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_J Immunol_ 2012; 188:3395-3403; Prepublished online 24 February 2012; doi: 10.4049/jimmunol.1103691
http://www.jimmunol.org/content/188/7/3395

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/02/24/jimmunol.1103691.DC1

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Obligate Symbionts Activate Immune System Development in the Tsetse Fly

Brian L. Weiss, Michele Maltz, and Serap Aksoy

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 [GmmApo]) 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 GmmApo 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: 3395–3403.

A ll 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 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 antimicrobial peptides (AMPs), including the production of phagocytic granulocytes, and directly generating antimalarial reactive oxygen species (4–6).

Tsetse flies (Glossina spp.) harbor three symbiotic bacteria that regulate important aspects of their host’s physiology. Two of these microbes, obligate Wigglesworthia and commensal Sodalis, are transferred during 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 immunocompromised during adulthood. This phenotype is characterized by a significantly reduced population of phagocytic sessile and circulating hemocytes, as well as an unusual susceptibility to infection with pathogenic trypanosomes and normally nonpathogenic Escherichia coli K12 (9–11). 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 current 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 apsymbiotic tsetse (Glossina morsitans morsitans; [GmmApo]) following microbial challenge, and investigated whether loss of immunity in GmmApo flies could be rescued through either transfer of immune cells from healthy individuals or symbiont provisioning. We obtained 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 insectory at 24°C with 50–55% relative humidity. These flies received deblininated bovine blood (Hemostat Laboratories) every 48 h 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 that they received are described in Table I.
Luciferase-expressing *E. coli* (recE. coli<sub>il</sub>) K12 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 recE. coli<sub/il</sub> cells in vivo was performed as described previously (13). GFP-expressing *E. coli* K12 (recE. coli<sub>GFP</sub>) were produced via electroporation with pGFP-UV plasmid DNA (Clontech). *Sodalis* were isolated from surface-sterilized G. moritans 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 (1 mM CaCl<sub>2</sub>, 0.2 mM MgCl<sub>2</sub>, 2.7 mM KCl, 120 mM NaCl, 1.4 mM NaHCO<sub>3</sub>, 1.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 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 CO<sub>2</sub> and subsequently injecting individuals with live bacterial cells using glass needles and a Narashige IM300 microinjector. Per os bacterial challenges were performed by adding 500 CFU *E. coli* per 20 μl (the approximate amount consumed by a fly) of the total blood meal. The vertebrate host complement system was heat inactivated (56°C for 1 h) 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.

**Homolymph collection and homocyte quantification**

Homolymph collection from wild-type G. moritans moritans (*Gmm*<sub>WT</sub>) and *Gmm*<sup>apo</sup> flies was performed using the high-injection/recovery method, as described previously (15). Subsequent determination of circulating homocyte abundance was performed using a Bright-Line hemocytometer (11). Sessile homocyte abundance was quantified by subjecting *Gmm*<sub>WT</sub> and *Gmm*<sup>apo</sup> flies (*n* = 3) to hemolymph 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 homocytes or any beads not engulfed by sessile homocytes. 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*<sub>WT</sub> and *Gmm*<sup>apo</sup> 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 are represented as a fraction of average normalized gene expression levels in bacteria-infected flies relative to expression levels in corresponding uninfected controls. Values represent mean ± SEM.

**Homolymph transplantation**

Undiluted homolymph 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 microtube on ice. Four cohorts of newly emerged aposymbiotic recipient flies were used, two of which were designated *Gmm*<sup>apo</sup>/*WT* or *Gmm*<sup>apo</sup>/apo based on whether they received homolymph transplanted from wild-type (WT) or aposymbiotic donors, respectively. *Gmm*<sup>apo</sup>/apo recipient flies received 1 μl donor homolymph (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*<sup>WT</sup> donor homolymph into soluble and cellular fractions, samples were centrifuged at 3000 g for 5 min. The cellular component was resuspended in chilled anticoagulant buffer (70% Mitsuhashi–Maramorosch medium, 30% anticoagulant citrate buffer [98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric acid (pH 4.5) ν/v]) (15) equal to the total amount of hemolymph from which they were collected. The remaining two cohorts of *Gmm*<sup>apo</sup> recipient flies were injected with either 1 μl cellular suspension (these flies are designated *Gmm*<sup>apo</sup>/WT or 1 μl the soluble hemolymph fraction (these flies are designated *Gmm*<sup>apo</sup>/apo)).

Aposymbiotic recipient flies were challenged with either 10<sup>3</sup> CFU live *E. coli*. Injections were performed using glass needles and a Narashige IM300 microinjector. Quantification of recE. coli<sub>il</sub> in recipient tsetse was performed as described above. Phagocytic capacity of transplanted hemocytes was determined by infecting *Gmm*<sup>apo</sup>/apo recipient flies with 10<sup>3</sup> CFU live recE. coli<sub>il</sub>. Twelve hours postchallenge, hemolymph was collected from three individuals, and homocytes 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).

**Bacterial complementation experiments**

A cartoon illustrating how bacterial complement experiments were performed is shown in Supplemental Fig. 1. Three cohorts (v = 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 bacterial complement, whereas those from the second and third GCs developed in the presence of bacterial complement. Offspring of these females were designated *Gmm*<sup>apo</sup>/WT and *Gmm*<sup>apo</sup>/apo, respectively.

*Wigglesworthia* was obtained by dissecting tsetse bacteriomes (an organ immediately adjacent to the midgut that houses this bacteria) from *Gmm*<sub>WT</sub> females (whereas *Sodalis* was maintained in culture as described above). *Gmm*<sup>apo</sup>/WT females were fed one bacteriome equivalent per four females, and *Gmm*<sup>apo</sup>/apo females were fed 4 × 10<sup>3</sup> *Sodalis/ml blood (thus, these flies ingested ~1 × 10<sup>6</sup> *Sodalis* every day) for 10 d. A third control cohort of symbiont-cured females received no bacterial complement (their offspring are designated *Gmm*<sup>apo</sup>/apo), and a fourth cohort of WT offspring (*Gmm*<sup>apo</sup>/WT) served as another control. To confirm the aposymbiotic status of offspring from symbiont-cured mothers (Supplemental Fig. 2), genomic DNA was extracted from larval offspring (third instar; *n* = 3) of all experimental cohorts using the Holmes–Bonner method (17). PCR (20-μl reactions) was performed in an MJ Research Thermal Cycler using
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 serpentine andloorzenge 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/WT and GmmApo/ghum individuals (n = 3) from the second GC of symbiont-cured mothers. Finally, all remaining mature adult offspring were challenged with 10^2 CFU live reE. 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 (9.0) 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

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 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 symbiotic bacteria (GmmApo) from the flies, whereas the vitamin-rich yeast extract with tetracycline and yeast. This antibiotic treatment clears all symbiotic bacteria (GmmApo) 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 GmmApo 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; GmmApo, 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 GmmApo 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 tep4, prophenoloxidase, and inducible NO synthase (iNOS), were expressed at significantly lower levels in GmmWgm 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 GmmWT and GmmApo 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
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\textsuperscript{Apo} flies compared with Gmm\textsuperscript{WT} 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\textsuperscript{WT} and Gmm\textsuperscript{Apo} 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\textsuperscript{Apo} individuals compared with Gmm\textsuperscript{WT} 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\textsuperscript{Apo} individuals are more susceptible to challenge with E. coli than are WT tsetse or tsetse that lack only Wigglesworthia (Gmm\textsuperscript{Wgm}–). 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\textsuperscript{WT} individuals and 59% of mature Gmm\textsuperscript{Wgm}– individuals survived systemic challenge with 10\textsuperscript{7} CFU of E. coli (Fig. 2A, top and middle panels). In contrast, all age-matched Gmm\textsuperscript{Apo} individuals perished by 12 dpc (Fig. 2A, bottom panel). We next challenged Gmm\textsuperscript{WT} and Gmm\textsuperscript{Apo} flies per os with 10\textsuperscript{3} and 10\textsuperscript{6} CFU of E. coli and found that all individuals survived this challenge (Fig. 2A, top and bottom panels). This finding suggests that mature Gmm\textsuperscript{Apo} flies are considerably more susceptible to systemic challenge with a foreign microbe than are age-matched Gmm\textsuperscript{WT} and Gmm\textsuperscript{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 that we observed among Gmm\textsuperscript{WT}, Gmm\textsuperscript{Wgm}–, and Gmm\textsuperscript{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\textsuperscript{7} CFU of E. coli, bacterial densities within mature Gmm\textsuperscript{WT} flies reached 8.3 × 10\textsuperscript{3} cells before being cleared. Interestingly, Gmm\textsuperscript{Wgm}– flies, which perish following challenge with 10\textsuperscript{6} 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\textsuperscript{Apo} flies peaked at 7.8 × 10\textsuperscript{6} 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\textsuperscript{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\textsuperscript{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\textsuperscript{WT} and Gmm\textsuperscript{Apo} individuals (donor flies) into the hemolocoel of Apo individuals (recipient flies) into the hemocoel of Apo individuals (donor flies) into the hemocoel of Apo individuals (recipient flies) designated Gmm\textsuperscript{Apo/WT} and Gmm\textsuperscript{Apo/Apo}, respectively). Five days after this procedure, we determined that Gmm\textsuperscript{Apo/WT} flies harbored 330 ± 20.4 hemocytes/μl of hemolymph, whereas Gmm\textsuperscript{Apo/Apo} flies harbored 5 ± 3.8 hemocytes/μl of hemolymph (Fig. 3A). We next investigated whether our hemolymph-transplantation procedure was able to rescue the Gmm\textsuperscript{Apo} flies. To do so, we challenged Gmm\textsuperscript{Apo/WT} and Gmm\textsuperscript{Apo/Apo}
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$^{Apo/WT}$ flies survived for 14 d following challenge. In comparison, only 2% of Gmm$^{Apo/Apo}$ flies survived their challenge (Fig. 3B). 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 separated hemolymph from WT donors (Gmm$^{Apo/Cell}$) perio exsary to recipient flies. Significantly more Gmm$^{Apo/Cell}$ flies showed that transplanted hemocytes engulfed the introduced E. coli that bacterial sepsis was the cause of high mortality we observed in this group of flies. In contrast, apsymbiotic recipients were able to clear all E. coli by 8 dpc when they had previously received a hemolymph transplant from Gmm$^{WT}$ donors (Fig. 3C). More so, microscopic examination of hemolymph from Gmm$^{Apo/WT}$ flies showed that transplanted hemocytes engulfed the introduced E. coli (Fig. 3D). Our results demonstrate that immune resistance can be restored in adult apsymbiotic tsetse if they harbor hemocytes transplanted from their WT counterparts.

**Supplementation of Wigglesworthia to symbiont-cured females restores immune system development in apsymbiotic 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 apsymbiotic flies with this bacterium. To circumvent this impediment, we tested whether we could restore the process of immune system development in Gmm$^{Apo}$ 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 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$^{WT}$ 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$^{Apo/Wig}$ and Gmm$^{Apo/Sod}$, respectively) was com-
pared with that of control cohort offspring from symbiont-cured mothers that received no bacterial supplement (Gmm\(^{\text{Apo/WT}}\)) and offspring from Gmm\(^{\text{VT}}\) mothers. 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 larvalogenesis (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\(^{\text{Apo/Wgm}}\), Gmm\(^{\text{Apo/Sgm}}\), and Gmm\(^{\text{Apo/NB}}\) larva from the first GC expressed significantly less serpent and lozenge than did Gmm\(^{\text{WT}}\) larva. However, after the onset of bacterial supplementation, Gmm\(^{\text{Apo/Wgm}}\) and Gmm\(^{\text{WT}}\) larva from the second and third GCs expressed comparable levels of serpent and lozenge, whereas Gmm\(^{\text{Apo/NB}}\) and Gmm\(^{\text{Apo/Sgm}}\) 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\(^{\text{Apo/Wgm}}\) WT, Gmm\(^{\text{Apo/NB}}\), and Gmm\(^{\text{Apo/Sgm}}\) 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\(^{\text{Apo/Wgm}}\) WT 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\(^{\text{Apo/NB}}\) (7 ± 3 and 9 ± 4 hemocytes/μl of hemolymph, respectively) and Gmm\(^{\text{Apo/Sgm}}\) flies (10 ± 4 and 4 ± 1 hemocytes/μl hemolymph, respectively), but it was significantly less than that of Gmm\(^{\text{WT}}\) 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\(^{\text{Apo/Wgm}}\) flies compared with adult Gmm\(^{\text{Apo/NB}}\) 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\(^{\text{Apo/Wgm}}\) and Gmm\(^{\text{Apo/Sgm}}\) 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 apsymbiotic offspring. Specifically, Gmm\(^{\text{Apo/Wgm}}\) 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\(^{\text{Apo/Wgm}}\) adults.

Gmm\(^{\text{Apo/Wgm}}\) flies are resistant to E. coli challenge

We observed that Gmm\(^{\text{Apo/Wgm}}\) offspring exhibit hallmarks of enhanced immunity. Thus, we next tested whether mature Gmm\(^{\text{Apo/Wgm}}\) adults would be resistant to systemic challenge with E. coli K12, whereas age-matched Gmm\(^{\text{Apo/Sgm}}\) and Gmm\(^{\text{Apo/NB}}\) flies would not. To this end, we observed that 38 and 43% of Gmm\(^{\text{Apo/Wgm}}\) adults from GCs 2 and 3, respectively, survived challenge with 10\(^5\) E. coli (Fig. 5A). Correspondingly, microscopic inspection of hemolymph from E. coli-resistant Gmm\(^{\text{Apo/Wgm}}\) adults revealed the presence of phagocytic hemocytes that harbored internalized E. coli cells (Fig. 5B). In contrast, Gmm\(^{\text{Apo/NB}}\) and Gmm\(^{\text{Apo/Sgm}}\) flies were highly susceptible to E. coli challenge.

![FIGURE 4. Dietary supplementation of Wigglesworthia cell extracts to symbiont-cured female tsetse induces immune system development in their apsymbiotic offspring. Three groups of pregnant female tsetse were provided four 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 complement the absence of these bacteria. The third group of symbiont-cured females received no bacterial complement. Finally, a fourth group of WT females received no tetracycline or bacterial complementation. Offspring of these females, which are designated Gmm\(^{\text{Apo/Wgm}}\), Gmm\(^{\text{Apo/Sgm}}\), and Gmm\(^{\text{Apo/NB}}\), respectively, were collected from three GCs and subsequently monitored to determine their immune system phenotype. GC1 is indicated in gray to signify that bacterial supplement of Gmm\(^{\text{Apo/Wgm}}\) and Gmm\(^{\text{Apo/Sgm}}\) mothers began after their first larval offspring were fully developed. (A) qPCR was performed on larval offspring (n = 3 larva/cohort/GC) to determine their levels of serpent and lozenge expression. (B) Circulating hemocyte abundance in adult offspring (n = 3 flies/cohoot/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\(^{\text{Apo/Wgm}}\) and Gmm\(^{\text{Apo/Sgm}}\) adults challenged with E. coli. Adult flies used for this experiment were from the second GC of symbiont-cured mothers. 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 three biological samples in duplicate. Values are presented as means. *p < 0.05, **p < 0.005.](http://www.jimmunol.org/Downloaded from)
and, like their Gmm$^{\text{Apo}}$ counterparts, all perished within the 14-d experimental period (Fig. 5A). This susceptible phenotype likely resulted from the fact that Gmm$^{\text{ApoNB}}$ and Gmm$^{\text{ApoSym}}$ adults are devoid of phagocytic hemocytes (Figs. 4B, 5B). These findings suggest that aposymbiotic tssete can survive infection with an otherwise lethal dose of E. coli if they completed intrauterine development while their mothers were fed a diet containing Wigglesworthia cell extracts. This immunocompetent phenotype exhibited by Gmm$^{\text{ApoWgm}}$ 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 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 (Gmm$^{\text{Apo}}$) flies derived from symbiont-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 Gmm$^{\text{Apo}}$ adults receive hemocytes transplanted from WT individuals. Furthermore, the process of immune system development in Gmm$^{\text{Apo}}$ larvae can be restored when their symbiont-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 $\approx 50$ million years (31).

Tsetse that undergo intrauterine larval development in the absence of only Wigglesworthia (Gmm$^{\text{Wgm}}$) exhibit a compromised immune system that, when compared with WT flies (Gmm$^{\text{WT}}$), 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, Gmm$^{\text{Apo}}$ adults harbor virtually no circulating (99% less than Gmm$^{\text{WT}}$ 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$^{\text{Wgm}}$ flies, which undergo intrauterine maturation in the presence of Sodalis and Wolbachia, house $\sim 40$-fold more circulating hemocytes than do their aposymbiotic counterparts and are more tolerant to E. coli challenge (11). The enhanced immunity exhibited by Gmm$^{\text{Wgm}}$ 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 their tsetse host. Although no experimental evidence exists that demonstrates a functional role of this nature for Sodalis, Wolbachia exhibits immunomodulatory properties in

![FIGURE 5. Gmm$^{\text{ApoWgm}}$ flies exhibit resistance to challenge with E. coli. (A) Percent survival of mature Gmm$^{\text{ApoWgm}}$, Gmm$^{\text{ApoSym}}$, Gmm$^{\text{ApoNB}}$, and Gmm$^{\text{WT}}$ adults from three GCs following challenge with 10$^3$ CFU of E. coli K12. Significantly more Gmm$^{\text{ApoWgm}}$ flies from the second GC survived this challenge than did age-matched Gmm$^{\text{ApoWgm}}$ and Gmm$^{\text{ApoNB}}$ individuals ($p < 0.01$). However, significantly fewer Gmm$^{\text{ApoWgm}}$ 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 Gmm$^{\text{WT}}$ and Gmm$^{\text{ApoWgm}}$ flies; $n = 20$ for Gmm$^{\text{ApoSym}}$ and Gmm$^{\text{ApoNB}}$ flies) infection experiments were performed in triplicate for all tsetse groups. GC3 is denoted with an asterisk because not enough Gmm$^{\text{ApoWgm}}$ flies 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. coli$^{\text{GFP}}$ hemolymph was collected from all individuals ($n = 3$ flies/group/GC) to monitor for the presence of phagocytic hemocytes. Samples were processed as previously described. Original magnification $\times 400$. In (A) and (B), GC1 is indicated in gray to signify that bacterial complement of Gmm$^{\text{ApoWgm}}$ and Gmm$^{\text{ApoSym}}$ mothers began after their first intrauterine larval offspring were approximately midway through their third developmental instar.](http://www.jimmunol.org/).
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 (wMelPop) (34). The presence of wMelPop 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 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 immunity that tsetse’s ability to overcome 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 immunity. First, E. coli kills GmmNpo 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 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 GmmNpo 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 activate 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 GmmNpo 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 reproducingly 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.

Acknowledgments
We thank Dr. Yineng Wu for assistance with qPCR and members of the Aksoy laboratory for critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

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