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Two C-type Lectins Cooperate to Defend *Anopheles gambiae* against Gram-negative Bacteria*

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C-type lectins (CTLs) are a family of proteins that share a common structural motif, the carbohydrate recognition domain, and may act as receptors in pathogen recognition. Indeed, some vertebrate CTLs, particularly the collectins, are unequivocally implicated in the innate immune response to certain microbes. Although studies in insects and other invertebrates have described CTL activation of effector immune responses *in vitro*, the contribution of these CTLs to immune defenses *in vivo* is still poorly understood. Here we report that two CTLs, *CTL4* and *CTLMA2*, which were shown previously to inhibit *Plasmodium berghei* ookinete melanization in the malaria vector *Anopheles gambiae*, are transcriptionally induced by bacterial challenge. Using *in vivo* reverse genetic analysis, we show that both CTLs are required for the clearance of *Escherichia coli*, but not *Staphylococcus aureus*, from adult female mosquitoes. Silencing either *CTL* dramatically reduces mosquito survival to Gram-negative but not to Gram-positive bacterial infections, suggesting a role in defense against Gram-negative bacteria. Furthermore, molecular characterization reveals that both CTLs are secreted into the mosquito hemolymph mainly in the form of a disulfide-linked heterodimer. This association explains the similar roles of these CTLs in bacterial defense as well as in the melanization response to *P. berghei* ookinetes. Apparently, CTL4 and CTLMA2 serve pleiotropic functions in the innate immune response of *A. gambiae*.

C-type lectins (CTLs)[3] constitute the largest and most diverse family of animal lectins. They function extracellularly and are either secreted or membrane-bound (1). CTLs bind carbohydrates in a Ca^{+}-dependent manner through a module designated as C-type carbohydrate-recognition domain, which belongs to a larger family of protein modules, the C-type lectin-like domains (CTLDs). Many CTLDs are Ca^{+}-independent and may not necessarily bind to sugar ligands (2). Carbohydrate/CTL interactions occur on cell surfaces, in the extracellular matrix, or on soluble secreted glycoproteins and may mediate processes such as cell adhesion, cell/cell interactions, glycoprotein turnover, and pathogen recognition leading to innate immune responses. In vertebrates, CTLs are important components of cellular as well as humoral innate immune responses. Membrane-bound, immune-related CTLs belong to several groups distinguished by their domain organization, whereas the soluble immune CTLs are mostly restricted to a single group, the collectins (collagenous lectins) (2). These are acute phase proteins mediating several functions, including opsonization and clearance of microbial agents (3, 4), and complement activation through the lectin pathway by the serum mannose-binding lectins (5, 6). The generation of collectin gene knock-out mice was fundamental in highlighting the importance of collectin/microbe interactions *in vivo* (7, 8).

Genomic analysis of invertebrate CTLD-containing proteins, in particular from insects (9, 10) and the nematode *Caenorhabditis elegans* (11), predicted most of them as soluble, often containing a single CTLD without accessory domains. To date, the role of insect CTLs in microbial defense has been investigated mainly in the *Lepidoptera*, *Bombyx mori* (12–15), *Manduca sexta* (16–19), and *Hyphantria cunea* (20). Very few reports have examined potential defense functions in other insect orders (21–23). Several immune functions have been proposed for insect CTLs, including activation of the prophenol oxidase cascade (16, 24), hemocyte-mediated encapsulation (25), nodule formation (12, 15), and opsonization (21, 23). However, with the exception of the *M. sexta* immulectin-2 (26), genetic evidence is missing on the possible contributions of insect CTLs to microbial defense *in vivo*. In this study we report an *in vivo* functional genetic analysis in the malaria vector, *Anopheles gambiae*, which specifically addresses this question.

The *A. gambiae* genome includes 23 genes of the CTL superfamily that encode proteins containing CTLDs. These genes have been classified into different subgroups according to sequence-specific signatures (9). Functional genetic analysis of selected *A. gambiae* CTL genes using RNA interference (RNAi) in adult female mosquitoes has allowed the identification of two CTLs, CTL4 and CTLMA2, which act as agonists of *Plasmodium* development in the vector (27); silencing of either of these genes induced massive melanization of *Plasmodium berghei* ookinetes in the basal labyrinth of the midgut epithelium, blocking their development to oocysts. Here we show, using *in*

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[3] The abbreviations used are: CTL, C-type lectin; CTLD, C-type lectin-like domain; RNAi, RNA interference; CFU, colony-forming unit; PBS, phosphate-buffered saline; dsRNA, double-stranded RNAs; qRT, quantitative real time reverse transcriptase; RT, reverse transcription; PO, phenol oxidase.

vivo RNAi, that CTL4 and CTLMA2 are also required for *A. gambiae* defense against Gram-negative but not Gram-positive bacteria. Mosquitoes in which either of these genes was silenced by injecting the corresponding double-stranded RNA (dsRNA) exhibited dramatic proliferation of *Escherichia coli* but not *Staphylococcus aureus*. Furthermore, we report that these CTLs exist in the mosquito hemolymph as a disulfide-linked heterodimer, thus explaining their similar roles in antibacterial defense as well as in the melanization response to *P. berghei*.

EXPERIMENTAL PROCEDURES

A. gambiae Strain and Gene Silencing by RNAi—All experiments were performed with *A. gambiae* G3 strain, which was reared as described previously (28). *In vivo* gene silencing by RNA interference was performed as reported (29). dsRNA for *lacZ* (control), *CTL4,* and *CTLMA2* were synthesized as described in Refs. 27, 30, 31, respectively.

Cell Lines and Bacterial Strains—Sf9 cells (Invitrogen) adapted to serum-free growth conditions, according to the manufacturer's recommendation, were maintained in Sf-900 II SFM (Invitrogen) supplemented with penicillin (100 units/μl) and streptomycin (100 μg/μl) (Invitrogen). The bacterial species used in this study include the following: ampicillin-resistant *E. coli* OP-50, a gift from J. J. Ewbank (INSERM, Marseille-Luminy, France); tetracycline-resistant *S. aureus*, *Enterococcus faecalis,* and *Micrococcus luteus,* kind gifts from Philippe Bulet (IBMC, Strasbourg, France); *Enterobacter cloacae* (ATCC 13047); and Pseudomonadaceae H2.26, a field isolate from *Anopheles gambiae sensu lato* (32) and a kind gift from Ingrid Faye (Stockholm University, Stockholm, Sweden). All strains were cultured in Luria-Bertani (LB) broth, harvested during the logarithmic growth phase, washed with phosphate-buffered saline (PBS), and resuspended in PBS to an absorbance at 600 nm ($A_{600\ nm}$) of 0.4 (*E. coli*, *S. aureus*, *E. faecalis*, and *M. luteus*), 0.004 (*E. cloacae*), and 0.001 (Pseudomonadaceae). $A_{600\ nm}$ of 0.4 corresponds approximately to 150,000, 200,000, 500,000, and 100,000 colony-forming units (CFU)/μl of *E. coli*, *S. aureus*, *E. faecalis,* and *M. luteus*, respectively. $A_{600\ nm}$ of 0.001 and 0.004 corresponds to 1500 and 1800 CFU/μl of Pseudomonadaceae and *E. cloacae*, respectively.

RNA Isolation, Quantitative Real Time Reverse Transcriptase PCR (qRT-PCR), and Semi-quantitative RT-PCR—Four-day-old adult female mosquitoes were challenged with *E. coli* or *S. aureus* either by injecting 69 nl of a bacterial suspension in PBS or by pricking with a needle dipped into a thick bacterial pellet. Mosquitoes injected or pricked with sterile PBS were used as controls. Total RNA was isolated from 15 whole mosquitoes at the indicated time points using TRIzol reagent (Invitrogen) according to the supplier's instructions, and contaminant genomic DNA was removed by DNase I treatment. First strand cDNA synthesis was primed from total RNA (3 μg) using oligo(dT)$_{12-18}$ and Superscript II, a modified Moloney murine leukemia virus reverse transcriptase, as described by the manufacturer (Invitrogen). qRT-PCR was performed in an ABI Prism 7000 sequence detection system using the SYBR Green PCR master mix kit (Applied Biosystems) according to the manufacturer's instructions. The primers used in qRT-PCR were described previously (27). Relative gene expression values were calculated using the comparative C_T method after checking for the efficiency of target amplification as described in the ABI Prism 7700 Sequence Detection System User Bulletin 2. For semiquantitative RT-PCR, cDNA synthesis was primed using oligo(dT)$_{25}$ magnetic beads as described previously (33). CTL4 primers (27) and CTLMA2 primers (forward, 5′-gcccatgcaaaccgttcgaggaga-3′; reverse, 5′-TGACagatgaacggcttctg-ctgcg-3′) were used to amplify 473- and 400-bp fragments, respectively, according to the following program (45 s at 95 °C; 60 s at 50 °C; and 60 s at 72 °C) for 25 cycles. The internal S7 control (34) was amplified for 20 cycles using the following program (45 s at 95 °C; 60 s at 59 °C; and 60 s at 72 °C). The linear range of all amplification reactions has been determined empirically. Amplicons were separated on a 1% agarose gel, stained with SYBR green dye (Molecular Probes), and analyzed with a fluorimager (Fuji).

Mosquito Survival and Bacterial Proliferation Assays—Bacterial injections and survival assays were done as described previously (29). Briefly, 4 days after dsRNA injection, individual mosquitoes were injected with 69 nl of the bacterial suspensions in PBS, and their survival was followed over a period of 10 days, except for Pseudomonadaceae-infected mosquitoes for which survival was examined during a 35-h period because of the strong pathogenicity of this bacterium. The Kaplan-Meier survival test was used to calculate the probability of survival over the indicated time scale. Statistical significance of the observed differences was calculated by the log-rank test. Differences were considered to be significant if p value was <0.05. For the bacterial proliferation assay, four batches of 10 mosquitoes each per genotype were ground in 500 μl of Luria-Bertani broth (LB) 48 h post-infection with *E. coli* or *S. aureus*. The mosquito homogenate was plated in serial dilutions onto LB-agar plates containing the appropriate antibiotic, and the number of CFU was determined. Statistical significance of the observed differences was calculated by the Kruskal-Wallis test followed by Dunn's post test. Differences were considered to be significant if $p < 0.05$.

Generation of Antiserum against CTL4 and CTLMA2—Partial sequences of the CTLMA2 and CTL4 open reading frames (including the CTLD) were amplified from whole adult female mosquito cDNA using the following primers: CTLMA2expF1, 5′-ggatcctgcccatgcaaaccgttcgaggaga-3′, and CTLMA2expR1, 5′-ctcgagttatcggaactgttgacagatgaacgg-3′); CTL4expF1, 5′-ggatccgttagcagcattgggattaccctcg-3′, and CTL4expR1, 5′-ctcgagttagaagtcgcaacccagctcattgtag-3′; BamHI and XhoI overhangs are underlined. A stop codon (TTA) was inserted in the reverse primer downstream of the XhoI overhang. Amplicons were subcloned into pGEM-T easy vector (Promega), sequenced, and then cloned into BamHI/XhoI sites of pGEX-4T-3 expression vector (GE Healthcare). The partial proteins were expressed as fusions with glutathione *S*-transferase in *E. coli* strain BL21 (DE3) and were mainly contained in inclusion bodies. The fusion proteins were re-solubilized from inclusion bodies as follows. Bacterial pellets were resuspended in 50 mM Tris buffer, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 10 mM dithiothreitol, 5 mM $MgCl_2$, lysozyme (1 mg/ml), and DNase I (1 μg/ml), and the lysate was incubated 30

min under constant agitation before centrifugation (20 min at 11,000 × *g*) in a refrigerated Sorvall centrifuge. The pellet was washed once with 50 mM Tris buffer, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol and once with the same buffer without detergent. The protein was extracted from the purified inclusion bodies using Tris buffer, pH 8.0, 8 M urea, and 1 mM dithiothreitol. The denatured fusion proteins were used to immunize two rabbits in Ribi Adjuvant (RAS, Ribi Immunochem). Rabbits were boosted every 4th week with 0.25 mg of antigen/rabbit until the final bleed. Monoclonal antibodies against CTLMA2 fusion protein were produced at the EMBL Monoclonal Antibody Core Facility as described (35).

Hemolymph Extraction and Western Blot Analysis—Hemolymph from 20 to 25 naive mosquitoes was extracted by proboscis clipping into sterile PBS containing EDTA-free protease inhibitor mixture (Roche Applied Science), at the indicated time points. Hemolymph proteins (~2 μg/well) were separated by SDS-PAGE and transferred to Hybond-P polyvinylidene difluoride (Amersham Biosciences) by semi-dry blotting (Bio-Rad). Blots were incubated with mouse anti-CTLMA2 monoclonal antibody (1/30), rabbit anti-CTLMA2 (1/200), rabbit anti-CTL4 (1/800), and rabbit anti-Serpin 3 (1/1000) polyclonal antibodies (36). Anti-mouse and anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibodies (Promega) were used at 1/10,000 and 1/30,000, respectively.

Expression of CTL4 and CTLMA2 in Sf9 Cells and Site-directed Mutagenesis—Full-length CTL4 and CTLMA2 coding sequences were amplified from mosquito cDNA using the following primers: CTL4_F, 5′-ggaattccaccatgcagatctcaaacatatttgtt-3′ and CTL4_R, 5′-gctctagactatattcacaaataaattgtctcggt-3′; CTLMA2_F, 5′-ggaattcaccatgaatttaatgatcacaattcta-3′ and CTLMA2_R, 5′-gctctagactatgttttcggaactgttgacagatgaa-3′) containing the restriction sites EcoRI and XbaI (underlined). Amplicons were cloned into EcoRI and XbaI restriction sites of the pIZT/V5His vector (Invitrogen) yielding pIZT/CTL4-V5His and pIZT/CTLMA2-V5His and sequenced. Sf9 cells adapted to serum-free conditions were seeded into 6-well plates (Nunc) at 50,000 cells per cm^2 and transfected with 0.5 μg of the respective plasmid using Cellfectin® as described by the manufacturer (Invitrogen). After 24 h, the medium was removed, and 1 ml of fresh serum-free medium was added to the cells. Equal volumes of conditioned media were harvested 5 days post-transfection and subjected to Western blot analysis using anti-V5 monoclonal antibody (Invitrogen) at 1/5000 and anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (Promega) at 1/10,000. For site-directed mutagenesis, the three cysteines present at the N terminus of CTL4 and CTLMA2 were substituted by alanines (CTL4, C39A, C41A, and C43A; CTLMA2, C34A, C36A, and C38A) using the QuikChange® mutagenesis kit (Stratagene). The following mutagenized primer pairs (changed bases are underlined) were used: CTL4_3cys_F, 5′-cgaaatgattacccaaaacctggccgttgctccggccggcaatccaagaggaggaaag-3′; CTL4_3cys_R, 5′-Ctttcctcctcttggattgccggccggagcaacggccaggttttgggtaatcatttcg-3′, and CTLMA2_3cys_F, 5′-ggatgatattcttcaacaaaacccagccccttgccccagccaaaccgttcgaggagaaagaatac-3′; CTLMA2_3Cys_R, 5′-gtattctttctcctcgaacggtttggctggggcaagggctgggttttgttgaagaatatcatcc-3′. The successful introduction of the mutations was confirmed by sequencing.

Two-color Western Blot Analysis—Sf9 cells were seeded into 6-well plates as described previously and transfected with 0.5 μg of each of pIZT/CTL4-V5His and pIZT/CTLMA2-V5His, either independently or simultaneously. Cells were incubated 24 h in presence of the plasmids, then washed with serum-free medium, and placed in 1 ml of serum-free medium. Cells were allowed to condition the medium for 7 days. Conditioned media were centrifuged to remove detached cells and cell debris. Proteins (~3 μg) from conditioned media were separated by 12% nonreducing SDS-PAGE and transferred to a low fluorescent polyvinylidene difluoride membrane (Li-COR Biosciences) by semi-dry blotting (Bio-Rad). Membranes were blocked for 1 h in Odyssey Blocking buffer (LI-COR Biosciences) and incubated with mouse anti-CTLMA2 monoclonal antibody (1/30) and rabbit anti-CTL4 (1/1000) polyclonal antibodies overnight in Odyssey Blocking buffer supplemented with 0.1% Tween 20. The Odyssey IRDye infrared secondary antibodies IRDye® 800CW-conjugated goat anti-mouse IgG (1:10,000) and IRDye® 680CW goat anti-rabbit (1:10,000) were used simultaneously to detect CTL4 and CTLMA2 protein bands, respectively. interaction using both 700 and 800 nm channels simultaneously at 169-μm resolution.

Affinity Purification of CTL4-CTLMA2 Complex from Sf9 Cells—Sf9 cells were seeded at 100,000 cells/cm2 in a 24-well plate. When 70–80% confluent, cells were transfected with 0.5 μg each of pIZT/CTL4-V5His and pIZT/CTLMA2-stopV5His (carrying a stop codon before the V5-His tag) either independently or simultaneously, using TransFectin lipid reagent (Bio-Rad) as described by the manufacturer. The stop codon was introduced by inserting a thymidine nucleotide between the last codon of the CTLMA2 coding sequence and the 2 bp preceding the XbaI site in pIZT/CTLMA2-V5His, using the QuikChange site-directed mutagenesis kit and the following mutagenized primers (XbaI site is underlined and the T insertion is in boldface): CTLMA2_stop_F, 5′-caacagttccgaaaacat**t**agtctagagcggcccg-3′, and CTLMA2_stop_R, 5′-cgggccgctctagact**a**atgttttcggaactgttg. Cells were incubated 24 h in presence of the plasmids, then washed with serum-free medium, and placed in 300 μl of serum-free medium. Cells were allowed to condition the medium for 3 days. Conditioned media were centrifuged to remove detached cells and cell debris. For each affinity purification reaction, 100 μl of conditioned medium supplemented with 20 mM imidazole was incubated with 25 μl of slurry of nickel-Sepharose 6 fast flow (GE Healthcare) at room temperature for 90 min. Following incubation, beads were centrifuged at 500 × *g* for 5 min, washed four times with PBS containing 10 mM imidazole and 0.05% Triton X-100, and eluted with 50 μl of 1× reducing protein sample buffer. Samples were heated at 100 °C for 7 min and analyzed by Western blot. Blots were incubated with mouse anti-CTLMA2 monoclonal antibody (1/30), followed by anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (Abcam) at 1/3000.

Phenol Oxidase (PO) Activity Assay—PO activity was assayed as described previously (37) except that 8 μg of mosquito

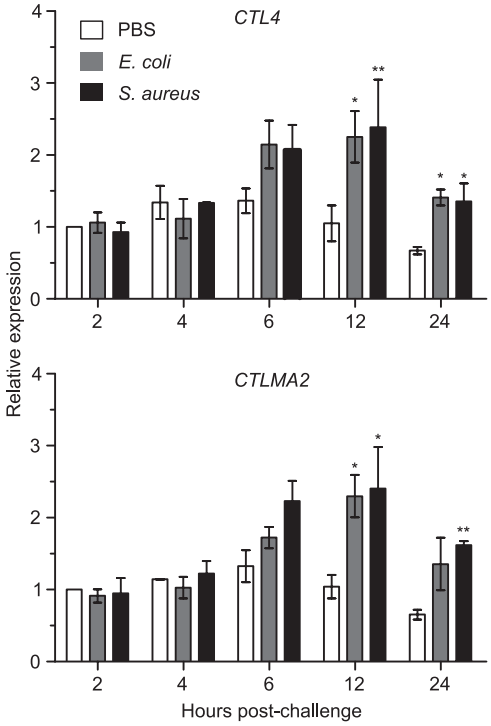

FIGURE 1. **Transcript levels of *CTL4* and *CTLMA2* in whole mosquitoes.** Analysis of transcript levels was performed by qRT-PCR in mosquitoes injected with *E. coli* (*gray*), *S. aureus* (*black*), or sterile PBS (control, *white*) at the indicated time points. The *ordinate* represents expression values relative to the PBS 2-h time point. Means are calculated from three independent biological experiments. *Error bars* represent the means ± S.E. Statistical significance was determined using the Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

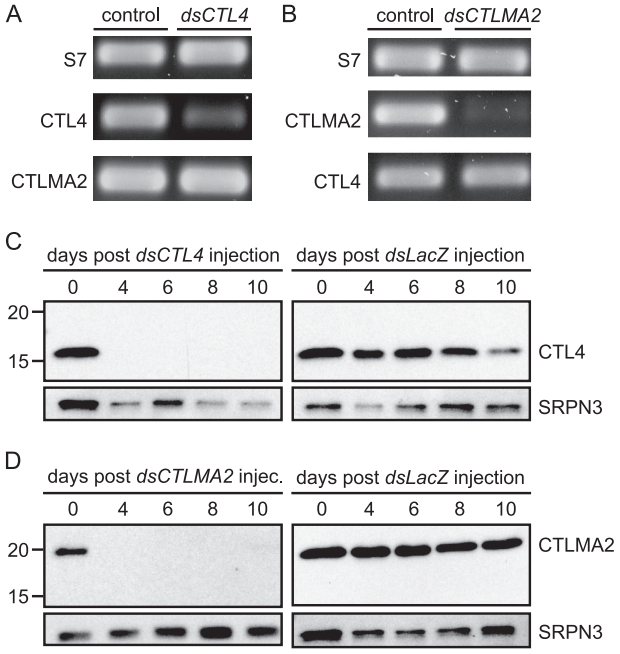

FIGURE 2. **Efficient silencing of *CTL4* and *CTLMA2 in vivo* using RNAi.** Semi-quantitative PCR showing specific reduction in *CTL4* (*A*) and *CTLMA2* transcripts (*B*) 4 days post-injection of naive mosquitoes with ds*CTL4* and ds*CTLMA2*, respectively. Transcripts of the ribosomal protein S7 were used as loading control. Western blots show the absence of CTL4 (*C*) and CTLMA2 proteins (*D*) from the hemolymph of ds*CTL4* and ds*CTLMA2* mosquitoes, respectively, relative to ds*lacZ* controls. Hemolymph samples were collected from naive mosquitoes at the indicated time points following dsRNA injection. Polyclonal rabbit anti-CTL4 and monoclonal mouse anti-CTLMA2 antibodies were used to detect CTL4 and CTLMA2, respectively. A polyclonal antibody against *A. gambiae* Serpin 3 (*SRPN3*) was used as loading control. *Injec.*, injection.

hemolymph were used per assay. The absorbance at 492 nm was measured at 10-min intervals during a 60-min period at room temperature in a microplate absorbance reader (Tecan).

RESULTS

CTL4 and CTLMA2 Expression Is Induced by Bacterial Infections—The involvement of several C-type lectins in vertebrate and putatively in invertebrate innate immune responses prompted us to test whether CTL4 and CTLMA2 are involved in the mosquito immune defense against bacterial infections. We first measured by qRT-PCR whether *CTL4* and *CTLMA2* expression is induced by bacterial challenge. *A. gambiae* female mosquitoes of the G3 strain were injected with a bacterial suspension of *E. coli* or *S. aureus* in PBS or with PBS alone, and the transcript levels of both genes were assessed in whole mosquitoes at several time points post-challenge (Fig. 1). CTL4 and CTLMA2 transcripts were significantly up-regulated in mosquitoes injected with bacterial suspensions as compared with the PBS-injected controls, at 6–24 h post-injection. The induced expression of both genes was maximal at 12 h after infection.

Mosquitoes Treated with dsCTL4 or dsCTLMA2 Are Susceptible to E. coli Infections—As both CTLs were induced following bacterial infections, we then tested by dsRNA-mediated gene silencing whether they are required for the mosquito antibacterial defense. To this aim, we treated adult female mosquitoes with ds*CTL4*, ds*CTLMA2*, or ds*lacZ* (control). Four days later, dsRNA-treated mosquitoes were injected with a suspension of *E. coli* or *S. aureus* in PBS, and their survival rates were compared over 10 days post-infection. To ensure that *CTL4* and *CTLMA2* were efficiently silenced during this period, we performed Western blot analysis on hemolymph extracted from mosquitoes at different time points post-ds*CTL4* (Fig. 2*C*) and -ds*CTLMA2* (Fig. 2*D*) injection. The results confirmed that both proteins were absent from the mosquito hemolymph at least through day 10 post-injection of the corresponding dsRNA. Furthermore, semi-quantitative RT-PCR analysis revealed the absence of cross-silencing at the mRNA levels between ds*CTL4* and ds*CTLMA2* as each dsRNA reduced specifically the transcript levels of its respective target gene (Fig. 2, *A* and *B*). Interestingly, both ds*CTL4*- and ds*CTLMA2*-treated mosquitoes extensively succumbed to *E. coli* infections (Fig. 3*A*); however, they survived *S. aureus* infections as effectively as did ds*lacZ* controls (Fig. 3*B*).

We then determined the number of viable *E. coli* (CFU) in experimentally infected mosquitoes of all three genotypes to investigate whether the compromised survival in ds*CTL4* and ds*CTLMA2* mosquitoes is because of increased bacterial proliferation. The results revealed that at 48 h post-infection, ds*CTL4* and ds*CTLMA2* mosquitoes indeed harbored much higher numbers of bacteria (4.6- and 4.3-fold, respectively) than ds*lacZ* controls (Fig. 3*C*). Mosquitoes in which both *CTLs* were silenced simultaneously did not show increased bacterial proliferation relative to those in which the individual genes were silenced, suggesting that *CTL4* and *CTLMA2* do not exhibit

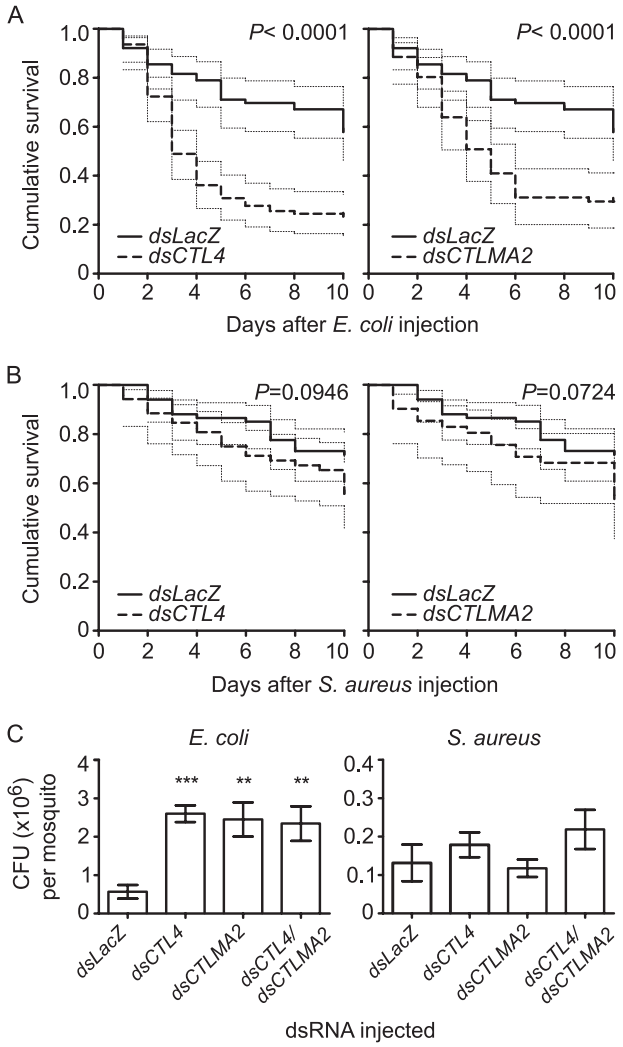

FIGURE 3. **ds*CTL4* and ds*CTLMA2* mosquitoes are susceptible to *E. coli* infections.** Four days post-injection of ds*lacZ* (control), ds*CTL4*, or ds*CTLMA2*, *A. gambiae* adult female mosquitoes were injected with *E. coli* ($A_{600\ nm} = 0.4$) (*A*) or *S. aureus* ($A_{600\ nm} = 0.4$) (*B*). Dead mosquitoes were counted daily over a period of 10 days post-bacterial challenge. *Graphs* represent the cumulative survival as calculated by the Kaplan-Meier method over the indicated time points for one representative experiment. The *thin dotted lines* represent the 95% confidence intervals. Statistical significance was calculated by the log rank test. Survival curves were considered to be significantly different if $p < 0.05$. *C*, ds*CTL4* and ds*CTLMA2* mosquitoes failed to clear *E. coli* but not *S. aureus*. dsRNA-treated mosquitoes were injected with ampicillin-resistant or tetracycline-resistant strains of *E. coli* or *S. aureus*, respectively. From each genotype, four batches of 10 mosquitoes each were ground in LB medium, 48 h post-bacterial challenge, and live bacterial counts were determined by plating serial dilutions on LB plates supplemented with the appropriate antibiotic. *Graphs* represent the mean CFU per mosquito from three independent biological experiments (except for ds*CTL4*/ds*CTLMA2* genotype where two independent biological experiments were performed). *Error bars* represent mean ± S.E. The Kruskal-Wallis test followed by the Dunn's post test was used to compare differences between the various ds*CTL* genotypes and the ds*lacZ* control. ***, $p < 0.001$; **, $p < 0.01$.

additive effects on antibacterial immunity. In accordance with the survival assays, ds*CTL4* and ds*CTLMA2* mosquitoes harbored similar numbers of *S. aureus* as ds*lacZ* controls (Fig. 3*C*). Taken together, our results suggest that both CTLs are required for defense against *E. coli* but not *S. aureus* bacteria.

dsCTL4-treated Mosquitoes Do Not Exhibit an Enhanced PO Activity—We have shown previously that ds*CTL4*- and ds*CTLMA2*-treated mosquitoes melanize and kill the majority

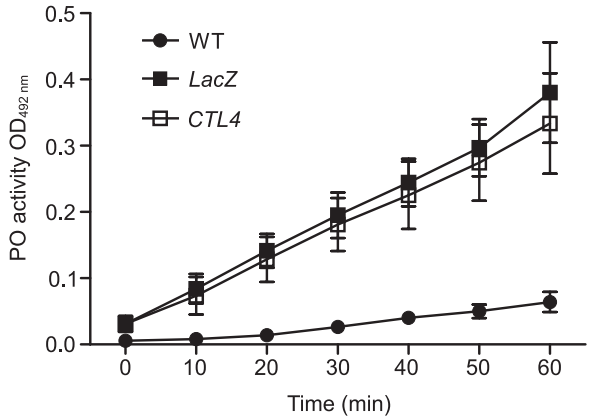

FIGURE 4. **PO activity in ds*CTL4* mosquitoes.** PO enzymatic activity (detected as absorbance at 492 nm, OD_{492}, after conversion of L-3,4-dihydroxyphenylalanine) was measured in hemolymph extracted from ds*lacZ* and ds*CTL4* mosquitoes 6 h post-injection of a mixture of *E. coli* and *S. aureus*. Noninjected wild type (*WT*) mosquitoes (controls) showed basal PO activity. The *graph* shows PO activity measured at 10-min intervals over a 1-h period. Means are calculated from three independent biological experiments. *Error bars* represent mean ± S.E.

of *P. berghei* ookinetes that invade the mosquito midgut epithelium, suggesting that CTL4 and CTLMA2 function as parasite agonists (27). The mechanism by which these CTLs function is not yet elucidated, but they seem to block the mosquito melanization response to invading ookinetes. To ensure that the death observed in ds*CTL4* and ds*CTLMA2* mosquitoes following infection with *E. coli* is not because of an enhanced melanotic response that could be toxic to the mosquitoes themselves, we measured the PO activity in ds*CTL4* mosquitoes, 6 h post-infection, with a mixture of *E. coli* and *S. aureus*, and we compared it with that in infected ds*lacZ* controls. The results clearly revealed that the PO activities in infected ds*CTL4* and ds*lacZ* mosquitoes were induced relative to noninfected controls, but to similar levels (Fig. 4), suggesting that the attrition observed in infected ds*CTL4* mosquitoes is indeed because of *E. coli* proliferation and not an enhanced PO activity.

CTL4 and CTLMA2 Form a Heterodimer in the Mosquito Hemolymph—Hemolymph from naive mosquitoes, extracted in reducing SDS protein sample buffer and immunoblotted with CTL4 and CTLMA2 antibodies, revealed two bands with apparent molecular masses of 17 (Fig. 5*B*) and 19 kDa (Fig. 5*C*) corresponding to CTL4 and CTLMA2 monomers, respectively. The theoretical molecular masses of CTL4 and CTLMA2 are 17.2 and 17.8 kDa, respectively, excluding the predicted N-terminal signal peptide (Fig. 5*A*). As we could not detect potential *N*-glycosylation sites in CTLMA2 by bioinformatic analysis, its low electrophoretic mobility in SDS-PAGE (19 kDa) as compared with its predicted molecular mass (17.8 kDa) is most convincingly attributed to lesser SDS binding due its predicted low isoelectric point (pI = 4.2). Anomalous migration of proteins rich in acidic amino acids on SDS-PAGE has been reported previously in the literature (38). Interestingly, in nonreducing conditions, both antibodies detected major bands of similar molecular masses (~28 kDa). The fact that these CTLs have different molecular masses under reducing conditions but display an identical molecular mass under nonreducing conditions suggests that they either form a disulfide-linked heterodimer or are part of two different complexes with fortuitously

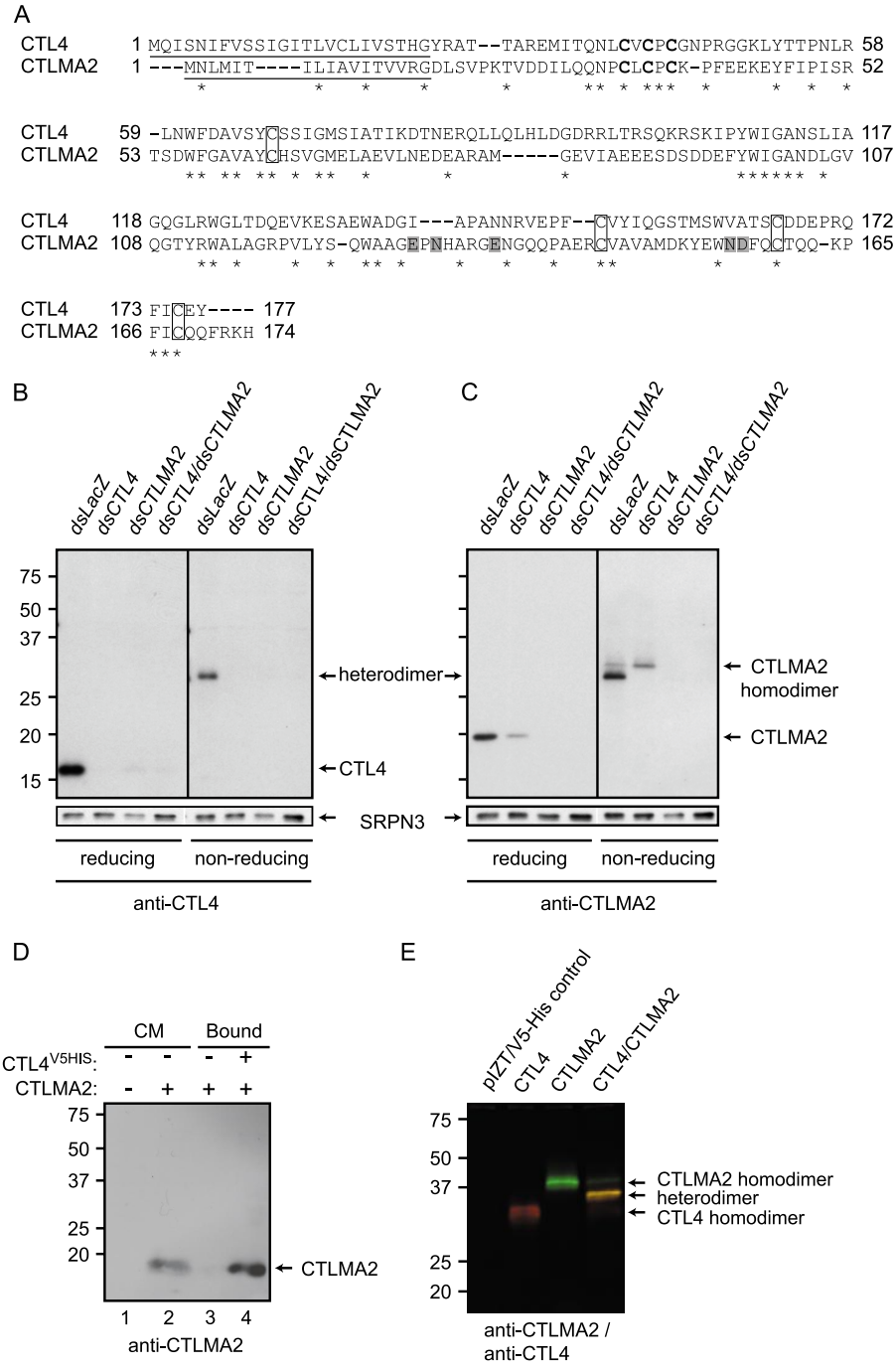

FIGURE 5. **CTL4 and CTLMA2 form a disulfide-linked heterodimer.** *A,* alignment of CTL4 and CTLMA2 protein sequences using T-COFFEE align software. The predicted signal peptides are *underlined*. The N-terminal cysteine residues involved in disulfide linkages between CTL4 and CTLMA2 are in *boldface*. The four cysteine residues that are diagnostic of the carbohydrate recognition domain are enclosed in *rectangles*. Amino acid residues that form the putative sugar-binding site were determined by sequence comparison with the rat mannose-binding protein (41) and are shaded in *gray*. The *numbers* refer to residues counted from the initiation methionine. Residues identical in both sequences are marked with an *asterisk*. *B* and *C,* Western blots of hemolymph proteins extracted 4 days after injecting naive mosquitoes with the indicated dsRNAs and separated by SDS-PAGE on a 13% gel. Under reducing conditions, CTL4 and CTLMA2 antibodies detect 17- and 19-kDa bands, respectively, and they both detect major bands with similar molecular masses of ~28 kDa in nonreducing conditions. Note that CTLMA2 exhibits a minor homodimeric form of 30 kDa. Polyclonal rabbit anti-CTL4 and monoclonal mouse anti-CTLMA2 antibodies were used to detect CTL4 and CTLMA2, respectively. A polyclonal antibody against Serpin 3 (*SRPN3*) was used as loading control. *D,* affinity purification of $CTL4^{V5His}$ on nickel-Sepharose captures nontagged CTLMA2. CTLMA2 was either singly or co-expressed with $CTL4^{V5His}$ in Sf9 cells. His tag capture from conditioned medium of cells co-expressing CTLMA2 and $CTL4^{V5His}$ co-purifies CTLMA2 (*lane 4*) is shown. CTLMA2 did not bind to nickel-Sepharose in the absence of $CTL4^{V5His}$ (*lane 3*). *CM,* conditioned medium of mock-transfected cells (*lane 1*) or cells expressing only CTLMA2 (*lane 2*). *E,* two-color Western blot analysis of Sf9-conditioned media expressing CTL^{V5His} and $CTLMA2^{V5His}$ either separately or simultaneously and subjected to nonreducing SDS-PAGE. The membrane was probed simultaneously with rabbit anti-CTL4 and mouse anti-CTLMA2 followed by simultaneous incubation with anti-mouse (*green*) and anti-rabbit (*red*) antibodies conjugated to infrared fluorescent dyes. CTL4 and CTLMA2 homodimers are in *red* and *green*, respectively, while the CTL4-CTLMA2 heterodimer appears *yellow*. Transfection with an empty pIZT/V5-His plasmid was used as a negative control.

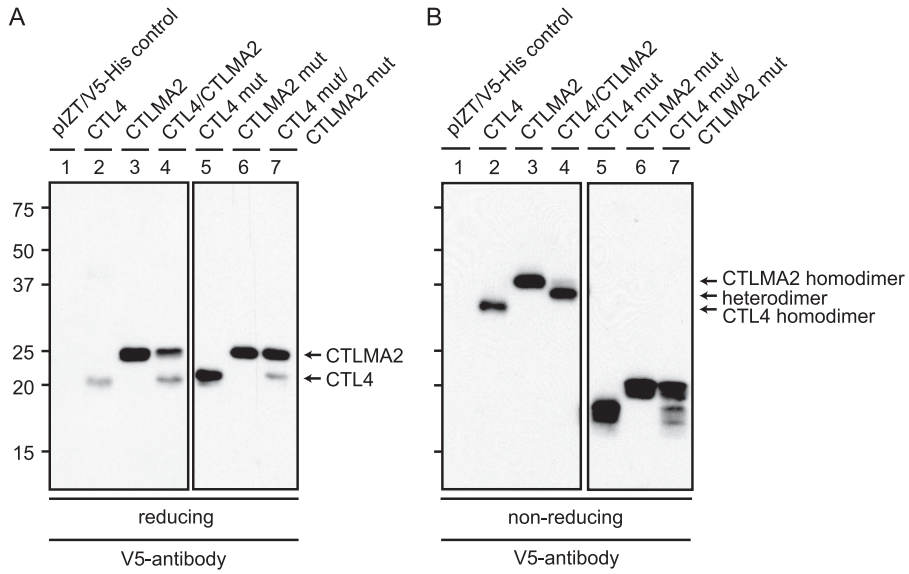

FIGURE 6. **CTLs with mutated N-terminal cysteines do not form dimers.** *A* and *B,* wild type CTLs and their mutants, CTL4 (C39A, C41A, and C43A) and CTLMA2 (C34A, C36A, and C38A), were expressed in Sf9 cells as C-terminal fusion proteins using the pIZT/V5His vector and separated by SDS-PAGE under reducing (*A*) and nonreducing conditions (*B*). Cells were transfected with the indicated plasmid constructs, and conditioned media were collected 5 days post-transfection. The blot was immunostained with anti-V5 tag monoclonal antibody. The apparent molecular masses of the bands are as follows: CTL4 monomer, 22 kDa; CTLMA2 monomer, 25 kDa; CTL4 homodimer, 32 kDa; CTLMA2 homodimer, 37 kDa; CTL4-CTLMA2 heterodimer, 34 kDa. *mut*, mutant.

identical molecular masses. Interestingly, when hemolymph was extracted from ds*CTLMA2* mosquitoes and immunoblotted with CTL4 antibody, both the CTL4 monomer (17 kDa) and the 28-kDa heterodimer were almost undetectable (Fig. 5*B*). Similarly, in hemolymph extracted from ds*CTL4* mosquitoes, the CTLMA2 monomer was barely detectable, and the 28-kDa heterodimer was completely absent (Fig. 5*C*). These results suggest that heterodimerization is required for efficient secretion of these CTLs *in vivo*. The apparent molecular mass of the CTL4-CTLMA2 heterodimer is lower than its expected 35-kDa value. This aberrant migration is probably because of a compact structure resulting from disulfide bonds within the CTLDs (two bonds in each domain) as well as between the N-terminal domains of CTL4 and CTLMA2. Under nonreducing conditions, a faint CTLMA2 band migrating slower than the heterodimer was present in ds*lacZ* control mosquitoes but absent from ds*CTLMA2* mosquitoes. This 30-kDa band is most convincingly interpreted as a minor CTLMA2 homodimer; it was slightly enhanced in the absence of the CTL4 partner (Fig. 5*C*), possibly because surplus CTLMA2 monomers associated to form homodimers. No CTL4 homodimer was detected in the mosquito hemolymph.

We used affinity purification to provide conclusive evidence for heterodimer formation. Because the amount of hemolymph extracts obtained from adult mosquitoes is very limiting, Sf9 cells were utilized instead to investigate CTL4-CTLMA2 complex formation. Recombinant nontagged CTLMA2 and recombinant CTL4 carrying a V5-His tag were co-expressed in Sf9 cells using pIZT/CTLMA2-stopV5His and pIZT/CTL4-V5His plasmids, respectively. Affinity purification of $CTL4^{V5His}$ over nickel-Sepharose from the conditioned medium of transfected Sf9 cells allowed the co-purification of the nontagged CTLMA2 (Fig. 5*D*) indicating that CTL4 and CTLMA2 form unambiguously a heterodimer. A similar conclusion was reached using two-color Western blot analysis. Here, conditioned media of Sf9 cells expressing $CTLMA2^{V5His}$ and $CTL4^{V5His}$ either separately or simultaneously were subjected to nonreducing SDS-PAGE. Following protein transfer, the membrane was probed simultaneously with rabbit anti-CTL4 and mouse anti-CTLMA2 antibodies followed by simultaneous incubation with anti-mouse (green) and anti-rabbit (red) secondary antibodies conjugated to infrared fluorescent dyes; in case of heterodimerization, a major yellow band should appear because of overlapping green and red colors, whereas the CTLMA2 and CTL4 homodimers should stain green and red, respectively. CTL4 and CTLMA2 formed homodimers (32 (*red*) and 37 kDa (*green*), respectively) when expressed separately in Sf9 cells (Fig. 5*E*). Importantly, when the CTLs were co-expressed, they formed almost exclusively a 34-kDa band (*yellow*) corresponding to the CTL4-CTLMA2 heterodimer (Fig. 5*E*). Taken together, our results strongly indicate that when CTL4 and CTLMA2 co-exist, they form a disulfide-linked heterodimer, whether in mosquito hemolymph or in conditioned medium of Sf9 cells.

Under nonreducing conditions, we did not detect any monomeric CTL4 or CTLMA2, neither in the hemolymph (Fig. 5, *B* and *C*) nor in conditioned medium of transfected Sf9 cells (Fig. 6*B*, *lanes 2–4*). A possible explanation could be that monomers are unstable *per se*. To answer this question unequivocally, dimerization was prevented by mutating all three cysteines at the N-terminal domain into alanines, in both proteins (Fig. 5*A*). These are the only cysteines that can form inter-chain disulfide linkages between the two CTLs; the four C-terminal cysteines are predicted to be key functional residues of the CTLD and do not form inter-protein disulfide bonds (39). We mutated all three N-terminal cysteines as we could not predict which ones actually contribute to disulfide bond formation in the dimers. Indeed, the CTL4 and CTLMA2 mutants lacking cysteines in the N-terminal domain were unable to produce homodimers when expressed separately or heterodimers when co-expressed in Sf9 cells (Fig. 6*B*, compare *lanes 2–4* with *lanes 5–7*); however, they were expressed and secreted into the supernatant as efficiently as the wild type proteins (Fig. 6*A*, compare *lanes 2–4* with *lanes 5–7*). The migration pattern of monomeric CTL4 and CTLMA2 mutants was different under reducing and nonreducing conditions; the mutants migrated faster and as double bands each under nonreducing relative to reducing conditions. This is probably because of the disulfide bonds in the CTLD that may influence proper protein migration under nonreducing conditions. In summary, our results indicate that preventing dimer formation does not inhibit the secretion of the individual monomers in Sf9 cells. This contradicts what

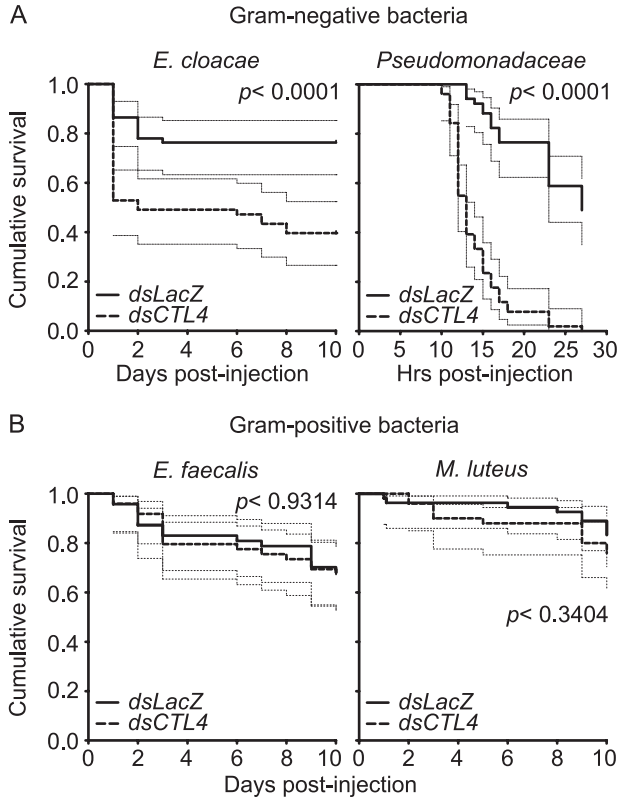

FIGURE 7. **dsCTL4 mosquitoes are susceptible to Gram-negative infections.** Four days post-injection of ds*lacZ* (control) or ds*CTL4*, *A. gambiae* adult female mosquitoes were challenged with the Gram-negative bacteria *E. cloacae* ($A_{600\,nm}$ = 0.004) and Pseudomonadaceae ($A_{600\,nm}$ = 0.001) (*A*) and Gram-positive bacteria *E. faecalis* ($A_{600\,nm}$ = 0.4) and *M. luteus* ($A_{600\,nm}$ = 0.4) (*B*). Dead mosquitoes were counted daily over a period of 10 days (all except Pseudomonadaceae) or 30 h (Pseudomonadaceae) post-bacterial challenge. *Graphs* represent the cumulative survival as calculated by the Kaplan-Meier method over the indicated time points for one representative experiment of three biological replicates. The *thin dotted lines* represent the 95% confidence intervals. Statistical significance was calculated by the log rank test. Survival curves were considered to be significantly different if $p < 0.05$.

was observed in the hemolymph whereby the depletion of one protein almost abolished the detection of the other partner (Fig. 5, *B* and *C*).

Depletion of the CTL4-CTLMA2 Heterodimer Increases Mosquito Susceptibility to Gram-negative Bacteria—We tested whether the CTL heterodimer is involved in defense against Gram-negative bacteria other than *E. coli*. Because the depletion of either CTL abolished heterodimer formation in the mosquito hemolymph, we used ds*CTL4* mosquitoes to address this question. Survival assays were performed as described previously for *E. coli*- and *S. aureus*-challenged dsRNA-treated mosquitoes. Interestingly, ds*CTL4* mosquitoes were also susceptible to two other Gram-negatives, *E. cloacae* and Pseudomonadaceae H2.26 (Fig. 7*A*), an isolate from field-caught *A. gambiae sensu lato* mosquitoes (32). Because the Pseudomonadaceae isolate was highly pathogenic, the survival of mosquitoes infected with this bacterium was scored over 35 h rather than 10 days. In contrast, ds*CTL4* mosquitoes survived infections with other Gram-positive bacteria, including *M. luteus* and *E. faecalis*, as efficiently as did ds*lacZ* controls (Fig. 7*B*). These results strongly suggest that the CTL4-CTLMA2 heterodimer is involved in defense against a broad range of Gram-negative bacteria.

DISCUSSION

C-type lectins are pattern recognition receptors that function in both mammalian and insect innate immunity. In insects, the contributions of CTLs to infection *in vivo* have not been addressed, except for *M. sexta* immulectin-2, which is required for the survival of larvae infected with *Serratia marcescens* (17) and *Photorhabdus* species (26). Indeed, the role of CTLs in insect immunity has been investigated mostly in the Lepidoptera, whereas in Diptera their functions are unknown. The obvious reason for favoring Lepidoptera is their relatively larger size that facilitates rigorous biochemical studies. Here we have used the model dipteran disease vector, *A. gambiae*, and showed, using *in vivo* reverse genetics, that two CTLs cooperate to defend the mosquito against Gram-negative but not Gram-positive bacterial infections.

Interestingly, CTL4 and CTLMA2 were both up-regulated following *E. coli* as well as *S. aureus* infections, suggesting the existence of a partial overlap in the transcriptional profiles of mosquito genes triggered by Gram-negative and Gram-positive bacterial infections. Such overlap has indeed been highlighted previously at the genomic scale using *A. gambiae* cDNA microarrays (40). We showed that these CTLs contribute to defense against Gram-negative but not Gram-positive bacteria, at least the strains tested herein. CTL4 and CTLMA2 were first identified as negative regulators of the mosquito melanization response to *P. berghei* ookinetes. However, in this study we showed that the compromised survival to a representative Gram-negative bacterium, *E. coli*, is due not to an enhanced phenol oxidase activity but rather to over-proliferation of bacteria in CTL-depleted mosquitoes.

Protein analysis of hemolymph CTL4 and CTLMA2 as well as their recombinant forms co-expressed in Sf9 cells, using Western blot and affinity purification, revealed that these CTLs form almost exclusively a disulfide-linked heterodimer. No monomeric forms were detected under any nonreducing conditions. Furthermore, *in vivo* RNAi revealed that silencing either *CTL* alone almost abolished detection of the individual monomers as well as the heterodimer in the mosquito hemolymph, suggesting that heterodimer formation is required for efficient secretion of these proteins into the hemolymph. Interestingly, when dimerization was inhibited by mutating the N-terminal cysteine residues in both CTLs, the monomers were still efficiently secreted into Sf9 conditioned medium. This discrepancy between Sf9 cells and hemolymph is due either to certain constraints on monomeric CTL secretion in adult mosquitoes that are absent from Sf9 cells or to the abnormally strong expression of CTLs in Sf9 cells (driven by the baculovirus OpIE2 promoter of pIZT vector) that may allow the secretion of mutant CTLs even if not properly folded or functional.

A minor CTLMA2 homodimer that migrated slower than the heterodimer was detected in mosquito hemolymph; slower homodimer migration is expected because CTLMA2 has a larger apparent molecular mass than CTL4. This homodimer is apparently not involved in defense against Gram-negative bacteria, as ds*CTL4* mosquitoes succumbed to *E. coli* infections, despite the fact that they are slightly enriched in this homodimer relative to ds*lacZ* controls. Whether CTLMA2 homodimer has distinct functional significance remains to be tested in future studies out-

side the scope of this work. Briefly, our results clearly show that the observed defensive role against Gram-negative bacteria is attributed to the CTL4-CTLMA2 heterodimer. This heterodimer also explains the similar melanotic phenotypes observed in ds*CTL* mosquitoes infected with *P. berghei* (27).

The protein sequences of CTL4 and CTLMA2 are significantly divergent. For instance, all the amino acid residues that are involved in putative sugar binding are conserved in the CTLMA2-CTLD but absent from that of CTL4 (Fig. 5*A*), suggesting that the latter might not necessarily bind sugars. Despite this divergence in the CTLD, an N-terminal cysteine-rich sequence (C*X*CPC) is conserved in both CTLs. We have shown by mutagenesis that these cysteines are essential for dimerization. Interestingly, the C*X*CPC sequence is not found in any other *A. gambiae* CTL, which suggests that CTL4 and CTLMA2 might have evolved from a common ancestral gene by duplication and diversification eventually leading to the formation of a heterodimeric gene product.

In conclusion, our work established that, in addition to their role as agonists of *Plasmodium* development, CTL4 and CTLMA2 significantly contribute to the mosquito defense against Gram-negative bacteria *in vivo*. Thus, they exhibit pleiotropic functions in mosquito innate immunity. This pleiotropy, and the wide diversity of members of the CTL family, which evolves rapidly in insects (9, 10), raises the intriguing possibility that CTLs might play a central role in more than one immune module in insects and invertebrates in general. In contrast to the convincing genetic evidence for the proposed immune functions of the CTL4-CTLMA2 heterodimer, its scope and mechanisms of action remain to be deciphered at the molecular level; this will be an essential step toward mechanistic understanding of the complex immunobiology of the malaria vector *A. gambiae*.

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