Excretions/secretions from medicinal larvae (*Lucilia sericata*) inhibit complement activation by two mechanisms

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ABSTRACT

Larvae of the blowfly *Lucilia sericata* facilitate wound healing by removing dead tissue and biofilms from non-healing and necrotic wounds. Another beneficial action of larvae and their excretions/secretions (ES) is down-regulation of excessive inflammation. As prolonged complement activation is key to excessive inflammation, the aim of this study was to elucidate the mechanisms underlying the anti-complement activities of ES. Results revealed that heat sensitive serine proteases in ES degrade multiple complement proteins in all steps of the three complement activation pathways. Importantly, C3a and C5a—major activators of inflammation—were also degraded by ES and pretreatment of these factors with ES completely blocked their ability to induce activation of human neutrophils. Pre-exposure of the neutrophils to ES did not affect their responsiveness to C3a/C5a and fMLP, indicating that the receptors for these activators on neutrophils were not affected by ES. Surprisingly, heat and serine protease inhibitor pretreatment did not affect the ability of ES to inhibit C5b-9 complex formation despite degrading complement proteins, indicating a second complement-inhibiting molecule in ES. Heated ES was as effective as intact ES in inhibiting C3 deposition upon activation of the alternative pathway, but was significantly less effective in wells with a classical or lectin pathway-specific coating. Unfortunately, the molecules affecting the complement system could not be identified due to an insufficient database for *L. sericata*. Together, larval ES inhibit complement activation by two different mechanisms and down-regulate the C3a/C5a-mediated neutrophil activation. This attenuates the inflammatory process, which may facilitate wound healing.

Chronic, non-healing wounds are often characterized by the presence of bacterial biofilms, slough, and cell debris in the wound bed.[1] The impaired wound healing is often a result of an inappropriate inflammatory process and/or deleterious composition of wound fluid. Prolonged influx and activation of neutrophils in the wound bed is responsible for high levels of proteolytic enzymes and reactive oxygen/nitrogen species leading to tissue damage rather than repair.[2] The number of patients suffering from chronic wounds has been increasing along with the rise in obese, diabetic, and immune-compromised patients. Non-healing wounds are responsible for patient morbidity as well as huge costs to the healthcare system.[3–6] Unfortunately, it remains difficult to treat such wounds as current treatment options often lack sufficient clinical efficacy and a scientific base.[7,8] Clearly, alternative treatment modalities are urgently needed in the field of wound care. In this connection, efficacy and safety of larval therapy, i.e., the application of sterile larvae of the blow fly *Lucilia sericata* to wounds according to a standardized protocol for clinical use, have been reported.[9,10] Larval therapy, which was approved in 2004 by the US Food and Drug Administration as a medical device to clean out wounds (510(k) #33391), is currently applied to over 15,000 patients annually in Europe and worldwide.[2] It has been shown that debridement, i.e., removal of infected and/or devitalized tissue from the wound, by larvae and their excretions/secretions (ES) facilitates and continues the process of wound healing.[11,12] *In vitro* many other beneficial effects of larvae and their ES on chronic wounds, such as disinfection, inhibition of complement activation, down-regulation of inflammation, and growth stimulation of the cells involved in wound healing, have been documented.[2,9,10,13]

The complement system plays an important role in the inflammatory reaction to tissue injury and invading microorganisms. Activation of complement cascades can be achieved through three pathways, i.e., the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP), resulting in the formation of the powerful pro-

inflammatory factors C3a and C5a, bacterial opsonins like C3b and C3bi, and C5b-9 (the membrane attack complex [MAC]), which is a highly effective bacteriolytic molecule.[14–16] The results of any of the three pathways are activation of the C3 convertase leading to the production of C3a and C3b and other C3 break-down products like C3c and C3d. However, prolonged complement activation, which is often seen in chronic wounds, causes tissue damage rather than repair and hence is detrimental to the wound healing process.[17,18] Therefore, the anti-complement and associated anti-inflammatory actions of larval ES may contribute to the down-regulation of the inflammatory processes in chronic wounds and facilitate wound healing, together with the debridement of the wound bed.

The main aim of this study was to elucidate the mechanisms underlying the anti-complement activities of ES from *L. sericata* larvae. Our results revealed two different mechanisms: one is achieved by serine proteases that degrade multiple complement proteins and the other by an unidentified heat-resistant molecule regulating the C3 amplification loop.

EXPERIMENTAL PROCEDURES

Preparation of larval ES

Larval ES were collected as previously described.[19] Briefly, sterile third instar larvae of the green bottle fly *L. sericata* (a kind gift from BioMonde GmbH, Barsbüttel, Germany) were washed 3 times with PBS (pH 7.4) and then incubated in PBS for 1 hour at 37°C in the dark. The collected ES were pooled and stored at −80°C until use. The protein concentration of the ES samples was determined by Bicinchonic Acid Protein Assay Kit (Pierce Biotechnology) according to manufacturer's instructions. One larva of third instar typically produces about 2 μg of protein/hour.[20] Where indicated, ES were heated for 15 minutes at 98°C in a Thermomixer Comfort (Eppendorf), further referred to as HES, or exposed to 1 mM of the serine protease inhibitor 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF; Enzo Life Sciences) for 1 hour at 37°C, further referred to as AES.

Measurement of complement activation by ELISA

For measurement of complement activation, the Wieslab Complement System Screen Kit (Euro Diagnostica), which detects C5b-9 deposition in wells pre-coated with pathway-specific activators, i.e., IgM for the CP, mannan for the LP, and LPS for the AP, was used. Briefly, lyophilized positive control serum (Wieslab kit) was reconstituted according to manufacturer's instructions and then diluted 101 times for analysis of the CP/LP and 18 times for the AP using diluents in the kit as recommended. Next, the diluted serum was mixed with ES (and HES/AES) or PBS as control at 4:1 ratio. The mixtures were immediately transferred to the pathway-specific coated wells (in the kit or in-house prepared plates, see below) and incubated for 1 hour at 37°C. Thereafter, the amount of C5b-9 formed in the wells was detected by alkaline phosphatase-labeled antibody directed against C5b-9 and the alkaline phosphatase substrate according to the manufacturer's instructions. Finally, the amount of C5b-9 in the wells was quantitated by measuring the optical density at 415 nm ($OD_{415\ nm}$) using an iMark Microplate Absorbance Reader (Bio-Rad). In addition to the Wieslab pre-coated wells, pathway-specific wells were prepared in-house. Briefly, Microlon 600 high binding 96-well plates (Greiner Bio-One) were coated with10 μg/ml purified human IgG (Jackson ImmunoResearch) for CP, 10 μg/ml mannan (from *Saccharomyces cerevisiae*, Sigma-Aldrich) for LP, both in coating buffer (Biosource), or with 2 μg/ml LPS (from *Salmonella enteritidis*, Hycult Biotech) in PBS supplemented with 10 mM $MgCl_2$ for AP. The IgG-coated plates were incubated for 1 hour at 37°C and the mannan and LPS-coated plates for 16 hours at room temperature. The non-specific binding sites in IgG- and mannan-coated plates were blocked with 1% (v/v) BSA in PBS for 1 hour at 37°C. Thereafter, the plates were washed with Wieslab washing buffer and after application of diluted serum the amount of C5b-9 formed in the wells was detected using Wieslab alkaline phosphatase-labeled antibody and substrate as described above.

Measurement of C3, C4, C1q, and mannan-binding lectin deposition on pathway-specific-coated wells by ELISA

The effect of larval ES on the amount of C3, C4, C1q, and mannan-binding lectin (MBL), deposited on pathway-specific coated wells was detected by ELISA as described above. In short, the Wieslab positive control serum was diluted 101 times for the detection of C3 (CP/LP), C4 (LP), and C1q (CP), 201 times for C4 (CP), 5 times for MBL (LP), and 18 times for C3 (AP). Next, the diluted serum was mixed with serially diluted ES (or PBS as control) at a 4:1 ratio for the detection of C3, C4, and MBL in mannan-coated wells, at a 1:1 ratio for the detection of C3 and C1q in IgM-coated wells, and at 2:1 for C3 detection in LPS-coated wells. After 1 hour at 37°C, the wells were washed and then the amount of the various complement proteins bound to the wells was detected using rabbit anti-human C3c (DAKO), goat anti-human C4 (Quidel), rabbit anti-human C1q (DAKO), or rabbit anti-MBL.[21] Next, the binding of these antibodies to the wells was detected using horse radish peroxidase-conjugated goat anti-rabbit IgG (DAKO) for C3c, C1q, and MBL, and horse radish peroxidase-conjugated rabbit anti-goat IgG (DAKO) for C4. Thereafter, the peroxidase substrate, i.e., 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma-Aldrich) solution supplemented with 0.015% (v/v) H_2O_2 (Sigma-Aldrich), was applied to each well. Finally, the amount of C3, C4, C1q, and MBL bound to the wells was quantitated by measuring the optical density at 405 nm ($OD_{405\ nm}$) using an iMark Microplate Absorbance Reader (Bio-Rad). In between subsequent incubation steps for 1 hour at 37°C, the wells were washed 3 times with Wieslab wash buffer.

Protein degradation assay

One microliter of 0.25 mg/ml of purified human C3, C1q, factor B, C3b, C5 (Quidel), C2 (Calbiochem), MBL,[21] C5a (Bachem), C3a (Alpha Diagnostic), human IgM (Thermo Scientific), IgG (Jackson ImmunoResearch), and BSA

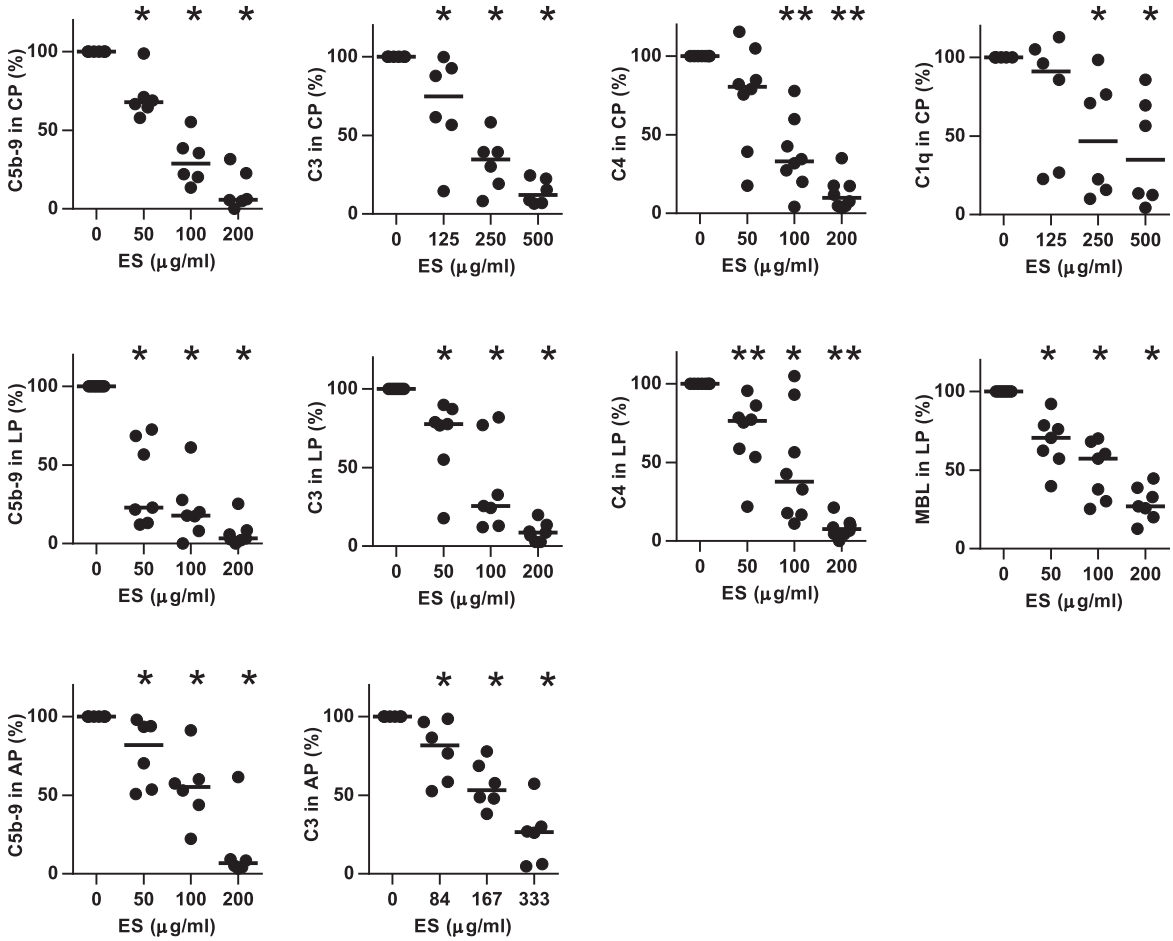

Figure 1. Effect of larval excretions/secretions (ES) on all steps of the three complement activation pathways. Diluted human serum was mixed with serially diluted larval ES in appropriate ratios, and pipetted into Wieslab wells coated with IgM for classical pathway (CP), mannan for the lectin pathway (LP), and LPS for the alternative pathway (AP) and then incubated for 1 hour at 37°C. Formation of C5b-9 and the deposition of C3, C4, C1q, and mannose-binding lectin (MBL) to the wells were quantitated by ELISAs. The results are expressed as % of the values found for serum mixed with PBS instead of ES. Data are from at least five independent experiments with lines representing median values. * $p < 0.05$, ** $p < 0.01$ for the differences between the results for ES-treated and control samples.

(Pierce) was mixed with 4 μl of 1 mg/ml larval ES (and where indicated 0.5 mg/ml), or PBS as control, for 1 hour at 37°C. After adding 4× laemmli sample buffer (Bio-Rad) supplemented with 10% (v/v) 2-mercaptoethanol (Merck), the mixtures were boiled for 5 minutes and then applied to precast gradient gels (4–20% Mini-PROTEAN TGX Gel, Bio-Rad). After SDS-PAGE using PowerPac200 (Bio-Rad) under constant voltage at 200V for 30 minutes in premixed Tris/Glycine/SDS electrophoresis buffer (Bio-Rad), the protein bands in the gel were visualized using SilverQuest Silver Stain kit (Invitrogen) according to manufacturer's instructions.

Measurement of the intracellular free Ca^{++} concentration in human neutrophils

This assay was performed as previously described[22] with minor modifications. First, human neutrophils were isolated from peripheral blood of healthy volunteers by Ficoll amidotrizoate (1.077 g/ml, Pharmacy of LUMC, the Netherlands) density centrifugation as described earlier.[23] Next, suspensions of 5×10^6 neutrophils/ml of RPMI 1640 medium (Gibco) containing 0.5% (v/v) human serum albumin (Sanquin), further referred to as RPMI/HSA, were prepared. Thereafter, the cells were loaded with 2 μM fluo-3AM (Invitrogen) for 20 minutes at room temperature in the dark, washed once, and then diluted 5 times in RPMI/HSA. Purified human C5a (Bachem) and C3a (Alpha Diagnostic International) at a concentration of 2 μM and 20 nM N-formyl methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich) were pretreated with 1 mg/ml ES (or HES/AES) or PBS as control at 1:1 ratio for 1 hour at 37°C before transferring to vials containing fluo-3AM-loaded neutrophils. The changes in fluorescence in the neutrophils after the stimuli were monitored by flow cytometry (FACS Calibur, Becton Dickinson). In short, 250 μl of fluo-3AM-loaded neutrophils (1×10^6 cells/ml)

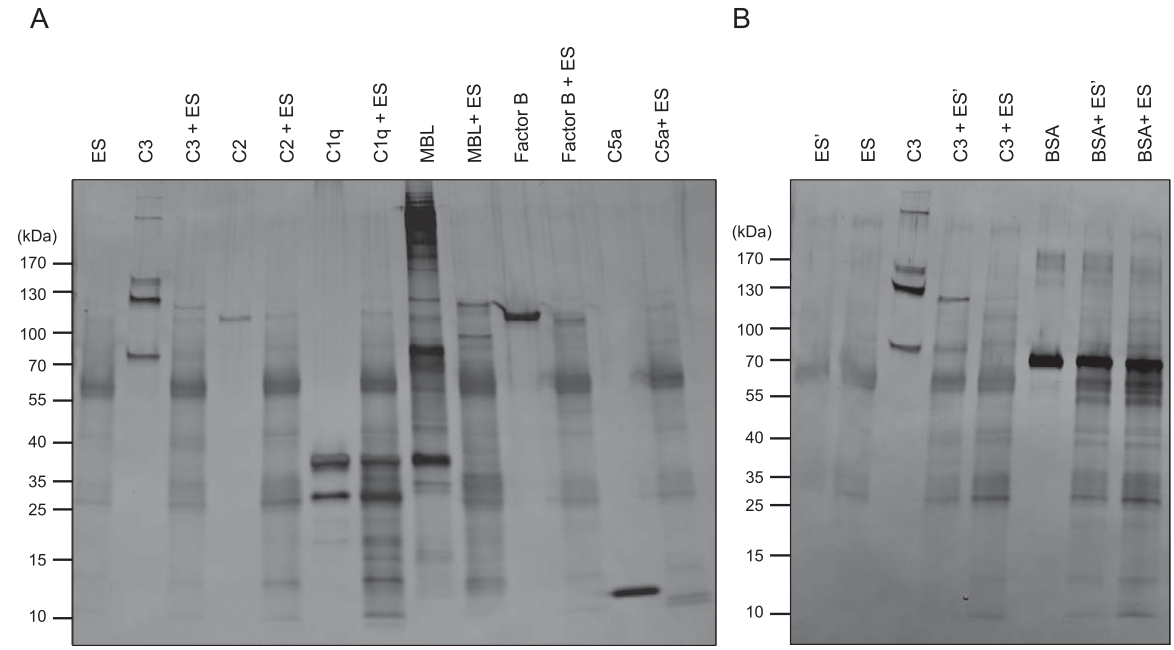

Figure 2. Degradation of complement proteins by larval ES. (A) Purified human C3, C2, C1q, MBL, factor B, and C5a proteins, were incubated with larval ES (0.8 mg/ml) or PBS as control for 1 hour at 37°C. Thereafter, the samples were subjected to SDS-PAGE, and the proteins in the gel were visualized by silver-staining. A representative image from at least 3 independent experiments is shown. (B) The selectivity of protein degradation by larval ES. C3 and BSA were incubated with ES (0.8 mg/ml) and ES′ (0.4 mg/ml) for 1 hour at 37°C. SDS-PAGE and the visualization of the proteins in the gel were performed as above. A representative image from at least 3 independent experiments is shown.

were aspirated briefly (10 seconds) to determine the baseline fluorescence intensity of the cells. Thereafter, 25 μl of the stimuli either or not pretreated with ES were added, vortexed immediately, and the subsequent changes in fluorescence intensity in the FL-1 channel were recorded over 120 seconds. The results were analyzed using the CellQuest Pro and FlowJo 10.1 software. The changes in the fluorescence intensity were plotted against time. For assessment of the effects of larval ES on the cells, fluo-3AM-loaded cells were pre-incubated with 1 mg/ml ES at 1:1 ratio for 5 and 60 minutes at room temperature, then washed and diluted 5 times in RPMI/HSA and finally exposed to C5a, C3a, fMLP, or PBS as control. Immediately thereafter the changes in fluorescence were monitored as described above.

Statistics

Wilcoxon signed-rank tests and Mann-Whitney U tests were used to calculate differences between the values for ES-pretreated and control samples using the GraphPad Prism 6.0 software package. p-Values of ≤ 0.05 were considered significant.

RESULTS

Larval ES inhibit each step of the three complement activation pathways

Previous research showed that larval ES can inhibit the main three complement activation pathways (the classical [CP], the lectin [LP], and the alternative [AP] pathways) in a dose-dependent fashion.[13] To obtain some insights into the mechanisms underlying these inhibitory actions, we first assessed the effects of ES on the deposition of various complement proteins on the pathway-specific coated wells by ELISAs. The results showed that deposition of all components that are located upstream from C5b-9, i.e., C3, C4, C1q, and MBL, on the wells was reduced by ES. These results were similar to those for the inhibitory effect of ES on C5b-9 formation (Figure 1). These findings indicate that ES inhibit complement activation in each step of the three activation pathways.

Degradation of proteins associated with complement activation by larval ES

As larval ES contain many kinds of proteases,[1] we hypothesized that complement inactivation was due to degradation of the complement proteins by ES. All proteins associated with complement activation, including C3, C4, C2, C1q, MBL, factor B, C5a, C3a, C3b, and C5 were degraded by ES (Figure 2A; data for C3a, C3b, and C5 are not shown). Interestingly, BSA (Figure 2B), human IgM and to a lesser degree IgG (Figure 3A) were less efficiently degraded by ES than complement-associated proteins like C3.

Effect of larval ES on the pathway-specific coatings

As ES degraded human immunoglobulins, we investigated the possibility that ES interfere with the pathway-specific

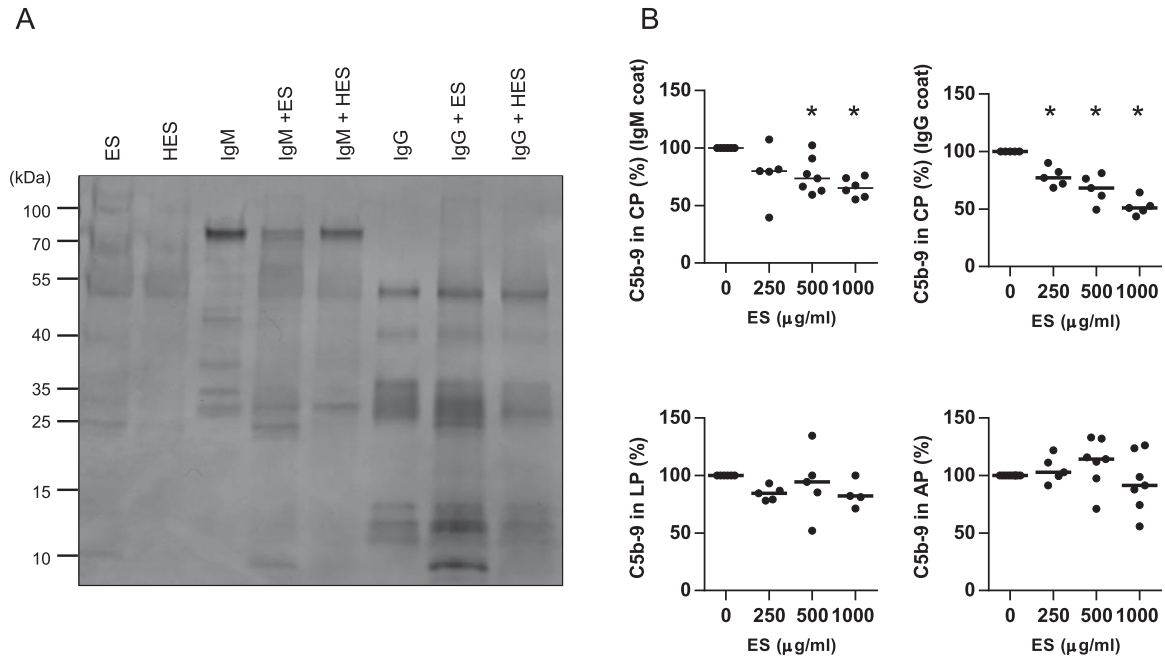

Figure 3. Effects of larval ES on human immunoglobulins. (A) Degradation of human immunoglobulins by larval ES. Purified human IgM and IgG were incubated with either 0.8 mg/ml ES, ES preheated for 15 minutes at 98°C (HES), or PBS as control for 1 hour at 37°C. Thereafter, the samples were subjected to SDS-PAGE, and the proteins in the gel were visualized by silver-staining. A representative image from at least 3 independent experiments is shown. (B) Effects of larval ES on the coating of the ELISA wells. Wieslab wells (coated with IgM for activation of the CP, with mannan for the LP, and with LPS for AP) and in-house prepared IgG-coated wells for the CP were pre-incubated with serial dilutions of ES or PBS as control for 1 hour at 37°C. After washing, appropriately diluted human serum was pipetted in the wells and incubated for 1 hour at 37°C. Thereafter, the formation of C5b-9 in the wells was measured by ELISA. Results are expressed as % of the values found for wells preincubated with PBS instead of ES. Data are from at least five independent experiments with lines representing median values. * $p < 0.05$ for the differences between the results for ES-preincubated and control samples.

coatings of the ELISA plates. In short, we exposed the coated plates to larval ES for 1 hour, washed the wells to remove remaining ES, and then added diluted human serum to the wells followed by detection of C5b-9 formation. The results showed that the degree of complement activation in IgM- and IgG-coated wells, but not mannan- and LPS-coated wells, was reduced by ES pretreatment in a dose-dependent fashion (Figure 3B). However, C5b-9 formation in these wells pretreated with ES was less than in immunoglobulin-coated wells incubated with serum that had been mixed with ES, indicating that degradation of human immunoglobulins by ES does not explain the inhibition of the CP activation by ES.

Larval ES inhibit complement-induced neutrophil activation

As C5a and C3a are well-known inducers of phagocyte activation and chemotaxis,[14–16] we determined the effect of ES on the changes in intracellular free Ca^{++} concentration in fluo-3AM-loaded human neutrophils upon exposure to C5a, C3a, and for comparison fMLP(24).[24] The results showed that C5a induced a rise in intracellular free Ca^{++} concentration (iCa^{++}) in neutrophils which did not occur after ES-pretreated C5a (Figure 4A and B). Similar results were found for C3a (data not shown), but importantly not for fMLP (Figure 4C and D). Furthermore, the rise in intracellular free Ca^{++} concentration in neutrophils upon exposure to C5a, C3a, and fMLP was not affected by pretreatment of the cells with ES for 5 and 60 minutes (Figure 4E–H, data for C3a not shown), which implies that ES did not impair the C5a-, C3a-, and fMLP-receptors on neutrophils and their down-stream signaling pathways. Together, these findings indicate that ES can attenuate neutrophil activation by degrading C5a and C3a.

Effects of heat and serine protease inhibitor on the anti-complement activities of larval ES

As the above results strongly implicate larval proteases to inhibit complement activation, we performed additional experiments to further characterize the proteases involved. The results revealed that heat-treated ES (HES) and the serine protease inhibitor AEBSF-treated ES (AES) were not as effective as ES in degrading C3 and C5a (Figure 5A). Likewise, HES and AES delayed the onset, but otherwise did not affect the C5a-induced rise in intracellular free Ca^{++} concentration in human neutrophils (Figure 5B). We cannot offer an explanation for the delayed onset, but this was also seen for the fMLP-induced rise in intracellular Ca^{++} (results not shown). Surprisingly, HES and AES inhibited C5b-9 formation in all pathway-specific

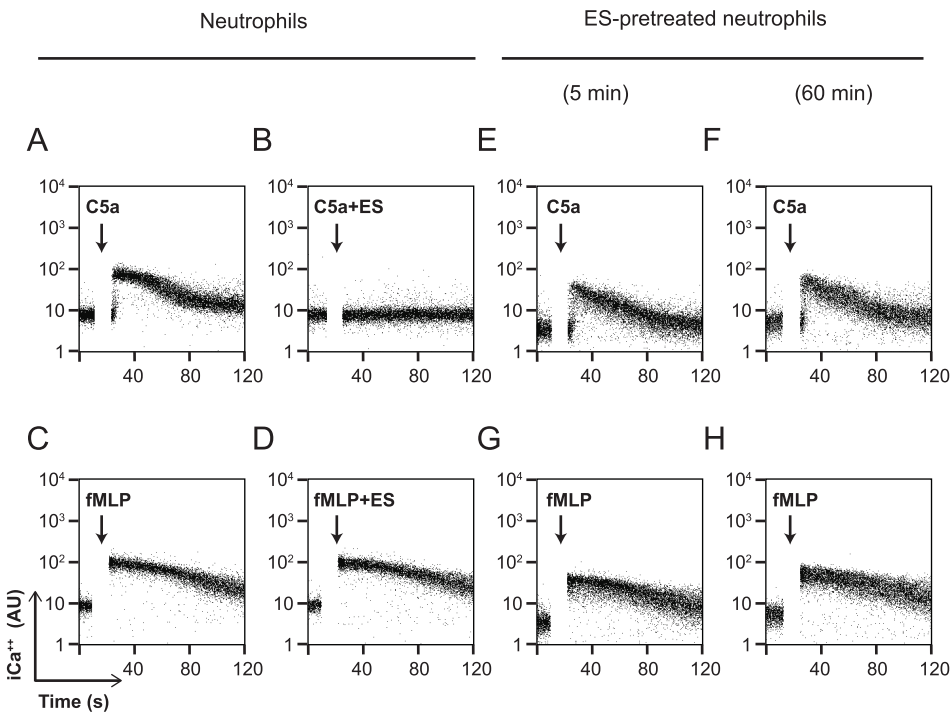

Figure 4. Effects of larval ES on neutrophil activation. The changes in the intracellular free Ca^{++} concentration (iCa^{++}) (AU; arbitrary unit) in fluo-3 AM-loaded human neutrophils upon addition (indicated by an arrow in every panel) of 2 μM C5a (A,B) and 20 nM fMLP (C,D) were monitored by flow cytometry. The stimuli were pretreated with either 0.5 mg/ml ES (B,D) or PBS as control (A,C) for 1 hour at 37°C. In addition, fluo-3 AM-loaded cells were pretreated with 0.5 mg/ml larval ES for 5 minutes (E,G) and 60 minutes (F,H), washed and then C5a (E,F) or fMLP (G,H) was applied to induce a rise in iCa^{++}. The results are from a representative experiment out of at least 3 independent experiments.

coated plates (Figure 5C). Interestingly, inhibition of C3 deposition on IgM-coated wells (the CP) and more remarkably on mannan-coated wells (the LP) was significantly less by HES than by ES, while C3 deposition on wells coated with LPS (the AP) was inhibited equally effective (Figure 6). These results indicate that ES possess heat-stable molecule(s) that regulate the C3 amplification loop in the AP and to a lesser degree in the CP/LP. Our finding that the ability of bovine chymotrypsin and proteinase K to inhibit the CP activation was sensitive to heat and AEBSF treatment (data not shown) confirms that these treatments were effective.

DISCUSSION

The primary aim of this study was to elucidate the mechanisms underlying the anti-complement effects of ES from medicinal larvae (*Lucilia sericata*). Our results show that ES can inhibit complement activation by two mechanisms, as schematically shown in Figure 7: serine proteases within ES degrade multiple complement proteins (indicated by full-lined circle) and an unknown heat-stable molecule down-regulates the C3 amplification loop (mainly in the AP, as indicated). Larval ES also degraded human immunoglobulins. Based on these *in vitro* data, it is likely that larval ES, by reducing the C3a and C5a production and subsequently degrading these important drivers of inflammation, down-regulate inflammation and thus facilitating wound healing. Although down-regulation of complement activation and other pro-inflammatory responses is beneficial for healing of chronic wounds, it could be that the antimicrobial potential of inflammatory phagocytes is also reduced. However, this is not likely as we reported earlier that *in vitro* phagocytosis and intracellularly killing of *Candida albicans* by human granulocytes[19] and of *Staphylococcus aureus* by human monocytes[25] were not reduced by ES.

The conclusion that serine proteases within ES are involved in the inhibition of complement activation is based on the following findings. First, larval ES inhibited the complement activation cascades at all steps investigated (Figure 1). The possibility that ES inhibit complement activation by degrading the coatings of the wells used to activate the different pathways can be excluded as ES did not affect the coatings for the LP/AP, and the inhibitory effects of ES on IgM and IgG coatings for the CP were considerably less than found to inhibit complement activation (Figure 3B). Second, larval ES degraded all complement proteins, including C3a and C5a in a dose-dependent fashion (Figure 2). In agreement, pretreatment of C5a and C3a with ES impaired the ability of these complement factors to activate human neutrophils (Figure 4). Third, the complement protein degrading activity of larval ES was heat-sensitive and affected by the serine protease inhibitor AEBSF (Figure 5A). Interestingly, degradation of the complement proteins by larval serine proteases results in

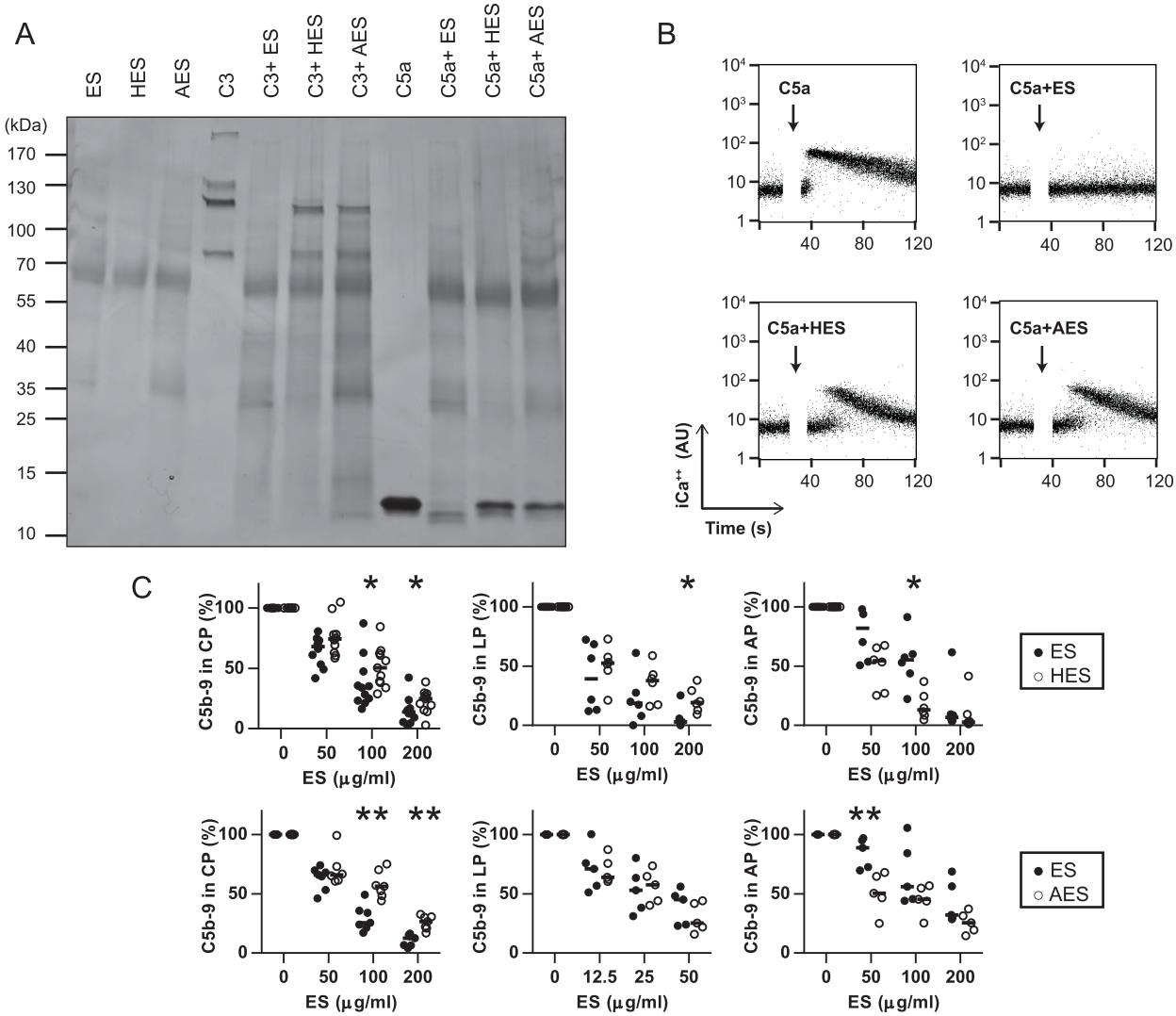

Figure 5. Involvement of heat-sensitive serine proteases in the anti-complement activities of larval ES. ES were pretreated for 15 minutes at 98°C (HES), or pre-incubated for 1 hour with 1 mM of the serine protease inhibitor AEBSF (AES). (A) Effect of heat and AEBSF on degradation of C3 and C5a by ES. These complement proteins were incubated with ES, HES, and AES (0.8 mg/ml) or PBS as control for 1 hour at 37°C. After SDS-PAGE, the proteins in the gel were visualized by silver-staining. A representative image from at least 3 independent experiments is shown. (B) Effect of heat and AEBSF on the inhibition of the C5a-induced rise in intracellular free Ca^{++} concentration (iCa^{++}) in human neutrophils by ES. C5a was incubated with ES, HES, and AES or PBS as control for 1 hour before addition to fluo-3 AM-loaded neutrophils, and the subsequent rise in iCa^{++} in the cells was monitored by flow cytometry. The results of a representative experiment out of at least 3 independent experiments are shown. (C) Effect of heat and AEBSF on the formation of C5b-9 complex. Diluted human serum was mixed with either ES (•) or HES/AES (○) at an appropriate ratio, and then applied to pathway-specific coated wells after which the amount of C5b-9 in the wells was detected by ELISA. The results are expressed as % of the values found for serum mixed with PBS instead of ES. Data are from at least five independent experiments with lines representing median values. * $p < 0.05$, ** $p < 0.01$ for the results for ES-treated vs. control samples.

complement inactivation, while most complement component proteins are known to work as endogenous serine proteases that cleave the next component into a more active form.[14–16]

Which serine proteases are responsible for the anti-complement activities of ES? It is known that larvae of *L. sericata* and related species secrete many kinds of serine proteases in rich quantities to facilitate the uptake of nutrients, i.e., liquefied necrotic tissue and bacteria.[1,2,9,10] Currently, three different serine proteases in ES from *L. sericata* larvae have been identified and produced by recombinant technology: chymotrypsin I degrades wound eschar/slough and inhibits staphylococcal biofilms,[26,27] sericase enhances plasminogen-activator

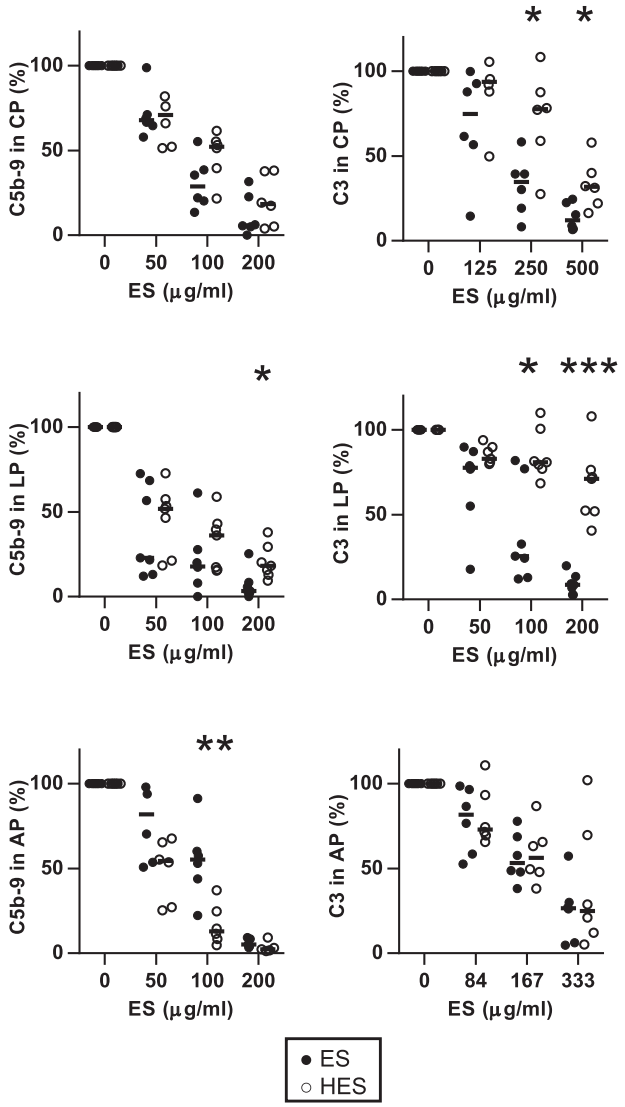

Figure 6. Involvement of a heat-stable molecule in the anti-complement activities of larval ES. Diluted human serum was mixed with increasing doses of ES (•) or HES (○) at an appropriate ratio prior to transfer to pathway-specifically coated wells. After incubation for 1 hour at 37°C, C5b-9 formation and bound C3 to the wells were quantitated using ELISAs. The results are expressed as % of formed/bound proteins as compared to controls, i.e., diluted serum mixed with PBS instead of ES. Data are from at least five independent experiments with lines representing median values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for the differences between the values for the results for ES-treated and control samples.

induced,[28] and the Jonah-like chymotrypsin activates the host contact/intrinsic pathway of coagulation.[29] Unfortunately, the serine protease inhibiting complement activation could not be identified yet due to the lack of a proper reference database for *L. sericata*. In addition, attempts to identify the complement inhibitory component(s) in larval ES using available data bases for flies like *Lucilia cuprina*,[30] *Musca domestica*,[31] and *Drosophila melanogaster*[32] as well as the Uniprot database were also unsuccessful. Currently, we are annotating the genome of *L. sericata* to develop a proper *L. sericata* data base to facilitate the identification of the active component(s) in larval ES.

The unexpected finding that pre-treatment by heat and AEBSF did not affect the ability of larval ES to inhibit C5b-9 formation (Figure 5C) indicates that a heat-resistant molecule in ES is also involved in inhibition of complement activation. Interestingly, ELISAs detecting C3 binding to pathway specific coated wells revealed that the C3 amplification loop (specifically in the AP) is inhibited by this heat-resistant regulator of complement in ES (Figure 6). In an attempt to identify this heat-stable complement inhibitor, we fractionated ES and HES by gel filtration using a Sephadex G100 column and the complement-inhibiting activity in the resulting fractions was assessed using the Wieslab ELISAs for the three complement activation pathways. Results revealed two clearly separated peaks with complement-inhibiting activity: one was associated with large molecular weight proteins and the other with middle/low molecular weight molecules. Comparison of the complement-inhibiting activity and the protein staining profiles of the various fractions indicated that a high molecular molecule within ES may be responsible for inhibiting complement. Further attempts to identify the protein(s) involved in complement inhibition using trypsin degradation of the proteins in the fractions followed by mass-spectrometry failed because of the lack of a proper reference database for *L. sericata*. Although the identity of this complement regulator could not be established, we found this activity within the relatively high molecular weight fractions of ES. In this connection, complement factor H (Mw 155 kDa) is worth to be mentioned since this regulatory glycoprotein acts as a cofactor for the factor I-mediated C3b cleavage in the AP and thus inhibits its amplification loop.[33] Moreover, factor H is known to be a highly heat-resistant protein[34] and to possess complement control protein (CCP) domains that are conserved among a wide range of species, including insects.[35] It is therefore attractive to hypothesize that larval ES comprise a factor H-like complement regulator.

Another import finding from this study pertains to the effects of ES on molecules other than complement proteins. We found ES to degrade human immunoglobulins and BSA less efficiently than complement proteins (Figures 2B and 3A). In addition, the receptors for C3a, C5a, and fMLP on neutrophils and the fMLP peptide (Figure 4) as well as mannan and LPS (Figure 3B) were not affected by ES (see also Figure 7). This data indicates that larval protease selectively degrade proteins, human complement proteins in particular.

Wound healing is a dynamic and complex process that can be explained by a three-stage strategy comprising of an inflammatory, proliferative, and remodeling phase.[1,2,7,17,18] Although a wound can be chronic when any of these phases is impaired, excessive inflammation is the most important to be addressed. While initial inflammatory responses are essential for wound healing, prolonged inflammation due to, for example, remnant devitalized tissue and microbes in the wound delays

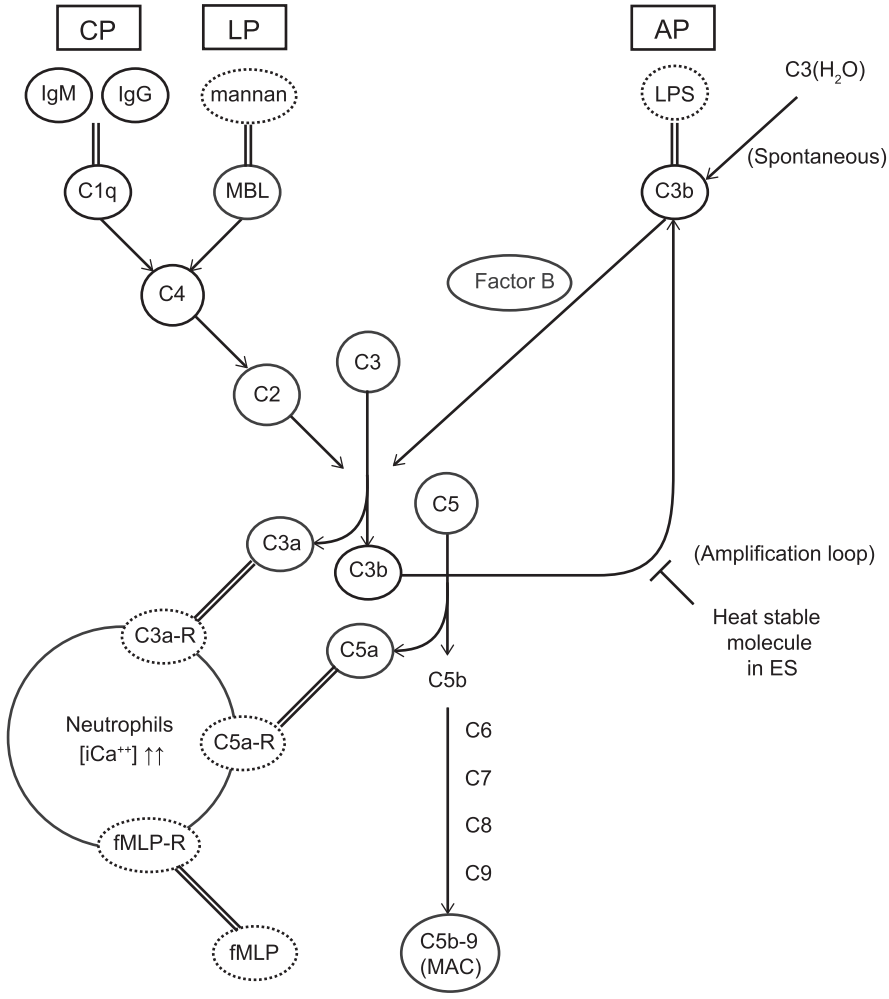

Figure 7. Schematic representation of the inhibitory effects of larval ES on the complement system. Complement activating components and its activators (used for pathway-specific coating for *in vitro* assays) are allocated according to the direction of complement activation in three pathways indicated by arrows. Double lines indicate the bindings of the molecules. Full-lined circles indicate proteins that are degraded by larval serine proteases, dash-lined circles are not affected by larval ES, and no circle indicates the proteins not tested. The possible target of the heat-stable molecule(s) in ES inhibiting the complement system is demonstrated. C3a-R/C5a-R/fMLP-R, C3a/C5a/fMLP receptors on neutrophils; $[Ca^{++}]i$, intracellular free Ca^{++} concentration; MAC, membrane attack complex.

initiation of the proliferative phase.[17,18] It has been reported that chronic wounds remain in a state of chronic inflammation which is characterized by abundant pro-inflammatory cell infiltration and increased generation of pro-inflammatory cytokines, proteases, and reactive oxygen species on chronic wounds.[2,18] Therefore, larval ES may modify the over-inflamed status of chronic wounds by inhibiting complement activation and—along with the debridement of the wounds—facilitate wound healing. Our observations provide new scientific insights in the beneficial actions of larval ES on wound healing and reveal potential new mechanisms to inhibit complement activation in other clinical conditions characterized by excessive complement activation,[36,37] including severe inflammatory response syndrome and multiple organ failure, ischemia-reperfusion injury, transplant surgery, rheumatic diseases, systemic lupus erythematosus, autoimmune-related nephritis, sepsis, and ischemia-reperfusion injury.

CONCLUSION

In conclusion, larval ES inhibit complement activation by two different mechanisms. The associated down-regulation of the C3a/C5a-mediated neutrophil activation attenuates the inflammatory process, which may facilitate wound healing.

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

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