{Apo/WT}$ or *Gmm*$^{Apo/Apo}$ recipient flies received 1 μl of donor hemolymph (this volume represents approximately 1/3 of the total volume collected from donor flies). On day 8 post-transplantation, 3 of these flies were sacrificed to quantify hemocyte number using a Bright-Line hemocytometer. To separate *Gmm*^{WT} donor hemolymph into soluble and cellular fractions, samples were centrifuged at 3000 × g for 5 min. The cellular component was resuspended in a volume of chilled anticoagulant buffer [70% MM medium, 30% anticoagulant citrate buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, buffer pH 4.5), vol/vol; 15] equal to the total amount of hemolymph from which they were collected. The remaining 2 cohorts of *Gmm*^{Apo} recipient flies were injected with either 1 μl of cellular suspension (these flies are designated *Gmm*$^{Apo/Cell}$), or 1 μl of the soluble hemolymph fraction (these flies are designated *Gmm*$^{Apo/Sol}$).

All aposymbiotic recipient flies were challenged with either 10^3 CFU of live rec*E. coli*$_{pIL}$ or *recE. coli*$_{GFP}$. Injections were performed using glass needles and a Narashige IM300 micro-injector. Quantification of rec*E. coli*$_{pIL}$ in recipient tsetse was performed as described above. Phagocytic capacity of transplanted hemocytes was determined by infecting *Gmm*$^{Apo/Apo}$ recipient flies with 10^3 CFU of live rec*E. coli*$_{GFP}$. Twelve hours post-challenge, hemolymph

NIH-PA Author Manuscript

was collected from 3 individuals and hemocytes monitored for the presence of engulfed GFP-expressing bacterial cells. Hemolymph samples were fixed on glass microscope slides via a 2 min incubation in 2% paraformaldehyde. Prior to visualization using a Zeiss Axioscope microscope, slides were overlayed with VectaShield hard set mounting medium containing DAPI (Vector Laboratories).

Bacterial complementation experiments

A cartoon illustrating in detail how bacterial compliment experiments were performed is shown in Supplemental Fig. 1. Three cohorts (*n*= 120 individuals/group) of pregnant female tsetse were fed a diet containing tetracycline (40 μg/ml of blood) every other day for 10 days. Additionally, throughout the course of the entire experiment, all blood meals (3 per week) also contained vitamin-rich yeast extract (1% w/v) to restore fertility associated with the absence of *Wigglesworthia* (16). Ten days post-copulation, 2 cohorts of symbiont-cured females were regularly fed a diet supplemented with *Wigglesworthia* and *Sodalis* cell extracts. By timing treatments in this manner, larvae from the 1st gonotrophic cycle (GC) went through most of their development in the absence of bacterial compliment while those from the 2nd and 3rd GCs developed in the presence of bacterial compliment. Offspring of these females were designated *Gmm*$^{Apo/Wgm}$ and *Gmm*$^{Apo/Sgm}$, respectively. *Wigglesworthia* was obtained by dissecting tsetse bacteriomes (an organ immediately adjacent to the midgut that houses this bacteria) from *Gmm*^{WT} females, while *Sodalis* was maintained in culture as describe above. *Gmm*$^{Apo/Wgm}$ females were fed 1 bacteriome equivalent per 4 females, and *Gmm*$^{Apo/Sgm}$ females were fed 4×10^7 *Sodalis* per ml of blood (these flies thus ingested ~1×10^6 *Sodalis* each time they fed). A 3rd control cohort of symbiont-cured females received no bacterial compliment (their offspring are designated *Gmm*$^{Apo/NB}$), and a 4th cohort of wild-type offspring (*Gmm*^{WT}) served as another control. To confirm the aposymbiotic status of offspring from symbiont-cured moms (Supplemental Fig. 2), genomic DNA was extracted from larval offspring (3rd instar; *n*= 3) of all experimental cohorts using the Holmes-Bonner method (17). PCR (20μl reactions) was performed in an MJ Research thermalcycler using bacteria-specific primers (Supplemental Table 1) and the following cycle program: 95°C for 5 min. followed by 30 cycles at 95°C, 55°C and 72°C, each for 1 min, and a final 7 min elongation/extension at 72°C.

To determine whether complimenting symbiont-cured moms with bacterial cell extracts impacted the immune system phenotype of their offspring, qPCR was used (as described above) to monitor the expression of *serpent* and *lozenge* in larvae (1st, 2nd and 3rd instar) from each of 3 gonotrophic cycles (GC; *n*=3 individuals per group per GC). All remaining offspring were allowed to mature to adulthood. At this time 3 individuals from each cohort and GC were taken to determine circulating hemocyte abundance (as described above). Furthermore, qPCR was used to compare immunity-related gene expression in *E. coli*-challenged *Gmm*$^{Apo/Wgm}$ and *Gmm*$^{Apo/Sgm}$ individuals (*n*=3) from the 2nd GC of symbiont-cured moms. Finally, all remaining mature adult offspring were challenged with 10^3 CFU of live *recE. coli*$_{GFP}$. Twelve hours post-challenge, hemolymph was collected and monitored to determine if hemocytes had engulfed GFP-expressing bacterial cells (*n*=3 individuals per group per GC). Hemolymph samples were fixed and visualized as described above.

Stats

Statistical significance between various treatments, and treatments and controls, is indicated in the corresponding figure legends. Survival curve comparisons were made by log-rank analysis using JMP (v9.0) software (www.jmp.com). Statistical analysis of qPCR data and hemocyte abundance was performed by Student's t test using Microsoft Excel software.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

RESULTS

Aposymbiotic tsetse exhibit atypical hallmarks of cellular and humoral immunity

A positive correlation exists between the proper function of an insect's immune system and the dynamics of it's microbiome (18). In an effort to better define the relationship between symbiosis and immunity in tsetse, we fed pregnant female's on a diet supplemented with tetracycline and yeast. This antibiotic treatment clears all symbionts from the flies, while the vitamin-rich yeast extract rescues the loss of fertility associated with the absence of obligate *Wigglesworthia* (9, 16). We then investigated whether offspring that underwent intrauterine development in the absence of all symbiotic bacteria (Gmm^{Apo}) exhibited an immune system phenotype during adulthood that was different from that of their WT counterparts that developed in the presence of their complete microbiome. To do so, we began by quantifying the number of circulating and sessile hemocytes present in 8 day old adult (hereafter referred to as `mature) Gmm^{WT} and Gmm^{Apo} flies. Our results indicate that mature WT tsetse harbor 113× more circulating hemocytes per μl of hemolymph than do their aposymbiotic counterparts (Gmm^{WT}, 793 ± 34 hemocytes per μl of hemolymph; Gmm^{Apo}, 7 ± 1 hemocytes per μl of hemolymph; Fig. 1A). To determine the functional relationship between symbiont status and sessile hemocyte abundance, we thoracically micro-injected WT and aposymbiotic adults with fluorescent microspheres. In both tsetse and *Drosophila* sessile hemocytes concentrate in large quantities around the anterior chamber of the fly's dorsal vessel (11, 19). Thus, we indirectly quantified sessile hemocyte number by measuring the fluorescent emission of injected microspheres that were found engulfed in this region. We observed that mature Gmm^{WT} flies engulfed 16× more microspheres than did age-matched Gmm^{Apo} individuals (Fig. 1B and Supplemental Fig. 3).

Previously we determined that several genes associated with humoral, cellular and epithelial immune pathways, including those that encode the antimicrobial peptides (AMPs) attacin and cecropin, as well as thioester-containing proteins (tep2 and tep 4), prophenoloxidase and inducible nitric oxide synthase (iNOS), were expressed at significantly lower levels in Gmm^{Wgm-} compared to Gmm^{WT} flies following infection with *E. coli* (11). In the present study we monitored expression of these same genes in age-matched Gmm^{WT} and Gmm^{Apo} flies that were either unchallenged or 3 days post-challenge (dpc) with *E. coli* K12. Furthermore, we also evaluated the expression of *peptidoglycan recognition protein LB* (*PGRP-LB*), *caudal*, *domeless* and *dual oxidase* (*DUOX*). In tsetse and closely-related *Drosophila*, PGRP-LB and caudal serve as negative regulators of NF-kappaB-dependent antimicrobial peptide expression (10, 20, 21), while domeless is a cytokine receptor that regulates expression of *tep4* through the `Janus Kinase Signal Transduction and Activator of Transcription' signaling pathway (22, 23). Finally, in *Drosophila* and mosquitoes, DUOX is involved in generating infection-induced antimicrobial reactive oxygen species (24, 25, 26).

Our expression analysis indicates that the presence of symbiotic bacteria during larval development induce basal immunity in tsetse. Specifically, we observed that *DUOX*, *domeless* and *caudal* are expressed at significantly lower levels in mature unchallenged Gmm^{Apo} compared to Gmm^{WT} flies (Fig. 1C, left graph). Following *per os* challenge with *E. coli*, no significant difference in immunity-related gene expression (with the exception of *iNOS*) was observed between Gmm^{WT} and Gmm^{Apo} flies (Fig. 1C, middle graph). However, systemic challenge resulted in a significant difference in the expression of all the genes we analyzed. Most notably, pathways associated with cellular immunity were significantly down-regulated in Gmm^{Apo} compared to Gmm^{WT} individuals, while those associated with humoral immune responses were significantly up-regulated (Fig. 1C, right graph). These findings indicate that tsetse's symbiotic bacteria are closely associated with the development of their host's immune system during larval maturation, and it's subsequent proper function in unchallenged and *E. coli* challenged adults.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Aposymbiotic tsetse are highly susceptible to normally non-pathogenic *E. coli*

We next determined whether *Gmm*^{Apo} individuals are more susceptible to challenge with *E. coli* than are WT tsetse or tsetse that lack only *Wigglesworthia* (*Gmm*$^{Wgm-}$). To do so we compared percent survival of mature adults from these 3 tsetse lines following systemic challenge with *E. coli* K12. We determined that 67% of mature *Gmm*^{WT} individuals, and 59% of mature *Gmm*$^{Wgm-}$ individuals, survived systemic challenge with 10^3 CFU of *E. coli* (Fig. 2A, top and middle panels). In contrast, all age-matched *Gmm*^{Apo} individuals perished by 12 dpc (Fig. 2A, bottom panel). We next challenged *Gmm*^{WT} and *Gmm*^{Apo} flies *per os* with 10^3 and 10^6 CFU of *E. coli*, and found that all individuals survived this challenge (Fig. 1A, top and bottom panels). This finding suggests that mature *Gmm*^{Apo} flies are considerably more susceptible to systemic challenge with a foreign microbe than are age-matched *Gmm*^{WT} and *Gmm*$^{Wgm-}$ individuals. Furthermore, tsetse's ability to overcome *per os* challenge with *E. coli* appears to be independent of symbiont status.

To determine a cause for the variation in survival we observed between *Gmm*^{WT}, *Gmm*$^{Wgm-}$ and *Gmm*^{Apo} individuals following challenge with *E. coli*, we monitored the dynamics of bacterial growth in each of these fly groups over time. When fed *E. coli*, both mature aposymbiotic and WT individuals cleared all *E. coli*. Following systemic challenge with 10^3 CFU of *E. coli*, bacterial densities within mature *Gmm*^{WT} flies reached 8.3×10^3 cells before being cleared. Interestingly, *Gmm*$^{Wgm-}$ flies, which perish following challenge with 10^6 CFU of *E. coli* (11), were able to clear all exogenous bacterial cells following challenge with this lower dose. On the other hand, bacterial density in *Gmm*^{Apo} flies peaked at 7.8×10^6 on day 6 post-challenge, after which all flies soon perished (Fig. 2B). This observation suggests that aposymbiotic tsetse were unable to control systemic infection with *E. coli* and thus likely perished as a result of their inability to tolerate high densities of this bacterium in their hemolymph. These findings taken together indicate that *Gmm*^{Apo} flies are significantly more susceptible to challenge with *E. coli* than are WT flies and flies that lack only *Wigglesworthia*.

Hemocyte transfer from WT tsetse restores the ability of *Gmm*^{Apo} adults to overcome infection with *E. coli*

We next set out to provide a definitive correlation between tsetse hemocytes and the fly's ability to overcome challenge with a foreign microbe. To do so we transplanted hemolymph from mature *Gmm*^{WT} and *Gmm*^{Apo} individuals (donor flies) into the hemocoel of susceptible *Gmm*^{Apo} flies (recipient flies are hereafter designated *Gmm*$^{Apo/WT}$ and *Gmm*$^{Apo/Apo}$, respectively). Five days after this procedure we determined that *Gmm*$^{Apo/WT}$ flies harbored 330 (± 20.4) hemocytes per μl of hemolymph, while *Gmm*$^{Apo/Apo}$ flies harbored 5 (± 3.8) hemocytes per μl of hemolymph (Fig. 3A). We next investigated whether our hemolymph transplantation procedure was able to rescue the *E. coli*-susceptible phenotype exhibited by *Gmm*^{Apo} flies. To do so we challenged *Gmm*$^{Apo/WT}$ and *Gmm*$^{Apo/Apo}$ individuals with 10^3 CFU of *E. coli* 3 days post-hemolymph transplantation, and subsequently monitored their survival over time. Our results indicate that 72% of *Gmm*$^{Apo/WT}$ flies survived for 14 days following challenge. In comparison, only 2% of *Gmm*$^{Apo/Apo}$ flies survived their challenge (Fig. 3B, top panel). These results demonstrate that *Gmm*^{Apo} flies are able to clear a systemic challenge with *E. coli* after they receive a transplant of hemolymph from WT donors.

We next investigated whether hemocytes, or a soluble antimicrobial or signaling molecule present in the transplanted hemolymph was responsible for restoring the resistant phenotype exhibited by recipient individuals. To address this issue we collected hemolymph from WT donors, separated it into soluble and cellular fractions by centrifugation, and then transplanted the separate fractions into 2 distinct groups of *Gmm*^{Apo} flies. Finally, 3 days

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

later we systemically challenged both groups of recipient flies with 10^3 CFU of *E. coli* K12. All aposymbiotic flies that received the soluble fraction of hemolymph from *Gmm*$^{\mathrm{WT}}$ donors (*Gmm*$^{\mathrm{Apo/Sol}}$) perished by day 12 post-challenge. In comparison, 62% of *Gmm*$^{\mathrm{Apo}}$ recipients that received the cellular fraction of hemolymph from *Gmm*$^{\mathrm{WT}}$ donors (*Gmm*$^{\mathrm{Apo/Cell}}$) survived for 14 days following bacterial challenge (Fig. 3B, bottom panel). These host survival curves indicate that *Gmm*$^{\mathrm{Apo}}$ flies survive challenge with *E. coli* when they had previously received a transplant of hemocytes, as opposed to soluble hemolymph molecules, from WT tsetse.

To determine a cause for the variation in survival we observed between these 2 groups, we monitored the dynamics of bacterial growth in each group over the course of the experiment. *E. coli* within *Gmm*$^{\mathrm{Apo/Apo}}$ flies replicated exponentially until a peak density of 2.1×10^7 was reached at 6 dpc. This finding suggests that bacterial sepsis was the cause of high mortality we observed in this group of flies. In contrast, aposymbiotic recipients were able to clear all *E. coli* by 8 dpc when they had previously received a hemolymph transplant from *Gmm*$^{\mathrm{WT}}$ donors (Fig. 3C). More so, microscopic examination of hemolymph from *Gmm*$^{\mathrm{Apo/WT}}$ flies showed that transplanted hemocytes engulfed the introduced *E. coli* (Fig. 3D). Our results demonstrate that immune resistance can be restored in adult aposymbiotic tsetse if they harbor hemocytes transplanted from their WT counterparts.

Supplementation of *Wigglesworthia* to symbiont-cured females restores immune system development in aposymbiotic offspring

Previous experiments revealed that the milk gland population of tsetse's obligate symbiont, *Wigglesworthia*, must be present during the development of immature stages in order for subsequent adults to exhibit a functional cellular immune system (11). To date we have been unable to culture *Wigglesworthia* and thus can not recolonize aposymbiotic flies with this bacterium. To circumvent this impediment we tested whether we could restore the process of immune system development in *Gmm*$^{\mathrm{Apo}}$ offspring by supplementing the diet of pregnant, symbiont-cured females with *Wigglesworthia*-containing extracts of bacteriome tissue collected from WT females. A detailed description of the experimental design we used to test this theory is provided in the Materials and Methods and Supplemental Fig. 1.

In brief, two treatment cohorts of pregnant *Gmm*$^{\mathrm{WT}}$ females were fed a diet supplemented with tetracycline and yeast extract (16). Ten days post-copulation, these symbiont-cured females began receiving either *Wigglesworthia* or *Sodalis* cell extracts in every blood meal. The immune system phenotype of offspring from these females (*Gmm*$^{\mathrm{Apo}/Wgm}$ and *Gmm*$^{\mathrm{Apo}/Sgm}$, respectively) was compared to that of control cohort offspring from symbiont-cured moms that received no bacterial supplement (*Gmm*$^{\mathrm{Apo/NB}}$) and offspring from *Gmm*$^{\mathrm{WT}}$ moms. We first evaluated the relative abundance of transcripts that encode the transcription factors `Serpent' and `Lozenge'. In *Drosophila*, these molecules direct hemocyte differentiation, or hematopoiesis, during embryogenesis and early larvagenesis (27). In tsetse, larvae that develop in the absence of *Wigglesworthia* express significantly less *serpent* and *lozenge* than do their WT counterparts (11). In the present study we found that *Gmm*$^{\mathrm{Apo}/Wgm}$, *Gmm*$^{\mathrm{Apo}/Sgm}$ and *Gmm*$^{\mathrm{Apo/NB}}$ larva from the 1$^{\mathrm{st}}$ gonotrophic cycle (GC) expressed significantly less *serpent* and *lozenge* than did *Gmm*$^{\mathrm{WT}}$ larva. However, after the onset of bacterial supplementation, *Gmm*$^{\mathrm{Apo}/Wgm}$ and *Gmm*$^{\mathrm{WT}}$ larva from the 2$^{\mathrm{nd}}$ and 3$^{\mathrm{rd}}$ GCs expressed comparable levels of *serpent* and *lozenge*, while *Gmm*$^{\mathrm{Apo/NB}}$ and *Gmm*$^{\mathrm{Apo}/Sgm}$ larva expressed less (Fig. 4A).

Because *serpent* and *lozenge* expression can be indicative of hematopoiesis, we next compared the number of hemocytes present in *Gmm*$^{\mathrm{Apo}/Wgm}$ adults to that found in age-matched *Gmm*$^{\mathrm{WT}}$, *Gmm*$^{\mathrm{Apo/NB}}$ and *Gmm*$^{\mathrm{Apo}/Sgm}$ flies. We found that the provisioning of *Wigglesworthia* extracts to symbiont-cured females resulted in an increase in the number of

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

circulating hemocytes present in their offspring. Specifically, hemocyte density in *Gmm*$^{Apo/Wgm}$ adults from GCs 2 and 3 was significantly greater (113 ± 33 and 127 ± 21 hemocytes/μl of hemolymph, respectively) than that found in age-matched *Gmm*$^{Apo/NB}$ (7 ± 3 and 9 ± 4 hemocytes/μl of hemolymph, respectively) and *Gmm*$^{Apo/Sgm}$ flies (10 ± 4 and 4 ± 1 hemocytes/μl hemolymph, respectively), but significantly less than that of *Gmm*^{WT} adults (733 ± 104 and 681 ± 68 hemocytes/μl hemolymph, respectively; Fig. 4B). Correspondingly, we observed that prophenoloxidase and *tep4*, which are expressed predominantly by hemocytes (28, 29), are found at significantly higher levels in adult *Gmm*$^{Apo/Wgm}$ compared to *Gmm*$^{Apo/Sgm}$ adults (from GC2) following systemic challenge with *E. coli* (Fig. 4C). A similar pattern was observed with genes involved in the generation of reactive oxygen species (*DUOX* and *iNOS*). Interestingly, humoral immunity-associated genes (AMPs and their regulators) were expressed at similar levels in *E. coli*-challenged *Gmm*$^{Apo/Wgm}$ and *Gmm*$^{Apo/Sgm}$ adults.

Our results suggest that feeding symbiont-cured moms a diet supplemented with *Wigglesworthia* cell extracts induces a physiological response that partially restores immune system development in their aposymbiotic offspring. Specifically, *Gmm*$^{Apo/Wgm}$ larvae exhibit increased expression of the hematopoietic transcription factors *serpent* and *lozenge*, and as adults these flies present a functional immune system characterized by the presence of circulating phagocytic hemocytes. Furthermore, the expression of genes involved in epithelial and cellular immunity is enhanced in *Gmm*$^{Apo/Wgm}$ adults.

Gmm$^{Apo/Wgm}$ flies are resistant to *E. coli* challenge

We observed that *Gmm*$^{Apo/Wgm}$ offspring exhibit hallmarks of enhanced immunity. Thus, we next tested whether mature *Gmm*$^{Apo/Wgm}$ adults would be resistant to systemic challenge with *E. coli* K12, while age-matched *Gmm*$^{Apo/Sgm}$ and *Gmm*$^{Apo/NB}$ flies, would not. To this end, we observed that 38% and 43% of *Gmm*$^{Apo/Wgm}$ adults from GCs 2 and 3, respectively, survived challenge with 10^3 *E. coli* (Fig. 5A). Correspondingly, microscopic inspection of hemolymph from *E. coli*-resistant *Gmm*$^{Apo/Wgm}$ adults revealed the presence of phagocytic hemocytes that harbored internalized *E. coli* cells (Fig. 5B). In contrast, *Gmm*$^{Apo/NB}$ and *Gmm*$^{Apo/Sgm}$ flies were highly susceptible to *E. coli* challenge, and like their *Gmm*^{Apo} counterparts, all perished within the 14 day experimental period (Fig. 5A). This susceptible phenotype likely resulted from the fact that *Gmm*$^{Apo/NB}$ and *Gmm*$^{Apo/Sgm}$ adults are devoid of phagocytic hemocytes (Figs. 4B and 5B). These findings suggest that aposymbiotic tsetse can survive infection with an otherwise lethal dose of *E. coli* if they complete intrauterine development while their moms were fed a diet containing *Wigglesworthia* cell extracts. This immuno-competent phenotype exhibited by *Gmm*$^{Apo/Wgm}$ adults likely results from the presence of phagocytic hemocytes in their hemolymph.

DISCUSSION

Symbiotic bacteria are gaining increased recognition as potent modulators of insect immunity (18, 30). In the present study we provide evidence that tsetse's symbiotic bacteria are intimately associated with the maturation of their host's immune system during juvenile development and it's subsequent proper function during adulthood. We determined that aposymbiotic (*Gmm*^{Apo}) flies derived from symbiont-cured moms present a severely compromised cellular immune system, and as such are highly susceptible to systemic infection with normally non-pathogenic *E. coli*. This immuno-compromised phenotype can be reversed when *Gmm*^{Apo} adults receive hemocytes transplanted from WT individuals. Furthermore, the process of immune system development in *Gmm*^{Apo} larvae can be restored when their symbiont-cured moms are fed a diet supplemented with *Wigglesworthia* cell extracts. Our results demonstrate that evolutionary time has stably anchored the obligate association between tsetse and *Wigglesworthia* such that this bacterium directly engenders

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

immunity, and thus ultimately the fecundity, of it's host. In return, tsetse provides *Wigglesworthia* with a protective and metabolite-rich niche that has enabled this bacterium to survive in this environment for at least 50 million years (31).

Tsetse that undergo intrauterine larval development in the absence of only *Wigglesworthia* (*Gmm*$^{Wgm-}$) exhibit a compromised immune system that, when compared to WT flies (*Gmm*^{WT}), is characterized by a 70% reduction in the number of phagocytic hemocytes (11). In the present study we found that eliminating all symbiotic bacteria from female tsetse markedly enhances the immuno-compromised phenotype of their offspring. In fact, *Gmm*^{Apo} adults harbor virtually no circulating (99% less than *Gmm*^{WT} adults) or sessile hemocytes and are correspondingly more susceptible to systemic infection with *E. coli* than are WT tsetse and tsetse that lack only *Wigglesworthia*. *Gmm*$^{Wgm-}$ flies, which undergo intrauterine maturation in the presence of *Sodalis* and *Wolbachia*, house approximately 40× more circulating hemocytes than do their aposymbiotic counterparts and are more tolerant to *E. coli* challenge. (11). The enhanced immunity exhibited by *Gmm*$^{Wgm-}$ individuals in comparison to their aposymbiotic counterparts suggests that the presence of *Sodalis* and *Wolbachia* during intrauterine development may induce a limited degree of immune system maturation in their tsetse host. Although no experimental evidence exists that demonstrates a functional role of this nature for *Sodalis, Wolbachia* exhibits immuno-modulatory properties in other insect models. For example, *Drosophila* treated with antibiotics to clear their *Wolbachia* infections are significantly more susceptible to a range of RNA viruses (32, 33). Furthermore, the mosquito *Aedes aegypti* can be stably transinfected with an exogenous strain of *Wolbachia* (*w*MelPop; 34). The presence of *w*MelPop appears to activate the immune system of offspring from transinfected females, which subsequently exhibit enhanced immunity against a range of pathogens (35, 36). Interestingly, unlike our laboratory colony, many natural populations of tsetse do not harbor *Sodalis* and/or *Wolbachia*, but are apparently still immuno-competent (37). It remains to be seen whether these symbionts play a role in stimulating immune system development in natural populations of tsetse.

Many insects, including *Drosophila, Anopheles* and *Manduca*, likely rely on their cellular immune systems as a potent first line of defense against systemic infection with pathogenic bacteria (38, 39, 40, 41). Similarly, tsetse become susceptible to infection with *E. coli* after their hemocyte function is abrogated via the uptake of polystyrene microspheres (11). In this study we provide further evidence that tsetse's ability to overcome systemic infection with *E. coli* also depends on the presence of a functional cellular immune system. First, *E. coli* kills *Gmm*^{Apo} adults despite the fact that they express dramatically more of the AMPs *cecropin* and *attacin* than do resistant WT flies. This finding suggests that AMPs alone are insufficient for tsetse to overcome systemic infection with *E. coli*. Secondly, *Gmm*^{Apo} adults survive this same infection if they had previously received hemolymph transplanted from WT donors. However, when WT donor hemolymph was separated into cellular and soluble fractions prior to transplantation, only *Gmm*^{Apo} recipients that received the cellular fraction (hemocytes) exhibited an *E. coli*-resistant phenotype. Thus, hemolymph-soluble factors such as an AMPs, hematopoeitic molecules or reactive oxygen species, presumably do not induce *E. coli* resistance when transplanted into *Gmm*^{Apo} flies. Instead resistance appears fixed to the cellular immunity-related activity of hemocyte-mediated phagocytosis.

Beneficial microbes in the human gut produce symbiosis factors that, unlike disease-causing virulence factors produced by pathogenic microbes, promote favorable health-related outcomes (42). For example, the human commensal, *Bacteroides fragalis*, produces one such molecule called polysaccharide A (PSA). Colonization of germ-free mice with this bacterium restores $CD4^+$ T cell populations to levels conventionally found in mice that house their native microbiota. This process is consistent with *B. fragalis* PSA-induced

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

development of secondary lymphoid tissues. *B. fragalis* PSA mutants fail to induce these systemic responses in germ-free animals (43). Similarly, mouse intestinal microbiota serve as a source of peptidoglycan (PGN) that enhances the efficacy of phagocytic neutrophils against pathogenic bacteria (44). To date no immuno-modulatory symbiosis factors have been characterized from insect-associated microbes. In this study we demonstrated that immune system development in Gmm^{Apo} larvae was activated when their moms were fed a diet supplemented with extracts of *Wigglesworthia* cells. This finding suggests that a molecular component of this obligate bacterium can actuate a trans-generational priming response in the intrauterine larvae of symbiont-cured females. This response restores the process of immune system maturation in larvae in the absence of milk gland-associated *Wigglesworthia*. Tsetse houses 2 distinct populations of *Wigglesworthia*, the first of which is found extracellularly in female milk gland secretions. These bacterial cells presumably colonize developing intrauterine larvae, which receive maternal milk for nourishment during tsetse's unique mode of viviparous reproduction (7). Tsetse's second population of *Wigglesworthia* resides within the cytosol of specialized bacteriocytes that collectively compromise an organ located immediately adjacent to midgut called the bacteriome (45). Interestingly, Gmm^{Wgm-} adults, which arise from female tsetse that house bacteriome-associated *Wigglesworthia* but lack their milk gland population, are immuno-compromised (11). Thus, this population of *Wigglesworthia* is insufficient to stimulate immune system development in intrauterine Gmm^{Wgm-} larvae. However, *Wigglesworthia*-containing bacteriome extracts supplemented in the diet of symbiont-cured moms can stimulate immune system development in Gmm^{Apo} larvae. Bacteriome-associated *Wigglesworthia* appear to produce the molecule(s) required to actuate immune system development in Gmm^{Apo} offspring, but they are concealed within the cytosol of bacteriocytes.

The mechanism by which *Wigglesworthia* extracts induce immune system development in Gmm^{Apo} larvae is currently unknown. In the mammalian system, symbiosis factors are translocated from the gut lumen to peripheral target immune tissues. In the mouse model *B. fragalis* cells, or *B. fragalis* PSA, is presumably taken up by gut-associated dendritic cells, which subsequently migrate to outlying lymphoid tissues where they signal for the differentiation of T cell lineages (43). In a similar manner, PGN shed by mouse intestinal microbiota is translocated from the luminal side of the gut epithelia into the circulatory system. A positive correlation exists between the concentration of PGN present in host sera and neutrophil function (44). Further experiments are required in the tsetse system to determine if immuno-stimulatory *Wigglesworthia* molecules are transported to the developing larvae where they exhibit direct activity, or if they act locally in the gut to induce a maternally-derived systemic response that subsequently induces larval immune system development.

Nutritional symbioses between bacteria and insects are well-documented (46, 47). The relationship between tsetse and *Wigglesworthia* presumably also has a nutritional component, as flies that lack this bacterium are reproductively sterile (48, 9). In fact, *Wigglesworthia's* highly reduced genome encodes many vitamins and cofactors that are missing from tsetse's vertebrate blood-specific diet (49). In this study we demonstrate that the tsetse-*Wigglesworthia* symbiosis is multi-dimensional in that this microbe is also intimately involved in activating the development of it's host's immune system. As such tsetse may be exploitable as relatively simple and efficient model for deciphering the basic molecular mechanisms that underlie symbiont-induced maturation of host immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Acknowledgments

We are grateful to Dr. Yineng Wu for assistance with qPCR, and members of the Aksoy lab for critical review of the manuscript.

Funding This work was generously funded by grants to S.A. from the National Institute of Allergy and Infectious Diseases (NIAID; AI051584), National Institute of General Medical Science (NIGMS; 069449) and the Ambrose Monell Foundation (http://www.monellvetlesen.org/).

Abbreviations

AMP	antimicrobial peptide
DV	dorsal vessel
qPCR	real-time quantitative PCR
WT	wild-type
GC	gonotrophic cycle
PGRP-LB	peptidoglycan recognition protein LB
tep	thioester-containing protein
iNOS	inducible nitric oxide synthase
DUOX	dual oxidase
dpc	days post-challenge

REFERENCES

1. Wernegreen JJ. Genome evolution in bacterial endosymbionts of insects. Nat. Rev. Genet. 2002; 3:850–861. [PubMed: 12415315]
2. Moran NA. Symbiosis. Curr. Biol. 2006; 16:R866–R871. [PubMed: 17055966]
3. Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 2008; 6:e2. [PubMed: 19222304]
4. Dong Y, Manfredini F, Dimopolous G. Implications of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 2009; 5:e1000423. [PubMed: 19424427]
5. Rodrigues J, Brayner FA, Alves LC, Dixit R, Barillas-Mury C. Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. Science. 2010; 329:1353–1355. [PubMed: 20829487]
6. Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto SL, Mulenga M, Dimopoulos G. Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. Science. 2011; 322:855–858. [PubMed: 21566196]
7. Attardo GM, Lohs C, Heddi A, Alam UH, Yildirim S, Aksoy S. Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. J. Insect Physiol. 2008; 54:1236–1242. [PubMed: 18647605]
8. Cheng Q, Ruel TD, Zhou W, Moloo SK, Majiwa P, O'Neill SL, Aksoy S. Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp. Med. Vet. Entomol. 2000; 14:44–50. [PubMed: 10759311]
9. Pais R, Lohs C, Wu Y, Wang J, Aksoy S. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. Appl. Environ. Microbiol. 2008; 74:5965–5974. [PubMed: 18689507]
10. Wang J, Wu Y, Yang G, Aksoy S. Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. Proc. Natl. Acad. Sci. USA. 2009; 106:12133–12138. [PubMed: 19587241]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

11. Weiss BL, Wang J, Aksoy S. Tsetse immune system maturation requires the presence of obligate symbionts in larvae. PLoS Biol. 2011; 9:e1000619. [PubMed: 21655301]
12. Moloo SK. An artificial feeding technique for *Glossina*. Parasitol. 1971; 68:507–512.
13. Weiss BL, Wu Y, Schwank JJ, Tolwinski NS, Aksoy S. An insect symbiosis is influenced by bacterium-specific polymorphisms in outer membrane protein A. Proc. Natl. Acad. Sci. USA. 2008; 105:15088–15093. [PubMed: 18815366]
14. Dale C, Maudlin I. *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. Int. J. Syst. Bacteriol. 1999; 49:267–27. [PubMed: 10028272]
15. Castillo JC, Robertson AE, Strand MR. Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. Insect Biochem. Mol. Biol. 2006; 36:891–903. [PubMed: 17098164]
16. Alam U, Medlock J, Brelsford C, Pais R, Lohs C, Balmand S, Carnogursky J, Heddi A, Takac P, Galvani A, Aksoy S. *Wolbachia* symbiont infections induce strong cytoplasmic incompatibility in the tsetse fly, *Glossina morsitans morsitans*. PLoS Pathog. 2011; 7:e1002415. [PubMed: 22174680]
17. Holmes DS, Bonner JB. Preparation, molecular weight, base composition and secondary structure of giant ribonucleic acid. Biochem. 1973; 12:2330–2338. [PubMed: 4710584]
18. Weiss BL, Aksoy S. Microbiome influences on insect host vector competence. Trends Parasitol. 2011; 27:514–522. [PubMed: 21697014]
19. Elrod-Erickson M, Mishra S, Schneider DS. Interactions between the cellular and humoral immune responses in *Drosophila*. Curr. Biol. 2000; 10:781–784. [PubMed: 10898983]
20. Zaidman-Rémy A, Hervé M, Poidevin M, Pili-Floury S, Kim MS, Blanot D, Oh BH, Ueda R, Mengin-Lecreulx D, Lemaitre B. The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. Immunity. 2006; 24:463–473. [PubMed: 16618604]
21. Ryu J, Kim S, Lee H, Bai J, Nam Y, Bae JW, Lee DG, Shin SC, Ha EM, Lee WJ. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science. 2008; 319:777–782. [PubMed: 18218863]
22. Krzemien J, Dubois L, Makki R, Meister M, Vincent A, Crozatier M. Control of blood cell homeostasis in *Drosophila* larvae by the posterior signaling centre. Nature. 2007; 446:325–328. [PubMed: 17361184]
23. Makki R, Meister M, Pennetier D, Ubeda JM, Braun A, Daburon V, Krzemien J, Bourbon HM, Zhou R, Vincent A, Crozatier M. A short receptor downregulates JAK/STAT signalling to control the *Drosophila* cellular immune response. PLoS Biol. 2010; 8:e1000441. [PubMed: 20689801]
24. Ha E, Oh C, Bae YS, Lee W. A direct role for dual oxidase in *Drosophila* gut immunity. Science. 2005; 310:847–850. [PubMed: 16272120]
25. Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C. A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. Science. 2010; 327:1644–1648. [PubMed: 20223948]
26. Oliveira JH, Gonçalves RL, Lara FA, Dias FA, Gandara AC, Menna-Barreto RF, Edwards MC, Laurindo FR, Silva-Neto MA, Sorgine MH, Oliviera PL. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. PLoS Pathog. 2011; 7:e1001320. [PubMed: 21445237]
27. Jung SH, Evans CJ, Uemura C, Banerjee U. The *Drosophila* lymph gland as a developmental model of hematopoiesis. Development. 2005; 132:2521–2533. [PubMed: 15857916]
28. Strand MR. The insect cellular immune response. Insect Sci. 2008; 15:1–15.
29. Bou-Aoun R, Hetru C, Troxler L, Doucet D, Ferrandon D, Matt N. Analysis of thioester-containing proteins during the immune response of *Drosophila melanogaster*. J. Innate Immunol. 2011; 3:52–64.
30. Cirimotich CM, Ramirez JL, Dimopoulos G. Native microbiota shape insect vector competence for human pathogens. Cell Host Microbe. 2011; 10:307–310. [PubMed: 22018231]
31. Chen X, Li S, Aksoy S. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. J. Mol. Evol. 1999; 48:49–58. [PubMed: 9873076]

NIH-PA Author Manuscript

32. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. *Wolbachia* and virus protection in insects. Science. 2008:322–702. [PubMed: 18420918]

33. Osborne SE, Leong YS, O'Neill SL, Johnson KN. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathog. 2009; 5:e1000656. [PubMed: 19911047]

34. McMeniman CJ, Lane RV, Cass BN, Fong AW, Sidhu M, Wang YF, O'Neill SL. Stable introduction of a life-shortening *Wolbachia* infection in the mosquito *Aedes aegypti*. Science. 2009; 323:141–144. [PubMed: 19119237]

35. Kambris K, Cook PE, Phuc HK, Sinkins SP. Immune activation by life-shortening *Wolbachia* and and reduced filarial competence in mosquitoes. Science. 2009; 326:134–136. [PubMed: 19797660]

36. Moriera LA, Iturbe-Ormaetxe I, Jeffrey JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. Cell. 2009; 139:1268–1278. [PubMed: 20064373]

37. Lindh JM, Lehane MJ. The tsetse fly *Glossina fuscipes fuscipes* (Diptera: Glossina) harbours a surprising diversity of bacteria other than symbionts. Antonie Van Leeuwenhoek. 2011; 99:711–720. [PubMed: 21203841]

38. Matova N, Anderson KV. Rel/NF-kappaB double mutants reveal that cellular immunity is central to *Drosophila* host defense. Proc. Natl. Acad. Sci. USA. 2006; 103:16424–16429. [PubMed: 17060622]

39. Haine ER, Moret Y, Siva-Jothy MT, Rolff J. Antimicrobial defense and persistent infection in insects. Science. 2008; 322:1257–1259. [PubMed: 19023083]

40. Blandin SA, Levashina EA. Phagocytosis in mosquito immune responses. Immunol. Rev. 2007; 219:8–16. [PubMed: 17850478]

41. Eleftherianos I, Xu M, Yadi H, Ffrench-Constant RH, Reynolds SE. Plasmatocyte-spreading peptide (PSP) plays a central role in insect cellular immune defenses against bacterial infection. J. Exp. Biol. 2009; 212:1840–1848. [PubMed: 19483002]

42. Mazmanian SK, Kasper DL. The love-hate relationship between bacterial polysaccharide and the host immune system. Nat. Rev. Immunol. 2006; 6:849–858. [PubMed: 17024229]

43. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005; 15:107–118. [PubMed: 16009137]

44. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. 2010; 16:228–231. [PubMed: 20081863]

45. Aksoy S. Tsetse – a haven for microorganisms. Parasitol. Today. 2000; 16:114–118. [PubMed: 10689331]

46. Hosokawa T, Koga R, Kikuchi Y, Meng X, Fukatsu T. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proc. Natl. Acad. Sci. USA. 2010; 107:769–774. [PubMed: 20080750]

47. Douglas AE. Lessons from studying insect symbioses. Cell Host Microbe. 2011; 10:359–367. [PubMed: 22018236]

48. Nogge G. Sterility in tsetse flies (*Glossina morsitans* Westwood) caused by loss of symbionts. Experientia. 1976; 32:995–996. [PubMed: 986317]

49. Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M, Aksoy S. Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglseworthia glossinidia*. Nat. Genet. 2002; 32:402–407. [PubMed: 12219091]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

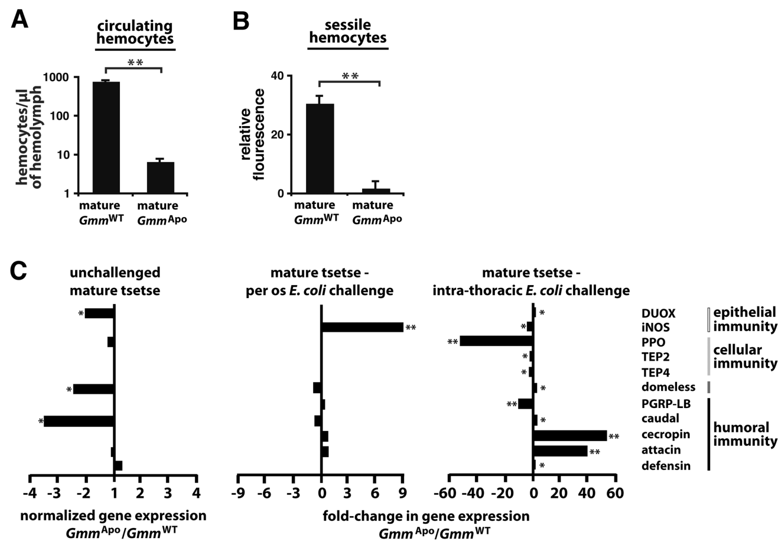

Figure 1. Aposymbiotic tsetse display atypical hallmarks of cellular and humoral immunity

(A) Number of circulating hemocytes per •l of hemolymph in mature *Gmm*^{WT} and *Gmm*^{Apo} flies (n=3 individuals from each tsetse line). (B) Quantitative analysis of sessile hemocyte abundance adjacent to the anterior chamber of the dorsal vessel of mature *Gmm*^{WT} and *Gmm*^{Apo} flies (n=3 individuals from each tsetse line). Relative fluorescence is proportional to the number of microspheres engulfed by sessile hemocytes and thus the number of these cells present in the region examined. (C) The effect of symbiont status and route of infection on the expression of selected immunity-related genes. Gene expression in uninfected *Gmm*^{Apo} and *Gmm*^{WT} individuals is normalized relative to constitutively-expressed tsetse β-*tubulin* (left panel). Fold-change in the expression of immunity-related genes in *Gmm*^{Apo} and *Gmm*^{WT} tsetse 3 d after *per os* (middle panel) and intra-thoracic (right panel) challenge with *E. coli* K12. All fold-change values are represented as a fraction of average normalized gene expression levels in bacteria-challenged flies relative to expression levels in PBS-injected controls. All quantitative measurements were performed on 3 biological samples in duplicate. Genes without a corresponding bar did not exhibit a fold-change in expression between samples compared, or their expression was undetectable via qPCR. Values are represented as means. * = $p < 0.05$, ** = $p < 0.005$ (Student's t-test).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

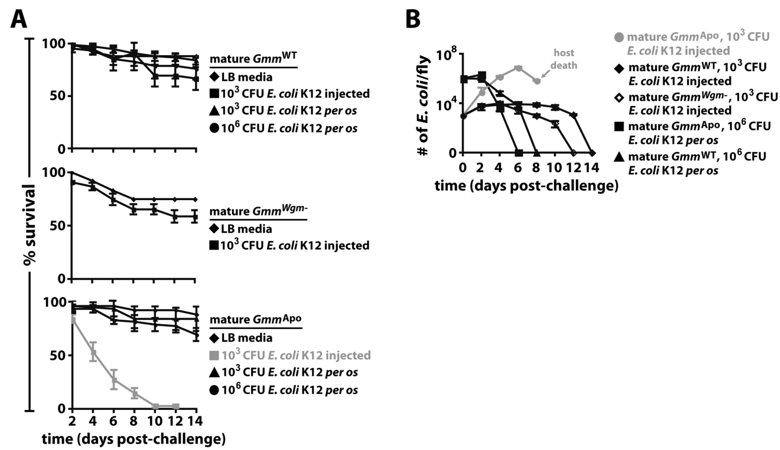

Figure 2. Symbiont status mediates tsetse's ability to survive challenge with *E. coli* K12

(A) The effect of symbiont status on the survival of tsetse following systemic and *per os* challenge with *E. coli* K12. Mature adult Gmm^{Apo} flies were significantly more susceptible to challenge with 10^3 CFU of *E. coli* than were age-matched Gmm^{WT} (bottom and top panels, $p < 0.001$) and Gmm^{Wgm-} flies (bottom and middle panels, $p < 0.001$). Both Gmm^{WT} and Gmm^{Apo} flies survived *per os* challenge with *E. coli.* Infection experiments were performed in triplicate, using 25 flies per replicate. (B) Average number (±SEM) of rec*E. coli*$_{\text{pIL}}$ per tsetse cohort over time (*n*=3 individuals per cohort per time point) following systemic and *per os* challenge with 10^3 CFU of bacteria. Values shown in grey represent lethal infections. By 8 dpc, not enough *E. coli*-injected Gmm^{Apo} flies remained to quantify bacterial density.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

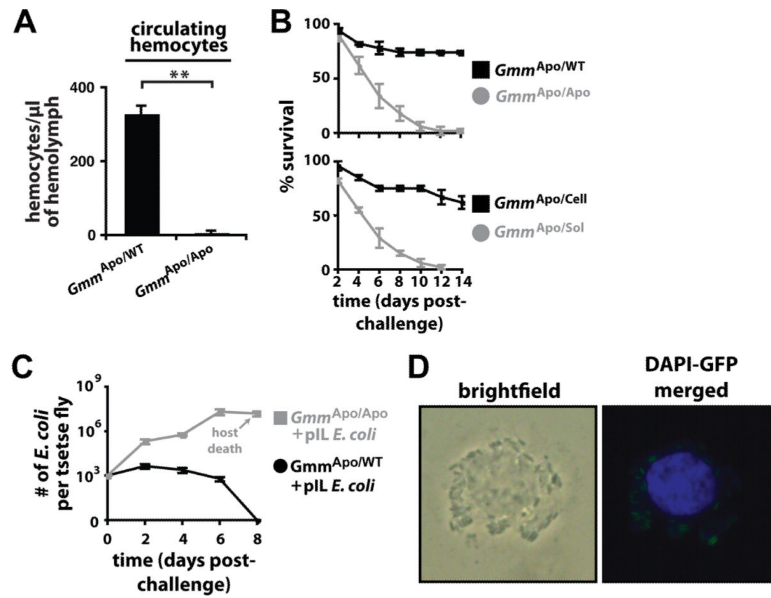

Figure 3. Hemocytes modulate tsetse's ability to overcome challenge with *E. coli* K12

(A) Hemolymph was collected from *Gmm*^{Apo} and *Gmm*^{WT} donor flies and immediately transplanted into *Gmm*^{Apo} recipients. Five days post-hemolymph transplantation, hemocyte abundance in *Gmm*$^{Apo/WT}$ and *Gmm*$^{Apo/Apo}$ recipient flies was quantified microscopically using a hemocytometer. *Gmm*$^{Apo/WT}$ flies housed significantly more circulating hemocytes than did *Gmm*$^{Apo/Apo}$ flies (** = $p < 0.005$). (B) *Gmm*$^{Apo/WT}$ and *Gmm*$^{Apo/Apo}$ recipient flies were challenged with *E. coli* 3 days after receiving a hemolymph transplant. Significantly more *Gmm*$^{Apo/WT}$ individuals survived *E. coli* challenge than did their *Gmm*$^{Apo/Apo}$ counterparts (top panel; $p < 0.001$). Donor hemolymph was then divided into cellular and soluble fractions via centrifugation. Significantly more *Gmm*$^{Apo/Cell}$ individuals survived *E. coli* challenge than did their *Gmm*$^{Apo/Sol}$ counterparts (bottom panel; $p < 0.001$). (C) Average number (±SEM) of rec*E. coli*$_{pIL}$ per *Gmm*$^{Apo/WT}$ and *Gmm*$^{Apo/Apo}$ recipient fly over time (n=3 individuals per treatment per time point) following systemic challenge with 10^3 CFU of bacteria. Values shown in grey represent lethal infections. (D) 12 hr post-challenge with *recE. coli*$_{GFP}$, hemolymph was collected, fixed on glass slides using 2% paraformaldehyde and microscopically examined for the presence of hemocyte-engulfed bacterial cells. *Gmm*$^{Apo/WT}$ recipient flies harbor engulfed bacterial cells.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

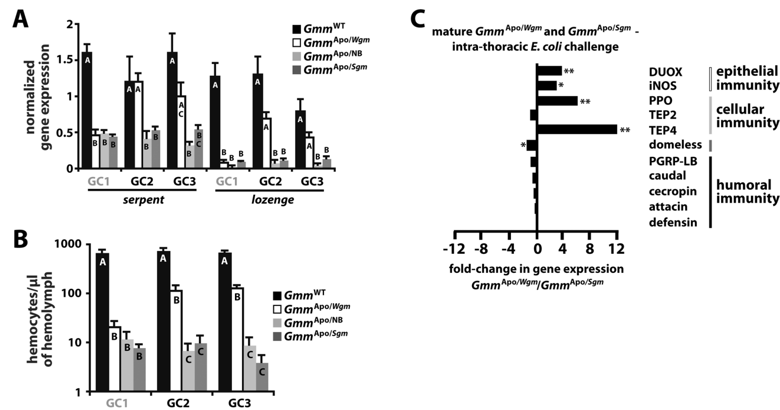

Figure 4. Dietary supplementation of *Wigglesworthia* cell extracts to symbiont-cured female tsetse induces immune system development in their aposymbiotic offspring

Three groups of pregnant female tsetse were provided 4 blood meals supplemented with the antibiotic tetracycline to clear all of their endogenous microbiota. Two cohorts of these symbiont-cured females then received diets supplemented with either *Wigglesworthia* or *Sodalis* cell extracts to compliment the absence of these bacteria. The third group of symbiont-cured females received no bacterial compliment. Finally, a fourth group of WT females received no tetracycline or bacterial complementation. Offspring of these females, which are designated $Gmm^{Apo/Wgm}$, $Gmm^{Apo/Sgm}$, $Gmm^{Apo/NB}$ and Gmm^{WT}, respectively, were collected from 3 gonotrophic cycles (GC) and subsequently monitored to determine their immune system phenotype. GC1 is indicated in grey to signify that bacterial compliment of $Gmm^{Apo/Wgm}$ and $Gmm^{Apo/Sgm}$ moms began after their first larval offspring were fully developed. (A) qPCR was performed on larval offspring (n=3 larva per cohort per GC) to determine their levels of *serpent* and *lozenge* expression. (B) Circulating hemocyte abundance in adult offspring (n=3 flies per cohort per GC) was quantified microscopically using a Bright-Line hemocytometer. In (A) and (B), bars with different letters indicate a statistically significant difference ($p < 0.05$) between samples. (C) Fold-change in the expression of immunity-related genes in $Gmm^{Apo/Wgm}$ and $Gmm^{Apo/Sgm}$ adults challenged with *E. coli*. Adult flies used for this experiment were from the 2nd GC of symbiont-cured moms. All fold-change values are represented as a fraction of average normalized gene expression levels in bacteria-challenged flies relative to expression levels in PBS-injected controls. Genes without a corresponding bar did not exhibit a fold-change in expression between samples compared, or their expression was undetectable via qPCR. All quantitative measurements were performed on 3 biological samples in duplicate. Values are represented as means. * = $p < 0.05$, ** = $p < 0.005$.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

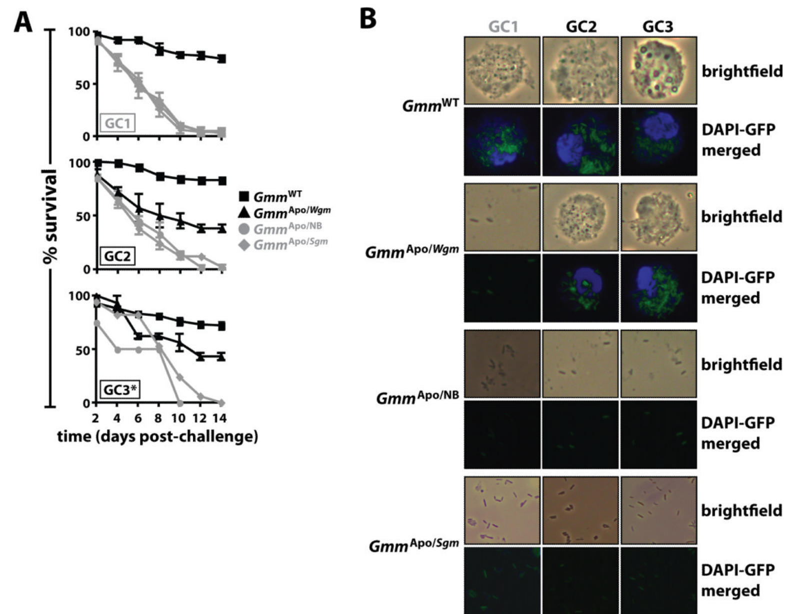

Figure 5. *Gmm*$^{\text{Apo/Wgm}}$ flies exhibit resistance to challenge with *E. coli*

(A) Percent survival of mature *Gmm*$^{\text{Apo/Wgm}}$, *Gmm*$^{\text{Apo/Sgm}}$, *Gmm*$^{\text{Apo/NB}}$ and *Gmm*$^{\text{WT}}$ adults from 3 GCs following challenge with 10^3 CFU of *E. coli* K12. Significantly more *Gmm*$^{\text{Apo/Wgm}}$ flies from the 2$^{\text{nd}}$ GC survived this challenge than did age-matched *Gmm*$^{\text{Apo/Sgm}}$ and *Gmm*$^{\text{Apo/NB}}$ individuals ($p < 0.01$). However, significantly fewer *Gmm*$^{\text{Apo/Wgm}}$ flies from these GCs survived this challenge than did their WT counterparts ($p < 0.01$). Values shown in grey represent lethal infections. Sample sizes are as follows: GC1 (*n*=25 flies per replicate for all tsetse cohorts) and GC2 (*n*=25 flies per replicate for *Gmm*$^{\text{WT}}$ and *Gmm*$^{\text{Apo/Wgm}}$ flies; *n*=20 for *Gmm*$^{\text{Apo/Sgm}}$ and *Gmm*$^{\text{Apo/NB}}$ flies) infection experiments were performed in triplicate for all tsetse groups. GC3 is denoted with an asterisk because not enough *Gmm*$^{\text{Apo/Sgm}}$ and *Gmm*$^{\text{Apo/NB}}$ offspring were produced to perform the experiment in triplicate (even in the presence of yeast extract, the fecundity of symbiont-cured females drops over time). Thus, statistical comparisons between these two groups were not performed. (B) Twelve hours post- challenge with *recE. coli*$_{\text{GFP}}$, hemolymph was collected from all individuals (*n*=3 flies per group per GC) to monitor for the presence of phagocytic hemocytes. Samples were processed as previously described. In (A) and (B), GC1 is indicated in grey to signify that bacterial compliment of *Gmm*$^{\text{Apo/Wgm}}$ and *Gmm*$^{\text{Apo/Sgm}}$ moms began after their first intrauterine larval offspring were approximately mid-way through their 3$^{\text{rd}}$ developmental instar.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Designation of tsetse cohorts used in this study, their symbiont status, and the treatment they received.

Tsetse designation	Symbiont status[a]	Origin/treatment[b]	Reference/source
Gmm ^{WT}	*Wgm, Sgm, Wol*	none	none
Gmm $^{Wgm-}$	*Sgm, Wol*	offspring of moms treated with Amp, yeast extract	9
Gmm ^{Apo}	apo	offspring of moms treated with Tet, yeast extract	16
Gmm $^{Apo/WT}$	apo	received hemolymph transplant from *Gmm*^{WT} donors	this study
Gmm $^{Apo/Apo}$	apo	received hemolymph transplant from *Gmm*^{Apo} donors	this study
Gmm $^{Apo/Sol}$	apo	received soluble fraction of *Gmm*^{WT} donor hemolymph	this study
Gmm $^{Apo/Cell}$	apo	received cellular fraction of *Gmm*^{WT} donor hemolymph	this study
Gmm $^{Apo/Wgm}$	apo	offspring of symbiont-cured moms complimented with *Wgm* cell extracts	this study
Gmm $^{Apo/Sgm}$	apo	offspring of symbiont-cured moms complimented with Sgm cell extracts	this study
Gmm $^{Apo/NB}$	apo	offspring of symbiont-cured moms that received no bacterial compliment	this study

[a] *Wgm*, *Wigglesworthia*; *Sgm*, *Sodalis*; *Wol*, *Wolbachia;* apo, aposymbiotic

[b] Amp, ampicillin; Tet, tetracycline