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A Novel Role for an Insect Apolipoprotein (Apolipophorin III) in β-1,3-Glucan Pattern Recognition and Cellular Encapsulation Reactions

Miranda M. A. Whitten,* Ian F. Tew,* Bok L. Lee,† and Norman A. Ratcliffe2*

Lipoproteins and molecules for pattern recognition are centrally important in the innate immune response of both vertebrates and invertebrates. Mammalian apolipoproteins such as apolipoprotein E (apoE) are involved in LPS detoxification, phagocytosis, and possibly pattern recognition. The multifunctional insect protein, apolipophorin III (apoLp-III), is homologous to apoE. In this study we describe novel roles for apoLp-III in pattern recognition and multicellular encapsulation reactions in the innate immune response, which may be of direct relevance to mammalian systems. It is known that apoLp-III stimulates antimicrobial peptide production in insect blood, enhances phagocytosis by insect blood cells (hemocytes), and binds and detoxifies LPS and lipoteichoic acid. In the present study we show that apoLp-III from the greater wax moth, Galleria mellonella, also binds to fungal conidia and β-1,3-glucan and therefore may act as a pattern recognition molecule for multiple microbial and parasitic invaders. This protein also stimulates increases in cellular encapsulation of nonself particles by the blood cells and exerts shorter term, time-dependent, modulatory effects on cell attachment and spreading. All these responses are dose dependent, occur within physiological levels, and, with the notable exception of β-glucan binding, are only observed with the lipid-associated form of apoLp-III. Preliminary studies also established a beneficial role for apoLp-III in the in vivo response to an entomopathogenic fungus. These data suggest a wide range of immune functions for a multiple specificity pattern recognition molecule and may provide a useful model for identifying further potential roles for homologous proteins in mammalian immunology, particularly in terms of fungal infections, pneumoconiosis, and granulomatous reactions. The Journal of Immunology, 2004, 172: 2177–2185.

Impressive similarities exist between the functional molecules and processes in the innate immune systems of invertebrates and mammals, and research on insects such as Drosophila provides vital details for mammalian studies (1, 2). One emerging area of interest concerns the roles of host apolipoproteins and pattern recognition molecules in cellular immune responses (3–5).

Mammalian lipoproteins, such as apolipoprotein E (apoE),3 are structurally and functionally homologous to insect apolipophorins (6) and perform important roles in innate immunity (7). For example, physiological levels of apoE bind LPS and prevent endotoxic shock (7), and apoE-deficient knockout mice are more susceptible to bacterial infections and LPS-induced toxic shock (4, 8). Additionally, mammalian low density lipoprotein (LDL; of which apoE is a component) stimulates a number of immune reactions, including promoting phagocytosis by macrophages (9), NO release by platelets (10), and trypanosome lysis (11). Modified LDLs bind to scavenger receptors (SRs), which function as pattern recognition receptors (12, 13).

Pattern recognition molecules also abound in insects (14), and recently, it has been shown that apolipophorin III (apoLp-III), a major exchangeable lipid transport molecule found in the blood (hemolymph), may also play a crucial role in the innate immune response and act in pattern recognition (15). ApoLp-III binds to Gram-positive bacteria and to lipoteichoic acid (LTA) (16, 17), and as in mammals, apoLp-III can also bind and detoxify LPS (18, 19) and promote phagocytosis (20). Furthermore, apoLp-III stimulates increases in hemolymph antibacterial activity (3) and superoxide production by blood cells (hemocytes) (15). Under normal conditions in larval hemolymph, apoLp-III exists abundantly in a folded, lipid-free state (21); however, recent studies (15, 22–24) strongly suggest that lipid-free apoLp-III must first convert to a lipid-associated conformation to function immunologically, as is the case for mammalian apoE (10). Until now, however, no role has been identified for apoLp-III in the binding of β-1,3-glucans released systemically from fungal cell walls. Glucan pattern recognition mechanisms are also not fully understood in mammals, although a number of glucan receptors, including SRs, have been identified associated with several cell types, including macrophages (25–27).

To date, the role of lipoproteins in cellular encapsulation or granulomatous reactions in vertebrate and invertebrate innate immunity has also been largely overlooked. In insects, as in other invertebrates, cellular encapsulation is an essential immune defense reaction that occurs when nonself materials, such as protozoans or fungi, are too large to be phagocytosed by individual blood cells (28). During encapsulation, hemocytes adhere to the surface of nonself particles and to each other, spreading to form...
layers of overlapping cells, which then destroy the particle by secreting cytotoxic compounds, including free radicals (28–30). Eventually, a black melanized nodule may form (31). Few studies have investigated the kinds of molecules mediating encapsulation and nodule formation in invertebrates or equivalent multicellular responses in vertebrates, such as pneumoniaonosis or granulomatous reactions to foreign bodies, parasites, or mycobacteria.

We have, however, recently identified and functionally implicated specific proteins in the early stage encapsulation reaction of insects. These include the highly conserved multifunctional Ca²⁺-transport protein, calreticulin (32); an 86-kDa protein with sequence homology to insect diapause protein 1 (33); as well as a novel 56-kDa protein (34). The role of calreticulin in mammalian immunity has only recently been reported, but it appears to be important for cell adhesion, Ag presentation, phagocytosis, and inflammation (35).

In the present paper we show that apoLp-III from the greater wax moth, Galleria mellonella, stimulates cellular encapsulation of foreign materials and modulates the behavior of hemocytes, performing differing immunological functions depending on its structural conformation. We also report for the first time that apoLp-III binds to a fungal β-1,3-glucan, thus acting as a pattern recognition receptor in encapsulation with a wider range of pathogen binding specificities than previously realized. Finally, we show that apoLp-III also has a protective role against fungal infections in vivo.

There are enormous similarities emerging between the immunological roles of mammalian and insect lipoproteins, and it is expected that continuing discoveries about insect encapsulation-related proteins will have direct relevance to mammalian immunity.

Materials and Methods

Chemicals and solutions
All chemicals were purchased from Sigma-Aldrich (Poole, U.K.), unless stated otherwise and were filter-sterilized (0.2 µm; Gelman, Ann Arbor, MI). All plastic materials and glassware were also sterilized before use.

Insect rearing and bleeding
Greater wax moth, Galleria mellonella (Lepidoptera: Pyralidae), were reared in large glass jars at 25 °C, with 12-h light, 12-h dark ratio, and were fed an artificial diet containing organic honey, glycerol, bees wax, organic wheat bran, and ground rat food ad libitum. Final instar larvae were used for all experiments, as these contain maximal levels of apoLp-III (36).

To collect hemolymph, larvae were anesthetized on ice and surface-sterilized with ethanol. A proleg was amputated, and hemolymph was allowed to drip into an ice-cold, sterile Eppendorf tube containing 1.5 ml of decoagulation buffer (Deco; pH 5.5) prepared as described previously (32) and saturated with phenylthiourea (PTU) to inhibit the prophenoloxidase decocoagulation buffer (Deco; pH 5.5) prepared as described previously (32) and saturated with phenylthiourea (PTU) to inhibit the prophenoloxidase decocoagulation buffer (Deco; pH 5.5) prepared as described previously (32) and saturated with phenylthiourea (PTU) to inhibit the prophenoloxidase de

Purification of native apoLp-III and lipid-associated apoLp-III complexes
Native apoLp-III was purified from heat-treated G. mellonella hemolymph, based on its inability to bind DEAE-Sepharose, using the method of Dettloff and Wiesner (15). For purification, hemolymph was collected as described above, except that each tube contained 10 µl of ultrapure water saturated with PTU instead of Deco. Pure apoLp-III fractions were suspended in Galleria physiological saline (GPS; 172 mM KCl, 68 mM NaCl, and 5 mM NaHCO₃, pH adjusted to 6.1 with HCl) (15).

To confirm the identity of the purified apoLp-III protein, 20 µg was electrophoresed onto a polyvinylidene difluoride membrane, and the N-terminal sequence was determined by automated Edman degradation, then compared with the protein sequence database of the National Center for Biotechnology Information (Bethesda, MD).

Lipid-associated apoLp-III complexes were synthesized for functional assays, as several reports indicated that apoLp-III functions immunologically only when bound to lipid (15, 22–24). To produce the complex, 1,2-dimyrystyl-rac-glycerol-3-phosphocholine (DMPC) was incubated with apoLp-III at a ratio of 2:1, respectively, as described by Dettloff and Wiesner (15).

β-1,3-Glucan binding by apoLp-III
To determine whether G. mellonella apoLp-III binds to β-1,3-glucan, pure and lipid-associated forms of apoLp-III and crude cell-free plasma samples were incubated with curdlan particles or curdlan-toyopearl beads (see below). Curdlan is a nonionic linear (1→3)-β-D-glucan, and toyopear is an uncharged vinyl polymer. Therefore, curdlan-toyopearl beads provide a good artificial model for fungal hyphae and an ideal target for encapsulation.

Crude cell-free plasma was prepared by pooling hemolymph from 15 G. mellonella larvae, into 3 µl of 0.1 M 4-amidinophenylmethanesulfonyl fluoride (a serine protease inhibitor). The hemolymph was then diluted 1/4 with imidazole buffer (50 mM imidazole, 0.2 M NaCl, 50 mM CaCl₂, 2H₂O, and 0.2% (w/v) n-mannitol), and hemocytes were removed by centrifugation at 14,000 × g for 10 min at 4°C. Eighty microliters of 0.05 mg/ml apoLp-III, 0.05 mg/ml DMPC/apoLp-III complex, or plasma were incubated for 1 h at 28°C with gentle agitation, with 80 µl of 10% (w/v) curdlan in GPS (curdlan is insoluble in saline solutions). 20% (v/v) curdlan-toyopearl beads suspended in GPS, 20% (v/v) control toyopearl beads (uncharged) suspended in GPS, or GPS only. Samples were then centrifuged at 15,000 × g for 10 min at room temperature, and 50 µl of supernatant (containing unbound protein) was removed and retained for SDS-PAGE. The remaining supernatant was discarded, and the pelleted beads or curdular particles were washed three times with 500 µl of imidazole buffer by centrifugation. Proteins that had bound to the surface of the beads or curdular particles were eluted three times by adding 80 µl of imidazole buffer saturated with NaCl, vortexed, and centrifuged at 21,000 × g for 1 min at 25°C. The eluates were pooled. All supernatants and eluates were processed for SDS-PAGE using a PAGEprep protein clean-up and enrichment kit (Perbio Science U.K., Cheshire, U.K.). Exactly 20 µl of each sample was run on 12% reducing SDS-PAGE gels (Bio-Rad Mini Protein III; Bio-Rad, Hemel Hempstead, U.K.) and stained with Coomassie Brilliant Blue.

Fungal binding by apoLp-III
To determine whether apoLp-III and DMPC/apoLp-III bind to live fungi, conidia from mutant strain V275 entomopathogenic M. anisopliae were treated as described in the glucan binding test above, but with the following modifications. Conidia were prepared as previously described by Wang et al. (37), and aliquots of 80 µl of conidia (1 × 10⁶/ml) were suspended in GPS containing 0.05% Tween 80. To obtain supernatant, conidia were centrifuged at 15,000 × g. Sodium chloride elution of bound apoLp-III was not performed, as too many other proteins would have been eluted from the conidial surface. Conidia were chosen in preference to hyphal bodies, as the former perform more reliably in G. mellonella infection systems. Conidia rapidly transform to hyphal bodies in vivo. Although still pathogenic, the mutant strain used lacks pigment and is less hydrophobic than its wild-type counterpart, making it easier to handle and more likely that β-glucans are accessible to immune factors.

Production of curdlan-toyopearl beads
Curdlan-toyopearl beads were made according to the method reported by Takaki et al. (38), using 10 mg of curdlan dissolved in 0.1 N NaOH/1 g of AF-aminotoyopearl 650M resin (TOSOH Biosep, Stuttgart, Germany). Control toyopearl beads were also produced as described above, but omitting curdlan.

Effects on plasmatocyte spreading on glass
Hemocyte spreading is a necessary step in cellular encapsulation (29), so the following assays were used to test the effects of apoLp-III and DMPC/apoLp-III complex on plasmatocyte spreading on glass.

For each of the functional assays described below, the treatments used were apoLp-III (native) or DMPC/apoLp-III complex (lipid-associated). The following controls were also included: inactivated apoLp-III, inactivated DMPC/apoLp-III complex, DMPC only, and BSA or GPS alone. All treatments were used at the same concentration (except DMPC, which was 2× concentrated) and diluted with GPS unless otherwise stated. Inactivation of apoLp-III and DMPC/apoLp-III was achieved by at least 20 freeze-thaw cycles (~ 80 to 100°C).

Four larvae were bled into 850 µl of ice-cold Grace’s insect medium (GIM; Invitrogen, Paisley, U.K.) plus 850 µl of Deco plus PTU, and the hemocytes were washed gently (200 × g) with GIM, placed on ice for 5 min, then washed and resuspended in 800 µl of GIM. Fifty microliters of
hemocyte suspension was added to wells of a flat-bottom, 96-well microtititer plate (Nunclon, Roskilde, Denmark), containing 5-mm glass coverslips (Menzel-Gläser, Braunschweig, Germany) plus 50 μl of one of the treatments or controls described above at 0.2 mg/ml.

The cells were incubated at 28°C for 20 min, and then the wells were aspirated and the tissue washed three times in GIM (1 h, 28°C). The coverslips were then mounted on slides with Kaiser’s glycercin jelly and examined under a phase contrast microscope to determine the percentage of fully spread plasmatocytes. At least 100 cells were counted in a randomly chosen field of view, and 10 monolayers (i.e., from 10 separate groups of insects) were counted per treatment.

To test the dose-response effect of DMPC/apoLp-III on hemocyte adhesion, the assay was conducted as described above using serial dilutions of DMPC/apoLp-III from 200 to 6.25 μg/ml (final concentrations). The control was GPS only.

A dose-response assay was also conducted using injected apoLp-III, which is expected to rapidly associate with lipid in vivo (22, 23). Pairs of insects were washed as described above, and 100 μl of hemocytes were transferred to wells containing glass coverslips. The hemocytes were incubated, fixed, and mounted as described above. Ten pairs of insects were tested per apoLp-III concentration. Finally, the effect of the length of the in vivo incubation time for injected apoLp-III was also tested using 20 μl of 1.0 mg/ml apoLp-III, and hemocyte samples were taken at 20, 40, or 60 min postinjection and treated as described above. All incubation times were selected on the basis of results from preliminary optimization experiments, and the apoLp-III concentrations used represent physiological levels (16).

Effects on hemocyte adhesion in vitro

Hemocyte adhesion was assessed based on the conversion of a nonfluorescent dye, calcine AM, to the fluorescent product calcine, by intracellular esterases. The dye can thus be used to quantify the total amount of cells in a well and the number remaining after nonadherent cells have been washed away (39).

Hemolymph from 30 insects was collected into 850 μl of GPS plus 850 μl of Deco with PTU, and the hemocytes were washed twice, then resuspended in GPS/Deco to give two 1.5-ml aliquots of 2 × 10⁵ ml⁻¹ cells. The first aliquot was mixed with 1.5 ml of 2 μM calcine AM (Molecular Probes, Leiden, The Netherlands) diluted with GPS. The second was diluted with 1.5 ml of GPS only (i.e., nondenuded cells for background readings). Both samples were incubated on a rocking platform at 28°C for 25 min, then washed twice and resuspended with GPS to a density of 2 × 10⁷ ml⁻¹. One hundred microliters of cells were introduced into the flat-bottom wells of a black, 96-well microfluor plate (Thermo Life Sciences, Basingstoke, U.K.) plus 100 μl of 0.1 mg/ml apoLp-III, DMPC/apoLp-III, or controls (described above). The plate was incubated at 28°C for 35 min to allow the hemocytes to adhere. An additional 100 μl of GPS was added to each well, and the prewash fluorescence was measured with a POLARStar Galaxy fluorescence plate reader (BMG Lab Technologies, Aylesbury, U.K.) at 490 nm excitation and 530 nm emission. Nonadherent cells were removed by gentle pipetting, and the wells were washed three times with GPS by pipetting, then filled with 200 μl of GPS. The postwash fluorescence of remaining adherent cells was then measured. To quantify dye leakage, wells were included that contained the supernatant of dyed cells, and the background for these wells was GPS alone.

The percent adhesion was calculated using the following formula: ([postwash dyed cells – postwash background]/[prewash dyed cells – prewash background] – [leakage – GPS background]) ×100.

To test the dose-response effect of DMPC/apoLp-III on hemocyte adhesion, the above experiment was repeated using serial dilutions of DMPC/apoLp-III complex from 250 to 1.95 μg/ml (final concentrations) and 0 μg/ml. The assays were repeated eight times. Hemocyte viability was checked based on the exclusion of 0.2% eosin and was always >90%.

Effects on cellular encapsulation

It was impossible to investigate the effects of apoLp-III on the early hemocyte encapsulation reaction to injected beads, because no beads could be recovered from the hemocoel despite attempting several extraction methods. This probably resulted from extremely rapid clumping or adherence of the beads to internal surfaces. Therefore, in vitro encapsulation assays were developed to assess the interactions of washed hemocytes with beads. Additionally, the gross, final stage cellular response to injected beads, which is characteristic by melanized nodule formation, was studied in dissected larvae.

Interactions of hemocytes with beads in vitro

Eleven G. mellonella larvae were bled into 1.8 ml of Deco saturated with PTU, and the hemocytes were washed twice in GIM and adjusted to 2 × 10⁷/ml. One hundred microliters of hemocyte suspension was transferred to one Eppendorf tube per treatment group, each containing 0.1 mg/ml apoLp-III or DMPC/apoLp-III in GIM or the controls described above. Each tube also contained 50 μl of 4 × 10⁶ ml⁻¹ curdian-toyopearl beads in GIM as the targets for encapsulation. Tubes were incubated in the dark for 20 h at 20°C with gentle constant rotation, and the contents were examined for the percentage of beads that had become encapsulated (defined as coverage of at least half the bead surface with hemocytes). The assay was repeated eight times.

Identical assays were performed with DEAE-Sepharse beads (positive charge) to assess the importance of the chemical charge of the encapsulation target.

To test the dose-response effect of DMPC/apoLp-III, assays with curdian-toyopearl were performed as described above using serial dilutions of DMPC/apoLp-III from 0.1 mg/ml to 3.125 μg/ml (final concentrations). Control tubes substituted DMPC/apoLp-III with GIM. This assay was repeated nine times.

Effects on the gross encapsulation and nodulation response in vivo

To assess the effects of apoLp-III on melanized nodule formation in vivo, larvae were injected with 40 μl of 1.2 × 10⁶ ml⁻¹ curdian-toyopearl beads suspended in one of the following solutions: 1 mg/ml apoLp-III or DMPC/apoLp-III complex in GPS or the controls described above. Nine insects weighing between 180 and 220 mg were tested per treatment group. Injected insects were kept at 25°C for 24 h, then killed by freezing. To observe nodules, the frozen larvae were boiled in water for 5 min to turn the tissues and hemolymph white, thus contrasting the black nodules. To quantify nodulation, bodies were cut in two longitudinally midway between the ventral and dorsal surface. Digital images were taken under a dissecting microscope and analyzed using Image Tool 3.0 image analysis software (University of Texas Health Science Center, San Antonio, TX).

The number of nodules present was counted, and the area of the nodules was calculated and expressed as a percentage of the total exposed body surface area.

The dose effect of apoLp-III was tested by injecting larvae with suspensions of 40 μl of apoLp-III at 1, 0.5, 0.1, or 0.01 mg/ml, each containing 1.2 × 10⁶ ml⁻¹ curdian-toyopearl beads.

Effects on mycosis in vivo

To establish a role for apoLp-III in the survival of fungus-infected insects, G. mellonella larvae were injected with mutant conidia of M. anisopliae that had been treated or untreated with apoLp-III. Sixty-four insects weighing 100 mg ± 10% were injected per treatment group. Sterile 10-μl suspensions of 500 conidia were incubated with 2 mg/ml apoLp-III in GPS and 0.025% Tween 80 for 20 min at 25°C before injection. Control insects received conidia pretreated with inactivated apoLp-III or untreated conidia only. An additional control group was injected with washed, formaldehyde-killed conidia to verify that the injection process itself was not responsible for mortalities. Previous binding experiments demonstrated that lipid-bound and lipid-free apoLp-III bind to conidia equally effectively, and thus the conidia described above were suspended using lipid-free apoLp-III. Injected insects were kept at 25°C and were examined at regular intervals for mortality, which was taken as a complete lack of movement. The time of death and the number of dead insects were recorded, and cadavers were removed and maintained in a moist environment until mycosis could be confirmed by the emergence of fungus from the cuticle. The experiment was terminated after 155 h.

Statistical analyses

Data were analyzed using PRISM (GraphPad, San Diego, CA). Differences between treatments and saline controls in dose-response and time-dependency assays were tested using repeated measures ANOVA with Dunnett’s post-test, whereas comparisons between other treatments and controls were made with Tukey’s multiple comparison post-test. Pearson’s correlation test was used to analyze dose-response data. Survival fractions were calculated for Metarhizium-infected larvae using the Kaplan-Meier method.
and differences between survival curves were analyzed by the log-rank test. Larvae that died in the absence of mycosis were censored from the study at the time of death. Results were considered significant at \( p < 0.05 \).

**Results**

**Confirmation of apoLp-III identity**

The sample gave an N-terminal amino acid sequence, DAST-PLQDLEKHAEFQKTF, that had 100% sequence identity with a published N-terminal sequence for *Galleria mellonella* apoLp-III (20).

**ApoLp-III binds β-glucan and fungal conidia**

Samples of pure apoLp-III, lipid-associated DMPC/apoLp-III, or apoLp-III in crude cell-free plasma were incubated with curdulan, either in suspension or in the form of curdulan-toyopearl beads, and also with *M. anisopliae* conidia. The results for purified apoLp-III are shown in Fig. 1. The 18.5-kDa apoLp-III band disappeared from the supernatant after contact with curdulan suspension and conidia, but not from any of the other treatments. Additionally, when adherent protein was eluted from the surface of curdulan, curdulan-toyopearl beads, or toyopearl beads, apoLp-III only appeared in the eluate from curdulan particles. Similar results were obtained using DMPC/apoLp-III and apoLp-III in crude plasma (data not shown). This shows that apoLp-III binds to free curdulan and live conidia regardless of whether the protein is in a lipid-associated conformation, but it does not bind effectively to toyopearl or curdulan-toyopearl beads.

**Lipid-associated apoLp-III inhibits plasmatocyte spreading**

Washed hemocytes were incubated in vitro with apoLp-III in its free state or as a lipid-bound complex or with controls and allowed to attach to glass coverslips. The percentage of plasmatocytes exhibiting spreading was then calculated. As shown in Fig. 2A and Fig. 3, although all treatments tested in vitro caused a decline in spreading relative to saline, DMPC/apoLp-III (lipid-associated) caused by far the greatest decrease, a response significantly lower than that to every other treatment tested, including the corresponding inactivated DMPC/apoLp-III control (\( p < 0.05 \) or less). The hemocytes appeared more rounded, and there were fewer plasmatocytes generally after treatment with lipid-associated apoLp-III (Fig. 3). This effect was dose dependent (Fig. 2B), causing a drop from 31 ± 3% spreading by saline-treated cells to 3 ± 0.7% in cells treated with 100 \( \mu \)g/ml DMPC/apoLp-III. These changes were significant at DMPC/apoLp-III concentrations of 12.5–200 \( \mu \)g/ml (\( p < 0.01 \)) compared with saline.

A similar dose response was seen using washed hemocytes from larvae injected with apoLp-III (Fig. 2C). In this study the percentage of spread plasmatocytes dropped rapidly from 24.5 ± 2.5% in saline-injected insects to 11.5 ± 1.6% after injection of just 5 \( \mu \)g of apoLp-III (\( p < 0.01 \)). Higher concentrations of apoLp-III had no additional inhibitory effect. The effects of injected apoLp-III on subsequent plasmatocyte spreading were inversely time dependent (Fig. 2D). After 40 min in vivo, the inhibitory effects of apoLp-III were significantly reduced (\( p < 0.01 \)) compared with the response after 20 min, and by 60 min spreading was at 29.7 ± 3.6% (\( p < 0.001 \)), which is very close to the level seen in hemocytes from saline-injected controls (31 ± 3%; Fig. 2A).
Very similar results were obtained when the spreading assay described above was repeated using preincubated hemocytes that had been washed before being added to the glass coverslips (data not shown), thus showing a minimal effect from any potential physicochemical alterations to the substrate.

**Lipid-associated apoLp-III inhibits hemocyte adhesion in vitro**

Washed hemocytes were tested for their ability to adhere to a plastic microtiter plate well in vitro by quantifying the background-corrected fluorescence of adherent cells. Adhesion was significantly inhibited by DMPC/apoLp-III (8.5 ± 1.8%) compared with cells in *Galleria* saline (16.0 ± 2.6%), but not by any of the other treatments tested (Fig. 4A; \( p < 0.05 \)). The difference between DMPC/apoLp-III and inactivated DMPC/apoLp-III (26.3 ± 3.5%) was also very significant (\( p < 0.001 \)). Fig. 4B illustrates the dose-response effects of various concentrations of DMPC/apoLp-III on hemocyte adhesion. There was a significant negative correlation between the concentration of DMPC/apoLp-III and hemocyte adhesion (\( r^2 = 0.63; \ p < 0.05 \)).

**Lipid-associated apoLp-III augments cellular encapsulation in vitro**

Washed hemocytes were tested for their ability to encapsulate curdlan-toyopearl beads and DEAE-Sepharose beads in vitro. In saline controls, only 7.9 ± 1.6% of curdlan-toyopearl beads were encapsulated after 20 h (Fig. 5A), whereas significantly more beads were encapsulated in the presence of lipid-associated apoLp-III compared with all the controls (36.5 ± 3.9%; \( p < 0.001 \)). To a lesser extent, encapsulation was also elevated in samples incubated with apoLp-III compared with the saline control (26.1 ± 3%; \( p < 0.001 \)); however, this difference was not significant when
comparing apoLP-III with inactivated apoLP-III. The other treatments, including the inactivated form of lipid-bound apoLP-III, failed to significantly alter the encapsulation response compared with saline.

Various concentrations of DMPC/apoLP-III were also used to test the dose-response effect on in vitro encapsulation (Fig. 5B). Increasing concentrations of DMPC/apoLP-III correlated with increases in encapsulation ($r^2 = 0.80; p < 0.01$), and these effects were significantly greater than those in saline controls from 12.5 $\mu$g/ml upwards ($p < 0.01$). Examples of nonencapsulated and encapsulated beads are shown in Fig. 6. Encapsulated beads often appeared clumped under the conditions of this assay, as in Fig. 6B; however, in cell-free systems the beads were not agglutinated by any of the above treatments (data not shown).

ApoLP-III augments in vivo nodule formation

Larvae were injected with curdlan-toyopearl beads plus 40 $\mu$g of various treatments to assess the effect on nodule formation in vivo (Fig. 7A). The area coverage by nodules in apoLP-III injected insects was 0.69 $\pm$ 0.11%, significantly greater ($p < 0.01$) than that in the control insects injected with saline (0.28 $\pm$ 0.07%), DMPC (0.27 $\pm$ 0.09%), BSA (0.17 $\pm$ 0.03%), and inactivated apoLP-III (0.25 $\pm$ 0.07%; $p < 0.001$). Lipid-associated apoLP-III also caused an enhanced level of nodulation (0.55 $\pm$ 0.1%) compared with inactivated DMPC/apoLP-III (0.17 $\pm$ 0.02%; $p < 0.05$). None of the other treatments provoked significant nodulation above the level of saline-injected insects (Fig. 7A). A dose-response effect was observed with various concentrations of apoLP-III (Fig. 7B), whereby increasing concentrations of apoLP-III correlated with increases in the area of nodules present in the insects ($r^2 = 0.95; p < 0.01$). This change was significantly greater than that in saline-injected insects after injection of 40 $\mu$g of apoLP-III ($p < 0.01$).

As shown in Fig. 8A, control insects injected with saline and beads produced only a few small black nodules that were widely dispersed. In contrast, increases in both the number and the size of nodules were observed in insects receiving apoLP-III suspended in one of the treatments as shown. After 24 h, the insects were cut in two longitudinally to expose black nodules. The area of the nodules was expressed as a percentage of the total exposed body surface area. A. Dose-response effect of native apoLP-III on nodule formation. B. Significance compared with saline control; $*$, significance compared with corresponding inactivated control.

Beneficial role for apoLP-III in fungal entomopathogenesis

Larvae were injected with mutant M. anisopliae conidia with or without apoLP-III pretreatment to investigate its potential role in the survival of fungus-infected insects (Fig. 9). Survival times for insects receiving apoLP-III treated conidia were significantly longer than those for controls injected with conidia plus inactivated apoLP-III ($p < 0.01$) or with conidia only ($p < 0.05$; 50% mortality occurred at 121, 89, and 86 h, respectively). Treatment with apoLP-III also resulted in a lower overall percent mortality by the end of the experiment (53% compared with 72 and 73%, respectively). A further control group injected with dead (formaldehyde-killed) conidia resulted in 100% survival (data not shown), indicating that injection injury itself did not cause mortality.

Discussion

This study highlights previously unreported roles for insect apoLP-III in three key areas of insect innate immunity, namely, fungal pattern recognition, hemocyte behavior central to multicellular encapsulation reactions, and response to an in vivo fungal infection. This is of particular relevance to mammalian immunology in light of the functional and structural similarities emerging between mammalian apolipoproteins and insect apolipophorins.

In addition to binding to LPS from Gram-negative bacteria and LTA from Gram-positive bacteria (16–19), the present study shows, for the first time, that apoLP-III binds to the particulate form of a $\beta$-1,3-glucan (a component of the fungal cell wall) and to fungal conidia. A. Saline control. B. Beads and hemocytes incubated with lipid-associated apoLP-III showing enhanced encapsulation responses, with beads coated by multiple layers of flattened cells. Bar = 50 $\mu$m.
recognition molecule with multiple specificities that can recognize fungal pathogens as well as bacteria.

Mammalian apolipoproteins such as ApoE exert several regulatory effects on immune responses, including LPS detoxification and phagocytosis (4, 7–10). A number of cell surface receptors, including dectin-1 and SRs, are known to mediate recognition of β-glucans in vertebrates (12, 13, 26, 40) and lead to cellular responses, such as phagocytosis and the respiratory burst (40). However, as far as we are aware, no plasma molecules recognizing β-glucans have been identified in mammals (40), and there have been no investigations to date on potential roles for mammalian apolipoproteins in either the recognition of fungal β-glucans or the multicellular encapsulation-like reactions in response to nonself particles. These latter reactions, which resemble encapsulation in invertebrates, occur during occupational lung diseases, such as pneumoconiosis, or granuloma formation in response to foreign bodies, β-glucans, parasites, or mycobacteria, where multicellular sheaths rich in macrophages are formed around the Ag (41). There also exists relatively little information about β-glucan receptors or β-glucan-binding proteins in invertebrates, with the majority of studies conducted on crustaceans (42, 43). However, it is known that β-glucan-binding protein stimulates hemocyte responses, including phagocytosis, spreading, and degranulation (42–44).

As detailed in the introduction, insects respond to a large foreign particle (such as a curdlan-toyopearl bead) by encapsulating it within a multicellular sheath of hemocytes. The events leading to encapsulation require nonself pattern recognition, hemocyte adhesion, and spreading, followed by addition of further cell layers, and finally the formation of end-stage melanized nodules (28–31). The present study has investigated the potential role of apoLp-III in each of these stages.

It is known that free apoLp-III injected into G. mellonella very rapidly associates with lipid to form lipophorin in vivo (22), and that to perform most of its immune stimulatory roles, apoLp-III must be in its lipid-associated structural conformation (15, 22, 24). In agreement, our present study shows that in vitro, significant effects on cellular immunity are generally only seen with lipid-bound apoLp-III, whereas injections of both free and lipid-bound apoLp-III significantly alter hemocyte activity in vivo. An exception was noted, however, in the case of β-1,3-glucan and conidial binding, which occur regardless of the structural conformation of apoLp-III. This feature has also been noted for the LPS binding activity of mammalian apoE, a structural homologue of apoLp-III (45). We therefore suggest that the primary role of lipid-free apoLp-III (the predominant natural form) may be in pattern recognition, functioning immediately in vivo as part of an effective pathogen recognition system, but that lipid-associated apoLp-III has some additional roles in activating or modulating hemocytes to perform several cell-mediated immune functions. These results also suggest that the change in conformation during lipid association does not affect the β-glucan binding site of the apoLp-III molecule. Zakarian et al. (46) showed that free apoLp-III binds to bacteria, but no other studies of the putative pattern recognition role of apoLp-III have compared lipid-free and lipid-associated forms. The lack of a β-1,3-glucan binding effect of apoLp-III seen with curdlan-toyopearl beads in our study can probably be explained by structural modifications caused by heat and acid treatment of the curdlan during bead production (47).

There have been several studies on the role of apoLp-III in immunity (15–17, 20–24), although this is the first to show temporal differences between inhibitory and stimulatory effects on hemocytes. Hemocyte adhesion and plasmatocyte spreading were dose-dependently inhibited by short term (20 min) exposures to lipid-associated apoLp-III, whereas longer term (20+ h) exposures enhanced encapsulation. The modulatory vs stimulatory effects of apoLp-III noted above are difficult to explain, because hemocyte adhesion and spreading are key early events in the encapsulation of foreign objects (28, 29). Mandato et al. (48) found that recombinant apoLp-III had no effect on hemocyte adhesion in Galleria in vitro, although they did not study the lipid-bound form. It could be that hemocytes are first rendered unresponsive to prevent a massive and possibly wasteful involvement of large numbers of hemocytes in a cellular clumping reaction, while the insect attempts to clear β-glucan from the hemolymph. Later, if this response proves insufficient, hemocytes may be triggered to begin encapsulation. Zakarian et al. (46) suggested that apoLp-III shuts down and conserves hemocytes for future infections. Our preliminary experiments investigating different incubation times for injected apoLp-III suggested that the inhibitory effects on spreading decrease with increasing time. Clearly, this is an area demanding further detailed investigation.

Inhibition of spreading in vitro by lipid-associated apoLp-III cannot simply be attributed to hydrophobic repulsion caused by lipid coating the glass substrate, because inhibition also occurred when hemocytes were preincubated with DMPC/apoLp-III and then washed before being introduced onto the glass slides, and plasmatocyte spreading improved in the presence of inactivated control treatments. Additionally, inhibition of plasmatocyte spreading occurred after free apoLp-III was injected into the
hemocoele of G. mellonella (where it rapidly associates with lipid).

The reduction of spreading induced by BSA could result from neutralization of charge. The mechanism by which DMPC/apoLp-III affects spreading is presently unknown, but it is possible that charge may also be a contributory factor.

We assessed the longer term effects of apoLp-III on cellular interactions with foreign particles by in vitro and in vivo encapsulation assays. Lipid-associated apoLp-III and, to a lesser extent, free apoLp-III enhanced the cellular encapsulation response by washed hemocytes in vitro to curdlan-toyopearl beads and DEAE-Sepharose, and the effect of lipid-associated apoLp-III was dose dependent. We also found that injections of apoLp-III dose-dependently enhanced melanized nodule formation (the end point of encapsulation) in response to curdlan-toyopearl beads. This enhancement of encapsulation is not surprising even though apoLp-III does not bind to the beads used, as the earlier spreading and attachment experiments show that lipid-bound apoLp-III can directly modify hemocyte behavior. As expected, the end-stage encapsulation response was also enhanced after injections of apoLp-III that was already lipid associated. This is the first report of the direct involvement of apoLp-III, or indeed any apolipoprotein, in cellular encapsulation reactions, which aim to efficiently remove injected foreign Ags from the blood. The mechanisms by which the apolipoprotein enhances this encapsulation process and whether apoLp-III mediates the induction of innate immune genes remain to be determined. We observed that encapsulated beads sometimes appeared clumped under the conditions of the in vitro encapsulation assay. However, apoLp-III does not agglutinate curdlan-toyopearl beads in a cell-free system (data not shown) with either the lipid-free or the lipid-associated form. Possibly, hemocytes themselves help mediate a moderate level of agglutination when in the presence of apoLp-III.

The survival assay comparing the times of death for larvae infected with the biological control agent M. anisopliae showed a significant increase in the survival time of insects infected with apoLp-III-treated conidia as well as a lower percent mortality overall. This provides firm, though preliminary, evidence for a beneficial role of apoLp-III during fungal entomopathogenesis in vivo. Further work is required to establish what that role may be, but one hypothesis could be that pretreatment of conidia may afford a stronger pattern recognition signal, triggering a more powerful or rapid antifungal response that may combine cellular (e.g., encapsulation) and humoral activities (e.g., gallerimycin antifungal peptide production) (49).

It would be interesting to investigate the nature of putative apoLp-III receptors in insects. Candidates could include Toll-like receptors or SRs on hemocytes or other cell types, as it is already known that apoLp-III is taken up by granular hemocytes (22), and that Drosophila cells appear to exhibit SR binding of β-glucans and other pathogen-associated molecular patterns (PAMPs) and endocytosis of modified LDL (50, 51). In mammals, bacterial lipoproteins bind the Toll-like receptor 2, which is itself a pattern recognition receptor (52, 53). In addition, modified LDLs of host origin are bound by SRs, which act as pattern recognition receptors, as they also bind many PAMPs, including β-glucans (13). In mammals, class AI and all SRs and dectin-1 are thought to mediate phagocytosis of bacteria by macrophages as well as NO production (12, 13, 40).

In conclusion, this is the first report to show that insect apoLp-III binds the PAMP, β-1,3-glucan, and fungal cells, and that it can improve the survival of fungus-infected insects, dose-dependently stimulate the encapsulation of foreign particles by immune cells at physiological levels, and modulate hemocyte spreading and adhesion in vitro. Possibly, the primary role of lipid-free apoLp-III may be in pattern recognition, but lipid-associated apoLp-III has additional roles in activating or modulating hemocytes to perform several cell-mediated immune functions. There are great similarities between the immunological roles of mammalian apolipoproteins and insect apoLp-III, and study of insect encapsulation-related proteins may have direct relevance to mammalian immunology and provide vital details for previously uninvestigated immune processes.

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References


