The role of apolipoprotein CI in lipid metabolism and bacterial sepsis

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Chapter 6

Apolipoprotein CI Modulates the Biological Response to Lipopolysaccharide: Analysis of the Structure and Function Relationship

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Abstract

Objective: Timely sensing of lipopolysaccharide (LPS) is critical for the host to fight invading Gram-negative bacteria by effectuating an efficient anti-bacterial attack. We recently found that full-length apoCI ($apoCI_{1-57}$) avidly binds to LPS, increases the serum residence of LPS, and enhances the LPS-induced proinflammatory response, all of which involve an LPS-binding motif in the C-terminus of apoCI (*i.e.* $apoCI_{48-54}$). The aim of this study was to further elucidate the structure-function relationship of apoCI with respect to these LPS-modulating effects.

Methods and Results: Since apoCI contains a number of alternating cationic/hydrophobic sequences throughout its structure that may be involved in binding LPS, we designed both N- and C-terminal apoCI-derived peptides containing varying numbers of such sequences. $ApoCI_{1-38}$, $apoCI_{1-30}$, and $apoCI_{35-57}$ were able to bind LPS, as evident from a ^{125}I-LPS mobility shift assay, indicating that apoCI indeed contains additional LPS-binding sites to the previously elucidated LPS-binding motif ($apoCI_{48-54}$). $ApoCI_{1-23}$ and $apoCI_{46-57}$ showed no LPS-binding capacity. The LPS-binding characteristics of the peptides were reflected by their effects on the kinetics of ^{125}I-LPS on intravenous injection in C57Bl/6 mice. $ApoCI_{1-38}$, $apoCI_{1-30}$, and $apoCI_{35-57}$ reduced the association of ^{125}I-LPS with the liver and prolonged its serum residence. Finally, both $apoCI_{1-30}$ and $apoCI_{35-57}$ enhanced the LPS-induced TNFα response *in vitro* (RAW 264.7 macrophages) and *in vivo* (C57Bl/6 mice).

Conclusions: In conclusion, we demonstrate that both the N- and C-terminal helix of apoCI contain structural elements to bind LPS, to affect the *in vivo* kinetics of LPS, and to enhance the proinflammatory response towards LPS. We anticipate that, besides the LPS-binding motif, highly conserved alternating cationic/hydrophobic sequences present throughout apoCI participate in the binding to LPS and modulation of the *in vivo* fate of LPS.

Introduction

Lipopolysaccharide (LPS) is the major constituent of the outer membrane of Gram-negative bacteria, and elicits an inflammatory response in monocytes/macrophages and endothelial cells via activation of the MD2-Toll-like receptor 4 (TLR4)-complex[1-6]. LPS molecules can be released from the outer membrane of Gram-negative bacteria upon death or rapid growth, and signal the presence of these bacteria in the circulation. Timely sensing of LPS by inflammatory cells to stimulate the inflammatory response towards LPS and bacteria is crucial for activation of the host defense system to fight Gram-negative infection[7]. If the

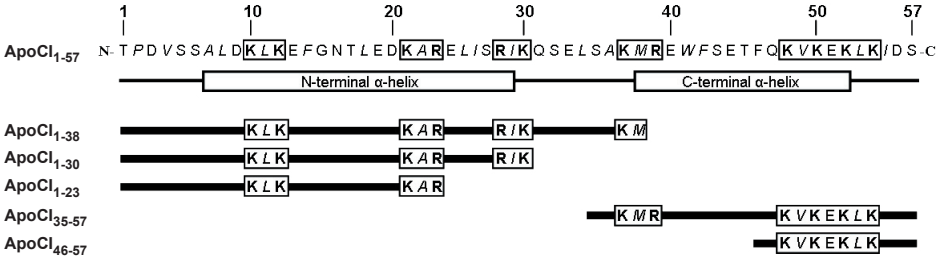

Figure 1. Sequence alignment of apoCI$_{1-57}$ and apoCI-derived peptides. Boldface residues represent basic amino acids and italic residues represent hydrophobic residues. The previously identified LPS-binding motif (residues 48-54) is boxed, as well as the alternating cationic/hydrophobic sequences (residues 10-12, 21-23, 28-30, and 37-39).

initial anti-bacterial inflammatory response is inadequate or too late, uncontrolled bacterial growth will occur with excessive release of LPS into the circulation. This will lead to an exaggerated systemic host response with respect to excessive production of inflammatory mediators, which can finally resulting in the serious and life-threatening symptoms of septic shock[8,9].

Apolipoprotein CI (apoCI) is the smallest identified plasma apolipoprotein (57 amino acids), and is unusually rich in lysine residues (9 residues; ~16 mol%). ApoCI circulates at relatively high concentrations of about 6 mg/dL as a constituent of primarily chylomicrons, very-low-density lipoproteins (VLDL), and high-density lipoproteins (HDL). ApoCI has classically been recognized as an inhibiting factor for lipoprotein clearance by affecting the lipoprotein lipase (LPL)-mediated processing[10,11], and subsequent receptor-mediated uptake of lipoproteins by the liver[12-15]. ApoCI contains two amphipathic α-helices (apoCI$_{7-29}$ and apoCI$_{38-52}$) that are separated by a flexible linker (apoCI$_{30-37}$), with no substantial intramolecular interactions (Fig. 1). By virtue of these properties, apoCI has a boomerang shape similar to the LPS-binding protein bactericidal/permeability increasing protein (BPI)[16,17].

We recently reported that apoCI protects mice against fatal Gram-negative infection[18]. We showed that apoCI strongly binds to LPS, thereby prolonging the residence time of LPS in the circulation. In addition, apoCI stimulated the LPS-induced production of TNFα by macrophages *in vitro* and in mice *in vivo*. By enhancing the biological response towards LPS and Gram-negative bacteria (*e.g.* production of TNFα, macrophage inhibitory factor (MIF), and E-selectin), apoCI improved the anti-bacterial attack, reduced bacterial outgrowth, and protected against intrapulmonal *Klebsiella pneumoniae*-induced fatal sepsis[18]. We previously noted that the lysine-rich *KVKEKLK* sequence (apoCI$_{48-54}$) was highly homologous to the sequences in the proposed LPS-binding regions of the LPS-binding proteins *Limulus* anti-LPS factor (LALF) (LALF$_{43-49}$; *KWKYKGK*)[19]

and cationic antimicrobial peptide-18 (CAP-18) (CAP-$18_{117-123}$; *KIKEKLK*)[20] (Table I). In fact, we have demonstrated that replacement of the lysine residues in this sequence by alanine residues (*i.e. AVAEALA*), which neutralizes positive charges without changing the protein structure, deteriorated the binding to LPS and decreased the ability of apoCI to stimulate the LPS-induced TNFα response[18]. However, in addition to the apparent LPS-binding motif *KVKEKLK*, apoCI contains

Table I: Comparison of the amino acid sequence of apoCI with proposed LPS/receptor-binding regions of several LPS-binding proteins (modified from De Haas *et al.*[30]).

LPS-binding proteins (aa)	Amino acid sequence[a]	Reference
BPI (85-100)	N *I* **K** *I* S *G* **K** *W* **K** *A* Q **K** **R** *F* *L* **K** *M*	**Little *et al.*[33]**
LBP (84-99)	S *I* **R** V Q *G* **R** *W* **K** V **R** **K** S *F* *F* **K** *L*	**Taylor *et al.*[21]**
Lf (28-34)	**R** **K** V **R** *G* *P* *P*	**Elass-Rochard[22]**
ApoE (133-149)[b]	*L* **R** V **R** *L* A S H *L* **R** **K** *L* **R** **K** **R** *L* *L*	**Lynch *et al.*[23]**
MD2 (119-132)	*F* S *F* **K** G *I* **K** *F* S **K** G **K** Y **K**	**Mancek *et al.*[6]**
CAP-18 (106-137)	*G* *L* **R** **K** **R** *L* **R** **K** *F* **R** N **K** *I* **K** E **K** *L* **K** **K** *I* *G* Q **K** *I* Q *G* *L* *L* *P* **K** *L* *A*	**Larrick *et al.*[20]**
LALF (32-50)	H Y **R** *I* **K** *P* T *F* **R** **R** *L* **K** *W* **K** *Y* **K** *G* **K** *F*	**Hoes *et al.*[19]**
ApoCI (1-30)	T *P* D V S S *A* *L* D **K** *L* **K** E *F* *G* N T *L* E D **K** *A* **R** E *L* *I* S **R** *I* **K**	
ApoCI (31-57)	Q S E *L* S *A* **K** *M* **R** E *W* *F* S E T *F* Q **K** *V* **K** E **K** *L* **K** *I* D	

[a] Boldface residues represent basic amino acids; italic residues represent hydrophobic amino acids; the underlined amino acids represent a comparable amino acid region.
[b] Receptor-binding domain not yet described as LPS-binding domain
Abbreviations used: aa, amino acid; apoE/CI, apolipoprotein E/CI; BPI, bactericidal/permeability increasing peptide; CAP-18, cationic antimicrobial protein-18; LALF, *Limulus* anti-LPS factor; LBP, LPS-binding protein; Lf, lactoferrin.

a number of alternating cationic/hydrophobic sequences throughout its structure. These sequences are also present in the proposed LPS-binding regions of other LPS-binding proteins, such as BPI, LPS-binding protein (LBP)[21], lactoferrin (Lf)[22], apoE[23], and MD2[6] (Table I). Similar to the previously identified LPS-binding motif of apoCI, these alternating cationic/hydrophobic sequences within apoCI are highly conserved during evolution[24].

In this study we aimed at analyzing the structure and function relationship of apoCI with respect to deaggregate LPS, prolong the serum residence time of LPS, and to stimulate the inflammatory effects of LPS. Hereto, we used an array of N- and C-terminal apoCI-derived peptides containing the apparent LPS-binding motif and/or varying amounts of highly conserved alternating cationic/hydrophobic sequences. We demonstrate that peptides containing either the full N-terminal helix or the full C-terminal helix are able to deaggregate LPS and enhance the inflammatory response towards LPS, although with somewhat reduced efficiency as compared to full-length apoCI. We thus conclude that in addition to the previously identified LPS-binding motif (apoCI_{48-54}) additional

structural elements present throughout the apoCI sequence cooperate in the binding of LPS and modulation of the *in vivo* fate of LPS.

Materials and Methods

Animals − Male C57Bl/6 mice from our own breeding were housed at the breeding facility of TNO-Quality of Life in a temperature- and humidity-controlled environment and were fed *ad libitum* with regular chow (Ssniff, Soest, Germany). All experiments were approved by the animal ethics committee of TNO. Experiments were conducted at 10-12 weeks of age.

Synthesis of ApoCI-derived Peptides − The synthesis of human apoCI-derived peptides was carried out by the Peptide Synthesis Facility of the Department of Immunohematology and Blood Transfusion at the Leiden University Medical Center (Leiden, The Netherlands) by solid phase peptide synthesis on a TentageIS-AC (Rap, Tübingen, Germany) using 9-fluorenylmethoxycarbonyl/t-Bu chemistry, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate/*N*-methylmorpholine for activation and 20% piperidine in *N*-methylpyrrolidone for fluorenylmethoxycarbonyl removal[25]. The peptides were cleaved from the resin, deprotected with trifluoroacetic acid/water, and purified on Vydac C18. The purified peptides were analyzed by RP-HPLC and the molecular masses were confirmed by MALDI-TOF mass spectrometry (purity > 95%). Synthesized full-length human apoCI (apoCI$_{1\text{-}57}$; purity > 95%) was obtained from Protein Chemistry Technology Center (UT Southwestern Medical Center, Dallas, TX).

Radiolabeling of LPS − *Salmonella Minnesota* Re595 LPS (Sigma-Aldrich co., St. Louis, EN) was radiolabeled as described for LPS by Ulevitch[26]. Briefly, 1 mg of LPS was incubated with 4.6 mg methyl 4-hydroxybenzimidate hydrochloride (Fluka Chemie, Buchs, Switzerland) in 1 mL of 50 mM borate buffer (pH 8.5) for 18 h. After dialysis against PBS, 0.5 mL of the derivatized product was radiolabeled using 10 µL of 4 mg/mL chloramine T (Merck, Darmstadt, Germany) and 10 µL (0.25 mCi) Na^{125}I (Amersham, Little Chalfont, UK). The reaction was stopped with 10 µL of 4 mg/mL NaS_2O_4 and the radioiodinated product was dialyzed extensively against PBS (pH 7.4). Prior to experiments the ^{125}I-LPS was sonicated 3 times for 30 sec, with 1 min intervals on ice in between, using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK) at 10 µm output. The quality of the ^{125}I-LPS was routinely checked by agarose gel electrophoresis and *in vivo*. The specific activity of ^{125}I-LPS was 1.0x10^3 cpm/ng.

Agarose Gel Electrophoresis − ^{125}I-LPS (150 ng) was incubated (30 min 37°C) in the absence or presence of apoCI-derived peptides or apoCI$_{1-57}$ at the indicated molar ratios. Aliquots of incubation mixtures were subjected to electrophoresis in 0.75% (w/v) agarose gels at pH 8.8 using 70 mM Tris-HCl, 80 mM hippuric acid, 0.6 mM EDTA, 260 mM NaOH buffer. Bromophenol blue (Merck) served as a front marker. For detection of ^{125}I-LPS, resulting gels were dried overnight at 65°C, exposed to a phosphorimaging plate (BAS-MS2040; Fuji Photo Film, Co. Ltd, Tokyo, Japan), and radioactivity was detected on a phosphor imaging analyzer (Fujix BAS-1000; Fuji Photo Film Co. Ltd.).

Kinetic Studies *in Vivo* − Mice were anesthetized by intraperitoneal injection of domitor (0.5 mg/kg; Pfizer, New York, NY), dormicum (5 mg/kg; Roche Netherlands, Mijdrecht, The Netherlands), and fentanyl (0.05 mg/kg; Janssen-Cilag B.V., Tilburg, The Netherlands) and the abdomens were opened. ^{125}I-LPS (10 µg/kg), preincubated (30 min 37°C) with or without apoCI-derived peptides, apoCI$_{1-57}$, or bovine serum albumin (BSA) at the indicated molar ratios, were injected via the vena cava inferior. Thirty minutes after injection, the serum residence of ^{125}I-LPS and the association of ^{125}I-LPS with the liver were determined. Blood samples (<50 µL) were taken from the vena cava inferior and allowed to clot for 30 min. The samples were centrifuged for 10 min at 7,000 rpm, and 20 µL serum samples were counted for ^{125}I-radioactivity. The total amount of radioactivity in the serum was calculated using the equation: serum volume (mL) = 0.04706 x body weight (g)[27]. At the same time, liver lobules were tied off, excised, weighed, and counted for radioactivity. Mice were killed and the remainder of the liver was excised and weighed. Uptake of ^{125}I-LPS by the liver was corrected for the radioactivity in the serum assumed to be present in the liver (84.7 µL serum per g wet weight)[28].

Challenging of RAW 264.7 Cells with LPS − RAW 264.7 cells, a murine macrophage cell line, were seeded into 24-well plates (1x10^6 cells/well) and cultured overnight at 37°C in DMEM with 10% FBS. Cells were washed with DMEM and incubated with LPS (1 ng/mL) preincubated (30 min 37°C) with or without apoCI-derived peptides or apoCI$_{1-57}$ at the indicated molar ratios in DMEM supplemented with 0.01% human serum albumin (4 h at 37°C). Incubation of cells with apoCI-derived peptides or apoCI alone was used as a control, in which the concentration of peptide was similar as the highest concentration used in combination with LPS in this assay. The medium was collected and TNFα was determined in the medium using the commercially available mouse TNFα-specific OptEIA™ ELISA (BD Biosciences Pharmingen) according to manufacturer's instructions.

Challenging of Mice with LPS − C57Bl/6 mice were injected intravenously with LPS (25 µg/kg) preincubated for 30 min at 37°C without or with apoCI-derived peptides or $apoCI_{1-57}$, in a 60-fold and 5-fold molar excess of peptide respectively, in the presence of 0.1% BSA (w/v). One minute after injection (t_0) and at the indicated times, blood samples were taken from the tail vein and put on ice. The samples were centrifuged for 4 min at 14,000 rpm, and TNFα levels were determined in the plasma samples using the commercially available mouse TNFα module set BMS607MST (Bender MedSystems, San Bruno, CA).

Statistical Analysis − Data were analyzed using non-parametric Mann-Whitney *U* tests. *P* values less than 0.05 were regarded as significant.

Results

Design of ApoCI-derived Peptides − To determine the structure-function relationship of apoCI with respect to LPS deaggregation and modulation of its *in vivo* kinetics, we designed five peptides derived from human apoCI. We generated three peptides containing the whole N-terminal α-helix (*i.e.* $apoCI_{1-38}$, $apoCI_{1-30}$) or a part thereof ($apoCI_{1-23}$), and two peptides containing the whole C-terminal α-helix (*i.e.* $apoCI_{35-57}$) or a part thereof ($apoCI_{46-57}$) (Fig. 1). The N-terminal peptides differ from each other by length and the amount of cationic/hydrophobic sequences that are present throughout the sequence of apoCI (residues 10-12, 21-23, and 28-30). The C-terminal peptides both contain the

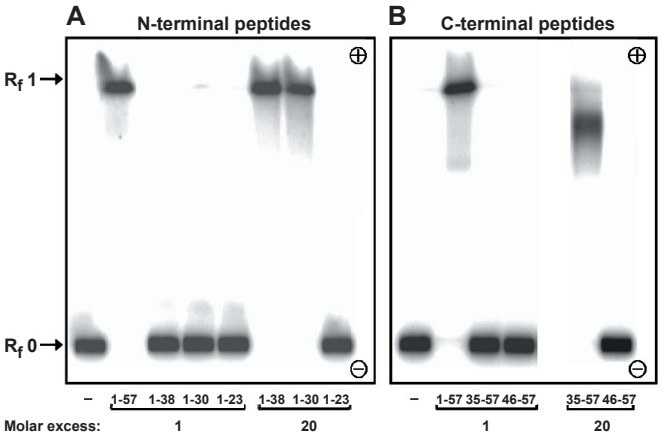

Figure 2. Effect of apoCI-derived peptides on the electrophoretic mobility of ^{125}I-LPS. ^{125}I-LPS (20 ng) was incubated without or with apoCI-peptides derived from the N-terminal helix (A) or the C-terminal helix (B) at a 1:1 and 1:20 molar ratio. As a positive control, ^{125}I-LPS was incubated with $apoCI_{1-57}$ at a 1:1 molar ratio. Aliquots of the incubation mixtures (approx. $1x10^5$ cpm) were subjected to electrophoresis in an 0.75% (w/v) agarose gel at pH 8.8. The resulting gel was dried, and assayed for radioactivity by autoradiography.

previously identified LPS-binding motif (residues 48-54) and differ by length and the presence of one cationic/hydrophobic sequence at position 37-39.

Effect of ApoCI-derived Peptides on the Electrophoretic Mobility of ^{125}I-LPS − To investigate the LPS-deaggregating properties of the apoCI-derived peptides *in vitro*, we incubated ^{125}I-LPS with the peptides and examined the electrophoretic mobility of the resulting complexes on agarose gel (Fig. 2). Whereas ^{125}I-LPS

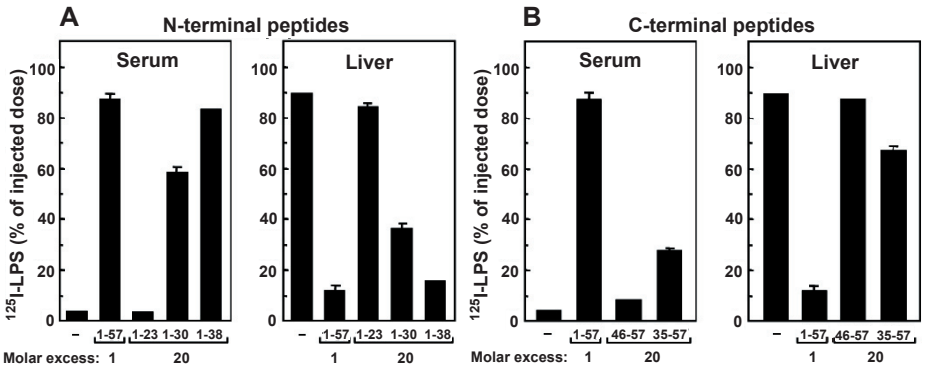

Figure 3. Effect of apoCI-derived peptides on the serum residence and liver association of ^{125}I-LPS *in vivo*. ^{125}I-LPS (10 µg/kg) was preincubated without or with a 20-fold molar excess of apoCI-derived peptides of the N-terminal helix (A) or of the C-terminal helix (B). The samples were injected via the vena cava inferior into anesthetized C57Bl/6 mice, and the serum residence (left panels) and liver association (right panels) of ^{125}I-LPS were determined after 30 min. As a positive control, ^{125}I-LPS was incubated with apoCI$_{1-57}$ in a 1:1 molar ratio. The data are expressed as percentage of injected dose ± S.D. (n=2-3).

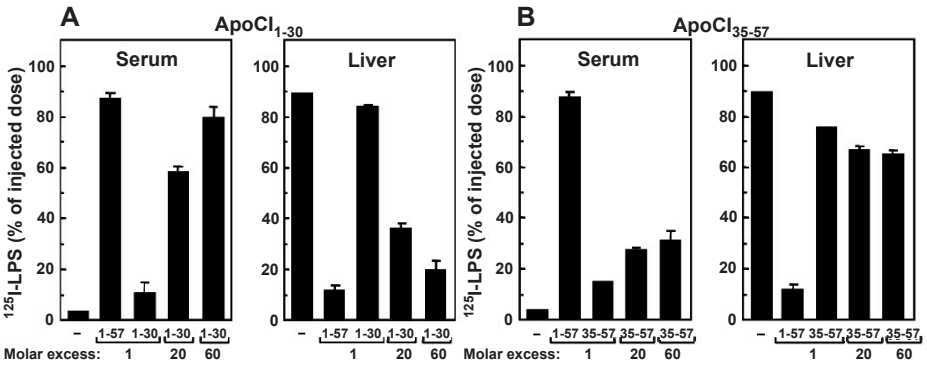

Figure 4. Dose-dependent effects of peptides containing the full N-terminal helix (apoCI$_{1-30}$) and full C-terminal helix (apoCI$_{35-57}$) on the serum residence and liver association of ^{125}I-LPS *in vivo*. ^{125}I-LPS (10 µg/kg) was preincubated without or with apoCI$_{1-30}$ (A) or apoCI$_{35-57}$ (B), at the indicated molar ratios. The samples were injected via the vena cava inferior into anesthetized C57Bl/6 mice, and the serum residence (left panels) and liver association (right panels) of ^{125}I-LPS were determined after 30 min. As a positive control, ^{125}I-LPS was incubated with apoCI$_{1-57}$ in a 1:1 molar ratio. The data are expressed as percentage of injected dose ± S.D. (n=2-3).

alone did not migrate (R_f = 0) due to formation of large micelles, incubation of ^{125}I-LPS with full-length apoCI (apoCI$_{1-57}$) at a 1:1 molar ratio resulted in a shift of all ^{125}I-LPS towards the front of the gel (R_f = 0.95), confirming our previous findings[18]. ApoCI$_{1-38}$, apoCI$_{1-30}$, and apoCI$_{35-57}$ showed reduced efficiency to deaggregate ^{125}I-LPS as compared to apoCI$_{1-57}$. These peptides were able to deaggregate ^{125}I-LPS at a 1:20 molar ratio, but not at a 1:1 molar ratio. In contrast, apoCI$_{1-23}$ and apoCI$_{46-57}$ did not deaggregate ^{125}I-LPS (Fig. 2), not even at a 60-fold molar excess (not shown).

Effect of ApoCI-derived Peptides on the Serum Residence and Liver Association of ^{125}I-LPS *in Vivo* − To examine whether the LPS-deaggregating characteristics of the various peptides would be reflected in their ability to modulate the kinetics of LPS *in vivo*, ^{125}I-LPS was incubated with the peptides and intravenously injected into C57Bl/6 mice (Fig. 3). ^{125}I-LPS alone rapidly cleared from serum and the majority associated with the liver. ApoCI$_{1-57}$ almost completely prevented the serum decay and liver association of ^{125}I-LPS at a 1:1 molar ratio, which is in line with our previous observation[18]. At a 20-fold molar excess, apoCI$_{1-23}$ was not able to affect the kinetics of ^{125}I-LPS. In contrast, apoCI$_{1-30}$ and apoCI$_{1-38}$ did cause a substantial increase in the serum residence of ^{125}I-LPS and a concomitant decrease in the liver association (Fig. 3A), indeed reflecting their relative LPS-deaggregating properties (Fig. 2). The C-terminal peptides, both of which contain the previously identified LPS-binding sequence, were less efficient with respect to modulating the kinetics of ^{125}I-LPS as compared to the N-terminal peptides (Fig. 3B). At a 20-fold molar excess, apoCI$_{46-57}$, the shortest peptide that contains the full LPS-binding motif, did not modulate the kinetics of ^{125}I-LPS. ApoCI$_{35-57}$, containing the full C-terminal helix, did show a modest increase of the serum residence and association to the liver of ^{125}I-LPS.

To further evaluate the dose-dependency of the ability of the peptides to modulate the kinetics of LPS, ^{125}I-LPS was incubated with increasing amounts of apoCI$_{1-30}$ (containing the N-terminal helix) (Fig. 4A) and apoCI$_{35-57}$ (containing the C-terminal helix) (Fig. 4B). At a 1:1 molar ratio, both peptides only minimally modulated the kinetics of ^{125}I-LPS. It indeed appeared that both peptides dose-dependently affected LPS-kinetics, but to a different extent. ApoCI$_{1-30}$ had approximately the same effect as full-length apoCI$_{1-57}$ at a 60-fold higher ratio (Fig. 4A). In contrast, at this 60-fold molar excess, apoCI$_{35-57}$ was still approximately 3-fold less active than apoCI$_{1-57}$ (Fig. 4B). Collectively, these results show that truncation of apoCI equally affects both the deaggregation of LPS (Fig. 3) and the ability to modulate the *in vivo* kinetics of LPS (Fig. 4). Importantly, both the N- and C-terminal helix contain structural components to deaggregate LPS and modulate the kinetics of LPS *in vivo*.

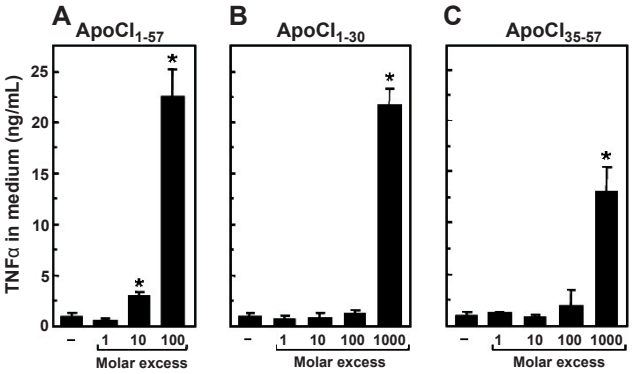

Figure 5. Effect of peptides containing the full N-terminal helix (apoCI$_{1-30}$) and full C-terminal helix (apoCI$_{35-57}$) on the LPS-induced TNFα response *in vitro*. RAW 264.7 cells were incubated in DMEM supplemented with 0.01% human serum albumin (4 h at 37°C) with LPS (1 ng/mL), preincubated (30 min at 37°C) without or with apoCI$_{1-57}$ (A), apoCI$_{1-30}$ (B), or apoCI$_{35-57}$ (C) at the indicated molar ratios. TNFα was determined in the medium by ELISA. The data are expressed as mean concentration TNFα in the medium ± S.D. (n=3-4). **P*<0.05 as compared to LPS alone.

Effect of ApoCI-derived Peptides on the LPS-induced TNFα Response *in Vitro* − Since we previously showed that full-length apoCI$_{1-57}$ is able to increase the LPS-induced TNFα response both *in vitro* and *in vivo*[18], we examined whether truncation of apoCI would affect the LPS-induced TNFα response in murine RAW 264.7 macrophages (Fig. 5). A 10-fold molar excess of apoCI$_{1-57}$ enhanced the LPS-induced TNFα response approximately 3-fold as compared to LPS alone (Fig. 5A), in line with our previous observations[18]. Increasing apoCI$_{1-57}$ concentrations to a 100-fold molar excess even further enhanced the LPS-induced TNFα response (approx. 24-fold). Both apoCI$_{1-30}$ (Fig. 5B) and apoCI$_{35-57}$ (Fig. 5C) were also able to increase the LPS-induced TNFα response. However, approximately 10-fold more peptide was required to achieve the level of stimulation as observed with apoCI$_{1-57}$. ApoCI$_{1-30}$ was more effective than apoCI$_{35-57}$, which is in line with its greater effects on LPS deaggregation and modulation of the kinetics of LPS. Incubation of macrophages with the peptides alone did not result in detectable TNFα secretion in the medium (not shown).

Effect of ApoCI-derived Peptides on the LPS-induced TNFα Response *in Vivo* − Finally, we studied whether the LPS-inducing characteristics of the peptides *in vitro* would be reflected in their ability to enhance the LPS-induced TNFα response *in vivo*. Hereto, plasma TNFα levels were measured in C57Bl/6 mice after injection of LPS alone or in the presence of apoCI$_{1-57}$ (Fig. 6A), apoCI$_{1-30}$, or apoCI$_{35-57}$ (Fig. 6B). We used a 5-fold molar excess of apoCI$_{1-57}$, to ensure effects on the LPS-induced TNFα response *in vivo*, since we found no effects on the *in vitro* LPS-stimulation studies with a 1:1 molar ratio. Indeed, apoCI$_{1-57}$

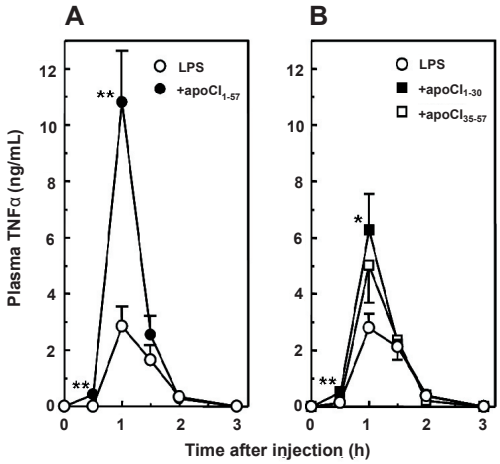

Figure 6. Effect of peptides containing the full N-terminal helix (apoCI$_{1-30}$) and full C-terminal helix (apoCI$_{35-57}$) on the LPS-induced TNFα response *in vivo*. LPS (25 µg/kg) preincubated without (white circles) or with (black circles) a 5-fold molar excess of apoCI$_{1-57}$ (A), or a 60-fold molar excess of apoCI$_{1-30}$ (black squares) or apoCI$_{35-57}$ (white squares) (B), were injected intravenously into C57Bl/6 mice in the presence of BSA (0.1% w/v). At the indicated time points, blood samples were taken and TNFα levels were determined in plasma by ELISA. Values represent means ± S.E.M. (n=6). Statistical differences were assessed as compared to LPS alone. **P*<0.05, ***P*<0.01.

markedly enhanced the LPS-induced plasma TNFα levels 3.8-fold at 1 h after injection (10.8 ± 1.8 vs. 2.8 ± 0.7 ng/mL; *P*<0.01). Similar as for the *in vitro* macrophage studies, both apoCI$_{1-30}$ and apoCI$_{35-57}$ were able to enhance the LPS-induced TNFα response, although with reduced efficiency as compared to apoCI$_{1-57}$. A 60-fold molar excess of apoCI$_{1-30}$ resulted in a 2.3-fold increased LPS-induced TNFα response (6.3 ± 1.3 vs. 2.8 ± 0.5 ng/mL; *P*<0.05), whereas apoCI$_{35-57}$ showed a non-significant trend towards a 1.8-fold increased LPS-induced TNFα response (5.0 ± 1.3 vs. 2.8 ± 0.5 ng/mL). Collectively, our findings indicate that both the N- and C-terminal helix contain structural components able to deaggregate LPS, modulate the *in vivo* kinetics of LPS, and enhance the LPS-induced TNFα response *in vitro* and *in vivo*.

Discussion

In addition to its classical function of inhibiting the uptake of lipoproteins by the liver, we recently identified apoCI as a biological enhancer of the proinflammatory response towards LPS and showed that apoCI efficiently bound to LPS involving an apparent LPS-binding motif in its C-terminal helix[18]. To further investigate the structure-function relationship of apoCI on its ability to deaggregate LPS and to stimulate the LPS-induced proinflammatory response, we now used an array of N- and C-terminal apoCI-derived peptides containing the apparent LPS-

binding motif and/or varying amounts of highly conserved alternating cationic/hydrophobic sequences that may cooperate in LPS binding. We demonstrate that, in addition to the LPS-binding motif, apoCI contains additional elements that enable binding of LPS and enhancing the inflammatory response towards LPS.

Our previous studies showed that the binding of apoCI to LPS was largely mediated by a lysine-rich LPS-binding motif in the C-terminus of apoCI (residues 48-54; *KVKEKLK*), which is highly homologous to the LPS-binding sequence of LALF[19] and CAP-18[20], as depicted in Table 1. Replacement of the lysines by alanines within this motif decreased the ability of apoCI to bind LPS[18], indicating the importance of this sequence in binding LPS. However, the binding to LPS was not completely abrogated and the mutant was still able to affect the *in vivo* kinetics of LPS to some extent[18], indicating that apoCI may contain additional elements that cooperate in LPS binding. Indeed, $apoCI_{1-30}$, $apoCI_{1-38}$, and $apoCI_{35-57}$ were all able to deaggregate LPS, prolong the residence time of LPS in the serum, and enhance the LPS-induced TNFα response. These findings indicate that both the N- and C-terminal helix contain additional structural elements able to deaggregate LPS and enhance the inflammatory response towards LPS.

We could not directly determine whether $apoCI_{1-30}$, $apoCI_{1-38}$, and $apoCI_{35-57}$ were able to actually bind LPS, and thus colocalized with LPS in the electrophoretic mobility shift assay, since we were not able to detect these peptides on the resulting gel. We have previously shown that full-length apoCI was able to firmly bind LPS and colocalize with LPS in this assay[18], indicating that the deaggregation potential of the various apoCI-derived peptides are most likely in line with their LPS-binding potential, and thus $apoCI_{1-30}$, $apoCI_{1-38}$, and $apoCI_{35-57}$ are able to bind LPS.

With the use of molecular modeling methods Frecer *et al.*[29] suggested that peptides containing cationic/hydrophobic motifs within their sequence are able to recognize and bind LPS with high affinity. Indeed, at least in some LPS-binding proteins (*e.g.* Lf and BPI), these domains are involved in the interaction with LPS[30]. Interestingly, besides the previously established LPS-binding motif, apoCI contains several of such amphipathic motifs throughout its sequence (Fig. 1). Our observation that peptides containing the N-terminal helix without the previously established LPS-binding motif (*i.e.* $apoCI_{1-30}$ and $apoCI_{1-38}$) were able to bind LPS and alter its *in vivo* fate, is thus likely explained by the presence of such amphipathic motifs.

The efficiencies of $apoCI_{1-30}$ and $apoCI_{35-57}$ to bind LPS and modulate the inflammatory response towards LPS were lower as compared to $apoCI_{1-57}$. This

knowledge, combined with our previous data where we showed that modification of the apparent LPS-binding motif diminished, but not completely abrogated the binding to LPS and modulation of the in vivo fate of LPS[18], suggests that the previously established LPS-binding motif together with alternating cationic/hydrophobic motifs within apoCI interact cooperatively with LPS. A similar interaction with LPS has previously been demonstrated for Lf[31] and SMAP-29[32], a sheep myeloid antimicrobial peptide, which both have two LPS-binding domains, and BPI[33] and serum amyloid P (SAP)[30], which both have three regions that contribute to binding LPS.

It is intriguing to speculate how apoCI enhances the LPS-induced proinflammatory response. All other proteins that are known to bind LPS (*e.g.* SAP, apoE, Lf and LALF), attenuate the LPS-induced inflammatory response, except for LBP and CD14, which are necessary in LPS signaling[34,35]. Part of the mechanism could involve the ability of apoCI to prolong the serum residence of LPS by transferring it to long-circulating lipoprotein particles such as HDL, resulting in longer exposure of LPS to the effector cells such as macrophages *in vivo*. However, we showed that apoCI also enhances the direct presentation of LPS to macrophages *in vitro* in the absence of lipoproteins or serum, suggesting that additional mechanism(s) are involved.

First, apoCI could be directly involved in enhancing the presentation of LPS to LBP and soluble CD14 (sCD14) in plasma, and/or to membrane CD14 (mCD14) and the MD2-TLR4-complex on effector cells, resulting in enhanced TLR4-mediated signaling. Second, apoCI may improve the stabilization of the LPS-MD2-TLR4 complex. Third, apoCI could modulate the binding and/or uptake of LPS by other receptors than TLR4, with an increased proinflammatory response as a result. For instance, the LDLr[36,37] and scavenger receptors, such as class A scavenger receptor (SR-A)[38-40] and SR-BI[41-43], have been shown to modulate inflammatory responses. Studies are ongoing to investigate the mechanism behind the apoCI-mediated enhanced LPS-induced inflammatory response.

In summary, we have demonstrated that both the N- and C-terminal helix of apoCI contain structural elements to bind LPS, to affect the *in vivo* kinetics of LPS, and to enhance the proinflammatory response towards LPS *in vitro* and *in vivo*. We anticipate that, beside the previously identified LPS-binding motif, highly conserved alternating cationic/hydrophobic motifs present throughout apoCI participate in the binding to LPS and modulation of the *in vivo* fate of LPS.

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