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Aggregation of thrombin-derived C-terminal fragments - a novel host defense mechanism

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Abstract

Effective control of endotoxins and bacteria is crucial for normal wound healing. During injury, the key enzyme thrombin is formed, leading to generation of fibrin. Here we show that human neutrophil elastase (HNE) cleaves thrombin, generating 11-kDa thrombin-derived C-terminal peptides (TCP), which bind to and form amorphous amyloid-like aggregates with both bacterial lipopolysaccharide (LPS) and Gram-negative bacteria. *In silico* molecular modeling employing atomic-resolution and coarse-grained simulations corroborates our experimental observations, altogether indicating increased aggregation through LPS-mediated intermolecular contacts between clusters of TCP molecules. Upon bacterial aggregation, recombinantly produced TCP induces permeabilization of *Escherichia coli* and phagocytic uptake. TCPs of about 11 kDa are present in acute wound fluids as well as in fibrin sloughs from patients with infected wounds. We noted aggregation and colocalization of LPS with TCPs in such fibrin material, which indicates the presence of LPS-TCP aggregates under physiological conditions. Apart from identifying a novel function of proteolysed thrombin and its fragments, our findings provide an interesting link between the coagulation system, innate immunity, LPS-scavenging and protein aggregation/amyloid formation.

Significance

The work summarized in this paper is based on the simple but unexpected observation that addition of LPS or bacteria to human wound fluids leads to precipitation of protein aggregates, a phenomenon not observed in plasma. Using a broad mix of technologies, from biophysical, biochemical and microbiological methods, fluorescence and electron microscopy, and in silico modeling, to studies on wound materials, we here demonstrate a previously undisclosed role of C-terminal thrombin fragments of about 11 kDa, involving LPS- and bacteria-induced aggregation and scavenging, facilitating clearance and microbial killing. Our findings provide a novel link between the major coagulation factor thrombin, innate immunity, and amyloid formation.

\body

Introduction

Skin wounds pose a potential threat for invasive infection and sepsis. It is therefore not surprising that multiple host defense systems have developed over the course of evolution that involve initial hemostasis, fibrin formation, and the subsequent action of multiple proteins and peptides of our innate immune system (1-3). In humans, such host defense systems include neutrophil-derived α-defensins and the cathelicidin LL-37 (3, 4) but also proteolytic products of plasma proteins, such as heparin cofactor II (5), antithrombin III (6), and thrombin (7-10). Lipopolysaccharide sensing by Toll-like receptor 4 (TLR4) is crucial in early responses to infection. However, an excessive LPS response gives rise to dysfunctionality, causing localized inflammation such as that found in infected wounds, but also severe systemic responses such as those seen in sepsis (11). Therefore, clearance and control of endotoxins is critical to maintaining a robust antibacterial response while maintaining control of inflammatory responses. Thrombin-derived C-terminal peptides of roughly 2 kDa (such as FYTHVFRLKKWIQKVIDQFGE and HVFRLKKWIQKVIDQFGE (12-14)), which are present in wounds, frequently form helices upon LPS-binding and exert anti-endotoxic functions *in vitro* and *in vivo* (10, 12). From a therapeutic standpoint, a prototypic thrombin-derived peptide, GKY25 (GKYGFYTHVFRLKKWIQKVIDQFGE) has been shown to protect against *P. aeruginosa* sepsis, mainly via reduction of both systemic cytokine responses and excessive coagulation (12, 15). Functional and structural studies have thus far focused on this and related low-molecular weight peptides (10, 12); the major 11-kDa TCP-form present in wound fluids (10) remains uncharacterized. During the course of our work, we noted that the 11-kDa TCP was notoriously prone to aggregation, which suggests that this structure may present some unique characteristics that differentiate

it from smaller TCPs. This finding prompted us to further investigate biological roles of TCPs.

Results

Purification and activity of rTCP. First, we generated a recombinant 96-amino acid TCP fragment (denoted here "$rTCP_{96}$") corresponding to the C-terminal fragment cleaved out from thrombin by HNE (Fig. S1A–B). Circular dichroism (CD) spectrometry analysis of $rTCP_{96}$ yielded secondary structure characteristics compatible with the corresponding thrombin region (similar to the B4 peptide). Furthermore, *in silico* modeling studies showed that an $rTCP_{96}$ structure analogous to the structure of the B4 chain of thrombin adopts a stable fold, with minimal structural drift over extended timescales, comparable with the X-ray structure of γ-thrombin, exhibiting compatible secondary structural content (Table S1 and Methods). Purified $rTCP_{96}$ was subjected to heparin-affinity chromatography and was found to elute at a similar salt strength as human γ-thrombin (Fig. S1C), compatible with a functional (heparin-binding) activity of $rTCP_{96}$. Consistent with previous findings of endogenous 11-kDa TCP (13), we found that the recombinant form was antimicrobial against *E. coli* in a gel-overlay assay. $rTCP_{96}$ also demonstrated antibacterial effects in radial diffusion assays (RDAs) against *E. coli* and *P. aeruginosa* and viable count assays (VCAs) against *E. coli* (Fig. S1D–H). The results demonstrate that $rTCP_{96}$, like the native form, and similarly to GKY25 comprising the C-terminal 25 amino acids of $rTCP_{96}$, kills Gram-negative bacteria *in vitro in the physiological concentration range 0.5 - 3 μM*. As GKY25 dose-dependently induces hemolysis, we explored whether TCPs could permeabilize human erythrocytes as well, but we did not detect any such effects (Fig. S1I).

TCPs interact with LPS. Based on these findings, we next explored possible LPS-$rTCP_{96}$ interactions and the structural implications of these interactions. Ellipsometry analysis of surface interactions revealed significant binding already at concentrations of 1–2 μM, for both $rTCP_{96}$ and GKY25 to surface-immobilized *E. coli* LPS, whereas the negative control IVE25 displayed much lower binding (Fig. 1A). As can be seen in Figure S1J, kinetics measurements of LPS binding showed comparable binding kinetics for $rTCP_{96}$ and GKY25, both displaying an initial mass-transport-limited phase, ranging less than ~200s, followed by a considerably slower binding, most likely as a consequence of structural re-arrangements in the LPS layer on peptide binding. Analogously, microscale thermophoresis (MST), a highly sensitive technique probing interactions between components in solution, demonstrated interactions of fluorescence-labeled $rTCP_{96}$ with *E. coli* LPS, with a K_D of 3.3 ± 1.8 μM (Fig. 1B). Furthermore, in a slot blot assay, using biotin-labeled *E. coli* LPS and immobilized $rTCP_{96}$, we observed binding of LPS to $rTCP_{96}$, which was eradicated by the addition of heparin (Fig. S1K). Since heparin binds to a region situated at the C-terminus of the thrombin molecule, which comprises a part of the exosite II site (16), these results indicate that the C-terminal helical end of $rTCP_{96}$ binds to LPS.

LPS induces aggregation of TCPs. Above observations were substantiated using CD spectrometry, and we found that LPS induced a helical conformation in the 25mer thrombin-derived C-terminal peptide GKY25, consistent with findings of previous studies (10, 17). For comparison, an increase in β-sheet content was observed for $rTCP_{96}$ after 30 min of incubation with LPS (Fig. 1C). Thioflavin T (ThT) has become amongst the most widely used "gold standards" for selectively staining and identifying amyloid fibrils characterized by β-strand-like interactions (18, 19). Unlike

149 the results observed for GKY25 after the addition of LPS, a significant increase in
150 ThT staining was noted after the addition of LPS to $rTCP_{96}$, (Fig. 1D), which is
151 consistent with CD data on β-sheet content (Fig. 1C). LPS-induced aggregation of
152 TCP_{96} was inhibited by an excess of the peptide GKY25 (Fig. S2A). Moreover, LPS-
153 induced ThT binding was observed using a dose range of 1-10 μM $rTCP_{96}$ in Tris
154 buffer and in presence of 1% citrated plasma (Fig. S2B). Likewise, LPS induced the
155 formation of amorphous aggregates of $rTCP_{96}$, of sizes 1–3 μm, as demonstrated by
156 negative-stain transmission electron microscopy (TEM; Fig. 1E). Furthermore,
157 $rTCP_{96}$ was added to a buffer containing citrated plasma. Again, we observed similar
158 LPS-induced aggregation that yielded aggregated forms with sizes ranging from 0.5–
159 30 μm (Fig. 1E). Moreover, co-aggregation of $rTCP_{96}$ with LPS was confirmed by
160 detection of significant differences in the hydrodynamic radii using dynamic light
161 scattering (Fig. S2C). Using CD spectrometry, ThT binding assay, and dynamic light
162 scattering (DLS) we did not detect a significant aggregation of pure $rTCP_{96}$ for a time
163 period up to 2 h, which contrasted to the LPS-induced aggregation, observed already
164 after an incubation period of 10 min (Fig. S2D-F).

165 Although $rTCP_{96}$ mimicked the antibacterial effects of native human TCP, it may be
166 argued that possible differences in folding patterns between the recombinant and
167 endogenous forms could have influenced the observed aggregation of the recombinant
168 peptide. To address this issue, we conducted CD, ThT, and TEM experiments
169 utilizing two fragmented thrombin forms containing TCPs in the form of HNE-
170 digested thrombin or γ-thrombin. The latter is a product of autoproteolysis and
171 contains a C-terminal peptide that is very similar to the HNE-generated fragment (9,
172 20). In all cases, the results using these endogenous TCPs were consistent with those

obtained with $rTCP_{96}$, and no significant aggregation was observed for intact thrombin (Fig. 3F-I).

Molecular simulations of the TCP-LPS interaction. Next, we performed *in silico* analyses of the TCP-LPS interaction using both atomic-resolution and CG molecular dynamics simulations. These simulations allowed us to assess the fold stability of the 11-kDa TCP structure, its conformational changes compared with those of intact thrombin, as well as the interaction modes of LPS with TCP. Our analysis revealed a newly exposed amphipathic helix, comprising residues 80–96, buried within intact thrombin. The hydrophobic portion of this amphipathic helix was observed to interact with a sheet region (residues 46–60); we also found that the hydrophobic portion was in a preferred interaction site for the lipid tails of LPS (Fig. 2A). We observed a twist in the β-sheet region in the absence of LPS that enabled the formation of a cluster of hydrophobic residues comprising of F52, Y57, M59, F81, W86, and F94 (Fig. 2A). Therefore, interactions with LPS may enable the relaxation of the sheet twist and accordingly explain the observed increase in β-content in the CD spectra. Coarse-grained models provide a pseudo-atomistic description of the system whilst enabling longer time- and length-scales to be attained, thereby enabling us to study the spontaneous self-assembly of TCP and LPS (Fig. 2B, S2 and Movie S1). These simulations revealed a significantly increased propensity of TCPs to aggregate in the presence of LPS (Fig. 2B and C). Pairwise distance analysis (Fig. 2C) indeed revealed that the aggregation coincides with the preferred LPS binding sites on TCP (Fig. 2A). Furthermore, inspection of the resultant aggregates revealed that LPS intercalates in the TCP clusters and thereby facilitates intermolecular interaction (Fig. 2C, right panel). Our *in silico* results thus correspond elegantly to the experimental data

198 presented above and highlight the unique capabilities of TCPs for structural
199 modulation and specific LPS-mediated intermolecular interactions that enable their
200 aggregation.

201

202 **TCPs mediate bacterial aggregation and phagocytosis.** Considering that $rTCP_{96}$
203 and degraded thrombin (10) exert antibacterial effects, we next explored whether
204 $rTCP_{96}$ could form aggregates together with Gram-negative (LPS-containing)
205 bacteria. Indeed, incubation of $rTCP_{96}$ with *E. coli* yielded similar amyloid-like ThT-
206 positive amorphous aggregates, as previously identified after the addition of LPS (Fig.
207 3A). Using negative-stain TEM, we identified aggregated bacteria, and we observed
208 signs of membrane perturbations in $rTCP_{96}$-treated cells only (Fig. 3B). The
209 impermeable marker FITC was taken up by these bacterial aggregates (Fig. 3C),
210 indicative of bacterial killing, which is consistent with the antibacterial effects by
211 $rTCP_{96}$ noted above (Fig. S1D–F). Figure 3D illustrates that while both GKY25 and
212 $rTCP_{96}$ were able to induce uptake of FITC, only $rTCP_{96}$ significantly increased the
213 aggregation of *E. coli* ($p<0.01$). In order to explore the functional significance of
214 TCP-mediated aggregation, we hypothesized that aggregate formation was linked to
215 phagocytic clearance. Indeed, we found that fluorescent *E. coli* particles, aggregated
216 with $rTCP_{96}$, were taken up by the macrophage cell line RAW 264.7. Notably, $rTCP_{96}$
217 at 0.5 μM yielded a roughly 25% increase in phagocytosis compared with *E. coli*
218 particles only *(*$p<0.01$; Fig. 3E). Phagocytized fluorescent *E. coli* particles (green
219 color), aggregated with $rTCP_{96}$ (visualized using fluorescently labeled antibodies),
220 were also detected in RAW 264.7 cells using fluorescence microscopy (Fig. 3F and
221 S3). Taken together, these results indicate that both the $rTCP_{96}$ and native TCPs are

222 able to aggregate and form amyloid structures, and that this phenomenon is promoted
223 in the presence of LPS or bacteria and followed by phagocytic uptake.

224

225 **TCPs are present in wound fluids and aggregate with *E. coli* and LPS.** Earlier
226 studies have shown that thrombin binds to fibrin clots and that fibrin acts as a
227 reservoir for active thrombin (21). Furthermore, human neutrophils release elastase
228 during the initial phases of wound healing, and the enzyme may cleave thrombin
229 present in soluble form, as well as when immobilized in fibrin (10). As expected, and
230 compatible with our previous findings, wound fluids from patients with non-infected
231 surgical wounds contained several TCPs (Fig. 4A). The concentrations of endogenous
232 TCPs (~11 kDa) in wound fluids have been estimated from calibration curves using
233 Western blot analysis. The calculated concentrations of peptides were from 0.6 to 2.7
234 μM (Fig S2G). As shown above, $rTCP_{96}$ forms aggregates in a plasma environment
235 (Fig. 1D). To address whether endogenous TCPs could also form aggregating amyloid
236 structures, we incubated human wound fluid with LPS and analyzed aggregate
237 formation. Similar to the *in vitro* studies described above, we were able to identify
238 LPS-induced aggregates in the wound fluids. By utilizing gold-labeled antibodies, we
239 detected C-terminal epitopes in these structures (Fig. 4B). When these experiments
240 were repeated with *E. coli,* we observed a significant aggregation with TEM after the
241 addition of the bacteria to the wound fluid (Fig. 4C). These results were supported by
242 confocal microscopy data (Fig. 4D and E), where the same initial experimental setup
243 was used, followed by specific staining for TCPs (red) and amyloids (green).
244 Therefore, TCP-containing aggregates were induced after the addition of LPS (Fig.
245 4D) or *E. coli* (Fig. 4E), yielding aggregates containing TCPs that overlapped with
246 ThT staining (green), thereby indicating the formation of TCP-containing amyloids.

TCPs are found with aggregated LPS *in vivo.* Non-healing venous ulcers are characterized by the presence of bacteria accompanied by the activation of neutrophils and the release of HNE (22, 23). We accordingly investigated the production of TCPs in fibrin slough from a patient with such a venous ulcer; we also studied the fluid from a wound dressing covering the ulcer. As shown in Figure 4F, we detected TCPs in both the fibrin and the wound exudate. We next investigated the fibrin slough from non-healing ulcers from two patients to search for the presence of TCP-LPS aggregates via negative-stain TEM utilizing specific gold-labeled antibodies. The antibodies against TCPs were detected by anti-rabbit IgG with labeled gold particles 10 nm in diameter (blue dots); for the detection of LPS we used IgG anti-LPS labeled with 5-nm gold particles (red dots, Fig. 4G). A colocalization of TCPs (10 nm particles) and LPS (5 nm particles) was observed in both patient samples, in the range 30-600 nm, with a peak in the lower range (below 50 nm) where the pair cross-correlation function (PCCF) exceeded ~2 (Fig. S2H). Antigens which do not cross-correlate have no PCCF peak in any interval, and the value is fluctuating around 1 (24).

Discussion

Endotoxin, a highly proinflammatory substance, as well as bacteria, need to be contained and controlled, and the present work discloses a simple but effective mechanism by which aggregation prone TCPs facilitate this clearance - of relevance for control of infection and inflammation during wounding. Apart from revealing a novel role for thrombin as a source for endotoxin- and bacteria-aggregating peptides during wounding, our results also raise interesting perspectives on the roles of, and relation between, antimicrobial and amyloidogenic peptides. It is clear that various

peptides from these two classes are able to bind and damage bacterial and eukaryotic cell membranes. Indeed, it has been proposed that some peptides that are simultaneously antimicrobial and amyloidogenic have separate sequence motifs encoding these two activities (25). Considering the observation that the shorter TCP GKY25 does not form any aggregates, this would imply that the sequence responsible for the aggregation behavior, and β-sheet formation, is found upstream of the GKY25 sequence, which is in agreement with *in silico* predictions. Although structurally separate, our data indicate that the antimicrobial and amyloidogenic properties are interdependent, indeed connecting the antimicrobial and LPS-aggregating functions within one single molecule.

A controlled and targeted proteinase action is crucial during wounding. Thrombin, initially formed by selective proteolysis by coagulation factor X, mediates fibrinogen degradation and clot formation (7) but also exerts other physiologic functions in innate defense and hemostasis (7) (Fig. 5). As previously reported, subsequent proteolysis leads to formation of smaller TCPs of about 2 kDa with anti-endotoxic functions (10, 14, 15) (Figure 5). Interestingly, *Pseudomonas aeruginosa* "hijacks" this mechanism by release of bacterial elastase, which cleaves out a TCP of 21 amino acids, thereby enabling modulation and circumvention of host responses (14). In many aspects, such TCPs act as "classical" host defense peptides like LL-37 (26), which also binds to LPS, leading to inhibition of NF-κB activation. The herein presented work on LPS and bacteria-induced aggregation of TCPs therefore adds a new, and previously undisclosed, dimension to the role of thrombin (Fig. 5). Furthermore, the finding that thrombin fragments, including ~11 kDa TCPs are present in acute, sterile wound fluids, together with the rapid aggregation of LPS and

297 bacteria observed in these wounds fluids *ex vivo*, indicates a fundamental role for
298 aggregation and amyloid formation in the containment and rapid clearance of
299 endotoxins and invading microbes.
300
301 This link between host defense and aggregation also suggests that chronic
302 inflammatory states may lead to dysfunctional activation of such host defense
303 pathways. Indeed, amyloidogenic aggregates are implicated in a broad range of
304 protein misfolding diseases such as Parkinson's, Huntington´s and Alzheimer's disease
305 (AD). Moreover, amyloidogenic amyloid-β-peptide variants have been shown to exert
306 antimicrobial activity (27, 28). Furthermore, peptides derived from the disulfide-
307 constrained loop region of human ß-amyloid precursor protein and the N-terminus of
308 human prion protein are antimicrobial (29, 30). Interestingly, neutrophils have
309 recently been implicated in the pathogenesis of amyloid deposits in AD of humans as
310 well as in mouse AD models (31), highlighting the importance of peripheral innate
311 immunity for amyloid formation. Thus, hypothetically, as both thrombin and
312 neutrophils are increased in AD (32, 33), high inflammatory activity could lead to
313 degradation of thrombin and generation of amyloidogenic complexes.
314 In summary, we have revealed here a previously unknown host defense action of
315 proteolysed thrombin and its fragments, based on LPS-induced
316 aggregation/scavenging, clearance, and microbial killing. Our findings add new
317 information to the broad spectra of thrombin functions and provide a novel link
318 among the coagulation system, host defense, and LPS- and bacteria-induced
319 aggregation and amyloid formation.
320
321

Materials and Methods

Ethics statement. The use of human wound materials was approved by the Ethics Committee at Lund University (LU 708-01, LU 509-01). Informed consent was obtained from all of the donors, and the use of human blood was approved by the Ethics Committee at Lund University, Lund, Sweden (permit no. 657-2008).

Biological materials. Fibrin sloughs from chronic non-healing wounds were collected using a sterile spatula and immediately either fixed in the solution for electron microscopy or frozen at -20°C for later extraction for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sterile wound fluids were obtained from surgical drainages after surgery. The collection of samples was performed 24 hours and 24–48 hours after surgery. The wound fluids were centrifuged, aliquoted, and stored at -20°C.

Microorganisms. The *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 bacterial strains were purchased from LGC (U.K.).

Cells. RAW 264.7 cells (American TYPE Culture Collection, USA) were cultured in DMEM (HyClone, GE Healthcare Life Science, USA) supplemented with 10% (v/v) heat-inactivated FBS (FBSi; Invitrogen, USA) and 1% (v/v) antibiotic-antimycotic solution (AA; Invitrogen).

Peptides. The thrombin-derived peptides GKY25 (GKYGFYTHVFRLKKWIQKVIDQFGE) and IVE25

(IVEGSDAEIGMSPWQVMLFRKSPQE) were synthesized by Biopeptide Co, Inc. (USA). We confirmed the purity (over 95%) via mass spectral analysis (MALDI-TOF Voyager, USA).

Digestion of α-thrombin and Western blotting. Digestion of α-thrombin (10 μM; Innovative Research, Inc., USA) was performed for 3 hours at 37°C using 0.8 μg/ml HNE (Calbiochem, USA). The protein and peptides were separated by electrophoresis on a 10–20% Tricine gel (Novex, Life Technologies, USA) and subsequently transferred to a PVDF membrane using the Trans-Blot Turbo (Bio-Rad, USA). Polyclonal rabbit antibodies against the C-terminal thrombin epitope VFR17 (VFRLKKWIQKVIDQFGE; diluted 1:800, Innovagen AB, Sweden), followed by porcine anti-rabbit HRP conjugated antibodies (1:1000, Dako, Denmark), were used to detect the C-terminal epitopes of thrombin. The C-terminal peptides were visualized using Chemiluminescent substrates (Thermo Scientific, USA) using a ChemiDoc MP imaging system (Bio-Rad).

Purification of $rTCP_{96}$. A bacterial expression system consisting of pGEX plasmid in *Escherichia coli* strain *BL21 codon plus (DE3) RIPL* (Invitrogen) was used to produce the $rTCP_{96}$. We cultivated the bacteria in LB broth (Sigma-Aldrich, USA) supplemented with 34 μg/ml chloramphenicol and 100 μg/ml carbomycin. IPTG (400 μM; VWR, USA), added at the mid-log phase, was used to induce peptide production in the bacterial system. The $rTCP_{96}$ peptides were extracted and purified by immobilized metal affinity chromatography (Ni-NTA Agarose, Invitrogen) under denaturing conditions (8 M Urea, 10 mM Tris, pH 7.4), extensively washed with 20 mM of imidazole in 8 M Urea, 10 mM Tris, pH 7.4, and then eluted by stepwise

increasing concentrations of imidazole (200 mM). $rTCP_{96}$ was desalted in 10 mM Tris, pH 7.4 by step-wise dialysis and concentrated using a 3-kDa molecular weight cut-off Amicon ultracentrifugal filter device (Millipore, Germany) and stored at 4°C prior to use (34). Peptide purity was confirmed via Tricine gel electrophoresis followed by Gel Code Blue Safe Protein staining (Thermo Scientific) and Western blotting, as noted above. The protein concentration was determined by Nanodrop (ND 1000, Thermo Scientific).

Heparin-affinity chromatography. We subjected $rTCP_{96}$ and γ-thrombin (10-30 μg) to fast protein liquid chromatography (ÄKTA purifier; GE Healthcare) using a HiTrap 1 ml Heparin HP column (GE Healthcare, Sweden). After injection, the samples were eluted with a linear gradient of 0–1M NaCl in 10 mM Tris, pH 7.4.

Extraction of thrombin fragments from fibrin sloughs and a wound dressing. The fibrin sloughs were freeze-dried using the freeze-drying system Freezone Plus 6 (Labconco, USA). The freeze-dried samples of the fibrin sloughs (≈7 mg) and 1 cm^2 of wound dressing (Allevyn, USA) were incubated with 300 μl of SDS loading buffer and homogenized using a MagNA lyser homogenizer (Roche, USA). The homogenized samples were boiled for 10 min at 95°C. We analyzed the protein/peptide composition of the extracts via electrophoresis and then Western blotting, as noted above.

Viable count assay. To determine the antibacterial activity of $rTCP_{96}$, we used *E. coli* ATCC 25922. The bacteria were grown to mid-logarithmic phase in 10 ml of Todd-Hewitt (TH) medium. The bacteria were centrifuged, washed, and suspended in 10 ml

of 10 mM of Tris buffer, pH 7.4. Next, *E. coli* ATCC 25922 (50 µl, 2×10^6 colony forming units; cfu/ml) were incubated with 3–6 µM of $rTCP_{96}$, GKY25, or buffer control (10 mM Tris buffer, pH 7.4) for 2 hours at 37°C. Dilution series of the incubated samples were plated on TH agar plates, incubated overnight at 37°C, and we calculated the cfu.

Radial diffusion assay. We used *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27852 for the RDA. The bacteria were grown to mid-log phase in 10 ml of TH medium, spun down, washed, and suspended in 10 ml of 10 mM Tris buffer, pH 7.4. This step was followed by the addition of bacteria (4×10^6) to 15 ml of under-lay agarose gel, consisting of 0.03% TH media, 1% (w/v) low-electroendosmosis-type (EEO) agarose (Sigma-Aldrich), and 0.02% (v/v) Tween 20 (Sigma-Aldrich). The underlay was poured into a 144-mm-diameter petri dish. After solidification, 4-mm-diameter wells were punched in the underlay, which were subsequently loaded with 6 µl of 10 µM of GKY25 or $rTCP_{96}$ in 10 mM Tris buffer, pH 7.4. The plates were thereafter incubated for 3 hours at 37°C. Molten over-lay gel (15 ml, 6% TH, and 1% low-EEO agarose in water) was added to the plate. We measured the antimicrobial activity of the peptides by measuring the radius of the clearing zone surrounding the wells after 18–24 hours of incubation at 37°C.

Gel-overlay assay. A gel-overlay assay was performed on duplicate samples that were run on a non-denaturing acid urea (AU-PAGE) gel in 5% acetic acid at 100 V for 75 min using reversed polarity. Bacterial pre-cultures were grown overnight in 10 ml of TH broth, inoculated the subsequent day and grown until their optical density (OD) reached 0.4. The bacteria were washed and the pellet re-suspended in 10 mM

Tris, pH 7.4. The bacteria (4×10^6) were added to 15 ml of melted underlay agarose (10 mM Tris, pH 7.4, 0.03% of TH broth, 1% of agarose type, and 1% low-EEO agarose) and poured into petri dishes. One AU gel was stained with Coomassie brilliant blue and the other was washed three times with 10 mM Tris, pH 7.4 for 4 min and then placed on the top of the underlay gel, followed by incubation for 3 hours at 37°C. Overlay agar (15 ml) was poured over underlay and left to solidify. The inhibition zones were measured using an electronic digital callipers (Perel, Belgium) after 16 hours of incubation at 37°C.

Slot-blot assay. We used a slot-blot assay to detect the interaction between $rTCP_{96}$ and LPS. $rTCP_{96}$ and GKY25 (2 and/or 5 μg per well) were bound to nitrocellulose membrane (Hybond-C, GE Healthcare, Biosciences) after pre-soaking in 10 mM Tris, pH 7.4. The membrane was incubated in the blocking solution (2% BSA in PBS, pH 7.4) for 1 hour at room temperature and subsequently incubated with 20 μg/ml biotinylated LPS (LPS-EB Biotin, InvivoGen, USA) in PBS for 1 hour at room temperature. Next, the membranes were washed three times for 10 min in PBS and incubated with streptavidin-horseradish peroxidase conjugate (Thermo Scientific). Binding was detected using peroxide solution and a luminol/enhancer solution (1:1 v/v) (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). To test for competitive inhibition of peptide binding to LPS, we also performed binding studies in the presence of unlabeled heparin (6 mg/ml).

Fluorescence microscopy. FITC is a fluorescent probe able to permeate the membranes of dead bacteria and bind to bacterial DNA, which is detected as a green fluorescent signal. *E. coli* ATCC 25922 were grown to mid-logarithmic phase in 10 ml

of TH medium. The bacteria were centrifuged, washed, and suspended in 10 ml of 10 mM Tris, pH 7.4. Next, *E. coli* ATCC 25922 (100 µl; 1×10^7 cfu/ml) were incubated with 10 µM of GKY25, $rTCP_{96}$ or control (10mM, Tris, pH 7.4) for 1 hour at 37°C. The microorganisms were incubated on poly (L-lysine)-coated glass slides via incubation for 45 min at 37°C. Next, 200 µl of FITC (6 µg/ml) was added to the top of each slide. The samples were thereafter fixed with 2% of paraformaldehyde for 15 min at 4°C and 45 min at room temperature. The cover slides were mounted on microscope slides with fluorescent mounting media (Daco, USA). A Nikon Eclipse TE300 inverted fluorescent microscope (Nikon, Japan) supplied with a Hamamatsu C4742-95 cooled CCD camera (Hamamatsu, Japan) and a Plan Apochromat x100 objective (Olympus, USA) were employed to acquire images of the FITC-stained bacteria. We used imaging software NIS-elements 3.1 (Nikon) for the image acquisition and processing.

Thioflavin T dye binding assays. Amyloid formation was determined using the dye Thioflavin T (ThT). Thioflavin T preferentially binds to the β-sheet structures of amyloidogenic proteins/peptides. For the assay, $rTCP_{96}$ (10 µM), GKY25 (10 µM), alpha thrombin (10 µM±HNE), γ-thrombin (10 µM), LPS (100 µg/ml), or buffer only (10 mM Tris, pH 7.4) were incubated with LPS for 30 min at 37°C. For examination of time dependence of the aggregation, $rTCP_{96}$ (10 µM) was incubated for 10, 30, 60 and 120 minutes at 37°C in absence or presence of LPS in 10 mM Tris, pH 7.4. For examination of concentration dependence of the aggregation, $rTCP_{96}$ (0.1, 0.5, 1, 2, 5 and 10 µM) was incubated for 30 minutes at 37°C in absence or presence of LPS in 10 mM Tris, pH 7.4. In one experiment, the buffer was supplemented with 1% citrated plasma. For evaluation of effects of GKY25 on the aggregation, GKY25 (1, 5,

472 10 and 20 μM) was added to $rTCP_{96}$ (10 μM) and LPS (100 μg/ml) in 10 mM Tris, pH
473 7.4, followed by a 30 min incubation period. Two hundred μl of the materials were
474 incubated with ThT (final concentration: 10 μM) for 15 min in the dark (ThT stock
475 was 1 mM stored in the dark at 4°C in glycine 0.1 M, pH 8.5). We measured ThT
476 fluorescence using a VICTOR3 Multilabel Plate Counter spectrofluorometer
477 (PerkinElmer, USA) at an excitation of 450 nm, with excitation and emission slit
478 widths of 10 nm. The baseline (10 mM Tris buffer, LPS or HNE) was subtracted from
479 the signal of each sample (18).
480
481 **Circular dichroism.** We performed circular dichroism (CD) measurements on a
482 Jasco J-810 spectropolarimeter (Jasco, USA) equipped with a Jasco CDF-426S Peltier
483 set to 25°C. The peptides/proteins were diluted to 10 μM in Tris buffer (10 mM, pH
484 7.4) and incubated with 10 μM of LPS for 1 hour at 37°C, placed in a 10 mm quartz
485 cuvette and, after extensive purging with nitrogen, scanned over the wavelength
486 interval 200–260 nm (scan speed: 20 nm/min). We calculated the averages of five
487 scans for each sample. For examination of time dependence, $rTCP_{96}$ (10 μM) was
488 incubated for 10 and 120 minutes at 37°C in absence or presence of LPS (100 μg/ml)
489 in 10 mM Tris, pH 7.4. The baseline (10 mM Tris buffer, LPS or HNE) was
490 subtracted from the spectra of each sample.
491
492 **Transmission electron microscopy**. We visualized the aggregates formed by $rTCP_{96}$
493 in plasma alone, or in the presence of LPS or *E. coli*, *of* HNE-treated α-thrombin (10
494 μM) or γ-thrombin alone (10 μM) after incubation with LPS using TEM (Jeol Jem
495 1230; Jeol, Japan) in combination with negative staining. The samples were adsorbed
496 onto carbon-coated grids for 60 s and stained with 7 microliters of 2% uranyl acetate

for 20 s. The grids were rendered hydrophilic via glow discharge at low pressure in air.

Furthermore, we used TEM to study the aggregation of endogenous thrombin fragments in acute wound fluids. Polyclonal rabbit antibodies (against the VFR17 epitope) were labeled with gold particles 20 nm in diameter and subsequently added to acute wound fluids and incubated for 30 min at 37°C. We also performed TEM analysis of TCPs and LPS in fibrin sloughs by incubating the material with gold-labeled VFR17 antibodies followed by incubation with gold-labeled anti-rabbit IgG (10 nm). Electron micrographs of the fibril sloughs were captured using an electron microscope Philips/FEI CM100 (Philips/FEI, USA). The detection of LPS was performed using IgG against LPS with labeled gold particles 5 nm in diameter. Analysis of the distribution and possible colocalization of large and small gold particles, representing TCPs and LPS, respectively, was performed using the plugin "Gold" in the Ellipse program (http://nucleus.img.cas.cz/gold/) (24).

Phagocytosis assay. The macrophage cell line RAW 264.7 (passage 8, 9, and 10), grown in DMEM media with 10% (v/v) of FBSi and 1% (v/v) AA, was seeded in 96-well tissue culture plates ($8x10^4$ cells per well) overnight at 37°C in a 5% CO_2 atmosphere. pHrodo Green *E. coli* BioParticles (Life Technologies, USA) were re-suspended in 2 ml Hanks' Balanced salt solution (Life Technologies, UK) and pre-treated with 1.0 and 0.5 μM of $rTCP_{96}$ in 10 mM of Tris for 1 hour at 37°C. The pre-treated suspension of *E. coli* BioParticles was added to the adherent RAW cells. To analyze phagocytosis, the particles were incubated with the cells for 1.5 hours at 37°C. We then measured fluorescence using a VICTOR3 Multilabel Plate Counter spectrofluorometer (PerkinElmer, USA) at excitation/emission wavelengths of

485/535 nm. The baseline uptake (of only *E. coli* BioParticles) was subtracted from the signal of each sample.

Confocal microscopy. We performed ThT staining to visualize amyloid formation of 10 µM $rTCP_{96}$ and endogenous TCPs in selected acute wound fluids, which were induced by LPS and/or *E. coli* ATCC 25922. The bacterial suspension was prepared as described above for RDA and VCA. The LPS/*E. coli*-treated samples of acute wound fluids/$rTCP_{96}$ were fixed and washed as described above (FITC staining). The samples were subsequently incubated with 200 µl of ThT (25 µM) on slides for 30 min at 37°C. The primary antibodies against the TCP sequence were added to the samples containing wound fluid, followed by the washing step and the addition of secondary antibodies. The incubation parameters were adopted from the Western blotting technique (see above), and the incubation duration of each antibody was 30 min at 37°C. The cover slides (Thermo Scientific, Germany) were mounted on microscope slides with fluorescent mounting media (Molecular probes, Life Technologies, USA).

RAW 264.7 cells (200000 cells/ml) were seeded on round cover glasses and incubated overnight to enable adherence. The next day, the *E. coli* particles were pre-incubated with 1 µM TCP as described in the phagocytosis assay. Fixed cells were permeabilized with 0.5% Triton diluted in PBS for 2 min at RT. The following staining method to visualize TCP was performed as described above. The samples were mounted with ProLong Gold with DAPI (Life Technologies, USA) to stain the nuclei.

We examined the mounted samples using an LSM 700 laser-scanning confocal microscope (Zeiss, Germany) with excitation wavelengths of 488 and 568 nm and a

C-Apochromat 63x/1.20W korr M27 glycerol immersion objective. Images were collected using Zen 2012 software and analyzed using ImageJ software (version 1.49q).

Hemolysis assay. Fresh citrated venous blood from healthy donors (2 females, 1 male) was centrifuged at 250*g*, and the pellet was washed three times with PBS. Next, the pellet was diluted five times, and 5 µl of this solution was transferred to each well of a 96-well plate containing 195 µl of $rTCP_{96}$ (0.1, 1, 2, 5, and 10 µM) in PBS. After 1 hour of incubation at 37°C and 5% CO_2, the plate was centrifuged at 800*g*. Next, 150 µl of each sample was transferred to a flat-bottom 96-well plate and the absorbance at 450 nm was measured. The results are expressed as a percentage of the erythrocyte lysis compared with the positive control (2.5% Tween-20). Values below 10% are regarded as non-hemolytic.

Ellipsometry. Peptide adsorption was studied *in situ* by null ellipsometry (35) using an Optrel Multiskop (Optrel, Germany) equipped with a 100 mW Nd:YAG laser (JDS Uniphase, USA). All of the measurements were carried out at 532 nm and an angle of incidence of 67.66° in a 5 ml cuvette under stirring (300 rpm). Both the principles of null ellipsometry and the procedures used have been previously described in the literature(36). In brief, by monitoring the change in the state of polarization of light reflected at a surface in the absence and presence of an adsorbed layer, the mean refractive index (n) and layer thickness (d) of the adsorbed layer can be obtained. Based on the thickness and the refractive index, we calculated the adsorbed amount (Γ) using the following equation:

$$\Gamma = \frac{(n - n_0)}{dn/dc} d \qquad (35)$$

where dn/dc (0.154 cm^3/g) is the refractive index increment and n_0 is the refractive index of the bulk solution. Corrections were routinely applied to the bulk refractive index to account for changes in temperature and excess electrolyte concentration.

LPS-coated surfaces were obtained by adsorbing *E. coli* LPS to methylated silica surfaces (surface potential -40 mV and contact angle 90° (37)) from 5 mg/ml LPS stock solution in water at a concentration of 0.4 mg/ml in 10 mM Tris, pH 7.4. This LPS concentration corresponds to saturation in the LPS adsorption isotherm (1.48±0.38 mg/m^2) under these conditions. Non-adsorbed LPS was removed by rinsing with Tris buffer at 5 ml/min for a period of 30 min and allowing to buffer to stabilize for 20 min. Peptide addition was subsequently performed to 0.01, 0.1, 0.5, and 1 μM, and adsorption monitored for at least one hour after each addition. All of the measurements were performed in at least triplicate at 25°C.

Molecular dynamics simulations. The crystal structure of human γ-thrombin was retrieved from the Protein Data Bank (PDB) (38). The 11-kDa TCP was modeled using a single chain of the γ-thrombin structure. The extracted chain was placed in a cubic box of TIP3P water molecules at a salt concentration of 0.1 M. An atomistic model of the 11-kDa TCP was derived using the Amber force field ff14SB (39). The 11-kDa TCP was simulated for 100 ns in the NpT ensemble at 300 K and 1 bar to assess the stability of the fold. In parallel, a CG model of TCP was derived from the same starting structure using the MARTINI force field (40). The CG model was incubated with a single lipid A molecule and simulated for 5 x 1 μs to determine the preferential interaction sites of TCP with LPS. Preferential sites comprise of the

596 newly exposed residues 44–62 and 80–96 for the lipid tails of LPS and the residues 1–
597 20 for the polar head of LPS. Next, the TCP molecules were incubated with and
598 without 8 LPS molecules in order to study aggregation behavior. We performed 5 x 1
599 μs of simulations for both the 8 TCP and the 8 TCP with 8 LPS systems. We
600 calculated the pairwise distances between all of the residues on the TCP molecules
601 and summed them over all of the intermolecular interactions. The resulting average
602 distances reveal increased aggregation at sites that had previously been identified to
603 preferentially interact with LPS.
604
605 ***In silico*** **modeling of TCP fold stability and secondary structure.** The region
606 corresponding to amino acids 527-622 (prothrombin numbering) was extracted from
607 the structure of full-length thrombin. It was simulated in explicit TIP3P water
608 molecules at a salt concentration of 0.1 M for 1 microsecond using the Amber force
609 field ff14SB (39), and exhibited a stable fold. After equilibration of the N-and C-
610 terminal segments, the backbone RMSD of the core domain with respect to the X-ray
611 structure of gamma-thrombin 0.25 nm respectively. Mean secondary structural
612 contents during the equilibrated simulation period were calculated using STRIDE
613 (10.1093/nar/gkh429) as implemented in *VMD 1.9.2 (*PMID: 8744570*)*.
614
615 **Microscale thermophoresis.** We performed MST analysis using a NanoTemper
616 Monolith NT.115 apparatus (Nano Temper Technologies, Germany). We used a
617 Monolith NT Protein labeling kit RED – NHS (Nano Temper Technologies) to label
618 10 μM of $rTCP_{96}$ according to the manufacture's protocol. A constant amount of 1.5
619 μM of $rTCP_{96}$ was mixed with increasing concentrations of LPS in Tris buffer (10
620 mM, pH 7.4). Next, 10 μl of the samples was loaded into standard glass capillaries

(Monolith NT Capillaries, Nano Temper Technologies), and we performed the MST analysis (settings for the light-emitting diode and infrared laser were 80%). Labeled IVE25 was added to LPS under the same conditions as above and used as negative control.

Dynamic light scattering. We performed dynamic light scattering (DLS) measurements to measure the hydrodynamic radii of $rTCP_{96}$, LPS and LPS-$rTCP_{96}$. Wyatt QELS (Quasi Elastic Light Scattering, Wyatt Technology Corporation, USA) and Dawn EOS (Enhance optical system, Wyatt Technology Corporation) equipped with a temperature-controlled microsampler instrument were used for DLS measurements. We incubated the samples for 1 hour at 37°C under reducing conditions and the scattered light was detected at 18 different angels simultaneously. Before the experiment, all samples were filtered through 0.22 μm pore sized micro-filters (Sartorius, Germany). Aliquots of samples were manually loaded into the flow cell and measured at 37°C. All samples (1 μM of $rTCP_{96}$, LPS, LPS-$rTCP_{96}$, respectively) were measured at least 15 times. GKY25 peptide was used as a negative control and analyzed under the same conditions. For evaluation of time dependence of the aggregation, $rTCP_{96}$ (1 μM) was incubated for 10, 30, 60 and 120 minutes at 37°C in absence or presence of LPS (10 μg/ml) in 10 mM Tris, pH 7.4. The hydrodynamic radii were analyzed by Astra V software using Zimm modeling.

Statistical analysis. The diagrams of RDA, VCA, ThT, DLS and hemolysis assay are presented as means and standard deviations from at least three independent experiments. We assessed differences in these assays using one-way ANOVA with Dunnett's multiple comparison tests and two-way ANOVA with Sidak's multiple

comparison tests. All of the data were analyzed using Graphpad Prism (GraphPad Software, Inc., USA). A p value less than 0.05 was considered to be statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

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References

1. Tennessen JA (2005) Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection. *Journal of Evolutionary Biology* 18(6):1387-1394.
2. Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 75(1):39-48.
3. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415(6870):389-395.
4. Huttner KM & Bevins CL (1999) Antimicrobial peptides as mediators of epithelial host defense. *Pediatric Research* 45(6):785-794.
5. Kalle M*, et al.* (2013) Proteolytic activation transforms heparin cofactor II into a host defense molecule. *J Immunol* 190(12):6303-6310.
6. Papareddy P*, et al.* (2014) Antimicrobial effects of helix D-derived peptides of human antithrombin III. *J Biol Chem* 289(43):29790-29800.
7. Bode W (2006) The structure of thrombin: a janus-headed proteinase. *Semin Thromb Hemost* 32 Suppl 1:16-31.
8. Davie EW & Kulman JD (2006) An overview of the structure and function of thrombin. *Semin Thromb Hemost* 32 Suppl 1:3-15.
9. Brower MS, Walz DA, Garry KE, & Fenton JW, 2nd (1987) Human neutrophil elastase alters human alpha-thrombin function: limited proteolysis near the gamma-cleavage site results in decreased fibrinogen clotting and platelet-stimulatory activity. *Blood* 69(3):813-819.
10. Papareddy P*, et al.* (2010) Proteolysis of human thrombin generates novel host defense peptides. *PLoS Pathog* 6(4):e1000857.
11. Angus DC & van der Poll T (2013) Severe sepsis and septic shock. *N Engl J Med* 369(21):2063.
12. Kalle M*, et al.* (2012) Host defense peptides of thrombin modulate inflammation and coagulation in endotoxin-mediated shock and Pseudomonas aeruginosa sepsis. *PLoS One* 7(12):e51313.
13. Papareddy P*, et al.* (2010) C-terminal peptides of tissue factor pathway inhibitor are novel host defense molecules. *J Biol Chem* 285(36):28387-28398.
14. van der Plas MJ*, et al.* (2016) Pseudomonas aeruginosa elastase cleaves a C-terminal peptide from human thrombin that inhibits host inflammatory responses. *Nat Commun* 7:11567.
15. Hansen FC*, et al.* (2015) The Thrombin-Derived Host Defense Peptide GKY25 Inhibits Endotoxin-Induced Responses through Interactions with Lipopolysaccharide and Macrophages/Monocytes. *J Immunol* 194(11):5397-5406.
16. Carter WJ, Cama E, & Huntington JA (2005) Crystal structure of thrombin bound to heparin. *J Biol Chem* 280(4):2745-2749.
17. Singh S, Kalle M, Papareddy P, Schmidtchen A, & Malmsten M (2013) Lipopolysaccharide interactions of C-terminal peptides from human thrombin. *Biomacromolecules* 14(5):1482-1492.
18. Petrlova J*, et al.* (2014) Conformational and aggregation properties of the 1-93 fragment of apolipoprotein A-I. *Protein Sci* 23(11):1559-1571.
19. Biancalana M & Koide S (2010) Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim Biophys Acta* 1804(7):1405-1412.
20. Fenton JW, Landis BH, Walz DA, & Finlayson JS (1977) *Human thrombin* (Ann Arbor, Ann Arbor Science Publishers).

21. Liu CY, Nossel HL, & Kaplan KL (1979) The binding of thrombin by fibrin. *J Biol Chem* 254(20):10421-10425.
22. Lundqvist K, Herwald H, Sonesson A, & Schmidtchen A (2004) Heparin binding protein is increased in chronic leg ulcer fluid and released from granulocytes by secreted products of Pseudomonas aeruginosa. *Thromb Haemost* 92(2):281-287.
23. Schmidtchen A (2000) Degradation of antiproteinases, complement and fibronectin in chronic leg ulcers. *Acta Derm Venereol* 80(3):179-184.
24. Philimonenko AA, Janacek J, & Hozak P (2000) Statistical evaluation of colocalization patterns in immunogold labeling experiments. *J Struct Biol* 132(3):201-210.
25. Landreh M, Johansson J, & Jornvall H (2014) Separate molecular determinants in amyloidogenic and antimicrobial peptides. *J Mol Biol* 426(11):2159-2166.
26. Scott MG, Davidson DJ, Gold MR, Bowdish D, & Hancock REW (2002) The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *Journal of Immunology* 169(7):3883-3891.
27. Soscia SJ, *et al.* (2010) The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS One* 5(3):e9505.
28. Spitzer P, *et al.* (2016) Amyloidogenic amyloid-beta-peptide variants induce microbial agglutination and exert antimicrobial activity. *Sci Rep* 6:32228.
29. Papareddy P, Morgelin M, Walse B, Schmidtchen A, & Malmsten M (2012) Antimicrobial activity of peptides derived from human ss-amyloid precursor protein. *J Pept Sci* 18(3):183-191.
30. Pasupuleti M, *et al.* (2009) Antimicrobial activity of human prion protein is mediated by its N-terminal region. *PLoS One* 4(10):e7358.
31. Zenaro E, *et al.* (2015) Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin. *Nat Med* 21(8):880-886.
32. Deng X, *et al.* (2014) Lipolysaccharide-Induced Neuroinflammation Is Associated with Alzheimer-Like Amyloidogenic Axonal Pathology and Dendritic Degeneration in Rats. *Adv Alzheimer Dis* 3(2):78-93.
33. Grammas P & Martinez JM (2014) Targeting thrombin: an inflammatory neurotoxin in Alzheimer's disease. *J Alzheimers Dis* 42 Suppl 4:S537-544.
34. Petrlova J, *et al.* (2011) A differential association of Apolipoprotein E isoforms with the amyloid-beta oligomer in solution. *Proteins* 79(2):402-416.
35. Azzam RMA & Bashara NM (1977) *Ellipsometry and polarized light* (North-Holland Pub. Co. ;sole distributors for the U.S.A. and Canada, Elsevier North-Holland, Amsterdam ; New York New York) pp xvii, 529 p.
36. Malmsten M (1994) Ellipsometry Studies of Protein Layers Adsorbed at Hydrophobic Surfaces. *J Colloid Interf Sci* 166(2):333-342.
37. Malmsten M, Burns N, & Veide A (1998) Electrostatic and hydrophobic effects of oligopeptide insertions on protein adsorption. *J Colloid Interf Sci* 204(1):104-111.
38. Rydel TJ, *et al.* (1994) Crystallographic structure of human gamma-thrombin. *J Biol Chem* 269(35):22000-22006.

39. Maier JA, *et al.* (2015) ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J Chem Theory Comput* 11(8):3696-3713.
40. Monticelli L, *et al.* (2008) The MARTINI coarse-grained force field: Extension to proteins. *Journal of Chemical Theory and Computation* 4(5):819-834.

Author Contributions: J.P. and A.S. conceived the project and designed the experiments. J.P. performed the experiments, including the peptide production, sample preparation, anti-microbial assays, and fluorescence microscopy. J.P. and F.H. performed the confocal microscopy and immunoblotting. J.P. and M.vd.P. carried out the hemolysis assay; J.P. and M.Mö performed the TEM. P.B. and R.H. contributed the *in silico* modeling data, and M.Ma performed the ellipsometry experiments. J.P. and A.S. wrote the manuscript. All of the authors discussed the results and commented on the final manuscript.

Author Information: Dr. Schmidtchen is a founder of in2cure AB, a company developing therapies based on thrombin-derived host defense peptides. The peptide GKY25 and variants are patent protected. The other authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.P. (jitka.petrlova@med.lu.se).

Figure Legends

Figure 1. Analysis of TCP-LPS interactions and LPS-mediated aggregation. (A) Ellipsometry analysis revealed adsorption of 1 μM of $rTCP_{96}$, GKY25, and IVE25 at LPS-coated surfaces from 10 mM Tris, pH 7.4 (transformation to mg peptide bound per mg LPS was achieved by dividing by the amount of LPS pre-adsorbed: 1.48±0.38 mg/m^2). (B) We used an MST assay to quantify the interaction of $rTCP_{96}$ with LPS. The peptide IVE25 was used as negative control. The mean values of four measurements ± their standard deviations are shown. (C) Changes in the secondary structure of $rTCP_{96}$ triggered by LPS binding were analyzed by CD spectroscopy. The data showed an increase in the β-sheet structure in $rTCP_{96}$ (10 μM) and an increase in the α-helical content in GKY25 (10 μM) after 30 min of incubation with LPS (100 μg/ml) at 37°C. (D) ThT assay, identifying a significant increase in amyloid formation in $rTCP_{96}$ (at 10 μM) after the addition of 100 μg/ml of LPS (n=3). (E) Negative-stain transmission electron microscopy revealed the formation of aggregates after incubation of $rTCP_{96}$ with LPS in 10 mM of Tris, pH 7.4 and supplemented with citrated plasma (CP). The insets show the same samples without LPS treatment using the same magnification. (F and G), Structural changes in digested α-thrombin (α-T, 10 μM) by HNE and γ-thrombin (γ-T) were recorded via (F) ThT assay and (G) CD spectroscopy. Thrombin was used as a control. (H and I), TEM analysis of aggregates (1–2 μm in length) present in digested α-thrombin and γ-thrombin (γ-T, 0.1–0.5 μm in length) after incubation with LPS. Only small amounts of aggregates, or none at all, were detected in α-thrombin (α-T) alone, or after addition of LPS, respectively.

Figure 2. *In silico* studies. (A) Structure of the 96aa TCP after 100 ns of atomic-resolution molecular dynamics simulations. The hydrophobic residues are shown in gray. The formation of a new hydrophobic cluster (yellow, labeled residues) of the

free TCP between the helix segment 80–96 (red) and the sheet residues 46–60 (brown) rely on a twist within the sheet. The extended tail residues formed by residues 46–60 and 80–96 preferentially interact with the lipid tails; the flat patch comprised of residues 1–20 (blue) of the 96aa TCP preferentially interacts with the polar head of LPS. (B) Snapshot from a TCP aggregation simulation showing a representative aggregated state that is mediated by LPS molecules (purple, thick). The TCP backbones are shown in various colors (thin lines). It is apparent that LPS intercalates within the TCP clusters and predominantly connects the preferential interaction sites. (C) Aggregation studies of TCP in the presence (+LPS) and absence (-LPS) of LPS. The panels indicate intermolecular distances of all 8 TCP molecules in the simulation box. Bright areas indicate contact with larger bright segments signifying larger contact areas between molecules. Dark areas indicate that the respective molecules do not interact. Increased aggregation is apparent through the appearance of additional bright areas in the system containing LPS.

Figure 3. Co-aggregation of *E. coli and* TCPs. (A) Confocal microscopy and ThT staining were used to detect *E. coli*-induced amyloid formation of $rTCP_{96}$ (10 μM) in 10 mM Tris buffer, pH 7.4. (B) TEM with negative stain analysis reveals the aggregation of $rTCP_{96}$-treated bacteria. (C) Fluorescence microscopy shows the agglutination of permeabilized $rTCP_{96}$-treated bacteria after the addition of FITC dye. The inset shows GKY25-treated *E. coli*. (D) A significant increase in the number of bacteria per aggregates was detected after treatment with 10 μM of $rTCP_{96}$ compared with GKY25 (n=3). (E) Phagocytosis assay using the macrophage cell line RAW 264.7 revealed a significant increase in the phagocytosis of *E. coli* particles pre-treated with $rTCP_{96}$ (0.5 μM; n=4). (F) Confocal microscopy analysis of phagocytized

E. coli particles (green) pre-treated with $rTCP_{96}$ (Alexa 568, red); we used RAW 264.7 cells (DAPI, blue).

Figure 4. C-terminal fragments of thrombin are found in human acute wound fluids, fibrin sloughs, and wound dressings. (A) Western blotting yielded 11-kDa TCPs in all wound fluid (WF) samples (arrow): 1) $rTCP_{96}$ (10 µM, 1 µl), 2) $rTCP_{96}$ in plasma (10 µM, 1 µl), 3–7) WF from patients no. 1–5 (adjusted to same amount of proteins). (B) *Ex vivo,* aggregates were detected by TEM and demonstrated aggregated endogenous TCPs peptides by using gold labeled anti-VFR17 epitope IgG (blue dots) in WF after incubation with LPS (left; 100 µg/ml) or without (right), the insets display a 50x lower magnified overview of the same samples. (C) Formation of aggregates in WF triggered by *E. coli* bacteria. The inset shows WF only, using the same magnification. (D and E) Confocal microscopy, using primary antibodies against TCPs followed by Alexa 568 labeled secondary antibodies and ThT staining, were used to determine amyloid aggregates containing TCPs in the WF in the presence of LPS (D) or *E. coli* (E). (F) Western blotting of extracts from fibrin slough (FS) and wound dressing revealed the presence of 11-kDa TCP fragments in both samples (arrow): 1) $rTCP_{96}$, 2) $rTCP_{96}$ in citrate plasma, 3) extract of FS, and 4) extract of wound dressing. (G) Colocalization of endogenous TCPs and LPS in the FS from two patients was detected via TEM analysis. The TCPs were recognized by anti-rabbit IgG with labeled gold particles 10 nm in diameter (blue dots). We detected LPS using IgG against LPS with labeled gold particles 5 nm in diameter (red dots). Colocalization exceeding 90% was observed in both samples. The insets show the same samples using a 4-fold higher magnification.

Figure 5. The multiple effects of thrombin and its fragments. Injury and infection activate the coagulation cascade resulting in Factor X (FX)-mediated cleavage of prothrombin. Thrombin is further cleaved by human neutrophil elastase (HNE), generating TCPs, or autoproteolysed (Trb) (38). Further proteolysis by endogenous HNE, or bacterial elastases such as lasB from *Pseudomonas aeruginosa* (PAE) gives rise to short TCPs. The red colored frame indicates the new findings presented in this work.

Supporting Information

Figure S1. Characteristics of rTCP$_{96}$. (A) Western blot analysis illustrating α-thrombin (α-T, 10 μM), α-thrombin digested for 3 h by HNE (α-T+HNE, 0.8 μg/ml), GKY25 (10 μM), and rTCP$_{96}$ (10 μM). The arrow indicates 11-kDa TCPs. This fragment has been previously characterized and is shown here for comparison and clarity (10). (B) The production and purity of recombinant thrombin C-terminal peptide (rTCP$_{96}$) was analyzed via SDS-PAGE and Western blotting. (C) Comparison of heparin binding of rTCP$_{96}$ and γ-thrombin using heparin-affinity fast protein liquid chromatography. Gel-based overlay assay and RDA (D - F) revealed inhibition zones using 10 μM of both GKY25 and rTCP$_{96}$ in *E. coli* and GKY25 in *E. coli* and *P. aeruginosa* (n=3). (G-H) A viable count assay demonstrated the antimicrobial activity of 3 μM rTCP$_{96}$, 3 μM of GKY25 (a positive control) and a concentration dependent manner from 0.1 to 3 μM rTCP$_{96}$ (n=4). (I) rTCP$_{96}$ (up to 10 μM) did not demonstrate any hemolytic effects. (J) Kinetic analyses of rTCP$_{96}$, GKY25, and IVE25 binding to surface-localized LPS are presented. (K) A slot-blot assay demonstrated binding of

895 $rTCP_{96}$ (2 and 5 µg) to biotin-labeled LPS. The binding was blocked after the addition
896 of heparin.
897
898 **Figure S2. Analyses of aggregation and colocalisation of TCPs.** (A) Effects of
899 GKY25 at the indicated doses on LPS-induced $rTCP_{96}$ aggregation (100 µg/ml of
900 LPS). The dotted line indicates the fluorescence of pure $rTCP_{96}$ (10 µM, n=4). (B)
901 Dose dependence of $rTCP_{96}$ aggregation, which was triggered by 100 µg/ml of LPS, is
902 demonstrated in presence of 10 mM Tris, pH 7.4 (n=4, left panel), or 10 mM Tris, pH
903 7.4 supplemented with 1% citrated human plasma (n=4, right panel). (C) Dynamic
904 light scattering (DLS) measurements were performed to determine the hydrodynamic
905 radii of 1 µM of $rTCP_{96}$, 10 µg/ml of LPS, and LPS-treated $rTCP_{96}$ (n=5). GKY25
906 was used as a negative control. (D) The far-UV CD spectra of $rTCP_{96}$ (10 µM) in the
907 presence and absence of LPS (100 µg/ml) in Tris buffer was recorded after incubation
908 for 10 and 120 min. ThT assay (E) and DLS analyses (F) were performed of $rTCP_{96}$
909 (10 µM) with or without LPS (100 µg/ml) addition after the indicated incubation
910 times (n=4). (G) The representative image of Western blot shows 1-4) the calibration
911 curve (0.1, 0.5, 1 and 2 µM of $rTCP_{96}$) and 5-6) the wound fluids of the same volumes
912 as the standards (n=4). (H) Analysis of clustering and colocalization patterns of TCPs
913 and LPS. The histogram depicts PCCF function values for intervals of distances. One
914 microscopic image from each sample was analyzed. A PCCF value above 1
915 corresponds to non-random mutual distribution-colocalization of two types of
916 particles (24).
917
918 **Figure S3. Computational prediction of co-aggregation of TCP and LPS.**
919 Interactions of eight 96aa TCP molecules and LPS from five random starting

orientations were predicted.

Figure S4. Control microscopy analysis. Control confocal microscopy analysis of phagocytized *E. coli* particles (green) and $rTCP_{96}$ (Alexa 568, red) incubated with RAW 264.7 cells (DAPI, blue).

Movie S1. TCP aggregation simulations in the presence of LPS. Simulations (1 μs) of eight 96aa TCP molecules incubated with LPS in a cubic box in one random starting orientation. The movie visualizes the observed trajectories in sequence. Lipopolysaccharide intercalates in the spaces between the TCP molecules (purple, thick), thereby bridging the gap between individual TCPs (backbones shown in various colors, thin lines.

Table S1

Secondary structure of TCP. Secondary structure of the TCP region (amino acids 527-622, prothrombin labeling) was deduced from the crystal structure of α- and γ-thrombin, computed from the CD spectra of $rTCP_{96}$, using k2D3 (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/), or derived from *in silico* modeling data (see Methods).