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Engagement of Platelet Toll-Like Receptor 9 by Novel Endogenous Ligands Promotes Platelet Hyper-Reactivity and Thrombosis

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Abstract

Rationale—A prothrombotic state and increased platelet reactivity are common in pathophysiological conditions associated with oxidative stress and infections. Such conditions are associated with an appearance of altered-self ligands in circulation that can be recognized by Toll-like receptors (TLR). Platelets express a number of TLR, including TLR9, however, the role of TLR in platelet function and thrombosis is poorly understood.

Objective—To investigate the biological activities of carboxy(alkylpyrrole) protein adducts (CAPs), an altered self-ligand generated in oxidative stress, on platelet function and thrombosis.

Methods and Results—In this study we show that CAPs represent novel unconventional ligands for TLR9. Furthermore, using human and murine platelets, we demonstrate that CAPs promote platelet activation, granule secretion, and aggregation in vitro and thrombosis in vivo via the TLR9/MyD88 pathway. Platelet activation by TLR9 ligands induces IRAK1 and AKT phosphorylation, and is Src kinase dependent. Physiological platelet agonists act synergistically with TLR9 ligands by inducing TLR9 expression on the platelet surface.

Conclusions—Our study demonstrates that platelet TLR9 is a functional platelet receptor that links oxidative stress, innate immunity, and thrombosis.

Keywords

Platelets; TLR9; carboxyalkylpyrrole protein adducts; oxidative stress

INTRODUCTION

Pathophysiological conditions associated with oxidative stress, such as dyslipidemia, diabetes, and acute or chronic infections are frequently associated with the prothrombotic state in which increased platelet responses to agonists play a significant role. The clinical

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DISCLOSURES
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importance of increased platelet reactivity is supported by the observation that subjects with elevations in various measures of platelet reactivity are at an increased prospective risk for coronary events and death.[1–3] A number of recent reports indicate that platelets may sense 'pathologic ligands' accumulating in circulation via a specific set of receptors and translate an activation signal into a prothrombotic state.[4] These receptors may include pattern recognition receptors such as scavenger receptors and Toll-like receptors (TLR). While the role of platelet scavenger receptors in sensing oxidative stress generated ligands has been recently highlighted,[5] studies on the function of platelet TLR is only starting to emerge. TLR recognize a number of pathogen-associated molecular patterns and damage associated molecular patterns of host origin that include altered-self ligands.[6] The recognition contributes to innate immune defense and a state of non-infectious inflammation, respectively. TLR 1, 2, 4, 6, 8, and 9 are expressed in megakaryocytes as well as in platelets, however, their role in platelet function is poorly understood.[7–9] It is believed that sensing various pathogens via different sets of receptors leads to the production of different profiles of pro-inflammatory cytokines by platelets contributing to the innate immunity, or modulates platelet function and directly contributes to cardiovascular pathology.[10, 11] Recent studies have shown that the presence of pathogen associated ligands for TLR2 and TLR4 (and their co-receptors TLR1 and TLR6) in circulation can induce a thromboinflammatory response in platelets.[12, 13] While evidences are accumulating that altered-self ligands can induce signaling via TLR in cells, such as endothelial and macrophages, there is a lack of data on the involvement of platelet TLR in the recognition of such ligands and translation of the signal into pathological responses. The role of platelet TLR9 is unknown.

Free-radical oxidation of targets containing polyunsaturated fatty acids generates a host of oxidative products, including the hydroxy-ω-oxoalkenoic acids (Online Scheme 1). Hydrolysis by phospholipase A_2 followed by the reaction of the resulting unesterified hydroxy-ω-oxoalkenoic acid with proteins, or the reaction of esterified hydroxy-ω-oxoalkenoic acid with proteins followed by hydrolysis, gives rise to a family of carboxyalkylpyrrole protein adducts (CAPs).[14, 15] CAPs belong to the so-called altered-self class of molecules. CAPs immunoreactivity was detected in atherosclerotic plaques, in tumors, and in healing wounds. Plasma levels of CAPs are significantly elevated in diabetes, atherosclerosis, renal failure, and degenerative diseases.[14–18] When incorporated into sn-2 position of oxidized phospholipids, hydroxy-ω-oxoalkenoic acid is recognized by scavenger receptors CD36 and SR-BI which belong to pattern recognition receptors, but are best known for their role in lipid metabolism.[19, 20] On the other hand, CAPs have been previously shown to be ligands for a member of TLR - another family of pattern recognition receptors.[16]

In this study, the effects of CAPs on platelets and molecular mechanisms of effects were examined. We found that CAPs represent a novel and unconventional ligand for TLR9. Moreover, CAPs can promote platelet activation and aggregation in vitro and accelerate thrombosis in vivo in TLR9/MyD88 dependent manner. Thus, our findings suggest that TLR9 on platelets is a functional receptor that links oxidative stress, innate immunity, and thrombosis.

METHODS

A detailed and expanded 'Material and Methods' section is available in the 'Online Supplemental Materials' at http://circres.ahajournals.org.

Preparation of 2-(ω-carboxyalkyl)pyrrole protein adducts and anti-CEP antibodies

The 2-(ω-carboxypropyl)pyrrole bovine serum albumin adduct (CPP-BSA), 2-(ω-carboxyethyle)pyrrole human serum albumin adduct (CEP-HSA), and other CAPs were generated by Paal-Knorr condensation reaction with γ-dicarbonyl compounds as described earlier.[15, 21] The polyclonal anti-CEP antibody was generated in a *Pasteurella*-free, New Zealand white rabbit by subcutaneous inoculation of CEP-KLH along with complete Freund's adjuvant, and the IgG fraction of anti-CEP antibodies was purified using immobilized protein G.[15]

Isolation of platelets

PRP and gel-filtered platelets were isolated as described.[5] Platelet counts were assessed by a Cellometer™ Auto-M10 (Nexcelom Bioscience Lawrence, MA).

Platelet aggregation

Platelet aggregation was monitored using a Chronolog Model 560VS aggregometer with AGGRRO/LINK version 5.1.9 software, at a stirring speed of 1000 rpm. Aliquots (200μL) of PRP were placed in cuvettes containing magnetic stirrer bars, warmed at 37°C, and stirred for 90 seconds to obtain a stable baseline. Platelet concentration in PRP was adjusted to 2×10^8 platelets/ml using platelet poor plasma of the same genotype.

Flowcytometry

Human and murine platelet suspensions (2.5×10^7/mL) prepared by gel-filtration in modified HEPES-Tyrode buffer. P-selectin expression and integrin-$\alpha_{IIb\beta3}$ activation were assessed as described previously.[5] Data was acquired using a FACS Calibur instrument (Becton Dickinson, San Jose, CA) and analyzed using the FlowJo 9.0.2 software (Tree Star, Ashland, OR).

Human TLR9 activation assay in HEK-Blue hTLR9 cells

The HEK-Blue™ hTLR9 cells were cultured in 96-well plate in the presence of indicated concentrations of agonists, and *NF-κB*-induced *SEAP* activity was assessed using QUANTI-Blue™, and a reading at OD 620 nm.

Surface Plasmon Resonance (SPR)

Real-time protein-protein interactions were analyzed using a Biacore3000 (BIAcore AB, GE Healthcare). Functional grade TLR9 peptides (Novus Biological) were bound to CM5 biosensor chips (Biacore) at pH 5.5. Analyte binding to the immobilized ligand was recorded by measuring the variation of the SPR angle, and the results are expressed in resonance units (RU).

Intravital Thrombosis

Intravital thrombosis study was performed using an acute carotid artery injury model as previously described.[5]

Statistical analysis

Values are expressed as means ± standard errors of mean (SEM). The statistical significance was evaluated between two groups of data using two-tailed unpaired Student's *t* test. We used two-tailed nonparametric Mann Whitney U test for analyzing the results of 'intravital carotid thrombosis', and 'tail cut bleeding time' studies. The *p* values less than 0.05 were considered as statistically significant.

RESULTS

2-(ω-carboxyalkyl)pyrrole protein adducts induce platelet activation

We first assessed the effects on platelets of 2-(ω-carboxyethyl)pyrrole protein (CEP) and 2-(ω-carboxypropyl)pyrrole (CPP) protein adducts (CAPs) of albumin as the most abundant target in plasma for modification by products of lipid peroxidation. In vitro studies demonstrated that these adducts induce significant integrin-$\alpha_{IIb}\beta_3$ activation and P-selectin expression in human platelets (Fig. 1a–b). In contrast, the sham-modified protein had no effect on platelets. In order to see whether the nature of the modified protein has any role in this effect, we studied adducts of a number of plasma proteins, including albumins from several species, IgG, as well as Keyhole limpet hemocyanin (KLH), a protein that is phylogenetically distant from mammalian proteins. 2-(ω-carboxyalkyl)pyrrole adducts (CAPs) of the tested proteins, but not native unmodified proteins, induced significant platelet activation responses (Fig. 1c–d), indicating that the nature of the modified protein plays no critical role. To test whether this effect is specific to 2-(ω-carboxyalkyl)pyrrole, we used platelet activation assay to test multiple protein adducts formed by oxidized lipids, which resembled hydroxy-ω-oxoalkenoic acids but were not capable of forming pyrroles (Online Table I). None of these non-pyrrole protein adducts had the ability to activate platelets, suggesting that the effect is specific for 2-(ω-carboxyalkyl) pyrrole modification of the proteins. We then tested whether the number of modification per protein plays a role in the effect of CAPs. CAPs with an increasing molar ratio of pyrrole per mole of protein were synthesized and tested in a platelet integrin-$\alpha_{IIb}\beta_3$ activation assay. We observed a direct correlation between the extent of protein modification by CAPs and the capacity to induce activation of human platelets (Online Figure I).

CAPs activate platelets via Toll-like receptors

CAPs belong to altered-self ligands that are commonly recognized by pattern recognition receptors.[5, 14, 16] Several pattern recognition receptors are expressed in platelets, including class B scavenger receptors CD36 and SR-BI.[4] Correspondingly, effects of CAPs were assessed using platelets from wild type mice (WT), CD36deficient ($CD36^{-/-}$) mice, and SR-BI deficient ($SR\text{-}BI^{-/-}$) mice. Platelets isolated from wild type mice were activated by CAPs similarly to human platelets (Fig. 2a). Activation of platelets of $CD36^{-/-}$ and $SR\text{-}BI^{-/-}$ mice was comparable to that of WT platelets (Fig. 2b, c), ruling out significant involvement of class B scavenger receptors in platelet activation by the CAPs. Platelets also express a number of TLR (Online Figure IIa) that can recognize various pathogen associated molecular patterns as well as altered-self ligands.[22] The adaptor protein MyD88 is a common mediator of TLR signaling in cells and is present in platelets (Online Figure IIa). Therefore, to test the general involvement of TLR in CAPs induced platelet activation; we first used platelets from $MyD88^{-/-}$ mice. CAPs failed to induce platelet activation in $MyD88^{-/-}$ platelets, demonstrating an absolute requirement for functional TLR-MyD88 signaling. This requirement was specific for CAPs, as integrin-$\alpha_{IIb}\beta_3$ activation response of $MyD88^{-/-}$ platelets to the physiological agonists like ADP (Fig. 2d) and thrombin (not shown) was similar to that of WT platelets.

CAPs induce platelet activation in a TLR9-dependent manner

We next tested the involvement of TLR 2, 4, 6, and 9, known to be expressed in platelets,[7, 9] in platelet responses to CAPs. We used Fab fragments of anti human TLR2, TLR4, TLR6, and TLR9 antibodies, or the chemical inhibitor of TLR4 (CLI 095, data not shown) to block a specific receptor. Unexpectedly, anti-human TLR9 Fab inhibited CAPs induced platelet activation, while no significant effects of Fab fragments to TLR2, TLR4, and TLR6 was observed (Fig. 3a, d–f). The effect of the anti-TLR9 Fab was specific, as it did not affect platelet activation by physiological agonists, such as thrombin or ADP (Fig. 3c). TLR9

expression on human platelets has been previously reported.[7, 8] Using RT-PCR and Western blot analyses, we detected the expression of TLR9 mRNA and protein in purified murine and human platelets, in the human megakaryocyte cell line Meg-01 and Meg-01 derived platelet like particles (Online Figure IIa). Immunofluorescence microscopy revealed granular staining of permeabilized human and murine platelets with anti-TLR9 antibody and lack of staining of platelets from TLR9 deficient mice (Online Figure IIb). Low levels of TLR9 expression were detected on non-permeabilized resting platelets; however, platelet activation by physiological agonists resulted in a significant increase of TLR9 expression in platelets (Online Figure IIc). To further demonstrate the specific involvement of TLR9 in response to CAPs, we used platelets from TLR9 deficient mice. Response of platelets from $TLR9^{-/-}$ mice to CAPs was negligible as compared to WT platelets (Fig. 3g–j, Online Figure III), demonstrating that TLR9 is a major mediator of the effects of CAPs on platelets. As an additional control, we tested the effects of CAPs using platelets from $TLR2^{-/-}$ and $TLR6^{-/-}$ mice and observed no significant effect on respective integrin-αIIbβ3 activation by CAPs (Online Figure IV).

CAPs are novel and unconventional ligands for TLR9

To demonstrate that CAPs represent new non-canonical ligands for TLR9, we used several approaches. We first studied direct interaction of CAPs with TLR9 by ‘surface plasmon resonance’ (SPR) using human TLR9 immobilized on the surface of a CM5 biosensor chip. As shown in Figure 4a, CAPs (CEP-BSA) interact with the immobilized TLR9 in a concentration dependent manner at high binding affinity ($KA = 8.53 \times 10^5$) and low dissociation ($KD = 1.17 \times 10^{-6}$) constants. Interaction increased with the increase in the extent of protein modification by CAPs, i.e the number of pyrroles per protein molecule (Fig. 4b). Pretreatment of immobilized TLR9 with anti-TLR9 antibody completely blocked the binding of CAPs to a TLR9 coated surface (Online Figure Va). To test whether TLR9 binding is specific for 2-(ω-carboxyalkyl)pyrrole modification, we used SPR assay to test control protein adducts formed by oxidized lipids, which resembled hydroxy-ω-oxoalkenoic acids but are not capable of forming a pyrrole. We observed very weak and completely reversible binding of control adducts in comparison to that of CAPs (Online Figure Vb, data for OHdiA-BSA are shown), suggesting that the effect is specific for 2-(ω-carboxyalkyl)pyrrole modification of the proteins. To further demonstrate direct interaction of CAPs and TLR9 in platelets, we incubated platelet lysate with CAPs and performed co-immunoprecipitation assay as described in the Methods section. TLR9 was specifically immunoprecipitated with CAPs, demonstrating the direct interaction of platelet TLR9 and CAPs (Fig. 4c). To demonstrate that CAPs can induce cellular responses via TLR9, even though this receptor is usually localized intracellularly, we used an alternative system - the HEK-Blue™ hTLR9 cell line that express the human TLR9 (Fig. 4d) and an NF-κB-inducible reporter gene. CAPs induced strong, concentration-dependent and TLR9-mediated signaling in this cell line. The magnitude of the response was comparable to that of established TLR9 specific ligand unmethylated CpG oligodeoxynucleotide (ODN2006) (Fig. 4e, f). Interestingly, while CAPs co-localize with platelet TLR9, we observed a lack of co-localization with the early endosomal marker Rab4 in platelets and partial co-localization with the late endosomal marker Rab9 (Online Figure VIa–c). These data are consistent with the recent finding of TLR9 localization to a novel electron-dense tubular system-related compartment.[23] In nucleated cells TLR9 undergoes proteolytic cleavage upon endosomal acidification, a step required for TLR9 signaling. We observed that an inhibitor of endosomal acidification chloroquine significantly inhibited activation of platelets by CAPs (Online Figure VId–e) but not by physiological platelet agonists ADP, Thrombin, or convulxin, suggesting that the key event leading to activation via TLR9 is similar in platelets and nucleated cells. Taken together, these results demonstrate that CAPs is a new and

unconventional ligand for TLR9 that could reach the endosomal TLR9, initiating the downstream signaling cascade.

CAPs induce platelet aggregation and promote platelet hypereactivity in vitro

We next tested whether CAPs can induce platelet aggregation in vitro. We observed that only protein with high degree of modification (≥ 5 pyrroles/per molecule of protein) can induce significant platelet aggregation (Fig 5a). Since TLR9 in platelets is expressed intracellularly in specialized granules, and since we observed that TLR9 expression can be induced by physiological agonists, we tested effects of proteins with level modification of less than 5 pyrroles per molecule on platelet activation and aggregation using platelets primed by either suboptimal concentrations of thrombin receptor activating peptide (TRAP), or weak physiological agonist ADP. Threshold concentrations of TRAP and ADP induced only reversible or no aggregation and activation responses (Fig. 5b, e, h). We observed irreversible and significantly accelerated platelet aggregation in TRAP and ADP primed human (Fig. 5b, c) and murine (Fig. 5e, f) platelets in response to proteins with lower level modification by CAPs. Correspondingly, while CAPs with lower level of modification induced only modest activation of platelets we observed a strong increase in P-selectin expression (Fig. 5d), and integrin-$\alpha_{IIb\beta3}$ activation (Fig. 5g) when platelets were primed with physiological agonist. This accelerated activation response was TLR9 dependent since pre-incubation of PRP with anti-human TLR9 Fab significantly reduced platelet aggregation induced by CAPs in ADP primed platelets (Fig. 5h, i). Taken together, these data suggest that presence of carboxyalkylpyrrole protein adducts in vivo may promote platelet hyperactivity and aggregation response, especially when physiological platelet agonists are present.

CAPs accelerate thrombosis in vivo in a MyD88 and TLR9 dependent manner

To test whether the presence of CAPs in circulation accelerates thrombosis in vivo and whether MyD88/TLR9 pathway is involved, we compared vessel occlusion times using a ferric chloride induced carotid artery thrombosis model on sex and age matched groups of MyD88$^{-/-}$, TLR9$^{-/-}$, and corresponding WT mice, which received intravenous injections of either CAPs or sham-modified proteins prior to the vascular damage. Occlusion times were similar in MyD88$^{-/-}$, TLR9$^{-/-}$, and corresponding WT mice receiving sham-modified proteins. In addition, tail cut bleeding time was similar in TLR9$^{-/-}$ and WT mice (Online Figure VII). These results and normal activation responses of platelets from MyD88$^{-/-}$ and TLR9$^{-/-}$ to physiological agonists in activation assay indicates that there are no major deficiencies in thrombosis in these two knockout mice. Nevertheless, time to complete thrombotic occlusion was significantly shortened in the WT mice that received CAPs as compared to the WT mice that received sham modified protein. In contrast, the presence of CAPs in circulation of MyD88$^{-/-}$ and TLR9$^{-/-}$ mice had no significant effect on the occlusion times (Fig. 6a, b). These results demonstrate that the presence of CAPs in vivo can accelerate thrombosis. They also demonstrate that TLR9 modulates thrombosis in vivo when specific ligands for TLR9 are present.

We then tested whether CAPs are present in hyperlipidemic ApoE$^{-/-}$ mice. Our data show that after intravenous injection, CAPs are rapidly removed from circulation (Online Figure VIII). Nevertheless, concentration of CAPs (data for CEP-adduct are shown) was increased in ApoE$^{-/-}$ mice on chow diet as compared to WT mice (Fig. 6c). Western diet feeding led to a significant accumulation of CAPs in the plasma of ApoE$^{-/-}$ mice (Fig. 6c). Platelets are capable of binding of CAPs; therefore, we tested whether platelets can accumulate CAPs in vivo. Indeed, we found a dramatic increase in platelet associated CAPs in ApoE$^{-/-}$ mice fed a Western type diet (Fig. 6d-e). Immunostaining of the atherosclerotic plaques of aortic root in hyperlipidemic ApoE$^{-/-}$ mice also revealed the presence of CEP in atherosclerotic

plaque, but not in surrounding tissue, (Fig. 6f) indicating that CAPs can also accumulate locally in hyperlipidemia. Taken together, this data confirm that CAPs accumulate in vivo in oxidative stress and demonstrate that presence of CAPs in circulation can modify platelet responses to physiological agonists and thrombosis.

CAPs induce TLR9/MyD88/IRAK1 signaling and require PI3K and Src kinases for platelet activation

Platelets express many components of a TLR signaling pathway downstream of MyD88[24], including IRAK4 (S. Panigrahi and E. Podrez unpublished). IRAK1 is one of the key mediators of the TLR signaling pathway downstream of MyD88 and IRAK4. CAPs alone and CAPs in ADP primed human platelets induced phosphorylation of IRAK1 (Fig. 7a). The connection between TLR9/MyD88/IRAK1 activation and platelet integrin activation is not known. MyD88 signaling may involve cGMP-dependent protein kinase. However, we found no effect of a PKG inhibitor DT2-oligopeptide in a concentration range of 50–500nM on CAPs induced platelet activation (Online Figure IX). The PI3K/AKT pathway plays a key role in platelet activation,[25–27] and has been linked to the TLR9 signaling in other cell types.[27–29] We found that in human platelets, CAPs could specifically induce AKT (Ser473) phosphorylation (Fig 7b). PI3K/AKT kinase inhibitor Ly294-002, Syc kinase inhibitor Bay61, Src kinase inhibitor PP2, but not the respective control PP3, also specifically blocked AKT1 phosphorylation in human platelets induced by CAPs at very low concentrations (Fig. 7c). Furthermore, CAPs induced P-selectin expression and integrin-$\alpha_{IIb}\beta_3$ activation of human platelets could be almost completely blocked by the Src kinase inhibitor PP2 (Fig. 7d). Three AKT isoforms are expressed in platelets. Deletions of specific AKT isoforms in the mouse suggest that they play distinct and redundant roles in platelet responses to physiological agonists.[26, 30, 31] We observed that CAPs induced platelet activation was significantly reduced in AKT1 and in AKT2, but not in AKT3 deficient platelets (Fig 7e), suggesting that, at least in murine platelets, AKT1 and AKT2 are the major isoforms involved in signaling events downstream of TLR9 and MyD88. Taken together, these findings demonstrate the role of PI3K/AKT and Src family kinases in platelet signaling induced by new unconventional ligands via TLR9/MyD88.

DISCUSSION

In this study, we have made several important observations. We have found, for the first time, that TLR9, a receptor, previously implicated only in immune cell responses to bacterial DNA, is a functional platelet receptor capable of modulating platelet function and thrombosis. Furthermore, while unmethylated CpG sequences of bacterial DNA are the only known ligands for TLR9, our study identifies 2-(ω-carboxyalkyl)pyrrole protein adducts(CAPs), a product of phospholipids oxidation generated in oxidative stress, as a novel non-canonical ligand for TLR9, which induces signaling events downstream of TLR9/MyD88 and promotes thrombosis.

The CAPs are present in circulation in a number of pathological conditions associated with oxidative stress, such as atherosclerosis, end stage renal disease, and age-related macular degeneration.[14, 32, 33] In the extracellular matrix of humans and animals, CAPs accumulate with age.[33] We also detected increased levels of CAPs in circulation and in the atherosclerotic plaques in hyperlipidemic ApoE$^{-/-}$ mice. We observed significant accumulation of CAPs in platelets in hyperlipidemia, suggesting that effects of CAPs can be disproportional to circulating levels.

One pathway of CAPs formation is via hydrolysis of oxidized phospholipids containing hydroxy-ω-oxoalkenoic acid by PLA_2. Unesterified hydroxy-ω-oxoalkenoic acids are chemically active and readily modify proteins, thus forming CAPs. When incorporated in

the sn-2 position of oxidized phospholipids, hydroxy-ω-oxoalkenoic acids are recognized by scavenger receptor CD36 on platelets and contribute to platelet hyperreactivity in hyperlipidemia.[5] Our finding demonstrates that the release of hydroxy-ω-oxoalkenoic acids from oxidized phospholipids also generates a product that promotes platelet activation, but via a different receptor and a different signaling pathway.

We demonstrated that CAPs can activate both platelets and HEK-293 cells that overexpress TLR9. In platelets, TLR9 is found in a recently described new electron-dense tubular system-related compartment.[23] How CAPs initially interact with TLR9 is not clear. It is possible that a small amount of TLR9 found on the resting platelet surface is responsible for this initial response. We observed that CAPs can induce an expression of TLR9 on platelets similar to thrombin, thus promoting its own binding to platelets. Alternatively, CAPs may enter the platelets via endocytosis and/or pinocytosis known to be active in platelets[34] with subsequent activation of TLR9. While the early steps of CAPs interaction with TLR9 are not clear, our finding of inhibition of CAPs induced activation by chloroquine suggests that the later steps are similar in platelets and nucleated cells.

Since proteins modified by CAPs are rapidly removed from circulation, it is not surprising that CAPs concentration in murine plasma is low. Nevertheless, this concentration is increased in conditions of mild oxidative stress present in ApoE$^{-/-}$ mice on chow diet, and further increased in conditions of severe oxidative stress when ApoE$^{-/-}$ mice are fed a Western type diet. We have previously shown that factors such as specific oxidized phospholipids that are present in circulation in hyperlipidemic ApoE$^{-/-}$ mice can directly activate platelets via receptors expressed on the surface of resting platelets.[5] While we did not show in this study that that endogenously produced CAPs contribute to platelet activation and thrombosis, our multiple in vitro and in vivo data suggest that CAPs are likely to potentiate in vivo platelet activation, aggregation, and thrombosis induced by other agonists.

Ligand binding induces TLR9 dimerization and subsequent recruitment of adaptor protein MyD88 via cytoplasmic domains TIRs, leading to an association with the IL-1R kinase (IRAK) via the death domains.[35] Platelets are shown to have several components of signaling pathway downstream of TLR, including MyD88, IRAK-1, and others.[24] We observed that CAPs induce IRAK1 phosphorylation, suggesting this pathway contributes to platelet activation. However, the molecular mechanism that links the TLR9/MyD88 pathway on one side, and platelet integrin-$\alpha_{IIb}\beta$ activation and accelerated aggregation response on the other side, needs further investigation. The PI3K/AKT pathway plays a key role in platelet activation,[25, 26] and is one downstream signaling cascade that has been linked to the TLR9 in other cell types.[27–29] Platelets contain both p110 catalytic and p85 regulatory subunit of PI3K involved in the inside-out signaling,[25] and MyD88 has a binding motif for the p85 that can make a functional association in LPS treated macrophages.[36] We found that CAPs are a strong inducer of AKT phosphorylation, and that the specific inhibition of the PI3K/AKT kinase pathway can partially block CAPs induced activation of human platelets. Moreover, response to CAPs was notably reduced in murine AKT1 and AKT2 deficient platelets. We have also found that the inhibition of the Src family kinases prevented platelet activation by CAPs and AKT phosphorylation, demonstrating critical involvement of Src family kinases in response to CAPs. TLR9 dependent activation of the Src family kinase, Lyn, has been shown in other cell types, but not in platelets.[37, 38] Whether Lyn is involved in signaling pathway induced by CAPs needs to be established.

Our finding that TLR9 is a receptor for such disparate ligands as unmethylated CpG oligonucleotide and CAPs is surprising, but it is not unparalleled. TLRs are known for their promiscuity. For example, a recent report showed that TLR4 could functionally bind to a

non-conventional TLR ligand Ni^{2+}.[39] Our study demonstrates that TLR9 activation is also not restricted to a specific interaction with its classical ligand, but has the additional function of sensing of a specific group of lipid peroxidation end products. Thus, TLR9 joins a growing number of pattern recognition receptors that modulate cell function in response to bacterial ligands and oxidative stress derived ligands, including the scavenger receptor CD36, TLR2, TLR4, and TLR6.[40–43] In endothelial cells, hydroxy-ω-oxoalkenoic acids associated with protein are recognized by TLR2 without the involvement of scavenger receptors.[16] Surprisingly, we did not find a contribution of TLR2 to platelet activation by CAPs, even though platelets do express low levels of functional TLR2. It is not clear why TLR2 does not apparently participate in CAPs induced platelet activation, but it has been shown that even such a specific and potent TLR2 ligand as Pam3CSK4 can induce platelet activation only at very high concentrations.[12, 44] Thus, one possibility is that the TLR9/MyD88 pathway in platelets is more sensitive to this type of ligand. Another possibility is that microvascular endothelial cells express (a) co-receptor(s) that is/are not expressed in platelets.

In conclusion, our study has demonstrated a novel connection between oxidative stress, innate immunity, and thrombosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations

CAPs	Carboxy(alkylpyrrole) protein adducts
CEP	2-(ω-carboxyethyl)pyrrole
CPP	2-(ω-carboxypropyl)pyrrole
CoIP	Co-immunoprecipitation
FACS	Fluorescent Activated Cell Sorter
PRP	Platelet rich plasma
PPP	Platelet poor plasma

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Novelty and Significance

What Is Known?

- Platelets may sense ‘pathologic ligands’ accumulating in circulation via a specific set of receptors and translate an activation signal into a prothrombotic state.
- TLR9, the canonical receptor for unmethylated CpG sequences of pathogenic DNA, is expressed at low levels in platelets, however, its role in platelet function is not known.

What New Information Does This Article Contribute?

- TLR9 is a functional platelet receptor capable of modulating platelet function and thrombosis.
- 2-(ω-carboxyalkyl)pyrrole protein adducts (CAPs), a product of phospholipids oxidation is generated in oxidative stress. It is a novel non-canonical ligand for TLR9, which induces platelet activation and promotes thrombosis via TLR9.

Control of platelet reactivity is regarded as critical for the prevention of coronary artery disease. While increased platelet reactivity and thrombogenic potential are common in pathophysiological conditions associated with oxidative stress, the mechanisms responsible are still poorly understood. We investigated the biological activities of CAPs, an altered-self ligand generated in oxidative stress, on platelet function and thrombosis. Using multiple complimentary approaches, we demonstrated that CAPs is a novel and unconventional ligand for TLR9. CAPs levels are increased in circulation in hyperlipidemic ApoE$^{-/-}$ mice leading to pronounced accumulation of CAPs in platelets. CAPs can promote platelet activation and aggregation in vitro and accelerate thrombosis in vivo in a TLR9/MyD88 dependent manner. Physiological platelet agonists act synergistically with TLR9 ligands by inducing TLR9 expression on the platelet surface. These findings suggest that TLR9 is a functional platelet receptor capable of modulating platelet function and thrombosis in response to oxidative stress, and establishes a novel connection between oxidative stress, innate immunity, and thrombosis.

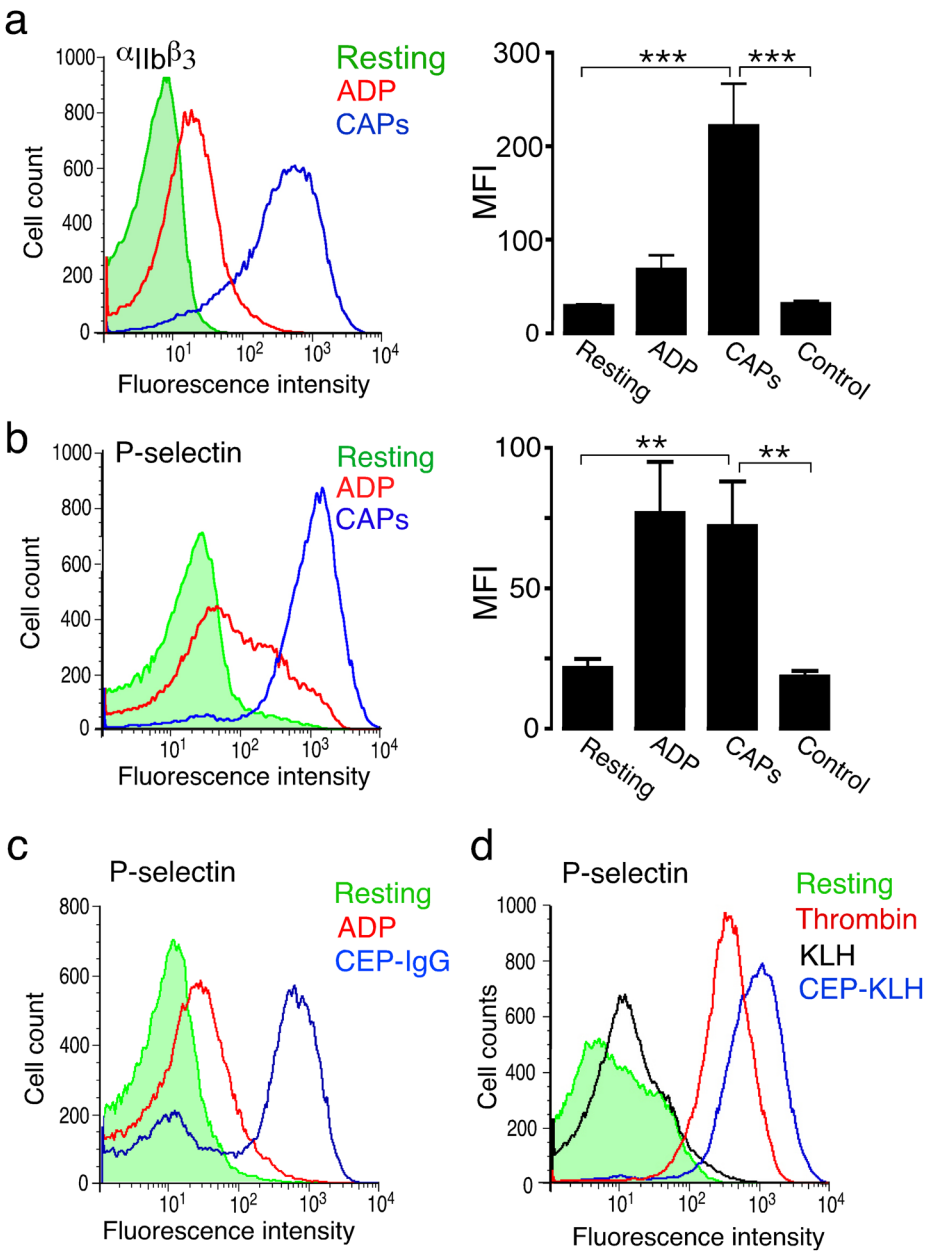

Figure 1. Carboxyethylpyrrole protein adducts (CAPs) activate platelet

Human platelets isolated by gel-filtration were incubated with indicated stimuli for 15 minutes and (a) integrin- $\alpha_{IIb}\beta_3$ activation and (b–d) P-selectin expression was assessed by flowcytometry using FITC- conjugated PAC-1 or PE-conjugated anti-CD62 antibody. (a, b) Right panels show quantification of the data presented as mean (SEM). Agonist concentrations are: ADP (5μM), Thrombin (0.05U/ml), CAPs (albumin 1.5μM, pyrrole 12.5μM). N ≥ 5. *p<0.03, ***p<0.0001.

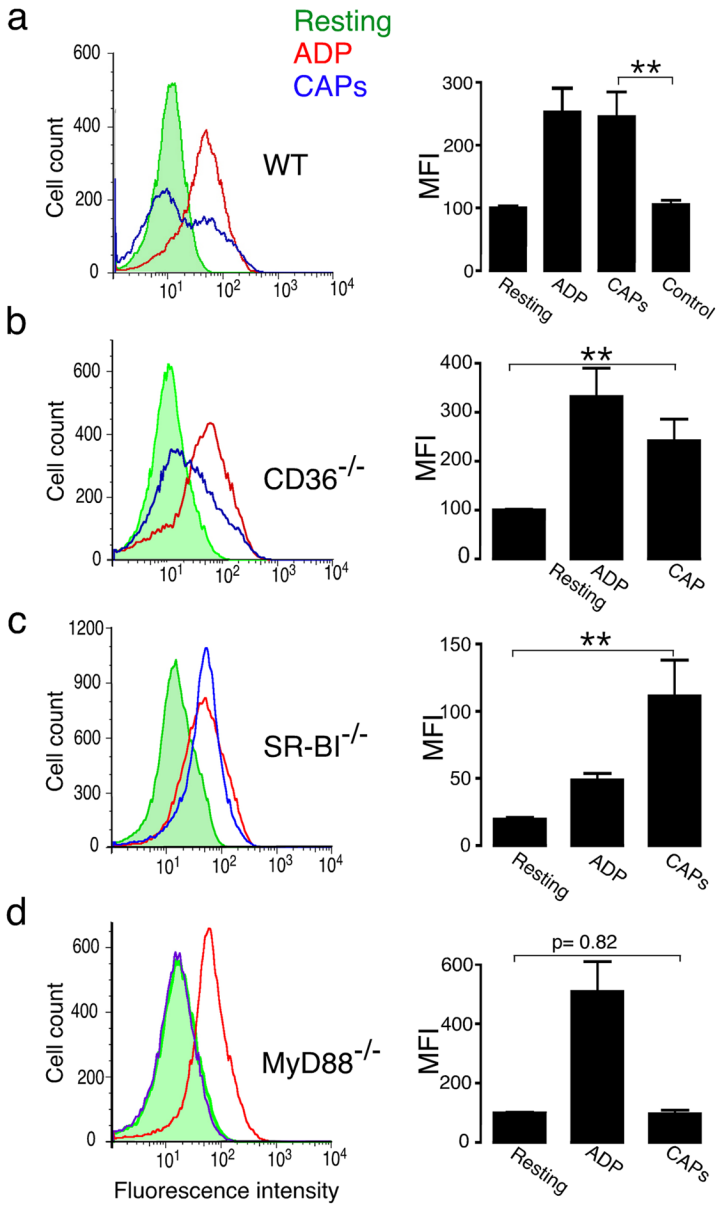

Figure 2. Carboxyalkylpyrrole protein adducts activate platelets via Toll-like receptors but not via class B scavenger receptors

Platelets from (a) wild type, (b) CD36$^{-/-}$, (c) SR-BI$^{-/-}$ and (d) MyD88$^{-/-}$ knockout mice were isolated by gel-filtration and stimulated with ADP 5μM or CAPs (CPP-BSA: albumin 3μM, pyrrole 25μM). The activation of platelets was assessed by FACS using activated conformation specific murine integrin-$\alpha_{IIb}\beta_3$ antibody (JON/A). Flowcytometry histograms (left) and corresponding mean fluorescence intensity (MFI) of at least five independent experiments (right) are shown. Data presented as mean (SEM). **p<0.001, ***p<0.0001.

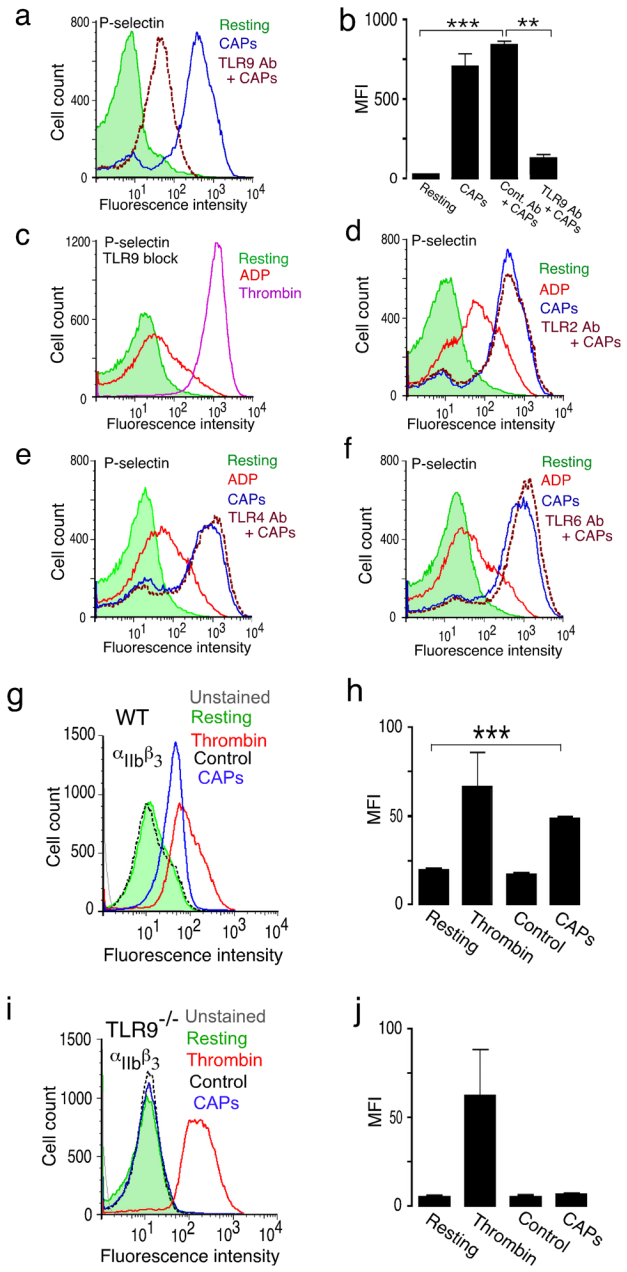

Figure 3. Carboxy-alkyl-pyrrole protein adducts induce platelet activation in a TLR9 dependent manner

(a–f) Human platelets isolated by gel-filtration were incubated with or without Fab fragments of specific anti-human TLR blocking monoclonal antibodies and stimulated by indicated agonists. P-selectin expression was assessed using FACS analysis. (g–j) Platelets from wild type and TLR9knockout mice were isolated and stimulated with α-thrombin 0.05U/mL, CAPs (albumin 1.5μM, pyrrole 12.5μM) and integrin-$\alpha_{IIb}\beta_3$ activation was assessed by FACS analysis and JON/A antibody. Right panels show the mean fluorescence intensity (MFI) values presented as (SEM). N = 3. ** p<0.001, *** p<0.0001.

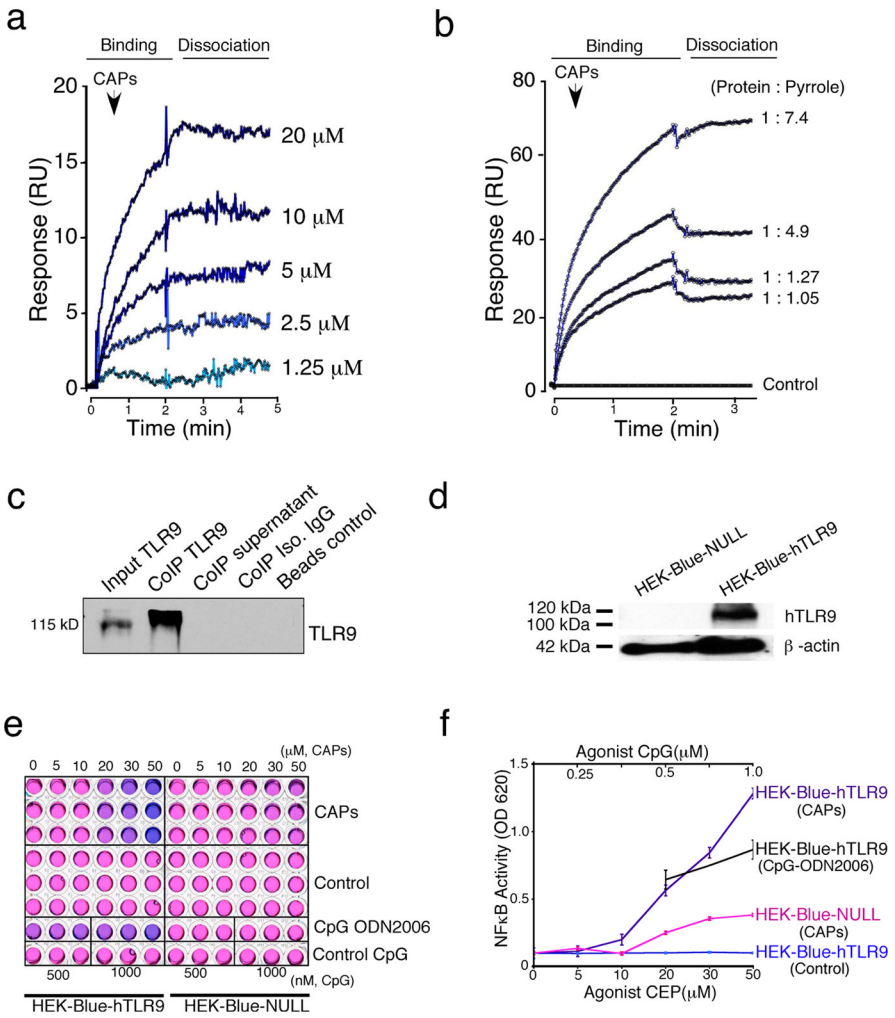

Figure 4. CAPs directly interact with TLR9 and can induce TLR9 dependent signaling

Surface plasmon resonance (SPR) assay: (a) Ascending concentrations of CAPs (CEP-BSA) or (b) CAPs with increasing molar ratio of CAPs per protein were passed at a flow rate of 20 μL/min over CM5 biosensor chip coated with functional grade TLR9 peptide with all active leucine rich regions (LRR). The binding and dissociation curves were recorded over time. (c) Human leukocyte depleted platelets were isolated by gel filtration, then incubated with CAPs for 15 minutes at 37°C, washed, lysed, and CEP-BSA was immunoprecipitated by anti-bovine serum albumin antibody as described in Methods. The immunoprecipitate was then separated by SDS PAGE, and TLR9 was detected by western blotting. (d) Confirmation of TLR9 over-expression in HEK-Blue™ hTLR9 cells by western blotting (e) HEK-Blue™ hTLR9 and HEK-Blue™ Null (control) cells expressing an NF-κB-inducible *SEAP* reporter gene were cultured in a 96-well plate for 18h in the presence of increasing concentrations of CAPs or human specific, type B CpG ODN2006 (positive control) or negative control CpG oligonucleotide, and NF-κB-induced *SEAP* activity was assessed in the culture supernatant using QUANTI-Blue™. (f) Quantification of the data presented as mean (SEM) by reading the OD at 620 nm. N = 3, *p<0.03

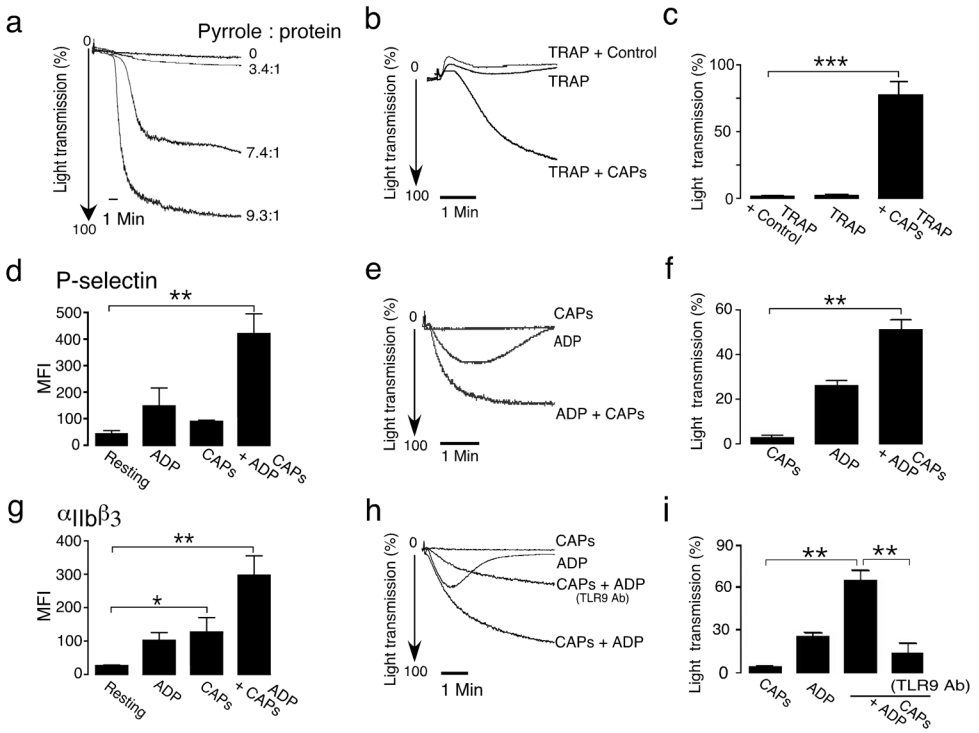

Figure 5. CAPs induce platelet aggregation and promote platelet hypereactivity in vitro

(a) Aggregation of human platelets stimulated with CAPs (CEP-BSA; albumin 1.5μM) with increasing degree of protein modifications (shown as molar ratio of pyrrole/protein). (b, c) CAPs (CEP-HSA; albumin 1.5μM, pyrrole 3.4 μM) induced aggregation, and (d) P-selectin expression of isolated human platelets primed with low dose ADP (5 μM) or TRAP (40 μM). (e, f) CAPs (CEP-HSA; albumin 1.5μM, pyrrole 3.4 μM) induced aggregation and (g) integrin-$\alpha_{IIb}\beta_3$ activation of isolated murine platelets primed with low dose ADP (3 μM). (h, i) Pretreatment of platelets with anti TLR9 blocking antibody inhibits synergism of CAPs and ADP. Quantification of the data shown as mean (SEM). N ≥ 3. * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

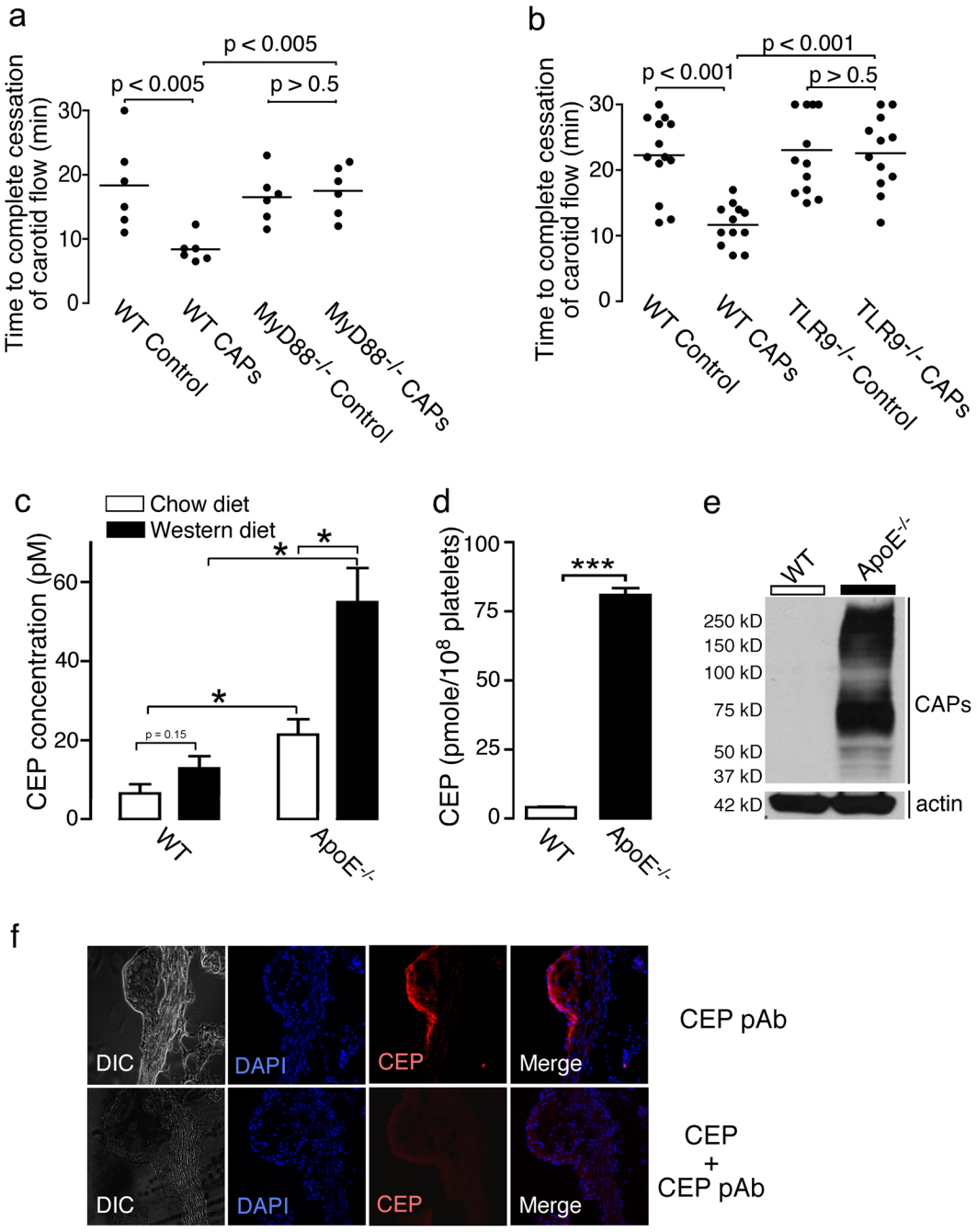

Figure 6. Carboxyalkylpyrrole protein adducts accelerate in vivo thrombosis in a MyD88/TLR9 dependent manner and are detected in ApoE$^{-/-}$ mice in vivo

(a, b) Anesthetized mice of indicated genotypes were injected with CAPs (albumin 3 μM, Pyrrole 25μM) or sham-modified control albumin (3 μM) as described in Methods, carotid arteries were exposed and thrombosis induced by application of $FeCl_3$. Thrombus formation in carotid arteries was visualized and the time to complete thrombotic occlusions were recorded. Data was analyzed by two-tailed nonparametric Mann Whitney test. (c) Plasma concentrations of CEP-adducts, measured by competitive ELISA in WT and ApoE$^{-/-}$ mice on chow or Western diet. Data are shown as mean (SEM) N=3. *p<0.05. (d) Concentrations of CEP-adducts in platelet lysates from WT mice fed chow diet and ApoE$^{-/-}$ mice fed

Western diet, measured by competitive ELISA. Data are shown as mean (SEM). N=3. ***p<0.0001. (e) CEP-adducts in WT, and ApoE$^{-/-}$ hyperlipidemic platelets, detected by western blotting. (f) Cryosections of aortic root of ApoE$^{-/-}$ mice with atherosclerotic lesions were immuno-stained with rabbit polyclonal anti-CEP antibody and DAPI. Anti-CEP antibody neutralized by incubating with excess of CAPs was used as control.

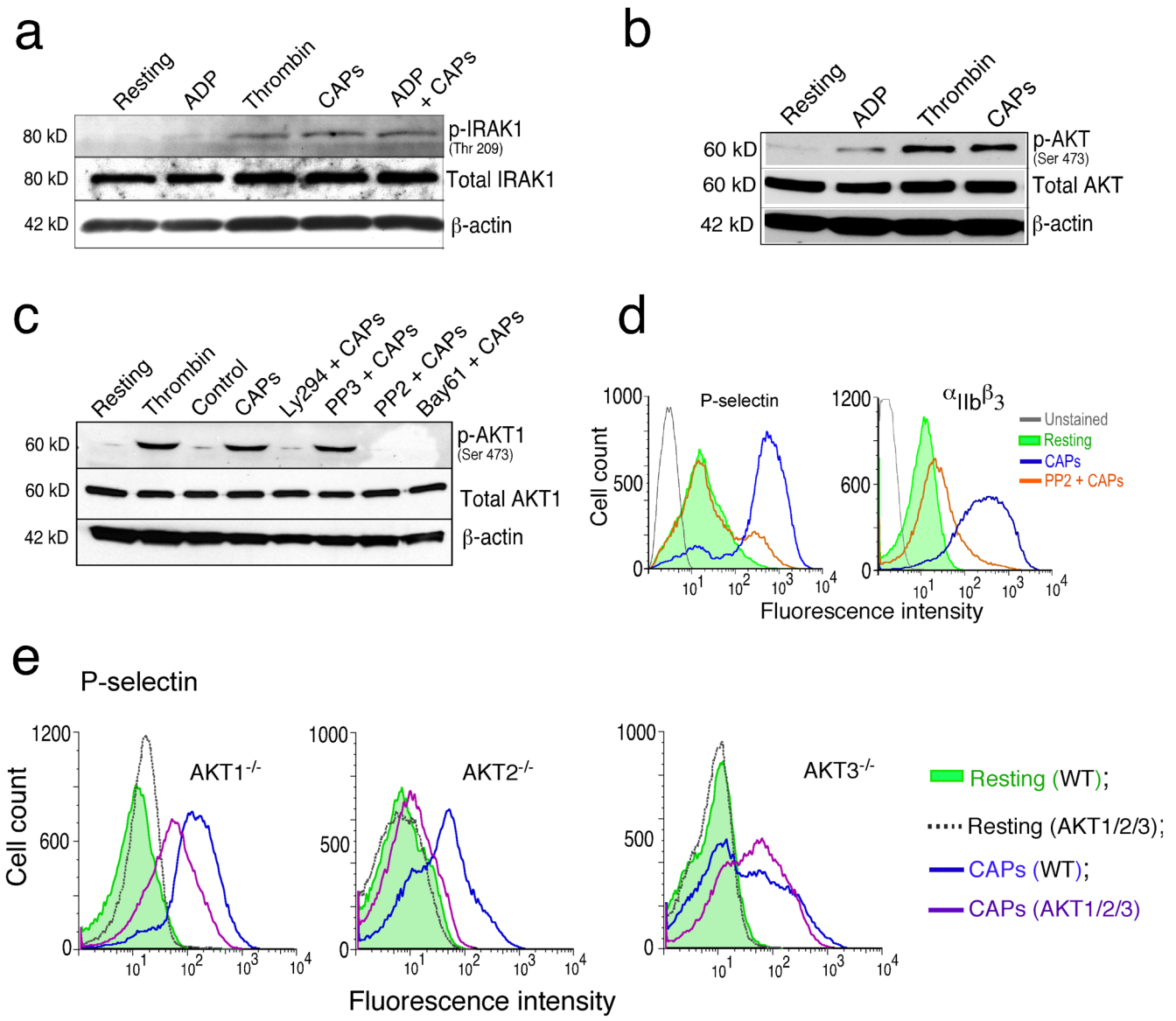

Figure 7. CAPs induce TLR9/MyD8/IRAK1 signaling and require PI3K and Src kinases for platelet activation

Human platelets were isolated by gel filtration and activated by indicated agonists, lysed and total protein expression and phosphorylation of indicated protein sites were detected using western blotting. (a) Phospho-IRAK1 (Thr209), (b) Phospho-AKT (Ser473) and (c) Phospho-AKT1 (Ser473) were detected in human platelets by rabbit monoclonal antibody with or without pretreatment of specific PI3K/AKT kinase inhibitor Ly294-002 (20μM), pan-Src kinase inhibitor PP2 (10μM) or control PP3 (10μM), and Syc kinase inhibitor Bay61 (5μM). Expression of total-IRAK1, total- AKT, total-AKT1 and β-actin were also estimated in the same blots as controls. (d) Effect of pan-Src kinase inhibitor PP2 (10μM) on P-selectin expression (left panel) and integrin-$\alpha_{IIb}\beta_3$(PAC1) activation (right panel) induced by CAPs in human platelets. (e)P-selectin expression induced by CAPs in AKT1/2/3 deficient murine platelets. Representative histograms from three independent experiments are shown. Concentrations of agonists are ADP (5μM), Thrombin (0.05U/mL), and CAPs (albumin 1.2μM, Pyrrole 10μM).