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Obligate Symbionts Activate Immune System Development in the Tsetse Fly

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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 three 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 (Glossina morsitans morsitans [Gmm40]) 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 nonpathogenic Escherichia coli. The susceptible phenotype exhibited by Gmm40 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 GmmApo larvae when their mothers 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 its host. The Journal of Immunology, 2012, 188: 000–000.
Luciferase-expressing *E. coli* (recE. coliag) K12 were produced via transformation with construct pBL, which encodes the firefly luciferase gene under transcriptional control of *Sodalis* insulinase promoter (13). The assay was used to quantify recE. coliag, cells in vivo was performed as described previously (13). GFP-expressing *E. coli* K12 (recE. coliagF) were produced via electroporation with pGFP-UV plasmid DNA (Clontech). *Sodalis* were isolated from surface-sterilized *G. morsitans* pupae and cultured on *Aedes albopictus* C6/36 cells, as described previously (14). *Sodalis*, which has a doubling time of ~24 h, was subsequently maintained in vitro, in the absence of C6/36 cells, at 25°C in Mitsuhashi–Maramorosch medium containing D (+) glucose, 6.5 g/l lactalbumin, 22 mM D (+) glucose, 6.5 g/l lactalbumin hydrolysate, and 5.0 g/l yeast extract) supplemented with 5% heat-inactivated FBS (14).

### Tsetse infections

Systemic challenge of tsetse was achieved by anesthetizing flies with CO2 and subsequently injecting individuals with live bacterial cells using glass needles and a Narashige IM300 microinjector. Per os bacterial challenges and subsequently injecting individuals with live bacterial cells using glass needles and a Narashige IM300 microinjector. Per os bacterial challenges were performed as described above. Phagocytic capacity of transplanted hemocytes was determined by infecting *Gmm* with 10^6 CFU live *E. coli* K12. Twelve hours postchallenge, hemolymph was collected from wild-type (WT) or aposymbiotic donors, and 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 overlaid with VECTASHIELD HardSet Mounting Medium containing DAPI (Vector Laboratories).

### Bacterial complementation experiments

A cartoon illustrating how bacterial complement 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 blood) every other day for 10 d. Additionally, throughout the course of the experiment, all blood meals (three/wk) also contained vitamin-rich yeast extract (1% w/v) to restore fertility associated with the absence of *Wigglesworthia* (16). Ten days postcopulation, two 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 first gonotrophic cycle (GC) went through most of their development in the absence of a bacterial complement, whereas those from the second and third GCs developed in the presence of bacterial complement. Offspring of these females were designated *Gmm*apoWgm and *Gmm*apoSgm, respectively. *Wigglesworthia* was obtained by dissecting tsetse bacteria (an organ immediately adjacent to the midgut that houses this bacteria) from *Gmm* females whereas *Sodalis* was maintained in cultured as described above. *Gmm*apoWgm females were fed one bacteriome equivalent per four females, and *Gmm*apoSgm females were fed 4 × 10^7 *Sodalis*/ml blood (thus, these flies ingested ~1 × 10^7 *Sodalis* each time they fed). A third control cohort of symbiont-cured females received no bacterial complement (their offspring are designated *Gmm*apoWgm and *Gmm*apoSgm, respectively). A fourth cohort of WT offspring (*Gmm* WT) served as another control. To confirm the aposymbiotic status of the abdomen. Hemolymph exuding from the wound was collected using a glass micropipette and placed into a microfuge tube on ice. Four cohorts of newly emerged aposymbiotic recipient flies were used, two of which were designated *Gmm*apoWT or *Gmm*apoapo based on whether they received hemolymph transplanted from wild-type (WT) or aposymbiotic donors, respectively. *Gmm*apoWT or *Gmm*apoapo recipient flies received 1 μl donor hemolymph (this volume represents approximately one third of the total volume collected from donor flies). On day 8 posttransplantation, three 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% Methuselah–Maramorosch medium, 30% anticoagulant citrate buffer [95 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric acid (pH 4.5)] (15) equal to the total amount of hemolymph from which they were collected. The remaining two cohorts of *Gmm*apoapo recipient flies were injected with either 1 μl cellular suspension (these flies are designated *Gmm*apoCell or 1 μl the soluble hemolymph fraction (these flies are designated *Gmm*apoSgm). Aposymbiotic recipient flies were challenged with either 10^7 CFU live *E. coli* K12 or recE. coliag. Injections were performed using glass needles and a Narashige IM300 microinjector. Quantification of recE. coliag, in recipient tsetse was performed as described above. Phagocytic capacity of transplanted hemocytes was determined by infecting *Gmm*apoapo recipient flies with 10^7 CFU live *E. coli* K12. Twelve hours postchallenge, hemolymph was collected from three individuals, and hemocytes were 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 overlaid with VECTASHIELD HardSet Mounting Medium containing DAPI (Vector Laboratories).

### Hemolymph collection and hemocyte quantification

Hemolymph collection from wild-type *G. morsitans* (Wgm) 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 hemocytometer (11). Sessile hemocyte abundance was quantified by subtracting *Gmm* WT and *Gmm*apo flies (n = 3) to hemocoeic injection with blue fluorescent microspheres. Twelve hours postinjection, flies were dissected to reveal tsetse’s dorsal vessel (DV). Exposed tissue was rinsed three times with PBS to remove contaminating circulating hemocytes or any beads not engulfed by sessile hemocytes. 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 three *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 extracted 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 I. Quantitative measurements were performed on three 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 were represented as a ratio of average normalized gene expression levels in bacteria-infected flies relative to expression levels in corresponding uninfected controls. Values represent 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 micropipette and placed into a microfuge tube on ice. Four cohorts of newly emerged aposymbiotic recipient flies were used, two of which were designated *Gmm*apoWT or *Gmm*apoapo based on whether they received hemolymph transplanted from wild-type (WT) or aposymbiotic donors, respectively. *Gmm*apoWT or *Gmm*apoapo recipient flies received 1 μl donor hemolymph (this volume represents approximately one third of the total volume collected from donor flies). On day 8 posttransplantation, three 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% Methuselah–Maramorosch medium, 30% anticoagulant citrate buffer [95 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric acid (pH 4.5)] (15) equal to the total amount of hemolymph from which they were collected. The remaining two cohorts of *Gmm*apoapo recipient flies were injected with either 1 μl cellular suspension (these flies are designated *Gmm*apoCell or 1 μl the soluble hemolymph fraction (these flies are designated *Gmm*apoSgm). Aposymbiotic recipient flies were challenged with either 10^7 CFU live *E. coli* K12 or recE. coliag. Injections were performed using glass needles and a Narashige IM300 microinjector. Quantification of recE. coliag, in recipient tsetse was performed as described above. Phagocytic capacity of transplanted hemocytes was determined by infecting *Gmm*apoapo recipient flies with 10^7 CFU live *E. coli* K12. Twelve hours postchallenge, hemolymph was collected from three individuals, and hemocytes were 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 overlaid with VECTASHIELD HardSet Mounting Medium containing DAPI (Vector Laboratories).

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

<table>
<thead>
<tr>
<th>Tsetse Designation</th>
<th>Symbiont Status</th>
<th>Origin/Treatment</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gmm WT</td>
<td>Wgm, Sgm, Wol</td>
<td>Offspring of mothers treated with Amp, yeast extract</td>
<td>(9)</td>
</tr>
<tr>
<td>Gmm Wgm apo</td>
<td>Sgm, Wol</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Gmm Apo</td>
<td>Apo</td>
<td>Offspring of mothers treated with Tet, yeast extract</td>
<td>(16)</td>
</tr>
<tr>
<td>Gmm Apo/WT</td>
<td>Apo</td>
<td>Received hemolymph transplant from Gmm Apo donors</td>
<td>This study</td>
</tr>
<tr>
<td>Gmm Apo/Sgm</td>
<td>Apo</td>
<td>Received soluble fraction of Gmm WT donor hemolymph</td>
<td>This study</td>
</tr>
<tr>
<td>Gmm Apo/Wgm</td>
<td>Apo</td>
<td>Received cellular fraction of Gmm WT donor hemolymph</td>
<td>This study</td>
</tr>
<tr>
<td>Gmm Apo/SgmWgm</td>
<td>Apo</td>
<td>Offspring of symbiont-cured mothers complemented with Wgm cell extracts</td>
<td>This study</td>
</tr>
<tr>
<td>Gmm Apo/NB</td>
<td>Apo</td>
<td>Offspring of symbiont-cured mothers that received no bacterial complement</td>
<td>This study</td>
</tr>
</tbody>
</table>

Amp, ampicillin; Apo, aposymbiotic; Sgm, Sodalis; Tet, tetracycline; Wgm, Wigglesworthia; Wol, Wolbachia.

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bacteria-specific primers (Supplemental Table I) and the following cycle program: 95 °C for 5 min, followed by 30 cycles at 95, 55, and 72 °C, each for 1 min, and a final 7-min elongation/extension at 72 °C.

To determine whether complementing symbiont-cured mothers 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 (first, second, and third instar) from each of three GCs (n = 3 individuals/group/GC). All remaining offspring were allowed to mature to adulthood. At this time, three 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 GmmApo/Wgm and GmmApo3IRD individuals (n = 3) from the second GC of symbiont-cured mothers. Finally, all remaining mature adult offspring were challenged with 10^9 CFU live recE. coliapp. Twelve hours postchallenge, hemolymph was collected and monitored to determine whether hemocytes had engulfed GFP-expressing bacterial cells (n = 3 individuals/group/GC). Hemolymph samples were fixed and visualized as described above.

Statistics

Statistical significance among various treatments, as well as treatments and controls, is indicated in the figure legends. Survival curve comparisons were made by log-rank analysis using JMP (v9.0) software (http://www.jmp.com). Statistical analysis of qPCR data and hemocyte abundance was performed by the Student t test using Microsoft Excel software.

Results

Aposeymbiotic 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 its microbiome (18). In an effort to better define the relationship between symbiosis and immunity in tsetse, we fed pregnant females a diet supplemented with tetracycline and yeast. This antibiotic treatment clears all symbionts from the flies, whereas 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-d-old adult (hereafter referred to as “mature”) Gmm^wt^ and Gmm^apo^ flies. Our results indicate that mature WT tsetse harbor 113-fold more circulating hemocytes/µl of hemolymph than do their aposymbiotic counterparts (Gmm^wt^, 793 ± 34 hemocytes/µl of hemolymph; Gmm^apo^, 7 ± 1 hemocytes/µl of hemolymph; Fig. 1A). To determine the functional relationship between symbiont status and sessile hemocyte abundance, we thoracically microinjected 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 DV (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-fold more microspheres than did age-matched Gmm^apo^ individuals (Fig. 1B, Supplemental Fig. 3).

Previously, we determined that several genes associated with humoral, cellular, and epithelial immune pathways, including those that encode the AMPs attacin and cecropin, as well as thioester-containing proteins (teps) tep2 and tep 4, prophenoloxidase, and inducible NO synthase (iNOS), were expressed at significantly lower levels in Gmm^wm^ flies compared with Gmm^wt^ flies following infection with E. coli (11). In the current study, we monitored expression of these same genes in age-matched Gmm^wt^ and Gmm^apo^ flies that were either unchallenged or 3 d postchallenge (dpc) with E. coli K12. Furthermore, we also evaluated the expression of PGRP-LB, caudal, domeless, and DUOX. In tsetse and closely related Drosophila, PGRP-LB and caudal serve as negative regulators of NF-κB–dependent antimicrobial peptide expression (10, 20, 21), whereas domeless is a cytokine receptor that

FIGURE 1. Aposeymbiotic tsetse display atypical hallmarks of cellular and humoral immunity. (A) Number of circulating hemocytes/µl 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 DV 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 intrathoracic (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 three 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 presented as means. *p < 0.05, **p < 0.005, Student t test.
regulates expression of *tep4* through the JAK/STAT-signaling pathway (22, 23). Finally, in *Drosophila* and mosquitoes, DUOX is involved in generating infection-induced antimicrobial reactive oxygen species (24–26).

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*<sup>Apo</sup> flies compared with *Gmm*<sup>WT</sup> flies (Fig. 1C, left panel). Following per os challenge with *E. coli*, no significant difference in immunity-related gene expression (with the exception of *iNOS*) was observed between *Gmm*<sup>WT</sup> and *Gmm*<sup>Apo</sup> flies (Fig. 1C, middle panel). However, systemic challenge resulted in a significant difference in the expression of all of the genes that we analyzed. Most notably, pathways associated with cellular immunity were significantly downregulated in *Gmm*<sup>Apo</sup> individuals compared with *Gmm*<sup>WT</sup> individuals, whereas those associated with humoral immune responses were significantly upregulated (Fig. 1C, right panel). These findings indicate that tsetse’s symbiotic bacteria are closely associated with the development of their host’s immune system during larval maturation and its subsequent proper function in unchallenged and *E. coli*-challenged adults.

Aposymbiotic tsetse are highly susceptible to normally nonpathogenic *E. coli*

We next determined whether *Gmm*<sup>Apo</sup> individuals are more susceptible to challenge with *E. coli* than are WT tsetse or tsetse that lack only *Wigglesworthia* (*Gmm*<sup>Wgm</sup>-). To do so, we compared percent survival of mature adults from these three tsetse lines following systemic challenge with *E. coli* K12. We determined that 67% of mature *Gmm*<sup>WT</sup> individuals and 59% of mature *Gmm*<sup>Wgm</sup>- individuals survived systemic challenge with 10<sup>8</sup> CFU of *E. coli* (Fig. 2A, top and middle panels). In contrast, all age-matched *Gmm*<sup>Apo</sup> individuals perished by 12 dpc (Fig. 2A, bottom panel). We next challenged *Gmm*<sup>Wgm</sup>- and *Gmm*<sup>Apo</sup> flies per os with 10<sup>3</sup> and 10<sup>6</sup> CFU of *E. coli* and found that all individuals survived this challenge (Fig. 2A, top and bottom panels). This finding suggests that mature *Gmm*<sup>Apo</sup> flies are considerably more susceptible to systemic challenge with a foreign microbe than are age-matched *Gmm*<sup>WT</sup> and *Gmm*<sup>Wgm</sup>- 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 that we observed among *Gmm*<sup>WT</sup>, *Gmm*<sup>Wgm</sup>-, and *Gmm*<sup>Apo</sup> 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<sup>8</sup> CFU of *E. coli*, bacterial densities within mature *Gmm*<sup>WT</sup> flies reached 8.3 × 10<sup>3</sup> cells before being cleared. Interestingly, *Gmm*<sup>Wgm</sup>- flies, which perish following challenge with 10<sup>6</sup> CFU of *E. coli* (11), were able to clear all exogenous bacterial cells following challenge with this lower dose. In contrast, bacterial density in *Gmm*<sup>Apo</sup> flies peaked at 7.8 × 10<sup>6</sup> on day 6 postchallenge, 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. Taken together, these findings indicate that *Gmm*<sup>Apo</sup> 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*<sup>Apo</sup> 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*<sup>WT</sup> and *Gmm*<sup>Apo</sup> individuals (donor flies) into the hemocoel of susceptible *Gmm*<sup>Apo</sup> flies (recipient flies are hereafter designated *Gmm*<sup>Apo*/WT* and *Gmm*<sup>Apo*/Apo*, respectively). Five days after this procedure, we determined that *Gmm*<sup>Apo*/WT* flies harbored 330 ± 20.4 hemocytes/μl of hemolymph, whereas *Gmm*<sup>Apo*/Apo*</sup> flies harbored 5 ± 3.8 hemocytes/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*<sup>Apo</sup> flies. To do so, we challenged *Gmm*<sup>Apo*/WT* and *Gmm*<sup>Apo*/Apo* flies with 10<sup>6</sup> CFU and 10<sup>3</sup> CFU of *E. coli* K12. Mature adult *Gmm*<sup>Apo*/WT* flies were significantly more susceptible to challenge with 10<sup>3</sup> CFU of *E. coli* than were age-matched *Gmm*<sup>WT</sup> flies (bottom and top panels, *p* < 0.001) and *Gmm*<sup>Wgm</sup>- flies (bottom and middle panels, *p* < 0.001). Both *Gmm*<sup>WT</sup> and *Gmm*<sup>Apo</sup> flies survived per os challenge with *E. coli*. Infection experiments were performed in triplicate, using 25 flies/replicate. (B) Average number (± SEM) of recE. coli<sub>μl</sub> per tsetse cohort over time (n = 3 individuals/cohort/time point) following systemic and per os challenge with 10<sup>8</sup> CFU of bacteria. Values shown in gray represent lethal infections. By 8 dpc, not enough *E. coli*-injected *Gmm*<sup>Apo</sup> flies remained to quantify bacterial density.
individuals with $10^3$ CFU of *E. coli* 3 d posthemolymph transplantation and subsequently monitored their survival over time. Our results indicate that 72% of *Gmm*<sup>Apo</sup>*WT* flies survived for 14 d following challenge. In comparison, only 2% of *Gmm*<sup>Apo</sup>*Apo* flies survived their challenge (Fig. 3B, *top panel*). These results demonstrate that *Gmm*<sup>WT</sup> 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 two distinct groups of *Gmm*<sup>Apo</sup> flies. Finally, 3 d 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*<sup>WT</sup> donors (*Gmm*<sup>Apo/Sol</sup>) perished by day 12 postchallenge. In comparison, 62% of *Gmm*<sup>Apo</sup> recipients that received the cellular fraction of hemolymph from *Gmm*<sup>WT</sup> donors (*Gmm*<sup>Apo/Cell</sup>) survived for 14 d following bacterial challenge (Fig. 3B, *bottom panel*). These host survival curves indicate that *Gmm*<sup>Apo</sup> flies survived 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 that we observed between these two groups, we monitored the dynamics of bacterial growth in each group over the course of the experiment. *E. coli* within *Gmm*<sup>Apo/Cell</sup> flies replicated exponentially, until a peak density of $2.1 \times 10^7$ 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*<sup>WT</sup> donors (Fig. 3C). More so, microscopic examination of hemolymph from *Gmm*<sup>Apo/WT</sup> 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 for subsequent adults to exhibit a functional cellular immune system (11). We have not been able to culture *Wigglesworthia* and, thus, cannot recolonize aposymbiotic flies with this bacterium. To circumvent this impediment, we tested whether we could restore the process of immune system development in *Gmm*<sup>Apo</sup> 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 that 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*<sup>WT</sup> females were fed a diet supplemented with tetracycline and yeast extract (16). Ten days postcopulation, 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*<sup>Apo/Wgm</sup> and *Gmm*<sup>Apo/Sgm</sup>, respectively) was com-
pared with that of control cohort offspring from symbiont-cured mothers that received no bacterial supplement (Gmm\(^{Noc/NB}\)) and offspring from Gmm\(^{WT}\) mothers. We first evaluated the relative abundance of transcripts that encode the transcription factors Spenrt 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 current study, we found that Gmm\(^{Apo/Wgm}\), Gmm\(^{Apo/Sgm}\), and Gmm\(^{Noc/NB}\) larva from the first GC expressed significantly less serpent and lozenge than did Gmm\(^{WT}\) larva. However, after the onset of bacterial supplementation, Gmm\(^{Apo/Wgm}\) and Gmm\(^{WT}\) larva from the second and third GCs expressed comparable levels of serpent and lozenge, whereas Gmm\(^{Apo/NB}\) and Gmm\(^{Apo/Sgm}\) 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\(^{Apo/Wgm}\) adults to that found in age-matched Gmm\(^{WT}\), Gmm\(^{Apo/NB}\), and Gmm\(^{Apo/Sgm}\) flies. We found that the provisioning of Wigglesworthia extracts to symbiont-cured females resulted in an increase in the number of 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 it was 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\(^{Apo/Wgm}\) flies compared with adult Gmm\(^{Apo/Sgm}\) flies (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 mothers 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, whereas 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\(^9\) 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.
FIGURE 5. GmmApo/Wgm flies exhibit resistance to challenge with E. coli. (A) Percent survival of mature GmmApo/Wgm, GmmApo/Sgm, GmmApo/NB, and GmmWT adults from three GCs following challenge with 10^3 CFU of E. coli K12. Significantly more GmmApo/Wgm flies from the second GC survived this challenge than did age-matched Gmmapo/Sgm and Gmmapo/NB individuals (p < 0.01). However, significantly fewer GmmApo/Wgm flies from these GCs survived this challenge than did their WT counterparts (p < 0.01). Values shown in gray represent lethal infections. Sample sizes are as follows: GC1 (n = 25 flies/replicate for all tsetse cohorts) and GC2 (n = 25 flies/replicate for GmmWT and GmmApo/Wgm flies; n = 20 for GmmApo/Sgm and GmmApo/NB flies) infection experiments were performed in triplicate for all tsetse groups. GC3 is denoted with an asterisk because not enough GmmApo/Wgm and GmmApo/NB offspring were produced to perform the experiment in triplicate (even in the presence of yeast extract, the fecundity of symbiont-cured females decreases over time). Thus, statistical comparisons between these two groups were not performed. (B) Twelve hours postchallenge with recE. coliGFP hemolymph was collected from all individuals (n = 3 flies/group/GC) to monitor for the presence of phagocytic hemocytes. Our results demonstrate that evolutionary time has stably anchored the obligate association between tsetse and Wigglesworthia such that this bacterium directly engenders immunity and, thus, ultimately the fecundity, of its host. In return, tsetse provides Wigglesworthia with a protective and metabolite-rich niche that has enabled this bacterium to survive in this environment for ≥50 million years (31).

Discussion

Symbiotic bacteria are gaining increased recognition as potent modulators of insect immunity (18, 30). In the current 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 its subsequent proper function during adulthood. We determined that aposymbiotic (GmmApo) flies derived from symbiotic-cured mothers present a severely compromised cellular immune system and, as such, are highly susceptible to systemic infection with normally nonpathogenic E. coli. This immunocompromised phenotype can be reversed when GmmApo adults receive hemocytes transplanted from WT individuals. Furthermore, the process of immune system development in GmmApo larvae can be restored when their symbiotic-cured mothers 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 immunity and, thus, ultimately the fecundity, of its host. In return, tsetse provides Wigglesworthia with a protective and metabolite-rich niche that has enabled this bacterium to survive in this environment for ≥50 million years (31).

Tsetse that undergo intrauterine larval development in the absence of only Wigglesworthia (GmmWgm−) exhibit a compromised immune system that, when compared with WT flies (GmmWT), is characterized by a 70% reduction in the number of phagocytic hemocytes (11). In the current study, we found that eliminating all symbiotic bacteria from female tsetse markedly enhances the immunocompromised phenotype of their offspring. In fact, GmmApo adults harbor virtually no circulating (99% less than GmmWT 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, GmmWgm− flies, which undergo intrauterine maturation in the presence of Sodalis and Wolbachia, house ∼40-fold more circulating hemocytes than do their aposymbiotic counterparts and are more tolerant to E. coli challenge (11). The enhanced immunity exhibited by GmmWgm− individuals in comparison with their aposymbiotic counterparts suggests that the presence of Sodalis and Wolbachia during intrauterine development may induce a limited degree of immune system maturation in the tsetse host. Although no experimental evidence exists that demonstrates a functional role of this nature for Sodalis, Wolbachia exhibits immunomodulatory 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 transfected with an exogenous strain of Wolbachia (wMelPop) (34). The presence of wMelPop appears to activate the immune system of offspring from transfected 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 immunocompetent (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 (38–41). 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 GmmWgm 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, GmmNpo adults survived this same infection if they previously received hemolymph transplanted from WT donors. However, when WT donor hemolymph was separated into cellular and soluble fractions prior to transplantation, only GmmNpo recipients that received the cellular fraction (hemocytes) exhibited an E. coli-resistant phenotype. Thus, hemolymph-soluble factors, such as an AMPs, hematopoietic molecules, or reactive oxygen species, presumably do not induce E. coli resistance when transplanted into GmmNpo 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 fragilis, 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. fragilis PSA-induced development of secondary lymphoid tissues. B. fragilis 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). No immunomodulatory symbiosis factors have been characterized from insect-associated microbes. In this study, we demonstrated that immune system development in GmmApo larvae was activated when their mothers were fed a diet supplemented with extracts of Wigglesworthia cells. This finding suggests that a molecular component of this obligate bacterium can actuate a transgenerational 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 two 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, GmmWgm adults, which arise from female tsetse that house bacteriome-associated Wigglesworthia but lack their milk gland population, are immunocompromised (11). Thus, this population of Wigglesworthia is insufficient to stimulate immune system development in intrauterine GmmWgm− larvae. However, Wigglesworthia-containing bacteriome extracts supplemented in the diet of symbiont-cured mothers can stimulate immune system development in GmmNpo larvae. Bacteriome-associated Wigglesworthia appear to produce the molecule(s) required to actuate immune system development in GmmNpo offspring, but they are concealed within the cytosol of bacteriocytes.

The mechanism by which Wigglesworthia extracts induce immune system development in GmmApo larvae is unknown. In the mammalian system, symbiosis factors are translocated from the gut lumen to peripheral target immune tissues. In the mouse model B. fragilis cells, or B. fragilis 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 whether immunostimulatory Wigglesworthia molecules are transported to the developing larvae where they exhibit direct activity, or whether 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, because 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 multidimensional in that this microbe is also intimately involved in activating the development of its host’s immune system. As such, tsetse may be exploitable as a relatively simple and efficient model for deciphering the basic molecular mechanisms that underlie symbiont-induced maturation of host immunity.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental material for

**Obligate symbionts activate immune system development in the tsetse fly**

Brian L. Weiss, Michele Maltz and Serap Aksoy
Supplemental Fig. 1. Cartoon illustrating the procedures used to perform bacterial compliment experiments.
Supplemental Fig. 2. Symbiont status of tsetse offspring used in bacterial compliment experiments.
Supplemental Fig. 3. Quantitative analysis of sessile hemocyte abundance in mature Gmm\textsuperscript{WT} and mature Gmm\textsuperscript{Apo} flies (n=3). The anterior-most chamber of tsetse’s dorsal vessel is indicated within a white circle (scale bar = 400 μm). The 2 remaining panels are close-ups of the anterior chamber (scale bar for both panels = 80 μm) visualized by excitation with UV light (365/415 nm).
## Supplemental Table 1. PCR primers used in this study.

<table>
<thead>
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\(^a\) Wgm, Wigglesworthia, Sgm, Sodalis